研究成果の刊行に関する一覧表

別紙 5-1

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M. Makino, Y. Maeda, M. Kai, T. Tamura, T. Mukai.	GM-CSF-mediated T-cell activation by macrophages infected with recombinant BCG that secretes major membrane protein-II of *Mycobacterium leprae*.	FEMS Immunol. Med. Microbiol.	55	39-46	2009
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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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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).

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,

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 μg 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 μg 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 $CD4^+$

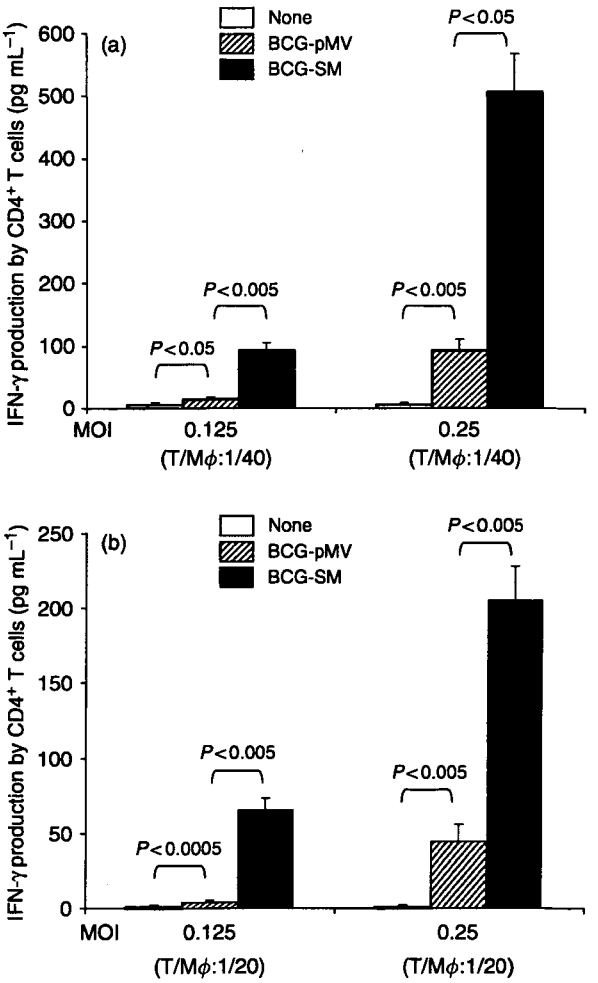

Fig. 1. Production of IFN-γ by $CD4^+$ T cells. (a) GM-Mϕ, differentiated by 3 days of culture with rGM-CSF from monocytes, were infected with BCG-pMV (vector control BCG) or BCG-SM (rBCG that secretes MMP-II) at the indicated MOI, and cultured for another 2 days. These GM-Mϕ were used as a stimulator 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.

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 μg 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.

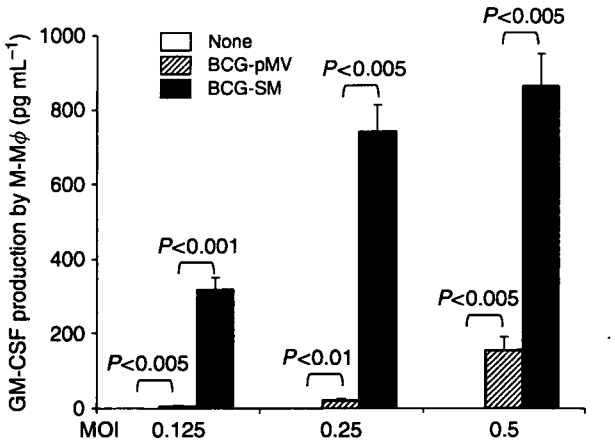

Fig. 2. Production of GM-CSF by M-Mϕ. M-Mϕ differentiated by 5 days of culture with rM-CSF from monocytes, were stimulated with BCG-pMV or BCG-SM for 24 h at the indicated MOI. A representative experiment based three separate experiments conducted using three separate PPD-positive individuals is shown.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

