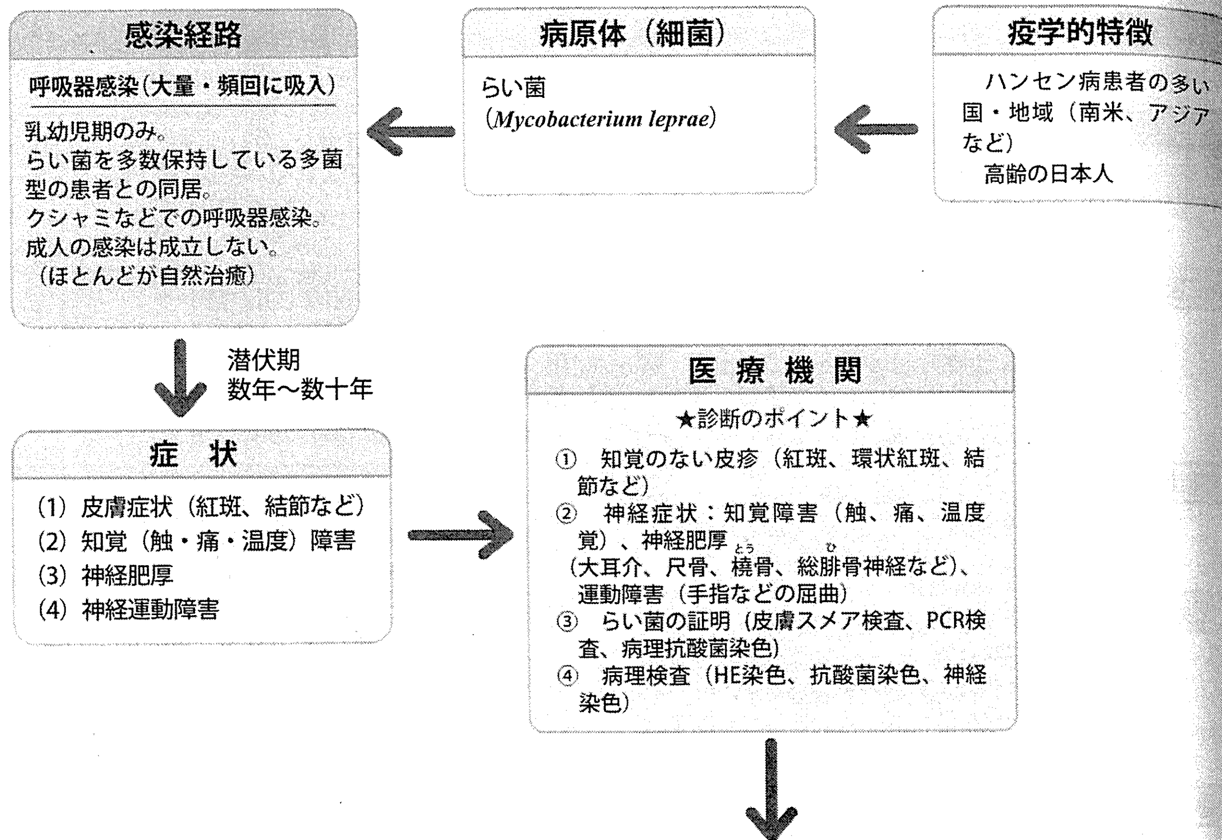
標準予防策

(17) ハンセン病
Hansen's disease (Leprosy)



ハンセン病の病型と治療

Ridley&Jopling 分類		LL	BL	BB	BT	TT	I
WHO 分類		多菌型(MB)			少菌型(PB)		
臨床	皮膚(皮疹)	概ね6個以上 紅斑、小結節、結節 ほぼ左右対称性			概ね1〜5個 紅斑、環状紅斑など 非対称性		
臨床	神経	知覚障害は軽度 / 正常			知覚(触・痛・温度)障害高度		
皮疹部の菌		陽性			陰性		
治療	(WHO/MDT を改変)	リファンピシン 600 mg 1×/月 クロファジミン 300 mg 1×/月 クロファジミン 50 mg / 日 DDS 100 mg / 日 } 1〜3年間			リファンピシン 600 mg 1×/月 DDS 100 mg / 日 } 6か月		

参考図書

(1) 石橋康正他監修『ハンセン病の外来診療』メジカルセンス 1997
(2) 厚生省『ハンセン病診断・治療指針』1997
(3) 後藤正道他『ハンセン病治療指針（第2版）』（日本ハンセン病学会雑誌）2006
(4) 小野友道他『ハンセン病アトラス』金原出版 2006
(5) 石井則久『皮膚抗酸菌症』金原出版 2008

発生状況

日本では、新規患者を毎年少数認める。日本人は数名で、高齢者の発症である。在日外国人は約8名で、ブラジルやアジア出身者が多い。2007年の全世界の新規患者数は約26万人と、減少している。

臨床症状

下記の診断ポイントが重要
① 知覚障害を伴う皮疹
② 手足の知覚麻痺（気づかずの火傷、外傷もおこる）
③ 末梢神経の肥厚（大耳介、尺骨神経など）

病型は、各個人のらい菌に対する特異的な免疫能の低下の程度と生体内のらい菌の菌数により、多菌型（multibacillary:MB）（従来のLLなどが相当）と、少菌型（paucibacillary:PB）（従来のTTなどが相当）に分類する。

ハンセン病の治療前、治療中、治療後に急性の炎症性反応が起こることがあり、「らい反応」という。急速に皮疹が増加したり再燃、また神経痛や末梢神経腫脹、眼の炎症などがおこる。

検査所見

皮膚スメア検査を実施し、らい菌の有無を検索
PCR検査（らい菌に特異的なDNAの検出）
鑑別：サルコイドーシス、皮膚リーシュマニア症、皮膚結核、リンパ腫、梅毒、環状紅斑、環状肉芽腫、尋常性白斑、レックリングハウゼン病、多発性単神経炎など種々の疾患がある。

病原体

らい菌（***Mycobacterium leprae***）。抗酸菌の一種で、現在まで人工培養できていない。

感染経路

多菌型(MB)未治療患者との乳幼児期における大量・頻回の接触（呼吸器感染）。

発病力は極めて弱い。らい菌に対して特異的に免疫の低下している乳幼児が、MB患者と長期間にわたる接触（呼吸器感染）により感染するが、多くは自然治癒する。ごく稀に長期間の潜伏を経て発病する。発病にいたるには、各個人の免疫能力のほかに、栄養状態、経済状態、衛生状態など多くの因子が関与する。

潜伏期

数年～数十年

治療方針

ハンセン病は感染症であり抗菌薬治療が基本であり、WHOの推奨する多剤併用療法（multidrug therapy:MDT）を改変して治療する。

多菌型(MB)では殺菌力の強いリファンピシンの他にクロファジミンとDDSの合計3剤を用いる。少菌型(PB)ではリファンピシンとDDSの2剤（クロファジミンを追加しても可）を用いる。治療期間は、MBでは臨床症状と菌の減少を勘案して1～3年間、PBでは6か月間とする。

らい反応では、ハンセン病の治療を継続したまま、ステロイド剤の内服を行う。

RESEARCH ARTICLE

GM-CSF-mediated T-cell activation by macrophages infected with recombinant BCG that secretes major membrane protein-II of *Mycobacterium leprae*

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Keywords
macrophage; *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG); *Mycobacterium leprae*; GM-CSF; T-cell activation; interleukin-10.

