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Induction of Cross-Priming of Naive CD8$^+$ T Lymphocytes by Recombinant Bacillus Calmette-Guérin That Secretes Heat Shock Protein 70-Major Membrane Protein-II Fusion Protein[1]

Tetsu Mukai, Yumi Maeda, Toshiki Tamura, Masanori Matsuoka, Yumiko Tsukamoto, and Masahiko Makino[2]

Because *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) unconvincingly activates human naive CD8$^+$ T cells, a rBCG (BCG-70M) that secretes a fusion protein comprising BCG-derived heat shock protein (HSP)70 and *Mycobacterium leprae*-derived major membrane protein (MMP)-II, one of the immunodominant Ags of *M. leprae*, was newly constructed to potentiate the ability of activating naive CD8$^+$ T cells through dendritic cells (DC). BCG-70M secreted HSP70-MMP-II fusion protein in vitro, which stimulated DC to produce IL-12p70 through TLR2. BCG-70M-infected DC activated not only memory and naive CD8$^+$ T cells, but also CD4$^+$ T cells of both types to produce IFN-γ. The activation of these naive T cells by BCG-70M was dependent on the MHC and CD86 molecules on BCG-70M-infected DC, and was significantly inhibited by pretreatment of DC with chloroquine. Both brefeldin A and lactacystin significantly inhibited the activation of naive CD8$^+$ T cells by BCG-70M through DC. Thus, the CD8$^+$ T cell activation may be induced by cross-presentation of Ags through a TAP- and proteosome-dependent cytosolic pathway. When naive CD8$^+$ T cells were stimulated by BCG-70M-infected DC in the presence of naive CD4$^+$ T cells, CD62L^{low}CD8$^+$ T cells and perforin-producing CD8$^+$ T cells were efficiently produced. MMP-II-reactive CD4$^+$ and CD8$^+$ memory T cells were efficiently produced in C57BL/6 mice by infection with BCG-70M. These results indicate that BCG-70M activated DC, CD4$^+$ T cells, and CD8$^+$ T cells, and the combination of HSP70 and MMP-II may be useful for inducing better T cell activation. *The Journal of Immunology,* 2009, 183: 6561–6568.

Leprosy is a chronic infectious disease induced by an intracellular infection with *Mycobacterium leprae* (1, 2). Host defense against *M. leprae* is chiefly conducted by adaptive immunity in which both IFN-γ-producing type 1 CD4$^+$ T cells and CD8$^+$ T cells play an important role, and the activation of these T cells inhibits the spread of *M. leprae* (3–5). The activation is induced by bacilli-loaded dendritic cells (DC),[3] which display one or more antigenic determinants of *M. leprae*. Previously, we identified major membrane protein (MMP)-II (gene name, bfrA or ML2038) as one of the immunodominant Ag of *M. leprae* (6). MMP-II activates dendritic cells (DC) by activating the NF-κB pathway as a consequence of TLR2's ligation, and DC pulsed with a rMMP-II protein activate both naive and memory-type CD4$^+$ and CD8$^+$ T cells to produce IFN-γ in an Ag-specific manner (6, 7). In the lesions of patients with paucibacillary leprosy, representative of clinical leprosy on one pole, the involvement of CD1a$^+$ DC and presence of substantially activated T cells have been observed (8, 9). Furthermore, MMP-II is thought to be recognized by both T cell subsets in *M. leprae*-infected individuals, including patients with paucibacillary leprosy (7). Therefore, MMP-II is considered to play essential roles in the induction of host defense activity against *M. leprae*. Also, we reported that T cells from lepromatous leprosy, representative of clinical leprosy on another pole, can be activated to produce IFN-γ when stimulated with MMP-II-pulsed autologous DC (7), although it is known that the T cells of lepromatous leprosy patients are usually unresponsive to *M. leprae*-derived Ags (2).

Mycobacterium bovis bacillus Calmette-Guérin (BCG) is the sole available vaccine against leprosy, and several reports have evaluated its efficacy. In some countries and endemic areas, BCG has effectively inhibited the development of leprosy, whereas in others, its efficacy is reported to be quite limited (10–12). These observations indicate that questions remain regarding the reliability of BCG as a vaccine, and, in fact, Setia et al. (13) elucidated the overall efficacy of BCG to be only 26% through meta-analyses of several studies and observations. Based on these findings, we previously produced a rBCG that secretes MMP-II intracytosolically (BCG-SM) (14). As expected, BCG-SM activated both naive CD4$^+$ and CD8$^+$ T cells (14) and inhibited *M. leprae* from multiplying to some extent, but not completely, in the footpads of C57BL/6 mice (Y. M., T. T., M. Mat., and M. Mak.; unpublished observations). It is known that the parental BCG activates chiefly CD4$^+$ T cells, and less efficiently activates naive CD8$^+$ T cells (15). That BCG-SM activated naive T cells of both subsets and, consequently, partially inhibited the multiplication of *M. leprae*,

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[3] Abbreviations used in this paper: DC, dendritic cell; BCC, *Mycobacterium bovis* bacillus Calmette-Guérin-derived cytosolic protein; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; BCG-SM, rBCG that secretes major membrane protein-II; HSP, heat shock protein; MMP, major membrane protein; MOI, multiplicity of infection.

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indicated that the secretion of an immunodominant Ag of *M. leprae* in phagosomes of APCs of host is a useful way to inhibit the growth of *M. leprae* through the activation of T cells by delivering the antigenic determinants on APCs. This point was also revealed in other intracellular infection systems such as *Mycobacterium tuberculosis*, in which the secretion of Ag85 complex, one of the immunogenic molecules of *M. tuberculosis*, from vaccinated BCG was revealed to be effective in inhibiting the replication of *M. tuberculosis* challenged subsequently (16). Although the mechanisms involved have not been fully clarified, the activation of CD8$^+$ T cells seems to be induced by Ag85 protein secreted from BCG (16).

In general, the most efficient immunological means of activating naive CD8$^+$ T cells using mycobacteria, including BCG, is to upregulate the activity of DC to cross-present mycobacteria-derived Ags to the CD8$^+$ T cells. In this respect, an active inducer of cross-presenting activity in APCs is heat shock protein (HSP)70 (17, 18). HSP70 may be closely associated with host defenses against intracellular pathogens such as mycobacteria (19, 20).

