In correlation with the survival of effector OT-I cells, Bcl-2 expression level in OT-I cells was significantly lower in IL-15 KO mice but higher in IL-15 Tg mice than in control mice during the contraction phase (Fig. 2, *C* and *D*). There was no significant difference between the levels of BcL-x_L expression in OT-I cells from IL-15 Tg and IL-15 KO mice on day 7 after infection (data not shown). Taken together, these results suggest that IL-15 plays a critical role in the survival but not in the proliferation of activated T cells during the contraction phase of immune response to *L. monocytogenes*.

We next examined intracellular expression of Bcl-2 in IL-7Rα^{low} and IL-7Rα^{high} OT-I cells on days 7 and 10 after *Listeria* infection. Bcl-2 expression levels in IL-7Rα^{low} effector OT-I cells of control mice and IL-15 Tg mice gradually increased from day 7 to 10 after infection, and the magnitude of increase was greater in IL-15 Tg mice (Fig. 2*E*). In contrast, Bcl-2 expression in IL-7Rα^{low} effector OT-I cells of IL-15 KO mice was not up-regulated from day 7 to 10, indicating that IL-15 plays a critical role in the up-regulation of Bcl-2 expression in effector CD8$^+$ T cells during the contraction phase. In contrast, in IL-7Rα^{high} memory OT-I cells from control mice, Bcl-2 expression was up-regulated, compared with that in IL-7Rα^{low} effector OT-I cells from day 7 to 10 after infection. In correlation with the survival of memory OT-I cells, Bcl-2 expression levels in IL-7Rα^{high} memory OT-I cells were significantly lower in IL-15 KO mice but higher in IL-15 Tg mice than in control mice on days 7 and 10 after infection. These results suggest that IL-15 plays a critical role in the survival of not only effector cells but also memory cell subsets during the contraction phase after *Listeria* infection.

*IL-15 during the contraction phase is essential for the survival of effector OT-I cells after r*LM*-OVA infection*

To determine whether CD8$^+$ T cell contraction is dependent on the presence of endogenous IL-15 during the expansion phase or contraction phase, we primed OT-I cells in C57BL/6 and IL-15 KO primary hosts with r*LM*-OVA and, on day 6 after infection, adoptively transferred equal numbers of purified CD8$^+$ cells containing effector OT-I cells into C57BL/6 and IL-15 KO secondary hosts that had been infected with r*LM*-OVA 6 days previously and examined OT-I cells recovered from the spleen on day 11 after infection (Fig. 3*A*). As expected, when effector OT-I cells generated in C57BL/6 primary hosts were transferred into infected C57BL/6 secondary hosts, OT-I cells were recovered from the spleen on day 11 after infection (Fig. 3*B*). In contrast, when effector OT-I cells generated in IL-15 KO primary hosts were transferred into infected IL-15 KO secondary hosts, OT-I cells were hardly detected in the spleen on day 11 after infection. This is consistent with data obtained from kinetics analysis of OT-I cells shown in Fig. 1*A*. In contrast, when effector OT-I cells generated in IL-15 KO and C57BL/6 primary hosts were transferred into C57BL/6 secondary hosts, the numbers of OT-I cells were similar. In contrast, the numbers of effector OT-I cells from C57BL/6 primary hosts were reduced in IL-15 KO secondary hosts to ~10% of those in C57BL/6 secondary hosts by day 5 after transfer, indicating that the presence of endogenous IL-15 during the contraction phase is essential for the survival of effector OT-I cells after *Listeria* infection. This was not caused by migration of effector OT-I cells to the spleen from other anatomical locations, as we observed a similar defect in OT-I cells isolated from PEC and peripheral LN (data not shown).

To further confirm the requirement of IL-15 for the survival of effector CD8$^+$ T cells during the contraction phase, we examined the effect of in vivo administration of exogenous IL-15 during either the expansion or contraction phase on the survival of effector

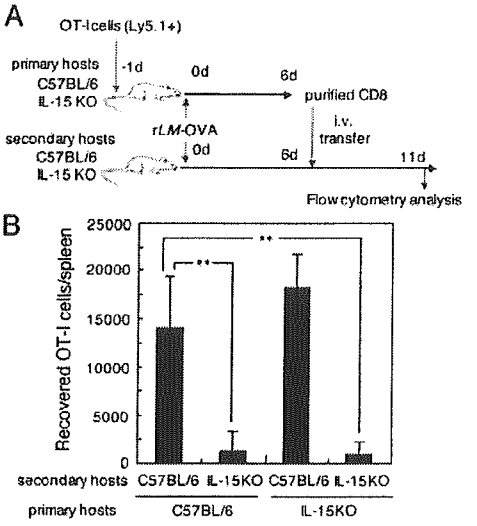

FIGURE 3. Endogenous IL-15 is required during the contraction phase but not the expansion phase for the survival of effector CD8$^+$ T cells after r*LM*-OVA infection. *A*, Effector OT-I cells from IL-15 KO primary hosts or C57BL/6 primary hosts on day 6 after infection were adoptively transferred into IL-15 KO secondary hosts or C57BL/6 secondary hosts that had been infected with r*LM*-OVA 6 days previously. Secondary recipients receiving effector OT-I cells were analyzed 5 days later by flow cytometry. *B*, Absolute number of transferred OT-I cells in the whole splenocyte population was calculated. Data were obtained from three separate experiments, and each value shown is the mean + SD for three mice. **, $p < 0.01$.

OT-I cells transferred into IL-15 KO hosts on day 11 after r*LM*-OVA infection. As shown in Fig. 4*A*, the administration of rIL-15 during the initial expansion phase (1–4 days) had no effect on contraction of the OT-I cells in IL-15 KO hosts on day 11 after infection. In contrast, rIL-15 administration during the initial contraction phase (7–10 days) prolonged the expansion of OT-I cells in IL-15 KO hosts and resulted in an increased number of OT-I cells after infection. This effect was seen in cells isolated from a variety of lymphoid and nonlymphoid compartments, including PBMC, peripheral LN, and PEC (data not shown). Although OT-I cells expressing low levels of CD127 were not detected in IL-15 KO mice treated with PBS during the contraction phase on day 11 after infection, these cells survived in IL-15 KO mice treated with rIL-15 during the contraction phase at the same time (Fig. 4*B*). We found that the increases in numbers of OT-I cells, especially CD127^{low} OT-I cells, occurred in a dose-dependent manner (data not shown). As shown in Fig. 4*C*, strong induction of Bcl-2 was found in OT-I cells from rIL-15-treated IL-15 KO hosts during the contraction phase but not during the expansion phase, compared with that in OT-I cells from PBS-treated IL-15 KO hosts on day 11 after infection. These results suggest that the presence of IL-15 during the contraction phase is essential for the survival of IL-7Rα^{low} effector CD8$^+$ T cells after *Listeria* infection.

To examine the generation of memory CD8$^+$ T cells in rIL-15-treated IL-15 KO mice at the memory phase, we determined the numbers of transferred OT-I cells in spleens from these mice on day 60 after r*LM*-OVA infection. As shown in Fig. 4, *D* and *E*, rIL-15 treatment during the contraction phase resulted in increased percentages and absolute numbers of memory OT-I cells in IL-15 KO mice on day 60 after r*LM*-OVA infection. Memory OT-I cells generated in rIL-15-treated IL-15 KO mice showed expression levels of CD127 and CD62L similar to those in memory OT-I cells

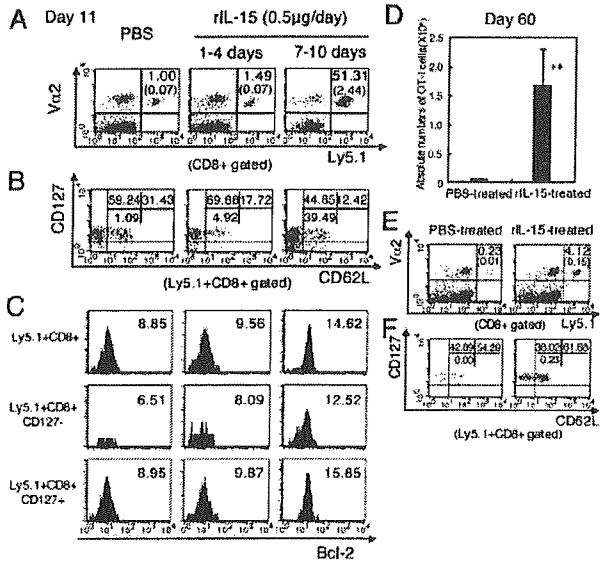

FIGURE 4. Exogenous IL-15 during the contraction phase increased the number of Ag-specific $CD8^+$ T cells in IL-15 KO mice after r*LM*-OVA infection. IL-15 KO mice that received naive OT-I cells were infected with r*LM*-OVA and were then treated with rIL-15 or PBS during the initial expansion phase (1–4 days) or contraction phase (7–10 days) of primary immune response. *A*, Proportion of the OT-I cells in the spleen after rIL-15 treatment was determined on day 11 after infection. The numbers represent the number of OT-I cells as a percentage of $CD8^+$ T cells and as a percentage of total lymphocytes (parentheses). *B*, Dot plots of $Ly5.1^+CD8^+$ cells stained for CD62L and CD127 on day 11 after infection are shown. *C*, Intracellular expression of Bcl-2 in OT-I cells after rIL-15 treatment. Histograms are gated on $Ly5.1^+CD8^+$, $Ly5.1^+CD8^+CD127^-$, or $Ly5.1^+CD8^+CD127^+$ cells in spleens from infected mice. The values in the *right corner* of each panel represent the mean fluorescence intensity of Bcl-2 expression in OT-I cells. *D*, IL-15 KO mice that received naive OT-I cells were infected with r*LM*-OVA and then treated with rIL-15 or PBS during the contraction phase (7–10 days) of primary immune responses. Total number of memory OT-I cells in the spleen on day 60 after infection was calculated. **, $p < 0.01$. *E*, Proportions of OT-I cells on day 60 after infection are shown. The numbers represent the number of OT-I cells as a percentage of $CD8^+$ T cells and as a percentage of total lymphocytes (parentheses). *F*, Dot plots of $Ly5.1^+CD8^+$ cells stained for CD62L and CD127 on day 60 after infection are shown.

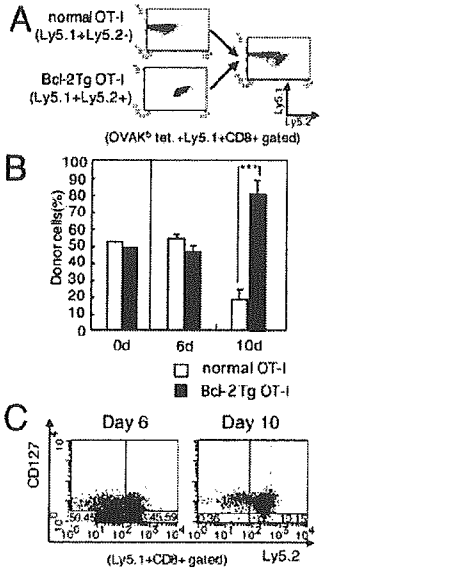

FIGURE 5. Enforced expression of Bcl-2 in OT-I cells prevented activated T cell death in IL-15 KO mice after r*LM*-OVA infection. *A*, Bcl-2 Tg OT-I cells ($Ly5.1^+Ly5.2^+$) and normal OT-I cells ($Ly5.1^+Ly5.2^-$) were mixed in equal proportions. The proportion of each donor cell population was determined before adoptive transfer. *B*, Mixtures of Bcl-2 Tg OT-I cells and normal OT-I cells were adoptively transferred into IL-15 KO mice ($Ly5.1^-Ly5.2^+$) that were then infected with r*LM*-OVA 24 h later. The graph depicts the ratio of normal OT-I cells to Bcl-2 Tg OT-I cells at indicated days before and after infection. Each value is the mean of three mice + SD. ***, $p < 0.005$. *C*, CD127 expression on either normal OT-I cells or Bcl-2 Tg OT-I cells transferred into IL-15 KO mice was examined on days 6 and 10 after infection. The numbers in the *lower corners* of each plot refer to the percentage of CD127-negative effector OT-I cells ($Ly5.1^+CD8^+$) that are derived from $Ly5.2^+$ (Bcl-2 Tg OT-I cells) or $Ly5.2^-$ (normal OT-I cells).

generated in PBS-treated IL-15 KO mice (Fig. 4*F*). Therefore, rIL-15 treatment during the contraction phase resulted in increased survival of not only IL-7Rα^{low} effector cells but also IL-7Rα^{high} memory cells after *Listeria* infection.

Effect of Bcl-2 overexpression in OT-I cells on $CD8^+$ T cell contraction in IL-15 KO mice

To determine whether up-regulation of Bcl-2 by IL-15 is involved in the prevention of activated T cell death in vivo, we performed transfer experiments using a mixed donor cell population containing equal numbers of normal OT-I cells ($Ly5.1^+Ly5.2^-$) and Bcl-2 Tg OT-I cells ($Ly5.1^+Ly5.2^+$) (Fig. 5*A*). The mixture was transferred into IL-15 KO hosts ($Ly5.1^-Ly5.2^+$), and then the hosts were infected with r*LM*-OVA 24 h later. We examined the ratio of normal OT-I cells to Bcl-2 Tg OT-I cells on days 6 and 10 after infection. As shown in Fig. 5*B*, substantial expansion of normal OT-I cells and Bcl-2 Tg OT-I cells had occurred in the spleens from IL-15 KO hosts on day 6 after infection. We confirmed by using an adoptive transfer system of CFSE-labeled OT-I cells that enforced expression of Bcl-2 did not affect expansion of OT-I cells during the expansion phase of immune response (data not shown). In contrast, the ratio of normal OT-I cells to Bcl-2 Tg OT-I cells was 15:85 in the spleens from IL-15 KO hosts on day 10 after infection. As shown in Fig. 5*C*, in IL-15 KO hosts, substantial numbers of IL-7Rα^{low} Bcl-2 Tg OT-I cells survived in the spleen, whereas IL-7Rα^{low} normal OT-I cells had disappeared on day 10 after infection. The numbers of IL-7Rα^{high} OT-I cells were also higher in Bcl-2 Tg OT-I cells, compared with those in normal OT-I cells in IL-15 KO hosts. There results suggest that up-regulation of Bcl-2 in activated T cells by IL-15 is essential for the survival of IL-7Rα^{low} effector and IL-7Rα^{high} memory $CD8^+$ T cells during the contraction phase after *Listeria* infection.