Abstract

The potential of *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) needs to be augmented to efficiently activate $CD4^+$ T cells through macrophages. *Mycobacterium leprae*-derived recombinant major membrane protein (MMP)-II induced GM-CSF production from macrophages. A recombinant BCG-SM that secretes MMP-II more efficiently produced GM-CSF and activated interferon (IFN)-γ-producing $CD4^+$ T cells than did vector control BCG when infected with macrophages. The T-cell activation by BCG-SM was dependent on the GM-CSF production by macrophages. Interleukin (IL)-10 production by macrophages stimulated with *M. leprae* was inhibited in a GM-CSF-dependent manner when the precursor monocytes were infected with BCG-SM. BCG inducing GM-CSF production was effective in macrophage-mediated T-cell activation partially through IL-10 inhibition.

Introduction

Leprosy is a chronic infectious disease induced by parasitic infection with *Mycobacterium leprae* (Stoner, 1979). Despite the marked reduction in the number of both registered leprosy cases and new cases, a significant number of new cases (254 525 for the year 2007) are still detected each year (World Health Organization, 2008). The emergence of multidrug-resistant *M. leprae* (Kai *et al.*, 2004), although still few in number, and the complexity of leprosy reactions are distressing (Moschella, 2004). These observations indicate the urgent need to develop an efficacious vaccine against leprosy. *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) has been known to provide partial protection against the development of leprosy (Ponnighaus *et al.*, 1992). However, meta-analyses conducted by Setia *et al.* (2006) demonstrated an overall protective effect of only 26% against leprosy. There seem to be several reasons why BCG is not as effective as previously predicted. One of them may be that the human immune cells most susceptible to BCG infection are macrophages (Grode *et al.*, 2005). On entry into macrophages, mycobacteria inhibit phagosome–lysosome fusion, which results in a less efficient stimulation of interferon (IFN)-γ-producing type 1 $CD4^+$ T cells (Ridley & Jopling, 1966; Frehel & Rastogi, 1987). Further, BCG as well as pathogenic mycobacteria can induce the production of an abundant amount of interleukin (IL)-10 from macrophages (Yamamura *et al.*, 1991), which inhibits activation of $CD4^+$ T cells (Jonuleit *et al.*, 2001; Granelli-Piperno *et al.*, 2004). Moreover, it has been demonstrated in a murine study that BCG primarily infects macrophages *in vivo*, and the active proliferation of T cells *in vivo* needs the enrolment of dendritic cells (DC). Further, DC are known to be the most professional antigen-presenting cells (APC) in terms of T-cell activation. Thus, the transfer to DC of antigens produced by the processing of intracellular BCG or of proteins secreted from the mycobacteria in macrophages, seems to be important (Winau *et al.*, 2006).

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We previously identified major membrane protein (MMP)-II (gene name, bfrA or ML2038), which is originally identified as bacterioferritin (Pessolani *et al.*, 1994) and localized in the cell membrane, as one of the dominant antigen of *M. leprae* (Maeda *et al.*, 2005; Makino *et al.*, 2005). Recombinant (r) MMP-II-pulsed DC activate naïve $CD4^+$ T cells to produce IFN-γ in an antigen-specific manner, and also stimulate T cells from not only paucibacillary leprosy, a representative clinical leprosy at one pole of the clinical spectrum, but also multibacillary leprosy, a representative leprosy at the opposite pole (Makino *et al.*, 2005). The activation of type 1 $CD4^+$ T cells is closely associated with the inhibition of the spread of *M. leprae in vivo* as observed in paucibacillary leprosy (Sieling *et al.*, 1999). In this respect, it was interesting to find that T cells from some paucibacillary leprosy patients seemed to be primed with MMP-II antigen *in vivo* (Makino *et al.*, 2005). Therefore, MMP-II was considered to be an immunodominant antigen of *M. leprae.* We constructed an rBCG strain (BCG-SM) that secretes MMP-II of *M. leprae* (Makino *et al.*, 2006). BCG-SM-infected DC stimulated quite efficiently both human naïve $CD4^+$ T cells and naïve $CD8^+$ T cells *in vitro*, and MMP-II-specific memory T cells were produced in mice inoculated with BCG-SM (Makino *et al.*, 2006).

Macrophages are heterogeneous in various aspects (Randolph *et al.*, 1999), and their differentiation is largely influenced by the cytokine milieu (Nakata *et al.*, 1991; Akagawa, 2002). Previously, we analysed the characteristics of two distinct macrophage subsets: rGM-CSF-mediated macrophages (GM-Mϕ) and rM-CSF-mediated macrophages (M-Mϕ) (Makino *et al.*, 2007). Both macrophages were equally susceptible to mycobacterial infection *in vitro*, but M-Mϕ infected with *M. leprae* did not activate $CD4^+$ T cells even after activation using both CD40 ligand and exogenous IFN-γ. Likewise, *Mycobacterium tuberculosis*-infected M-Mϕ failed to stimulate T cells (Verreck *et al.*, 2004). Further, a large amount of IL-10 was produced from M-Mϕ on stimulation with mycobacteria. Therefore, the fact that mycobacteria are highly susceptible to phagocytosis by M-Mϕ and poorly stimulate T cells through M-Mϕ, may be closely associated with the affinity of mycobacteria to macrophages, the induction of a latent infection and, in some cases, the development of disease. Likewise, M-Mϕ is one of the major target immune cells of BCG infection. However, to control the subsequently invading pathogenic mycobacteria, such as *M. leprae*, by producing memory T cells, modified BCG including the newly developed recombinant BCG-SM is required to be able to fully stimulate T cells even if M-Mϕ are the initial target host cells.

In this report, we examined the T-cell-stimulating ability of BCG-SM-infected M-Mϕ, and further assessed the influence of BCG-SM on the IL-10-producing activity of Mϕ upon a challenge with *M. leprae.*

Materials and methods

Preparation of cells and bacteria

Peripheral blood was obtained from healthy purified protein derivative (PPD)-positive individuals with informed consent. In Japan, most healthy individuals are PPD-positive due to a compulsory BCG vaccination for children (0–4 years old). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use as previously described (Makino & Baba, 1997). For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from freshly isolated heparinized blood or from cryopreserved PBMC using immunomagnetic beads coated with anti-CD3 monoclonal antiboby (mAb) (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates and nonadherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (Makino & Baba, 1997). Macrophages were generated by culturing monocytes in the presence of 20% foetal calf serum and either rM-CSF (R and D Systems, Abingdon, UK) (M-Mϕ) or rGM-CSF (PeproTech EC Ltd, London, UK) (GM-Mϕ) (Makino *et al.*, 2007). Both GM-Mϕ and M-Mϕ were pulsed with rBCGs on day 3 or 5 of culture, and were used as a stimulator of T cells on day 5 or 7 (Makino *et al.*, 2007). A recombinant BCG that secretes *M. leprae*-derived MMP-II was constructed as described previously (Makino *et al.*, 2006). In brief, a shuttle vector, pMV-261, having a kanamycin resistance gene and origins of replication for *Escherichia coli* and mycobacteria was used to construct pMV-SM (Secreting MMP-II) having the MMP-II cDNA fragment. The BCG substrain Pasteur was cultured *in vitro* using Middlebrook 7H9 broth (BD Biosciences Pharmingen, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin–dextrose–catalase (BD). Expression vectors were introduced into BCG by electroporation (Snapper *et al.*, 1988). Transformants were selected on Middlebrook 7H10 agar (BD) plates supplemented with 10% OADC (BD) and 25 $\mu g\,mL^{-1}$ kanamycin. Mycobacteria were subsequently grown in Middlebrook 7H9 broth containing 25 $\mu g\,mL^{-1}$ of kanamycin. BCG containing pMV-SM as an extrachromosomal plasmid is referred to as BCG-SM, and BCG containing pMV-261 is referred to as BCG-pMV (vector control BCG). *Mycobacterium leprae* (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice (McDermott-Lancaster *et al.*, 1987). The isolated bacteria were counted using Shepard's method (McDermott-Lancaster *et al.*, 1987). The multiplicity of infection (MOI) was determined based on the assumption that macrophages were equally susceptible to infection with BCG or *M. leprae* (Hashimoto *et al.*, 2002). A recombinant MMP-II protein was produced as reported previously (Maeda *et al.*, 2005). Briefly,

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the MMP-II gene (ML2038) was inserted into the expression plasmid pET28 (Novagen, Madison, WI) and transformed into *E. coli* strain ER2566 (New England BioLabs, Ipswich, MA). The expressed protein was eluted using Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA). As a control for *M. leprae* antigen, we have purified hsp18 (ML1795) in *E. coli* using the PET expression system. The cytosolic fraction of the parental BCG was obtained as described previously (Maeda *et al.*, 2003).

