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 proteasome-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 defense 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 defense 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 phagosomes 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) are 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 BCG (BCG-ΔUT-11-3) by deleting 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-
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 ML0238) 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-7OM, having a BCG-derived heat shock protein 70 (HSP70)-MMP-II fusion gene, and subcutaneous single BCG-70M vaccine 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 (rMMP-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., Turkua, 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 strain H37Rv and from M. tuberculosis strain H37Rv. The oligonucleotide primers used for amplification of the hsp70 gene were F-Mb70Bal (5′-aaTGTTGCTTACTGTTGCG-3′) and R-Mb70Eco (5′-aaaGAATTCTCTGCCGAGACTCA-3′). The MMP-II sequence from M. tuberculosis genomic DNA was amplified with primers F-MMPTB-Eco (5′-attGATTCTCAACTGTTGCG-3′) and R-MMPTB-Bsal (5′-attGCTGCACTTGTTGCGGAGA-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 an antibiotic resistance gene (hygromycin) cassette, the BxaI-Nhel fragment from pYUB854 (36) was cloned into the SpeI-Nhel 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 pYUB710 encoding ybf-resolvase (ybf

- 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 × 10^6 live cells were analyzed. For the analysis of cell surface Ags, the following MAb s were used: fluorescein isothiocyanate (FITC)-conjugated MAb s 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 conjugated MAb s 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 antihuman immunoglobulin (Ig) MAb s (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 processing 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 (BG9; 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). Naïve 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 CD25 molecule. Memory-type T cells were similarly produced by the treatment of cells with MAb to CD45RA Ag. The purified responder cells (1 × 10⁶ 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 10⁶ CFU/ml. Before the aliquots were used for inoculation, the concentrations of viable bacilli were determined by plating on Middlebrook 7H110 agar plates. Three 5-week-old C57BL/6 mice (Clea Japan Inc., Tokyo, Japan) per group were inoculated subcutaneously with 0.1 ml of phosphate-buffered saline (PBS) or PBS containing 1 × 10⁴ or 1 × 10⁵ rBCGs. 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 × 10⁶ cells/ml 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 × 10⁶ 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 A212; 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 naïve 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 produced 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 naïve 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 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 naïve 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 naïve CD4+ T cells or CD8+ T cells (data not shown).

Activation of memory-type and naïve 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-
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). 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 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 MAb. 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 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. Titer 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 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 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 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.
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 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.

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 × 10^5 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 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 × 10^5 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 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 × 10^5 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 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 × 10^5 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 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 × 10^5 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 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 × 10^5 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 BCG-261H. Similar differences between BCG-261H and BCG-DHTM were observed at different MOIs (data not shown).
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 (1×10^6 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 (1×10^6 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 (36). The activation of both CD4+ T cells and CD8+ T cells is required for inhibition of the replication
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 urine-deficient rBCG feasibly translocated into lysosomes, where abundant enzymes are available (18). Although urine-deficient BCG-DUT-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-DUT-11-3. Previously, we reported that urine-deficient rBCG that secretes a fusion protein composed of HSP70 and MMP-II from M. leprae was superior to urine-deficient BCG-DUT-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 helped 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 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 CD162high T cell population among the indicated T cells. A representative of three separate experiments is shown.
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 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 $1 \times 10^3$ CFU of either BCG-261H or BCG-DHTM. Four weeks (a) or 12 weeks (b) after inoculation, splenocytes ($2 \times 10^5$ 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.
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 naïve T cells produced CD27low and CCR7low 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.