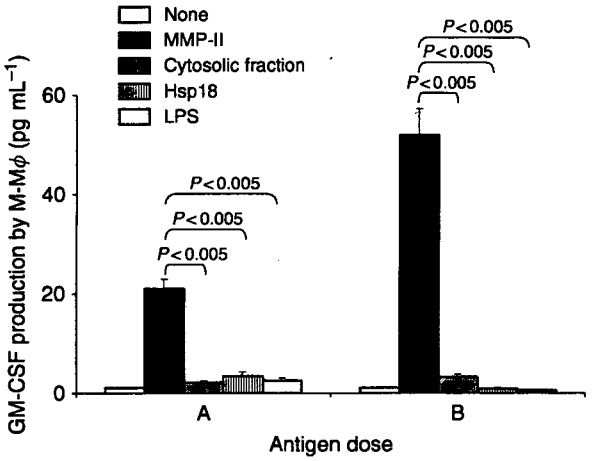

Fig. 3. GM-CSF production by M-Mϕ. M-Mϕ obtained after 5 days of culture with rM-CSF were stimulated for 24 h with rMMP-II, the BCG-derived cytosolic fraction or *Mycobacterium leprae*-derived hsp18 antigen [dose of antigen in (a) 5 μg mL^{-1} and that in (b) 10 μg mL^{-1}]. Lipopolysaccharide, assumed 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

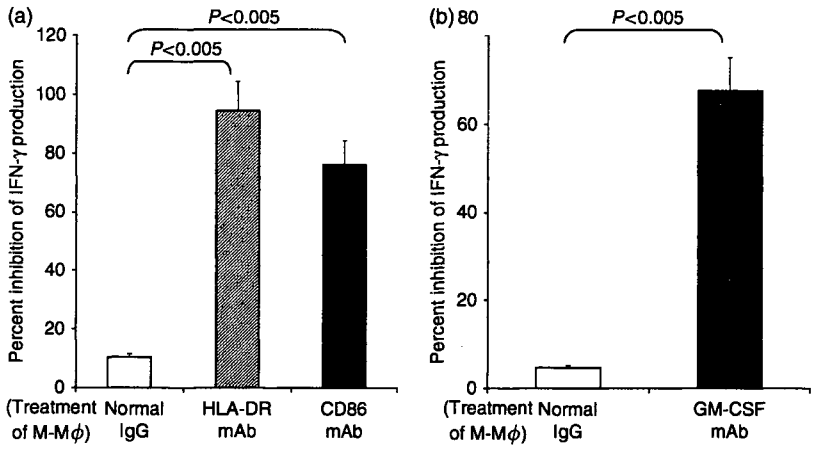

Fig. 4. (a) Inhibition of IFN-γ production by $CD4^+$ T cells by pretreatment of BCG-SM-infected M-Mϕ with mAb to HLA-DR or CD86. M-Mϕ differentiated from monocytes using rM-CSF were infected with BCG-SM at an MOI of 0.25 on day 5 of culture and cultured for another 2 days. The BCG-SM-infected M-Mϕ were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