Antigen-presenting function of rBCG-infected macrophages

The ability of rBCG-infected macrophages to stimulate T cells was assessed using an autologous macrophage-T cell coculture system as previously described (Wakamatsu *et al.*, 1999; Hashimoto *et al.*, 2002). The responder $CD4^+$ T cells were purified from freshly thawed PBMC using a CD4-negative isolation kit (Dynabeads 450) (Wakamatsu *et al.*, 1999). The purity of $CD4^+$ T cells was $> 95\%$ as assessed by FACS. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates and macrophages were added to give the indicated macrophage : $CD4^+$ T-cell ratio. Supernatants of macrophage–T cell cocultures were collected on day 4. To identify molecules restricting T-cell activation, the following purified mAbs were used: anti-HLA-DR Ab (L243) and anti-CD86 Ab [IT2.2, Becton Dickinson (BD), San Jose, CA]. The concentration of IFN-γ produced by $CD4^+$ T cells was quantified using an enzyme assay kit, OptEIA Human enzyme-linked immunosorbent assay(ELISA) Set (BD). In some cases, M-Mϕ were pulsed with BCG-SM in the presence of $10\,\mu g\,mL^{-1}$ of either normal rat IgG or neutralizing mAb to GM-CSF (rat IgG2a) (BD).

Production of IL-10 and GM-CSF by macrophages

The ability of M-Mϕ to produce IL-10 on stimulation with *M. leprae* was assessed. The monocytes were pretreated with the indicated dose of rBCG and subsequently made to differentiate into M-Mϕ by culturing for 5 days in the presence of rBCG and M-CSF. These macrophages were stimulated with *M. leprae* at the indicated MOI for 24 h. In some cases, monocytes were infected with BCG-SM in the presence of $10\,\mu g\,mL^{-1}$ of neutralizing mAb to GM-CSF. Also, the ability of M-Mϕ to produce GM-CSF on stimulation with rBCG for 24 h was assessed. The concentration of these cytokines was quantified using OptEIA Human ELISA Set (BD).

Statistical analysis

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

Results

Effect of rBCG-infected macrophages on T-cell-stimulating activity

We analysed the T-cell-stimulating activity of rBCG-infected GM-Mϕ and M-Mϕ (Fig. 1). GM-Mϕ infected with either BCG-SM or BCG-pMV significantly stimulated $CD4^+$

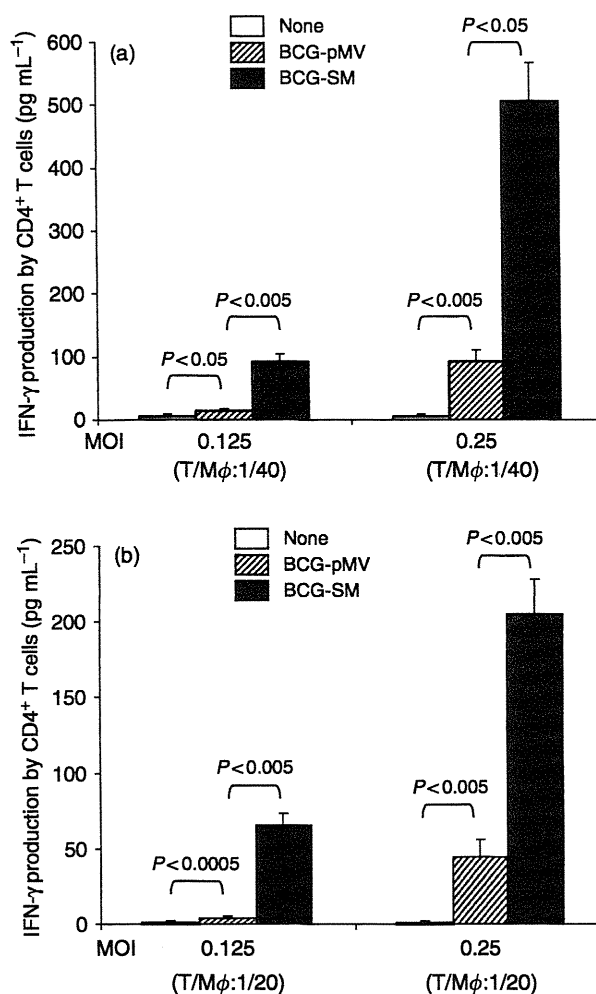

Fig. 1. Production of IFN-γ by $CD4^+$ T cells. (a) GM-Mϕ, differentiated by 3 days of culture with rGM-CSF from monocytes, were infected with BCG-pMV (vector control BCG) or BCG-SM (rBCG that secretes MMP-II) at the indicated MOI, and cultured for another 2 days. These GM-Mϕ were used as a stimulator of $CD4^+$ T cells (1×10^5 cells per well) at a T cell : GM-Mϕ ratio of 40 : 1 in a 4-day culture. (b) M-Mϕ, differentiated by 5 days of culture with rM-CSF from monocytes, were infected with BCG-pMV or BCG-SM at the indicated MOI, and cultured for another 2 days. M-Mϕ were then used as a stimulator of $CD4^+$ T cells (1×10^5 cells per well) at a T cell : M-Mϕ ratio of 20 : 1 in a 4-day culture. 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.

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T cells. However, a larger amount of IFN-γ was produced by the T cells when GM-Mϕ were infected with BCG-SM (Fig. 1a), although BCG vaccination did not prime for MMP-II-specific T-cell response (not shown). We then analysed the T-cell-stimulating activity of BCG-infected M-Mϕ (Fig. 1b). Again, M-Mϕ infected with BCG-SM induced a higher amount of IFN-γ production by T cells than did BCG-pMV-infected M-Mϕ, although the IFN-γ production was less efficient than that induced by rBCG-infected GM-Mϕ even though higher doses of BCG-infected M-Mϕ were used as a stimulator.

Factors associated with the induction of the T-cell-stimulating activity of M-Mϕ

To define the factors associated with the $CD4^+$ T-cell activation by BCG-SM-infected M-Mϕ, we phenotypically analysed M-Mϕ infected with either BCG-pMV or BCG-SM. There was no significant difference between BCG-pMV-infected M-Mϕ and BCG-SM-infected M-Mϕ in the expression of HLA-DR, CD86 or CD40 molecules (not shown). The cytokines produced by M-Mϕ stimulated with rBCGs, including GM-CSF and IL-23, were examined. Both rBCGs induced GM-CSF production, but BCG-SM did so more efficiently than BCG-pMV (Fig. 2). However, IL-23 was not produced by M-Mϕ on stimulation with either BCG-pMV or BCG-SM. We also assessed whether rMMP-II protein can induce GM-CSF production in macrophages. Whereas *M. leprae*-derived cytosolic protein (not shown), other mycobacterial proteins such as BCG-derived cytosolic protein (5–10 $\mu g\,mL^{-1}$), control recombinant *M. leprae* antigen (hsp18), and lipopolysaccharide (amount present with rMMP-II protein) did not stimulate Mϕ, MMP-II induced GM-CSF production in a concentration-dependent manner (Fig. 3). rMMP-II also efficiently induced the production of other cytokines including tumour necrosis factor (TNF)α and IL-12p40 from Mϕ (not shown).