IL-15Rα expression on host cells is essential for the survival of effector OT-I cells during the contraction phase

Recent studies have demonstrated that IL-15Rα-mediated transpresentation of IL-15 is a major mechanism of IL-15-mediated actions (22, 31–33). To determine whether expression of IL-15Rα by effector OT-I cells or by host cells is required for the survival of effector OT-I cells, we adoptively transferred IL-15R$\alpha^{-/+}$ OT-I cells or IL-15R$\alpha^{-/-}$ OT-I cells into IL-15R$\alpha^{+/+}$ or IL-15R$\alpha^{-/-}$ hosts followed by challenge with r*LM*-OVA. As shown in Fig. 6*A*, nearly identical numbers of IL-15R$\alpha^{+/+}$ OT-I cells and IL-15R$\alpha^{-/-}$ OT-I cells were obtained from spleens of IL-15R$\alpha^{+/+}$ hosts on days 7 and 10 after primary infection. In contrast, the numbers of IL-15R$\alpha^{+/+}$ OT-I cells in IL-15R$\alpha^{-/-}$ hosts on day 7 after infection were almost same as those in IL-15R$\alpha^{+/+}$ hosts,

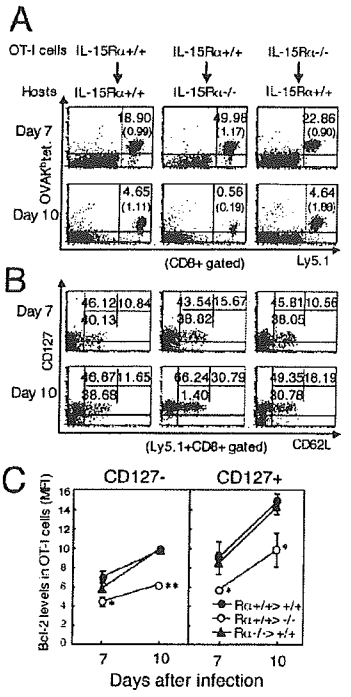

FIGURE 6. IL-15Rα expression on host cells but not on CD8+ T cells was required for the survival of effector CD8+ T cells during the contraction phase after r*LM*-OVA infection. IL-15Rα$^{+/+}$ or IL-15Rα$^{-/-}$ OT-I cells (Ly5.1+) were adoptively transferred into IL-15Rα$^{+/+}$ or IL-15Rα$^{-/-}$ hosts that were then infected with r*LM*-OVA 24 h later. *A*, Proportions of OT-I cells in the spleen from OT-I chimeric mice on days 7 and 10 after infection. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on CD8+ cells. The numbers represent the number of OT-I cells as a percentage of CD8+ T cells and as a percentage of total lymphocytes (parentheses). *B*, Dot plots of Ly5.1+CD8+ cells stained for CD62L and CD127 on days 7 and 10 after infection are shown. *C*, Changes in mean fluorescence intensity (MFI) of Bcl-2 staining in CD127− and CD127+ OT-I cells from IL-15Rα$^{+/+}$OT-I>$^{+/+}$, IL-15Rα$^{+/+}$OT-I>$^{-/-}$, and IL-15Rα$^{-/-}$OT-I>$^{+/+}$ chimeric mice on days 7 and 10 after r*LM*-OVA infection. *, $p < 0.05$; **, $p < 0.01$.

whereas the number of IL-15Rα$^{+/+}$ OT-I cells in IL-15Rα$^{-/-}$ hosts was significantly lower on day 10 after infection. The numbers of IL-7Rα^{low}IL-15Rα$^{+/+}$ and IL-7Rα^{low}IL-15Rα$^{-/-}$ OT-I cells in IL-15Rα$^{+/+}$ hosts on day 10 after *Listeria* infection were comparable, whereas IL-7Rα^{low}IL-15Rα$^{+/+}$ OT-I cells transferred into IL-15Rα$^{-/-}$ hosts had disappeared on day 10 after infection (Fig. 6*B*). As shown in Fig. 6*C*, intracellular expression levels of Bcl-2 in IL-7Rα^{low} OT-I cells among IL-15Rα$^{+/+}$ and IL-15Rα$^{-/-}$ cells had increased equally during the contraction phase after infection in IL-15Rα$^{+/+}$ hosts. In contrast, IL-7Rα^{low}IL-15Rα$^{+/+}$ OT-I cells could not up-regulate expression of Bcl-2 by day 10 after *Listeria* infection in IL-15Rα$^{-/-}$ hosts. These results suggest that IL-15Rα expression on host cells but not on OT-I cells is crucial for the survival of effector OT-I cells during the contraction phase after *Listeria* infection.

Effect of in vivo administration of rIL-15 during the contraction phase on survival of Ag-specific CD8+ T cells after rLM-OVA infection in normal mice

To investigate the therapeutic efficacy of rIL-15 in promoting memory T cell generation in normal mice, we administrated rIL-15 to r*LM*-OVA-infected C57BL/6 mice during the contraction phase (7–10 days). As shown in Fig. 7*A*, the numbers of $OVA_{257-264}$-specific CD8+ T cells in both peripheral lymphoid and nonlymphoid tissues were significantly increased in rIL-15-treated normal mice on day 11 after infection. Staining for T cell subpopulations revealed that the CD127^{low} subset in rIL-15-treated normal mice was much larger than other subsets on day 11 after r*LM*-OVA infection (Fig. 7*B*); absolute numbers of CD127^{high} subsets were also increased in rIL-15-treated normal mice. Similarly, treatment

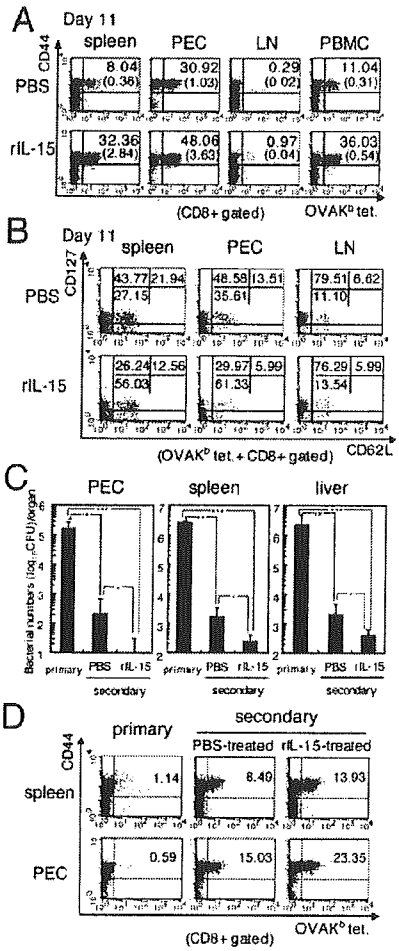

FIGURE 7. rIL-15 treatment during the contraction phase resulted in an increased number of Ag-specific memory CD8+ T cells and provided robust protective immunity against secondary *Listeria* infection. *A*, r*LM*-OVA-infected C57BL/6 mice were injected with 0.5 μg of rIL-15 or PBS during the contraction phase (7–10 days). Proportions of the $OVA_{257-264}$-specific CD8+ T cells in the spleen, PEC, peripheral LN, and PBMC were examined after rIL-15 treatment. The numbers represent the number of MHC tetramer binding cells as a percentage of CD8+ T cells and as a percentage of total lymphocytes (parentheses). *B*, Dot plots of CD8+ and $OVA_{257-264}$ K^b tetramer staining-positive cells stained for CD62L and CD127 11 days after infection are shown. *C*, On day 60 after initial infection, rIL-15-treated or PBS-treated mice were challenged with a lethal dose of r*LM*-OVA. Naive C57BL/6 mice were introduced into the experiment at that time point. Bacterial numbers in the PEC, spleen, and liver on day 3 after the secondary challenge were determined. Data were obtained from three separate experiments, and each value shown is the mean + SD for five mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$. *D*, Proportions of $OVA_{257-264}$-specific CD8+ T cells in the PEC and spleen from representative mice on day 5 after primary or secondary infection. Analysis gate had been set on CD8+ cells.

of BALB/c mice with rIL-15 during the contraction phase resulted in an increase in the number of listeriolysin O_{91-99}-specific $CD8^+$ T cells on day 14 after *L. monocytogenes* infection (data not shown). Thus, in vivo administration of rIL-15 during the contraction phase enhanced survival of Ag-specific $CD8^+$ T cells in two different mouse strains.

To determine the protective capacity of memory $CD8^+$ T cells generated in rIL-15-treated normal mice, we administrated rIL-15 or PBS to r*LM*-OVA-immune C57BL/6 mice during the contraction phase (7–10 days). On day 60 after initial infection, we rechallenged these previously infected mice and naive mice with a lethal dose of r*LM*-OVA and determined the number of bacteria in the PEC, spleen, and liver on day 3 after rechallenge. We also compared expansion of $OVA_{257-264}$-specific memory $CD8^+$ T cells in these mice on day 5 after rechallenge. As expected, memory $CD8^+$ T cells in PBS-treated mice provided substantial protective immunity against rechallenge with r*LM*-OVA (Fig. 7*C*). Administration of rIL-15 during the contraction phase resulted in 10-fold reductions in bacterial loads in the PEC, spleen, and liver of immunized mice, compared with those in the case of PBS administration on day 3 after reinfection. The proportions of $OVA_{257-264}$-specific $CD8^+$ T cells in the PEC and spleen were substantially increased in rIL-15-treated immune mice, compared with those in PBS-treated immune mice on day 5 after reinfection (Fig. 7*D*). These results suggest that rIL-15 treatment during the contraction phase provides robust protective immunity against reinfection.

Discussion

In this study, we examined the roles of IL-15 in activated T cell death using a system of adoptive transfer of OT-I cells into naive IL-15 KO or IL-15 Tg hosts that were subsequently infected with r*LM*-OVA. We found that the survival of effector $CD8^+$ T cells during the contraction phase is critically dependent on transpresentation of IL-15 by IL-15Rα. Enforced expression of Bcl-2 in Ag-specific $CD8^+$ T cells protected the majority of activated T cells from death in an IL-15-deficient environment. These results suggest that IL-15 plays an important role in the survival of effector $CD8^+$ T cells after *Listeria* infection via up-regulation of Bcl-2 expression.

There are several possible explanations of how IL-15 serves to increase numbers of effector $CD8^+$ T cells during the contraction phase of immune response. The first possibility is that different kinetics of bacterial burden may affect the number of effector $CD8^+$ T cells during the course of infection. However, we found that the kinetics of bacterial growth was similar in all groups, and no difference in the number of bacteria was detected in the spleen between control and IL-15KO mice during the course of infection. IL-15 is important in maturation of dendritic cells for Ag presentation (34), raising the second possibility that generation of effector cells or memory cell precursors may be affected during the expansion phase. Schluns et al. (24) suggested that IL-15 was required for the generation of Ag-specific $CD8^+$ T cells after VSV infection, whereas we found in the present study that the numbers of both IL-7Rα^{low} and IL-7Rα^{high} OT-I cells in IL-15 KO or IL-15 Tg mice were comparable to those in control mice at the peak of immune response to r*LM*-OVA. Therefore, IL-15 may not be required for generation of effector OT-I cells during the expansion phase of r*LM*-OVA infection. Becker et al. (21) have reported that the $CD8^+$ T cell responses to most of epitopes, including NP396, were only slightly, if any, affected after primary infection, whereas $CD8^+$ T cell response to one epitope (GP33) did show a rather dramatic requirement for IL-15. Wang et al. (35) showed that $CD8^+$ T cells specific to LCMV epitopes NP396 and GP33 differed in their preapoptotic state, with NP396-specific T cells binding more annexin V than GP33-specifc T cells during the expansion phase on day 8 after LCMV infection. These results suggest that population of T cells specific to different epitopes can have different properties such as IL-15 dependency during the expansion phase after acute infection. The difference in IL-15 dependency may be linked to intrinsic properties of the epitopes such as avidity of epitope and T cells. The discrepancy between VSV and r*LM*-OVA infections may be at least partly explained by a difference in intrinsic properties of the epitopes. Several studies have shown that IL-15 has the potential roles in maintenance of Ag-specific memory $CD8^+$ T cells, which are capable of slowly dividing without Ag stimulation, during the memory phase (21–24). Therefore, another possibility is that IL-15 may induce proliferation of effector/memory $CD8^+$ T cells during the contraction phase. However, we found that endogenous IL-15 did not affect proliferation of OT-I cells during the contraction phase after *Listeria* infection, thus excluding this possibility. IL-15 has been shown to be a potent inhibitor of several apoptosis pathways in several lymphocytes via induction of antiapoptotic molecules (36–40). Hence, it is also possible that IL-15 protects activated T cells from apoptosis during the contraction phase. We favor this possibility because we found that intracellular expression level of active caspase-3 in OT-I cells was increased significantly in IL-15 KO mice but decreased in IL-15 Tg mice during the contraction phase, compared with control mice. In the present study, we examined the protective role of IL-15 in contraction of $CD8^+$ T cells specific to only one immunodominant epitope. The generality of this finding and its possible implications awaits further analysis with different epitopes. The generality of this finding and its possible implications awaits further analysis with different epitopes. It has been shown that up-regulation of Bcl-2 expression in activated T cells plays a central role in prevention of activated T cell death in vivo (4, 5). We found that IL-7Rα^{low} effector OT-I cells in IL-15 KO mice failed to up-regulate Bcl-2 expression during the contraction phase and resulted in disappearance of their subsets and that their death can be inhibited by Bcl-2 overexpression. Moreover, in vivo administration of rIL-15 during the contraction phase in IL-15 KO mice restored expression of Bcl-2 in OT-I cells and inhibited the abolishment of effector OT-I cells. Therefore, it is most likely that IL-15 serves to protect effector $CD8^+$ T cells from apoptosis by inducing up-regulation of Bcl-2 expression. Bcl-2 expression is induced via signaling from cγ-chain (15, 16), which is used by IL-2, IL-4, IL-7, IL-9, and IL-21, as well as IL-15. It has been shown that rIL-2 treatment during the contraction phase increased the expression of Bcl-2 in effector $CD8^+$ T cells after LCMV infection, resulting in increased survival of Ag-specific $CD8^+$ T cells (41). We found that the expression of Bcl-2 in effector OT-I cells could be induced not only upon stimulation with rIL-15 but also upon stimulation with rIL-2 in vitro in a dose-dependent manner (our unpublished data). Therefore, IL-2 may also increase the survival of effector $CD8^+$ T cells during the contraction phase via inducing Bcl-2 expression. Activated T cells capable of producing IL-2 disappear rapidly during the contraction phase of primary immune response, whereas IL-15 may be constitutively produced by non-T cells during the contraction phase. This may explain why effector $CD8^+$ T cells use only IL-15 rather than the other common γ cytokines, including IL-2, during the contraction phase under physiological conditions. There has been an accumulation of evidence supporting the idea that transpresentation of IL-15 is a major mechanism of IL-15-mediated actions (22, 31–33). We also found that transpresentation of IL-15 by IL-15Rα plays a major role in up-regulation of Bcl-2 expression in effector $CD8^+$ T cells during the contraction phase. We speculate that IL-15Rα^+ host cells, after

binding IL-15, may retain the complex and act as a reservoir for IL-15 after reduction of survival cytokines production during the contraction phase. Although a previous study suggested that IL-2Rα could present IL-2 in *trans* (42), IL-2Rα alone binds IL-2 with low affinity, and in vivo studies with IL-2Rα KO T cells indicated that IL-2Rα plays a cell-autonomous role in supporting T cells (43, 44). Taken together, transpresentation of IL-15 but not IL-2 by host cells were critical for the survival of effector CD8$^+$ T cells during the contraction phase of immune response via up-regulation of Bcl-2 expression.