In this study, in the search for another tool capable of stimulating naive CD8$^+$ T cells efficiently, we newly constructed a rBCG having an extrachromosomal BCG-derived HSP70 gene linked to the gene encoding MMP-II of *M. leprae* (BCG-70M), and evaluated its immunostimulatory activities. The BCG-70M secreted the HSP70-MMP-II fusion protein in vitro, and DC infected with BCG-70M more efficiently activated not only naive CD8$^+$ T cells by cross-presentation, but also naive CD4$^+$ T cells. Furthermore, BCG-70M produced memory T cells, of both CD4$^+$ and CD8$^+$ subsets in mice, capable of responding to MMP-II.

Materials and Methods

Preparation of cells and Ags

Peripheral blood was obtained from healthy purified protein derivative-positive individuals under informed consent using a double-blind system. In Japan, a BCG vaccination is compulsory for children (0~4 years old). PBMCs were isolated using Ficoll-Paque PLUS (Pharmacia) and cryopreserved in liquid nitrogen until used, as described previously (21). 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 mAb (Dynabeads 450; Dynal Biotech). The CD3$^-$ PBMC fraction was plated on collagen-coated plates, and the nonplastic-adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (22). Monocyte-derived DC were differentiated, as described previously (21, 23). Briefly, monocytes were cultured in the presence of 50 ng of rGM-CSF (PeproTech) and 10 ng of rIL-4 (PeproTech) per ml (23). 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. The rMMP-II protein and BCG-derived cytosolic protein (BCC) were produced, as described previously (6, 24).

Vector construction and preparation of rBCG

For the preparation of rBCG that secretes HSP70-MMP-II fusion protein, a plasmid pMV-70M was constructed having a hygromycin resistance gene and origins of replication for *Escherichia coli* and mycobacteria. Briefly, the genomic DNA from BCG substrain Tokyo or *M. leprae* strain Thai-53 was purified by proteinase K digestion and phenol-chloroform extraction. The oligonucleotide primers for the amplication of the *hsp70* gene were FMb70Bal (5′-aaaTGGCCAtggctcgtgcggtcggg-3′; capital letters indicate a *Bal*I site) and RMb70Eco (5′-aaaGAATTCcttggcctcccggccg-3′; capital letters indicate an *Eco*RI site). The primers for the Ag85B signal sequence of BCG were FMbAg85Bal (5′-tttTGGCCAtgacagacgtgagccgaaa-3′; capital letters indicate a *Bal*I site) and RMbAg85 Eco120 (5′-aaaGAATTC cgcgcccgcggttgcc-3′; capital letters indicate an *Eco*RI site). The MMP-II sequence from *M. leprae* genomic DNA was amplified with FMMPEco4 (5′-aaaGAATTCcaaggtgatccggatgt-3′; capital letters indicate an *Eco*RI site) and RMMP Sal (5′-tgaGTCGACttaactcggcggccggga-3′; capital letters indicate a *Sal*I site). The amplified products were digested with appropriate restriction enzymes and cloned into a *Bal*I-*Sal*I-digested parental pMV261 plasmid. For replacing the kanamycin resistance gene with a hygromycin resistance cassette, the *Xba*I-*Nhe*I fragment from pYUB854 (25) was cloned into *Spe*I-*Nhe*I-digested plasmids.

BCG substrain Tokyo was cultured in vitro using Middlebrook 7H9 broth (BD Biosciences) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (BD Biosciences) or Sauton medium containing 0.05% Tween 80. Expression vectors were introduced into BCG by electroporation (26). Transformants were selected on Middlebrook 7H10 agar (BD Biosciences) plates. The BCG containing pMV-HSP70-MMP-II as an extrachromosomal plasmid is referred to as BCG-70M, and that containing pMV-261 is referred to as BCG-261H (BCG vector control). rBCGs were grown to a log phase, and stored at 10^8 CFU/ml at −80°C. Before the infection of DC, BCGs were counted by the colony assay method. There was no significant difference in growth in vitro between BCG-261H and BCG-70M.

Expression of the fusion protein HSP70-MMP-II

To verify the secretion of MMP-II and HSP70 from BCG-70M, the culture supernatant of BCG-70M, cultured for 20 days in Sauton medium, was collected, and concentrated using the Labscale TFF system (Millipore), after the supernatant was depleted of the cells by centrifugation. rMMP-II protein was used as a control for Western blotting. SDS-PAGE and electroblotting were conducted using standard methods (27). Western blotting was performed, as follows: a membrane having the transferred protein was blocked in 5% skim milk and then incubated with anti-MMP-II mAb 202-3 (IgG2a) or anti-mycobacterial HSP70 mAb (HyTest), which is not cross-reactive to mammalian HSP70 homologues. An alkaline-phosphatase-conjugated anti-mouse IgG Ab (BioSource International) was used as the secondary Ab. Color development was performed using NBT/5-bromo-4-chloro-3-indolyl phosphate detection reagent (Calbiochem).

Analysis of cell surface Ag

The expression of cell surface Ag on DC was analyzed using FACS Calibur. Dead cells were eliminated from the analysis based on staining with propidium iodide (Sigma-Aldrich), and 1×10^4 live cells were analyzed. For the analysis of the cell surface Ag, the following mAbs were used: a FITC-conjugated mAb against HLA-ABC (G46-2.6; BD Pharmingen), HLA-DR (L243; BD Biosciences), CD86 (FUN-1; BD Biosciences), CD83 (HB15a; Immunotech), and CD62L (Dreg 56; BD Biosciences), and a PE-conjugated mAb to CD8 (RPA-T8; BD Biosciences).

The expression of MMP-II on rBCG-infected DC was determined using a mAb (M270-13, IgM, κ) against MMP-II, which probably detects MMP-II in a complex with MHC molecules on the surface of DC (7), followed by a FITC-conjugated anti-mouse Igs Ab (Tago-immunologicals). For the inhibition of the intracellular processing of phagocytosed bacteria, DC were treated with 50 μM chloroquine (Sigma-Aldrich) for 2 h, washed, subsequently infected with BCG, and subjected to analyses of MMP-II surface expression. The intracellular production of perforin was assessed, as follows: unseparated naive T cells were stimulated with rBCG-infected DC for 5 days, and CD8$^+$ T cells were surface stained with a PE-labeled mAb to CD8, and fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using permeabilizing solution (BD Biosciences), and stained with a FITC-conjugated mAb to perforin (δG9; BD Biosciences).