We examined the influence of surface antigens on M-Mϕ. The T-cell-stimulating activity of BCG-SM-infected M-Mϕ was significantly inhibited when the infected M-Mϕ were pretreated with the mAb to HLA-DR or CD86 antigens, whereas the control IgG did not affect IFN-γ production by T cells (Fig. 4a). However, IFN-γ production was partially inhibited when BCG-SM-infected M-Mϕ were treated with the mAb to MMP-II (not shown). Next, we examined the effect on T-cell activation of GM-CSF produced by M-Mϕ stimulated with BCG-SM (Fig. 4b). When M-Mϕ were infected with BCG-SM in the presence of the neutralizing mAb to GM-CSF, IFN-γ production by $CD4^+$ T cells was significantly inhibited. The T-cell-stimulating activity of BCG-SM-infected M-Mϕ was not affected by normal rat IgG.

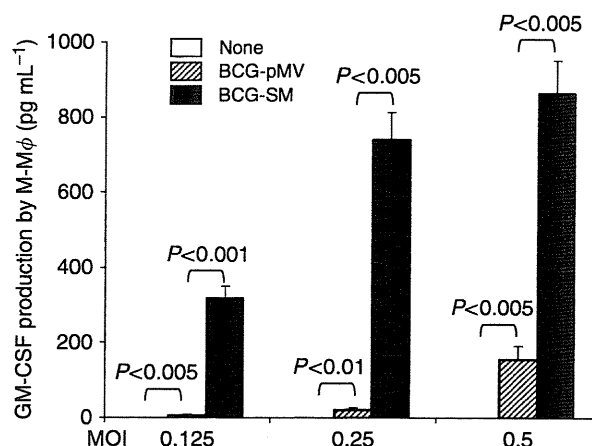

Fig. 2. Production of GM-CSF by M-Mϕ. M-Mϕ differentiated by 5 days of culture with rM-CSF from monocytes, were stimulated with BCG-pMV or BCG-SM for 24 h at the indicated MOI. A representative experiment based three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

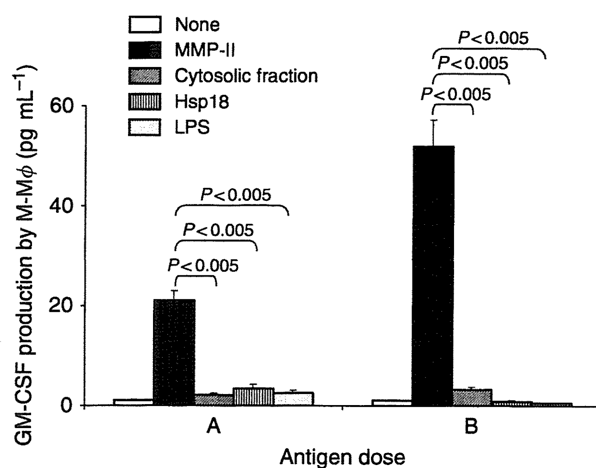

Fig. 3. GM-CSF production by M-Mϕ. M-Mϕ obtained after 5 days of culture with rM-CSF were stimulated for 24 h with rMMP-II, the BCG-derived cytosolic fraction or *Mycobacterium leprae*-derived hsp18 antigen [dose of antigen in (a) 5 $\mu g\,mL^{-1}$ and that in (b) 10 $\mu g\,mL^{-1}$]. Lipopolysaccharide, assumed to be present with rMMP-II protein (660 $ng\,mg^{-1}$ MMP-II protein), was used as a negative control (lipopolysaccharide in (a) 3.3 $ng\,mL^{-1}$ and that in (b) 6.6 $ng\,mL^{-1}$). 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.

Effect of infection of monocytes with BCG on IL-10 production by M-Mϕ

Macrophages are one of the cells most sensitive to *M. leprae* infection and M-Mϕ produce abundant IL-10 when infected with the bacteria (Makino *et al.*, 2007). As precursor

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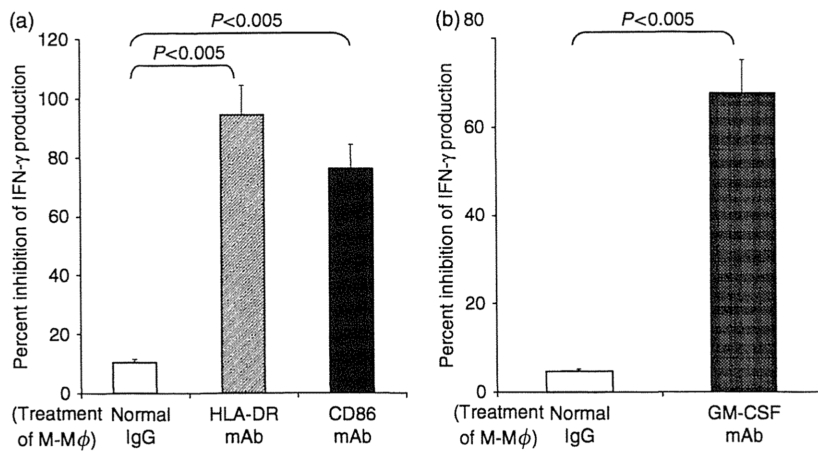

Fig. 4. (a) Inhibition of IFN-γ production by $CD4^+$ T cells by pretreatment of BCG-SM-infected M-Mϕ with mAb to HLA-DR or CD86. M-Mϕ differentiated from monocytes using rM-CSF were infected with BCG-SM at an MOI of 0.25 on day 5 of culture and cultured for another 2 days. The BCG-SM-infected M-Mϕ were 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.

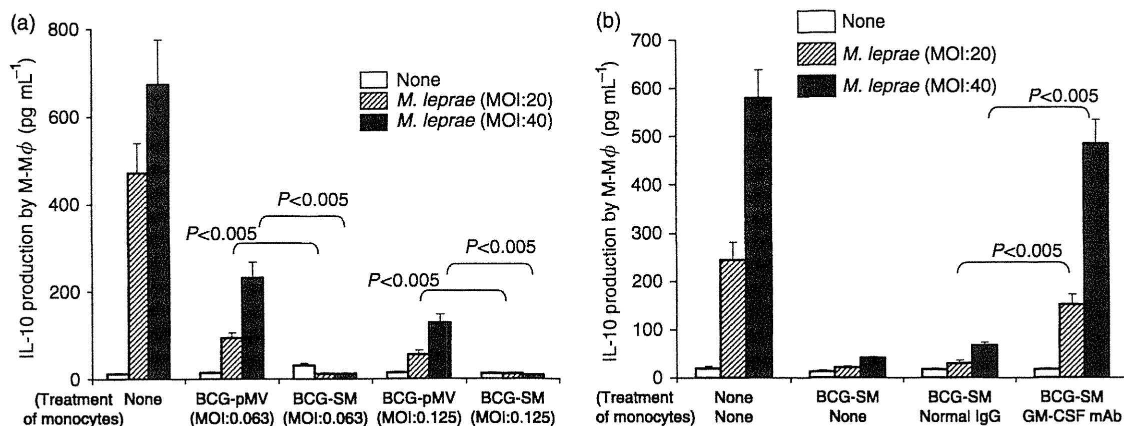

Fig. 5. (a) Production of IL-10 by M-Mϕ. Monocytes were infected with the indicated dose of BCG-pMV or BCG-SM, and subsequently differentiated into M-Mϕ by culturing for 5 days in the presence of M-CSF and rBCGs. These rBCG-preinfected M-Mϕ were stimulated with *Mycobacterium leprae* at the indicated 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

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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

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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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References

Akagawa KS (2002) Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages. *Int J Hematol* **76**: 27–34.

Frehel C & Rastogi N (1987) *Mycobacterium leprae* surface components intervene in the early phagosome–lysosome fusion inhibition event. *Infect Immun* **55**: 2916–2921.

