Protection against *Mycobacterium tuberculosis* not only depends on CD4+ T helper type 1 (Th1) cells but also, on CD8+ T cells. Interleukin (IL)–15 has an important function in the maintenance of memory CD8+ T cells. In the present study, we examined the efficacy of recombinant *Mycobacterium bovis* bacille Calmette-Guérin (rBCG) secreting fusion protein antigen (Ag) 85B murine IL-15 (rBCG-Ag85B-IL15) in providing protection against *M. tuberculosis* infection. The levels of major histocompatibility (MHC) class I b (H2-M3)–binding TB2– or MHC class I a (H-2Dβ)–binding MPT64-specific CD8+ T cells producing interferon (IFN)–γ were significantly higher after immunization with rBCG-Ag85B-IL15 than after immunization with rBCG secreting Ag85B (rBCG-Ag85B). The levels of purified protein derivative– or Ag85B-specific CD4+ T cells producing IFN–γ were also higher in mice immunized with rBCG-Ag85B-IL15 than in mice immunized with rBCG-Ag85B. Mice immunized with rBCG-Ag85B-IL15 exhibited CD8+ and CD4+ T cells responses that were stronger than those in mice immunized with rBCG-Ag85B, as well as robust protection in the lung against intratracheal challenge of *M. tuberculosis*. Thus, rBCG-Ag85B-IL15 vaccination capable of inducing efficient cell-mediated immunity might be used as an effective vaccine for tuberculosis.

Approximately one-third of the world population has been latently infected with *Mycobacterium tuberculosis* [1]. Although *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine efficiently protects children against the early manifestations of tuberculosis (TB) [2, 3], especially meningeal TB [4], it confers incomplete protection against TB in adults, because BCG is not effective for inducing long-term cellular immunity [5]. Therefore, it is urgently required that improved vaccines for TB be developed to take the place of BCG [6]. Several strategies have been adopted to develop recombinant BCG (rBCG) for protection against TB. One strategy involves the development of an rBCG strain that produces the immunodominant or *M. tuberculosis*–specific antigen (Ag), such as Ag85 complex, 38 kDa, or region of difference–1 (RD-1), which showed enhanced protective efficacy [7, 8]. Another strategy involves the development of an rBCG strain producing cytokine, such as interleukin (IL)–2, IL-18, or interferon (IFN)–γ, which is reported to up-regulate T helper type 1 (Th1)–type immunity in vitro [9–12]. However, there are few reports indicating that rBCG expressing cytokine provided stronger protection against TB.

Although protection against infection by *M. tuberculosis* depends mainly on CD4+ Th1 cells, there are substantial lines of evidence that CD8+ T cells play a requi-
A recent study suggested that CD8/H11001 T cells are more important than CD4/H11001 T cells in controlling TB in the latent phase [15, 16]. We recently reported that vaccination of bone marrow–derived dendritic cells pulsed with H2-M3–binding peptide TB2 [17] or with major histocompatibility class (MHC) class Ia (H-2Db)–binding peptide MPT64190–198 [18] elicited Ag-specific CD8/H11001 T cells, leading to protection against intratracheal infection with M. tuberculosis [19]. Thus, several vaccination strategies can be determined to induce protective memory CD8/H11001 T cells.

IL-15 has an important function in the proliferation and survival of memory-phenotype CD8/H11001 T cells [20, 21]. We previously found that IL-15 transgenic mice expressing IL-15 cDNA encoding a secretable isoform showed enhanced protection against infection with M. bovis BCG via activation of NK and CD8/H11001 T cells [22–24]. We recently showed, with the use of IL-15−/− mice, that IL-15 plays an important role in the development of protective immunity to BCG infection by sustaining CD8/H11001 T cell responses in lung. In the present study, we constructed an rBCG strain expressing the fusion protein of IL-15 and Ag85B (rBCG-Ag85B-IL15) and examined its efficacy as a vaccine against M. tuberculosis infection.

**MATERIALS AND METHODS**

**Mice.** C57BL/6 mice (age, 6–8 weeks) were obtained from Charles River Japan. Mice were maintained under specific pathogen-free conditions and were offered food and water ad libitum. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences [25].