A notable finding in the present study is that IL-15 could increase the survival of not only IL-7Rα^{low} effector cells but also IL-7Rα^{high} memory cells during the contraction phase. A recent study has shown that only those IL-7Rα-expressing Ag-specific CD8$^+$ T cells at the peak of primary immune response give rise to memory cells after adoptive transfer into naive hosts (29, 30), suggesting that only IL-7Rα^{high} cells are capable of surviving CD8$^+$ T cell contraction. It has been shown that expression of Bcl-2 was partially impaired in IL-7Rα-deficient OT-I cells during the contraction phase after VSV-OVA infection, resulting in a decrease in the number of memory CD8$^+$ T cells (45). These findings suggest that IL-7 is required for the survival of IL-7Rα^{high} memory CD8$^+$ T cells during the contraction phase. In the present study, we found that IL-15 is also involved in the protection IL-7Rα^{high}CD8$^+$ T cells from apoptosis during the contraction phase via inducing Bcl-2 expression. Collectively, these findings suggest that both IL-15 and IL-7 participate in the increased survival of memory CD8$^+$ T cells during the contraction phase via inducing Bcl-2 expression.

Administration of rIL-15 during the contraction phase may be useful for enhancing vaccination. However, IL-15 may have prolonged effector responses rather than increasing memory cells because IL-15 therapy during the contraction phase resulted in a greater increase in the number of IL-7Rα^{low} effector cells than the number of IL-7Rα^{high} memory subsets and resulted in a gradual decreases in the number of Ag-specific CD8$^+$ T cells over a period of several months. Therefore, IL-15 may be more useful in strategies for treating chronic infections, including *Mycobacterium tuberculosis* infection, in which transient prolongation of T cell responses may be required to reduce the bacterial burden. Additional experiments on *M. tuberculosis* infection may enable us to determine conclusively whether IL-15 is useful for the development of new immunoprotective approaches against chronic infection.

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Disclosures

The authors have no financial conflict of interest.

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Impaired Protection against *Mycobacterium bovis* Bacillus Calmette-Guérin Infection in IL-15-Deficient Mice[1]

Kimika Saito,* Toshiki Yajima,* Shino Kumabe,* Takehiko Doi,* Hisakata Yamada,* Subash Sad,† Hao Shen,‡ and Yasunobu Yoshikai[2]*

To investigate the potential role of endogenous IL-15 in mycobacterial infection, we examined protective immunity in IL-15-deficient (IL-15$^{-/-}$) mice after infection with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) or recombinant OVA-expressing BCG (rBCG-OVA). IL-15$^{-/-}$ mice exhibited an impaired protection in the lung on day 120 after BCG infection as assessed by bacterial growth. CD4$^+$ Th1 response capable of producing IFN-γ was normally detected in spleen and lung of IL-15$^{-/-}$ mice on day 120 after infection. Although Ag-specific CD8 responses capable of producing IFN-γ and exhibiting cytotoxic activity were detected in the lung on day 21 after infection with rBCG-OVA, the responses were severely impaired on days 70 and 120 in IL-15$^{-/-}$ mice. The degree of proliferation of Ag-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice was similar to that in wild-type mice during the course of infection with rBCG-OVA, whereas sensitivity to apoptosis of Ag-specific CD8$^+$ T cells significantly increased in IL-15$^{-/-}$ mice. These results suggest that IL-15 plays an important role in the development of long-lasting protective immunity to BCG infection via sustaining CD8 responses in the lung. *The Journal of Immunology*, **2006, 176: 2496–2504.**

It is widely accepted that protection against infection with mycobacteria such as *Mycobacterium bovis* and *M. tuberculosis* depends mainly on IFN-γ produced by CD4$^+$ Th1 cells (1–3). There are several lines of evidence that CD8$^+$ T cells producing IFN-γ and exhibiting cytotoxicity play a requisite role in resistance to mycobacterial infection (3–5). β_2-Microgloburin-deficient mice and TAP-deficient (TAP$^{-/-}$) mice, which lack functional CD8$^+$ T cells, are susceptible to infection with *M. tuberculosis* (6, 7). Adoptive transfer of immunized CD8$^+$ T cells can confer protection against subsequent challenge with *M. tuberculosis* (8). It has been suggested that CD8$^+$ CTLs releasing perforin and granulysin play a role in protection against *M. tuberculosis* infection via a cytolysis mechanism (9, 10). In contrast, the resistance to *M. tuberculosis* infection in both perforin- and Fas-deficient mice was unaltered, suggesting that the cytotoxic function of CD8$^+$ T cells may not be critical in protection against tuberculosis (11). A recent study has suggested that CD8$^+$ T cells are more important than CD4$^+$ T cells in controlling the latent phase of tuberculosis infection, which comprises the majority of human infections (12–14). Thus, it appears that protection against chronic pulmonary tuberculosis requires a sustained cellular immunity mediated by CD8$^+$ T cells and that Ag-specific CD8$^+$ T cells are a major target for vaccine design.

IL-15 uses β- and γ-chains of the IL-2R for signal transduction and thus shares many properties of IL-2 despite having no sequence homology with IL-2 (15, 16). IL-15 has been reported to stimulate NK cells and TCR$\gamma\delta$ intestinal intraepithelial lymphocytes to produce IFN-γ and enhance their cytotoxicity (17, 18). Furthermore, IL-15 has an important function in the proliferation and survival of memory-phenotype CD8$^+$ T cells. We previously found that IL-15 transgenic (Tg)[3] mice expressing IL-15 cDNA encoding a secretable isoform had an increased number of memory CD8$^+$ T cells in a naive state and showed enhanced protection against infection with *Listeria monocytogenes* and against infection with *M. bovis* bacillus Calmette-Guérin (BCG) via activation of NK cells and CD8$^+$ T cells (19–21). In contrast, IL-15R$\alpha^{-/-}$ and IL-15$^{-/-}$ mice have been reported to be deficient in memory-phenotype CD8$^+$ T cells in addition to NK cells, NKT cells, and TCR$\gamma\delta$ intestinal intraepithelial lymphocytes (22, 23). However, IL-15$^{-/-}$ mice have been reported not to be susceptible to infection with lymphocytic choriomeningitis virus (LCMV) (24) or *Toxoplasma gondii* (25), although soluble IL-15Rα treatment exacerbated *Toxoplasma* infection (26). IL-15$^{-/-}$ mice may compensate for the defect in the host defense system by IL-15-dependent cell populations by alternative mechanisms.

With the aim of elucidating the roles of endogenous IL-15 in vivo, we examined susceptibility and T cell-mediated immunity against BCG and recombinant OVA-expressing BCG (rBCG-OVA) in IL-15$^{-/-}$ mice. We found that bacterial growth was increased in lungs of IL-15$^{-/-}$ mice on day 120 after i.p. infection with BCG. CD4$^+$ Th1 response capable of producing IFN-γ in response to purified protein derivative (PPD) was normally detected, but the model experiments performed in mice infected with rBCG-OVA suggest that Ag-specific CD8 responses capable of

*Division of Host Defense, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan; †Laboratory of Cellular Immunology, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada; and ‡Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

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[2] Address correspondence and reprint requests to Dr. Yasunobu Yoshikai, Division of Host Defense, Medical Institute of Bioregulation, Kyushu University, Fukuoka, 812-8582, Japan. E-mail address: yoshikai@bioreg.kyushu-u.ac.jp

[3] Abbreviations used in this paper: Tg, transgenic; BCG, bacillus Calmette-Guérin; LCMV, lymphocytic choriomeningitis virus; rBCG-OVA, recombinant OVA-expressing BCG; PPD, purified protein derivative; WT, wild type; Tc1, T cytotoxic 1; MNC, mononuclear cell; DC, dendritic cell; CD62L, CD62 ligand.

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producing IFN-γ and exhibiting cytotoxic activity were severely impaired in IL-15$^{-/-}$ mice on days 70 and 120 after rBCG-OVA infection. Although IL-15-dependent cell proliferation was important for maintenance of Ag-specific memory CD8$^+$ T cells after acute infection with microbes, proliferation of memory CD8$^+$ T cells in IL-15$^{-/-}$ mice was almost the same as that in wild-type (WT) mice during the course of rBCG-OVA infection. In contrast, Ag-specific CD8$^+$ T cells from infected IL-15$^{-/-}$ mice were more susceptible to apoptosis than were those from infected WT mice. Thus, these results suggest that IL-15 plays an important role in the development of long-lasting protective immunity to BCG infection via sustaining T cytotoxic 1 (Tc1) responses in the lung.

Materials and Methods

Mice

C57BL/6-background IL-15$^{-/-}$ mice were purchased from Taconic. In each experiment, age- and sex-matched C57BL/6 mice, purchased from Charles River Japan, were used as controls. Mice were maintained under specific pathogen-free conditions and offered food and water ad libitum. All mice were used at 6–8 wk of age.

Microorganisms

Lyophilized *M. bovis* BCG (Tokyo strain) was purchased from Kyowa Pharmaceuticals. rBCG-OVA was previously described (27, 28). Briefly, BCG (Pasteur strain) was grown on Middlebrook 7H10 solid medium (Difco). A partial sequence of the OVA gene 230–359, which encodes the SIINFEKL epitope and its flanking sequences, was cloned in the pMV261 vector, downstream of the Ag 85B secretion signal and under the control of heat shock protein 60 promoter. BCG and rBCG-OVA were dissolved in 7H9 medium (Difco) supplemented with albumin-dextrose-catalase enrichment (Difco). The viable bacterial numbers were determined by a 7H10 (Difco) plate supplemented with oleic acid-albumin-dextrose-catalase enrichment (Difco). Small aliquots of BCG suspended in 7H9 medium containing 10% glycerol were stored at −80°C until use. The concentration of bacteria was quantified by plate counting. Before use, the bacteria were washed three times with PBS and resuspended in PBS. Mice were inoculated i.p. with 2–10 × 10^6 CFU (depending on the experiment) of BCG or rBCG-OVA in a volume of 200 μl of PBS.

Abs and reagents

FITC-conjugated anti-CD3ε (145-2C11), CD44 (IM7), and IFN-γ (XMG1.2) mAbs; PE-conjugated anti-NK1.1 (PK136), TCRγδ (UC7), and CD8α (53-6.7) mAbs; CyChrome-conjugated anti-CD4 (GK1.5), CD8α (53-6.7), and TCRβ (H57-597) mAbs; biotin-conjugated anti-CD44 (IM7) mAb; allophycocyanin-conjugated streptavidin; and biotin-conjugated anti-Ly5.1 mAb (A20) were purchased from BD Pharmingen. FITC-conjugated hamster anti-mouse activated form of caspase-3 (C92-605), Bcl-2 mAb (3F11), and hamster anti-mouse isotype control were obtained from BD Pharmingen. Annexin V^{FITC} apoptosis detection kit was purchased from Sigma-Aldrich. $OVA_{257-264}$ H-2K^b tetramers were purchased from MBL.

Cell preparation

Splenocytes from BCG-infected mice were prepared by centrifugation and resuspended in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES. Lung mononuclear cells (MNCs) were prepared as described previously (29). Briefly, lung tissue was minced and incubated with stirring at 37°C for 30 min in HBSS with 1.3 mM EDTA, followed by treatment at 37°C for 1 h with collagenase (150 U/ml; Invitrogen Life Technologies) in RPMI 1640 with 10% FBS. The resulting suspension was pelleted by centrifugation, resuspended in 44% Percoll (Pharmacia) layered on 66.6% Percoll, and centrifuged at 600 × *g*. Cells at the gradient interface were harvested and washed extensively before use.

Flow cytometric analysis

Splenocytes or lung MNCs were preincubated with a culture supernatant from 2.4 G_2 to prevent nonspecific staining. After washing, cells were stained with various combinations of mAbs. Staining with biotin-conjugated mAb was followed by treatment with streptavidin-CyChrome or -allophycocyanin. The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). The data were analyzed with CellQuest software (BD Biosciences).