REFERENCES

1. Flynn JL, Chan J. 2001. Immunology of tuberculosis. Annu. Rev. Immunol. 19:93–129. http://dx.doi.org/10.1146/annurev.immunol.19.1.93.
2. North RJ, Jung YJ. 2004. Immunity to tuberculosis. Annu. Rev. Immunol. 22:599–623. http://dx.doi.org/10.1146/annurev.immunol.22.012703.104635.
3. World Health Organization. 2012. Global tuberculosis report 2012. World Health Organization, Geneva, Switzerland.
4. Kaufmann SH, McMichael AJ. 2005. Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. Nat. Med. 11(Suppl):S33–S54. http://dx.doi.org/10.1038/nm1221.
5. World Health Organization. 2007. Global MDR-TB and XDR-TB response plan 2007–2008, p 1–48. In WHO report 2007. World Health Organization, Geneva, Switzerland.
6. Mittrucker H-W, Steinhoff U, Köhler A, Krause M, Lazar D, Mex P, Miekley D, Kaufmann SH. 2007. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. Proc. Natl. Acad. Sci. U. S. A. 104:12434–12439. http://dx.doi.org/10.1073/pnas.0703510104.
7. Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection. Proc. Natl. Acad. Sci. U. S. A. 89:12013–12017.
8. Hobe K, Janssen E, Beutler B. 2004. The interface between innate and adaptive immunity. Nat. Immunol. 5:971–974. http://dx.doi.org/10.1038/nn1004-971.
9. Aagaard CS, Hoang TTTK, Vingsbo-Lundberg C, Dietrich J, Andersen P. 2009. Quality and vaccine efficacy of CD4+ T cell responses directed to dominant and subdominant epitopes in ESAT-6 from Mycobacterium tuberculosis. J. Immunol. 183:2659–2668. http://dx.doi.org/10.4049/jimmunol.0900947.
10. Forbes EK, Sander C, Ronan EO, McShane H, Hill AVS, Beverley PCL, Tchilian EZ. 2008. Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice. J. Immunol. 181:4955–4964.
11. Kaufmann SH. 1988. CD8+ T lymphocytes in intracellular microbial infections. Immunol. Today 9:168–174.
12. Caccamo N, Meraaviglia S, Mendola CL, Guggino G, Dieli F, Salerno A. 2006. Phenotypical and functional analysis of memory and effector human CD8 T cells specific for mycobacterial antigens. J. Immunol. 177:1780–1785.
13. Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Kaufmann SH, McMichael AJ. 2004. Immunity to tuberculosis. Annu. Rev. Immunol. 22:1243–1265. http://dx.doi.org/10.1146/annurev.immunol.22.012703.124343.
14. Woodworth JS, Wu Y, Behar SM. 2008. Mycobacterium tuberculosis-specific CD8+ T cells require perforin to kill target cells and provide protection in vivo. J. Immunol. 181:8595–8603.
15. Pancholi P, Mirra A, Bhardwaj N, Steinman RM. 1993. Sequestration from immune CD4+ T cells of mycobacteria growing in human macrophages. Science 260:984–986. http://dx.doi.org/10.1126/science.8098850.
16. Soualhine H, Dehghani A-E, Sun J, Mak K, Talal A, AvGay Y, Hmama Z. 2007. Mycobacterium bovis bacillus Calmette-Guérin secreting active cathepsin S stimulates expression of mature MHC class II molecules and antigen presentation in human macrophages. J. Immunol. 179:5137–5145.
Reyrat JM, Berthet FX, Gicquel B. 1995. The urease locus of Mycobacterium tuberculosis and its utilization for allelic exchange in Mycobacterium bovis bacillus Calmette-Guérin. Proc. Natl. Acad. Sci. U. S. A. 92:8768–8772.

Grose L, Seiler P, Baumann S, Hess J, Brinkmann V, Eddine AN, Mann P, Goosmann C, Bandermann S, Smith D, Bancroft GJ, Reyrat JM, van Soolingen D, Raupach B, Kaufmann SHE. 2005. Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guérin mutants that secrete listeriolysin. J. Clin. Invest. 115: 2472–2479. http://dx.doi.org/10.1172/JCI24617.

Mukai T, Maeda Y, Tamura T, Miyamoto Y, Makino M. 2008. CD4+ T-cell activation by antigen-presenting cells infected with urease-deficient recombinant Mycobacterium bovis bacillus Calmette-Guérin, FEMS Immunol. Med. Microbiol. 53:96–106. http://dx.doi.org/10.1111/j.1574-695X.2008.00407.x.

Horwitz MA, Lee BW, Dillon BJ, Harth G. 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. U. S. A. 92: 1530–1534.

Skeikey YA, Alderson MR, Ovendale PJ, Guderian JA, Brandt L, Dillon DC, Campos-Neto A, Lobet Y, Dalemans W, Orme IM, Reed SG. 2004. Differential immune responses and protective efficacy induced by components of a tuberculosis polyprotein vaccine, Mtbb72F, delivered as naked DNA or recombinant protein. J. Immunol. 172:7618–7628.

Andersen P. 2007. Tuberculosis vaccines: an update. Nat. Rev. Microbiol. 5:484–487.