**Construction of expression plasmid.** The construction of the plasmid used for expression of murine IL-15 (mIL-15) is shown in figure 1A. After the mIL-15 sequence was confirmed by DNA sequencing, the DNA fragment was cloned into the pKH20 [26] XhoI sites located in the Ag85B region [27]. pKH20 is the vector in pUC18 that contains the Ag85B gene. The DNA fragment of Ag85B and IL-15 were digested with XhoI and HindIII and inserted into the pNN2 BamHI/HindIII site. This vector is the Escherichia coli–mycobacteria shuttle vector that possesses a kanamycin resistance gene as a selection marker [28].

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**Figure 1.** Construction of pNN2 murine interleukin (mIL-15) (A) and detection of antigen (Ag) 85B–interleukin-15 (IL-15) in the lysate of rBCG-Ag85B-IL15 (recombinant bacille Calmette-Guérin [rBCG] secreting Ag85B-fused murine IL-15) by Western blot analysis (B). The pellet of rBCG-Ag85B-IL15, rBCG secreting Ag85B (rBCG-Ag85B), or bacille Calmette-Guérin (BCG)–plasmid vector was suspended in lysis buffer for 30 min, or the bacteria were cultured in 7H9 medium for 1 week. After centrifugation, the supernatants were harvested. After protein quantitation of the supernatants was performed, samples were applied to SDS-PAGE and then were blotted and reacted with anti–IL-15 antibody or anti-Ag85B serum.
mIL15-Ag85B was then introduced into BCG (Tokyo 172 strain) by electroporation. The transformed BCG was plated on Middlebrook 7H10 agar supplemented with 10% oleic acid dextrose catalase, 20 μg/mL kanamycin, 100 U/mL penicillin G, and 100 μg/mL cycloheximide. After growing for 3 weeks at 37°C, some single colonies were picked up and grown in Sauton’s medium for 3 weeks at 37°C.

**Microorganisms and infection.** rBCG-Ag85B-IL15, rBCG secreting Ag85B (rBCG-Ag85B), rBCG only with plasmid vector (BCG–plasmid vector), or M. tuberculosis H37Rv was cultured and stored using the same method, with use of the BCG Tokyo strain previously described [29]. Naïve mice were inoculated intraperitoneally with 5 × 10⁶ cfu of rBCG-Ag85B-IL15 or rBCG-Ag85B. On day 90 after inoculation, the mice were treated for 4 weeks with isoniazid (0.1 g/L) added to drinking water, to clear live BCG bacteria. Then, on day 127, the mice were intratracheally infected with 2 × 10⁷ cfu of M. tuberculosis H37Rv. Infections were performed in groups of ≥4 mice in 1 experiment, and each data point represents the mean value for 3–5 infected mice after ≥3 independent experiments.

**Detection of Ag85B-mIL15 fusion protein.** rBCG-Ag85B-IL15, rBCG-Ag85B, or BCG–plasmid vector was lysed in lysis buffer for 30 min or cultured in 7H9 medium for 1 week. After centrifugation, supernatants were harvested, and quantitation of protein in the supernatants was performed using Ultraspec 3300 pro (Amersham Biosciences). Then, samples reconstituted to the same dose of protein were applied to SDS-PAGE. After naive PAGE was performed, proteins were blotted and reacted with anti–IL-15 antibodies (Abs) or anti-Ag85B serum.

**Abs and reagents.** Fluorescein isothiocyanate–conjugated anti-CD3ε (145–2C11), anti-IFN-γ (XMGI1.2); phycoerythrin-conjugated anti-CD8α (53–6.7), anti-CD62L (MEL-14); peridinin chlorophyll protein–cytochrome 5.5–labeled anti-CD4 (RM4–5), CD8α (53–6.7); and allophyocyanin-conjugated anti-CD44 (IM7) monoclonal antibodies (MAbs) were purchased from BD Pharmingen. H2-D^d-binding peptide MPT64190–198 (FAVTNDGVI) [18], H2-M3–binding peptide TB2 (f-MLVLLV) [17], and I-A^d–binding peptide Peptide-25 at 37°C for 48 h in the presence of mitomycin C–treated splenocytes (1 × 10⁶ cells/well) obtained from naive C57BL/6 mice. Culture supernatants were collected, and concentrations of IFN-γ in supernatants were measured using ELISA kits (Genzyme Diagnostics).

