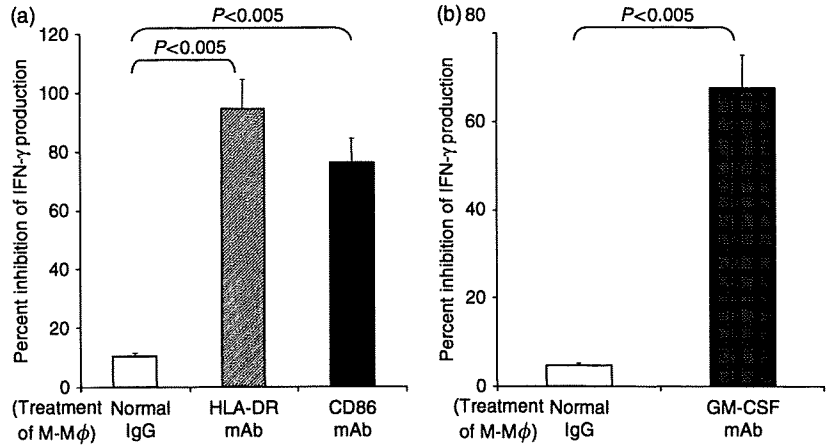

Fig. 4. (a) Inhibition of IFN-γ production by $CD4^+$ T cells by pretreatment of BCG-SM-infected M-Mϕ with mAb to HLA-DR or CD86. M-Mϕ differentiated from monocytes using rM-CSF were infected with BCG-SM at an MOI of 0.25 on day 5 of culture and cultured for another 2 days. The BCG-SM-infected M-Mϕ were treated with mAb to HLA-DR or CD86, or isotype-matched control IgG (10 μg mL^{-1}), and used as a stimulator of $CD4^+$ T cells, at a T cell : M-Mϕ ratio of 20 : 1 and cultured for another 4 days. The optimal concentration of mAb was determined in advance. Non-pretreated BCG-SM-infected M-Mϕ induced the production of 220.8 pg mL^{-1} of IFN-γ by $CD4^+$ T cells. This titre was taken as 0% inhibition. (b) Inhibition of IFN-γ production by $CD4^+$ T cells by neutralizing GM-CSF produced from BCG-SM-infected M-Mϕ. M-Mϕ, differentiated from monocytes by culturing for 5 days with rM-CSF, were infected with BCG-SM (MOI 0.25) in the presence of neutralizing mAb to GM-CSF or isotype-matched control IgG (10 μg mL^{-1}). These M-Mϕ were used as a stimulator of $CD4^+$ T cells as in (a). The optimal concentration of mAb was determined in advance. M-Mϕ infected with BCG-SM in the absence of any Ab induced the production of 168.3 pg mL^{-1} of IFN-γ by $CD4^+$ T cells. This titre was taken as 0% inhibition. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

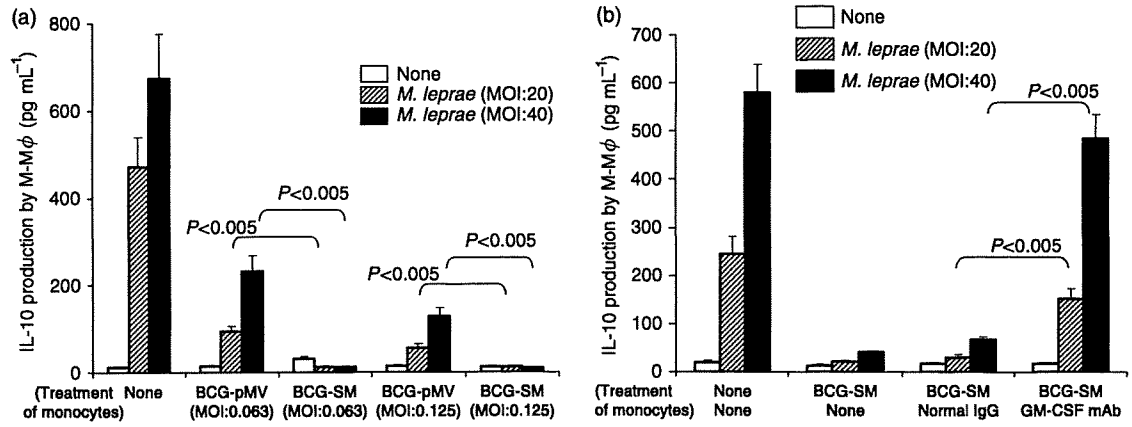

Fig. 5. (a) Production of IL-10 by M-Mϕ. Monocytes were infected with the indicated dose of BCG-pMV or BCG-SM, and subsequently differentiated into M-Mϕ by culturing for 5 days in the presence of M-CSF and rBCGs. These rBCG-preinfected M-Mϕ were stimulated with *Mycobacterium leprae* at the indicated MOI for 24 h. (b) Recovery of IL-10 production by M-Mϕ. Monocytes were infected with BCG-SM (MOI 0.063) in the presence of neutralizing GM-CSF mAb or isotype-matched control IgG and were subsequently differentiated into M-Mϕ by culturing for 5 days. These BCG-SM-preinfected M-Mϕ were stimulated with *M. leprae* at the indicated MOI for 24 h. The optimal concentration of mAb was determined in advance. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is show. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

monocytes also produced GM-CSF on stimulation with BCG in a BCG-SM-predominant fashion (not shown), we examined the effect of infection with rBCGs in monocytes on IL-10 production by M-Mϕ challenged with *M. leprae* (Fig. 5). M-Mϕ differentiated from monocytes untreated with any bacteria produced > 400 pg mL^{-1} of IL-10 on stimulation with *M. leprae*; however, the production of cytokine by M-Mϕ pretreated with rBCGs was significantly inhibited (Fig. 5a). The inhibition was more significant when BCG-SM was used as a stimulator of monocytes, and IL-10 production by M-Mϕ was almost completely inhibited. The inhibition was dependent on the dose of BCGs

used for pretreatment. In addition, pretreatment of monocytes with BCG-SM inhibited the IL-10 production induced even by lipopolysaccharide (not shown).

Furthermore, M-Mϕ differentiated from monocytes infected with BCG-SM in the presence of normal IgG did not produce IL-10 on stimulation with *M. leprae* (Fig. 5b). However, a significant level of IL-10 was produced when monocytes were infected with BCG-SM in the presence of the neutralizing mAb to GM-CSF. These results indicate that endogenously produced GM-CSF can inhibit IL-10 production.

Discussion

The host defence against intracellular parasitic pathogens such as *M. leprae* is closely associated with the activation of IFN-γ-producing type 1 T cells (Hashimoto *et al.*, 2002). In fact, in patients with paucibacillary leprosy, the activation of $CD4^+$ T cells results in inhibition of the intracellular multiplication and intercellular spread of *M. leprae* (Sieling *et al.*, 1999). The T-cell activation largely depends on the extent of the activation of APC, in which DC play an extremely important role, as DC are the most powerful T-cell stimulators among the APC (Hashimoto *et al.*, 2002). However, if T cells are not efficiently activated due to poor participation of DC, *M. leprae* may be predominantly retained in macrophages. In fact, multibacillary leprosy patients retain numerous *M. leprae* in their macrophages which, in some cases, allow the multiplication and intercellular spread of the bacteria (Ridley & Jopling, 1966).

