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Mutant *Escherichia coli* enterotoxin as a mucosal adjuvant induces specific Th1 responses of $CD4^+$ and $CD8^+$ T cells to nasal killed-bacillus calmette–guerin in mice

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Abstract

On single nasal immunization of mice with killed-bacillus calmette–guerin (BCG) plus a mutant *Escherichia coli* enterotoxin, delayed-type hypersensitivity was induced and BCG-infection decreased. Spleen cells, particularly $CD4^+$ T cells among them produced IL-2, IFNγ and TNFα in response to the killed-BCG or purified protein derivatives. $CD8^+$ T cells including cytotoxic T lymphocytes produced IFNγ and TNFα. However, both types of T cells reacted a little to Ag85B.

The mutant induces cellular immunity to nasal killed-BCG vaccine and decreases BCG-infection. $CD4^+$ and $CD8^+$ T cells produce cytokines effective for tuberculosis. Although killed-BCG loses some antigens like Ag85B, nasal killed-BCG plus the mutant is useful for tuberculosis.

Keywords: Killed-BCG; Mutant; Heat-labile enterotoxin; Mucosal adjuvant

1. Introduction

Tuberculosis (TB) remains a major public health problem in the world. Despite intramuscular administration of bacillus calmette–guerin (BCG) vaccine throughout the world, tuberculosis remains the leading cause of death due to a single pathogen [1]. The efficacy of BCG vaccine is controversial and exhibits extremely variable levels of protection in different populations [2]. The development of subunit tuberculosis vaccines has been spurred on by the finding that partial immunity is conferred by intramuscular vaccination of animals with culture filtrate proteins [3,4], purified antigen 85B (A858B) [2], and naked DNA-encoding mycobacterial antigens [5,6]. The cell-mediated immune (CMI) response of the Th1 type, characterized by elevated production of IL-2, gamma interferon (IFN)γ and TNFα, has been determined to be essential for protective immunity against acute and chronic *M. tuberculosis* infection [7–11]. Although killed-BCG vaccine is also administered intramuscularly but is not effective for diminishing tuberculosis, one of the major problems with killed-BCG vaccine is that it loses some important antigens on killing and thus does not provide good effectiveness as to induction of cellular immunity [12,13].

It has been reported that cholera toxin and *Escherichia coli* heat-labile enterotoxin (LT) exhibit mucosal adjuvant actions with co-administered antigens [14–18]. In a previous study [19], we demonstrated that a mutant of LT exhibits strong adjuvant activity toward varicella-zoster virus (VZV), causing a high cellular immune response when co-administered with live VZV vaccine per-nasally [19,20]. Thus, the mutant is effective in inducing a specific protective response of Th1 type T cells to nasal antigens.

Abbreviations: LT, heat-labile enterotoxin; BCG, bacillus calmette–guerin; VZV, varicella-zoster virus

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Moreover, although live BCG vaccine is intramuscularly administered, nasal vaccination is more convenient and can to be performed repeatedly. However, as nasal administration of live BCG might cause lung infection in a compromised host [21] and is not effective without an adjuvant, nasal administration of live BCG may be dangerous. Some kinds of materials are necessary as a mucosal adjuvant [22]. In the present study, we examined whether or not nasal killed-BCG vaccine plus a mutant induces effective cellular immunity to BCG and the production of cytokines affecting acute and chronic tuberculosis infection in mice.

2. Materials and methods

2.1. BCG, PPD and mice

M. bovis BCG (substrain Tokyo) was purchased from Japan BCG Inc. (Tokyo, Japan). Purified protein derivatives (PPD) were purchased from Staten's Serum Institute (Tuberculin PPD Batch RT49). All experiments were performed using ICR or C3H/He female mice (Shizuoka Animal Co. Ltd.). Mice raised and maintained in a specific pathogen-free condition were used at ages of 8–9 weeks. The controls were age-matched female mice.

2.2. Bacteria and plasmids, and preparation of a mutant

Mutant 135-5, whose A subunit does not have the peptide Arg192–Thr193–Ile194, was constructed by means of polymerase chain reaction (PCR) as described [23]. After the plasmid in the mutant had been transformed into *E. coli* MV1184, the mutant was purified by immobilized-D-galactose affinity column chromatography with TEAN buffer (50 mM Tris–HCl, 1 mM EDTA, 3 mM NaN_3 and 0.2 M NaCl, pH 6.8) as described [23]. The conditions for cell culture in CAYE medium, the isolation of crude cell extracts and LT purification by successive chromatographies have been described previously [23].

2.3. Preparation of recombinant Ag85B

The Ag85B gene was prepared as described previously [24,25]. After the gene had been amplified by PCR with two primers, TGGGATCCTTCTCCCGGCCGGGGC TGCCGGTC and ACAGGAAACAGCTATGACCATGATTAC, it was ligated with the gluthatione *S*-transferase (GST) gene on the 4T-1 plasmid (Pharmacia) at the Bam-H1 and Eco-R1 sites. After the plasmid had been transformed to the BL21d strain, the bacteria were cultured in 2YT broth (1.6% tryptone, 1% yeast extract and 0.5% NaCl) containing 2% glucose and then stimulated with 0.1 mM IPTG. They were collected by centrifugation and sonicated in PBS containing 0.3% sarcocirate. After centrifugation, the supernatant was applied to a gluthatione Sepharose-4B column. The column was washed with PBS and then equilibrated with a buffer (2 mM $CaCl_2$, 140 mM NaCl and 50 mM Tris–HCl, pH 7.2). The Sepharose-4B gel was digested with thrombin (1 U/0.7 ml) for 18 h at 23 °C.

Recombinant Ag85B (rAg85B) eluted from the gel was analyzed by SDS-PAGE. As the sample gave one band corresponding to 32 kDa, it was used in this experiment.

2.4. Nasal immunization of mice

The purified toxin, 10 μg/dose, was mixed with live or killed-BCG (10^4, 10^5 or 10^6 BCG cells) in PBS, and then 20 μl of the mixture was per-nasally administered to mice under light pentobarbital sodium anesthesia as described previously [19,20]. Heating at 70 °C for 2 h performed killing of BCG. After a single nasal administration, experiments were performed.

2.5. Footpad test and titers of anti-killed-BCG antibodies

ICR mice were per-nasally immunized with live or killed-BCG vaccine with or without the mutant (10 μg). Mice were fed over 4 weeks and then killed-BCG cells (10^5 cells) in 100 μl of PBS were given to the mice by injection into one hind footpad. The footpad thickness was determined with a dial thickness gauge before and 24 h after the injection. After the measurements, sera were prepared.

Killed-BCG in PBS was sonicated in PBS and then centrifuged at 30,000 rpm for 2 h. The supernatant (50 μg/ml) and PPD (10 μg/ml) in PBS were used for coating enzyme-linked immunosorbent assay (ELISA) plates to determine the anti-BCG or anti-PPD titers. Other procedures were performed as described previously [23].

2.6. Inhibition test of BCG-infection in spleens of mice

According to the method as described previously [26], inhibition test of BCG-infection in spleens was performed with C3H/He mice. One month after C3H/He mice had been per-nasally immunized with live or killed-BCG (10^6 killed-BCG which contained 15 μg of protein) with or without the mutant (10 μg), 10^6 live BCG cells were intravenously injected into the mice. Four weeks later, the spleens from the mice were homogenized in 1 ml of PBS, and 100 μl aliquots of the homogenates were plated on Middlebrook 7H11 agar plates containing 5 mg/ml bovine albumin, 4 μg/ml catalase, 0.2% glycerol, 2 mg/ml glucose, 0.85% NaCl and 0.05 mg/ml olenic acid agar. Two months later, the numbers of colonies formed on agar were determined.

2.7. Cytokine production by spleen cells from mice per-nasally immunized with the vaccine

One month after C3H/He mice had been per-nasally immunized with live or killed-BCG (10^6 BCG) with or without the mutant (10 μg), spleen cells from three mice were

prepared in RPMI containing 10% FCS and 10 U/ml heparin, and then mixed. Mixed cells (10^7)/ml were incubated for 24 h with or without killed-BCG or PPD at each concentration. The cytokine level in the culture supernatant was determined by ELISA. ELISA for cytokines i.e., IL-2, IL-4, IFNγ and TNFα, was performed with a Biotrak™ kit (Amersham Life Science, Co. Ltd.) in duplicate.

2.8. Preparation of a $CD4^+$ or $CD8^+$ T cell fraction from spleen cells

One month after C3H/He mice had been per-nasally immunized with live or killed-BCG (10^6 BCG) with or without the mutant (10 μg), spleen cells from three mice were prepared in RPMI containing 10% FCS and 10 U/ml heparin, and then mixed.

Immunomagnetic separation was performed for spleen cells to prepare $CD4^+$ and $CD8^+$ T cells. For the selection of $CD4^+$ T and $CD8^+$ T cells, $CD4^+$ and $CD8^+$ T cell isolation kits (Mitenyi Biotec. Co. Ltd.), respectively, were used. The kits comprised indirect magnetic labeling systems for the isolation of $CD4^+$ and $CD8^+$ T cells from a cell suspension. Non-$CD4^+$ or -$CD8^+$ T cells were indirectly labeled with a cocktail of biotin-conjugated antibodies against each cell type and anti-biotin microbeads. According to the manufacture's guidelines, $CD4^+$ and $CD8^+$ T ells were isolated.

The T cells prepared were analyzed by two-color flow cytometry as described previously [18]. Primary and activated $CD4^+$ and $CD8^+$ T cells comprised at least 95% $CD3^+/CD4^+$ and $CD3^+/CD8^+$, respectively.

2.9. FCM

The following mAbs, coupled to FITC, PE or biotin, were used for flow cytometry: biotin-anti-CD3ε (clone 145-2C11) from Bioscinence Co. Ltd.; FITC anti-mouse CD4 (clone YTS191.1.2) from Accurate Chemical and Scientific Corporation; FITC anti-mouse CD8 (clone CT-CD8a) from Caltag Lab., Burlingand; PE anti-mouse IFNγ (clone XMG1.2) and Biotin-FasL (clone MFL3) from Pharmingen Co. Ltd. Anti-perforin (clone CB5.4) was purchased from Alexis Biochemicals, SanDiego, and biotinated with a protein biotinylation module (Amersham Life Science).

To stain surface antigens on spleen cells, 1×10^5 cells were treated with 5% skim milk in PBS at 4 °C for 30 min. Samples were then incubated with a mixture containing the designated anti-CD mIgG at 4 °C for 30 min as described [18]. For intracellular cytokine or perforin staining, we added 2 ml of an FACS lysing solution (Becton Dickenson, FACSTM lysing solution) to the cells, followed by blocking in 5% skim milk. After incubation at room temperature for 10 min, the cells were re-suspended in 500 μl of an FACS permeabilizing solution (Becton Dickenson, FACSTM permeabilizing solution) and then incubated for 10 min at room temperature. Following washing in PBS containing 2% FCS, samples were stained with the designated mAb at 4 °C for 30 min. Flow cytometric analysis was performed with a FACScan (Becton Dickenson, FACSCaliber); data were analyzed using Lysis II software.

