tion of $CD8^+$ T cells after inoculation with *M. bovis* BCG and on the efficacy of BCG vaccination against *M. tuberculosis* infection. In vivo administration of rIL-15 from Days 22 to 42 after BCG inoculation inhibited the contraction of effector $CD8^+$ T cells, resulting in a significant increase in the number of antigen-specific memory $CD8^+$ T cells producing IFN-γ. However, the treatment of rIL-15 did not provide efficient memory T cell responses against subsequent infection with *M. tuberculosis*.

MATERIALS AND METHODS

Mice

C57BL/6 mice, used at 6–8 weeks of age, were obtained from Charles River Laboratories (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 [15].

Microorganisms

rBCG-OVA were described previously [16, 17]. Briefly, BCG (Pasteur strain) was grown on Middlebrook 7H10 solid medium (Difco, Detroit, MI, USA). A partial sequence of the OVA gene 230-359, which encodes the SIINFEKL epitope and its flanking sequences, was cloned in the pMV261 vector, downstream of the Ag85B secretion signal and under the control of the heat shock protein 60 promoter. rBCG-OVA were dissolved in 7H9 medium (Difco) supplemented with oleic acid-albumin-dextrose-catalase enrichment (Difco). Small aliquots of rBCG-OVA suspended in 7H9 medium containing 10% glycerol were stored at −80°C until use. *M. tuberculosis* H37Rv were cultured and stored by the same method with rBCG-OVA. The concentration of bacteria was quantified by plate-counting. Before use, the bacteria were washed twice with PBS containing 0.05% Tween 80 and resuspended in PBS.

Bacterial infection and rIL-15 treatment

Naïve mice were i.p.-inoculated with 5×10^6 CFU rBCG-OVA in a volume of 200 μl PBS. From Day 22 after preimmunization, mice were i.v.-treated with rIL-15 (Takeda Chemical, Osaka, Japan; 0.5 μg/day) or PBS every day until Day 42, and on Day 91 after preimmunization (49 days after the end of rIL-15 treatment), mice were treated with isoniazid (0.1 g/L) in drinking water for 4 weeks to clear the live BCG and then on Day 127, were i.t.-infected with 2×10^5 CFU *M. tuberculosis* H37Rv in a volume of 50 μl PBS under the Biohazard Level-3 procedures.

Antibodies and reagents

FITC-conjugated anti-CD3ε (145-2C11), CD62L (MEL-14), CD127 (A7R34), IFN-γ (XMG1.2); PE-conjugated anti-CD8α (53-6.7); PerCP-Cy5.5-labeled anti-CD4 (RM4-5) and CD8α (53-6.7); and allophycocyanin-conjugated anti-CD44 (IM7) and CD62L (MEL-14) mAb were purchased from BD PharMingen (San Diego, CA, USA). FITC-conjugated hamster anti-mouse activated form of Caspase-3 (C92-605), Bcl-2 mAb (3F11), and hamster anti-mouse isotype control were obtained from BD PharMingen. Annexin-V^{FITC} apoptosis detection kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). PE-conjugated $OVA_{257-264}$ H-$2K^b$ tetramers were purchased from MBL (Nagoya, Japan).

Cell preparation

PECs were obtained by lavage of the peritoneal cavity with 5 ml 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. Lung MNCs were prepared as described previously [18]. Briefly, lung tissue was minced and incubated with stirring at 37°C for 30 min in HBSS with 1.3 mM EDTA, followed by treatment at 37°C for 1.5 h with collagenase (150 U/ml, Invitrogen Life Technologies, Carlsbad, CA, USA) in RPMI 1640 with 10% FBS. The resulting suspension was pelleted by centrifugation, resuspended in 45% Percoll (Pharmacia, Uppsala, Sweden), layered on 66.6% Percoll, and centrifuged at 600 *g*. Cells at the gradient interface were harvested and washed extensively before use.

Flow cytometric analysis

Splenocytes, PECs, or lung MNCs were preincubated with a culture supernatant from 2.4 G_2 to prevent nonspecific staining. After washing, cells were stained with various combinations of mAb. The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed with CellQuest software (BD Biosciences).

Intracellular staining for cytokine, Bcl-2, or Caspase-3

Splenocytes and lung MNCs were harvested, washed, and incubated without any stimulation or with 5 μg/ml PPD (Japan BCG Association, Tokyo, Japan) and 100 pg/ml rIL-2 (Takeda Chemical) for 6 h at 37°C and 5% CO_2, with 10 μg/ml brefeldin A (Sigma-Aldrich) added in the last 2 h or with 5 μg/ml $OVA_{257-264}$ peptide for 4 h in the presence of brefeldin A at a concentration of 5×10^6 in RPMI containing 10% FCS. After culture, cells were preincubated with 2.4 G_2 and stained with various combinations of mAb. After surface staining, cells were subjected to intracellular cytokine staining using a Fast Immune Cytokine System (BD Biosciences), according to the manufacturer's instructions. Briefly, cells were washed and fixed in 1000 μl FACS lysing solution (BD Biosciences) for 10 min at room temperature and were then washed again, resuspended in 500 μl FACS permeabilizing solution (BD Biosciences), and incubated for 10 min at room temperature. After washing, the cells were stained with FITC-conjugated IFN-γ mAb or isotype control rat IgG (BD PharMingen).

Before staining for intracellular Bcl-2, splenocytes were stained for surface markers in FACS buffer with PerCP-Cy5.5-conjugated anti-CD8 mAb and PE-conjugated $OVA_{257-264}$ H-$2K^b$ tetramers for 60 min at 4°C and then were fixed, permeabilized, and stained with FITC-conjugated hamster anti-mouse Bcl-2 or its isotype control. For the staining of activated Caspase-3, cells were first cultured in 48-well flat-bottom plates without any stimulation for 24 h at 37°C and 5% CO_2 and then were stained for surface markers. Then, the cells were washed, fixed, and permeabilized using the Cytofix/Cytoperm intracellular staining kit (BD Biosciences) and were finally incubated with anti-Caspase-3 at a 1/100 dilution in perm/wash buffer for 30 min at room temperature. After intracellular staining, fluorescence of the cells was analyzed by a FACSCalibur flow cytometer (BD Biosciences).

Annexin-V staining

Splenocytes from mice were stained for surface markers, and the amount of apoptosis was determined by staining these cells with Annexin-V^{FITC}, according to the manufacturer's instructions. Briefly, cells were suspended in 1× binding buffer at 500 μl/tube and incubated with 5 μl/tube Annexin-V^{FITC} for 10 min in the dark at room temperature. Cells were washed twice with 1× binding buffer to remove any unbound mAb. Samples were analyzed within 30 min.

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

Expansion and contraction of $OVA_{257-264}$-specific $CD8^+$ T cells in C57BL/6 mice after inoculation with rBCG-OVA

To identify the timing of the contraction phase of antigen-specific $CD8^+$ T cells after BCG infection, we examined the number of $OVA_{257-264}$-tetramer$^+CD8^+$ T cells, as assessed by staining with an H-$2K^b$ tetramer coupled with an OVA-derived

SIINFEKL peptide or $OVA_{257-264}$-specific IFN-γ-producing-$CD8^+$ T cells, as assessed by intracellular staining with anti-IFN-γ mAb after stimulating the cells with $OVA_{257-264}$ peptide every 7 days after an i.p. inoculation with rBCG-OVA. We found that the bacterial number increased to a peak at approximately the 2nd week and thereafter, decreased in the spleen after rBCG-OVA inoculation (data not shown), and $OVA_{257-264}$-tetramer$^+CD8^+$ T cells and $OVA_{257-264}$-specific IFN-γ-producing-$CD8^+$ T cells in the spleen or PEC expanded and reached a peak at approximately Day 21 after rBCG-OVA inoculation, followed by contraction until Day 42, and thereafter, were maintained at relatively stable cell numbers (data not shown; the profiles of cell kinetics were quite similar to that of rBCG-OVA-preinoculated PBS-treated mice in **Fig. 1**). Thus, the contraction phase of antigen-specific $CD8^+$ T cells in normal C57BL/6 mice was from Day 22 to Day 42 following rBCG-OVA inoculation.

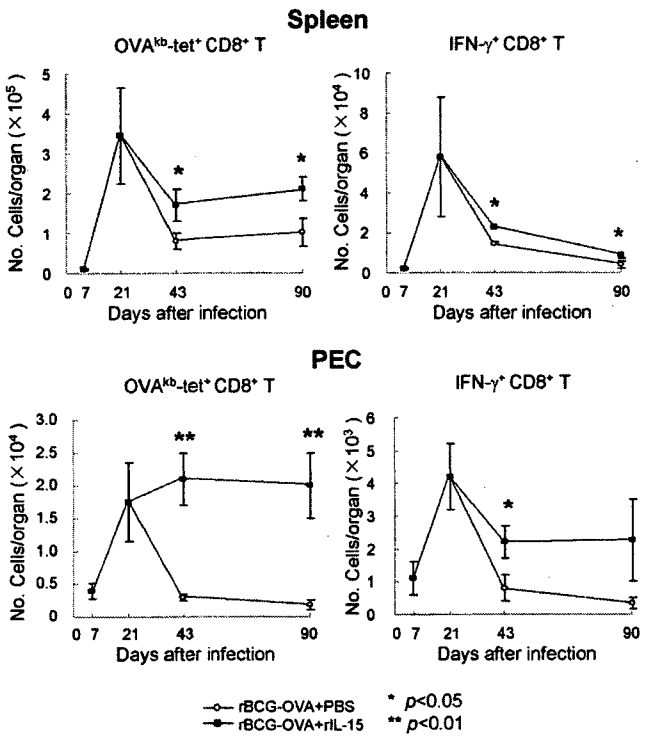

Figure 1. Kinetics of $OVA_{257-264}$-specific $CD8^+$ T cells in C57BL/6 mice after rBCG-OVA inoculation. Mice were treated with rIL-15 or PBS from Day 22 to Day 42 after rBCG-OVA i.p. preinoculation. Splenocytes and PECs were harvested on Days 7, 21, 43, and 90 after the inoculation and were stained with anti-CD8 mAb, anti-CD44 mAb, and $OVA_{257-264}$ H-$2K^b$ tetramer or were cultured with $OVA_{257-264}$ peptide for 4 h at 37°C and then were subjected to intercellular cytokine staining for IFN-γ. The absolute numbers of $OVA_{257-264}$ H-$2K^b$ tetramer staining-positive $CD44^+CD8^+$ and IFN-$\gamma^+CD44^+CD8^+$ T cells were calculated by multiplying total splenocytes or PECs by the percentages of each subset in the spleen or PEC. Data of a representative are shown from three separate experiments and are expressed as means ± SD of three to four mice of each group. *, $P < 0.05$, **, $P < 0.01$, significantly different from the value for PBS-treated mice.

Effect of in vivo administration of rIL-15 during the contraction phase on generation of $OVA_{257-264}$-specific memory phenotype $CD8^+$ T cells in mice

To determine the effect of in vivo administration of exogenous IL-15 on the generation of memory-phenotype $CD8^+$ T cells, we injected rIL-15 into mice every day from Day 22 to Day 42 during the contraction phase after rBCG-OVA inoculation and examined $OVA_{257-264}$-specific $CD8^+$ T cells by FACS analysis. Activated $CD44^+CD8^+$ T cells were maintained at a significantly higher level in rIL15-treated mice than in PBS-treated mice in the spleen and lung ($P<0.05$), and $CD44^+CD4^+$ T cells did not change in rIL-15-treated mice on Days 43 (data not shown) or 90 after inoculation (**Fig. 2, A** and **B**). The $OVA_{257-264}$-tetramer$^+CD44^+CD8^+$ T cells in the spleen, PEC, or lung of rIL-15-treated mice were significantly more than those in PBS-treated mice on Day 43 (Fig. 1, $P<0.05$ or 0.01) or Day 90 (Figs. 1 and 2, C and D, $P<0.05$ or 0.01). We further stained the $OVA_{257-264}$-tetramer$^+CD8^+$ T cells for expression of CD62L and CD44/CD127 to identify which populations in antigen-specific $CD8^+$ T cells were increased in the spleen of rIL-15-treated mice on Day 90. As shown in Figure 2E, $OVA_{257-264}$-specific $CD62L^+CD44^+$ (or $CD62L^+CD127^+$) $CD8^+$ T cells, corresponding to central memory type, showed a twofold higher population than those from PBS-treated mice ($P<0.05$). Thus, rIL-15 treatment during the contraction phase resulted in a higher level of memory $CD8^+$ T cell maintenance, especially an increase of the number of central memory $CD8^+$ T cells in the lymphoid organ.