APC functions of DC

The ability of BCG-infected DC to stimulate T cells was assessed using an autologous DC-T cell coculture, as described previously (5, 23). Purification of CD4$^+$ and CD8$^+$ T cells was conducted by using negative isolation kits (Dynabeads 450; Dynal Biotech) (23). The purity of the CD4$^+$ and CD8$^+$ T cells was more than 95% when assessed using FACSCalibur. Naive CD4$^+$ and CD8$^+$ T cells were produced by further treatment of these T cells with a mAb to CD45RO, followed by beads coated with a mAb to goat anti-mouse IgGs (Dynal Biotech). The purity of both subsets of naive T cells was more than 97%. More than 98% of CD45RA$^+$ T cells expressed CCR7 molecule. Memory-type T cells were similarly produced by the treatment of cells with a mAb to CD45RA Ag. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates, and DC were added to give the indicated DC:T cell ratio. Supernatants of DC-T cell cocultures were collected on day 4, and the cytokine levels were determined. In some cases, rBCG-infected DC were treated with a mAb to HLA-ABC (W6/32, mouse IgG2a, κ), HLA-DR (L243, mouse IgG2a, κ), CD86 (IT2.2, mouse IgG2b, κ; BD Biosciences), or MMP-II (M270-13), or with normal mouse IgG or IgM. The optimal concentration was determined in advance. Also, in some cases, immature DC

were treated with the indicated dose of chloroquine, brefeldin A (Sigma-Aldrich), or lactacystin (Sigma-Aldrich), and subsequently infected with BCG-70M. The optimal dose of these 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, TNF-α, and IL-1β produced by DC 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 Biosciences). The murine mAb against TLR2 (clone 2392; IgG1) with antagonistic activity was obtained from Genentech. The optimal concentration of these mAbs was determined in advance.

Animal experiments

For the inoculation of mice, rBCG was cultured in Middlebrook 7H9 medium to a log phase of growth 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 a Middlebrook 7H10 agar plate. Three 5-wk-old C57BL/6J mice (Japan CLEA) per group were inoculated s.c. with 0.1 ml of PBS or PBS containing 1×10^2 or 1×10^3 rBCGs. The animals were kept in specific pathogen-free conditions and supplied with sterilized food and water. Four weeks after the inoculation, the spleens were removed and the splenocytes were suspended at a concentration of 2×10^6 cells/ml in culture medium. The splenocytes were stimulated with an indicated concentration of rMMP-II, rHSP70 (HyTest), or BCC in triplicate in 96-well round-bottom microplates (14, 28). The individual culture supernatants were collected 3–4 days after the stimulation, and IFN-γ was measured using Opt EIA Mouse ELISA Set (BD Biosciences). The splenocytes obtained from C57BL/6 mice infected with rBCG were also subjected to the identification of T cell subsets responsible for IFN-γ production. The intracellular production of IFN-γ by $CD4^+$ T cells and $CD8^+$ T cells that were restimulated for 3 days in vitro with rMMP-II protein was assessed, as follows: cells were treated with Golgi Stop, and Golgi transport was inhibited for 4 h. Then the cells were surface stained with an allophycocyanin-labeled mAb to CD4 (RM4-5; BD Biosciences) and a PE-labeled mAb to CD8 (H35.17-2; BD Biosciences) in the presence of 7-aminoactinomycin D, after which the cells were washed with PBS containing 1% FCS and fixed in 1.6% formaldehyde. Subsequently, they were permeabilized using 0.1% saponin, and stained with a FITC-conjugated mAb to IFN-γ (XMG1.2; BD Biosciences) or isotype control IgG. Eight C57BL/6 mice per group were vaccinated with the indicated dose of BCG-261H or BCG-70M for 4 wk, and were challenged with 5×10^3/mouse *M. leprae* in footpad. Thirty-two weeks later, footpad was removed. The number of *M. leprae* grown in footpad was enumerated by Shepard method (29). 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.

Results

Secretion of HSP70-MMP-II fusion protein from the rBCG (BCG-70M)

To verify the secretion of MMP-II protein from BCG-70M, culture filtrates of BCG transformants including BCG-261H (vector control) and BCG-70M were concentrated and examined by Western blotting analysis using mAbs to MMP-II and HSP70 (Fig. 1*A*). When probed by the MMP-II mAb, BCG-70M showed distinct band at 90-kDa equivalent to the molecular mass of the fusion protein comprising HSP70 and MMP-II, and control rMMP-II showed a 22-kDa band. Because BCG-Tokyo, a parental strain of BCG-70M and BCG-261H, has the gene encoding BCG-derived MMP-II, a faint 22-kDa band was observed in the culture filtrate of BCG-261H. In addition, when the culture filtrates were examined using the mAb to HSP70, the BCG-70M-derived filtrates expressed the 90-kDa protein, whereas the filtrates obtained from BCG-261H and rMMP-II protein did not express any obvious band. These results indicate that BCG-70M efficiently secreted the fusion protein comprising HSP70 and MMP-II. Furthermore, the HSP70-MMP-II fusion protein stimulated DC and induced a significant level of IL-12p70 production (Fig. 1*B*). To address the

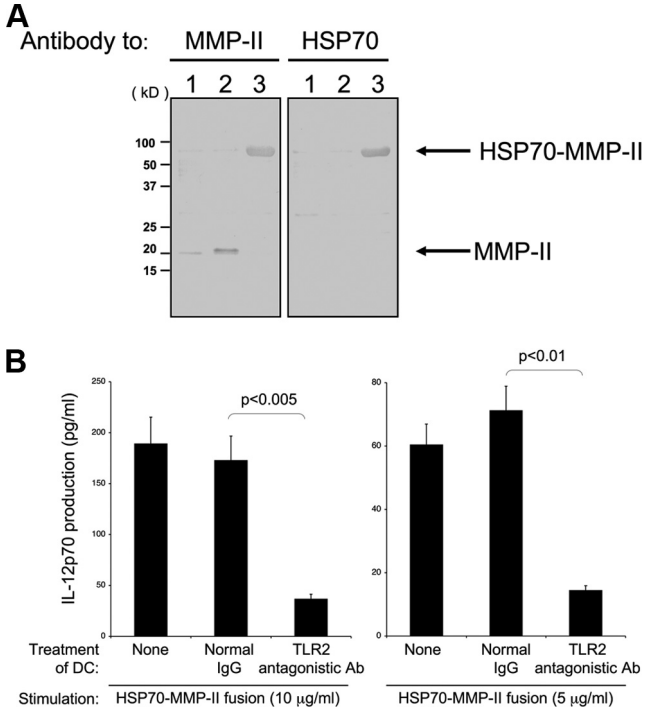

FIGURE 1. *A*, Western blotting analysis of protein secreted from BCG-70M. A mAb to either MMP-II or HSP70 was used to detect HSP70-MMP-II fusion protein. *Lane 1*, Culture filtrates of BCG-261H. *Lane 2*, rMMP-II protein. *Lane 3*, Culture filtrates of BCG-70M. *B*, Contribution of TLR2 to IL-12p70 production by DC by stimulation with HSP70-MMP-II fusion protein. PBMCs were obtained from one donor. Monocyte-derived DC were pretreated with either normal murine IgG or TLR2 antagonistic Ab (10 μg/ml) and subsequently stimulated with BCG-70M-derived HSP70-MMP-II fusion protein (10 or 5 μg/ml) for 24 h. The concentration of IL-12p70 was determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean $\pm$ SD. Titers were statistically compared using Student's *t* test.

contribution of TLR2 expressed on DC to the IL-12p70 production, DC were pretreated with an antagonistic Ab to TLR2 and subsequently stimulated with the fusion protein. More than 80% of IL-12p70 production was inhibited by the anti-TLR2 antagonistic Ab, whereas pretreatment of DC with normal murine IgG did not affect the level of production. Although BCG-261H induced IL-12p70 production from DC, production was only partially inhibited by the antagonistic Ab to TLR2 (data not shown).