Granelli-Piperno A, Golebiowska A, Trumpfheller C, Siegal FP & Steinman RM (2004) HIV-1-infected monocyte-derived dendritic cells do not undergo maturation but can elicit IL-10 production and T cell regulation. *P Natl Acad Sci USA* **101**: 7669–7674.

Grode L, Seiler P, Baumann S *et al.* (2005) Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* Bacille Calmette–Guérin mutants that secrete listeriolysin. *J Clin Invest* **115**: 2472–2479.

Hashimoto K, Maeda Y, Kimura H, Suzuki K, Masuda A, Matsuoka M & Makino M (2002) Infection of *M. leprae* to monocyte derived dendritic cells and its influence on antigen presenting function. *Infect Immun* **70**: 5167–5176.

Jonuleit H, Schmitt E, Steinbrink K & Enk AH (2001) Dendritic cells as a tool to induce anergic and regulatory T cells. *Trends Immunol* **22**: 394–400.

Kai M, Maeda Y, Maeda S, Fukutomi Y, Kobayashi K, Kashiwabara Y, Makino M, Abbasi MA, Khan MZ & Shah PA (2004) Active surveillance of leprosy contacts in country with low prevalence rate. *Int J Leprosy* **72**: 50–53.

Maeda Y, Gidoh M, Ishii N, Mukai C & Makino M (2003) Assessment of cell mediated immunogenicity of *Mycobacterium leprae*-derived antigens. *Cell Immunol* **222**: 69–77.

Maeda Y, Mukai T, Spencer J & Makino M (2005) Identification of immunomodulating agent from *Mycobacterium leprae*. *Infect Immun* **73**: 2744–2750.

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

Makino M, Maeda Y & Ishii N (2005) Immunostimulatory activity of major membrane protein-II from *Mycobacterium leprae*. *Cell Immunol* **233**: 53–60.

Makino M, Maeda Y & Inagaki K (2006) Immunostimulatory activity of recombinant *Mycobacterium bovis* BCG that secretes Major Membrane Protein II of *Mycobacterium leprae*. *Infect Immun* **74**: 6264–6271.

Makino M, Maeda Y, Fukutomi Y & Mukai T (2007) Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages. *Microbes Infect* **9**: 70–77.

McDermott-Lancaster RD, Ito T, Kohsaka K, Guelpa-Lauras CC & Grosset JH (1987) Multiplication of *Mycobacterium leprae* in the nude mouse, and some applications of nude mice to experimental leprosy. *Int J Leprosy* **55**: 889–895.

Mochida-Nishimura K, Akagawa KS & Rich EA (2001) Interleukin-10 contributes development of macrophage suppressor activities by macrophage colony-stimulating factor, but not by granulocyte-macrophage colony-stimulating factor. *Cell Immunol* **214**: 81–88.

Moschella SL (2004) An update on the diagnosis and treatment of leprosy. *J Am Acad Dermatol* **51**: 417–426.

Murray PJ & Young RA (1999) Increased antimycobacterial immunity in interleukin-10-deficient mice. *Infect Immun* **67**: 3087–3095.

Nakata K, Akagawa KS, Fukayama M, Hayashi Y, Kadokura M & Tokunaga T (1991) Granulocyte-macrophage colony-stimulating factor promotes the proliferation of human alveolar macrophages *in vitro*. *J Immunol* **147**: 1266–1272.

Pessolani MC, Smith DR, Rivoire B, McCormick J, Hefta SA, Cole ST & Brennan PJ (1994) Purification, characterization, gene sequence, and significance of a bacterioferritin from *Mycobacterium leprae*. *J Exp Med* **180**: 319–327.

Ponnighaus JM, Fine PE, Sterne JA, Wilson RJ, Msosa E, Gruer PJ, Jenkins PA, Lucas SB, Liomba NG & Bliss L (1992) Efficacy of BCG vaccine against leprosy and tuberculosis in northern Malawi. *Lancet* **14**: 636–639.

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Randolph GJ, Inaba K, Robbiani DF, Steinman RM & Muller WA (1999) Differentiation of phagocytic monocytes into lymph node dendritic cells *in vivo*. *Immunity* **11**: 753–761.

Ridley DS & Jopling WH (1966) Classification of leprosy according to immunity. A five-group system. *Int J Leprosy* **34**: 255–273.

Setia MS, Steinmaus C, Ho CH & Rutherford GW (2006) The role of BCG in prevention of leprosy: a meta-analysis. *Lancet Infect Dis* **6**: 162–170.

Sieling PA, Jullien D, Dahlem M, Tedder TF, Rea TH, Modlin RL & Porcelli SA (1999) CD1 expression by dendritic cells in human leprosy lesions: correlation with effective host immunity. *J Immunol* **162**: 1851–1858.

Snapper SB, lugosi L, Jekkel A, Melton RE, Kieser T, Bloom BR & Jacobs WR Jr (1988) Lysogeny and transformation in mycobacteria: stable expression of foreign genes. *P Natl Acad Sci USA* **85**: 6987–6991.

Stoner GL (1979) Importance of the neural predilection of *Mycobacterium leprae* in leprosy. *Lancet* **2**: 994–996.

Verreck FA, de Boer T, Langenberg DM, Hoeve MA, Kramer M, Vaisberg E, Kastelein R, Kolk A, de Waal-Malefyt R & Ottenhoff TH (2004) Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *P Natl Acad Sci USA* **101**: 4560–4565.

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

Winau F, Weber S, Sad S, de Diego J, Hoops SL, Breiden B, Sandhoff K, Brinkmann V, Kaufmann SHE & Schaible UE (2006) Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. *Immunity* **24**: 105–117.

World Health Organization (2008) Global leprosy situation, 2008. *Wkly Epidemiol Rec* **83**: 293–300.

Yamamura M, Uyemura K, Deans RJ, Weinberg K, Rea TH, Bloom BR & Modlin RL (1991) Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* **254**: 277–279.