3. Results

3.1. Delayed-type hypersensitivity (DTH) to live or killed-BCG vaccine co-administered with the mutant on footpad test

As described [19], mutant 135-5 exhibits stronger adjuvant ability to induce cellular immunity to VZV than normal LT in mice. Thus, we examined whether or not this mutant also had mucosal adjuvant ability to induce cellular immunity to live or killed-BCG vaccine on nasal administration. To determine whether or not delayed-type hypersensitivity (DTH) to live or killed-BCG vaccine was induced by intranasal co-administration of the mutant, a footpad test was performed in ICR mice as described previously [19]. Table 1 shows that the footpads of mice immunized with both a vaccine plus the mutant were significantly thicker than those of mice immunized with just each vaccine ($p < 0.05$), suggesting that the mutant has an adjuvant action for DTH to live or killed-BCG vaccine.

Sera from mice were prepared and the titers of anti-killed-BCG were determined by ELISA. The titers of anti-killed-BCG or anti-PPD antibodies in sera from mice immunized with live or killed-BCG plus the mutant were not different from those with killed-BCG alone, and were under eight.

Moreover, the data of DTH with C3H/He mice showed nearly the same results as those with ICR mice on footpad test. Then, as described previously [26], we did other experiments with C3H/He mice.

3.2. Inhibition test for BCG-infection in spleens of mice

Whether or not immune responses induced by live or killed-BCG vaccine plus the mutant was effective as to bacterial infection was determined as described previously [26]. Table 2 shows that bacteria in the spleens of mice immunized with killed-BCG plus the mutant were significantly less than in C3H/He mice immunized with killed-BCG, the mutant or PBS.

3.3. Cytokine induction in spleen cells from mice immunized with live or killed-BCG vaccine plus the mutant

To determine the function of the response of spleen cells, we examined cytokine production by spleen cells from mice co-immunized with live or killed-BCG vaccine plus the mutant. As shown in Fig. 1, the concentration of each cytokine in the culture supernatant was determined 24 h after stimulation of the spleen cells with killed-BCG or PPD in vitro. When the spleen cells from mice immunized with live or

Table 1
Delayed-type hypersensitivity (DTH) to live or killed-BCG vaccine co-administrated with mutant on footpad test

Immunization	Antigen BCG (PFU)	Adjuvant Mutant (μg)	Thickness of footpads (mm)
PBS	–	–	2.75 ± 0.44[a]
Mutant (mLT)	–	10	2.70 ± 0.37[a]
Killed-BCG	10^5	–	2.67 ± 0.20[a]
	10^6	–	2.76 ± 0.11[a]
	10^7	–	2.84 ± 0.20[a]
Killed-BCG + mLT	10^5	10	3.16 ± 0.38[a]
	10^6	10	3.97 ± 0.47[a,*]
	10^7	10	4.04 ± 0.41[a,*]
Live BCG	10^5	–	2.75 ± 0.38[b]
	10^6	–	2.90 ± 0.24[b]
Live BCG + mLT	10^5	10	3.06 ± 0.43[b]
	10^6	10	4.79 ± 0.31[b,**]

[a] Killed-BCG (10^6) was injected into footpads.
[b] Live BCG (10^6) was injected into footpads.
[*] A value (mean ± S.D.) significantly smaller than for killed-BCG alone ($p < 0.05$).
[**] A value (mean ± S.D.) significantly smaller than for live BCG alone ($p < 0.05$).

killed-BCG vaccine plus the mutant were stimulated with killed-BCG or PPD, they produced from 2- to 5-fold the levels of IL-2, IFNγ and TNFα in mice immunized with a vaccine alone, suggesting that Th1 type helper T cells in the spleen was stimulated, and was IL-2, IFNγ and TNFα production augmented in vitro.

On other hand, in all cases, no IL-4 response was detected.

3.4. Cytokine induction in $CD4^+$ and $CD8^+$ T cells among spleen cells of mice immunized with killed-BCG vaccine plus the mutant

It has been reported that $CD4^+$ and $CD8^+$ T cells producing IFNγ and TNFα are important for host-defense against tuberculosis [7–13]. Therefore, to determine the functions of $CD4^+$ and $CD8^+$ T cell responses, we examined the cytokine production by purified $CD4^+$ and $CD8^+$ T cells (1×10^6 cells) from mice co-immunized with killed-BCG plus the mutant.

The concentrations of cytokines in culture supernatants were determined 24 h after killed-BCG, PPD or purified recombinant Ag85B (rAg85B) had been used to stimulate purified $CD4^+$ T cells in vitro (Fig. 2). $CD4^+$ T cells produced IL-2, IFNγ and TNFα in a dose-dependent manner as to killed-BCG and PPD, suggesting that specific $CD4^+$ T cells producing IFNγ and TNFα were induced on immunization with killed-BCG plus the mutant. However, the responses of these cytokines to rAg85B were lower than those of killed-BCG and PPD.

Moreover, it was examined whether or not purified $CD8^+$ T cells produced each cytokine (Fig. 3). $CD8^+$ T cells from mice immunized with killed-BCG plus the mutant also produced IFNγ and TNFα in a dose-dependent manner as to killed-BCG and PPD. On other hand, the responses of these cytokines to rAg85B were also lower than those of killed-BCG and PPD.

3.5. Perforin or FasL production by $CD8^+$ T cells producing IFNγ on induction by killed-BCG stimulation

It has been reported that that the principal mechanisms of cytotoxic T lymphocytes (CTL)-mediated cytolysis are

Table 2
Inhibition test of BCG-infection in spleen of mice

Nasal immunization	Antigen BCG (PFU)	Adjuvant Mutant (μg)	Amount of bacteria in spleen ($\times 10^4$)[a]
PBS	–	–	6.13 ± 2.87
Mutant (mLT)	–	10	5.02 ± 0.47
Killed-BCG	10^6	–	4.86 ± 1.86
Killed-BCG + mLT	10^6	10	2.25 ± 2.36[*]
Live BCG	10^6	–	4.55 ± 1.87
Live BCG + mLT	10^6	10	2.57 ± 1.15[**]

[a] Values are means for six or eight mice.
[*] A value (mean ± S.D.) significantly smaller than for killed-BCG alone ($p < 0.05$).
[**] A value (mean ± S.D.) significantly smaller than for live BCG alone ($p < 0.05$).

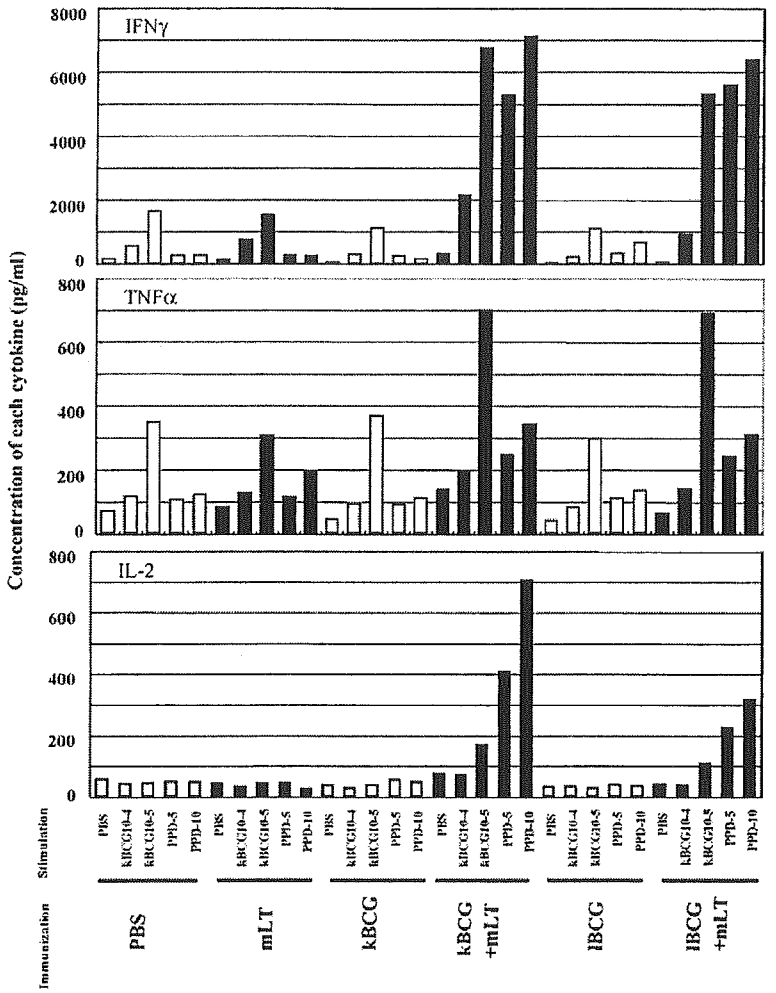

Fig. 1. Cytokine induction in spleen cells from mice immunized with live or killed-BCG vaccine plus the mutant. Three C3H/He mice were immunized intranasally once with live or killed-BCG vaccine with or without the mutant for each experiment. One month later, mixture of spleen cells from three mice for each experiment was prepared. The spleen cell fraction (10^7/ml) was stimulated with each dose of killed-BCG or PPD in vitro as described under Section 2. The concentration of each cytokine was determined with an ELISA kit. The data shown are representative of two independent analyses with nearly the same results. Immunization of C3H/He mice: PBS, 1 × PBS; mLT, 10 μg of the mutant/mouse; killed-BCG, 10^6 killed-BCG cells/mouse; killed-BCG + mLT, 10^6 killed-BCG cells + 10 μg of the mutant/mouse; l-BCG, 10^6 live BCG cells/mouse; live BCG + mLT, 10^6 live BCG cells + 10 μg of the mutant/mouse. Stimulation in vitro — PBS: 1 × PBS; kBCG 10-4 or 10-5: 10^4 or 10^5 killed-BCG cells/ml; PPD-5 or -10: 5 or 10 μg/ml of PPD.

the delivery of cytotoxic granular proteins, i.e., perforin and granzymes, to the target cells recognized by CTL, and interaction between Fas ligands (FasL) on CTLs and Fas on target cells [27,28]. Then, to elucidate the function of $CD8^+$ T cells producing IFNγ on induction by killed-BCG, we examined the production of perforin and FasL by $CD8^+$ T cells. Following intracellular perforin staining, small numbers of $CD8^+$ T cells were stained with anti-perforin and anti-IFN γ antibodies at 24 and 48 h. However, it was impossible to say that perforin increased in $CD8^+$ T cells producing IFNγ. On the other hand, $CD8^+$ T cells producing IFNγ were stained with anti-FasL and anti-IFNγ antibodies at 24 h (Fig. 4), demonstrating that some of the $CD8^+$ T cells producing IFNγ on induction by killed-BCG may contain specific cytotoxic T lymphocytes.