Hoft DF. 2008. Tuberculosis vaccine development: goals, immunological design, and evaluation. Lancet 372:164–175. http://dx.doi.org/10.1016/S0140-6736(08)61036-3.

Mukai T, Maeda Y, Tamura T, Miyamoto Y, Makino M. 2005. Identification of immunomodulating agent from Mycobacterium leprae. Infect. Immun. 73: 2744–2750. http://dx.doi.org/10.1128/IAI.73.5.2744-2750.2005.

Makino M, Maeda Y, Ishii N. 2005. Immunostimulatory activity of major membrane protein-II from Mycobacterium tuberculosis. Clin. Vaccine Immunol. 12:115–123. doi.org/10.1128/CVI.00459-10.

Makino M, Baba Y. 1997. A cryopreservation method of human peripheral blood mononuclear cells for efficient production of dendritic cells. Scand. J. Immunol. 45:618–622.

Wakamatsu S, Makino M, Tei C, Baba M. 1999. Monocyte-driven activation-induced apoptotic cell death of human T-lymphotropic virus type I-infected T cells. J. Immunol. 163:3914–3919.

Makino M, Shimokubo S, Wakamatsu S, Izumo S, Baba M. 1999. The role of human T-lymphotropic virus type 1 (HTLV-1)-infected dendritic cells in the development of HTLV-I-associated myelopathy/tropical spastic paraparesis. J. Virol. 73:4579–4581.

Makino M, Maeda Y, Fukutomi Y, Mukai T. 2007. Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages. Microbes Infect. 9:70–77. http://dx.doi.org/10.1016/j.micinf.2006.10.011.

Makino M, Maeda Y, Kai M, Tamura T, Mukai T. 2009. GM-CSF-mediated T-cell activation by macrophages infected with recombinant BCG that secretes major membrane protein-II of Mycobacterium leprae. FEMS Immunol. Med. Microbiol. 55:39–46. http://dx.doi.org/10.1111/j.1574-695X.2008.00495.x.

Maeda Y, Gidoh M, Ishii N, Mukai C, Makino M. 2003. Assessment of cell mediated immunogenicity of Mycobacterium leprae-derived antigens. Cell. Immunol. 222:69–77. http://dx.doi.org/10.1016/S0008-8749(03)00078-9.

Barbarow S, Barbarow S, Jr, Pavelka MS, Jr, Sambandamurthy V, Larsen M, Tufariello J, Chan J, Hatfull G, Jacobs WR, Jr. 2002. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in Mycobacterium tuberculosis, M. bovis BCG and M. smegmatis. Microbiology 148:3007–3017.

Hashimoto K, Maeda Y, Kimura H, Suzuki K, Masuda A, Matsuoka M, Makino M. 2002. Mycobacterium leprae infection in monocyte-derived dendritic cells and its influence on antigen-presenting function. Infect. Immun. 70:5167–5176. http://dx.doi.org/10.1128/IAI.70.9.5167-5176.2002.

Wolf AJ, Desvignes L, Linas B, Banaïe N, Tamura T, Takatsu K, Ernst JD. 2008. Initiation of the adaptive immune response to Mycobacterium tuberculosis depends on antigen production in the local lymph node, not the lungs. J. Exp. Med. 205:105–115. http://dx.doi.org/10.1084/jem.20071367.

Wolf AJ, Linas B, Treveño-Núñez GJ, Kincaid E, Tamura T, Takatsu K, Ernst JD. 2007. Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo. J. Immunol. 179:2509–2519.

Mukai T, Maeda Y, Tamura T, Matsuoka M, Tsukamoto Y, Makino M. 2010. Enhanced activation of T lymphocytes by urease-deficient recombinant bacillus Calmette-Guérin producing heat shock protein 70-major membrane protein-II fusion protein. J. Immunol. 185:6234–6243. http://dx.doi.org/10.4049/jimmunol.1000198.

Baldwin SL, Ching LK, Pike SO, Moutaftsi M, Lucas E, Vallur A, Orr MT, Bertholet S, Reed SG, Coler RN. 2013. Protection against tuberculosis with homologous or heterologous protein/vector vaccine approaches is not dependent on CD8+ T cells. J. Immunol. 191:2514–2525. http://dx.doi.org/10.4049/jimmunol.1301161.