Introduction

About one third of the world population has been latently infected with *Mycobacterium tuberculosis* [1]. Although BCG vaccine protects children efficiently against the early manifestations of 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 to develop improved vaccines for TB in place of BCG [6]. Several strategies have been adopted to develop recombinant (r)BCG against TB. One is a BCG strain producing the immunodominant or *M. tuberculosis*-specific antigen (Ag) such as Ag85 complex, 38kDa or RD-1, which showed an enhanced protective efficacy [7, 8]. Another type is an rBCG strain producing cytokine such as IL-2, IL-18 or IFN-γ, which is reported to up-regulate Th1 type immunity *in vitro* [9-12]. However, there are few reports that rBCG expressing cytokine provided a 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 requisite role in this protection [13, 14]. A recent study has suggested that $CD8^+$ T cells are more important than $CD4^+$ T cells in controlling the latent phase of TB. [15, 16]. We have recently reported that vaccination of bone marrow-derived dendritic cell (BMDC) pulsed with H2-M3- binding peptide, TB2 [17] or MHC class Ia ($H\text{-}2D^b$)-binding peptide, $MPT64_{190\text{-}198}$ [18] elicited Ag-specific $CD8^+$ T cells, leading to protection against intratracheal infection with *M. tuberculosis* infection[19]. Thus, several vaccination strategies can be settled to induce protective memory $CD8^+$ T cells.

IL-15 has an important function in the proliferation and survival of memory-phenotype $CD8^+$ 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 *Mycobacterium bovis* BCG via activation of NK and $CD8^+$ T cells [22-24]. We have recently showed with $IL\text{-}15^{-/-}$ mice that IL-15 plays an important role in the development of protective immunity to BCG infection by sustaining $CD8^+$ 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 used at 6-8 weeks of age were obtained from Charles River Japan. Mice were maintained under specific pathogen-free conditions and offered food and water *ad libitum*. All experiments were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences.

Construction of expression plasmid

The construction of plasmid used for expression of mIL-15 is shown in Fig. 1A. After the mIL-15 sequence was confirmed by DNA sequencing, the DNA fragment was cloned into the pKH20 [26] Xho I sites located in the Ag85B region [27]. pKH20 is the vector in pUC18 that contains Ag85B gene. The DNA fragment of Ag85B and IL-15 was digested with Bam HI/HindIII and was inserted into the pNN2 Bam HI/HindIII site. This vector is the *E. coli*-mycobacteria shuttle vector which possesses a kanamycin-resistance gene as a selection marker [28]. pNN2-mIL-15-Ag85B were then introduced into BCG (Tokyo 172 strain) by electroporation. The transformed BCG was plated on Middlebrook 7H10 agar supplemented with 10% OADC, 20 μg/ml kanamycin, 100U/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 media for 3 weeks at 37°C.

Microorganisms and infection

rBCG-Ag85B-IL15, rBCG-Ag85B, rBCG only with plasmid vector (BCG-plasmid-vector) or *Mycobacterium tuberculosis* H37Rv were cultured and stored by the same method with BCG Tokyo strain previously described [25]. Naïve mice were inoculated intraperitoneally (i.p.) with 5×10^6 CFU of rBCG-Ag85B-IL15/rBCG-Ag85B. On day 90 after the inoculation, mice were treated with isoniazid (0.1g/L) in drinking water for 4 weeks to clear the live BCG bacteria and then on day 127 rmice were intratracheally (i.t.) infected with 2×10^5 CFU of *M. tuberculosis* H37Rv. Infections were performed in groups of at least 4 mice in one experiment and each data point is the mean of 3-5 infected mice in a representative from at least three independent experiments.

Detection of Ag85B-mIL-15 fusion protein

rBCG-Ag85B-IL15, rBCG-Ag85B or BCG-plasmid-vector were lyzed in lysis buffer for 30 min or cultured in 7H9 medium for 1 week. After centrifugation, supernatants were harvested and quantitation of protein in supernatants was done by Ultrospec 3300 pro (Amersham Biosciences). Then samples reconstituted to the same dose of protein were applied to SDS-PAGE. After naïve PAGE, proteins were blotted and reacted with anti-IL15 antibody (Ab) or anti-Ag85B serum.