The tissue resident macrophages, represented by GM-Mϕ and M-Mϕ, are heterogeneous in terms of function (Nakata *et al.*, 1991; Randolph *et al.*, 1999; Akagawa, 2002), despite being similarly susceptible to mycobacterial infection (Makino *et al.*, 2007). GM-Mϕ infected with *M. tuberculosis* or *M. leprae* significantly stimulated $CD4^+$ T cells, whereas M-Mϕ failed to stimulate $CD4^+$ T cells (Verreck *et al.*, 2004; Makino *et al.*, 2007). In this study, we found that, similar to those pathogenic mycobacteria, vector control BCG (BCG-pMV)-infected GM-Mϕ significantly stimulated $CD4^+$ T cells, whereas the BCG-pMV-infected M-Mϕ were less efficient in stimulating these cells. These results indicate the possibility that parental BCG may long reside in M-Mϕ and stimulate T cells inadequately, like the *M. leprae*-infected resident macrophages in multibacillary leprosy. In contrast to BCG-pMV, rBCG that secretes MMP-II (BCG-SM) has the ability to enlist not only GM-Mϕ, but also M-Mϕ, for T-cell activation. Further, the production of IFN-γ by $CD4^+$ T cells stimulated with BCG-SM-infected M-Mϕ was significantly inhibited by pretreatment of the M-Mϕ with the mAb to HLA-DR or CD86 antigens. In addition, the pretreatment of M-Mϕ infected with both BCG-SM and BCG-pMV effectively inhibited $CD4^+$ T-cell activation (not shown). Therefore, the BCG-SM-infected M-Mϕ seemed to stimulate $CD4^+$ T cells in an antigen-specific manner. Furthermore, there was a striking difference between BCG-pMV and BCG-SM in the induction of GM-CSF production. Not only from M-Mϕ, but also from GM-Mϕ, BCG-SM more efficiently induced GM-CSF production than BCG-pMV, and, further, rMMP-II protein, though less efficient, induced significant GM-CSF production. Previously, we reported that rMMP-II is highly immunogenic and induces production of various cytokines, including IL-12 and TNF-α, from APCs such as macrophages and DC (Maeda *et al.*, 2005). These findings indicate that the enhanced production of GM-CSF on stimulation by BCG-SM was at least partially associated with the secretion of MMP-II from BCG-SM.

As the activation of T cells by BCG-SM-infected M-Mϕ was largely inhibited when endogenously produced GM-CSF was neutralized by the mAb to GM-CSF, the endogenously produced GM-CSF may be closely associated with the enhanced T-cell activation by BCG-SM. Although we could not identify the most relevant antigen for T-cell activation, GM-CSF may change the activation status of macrophages or may at least partially transform the BCG-SM-infected M-Mϕ to GM-Mϕ (Makino *et al.*, 2007). Therefore, BCG-SM seems to be a unique rBCG capable of producing GM-CSF and utilizing M-Mϕ for T-cell stimulation.

Another important characteristic of mycobacteria which contributes to the inhibition of T-cell activation is the abundant production of IL-10 by M-Mϕ (Jonuleit *et al.*, 2001; Mochida-Nishimura *et al.*, 2001; Granelli-Piperno *et al.*, 2004). The major purpose of a vaccination is the production of memory T cells which can rapidly respond to subsequently invading pathogenic mycobacteria. However, IL-10 inhibits the re-activation of memory T cells *in vitro*. We found that the ability of BCG-SM to induce production of GM-CSF is useful to inhibit IL-10 production. Monocytes were quite sensitive in the production of GM-CSF, and both BCG-pMV and BCG-SM induced cytokine production by monocytes, although BCG-SM predominated at lower doses (not shown). Thus, even BCG-pMV inhibited IL-10 production at higher doses. However, macrophages differentiated from monocytes which were infected with a small dose of BCG-SM completely inhibited IL-10 production upon subsequent challenge with *M. leprae*, and the inhibitory activity was at least partially cancelled out by the neutralization of endogenously produced GM-CSF. Further, heat-killed BCG-SM, which does not secrete MMP-II (Makino *et al.*, 2006), did not inhibit IL-10 production. These observations indicate that macrophages treated with GM-CSF endogenously diminished the ability to produce IL-10 upon stimulation with *M. leprae*. Previously, we observed that addition of GM-CSF exogenously blocked the ability to produce IL-10 (Makino *et al.*, 2007), which agrees with the

present data. The benefit of inhibition of IL-10 production for host defence has been previously demonstrated *in vivo*. IL-10-deficient mice displayed increased anti-mycobacterial immune responses and decreased bacterial burden (Murray & Young, 1999). In the absence of IL-10, antigen-specific memory T cells, which are efficiently produced by vaccination with BCG-SM for instance, may be fully activated for elimination of *M. leprae*. Although these are still preliminary findings, in one experiment BCG-SM more efficiently inhibited the multiplication of *M. leprae* in footpads of mice than in parent BCG. Therefore, BCG-SM may wipe out favourable conditions for the survival of *M. leprae*. The molecules that are present in the parental BCG and are associated with GM-CSF production remain undefined in the present study, but identification of these molecules may be useful to further enhance the T-cell-stimulating activity of BCG-SM. Also, the identification of such molecules may contribute greatly to the control of the pathogenic mycobacterial diseases using modified BCG.

In this study, we demonstrated that BCG-SM which can induce abundant GM-CSF production, may be more potent than parent BCG in immunostimulation and in the inhibition of IL-10 production, for preventing the survival of *M. leprae*.

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Inhibition of the Multiplication of *Mycobacterium leprae* by Vaccination with a Recombinant *M. bovis* BCG Strain That Secretes Major Membrane Protein II in Mice[▽]

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The ability of a recombinant *Mycobacterium bovis* BCG strain that secretes major membrane protein II (MMP-II) of *Mycobacterium leprae* (BCG-SM) to confer protection against leprosy was evaluated by use of a mouse footpad model. C57BL/6J mice intradermally inoculated with BCG-SM produced splenic T cells which secreted significant amounts of gamma interferon (IFN-γ) in response to either the recombinant MMP-II, the *M. leprae*-derived membrane fraction, or the BCG-derived cytosolic fraction in vitro more efficiently than those from the mice infected with the vector control BCG strain (BCG-pMV, a BCG strain containing pMV-261). A higher percentage of $CD8^+$ T cells obtained from BCG-SM-inoculated mice than those obtained from BCG-pMV-inoculated mice produced intracellular IFN-γ on restimulation with the *M. leprae* antigens. BCG-SM inhibited the multiplication of *M. leprae* in the footpads of C57BL/6J mice more efficiently than BCG-pMV. These results indicate that a BCG strain that secretes MMP-II could be a better vaccine candidate for leprosy.

Leprosy, which is caused by *Mycobacterium leprae*, is an infectious disease that still affects thousands of people worldwide. According to WHO's weekly epidemiological report, 254,525 new cases were detected in 2007 (25). One reason why leprosy is still prevalent may be due to the inherent characteristics of *M. leprae*, i.e., slow growth and weak pathogenicity. It takes 12 to 14 days for *M. leprae* to replicate, so it is predicted that 2 to 5 years are necessary for the clinical manifestations to appear after an infection (1, 18). Likewise, it takes 6 to 8 months for the recognizable swelling of the footpad to appear in nude mice (22).

Leprosy is clinically divided into two major categories: multibacillary (MB) leprosy and paucibacillary (PB) leprosy. In the lesions of patients with PB leprosy, dendritic cells (DCs) and activated T cells are involved with confining *M. leprae* to a localized area. These pathological observations indicate that cell-mediated reactions are triggered and that the activation of both $CD4^+$ and $CD8^+$ T cells is closely associated with inhibition of the spread of the bacilli. In contrast, abundant foamy macrophages loaded with bacilli but not DCs appear in the lesions of MB patients (11). It can be speculated that antigen (Ag)-presenting cells such as DCs recognize the immunodominant Ags of *M. leprae* and express those derivatives on their surfaces, thereby activating T cells. Previously, using T cells from patients with PB leprosy, we have identified major membrane protein II (MMP-II), also known as bacterioferritin (ML2038), as one of the immunodominant Ags (8). We found that MMP-II activates DCs through Toll-like receptor 2, leading to higher levels of expression of major histocompatibility complex class I and class II, CD86, and CD83 Ags and increased levels of production of interleukin-12 p70. Furthermore, MMP-II-pulsed DCs derived from patients with PB leprosy activated both autologous $CD4^+$ T cells and $CD8^+$ T cells to produce gamma interferon (IFN-γ) in amounts larger than the amounts produced by T cells from patients with MB leprosy and *M. bovis* BCG-vaccinated healthy individuals, indicating that T cells from patients with PB leprosy may be primed with MMP-II in vivo.