The *M. tuberculosis*-derived antigen-specific $CD8^+$ and $CD4^+$ T cells in rIL-15-treated mice were examined by intracellular cytokine FACS analysis for IFN-γ expression. As shown in **Figure 3, A** and **B**, the number of PPD-specific IFN-γ-producing-$CD8^+$ T cells in the spleen from rIL-15-treated mice was significantly more than those in PBS-treated mice on Day 90 after inocuation ($P<0.05$), and the percentage of PPD-specific IFN-γ-producing-$CD4^+$ Th cells in the spleen was much the same in rIL-15-treated mice as in PBS-treated mice. Detectable levels of IFN-γ^+ cells could not be observed in the lung of rIL-15- and PBS-treated groups (Fig. 3A). We next stimulated the cells from the spleen, PEC, or lung with $OVA_{257-264}$ peptide for cytokine FACS. There were significantly more $OVA_{257-264}$-specific IFN-γ-producing-$CD8^+$ T cells in the spleen and lung from rIL-15-treated mice than from PBS-treated mice on Day 43 or Day 90 (Fig. 1, $P<0.05$). Thus, rIL-15 treatment during the contraction phase resulted in a significant increase in the number of $CD8^+$ T cells producing IFN-γ in lymphoid and nonlymphoid organs.

Impaired apoptosis in $OVA_{257-264}$-specific $CD8^+$ T cells in rIL-15-treated mice

To determine the apoptotic potential of OVA-specific $CD8^+$ T cells in the spleen from IL-15-treated mice on Day 90 after rBCG-OVA inoculation, we examined the expression level of Annexin-V, an early marker of apoptotic cells, or active Caspase-3, a terminal effector for apoptosis, in the $OVA_{257-264}$-tetramer$^+CD8^+$ T cells. The expression level of both of these apoptotic markers was significantly lower in rIL-15-treated mice

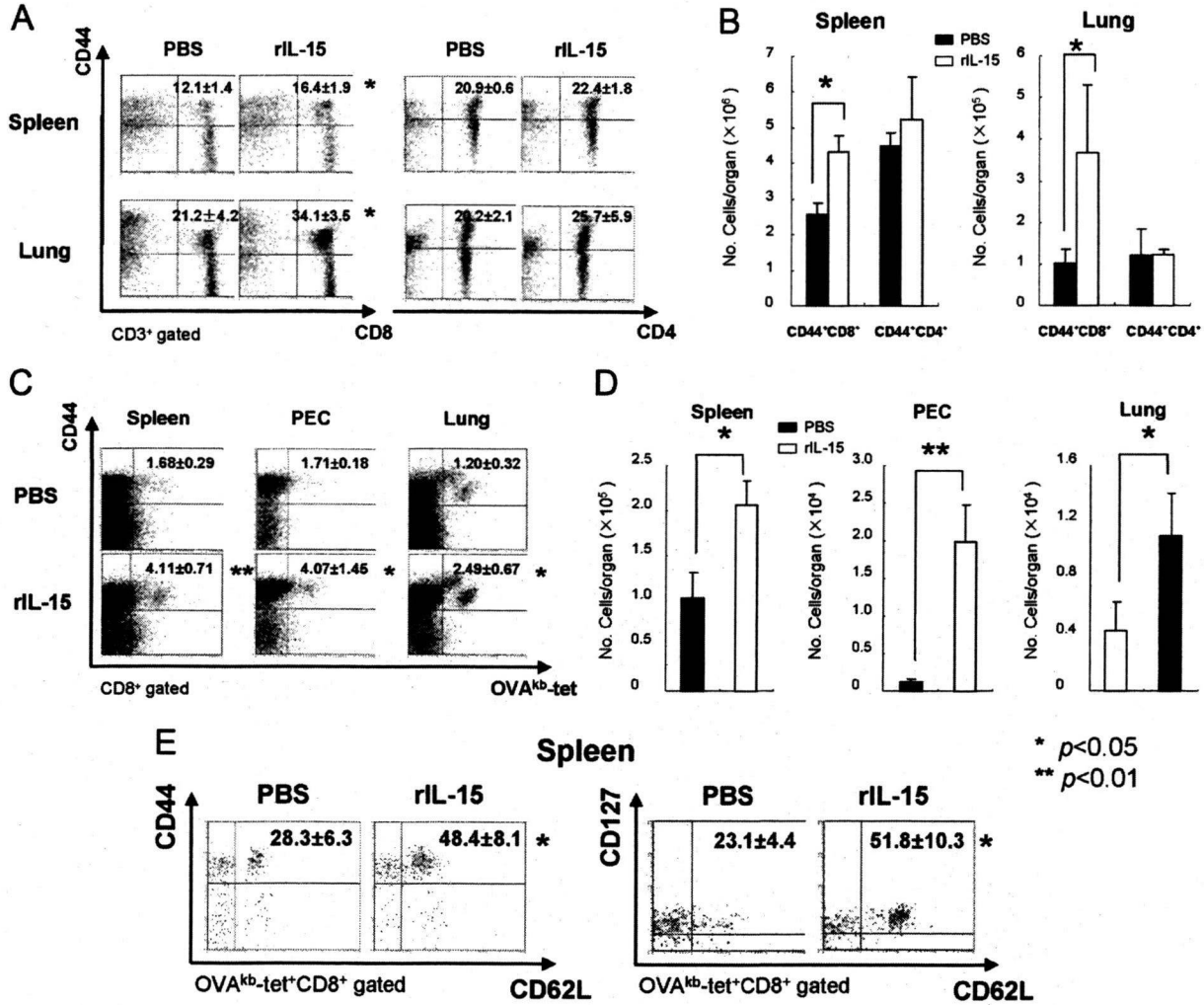

Figure 2. Exogenous IL-15 during the contraction phase increased the number of memory phenotype CD8$^+$ T cells in C57BL/6 mice after rBCG-OVA inoculation. C57BL/6 mice were treated with rIL-15 or PBS during the contraction phase (22–42 days) of primary immune response to rBCG-OVA. Proportions of the CD44$^+$CD8$^+$ or CD44$^+$CD4$^+$ T cells in spleen or lung (A), proportions of $OVA_{257-264}$ H-2K^b tetramer staining-positive CD44$^+$CD8$^+$ T cells in spleen, PEC, or lung (C), and $OVA_{257-264}$ H-2K^b tetramer$^+$CD44$^+$CD62L$^+$ or CD127$^+$CD62L$^+$ CD8$^+$ central memory T cells in spleen (E) from rIL-15- or PBS-treated mice were determined on Day 90 after rBCG-OVA inoculation. The absolute numbers of CD44$^+$CD8$^+$, CD44$^+$CD4$^+$ T cells (B) and $OVA_{257-264}$-tetramer$^+$CD44$^+$CD8$^+$ T cells (D) were calculated by multiplying total number of splenocytes, PECs, or lung MNCs by the percentages of each subset in the spleen, PEC, or lung. Data of a representative are shown from three separate experiments and are expressed as means ± SD of three to four mice of each group. *, $P<0.05$; **, $P<0.01$.

than in control mice (Fig. 3E, $P<0.05$). The intracellular expression levels of the antiapoptotic protein Bcl-2 in the OVA-specific CD8$^+$ T cells from rIL-15-treated mice were significantly higher compared with those in PBS-treated mice (Fig. 3E, $P<0.05$). Thus, in vivo administration of rIL-15 during the contraction phase after BCG infection prevented significantly more antigen-specific CD8$^+$ T cells from undergoing apoptosis.

Effect of rIL-15 administration during the contraction phase on the efficacy of BCG vaccination against *M. tuberculosis* infection

We investigate the effect of rIL-15 administration on the efficacy of BCG vaccination against *M. tuberculosis* infection. Mice were treated with rIL-15 or PBS during the contraction phase (Days 22–42) after BCG inoculation, treated with isoniazid in drinking water from Day 91 for 4 weeks to clear live BCG bacteria, and subsequently, challenged i.t. with *M. tuberculosis* H37Rv on Day 127. The $OVA_{257-264}$-specific CD8$^+$ T cells were examined just before challenge of *M. tuberculosis*. The numbers of OVA-tetramer$^+$CD8$^+$ T cells in the spleen and $OVA_{257-264}$-specific IFN-γ-producing CD8$^+$ T cells in the lung are still greater in rIL-15-treated mice ($P<0.05$), although the cell levels declined as compared with that on Day 90 after BCG inoculation (**Fig. 4A**). The PPD-specific IFN-γ-producing CD8$^+$ or CD4$^+$ T cells in the lung were examined on Day 28 or Day 70 after the challenge with *M. tuberculosis*. As shown in Figure 4B, the larger numbers of CD8$^+$ T cells in the lungs from rIL-15-treated mice produced IFN-γ in response to PPD compared

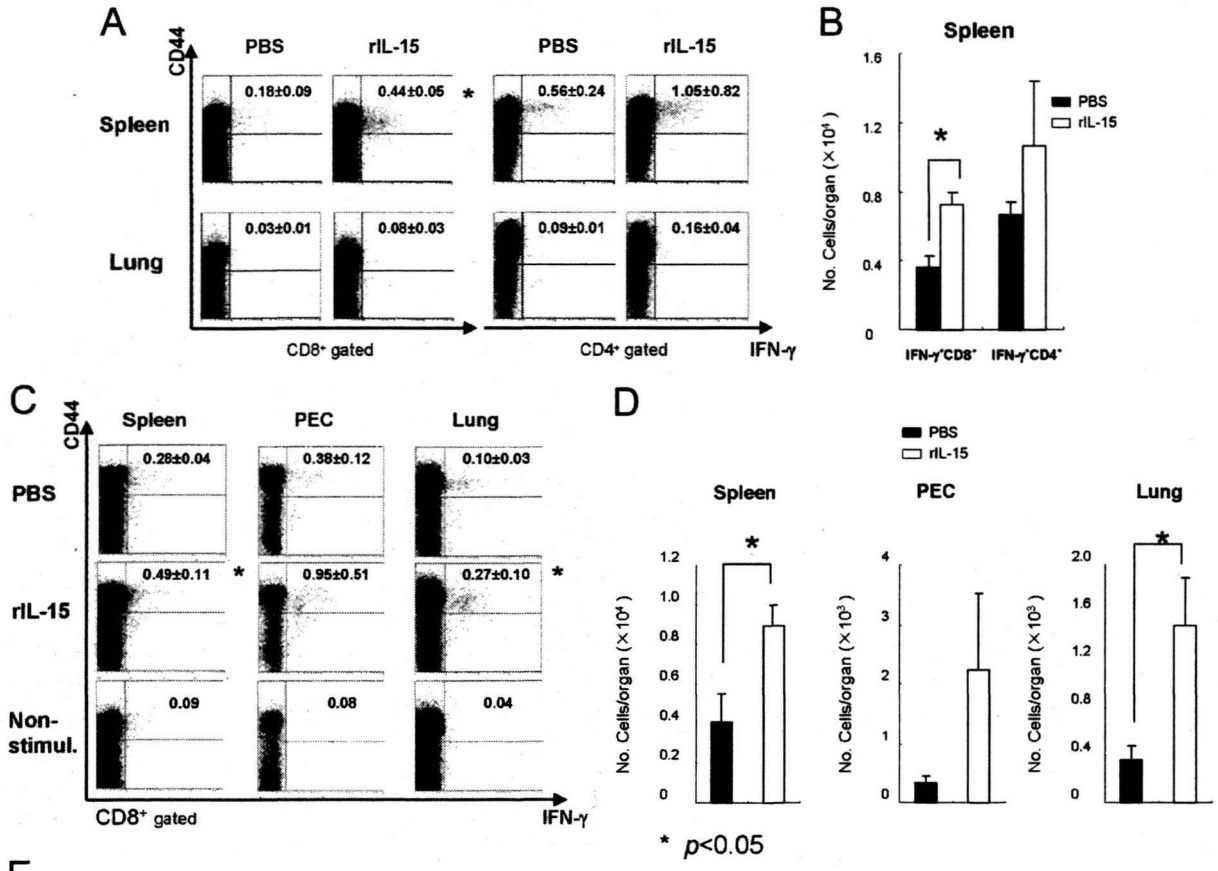

Figure 3. The number of IFN-γ-producing-CD44$^+$CD8$^+$ T cells and the expression of Annexin-V, active Caspase-3, and Bcl-2 on OVA$_{257-264}$-specific CD8$^+$ T cells in rIL-15-treated C57BL/6 mice after rBCG-OVA inoculation. C57BL/6 mice were treated with rIL-15 or PBS during the contraction phase (22–42 days) of primary immune response to rBCG-OVA. On Day 90 after the inoculation, cells were harvested from the spleen, PEC, or lung and were stimulated with PPD (A, B) or OVA$_{257-264}$ peptide (C, D) for 6 or 4 h and then were stained intracellularly for IFN-γ. An analysis gate was set on CD8$^+$ or CD4$^+$ T cells (A) or on CD8$^+$ T cells (C), and background levels of CD4$^+$ T cells responding to no stimulation were 0.21% in the spleen and 0.08% in the lung. The absolute numbers of IFN-γ$^+$CD44$^+$CD8$^+$ (B, D) or IFN-γ$^+$CD44$^+$CD4$^+$ (B) T cells were calculated by multiplying total numbers of splenocytes, PECs, or lung MNCs by the percentages of each subset in the spleen, PEC, or lung. (E) Spenocytes from rIL-15- or PBS-treated mice were stained with anti-CD8 and OVA$_{257-264}$ K^b tetramer, followed by Annexin-V antibodies or intracellular staining of anti-active Caspase-3 antibody or Bcl-2 with its isotype control IgG staining. The numbers indicate the percentage of OVA-tet$^+$CD8$^+$ T cells stained negative for anti-Annexin-V antibody or positive for antiactive Caspase-3 antibody, and for Bcl-2 expression, the numbers showed the mean fluorescence intensity of OVA-tet$^+$CD8$^+$ IgG$^-$Bcl-2$^+$. Data of a representative are shown from three separate experiments and are expressed as means ± SD of three mice of each group. *, $P < 0.05$.

