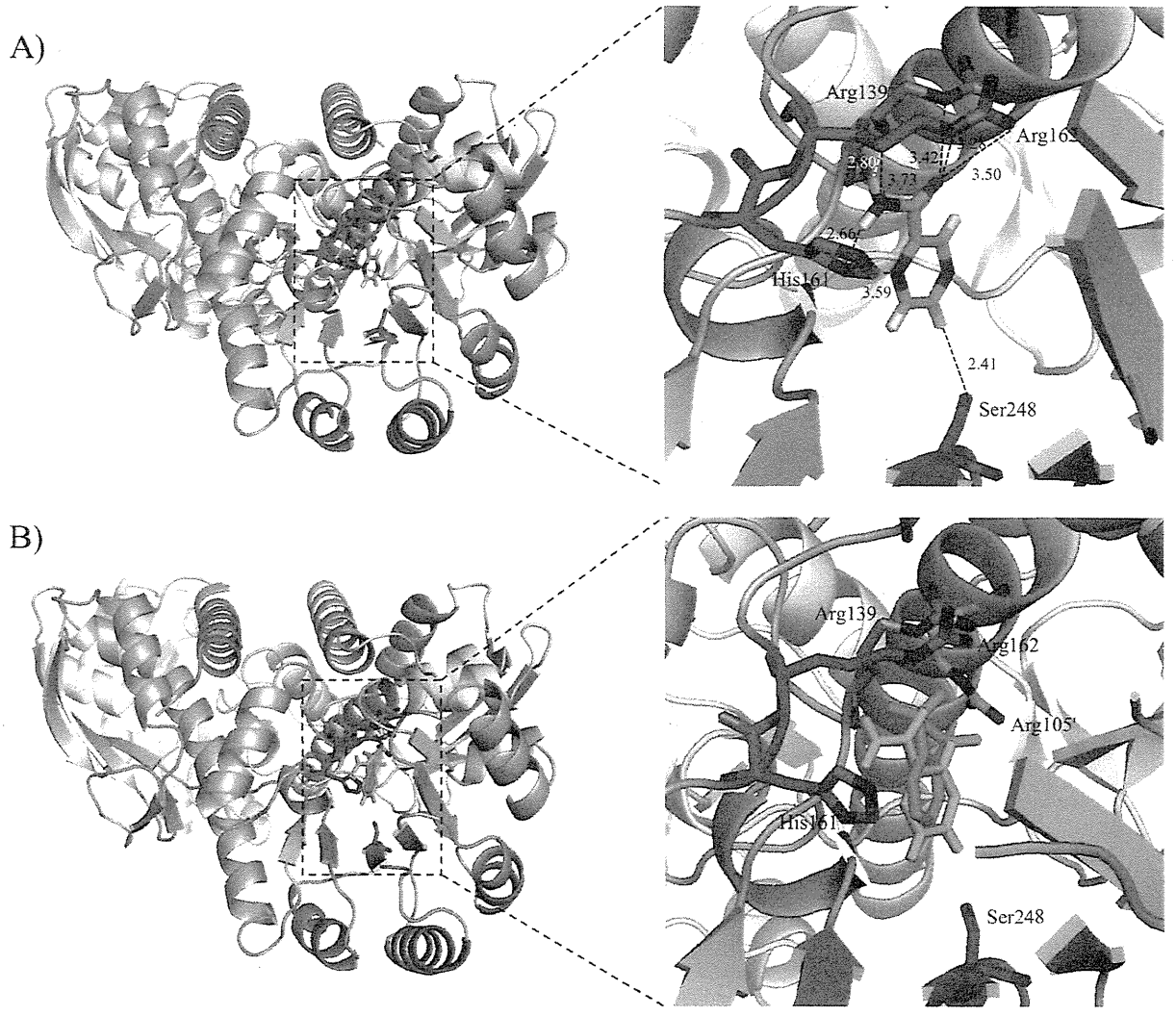

Figure 8. Molecular docking studies. The binding mode of PZA or QA was shown within the catalytic site of MtQAPRTase. The A subunit of MtQAPRTase is depicted in light orange while the B subunit is depicted in pale cyan. The overall structures and the catalytic site on MtQAPRTase are shown on the left and right, respectively. The models of complex structures are indicated as follows: (A) WT MtQAPRTase–PZA and (B) WT MtQAPRTase–merging with PZA [cyan] and QA [green]. The dotted line indicates hydrogen bonding (A and B), and the distance between amino acid residues and PZA/QA is indicated as well.
doi:10.1371/journal.pone.0100062.g008

Molecular docking study

According to the values of the protein-ligand binding free energy and the score, the optimum docking model was selected. When the PZA was used as the ligand in molecular docking, the values of the protein-ligand binding free energy and the score were −5.22 and −1.80, respectively. On the other hand, when the QA was used as the ligand, the values of the protein-ligand binding free energy and the score were −5.87 and −2.07, respectively. There was no significant difference between the values of the protein-ligand binding free energy and the score of PZA and QA. Molecular docking results are shown in Fig. 8. Although the structure of QAPRTase has been reported as a three-dimer form [5,16], we used dimeric forms of A and B subunits for the molecular docking study. Our docking results showed that PZA binds to Arg139, His161, Arg162, and Ser248 through a hydrogen-bonding network (Fig. 8A and Table 4). Arg139 and Arg162 are involved in QA binding too (Fig. 6) [5]. Therefore, we suggest that the PZA-binding site of MtQAPRTase overlaps the QA-binding site (Fig. 8B). Arg139, His161, Arg162, and Ser248 were changed to Ala after PZA was docked to MtQAPRTase by using the WinCoot-0.7.2 program. We found that Ala139, Ala161, Ala162, and Ala248 did not interact with PZA (data not shown); thus, we believe that these amino acid residues play an important role in the interaction between MtQAPRTase and PZA (Fig. 8)

Discussion

We describe here the molecular cloning, expression, and purification of MtQAPRTase to determine its biochemical properties. MtQAPRTase forms a dimer in solution, which is consistent with its crystal structure [5]. The optimum temperature

Table 4. Selected contact between MtQAPRTase and PZA.

PZA atom	Protein atom	Distance (Å)
O	Arg139 NE	3.42
N1	His161 NE2	3.75
N1	His161 ND1	3.56
H2	His161 ND1	2.66
N3	His161 ND1	3.59
O	Arg162 NH2	3.50
O	Arg162 NE	3.28
H1	Arg162 CG	2.80
N1	Arg162 CG	3.73
H5	Ser248 OG	2.41

doi:10.1371/journal.pone.0100062.t004

and pH of MtQAPRTase were 60°C and pH 9.2 and are similar to those of the QAPRTase from *Alcaligenes eutrophus nov.* subsp. *Quinolinicus* [42]. Because the enzymatic activity of MtQAPRTase was detected at pH 7.2 and 37°C (Fig. 4), we suggest that MtQAPRTase functions as a QAPRTase in *M. tuberculosis*.

Liu H. *et al.* [16] reported the kinetic characterization of QAPRTase (hQAPRTase) from *Homo sapiens*. We compared the kinetic studies of MtQAPRTase with those of hQAPRTase and recalculated the values of k_{cat} from V_{max} values. The k_{cat} value for QA determined using the MtQAPRTase assay was 0.12 [s^{-1}] and that reported for hQAPRTase was 0.04 [s^{-1}]. The K_m values for QA when evaluating MtQAPRTase and hQAPRTase were 80 and 22 μM, respectively. Therefore, we suggest that the enzymatic activity of MtQAPRTase is similar to that of hQAPRTase.

The inhibitory effects of PZA or POA on MtQAPRTase activity were determined at neutral (7.2) and weak acidic (6.2) pH, because it was reported that in *M. tuberculosis*, at an acidic external pH. In *M. tuberculosis*, at an acidic external pH, the rate of passive transmembrane equilibrium of POA apparently overwhelms that of active efflux, resulting in a huge accumulation of POA in the cells [31,43]. Although the internal pH in *M. tuberculosis* exposed to an acidic external pH is not known, we speculate that the internal pH is weakly acidic. Therefore, we evaluated the inhibitory effects at pH 6.2. Our results show that the inhibitory effect of PZA at neutral pH condition was dramatically higher than the inhibitory effect of POA at any pH value (Fig. 7B). Therefore, we suggest that PZA may inhibit the MtQAPRTase activity at neutral pH.

Structural studies indicate that Arg105′, Arg139, Arg162, Lys172, and His175 residues interact with QA, suggesting that the C3 carboxylate group of QA forms hydrogen bonds with the side-chain atoms of Arg162 and Arg139, whereas the C2 carboxylate group is within hydrogen-bonding distance of the main chain of Arg139 and the side chains of Arg105′ and Lys172 (Fig. 6) [5]. Further, the side chain of His161 is within van der Waals distance of the substrate [5]. We found that the four mutated amino acids play an important role in enzymatic activity of MtQAPRTase. QA binds tightly to QA-binding site (Arg105′, Arg139, Arg162, and Lys172) through hydrogen bonds (H-N-H_O-H and H-N_O-H) and other bonds (Fig. 6) [5], but not to alanine mutants (Ala105′, Ala139, Ala162, and Ala172) because of the absence of hydrogen-bonding (data not shown). The subsequent loss of hydrogen bonding could induce a substantial conformational change, which disrupts enzymatic activity of MtQAPRTase.

We further demonstrated that PZA strongly inhibited MtQAPRTase activity (Fig. 7). Based on the crystal structure and data on the interactions between QA and QAPRTase [5,13], we hypothesize that the PZA-binding site of QAPRTase overlaps the QA-binding site, because the structures of PZA and QA are very similar (Fig. 7A). Therefore, we investigated the binding mode of a PZA instead of a QA within the active site of MtQAPRTase using molecular docking analysis (Fig. 8). Our molecular docking results show that the PZA binds to amino acid residues (Arg139, His161, Arg162, and Ser248) through a hydrogen-bonding network (Fig. 8A and Table 4), and QA binds to amino acid residues (Arg105′, Arg139, Arg162, and Lys172) on MtQAPRTase (Fig. 8B) [5]. We suggest further that Arg139 and Arg162 are essential amino acid residues for the formation of QA- and PZA-binding sites, and His161 and Ser248 may be important only for recognition of PZA.

In conclusion, the present study identifies PZA as an inhibitor of MtQAPRTase. Base on the structure of PZA new inhibitors of MtQAPRTase could be designed. Because MtQAPRTase is a potential candidate target of new anti-TB drugs [5,10–12], we expect that our present findings will contribute to their development.

Supporting Information

Figure S1 The possible confirmations of His tag. The ribbon model (A) and surface model (B) of WT MtQAPRTase (PDB ID:1QPO) and 3 calculated structures, after elongating six histidines in C-terminal and molecular dynamics simulation, were shown. The one subunit is depicted in pale cyan while the other subunit is depicted in green. The additional six histidines are depicted in red. The active site of MtQAPRTase is shown in a broken line.
(TIF)

Author Contributions

Conceived and designed the experiments: HK KS SM. Performed the experiments: HK ER. Analyzed the data: HK SM. Contributed reagents/materials/analysis tools: HK SM. Wrote the paper: HK KS ER SM.

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Figure S1

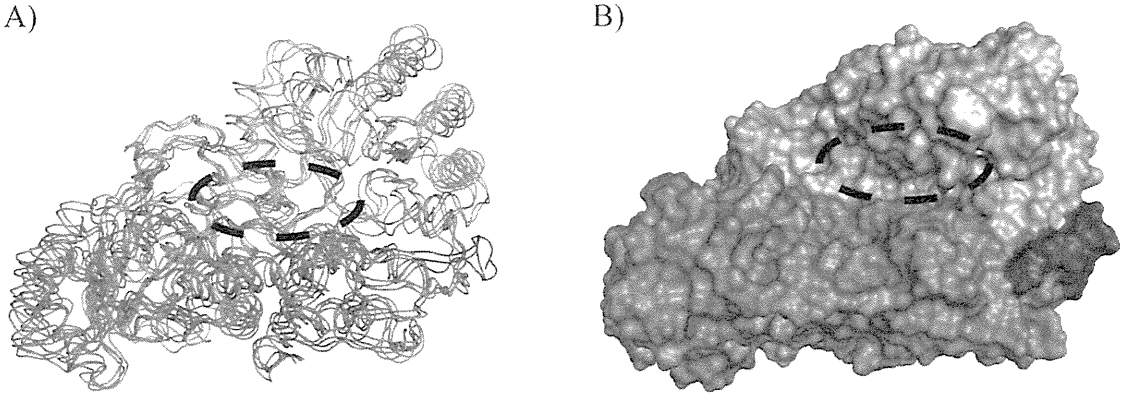

Figure S1. The possible confirmations of His tag.
The ribbon model (A) and surface model (B) of WT MtQAPRTase (PDB ID:1QPO) and 3 calculated structures, after elongating six histidines in C-terminal and molecular dynamics simulation, were shown. The one subunit is depicted in pale cyan while the other subunit is depicted in green. The additional six histidines are depicted in red. The active site of MtQAPRTase is shown in a broken line.

