higher level of IL-12p40, IL-1β, TNFα and GM-CSF than that with BCG-261H at both MOIs: 0.25 and 0.50 (Figure 2c).

Activation of naïve and memory type T cells by BCG-dHCM

The ability of BCG-dHCM to activate naïve $CD4^+$ T cells through DC was evaluated using BCG-261H as a control (Figure 3). BCG-dHCM more efficiently activated naïve $CD4^+$ T cells than BCG-261H to produce IFN-γ (Figure 3a). The IFN-γ production from naïve $CD4^+$ T cells by BCG-dHCM stimulation seems to be induced by an Ag-specific manner, since the treatment of the surface of BCG-dHCM-infected DC with mAb to HLA-DR or CD86 showed 90% inhibition (Figure 3b). Further, pretreatment of immature DC with chloroquine prior to infection with BCG-dHCM, inhibited the production of IFN-γ from naïve $CD4^+$ T cells (Figure 3c). These results suggest that the phagosomal maturation is induced by BCG-dHCM infection and is closely associated with the activation of naïve $CD4^+$ T cells. Previously, we reported that only when the urease-deficient rBCG, but not normal BCG, was used as a host BCG and introduced with genes encoding antigenic molecules, the rBCG could activate memory type $CD4^+$ T cells through M-CSF-dependent macrophages [27]. We checked whether BCG-dHCM activates memory type $CD4^+$ T cells, and found that BCG-dHCM induced the significant production of IFN-γ from the responder population (Figure 4a). Again, the activation of memory type $CD4^+$ T cells by BCG-dHCM-infected macrophages seems to be dependent on the expression of MHC class II and CD86 Ags, since the treatment of rBCG-infected macrophages with mAb to HLA-DR or CD86 inhibited the IFN-γ production significantly (Figure 4b). Further, the treatment of macrophages with chloroquine prior to BCG-dHCM infection, inhibited IFN-γ production by memory type $CD4^+$ T cells (Figure 4c). Next, we assessed whether BCG-dHCM could activate naïve $CD8^+$ T cells when DC were enrolled as APC (Figure 5). While vector control BCG did not induce the activation of naïve $CD8^+$ T cells as reported [19,20], BCG-dHCM induced significant level of activation of naïve $CD8^+$ T cells (Figure 5a). A significant concentration of IFN-γ can be released from naïve $CD8^+$ T cells. Also the mAb treatment of BCG-dHCM-infected DC with anti-HLA-ABC Ab or anti-CD86 Ab significantly inhibited the IFN-γ production from naïve $CD8^+$ T cells (Figure 5b), and pre-treatment of immature DC with chloroquine, again, inhibited the cytokine production from the responder (Figure 5c). These results suggest that BCG-dHCM can activate not only naïve $CD4^+$ T cells, but also naïve $CD8^+$ T cells through DC, and also activate memory $CD4^+$ T cells through macrophages in an Ag-dependent manner. Further, the phagosomal maturation is closely associated with the T cell activation.