4. Discussion

From the findings described in this paper, we drew the following conclusions concerning nasal killed-BCG vaccine with the mutant against *M. tuberculosis*. (i) Nasal killed-BCG vaccine plus the mutant is able to induce specific type 1 immune responses (Table 1 and Figs. 1–3), which are effective for acute tuberculosis (Table 2). (ii) Although cholera toxin (CT) has mucosal adjuvant action like LT, some papers

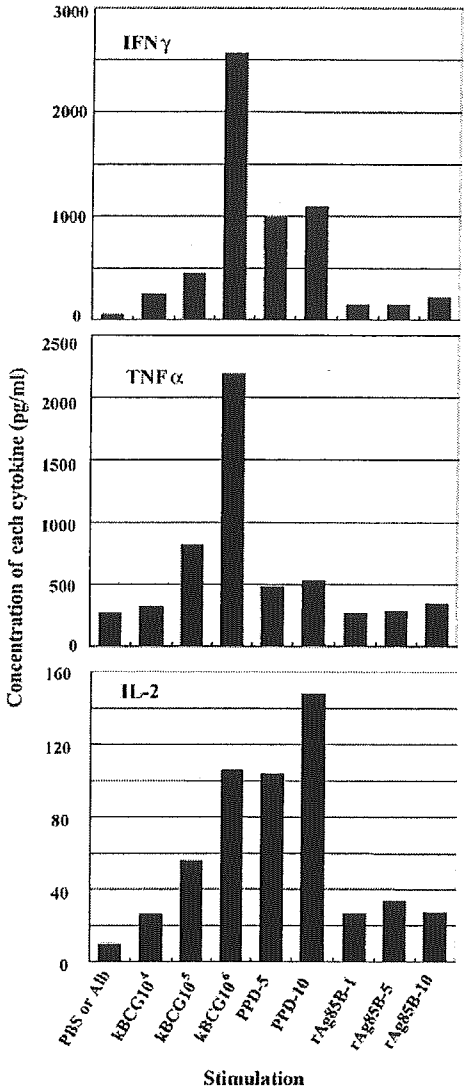

Fig. 2. Cytokine induction in $CD4^+$ T cells from spleen cells of mice immunized with killed-BCG vaccine plus the mutant. Three mice were immunized per-nasally once with killed-BCG (10^6 cells) with the mutant (10 μg). One month later, $CD4^+$ T cells from three C3H/He mice were prepared and APCs were also prepared from mice without immunization. A mixture of the $CD4^+$ T cell fraction (10^6 cells/ml) and APCs (5×10^6 cells/ml) was stimulated with killed-BCG (10^6 cells/ml) in vitro as described under Section 2. As 10^6 killed-BCG contained 15 μg of protein, 20 μg of albumin was uses as negative control. Twenty-four hours later, the concentration of each cytokine was determined with an ELISA kit. The data shown are representative of two independent analyses with nearly the same results. Stimulation in vitro — PBS or Alb: 1× PBS or 20 μg/ml of albumin; kBCG 10-4, 10-5 or 10-6: 10^4, 10^5 or 10^6 killed-BCG cells/ml; PPD-5 or -10: 5 or 10 μg/ml of PPD; rAg85B-1, -5 or -10: 1, 5 or 10 μg/ml of rAg85B.

suggested that CT or its B subunit had an adjuvant action to live nasal BCG vaccine [22,29]. However, there has been no paper showing that nasal killed-BCG with a toxin is effective for tuberculosis.

We detected significant levels of specific IL-2, IFNγ and TNFα production in response to killed-BCG and PPD in spleen cells of mice immunized with nasal killed-BCG vaccine plus the mutant (Fig. 1), but not of IL-4. IL-2, IFNγ and TNFα are hallmarks of type 1 immune responses and are considered important for the protective immunity against acute

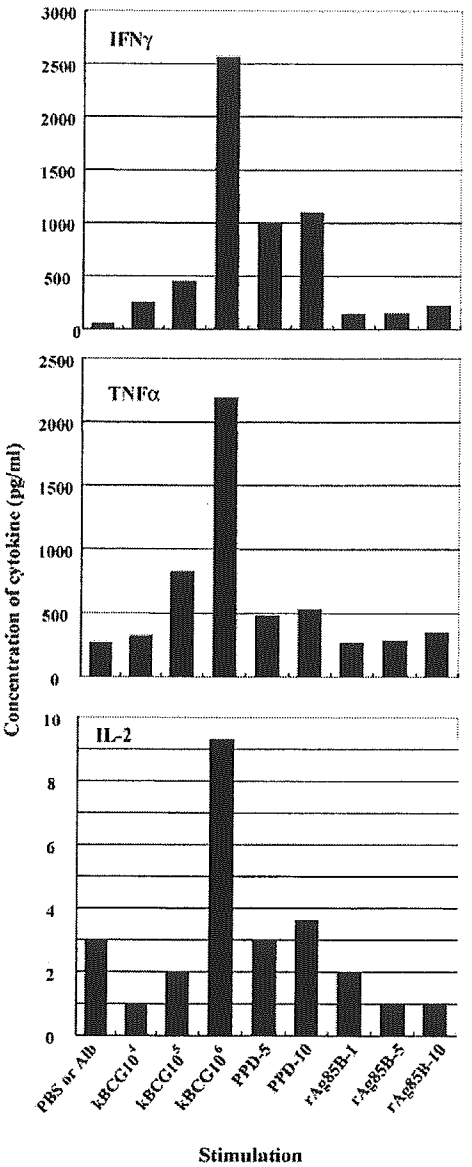

Fig. 3. Cytokine induction in $CD8^+$ T cells from spleen cells of mice immunized with killed-BCG vaccine plus the mutant. Three C3H/He mice were immunized per-nasally once with killed-BCG (10^6 cells) plus the mutant (10 μg). One month later, $CD8^+$ T cells from three mice were prepared and APCs were also prepared from mice without immunization. A mixture of the $CD8^+$ T cell fraction (10^6 cells/ml) and APCs (5×10^6 cells/ml) was stimulated with killed-BCG (10^6 cells/ml) in vitro as described under Section 2. Twenty-four hours later, the concentration of each cytokine was determined with an ELISA kit. The data shown are representative of two independent analyses with nearly the same results. Stimulation in vitro — PBS or Alb: 1× PBS or 20 μg/ml of albumin; kBCG 10-4, 10-5 or 10-6: 10^4, 10^5 or 10^6 killed-BCG cells/ml; PPD-5 or -10: 5 or 10 μg/ml of PPD; rAg85B-1, -5 or -10: 1, 5 or 10 μg/ml of rAg85B.

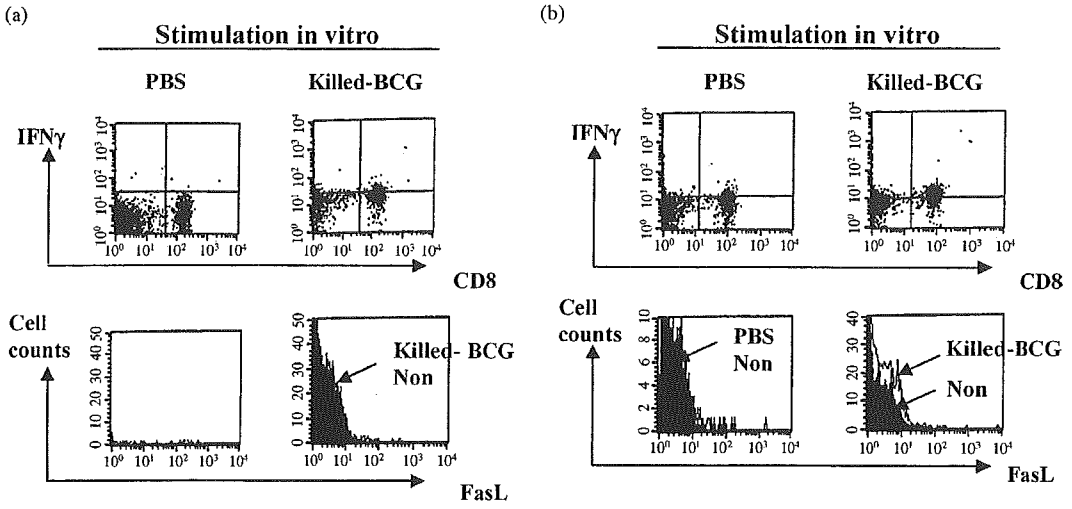

Fig. 4. FasL production by $CD8^+$ T cells producing IFNγ on induction by killed-BCG stimulation. Three C3H/He mice were immunized per-nasally once with PBS (a) or killed-BCG (10^6 cells) plus the mutant (10 μg) (b). One month later, spleen cells from three mice were prepared and 10^6 cells/ml were stimulated with PBS or killed-BCG (10^6 cells/ml) in vitro as described under Section 2. Twenty-four hours later, each antigen of IFNγ, CD8 or FasL was stained. FasL in double positive fraction of IFNγ and CD8 antigen was analyzed as described under Section 2. The data shown are representative of two independent analyses with nearly the same results.

and chronic infection by *M. tuberculosis* [7–13]. Moreover, purified $CD4^+$ T cells also produced IL-2, IFNγ and TNFα on their stimulation (Fig. 2). It has been reported that $CD4^+$ T cells producing IFNγ and TNFα are primary effectors sufficient to activate macrophages, which can control or eliminate intracellular bacteria. In $CD4^+$ T cell-depleted mice or mice pretreated with anti-CD4 antibodies, tuberculosis infection is rapidly reactivated. Moreover, in mice in which IFNγ, the IFNγ receptor, TNFα or the TNF receptor is deleted, tuberculosis infection results in rapid death, with higher bacterial burdens compared to in control mice [12]. Therefore, $CD4^+$ T cells, and their production of IFNγ and TNFα have the potential to affect anti-mycobacterial immunity. However, the IFNγ levels in the lungs of $CD4^+$ T cell-deleted mice overall were similar to those in control mice, due to IFNγ production by specific $CD8^+$ T cells responding the tuberculosis. $CD8^+$ T cells are also important as an IFNγ source in *M. tuberculosis* infection [12]. As shown in Fig. 3, $CD8^+$ T cells from immunized mice also produced IFNγ and TFNα on in vitro stimulation with killed-BCG or PPD. Specific $CD8^+$ T cells reacting to tuberculosis antigens are induced on nasal vaccination of killed-BCG plus the mutant and produce IFNγ and TFNα, which activate macrophages [13]. These data also suggest that the mutant strongly polarizes the activation balance of Th1/Th2 type T cells for killed-BCG to the Th1 type side like for VZV [19]. On the other hand, Haile et al. recently [30] suggested that intranasal priming with heat killed-BCG in Eurocine^{TM} L3 adjuvant followed by an intranasal booster is effective for *M tuberculosis* infection. With their booster, mycobacterial antigen-specific serum IgG and IFNγ responses are elicited, as Eurocine^{TM} L3 adjuvant induces a relatively Th2 response. Although killed-BCG plus the mutant was once successfully administered to mice per-nasally without a following booster, the adjuvant action of the mutant is different from that of Eurocine^{TM} L3, which activates both types of T cells with killed-BCG.