In vitro culture and cytokine ELISA

Spleen cells were subjected to an Ag stimulation assay for cytokine production. Nylon-wool-passed spleen cells from BCG-infected mice on days 14 and 120 after infection were resuspended in RPMI 1640 and added to 96-well plates at a concentration of 2 × 10^5 cells/well. Cells were cultured without any stimulation or with 5 μg/ml PPD (Japan BCG Association) in the presence of mitomycin C-treated splenocytes (1 × 10^6) from naive mice for 48 h at 37°C. Supernatants were collected and stored at −80°C until the cytokine assay. IFN-γ, IL-10, and IL-4 concentrations in the culture supernatant were measured using ELISA development kits (Genzyme Diagnostics).

Intracellular cytokine, caspase-3, or Bcl-2 staining

Splenocytes or lung MNCs were incubated without any stimulation or with 5 μg/ml PPD or 5 μg/ml $OVA_{257-264}$ peptide and 100 pg/ml rIL-2 (Takeda Chemical) for 6 h at 37°C and 5% CO_2 with 10 μg/ml brefeldin A (Sigma-Aldrich) added for the last 2 h in 48-well flat-bottom plates at a concentration of 5 × 10^6 cells/well (Falcon; BD Biosciences) in a volume of 0.5 ml of RPMI 1640 containing 10% FCS. After 6 h of culture, the cells were harvested and surface stained in staining buffer with CyChrome-conjugated anti-CD4 mAb, PE-conjugated anti-CD8 mAb, and allophycocyanin anti-CD44 mAb. After surface staining, cells were subjected to intracellular cytokine staining using a Fast Immune Cytokine System (BD Biosciences) according to the manufacturer's instructions. The cells were washed and fixed in 1000 μl of FACS lysing solution (BD Biosciences) for 10 min at room temperature and were then washed again, resuspended in 500 μl of FACS permeabilizing solution (BD Biosciences), and incubated for 10 min at room temperature. After washing, the cells were stained with FITC-conjugated IFN-γ mAb or FITC-conjugated isotype control rat IgG (BD Pharmingen).

Before staining for intracellular activated caspase-3 and Bcl-2, splenocytes or lung MNCs from infected mice were stained for surface markers in staining buffer with CyChrome-conjugated anti-CD8 mAb and PE-conjugated $OVA_{257-264}$ H-2K^b tetramers for 60 min at 4°C. After that, half of the cells were fixed and permeabilized with the above solution and then were stained with FITC-conjugated hamster anti-mouse Bcl-2 or its isotype control. The other half of the cells were washed, fixed, and permeabilized using the Cytofix/Cytoperm intracellular staining kit (BD Biosciences). The cells were incubated with anti-caspase-3 at 1/100 dilution in perm/wash buffer for 30 min at room temperature. After intracellular staining, fluorescence of the cells was analyzed using a flow cytometer.

Annexin V staining

Splenocytes and lung MNCs from uninfected WT and IL-15$^{-/-}$ mice were stained for surface markers in staining buffer with CyChrome-conjugated anti-CD8 mAb and PE-conjugated $OVA_{257-264}$ H-2K^b tetramers for 60 min. Then the amount of apoptosis was determined by staining with Annexin V^{FITC} conjugate according to the manufacturer's instructions. Briefly, cells were suspended in 500 μl/tube 1× binding buffer and incubated with 5 μl/tube Annexin V^{FITC} for 10 min in the dark at room temperature. Cells were washed twice with 1× binding buffer to remove any unbound Annexin V^{FITC}. Samples were analyzed within 30 min.

In vivo cytotoxicity assay

Analysis of in vivo cytolytic activity was carried by a protocol similar to those previously reported (29). B6-Ly5.1$^+$ 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^{high} cells were pulsed with 5 μg/ml $OVA_{257-264}$ peptide for 1 h at 37°C, whereas CFSE^{low} cells remained nonpulsed. After washing, these groups were mixed in equal proportions and then injected i.v. into mice infected with rBCG-OVA 21, 70, or 120 days previously. Spleens or lungs were obtained from recipients 24 h later for flow cytometric analysis to measure in vivo killing activities. Percent specific lysis was calculated according to the following formula: 1 − (ratio primed/ratio unprimed) × 100, where the ratio unprimed = % CFSE^{low}/% CFSE^{high} cells remaining in noninfected recipients and ratio primed = % CFSE^{low}/% CFSE^{high} cells remaining in infected recipients.

Analysis of T cell proliferation after rBCG-OVA infection in vivo

Mice infected with rBCG-OVA 14 or 113 days previously were given water containing 0.8 mg/ml BrdU for 7 days. Splenocytes or lung MNCs were stained with $OVA_{257-264}$ K^b tetramer and anti-CD8 mAb for 30 min at 4°C and then were subjected to intracellular BrdU staining using a BrdU flow kit according to the instructions of the manufacturer (BD Biosciences).

Statistical analysis

The statistical significance of the data was determined by Student's t test; a value of $p < 0.05$ was considered significant.

Results

Bacterial growth in IL-15$^{-/-}$ mice after BCG infection

To elucidate the roles of endogenous IL-15 in protection against BCG infection, we examined the kinetics of bacterial growth in the peritoneal cavity, liver, lung, and spleen of IL-15$^{-/-}$ mice after i.p. infection with BCG. As shown in Fig. 1, number of bacteria in the spleen and peritoneal cavity decreased with time both in WT and IL-15$^{-/-}$ mice. However, the number of bacteria in the lung on day 120 after BCG infection was significantly higher in IL-15$^{-/-}$ mice than in WT mice ($p < 0.005$). A similar tendency in bacterial growth was observed in the liver ($p < 0.05$). Thus, IL-15$^{-/-}$ mice were susceptible to BCG infection, especially in the lung and liver at the later stage after infection as assessed by bacterial growth.

Lymphocyte populations in the spleen and lung of IL-15$^{-/-}$ mice after BCG infection

We next examined the kinetics of lymphocytes in the spleen and lung of IL-15$^{-/-}$ mice and WT mice after BCG infection. Flow cytometry analysis for expression of CD3ϵ, TCR$\alpha\beta$, TCR$\gamma\delta$, NK1.1, CD4, and CD8 was conducted on cells of the spleen and lung on days 0, 14, 70, and 120 after infection. The numbers of CD8$^+$cells, CD3$^+$NK1.1$^+$ cells, and $\gamma\delta$ T cells in the spleen and lung were significantly decreased in IL-15$^{-/-}$ mice before infection, whereas the spleen and lung of IL-15$^{-/-}$ mice were almost devoid of CD3$^-$NK1.1$^+$ cells (Fig. 2 and data not shown). CD4$^+$

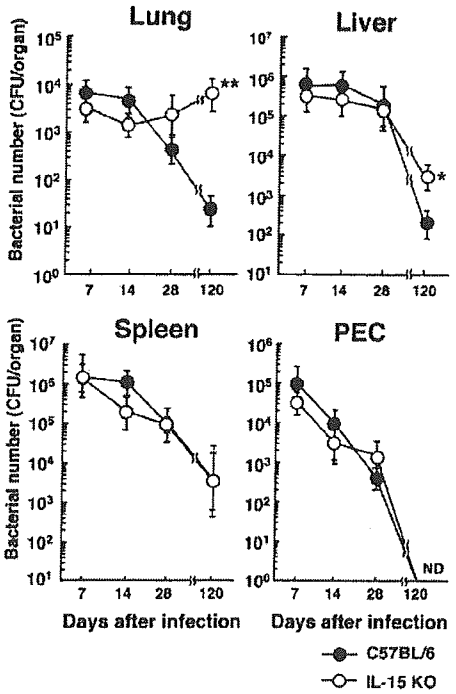

FIGURE 1. Bacterial growth in IL-15$^{-/-}$ mice after infection with BCG. IL-15$^{-/-}$ mice and age-matched WT mice were infected i.p. with 1.0×10^6 CFU of BCG (Tokyo strain). The numbers of bacteria recovered from peritoneal cavity, liver, spleen, or lung of infected mice were determined at the indicated days. Data of a representative are shown from three separate experiments and are expressed as means ± SD of five mice of each group. ND, Not detectable. *, $p < 0.05$; **, $p < 0.005$.

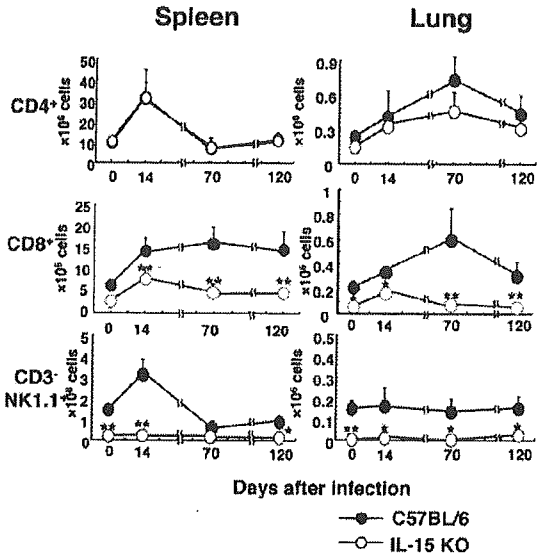

FIGURE 2. Kinetics of absolute number of lymphocyte subsets in IL-15$^{-/-}$ mice after BCG infection. The cells of spleen and the lung on day 0, 14, 70, or 120 after BCG infection were stained with various mAbs, and the absolute numbers of CD4$^+$, CD8$^+$, or NK1.1$^+$CD3$^-$ cells were calculated by multiplying total splenocytes or lung MNCs by the percentage of each subset in spleen or lung. Data of a representative are shown from three separate experiments and are expressed as means ± SD of three mice of each group. *, $p < 0.05$; **, $p < 0.01$.

T cells had increased until day 14 and gradually decreased by day 70 after infection in the spleen. The number of CD4$^+$ T cells in lung showed slower time kinetics. There were no significant differences between WT and IL-15$^{-/-}$ mice. In contrast, the numbers of CD8$^+$ T cells in the spleen and lung were increased in WT mice on day 14 and remained at increased levels on day 120. Similar to WT mice, the numbers of CD8$^+$ T cells were substantially increased in the spleen of IL-15$^{-/-}$ mice on day 14 after BCG infection but, in contrast with WT mice, the numbers of CD8$^+$ T cells were gradually decreased by day 70 after infection. The number of CD3$^-$NK1.1$^+$ cells was increased in the spleens of WT mice on day 14 after infection and then decreased by day 120 after infection, whereas CD3$^-$NK1.1$^+$ cells were undetectable in the spleens and lungs of IL-15$^{-/-}$ mice during the course of BCG infection. The numbers of CD3$^+\gamma\delta^+$ and CD3$^+$NK1.1$^+$ cells were increased in the spleens of IL-15$^{-/-}$ mice and WT mice on day 14, and the increase was more prominent in WT mice (data not shown). Taken together, the results indicate that only a few, if any, CD8$^+$ T cells remained in lung and spleen of IL-15$^{-/-}$ mice for a long time after BCG infection.

Cytokine production by Ag-stimulated T cells in the spleen and lung of IL-15$^{-/-}$ mice after BCG infection

To investigate whether Ag-specific T cells were able to be generated in IL-15$^{-/-}$ mice during the course of BCG infection, T cells were isolated from spleens of the mice on days 14 and 120 after BCG infection and were cultured with or without PPD in the presence of APC, and the culture supernatants were examined by ELISA for IFN-γ, IL-4, or IL-10 release. T cells from IL-15$^{-/-}$ mice on day 14 after infection produced higher levels of IFN-γ in response to PPD than did those from WT mice (Fig. 3*A*; $p < 0.05$). The same tendency was observed in T cells from IL-15$^{-/-}$ mice infected with BCG 120 days previously, although the difference was not statistically significant. These results suggest that BCG-specific Th1 cell responses are normally generated in IL-15$^{-/-}$

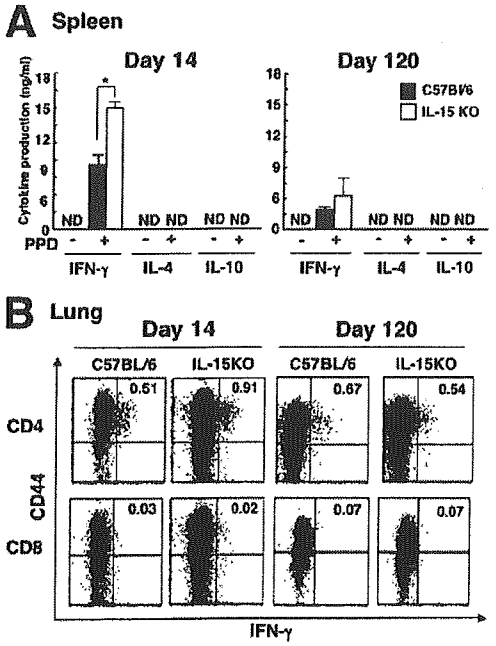

FIGURE 3. Cytokine production of T cells from IL-15$^{-/-}$ mice infected with BCG. *A*, T cells on day 14 or 120 after BCG infection were cultured with PPD for 48 h, and IFN-γ, IL-4, or IL-10 production in the supernatants was assayed by ELISA. The data are representative of two separate experiments and are expressed as means of triplicates ± SD; ND, not detectable; *, $p < 0.05$. *B*, Intracellular expression of IFN-γ by CD4$^+$ T cells and CD8$^+$ T cells from IL-15$^{-/-}$ mice infected with BCG. Lung MNCs were pooled from three mice of each group on day 14 or 120 after BCG infection and were cultured with PPD and surface-stained with anti-CD4, -CD8, and -CD44 mAbs. Intracellular cytokine-producing cells were examined using a flow cytometer and were analyzed by gating on CD4$^+$ or CD8$^+$ T cells. Data are representative of two independent experiments and are shown as typical two-color profiles.

mice after BCG infection. We further examined the production of IL-4 and IL-10 by CD4$^+$ T cells from WT and IL-15$^{-/-}$ mice infected with BCG. Neither T cells from WT mice nor those from IL-15$^{-/-}$ mice produced IL-4 or IL-10 in response to PPD, suggesting that PPD-specific Th2 or regulatory T cells are not generated in IL-15$^{-/-}$ mice.