**Histologic examination.** The left upper lobes of lungs were preserved in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Samples from 4 mice per group were examined.

**Statistical analysis.** The statistical significance of the data was determined by Student’s t test. P < .05 was considered to be statistically significant.

**RESULTS**

**Detection of Ag85B-IL15 fusion protein from rBCG-Ag85B-IL15.** To detect the fusion protein of Ag85B-IL15, lysates or culture supernatants of rBCG-Ag85B-IL15, rBCG-Ag85B, or BCG–plasmid vector were blotted and reacted with anti–IL-15 Abs or anti-Ag85B serum. As shown in figure 1B, the fusion protein, which reacted with anti–IL-15 Abs and anti-Ag85B serum, was detected at ~40 kDa in both bacterial lysate and culture supernatant of the rBCG-Ag85B-IL15 lane but in neither the rBCG-Ag85B nor the BCG–plasmid vector lane, in which only 30 kDa corresponding to Ag85B were detected. Thus, Ag85B plus IL-15 fusion protein was confirmed to be existent in rBCG-Ag85B-IL15.

**Bacterial growth in mice after immunization with rBCG-Ag85B-IL15.** We first examined the kinetics of in vivo bacterial growth in the spleen, lungs, and PECs of C57BL/6 mice after...
intraperitoneal immunization with rBCG-Ag85B-IL15 or rBCG-Ag85B. The numbers of bacteria in PECs and lungs were significant lower on day 21 after rBCG-Ag85B-IL15 inoculation than after rBCG-Ag85B inoculation (P < .05), and the number of bacteria in spleen was significantly lower on days 42 and 70 after rBCG-Ag85B-IL15 immunization (P < .01) (figure 2). The difference in bacterial growth may not be due to a difference in duplication ability between rBCG-Ag85B-IL15 and rBCG-Ag85B, because both types of rBCG grew equally in vitro. This observation suggests that the cell-mediated immune responses induced by rBCG-Ag85B-IL15 in vivo might be stronger than those induced by rBCG-Ag85B.

**T cell populations in mice after immunization with rBCG-Ag85B-IL15.** We next examined the kinetics of T cell subpopulations in the spleen, PECs, and lungs of mice after rBCG-Ag85B-IL15 immunization. The numbers of CD44^+CD8^+ T cells in the PECs and lungs of mice immunized with rBCG-Ag85B-IL15 on day 21 increased to nearly twice the number of such cells noted in mice immunized with rBCG-Ag85B (P < .05) (figure 3). The numbers of bacteria in the spleen and lungs also significantly increased on day 70 after immunization with rBCG-Ag85B-IL15 (P < .01 or P < .05). Surprisingly, the levels of CD44^+CD4^+ T cells in the spleen and lungs of mice immunized with rBCG-Ag85B-IL15 were also significantly higher than such levels in mice immunized with rBCG-Ag85B, during the course of immunization (P < .05). Thus, in both lymphoid and nonlymphoid organs, vaccination with rBCG-Ag85B-IL15 induced higher levels of CD44^+CD8^+ and CD44^+CD4^+ T cells than did rBCG-Ag85B, during the course of immunization.