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Efficient Activation of Human T Cells of Both CD4 and CD8 Subsets by Urease-Deficient Recombinant *Mycobacterium bovis* BCG That Produced a Heat Shock Protein 70-*M. tuberculosis*-Derived Major Membrane Protein II Fusion Protein

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For the purpose of obtaining *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) capable of activating human naive T cells, urease-deficient BCG expressing a fusion protein composed of *Mycobacterium tuberculosis*-derived major membrane protein II (MMP-II) and heat shock protein 70 (HSP70) of BCG (BCG-DHTM) was produced. BCG-DHTM secreted the HSP70-MMP-II fusion protein and effectively activated human monocyte-derived dendritic cells (DCs) by inducing phenotypic changes and enhanced cytokine production. BCG-DHTM-infected DCs activated naive T cells of both CD4 and naive CD8 subsets, in an antigen (Ag)-dependent manner. The T cell activation induced by BCG-DHTM was inhibited by the pretreatment of DCs with chloroquine. The naive $CD8^+$ T cell activation was mediated by the transporter associated with antigen presentation (TAP) and the proteosome-dependent cytosolic cross-priming pathway. Memory $CD8^+$ T cells and perforin-producing effector $CD8^+$ T cells were efficiently produced from the naive T cell population by BCG-DHTM stimulation. Single primary infection with BCG-DHTM in C57BL/6 mice efficiently produced T cells responsive to *in vitro* secondary stimulation with HSP70, MMP-II, and *M. tuberculosis*-derived cytosolic protein and inhibited the multiplication of subsequently aerosol-challenged *M. tuberculosis* more efficiently than did vector control BCG. These results indicate that the introduction of MMP-II and HSP70 into urease-deficient BCG may be useful for improving BCG for control of tuberculosis.

Mycobacterium tuberculosis is a causative bacterium of tuberculosis. One-third of the global population is latently infected with *M. tuberculosis*, which is responsible for 1.4 million deaths worldwide each year (1–4). Recently, multidrug-resistant strains of *M. tuberculosis* have emerged and spread worldwide (5), which mandates the development of reliable preventive measures and therapeutic tools. The manifestation of adult lung tuberculosis cannot be prevented by currently used *Mycobacterium bovis* BCG (6); therefore, the development of more-effective single-injection vaccines is strongly desired.

Host defenses against *M. tuberculosis* are conducted largely by type 1 T cells of both CD4 and CD8 subsets (7–9). As one of the most important effector elements, gamma interferon (IFN-γ) is well known (10). IFN-γ can be produced chiefly by $CD4^+$ T cells and $CD8^+$ T cells. $CD8^+$ T cells are also required to differentiate into cytotoxic T lymphocytes capable of killing *M. tuberculosis*-infected macrophages and dendritic cells (DCs) (11, 12). The killing process is via a granule-dependent mechanism involving perforin, which is produced in activated T cells (13, 14). Therefore, both $CD4^+$ T cells and $CD8^+$ T cells, specifically of the memory phenotype capable of responding immediately to *M. tuberculosis*-infected cells, are key elements in host defenses against *M. tuberculosis*, and vaccinating agents are required to have the ability to activate T cells to produce memory subpopulations. BCG activates naive $CD4^+$ T cells substantially but not convincingly and activates naive $CD8^+$ T cells poorly (15, 16). The reasons why BCG cannot prevent the development of tuberculosis have not been elucidated fully, but one of the major reasons is its poor immunostimulatory activities, based on the lack of ability to induce phagosomal maturation (17–19). Therefore, BCG-derived antigens (Ags) cannot be fully processed in the Ag-presenting cells (APCs) and cannot be efficiently presented to $CD4^+$ T cells and $CD8^+$ T cells. These observations indicate that improvement of BCG in terms of activation ability is necessary.

Various molecules, including early secretory antigenic target 6 (ESAT6), the Ag85 family proteins, and polyprotein Mtb72F, have been identified as good candidates for component vaccines against tuberculosis (9, 20–24). However, the development of a fully reliable vaccine using these component molecules has not been successful. Furthermore, the strategy that is necessary to improve BCG is still not fully determined, although some candidate recombinant BCG (rBCG) is already available (17–19). Previously, Grode et al. produced urease-deficient rBCG that produced acidic phagosomes due to lack of ammonium production and effectively translocated into lysosomes (18). However, the urease depletion alone potentiated the immunostimulatory activity of BCG but did not effectively inhibit the multiplication of *M. tuberculosis* in lung. This requires secretion of another foreign Ag, listeriolysin. We independently produced urease-deficient rBCG (BCG-ΔUT-11-3) by depleting the *ureC* gene, which encodes urease, from parent BCG (19). BCG-ΔUT-11-3 strongly activated naive human $CD4^+$ T cells to produce IFN-γ but failed to stimu-

Received 8 September 2013 Returned for modification 1 October 2013
Accepted 18 October 2013
Published ahead of print 23 October 2013
Editor: W. R. Waters
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doi:10.1128/CVI.00564-13

late naive human $CD8^+$ T cells, which indicates that another modification of BCG is necessary.

Similarly, a new reliable vaccine is needed for prevention of leprosy, which is caused by infection with *Mycobacterium leprae*. We are currently developing a new rBCG capable of inhibiting the multiplication of *M. leprae in vivo*. First, we identified major membrane protein II (MMP-II) (gene name, *bfrA* or ML2038) as one of the immunodominant Ags of *M. leprae* (25). MMP-II can ligate Toll-like receptor 2 (TLR2) and consequently activates the NF-κB pathway of APCs (25), and MMP-II-pulsed DCs activate both naive $CD4^+$ T cells and naive $CD8^+$ T cells (25, 26). Second, we tried to improve BCG by overexpressing MMP-II. When we introduced the MMP-II gene into BCG extrachromosomally, the rBCG showed enhanced activity to stimulate naive T cells of both CD4 and CD8 subsets (27). The second rBCG that we produced was BCG-70M, having a BCG-derived heat shock protein 70 (HSP70)-MMP-II fusion gene, and subcutaneous single BCG-70M vaccination inhibited the multiplication of *M. leprae* in C57BL/6 mice (28). Therefore, the secretion of the HSP70-MMP-II fusion protein was useful for enhancing the T cell-stimulating activity of BCG.

Overall, these results suggest that the combination of urease depletion and intraphagosomal secretion of antigenic protein is useful for construction of a new rBCG. We found that *M. tuberculosis* has an MMP-II gene (gene name, Rv1876) that is 100% homologous to the MMP-II gene of BCG and 90% homologous to that of *M. leprae* at the amino acid level. Previously, we purified the recombinant MMP-II (rMMP-II) protein of *M. tuberculosis* using *Mycobacterium smegmatis* and evaluated its immunostimulatory activities (29). Similar to *M. leprae*-derived MMP-II, *M. tuberculosis*-derived MMP-II ligates TLR2 and activates DCs, and the MMP-II-pulsed DCs activate both subsets of naive T cells (29). Furthermore, both human DCs and macrophages infected with *M. tuberculosis* strains such as H37Rv and H37Ra expressed MMP-II derivatives on their surfaces (29). These results indicate that the MMP-II of *M. tuberculosis* is highly immunogenic and might be a good candidate for vaccine development. Therefore, in this study, we produced a new rBCG, termed BCG-DHTM, in which urease-deficient BCG-ΔUT-11-3 was introduced with a fusion gene composed of the *M. tuberculosis*-derived MMP-II gene and the HSP70 gene of *M. tuberculosis* (Rv0350), and we evaluated its immunostimulatory activities.

MATERIALS AND METHODS

Preparation of cells and Ags. Peripheral blood samples were obtained from healthy purified protein derivative (PPD)-positive individuals, with informed consent. In Japan, BCG vaccination is compulsory for children (0 to 1 year of age). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as described previously (30). For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMCs by using immunomagnetic beads coated with anti-CD3 monoclonal antibody (MAb) (Dynabeads 450; Dynal Biotech, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on tissue culture plates, and adherent cells were used as monocytes (31). DCs were differentiated as described previously (30, 32). Briefly, monocytes were cultured in the presence of 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (Pepro Tech EC Ltd., London, England) and 10 ng of recombinant interleukin-4 (rIL-4) (Pepro Tech) per ml (32). On day 4 of culture, immature DCs were infected with rBCG at the indicated multiplicity of infection (MOI) and, on day 6 of culture, DCs were used for further analyses of surface Ag and for mixed lymphocyte assays. Macrophages were differentiated as described previously (33, 34). In brief, monocytes were cultured in the presence of 10 ng of recombinant macrophage colony-stimulating factor (rM-CSF) (R&D Systems, Inc., Minneapolis, MN) per ml. On day 5 of culture, macrophages were infected with rBCG at the indicated MOIs and, on day 7 of culture, they were used for further analyses of surface Ag and for mixed lymphocyte assays. The rMMP-II protein was produced as described previously (25, 35). The rHSP70 protein was purchased (Hy Test Ltd., Turku, Finland), and H37Rv-derived cytosolic protein was produced as described previously (35).

Vector construction and preparation of rBCG. The genomic DNAs were obtained from BCG substrain Tokyo and from *M. tuberculosis* strain H37Rv. The oligonucleotide primers used for amplification of the *hsp70* gene were F-Mb70Bal (5′-aaaTGGCCAtggctcgtgcggtcggg-3′) and R-Mb70Eco (5′-aaaGAATTCcttggcctcccggccg-3′). The MMP-II sequence from *M. tuberculosis* genomic DNA was amplified with primers F-MMPTBEco (5′-aattGAATTCatgcaaggtgatcccgatgt-3′) and R-MMPT-BSal (5′-aattGTCGACtcaggtcggtgggcgaga-3′). In all primer sequences, capital letters indicate restriction sites. The amplified products were digested with appropriate restriction enzymes and cloned into the parental pMV261 plasmid. For replacement of the kanamycin resistance gene with the hygromycin resistance cassette, the XbaI-NheI fragment from pYUB854 (36) was cloned into the SpeI-NheI fragment of the plasmid (36). The rBCG in which the *ureC* gene was disrupted (BCG-ΔUT-11) was produced as described previously (19). The hygromycin cassette in BCG-ΔUT-11 was removed by using pYUB870 encoding γδ-resolvase (γδ-*tnp*R) (36). The unmarked BCG was named BCG-ΔUT-11-3. The HSP70-MMP II fusion protein-expressing vector was introduced into BCG-ΔUT-11-3 by electroporation. BCG-ΔUT-11-3 containing pMV-HSP70-MMP-II as an extrachromosomal plasmid is referred to as BCG-DHTM, and BCG-Tokyo containing pMV-261-hygromycin is referred to as BCG-261H (BCG vector control). Recombinant BCGs and *M. tuberculosis* strain H37Rv were grown to log phase and stored at −80°C, at 10^8 CFU/ml. Before infection of DCs and macrophages, BCG levels were counted by the colony assay method. There was no significant difference in *in vitro* culture growth between BCG-261H and BCG-DHTM.

Analysis of cell surface Ags. The expression of cell surface Ags on DCs and lymphocytes was analyzed using a FACSCalibur system (BD Biosciences, San Jose, CA). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich, St. Louis, MO), and 1×10^4 live cells were analyzed. For the analysis of cell surface Ags, the following MAbs were used: fluorescein isothiocyanate (FITC)-conjugated MAbs against HLA-ABC (G46-2.6; BD Biosciences), HLA-DR (L243; BD Biosciences), CD86 (FUN-1; BD Biosciences), CD83 (HB15a; Immunotech, Marseille, France), CD62L (Dreg 56; BD Biosciences), CCR7 (clone 150503; R&D Systems), and CD27 (M-T271; BD Biosciences) and phycoerythrin-conjugated MAbs to CD162 (TB5; Exbio, Prague, Czech Republic), CD8 (RPA-T8; BD Biosciences), and CD4 (RPA-T4; BD Biosciences).