However, it has been reported that some important antigens like Ag85B disappear or are antigenically inactivated in BCG-killed by heating [12,25]. In nasal killed-BCG vaccine plus the mutant, small numbers of $CD4^+$ and $CD8^+$ T cells responding to Ag85B were detected (Figs. 2 and 3). Therefore, nasal killed-BCG vaccine plus the mutant is effective as a subunit vaccine for inducing cellular immunity, may become more valuable with the addition of some important antigens, and can possibly be administered repeatedly and safely to maintain effective cellular immunity to tuberculosis.

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A Novel Role of IL-15 in Early Activation of Memory CD8$^+$ CTL after Reinfection[1]

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A rapid induction of effector functions in memory T cells provides rapid and intensified protection against reinfection. To determine potential roles of IL-15 in early expansion and activation of memory CD8$^+$ T cells in secondary immune response, we examined the cell division and cytotoxicity of memory CD8$^+$ T cells expressing $OVA_{257-264}/K^b$-specific TCR that were transferred into IL-15-transgenic (Tg) mice, IL-15 knockout (KO) mice, or control C57BL/6 mice followed by challenge with recombinant *Listeria monocytogenes* expressing OVA (rLM-OVA). In vivo CTL activities and expression of granzyme B of the transferred CD8$^+$ T cells were significantly higher in the IL-15 Tg mice but lower in the IL-15 KO mice than those in control mice at the early stage after challenge with rLM-OVA. In contrast, there was no difference in the cell division in IL-15 Tg mice and IL-15 KO mice compared with those in control mice. In vivo administration of rIL-15 conferred robust protection against reinfection via induction of granzyme B in the memory CD8$^+$ T cells. These results suggest that IL-15 plays an important role in early activation of memory CD8$^+$ T cells. *The Journal of Immunology,* 2005, 174: 3590–3597.

Antigen-specific memory CD8$^+$ T cell responses are of vital importance in protective immunity against re-exposure to intracellular pathogens (1–6). Memory CD8$^+$ T cells provided faster, more effective functions, including the secretion of cytokines and lysis of infected target cells, during secondary immune responses. The enhanced secondary immune response is thought to be due both to increased frequency of Ag-specific CD8$^+$ T cell precursors and to increased functional activities of memory T cells that respond to Ag in a manner qualitatively different from naive T cells (7–9). Studies using mitotic inhibitors have revealed that memory CD8$^+$ T cells can become cytotoxic without undergoing cell division after restimulation with Ag (10). Memory CD8$^+$ T cells were shown to become cytolytic more rapidly and to secret a greater amount of IFN-γ than naive T cells after in vitro Ag stimulation, although no significant difference was found between naive and memory CD8$^+$ T cells in their proliferative capacities (11). It has also been reported that memory CD8$^+$ T cells under in vitro conditions were more efficient and precious cytokine secretors, although they proliferated poorly (12). Thus, memory CD8$^+$ T cells can be reactivated to become cytotoxic without undergoing cell division.

IL-15 belongs to the four-helix bundle cytokine family and uses β- and γ-chains of IL-2R for signal transduction, and it thus has many properties that are the same as those of IL-2, despite the fact that it has no sequence homology with IL-2 (13–15). In contrast to IL-2, which is produced mainly by activated T cells, IL-15 is produced by a wide variety of tissues, including placenta, skeletal muscle, kidney, and macrophages upon stimulation with LPS (16, 17). IL-2 is known to be important in clonal expansion of Ag-specific CD8$^+$ T cells during primary immune response, whereas IL-15 is not mandatory for the expansion of CD8$^+$ T cells in the immune response. Recent studies have demonstrated that primary responses to lymphocytic choriomeningitis virus (LCMV)[3] and OVA were readily generated in IL-15 knockout (KO) mice or IL-15Rα KO mice to a level equal to that in control mice (18, 19). We have also reported that generation of listeriolysin O $(LLO)_{91-99}$-specific CD8$^+$ T cells in IL-15-transgenic (Tg) mice normally occurred after primary infection with *Listeria monocytogenes* (20). In contrast, IL-15 has potential roles in maintenance of memory phenotype CD8$^+$ T cells, which are capable of slowly dividing without Ag stimulation (18, 19). We have demonstrated that the number of LLO_{91-99}-positive memory CD8$^+$ T cells was significantly higher 6 wk after primary infection with *L. monocytogenes*, which resulted in increased levels of Bcl-2 expression and cell division without Ag stimulation (20). Thus, IL-15 plays an important role in long-term maintenance of Ag-specific memory CD8$^+$ T cells in an Ag-independent manner. In addition, there are several lines of evidence that IL-15 is capable of stimulating NK cells and CD8$^+$ CTL to exhibit increased cytotoxicities (21–24). IL-15 has been reported to directly up-regulate expression of cytotoxic molecules such as granzyme B and perforin, mimicking TCR cross-linking in the induction of cytotoxic molecules and cytotoxicity of memory CD8$^+$ T cells (25). These findings raise the possibility that IL-15 plays an important role in rapid elicitation of effector functions in

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[3] Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; KO, knockout; LLO, listeriolysin O; Tg, transgenic; rLM-OVA, recombinant *L. monocytogenes* expressing OVA; PEC, peritoneal exudate cell; LN, lymph node; T_{CM}, central memory cell; T_{EM}, effector memory cell.

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memory $CD8^+$ T cells in re-exposure to microbial invasion, providing robust protection against reinfection.

In the present study, to elucidate potential roles of IL-15 in elicitation of effector functions of microbial Ag-specific memory $CD8^+$ T cells in secondary immune response, we examined the effector functions of Ag-specific memory $CD8^+$ T cells transferred into IL-15 Tg mice or IL-15 KO mice followed by infection with recombinant *L. monocytogenes* expressing OVA (rLM-OVA). We found that in vivo CTL activities and expression of granzyme B of the transferred $CD8^+$ T cells were significantly higher in the IL-15 Tg mice but lower in the IL-15 KO mice than those in control mice at the early stage after challenge with rLM-OVA. Furthermore, in vivo administration of exogenous IL-15 conferred protection against secondary infection via induction of cytotoxic molecules in the Ag-specific memory $CD8^+$ T cells. These results suggest that IL-15 plays an important role in early activation of Ag-specific memory $CD8^+$ T cells following secondary infection with microbes.

Materials and Methods

Mice

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

Microorganism

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

Abs and reagents

FITC-conjugated anti-CD44 (IM7), anti-CD69 (H1.2F3), anti-CD25 (7D4), anti-Ly6C (AL-21), and anti-IFN-γ (XMG1.2); PE-conjugated anti-Vα2(B20.1), anti-CD62L (MEL-14), CD122 (5H4), and CD132 (4G3); CyChrome-conjugated anti-CD8α (53-6.7) and streptavidin; allophycocyanin-conjugated streptavidin; and biotin-conjugated anti-Ly5.1 (A20) were purchased from BD Biosciences. PE-labeled anti-human granzyme B mAb and isotype control Ab were obtained from Caltag Laboratories. CFSE was purchased from Molecular Probes. $OVA_{257-264}$ H-$2K^b$ tetramers were purchased from MBL.

Analysis of intracellular granzyme B synthesis

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

Generation of memory OT-I cells

Memory OT-I cells were generated as follows. Purified $CD8^+$ T cells (2×10^6) of splenocytes from naive OT-I mice were injected i.v. into naive C57BL/6 hosts. Twenty-four hours later, these mice were infected with 5×10^5 CFU rLM-OVA. After 40 or more days, these mice were sacrificed, and the number of transgenic memory cells was determined by staining with anti-CD8 mAb, anti-Ly5.1 mAb, and $OVA_{257-264}$ H-$2K^b$ tetramer. In some experiments, mice harboring memory OT-I cells were injected i.p. with various doses of rIL-15 or PBS for control.

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

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

In vivo cytotoxicity assay

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

Results

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

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

To determine the role of IL-15 in activation of effector $CD8^+$ T cells during primary immune response, we examined the expression levels of activation markers and granzyme B of OT-I cells after primary infection with rLM-OVA. The OT-I cells, of $CD44^{low}$, CD122 (IL-2 Rβ)^{low}, and CD132(cγ)^{low} phenotypes, did not contain intracellular granzyme B in IL-15 KO and IL-15 Tg mice on day 3 and were equivalently increased on OT-I cells on day 5 after rLM-OVA infection (Fig. 1*C* and data not shown). Only the CFSE-negative OT-I cells from each mouse contained

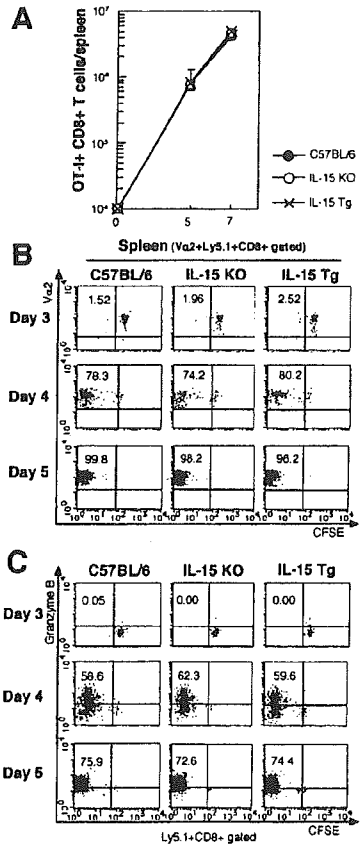

FIGURE 1. IL-15 is not required for expansion during primary CD8$^+$ T cell response. *A*, Purified naive OT-I cells (Ly5.1$^+$) from splenocytes were adoptively transferred i.v. into naive IL-15 KO mice, IL-15 Tg mice, or control C57BL/6 mice (Ly5.2$^+$) that were infected with rLM-OVA 24 h later. On days 5 and 7 after rLM-OVA infection, splenocytes from these mice were stained with $OVA_{257-264}$ MHC class I tetramer or anti-Vα2 mAb, anti-Ly5.1 mAb, and anti-CD8mAb. Absolute numbers of OVA-specific CD8$^+$ T cells in the spleen were calculated on days 5 and 7 after rLM-OVA infection. Data are presented as mean ± SD for five mice. *B*, CFSE-labeled naive OT-I cells (Ly5.1$^+$) were adoptively transferred into naive IL-15 KO, IL-15 Tg, and control C57BL/6 mice 1 day before rLM-OVA infection. Proliferation of specific T cells (Vα2$^+$Ly5.1$^+$CD8$^+$) was assessed in the spleen by flow cytometry at indicated time points. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on Vα2$^+$Ly5.1$^+$CD8$^+$ cells. *C*, Splenocytes were isolated and analyzed for the expression of CFSE and intracellular granzyme B direct ex vivo on the indicated days after infection. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on Ly5.1$^+$CD8$^+$ cells.