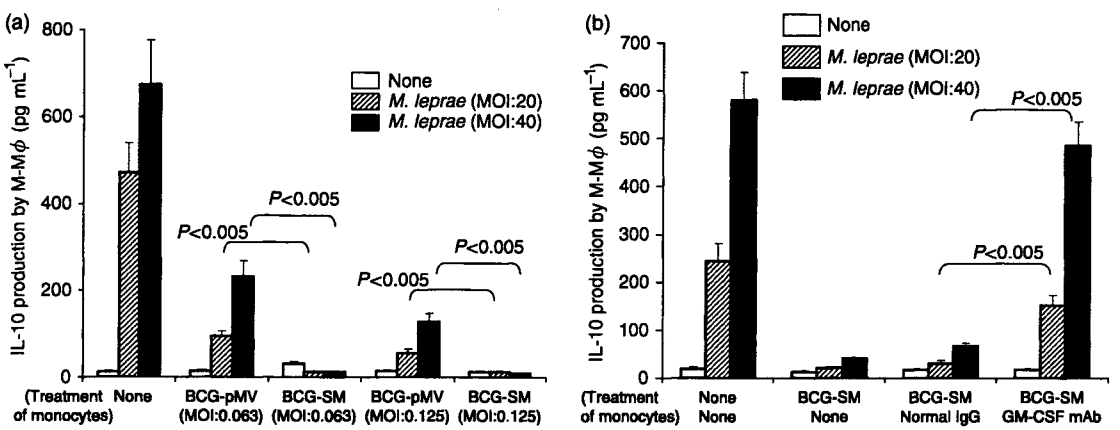

Fig. 5. (a) Production of IL-10 by M-Mϕ. Monocytes were infected with the indicated dose of BCG-pMV or BCG-SM, and subsequently differentiated into M-Mϕ by culturing for 5 days in the presence of M-CSF and rBCGs. These rBCG-preinfected M-Mϕ were stimulated with *Mycobacterium leprae* at the indicated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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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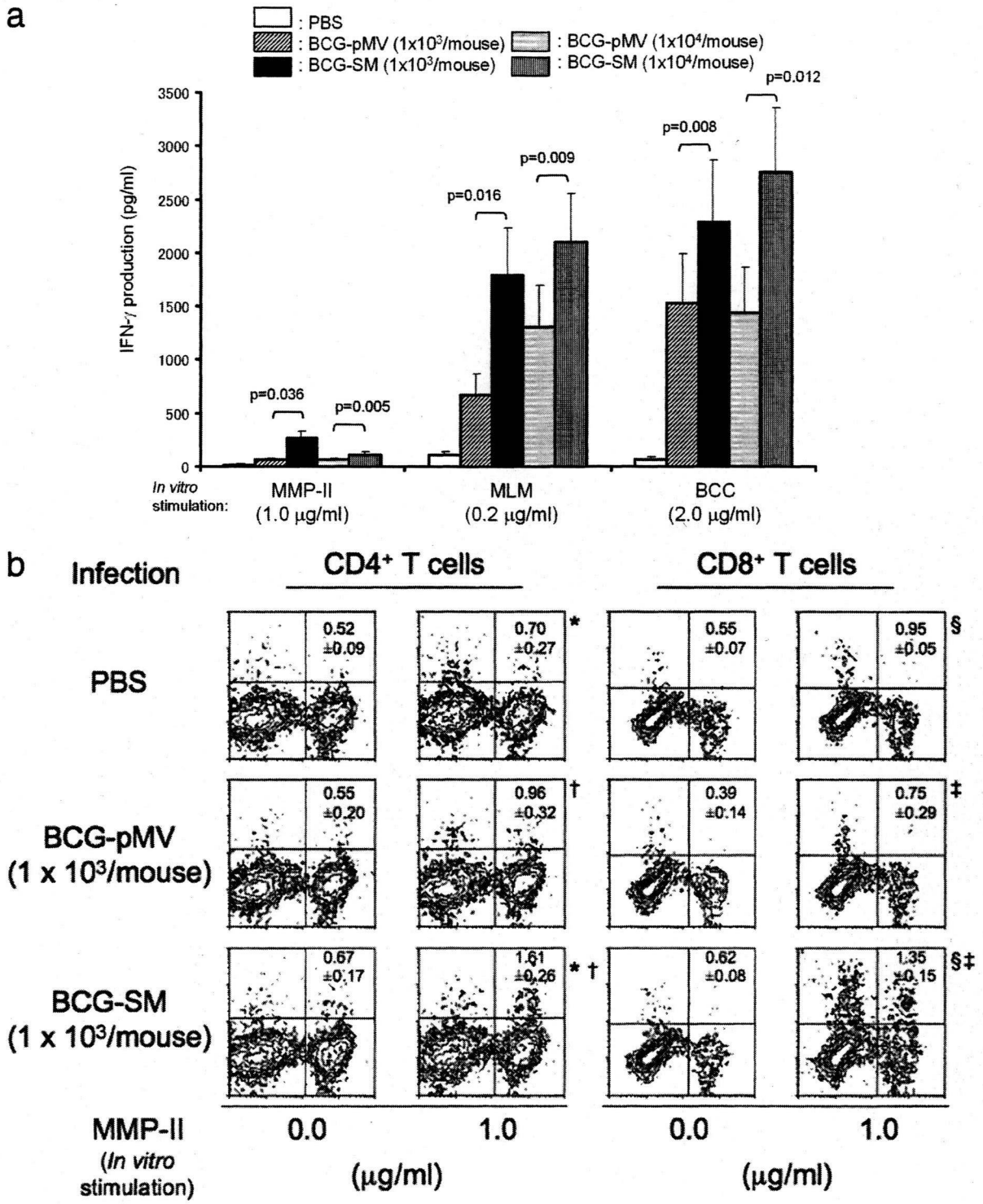