Abs and reagents

FITC-conjugated anti-CD3ε (145-2C11), IFN-γ (XMG1.2); PE-conjugated anti-CD8α (53-6.7), anti-CD62L (MEL-14); PerCP-Cy5.5 labeled anti-CD4 (RM4-5), CD8α (53-6.7) and APC conjugated anti-CD44 (IM7) mAbs were purchased from BD Pharmingen. The H2-D^b-binding

peptide, $MPT64_{190-198}$ (FAVTNDGVI) [18], H_2-M_3-binding peptide, TB2 (f-MLVLLV) [17], and I-A^b-binding peptide, $Pepetide25_{aa240-254}$ (FQDAYNAAGGHNAVF) [29] were purchased from Greiner Bio-One.

Cell preparation

Peritoneal exudates cells (PECs) were obtained by lavage of the peritoneal cavity with 5ml HBSS. PECs and splenocytes were prepared by centrifugation and resuspended in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100 μl/ml streptomycin, and 10 mM HEPES. For the lung mononuclear cells (MNCs), lung tissue was minced and incubated with stirring at 37°C for 30 min in HBSS with 1.3mM EDTA, followed by treatment at 37°C for 1.5 h with collagenase (150 U/ml; Invitrogen Life Technologies) in RPMI 1640 with 10% FBS. The resulting suspension was pelleted by centrifugation, resuspended in 45% Percoll (Pharmacia) layered on 66.6% Percoll, and centrifuged at 600×*g*. Cells at the gradient interface were harvested and washed extensively before use.

Intracellular cytokine staining

Splenocytes, PECs and lung MNCs were harvested, washed and incubated without any stimulation or with 5μg/ml purified protein derivative (PPD) (Japan BCG Association) for 6 hrs at 37°C and 5% CO_2, with 10 μg/ml befeldin A (Sigma) added in the last 2 h, or with 10μM/ml MPT-64, TB2 or Peptide-25 for 4 hrs in the presence of befeldin A at a concentration of 5×10^6 in RPMI containing 10% FCS. After culture, cells were preincubated with 2.4G2 to prevent nonspecific staining, and

stained with various combinations of mAbs. Cells were then permeabilized and stained with IFN-γ mAb. Samples were analyzed using a FACSCalibur flow cytometer, and data were analyzed with CellQuest software (BD Biosciences).

In vitro culture and cytokine ELISA

Nylon-wool-passed T cells from splenocytes were incubated with anti-CD4 or CD8 microbeads, and $CD4^+$ or $CD8^+$ T cells were purified to >95% by positive selection using autoMACS (201-01) and cultured at a concentration of 2×10^5 cells/well without any simulation or with 10μM/ml TB2 or Peptide-25 in the presence of mitomycin C-treated splenocytes (1×10^6) from naïve C57BL/6 mice for 48 h at 37°C. Culture supernatants were collected and concentrations of IFN-γ in supernatants were measured using ELISA kits (Genzyme Diagnostics).

Histological examination

Upper-left lobes of lungs were preserved in 10% buffered formalin, embedded in paraffin, sectioned and stained with H&E. Samples from four mice per group were examined.

Statistical analysis

The statistical significance of the data was determined by the Student's *t*-test; a value of $p<0.05$ was considered 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 Ab or anti-Ag85B serum. As shown in Fig. 1B, the fusion protein, which reacted with anti-IL-15 Ab and anti-Ag85B serum, was detected at about 40 kDa in both bacterial lysate and culture supernatant of rBCG-Ag85B-IL15 lane but neither in the rBCG-Ag85B nor in the BCG-plasmid vector lane, in which only 30 kDa corresponding to Ag85B was detected. Thus, Ag85B + IL-15 fusion protein was confirmed to be existent in rBCG-Ag85B-IL15.