The expression of MMP-II on rBCG-infected DCs was determined using the MAb against MMP-II of *M. leprae* (M270-13, IgM, kappa), which may detect MMP-II associated with major histocompatibility complex (MHC) molecules (26), followed by FITC-conjugated anti-mouse immunoglobulin (Ig) MAb (Tago Immunologicals, Camarillo, CA). For inhibition of the intracellular processing of phagocytosed bacteria, DCs were treated with 50 μM chloroquine (Sigma-Aldrich) for 2 h, washed, infected with rBCG, and subjected to analyses of MMP-II surface expression. The intracellular production of perforin was assessed as follows: naive $CD8^+$ T cells were stimulated with rBCG-infected DCs for 5 days in the presence of naive $CD4^+$ T cells, and $CD8^+$ T cells were surface stained with phycoerythrin-labeled MAb to CD8 and fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using permeabilizing solution (BD Biosciences) and were stained with FITC-conjugated MAb to perforin (δG9; BD Biosciences) or FITC-labeled isotype control.

APC functions of DCs. The ability of BCG-infected DCs and macrophages to stimulate T cells was assessed using an autologous APC-T cell coculture, as previously described (32, 37). Purification of $CD4^+$ and $CD8^+$ T cells was conducted by using negative-isolation kits (Dynabeads 450; Dynal Biotech) (32). Naive $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with MAb to CD45RO, followed by beads coated with goat anti-mouse IgG MAb (Dynal Biotech). More than 98% of $CD45RA^+$ T cells were positive for the expression of CCR7 molecules. Memory-type T cells were similarly produced by the treatment of cells with MAb to CD45RA Ag. The purified responder cells (1×10^5 cells per well) were plated in 96-well round-bottom tissue culture plates, and DCs or macrophages infected with rBCG were added to give the indicated APC/T cell ratio. Supernatants of APC-T cell cocultures were collected on day 4, and cytokine levels were determined. In some cases, rBCG-infected DCs and macrophages were treated with MAb to HLA-ABC (W6/32, mouse IgG2a, kappa), HLA-DR (L243, mouse IgG2a, kappa), or CD86 (IT2.2, mouse IgG2b, kappa; BD Biosciences) or normal mouse IgG. The optimal concentrations of the MAbs were determined in advance. Also, in some cases, immature DCs and macrophages were treated with the indicated doses of chloroquine, brefeldin A (Sigma-Aldrich), or lactacystin (Sigma-Aldrich) and subsequently infected with BCG-DHTM. The optimal doses of these reagents were determined in advance.

Measurement of cytokine production. Levels of the following cytokines were measured: IFN-γ produced by $CD4^+$ and $CD8^+$ T cells and interleukin 12p70 (IL-12p70), tumor necrosis factor alpha (TNF-α), IL-1β, and GM-CSF produced by DCs or macrophages stimulated for 24 or 48 h with rBCGs. The concentrations of these cytokines were quantified using enzyme assay kits (Opt EIA human enzyme-linked immunosorbent assay [ELISA] set; BD Biosciences).

Animal studies. For inoculation into mice, rBCG and *M. tuberculosis* strain H37Rv were cultured in Middlebrook 7H9 medium to log phase and stored at −80°C, at 10^8 CFU/ml. Before the aliquots were used for inoculation, the concentrations of viable bacilli were determined by plating on Middlebrook 7H10 agar plates. Three 5-week-old C57BL/6J mice (Clea Japan Inc., Tokyo, Japan) per group were inoculated subcutaneously with 0.1 ml of phosphate-buffered saline (PBS) or PBS containing 1×10^3 or 1×10^4 rBCG. The animals were kept under specific pathogen-free conditions and were supplied with sterilized food and water. Four or 12 weeks after inoculation, the spleens were removed and splenocytes were suspended in culture medium at a concentration of 2×10^6 cells per ml. The splenocytes were stimulated with the indicated concentrations of rMMP-II, rHSP70 (HyTest), or H37Rv-derived cytosolic protein, in triplicate, in 96-well round-bottom microplates (19, 27). The individual culture supernatants were collected 3 to 4 days after stimulation. For observation of the effect of BCG vaccination on *M. tuberculosis* infection, five C57BL/6 mice per group were vaccinated with either BCG-261H or BCG-DHTM, at 1×10^4 CFU/mouse, for 6 weeks and were challenged with H37Rv at 100 CFU/lung by aerosol infection using an automated inhalation exposure apparatus (model 099C A4212; Glas-Col Corp.). Six weeks later, bacterial burdens in the lung and spleen were assessed by mechanical disruption in PBS with 0.5% (vol/vol) Tween 80 and enumerated by colony assay. Animal studies were reviewed and approved by the Animal Research Committee of Experimental Animals of the National Institute of Infectious Diseases and were conducted according to their guidelines.

Statistical analysis. Student's *t* test was applied to determine statistical differences. Throughout the *in vitro* experiments, we included 1 or 2 technical replicates in each individual experiment and used at least 3 separate PBMC donors.

RESULTS

Activation of Ag-presenting cells by BCG-DHTM. The purpose of rBCG production is to activate naive T cells effectively and, consequently, to produce memory-type T cells efficiently. In order to stimulate responder T cells, APCs susceptible to BCG infection should be adequately activated by infection with rBCG. We assessed the ability of newly produced rBCG (BCG-DHTM) to activate APCs with respect to phenotypic changes and cytokine production (Fig. 1). To assess phenotypic changes, we examined the expression of MHC, CD86, and CD83 molecules on DCs (Fig. 1a). Both vector control BCG (BCG-261H) and BCG-DHTM upregulated the expression of these molecules, but BCG-DHTM infection induced upregulation more efficiently than did infection with BCG-261H. We measured the production of cytokines, including IL-12p70, TNF-α, and IL-1β, from DCs with stimulation with rBCGs (Fig. 1b). Significantly higher levels of all of these cytokines were produced by BCG-DHTM stimulation. Further, when M-CSF-dependent macrophages were stimulated with either BCG-261H or BCG-DHTM, BCG-DHTM stimulation induced production of significantly higher levels of TNF-α and GM-CSF (Fig. 1c). In all of these experiments, we used various doses of rBCGs for the assessments, and similar changes were observed (data not shown). These results indicated that BCG-DHTM activated DCs and macrophages more efficiently than did BCG-261H.

Activation of memory-type and naive $CD4^+$ T cells by BCG-DHTM. Previously, we reported that the enhanced activation of both $CD4^+$ T cells and $CD8^+$ T cells induced by rBCG that was introduced with the *M. leprae*-derived MMP-II-HSP70 fusion gene was dependent on secretion of the HSP70-MMP-II fusion protein. Since we also confirmed that newly produced BCG-DHTM secreted the fusion protein composed of *M. tuberculosis*-derived MMP-II and HSP70 (data not shown), we assessed the $CD4^+$ T cell-stimulating ability of BCG-DHTM (Fig. 2). When either BCG-261H or BCG-DHTM was used to infect DCs and was used as a stimulator, autologous memory-type $CD4^+$ T cells produced significantly higher levels of IFN-γ by stimulation of BCG-DHTM-infected DCs than BCG-261H-infected DCs (Fig. 2a); around 300 pg/ml of IFN-γ was secreted by stimulation with very small numbers of rBCG-infected DCs (MOI, 0.063) and with very small numbers of DCs (T cell/DC ratio, 80:1). At different T cell/DC ratios, BCG-DHTM exhibited higher activity (data not shown). In addition to IFN-γ, TNF-α and IL-2 were efficiently produced with BCG-DHTM stimulation (data not shown). Then, we assessed rBCG-infected macrophages as stimulators. Compared to DCs, macrophages needed to be infected with higher doses of rBCGs to stimulate memory-type $CD4^+$ T cells; however, more than 100 pg/ml of IFN-γ could be produced by responder $CD4^+$ T cells when BCG-DHTM was used at an MOI of 0.5 to infect macrophages. Further, a larger quantity of macrophages (T cell/macrophage ratio, 10:1) was needed to activate $CD4^+$ T cells convincingly (Fig. 2b). It should be noted that the BCG vector control could not induce the apparent activation of $CD4^+$ T cells. BCG-DHTM did not induce IFN-γ production from either DCs or macrophages (data not shown). Then, we assessed the activity of BCG-DHTM to stimulate naive $CD4^+$ T cells (Fig. 2c). BCG-DHTM induced the production of significantly higher levels of IFN-γ than did BCG-261H at MOIs of 0.063 to 0.25 (T cell/DC ratio, 20:1). Increasing IFN-γ levels could be produced, depending on the dose of BCG-DHTM used to infect DCs. Also, at different T cell/DC ratios, BCG-DHTM showed greater activity (data not shown). However, BCG-DHTM-infected macrophages failed to induce the production of significant levels of IFN-γ from naive $CD4^+$ T cells or $CD8^+$ T cells (data not shown).

Activation of memory-type and naive $CD8^+$ T cells by BCG-DHTM. The effects of BCG-DHTM-infected DCs on $CD8^+$ T cell activation were examined (Fig. 3). While BCG-261H did not ac-

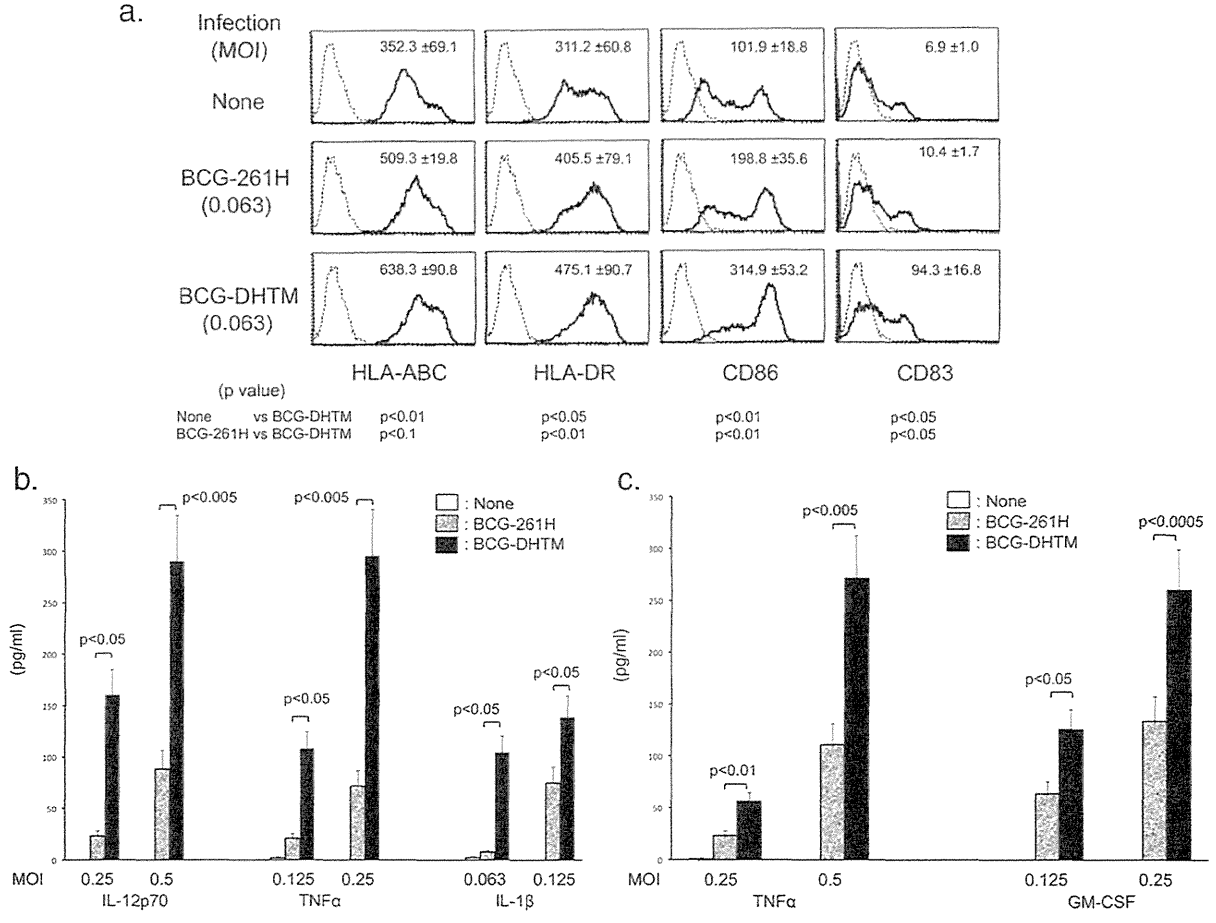

FIG 1 (a) Upregulation of APC-associated molecules and activation markers on DCs by infection with BCG-DHTM. Monocyte-derived immature DCs were infected with either BCG-261H or BCG-DHTM at an MOI of 0.063 and were cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DCs from day 6 of culture were gated and analyzed. Dashed lines, isotype-matched control IgG; solid lines, indicated test MAbs. Representative results of three separate experiments are shown. The numbers in the top right corners of the panels represent the differences in fluorescence intensity (mean ± standard deviation [SD]) between the control IgG and the test MAb in three independent experiments. (b) Cytokine production from DCs by stimulation with BCG-DHTM. DCs produced using rGM-CSF and rIL-4 were stimulated with either BCG-261H or BCG-DHTM for 24 h. (c) Cytokine production from macrophages by stimulation with BCG-DHTM. Macrophages differentiated from monocytes by using M-CSF were stimulated with either BCG-261H or BCG-DHTM for 24 h. The concentrations of the indicated cytokines were determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as mean ± SD. Titers were statistically compared using Student's *t* test.