The BCG vaccine has been used for the prevention of tuberculosis, although its role in the prevention of leprosy is still being debated. The protective efficacy of BCG against leprosy has been tested in several trials, including studies in the Karonga District of northern Malawi, in which 50% protection was observed (17). Through combined systematic analyses of experimental studies, Setia et al. found that the BCG vaccine had an overall level of protective efficacy of 26% against human leprosy (19). Their observational studies overestimated the protective effect at 61%. In another review of 29 studies, Zodpey reported that 44.8% of the reports indicated that the BCG vaccine had a level of efficacy of 50% or more (26). These observations indicate that improvements to the BCG vaccine are necessary to increase its protective effect. Recently, we produced a recombinant BCG strain that secretes MMP-II (strain BCG-SM, where SM indicates secreting MMP-II). Since MMP-II has the ability to ligate Toll-like receptor 2, we expected BCG-SM to highly activate human T cells. In fact, BCG-SM activated not only naïve $CD4^+$ T cells but also naïve $CD8^+$ T cells through DCs (9). The fact that BCG-SM was more efficient than the parental BCG strain at the activation of both subsets of naïve T cells led us to seek further insights into the protective activity of BCG-SM. In the present study, we investigated the effect of vaccination of BCG-SM on the multiplication of *M. leprae* in mice.

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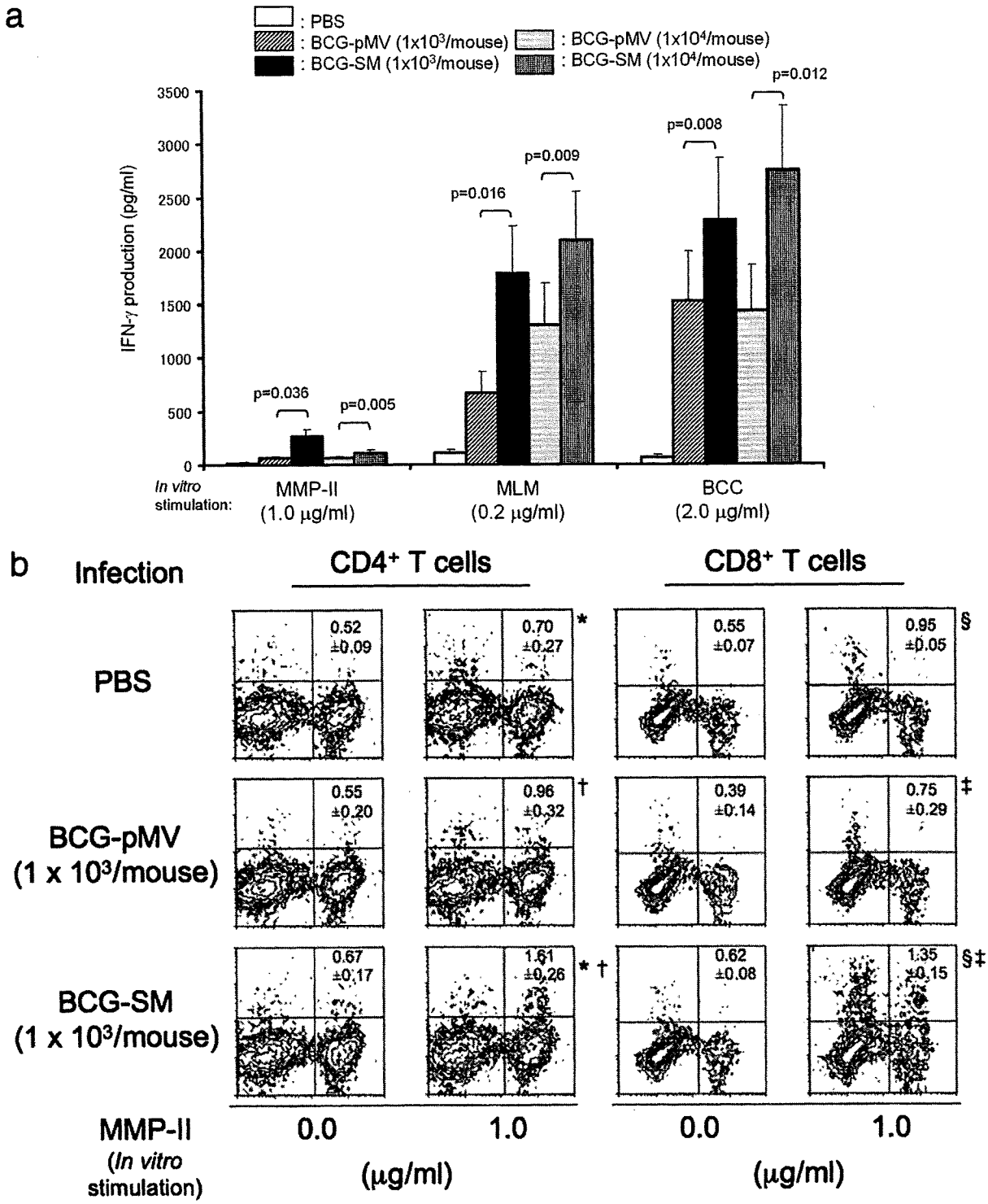


FIG. 1. (a) Production of T cells responsive to *M. leprae*-derived Ags by inoculation with recombinant BCG in mice. Five-week-old C57BL/6J mice were intradermally inoculated with the indicated dose of either BCG-pMV (the vector control BCG strain) or BCG-SM (an MMP-II-secreting BCG strain). Four weeks after the inoculation, splenocytes were restimulated in vitro with the indicated doses of various Ags for 4 days in vitro, and the level of IFN-γ production in the cell supernatant was measured by ELISA. The assays were performed in triplicate for each mouse, and the results for three mice per group are shown as the means ± standard deviations. The representative results of one of three separate experiments are shown. The titers were compared statistically by Student's *t* test. (b) Intracellular IFN-γ production by $CD4^+$ and $CD8^+$ T cells in mice intradermally inoculated with BCG by secondary stimulation. Five-week-old C57BL/6J mice were intradermally infected with 1×10^3 CFU of either BCG-pMV or BCG-SM per mouse. Four weeks after the inoculation, splenocytes (2×10^5/well) were stimulated with 1.0 μg/ml of recombinant MMP-II for 3 days in vitro. The $CD4^+$ T cells and $CD8^+$ T cells were gated separately and analyzed for the intracellular production of IFN-γ. The number at the top right-hand corner of each panel represents the mean percentage of IFN-γ-producing cells ± standard deviation (for three mice) among the gated T-cell population. A representative plot of one of three separate experiments is shown. The titers were compared statistically by Student's *t* test. *, $P < 0.0001$; †, $P < 0.005$; §, $P < 0.05$; ‡, $P < 0.05$.