Bacterial growth in mice after rBCG-Ag85B-IL15 immunization

We first examined the kinetics of *in vivo* bacterial growth in spleen, lung and PEC of C57BL/6 mice after i.p. immunization with rBCG-Ag85B-IL15 or rBCG-Ag85B. The numbers of bacteria in PEC and lung were significant lower on day 21 after rBCG-Ag85B-IL15 inoculation than rBCG-Ag85B ($p<0.05$) and the bacterial number in spleen was significantly lower on days 42 and 70 after rBCG-Ag85B-IL15 immunization ($p<0.01$, Fig. 2). The difference in bacterial growth may not be due to a difference in duplication ability between them because both rBCG equally grew *in vitro*. It was implicated that rBCG-Ag85B-IL15 might induce stronger cell-mediated immune responses *in vivo* than rBCG-Ag85B.

T cell populations in mice after rBCG-Ag85B-IL15 immunization

We next examined the kinetics of T cell subpopulations in the spleen, PEC and lung of mice after rBCG-Ag85B-IL15 immunization. The numbers of $CD44^+CD8^+$ T cells in the PEC and lung of rBCG-Ag85B-IL15-immunized mice on day 21 reached to nearly twice more than those in rBCG-Ag85B-immunized mice (Fig. 3, $p<0.05$). The numbers in the spleen and lung also significantly increased on day 70 after immunization with rBCG-Ag85B-IL15 ($p<0.01$ or 0.05). Surprisingly, the levels of $CD44^+CD4^+$ T cells in the spleen and lung from rBCG-Ag85B-IL15-immunized mice were also significantly higher than those in rBCG-Ag85B-immunized mice during the course of immunization ($p<0.05$). Thus, in both lymphoid and nonlymphoid organs, rBCG-Ag85B-IL15 vaccination induced higher levels of $CD44^+CD8^+$ and $CD44^+CD4^+$ T cells than rBCG-Ag85B during the course of immunization.

IFN-γ production by Ag-specific T cells after rBCG-Ag85B-IL15 immunization

To investigate Ag-specific $CD8^+$ and $CD4^+$ T cell responses in the spleens, PEC and lungs of rBCG-Ag85B-IL15-immunized C57BL/6 mice, we used cytokine 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 PPD were detected in these organs on day 21 after rBCG immunization and the levels of IFN-γ-producing-T cells in the $CD4^+$ and $CD8^+$ populations were significantly higher in the PEC and lung of rBCG-Ag85B-IL-15-immunized mice than rBCG-Ag85B-immunized mice (Fig. 4A, $p<0.05$). The levels of IFN-γ-producing-$CD8^+$ T cells in response to MPT-64 or TB2, and IFN-γ-producing-$CD4^+$ T cells in response to Peptide-25 in spleens of

rBCG-Ag85B-IL15-immunized mice were significantly higher than in rBCG-Ag85B-immunized mice on day 21 after immunization (Fig. 4B, $p<0.05$). 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 rBCG-Ag85B.

Appreciable levels of both $CD8^+$ and $CD4^+$ responses capable of producing IFN-γ in response to mycobacterial-antigens were detected in the spleens and lungs on day 70 after rBCG infection (Fig. 5A). The numbers of PPD-specific IFN-$\gamma^+CD44^+CD8^+$ T cells in the lung and TB2-specific IFN-$\gamma^+CD44^+CD8^+$ T cells in the spleen were significantly more in rBCG-Ag85B-IL15-immunized mice than in rBCG-Ag85B-immunized mice ($p<0.05$). PPD- or Peptide25-specific IFN-$\gamma^+CD44^+CD4^+$ T cells in the spleens or lungs were also higher in rBCG-Ag85B-IL15-immunized mice than in rBCG-Ag85B-immunized mice ($p<0.05$). 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 APC, and culture supernatants were examined by ELISA for IFN-γ release. As shown in Fig. 5B, the levels of IFN-γ production by $CD8^+$ or $CD4^+$ T cells in rBCG-Ag85B-IL15-immunized mice were significantly higher than those from rBCG-Ag85B-immunized mice ($p<0.05$). Taken together, these data suggested that rBCG-Ag85B-IL15 vaccination enhanced not only the expansion but also the maintenance of Ag-specific $CD8^+$ and $CD4^+$ T cells.