Characteristics of BCG-70M

To define infectivity and survival in APCs, we examined the recovery rate of BCG-261H and BCG-70M. There was no significant difference between the two strains, and similar amounts of BCG were recovered as that of infected number (data not shown). Both HSP70 and MMP-II are known to be immunostimulators (6, 30). To see the effect of the secretion of HSP70-MMP-II fusion protein from BCG on the activation of DC, we analyzed the expression of surface Ags of BCG-infected DC (Fig. 2*A*). Both BCG-261H and BCG-70M enhanced the expression of HLA-ABC, HLA-DR, CD86, and CD83 Ags, but BCG-70M was significantly more efficient in up-regulating the expression of these molecules than BCG-261H. Furthermore, when various MOIs of BCG were used, a similar difference between BCG-261H and BCG-70M was observed (data not shown). Thus, BCG-70M phenotypically activated DC. Furthermroe, BCG-70M-infected DC significantly,

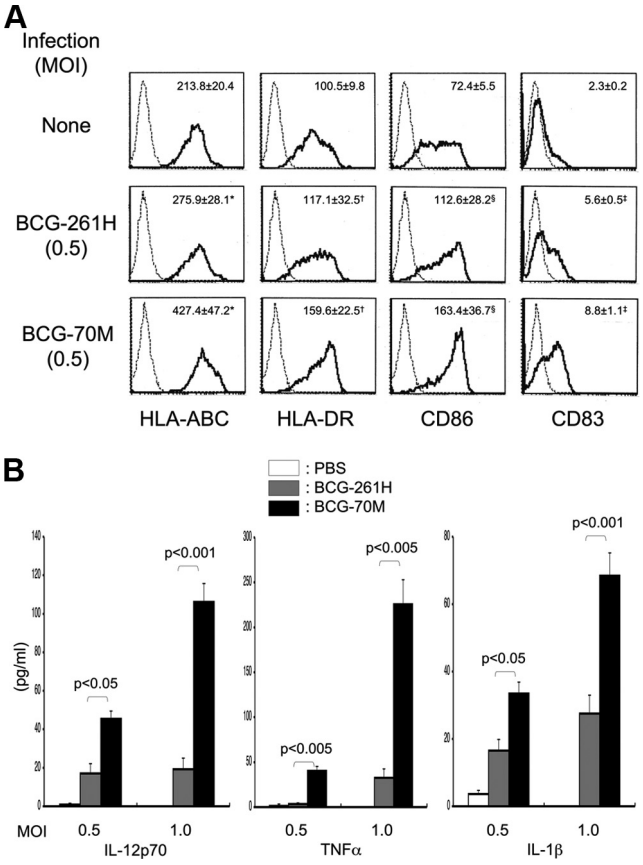

FIGURE 2. *A*, Up-regulated expression of APC-associated molecules on DC by infection with BCG-70M. PBMCs were obtained from one donor. Monocyte-derived immature DC were infected with either BCG-261H or BCG-70M at a MOI of 0.5 and cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DC from day 5 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 in the *top right-hand corner* of each panel represents the mean ± SD for three independent experiments of the difference in mean fluorescence intensity between the control IgG and the test mAb. Titers were statistically compared using Student's *t* test. *, $p < 0.01$; †, $p < 0.05$; §, $p < 0.01$; ‡, $p < 0.05$. *B*, Cytokine production from DC stimulated with BCG. PBMCs were obtained from one donor. Monocyte-derived DC from 4 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated dose of either BCG-261H or BCG-70M for 24 h. The concentration of the indicated cytokine was determined by the ELISA method. A representative of three separate experiments 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.

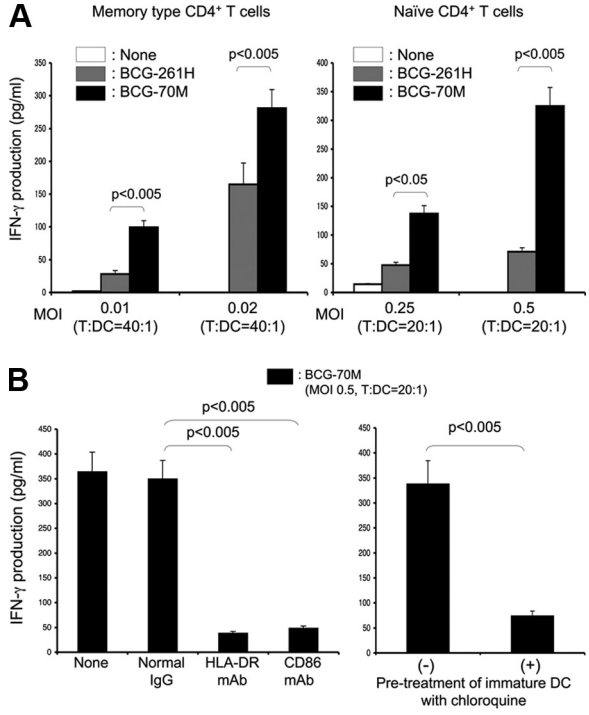

FIGURE 3. *A*, IFN-γ production from CD4+ T cells by stimulation with BCG. PBMCs were obtained from one donor. Monocyte-derived DC were infected with either BCG-261H or BCG-70M at the indicated MOI, and used as a stimulator of memory-type or naive CD4+ T cells in a 4-day culture. Responder CD4+ T cells (1×10^5) were stimulated with the indicated dose of BCG-infected DC. A representative of three separate experiments 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. *B*, Inhibition of naive CD4+ T cell activation by the treatment of BCG-70M-infected DC with mAb or the treatment of immature DC with chloroquine. PBMCs were obtained from one donor. Monocyte-derived DC were infected with BCG-70M at a MOI of 0.5, and subsequently treated with 10 μg/ml mAb to HLA-DR, CD86 Ags, or normal murine IgG. Immature DC were treated with 50 μM chloroquine for 2 h and subsequently infected with BCG-70M at a MOI of 0.5. These DC were used as the stimulator of naive CD4+ T cells (1×10^5/well) at T:DC = 20:1. IFN-γ produced from T cells was measured. A representative of three separate experiments 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.

though faintly, expressed MMP-II on their surface, and the MMP-II expression was inhibited by the pretreatment of DC with chloroquine, an inhibitor of phagosomal acidification, before BCG-70M infection (data not shown). Moreover, when we examined the influence of BCG-70M infection in DC in terms of the production of proinflammatory cytokines, BCG-70M was superior to BCG-261H in the production of IL-12p70, TNF-α, and IL-1β (Fig. 2*B*). These results indicate again that BCG-70M more efficiently activated DC than did the parental BCG.