tivate memory $CD8^+$ T cells efficiently, BCG-DHTM activated the T cells and induced the production of more than 100 pg/ml of IFN-γ (Fig. 3a). Compared to the dose of rBCG required for activation of memory-type $CD4^+$ T cells, a higher dose of rBCG was needed, which may be based on the fact that parent BCG did not activate naive $CD8^+$ T cells and did not produce BCG-specific memory-type $CD8^+$ T cells efficiently. When autologous naive $CD8^+$ T cells were stimulated by rBCG as a responder population, only BCG-DHTM efficiently activated naive $CD8^+$ cells to produce IFN-γ (Fig. 3b). Efficient concentrations of IFN-γ could be produced from naive $CD8^+$ T cells by stimulation with DCs infected with BCG-DHTM. As observed previously (18, 32), BCG-261H did not activate naive $CD8^+$ T cells. These phenomena were observed consistently under various conditions, including different MOIs and T cell/DC ratios. In order to confirm the activation of naive $CD8^+$ T cells by BCG-DHTM, the expression of activation markers on naive $CD8^+$ T cells was examined (Fig. 3c). When autologous naive $CD8^+$ T cells were stimulated with DCs infected with either BCG-261H or BCG-DHTM in the presence of naive $CD4^+$ T cells, more-efficient downregulation of CD62L expression on $CD8^+$ T cells was induced by BCG-DHTM stimulation. These phenomena were observed at different MOIs and, even at lower MOIs such as 0.031, efficient downregulation was observed in BCG-DHTM-stimulated $CD8^+$ T cells.

Characteristics of BCG-DHTM. Previously, we reported that BCG-70M induced expression of MMP-II on the surface of DCs infected with BCG-70 M (28). Thus, we analyzed the DCs infected with BCG-DHTM in terms of MMP-II expression (Fig. 4a). Whereas both uninfected DCs and DCs infected with BCG-261H did not express MMP-II derivatives on the surface, BCG-DHTM induced expression of MMP-II derivatives (6.8-fold increase in MMP-II expression in BCG-261H-infected DCs). Higher levels of expression were observed when higher MOIs of rBCG were used (data not shown). Further, MMP-II expression was inhibited by the treatment of immature DCs with chloroquine, an inhibitor of phagosomal acidification, prior to infection with BCG-DHTM.

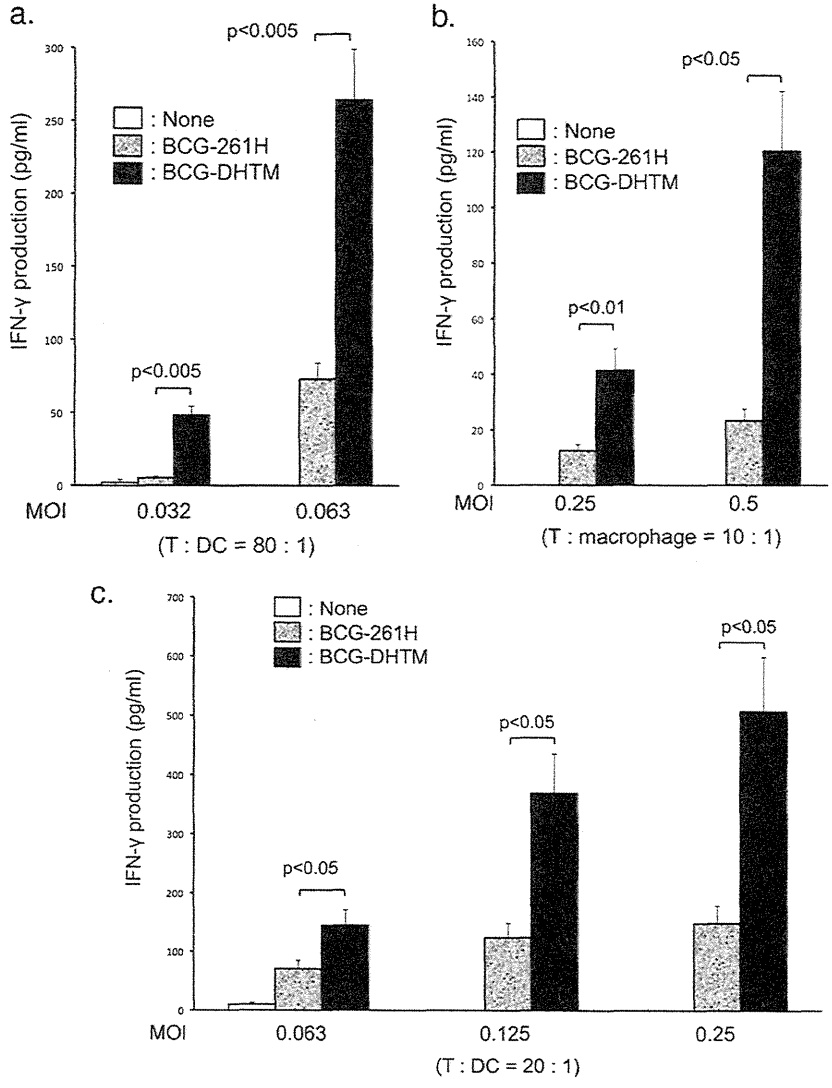

FIG 2 (a) IFN-γ production from memory-type $CD4^+$ T cells by stimulation with BCG-DHTM-infected DCs. Monocyte-derived DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators of memory-type $CD4^+$ T cells in a 4-day culture; 10^5 responder T cells were stimulated with the rBCG-infected DCs at a T cell/DC ratio of 80:1. (b) IFN-γ production from memory-type $CD4^+$ T cells by stimulation with BCG-DHTM-infected macrophages. Macrophages produced by using M-CSF were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators of responder $CD4^+$ T cells in a 4-day culture; 10^5 responder T cells were stimulated with the rBCG-infected macrophages at a T cell/macrophage ratio of 10:1. (c) IFN-γ production from naive $CD4^+$ T cells by stimulation with BCG-DHTM-infected DCs. Monocyte-derived DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators; 10^5 responder naive $CD4^+$ T cells were stimulated with the rBCG-infected DCs at a T cell/DC ratio of 20:1. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as mean ± SD. Titers were statistically compared using Student's *t* test.

According to these results, we analyzed the effect of chloroquine treatment of immature DCs on the activation of T cells by BCG-DHTM (Fig. 4b). IFN-γ production from naive $CD4^+$ T cells and naive $CD8^+$ T cells by stimulation with BCG-DHTM-infected DCs and that from memory-type $CD4^+$ T cells by stimulation with BCG-DHTM-infected macrophages were significantly inhibited by chloroquine treatment of these APCs. These results suggest the possibility that the secreted fusion protein is one of the elements responsible for the activation of both $CD4^+$ T cells and $CD8^+$ T cells. BCG-DHTM-infected DCs or macrophages were treated with MAbs to HLA and CD86 molecules prior to being used as stimulators of responder T cells (Fig. 4c). Treatment of BCG-DHTM-infected APCs with MAbs to HLA-DR, CD86, and MMP-II (data not shown) significantly inhibited IFN-γ production from naive $CD4^+$ T cells and memory-type $CD4^+$ T cells. Also, the treatment of BCG-DHTM-infected DCs with MAbs to HLA-ABC and CD86 molecules significantly inhibited the production of IFN-γ from naive $CD8^+$ T cells. These results suggested that BCG-DHTM activated T cells in an Ag-specific manner, at least partially. In general, for activation of naive $CD8^+$ T cells by bacteria, the activation of cross-presenting pathways in APCs is required. We examined whether BCG-DHTM utilized the cytosolic cross-presentation pathway for the activation of naive $CD8^+$ T cells (Fig. 4d). To this end, we treated immature DCs with either

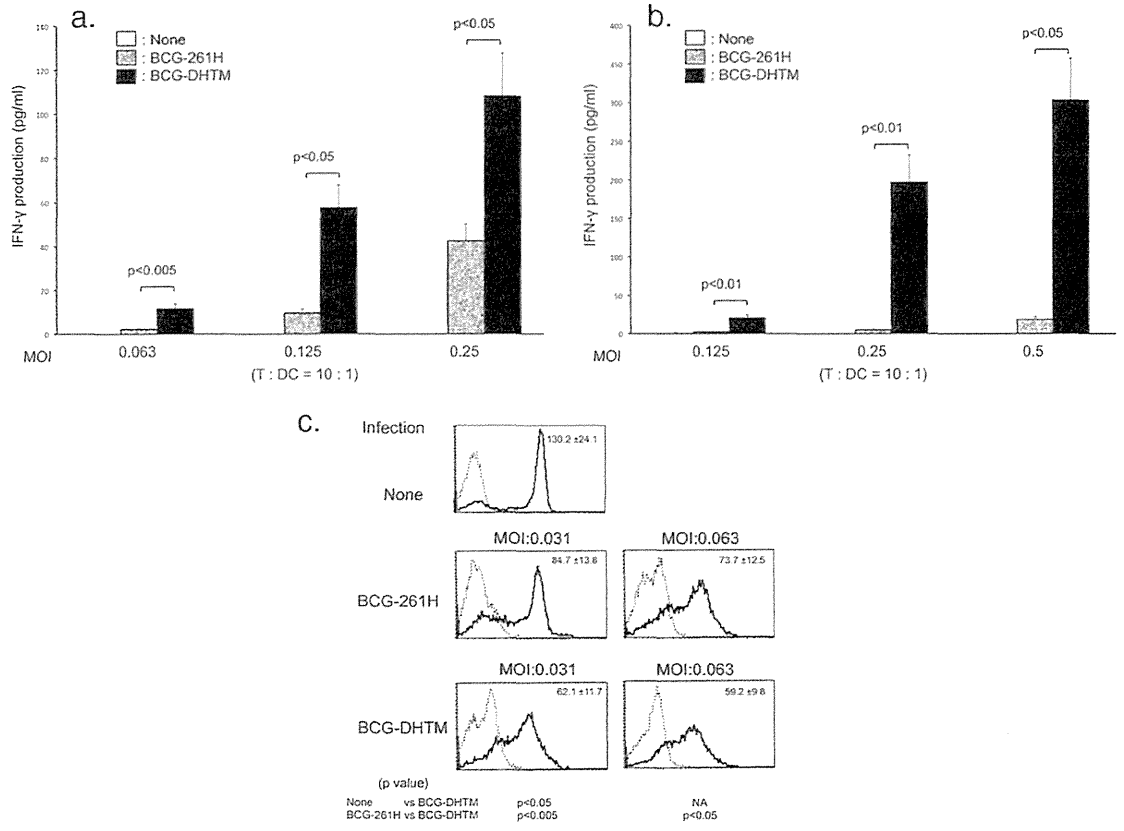

FIG 3 (a) IFN-γ production from memory-type $CD8^+$ T cells by stimulation with BCG-DHTM. Monocyte-derived DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators; 10^5 responder memory-type $CD8^+$ T cells were stimulated for 4 days with rBCG-infected DCs at a T cell/DC ratio of 10:1. (b) IFN-γ production from naive $CD8^+$ T cells by stimulation with BCG-DHTM. DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators; 10^5 responder T cells were stimulated for 4 days with rBCG-infected DCs at a T cell/DC ratio of 10:1. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as mean ± SD. Titers were statistically compared using Student's *t* test. (c) Downregulation of CD62L expression on naive $CD8^+$ T cells by BCG-DHTM stimulation. DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were cocultured with unseparated naive T cells for 5 days at a T cell/DC ratio of 20:1. The stimulated $CD8^+$ T cells were gated and analyzed for expression of CD62L molecules. Dashed lines, isotype-matched control IgG; solid lines, anti-CD62L MAb. The numbers in the top right corners of the panels represent the differences in fluorescence intensity (mean ± standard deviation) between the control IgG and anti-CD62L MAb in three independent experiments. A representative of three separate experiments is shown.

brefeldin A or lactacystin and subsequently infected them with BCG-DHTM at an MOI of 0.25. These pretreatments of DCs significantly inhibited IFN-γ production from naive $CD8^+$ T cells.