To determine CD4$^+$ Th1 and CD8$^+$ Tc1 responses in the spleen and lung from infected IL-15$^{-/-}$ mice, we used cytokine FACS analysis for determination of expression of CD4 or CD8 and CD44 and for determination of intracellular IFN-γ after stimulation with PPD. As shown in Fig. 3*B*, CD4$^+$ Th1 response capable of producing IFN-γ in response to PPD was substantially detected in the lungs of IL-15$^{-/-}$ mice on days 14 and 120 after BCG infection. In contrast, IFN-γ production in response to PPD was not detected in the CD8$^+$ T cell population from either IL-15$^{-/-}$ mice or WT mice infected with BCG 14 or 120 days previously. Similar results were obtained for the spleen, although the differences were less pronounced (data not shown).

Ag-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice infected with rBCG-OVA

Ag-specific CD8$^+$ T cells are an important T cell subset for controlling the late phase of tuberculosis infection (13, 14). Unfortunately, immunostimulatory BCG-derived peptides recognized by CD8$^+$ T cells have not been defined. Therefore, to examine more carefully the kinetics of the Ag-specific CD8$^+$ T cell response after BCG infection, we decided to use rBCG-OVA for detection of Ag-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice. OVA expressed by rBCG-OVA was at ~0.01% of total protein of rBCG-OVA. The timing of OVA expression was as early as 7 days, because OVA-specific immune responses were detected at this stage after infection (data not shown). The numbers of OVA-specific CD8$^+$ T cells were assessed by staining with an H-2K^b tetramer coupled with an OVA-derived SIINFEKL peptide on days 21, 70, and 120 after rBCG-OVA infection (27). The kinetics of bacterial growth of rBCG-OVA were similar to those of BCG Tokyo strain (data not shown). As shown in Fig. 4*A*, the numbers of OVA-specific CD8$^+$ T cells in both the spleens and lungs of IL-15$^{-/-}$ mice on day 21

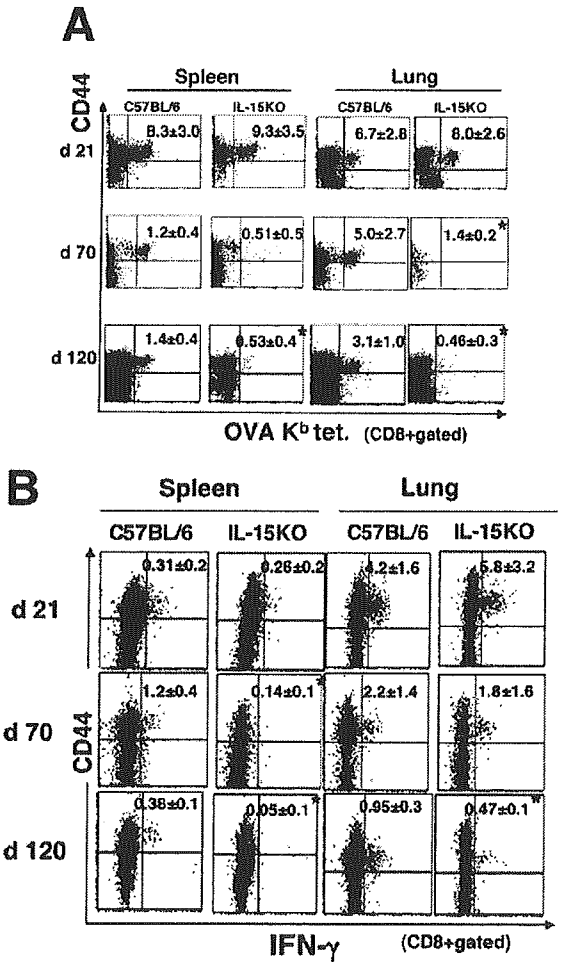

FIGURE 4. Generation of OVA$_{257-264}$-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice infected with rBCG-OVA. *A*, Splenocytes and lung MNCs on day 21, 70, or 120 after rBCG-OVA infection were stained with anti-CD8 mAb, anti-CD44 mAb, and OVA$_{257-264}$ MHC class I tetramer. Samples were analyzed by flow cytometry and analyzed by gating on CD8$^+$ T cells. Data were obtained from three separate experiments, and each value shown is the mean ± SD for three experiments. Statistically significant differences between IL-15$^{-/-}$ mice and WT mice are shown (*, $p < 0.05$). *B*, Intracellular expression of IFN-γ in the OVA$_{257-264}$-specific CD8$^+$ T cells from IL-15$^{-/-}$ mice after rBCG-OVA infection. Cells pooled from three mice of each group on day 21, 70, or 120 after infection were cultured with OVA$_{257-264}$ peptide and then were subjected to intercellular cytokine staining. Analysis gate was set on CD8$^+$ T cells. Data were obtained from three separate experiments, and each value shown is the mean ± SD for three experiments. Statistically significant differences between IL-15$^{-/-}$ mice and WT mice are shown (*, $p < 0.05$).

after infection were comparable with those in WT mice, whereas the numbers were significantly lower in IL-15$^{-/-}$ mice, especially in the lung, on days 70 and 120 after infection ($p < 0.05$). Thus, Ag-specific effector CD8$^+$ T cells can be generated in the spleen and lung at the early stage after infection, but they do not remain for a long time after BCG infection in IL-15$^{-/-}$ mice.

To further confirm the degree of generation of Ag-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice infected with rBCG-OVA, we used cytokine FACS analysis for determination of the expression of CD4 or CD8 and CD44 and for determination of the intracellular IFN-γ after stimulation with $OVA_{257-264}$ peptide. The levels of CD44$^+$CD8$^+$ T cells producing IFN-γ in response to OVA peptide in the spleens and lungs from IL-15$^{-/-}$ mice on day 21 were comparable with those in the spleens and lungs from WT mice (Fig. 4*B*). An appreciable number of CD8$^+$ T cells in the spleens and lungs from WT mice on day 120 produced IFN-γ in response to OVA peptide, whereas the numbers of such CD8$^+$ T cells were greatly reduced in the spleens and lungs from IL-15$^{-/-}$ mice ($p < 0.05$). Thus, Ag-specific CD8$^+$ T cells may not be able to be sustained for a long time in the lung in the absence of IL-15.

In vivo cytotoxicity of CD8$^+$ T cells in IL-15$^{-/-}$ mice infected with rBCG-OVA

To directly detect cytotoxic activity of CD8$^+$ T cells in vivo, we measured the ability of CD8$^+$ T cells to eliminate fluorescent-labeled spleen cells pulsed with $OVA_{257-264}$ peptides after rBCG-OVA infection. B6-Ly5.1$^+$ splenocytes were divided into two populations and labeled with a high concentration and a low concentration of CFSE. Next, CFSE^{high} cells were pulsed with $OVA_{257-264}$ peptide, whereas CFSE^{low} cells remained nonpulsed. These groups were mixed in equal proportions and then injected i.v. into mice infected with rBCG-OVA 21, 70, or 120 days previously. Splenocytes or lung MNCs were obtained from recipients 24 h later for flow cytometric analysis to measure in vivo killing activities. As shown in Fig. 5, OVA-pulsed target cells had been eliminated equally in the spleen or lung of IL-15$^{-/-}$ mice and WT mice on day 21 after infection, indicating that CD8 effector CTLs can be generated in IL-15$^{-/-}$ mice at the early stage after BCG infection. In contrast, the elimination of OVA-pulsed target cells was severely impaired in the spleen and lung of IL-15$^{-/-}$ mice on days 70 and 120 after infection. Thus, in vivo CTL activity was severely reduced in IL-15$^{-/-}$ mice at the late stage after BCG infection.

Cell division of OVA-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice infected with rBCG-OVA

The number of memory CD8$^+$ T cells is maintained by a balance among cell survival, apoptosis, and proliferation. It is well established that Ag-specific memory CD8$^+$ T cells undergo homeostatic proliferation, which is believed to be an important mechanism promoting survival by avoiding cell attribution over time. To elucidate whether the proliferation is involved in the decreases in OVA-specific memory CD8$^+$ T cells in IL-15$^{-/-}$ mice, we examined the cell proliferation of Ag-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice at the early and late stages after rBCG-OVA infection. IL-15$^{-/-}$ mice and WT mice were infected with rBCG-OVA, and then they were given BrdU in their drinking water during the early stage (days 14–21) or late stage (days 113–120) after infection. On day 21 or 120 after infection, these mice were sacrificed and BrdU incorporation was examined in OVA-specific CD8$^+$ T cells from the lung and spleen. As shown in Fig. 6*B*, proliferation of OVA-specific CD8$^+$ T cells in the spleen and lung of IL-15$^{-/-}$ mice was comparable with that in the spleen and lung of WT mice on day 21 after rBCG-OVA infection, indicating that IL-15 is not essential for expansion of Ag-specific effector CD8$^+$ T cells at the early stage of BCG infection. Surprisingly, we found that OVA-specific memory CD8$^+$ T cells in the spleen and lung were also able to undergo cell division in the absence of IL-15 at the later stage of rBCG-OVA infection. Cell division of whole CD8$^+$ T cells in

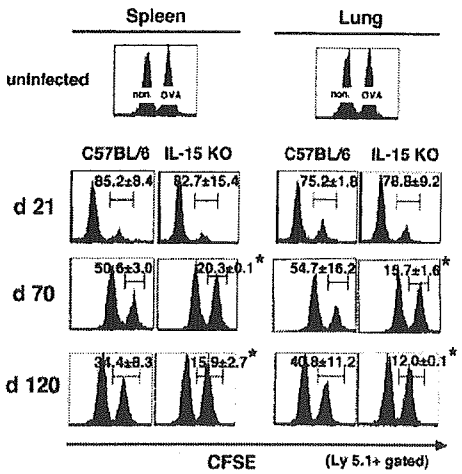

FIGURE 5. In vivo cytotoxic activities in IL-15$^{-/-}$ mice infected with rBCG-OVA. Histograms are gated on Ly5.1$^+$ cells in the spleen or lung 24 h after coinjection with equal numbers of CFSE^{high}-labeled and $OVA_{257-264}$-pulsed and CFSE^{low}-labeled and nonpulsed Ly5.1$^+$ splenocytes into mice infected with rBCG-OVA 21, 70, or 120 days previously. The values at the *upper right* 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. Statistically significant differences between IL-15$^{-/-}$ mice and WT mice are shown (*, $p < 0.05$).

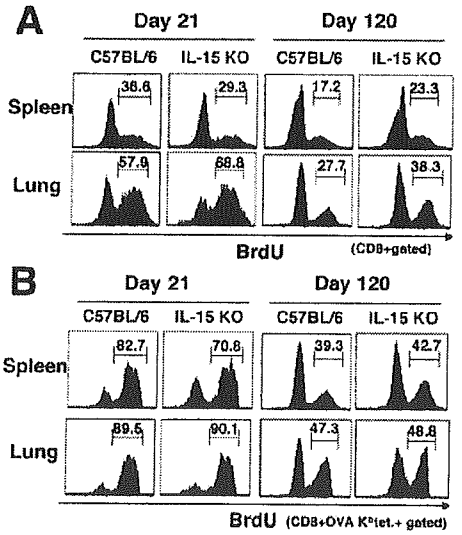

FIGURE 6. Analysis of cell proliferation of Ag-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice infected with rBCG-OVA. WT or IL-15$^{-/-}$ mice infected with rBCG-OVA 14 or 113 days previously were given water containing 0.8 mg/ml BrdU for 7 days. On days 21 or 120 after infection, splenocytes or lung MNCs were stained with $OVA_{257-264}$ K^b tetramer and anti-CD8 mAb for 30 min at 4°C and then subjected to intracellular BrdU staining. Histograms are gated on CD8$^+$ (*A*) or OVA K^b $^+$ CD8$^+$ (*B*) in the spleen or lung, and the number indicated is the percentage of whole CD8$^+$ T cells (CD8$^+$) or OVA-specific CD8$^+$ T cells ($OVA_{257-264}$ K^b tet$^+$ CD8$^+$) stained positive for anti-BrdU mAb.

IL-15$^{-/-}$ mice was also equivalently increased compared with that in WT mice on days 21 and 120 after infection (Fig. 6*A*). Taken together, the results suggest that Ag-specific CD8$^+$ T cells generated in IL-15$^{-/-}$ mice persist by cell division after BCG infection in an IL-15-independent manner.

Sensitivity to apoptosis and Bcl-2 protein in OVA-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice after rBCG-OVA infection

It is well known that antiapoptotic molecules play a critical role in the regulation of survival of memory CD8$^+$ T cells. Based on the finding of a gradual decline in the number of OVA-specific CD8$^+$ T cells during the late stage of rBCG-OVA infection, we hypothesized that Ag-specific CD8$^+$ T cells might be more sensitive to apoptosis in an IL-15-deficient environment. To address this, we examined their binding of annexin V, an early marker of apoptotic cells, and expression of active caspase-3, a terminal effector for apoptosis. Splenocytes or lung MNCs from IL-15$^{-/-}$ and WT mice infected with rBCG-OVA were stained with anti-CD8 and $OVA_{257-264}$ K^b tetramer and then with annexin V and active caspase-3 mAb. As shown in Fig. 7, *A* and *B*, the frequency of annexin V-positive cells in OVA-specific CD8$^+$ T cells from the lung was markedly increased in infected IL-15$^{-/-}$ mice compared with those in WT mice on days 21 and 120 after infection, whereas the frequency of active caspase-3-positive in OVA-specific CD8$^+$ T cells from the spleen and lung was markedly increased on day 70. We also evaluated intracellular Bcl-2 levels in OVA-specific CD8$^+$ T cells on days 21 and 120 after rBCG-OVA infection. As shown in Fig. 7*C*, the levels of Bcl-2 expression in the spleen and lung of WT mice are the same as those of IL-15$^{-/-}$ mice on day 21 after rBCG-OVA infection. However, the expression levels were significantly lower in the lung of IL-15$^{-/-}$ mice compared with those in WT mice on day 120 after infection ($p < 0.05$; Fig. 7*C*). These results indicate that Ag-specific CD8$^+$ T cells generated in IL-15$^{-/-}$ mice are more sensitive to apoptosis due to decreased Bcl-2 levels in those cells.