Protection against M. tuberculosis infection in rBCG-Ag85B-IL15-immunized mice

To investigate the efficacy of rBCG-Ag85B-IL15 vaccination for protection against *M. tuberculosis* infection, mice were challenged i.t. with *M tuberculosis* H37Rv on day 127 after rBCG-Ag85B/rBCG-Ag85B-IL15-immunization. The Ag-specific IFN-γ-producing-$CD8^+$ or $CD4^+$ T cells were examined by cytokine FACS on day 28 after the challenge, on which *M. tuberculosis*-specific T cells reached their peak of expansion in mice. The larger numbers of $CD8^+$ T cells in the lungs from rBCG-Ag85B-IL15-immunized mice produced IFN-γ in response to PPD compared with those from rBCG-Ag85B-immunized or non-immunized mice ($p<0.05$, Table 1). TB2- or MTP64-specific $CD8^+$ T cells in the lungs or spleens from rBCG-Ag85B-IL15-immunized mice also increased ($p<0.05$, Table 1). In MTP64- or TB2-specific IFN-γ^+CD8^+ T cells, the numbers of $CD44^+CD62L^+$ $CD8^+$ T cells corresponding to central memory T cells were more in rBCG-Ag85B-IL15-immunized mice than rBCG-Ag85B-immunized or non-immunized mice ($p<0.05$, Table 1). PPD-specific IFN-γ-producing-$CD4^+$ T cells were markedly increased in rBCG-Ag85B-IL15-immunized mice ($p<0.05$, Table 1). Peptide25-specific IFN-γ-producing-$CD4^+$ or $CD44^+CD62L^+$IFN-γ^+CD4^+ T cells in rBCG-Ag85B-IL15-immunized mice were also significantly more than in rBCG-Ag85B-immunized mice ($p<0.05$, Table 1). The numbers of IFN-γ-producing-$CD4^+$ T cells in non-immunized mice were sometimes more than in immunized mice, which may be due to the 10 times higher bacterial burden in the organs(data not shown). These data indicated that memory $CD8^+$ and $CD4^+$ T cells from rBCG-Ag85B-IL15-immunized mice re-expanded to greater levels than those from rBCG-Ag85B-immunized mice after challenge with *M. tuberculosis*.

We then examined the bacterial burden in rBCG-Ag85B-IL15- or rBCG-Ag85B-immunized mice 10 weeks after infection with *M. tuberculosis*. As shown in Fig. 6A, the bacterial numbers in lungs were significantly lower in rBCG-Ag85B-IL15-immunized mice than in rBCG-Ag85B-immunized mice ($p<0.05$). Non-immunized mice showed 10 times higher bacterial numbers than rBCG- immunized mice.

Histological pictures of ×50 and ×200 magnification showed that in non-immunized mice, little lymphoid infiltration was found and the whole pulmonary lobe was filled with lowly 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 were found, but the whole lung of rBCG-Ag85B-immunized mice were also full of granulomas, whereas histopathological changes were quite light and very localized in the lung of rBCG-Ag85B-IL15-immunized mice (Fig. 6B).

Discussion

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

IL-15 was reported to have an important function in the proliferation and survival of memory $CD8^+$ T cells [30, 31], and we have recently demonstrated that IL-15 played an important role in

preventing effector $CD8^+$ T cells from apoptosis during the contraction phase after infection with intracellular bacteria such as *Listeria monocytogenes* [32]. In the present study, we found that mice immunized with rBCG-Ag85B-IL15 showed more $CD44^+CD8^+$ 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 generation of long-lived $CD8^+$ T cells and in maintaining memory $CD8^+$ T cells after rBCG-Ag85B-IL15 immunization.