with those from PBS-treated mice on Days 28 and 70 ($P<0.05$). Frequencies of IFN-γ$^+$ CD4$^+$ T cells in rIL-15 and PBS-treated mice had no differences. The number of IFN-γ-producing-CD4$^+$ T cells in nonimmunized mice was sometimes higher than immunized mice, which may be a result of the 10 times higher bacterial burden in the organs (**Fig. 5**). These data indicate that memory CD8$^+$ but not CD4$^+$ T cells from rIL-15-treated mice re-expanded to greater levels than those from PBS-treated mice after challenge with *M. tuberculosis*.

The bacterial burden in rIL-15- or PBS-treated mice was determined on Day 28 or Day 70 after challenge with *M. tuberculosis*. As shown in Figure 5, A and B, the bacterial numbers in lungs and spleens were significantly lower in rIL-15-treated or PBS-treated mice than in nonimmunized mice ($P<0.05$). However, there was no significant difference between rIL-15-treated mice and PBS-treated mice in these organs. We also examined the histopathologic changes in the lungs of rBCG-OVA-immunized rIL-15-treated; rBCG-OVA-immunized PBS-treated; or nonimmunized

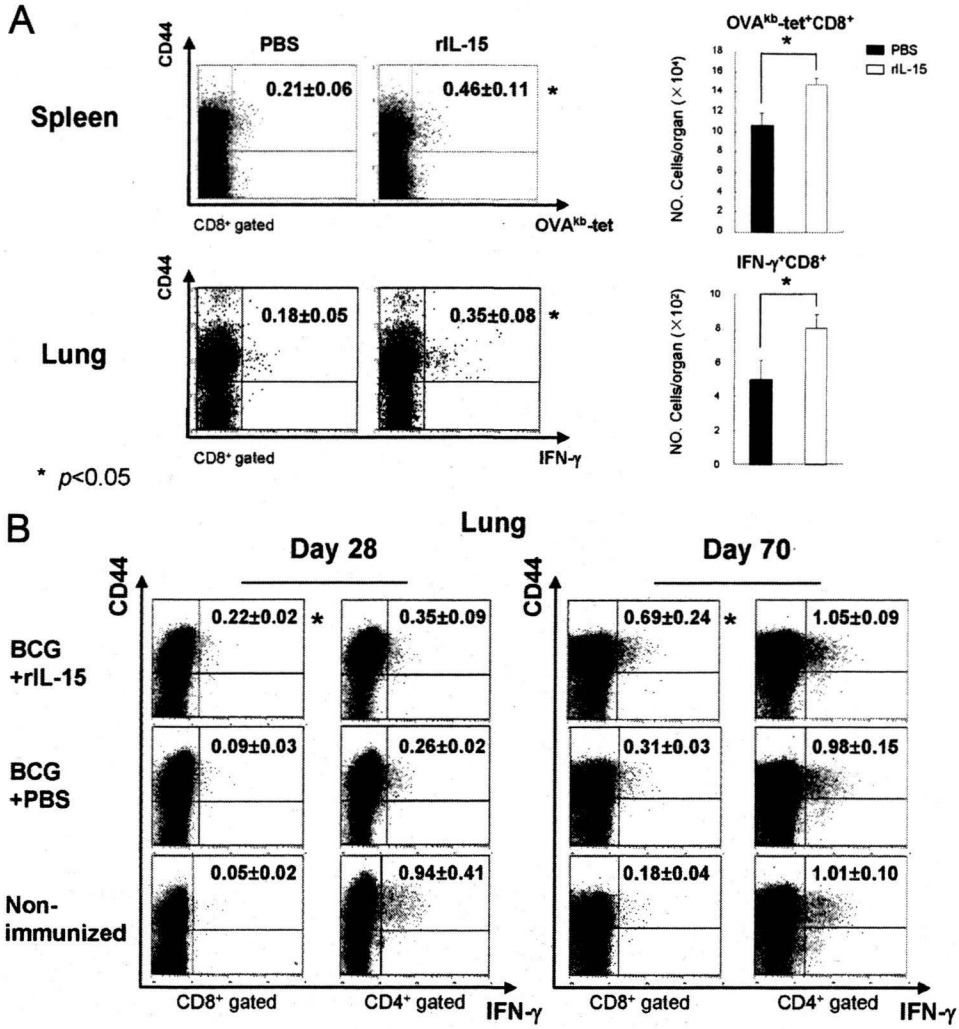

Figure 4. Intracellular expression of IFN-γ by T cells in the lung or spleen from rIL-15-treated mice after i.t. infection with *M. tuberculosis*. C57BL/6 mice were treated with rIL-15 or PBS during the contraction phase (22–42 days) of primary immune response to rBCG-OVA; on Day 91 after the inoculation, mice were treated with isoniazid (0.1 g/L) in drinking water for 4 weeks to clear the live BCG bacteria, and then on Day 127, mice were i.t.-infected with 2×10^5 CFU of *M. tuberculosis* H37Rv. (A) Splenocytes or lung MNCs were harvested from rIL-15 or PBS-treated mice on Day 126, just before *M. tuberculosis* challenge and were stained with $OVA_{257\text{-}264}$ K^b tetramer or were cultured with $OVA_{257\text{-}264}$ peptide for 4 h and then were subjected to intercellular cytokine staining for IFN-γ. (B) Lung MNCs were harvested from mice of each group 4 weeks and 10 weeks after *M. tuberculosis* infection and stimulated with PPD for 6 h. After stimulation, cells were stained with anti-CD8/CD4 and anti-CD44 mAb and then intracellularly stained with anti-IFN-γ mAb. Intracellular cytokine-producing cells were examined by FACS and were analyzed by gating on $CD8^+$ or $CD4^+$ T cells. Each number indicates mean percentage of IFN-γ^+ $CD44^+$ cells in $CD8^+$ T cells ± SD of three to four mice of each group. Data of a representative are shown from three separate experiments. *, $P < 0.05$, significantly different from the value for PBS-treated mice pre-infected with rBCG-OVA.

mice on Day 70 after *M. tuberculosis* challenge. In nonimmunized mice, little lymphoid infiltration was found, and the whole pulmonary lobe was filled with lowly structured, loose granulomas. In rBCG-OVA-immunized IL-15- and PBS-treated mice, a great deal of lymphoid follicle formations was found, but there was no significant difference between these two groups (**Fig. 6**).

DISCUSSION

It has been reported that treatment of members of the cγ-chain cytokine family, including IL-2, IL-7, or IL-15 from the beginning of immunization or infection, did not affect the effector/memory $CD8^+$ T cell population quanti- and qualitatively, resulting in no efficacy on treatment of infectious diseases [13, 19]. We here show that in vivo administration of rIL-15 from Days 22 to 42 after BCG inoculation inhibited the contraction of effector $CD8^+$ T cells, resulting in a significant increase of antigen-specific memory $CD8^+$ T cells. However, the IL-15 treatment, only at the contraction phase after BCG vaccination, failed to elicit improved efficacy of vaccination against *M. tuberculosis* as compared with BCG alone, presumably as the higher level of memory $CD8^+$ T cells did not last for a long time.

Unlike acute infection with *L. monocytogenes*, priming of $CD8^+$ T cells during infection of mice with *M. bovis* BCG was delayed, weaker in magnitude, which peaked at approximately the 3rd week after infection, and subsequently, declined to a low level [20]. During acute infection with *L. monocytogenes*, antigen-specific $CD8^+$ T cells differentiate rapidly into effectors ($CD62L^{low}CD44^{high}$), most of which subsequently die by apoptosis during the contraction phase, and $CD8^+$ T cells primed during chronic BCG infection differentiated primarily into the central memory phenotype ($CD62L^{high}CD44^{high}$) that correlated with subsequent reduced attrition of the primed cells [17]. It has been found recently that thymic emigrants masked the contraction phase of the $CD8^+$ T cell response in the mouse model of chronic lymphocytic choriomeningitis virus infection [21]. Therefore, $CD8^+$ T cell contraction may be more obscure during chronic infection with BCG than during acute infection with *L. monocytogenes* infection. However, we found in the present study that although priming of $CD8^+$ T cells of mice during BCG infection was delayed and weaker in magnitude, peaking at approximately the 3rd week of infection, the $CD8^+$ T cells declined to a low level subsequently by Day 42 after BCG infection. Furthermore, in vivo administra-

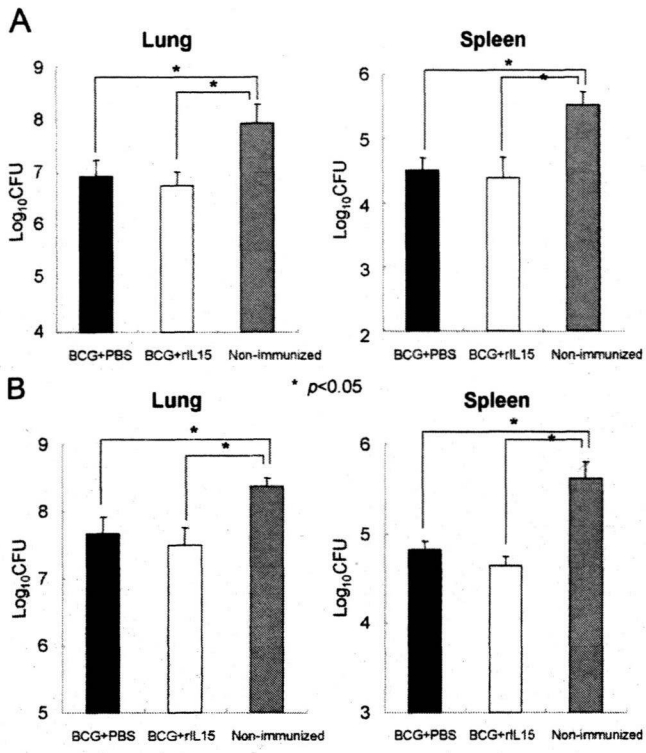

Figure 5. The bacterial growth in the lung or spleen from rIL-15-treated mice after i.t. infection with *M. tuberculosis* H37Rv. C57BL/6 mice were treated with rIL-15 or PBS during the contraction phase (22–42 days) of primary immune response to rBCG-OVA; on Day 91 after the inoculation, mice were treated with isoniazid in drinking water for 4 weeks to clear the live BCG bacteria, and then on Day 127, mice were i.t.-infected with 2×10^5 CFU of *M. tuberculosis* H37Rv. Mice were killed 4 weeks (A) or 10 weeks (B) after the infection, and the number of bacteria recovered from the lung and spleen was determined. The data are representative of three separate experiments and are expressed as means ± SD of five mice of each group. *, $P < 0.05$, significantly different from the value for nonimmunized mice.

tion of rIL-15 during the contraction phase enhanced the survival of antigen-specific $CD8^+$ T cells, especially the central memory phenotype ($CD62L^{high}CD44^{high}$), resulting in providing an increase of memory $CD8^+$ T cells. We have reported previously that in vivo administration of rIL-15 during the contraction phase during acute infection with *L. monocytogenes* enhanced survival of antigen-specific $CD8^+$ T cells, especially effectors ($CD62L^{low}CD44^{high}$), resulting in providing strong memory $CD8^+$ T cell responses [14]. These results are consistent with a previous report that during acute infection with *L. monocytogenes*, antigen-specific $CD8^+$ T cells differentiate rapidly into effectors ($CD62L^{low}CD44^{high}$), and $CD8^+$ T cells primed during infection with the attenuated pathogen rBCG-OVA differentiated primarily into the central memory phenotype ($CD62L^{high}CD44^{high}$) [17]. Recently, it has been reported that $CD8^+$ T contraction in acute infection involved only Bim, a preapoptic Bcl-2 homology 3-only protein, with no contribution by Fas, whereas both pathways were involved in $CD8^+$ T cell contraction in chronic infection [22]. Thus, the potency of IL-15 at the contraction phase may influence the fate of $CD8^+$ T cells in a different way between acute and chronic infection.