**IFN-γ production by Ag-specific T cells after immunization with rBCG-Ag85B-IL15.** To investigate Ag-specific CD8^+ and CD4^+ T cell responses in the spleen, PECs, and lungs of C57BL/6 mice immunized with rBCG-Ag85B-IL15, we used cytokine fluorescence-activated cell sorter (FACS) analysis for intracellular IFN-γ after stimulation with mycobacterial Ag. The CD8^+ and CD4^+ T cells capable of producing IFN-γ in response to purified protein derivative (PPD) were detected in these organs on day 21 after rBCG immunization, and the levels of T cells producing IFN-γ in the CD4^+ and CD8^+ populations were significantly higher in the PECs and lungs of mice immunized with rBCG-Ag85B-IL15 than in those of mice immunized with rBCG-Ag85B (figure 4A) (P < .05). The levels of IFN-γ-producing CD8^+ T cells generated in response to MPT64 or TB2, as well as the levels of IFN-γ-producing CD4^+ T cells generated in response to Peptide-25 in the spleens of mice immunized with rBCG-Ag85B-IL15 were significantly higher than those noted in mice immunized with rBCG-Ag85B on day 21 after immunization (P < .05) (figure 4B). These results suggested that rBCG-Ag85B-IL15 vaccination could induce greater levels of Ag-specific IFN-γ-producing CD8^+ and CD4^+ T cell responses than could rBCG-Ag85B vaccination.
rBCG infection (figure 5A). The numbers of PPD-specific IFN-γ+CD44+CD8+ T cells in the lungs and TB2-specific IFN-γ+CD44+CD8+ T cells in the spleen were significantly greater in mice immunized with rBCG-Ag85B-IL15 than in mice immunized with rBCG-Ag85B (P < .05). Numbers of PPD- or Peptide-25–specific IFN-γ+CD44+CD4+ T cells in the spleens or lungs were also higher in mice immunized with rBCG-Ag85B-IL15 than in mice immunized with rBCG-Ag85B (P < .05 or P < .01). To further confirm the levels of T cell responses at this stage, we isolated CD8+ or CD4+ T cells from the spleens of rBCG-immunized mice and cultured them with TB2 or Peptide-25 in the presence of antigen-presenting cells, and culture supernatants were examined by ELISA for IFN-γ release. As shown in figure 5B, the levels of IFN-γ production by CD8+ or CD4+ T cells in mice immunized with rBCG-Ag85B-IL15 were significantly higher than those in mice immunized with rBCG-Ag85B (P < .05). Taken together, these data suggest that rBCG-Ag85B-IL15 vaccination enhanced not only the ex-

**Figure 3.** Kinetics of absolute numbers of T cell subsets in mice after immunization with rBCG-Ag85B-IL15 (recombinant bacille Calmette-Guérin [rBCG] secreting antigen [Ag] 85B–fused murine interleukin-15 [IL-15]). The cells in the spleen, peritoneal exudate cells (PECs), and lungs on days 0, 7, 21, 42, or 70 after immunization with rBCG-Ag85B-IL15 or rBCG secreting Ag85B (rBCG-Ag85B) were stained with various monoclonal antibodies, and the absolute numbers of CD44+CD8+ and CD44+CD4+ T cells were calculated by multiplying the total number of splenocytes, PECs, or lung mononuclear cells by the percentages of each subset in the spleen, PECs, or lungs. Data are representative of 3 separate experiments and are expressed as the mean value (±SD) for 3 mice per group. *, P < .05; **, P < .01.
pansion but, also, the maintenance of Ag-specific CD8\(^+\) and CD4\(^+\) T cells.

Protection against \textit{M. tuberculosis} infection in mice immunized with rBCG-Ag85B-IL15. To investigate the efficacy of rBCG-Ag85B-IL15 vaccination in providing protection against \textit{M. tuberculosis} infection, mice were challenged intratracheally with \textit{M. tuberculosis} H37Rv on day 127 after rBCG-Ag85B or rBCG-Ag85B-IL15 immunization. The Ag-specific IFN-\(\gamma\)-pro-
Producing CD8\(^+\) or CD4\(^+\) T cells were examined by cytokine FACS on day 28 after the challenge, at which time *M. tuberculosis*-specific T cells reached their peak of expansion in mice. As shown in table 1, the larger numbers of CD8\(^+\) T cells in the lungs of mice immunized with rBCG-Ag85B-IL15, compared with the numbers of such cells in the lungs of mice immunized with rBCG-Ag85B or in nonimmunized mice, produced IFN-\(\gamma\) in response to PPD (\(P < .05\)). The number of TB2- or MPT64-specific CD8\(^+\) T cells in the lungs or spleen of mice immunized with rBCG-Ag85B-IL15 also increased (\(P < .05\)). In MPT64-
or TB2-specific IFN-γ+CD8+ T cells, the numbers of CD4+CD62L+CD8+ T cells corresponding to central memory T cells were higher in mice immunized with rBCG-Ag85B-IL15 than in mice immunized with rBCG-Ag85B or in nonimmunized mice (P < .05). The number of PPD-specific IFN-γ-producing CD4+ T cells was markedly increased in mice immunized with rBCG-Ag85B-IL15 (P < .05). The number of Peptide-25–specific IFN-γ-producing CD4+ or CD4+CD62L+IFN-γ+CD4+ T cells in mice immunized with rBCG-Ag85B-IL15 was also significantly greater than that in mice immunized with rBCG-Ag85B (P < .05). The number of CD4+ T cells producing IFN-γ in nonimmunized mice was sometimes greater than the number of such cells observed in immunized mice, which may be the result of a 10-fold greater bacterial burden in the organs of nonimmunized mice (data not shown). These data indicate that memory CD8+ and CD4+ T cells from mice immunized with rBCG-Ag85B-IL15 reexpanded to greater levels than did those from mice immunized with rBCG-Ag85B after challenge with M. tuberculosis.