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

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

We next examined the role of IL-15 in expansion and activation of memory CD8$^+$ T cells during secondary immune responses using a system of adoptive transfer of memory OT-I cells into naive IL-15 Tg, IL-15 KO, and C57BL/6 hosts. Because the magnitude of the secondary expansion of memory CD8$^+$ T cells is related to the precursor frequency of Ag-specific memory CD8$^+$ T cells and because numbers of endogenous $OVA_{257-264}$-specific memory CD8$^+$ T cells in the spleen were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice than those in control mice >40 days after primary infection (our unpublished data), we used an adoptive transfer system in which the numbers of Ag-specific cells could be defined precisely before and during immune responses. We generated memory CD8$^+$ T cells in vivo by adoptive transfer of Ly5.1$^+$ naive OT-I cells into C57BL/6 mice (Ly5.2$^+$) followed by infection with rLM-OVA and purified the memory OT-I cells at 40 or more days after primary rLM-OVA infection. Memory OT-I cells were of CD44^{high}, CD122^{high}, CD132$^+$, CD25$^-$, and CD69$^-$ phenotypes (data not shown), representing resting memory CD8$^+$ T cells. Two subsets of memory CD8$^+$ T cells based on their anatomical location, expression of cell surface markers, and effector functions have been described (30, 31). Approximately 60% of the memory OT-I cells purified from spleens of recipients expressed CD62L, representing central memory cells (T_{CM}), and 40% were of CD62L$^-$ phenotype, representing effector memory cells (T_{EM}).

To determine the role of IL-15 in expansion of memory OT-I cells during the secondary immune response, memory OT-I cells were labeled with CFSE and adoptively transferred i.v. into naive IL-15 KO, IL-15 Tg, and C57BL/6 hosts. To compare expansion of memory CD8$^+$ T cell subsets after secondary infection, it was critical to demonstrate that total number of memory CD8$^+$ T cells was the same after adoptive transfer to recipient mice. At 24 h after adoptive transfer, total number of memory OT-I cells recovered from spleen in IL-15 Tg and IL-15 KO mice were almost the same as those in control mice (data not shown). Then these mice were challenged with a lethal dose of rLM-OVA and the proliferation of memory OT-I cells was analyzed after infection (Fig. 2*A*). As shown in Fig. 2*B*, none of the memory OT-I cells had divided by day 3 infection when transferred into naive IL-15 Tg, IL-15 KO, or C57BL/6 mice, showing kinetics similar to the kinetics of naive OT-1 cells after primary infection (Figs. 1*B* and 2*B*). A massive expansion of OT-I cells occurred in all groups on day 5 after *Listeria* infection. Thus, during the secondary response, there was no obvious difference in the CFSE profiles of memory OT-I cells in IL-15 Tg and IL-15 KO mice compared with those of control mice. These results suggest that IL-15 is not essential for proliferation of memory CD8$^+$ T cells during secondary immune response.

To determine the role of IL-15 in activation of memory CD8$^+$ T cells during secondary immune response, we analyzed the expression of granzyme B in memory OT-I cells in naive IL-15 Tg and IL-15 KO mice after rLM-OVA infection. The expression levels of intracellular granzyme B in memory OT-I cells were significantly lower in IL-15 KO mice but higher in IL-15 Tg mice than those in control mice on day 2 after rLM-OVA infection ($p < 0.05$, Fig. 2, *C* and *D*). On day 5 after reinfection, intracellular expression levels of granzyme B in the dividing memory OT-I cells in IL-15 Tg and IL-15 KO mice became comparable to those in control mice. We further analyzed the ability of transferred memory OT-I cells to produce IFN-γ upon restimulation with peptide on days 2 and 5 after rLM-OVA infection. The proportions of

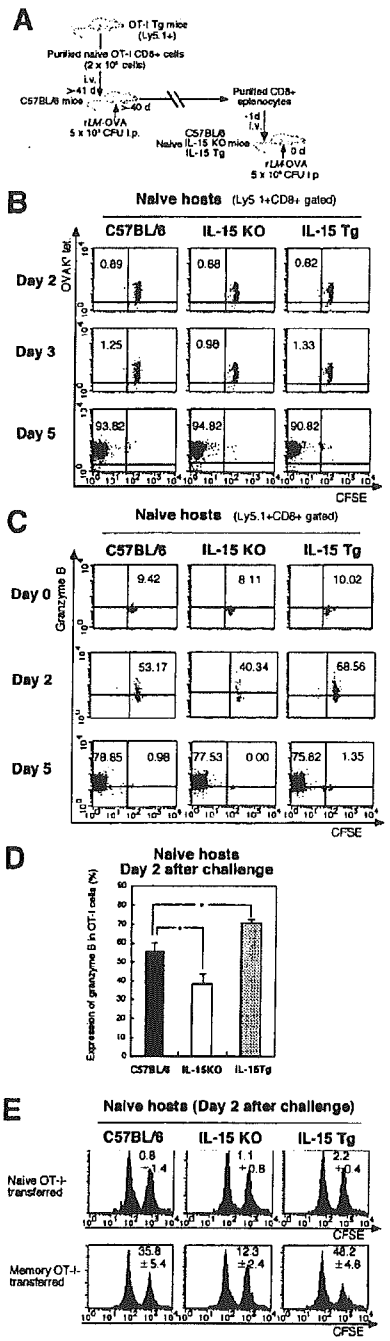

FIGURE 2. IL-15 plays an important role in the induction of cytotoxic molecules of Ag-specific memory $CD8^+$ T cells during secondary immune response in naive hosts. *A*, Purified naive OT-I cells ($Ly5.1^+$) were adoptively transferred into naive C57BL/6 hosts that were infected with rLM-OVA 24 h later. At 40 or more days after primary infection, $CD8^+$ T cells from these mice were purified by negative selection using MACS and labeled with CFSE. CFSE-labeled $CD8^+$ T cells containing 5×10^5 memory OT-I cells were adoptively transferred i.v. into naive IL-15 KO, IL-15 Tg, and control mice that were infected with rLM-OVA 24 h later. *B*, CFSE fluorescence of splenic OT-I cells was analyzed by flow cytometry by staining with anti-CD8 mAb, anti-Ly5.1 mAb, and OVA K^b tetramer at indicated time points. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on $Ly5.1^+CD8^+$ cells. *C*, Splenocytes from IL-15 KO, IL-15 Tg, or control mice harboring CFSE-labeled memory OT-I cells were isolated at indicated days before and after reinfection and stained for expression of CFSE and intracellular granzyme B of memory OT-I cells. Dot plots are gated on donor cells ($Ly5.1^+CD8^+$), and the number indicated is the percentage of donor cells stained positive for granzyme B. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles. *D*, Intracellular expression of granzyme B in OT-I cells transferred into naive IL-15 KO, IL-15 Tg, or control mice on day 2 after infection. Data were obtained from three separate experiments, and each value shown is the mean + SD for five mice (*, $p < 0.05$). *E*, Memory or naive OT-I cells (5×10^5) were adoptively transferred into naive IL-15 KO mice, IL-15 Tg mice, or control C57BL/6 mice that were infected with rLM-OVA 24 h later. As target cells, spleen cells from naive mice ($Ly5.1^+Ly5.2+$) were pulsed with OVA peptides or left unpulsed and then injected i.v. into each infected mouse harboring memory or naive OT-I cells on day 2 after rLM-OVA infection. Histograms are gated on $Ly5.1^+Ly5.2^+$ cells in the spleen from infected mice. The values in the *right corner* of each panel represent the percentage of specific killing compared with nonpulsed cells. Data were obtained from three separate experiments, and each value shown is the mean ± SD for three experiments.

intracellular IFN-γ production by OT-I cells in the spleens from IL-15 Tg and IL-15 KO mice were almost the same as those in control mice (data not shown). These results demonstrate that IL-15 are involved in the induction of granzyme B in nondividing memory $CD8^+$ T cells at the early stage of secondary immune response.

To directly detect cytotoxic activity of OT-I cells in vivo, we measured the ability of naive and memory OT-I cells to eliminate fluorescent-labeled spleen cells pulsed with $OVA_{257-264}$ peptides at the early stage after *Listeria* infection. Naive or memory OT-I cells were adoptively transferred into naive IL-15 Tg mice, IL-15 KO mice, and control C57BL/6 mice that were infected with rLM-OVA 24 h later. We confirmed that naive IL-15 Tg mice, IL-15 KO mice, and control C57BL/6 mice had not developed any in vivo CTL activities on day 2 after rLM-OVA (data not shown). Consistently, none of the $OVA_{257-264}$ peptide-pulsed donor cells in any group of mice into which naive OT-I cells had been transferred were cleared from the spleens on day 2 after infection (Fig. 2*E*). In contrast, on day 2 after infection ~35% of $OVA_{257-264}$ peptide-pulsed donor cells were cleared from the spleens of C57BL/6 mice into which memory OT-1 cells had been transferred. In correlation with the expression of intracellular granzyme B of memory OT-I cells, in vivo CTL activity levels of memory OT-I cells were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice than those in control mice (Fig. 2*E*). These results suggest that IL-15 provides early activation of Ag-specific memory $CD8^+$ CTL via induction of cytotoxic molecules.