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 [23, 24]. Several vaccination strategies can be settled to induce protective memory $CD8^+$ T cells. We have reported recently that vaccination of bone marrow-derived dendritic cells pulsed with H2-M3 binding peptide TB2 [25] or MHC class Ia (H-$2D^b$)-binding peptide $MPT64_{190-198}$ [26] elicited antigen-specific $CD8^+$ T cells, leading to protection against i.t. infection with *M. tuberculosis* infection [27]. We reported previously that BCG immunization in IL-15 Tg mice showed an increase of antigen-specific $CD8^+$ T cells and elicited improved protection against *M. tuberculosis* [10]. Furthermore, we have reported that rBCG-Ag85B-IL15 induced stronger responses by antigen-specific $CD8^+$ T cells and robust protection in lung against i.t. challenge of *M. tuberculosis* [28]. In the present study, we show that in vivo administration of rIL-15 from Days 22 to 42 after BCG inoculation inhibited the contraction of effector $CD8^+$ T cells, resulting in an increase of antigen-specific memory $CD8^+$ T cells. However, it could not induce a long-lasting increase and consequently failed to elicit improved efficacy of vaccination against *M. tuberculosis* as compared with

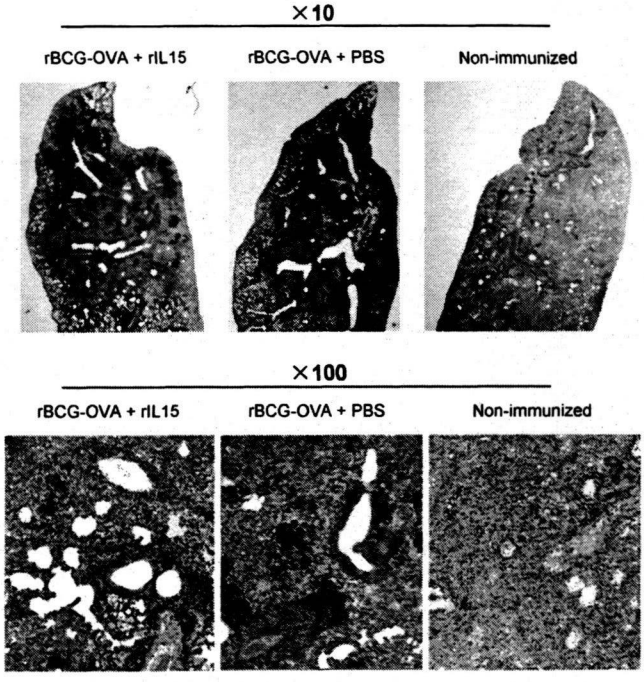

Figure 6. Histologic examination of lung tissues from rIL-15-treated mice after i.t. infection with *M. tuberculosis* H37Rv. C57BL/6 mice were treated with rIL-15 or PBS during the contraction phase (22–42 days) of primary immune response to rBCG-OVA; on Day 91 after the inoculation, mice were treated with isoniazid in drinking water for 4 weeks to clear the live BCG bacteria, and then on Day 127, mice were i.t.-infected with 2×10^5 CFU of *M. tuberculosis* H37Rv. Mice were killed 10 weeks after the infection, and histopathological changes of lung from rBCG-OVA-preinoculated rIL-15-treated; rBCG-OVA-preinoculated PBS-treated; or nonimmunized mice were examined. Formalin-fixed sections were stained with H&E. Original magnification, ×10 or ×100. An example from one of two separate experiments is shown.

BCG alone. Continuous release of IL-15 may be responsible for long-lasting CD8$^+$ T cell immunological memory in IL-15 Tg mice and mice inoculated with rBCG-Ag85B-IL15.

There are several lines of evidence in which IL-15 plays an important role in the homeostatic proliferation of antigen-specific memory CD8$^+$ T cells [29–31]. Therefore, it is possible that only short-term administration of IL-15 for inhibition of CD8$^+$ T cell contraction in the present study may not be enough for maintenance of long-lasting CD8$^+$ T cells providing improved protection against *M. tuberculosis*. Continuous release of IL-15 may be required not only for inhibition of contraction but also for homeostatic proliferation of memory CD8$^+$ T cells to induce long-lasting CD8$^+$ T cell immunological memory.

In conclusion, our results in the present study with in vivo administration of rIL-15 during the T cell contraction phase may reflect the important role of IL-15 in protecting antigen-specific CD8$^+$ T cell contraction during chronic infection with BCG. However, continuous expression of IL-15 after BCG immunization may be required to augment the efficacy of the vaccination against protection against *M. tuberculosis*.

ACKNOWLEDGMENTS

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ORIGINAL ARTICLE

A host-vector system for molecular study of the intracellular growth of *Mycobacterium tuberculosis* in phagocytic cells

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ABSTRACT

The mechanisms by which *Mycobacterium tuberculosis* survives and persists in phagocytic cells remain poorly understood. To study the question, a convenient and safe host-vector system is indispensable. In this study it has been shown that, in contrast with *M. smegmatis* strain mc^{2}155 which has been widely used for molecular analysis, *M. smegmatis* strain J15cs is able to survive even at day 6 post-infection in a murine macrophage cell line, J774. The survivability of J15cs was found to depend on the culture medium used for the bacteria prior to infection. Bacteria precultured on nutrient agar medium showed a high survivability and a characteristic cell wall ultrastructure. A plasmid vector, pYT923hyg, was developed from an *Escherichia coli*- mycobacterium shuttle vector pYT923 (previously constructed in our laboratory) to obtain three drug resistant genes (amp-, hyg- and km-resistant gene) and cloning sites in the km resistant gene. The vector pYT923hyg exerted no influence on *in vitro* growth of J15cs and intracellular survival in J774 cells, and was stably retained in J15cs after serial subculturing (three subcultures) in Luria-Bertani broth and at day 5 post-infection into J774 cells. Furthermore, using this system, the possibility of a relationship between some seemingly essential genes of *M. tuberculosis* and intracellular growth was demonstrated.

In this study, *M. smegmatis* strain J15cs and pYT923hyg were found to be capable of serving as an appropriate host-vector system for molecular study of the intracellular growth of *M. tuberculosis* in phagocytic cells; this system may be useful as a screening tool for *M. tuberculosis* genes.

Key words host-vector system, intracellular growth, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*.

M. tuberculosis is an important pathogen and remains a major cause of mortality worldwide. A problematic point in the eradication of tuberculosis is reactivation of pre-existing infection (1). It is known that the tubercle bacillus survives in phagocytic cells during the dormant stage of infection, and that the persistent form of the bacilli is not effectively killed by anti-tuberculosis agents (2). Therefore, in order to succeed in controlling tuberculosis, the mechanism of reciprocal conversion between resting and active forms must be understood. Many studies have attempted to develop *in vitro* models of *M. tuberculosis* persistent infection. Nutrient and oxygen starvation are used as models of persistent infection; however, these do not sufficiently reflect the intracellular environment (3). *M. tuberculosis* is a strong and slow-growing pathogen which spreads by airborne transmission. Accordingly, a safe and handy host-vector system is required for study of its intracellular growth. *M. bovis* BCG, *M. avium* and

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List of Abbreviations: Amp, ampicillin; Amp^r, ampicillin resistant; CFU, colony forming unit; *E. coli Escherichia coli;* EDL, electron-dense layer; EEDL, extra electron-dense layer; FCS, fetal calf serum; Hyg, hygromycin; Hyg^r, hygromycin resistant; Km, kanamycin; Km^r, kanamycin resistant; LB, Luria-Bertani; *M. Mycobacterium;* PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SDS, sodium dodecylsulfate; TEM, transmission electron microscope.

M. marinum, which are able to survive within phagocytes, have been used as host strains (4–6). However, they are also slow-growers. *M. smegmatis* strain mc^{2}155 (7), a rapid-growing mycobacterium, has been widely used as a host strain for molecular analysis of mycobacteria. However, the strain mostly lasts only a few days in phagocytic cells (8–10). A proper host-vector system for study of intracellular growth of, and persistent infection with, *M. tuberculosis* has never been proposed.

Previously, we have reported construction of a host-vector system (and variations) in mycobacteria. The vectors (pYT series) of this system originate from a plasmid pMSC262 (5, 11). Qin *et al.* have reported that pYT937 replicates and is stable in *M. bovis* BCG, and pYT923 replicates in both *M. bovis* BCG and *M. smegmatis* strain J15cs but not in mc^{2}155 (11). In the course of studies using host-vector systems, we found that the strain J15cs had a tendency to survive in the murine macrophage cell line, J774. The plasmid pYT923, which can replicate in J15cs, has only one drug resistant gene (Km^r), thus it is inappropriate as a cloning vector. Furthermore, retention of a vector plasmid in a host strain is necessary for genetic study (12, 13). However, the stability of pYT923 in J15cs has never been examined.

In this paper, we have demonstrated intracellular survival of J15cs in J774 cells, developed pYT923 into a useful vector, pYT923hyg, and examined the stability of pYT923hyg in liquid media and in J774 cells. We have also shown that some genes of *M. tuberculosis* may be related to intracellular growth using this host-vector system.

MATERIALS AND METHODS

Bacterial strains and vector plasmids

M. smegmatis strain J15cs (11) derived from strain Jucho (14, 15), and strain mc^{2}155 derived from ATCC607 (7) were used in this study. *M. tuberculosis* strain H37Rv was used as a donor of the genes of Rv1388, Rv1389 and Rv1390. *E. coli* K12 strain KP7600 (16) and *M. smegmatis* strain J15cs were used as recipient strains for transformation.

An *E. coli*-mycobacterium shuttle vector pYT923 (Km resistant: Km^r, *kan* gene) (5) and an *E. coli* plasmid pHP45Ωhyg (Amp and Hyg resistant: Amp^r and Hyg^r, *amp* and *hyg* genes) (17) were used for construction of a modified shuttle vector pYT923hyg. A shuttle vector pYUB76 (Km^r) (18), which was derived from pAL5000, was used as a control for plasmid stability.

In vitro phenotypes and preparation of bacterial single cell suspensions

Colony morphology was observed after culturing on nutrient agar medium (Nissui Pharmaceutical, Tokyo, Japan) for 3 days at 37°C. The tendency to aggregation was monitored by culture in LB broth for 3 days without shaking. Hydrophobicity of the strain was examined by applying droplets of either water or oil to bacterial lawns, as previously reported (19).

Bacterial single cell suspensions were prepared as follows. *M. smegmatis* strains were precultured on either nutrient agar or 1% Ogawa egg medium (Nissui Pharmaceutical) for 7 days. Bacterial cells were suspended in PBS, and passed through a 5-μm-pore-sized syringe filter (Millipore, Billerico, MA, USA) in order to remove clumped cells. Then, single cell suspensions were prepared at a concentration of approximately 1×10^8 CFU/ml.

Intracellular growth

A J774 murine macrophage-like cell line, J774–1, was donated by the Center for Biomedical Research Institute of Development, Aging, and Cancer of Tohoku University, Japan. The cells were maintained in RPMI 1640 medium (Nissui Pharmaceutical) supplemented with 10% inactivated FCS (GIBCO, Grand Island, NY, USA) at 37°C in 5% CO_2.

For the infection experiments, the J774 cells were grown to semi-confluence for 48 hr on Lab-Tek chamber slides (Nunc, Naperville, IL, USA) and 24-well flat-bottom tissue culture plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA), which were used for Ziehl-Neelsen staining and counting CFU, respectively. A bacterial single-cell suspension of approximately 1×10^7 CFU/well was inoculated into the J774 cells at an approximate multiplicity of infection of 10. After co-cultivation for 3 hr, the infected J774 cells were washed twice with PBS in order to remove nonphagocytized bacteria (20). In order to kill any remaining extracellular bacteria, the infected J774 cells were further incubated for 2 hr with fresh culture medium supplemented with 5% inactivated FCS and 200 μg/ml of amikacin (Sigma Chemicals, St. Louis, MO, USA) (6). The culture medium was then replaced with fresh culture medium with 2 μg/ml of amikacin (this time point was deemed day 0), and the infected cells cultured for 6 days.