We then examined the bacterial burden in mice immunized with rBCG-Ag85B-IL15 or rBCG-Ag85B 10 weeks after infection with M. tuberculosis. As shown in figure 6A, the bacterial counts in the lungs were significantly lower in mice immunized with rBCG-Ag85B-IL15 than in mice immunized with rBCG-Ag85B (P < .05). Nonimmunized mice had bacterial counts that were 10-fold higher than those in mice immunized with rBCG.

Histologic images (magnification, ×50 and ×200) showed that, in nonimmunized mice, little lymphoid infiltration was found, and the whole pulmonary lobe was filled with loosely structured, loose granulomas. In both rBCG-Ag85B– and rBCG-Ag85B-IL15–immunized mice, a great deal of lymphoid follicle formations and highly structured, compact granulomas was found. However, the whole lungs of mice immunized with rBCG-Ag85B were also full of granulomas, whereas histopathologic changes were fewer and were very localized in the lungs of mice immunized with rBCG-Ag85B-IL15 (figure 6B).

**DISCUSSION**

In the present study, we constructed rBCG secreting murine IL-15 and studied its biological functions in vivo as a vaccine to
protect against M. tuberculosis. We showed the first evidence that rBCG-Ag85B-IL15 vaccination generated long-lived memory CD8+ and CD4+ T cells and conferred strong protection against M. tuberculosis in the lung.

IL-15 was reported to have an important function in the proliferation and survival of memory CD8+ T cells [31, 32], and we recently demonstrated that IL-15 played an important role in preventing apoptosis of effector CD8+ T cells during the con-
traction phase after infection with intracellular bacteria, such as *Listeria monocytogenes* [33]. In the present study, we found that mice immunized with rBCG-Ag85B-IL15 showed more CD4+ CD8+ T cells and higher frequencies of Ag-specific IFN-γ–producing CD8+ T cells on day 21, when expansion of effector T cells reaches the peak, and on day 70, when the T cells go into the memory phase. These results suggest that IL-15 plays important roles in the generation of long-lived CD8+ T cells and in maintaining memory CD8+ T cells after immunization with rBCG-Ag85B-IL15.

Ag85B, a fibronectin-binding protein [34], has an immunopotentiating effect by inducing T cell proliferation and synthesis of IFN-γ [35] and TNF-α [36]. Recent studies showed that, by increasing the number of CD4+ T cells producing IFN-γ, rBCG expressing Ag85B could significantly augment a Th1 response more than could the original BCG strain [37, 38]. Surprisingly, we found that not only the number of CD44+CD8+ T cells but, also, the number of CD44+CD8+ T cells was significantly increased in mice immunized with rBCG-Ag85B-IL15, compared with mice immunized with rBCG-Ag85B. The levels of mycobacterial Ag-specific CD4+ T cells in mice immunized with rBCG-Ag85B-IL15 were significantly higher on day 21, and these higher frequencies were also maintained 70 days after immunization. This notable finding is discrepant with the notion that IL-15 is irrelevant for the homeostatic proliferation of memory CD4+ T cells [39–41], and Ag-specific CD4+ T cells appear to be mainly controlled by IL-7 [42, 43]. CD4+ T cell counts in the range considered to be normal are present in naïve IL-15−/− mice [31] and after infection of the BCG Tokyo strain [29].