T cell activation by BCG-70M

Enhanced activation of DC may be closely associated with the efficient activation of both CD4+ and CD8+ T cell subsets. Because BCG-infected mitomycin C-treated DC were confirmed not to produce IFN-γ (data not shown), the T cell-activating ability of BCG-70M was assessed by using DC as APCs. Memory-type and naive CD4+ T cells were purified from healthy BCG-vaccinated individuals, and were stimulated with DC infected with rBCG (Fig. 3*A*). As expected, memory T cells responded more vigorously to DC infected with smaller dose of BCG than naive CD4+ T cells. Although both BCG-261H and BCG-70M stimulated memory and naive CD4+ T cells, BCG-70M induced a significantly higher level of IFN-γ production in both types of CD4+ T cells than BCG-261H. Note that high levels of IFN-γ could be produced from naive CD4+ T cells by BCG-70M. In addition, various MOIs and T:DC ratios were assessed, and a similar difference between BCG-261H and BCG-70M was observed (data not shown). To address the mechanism leading to the activation of naive CD4+ T cells, BCG-70M-infected DC were treated with mAbs to HLA-DR and CD86 molecules and subsequently used as a stimulator of the T cells. The IFN-γ production from naive CD4+ T cells was significantly inhibited by the surface treatment of BCG-70M-infected DC with the mAb to HLA-DR or CD86 (Fig. 3*B*), and similarly, IL-2 production from naive CD4+ T cells

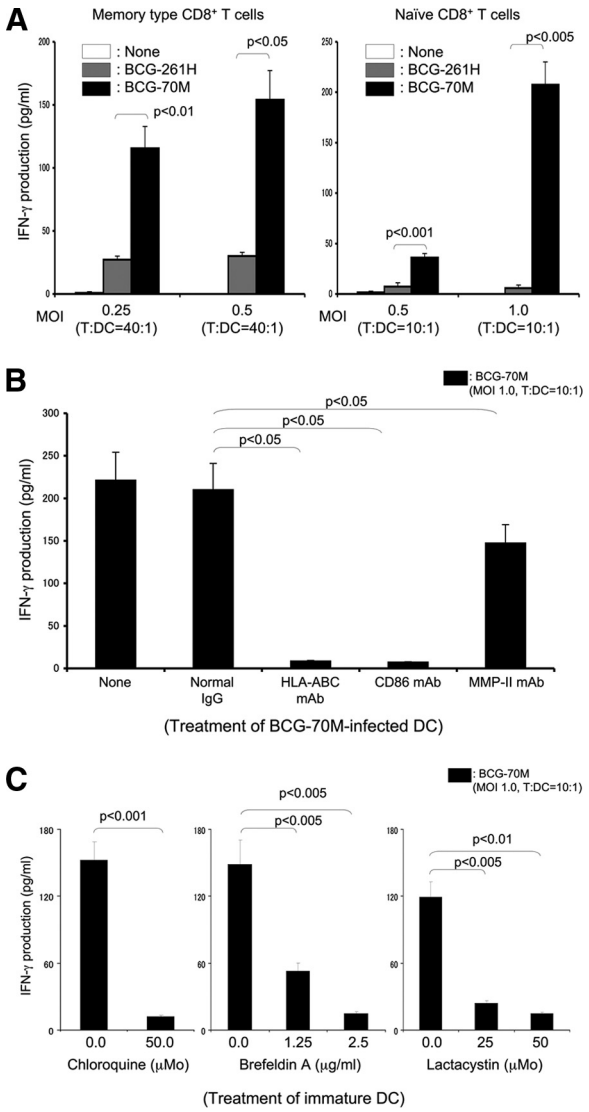

FIGURE 4. *A*, IFN-γ production from CD8$^+$ T cells by stimulation with BCG. PBMCs were obtained from one donor. Monocyte-derived DC were infected with either BCG-261H or BCG-70M at the indicated MOI, and used as a stimulator of memory-type or naive CD8$^+$ T cells in a 4-day culture. Responder CD8$^+$ T cells (1×10^5) were stimulated with the indicated dose of BCG-infected DC. A representative of three separate experiments 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. *B*, Inhibition of naive CD8$^+$ T cell activation by the treatment of BCG-70M-infected DC with mAb. PBMCs were obtained from one donor. Monocyte-derived DC were infected with BCG-70M at a MOI of 1.0, and subsequently treated with 10 μg/ml mAb to HLA-ABC, CD86, or MMP-II (M270-13). The DC were used as the stimulator of naive CD8$^+$ T cells (1×10^5/well) at T:DC = 10:1. IFN-γ produced from the T cells was measured by the ELISA method. A representative of three separate experiments 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. *C*, Effect of treatment of immature DC with reagents on the activation of naive CD8$^+$ T cells. PBMCs were obtained from one donor. Monocyte-derived immature DC were treated with the indicated dose of either chloroquine, brefeldin A, or lactacystin, and subsequently infected with BCG-70M at a MOI of 1.0. These DC were used as the stimulator of naive CD8$^+$ T cells (1×10^5/well) at T:DC = 10:1. IFN-γ produced from the T cells was measured. A representative of three separate experiments 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.