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

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

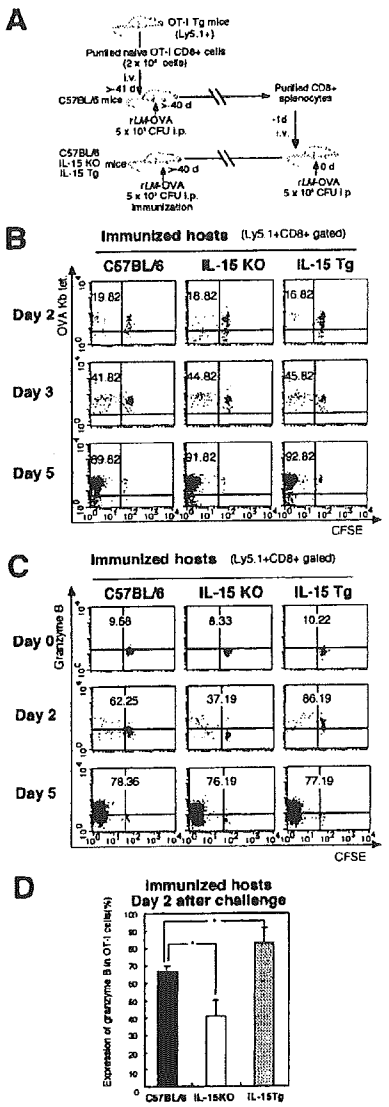

FIGURE 3. IL-15 plays an important role in the induction of cytotoxic molecules of Ag-specific memory CD8$^+$ T cells during secondary immune response in immunized hosts. *A*, CFSE-labeled memory OT-I cells (Ly5.1$^+$) generated in C57BL/6 mice were adoptively transferred i.v. into IL-15 KO, IL-15 Tg,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

occurred in all groups on day 5 after *Listeria* infection. Thus, during secondary *Listeria* infection, there was no obvious difference in the CFSE profiles of memory OT-I cells in IL-15 Tg and IL-15 KO mice compared with those of control mice. These results suggest that not only in the specific situation in naive hosts but also in a physiological situation in immunized hosts, IL-15 is not essential for proliferation of memory CD8$^+$ T cells during secondary infection.

We next analyzed the expression of granzyme B in memory OT-I cells in immunized IL-15 Tg and IL-15 KO hosts after rLM-OVA re-infection. The expression levels of intracellular granzyme B in memory OT-I cells not dividing were significantly lower in IL-15 KO mice but higher in IL-15 Tg mice than those in control mice on day 2 after rLM-OVA reinfection ($p < 0.05$, Fig. 3, *C* and *D*). Intracellular expression levels of granzyme B in the dividing memory OT-I cells in immunized IL-15 Tg and IL-15 KO mice became comparable to those in control mice on day 5 after reinfection. Taken together, these results demonstrate that IL-15 plays an important role in rapid elicitation of effector functions in nondividing memory CD8$^+$ T cells at the early stage after secondary infection.

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

On day 40 after rLM-OVA infection, memory OT-I cells could be generated and maintained in IL-15 KO mice into which naive OT-1 cells had been transferred, albeit at lower degrees than those in control mice (our unpublished data). To test directly whether IL-15 is involved in the induction of granzyme B in memory CD8$^+$ CTL after reinfection, we examined the effects of in vivo administration of rIL-15 on expression of intracellular granzyme B in memory CD8$^+$ T cells after secondary infection in an IL-15-deficient environment. IL-15 KO mice were subjected to transfer of naive OT-I cells followed by rLM-OVA infection, and the IL-15 KO mice were rechallenged 40 days later with a lethal dose of rLM-OVA. rIL-15 was injected i.p. at 0 and 24 h after rechallenge with rLM-OVA (Fig. 4*A*). On day 2 after secondary infection, splenocytes were prepared and intracellular granzyme B staining was performed. As shown in Fig. 4*B*, strong induction of granzyme B was found in memory OT-I CTL from rIL-15-treated IL-15 KO mice compared with that in memory OT-I CTL from PBS-treated IL-15 KO mice after secondary infection. In contrast, there were no significant differences in the proportions of memory OT-I CTL between rIL-15-treated IL-15 KO mice and PBS-treated IL-15 KO mice on day 2 after reinfection (data not shown). Intracellular expression levels of granzyme B in endogenous CD8$^+$ T cells were also significantly increased in rIL-15-treated IL-15 KO mice compared with those in PBS-treated IL-15 KO mice on day 2 after secondary infection. We next measured the in vivo cytolytic activities of memory OT-I cells in rIL-15-treated IL-15 KO mice on day 2 after infection. In vivo CTL activity levels were significantly higher in rIL-15-treated IL-15 KO mice than in PBS-treated IL-15 KO mice after secondary infection (Fig. 4*C*). Moreover, to evaluate antilisterial immunity of memory CTL after treatment with rIL-15, the bacterial growth was determined in the spleen and liver from both rIL-15-treated IL-15 KO mice and PBS-treated IL-15 KO mice after secondary infection. As shown in Fig. 4*D*, both PBS-treated naive IL-15 KO mice and rIL-15-treated naive IL-15 KO mice had high bacterial loads in the spleens and livers on day 2 after infection. In contrast, immunized IL-15 KO mice exhibited antilisterial immunity, as shown by a reduction in bacterial counts in the spleens and livers after rechallenge. Administration of rIL-15 resulted in 10- to 30-fold reduction in bacterial loads in the spleens and livers of immunized IL-15 KO mice compared with

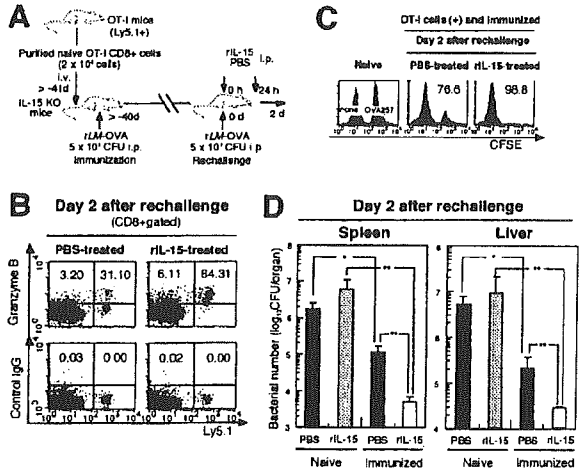

FIGURE 4. In vivo administration of exogenous IL-15 directly induced the expression of cytotoxic effector molecules in memory $CD8^+$ T cells after secondary infection. *A*, Purified naive OT-I cells were adoptively transferred into naive IL-15 KO hosts that were immunized with rLM-OVA 24 h later. At 40 or more days after immunization, IL-15 KO mice harboring memory OT-I cells were rechallenged with a lethal dose of rLM-OVA. rIL-15 (2 μg) or PBS for control was injected i.p. at 0 and 24 h after rechallenge with rLM-OVA. *B*, On day 2 after rechallenge with rLM-OVA, splenocytes from rIL-15-treated and PBS-treated mice harboring memory OT-I cells were prepared and intracellular granzyme B staining was performed. Dot plots are gated on $CD8^+$ cells, and the number indicated is the percentage of donor cells ($Ly5.1^+$) or recipient cells ($Ly5.1^-$) stained positive for granzyme B or the isotype control. Data are representative of three independent experiments using pooled cells from three mice and are shown as typical two-color profiles. *C*, Spleen cells from naive mice ($Ly5.1^+Ly5.2^+$) were pulsed with OVA peptides or left unpulsed and then injected i.v. into rIL-15-treated or PBS-treated IL-15 KO mice rechallenged with rLM-OVA 2 days previously, and then in vivo CTL activity was examined at 5 h after adoptive transfer in target cells. Histograms are gated on $Ly5.1^+Ly5.2^+$ cells in the spleen from infected mice. The values in the *right corner* of each panel represent the percentage of specific killing compared with nonpulsed cells. *D*, The numbers of bacteria in the spleens and livers from rIL-15-treated or PBS-treated IL-15 KO mice harboring memory OT-I cells were determined on day 2 after secondary infection. Data were obtained from three separate experiments, and each value shown is the mean +SD for five mice. *, $p < 0.05$; **, $p < 0.01$.

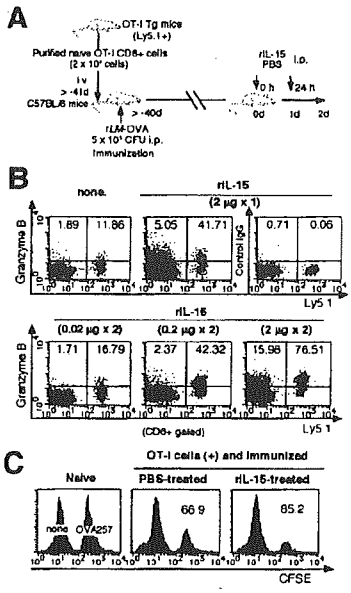

FIGURE 5. In vivo administration of exogenous IL-15 alone can induce the expression of cytotoxic effector molecules and in vivo CTL activities in memory $CD8^+$ T cells. *A*, Purified naive OT-I cells were adoptively transferred into naive C57BL/6 hosts that were immunized with rLM-OVA 24 h later. At 40 or more days after immunization, C57BL/6 mice harboring memory OT-I cells were injected i.p. with various doses of rIL-15 or PBS for control. *B*, At 24 h after administration of various doses of rIL-15 once or twice, mice were sacrificed and expression of intracellular granzyme B was analyzed. Dot plots are gated on $CD8^+$ cells, and the number indicated is the percentage of donor cells ($Ly5.1^+$) or recipient cells ($Ly5.1^-$) stained positive for granzyme B or the isotype control IgG. *C*, C57BL/6 mice harboring memory OT-I cells were injected i.p. with 2 μg rIL-15 or PBS for control. At 24 h after a single injection of rIL-15, spleen cells from naive mice ($Ly5.1^+Ly5.2^+$) were pulsed with OVA peptides or left unpulsed and then injected i.v. into each mouse. Then in vivo killer activity at 5 h after adoptive transfer in targets cells was examined. Histograms are gated on $Ly5.1^+Ly5.2^+$ cells in the spleen. The values in the *right corner* of each panel represent the percentage of specific killing compared with nonpulsed cells.

those in the case of PBS administration. These results suggest that IL-15 plays an important role in the induction of effector functions in Ag-specific memory $CD8^+$ CTL following re-exposure to microbes.

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

Discussion

In the present study, we examined the roles of IL-15 in expansion and activation of Ag-specific naive and memory $CD8^+$ T cells by direct comparison of naive and memory $CD8^+$ T cells that exhibit the same Ag specificity for $OVA_{257-264}/K^b$ in experiments on adoptive transfer into IL-15 Tg mice and IL-15 KO mice after infection with rLM-OVA. The absolute numbers of and the frequencies of division of naive $OVA_{257-264}/K^b$-specific $CD8^+$ T cells in IL-15 Tg mice and IL-15 KO mice were almost the same as those in control C57BL/6 mice after primary infection with

rLM-OVA, confirming that IL-15 is not essential in priming naive CD8$^+$ T cells for expansion and differentiation into effector CTL following microbial infection. In contrast, in vivo CTL activity levels of memory $OVA_{257-264}/K^b$-specific CD8$^+$ T cells were significantly higher in IL-15 Tg mice but lower in IL-15 KO mice at the early stage of secondary immune response, well before the division of memory CD8$^+$ T cells occurred. Moreover, in vivo administration of exogenous IL-15 confers robust protection against reinfection via induction of a cytotoxic molecule in memory CD8$^+$ T cells. These results suggest that IL-15 plays an important role in early activation of Ag-specific memory CD8$^+$ T cells following secondary infection with microbes.