On the other hand, there are several lines of evidence that IL-15 plays an important role in the homeostatic proliferation of Ag-specific memory CD4+ T cells. IL-15 treatment promoted the proliferation of human memory CD4+ T cells in vitro and mouse Ag-specific memory CD4+ T cells in vivo [42, 44]. Recently, in their study of lymphocytic choriomeningitis virus (LCMV)–specific T cell–receptor transgenic mice, Purton et al. [45] reported that, under normal physiologic conditions, IL-15 was crucial for both survival and homeostatic proliferation than were analogous cells produced in wild-type mice [45, 46]. Thus, the discrepancy in the role of IL-15 in memory CD4+ T cells may be explained by a different nature of CD4+ T cells generated de novo in IL-15−/− mice and wild-type mice. CD4+ T cells generated de novo in IL-15−/− mice may become permanently conditioned to cope with IL-15 deficiency, and they appear to have found an alternative way to sustain their homeostasis in the absence of IL-15. In the present study, our results with normal CD4+ T cells from C56BL/6 mice may reflect the role of IL-15 in the generation and maintenance of memory CD4+ T cells under physiological conditions.

Saito et al. [29] recently showed, in IL-15−/− mice, that IL-15 plays an important role in the development of long-lasting protective immunity to BCG infection by sustaining CD8+ T cell responses in the lungs. In the present study, 10 weeks after *M. tuberculosis* infection, the bacterial burden in the lungs of mice immunized with rBCG-Ag85B-IL15 was significantly lower than that in mice immunized with rBCG-Ag85B. Lazarevic et al. [47] recently reported that a significant increase in the bacterial count was seen in the lungs of IL-15−/− mice at 12 weeks but not at 15 weeks after aerosol infection with a very low dose (<30 cfu/mouse) of *M. tuberculosis* (Erdman strain). Rausch et al. [48] showed that IL-15 was critical for the development of full-blown protective immunity to experimental *M. tuberculosis* infection in mice, because (1) the lack of IL-15 was associated with a dramatic reduction in the numbers and function of CD8+ T cells and (2), in IL-15−/− mice, the bacterial burden in the lung on day 42 or 105 after aerosol infection with *M. tuberculosis* (H37Rv strain; 300 cfu/mouse) was 7–8 times higher than that noted in control mice. Thus, although the efficacy of IL-15 in providing protection against mycobacterial infection varied depending on the initial bacterial load and the virulence of *M. tuberculosis*, IL-15 may play a role in providing protection against *M. tuberculosis* infection.

Using administration of recombinant IL-15, Lazarevic et al. [47] could not find a significantly lower bacterial burden or increased numbers of CD8+ or CD4+ T cells in mice infected with *M. tuberculosis*, compared with mice that were not administered IL-15. In contrast, in our study, IL-15 secreted by rBCG was demonstrated to be markedly helpful both in clearance of bacteria and in creating increases in the number of CD8+ T cells. This comparison indicates that endogenously secreted IL-15 from rBCG is a reasonable method for the generation of T cell immunologic memory induced by BCG, because continuous release of IL-15 is an important factor for success in memory T cell maintenance, whereas cytokine activities are rapidly reduced when cytokines alone are administrated in the host.

It is widely accepted that protection against infection with *M. tuberculosis* depends on both CD4+ Th1 and CD8+ T cytotoxic 1 cells [49, 50]. The success of rBCG-Ag85B-IL15 as a vaccine is associated with the finding that, with the help of IL-15 plus Ag85B protein as adjuvant, BCG can generate high levels of Ag-specific memory CD8+ T cells, as well as CD4+ T cells, which conferred much stronger protection against *M. tuberculosis* challenge in the lung. The abundant generation of long-lived memory CD8+ and CD4+T cells after BCG immunization, the marked production of IFN-γ from T cells, and the lower bacterial burden in the lungs of immunized mice, compared with that noted in the lungs of nonimmunized mice, after *M. tuberculosis* challenge indicate that IL-15 does
play an important role in the proliferation and maintenance of memory T cells and that it can be used as an immune adjuvant to increase the efficacy of BCG vaccination.

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References


40. Robertson JM, MacLeod M, Marsden VS, Kappler JW, Marrack P. Not all CD4⁺ memory T cells are long lived. Immunol Rev 2006; 211:49–57.