Discussion

It is widely accepted that CD8$^+$ T cells play a requisite role in resistance to mycobacterial infection (3–5). In this study, we investigated cell-mediated immunity against primary infection with an avirulent strain of BCG (Tokyo strain) and rBCG-OVA in IL-15$^{-/-}$ mice and found that the IL-15$^{-/-}$ mice showed impaired resistance in the lung at the late stage of primary BCG infection accompanied by marked decreases in Ag-specific CD8$^+$ T cells producing IFN-γ and exhibiting cytotoxicity. Although the degree of proliferation of Ag-specific memory CD8$^+$ T cells was similar to that in WT mice, sensitivity to apoptosis of OVA-specific CD8$^+$ T cells significantly increased in IL-15$^{-/-}$ mice during the course of BCG infection. These results suggest that IL-15 plays an important role in the development of long-lasting protective immunity to BCG infection via sustaining CD8 responses in the lung. The i.p. route of BCG administration we used in the present study does not model for mycobacterial infections, which usually enter the body through respiratory tract, or for BCG vaccination in human, which is s.c. delivered. However, there is evidence that CD8$^+$ CTL cells might not be able to migrate to lung efficiently after s.c. vaccination (30). Furthermore, i.p. BCG vaccination is reported to be able to elicit CD8$^+$ CTL cells in lung more effectively than the s.c. vaccination (31). The role of IL-15 in sustaining CD8 responses in the lung may be conspicuous only in i.p. route for BCG infection; however, Lazarevic et al. (32) have recently reported the induction of *M. tuberculosis*-specific primary and secondary T cell responses in IL-15$^{-/-}$ mice, which shows a significant increase in bacterial number in lung but not in spleen of

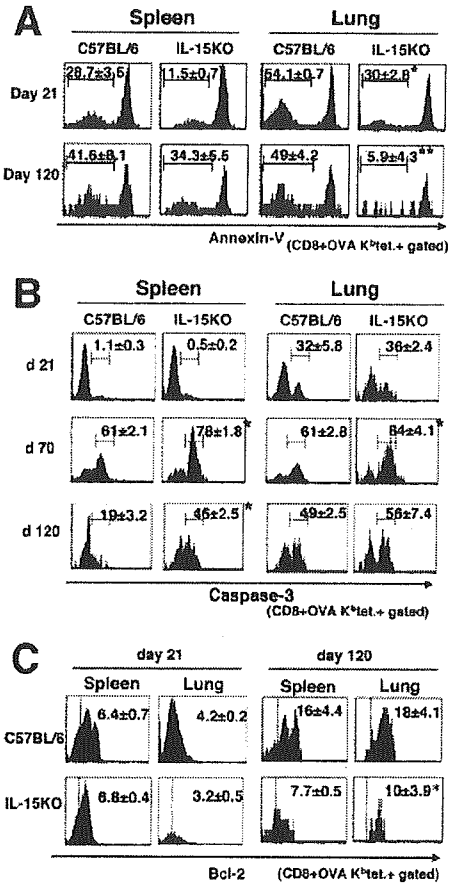

FIGURE 7. Sensitivity to apoptosis and Bcl-2 expression in OVA-specific CD8$^+$ T cells in IL-15$^{-/-}$ mice infected with rBCG-OVA. Splenocytes or lung MNCs from IL-15$^{-/-}$ and WT mice infected with rBCG-OVA were stained with anti-CD8 and $OVA_{257-264}$ K^b tetramer, followed by annexin V (*A*), anti-active caspase-3 Ab (*B*), or Bcl-2 with its isotype control staining (*C*). The number indicated in *A* and *B* is the percentage of OVA-specific CD8$^+$ T cells (OVA K^b tet$^+$ CD8$^+$) stained negative for anti-annexin V mAb or positive for anti-active caspase-3 Ab, and in *C* it is the mean fluorescence intensity. The level of isotype control was drawn on each histogram of Bcl-2 as a vertical dotted line. Data of representative are shown from two separate experiments and are expressed as means ± SD of three mice of each group. Statistically significant differences between IL-15$^{-/-}$ mice and WT mice are shown (*, $p < 0.05$; **, $p < 0.01$).

IL-15$^{-/-}$ mice at late stage (12 wk) after aerosol infection with *M. tuberculosis*. These results are consistent with our results, although they did not elucidate the mechanisms for impaired protection in lung of IL-15$^{-/-}$ mice. Our findings showing the roles of IL-15 in protection in lung against BCG infection may be able to apply to infection with all mycobacteria species usually penetrating through respiratory tract.

A notable finding in the present study is that Ag-specific CD8$^+$ T cells could not be sustained for a long time in lung in the absence of IL-15. Upon encounter with a pathogenic microbe, Ag-specific T cells proliferate and differentiate into activated effector T cells at the expansion phase. Most of the activated T cells die by apoptosis at the contraction phase, but the few that survive become memory cells and persist for a long period of time at the maintenance stage, sometimes throughout the life of an animal (33–35). A key issue is at which stage IL-15 is involved in determining the size of Ag-driven CD8$^+$ T cells in the lung after BCG infection. IL-15 is

important in maturation of dendritic cells (DCs) for Ag presentation (36), suggesting that generation of effector $CD8^+$ T cells may be impaired at the expansion phase after BCG infection, resulting in a decrease in the number of Ag-specific $CD8^+$ T cells in the lung at the late stage of infection. Recent studies have demonstrated that primary responses to LCMV and OVA were readily generated in IL-$15^{-/-}$ mice or IL-$15R\alpha^{-/-}$ mice to a level equal to that in control mice (24, 37). We have also reported that generation of Ag-specific $CD8^+$ T cells in IL-15 Tg mice and IL-$15^{-/-}$ mice normally occurred after primary infection with *L. monocytogenes* (38, 39). In contrast with acute infection such as infection with *L. monocytogenes* or LCMV, in which effector $CD8^+$ T cell response peaks at ~5–7 days postinfection, BCG causes chronic infection, in which the bacterial burden persists for a long time. Peak response by Ag-specific $CD8^+$ T cells is delayed at ~21–30 days after BCG infection compared with that in the case of acute infection (27). We have shown in this study that levels of effector $CD8^+$ T cells in the spleen and lung of IL-$15^{-/-}$ mice were normal on day 21 after rBCG-OVA infection. This may explain why IL-$15^{-/-}$ mice showed the same level of resistance in the lung at the early stage of BCG infection. IL-15 is not mandatory for the expansion of $CD8^+$ T cells in the immune response against BCG.

IL-15 has been shown to be a potent inhibitor of several apoptosis pathways in several lymphocytes via induction of antiapoptotic molecules (40–43). Similar to acute infection with *L. monocytogenes* and LCMV, the number of effector $CD8^+$ T cells was gradually decreased by apoptosis from day 21 to day 120 after BCG infection. In the present study, we found that the levels of annexin V expression in OVA-specific $CD8^+$ T cells of IL-$15^{-/-}$ mice were significantly higher on days 21 and 120 after rBCG-OVA infection than those in WT mice. We further confirmed that the number of apoptotic cells in Ag-specific $CD8^+$ T cells was increased in lung of IL-$15^{-/-}$ mice at the late stage after infection as assessed by active caspase-3 expression. Therefore, it is possible that IL-15 protects effector $CD8^+$ T cells from apoptosis during the contraction phase of immune responses after BCG infection. Most of $CD8^+$ effector T cells down-regulate the expression of Bcl-2 as compared with those in naive $CD8^+$ T cells in the contraction phase. Bcl-2 expression is induced via signaling from the common cytokine receptor γ-chain, which is used by IL-15 (15, 38–40) and prevents apoptosis by withdrawal of growth factors. We found that the expression level of Bcl-2 in Ag-specific $CD8^+$ T cells was significantly decreased in IL-$15^{-/-}$ mice compared with that in WT mice on day 120 after rBCG-OVA infection. This indicates that the absence of IL-15 makes the Ag-specific $CD8^+$ T cells more sensitive to apoptosis due to decreased Bcl-2 levels in those cells. However, recent studies have demonstrated that Fas-Fas ligand signaling is responsible for apoptosis induced by repetitive Ag exposure, high doses of a persistent Ag, or an Ag expressed systemically (44–46). Fas-Fas ligand signaling may also be involved in the death of activated T cells after BCG infection, because BCG causes chronic infection in which the bacterial burden persists for a long time. It has been shown that IL-15 blocks TNFR1-mediated cell death of fibroblasts by inhibition of an early step in the apoptosis signal cascade (47). Therefore, IL-15 may protect Ag-specific effector $CD8^+$ T cells from Fas-mediated activation-induced cell death in response to a persistent BCG Ag. Further investigation is needed to elucidate these possibilities. We have recently reported that IL-15 Tg mice showed augmented Tc1 responses against BCG infection (21), and these augmented Tc1 responses may be explained by inhibition of apoptosis of Ag-specific $CD8^+$ T cells after BCG immunization. The decrease in the number of Ag-specific $CD8^+$ T cells at this stage may also be due to T cell exhaustion caused by the persistence of high Ag levels. IL-15 may prevent Ag-specific $CD8^+$ T cells from T cell exhaustion. Alternatively, DCs infected with mycobacteria often sequester Ag or production of suppressive cytokines such as IL-10 (48). DCs in IL-$15^{-/-}$ mice may have such characteristics during BCG infection, resulting in impaired APC activity to sustain the $CD8^+$ T cells.

T cells undergo two distinct types of proliferation: Ag-driven (Ag-dependent) proliferation and homeostatic (Ag-independent) proliferation. Ag-independent proliferation is thought to be required for the long-term maintenance of Ag-specific memory $CD8^+$ T cells after acute infection with microbes (33–35). IL-15 may play a role in the long-term survival of T cells in vivo by inducing proliferation of memory $CD8^+$ T cells in addition to protecting $CD8^+$ T cells against activation-induced apoptosis. We have demonstrated that the number of listeriolysin O 91–99-positive memory $CD8^+$ T cells was significantly higher in IL-15 Tg mice 6 wk after primary infection with *L. monocytogenes*, which resulted in Ag-independent cell proliferation (38). Thus, IL-15 plays an important role in long-term maintenance of Ag-specific memory $CD8^+$ T cells in an Ag-independent manner. However, we found in the present study that Ag-specific memory $CD8^+$ T cells were still able to proliferate in the absence of IL-15, unlike what was found for acute infection with microbes that are completely cleared. We previously reported that IL-15 is not required for Ag-dependent proliferation of memory $CD8^+$ T cells after secondary infection with *L. monocytogenes* (39). Wherry et al. reported that Ag-specific $CD8^+$ T cells failed to acquire the cardinal memory T cell properties of long-term Ag-independent persistence during chronic infection with LCMV (49). A recent study has demonstrated that Ag-specific memory $CD8^+$ T cells in IL-$15^{-/-}$ mice were able to undergo cell proliferation in an Ag-dependent manner during latent gammaherpesvirus infection (50). Therefore, during chronic infection with BCG, Ag-dependent, but not IL-15-dependent, proliferation may be important in the maintenance of Ag-specific $CD8^+$ T cells due to the persistent low levels of Ags. Two subsets of memory $CD8^+$ T cells based on their anatomical location, expression of cell surface markers, and effector functions have been described (51). Memory $CD8^+$ T cells expressing homing receptors such as CD62 ligand (CD62L) and CCR7, which allow efficient homing to lymph nodes, are termed central memory cells, whereas memory T cells lacking these lymph node homing receptors, which are located in nonlymphoid tissues, are termed effector memory cells. During chronic infection, Ag-specific $CD8^+$ T cells often retain a $CD62L^{low}CCR7^{low}$ phenotype that favors homing to nonlymphoid tissues (52, 53). We found in this study that the numbers of Ag-specific memory $CD8^+$ T cells in IL-$15^{-/-}$ mice were more decreased in nonlymphoid tissue than were those in lymphoid tissue on day 120 after rBCG-OVA infection. In correlation with the number of memory $CD8^+$ T cells, the number of annexin V-negative OVA-specific $CD8^+$ T cells of IL-$15^{-/-}$ mice was significantly decreased in the lung compared with that in the spleen on day 120 after rBCG-OVA infection. We recently found that IL-15 affects mainly the survival of Ag-specific $CD62L^{low}$ $CD8^+$ T cells during the contraction phase after *Listeria* infection (our unpublished data). Therefore, IL-15 may affect mainly the survival of effector cells or effector memory cells, which reside mainly in nonlymphoid tissues such as the lung and serve as the fist line of host defense against microbial invasion.

In addition, there are several lines of evidence that IL-15 is capable of stimulating $CD8^+$ CTLs to exhibit increased cytotoxicity (54, 55). IL-15 has been reported to directly up-regulate the expression of cytotoxic molecules such as granzyme B and perforin, mimicking TCR cross-linking in the induction of cytotoxic

molecules and cytotoxicity of effector $CD8^+$ T cells (56). Recent studies have suggested that $CD8^+$ CTLs releasing perforin and granulysin play a role in protection against *M. tuberculosis* infection via a cytolysis mechanism (9–11). We showed in this study that in vivo killer activity of Ag-specific $CD8^+$ T cells was significantly impaired in the lung at the late stage of rBCG-OVA infection. These findings raise the possibility that IL-15 plays an important role in rapid elicitation of cytotoxic functions in effector $CD8^+$ T cells in microbial invasion, providing robust protection against chronic infection; however, at present we do not know the relative contribution of cytotoxicity by $CD8^+$ T cells to protection against BCG infection.

Infection of mice with less virulent BCG consistently showed that $CD8^+$ T cells made no contribution to immunity in normal mice (57, 58). Although $CD8^+$ T cells play a crucial role in protection in the lung at the late stage of BCG infection, the relative contribution of cells other than $CD8^+$ T cells to protection against BCG infection remains to be elucidated. IL-15 is known to play important roles in proliferation, accumulation, and maintenance of NK cells (22, 23). The results of the present study revealed that IL-$15^{-/-}$ mice have greatly reduced numbers of NK cells in peripheral lymphoid tissues and that NK cells remained at undetectable levels on day 120 after BCG infection. We previously reported that in vivo administration of either anti-Asialo GM1Ab or anti-CD8 mAb abrogated antibacterial activity, suggesting that both NK cells and $CD8^+$ T cells are required for protection against BCG infection in IL-15 Tg mice (21). Therefore, NK cells may also contribute to protection in the lung at the late stage of infection in WT mice; however, in WT mice, in vivo depletion of NK cells did not have an obvious effect on the growth of bacteria at the early stage of BCG infection (21, 59). In the present study, we found that IL-$15^{-/-}$ mice lacking NK cells exhibited the same level of resistance as that shown by WT mice by day 14 after BCG infection. Therefore, NK cells do not appear to be important for the control of BCG infection in WT mice. Taken together, it appears that IL-15 may serve to induce proliferation and/or accumulation of NK cells during BCG infection and that an increase in the number of NK cells is not essential for enhanced resistance against BCG infection in WT mice.