FIG. 1. (a) Production of T cells responsive to *M. leprae*-derived Ags by inoculation with recombinant BCG in mice. Five-week-old C57BL/6J mice were intradermally inoculated with the indicated dose of either BCG-pMV (the vector control BCG strain) or BCG-SM (an MMP-II-secreting BCG strain). Four weeks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

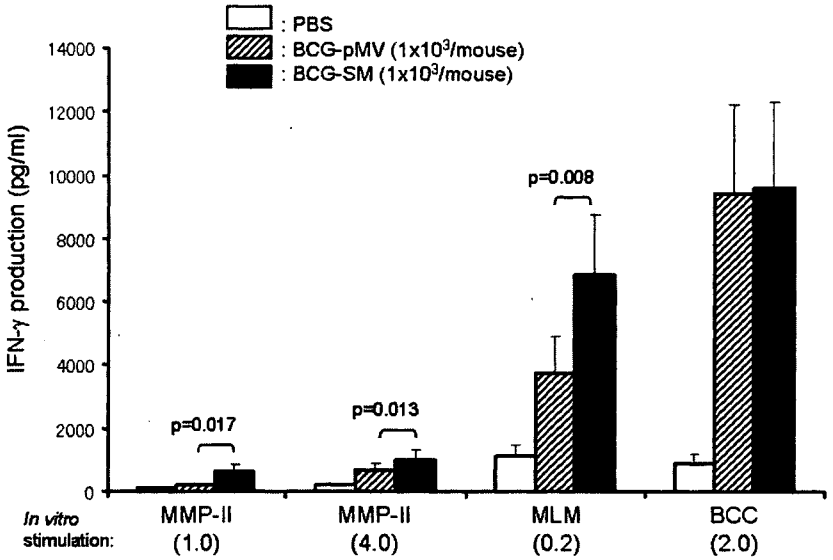


FIG. 2. Long-term effects of vaccination with recombinant BCG on the production of T cells responsive to *M. leprae*-derived Ags. C57BL/6J mice were intradermally infected with 1×10^3 CFU of either BCG-pMV or BCG-SM per mouse. Thirty-four weeks after the inoculation, splenocytes were stimulated with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

MATERIALS AND METHODS

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

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

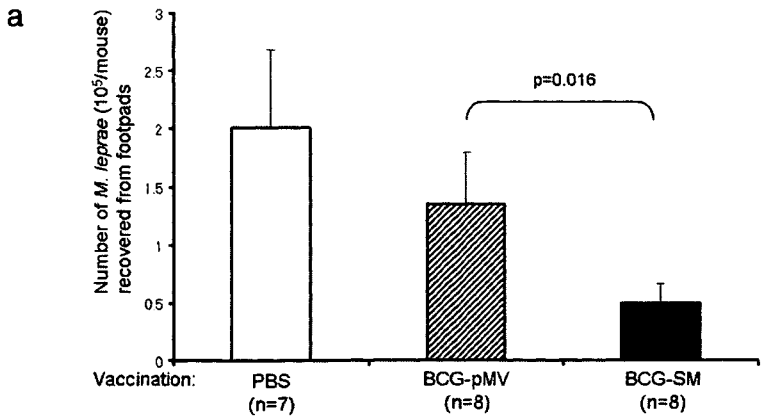
Animal studies. For inoculation into mice, recombinant BCG strains were cultured in Middlebrook 7H9 medium to the log phase of growth and were stored at 10^8 CFU/ml at −80°C. Before the aliquots were used for inoculation, the concentration of viable bacilli was determined by plating the bacilli on Middlebrook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