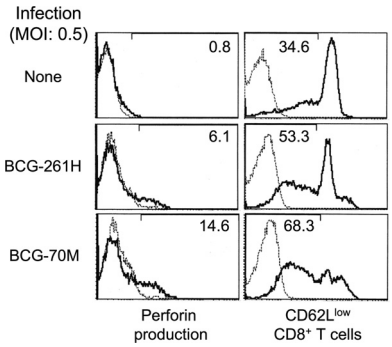

FIGURE 5. Influence of naive CD4$^+$ T cells on the activation of naive CD8$^+$ T cells. PBMCs were obtained from one donor. Monocyte-derived DC were infected with BCG (MOI 0.5) and cocultured with unseparated naive T cells (T:DC = 10:1) for 7 days. The stimulated CD8$^+$ T cells were gated and analyzed for perforin production and for expression of CD62L Ag. Numbers indicate either percentage of perforin-positive CD8$^+$ T cells or CD62L^{low} CD8$^+$ T cells among CD8$^+$ T cell population. A representative of three separate experiments is shown.

was inhibited (data not shown). Furthermore, pretreatment of immature DC with chloroquine before infection with BCG-70M significantly inhibited the IFN-γ production from naive CD4$^+$ T cells (Fig. 3*B*). These results indicated that the secreted HSP70-MMP-II protein or BCG-70M itself may be processed in the DC, and some of the antigenic peptides were used for the stimulation of autologous Ag-specific naive CD4$^+$ T cells. Similarly, BCG-70M-infected DC stimulated memory CD8$^+$ T cells more efficiently than BCG-261H-infected DC, although a higher dose of BCG-70M was necessary to induce a similar level of IFN-γ production from CD8$^+$ T cells than the dose of BCG-70M required to produce the cytokine from memory CD4$^+$ T cells. As reported, BCG-261H did not activate naive CD8$^+$ T cells efficiently (15); however, BCG-70M-infected DC induced a significant level of IFN-γ production from naive CD8$^+$ T cells (Fig. 4*A*). Using a higher dose of BCG-70M (MOI 1.0) and a larger number of BCG-70M-infected DC (T:DC = 10:1), a high concentration (~200 pg/ml) of IFN-γ could be produced from naive CD8$^+$ T cells. In addition, various MOIs and T:DC ratios were assessed, and a similar difference between BCG-261H and BCG-70M was observed (data not shown). To clarify the mechanism leading to the activation of naive CD8$^+$ T cells by BCG-70M, BCG-70M-infected DC were treated with mAbs. Again, the activation of naive CD8$^+$ T cells by BCG-70M-infected DC was significantly inhibited by the treatment of the DC with the mAb to HLA-ABC or CD86. However, surface treatment of the DC with the mAb to MMP-II significantly, but only partially, inhibited the T cell activation (Fig. 4*B*). These results may indicate that BCG-70M-infected DC cross-primed naive CD8$^+$ T cells in an Ag-specific manner. To elucidate the mechanisms leading to the cross-presentation by BCG-70M-infected DC, we treated immature DC with various reagents in advance of the BCG-70M infection (Fig. 4*C*). On the pretreatment of DC with chloroquine, IFN-γ production from naive CD8$^+$ T cells was significantly inhibited, indicating that protein derived from BCG-70M was degraded in presumably the phagolysosome. Furthermore, on the pretreatment of DC with brefeldin A, an inhibitor of antegrade Golgi transportation, and lactacystin, an inhibitor of proteosomal protein degradation, IFN-γ production from naive CD8$^+$ T cells was inhibited significantly in a manner dependent on the concentration of the reagents. Because BCG-70M activated both naive CD4$^+$ T cells and naive CD8$^+$ T cells, we stimulated naive CD8$^+$ T cells with BCG-70M in the presence of the CD4$^+$ T cells (Fig. 5). The expression level of CD62L on some CD8$^+$ T cells was

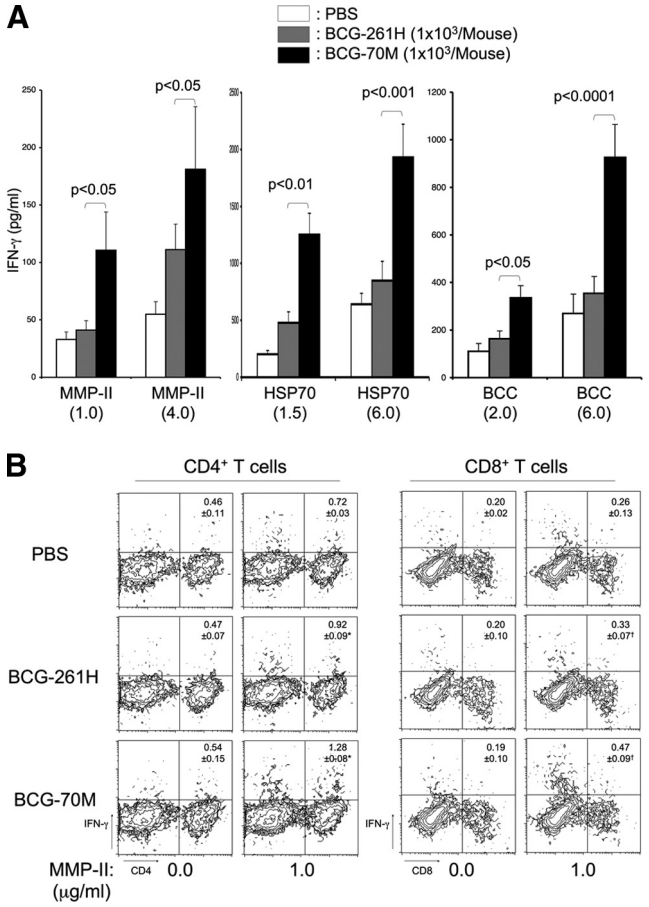

FIGURE 6. *A*, Production of memory-type T cells in C57BL/6 mice by infection with BCG. Five-week-old C57BL/6 mice were infected with 1×10^3 CFU/mouse of either BCG-261H or BCG-70M s.c. Four weeks after the inoculation, splenocytes (2×10^5 cells/well) were stimulated with the indicated dose of either MMP-II-, HSP70-, or BCG-derived cytosolic protein for 4 days, and IFN-γ produced in the cell supernatant was measured. Assays were performed in triplicate for each mouse, and the results of three mice per group are shown as the mean ± SD. Representative results of four separate experiments are shown. Titers were statistically compared using Student's *t* test. *B*, Intracellular IFN-γ production from $CD4^+$ T cells and $CD8^+$ T cells in C57BL/6 mice s.c. inoculated with BCG by secondary stimulation. Groups of 5-wk-old C57BL/6 mice were infected with 1×10^3/mouse BCG-261H or BCG-70M intradermally. Four weeks after the inoculation, splenocytes (2×10^5 cells/well) were stimulated with 1.0 μg/ml rMMP-II for 3 days. The $CD4^+$ T cells and $CD8^+$ T cells were gated separately and analyzed for intracellular production of IFN-γ. The number in the *top right-hand corner* of each panel represents the mean ± SD for three mice in the percentage of IFN-γ-producing cells among the gated T cell population. Representative results of four separate experiments are shown. Titers were statistically compared using Student's *t* test. *, $p < 0.05$; †, $p < 0.01$.