The intracellular morphology and survival of mycobacteria were evaluated by Ziehl-Neelsen staining and counting CFU, respectively. The adherent cells were treated with 1% Triton-X100 and a rubber policeman and the lysates plated on nutrient agar medium. The same experiments were repeated five times, and statistical analysis was carried out using Dunnett's test. The survival rates at appropriate

time points were calculated as follows: day 0 CFU mean was deemed 100% and the following days' CFU means were calculated as percentages of the day 0 mean.

Transmission electron microscopy

The bacteria cultured on nutrient agar and Ogawa egg mediums for 7 days were then suspended in PBS. These suspensions, and the infected J774 cells at day 3 post-infection, were fixed with 2% glutaraldehyde and 1% osmium tetroxide. After dehydration, embedding and the sectioning process, the sections were stained with uranyl acetate and lead citrate, and then observed using a JEOL JEM-1200EX (21).

Construction of pYT923hyg, pYT923hyg-Rv1388 and pYT923hyg-Rv1388-Rv1389-Rv1390

A modified shuttle vector, pYT923hyg, was constructed as follows. A vector pYT923 (Km^r) was digested with *Pst*I restriction enzyme and ligated into the *Pst*I site in the *amp* gene of an *E. coli* plasmid pHP45Ωhyg (Hyg^r, Amp^r). Ligation mixture transformed *E. coli* KP7600, and transformants with Km (50 μg/ml), Hyg (200 μg/ml), and Amp (50 μg/ml) resistance were selected. The modified vector plasmid which was purified from *E. coli* transformants and designated as pYT923hyg, had *kan* and *hyg* genes and a regenerated *amp* gene.

A recombinant plasmid, pYT923hyg-Rv1388, was constructed as follows. A 916-bp fragment containing the Rv1388 gene and the putative promoter from *M. tuberculosis* H37Rv was amplified by the PCR method using a primer set involving nucleotide sequence of restriction enzyme *Eco*T22I site (Sf: 5′-TGTCCGATGATGCATAGCAC-3′ and Sr:5′-TTCGGCATGCATTGTGAGCT-3′ [*Eco*T22I sites are underlined]) and digested by the *Eco*T22I enzyme. The digested PCR product was ligated into the *Eco*T22I site in the *kan* gene of pYT923hyg. Ligation mixture transformed *E. coli* KP7600 and transformants were selected by Amp^r, Hyg^r and Km^s. With the same protocol, a recombinant plasmid pYT923hyg-Rv1388-Rv1389-Rv1390 was constructed to clone a 2090-bp fragment containing the Rv1388, Rv1389, and Rv1390 genes and the putative promoter was amplified using primers Lf and Lr; Lf (5′-GTCAGGGCAATGTCCGATGATGCATAGCAC-3′) and Lr (5′-GTTGACGAACAACCATGCATGCCTTGTAGG-3′).

The transformants, J15cs/pYT923hyg, J15cs/pYT923hyg-Rv1388, J15cs/pYT923hyg-Rv1388-Rv1389-Rv1390 and J15cs/pYUB76, were constructed by electroporation (5) using a Gene Pulser electroporator (Bio-Rad, Richmond, CA, USA) and selected with appropriate antibiotics. In order to exclude spontaneous resistant mutants and confirm the existence of the vector pYT923hyg, the PCR method was examined by using the primers Hf (5′-CCGCGCAGATCACTTCCAACAATT-3′) and Hr (5′-GTCATTCAAAAGGTCATCCACCGG-3′), which were engineered to amplify a 350-bp fragment including a part of *hyg* gene and the neighbor region of pHP45Ωhyg.

Plasmid stability assay

Plasmid stability was determined according to the method reported by Chiba (22) with modifications. J15cs/pYT923hyg, J15cs/pYT923hyg-Rv1388, and J15cs/pYUB76 were grown on nutrient agar medium with appropriate antibiotics for 72 hr, and then the bacterial cells were suspended in PBS. The cell suspensions were filtrated through 5-μm filter and prepared at a concentration of approximately 1×10^8 CFU/ml. Each cell suspension was diluted 1:100 in antibiotic-free LB broth supplemented with 0.05% Tween80, and incubated for 48 hr. The culture broth was filtrated through a 5-μm filter, and 0.1 ml of the filtrate inoculated into 10 ml antibiotic-free LB-broth with 0.05% Tween80 and incubated for another 48 hr. Serial subculturing was conducted three times. CFU were counted for each culture on nutrient agar medium either with or without appropriate antibiotics. Plasmid stability was determined as the proportion of CFU on nutrient agar medium with antibiotics to that on the medium without antibiotics. Stability of the plasmids within J774 cells was examined at day 5 after infection. The examination was conducted using the same method as described in the intracellular growth experiment.

RESULTS

Hydrophobic properties of *M. smegmatis* strain J15cs

After being on nutrient agar medium for 3 days at 37°C, strain J15cs formed rough and dry colonies, in contrast to the smooth and wet colonies of mc^2155 (Fig. 1a). The edges of the J15cs colonies were irregular, while those of mc^2155 were smoothly curved. The morphological phenotype of J15cs colonies was more obvious on nutrient agar than on Ogawa egg medium. Also in LB broth, J15cs easily formed aggregative clumps and in PBS it was hard to make a single cell suspension of it, whereas mc^2155 did not aggregate (Fig. 1b). When a droplet of water and a droplet of oil were applied to bacterial lawns, the drop of water on the lawn of J15cs formed a spherical bead while the drop of oil spread over the surface (Fig. 1c). In contrast, on the lawn of mc^2155, water did not make a spherical bead and

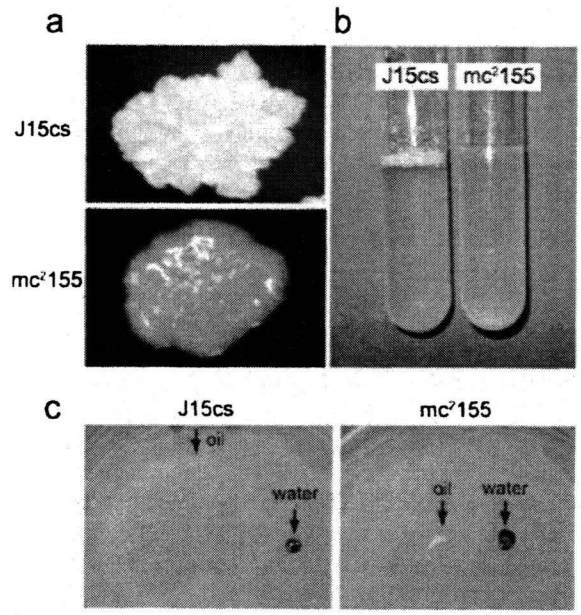

Fig. 1. Comparison between *M. smegmatis* strains J15cs and mc²155 of phenotypic and hydrophobic characteristics. Bacteria were grown aerobically (a) on nutrient agar medium and (b) in LB broth for 3 days at 37°C. (c) To examine hydrophobicity, a droplet (10 μl) of oil and a droplet of water containing trypan blue were applied to the surface of bacterial lawn. Arrows indicate the rims of the droplets.

oil did not spread. These results showed that J15cs has hydrophobic characteristics.

Intracellular survival of strains J15cs and effect of the preculture medium

The intracellular survival of strain J15cs was examined using a murine macrophage cell line, J774, and mc²155 as the control (Fig. 2). J15cs and mc²155 were cultured on nutrient agar medium (J15cs-NA and mc²155-NA) and Ogawa egg medium (J15cs-Ogawa and mc²155-Ogawa) for 7 days, and single cell suspensions (approximately 1×10^7 CFU/well) were infected into J774 cells. To kill all extracellular bacilli we used a high concentration of amikacin (200 μg/ml) for 2 hr, and to prevent extracellular growth a low concentration of amikacin (2 μg/ml) (6). This drug and these concentrations were chosen based on tests of the susceptibility of J15cs to seven antibiotics, namely arbekacin, ribostamycin, spectinomycin, tobramycin, amikacin, gentamicin, and streptomycin. Amikacin prevented bacterial growth on nutrient agar medium at 50 μg/ml and in RPMI1640 medium at 2 μg/ml, and had no morphological effects on intracellular bacilli (data not shown). CFU of intracellular bacilli at 0, 2, 4, and 6 days post-infection are shown in Fig. 2a. When J15cs-NA was inoculated, intracellular CFU were 3.5×10^5, 2.8×10^5, 1.2×10^5, and 7.7×10^4 CFU/well at 0, 2, 4, and 6 days post-infection, respectively. In the case of mc²155-NA, intracellular CFU were 2.5×10^5, 4.2×10^4, 9.0×10^3, and 6.2×10^2 CFU/well. On the other hand, when J15cs-Ogawa was inoculated, the intracellular CFU were 1.7×10^5, 1.3×10^5, 4.6×10^4, and 1.6×10^3 CFU/well. In the case of mc²155-Ogawa, intracellular CFU were 1.2×10^5, 1.5×10^4, 7.5×10^2, and 3.4×10 CFU/well.

The percentage ratios of the intracellular CFU to that of day 0 are shown in Fig. 2b. Those of J15cs-NA were 80.0%, 35.0% and 22.0% at 2, 4 and 6 days post-infection, and those of mc²155-NA 17.0%, 3.6% and 0.2%, respectively. The ratios of J15cs-Ogawa were 73.0%, 25.9%, and 0.9%, and those of mc²155-Ogawa 12.0%, 0.6%, and 0.02%, respectively. At 2 days post-infection, a significant difference was observed between J15cs and mc²155. Furthermore, at 6 days post-infection, strain J15cs-NA was the only one with any outstanding survivors.

The intracellular morphologies of J15cs-NA and mc²155-NA in J774 cells were observed by Ziehl-Neelsen staining (Fig. 2c). At day 0, J15cs-NA showed its typical rod shape and was distributed unevenly throughout the phagocytic cells. However, by day 3 J15cs-NA had elongated and it continued to elongate through day 6, displaying mass formations by day 6. This observation suggests that despite the decrease in CFU, J15cs may continue to multiply to a degree in J774 cells. In contrast, at day 3 fewer mc²155 were seen under light microscopic observation and they had gone from the J774 cells by day 6.

Ultrastructure of the cell wall after preculture on nutrient agar and Ogawa egg mediums

The ultrastructure of the cell walls of J15cs and mc²155 precultured on nutrient agar and Ogawa egg mediums for 7 days were observed by TEM (Fig. 3). J15cs-NA showed an EEDL (Fig. 3a) compared to mc²155-NA possessing one thick EDL (Fig. 3b). When the bacteria were cultured on Ogawa egg medium, neither strain showed any EEDL (Fig. 3c and d).