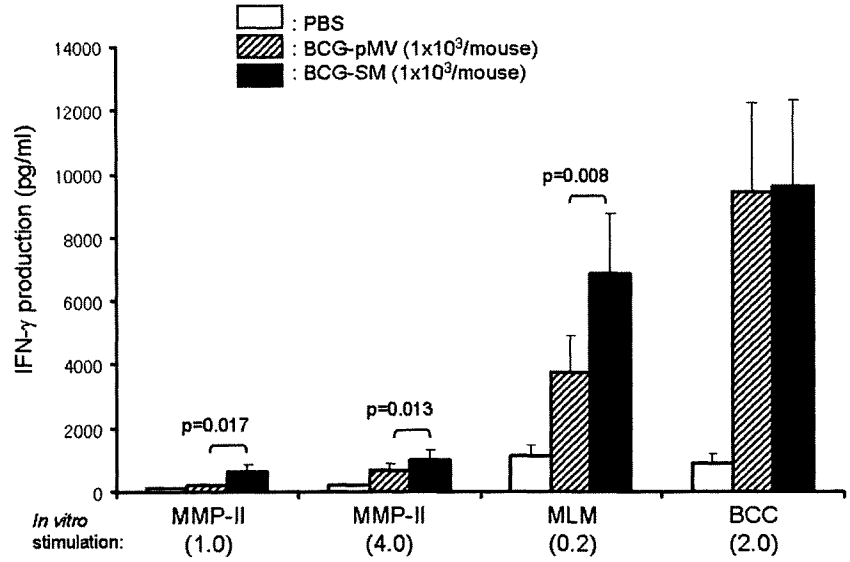


FIG. 2. Long-term effects of vaccination with recombinant BCG on the production of T cells responsive to *M. leprae*-derived Ags. C57BL/6J mice were intradermally infected with 1×10^3 CFU of either BCG-pMV or BCG-SM per mouse. Thirty-four weeks after the inoculation, splenocytes were stimulated with the indicated dose of various Ags for 4 days in vitro, and the amount of IFN-γ produced in the cell supernatant was measured. Assays were carried out in triplicate for each mouse, and the results for three mice per group are shown as the means ± standard deviations. The titers were compared statistically by Student's *t* test.

MATERIALS AND METHODS

Preparation of *M. leprae*, the recombinant BCG strain, and Ags. *M. leprae* (strain Thai-53) was maintained by serial passage in athymic BALB/c *nu/nu* mice (Clea Japan, Inc., Tokyo, Japan) by inoculation of the bacilli into both hind footpads. At 8 months postinoculation, the footpads were processed to recover *M. leprae* bacilli by a previously described method (12, 22). The isolated bacteria were counted by a previously described method (10, 21). Nonfrozen, freshly prepared bacteria were used for inoculation of the mice.

A recombinant BCG strain that secretes *M. leprae*-derived MMP-II was constructed as described previously (9). In brief, a shuttle vector, pMV-261, was used to construct pMV-SM with the MMP-II cDNA fragment. BCG substrain Pasteur was cultured in vitro in Middlebrook 7H9 broth (BD Biosciences–Pharmingen, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (BD Biosciences). Expression vectors were introduced into the BCG strain by electroporation. Transformants were selected on Middlebrook 7H10 agar (BD Biosciences) plates supplemented with 10% oleic acid-albumin-dextrose-catalase (BD Biosciences) and 25 μg/ml kanamycin. The mycobacteria were subsequently grown in Middlebrook 7H9 broth containing 25 μg/ml of kanamycin. The BCG strain containing pMV-SM as an extrachromosomal plasmid is referred to as BCG-SM, while the BCG strain containing pMV-261 is referred to as BCG-pMV. In terms of in vitro growth and infectivity, there was no difference between the two strains. The recombinant MMP-II, the *M. leprae*-derived membrane fraction (MLM), and the cytosolic fraction of BCG (BCC) were obtained as described previously (8, 13).

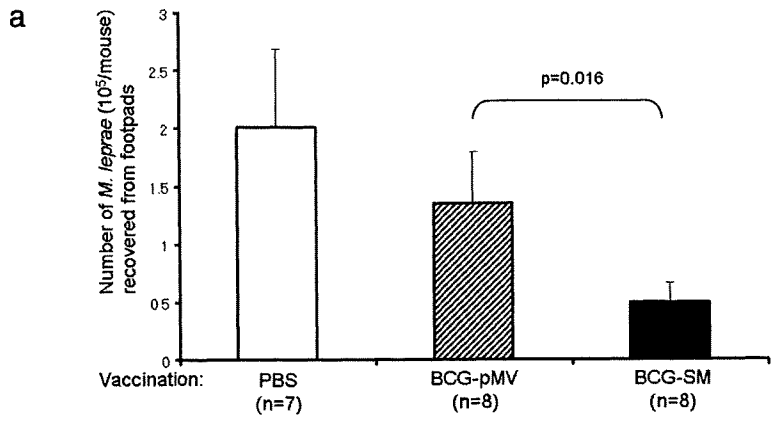
Animal studies. For inoculation into mice, recombinant BCG strains were cultured in Middlebrook 7H9 medium to the log phase of growth and were stored at 10^8 CFU/ml at −80°C. Before the aliquots were used for inoculation, the concentration of viable bacilli was determined by plating the bacilli on Middlebrook 7H11 agar. The indicated numbers of 5-week-old C57BL/6J mice (Clea Japan, Inc.) per group were inoculated intradermally with 0.1 ml of phosphate-buffered saline (PBS) or PBS containing 1×10^3 or 1×10^4 CFU of recombinant BCG per mouse. The animals were kept under specific-pathogen-free conditions and were supplied with sterilized food and water. Four or 34 weeks after inoculation, the spleens were removed and the splenocytes were suspended at a concentration of 2×10^6 cells per ml in culture medium. The splenocytes were stimulated with the indicated concentration of recombinant MMP-II, MLM, or BCC in triplicate in 96-well round-bottom microplates (8). The individual culture supernatants were collected 3 to 4 days after stimulation, and the level of IFN-γ was measured with an Opt EIA mouse enzyme-linked immunosorbent assay (ELISA) set (BD Biosciences). For the recovery of BCG in the spleen 3 weeks after inoculation, the cells were lysed with 0.2% saponin and plated on Middlebrook 7H10 agar for colony counting.

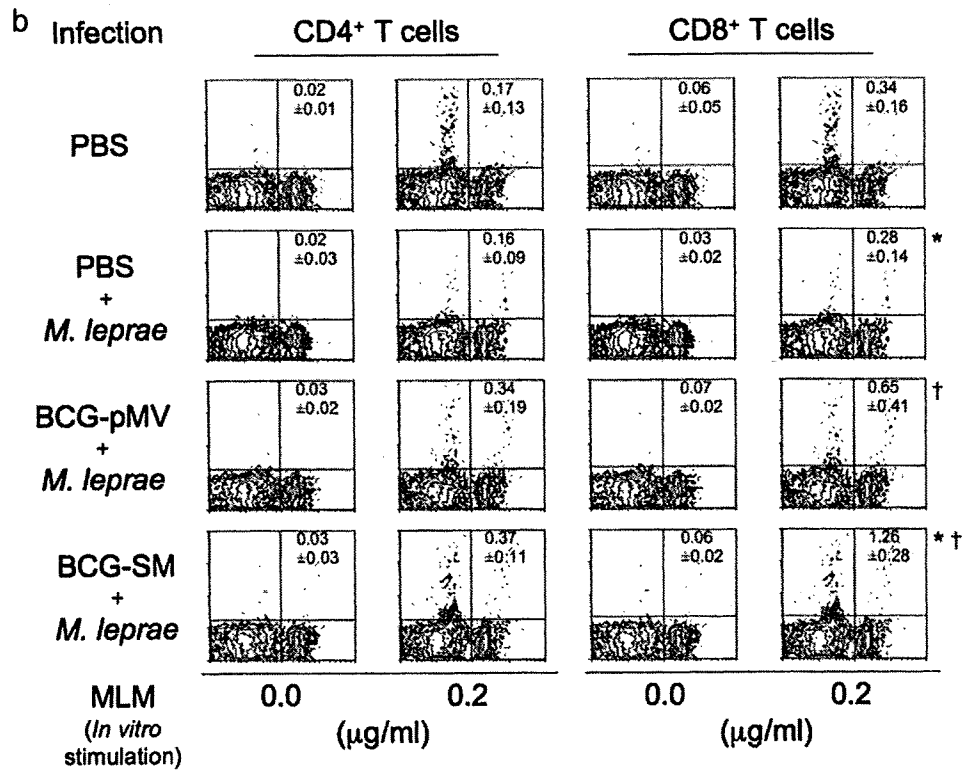
The splenocytes obtained from C57BL/6J mice infected with the recombinant BCG strains were also subjected to the identification of the T-cell subsets responsible for IFN-γ production. The level of intracellular production of IFN-γ by $CD4^+$ T cells and $CD8^+$ T cells, which were restimulated for 3 days in vitro with recombinant MMP-II or MLM, was assessed as follows: cells were treated with Golgi Stop (BD Biosciences), and Golgi transport was inhibited for 4 h. The cells were then surface stained with an allophycocyanin-labeled monoclonal antibody (MAb) to CD4 (MAb RM4-5; BD Biosciences) and a phycoerythrin-labeled MAb to CD8 (MAb H35.17-2; BD Biosciences) in the presence of 7-amino actinomycin D, after which they were washed with PBS containing 1% fetal calf serum and fixed in 1.6% formaldehyde. Subsequently, they were permeabilized with 0.1% saponin and stained with a fluorescein isothiocyanate-conjugated MAb to IFN-γ (MAb XMG1.2; BD Biosciences) or isotype control immunoglobulin G. In another set of experiments, C57BL/6J mice vaccinated intradermally with the indicated dose of BCG-pMV or BCG-SM for 4 weeks were challenged in the footpad with 5×10^3 of *M. leprae* per mouse. Thirty or 31 weeks later, the footpads and spleens were processed for further analyses. The number of *M. leprae* bacilli that grew in the footpads was enumerated by the method of Shepard and McRae (21), and the splenocytes were used to assess the level of IFN-γ production by the ELISA method and for intracellular staining for IFN-γ by flow cytometry (FACSCalibur flow cytometer; BD Biosciences). The animal experiments were reviewed and approved by the Animal Research Committee of Experimental Animals of the National Institute of Infectious Diseases and were conducted according to established guidelines.