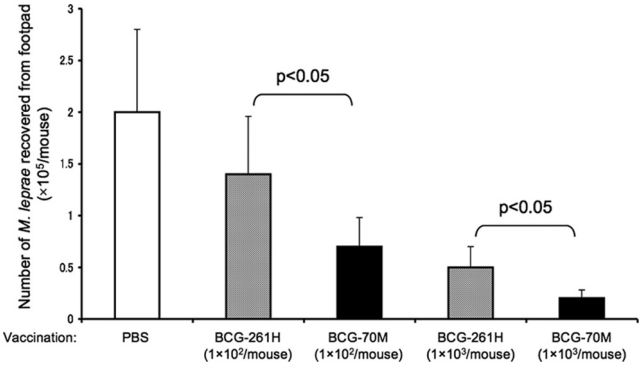

FIGURE 7. Inhibition of *M. leprae* multiplication by s.c. vaccination with BCG-70M. Five-week-old C57BL/6 mice (8 mice per group) were vaccinated with 1×10^2 or 1×10^3 CFU/mouse either BCG-261H or BCG-70M s.c., and were challenged with 5×10^3 bacilli/mouse *M. leprae* in footpad 4 wk after the vaccination. The number of *M. leprae* recovered from the footpad at 32 wk after the challenge was enumerated by Shepard's methods (29). Representative results of two separate experiments are shown. Titers were statistically compared using Student's *t* test.

significantly reduced by stimulation with BCG-70M-infected DC, and a significant amount of intracellular perforin was produced in naive $CD8^+$ T cells by the stimulation. These changes were more efficiently induced by BCG-70M-infected DC than by BCG-261H-infected DC (Fig. 5). The $CD62L^{low}CD8^+$ T cells and perforin-producing $CD8^+$ T cells were not produced when naive $CD8^+$ T cells were stimulated in the absence of naive $CD4^+$ T cells (data not shown).

Memory T cell production by BCG-70M in vivo

Another important aspect to be studied is the production of Ag-specific memory T cells in vivo. C57BL/6 mice were infected with 1000 CFU/mouse rBCG for 4 wk, and their splenocytes were secondarily stimulated in vitro with rMMP-II protein, or recall Ags, like BCC (Fig. 6*A*). When a lower dose of MMP-II was used as a stimulator, only T cells obtained from BCG-70M-infected mice responded to the stimulator. Because BCG-Tokyo, the parental strain of the rBCGs, has the gene encoding MMP-II, a higher dose of *M. leprae*-derived MMP-II induced IFN-γ production from both T cells obtained from BCG-261H- and BCG-70M-inoculated mice; however, T cells from BCG-70M-infected mice more efficiently produced the cytokine than those from BCG-261H-infected mice. Also, T cells from BCG-70M-inoculated mice produced significantly higher level of IFN-γ than T cells from mice inoculated with BCG-261H on stimulation with HSP70 in vitro. The efficient generation of memory T cells responding to mycobacteria-derived Ags in mice infected with BCG-70M was confirmed, because only T cells from mice infected with BCG-70M significantly responded to BCC (Fig. 6*A*). To clarify the T cell subsets responsible for the IFN-γ production on secondary MMP-II stimulation, T cells producing the cytokine intracellulary were analyzed (Fig. 6*B*). Both $CD4^+$ T cells and $CD8^+$ T cells derived from not only non-BCG-inoculated mice, but also BCG-infected mice, produced intracellular IFN-γ by MMP-II stimulation. Both noninoculated and PBS-inoculated mice showed the similar responses (data not shown). However, significantly larger populations of both $CD4^+$ T cells (~1.3%) and $CD8^+$ T cells (~0.5%) obtained from BCG-70M-infected mice produced the cytokine. There were no $CD4^+$ T cells or $CD8^+$ T cells that were positively stained with the isotype control IgG (data not shown).

Effect of BCG-70M vaccination on the multiplication of M. leprae *in vivo*

C57BL/6 mice vaccinated with either BCG-261H or BCG-70M (1×10^2 or 1×10^3 CFU/mouse) for 4 wk were challenged with 5×10^3 of *M. leprae* in footpad. Thirty-two weeks later, footpad was removed and *M. leprae* recovered from the footpad was enumerated (Fig. 7). A total of 2×10^5 *M. leprae* was recovered from mice inoculated with PBS and challenged with *M. leprae*. Both mice inoculated with BCG-261H or BCG-70M inhibited the multiplication of *M. leprae* in the manner dependent on the dose of rBCG vaccinated; however, BCG-70M vaccination was significantly more efficient than BCG-261H vaccination in inhibiting the

multiplication, and only 2×10^4 *M. leprae* was recovered from mice vaccinated with 1×10^3 CFU/mouse BCG-70M.

Discussion

M. leprae is a representative mycobacterium among slow-growing prokaryotes, which needs 12–14 days for one division and 10–70 years for the manifestation of the disease, depending on the dose of bacilli entering the hosts. Host defense against *M. leprae* is chiefly conducted by adaptive immunity involving both $CD4^+$ and $CD8^+$ T cell subsets (3–5). Although $CD4^+$ T cells usually act at the initial phase of infection, $CD8^+$ T cells inhibit the multiplication of *M. leprae* in the chronic phase or when it is reactivated from a dormant state (31). Therefore, the vaccine should have an ability to competently activate not only $CD4^+$ T cells, but also $CD8^+$ T cells to produce memory T cells. To date, BCG is used as sole, but safe, vaccine against leprosy; however, nowadays, its efficacy is considered not so convincing (13). We have made several attempts to improve the potency of BCG, especially its immunostimulatory activities. We chiefly focused on achieving the fusion of BCG-infected phagosomes with lysosomes, without which the full and polyclonal activation of Ag-specific T cells cannot be expected. One approach we tried was the production of an *ure C*-deficient rBCG that successfully produces acidic phagosomes and facilitates their fusion with lysosomes (15, 28, 32, 33). Actually, the rBCG efficiently colocalized with lysosomes and effectively stimulated $CD4^+$ T cells when DC were targeted (28). However, it did not activate naive $CD8^+$ T cells. Then, we produced a second rBCG that secretes MMP-II (BCG-SM) in the phagosome (14). BCG-SM was useful in the activation of not only naive $CD4^+$ T cells, but also naive $CD8^+$ T cells to some extent (14). The T cell activation presumably occurs as a consequence of the translocation of the antigenic determinants of the secreted MMP-II to the cell surface, although the precise mechanism has not been clarified. Therefore, the intracellular secretion of immunodominant Ag by BCG is thought necessary to enhance the T cell-stimulating activity of BCG. However, BCG-SM vaccinations only partially inhibited the multiplication of *M. leprae* in the footpads of mice (our unpublished observation). These observations indicate the need for another rBCG capable of activating both naive $CD4^+$ and $CD8^+$ T cells more strongly.