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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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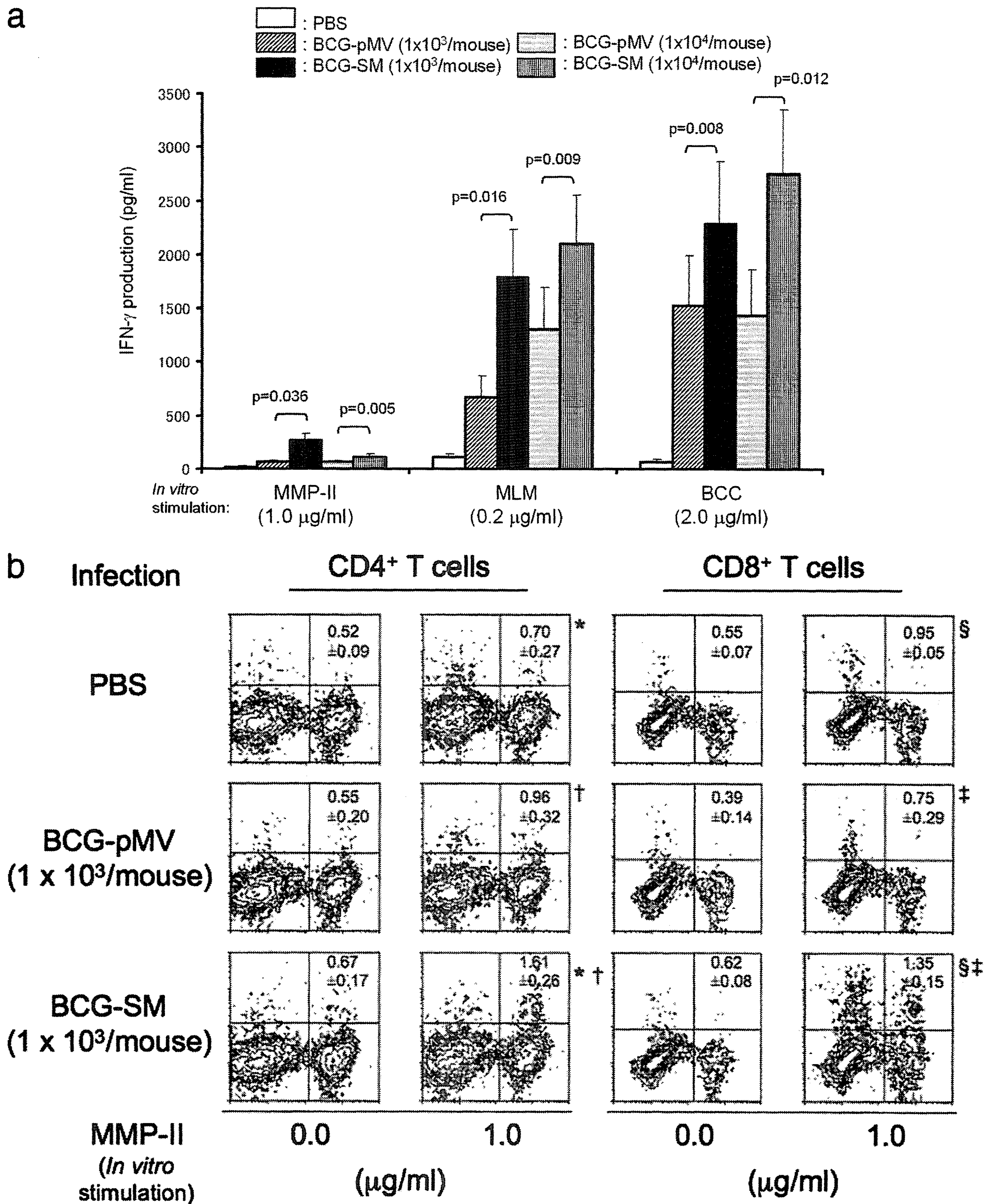


FIG. 1. (a) Production of T cells responsive to *M. leprae*-derived Ags by inoculation with recombinant BCG in mice. Five-week-old C57BL/6J mice were intradermally inoculated with the indicated dose of either BCG-pMV (the vector control BCG strain) or BCG-SM (an MMP-II-secreting BCG strain). Four weeks 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. *, $P < 0.0001$; †, $P < 0.005$; §, $P < 0.05$; ‡, $P < 0.05$.

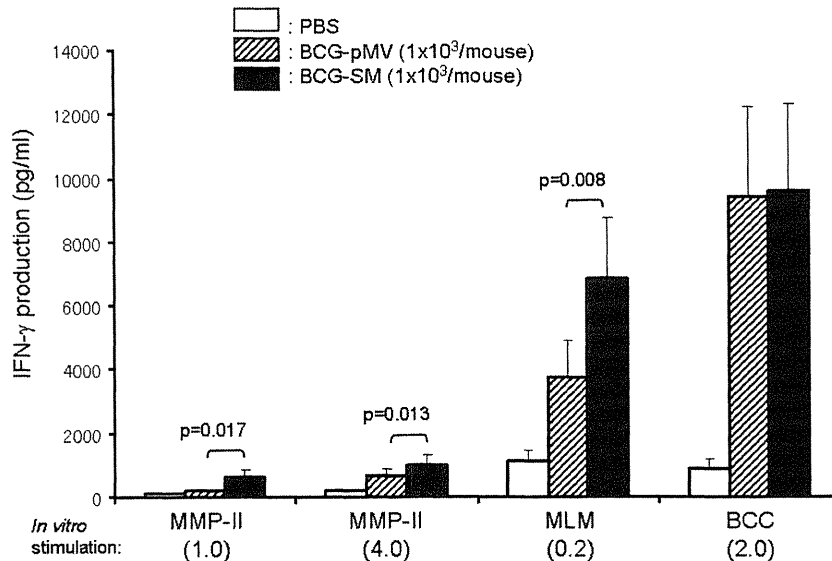


FIG. 2. Long-term effects of vaccination with recombinant BCG on the production of T cells responsive to *M. leprae*-derived Ags. C57BL/6J mice were intradermally infected with 1×10^3 CFU of either BCG-pMV or BCG-SM per mouse. Thirty-four weeks after the inoculation, splenocytes were stimulated with 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).

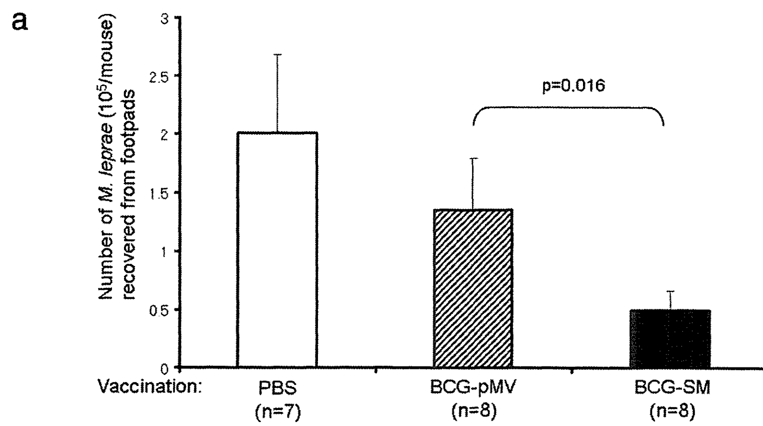
Animal studies. For inoculation into mice, recombinant BCG strains were cultured in Middlebrook 7H9 medium to the log phase of growth and were stored at 10^8 CFU/ml at −80°C. Before the aliquots were used for inoculation, the concentration of viable bacilli was determined by plating the bacilli on Middlebrook 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.

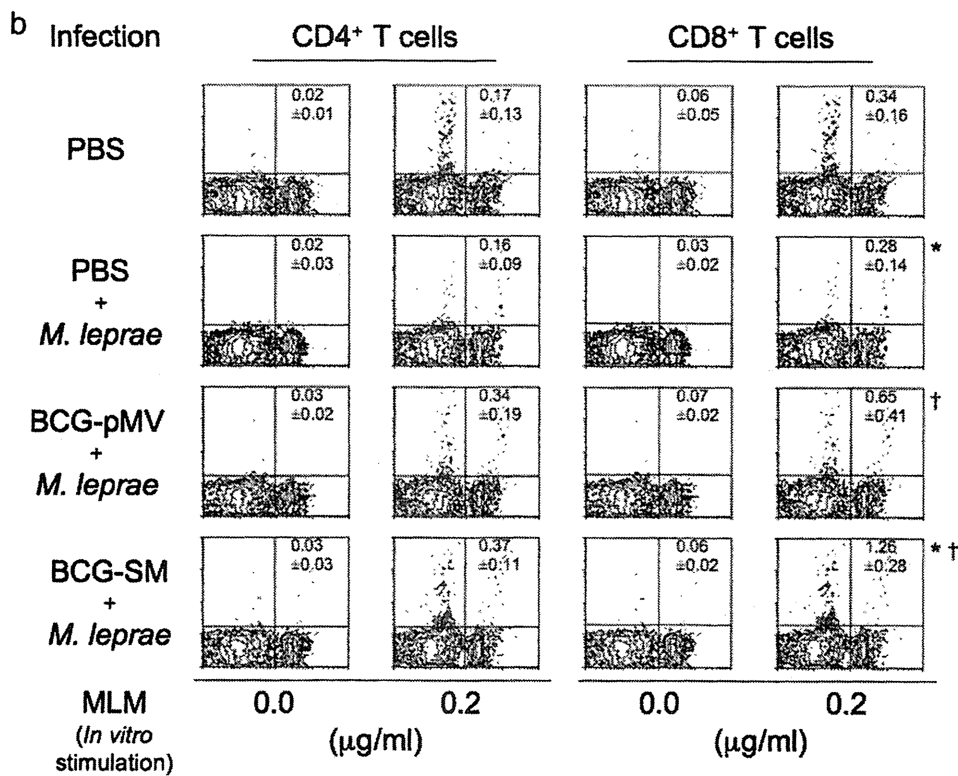
The splenocytes obtained from C57BL/6J mice infected with the recombinant BCG strains were also subjected to the identification of the T-cell subsets responsible for IFN-γ production. The level of intracellular production of IFN-γ by $CD4^+$ T cells and $CD8^+$ T cells, which were restimulated for 3 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.