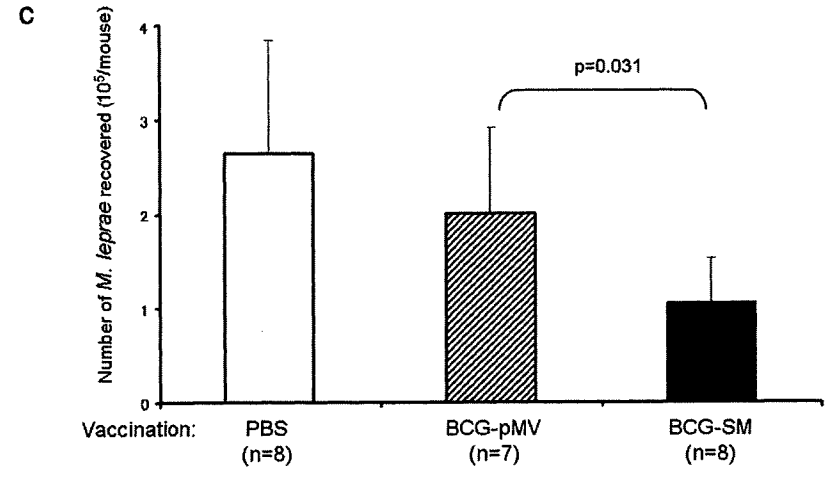
Statistical analysis. Student's *t* test and the Mann-Whitney-Wilcoxon test were used to determine statistical differences.

RESULTS

Production of *M. leprae*-derived Ag-responsive T cells in C57BL/6J mice vaccinated with BCG-SM. The purpose of vaccination is to produce T cells which can respond to *M. leprae* or *M. leprae*-derived Ags. C57BL/6J mice were intradermally infected with either BCG-pMV or BCG-SM; and their splenocytes were restimulated in vitro with the recombinant MMP-II, the MLM protein, or the BCC protein (Fig. 1a). While the


a
Number of M. leprae (10⁵/mouse) recovered from footpads
3
2.5
2
1.5
1
0.5
0
p=0.016
Vaccination:
PBS (n=7)
BCG-pMV (n=8)
BCG-SM (n=8)


b
Infection
CD4+ T cells
CD8+ T cells
PBS
0.02 ±0.01
0.17 ±0.13
0.06 ±0.05
0.34 ±0.16
PBS + M. leprae
0.02 ±0.03
0.16 ±0.09
0.03 ±0.02
0.28 ±0.14
*
BCG-pMV + M. leprae
0.03 ±0.02
0.34 ±0.19
0.07 ±0.02
0.65 ±0.41
†
BCG-SM + M. leprae
0.03 ±0.03
0.37 ±0.11
0.06 ±0.02
1.26 ±0.28
* †
MLM (In vitro stimulation)
0.0
0.2
(μg/ml)
0.0
0.2
(μg/ml)


c
Number of M. leprae recovered (10⁵/mouse)
4
3
2
1
0
p=0.031
Vaccination:
PBS (n=8)
BCG-pMV (n=7)
BCG-SM (n=8)

splenocytes obtained from C57BL/6J mice inoculated with PBS minimally responded to these Ags, mice infected with either BCG-pMV or BCG-SM significantly responded to the Ags. However, splenocytes from the BCG-SM-vaccinated mice responded to all Ags more strongly and produced levels of IFN-γ higher than those from BCG-pMV-vaccinated mice. In order to define the T cells responsible for IFN-γ production, the T cells producing the intracellular cytokines were determined (Fig. 1b). Both $CD4^+$ T cells and $CD8^+$ T cells obtained from mice inoculated with PBS, BCG-pMV, or BCG-SM produced IFN-γ on stimulation with MMP-II. However, the two subsets of T cells from BCG-SM-infected mice responded to the stimulation more strongly than T cells from BCG-pMV-infected mice, and more than 1.0% of both $CD4^+$ T cells and $CD8^+$ T cells produced IFN-γ.

Human leprosy usually manifests long after the infection with *M. leprae*. Therefore, we evaluated the long-term effect of vaccination with BCG-SM (Fig. 2). C57BL/6J mice were vaccinated intradermally with the recombinant BCG strain for 34 weeks, and their splenocytes were examined for a secondary response to *M. leprae*-derived Ags. While the response to BCC did not differ between BCG-pMV- and BCG-SM-infected mice, significantly higher levels of IFN-γ were produced in splenocytes from BCG-SM-vaccinated mice than in those from BCG-pMV-vaccinated mice on in vitro restimulation with both the recombinant MMP-II and MLM. These results indicate that the effect of the BCG-SM vaccination persisted for a long time.

Effect of BCG-SM vaccination on multiplication of *M. leprae* in vivo. C57BL/6J mice that had been vaccinated 4 weeks earlier with either BCG-pMV or BCG-SM (1×10^4/mouse) intradermally were challenged with 5×10^3 *M. leprae* bacilli in the footpad. Thirty or 31 weeks later, the footpads were removed and the *M. leprae* bacilli recovered were enumerated (Fig. 3a). A total of 2×10^5 *M. leprae* were recovered from the mice inoculated with PBS and challenged with *M. leprae*, and BCG-pMV partially inhibited the multiplication of *M. leprae*. However, only 5×10^4 *M. leprae* bacilli were recovered from the BCG-SM-vaccinated mice, showing that BCG-SM is more effective than BCG-pMV at inhibiting the growth of *M. leprae*. In order to clarify the T-cell population responsible for the inhibition of *M. leprae* growth, $CD4^+$ T cells and $CD8^+$ T cells from BCG-vaccinated and *M. leprae*-challenged mice were restimulated with MMP-II (data not shown) or MLM (Fig. 3b) in vitro. There was no significant difference in the percentage of IFN-γ-producing $CD4^+$ T cells among uninfected *M. leprae*-challenged, BCG-pMV-vaccinated *M. leprae*-challenged, and BCG-SM-vaccinated *M. leprae*-challenged mice. However, significantly higher numbers of $CD8^+$ T cells from BCG-SM-vaccinated *M. leprae*-challenged mice than T cells from the other groups of mice produced intracellular IFN-γ in response to MMP-II. We then examined the effect of a lower dose of recombinant BCG on the multiplication of *M. leprae* in the footpads of mice. Again, a 40-fold increase in the number of *M. leprae* bacilli was observed in *M. leprae*-challenged (5×10^3/mouse) nonvaccinated mice. Also, vaccination with BCG-SM was more effective in inhibiting the growth of *M. leprae* than vaccination with BCG-pMV (Fig. 3c).