Because the strong activation of naive $CD8^+$ T cells by mycobacteria required the cross-presentation of mycobacteria-derived Ags to $CD8^+$ T cells, in this study, we used BCG-derived HSP70 as a mediator facilitating the cross-presentation by DC, because HSPs of both mammalian host cells and bacterial origin are reported to have chaperone activity (34) and can effectively prime a cytolytic response (35). The usefulness of HSP65 as a convincing stimulator of $CD8^+$ T cells was revealed in animal studies to develop vaccine against *M. tuberculosis* (36). A DNA vaccine containing the *hsp65* gene activated naive $CD8^+$ T cells, and consequently inhibited the development of tuberculosis, although precisely how was not clarified. Our newly constructed rBCG (BCG-70M) secreted a fusion protein comprising HSP70 and MMP-II in vitro in the absence of any exogenous secretion signal. The secretion of HSP70-MMP-II fusion was confirmed by Western blotting analyses (Fig. 1*A*) and by the surface expression of MMP-II on DC (data not shown). The exact mechanism leading to the secretion of the fusion protein from BCG-70M is not known, but the secretion could be due to the inherent characteristics of HSP70 to be secreted (16, 37). Although we tried to enhance the secreting activity of BCG-70M by additionally inserting *M. tuberculosis*-derived secretion signal to BCG-70M, the secretion efficacy was rather inhibited and the construct stimulated naive T cells less efficiently than BCG-70M (data not shown).

BCG-70M secreted a 90-kDa fusion protein composed of HSP70 and MMP-II (14). The activation of naive $CD8^+$ T cells by BCG-70M was only partially inhibited by the treatment of DC with the mAb to MMP-II. Therefore, it can be speculated that BCG-70M activated $CD8^+$ T cells polyclonally by using various epitopes, originating from MMP-II, HSP70, or other Ags of BCG, because T cells from BCG-70M-infected mice vigorously responded to MMP-II, HSP70, and BCG-derived cytosolic protein (Fig. 6*A*). Therefore, HSP70 may alter the clonality of responding $CD8^+$ T cells, and the production of such polyclonal Ag-specific $CD8^+$ T cells might be beneficial for the broad coverage of a heterogenous MHC population.

BCG-70M induced higher level of cytokine production, including IL-12p70, TNF-α, and IL-1β, than the vector control BCG. The enhanced cytokine production by BCG-70M could be due to the intracellular secretion of HSP70 as a part of the fusion protein by the BCG. MMP-II can ligate TLR2 (6), but HSP70 is also known to bind TLRs (38); thus, the secreted HSP70-MMP-II protein seems to activate DC strongly. The contribution of TLR2 to cytokine production was confirmed by the inhibition of IL-12p70 production by antagonistic Ab to TLR2. The cytokines released from DC by BCG-70M stimulation could facilitate skewing of the direction of T cell activation to type 1 and induce the efficient and strong production of IFN-γ from naive $CD8^+$ T cells.

HSPs play a varied role in enhancing the ability of APCs to stimulate T cells (39). For the activation of Ag-specific $CD4^+$ and $CD8^+$ T cells, peptides should be loaded onto the corresponding MHC pathways. For the loading of BCG-derived Ags on these pathways, the proteins secreted from phagocytozed BCG should be transported to functional lysosomes. In the phagolysosome, some portions of HSP70-MMP-II fusion protein could be degraded, but the rest would be sequestrated into the cytosol, where they are degraded and used for cross-priming $CD8^+$ T cells. BCG-70M-infected DC expressed derivatives of MMP-II and the other proteins on their surface, and they activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells. However, both MMP-II expression on DC and the activation of the T cells by DC were inhibited by the pretreatment of DC with chloroquine (24, 40). These results indicate that secreted HSP70-MMP-II fusion protein was efficiently processed in lysosomes and its derivatives are used for the activation of both subsets of naive T cells. When naive $CD8^+$ T cells were stimulated by BCG-70M in the presence of naive $CD4^+$ T cells, $CD62L^{low}CD8^+$ T cells and perforin-producing $CD8^+$ T cells were efficiently produced. The activation of naive T cells was confirmed by the production of memory-type T cells by BCG-70M infection to unprimed mice, because both $CD4^+$ and $CD8^+$ T cell subsets from BCG-70M-infected mice responded to the restimulation with MMP-II in vitro. Furthermore, BCG-70M significantly and more efficiently inhibited the multiplication of *M. leprae*, which were challenged in footpad of mice, than BCG-261H.

There are two pathways of cross-presentation, as follows: cytosolic (ER-Golgi-dependent) and vacuolar pathways (20). It is known that HSP can enhance both pathways (20). In the present study, IFN-γ production from naive $CD8^+$ T cells was largely blocked by the treatment of DC with brefeldin A, an inhibitior of antegrade Golgi transportation and of TAP-dependent transportation, and also with lactacystin, a proteosomal protein degradation blocker (20, 40). Therefore, it can be presumed that the fusion protein was sequestered into the cytosol from the lysosome, degraded in the proteosome, and used for loading on MHC class I molecules through the TAP-dependent pathway. Furthermore, it has been reported that proteins that are intracellularly secreted are usually processed by a cytosolic (ER-Golgi-dependent) pathway, and DC prefer this pathway for cross-priming $CD8^+$ T cells with

protein Ag (20). Our present observations seem to fit well with these previous findings. Therefore, we concluded that BCG-70M activates naive CD8$^+$ T cells through the ER-Golgi-dependent cytosolic cross-presentation pathway. However, *M. tuberculosis*-derived HSP70 activated T cells through a post-Golgi, proteosomal-independent mechanism, and both brefeldin A and lactacystin may inhibit vacuolar pathway in some cases (20). Also, the vacuolar pathway is used more frequently by macrophages (20). Therefore, the possibility that BCG-70M may also use the post-Golgi pathway in vivo cannot be ruled out.

Taken together, in this study, we constructed a rBCG that secretes HSP70-MMP-II fusion protein, which effectively activates not only DC, but also naive T cells. Therefore, the combination of HSP70 and MMP-II may be useful for stimulating both subsets of naive T cells.

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Disclosures

The authors have no financial conflict of interest.

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