Production of memory and effector T cells from naive $CD8^+$ T cells by BCG-DHTM. Since it is well documented that the production of long-lasting memory $CD8^+$ T cells from naive $CD8^+$ T cells requires help from $CD4^+$ T cells and since BCG-DHTM activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells, naive unseparated T cells were stimulated with DCs infected with either BCG-261H or BCG-DHTM and the stimulated $CD8^+$ T cells were gated and analyzed (Fig. 5a). Stimulation with BCG-DHTM more efficiently produced $CD27^{low}$ or $CCR7^{low}$ memory-type T cells from naive T cells. Further, BCG-DHTM produced perforin-producing $CD8^+$ T cells more efficiently than did BCG-261H. Efficient production of these $CD8^+$ T cells was observed with different doses of BCG; however, in the absence of $CD4^+$ T cells, production of memory and effector T cells was not observed (data not shown). Furthermore, it is necessary to produce T cells with high migratory function. Thus, we assessed the expression of CD162 on both $CD4^+$ T cells and $CD8^+$ T cells stimulated with DCs infected with BCG-DHTM (Fig. 5b). BCG-DHTM produced $CD162^{high}$ $CD4^+$ T cells and $CD162^{high}$ $CD8^+$ T cells more efficiently than did BCG-261H. Similar differences between BCG-261H and BCG-DHTM were observed at different MOIs (data not shown).

Production of T cells responsive to secondary stimulation by BCG-DHTM infection *in vivo*. Functional studies using C57BL/6 mice were conducted to examine the ability of BCG-DHTM to produce T cells that were highly responsive to secondary *in vitro* stimulation (Fig. 6). The mice were subcutaneously inoculated with 1×10^3 CFU/mouse of rBCGs 4 weeks (Fig. 6a) or 12 weeks (Fig. 6b) before *in vitro* stimulation. As secondary stimulators, recombinant MMP-II, recombinant HSP70 protein, and H37Rv-derived cytosolic protein were used. Some of these proteins are highly immunogenic and thus they induced substantial IFN-γ production from T cells of uninfected mice; however, splenic T cells from mice inoculated with BCG-DHTM 4 weeks previously produced significantly higher levels of IFN-γ and IL-2 (data not shown) than did T cells from uninoculated mice and those from BCG-261H-infected mice, by responding to all of the secondary stimulators (Fig. 6a). To examine the long-term effects of single inoculations of BCG-DHTM, T cells from C57BL/6 mice similarly

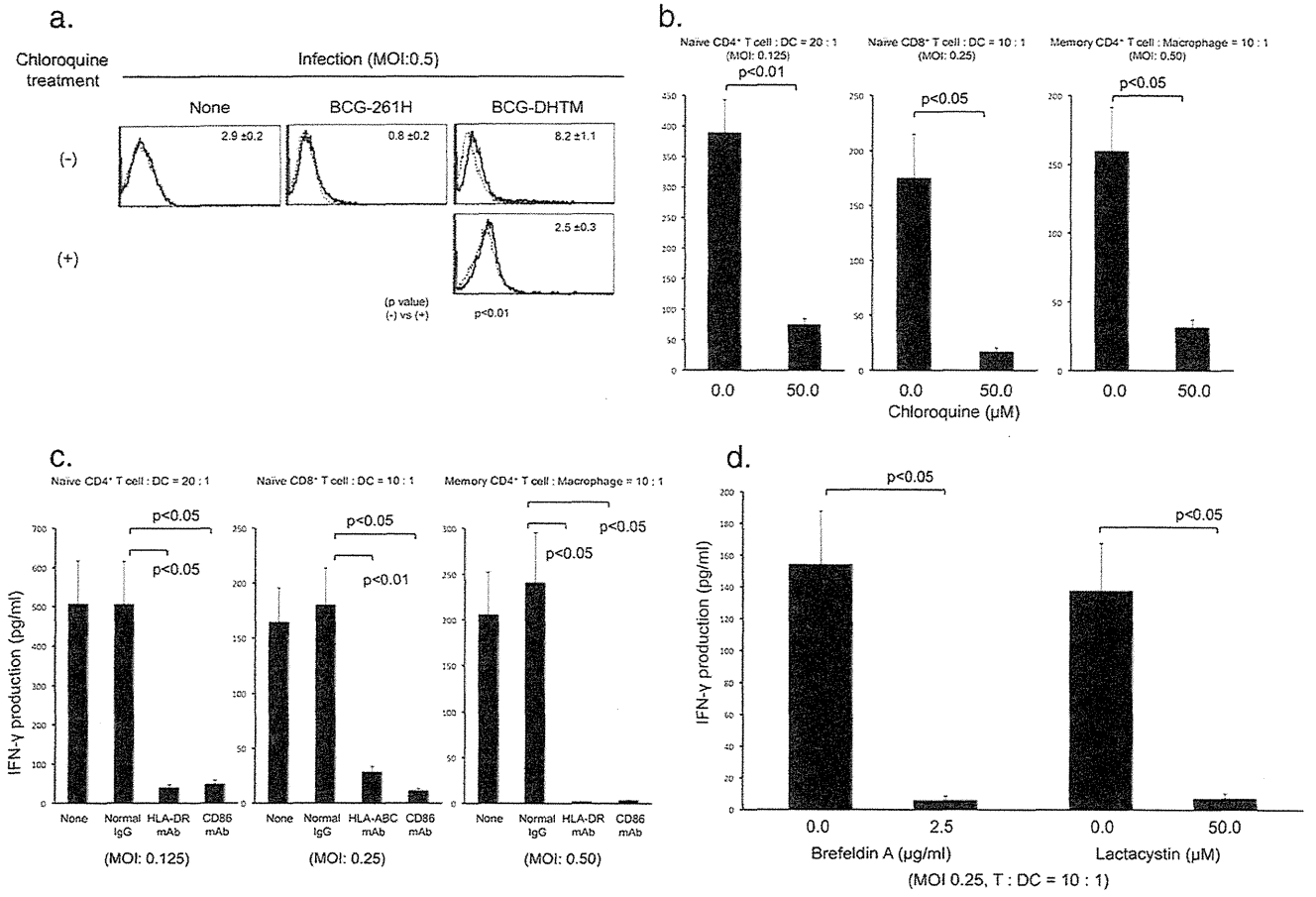

FIG 4 (a) Expression of MMP-II on DCs. Immature DCs were either treated with 50 μM chloroquine for 2 h or not treated and subsequently were infected with either BCG-261H or BCG-DHTM at an MOI of 0.5. After 2 days of culture in the presence of rGM-CSF and rIL-4, DCs were gated and analyzed. Dashed lines, control normal IgM; solid lines, anti-MMP-II MAb (IgM). The numbers in the top right corners of the panels represent the differences in fluorescence intensity (mean ± standard deviation) between the control IgM and MMP-II MAb in three independent experiments. Representative results of three separate experiments are shown. (b) Effects of chloroquine treatment of DCs and macrophages on the activation of T cells. Immature DCs and macrophages were treated with chloroquine (50 μM for 2 h) or not treated and subsequently were infected with BCG-DHTM at the indicated MOIs. These DCs and macrophages were used as stimulators of the indicated responder T cells at the indicated responder/stimulator ratios. IFN-γ produced by T cells was measured. (c) Inhibition of T cell activation by treatment of BCG-DHTM-infected DCs and macrophages with MAbs. DCs and macrophages were infected with BCG-DHTM at the indicated MOIs and subsequently were treated with 10 μg/ml of the MAb or normal murine IgG. These APCs were used as the stimulators of the indicated responder T cells (1×10^5 cells/well), at the indicated T cell/APC ratios, for 4 days. IFN-γ produced by T cells was measured. (d) Effects of treatment of immature DCs with brefeldin A or lactacystin on the activation of naive $CD8^+$ T cells. Immature DCs from 4 days of culture were treated with either brefeldin A (2.5 μg/ml) or lactacystin (50 μM) or not treated and subsequently were infected with BCG-DHTM at an MOI of 0.25. These DCs were used as stimulators of responder autologous naive $CD8^+$ T cells (1×10^5/well) at a T cell/DC ratio of 10:1. IFN-γ produced by T cells was measured. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as mean ± SD. Titers were statistically compared using Student's *t* test.

inoculated with rBCGs 12 weeks previously were examined (Fig. 6b). Again, significantly higher levels of IFN-γ were produced from T cells obtained from mice inoculated with BCG-DHTM with secondary stimulation, although we could not recover BCG from spleen. In a separate experiment, a different dose (1×10^2 CFU/mouse) of BCG was examined, and similar results were obtained (data not shown).

Effect of BCG-DHTM vaccination on the multiplication of H37Rv *in vivo*. C57BL/6 mice vaccinated with either BCG-261H or BCG-DHTM (1×10^4 CFU/mouse) for 6 weeks were challenged with 100 CFU per lung of H37Rv by aerosol infection. Six weeks later, the *M. tuberculosis* recovered from both lungs and spleen was enumerated (Fig. 7). Mice vaccinated with either BCG-261H or BCG-DHTM demonstrated inhibited multiplication of *M. tuberculosis* in the lung, and BCG-DHTM vaccination inhibited *M. tuberculosis* multiplication more strongly than did BCG-261H vaccination (Fig. 7a). Similar results were observed in the spleen (Fig. 7b). Similar protective effects of BCG-DHTM on *M. tuberculosis* recovery were observed in mice 12 weeks after vaccination (data not shown).

DISCUSSION

Studies using the T cell receptor-transgenic *M. tuberculosis* mouse model clearly demonstrated that the most susceptible APCs, including DCs and macrophages, need at least 7 to 10 days to initiate stimulation of $CD4^+$ T cells and $CD8^+$ T cells in regional lymph nodes on aerosol infection with *M. tuberculosis* and the stimulated T cells need 4 to 5 weeks to initiate inhibition of the multiplication of *M. tuberculosis* in lungs (38). The activation of both $CD4^+$ T cells and $CD8^+$ T cells is required for inhibition of the replication

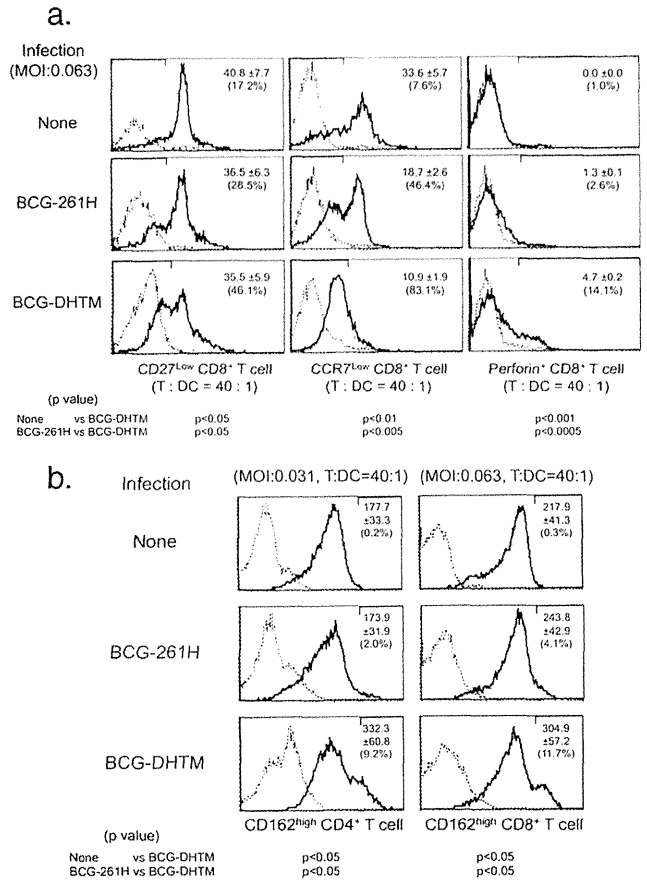

FIG 5 (a) Expression of memory markers and perforin production on naive $CD8^+$ T cells stimulated with DCs infected with BCG-DHTM. DCs were infected with either BCG-261H or BCG-DHTM at an MOI of 0.063 and were cocultured with unseparated naive T cells (T cell/DC ratio of 40:1) for 5 days. The stimulated $CD8^+$ T cells were gated and analyzed for expression of the indicated molecules and for perforin production. (b) Expression of migration markers on naive T cells. DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were cocultured with naive T cells (T cell/DC ratio of 40:1) for 5 days. Stimulated T cells of the CD4 or CD8 subset were gated and analyzed for expression of CD162 molecules. Dashed lines, isotype-matched control IgG; solid lines, anti-CD162 MAb. The numbers in the top right corners of the panels represent the differences in fluorescence intensity (mean ± standard deviation) between the control IgG and the test MAb in three independent experiments. The numbers in parentheses indicate the percentages of the $CD162^{high}$ T cell population among the indicated T cells. A representative of three separate experiments is shown.

of *M. tuberculosis* or killing of *M. tuberculosis* (7–9), and DCs play a central role in activating T cells (39). It has been reported that $CD4^+$ T cells act at the initial stage of *M. tuberculosis* infection and $CD8^+$ T cells work chiefly at the chronic stage (4). The purpose of vaccination aimed at controlling tuberculosis manifestation is to produce T cells that can immediately respond to antigenic molecules expressed on the surface of *M. tuberculosis*-infected APCs in the regional lymph nodes. BCG is essentially capable of activating naive $CD4^+$ T cells, but its potency is not convincing and is not suitable for stimulation of naive $CD8^+$ T cells (15, 16). Further, macrophages infected with BCG inefficiently activate $CD4^+$ T cells (15). Therefore, BCG is not an excellent vaccine in terms of producing abundant T cells capable of responding to secondary stimulation, and improvement of BCG is necessary.