IL-15 also plays important roles in proliferation, accumulation, and maintenance of NKT cells and a subset of $\gamma\delta$ T cells (18, 22, 23). The results of the present study revealed that the numbers of NKT cells and $\gamma\delta$ T cells were remarkably increased in WT mice after BCG infection (data not shown). It has been reported that host defense and delayed-type hypersensitivity response to *M. bovis* BCG in $NKT^{-/-}$ mice were not different from those in WT mice after pulmonary infection (60). Studies with TCR$\gamma\delta$ gene-knockout mice suggested that TCR$\gamma\delta$ T cells play a role in granuloma formation to *Mycobacteria* but not in protection as assessed by bacterial growth (61). These results suggest that Vα14 NKT cells and $\gamma\delta$ T cells play only a marginal role, if any, in host resistance to mycobacterial infection.

Th1 cells secreting IFN-γ and TNF-α play a crucial role in protection against mycobacterial infection (1–3). The results of the present study reveal that there is no difference in Th1 responses in the spleen and lung of IL-$15^{-/-}$ mice and WT mice. This may explain why no difference was found between numbers of bacteria in the spleen at the early and late stages of BCG infection. $CD8^+$ T cells are more important for protection in the lung at the late stage of BCG infection. This speculation warrants further examination with deletion of Ag-specific $CD4^+$ T cells in IL-$15^{-/-}$ mice infected with BCG.

In conclusion, we found that the IL-$15^{-/-}$ mice showed impaired resistance in the lung at the late stage of primary BCG infection accompanied by marked decreases in Ag-specific $CD8^+$ T cells producing IFN-γ and exhibiting cytotoxicity. Although BCG has been used as a vaccine, it confers incomplete protection against tuberculosis, at least in adults. Because the results of this study using IL-$15^{-/-}$ mice indicate that IL-15 is important for long-lasting protective immunity in the lung mediated by $CD8^+$ Tc1/CTL, it is thought that IL-15 can be used as an immune adjuvant to increase the efficacy of BCG vaccination via enhancing CD8 response in the lung. Additional experiments with aerosol challenge with *M. tuberculosis* might enable us to determine conclusively whether IL-15 is useful for the development of new immunoprotective approaches against mycobacterial infection.

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Disclosures

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Augmented induction of CD8$^+$ cytotoxic T-cell response and antitumour resistance by T helper type 1-inducing peptide

Takeshi Kikuchi,[1,2] Shuichiro Uehara,[1,2] Haruyuki Ariga,[1,3] Takeshi Tokunaga,[1] Ai Kariyone,[1] Toshiki Tamura[1] and Kiyoshi Takatsu[1]

[1]*Division of Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo,* [2]*Department of Pediatric Surgery, Nihon University School of Medicine, Tokyo, and* [3]*First Department of Internal Medicine, Kyorin University School of Medicine, Tokyo, Japan*

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Correspondence: Dr Kiyoshi Takatsu, Division of Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-Ku, Tokyo 108–8639, Japan. Email: takatsuk@ims.u-tokyo.ac.jp
Senior author: Takeshi Kikuchi, email: kikuchiT@ims.u-tokyo.ac.jp

Summary

The effector CD8$^+$ T cells recognize major histocompatibility complex (MHC) class I binding altered self-peptides expressed in tumour cells. Although the requirement for CD4$^+$ T helper type 1 (Th1) cells in regulating CD8$^+$ T cells has been documented, their target epitopes and functional impact in antitumour responses remain unclear. We examined whether a potent immunogenic peptide of *Mycobacterium tuberculosis* eliciting Th1 immunity contributes to the generation of CD8$^+$ T cells and to protective antitumour immune responses to unrelated tumour-specific antigens. Peptide-25, a major Th epitope of Ag85B from *M. tuberculosis* preferentially induced CD4$^+$ Th1 cells in C57BL/6 mice and had an augmenting effect on Th1 generation for coimmunized unrelated antigenic peptides. Coimmunization of mice with Peptide-25 and ovalbumin (OVA) or Peptide-25 and B16 melanoma peptide [tyrosinase-related protein-2 (TRP-2)] for MHC class I led to a profound increase in CD8$^+$ T cells specific for OVA and TRP-2 peptides, respectively. This heightened response depended on Peptide-25-specific CD4$^+$ T cells and interferon-γ-producing T cells. In tumour protection assays, immunization with Peptide-25 and OVA resulted in the enhancement of CD8$^+$ cytotoxic cell generation specific for OVA and the growth inhibition of EL-4 thymoma expressing OVA peptide leading to the tumour rejection. These phenomena were not achieved by immunization with OVA alone. Peptide-25-reactive Th1 cells counteractivated dendritic cells in the presence of Peptide-25 leading them to activate and present OVA peptide to CD8$^+$ cytotoxic T cells.

Keywords: antigen presentation; cytotoxic T cells; peptide; T helper 1 cells; tumour immunity

Introduction

The identification of tumour antigens has renewed interest in immunotherapy for cancer. There is a body of evidence that tumour-specific T cells recognize tumour-associated antigens on the cancer cells and play an essential role in inhibiting tumour growth and eradicating cancer cells.[1–3] CD8$^+$ cytotoxic T lymphocytes (CTL) from specifically immunized mice are capable of destroying tumour target cells *in vitro*[4] and adoptive transfer of CD8$^+$ T cells from immunized donors confers resistance to tumour transplants on naive mice.[5–7] As CD8$^+$ CTL can lyse tumour cells directly and destroy large tumour masses *in vivo*, much attention has focused on the role of CD8$^+$ T cells in the immunotherapy of cancer. Over the past two decades, a wide range of peptides derived from tumour cells of mice and humans that bind major histocompatibility complex (MHC) class I and are recognized by CD8$^+$ T cells has been defined.[1,8,9] However, in both clinical and animal studies, therapeutic strategies focused on the use of CD8$^+$ T cells and MHC class I-restricted tumour antigens have not been effective in eliminating cancer cells.

There has been a recent reappraisal of the role and importance of CD4$^+$ T helper (Th) cells in antitumour responses, because CD4$^+$ Th cells are required for generating and maintaining potent antitumour immunity.[5,6,10] The role of CD8$^+$ and CD4$^+$ T cells in tumour systems has been the object of intense interest. A major obstacle for the development of optimal cancer vaccines is the lack of effective methods for identifying MHC class

II-restricted tumour antigens that can stimulate $CD4^+$ T cells.[11,12] Identification of such antigens would provide new opportunities for developing effective $CD8^+$ CTL and would improve our understanding of the mechanisms by which $CD4^+$ T cells regulate the host immune system.

A variety of tumour-derived antigens have been defined by immunoglobulin G (IgG) antibodies in sera taken from tumour bearers with serological identification of antigens by recombinant expression cloning (SEREX).[13–16] The SEREX repertoire can be considered a reflection of the $CD4^+$ T-cell repertoire. Shiku and his colleagues reported that coimmunization of mice with plasmids encoding these SEREX-defined wild-type antigens and mutated mitogen-activated protein kinase 2 (mERK2; containing tumour-specific CTL epitope 9m of CMS5) led to a profound increase in $CD8^+$ T cells specific for mERK2.[13] This heightened response depends on $CD4^+$ T cells and on the copresentation of SEREX-defined wild-type antigens and the CTL epitope. Their results indicate the essential role of $CD4^+$ T cells in mediating the increased $CD8^+$ T-cell response and tumour inhibition induced by coimmunization with SEREX-defined antigens.

We have reported that immunization of *Mycobacterium tuberculosis*-primed mice with purified protein derivative (PPD)-modified attenuated X5563 myeloma cells induces an X5563-specific $CD8^+$ CTL response and antitumour immunity.[17–19] We infer from these results that *M. tuberculosis*-derived proteins or peptides may enhance the $CD8^+$ CTL response and antitumour immunity by coimmunization with tumour antigen or neo-tumour antigen. Ag85B, one of the major proteins secreted by *M. tuberculosis*, elicits a strong Th1 response *in vitro* in T cells from both PPD-positive asymptomatic human subjects and Ag85B-primed cells of C57BL/6 ($I\text{-}A^b$) mice. Peptide-25 (amino acids 240–254) of Ag85B, which is the most potent antigen species yet purified for both humans and mice, is a major Th1 cell epitope of Ag85B. Active immunization of C57BL/6 mice with Peptide-25 induces the differentiation of $CD4^+$ T-cell receptor (TCR) $V\beta11^+$ T cells that produce interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α)[20–23].

We investigated whether Th1-inducible Peptide-25 intensifies the $CD8^+$ CTL response to unrelated tumour-specific antigens through stimulation of a $CD4^+$ Th1 cell response leading to the induction of antitumour immunity that is effective in eliminating cancer cells. We also discuss the possible mechanisms of Peptide-25-induced enhancement of the $CD8^+$ CTL response.

Materials and methods

Mice

C57BL/6 mice were purchased from Charles River Japan (Tokyo, Japan). Peptide-25-reactive TCR transgenic (Tg) (P25 TCR-Tg) mice were generated and maintained as described previously.[24] IFN-γ deficient ($IFN\text{-}\gamma^{-/-}$) mice[25] were kindly provided by Dr Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Ovalbumin (OVA)-specific TCR-Tg (OT-1) mice were kindly provided by Dr T. Hirano (Osaka University, Suita, Japan). These mice were housed in the animal facility at the Institute of Medical Science, University of Tokyo, under specific pathogen-free conditions, and were used at 8–12 weeks of age.

Antigens and reagents

Peptide-25 (FQDAYNAAGGHNAVF), Peptide-9 (DWY SPACGKAGCQTY), and Peptide-18 (AGGYKAADM WGPSSD) of Ag85B were synthesized by Funakoshi Co., Ltd (Tokyo, Japan). Purified chicken OVA was purchased from Sigma-Aldrich, Co. (St Louis, MO). MHC class I-binding OVA Peptide (SIINFEKL) and B16 melanoma peptide tyrosinase-related protein-2 (TRP-2) (VYDF FVWL)[26] were also synthesized by Funakoshi Co., Ltd.

Culture medium

RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Sigma-Aldrich, Co.), 50 μM 2-mercaptoethanol, 100 IU/ml penicillin G and 50 μg/ml streptomycin was used as the complete medium for cultures throughout the present experiment.

Cell lines

The murine thymoma line, EL-4 ($H\text{-}2K^b$) was purchased from the American Type Culture Collection (Rockville, MD). EL-4 transfectant of the OVA gene (E.G7 cells) was kindly provided by Dr H. Udono (Nagasaki University School of Medicine, Nagasaki, Japan) and the B16 melanoma cell line was kindly provided by Dr H. Tahara (Institute of Medical Science, University of Tokyo, Tokyo, Japan).

Immunization

Mice were immunized by subcutaneous injection on the abdomen with OVA (10 μg/mouse) emulsified in incomplete Freund's adjuvant (IFA), Peptide-25, or its related peptide (10 μg/mouse) in IFA or a mixture of OVA (10 μg/mouse) and Peptide-25 (10 μg/mouse) in IFA as described previously.[21] In some experiments, mice were immunized with OVA (10 μg/mouse) in IFA on the left-hand side of the abdomen and with Peptide-25 (10 μg/mouse) in IFA on the right-hand side of the abdomen. We also immunized mice with MHC class I-binding TRP-2 peptide (10 μg/mouse) in place of OVA.

In vivo *and* in vitro *T-cell depletion*

$CD4^+$ T cells were depleted *in vivo* by the administration of 0·3 mg monoclonal antibodies (mAbs) against CD4 (GK1.5) on days −13, −12, −11, −6, −5, −4, +1, +2 and +3 relative to immunization. Fluorescence-activated cell sorter (FACS) analysis of blood mononuclear cells from GK1.5-treated mice at the time of immunization confirmed the effectiveness of the $CD4^+$ T-cell depletion. *In vitro* T-cell depletion was achieved by the incubation of spleen cells with either the IgM subclass of mAb against CD4 or CD8 and guinea-pig complement. FACS analysis of the treated spleen cells confirmed the effectiveness of the depletion.

In vitro *CTL induction and $CD8^+$ cytotoxic T-cell assay*

In vitro CTL induction and $CD8^+$ CTL assay were carried out according to previously described methods[17,18] with slight modification. Ten days after immunization with OVA in IFA or OVA and Peptide-25 in IFA, spleen cells (1×10^7) were cultured *in vitro* with γ-irradiated (20 000 rad) E.G7 cells (8×10^5). Spleen cells from TRP-2-immunized mice were stimulated *in vitro* with TRP-2 (10 μg/ml). After 5 days in culture, the CTL activity of the resulting effector cells was assayed. Target cells (E.G7, EL-4, and B16 melanoma cells) were labelled with ^{51}Cr (Perkin Elmer Life Science, Boston, MA) at 37° for 40 min. After washing, ^{51}Cr-labelled target cells (1×10^4) were incubated with effector cells at various effector cell to target cell ratios. Release of ^{51}Cr was measured in the supernatants that were harvested after 4 hr incubation. Maximum release was measured by resuspending the target cells in lysis buffer containing 0·1% Triton-X-100. Spontaneous release was obtained from target cells incubated with medium alone and was less than 10% of maximum ^{51}Cr release. The percentage specific lysis was calculated according to the following formula, where c.p.m. represents counts per minute: percentage specific lysis = $[(c.p.m._{experimental\ release} - c.p.m._{spontaneous\ release})/(c.p.m._{maximum\ release} - c.p.m._{spontaneous\ release})] \times 100$.

A dose–response curve of effector cells was established in all experiments and the number of lytic units (LU) was calculated as previously described.[19] In these calculations 1 LU was arbitrarily defined as the number of spleen cells required to achieve 50% lysis of 1×10^4 ^{51}Cr-labelled target cells during a 4-hr incubation.