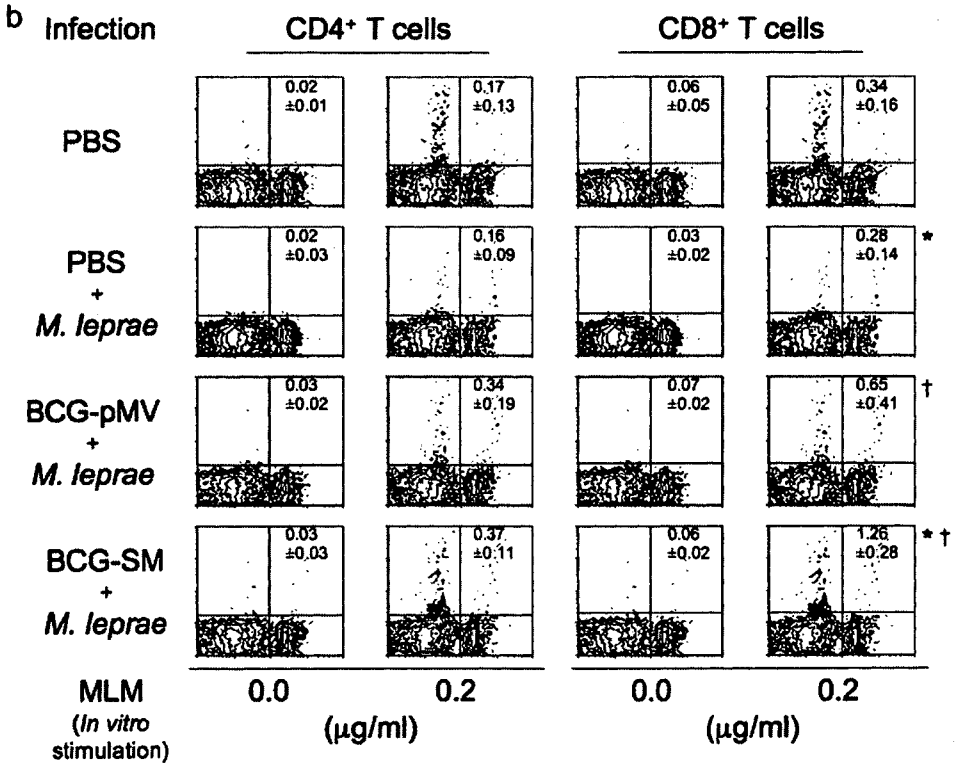
The splenocytes obtained from C57BL/6J mice infected with the recombinant BCG strains were also subjected to the identification of the T-cell subsets responsible for IFN-γ production. The level of intracellular production of IFN-γ by $CD4^+$ T cells and $CD8^+$ T cells, which were restimulated for 3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

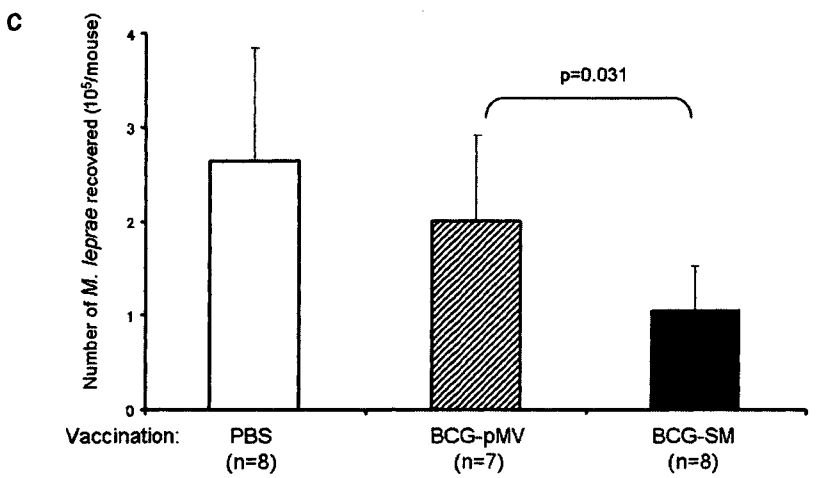
Statistical analysis. Student's *t* test and the Mann-Whitney-Wilcoxon test were used to determine statistical differences.

RESULTS

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


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


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


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

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

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

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

DISCUSSION

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

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

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

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

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

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