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

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

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

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

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Disclosures

The authors have no financial conflict of interest.

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IL-15 Regulates CD8$^+$ T Cell Contraction during Primary Infection[1]

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During the course of acute infection with an intracellular pathogen, Ag-specific T cells proliferate in the expansion phase, and then most of the T cells die by apoptosis in the following contraction phase, but the few that survive become memory cells and persist for a long period of time. Although IL-15 is known to play an important role in long-term maintenance of memory CD8$^+$ T cells, the potential roles of IL-15 in CD8$^+$ T cell contraction are not known. Using an adoptive transfer system of OT-I cells expressing OVA$_{257-264}$/K^b-specific TCR into control, IL-15 knockout (KO) and IL-15 transgenic (Tg) mice followed by challenge with recombinant *Listeria monocytogenes* expressing OVA, we found that the survival of CD44$^+$CD62L$^-$CD127$^-$ effector OT-I cells during the contraction phase is critically dependent on IL-15. In correlation with the expression level of Bcl-2 in OT-I cells, the number of OT-I cells was markedly reduced in IL-15 KO mice but remained at a high level in IL-15 Tg mice during the contraction phase, compared with control mice. In vivo administration of rIL-15 during the contraction phase in IL-15 KO mice inhibited the contraction of effector OT-I cells accompanied by up-regulation of Bcl-2 expression. Furthermore, enforced expression of Bcl-2 protected the majority of effector OT-I cells from death in IL-15 KO mice after infection. These results suggest that IL-15 plays a critical role in protecting effector CD8$^+$ T cells from apoptosis during the contraction phase following a microbial infection via inducing antiapoptotic molecules. *The Journal of Immunology,* **2006, 176: 507–515.**

Upon encounter with a pathogenic microbe, naive Ag-specific CD8$^+$ T cells proliferate and differentiate into effector CD8$^+$ T cells during the expansion phase. Most of the activated T cells die by apoptosis during the contraction phase, but the few that survive become memory cells and persist for a long period of time (1–3). The size of memory CD8$^+$ T cell pool is dependent on the amounts of surviving T cells from T cell contraction by apoptosis after primary TCR-mediated activation. Therefore, identification of molecular mechanisms responsible for activated T cell death during the contraction phase is important for our understanding how memory develops after infection with microbes. At least two types of cell death can occur in activated T cells during the contraction phase: activation-induced cell death (3), also called Ag-driven apoptosis, and activated T cell autonomous cell death (ACAD)[3], also called growth factor withdrawal-induced apoptosis (4–6). Activation-induced cell death is triggered mostly through cell surface proteins of the TNFR family, including Fas (CD95) (7–9). However, recent studies using Fas/Fas ligand mutant or Fas/TNFRI-deficient mice have demonstrated that neither Fas/Fas ligand nor TNFRI is required for T cell death during the contraction phase (10–14). In contrast, evidence that ACAD is responsible for the death of the majority of activated T cells responding to a foreign Ag has been obtained (10, 11, 14). Several studies have shown that the death of the majority of activated T cells responding to a foreign Ag in vivo can be prevented by enforced expression of Bcl-2, indicating that Bcl-2 up-regulation in effector T cells plays a critical role in preventing activated T cell death by ACAD during the contraction phase (10, 11, 14). Several studies have demonstrated that Bcl-2 expression was induced via signaling from the common cytokine receptor γ (cγ)-chain (15, 16). Gett et al. (17) have reported recently that naive CD8$^+$ T cells receiving prolonged or strong stimulation of TCR can differentiate into effector cells and survive as memory T cells by enhancing IL-15/IL-7 responsiveness. Thus, IL-15 and IL-7 may play a critical role in up-regulation of Bcl-2 and the survival of effector CD8$^+$ T cells during the contraction phase.

IL-15 belongs to the four-helix bundle cytokine family and uses IL-2/-15Rβ- and cγ-chains for signal transduction (18–20) and has the potential roles in maintenance of Ag-specific memory CD8$^+$ T cells during the memory phase via inducing slowly dividing and/or via inducing antiapoptotic molecules such as Bcl-2 without Ag stimulation (21–23). In contrast, potential roles of IL-15 during the expansion and contraction phases after acute infection remained to be controversial. There are several lines of evidence that IL-15 is not mandatory for expansion of effector CD8$^+$ T cells during the expansion phase following acute infection with lymphocytic choriomeningitis virus (LCMV) or *Listeria monocytogenes* (21–23), while IL-15 were reported to play a critical role in CD8$^+$ T cell

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[3] Abbreviations used in this paper: ACAD, activated T cell autonomous cell death; cγ, common cytokine receptor γ; LCMV, lymphocytic choriomeningitis; VSV, vesicular stomatitis virus; KO, knockout; Tg, transgenic; r*LM*-OVA, recombinant *Listeria monocytogenes* expressing OVA; PEC, peritoneal exudate cell; LN, lymph node.

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response to one epitope of LCMV (21) or vesicular stomatitis virus (VSV) during the expansion phase after infection (24).

In the present study, we examined the roles of IL-15 in activated T cell death using a system of adoptive transfer of OT-I cells expressing $OVA_{257-264}/K^b$-specific TCR into naive IL-15 knockout (KO) and IL-15 transgenic (Tg) hosts that were subsequently infected with recombinant *Listeria monocytogenes* expressing OVA (r*LM*-OVA). Our results demonstrate that the survival of effector CD8$^+$ T cells during the contraction phase is critically dependent on IL-15. These findings provide an insight into approach to develop effective vaccination for generation of long-lived CD8$^+$ T cell memory.

Materials and Methods

Mice

C57BL/6-background IL-15 Tg mice, which were constructed using originally described IL-15 cDNA, have been described previously (25). C57BL/6-background IL-15 KO mice were purchased from Taconic Farms. IL-15Rα KO mice were purchased from The Jackson Laboratory and were >10 generations backcrossed to C57BL/6 mice. OT-I mice expressing the $OVA_{257-264}/K^b$-specific TCR and C57BL/6 Ly5.1-congenic mice were also obtained from The Jackson Laboratory. Eμ-bcl-2-25 Tg mice, which express Bcl-2 under the control of the 5′ IgH enhancer (Eμ) in T cells, were described previously (26). Bcl-2 Tg OT-I mice were generated by crossing OT-I (Ly5.1$^+$) mice with the Eμ-bcl-2-25 line. IL-15Rα KO OT-I mice were generated by intercrossing OT-I mice with the IL-15Rα KO mice.

Microorganism

r*LM*-OVA were described previously (27). Bacterial virulence was maintained by serial passages in C57BL/6 mice as described previously (28). Mice were infected i.p. with a sublethal dose of 5×10^5 CFU (~0.1 LD_{50}) or a lethal dose of 5×10^7 CFU (~10 LD_{50}) of r*LM*-OVA in 0.2 ml of PBS on day 0. The spleen and liver were removed from each mouse and separately placed in homogenizers containing 3 ml of HBSS. These samples were spread on trypto-soya agar plates, and colonies were counted after incubation for 24 h at 37°C.

Abs and reagents

FITC-conjugated anti-CD44 (IM7), Ly5.1 (A20), CD62L (MEL-14), and IFN-γ (XMG1.2) mAbs; PE-conjugated anti-Vα2 (B20.1) and CD127 (A7R34) mAbs; CyChrome-conjugated anti-CD8α (53-6.7) mAb and streptavidin; allophycocyanin-conjugated streptavidin; and biotin-conjugated anti-Ly5.1 (A20) and Ly5.2 (104) mAbs were purchased from eBioscience. FITC-conjugated hamster anti-mouse Bcl-2 mAb (3F11) and its isotype FITC-conjugated control Ab to hamster were also obtained from BD Biosciences. FITC-conjugated anti-active caspase-3 mAb were also obtained from BD Biosciences. CFSE was purchased from Molecular Probes. $OVA_{257-264}$ H-2K^b tetramers were purchased from MBL.

Flow cytometry analysis

Splenocytes were preincubated with a culture supernatant from 2.4 G2 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. In some experiments, splenocytes were subjected to intracellular staining using a Fast Immune Cytokine System according to the instructions of the manufacturer (BD Biosciences), and the fluorescence of the cells was analyzed using a flow cytometer.

Adoptive transfer

OT-I chimeras were generated by adoptive transfer of $1–2 \times 10^6$ naive OT-I cells into naive mice followed by r*LM*-OVA infection. Transferred OT-I cells were identified by staining with an mAb to Ly5.1, CD8, and Vα2 or $OVA_{257-264}$ K^b tetramer. In some experiments, a mixture containing an equal number of OT-I cells (Ly5.1$^+$Ly5.2$^-$) and Bcl-2 Tg OT-I cells (Ly5.1$^+$Ly5.2$^+$) from the spleen was injected i.v. into IL-15 KO hosts (Ly5.1$^-$Ly5.2$^+$) that were subsequently infected with r*LM*-OVA 24 h later. The mixtures were analyzed to determine the actual ratio of each subset of donor cells by staining with Ly5.1, Ly5.2, Vα2, and CD8 mAbs.

Analysis of T cell proliferation

Effector OT-I cells were labeled with CFSE as described previously (28). Proliferation of transferred OT-I cells was visualized by flow cytometry analysis of their CFSE profile.

Statistical analysis

The significance of all data was determined by Student's *t* test. A value of $p < 0.05$ was taken as the level of significance. Analysis was conducted using Stat-View 5.0 software (Abacus Concepts).