Ag85B, a fibronectin-binding protein [33], has an immunopotentiating effect by inducing T cell proliferation and synthesis of IFN-γ [34] and TNF-α [35]. Recent studies showed that rBCG expressing Ag85B could significantly augment Th1 response more than normal BCG by increasing IFN-γ-producing-$CD4^+$ T cells [36, 37]. Surprisingly, we found that not only the $CD44^+CD8^+$, but also the $CD44^+CD4^+$ T cells were significantly increased in rBCG-Ag85B-IL15-immunized mice than in rBCG-Ag85B-immunized mice. The levels of mycobacterial-Ag-specific $CD4^+$ T cells in rBCG-Ag85B-IL15-immunized mice 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 [38-40], and Ag-specific $CD4^+$ T cells appear to be mainly controlled by IL-7 [41, 42]. Normal numbers of $CD4^+$ T cells are present in naïve IL-$15^{-/-}$ mice [30], and after infection of the BCG Tokyo strain [25]. 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* [41, 43]. Recently, by studying lymphocytic choriomeningitis virus (LCMV)-specific TCR transgenic mice, Purton et al. reported that under normal physiological conditions, IL-15 was crucial for both survival and homeostatic proliferation of memory $CD4^+$ T cells [44], and that, in a normal environment, memory $CD4^+$ T cells closely resembled memory $CD8^+$ T cells in their dependency on both IL-7 and IL-15 for their homeostasis. It was reported that memory $CD8^+$ T cells produced *de novo* in IL-$15^{-/-}$ mice were considerably less dependent on IL-15 for homeostatic proliferation than analogous cells produced in normal mice [44, 45]. Thus, the discrepancy in the role of IL-15 on memory $CD4^+$ T cells may be explained by a different nature of $CD4^+$ T cells generated *de novo* in IL-$15^{-/-}$ mice and normal mice. $CD4^+$ T cells generated *de novo* in IL-$15^{-/-}$ mice may become permanently conditioned to cope with IL-15 deficiency and appear to have found an alternative way to sustain their homeostasis in the absence of IL-15. Our results in the present study with normal $CD4^+$ T cells from C56BL/6 mice may reflect the role of IL-15 in generation and maintenance of memory $CD4^+$ T cells in physiological condition.

We have recently showed with 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 lung (25). In the present study, ten weeks after *M. tuberculosis* infection, the bacterial burden in the lung of rBCG-Ag85B-IL15-immunized mice was significantly lower than in rBCG-Ag85B-immunized mice. Lazarevic et al. recently reported that a significant increase in bacterial number 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 (less than 30 CFU/mouse) of *M. tuberculosis* (Erdman strain)[46]. Rausch et al. showed that IL-15 was critical for the development of full-blown protective immunity against experimental *M. tuberculosis* in mice, because the lack of IL-15 was associated with a dramatic reduction in the numbers and function of CD8+ T cells, and in IL-15$^{-/-}$ mice, 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 control mice [47]. Thus, although the efficacy of IL-15 in protection against mycobacterial infection varied depending on initial bacterial load and virulence of *M. tuberculosis*, IL-15 may play a role in protection against *M. tuberculosis* infection. Using the recombinant IL-15 administration, Lazarevic et al. could not find a significantly lower bacterial burden or increased numbers of $CD8^+$ or $CD4^+$ T cells in *M. tuberculosis*-infected mice compared with non-administrated mice. In contrast, in our study, IL-15 secreted by rBCG showed to be markedly helpful both in the bacterial clearance and the increases in $CD8^+$ T cells. This comparison indicates that endogenously secreted IL-15 from rBCG is a reasonable method for the generation of T cell immunological 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 $CD8^+$ Tc1 cells [48, 49]. The success of rBCG-Ag85B-IL15 as a vaccine is that with the help of IL-15+Ag85B protein as adjuvant, BCG can generate high levels of Ag-specific memory $CD8^+$ as well as $CD4^+$ T cells, which conferred much stronger protection in the lung against *M. tuberculosis*

challenge. 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 lung of immunized mice after *M. tuberculosis* challenge indicate that IL-15 does play an important role in the proliferation and maintenance of memory T cells, and can be used as an immune adjuvant to increase the efficacy of BCG vaccination.

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