Tumour challenge experiments

Three groups of 12 mice were immunized by subcutaneous injection of the abdomen with OVA (10 μg/mouse) in IFA, Peptide-25 (10 μg/mouse) in IFA, or a mixture of OVA (10 μg/mouse) and Peptide-25 (10 μg/mouse) in IFA. Twelve mice were injected with IFA without any protein or peptide to act as a control group. Ten days after the immunization, all mice were challenged by subcutaneous injection with E.G7 (5×10^5 cells/mouse) on their backs. In some experiments, B16 melanoma cells (5×10^5 cells/mouse) were transplanted in TRP-2-immunized mice. Tumour size was assessed using a microcaliper a 2-day to 3-day intervals and was expressed as the square of the smallest diameter of the tumour multiplied by its largest diameter. The survival of the mice was also monitored periodically.

Frequency analysis of OVA-specific CTL

The frequency of OVA-specific CTL in spleen cells after immunization was measured using OVA peptide-loaded $H\text{-}2K^b$:Ig protein (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions. Spleen cells prepared from mice 10 days after immunization, were stained with 4 μg OVA peptide-loaded $H\text{-}2K^b$:Ig protein and incubated for 60 min at 4°. After washing, cells were stained with anti-mouse IgG1 (A85-1)–phycoerythrin (PE; BD Biosciences Pharmingen) and anti-CD8 (53-6.7)–fluorescein isothiocyanate (FITC; BD Biosciences Pharmingen) and incubated for 30 min at 4°. After washing, cells were analysed using FACSCalibur (Becton Dickinson, Mountain View, CA).

Assay for dendritic cell activation

Immature dendritic cells (DCs) were propagated *in vitro* by culturing $CD11c^+$ bone marrow cells with granulocyte–macrophage colony-stimulating factor (GM-CSF) (20 ng/ml) and interleukin-3 (IL-3) (20 ng/ml) for 6 days. To assess the expression of surface molecules and IL-12 production of DCs after Peptide-25 treatment, the cells obtained (5×10^5) were cocultured with Peptide-25 (10 μg/ml) in the presence of $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice for 48 hr. The expression of surface molecules on DCs was analysed by FACS. The IL-12 production was assessed by enzyme-linked immunosorbent assay (ELISA). To assess the antigen-presenting activity of DCs after Peptide-25 treatment, the cells obtained (5×10^5) were cocultured with $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice and 5-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled $CD8^+$ T cells (5×10^5) from OT-1 mice for 96 hr in the presence of Peptide-25 (10 μg/ml) and OVA (10 μg/ml). After the culture, cell division cycles were determined by FACS analysis.[27]

Assay for cytokine production by intracellular cytokine staining and ELISA

For assessment of cytokine production of spleen cells from OVA- or TRP-2-immunized mice, spleen cells

(1×10^6/ml) prepared from mice 10 days after immunization, were stimulated with 10 μg/ml OVA or 10 μg/ml TRP-2. After the stimulation, IFN-γ- and IL-4-producing cells were examined by intracellular staining according to previously described methods.[22,24] In brief, 2 μM of monensin (BD Biosciences Pharmingen) was added for the last 4 hr of the culture. The cells were harvested and stained with 7-amino-actinomycin D and anti-CD4 (GK1·5)- or anti-CD8- allophycocyanin (BD Biosciences Pharmingen). Then, the cells were washed in 0·05% azide–1%FCS–phosphate-buffered saline, fixed with 1·6% formaldehyde, made permeable with 0·1% saponin and stained with anti-IFN-γ-FITC (XMG1.2) (BD Biosciences Pharmingen) and anti-IL-4-PE (11B11) (BD Biosciences Pharmingen) or isotype control antibodies. Stained cells were gated on live $CD4^+$ or $CD8^+$ cells and analysed by FACSCalibur. The amounts of IFN-γ and IL-4 in the culture supernatant after OVA or TRP-2 stimulation *in vitro* were quantified by ELISA following the manufacturer's instructions. The mAbs specific for mouse IFN-γ and IL-4 that were used for capture and detection of cytokines were purchased from BD Biosciences Pharmingen. ELISA of IL-12p40 was conducted using a murine IL-12 p40 OptEIA™ ELISA kit (BD Biosciences Pharmingen).

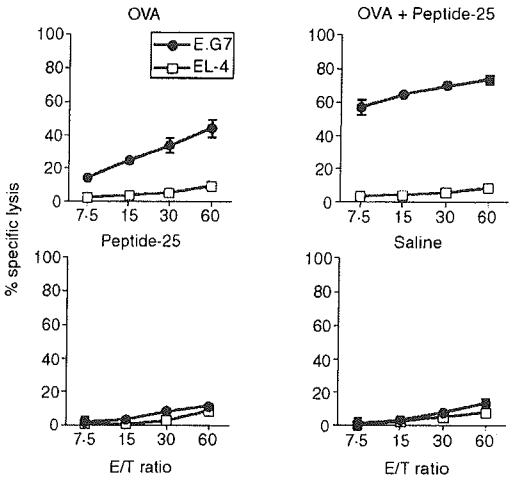
ELISA for anti-OVA antibody titration

For assessment of anti-OVA IgG1 and IgG2a levels, serum was collected from the immunized mice at 10 days after immunization and added to the OVA-coated plate. Biotinylated goat anti-mouse IgG1 antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) or biotinylated goat anti-mouse IgG2a (5.7.2) was applied and detection was performed using streptavidin-peroxidase (Zymed Laboratories Inc., San Francisco, CA).

Results

Peptide-25 enhances the generation of OVA-specific $CD8^+$ CTL response

Peptide-25, a 15-mer peptide of Ag85B is a major T-cell epitope recognized by $CD4^+$ I-A^b-restricted Th1 cells specific for Ag85B of *M. tuberculosis*.[20,21] Immunization of C57BL/6 mice with Peptide-25 induced the generation of IFN-γ- and TNF-α-producing Th1 cells that preferentially express TCRVβ11.[21] As $CD4^+$ Th1 cells can augment the $CD8^+$ CTL response[28,29] we examined whether immunization with a mixture of OVA and Peptide-25 can enhance the generation of an OVA-specific CTL response compared to OVA immunization. Three groups of mice were immunized with OVA in IFA, Peptide-25 in IFA, or a mixture of OVA and Peptide-25 in IFA. As a control, a group of mice was treated with IFA. Ten days after the immunization, spleen cells from each group of mice were



Lytic unit (/10^6)	OVA	OVA + Peptide-25	Peptide-25	Saline
E.G7	1·0	29·1	<0·01	<0·01
EL-4	<0·01	<0·01	<0·01	<0·01

Figure 1. Enhanced induction of OVA-specific $CD8^+$ CTL response in spleen by coimmunization with OVA and Peptide-25. Three groups of mice were immunized with OVA (10 μg) in IFA, OVA (10 μg) and Peptide-25 (10 μg) in IFA or Peptide-25 (10 μg) in IFA subcutaneously. Spleen cells from each group of mice 10 days after the immunization were subjected to OVA-specific CTL assay. CTL assay was conducted by incubating sensitized cells with ^{51}Cr-labelled E.G7 or ^{51}Cr-labelled EL-4 cells of various effector to target ratios at 37° for 4 hr. The values represent the geometric means and standard deviation of percentage specific lysis in triplicate cultures. Background lysis of the target cells was less than 10% of maximum ^{51}Cr-release. The number of LU was calculated from the dose–response curve obtained for each group. A representative result of a series of five experiments is shown.

stimulated *in vitro* for 5 days with heavily irradiated E.G7 as stimulator cells that express OVA linked to the MHC class I molecule. The responding cells recovered after the culture were subjected to OVA-specific CTL assay as effector cells. The CTL activity was assessed on a 4-hr ^{51}Cr-release assay using ^{51}Cr-labelled E.G7 or EL-4. Results clearly revealed that the spleen cells from OVA-immunized mice mounted a significant CTL response to E.G7, but not to EL-4 upon *in vitro* stimulation with irradiated E.G7 (Fig. 1). Interestingly, a robust OVA-specific CTL response was induced in the culture of the spleen cells from mice immunized with a mixture of OVA and Peptide-25 in IFA upon E.G7 stimulation. The enhancement of the OVA-specific CTL response by coimmunization of OVA with Peptide-25 was quantitatively confirmed by calculating the lytic unit. Spleen cells from Peptide-25-immunized mice or from IFA-treated mice did not mount a significant CTL response to E.G7 upon E.G7 re-stimulation *in vitro*, indicating that Peptide-25 immunization does not induce a polyclonal CTL response.

We confirmed that an enhanced OVA-specific CTL response was observed after coimmunization with Peptide-25 and MHC class I-binding OVA peptide (data not shown). The OVA-specific CTL activity in effector cells was abrogated completely by the depletion of $CD8^+$ T cells using anti-CD8 mAb plus complement treatment before CTL assay, while the CTL activity remained the same in the treatment of spleen cells with anti-CD4 mAb plus complement (data not shown).

To examine the enhancing effect of I-A^b-binding peptides other than Peptide-25 on OVA-specific CTL generation, three groups of mice were immunized with OVA in IFA, a mixture of OVA and Peptide-25 in IFA, or a mixture of OVA and Peptide-9 of Ag85B in IFA. As a control, a group of mice was immunized with OVA and Peptide-18 (non-I-A^b-binding peptide of Ag85B) in IFA. The generation of an OVA-specific CTL response in spleen cells was assessed 10 days after the immunization. While immunization with OVA and Peptide-25 induced a potent OVA-specific CTL response, the CTL response observed in spleens from mice immunized with OVA and Peptide-9 was much less, if present at all (data not shown). Immunization with a mixture of OVA and Peptide-18 did not show enhancement of the OVA-specific CTL response.

In separate experiments, we analysed the augmenting effect of Peptide-25 on the CTL response specific for TRP-2 peptide, which is an MHC class I-binding peptide of murine melanoma. We immunized C57BL/6 mice with TRP-2 in IFA or a mixture of TRP-2 and Peptide-25 in IFA twice with a 10-day interval. Spleen cells from each group of mice were stimulated *in vitro* with TRP-2 for 5 days and TRP-2-specific CTL assay was conducted. Co-immunization with TRP-2 and Peptide-25 induced a significant $CD8^+$ CTL response in T cells to TRP-2 and IFN-γ production, while TRP-2 immunization was ineffective (Fig. 2a,b).

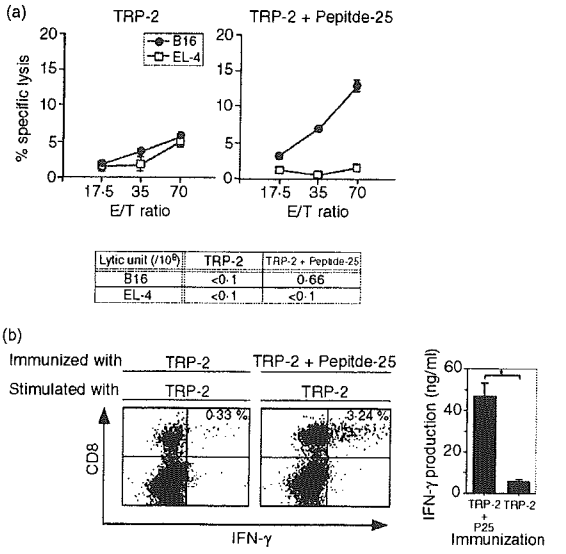


Figure 2. Enhancing effect of Peptide-25 on TRP-2-specific $CD8^+$ CTL response by coimmunization with TRP-2. (a) Two groups of mice were immunized subcutaneously with TRP-2 (10 μg) in IFA or TRP-2 (10 μg) and Peptide-25 (P25) (10 μg) in IFA. Spleen cells from each group of mice were subjected to TRP-2-specific CTL assay 10 days after the immunization. CTL assay was conducted by incubating sensitized cells with ^{51}Cr-labelled B16 melanoma or ^{51}Cr-labelled EL-4 cells of various effector to target ratios at 37° for 4 hr. The values represent the geometric means and standard deviation of percentage specific lysis in triplicate cultures. Background lysis of the target cells was less than 9% of maximum ^{51}Cr-release. The number of LU was calculated from the dose–response curve obtained with each group. A representative result of a series of three experiments is shown. (b) Ten days after immunization, spleen cells were stimulated with TRP-2 (10 μg/ml) for 2 days. Intracellular staining of IFN-γ was carried out on the recovered cells and they were examined by FACSCalibur. The percentages of IFN-γ-producing $CD8^+$ cells are presented in the upper right region. IFN-γ production of spleen cells in the culture supernatants was quantified by ELISA. The values represent the mean and standard deviation of the triplicate cultures. *$P < 0{\cdot}01$ by Student's *t*-test.

Co-immunization of a mixture of OVA and Peptide-25 at the same site is required for the enhanced $CD8^+$ CTL response

We examined whether enhanced OVA-specific CTL generation by coimmunization with Peptide-25 can be induced when OVA and Peptide-25 are immunized separately. A group of mice was immunized with a mixture of OVA and Peptide-25 in IFA subcutaneously at the same site on the right-hand side of the abdomen. A group of mice was immunized with OVA in IFA and Peptide-25 in IFA separately (left and right sides of the abdomen, respectively). The CTL assay was conducted using spleen cells from each group of mice 10 days after the immunization. As shown in Fig. 3, the enhancement of the OVA-specific CTL response by Peptide-25 was observed only when a mixture of OVA and Peptide-25 in IFA was immunized at the same site. These results suggest that OVA and Peptide-25 need to be presented by the same antigen-presenting cells (APCs) for antigen processing to occur.

The enhancement of the OVA-specific cytolytic T-cell response by Peptide-25 depends on $CD4^+$ T cells and IFN-γ

To understand the efficacy of Peptide-25 to enhance OVA-specific cytotoxic activity, we examined whether IFN-γ-producing $CD4^+$ T cells contribute to the above enhancing effect of Peptide-25 on OVA-specific CTL generation. First, we depleted $CD4^+$ T cells *in vivo* by administering anti-CD4 mAb (GK1.5) to two groups of mice as