Results

*Expansion and contraction of OT-I cells in IL-15 KO or IL-15 Tg mice in primary response to r*LM*-OVA*

To determine the potential roles of IL-15 in CD8$^+$ T cell contraction, we examined the fate of OT-I cells that were transferred into IL-15 Tg mice, IL-15 KO mice, or control mice followed by challenge with r*LM*-OVA. We found that the bacterial number increased to a maximal level on day 3 in the spleen and thereafter cleared completely by day 7 after infection in all groups. There were no differences in the bacterial numbers in the spleen between control ($1.6 \times 10^2 \pm 1.3$ CFU, $n = 6$) and IL-15 KO mice ($1.8 \times 10^2 \pm 1.7$ CFU, $n = 6$) or IL-15Rα KO mice ($1.3 \times 10^2 \pm 1.5$ CFU, $n = 5$) on day 6 after infection, whereas the number was significantly lower at the peak level in IL-15 Tg mice ($8.2 \times 10^5 \pm 2.3$ CFU in control mice and $0.9 \times 10^5 \pm 0.6$ CFU in IL-15 Tg mice, $n = 6$). Consistent with our previous findings (28), the absolute numbers of OT-I cells in the spleen of IL-15 KO and IL-15 Tg mice were almost the same as those in control mice on days 5 and 7 after primary infection (Fig. 1*A*). In contrast, the number of OT-I cells was markedly reduced in IL-15 KO mice but remained at a high level in IL-15 Tg mice on days 10 and 14 after infection (Fig. 1, *A* and *B*). The absolute numbers of OT-I cells in the peritoneal exudate cells (PEC) and peripheral lymph nodes (LN) were also decreased in IL-15 KO mice but remained stable in IL-15 Tg mice during the contraction phase (data not shown). These results suggest that IL-15 plays an important role in maintaining numbers of Ag-specific CD8$^+$ T cells during the contraction phase of immune response.

*IL-7Rα and CD62L expression on OT-I cells during the contraction phase after r*LM*-OVA infection*

Recent studies have demonstrated that the surface expression of IL-7R α-chain as a memory T cell marker enables effector T cells to be distinguished from memory T cells at peak time points of primary immune responses (29). Furthermore, Huster et al. (30) reported that a combination of surface staining for CD127 (IL-7Rα) and CD62L enables separation of two functionally distinct memory cell subsets, which are similar to cell subsets recently described as central memory cell subsets (CD127^{high} and CD62L^{high}) and effector memory cell subsets (CD127^{high} and CD62L^{low}). To determine the requirement of IL-15 for the survival of effector cells or memory cell subsets during the contraction phase, we examined the kinetics of IL-7Rα and CD62L expression on OT-I cells transferred into IL-15 KO mice, IL-15 Tg mice, or control mice after r*LM*-OVA infection. As shown in Fig. 1*C*, proportions of IL-7Rα^{low} OT-I cells in IL-15 Tg mice and IL-15 KO mice were comparable to those in control mice on day 5 after infection, indicating that IL-15 did not affect the generation of effector CD8$^+$ T cells during the expansion phase of primary response. On day 10 after infection, the number of IL-7Rα^{low} OT-I cells was significantly greater in IL-15 Tg mice (Fig. 1, *C* and *D*). In contrast, IL-7Rα^{low} OT-I cells had almost completely vanished in IL-15 KO mice on day 10 after infection, indicating that IL-15

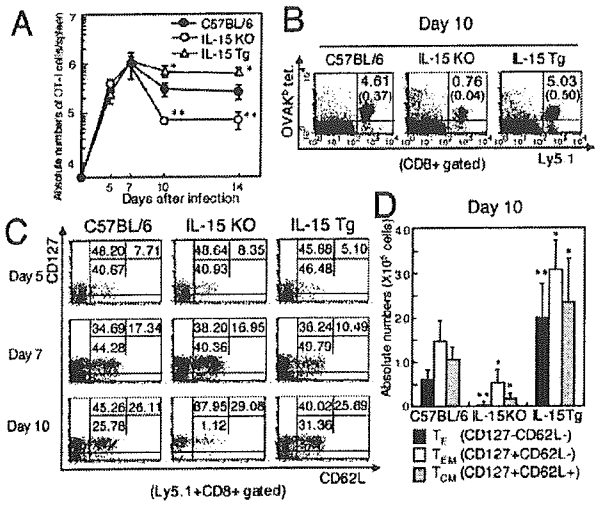

FIGURE 1. IL-15 is essential for the survival of effector $CD8^+$ T cells during the contraction phase after r*LM*-OVA infection. Purified naive OT-I cells ($Ly5.1^+$) were adoptively transferred i.v. into naive IL-15 KO mice, IL-15 Tg mice, or control C57BL/6 mice ($Ly5.2^+$) that were infected with r*LM*-OVA 24 h later. *A*, On days 5, 7, 10, and 14 after infection, total number of OT-I cells was calculated by multiplying the total number of spleen cells by the percentage of $V\alpha2^+Ly5.1^+CD8^+$ cells in the spleen. Data are presented as means ± SDs for five mice. *, $p < 0.05$; **, $p < 0.01$. *B*, Proportions of OT-I cells in the spleens in recipient mice on day 10 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). *C*, On days 5, 7, and 10 after r*LM*-OVA infection, double staining of activated OT-I cells for surface expression of CD62L and CD127 was performed. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on $Ly5.1^+CD8^+$ cells. *D*, Absolute number of effector cells (T_E), effector memory cells (T_{EM}), or central memory cells (T_{CM}) of OT-I cells from IL-15 KO mice, IL-15 Tg mice, and C57BL/6 mice on day 10 after infection was calculated. Data were obtained from three separate experiments, and each value shown is the mean ± SD for three experiments. *, $p < 0.05$; **, $p < 0.01$.

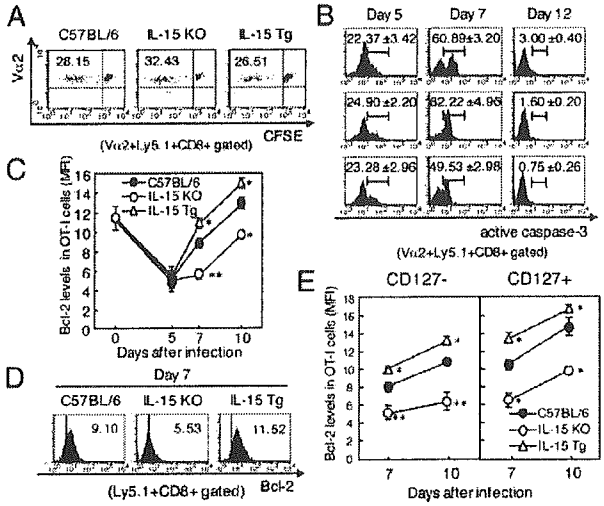

FIGURE 2. IL-15 plays a critical role in the survival but not in the proliferation of activated T cells during the contraction phase after r*LM*-OVA infection. *A*, CFSE-labeled effector OT-I cells from C57BL/6 mice on day 6 after infection were adoptively transferred into IL-15 KO mice, IL-15 Tg mice, or C57BL/6 mice that had been infected with r*LM*-OVA 6 days previously. Recipients receiving effector OT-I cells were analyzed 4 days later. Data are shown as the percentage of OT-I cells ($V\alpha2^+Ly5.1^+CD8^+$) that have undergone one or more proliferations and are representative of three independent experiments, each containing three to four mice per group. *B*, Apoptosis of ex vivo-cultured (24 h) OT-I cells from IL-15 KO mice, IL-15 Tg mice, and C57BL/6 mice on days 5, 7, and 12 after infection was determined by intracellular active caspase-3 staining. An analysis gate was set on $V\alpha2^+Ly5.1^+CD8^+$ cells. Data are presented as means ± SDs for three mice at indicated days after infection. *C*, Changes in mean fluorescence intensity (MFI) of Bcl-2 staining in OT-I cells from IL-15 KO mice, IL-15 Tg mice, and control mice as indicated after r*LM*-OVA infection are shown. Data are presented as means ± SDs for five mice at indicated days after infection. *, $p < 0.05$; **, $p < 0.01$. *D*, Representative flow cytometric histograms showing the intracellular expression of Bcl-2 in OT-I cells from IL-15 KO mice, IL-15 Tg mice, and control mice on day 7 after infection. Histograms are gated on $Ly5.1^+CD8^+$ cells in spleens from infected mice. The values in the *right corner* of each panel represent the MFI of Bcl-2 expression in OT-I cells (vertical line, negative control). *E*, Changes in MFI of Bcl-2 staining in $CD127^-$ and $CD127^+$ OT-I cells from IL-15 KO mice, IL-15 Tg mice, and control mice on days 7 and 10 after r*LM*-OVA infection. *, $p < 0.05$; **, $p < 0.01$.

is crucial in preventing effector $CD8^+$ T cells from apoptosis during the contraction phase. The numbers of IL-$7R\alpha^{high}CD62L^{low}$ and IL-$7R\alpha^{high}CD62L^{high}$ memory OT-I cells were also decreased in IL-15 KO mice but significantly increased in IL-15 Tg mice on day 10 after *Listeria* infection (Fig. 1*D*). Thus, these results suggest that IL-15 plays an important role in maintaining numbers of not only effector cells but also memory cell subsets during the contraction phase after *Listeria* infection.

Proliferation and survival of OT-I cells during the contraction phase after rLM-*OVA infection*

The number of Ag-specific $CD8^+$ T cells is regulated by a balance among cell survival, apoptosis, and proliferation. To determine whether endogenous IL-15 induces proliferation of OT-I cells during the contraction phase, we transferred CFSE-labeled OT-I cells ($Ly5.1^+$) from r*LM*-OVA-infected normal mice (6 days after infection) into infected IL-15 KO mice, IL-15 Tg mice, or control mice (6 days after infection) and then analyzed the proliferation of OT-I cells on day 4 after transfer. Although the number of effector OT-I cells recovered from the spleen was smaller in IL-15 KO hosts but larger in IL-15 Tg hosts than in control hosts, the effector OT-I cells proliferated equally in all groups by day 4 after adoptive transfer (Fig. 2*A* and data not shown). Similarly, when the proliferation of OT-I cells was measured by inoculation of BrdU 4 days during the contraction phase after *Listeria* infection, no differences were observed in all groups by day 12 after infection (data not shown). These results indicate that endogenous IL-15 is dispensable in the proliferation of effector T cells during the contraction phase.

To determine whether IL-15 is involved in the survival of effector $CD8^+$ T cells, we next examined the apoptotic potential of OT-I cells from IL-15 KO, IL-15 Tg, and control mice on days 5, 7 and 12 after r*LM*-OVA infection. On day 7 after infection, the expression level of active caspase-3 in OT-I cells was significantly higher in IL-15 KO mice but lower in IL-15 Tg mice than in control mice after 24 h after of culture (Fig. 2*B*), indicating that IL-15 may protect the effector $CD8^+$ T cells from apoptosis. We examined the intracellular expression levels of Bcl-2 in OT-I cells from these mice during the expansion phase and contraction phase. As shown in Fig. 2*C*, the levels of the antiapoptotic protein Bcl-2 in effector OT-I cells from control mice were rapidly down-regulated, compared with those in naive OT-I cells on day 5 after *Listeria* infection, and Bcl-2 expression was up-regulated gradually during the contraction phase from day 5 to 10 after infection.