We have previously made efforts to improve the ability of BCG to stimulate T cells, chiefly aiming to produce better vaccines against leprosy. We used MMP-II protein to improve the function of BCG, as the most important element of vaccines (25), and we found that intraphagosomal secretion of HSP70-MMP-II fusion protein is quite useful to stimulate naive T cells of both CD4 and CD8 subsets. Further, we and others showed that urease-deficient rBCG feasibly translocated into lysosomes, where abundant enzymes are available (18). Although urease-deficient BCG-ΔUT-11-3 activated naive $CD4^+$ T cells, it failed to activate naive $CD8^+$ T cells to produce IFN-γ (19). Furthermore, Grode et al. showed that depletion of urease activity in BCG is not sufficient to inhibit the multiplication of *M. tuberculosis* in lung (18). Therefore, it could be speculated that, in order to overcome fully the intrinsic defect of BCG, that is, a lack of phagosome and lysosome fusion, the combination of urease depletion and intraphagosomal secretion of antigenic molecules would be useful.

Our previous study indicated that the rMMP-II protein of *M. tuberculosis* is highly immunogenic and DCs pulsed with MMP-II proteins activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells, but *M. tuberculosis*-derived MMP-II was superior to *M. leprae*-derived MMP-II in all of these functions (29). Furthermore, individuals who were vaccinated with BCG possessing MMP-II 100% homologous to that of *M. tuberculosis* were assumed to be primed with MMP-II *in vivo* (29). Thus, MMP-II of *M. tuberculosis* was considered to have T cell-stimulating activity, and these activated T cells, which subsequently differentiated into the memory state, may be able to respond to *M. tuberculosis*-infected APCs immediately. Therefore, MMP-II could be a useful candidate as a component of vaccines against tuberculosis.

Based on these previous findings and speculation, we produced a new rBCG termed BCG-DHTM, using the MMP-II of *M. tuberculosis*. BCG-DHTM was produced by introducing the HSP70-MMP-II fusion gene into urease-deficient BCG-ΔUT-11-3. Previously, we reported that urease-deficient rBCG that secretes a fusion protein composed of HSP70 and MMP-II from *M. leprae* was superior to urease-deficient BCG-ΔUT-11-3 and normal BCG that secretes the fusion protein in the activation of APCs and naive T cells and the production of memory-type T cells in mice (40). Therefore, we used only vector control BCG-261H as a control BCG in this study. BCG-DHTM induced enhanced activation of naive $CD4^+$ T cells and convincingly activated naive $CD8^+$ T cells to produce IFN-γ, although the availability of non-BCG-vaccinated naive PBMCs would help to confirm these observations. Naive $CD8^+$ T cell activation was confirmed by the observation of phenotypic changes such as expression of activation markers. The activation of naive $CD8^+$ T cells was induced by using the transporter associated with antigen presentation (TAP) and the proteosome-dependent cytosolic cross-presenting pathway, because IFN-γ production from naive $CD8^+$ T cells was largely inhibited by pretreatment of immature DCs with either brefeldin A, an inhibitor of TAP-dependent transportation, or lactacystin, a proteosomal protein degradation blocker. The activation of naive T cells of the CD4 and CD8 subsets by BCG-DHTM was carried out in an Ag-specific manner, since treatment of BCG-DHTM-infected DCs with MAbs to MHC or CD86 molecules significantly inhibited IFN-γ production from naive T cells. Further, BCG-DHTM could activate $CD4^+$ T cells even when macrophages were used as APCs, the function of which is important, because the parent BCG possesses the solid defect of the

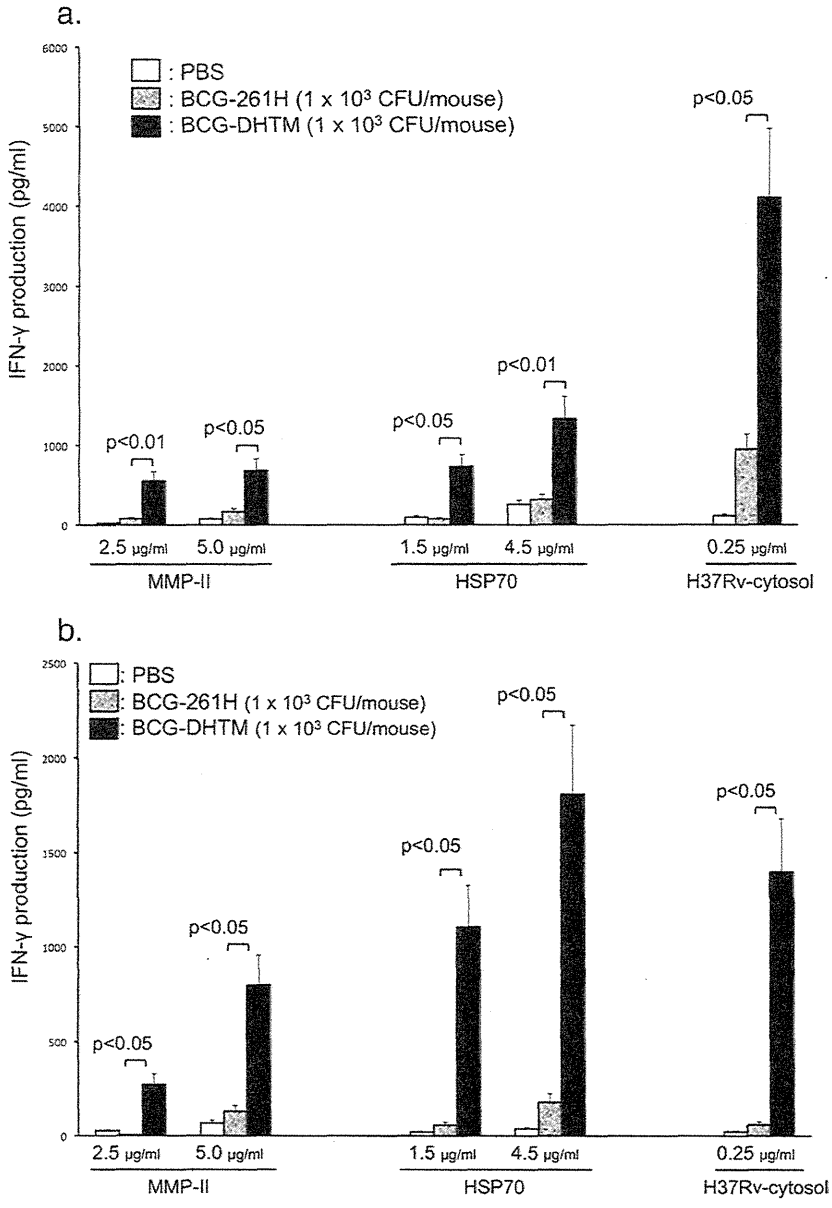

FIG 6 Production of T cells responsive to secondary *in vitro* stimulation in C57BL/6 mice by infection with BCG-DHTM. Three 5-week-old C57BL/6 mice per group were each infected subcutaneously with 1×10^3 CFU of either BCG-261H or BCG-DHTM. Four weeks (a) or 12 weeks (b) after inoculation, splenocytes (2×10^5 cells/well) were stimulated *in vitro* with the indicated stimulators for 4 days, and IFN-γ levels in the cell supernatants were measured. Assays were performed in triplicate for each mouse, and the results for three mice per group are shown as mean ± SD. Representative results of three separate experiments are shown. Concentrations of IFN-γ were statistically compared using Student's *t* test.

inability to activate $CD4^+$ T cells via macrophages. Activation of these naive T cells by DCs and activation of $CD4^+$ T cells by macrophages are closely associated with phagosomal maturation. This conclusion is supported by the observation that pretreatment of DCs and macrophages with chloroquine, an inhibitor of phagosomal acidification, blocked the activation of responder T cells. The HSP70-MMP-II fusion protein secreted in phagosomes can contribute to the activation of naive T cells but, in addition, the fusion protein can be secreted in lysosomes owing to urease deficiency. The protein secreted in lysosomes could be more efficiently degraded into antigenic determinants than that secreted in phagosomes, because lysosomes contain abundant enzymes. The frequency of T cells specific for HSP70-MMP-II fusion protein is low; thus, it seems difficult to inhibit the multiplication of *M. tuberculosis* by the actions of only the fusion protein secreted from normal rBCG. Thus, we need Ag-specific polyclonal T cells. In this respect, urease depletion seems to be useful, because rBCG itself might be processed by the enzyme present in lysosomes. Therefore, BCG-DHTM may be able to activate not only fusion protein-specific T cells but also other T cells polyclonally by using parent BCG-derived Ags. These speculations seem to be supported by animal studies, at least partially. C57BL/6 mice injected with

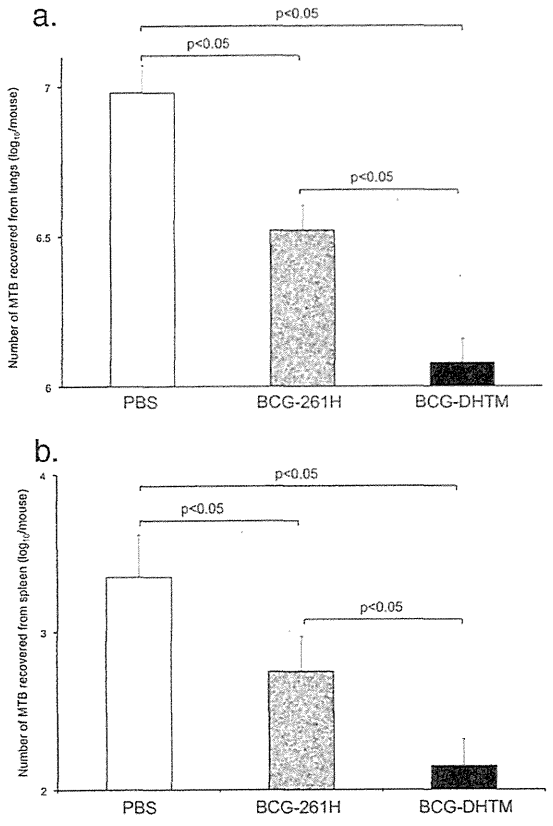

FIG 7 Inhibition of *M. tuberculosis* multiplication by subcutaneous vaccination with BCG-DHTM. Five-week-old C57BL/6 mice (5 mice/group) were subcutaneously vaccinated with either BCG-261H or BCG-DHTM at 1×10^4 CFU/mouse and were challenged with 100 CFU/lung of H37Rv by aerosol infection 6 weeks postvaccination. *M. tuberculosis* (MTB) isolates recovered from the lungs (a) and spleen (b) at 6 weeks postchallenge were enumerated by the colony assay method. Titers were statistically compared using Student's *t* test. A representative of three separate experiments is shown.

BCG-DHTM produced T cells that responded strongly to *in vitro* secondary stimulation with MMP-II and HSP70, as well as *M. tuberculosis*-derived cytosolic protein.

The BCG-DHTM stimulation of naive T cells produced $CD27^{low}$ and $CCR7^{low}$ memory-type $CD8^+$ T cells *in vitro*, and both $CD4^+$ T cells and $CD8^+$ T cells highly expressed migration markers. These observations seem to be important because both subsets of T cells have to migrate immediately and alternatively between lung and regional lymph nodes to react with *M. tuberculosis*-infected APCs efficiently. Production of these T cells may be associated with the partial inhibition of *M. tuberculosis* multiplication in lungs and spleen of mice vaccinated with BCG-DHTM. Although BCG-DHTM showed high immunostimulating activities, it only partially inhibited the growth of *M. tuberculosis* in lungs. There might be several reasons for this unconvincing inhibition. One reason might be the lack of pathogenic features of *M. tuberculosis* in BCG-DHTM, and the second is the relatively small dose of BCG used for vaccination. The third reason may be that we tested bacterial burdens in lungs and spleens at 6 weeks and not 4 weeks, a frequently used time point (41), after *M. tuberculosis* challenge, because 4 to 5 weeks are necessary to reach stable levels of pulmonary bacterial burdens even in naive mice (38). Also, due to BCG strain differences, there may be differences in protective effects in experiments with mice. Therefore, another effort is absolutely required for the production of a more potent BCG to inhibit *M. tuberculosis*. However, the present study may indicate that the HSP70-MMP-II fusion protein could be a candidate vaccine component for the control of tuberculosis.