Construction and stability of a shuttle vector and recombinant plasmids

A shuttle vector, pYT923hyg (11.9 kb), was constructed by combining a shuttle vector pYT923 (7.3 kb) and an *E. coli* plasmid pHP45Ωhyg (4.6 kb) to get additional selection markers and useful cloning sites (Fig. 4). As a result, pYT923hyg possessed a *kan* gene, a *hyg* gene, and a regenerated *amp* gene. The *Eco*T22I restriction sites in the *kan* gene are useful for cloning of foreign DNA. Using

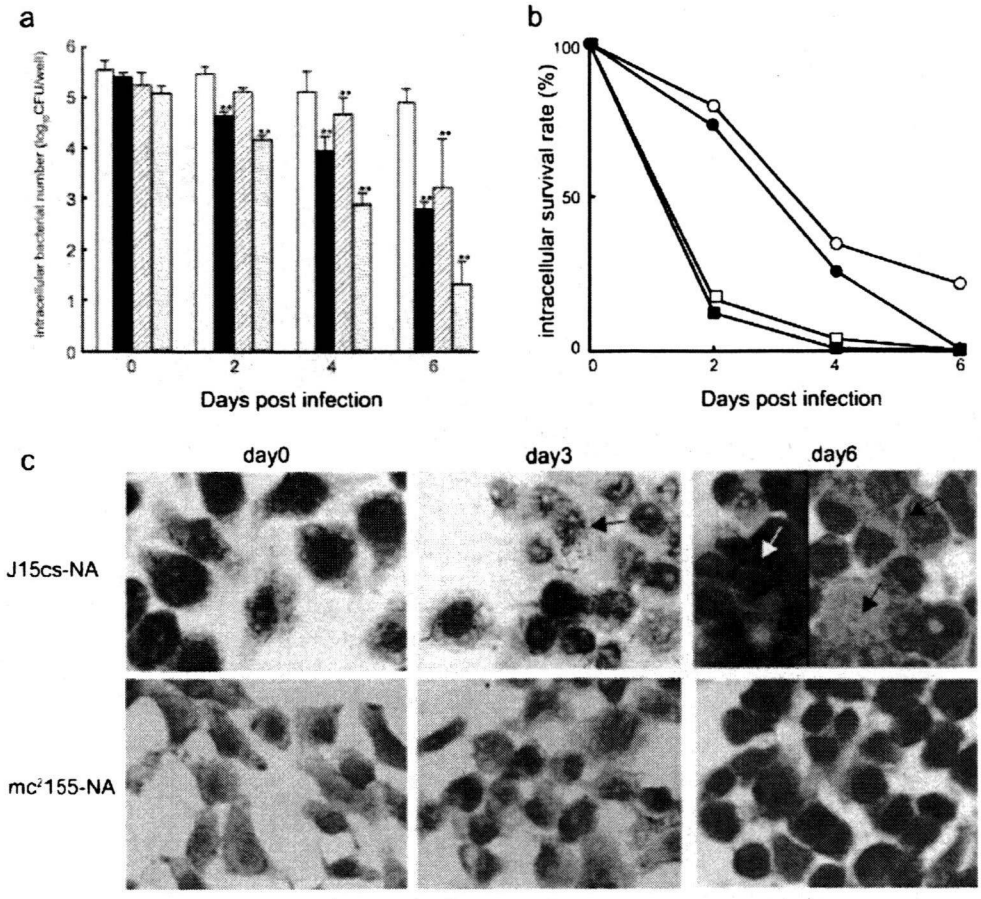

Fig. 2. Intracellular survival of J15cs and mc²155 within J774 cells. Bacteria were precultured on either nutrient agar or Ogawa egg medium for 7 days, and inoculated into J774 cells. (a) CFU/well at day 0, 2, 4, 6 post-infection. Data represent the means ± one standard deviation of five independent experiments. The double asterisks indicate statistically significant differences between J15cs-NA and others ($P < 0.01$). Open bars, J15cs-NA; closed bars, mc²155-NA; slashed bars, J15cs-Ogawa; dotted bars, mc²155-Ogawa. (b) The percentage ratios of CFU compared to that of day 0. Data represent the mean of five independent experiments. Open circles, J15cs-NA; open squares, mc²155-NA; closed circles, J15cs-Ogawa; closed squares, mc²155-Ogawa. (c) Ziehl-Neelsen staining of J15cs-NA and mc²155-NA infected J774 cells at day 0, 3, 6 post-infection. The white arrow indicates a bacterial mass. Black arrows indicate long rod-shaped bacteria.

this vector, we constructed a recombinant plasmid, pYT923hyg-Rv1388, by cloning a 916 bp-fragment containing the Rv1388 gene and the putative promoter region of *M. tuberculosis*. The Rv1388 gene is correspondent to the *mIHF* gene of *M. smegmatis*.

The vector pYT923hyg and the recombinant plasmid pYT923hyg-Rv1388 had no influence on the growth rate of J15cs in LB broth. The doubling times of the transformants, J15cs/pYT923hyg and J15cs/pYT923hyg-Rv1388, were approximately 4 hr, similar to that of the host strain J15cs (data not shown). After serial subculturing (three subcultures) in LB broth, populations of pYT923hyg and pYT923hyg-Rv1388 carrying cells were 99.3% and 96.3% of the total cell number, respectively (Fig. 5a). However, pYUB76 was retained at less than 0.1%. Furthermore, pYT923hyg and pYT923hyg-Rv1388 had no effect on intracellular survival of J15cs in J774 cells (data not shown). The stability of the plasmids in J774 cells was examined at day 5 post-infection (Fig. 5b). The percentages of plasmid-carrying cells of J15cs/pYT923hyg and J15cs/pYT923hyg-Rv1388 were 100% and 98%, respectively. On the other hand, that of J15cs/pYUB76 was only 7.2%. Thus, pYT923hyg and pYT923hyg-Rv1388 in J15cs were stable both in liquid medium and in J774 cells, in contrast to pYUB76.

In addition, TEM revealed that J15cs/pYT923hyg was indeed phagocytosed in cytoplasmic vacuoles and showed an EEDL within J774 cells (Fig. 5c).

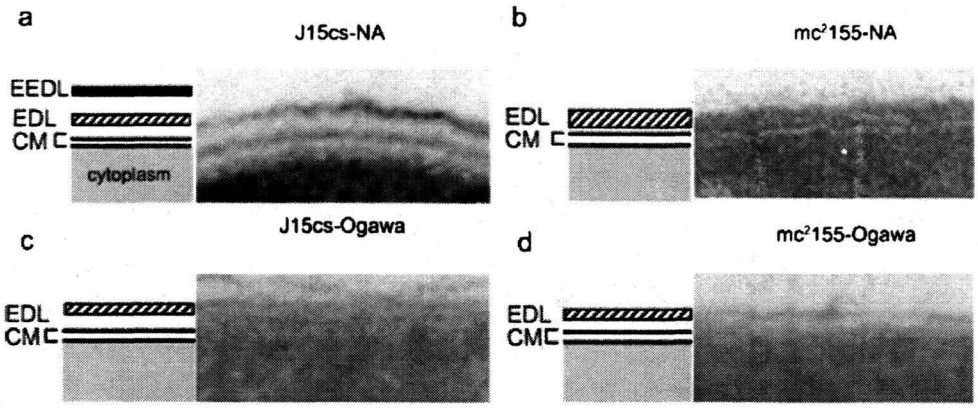

Fig. 3. Transmission electron micrographs of the cell wall structures of J15cs and mc²155. (a) J15cs-NA, (b) mc²155-NA (c) J15cs-Ogawa and (d) mc²155-Ogawa were cultured for 7 days at 37°C. CM, cytoplasmic membrane; EDL, electron dense layer; EEDL, extra electron dense layer.

Genes of *M. tuberculosis* which could affect intracellular survival

When we cloned a fragment of size 2090-bp containing Rv1388-Rv1389-Rv1390 genes and the putative promoter, the transformant J15cs/pYT923hyg-Rv1388-Rv1389-Rv1390 showed peculiar features in J774 cells. The Rv1390 gene is probable *rpoZ*. The Rv1388, Rv1389 and Rv1390 genes may form one putative operon. In *in vitro* growth the transformant showed no differences from other J15cs transformant, such as colony morphology on agar media and growth rate in the LB broth (data not shown). The intracellular bacteria at day 0 showed similar cell morphology and acid-fastness in all cases (Fig. 6). However, after 3 days of incubation, intracellular bacilli of J15cs/pYT923hyg-Rv1388-Rv1389-Rv1390 showed degeneration in some features, developing for example shortness and weak acid-fastness; while J15cs/pYT923hyg and J15cs/pYT923hyg-Rv1388 exhibited elongation and pronounced acid-fastness. Using this host-vector system, it has become possible to observe the effect of the tuberculosis genes on J15cs within phagocytic cells.

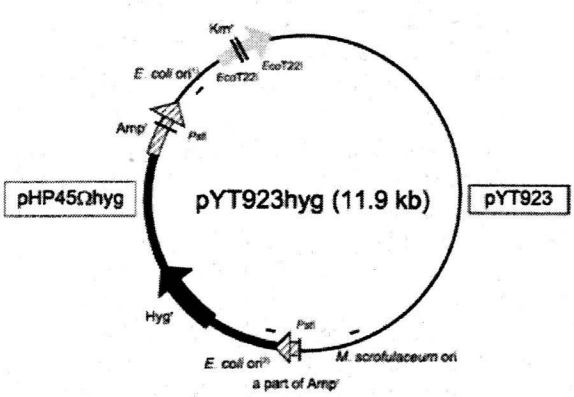

Fig. 4. Construction of a shuttle vector pYT923hyg. A vector pYT923hyg was constructed by ligation at the *PstI* site of pYT923 and pHP45Ωhyg. The vector carried Amp^r, Km^r and Hyg^r genes, and had cloning sites of *Eco*T22I restriction site in Km^r gene. *E. coli* ori [1] and *E. coli* ori [2] are the replication origins of pACYC177 and pHP45Ωhyg, respectively.

DISCUSSION

In this paper, we have demonstrated that *M. smegmatis* strain J15cs and pYT923hyg is a safe and convenient host-vector system for the study of molecular mechanisms of intracellular survival and persistent infection of *M. tuberculosis* in phagocytic cells.

We found the host strain J15cs to be highly hydrophobic and to easily aggregate in comparison to other *M. smegmatis* strains such as ATCC607 (i.e., the original strain of mc²155), PM5, and Rabinowitchi (23). Besides, it was able to survive well in phagocytic cells J774, unlike other strains of *M. smegmatis*. We originally obtained it as a highly transformable mutant of the *M. smegmatis* strain Jucho. The strain Jucho has a complicated identification history. It was initially reported as *M. avium* (14, 15) and later identified to be *M. smegmatis* by biochemical characterization (24). As genetic identification has not been performed until now, we conducted target PCR using *Mycobacterium* genus-specific primers of the 16S rRNA gene (25) and PCR-RFLP analysis of the *hsp*65 gene (26). This data signifies that J15cs is *M. smegmatis* (data not shown).

In contrast, the *M. smegmatis* strain mc²155, which has been commonly used for molecular study of mycobacteria, has been reported to disappear from phagocytic cells within a few days (8–10). Sharbati-Tehrani *et al.* have reported that a deletion mutant of *mspA* together

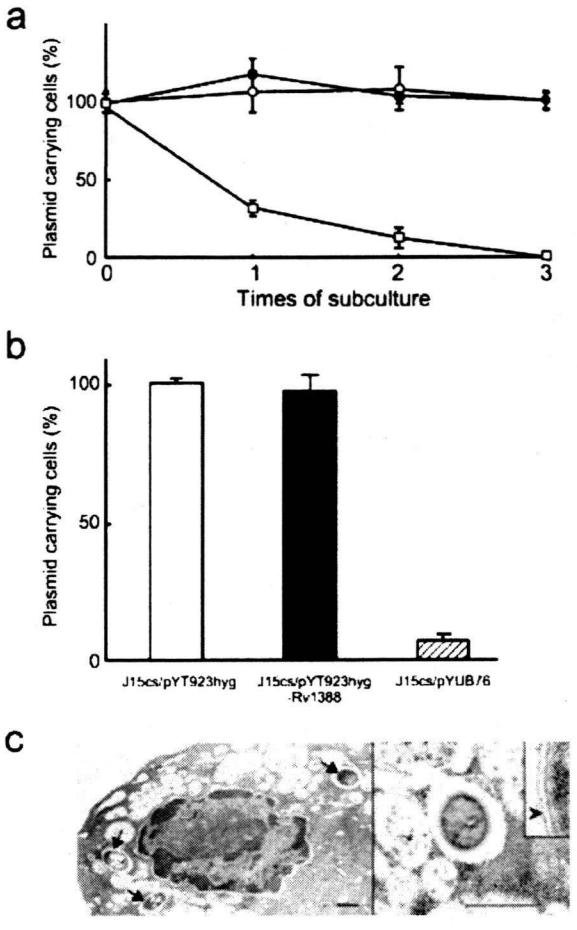

Fig. 5. Stability of plasmids in strain J15cs. (a). In liquid medium: J15cs/pYT923hyg, J15cs/pYT923hyg-Rv1388 and J15cs/pYUB76 were grown in LB broth without antibiotic selection during serial subculturing (three subcultures). Open circles, J15cs/pYT923hyg; closed circles, J15cs/pYT923hyg-Rv1388; open squares, J15cs/pYUB76. (b) Within J774 cells: J15cs/pYT923hyg, J15cs/pYT923hyg-Rv1388 and J15cs/pYUB76 were infected into J774 cells and incubated for 5 days. Open bar, J15cs/pYT923hyg; closed bar, J15cs/pYT923hyg-Rv1388; slashed bar, J15cs/pYUB76. (c) TEM of J15cs/pYT923hyg within J774 cells. The percentage of plasmid-carrying cells means the ratio of numbers antibiotic-resistant bacteria to numbers of total bacteria. Data represent the means ± one standard deviation of three independent experiments. J15cs/pYT923hyg in J774 cells at day 3 post-infection. Arrows show phagocytosed bacteria in the vacuoles. Arrow head indicates EEDL. Bars, 0.5 μm.

with *mspC* of *M. smegmatis* mc^{2}155 enhances intracellular persistence in the mouse macrophage cell line J774A.1 (9). At 54 hr post infection, the mutant strain showed 12.7% of bacterial load compared to that at 6 hr, while the wild type mc^{2}155 showed 2.7%. In this study, when the bacteria were precultured on nutrient agar medium, 80% of the strain J15cs had survived at day 2, and 22% even at day 6. Moreover, though the survival ratio of day 6 was only 22%, Ziehl-Neelsen staining showed significantly elongated and multiplicated intracellular bacilli forming masses (Fig. 2c). The gap between the CFU counts and this microscopic observation seems to be partly attributable to the aggregation characteristics of J15cs. In order to clarify this inconsistency, we used sonication and 0.05% Tween80 after treatment with Triton-X100 to break the aggregates into single cells. However, as bacterial clumps were still observed by phase contrast microscopy (data not shown) after this procedure, we could not exclude the possibility that CFU were underestimated.