DISCUSSION

In 1991, the World Health Assembly proposed the elimination of leprosy as a public health problem by the year 2000, since the multidrug therapy was drastically effective in reducing the number of registered leprosy cases. However, at present, more than 200,000 newly manifested leprosy cases are still reported annually (25). Therefore, in order to eliminate the disease, an effective and safe vaccine is needed. The vaccine should also be widely available at a low cost. An assessment of the cost-effectiveness of BCG vaccination on childhood tuberculosis was conducted and was found to be a highly cost-effective intervention (23). However, human immunodeficiency virus (HIV)-infected infants who were vaccinated with BCG at birth were at high risk of developing disseminated BCG disease (5, 6). Therefore, care should be taken to prevent the vaccination of HIV-exposed infants with BCG. For the prevention of leprosy, a number of field trials as well as animal experiments have been conducted to test the efficacies of heat-killed *Mycobacterium leprae*, *Mycobacterium* sp. strain w, the combination of *M. leprae* and *Mycobacterium* sp. strain w, and recombinant *M. bovis* BCG as candidate vaccines in regions of endemicity (1–4, 7, 15, 20, 24). Although *M. bovis* BCG offered a certain level of protection against leprosy, its effect needs to be bolstered (19, 26). To improve BCG, its immunostimulatory activity needs to be enhanced. It is generally believed that in the host defense against mycobacteria, including *M. leprae*, both $CD4^+$ T cells and $CD8^+$ T cells play a central role. In the initial stage of a mycobacterial infection, the cells that mainly participate are the IFN-γ-producing $CD4^+$ T cells. The IFN-γ produced from $CD4^+$ T cells may activate macrophages infected with the mycobacteria, and the activated macrophages may induce the intracellular killing of the mycobacteria. In

FIG. 3. (a) Effect of vaccination with recombinant BCG on *M. leprae* multiplication. Five-week-old C57BL/6J mice were intradermally inoculated with 1×10^4 CFU of BCG-SM or BCG-pMV per mouse 4 weeks prior to challenge in the footpad with 5×10^3 of *M. leprae*. Thirty to 31 weeks later, the number of *M. leprae* bacilli recovered from the footpad was enumerated by the method of Shepard (22). The indicated number of the mice per group was used, and the numbers of bacilli recovered were compared statistically by the Mann-Whitney-Wilcoxon test. (b) Intracellular production of IFN-γ by $CD4^+$ T cells and $CD8^+$ T cells in mice vaccinated with BCG and challenged with *M. leprae*. C57BL/6J mice were vaccinated with 1×10^4 CFU of either BCG-SM or BCG-pMV per mouse for 4 weeks and challenged with 5×10^3 bacilli of *M. leprae* for 30 to 31 weeks. Splenocytes (2×10^5/well) were obtained from these mice and were restimulated with 0.2 μg/ml of MLM for 3 days in vitro. The $CD4^+$ T cells and $CD8^+$ T cells were gated separately and were analyzed for the intracellular production of IFN-γ. The number in the top right-hand corner of each panel represents the mean percentage of IFN-γ-producing cells ± standard deviation (for three mice) among the gated T-cell population. The titers were compared statistically by Student's *t* test. *, $P < 0.01$; †, $P < 0.05$. (c) Effect of vaccination with a low dose of BCG on the multiplication of *M. leprae*. Again, as described for panel a, C57BL/6J mice were inoculated but they were inoculated with a lower dose of recombinant BCG (1×10^3 CFU per mouse), and the effect on the multiplication of *M. leprae* was observed. The numbers of bacilli recovered were compared statistically by the Mann-Whitney-Wilcoxon test.

contrast, in the chronic stage, cytotoxic T lymphocytes differentiated from the activated type 1 $CD8^+$ T cells mainly act to inhibit the growth of the intracellular mycobacteria (11, 16). Thus, the activation of both $CD4^+$ T cells and $CD8^+$ T cells is essential for inhibiting the multiplication of mycobacteria.

We previously screened for *M. leprae* antigens with immunostimulatory properties and observed that a membrane protein, namely, MMP-II, stimulated human monocyte-derived DCs to produce the active form of interleukin-12 and tumor necrosis factor alpha (8). DCs pulsed with MMP-II stimulated both $CD4^+$ and $CD8^+$ T cells to produce IFN-γ. Therefore, we produced a recombinant BCG strain that secretes *M. leprae*-derived MMP-II (strain BCG-SM). The DCs infected with BCG-SM activated both human naïve $CD4^+$ T cells and naïve $CD8^+$ T cells more efficiently than the vector control BCG (9). T cells of both subsets which can respond to MLM as well as recombinant MMP-II were more efficiently produced from unprimed mice by inoculation with BCG-SM (Fig. 1). At 3 weeks postinoculation, no BCG could be recovered from the spleen. Moreover, it was found that BCG-SM effectively inhibited the multiplication of *M. leprae* in the footpads of C57BL/6J mice, possibly due to the efficient production of T cells responsive to *M. leprae*-derived Ags. It may be difficult to determine the T-cell subset responsible for the inhibition; however, $CD8^+$ T cells from mice vaccinated with BCG-SM and challenged with *M. leprae* for 30 weeks still had the ability to produce IFN-γ after stimulation with *M. leprae*-derived Ag. Furthermore, *M. leprae*-responsive $CD4^+$ and $CD8^+$ T cells persisted for 34 weeks after infection with BCG-SM. Therefore, it is possible that $CD8^+$ T cells at least partially contribute to inhibiting the growth of *M. leprae* in vivo.

Earlier efforts to produce a vaccine against leprosy have not been particularly successful. Some reports indicated that a mixture of refined components of *M. leprae* was protective, while others emphasized DNA-based vaccines (13, 14). To date, BCG-based vaccines seem to be more promising in terms of their applicability in the field due to the safety and history of global usage of BCG. Taken together, the present study indicates that a recombinant BCG strain that secretes MMP-II could be a useful candidate as a vaccine against leprosy.

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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]

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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

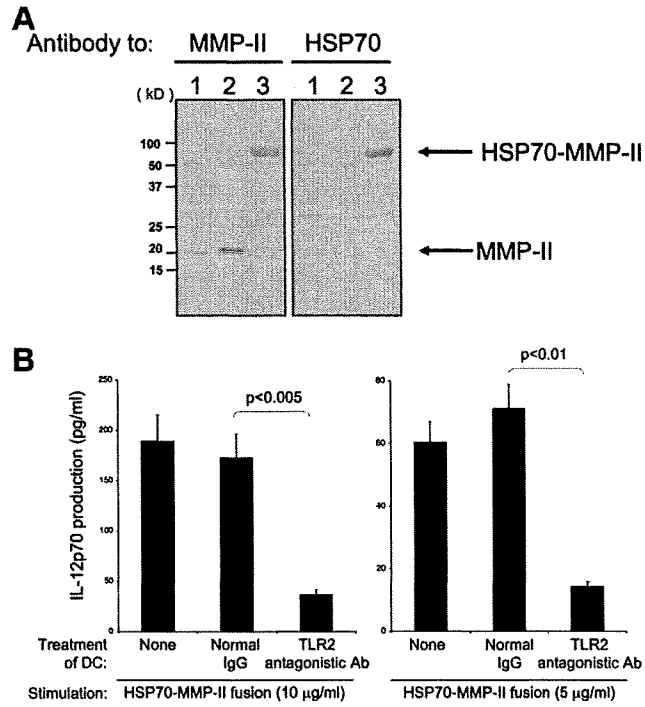

FIGURE 1. *A*, Western blotting analysis of protein secreted from BCG-70M. A mAb to either MMP-II or HSP70 was used to detect HSP70-MMP-II fusion protein. *Lane 1*, Culture filtrates of BCG-261H. *Lane 2*, rMMP-II protein. *Lane 3*, Culture filtrates of BCG-70M. *B*, Contribution of TLR2 to IL-12p70 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 ± 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,