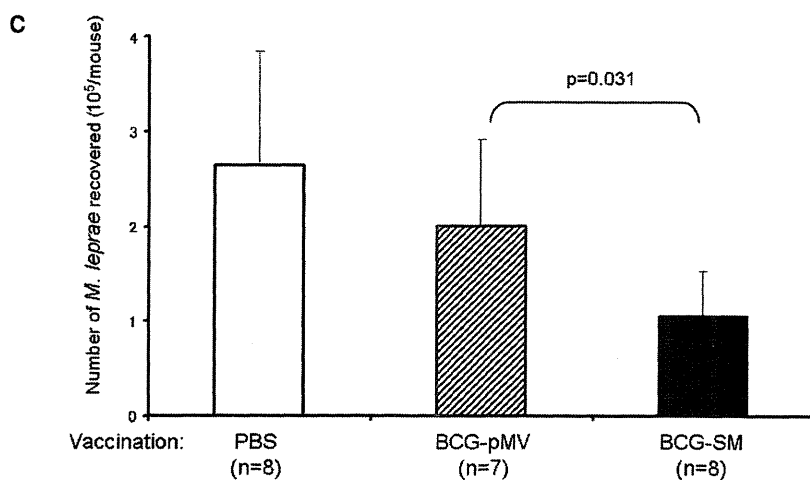
Statistical analysis. Student's *t* test and the Mann-Whitney-Wilcoxon test were used to determine statistical differences.

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
p=0.016
Vaccination:
PBS (n=7)
BCG-pMV (n=8)
BCG-SM (n=8)


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


c
Number of M. leprae recovered (10⁵/mouse)
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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REFERENCES

1. **Cho, S. N., and P. J. Brennan.** 1999. New biological tools for leprosy surveillance. Int. J. Lepr. Other Mycobact. Dis. **67:**59–62.
2. **Convit, J., C. Sampson, M. Zúñiga, P. G. Smith, J. Plata, J. Silva, J. Molina, M. E. Pinardi, B. R. Bloom, and A. Salgado.** 1992. Immunoprophylactic trial with combined *Mycobacterium leprae*/BCG vaccine against leprosy: preliminary results. Lancet **339:**446–450.
3. **Gelber, R. H., V. Mehra, B. Bloom, L. P. Murray, P. Siu, M. Tsang, and P. J. Brennan.** 1994. Vaccination with pure *Mycobacterium leprae* proteins inhibits *M. leprae* multiplication in mouse footpads. Infect. Immun. **62:**4250–4255.
4. **Gormus, B. J., G. B. Baskin, K. Xu, M. S. Ratterree, L. N. Martin, P. A. Mack, R. P. Bohm, Jr., W. M. Meyers, and G. P. Walsh.** 2000. Antileprosy protective vaccination of sooty mangabey monkeys with BCG or BCG plus heat-killed *Mycobacterium leprae*: immunologic observations. Int. J. Lepr. Other Mycobact. Dis. **68:**434–443.
5. **Hesseling, A. C., M. F. Cotton, C. Fordham von Reyn, S. M. Graham, R. P. Gie, and G. D. Hussey.** 2008. Consensus statement on the revised World Health Organization recommendations for BCG vaccination in HIV-infected infants. Int. J. Tuberc. Lung Dis. **12:**1376–1379.
6. **Hesseling, A. C., B. J. Marais, R. P. Gie, H. S. Schaaf, P. E. Fine, P. Godfrey-Faussett, and N. Beyers.** 2007. The risk of disseminated bacille Calmette-Guerin (BCG) disease in HIV-infected children. Vaccine **25:**14–18.
7. **Karonga Prevention Trial Group.** 1996. Randomised controlled trial of single BCG, repeated BCG, or combined BCG and killed *Mycobacterium leprae* vaccine for prevention of leprosy and tuberculosis in Malawi. Lancet **348:**17–24.
8. **Maeda, Y., T. Mukai, J. Spencer, and M. Makino.** 2005. Identification of an immunomodulating agent from *Mycobacterium leprae*. Infect. Immun. **73:**2744–2750.
9. **Makino, M., Y. Maeda, and K. Inagaki.** 2006. Immunostimulatory activity of recombinant *Mycobacterium bovis* BCG that secretes major membrane protein II of *Mycobacterium leprae*. Infect. Immun. **74:**6264–6271.
10. **McDermott-Lancaster, R. D., T. Ito, K. Kohsaka, C. C. Guelpa-Lauras, and J. H. Grosset.** 1987. Multiplication of *Mycobacterium leprae* in the nude mouse, and some applications of nude mice to experimental leprosy. Int. J. Lepr. Other Mycobact. Dis. **55:**889–895.
11. **Modlin, R. L.** 1994. Thl-Th2 paradigm: insights from leprosy. J. Investig. Dermatol. **102:**828–832.
12. **Nakamura, M.** 1994. Elimination of contaminants in a homogenate of nude-mouse footpad experimentally infected with *Mycobacterium leprae*. Jpn. J. Lepr. **64:**47–50.
13. **Ngamying, M., P. Sawanpanyalert, R. Butraporn, J. Nikasri, S. N. Cho, L. Levy, and P. J. Brennan.** 2003. Effect of vaccination with refined components of the organism on infection of mice with *Mycobacterium leprae*. Infect. Immun. **71:**1596–1598.
14. **Nomaguchi, H., T. Mukai, F. Takeshita, M. Matsuoka, Y. Maeda, T. M. Aye, N. Jahan, Y. Yogi, M. Endo, Y. Sato, and M. Makino.** 2002. Effect of *hsp65* DNA vaccination carrying immunostimulatory DNA sequences (CpG motifs) against *Mycobacterium leprae* multiplication in mice. Int. J. Lepr. Other Mycobact. Dis. **70:**182–190.
15. **Ohara, N., M. Matsuoka, H. Nomaguchi, M. Naito, and T. Yamada.** 2001. Protective responses against experimental *Mycobacterium leprae* infection in mice induced by recombinant bacillus Calmette-Guérin over-producing three putative protective antigen candidates. Vaccine **19:**1906–1910.
16. **Ottenhoff, T. H., and T. Mutis.** 1990. Specific killing of cytotoxic T cells and antigen-presenting cells by $CD4^+$ cytotoxic T cell clones. A novel potentially immunoregulatory T-T cell interaction in man. J. Exp. Med. **171:**2011–2024.
17. **Pönnighaus, J. M., P. E. Fine, J. A. Sterne, R. J. Wilson, E. Msosa, P. J. Gruer, P. A. Jenkins, S. B. Lucas, N. G. Liomba, and L. Bliss.** 1992. Efficacy of BCG vaccine against leprosy and tuberculosis in northern Malawi. Lancet **339:**636–639.
18. **Salazar, L., H. Fsihi, E. de Rossi, G. Riccardi, C. Rios, S. T. Cole, and H. E. Takiff.** 1996. Organization of the origins of replication of the chromosomes of *Mycobacterium smegmatis*, *Mycobacterium leprae* and *Mycobacterium tuberculosis* and isolation of a functional origin from *M. smegmatis*. Mol. Microbiol. **20:**283–293.
19. **Setia, M. S., C. Steinmaus, C. S. Ho, and G. W. Rutherford.** 2006. The role of BCG in prevention of leprosy: a meta-analysis. Lancet Infect. Dis. **6:**162–170.
20. **Sharma, P., R. Mukherjee, G. P. Talwar, K. G. Sarathchandra, R. Walia, S. K. Parida, R. M. Pandey, R. Rani, H. Kar, A. Mukherjee, K. Katoch, S. K. Benara, T. Singh, and P. Singh.** 2005. Immunoprophylactic effects of the anti-leprosy Mw vaccine in household contacts of leprosy patients: clinical field trials with a follow up of 8–10 years. Lepr. Rev. **76:**127–143.
21. **Shepard, C. C., and D. H. McRae.** 1968. A method for counting acid-fast bacteria. Int. J. Lepr. Other Mycobact. Dis. **36:**78–82.
22. **Shepard, C. C.** 1983. Animal vaccination studies with *Mycobacterium leprae*. Int. J. Lepr. Other Mycobact. Dis. **51:**519–523.
23. **Trunz, B. B., P. Fine, and C. Dye.** 2006. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet **367:**1173–1180.
24. **Walia, R., K. G. Sarathchandra, R. M. Pandey, S. K. Parida, S. A. Zaheer, H. K. Kar, A. Mukherjee, R. Mukherjee, and G. P. Talwar.** 1993. Field trials on the use of Mycobacterium w vaccine in conjunction with multidrug therapy in leprosy patients for immunotherapeutic and immunoprophylactic purposes. Lepr. Rev. **64:**302–311.
25. **World Health Organization.** 2008. Global leprosy situation, beginning of 2008. Wkly. Epidemiol. Rec. **33:**293–300.
26. **Zodpey, S. P.** 2007. Protective effect of bacillus Calmette Guerin (BCG) vaccine in the prevention of leprosy: a meta-analysis. Indian J. Dermatol. Venereol. Leprol. **73:**86–93.