ACKNOWLEDGMENTS

We thank M. Kujiraoka for her technical support and the Japanese Red Cross Society for kindly providing PBMCs from healthy donors.

This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare of Japan.

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Tsukamoto *et al. BMC Infectious Diseases* 2014, **14**:179
http://www.biomedcentral.com/1471-2334/14/179

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Polyclonal activation of naïve T cells by urease deficient-recombinant BCG that produced protein complex composed of heat shock protein 70, CysO and major membrane protein-II

Yumiko Tsukamoto*, Yumi Maeda, Toshiki Tamura, Tetsu Mukai and Masahiko Makino

Abstract

Background: *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is known to be only partially effective in inhibiting *M. tuberculosis* (MTB) multiplication in human. A new recombinant (r) urease-deficient BCG (BCG-dHCM) that secretes protein composed of heat shock protein (HSP)70, MTB-derived CysO and major membrane protein (MMP)-II was produced for the efficient production of interferon gamma (IFN-γ) which is an essential element for mycobacteriocidal action and inhibition of neutrophil accumulation in lungs.

Methods: Human monocyte-derived dendritic cells (DC) and macrophages were differentiated from human monocytes, infected with BCG and autologous T cells-stimulating activity of different constructs of BCG was assessed. C57BL/6 mice were used to test the effectiveness of BCG for the production of T cells responsive to MTB-derived antigens (Ags).

Results: BCG-dHCM intracellularly secreted HSP70-CysO-MMP-II fusion protein, and activated DC by up-regulating Major Histcompatibility Complex (MHC), CD86 and CD83 molecules and enhanced various cytokines production from DC and macrophages. BCG-dHCM activated naïve T cells of both CD4 and CD8 subsets through DC, and memory type $CD4^+$ T cells through macrophages in a manner dependent on MHC and CD86 molecules. These T cell activations were inhibited by the pre-treatment of Ag-presenting cells (APCs) with chloroquine. The single and primary BCG-dHCM-inoculation produced long lasting T cells responsive to *in vitro* secondarily stimulation with HSP70, CysO, MMP-II and H37Rv-derived cytosolic protein, and partially inhibited the replication of aerosol-challenged MTB.

Conclusions: The results indicate that introduction of different type of immunogenic molecules into a urease-deficient rBCG is useful for providing polyclonal T cell activating ability to BCG and for production of T cells responsive to secondary stimulation.

Keywords: Tuberculosis, BCG, T cell activation

Background

Tuberculosis is a chronic infectious disease, induced by intracellular infection with MTB and is responsible for around 1.4 million deaths yearly worldwide [1-3], and it is estimated that one-third of the global populations is latently infected with MTB. The emergence and worldwide spread of multidrug-resistant strains of MTB mandates the development of more effective preventive and therapeutic tools [4]. Studies using T cell receptor transgenic mice specific for Ag85B-derived $CD4^+$ T cells epitope which is one of the most useful animal model for understanding host defense mechanisms against MTB clearly demonstrated that the MTB-susceptible APCs including DC and macrophages need 7–10 days to initiate activating type 1 $CD4^+$ T cells and $CD8^+$ T cells in regional lymph nodes after aerosol MTB infection, and the stimulated T cells need

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4–5 weeks to initiate inhibiting the growth of MTB in primary mice [5,6]. However, after the adequate activation of both subsets of T cells, the number of MTB residing in the lung remains stable [5,6]. These observations clearly demonstrate that type 1 T cells are essential elements in inhibiting the multiplication of MTB and also that lack of memory type T cells capable of reacting to MTB-infected APCs strongly and immediately allows MTB to multiply. In addition to the animal studies, *in vitro* studies using human APCs or T cells reveal that host defense against MTB is conducted chiefly by type 1 $CD4^+$ T cells and $CD8^+$ T cells [7-9]. Among them, IFN-γ produced from both subsets of T cells is considered as one of the most important element for mycobacteriocidal action [10], and cytotoxic T lymphocytes differentiated from the activated $CD8^+$ T cells act chiefly at late stage of MTB infection [1,11,12]. The killing process of MTB-infected APCs is via a granule-dependent mechanism [13,14]. Although BCG has been used as a vaccine against tuberculosis widely, BCG cannot prevent the manifestation of adult lung tuberculosis [15]. The major reason why BCG is not fully functional, remains to be elucidated. BCG, as a vaccine, is not convincing in terms of T cell activation, since BCG activates naïve $CD4^+$ T cells substantially, but poorly activates naïve $CD8^+$ T cells [16,17]. The reason why BCG cannot activate naïve T cells fully, may be based on the lack of the ability to induce phagosomal maturation [18-20]. Therefore, improvement of T cell-stimulating ability of BCG is strongly required. Presently various new protein vaccine candidates have been selected for clinical trials [18-20]. Actually the vaccine candidates including early secretory antigenic target-6, culture filtrate protein 10, Ag85 family and polyprotein Ag designated Mtb72F and a fusion protein ID93 [9,21-26] are produced based on Ags that are recognized in infected individuals. However, fully reliable new vaccine has not been established yet.

Recently, we have produced recombinant BCG, termed BCG-DHTM which strongly activated human naïve $CD4^+$ T cells and naïve $CD8^+$ T cells, and, in mice, produced T cells responsive to H37Rv-derived cytosolic protein [27]. In the production of BCG-DHTM, we employed two independent strategies in order to overcome the intrinsic defect of BCG, that is an ability to block phagosome maturation to inhibit processing of Ag and presentation to type 1 T cells. One of the strategies is inactivation of *ureC* gene of BCG, which encodes urease, from BCG [19,20]. The urease produces ammonia from urea and inhibits the phagosomal acidification. The urease depletion facilitates the translocation of BCG to lysosome, and enhanced the ability of BCG to activate human naïve $CD4^+$ T cells [19,20]. The other one is the intracellular secretion of antigenic molecule. As the key antigen, we used MMP-II, since MMP-II is recognized by human T cells after infection with *M. leprae* or BCG, and can ligate Toll like receptor (TLR)2 and consequently activate both DC and macrophages [28-31]. Also we used HSP70, since HSP70 has a chaperon activity and can prime cytotoxic T lymphocytes. The intraphagosomal secretion of HSP70-MMP-II fusion protein induced strong activation of naïve $CD4^+$ T cells and $CD8^+$ T cells [32]. Since both strategies, that is urease depletion and an intracellular secretion of HSP70-MMP-II fusion protein, worked synergistically in terms of T cell activation, the gene encoding the HSP70-MMP-II fusion protein was introduced into urease-depleted rBCG (BCG-ΔUT-11-3) for production of BCG-DHTM [27].

Although BCG-DHTM activated both subsets of T cells to produce IFN-γ, production of memory T cells capable of responding to MTB-derived molecules which can be induced chiefly in the activating phase of MTB growth, is needed for the tuberculosis protection. To address this point, we selected CysO (Rv1335 or CFP10A) as the target gene, since CysO also participates in cysteine biosynthesis pathway in MTB [33]. MTB has two independent cysteine biosynthesis pathways, namely the conventional pathway and the alternative pathway. CysO is engaged in the alternative pathway. This pathway is more advantageous under the oxidative conditions. Indeed, CysO expression is induced under diamide stress, one of the oxidative stress conditions [34]. This implies that CysO may be essential for MTB to survive within macrophages, since MTB is exposed to oxidative stress in macrophages [35]. Furthermore, CysO expression is repressed in hypoxic condition and induced in reaeration condition, indicating that CysO may be essential for MTB in the growth phase [36]. Moreover CysO is categorized under ubiquitin superfamily, and may direct protein towards proteasome degradation pathway, which is essential for many cellular processes [37]. Therefore, the CysO involvement in improved cellular response against MTB within macrophages or in growth phase was considered.

In this study, we introduced CysO gene in combination with HSP70-MMP-II fusion gene into urease-deficient BCG-ΔUT-11-3, and produced new rBCG termed BCG-dHCM, and evaluated its T-cell stimulating activities.

Methods

Preparation of cells and Ags

Peripheral blood was obtained from healthy PPD-positive individuals under informed consent. The study was approved by the ethics committee of the National Institute of Infectious Diseases, Tokyo. In Japan, BCG vaccination is compulsory for children (0 ~ 1 year-old). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [38]. The viability of T cells obtained from cryopreserved PBMCs was more than 90% and no selection in terms of functionality was induced in both

monocytes, a precursor of DC and macrophages, and T cells. For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal antibody (mAb) (Dynabeads 450; Dynal Biotech, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on tissue culture plates and the non-plastic adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes [39]. Monocyte-derived DC were differentiated as described previously [38,40]. Briefly, monocytes were cultured in the presence of 50 ng of rGM-CSF (Pepro Tech EC LTD, London, England) and 10 ng of rIL-4 (Pepro Tech) per ml [40]. On day 4 of culture, immature DC were infected with rBCG at an indicated multiplicity of infection (MOI) and, on day 6 of culture, DC were used for further analyses of surface Ag and for mixed lymphocyte assays. Macrophages were differentiated as described previously [41,42]. In brief, monocytes were cultured in the presence of 10 ng of rM-CSF (R & D Systems, Inc., Minneapolis, MN) per ml. On day 5 of culture, macrophages were infected with rBCG at an indicated MOI and, on day 7 of culture, they were used for further analyses of surface Ag and mixed lymphocyte assay. The rMMP-II protein was produced as described previously [28,43], and the CysO protein was produced in an LPS-free condition by using *M. smegmatis*. Polyclonal Ab against CysO was produced by immunizing rabbit with the recombinant protein. The rHSP70 protein was purchased (Hy Test Ltd., Turku, Finland) and H37Rv-derived cytosolic protein was produced as described previously [43].

Vector construction and preparation of rBCG

The genomic DNAs were obtained from BCG substrain Tokyo and from MTB H37Rv strain. The oligonucleotide primers used for the amplication of *hsp70* gene were F-Mb70Bal (5′-aaaTGGCCAtggctcgtgcggtcggg-3′) and R-Mb70Eco (5′-aaaGAATTCcttggcctcccggccg-3′). MMP-II sequence from MTB genomic DNA was amplified with primers: F-MMP TB Eco (aattGAATTCatgca aggtgatcccgatgt) and R-MMP TB Sal (5′-aattGTCGACtcaggtcggtgggcgaga). CysO sequence from MTB genomic DNA was amplified with primers: F-CysO (5′-ggccg ggaggccaagaacgtcaccgtatccattcc-3′) and R-CysO: (5′-at cgggatcaccttgcccaccggccacggcgggga-3′). The amplified HSP70 and MMP-II sequence were digested with appropriate restriction enzymes and cloned into parental pMV261H plasmid. For the cloning of CysO sequence into pMV261, In-Fusion HD cloning kit (Clontech laboratories, Mountain View, CA) was used. For replacing kanamycin resistance gene to hygromycin resistance cassette, the *Xba* I-*Nhe* I fragment from pYUB854 [44] was cloned into *Spe* I-*Nhe* I fragment of the plasmid [44]. The rBCG (BCG-ΔUT-11) of which *ureC* gene was disrupted, was produced as described previously [20]. The hygromycin cassette in the BCG-ΔUT-11 was removed by using pYUB870 encoding γδ-resolvase (γδ-*tnp*R) [44]. The unmarked BCG was named BCG-ΔUT-11-3. The HSP70-CysO-MMP II fusion protein expressing vector was introduced into BCG-ΔUT-11-3 by electroporation method. BCG-ΔUT-11-3 containing pMV-HSP70-CysO-MMP-II as an extrachromosomal plasmid is referred to as BCG-dHCM, and that containing pMV-261-hygromycin is referred to as BCG-261H (BCG vector control). Recombinant BCGs and MTB H37Rv strain were grown to log phase, and stored at 10^8 colony forming unit (CFU)/ml at −80°C. Before infection to DC and macrophages, BCGs were counted by colony assay method. There is no significant difference in the *in vitro* culture growth between BCG-261H and BCG-dHCM.