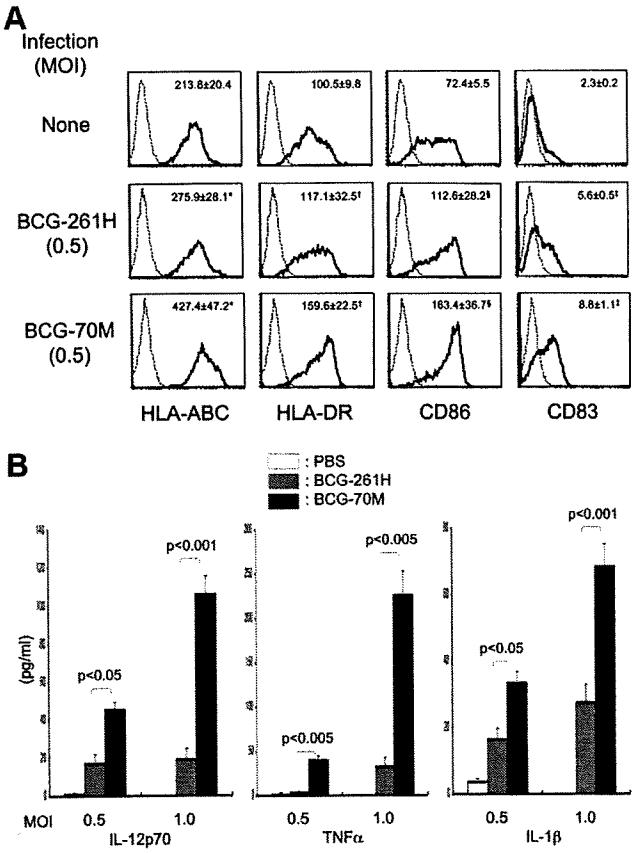

FIGURE 2. *A*, Up-regulated expression of APC-associated molecules on DC by infection with BCG-70M. PBMCs were obtained from one donor. Monocyte-derived immature DC were infected with either BCG-261H or BCG-70M at a MOI of 0.5 and cultured for another 2 days in the presence of rGM-CSF and rIL-4. The 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.

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

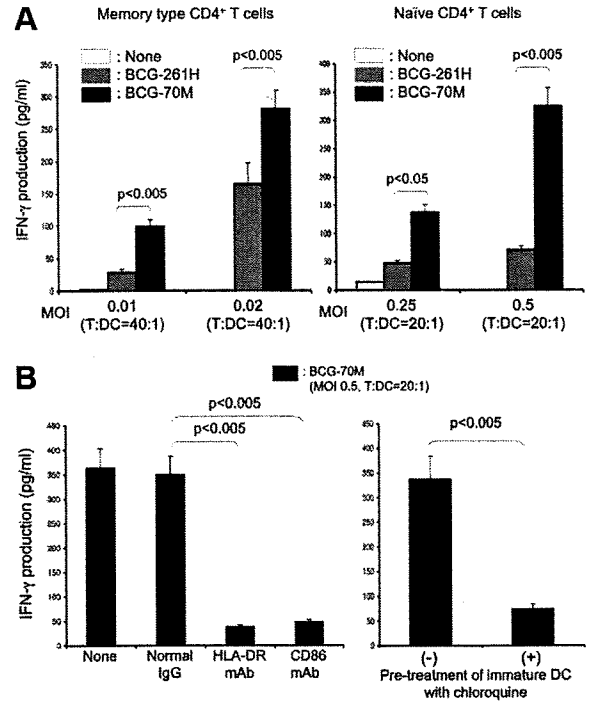

FIGURE 3. *A*, IFN-γ production from $CD4^+$ T cells by stimulation with BCG. PBMCs were obtained from one donor. Monocyte-derived DC were infected with either BCG-261H or BCG-70M at the indicated MOI, and used as a stimulator of memory-type or naive $CD4^+$ T cells in a 4-day culture. Responder $CD4^+$ 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.

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

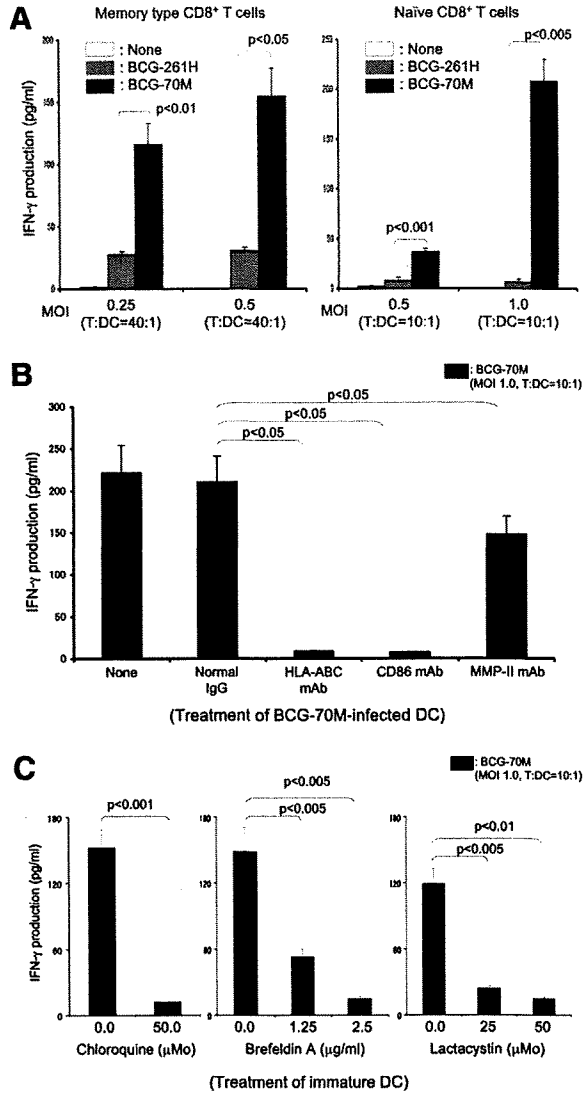

FIGURE 4. *A*, IFN-γ production from $CD8^+$ T cells by stimulation with BCG. PBMCs were obtained from one donor. Monocyte-derived DC were infected with either BCG-261H or BCG-70M at the indicated MOI, and used as a stimulator of memory-type or naive $CD8^+$ T cells in a 4-day culture. Responder $CD8^+$ 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.

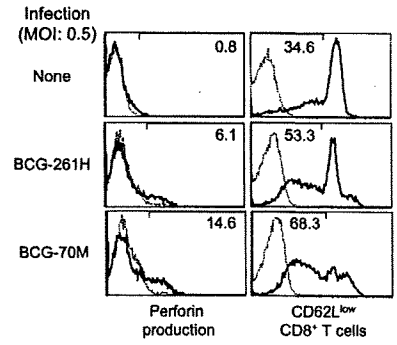

FIGURE 5. Influence of naive $CD4^+$ T cells on the activation of naive $CD8^+$ T cells. PBMCs were obtained from one donor. Monocyte-derived DC were infected with BCG (MOI 0.5) and cocultured with unseparated naive T cells (T:DC = 10:1) for 7 days. The stimulated $CD8^+$ T cells were gated and analyzed 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