It is noteworthy that we found that culture conditions prior to infection are significant with respect to subsequent intracellular survival. Nutrient agar medium was more appropriate than Ogawa egg medium, and 7 day- and the 14 day-culture on nutrient agar medium was more successful than 2 and 3 day-cultures (data not shown). Additionally, J15cs precultured on 7H11 agar medium, in 7H9 and in LB broth also showed similar time courses to bacilli on Ogawa medium (data not shown).

TEM showed that, like *M. tuberculosis* (27) but unlike mc^{2}155 (Fig. 3), only J15cs-NA had an EEDL in the outmost layer of the cell wall structure,. The relationship between cell wall structure and intracellular survival is an interesting topic. Singh *et al.* have reported that the deletion mutant of the *mymA* gene of *M. tuberculosis* shows (i) increased sensitivity to SDS, (ii) alteration in the cell wall ultrastructure and (iii) reduced ability to survive in activated macrophages (28). J15cs also presents extreme SDS resistance, being able to thrive on nutrient agar medium supplemented with 0.0075% SDS, whereas mc^{2}155 hardly grew (data not shown). In addition, J15cs showed restricted sliding motility on the surface of 0.3% agarose plates and pellicle formation on the surface of liquid mediums similarly to *M. tuberculosis*, and differently from mc^{2}155 (data not shown) (29, 30).

Regarding the vector, pYT923hyg has two *E. coli* replication regions: the p15A replicon, carried by pACYC177 and the pMB1 replicon, carried by pHP45Ωhyg. These replicons are compatible (31). Some shuttle vectors derived from pMSC262 have been reported to delay the growth of transformants containing the vectors (12). However, such a phenomenon was not observed in our study. The vector pYT923 has been reported to be unable to replicate in mc^{2}155 (11), and the vector pYT923hyg to be stably maintained in J15cs in a liquid medium and in J774 cells. Fortunately, recombinant plasmids containing the Rv1388 and Rv1388-Rv1389-Rv1390 genes (approximately 1kb and 2kb) of *M. tuberculosis* H37Rv are also stable compared to pYUB76 in spite of their large size. The Rv1388 gene is correspondent to the *mIHF* gene of *M. smegmatis*; the protein mIHF is expressed during a

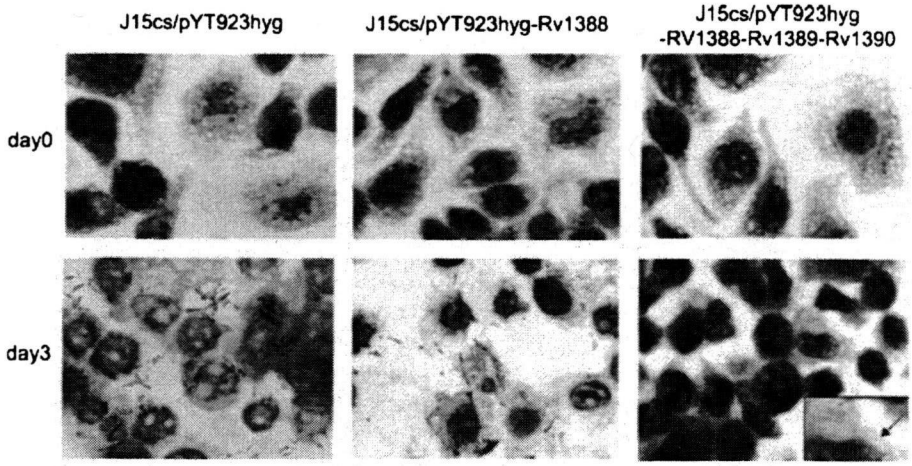

Fig. 6. Intracellular survival as shown by light microscopy of Ziehl-Neelsen stained slides. J15cs/pYT923hyg, J15cs/pYT923hyg-Rv1388, and J15cs/pYT923hyg-Rv1388-Rv1389-Rv1390 within J774 cells at day 0 and day 3 post-infection. Arrow indicates degenerated morphology of the transformant J15cs/pYT923hyg-Rv1388-Rv1389-Rv1390.

period of phase conversion from exponential to stationary growth (32). Moreover, as determined by DNA microarray analysis the Rv1388 gene is up-and down-regulated during nutrient starvation (33). It seems that the genes may be related to growth phase variation. The functions have not been clarified yet, because they are essential (32, 34, 35) and the knockout mutant has thus far not been obtained. Furthermore, Rv1388 may form one putative operon with Rv1389 and Rv1390 genes, whose functions are still unknown.

Our experiment using our host-vector system has shown that the putative operon consisting of the Rv1388-Rv1389-Rv1390 genes influences the intracellular morphology of J15cs in J774 cells, while it has no obvious effect on *in vitro* growth. Although further studies are necessary to establish the precise role of each gene, we were able to screen the genes easily using our host-vector system.

Even though clumping may be common with *M. smegmatis* J15cs, it is a rapid-growing mycobacterium, an extremely weak pathogen, and can survive in phagocytic cells. Therefore, the host-vector system consisting of *M. smegmatis* J15cs and pYT923hyg provides a helpful tool for molecular study of the tuberculosis genes which may be related to intracellular growth in phagocytic cells. In addition, this system may be especially useful for the screening of essential genes expressed in the intracellular environment.

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RESEARCH ARTICLE

Comparable studies of immunostimulating activities *in vitro* among *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) substrains

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bacillus Calmette–Guérin; cytokine; trehalose 6,6′ dimycolate; tuberculosis.

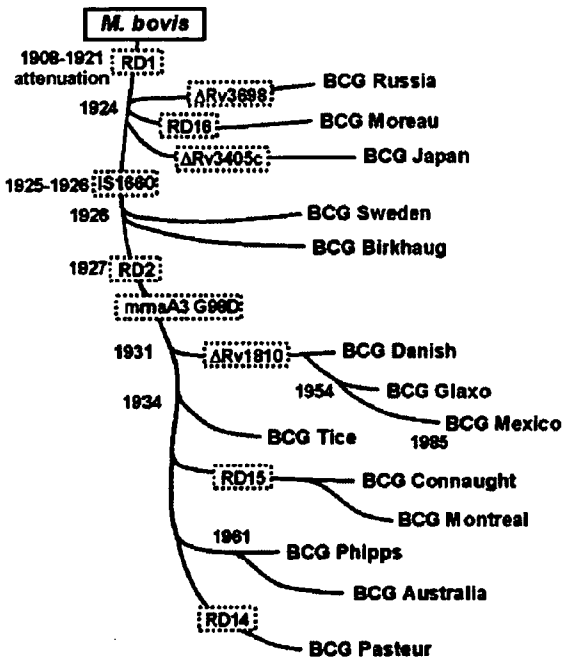
Abstract

During the serial passage of *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) in different countries after initial seed distribution from the Pasteur Institute, specific insertions and deletions in the genome among BCG substrains were observed and speculated to result in differences in immunological activities. 'Early-shared strains' of BCG (Russia, Moreau, Japan, Sweden, Birkhaug), distributed by the Pasteur Institute, which conserve three types of mycolate (α, methoxy, keto) in cell wall, exhibited stronger activities of induction of nitric oxide, interleukin-1β (IL-1β), IL-6, IL-8, IL-12 and tumor necrosis factor (TNF)-α, from human epithelial cell line A549, human myelomonocytic cell line THP-1 and mouse bone marrow cells in the presence of interferon-γ (IFN-γ) than did 'late-shared strains' of BCG (Danish, Glaxo, Mexico, Tice, Connaught, Montreal, Phipps, Australia, Pasteur). The stronger induction of IL-12 and TNF-α in the presence of IFN-γ was also observed by trehalose 6,6′-dimycolate (TDM) extracted from BCG-Japan than by TDM from BCG-Connaught, which lacks the methoxymycolate residue. These results suggest that 'early-shared strains' are more potent immunostimulating agents than 'late-shared strains', which could be attributed partially to methoxymycolate. Our study provides the basic information for immunological characterization of various BCG strains and may contribute to a re-evaluation of them as a reference strain for vaccination against tuberculosis.

Introduction

Bacillus Calmette–Guérin (BCG) is an attenuated strain of *Mycobacterium bovis*. After extensive tests in animals, BCG was first used as a vaccine in 1921. Today, it is estimated that > 3 billion people have been vaccinated with BCG (Andersen & Doherty, 2005). BCG vaccine strains were formerly distributed by the Pasteur Institute and subcultured in different countries. More than 14 strains of BCG are subcultured and used as BCG vaccine in different parts of the world (Scheme 1). Recently, Brosch *et al.* (2007) analyzed the genetic differences among BCG strains. They classified BCG-Russia, -Moreau and -Tokyo as Group I strains. Group I strains consist of 'early-shared strains,' in which the original characteristics of 'authentic Pasteur' were expected to be conserved because there is less insertion and mutation in the genome of the bacilli. Of note, among 'early-shared strains', BCG-Moreau lacks the region of difference (RD) 16 region in the genome, BCG-Russia lacks ΔRv3698 and *recA* regions (Keller *et al.*, 2008) and BCG-Tokyo lacks Rv3405c (Honda *et al.*, 2006). BCG-Tokyo induces a significantly higher level of Th1-cytokines [interferon-γ (IFN-γ), tumor necrosis factor (TNF)-α, interleukin-12 (IL-12)] and a lower secretion of the IL-4, a Th2-cytokine, accompanied by greater $CD4^+$ and $CD8^+$ T-cell proliferation, than did BCG-Danish (Davids *et al.*, 2006).

The World Health Organization (WHO) recommends a prime/boost strategy for tuberculosis vaccine. In a field



Scheme 1. Genealogy of BCG vaccine substrains. The scheme displays *Mycobacterium bovis*, the original strain of BCG vaccine, and the series of the genomic alternation of BCG substrains including the deletions of RD, single-nucleotide polymorphisms and duplications of genomic regions. The figure has been revised from Behr *et al.* (1999), Brosch *et al.* (2007) and Keller *et al.* (2008).

study, the efficacy of BCG vaccination has been controversial. According to many reports of a field trial of vaccine efficacy of BCG against tuberculosis, WHO does not recommend the revaccination of BCG for tuberculin-negative individuals and the vaccine efficacy against adult-type tuberculosis has been debated (WHO, 1995, 1999). However, WHO recommends vaccination of BCG for newborn babies to prevent tuberculosis, especially meningitis and non-lung-type tuberculosis. WHO also recommends replacement of the international reference strain of BCG vaccine; therefore, in 2005 in Paris, three candidates (Danish-1331, Tokyo-172 and Russia) were selected (WHO, 2005). For the re-evaluation of BCG vaccine for use as an international standard, not only an animal model but also an *in vitro* system is needed, because it would contribute to the quality control of BCG vaccine and production of vaccine in good manufacture practice grade condition. We have previously reported an *in vitro* evaluation system of mycobacterial virulence using human origin cell line (Takii *et al.*, 2001).

An important and defining characteristic of mycobacteria is their capacity to synthesize long-chain β-hydroxy, α-alkyl fatty acids, known as mycolic acids. One type of mycolic acid, containing an α-methyl branched methyl ether, is known as methoxymycolic acid (Barry *et al.*, 1998). Belley *et al.* (2004) proved the importance of *mma3* gene in methoxymycolate synthesis by complementing BCG-Pasteur with the wild-type *mma3* gene from *Mycobacterium tuberculosis*. Behr *et al.* (2000) reported that a single nucleotide polymorphism occurred in *mma3* between 1927 and 1931, resulting in loss of methoxymycolic acid production in BCG substrains obtained after that period.