Analysis of cell surface Ag

The expression of cell surface Ag on DC was analyzed using FACSCalibur (BD Bioscience, San Jose, CA). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich, St. Louis, MO) and 1×10^4 live cells were analyzed. For the analysis of the cell surface Ag, the following mAbs were used: FITC-conjugated mAb against HLA-ABC (G46-2.6, BD Bioscience), HLA-DR (L243, BD Bioscience), CD86 (FUN-1, BD Bioscience), and CD83 (HB15a, Immunotech, Marseille, France).

APC function of DC

The ability of DC infected with BCG or pulsed with recombinant protein and BCG-infected macrophages to stimulate T cells was assessed using an autologous APC-T cell co-culture as previously described [40,45]. Purification of $CD4^+$ and $CD8^+$ T cells was conducted by using negative-isolation kits (Dynabeads 450, Dynal Biotech) [40]. The purity of the $CD4^+$ and $CD8^+$ T cells was more than 95% when assessed using FACSCalibur. Naïve $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with mAb to CD45RO, which were followed by beads coated with goat anti-mouse IgGs Ab (Dynal Biotech). The purity of both subsets of naïve T cells was more than 97%. However, there was no contamination of memory type T cells in the naïve T cell preparations. More than 98% of $CD45RA^+$ T cells was positive in the expression of CCR7 molecule. Memory type T cells were similarly produced by the treatment of cells with mAb to CD45RA Ag. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates, and APCs infected with rBCG or pulsed with protein were added to give the indicated APC: T cell ratio. Supernatants of APC-T cell co-cultures were collected on day 4 and the cytokine levels

were determined. In some cases, rBCG-infected DC and macrophages were treated with mAb to HLA-ABC (W6/32, Mouse IgG2a, kappa), HLA-DR (L243, Mouse IgG2a, kappa), CD86 (IT2.2, Mouse IgG2b, kappa, BD Biosciences) or normal mouse IgG. Also, in some cases, immature DC and macrophages were treated with 50 μM of chloroquine (Sigma-Aldrich) for 2 h and subsequently infected with BCG-dHCM. The optimal dose of the Abs and reagents was determined in advance.

Measurement of cytokine production

Levels of the following cytokines were measured; IFN-γ produced by $CD4^+$ and $CD8^+$ T cells, and IL-12p70, IL-12p40, TNFα, IL-1β and GM-CSF produced by DC or macrophages stimulated for 24 or 48 h with rBCGs. The concentrations of these cytokines were quantified using the enzyme assay kits, Opt EIA Human ELISA Set (BD Bioscience).

Animal studies

For inoculation into mice, rBCG and MTB H37Rv strain were cultured in Middlebrook 7H9 medium supplemented with Middlebrook ADC enrichment to log phase and stored at 10^8 CFU/ml at −80°C. Before the aliquots were used for inoculation, the concentration of viable bacilli was determined by plating on Middlebrook 7H10 agar plate supplemented with Middlebrook OADC enrichment. Three 5-week-old C57BL/6 J mice (Clea Japan Inc., Tokyo, Japan) per group were inoculated subcutaneously with 0.1 ml of PBS or PBS containing 1×10^3 rBCGs. The animals were kept in specific pathogen free conditions and were supplied with sterilized food and water. Four or 12 weeks after inoculation, the spleens were removed and the splenocytes were suspended at a concentration of 2×10^6 cells per ml in culture medium. The splenocytes were stimulated with an indicated concentration of rMMP-II, rHSP70 (HyTest), rCysO or H37Rv-derived cytosolic protein in triplicates in 96-well round bottom microplates [20,30]. The individual culture supernatants were collected 3–4 days after stimulation and IFN-γ was measured using Opt EIA Mouse ELISA Set (BD Bioscience). For observing the effect of BCG vaccination on MTB infection, five C57BL/6 mice per group were vaccinated with 1×10^3 CFU/mouse either BCG-261H or BCG-dHCM for 6 weeks, and were challenged with 100 CFU/lungs of H37Rv by aerosol infection using an automated inhalation exposure apparatus (Glas-Col Corp., IN, Model 099C A4212). Six weeks later, bacterial burden in lungs and spleen was assessed by mechanical disruption in PBS with 0.05% v/v Tween 80 and enumerated by colony assay. Animal studies were reviewed and approved by the Animal Research Committee of Experimental Animals of the National Institute of Infectious Diseases, and were conducted according to their guidelines.

Statistical analysis

Student's *t*-test was applied to determine the statistical differences.

Results

Immunological characterization of rCysO protein

Since rCysO protein produced in *M. smegmatis* revealed a single band on SDS-PAGE electrophoresis and confirmed by Western blot analysis using polyclonal Ab to CysO (not shown), the immunostimulatory activities of rCysO protein were assessed using rMMP-II of MTB as a positive control. We assessed the activation of APCs from the aspect of phenotypic changes and cytokine production (Figure 1). The stimulation of immature DC with rCysO protein up-regulated the expression of MHC molecules, CD86, and CD83 Ags, comparable to rMMP-II protein (Figure 1a). Further, both rMMP-II and rCysO proteins induced IL-12p40 production from DC and TNFα production from macrophages to a similar level (Figure 1b). These results indicate that CysO protein has an ability to activate APCs. When we pulsed immature DC with rMMP-II or rCysO protein and used as a stimulator of autologous $CD4^+$ T cells, both proteins induced IFN-γ production from not only memory type $CD4^+$ T cells, but also from naïve $CD4^+$ T cells with the help of CD40-CD40L interaction. The concentration of IFN-γ released from $CD4^+$ T cells by the stimulation with rMMP-II and rCysO was comparable (Figure 1c). Therefore, rCysO protein was found to possess immunostimulatory. We also examined if the newly produced recombinant BCG-dHCM secretes the HSP70-CysO-MMP-II fusion protein by using Western blot analyses. When probed by the Ab to either of HSP70, CysO or MMP-II, BCG-dHCM showed distinct band at 95 kD equivalent to the molecular mass of the fusion protein comprising HSP70, CysO and MMP-II (not shown).

Activation of Ag-presenting cells by BCG-dHCM

In order to activate naïve T cells and produce memory type T cells efficiently, rBCG should also activate APC adequately. We assessed the activation of DC from the aspects of cytokine production and phenotypic changes (Figure 2). BCG-dHCM stimulated DC to produce IL-12p70, TNFα and IL-1β more efficiently than BCG-261H at both MOIs: 0.25 and 0.50 (Figure 2a). To assess the phenotypic changes induced by BCG-dHCM infection, we assessed the expression of MHC, CD86 and CD83 molecules on DC (Figure 2b). Both BCG-261H and BCG-dHCM up-regulated the expression of these molecules, but the infection with BCG-dHCM induced higher level of the expression. We used various dose of rBCGs for the assessment, and the similar changes were observed (not shown). Further, the stimulation of macrophages with BCG-dHCM induced the production of

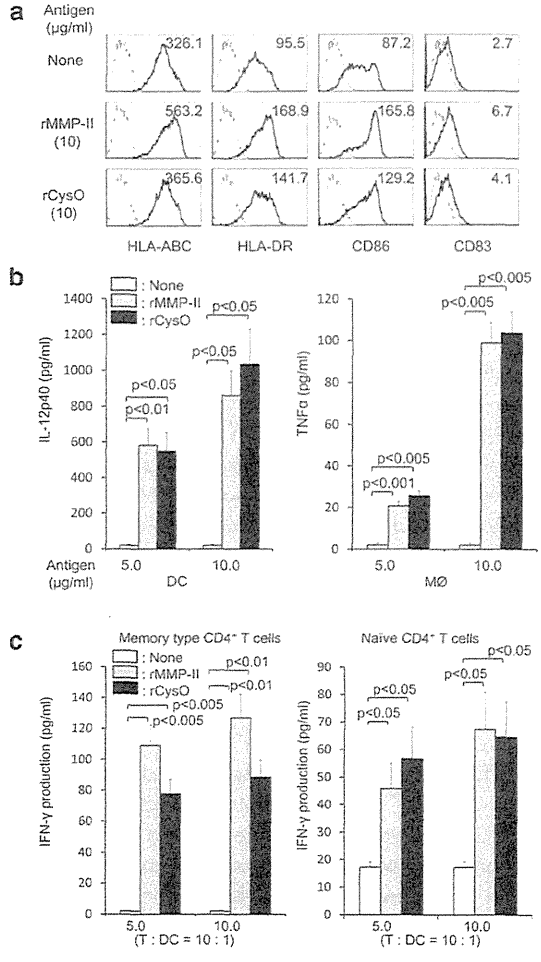

Figure 1 Activation of APCs by rCysO in terms of phenotypic changes and cytokine production. a. Up-regulated expression of APC-associated molecules on DC by stimulation with rCysO protein. Monocyte-derived immature DC were stimulated with either rMMP-II or rCysO protein at 10 μg/ml, and cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DC from day 6 were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. Representative results of three separate experiments are shown. The number at the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. **b.** Cytokine production from APCs by stimulation with rCysO protein. DC produced using rGM-CSF and rIL-4 and macrophages differentiated from monocytes by using M-CSF were stimulated with either rMMP-II or rCysO protein for 24 h. **c.** IFN-γ production from $CD4^+$ T cells by stimulation with rCysO protein. Monocyte-derived DC were stimulated with either rMMP-II or rCysO protein, and were used as a stimulator of memory type or naïve $CD4^+$ T cells in a 4-day culture. 10^5 responder T cells were stimulated with the protein-pulsed DC at T: DC = 10: 1. In case of the stimulation of naïve $CD4^+$ T cells, the protein-pulsed DC were further treated with 1.0 μg/ml of rCD40 ligand for 24 h. The concentration of the indicated cytokine was determined by the ELISA method. A representative of three separate experiments conducted by using 3 different PBMCs-donors is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t*-test.

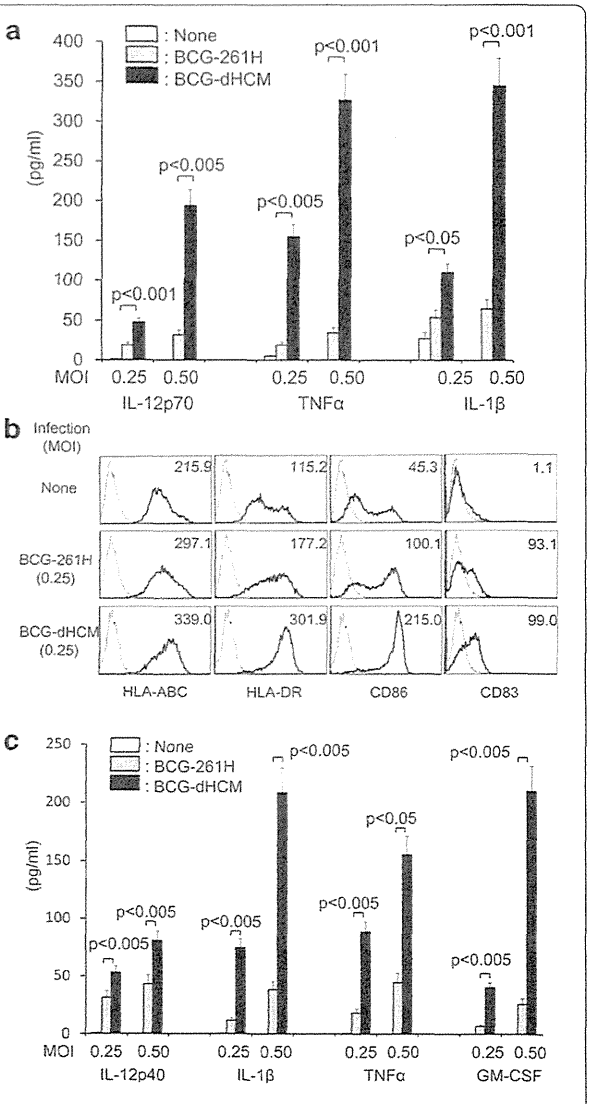

Figure 2 Activation of APCs by BCG-dHCM. a. Cytokine production from DC by stimulation with BCG-dHCM. DC produced using rGM-CSF and rIL-4 were stimulated with either BCG-261H or BCG-dHCM for 24 h. **b.** Up-regulation of APC-associated molecules and activation marker on DC by infection with BCG-dHCM. Monocyte-derived immature DC were infected with either BCG-261H or BCG-dHCM at an MOI 0.25 and cultured for another two days in the presence of rGM-CSF and rIL-4. The DC from day 6 of culture were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number at the top right corner of each panel represents the difference in the fluorescence intensity between the control IgG and the test mAb. **c.** Cytokine production from macrophages by stimulation with BCG-dHCM. Macrophages differentiated from monocytes by using M-CSF were stimulated with either BCG-261H or BCG-dHCM for 24 h. The concentration of the indicated cytokine was determined by the ELISA method. A representative of three separate experiments conducted by using 3 different PBMCs-donors is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t*-test.