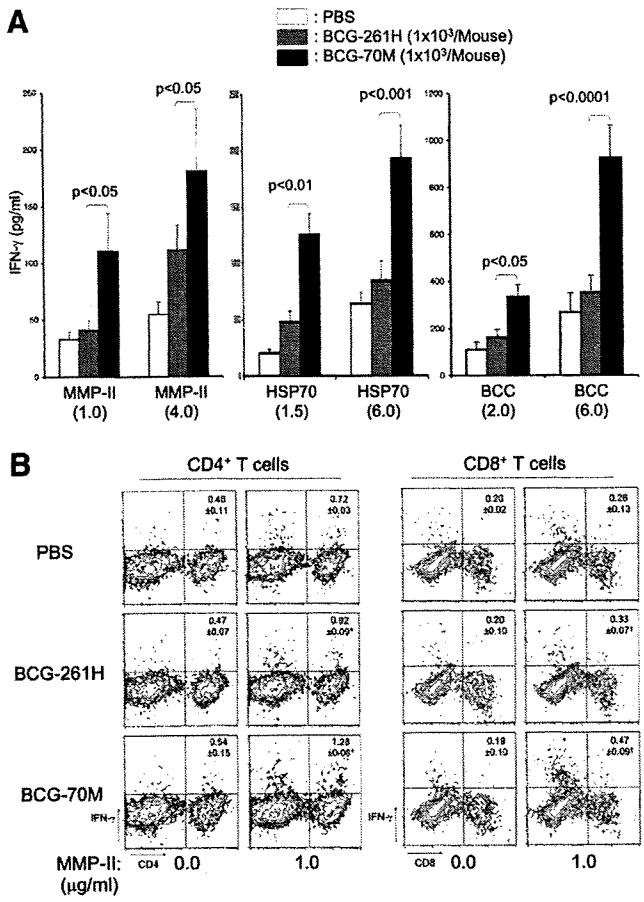

FIGURE 6. *A*, Production of memory-type T cells in C57BL/6 mice by infection with BCG. Five-week-old C57BL/6 mice were infected with 1×10^3 CFU/mouse of either BCG-261H or BCG-70M s.c. Four weeks after the inoculation, splenocytes (2×10^5 cells/well) were stimulated with the indicated dose of either 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. *, $p < 0.05$; †, $p < 0.01$.

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

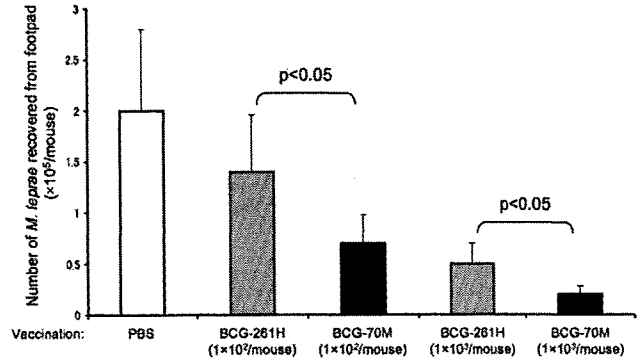

FIGURE 7. Inhibition of *M. leprae* multiplication by s.c. vaccination with BCG-70M. Five-week-old C57BL/6 mice (8 mice per group) were vaccinated with 1×10^2 or 1×10^3 CFU/mouse either BCG-261H or BCG-70M s.c., and were challenged with 5×10^3 bacilli/mouse *M. leprae* in footpad 4 wk after the vaccination. 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.

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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Temperature dependency for survival of *Mycobacterium leprae* in macrophages

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Hansen's disease is caused by an infection with an intracellular pathogen, *Mycobacterium leprae*, which mainly inhabits macrophages and Schwann cells. However, little is known about the survival or growth mechanisms of the bacilli in mouse and human macrophages. In the present study, by using radiorespirometry analysis for the evaluation of the viability of *M.leprae*, we observed that *in vitro* incubation of *M.leprae*-infected macrophages at 35°C was more growth permissive than at 37°C, and supplementation with the immunosuppressive cytokine IL-10 supported the survival of the bacilli in the macrophages for 3 weeks, whereas viability of the bacilli was gradually lost if cultured without IL-10. In human macrophages, *M.leprae* retained its viability when cultured at 35°C for at least 4 weeks without IL-10. However, the viability of *M.leprae* was almost lost within 2 weeks if cultured at 37°C. These data suggest that temperature is a crucial factor for the survival of *M.leprae* in host cells.

Introduction

Hansen's disease is caused by an infection with *Mycobacterium leprae*. *M.leprae* is an intracellular pathogen, mainly residing in macrophages and Schwann cells. In patients, *M.leprae* is predominantly observed in the skin, nasal mucosa and peripheral nerves, particularly the more superficial ones. This clinical observation suggests that the optimal temperature of *M.leprae* for survival in human cells is less than 37°C [1]. In animal models, *M.leprae* multiplies in the mouse footpad where the temperature is lower than the core temperature, and the optimal temperature for the growth of *M.leprae* is reported to be in the range of several degrees above and below 30°C [2].

From another aspect, the growth of *M.leprae* seems to be largely affected by the host immune

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response. Hansen's disease is characterized by a broad spectrum of the host immune response, such as lepromatous type (towards the increased load of bacteria) and tuberculoid type (towards the decreased bacterial load). In lepromatous type leprosy, Th-2 cytokines (IL-4, IL-5 and IL-10) are predominantly expressed in local lesions. In contrast, in tuberculoid type, Th-1 cytokines (IFN-γ, IL-2) are predominantly expressed [3]. Among cytokines, IFN-γ has been demonstrated to play a central role in activating macrophages to kill intracellular pathogens including *M.leprae*, whereas IL-10 is reported to inhibit the microbicidal activity of macrophages, resulting in the survival of the intracellular pathogen [4]. However, little is known about the survival and growth mechanisms of *M.leprae* in human macrophages since the viability of these uncultivable bacilli cannot be easily measured by in vitro study.

Previously we reported that metabolically active *M.leprae* were maintained in monolayer cultures of mouse peritoneal macrophages and supplemental IL-10 bolstered *M.leprae* metabolism in the macrophages for as long as 8 weeks. In the cell culture system temperature is extremely important and 31-33°C incubation temperature is more growth permissive than 37°C [5]. In the present study, we observed that incubation of mouse macrophages at 35°C was also more permissive than at 37°C and supplemental IL-10, but not TGF-β, supported the metabolic activity of *M.leprae* in the macrophages for several weeks. Moreover, *M.leprae* from infected human macrophages cultured in vitro sustained metabolic activity for at least 4 weeks if cultured at 35°C but not at 37°C. Collectively, these data demonstrate that temperature is one of the crucial factors for *M.leprae* survival in human host cells.

Materials and Methods

M.leprae inoculum: The Thai-53 strain of *M.leprae* [6] was maintained in continuous passage in athymic *nu/nu* mice (Clea Co, Tokyo, Japan) by inoculation of bacilli into both hind foot pads. Experiments with mice were performed in compliance with the guidelines of the Experimental Animal Committee of the National Institute of Infectious Diseases. At approximately one year post inoculation, the foot pads were processed to recover *M.leprae* by Nakamura's method with a slight modification [7]. Briefly, tissue was minced and homogenized with Hanks' balanced salt solution (HBSS) containing 0.05% Tween 80. The homogenate was centrifuged at 150×g for 10 min and supernatant of the sample homogenate was treated with 0.05% trypsin at 37°C for 60min. The suspension was centrifuged at 4,000×g for 20min and sediment was re-suspended in HBSS followed by treatment with 1% sodium hydroxide at 37°C for 15min. The treated material was washed and re-suspended in HBSS at the desired bacillary concentration. Bacillary number in each foot pad was enumerated individually according to standard techniques [8].

Cytokines: Murine recombinant IL-10 was obtained from Genzyme Corp. TGF-β was obtained from Kurashiki Bouseki (Kurashiki, Japan). Both cytokines were stored at -80°C until use.

Mouse macrophage culture: Mouse peritoneal resident cells (approximately 50% macrophages) were harvested from retired ICR mice and suspended as previously described [9] at a concentration of 2×10^6/ml in RPMI 1640 (Gibco BRL, Invitrogen Corp., Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS, HyClone Laboratories, Logan UT), 25 mM N-2-