Specific deletion of the RD region was reported in the genome of BCG (Behr *et al.*, 1999) and recently Brosch *et al.* (2007) reported the comprehensive analysis of the genome in BCG vaccine strains. However, the genetic deletions described have not been directly linked to phenotypic changes and immunological characteristics, and therefore their implications in the attenuation process have not been well elucidated. As we expected that the innate immunity and adjuvant characteristics would be different among BCG substrains, in this study we measured the immunostimulating activities of BCG substrains and investigated the relationship *in vitro* between genetic background and immunological activities of the strains.

Materials and methods

Bacterial strains

Mycobacterium bovis BCG strains, Australia (ATCC 35739), Birkhaug (ATCC 35731), Connaught (ATCC 35745), Danish (ATCC 35733), Glaxo (ATCC 35741), Mexican (ATCC 35738), Montreal (ATCC 35735), Pasteur (ATCC 35734), Phipps (ATCC 35744), Tice (ATCC 35743), Russia (ATCC 35740) and *Mycobacterium phlei* (ATCC 11758) were purchased from America Type Culture Collection (ATCC, Manassas, VA). BCG-Moreau was provided by Dr Takahashi [The Research Institute of Tuberculosis Japan Anti-tuberculosis Association (Kiyose, Tokyo, Japan)]. BCG-Sweden (vaccine seed) was provided from National Institute of Infectious Disease Japan, Tokyo, Japan. BCG-Japan (Tokyo-172) was purchased from BCG Japan Co. Ltd (Tokyo, Japan).

Bacterial culture and freeze stock

Bacterial cultures were grown in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 10% ADC [5% bovine serum albumin (fraction V), 2% dextrose and 0.005% bovine liver catalase] and 0.05% Tween 80 at 37 °C under static conditions. Bacteria were grown at an optimal density at 530 nm of 0.6–0.8. Then, cultures were aliquoted and stored at −70 °C until needed. The CFU of the aliquots were determined by colony assay on 7H11 agar. Bacterial culture medium was prepared in endotoxin-free materials. Heat-killed bacteria were prepared by heating to 60 °C for 30 min.

Cell culture

The human pulmonary type II alveolar epithelial cell line A549 (ATCC CCL-185), the human acute monocyte leukemia cell line THP-1 (ATCC TIB-202) and mouse leukemia macrophage cell line RAW264.7 (ATCC TIB-71) were purchased from ATCC. A549 was maintained in Dulbecco's Modified Eagle's medium (DMEM), THP-1 and RAW264.7 were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), containing 100 U mL^{-1} penicillin G and 100 μg mL^{-1} of streptomycin, in a humidified 5% CO_2 atmosphere at 37 °C. The medium was replaced by the medium containing 100 U mL^{-1} penicillin G, an antibiotic with no significant antimycobacterial activity at this concentration, before performing the assay.

Bone marrow cell (BMC) isolation

Bone marrow was isolated from the tibias and femurs of C57BL/6J female mice 4–8 weeks of age. The bone marrow was triturated using an 18-G needle and passed through a 70-μm nylon mesh to make a single cell suspension in RPMI-1640 medium. Following the centrifugation at 200 *g* for 5 min, BMCs were hemolyzed in 0.83% NH_4Cl solution at room temperature for 5 min. After the centrifugation, the cells were suspended in phenol red-free RPMI-1640 supplemented with 5% FBS and 100 U mL^{-1} of penicillin G.

Extraction, isolation and purification of trehalose 6,6′-dimycolate (TDM)

Bacterial culture was centrifuged after autoclaving at 121 °C for 15 min. Lipids were extracted from heat-killed cells, with 20 volumes of chloroform/methanol (2 : 1, v/v) ground three times. The two phases were separated in a funnel. After the lower phase containing major glycolipid was collected, the solvent was evaporated off with a rotary evaporator. The total lipids were first separated by solvent fractionation and then the fractions that were acetone-soluble, chloroform-soluble, tetrahydrofuran-soluble or tetrahydrofuran-insoluble were further separated by thin-layer chromatography (TLC) on silica plates (Uniplate; Analtech) using chloroform/methanol/water (90 : 10 : 1, v/v/v) or chloroform/methanol/acetone/acetic acid (90 : 10 : 6 : 1, v/v/v/v) as the solvent system. Glycolipid spots were visualized with a 9 M H_2SO_4 spray following by charring at 200 °C for analytical purposes or with iodine vapor for a few minutes for preparative purposes. TDM was recovered from the plate immediately after the iodine color had disappeared by passing through a small glass column with the solvent chloroform/methanol (2 : 1, v/v). Finally, TDM was purified until a single spot was obtained by repeating TLC (Fujita *et al.*, 2005a, b).

Preparation of TDM-coated microplates

To prepare the TDM coating solution for plate coating, the purified TDM was dissolved at a concentration of 1 mg mL^{-1} in *n*-hexane. A 100-μL aliquot of the TDM solution, diluted to the appropriate concentration, was deposited in a polystyrene microplate well (Nunc-Immunoplate; Nalge Nunc International), and the plates allowed to dry at room temperature overnight.

Coating of TDM on polystyrene microbeads

Microbeads were coated with TDM derived from BCG-Tokyo, -Connaught or *M. phlei* according to a published protocol [coating TDM on microbeads, 0.98-μm mean diameter polystyrene beads were obtained from Polyscience Inc., polybead polystyrene microspheres (2.58% solids–latex)]. Beads were washed three times with carbonate–bicarbonate buffer (8 mM sodium carbonate, 17 mM sodium bicarbonate, pH 9.6) with centrifugation at 2200 *g* for 15 min. Beads to be coated with lipids were further washed once in 30% ethanol and once in 1% ethanol. The purified TDM dissolved to the appropriate concentration in ethanol was added to the beads. Beads were incubated at 37 °C for 12 h with constant gentle mixing. The beads were finally washed three times with phosphate-buffered saline and suspended in culture medium for further use (Indrigo *et al.*, 2003).

Nitric oxide (NO) assay

For the NO assay, 2×10^5 cells of A549 and RAW264.7 were suspended in 450 μL of DMEM supplemented with 1% FBS, and 5×10^5 of mouse primary BMCs was suspended in 450 μL of RPMI-1640 supplemented with 5% FBS. Aliquots of 450 μL of cell suspensions and 50 μL of BCG suspension containing multiplicity of infection (MOI) 10 of intact BCG were mixed together and cultured in a 24-well plate. A549 and RAW264.7 cells were cultured with or without the inflammatory cytokine mix, IL-1α (100 U mL^{-1}) and TNF-α (100 U mL^{-1}) or IFN-γ (100 U mL^{-1}), and BMCs were cultured with or without IFN-γ. The culture mediums used for NO assay were phenol red free and supplemented with 100 U mL^{-1} of penicillin G.

In a parallel set of experiments, the A549 and RAW264.7 cells were stimulated with heat-killed BCG strains (10 μg mL^{-1}), TDM-coated microbeads (at a ratio of 10 beads per cell) or TDM-coated microplates in the presence or absence of cytokine mix. Following incubation for 48 h, the supernatants were centrifuged at 400 *g* for 10 min to make it cell free. Ten microliters of 0.05 mg mL^{-1} 2,3-diaminonaphthalene (in 0.62 M HCl) was mixed with 100 μL of the culture supernatants and incubated at room temperature in the dark for 10 min. The reaction was

terminated with 10 μL of 1.4 N NaOH and the fluorescence was measured at 365/450 nm using Fluoroskan Ascent (Thermo Scientific) (Jagannath *et al.*, 1998). The concentration of nitrite (NO_2) was determined using sodium nitrite as standard. Cell-free medium was used as a blank for the assay. The viability of the A549 and RAW264.7 cells after infection or stimulation was examined by staining with crystal violet.

Cytokine assay

For the cytokine assay, 5×10^5 cells 450 μL^{-1} of THP-1 or BMCs were incubated with 50 μL of intact bacteria suspensions containing MOI 100 (THP-1) or MOI 10 (BMCs) of BCG. In addition, THP-1 were incubated with heat-killed bacteria (1 $\mu g\,mL^{-1}$), TDM-coated microbeads at a ratio of 1:10 (cells:microbeads), or cultured on the TDM-coated microplates in RPMI-1640 medium supplemented with 1% (THP-1) or 5% (BMCs) FBS at 37 °C in the presence or absence of IFN-γ (100 U mL^{-1}). Following incubation for 72 h, the cells and the bacteria were removed by centrifugation and filtration using an Advantec 0.20-μm syringe filter unit (Toyo Roshi Kaisya Ltd). The cytokines IL-1β, IL-6, IL-8, IL-12p40 and TNF-α in the supernatants of THP-1, and IL-12p40 and TNF-α in the supernatants of BMCs were determined by enzyme-linked immunosorbent assay (ELISA), using a BD OptEIA™ ELISA set (BD Bioscience). The assay was performed in accordance with the manufacturer's instruction.

Reporter gene assay

The nuclear factor-κB (NF-κB) reporter plasmid pGL3-4κB-Luc and the pCMV-βgal plasmid (Matsumura *et al.*, 2003) (for normalization of transfection efficiency) were transiently transfected into A549 cells using the calcium phosphate–DNA coprecipitation method. After 15 h of transfection, cells were washed twice with culture medium and stimulated with MOI 10 of BCG strains for 48 h. Luciferase assays were performed with the luciferase reporter gene assay kit (Roche) according to the manufacturer's instructions. The light emission was measured using the Fluoroskan Ascent (Thermo Scientific). Luciferase activity was expressed after normalization with the β-galactosidase value in the same sample.

Statistical analysis

Each experiment was repeated three times and results are expressed as mean ± SD. In Figs 1, 2 and 4–7, the data of the BCG-Russia, -Moreau, -Japan, -Sweden and -Birkhaug strains (Figs 1 and 2) or the BCG-Russia, -Moreau, -Japan and -Sweden strains (Figs 4–7) were sorted into an 'early-shared strains' group, and BCG-Danish, -Glaxo, -Mexico, -Tice, -Connaught, -Montreal, -Phipps, -Australia and -Pasteur strains (Figs 1 and 2) or the BCG-Danish, -Connaught and -Pasteur (Figs 4–7) strains into a 'late-shared' strains group in the statistical analysis. Following the assessment of variance by *F*-test, statistically significant differences between two series were assessed using Student's *t*-test or Welch's *t*-test. Turkey's or Turkey–Kramer multiple pairwise comparison test was applied for assessment of significant differences among three series. Statistical analysis was performed using KYPLOT ver. 2.0 (KyensLab, Tokyo, Japan).

Results

NO production from human lung epithelial cell line, A549 and mouse primary BMCs stimulated with BCG strains

Production of NO by BCG-infected A549 cells indicates active participation of alveolar epithelium in the innate immune response to tuberculosis (Roy *et al.*, 2004). The kinetics of secretion was optimal at 48 h postinfection (data not shown). Therefore, this time point was chosen for all further experiments. When A549 cells were infected with BCG strains, NO production by the cells in the presence of cytokines, as measured by the conversion of 2,3-diaminonaphthalene to its fluorescent product, 2,3-naphthotriazole, was higher in the 'early-shared strains' of BCG, BCG-Russia, -Moreau, -Japan, -Sweden and -Birkhaug, than in the 'late-shared strains' of BCG, BCG-Danish, -Glaxo, -Mexico, -Tice, -Connaught, -Montreal, -Phipps, -Australia and -Pasteur (Fig. 1a). Human macrophages cannot produce NO (Weinberg *et al.*, 1995; Arias *et al.*, 1997; Thoma-Uszynski *et al.*, 2001). Mouse primary BMCs were also investigated for NO production. 'Early-shared strains' of BCG, BCG-Russia, -Moreau, -Japan, -Sweden and -Birkhaug, exhibited higher inducing activity of NO with or without IFN-γ from BMCs (Fig. 1b). The numbers of bacilli did not differ significantly during assay periods (data not shown). The activation of the NF-κB pathway, which is involved in inducing NO synthase activity (iNOS), correlated with the pattern of NO production from A549 (Figs 1a and 2) (Taylor *et al.*, 1998). The viability of the cells was not affected by BCG infection as measured by the clearance of A549 and BMCs from the culture plates and staining the cells with crystal violet (data not shown). The genetic backgrounds from BCG-Russia to BCG-Birkhaug are very close, therefore, the original characteristics of the 'authentic' BCG-Pasteur might be still conserved in the 'early-shared strains' (Scheme 1).

Subclass of mycolate among BCG strains differentially induce NO production and inflammatory cytokines

Three types of mycolic acids (α-, keto-, methoxy-) are found in the cell wall of *Mycobacterium* species and they contribute