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

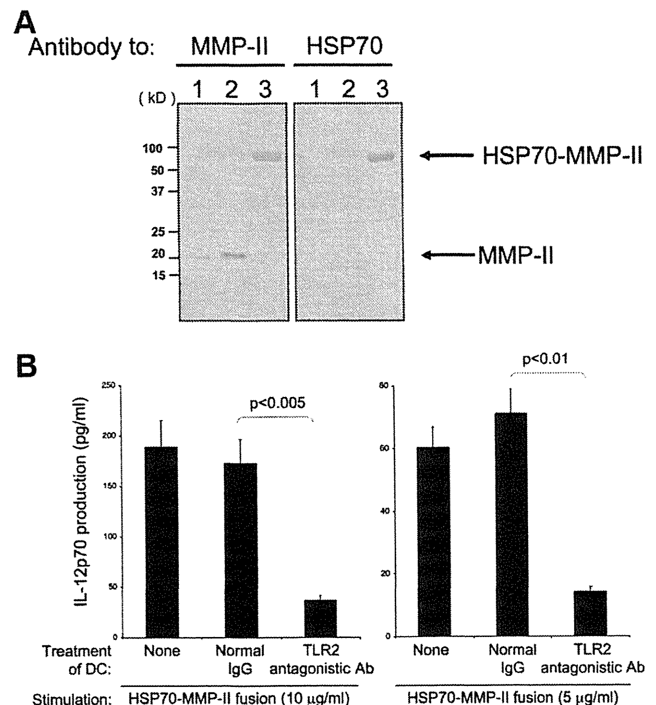

FIGURE 1. *A*, Western blotting analysis of protein secreted from BCG-70M. A mAb to either MMP-II or HSP70 was used to detect HSP70-MMP-II fusion protein. *Lane 1*, Culture filtrates of BCG-261H. *Lane 2*, rMMP-II protein. *Lane 3*, Culture filtrates of BCG-70M. *B*, Contribution of TLR2 to IL-12p70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

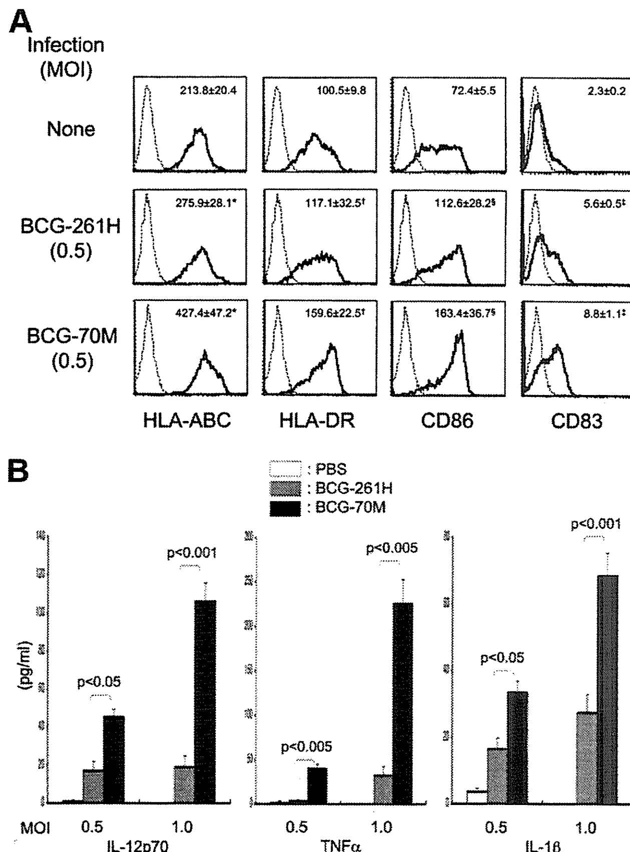

FIGURE 2. *A*, Up-regulated expression of APC-associated molecules on DC by infection with BCG-70M. PBMCs were obtained from one donor. Monocyte-derived immature DC were infected with either BCG-261H or BCG-70M at a MOI of 0.5 and cultured for another 2 days in the presence of rGM-CSF and rIL-4. The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

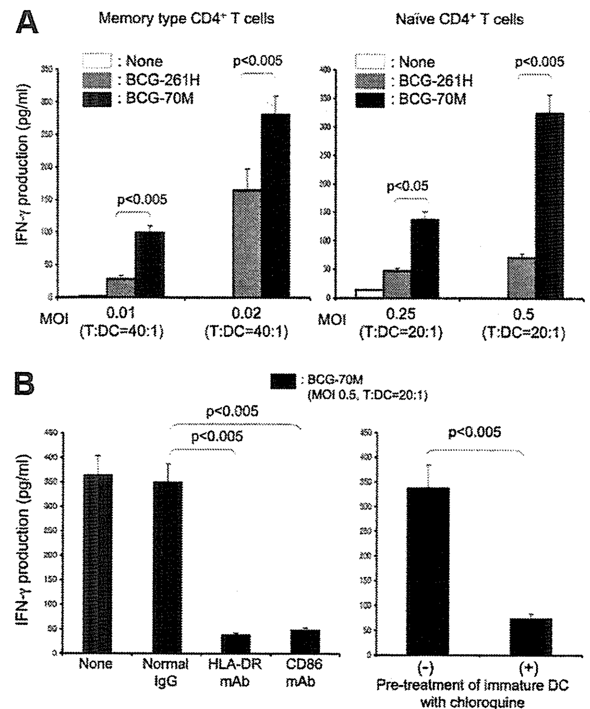

FIGURE 3. *A*, IFN-γ production from $CD4^+$ T cells by stimulation with BCG. PBMCs were obtained from one donor. Monocyte-derived DC were infected with either BCG-261H or BCG-70M at the indicated MOI, and used as a stimulator of memory-type or naive $CD4^+$ T cells in a 4-day culture. Responder $CD4^+$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