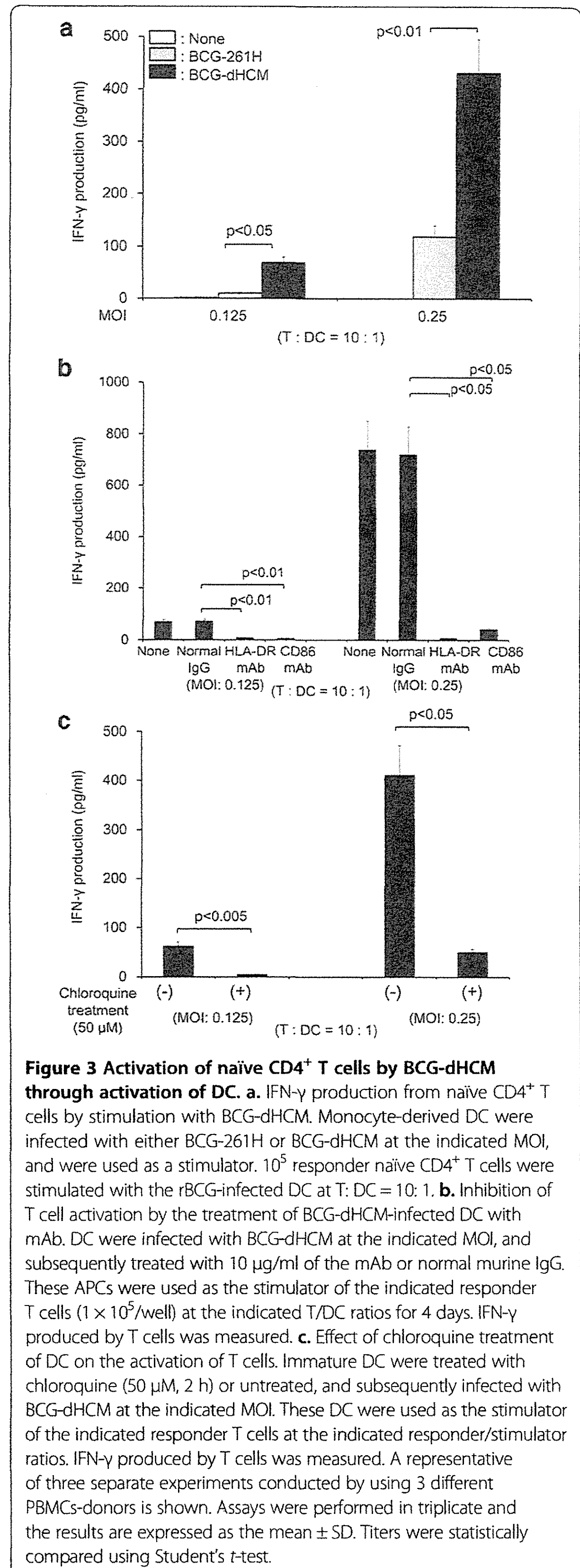

Figure 3 Activation of naïve $CD4^+$ T cells by BCG-dHCM through activation of DC. a. IFN-γ production from naïve $CD4^+$ T cells by stimulation with BCG-dHCM. Monocyte-derived DC were infected with either BCG-261H or BCG-dHCM at the indicated MOI, and were used as a stimulator. 10^5 responder naïve $CD4^+$ T cells were stimulated with the rBCG-infected DC at T: DC = 10: 1. **b.** Inhibition of T cell activation by the treatment of BCG-dHCM-infected DC with mAb. DC were infected with BCG-dHCM at the indicated MOI, and subsequently treated with 10 μg/ml of the mAb or normal murine IgG. These APCs were used as the stimulator of the indicated responder T cells (1×10^5/well) at the indicated T/DC ratios for 4 days. IFN-γ produced by T cells was measured. **c.** Effect of chloroquine treatment of DC on the activation of T cells. Immature DC were treated with chloroquine (50 μM, 2 h) or untreated, and subsequently infected with BCG-dHCM at the indicated MOI. These DC were used as the stimulator of the indicated responder T cells at the indicated responder/stimulator ratios. IFN-γ produced by T cells was measured. A representative of three separate experiments conducted by using 3 different PBMCs-donors is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t*-test.

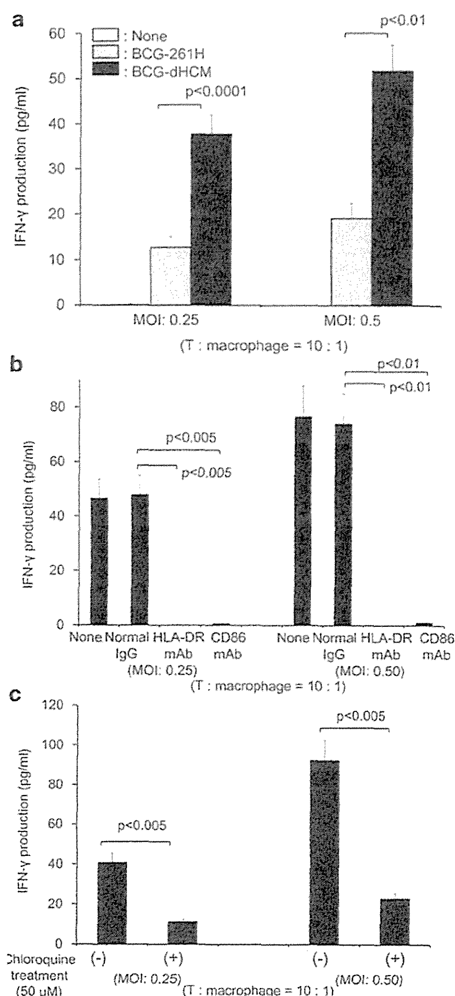

Figure 4 Activation of memory-type CD4^{+} T cells by BCG-dHCM through activation of macrophages. a. IFN-γ production from memory type CD4^{+} T cells by stimulation with BCG-dHCM. Macrophages differentiated by using M-CSF were infected with either BCG-261H or BCG-dHCM at the indicated MOI, and were used as a stimulator of responder CD4^{+} T cells in a 4-day culture. 10^5 responder T cells were stimulated with the rBCG-infected macrophages at T: macrophage = 10: 1. **b.** Inhibition of T cell activation by the treatment of BCG-dHCM-infected macrophages with mAb. Macrophages were infected with BCG-dHCM at the indicated MOI, and subsequently treated with 10 μg/ml of the mAb or normal murine IgG. These APCs were used as the stimulator of the indicated responder T cells (1 x 10^5/well) at the indicated T/macrophages ratios for 4 days. IFN-γ produced by T cells was measured. **c.** Effect of chloroquine treatment of macrophages on the activation of T cells. Macrophages were treated with chloroquine (50 μM, 2 h) or untreated, and subsequently infected with BCG-dHCM at the indicated MOI. These macrophages were used as the stimulator of the indicated responder T cells at the indicated responder/stimulator ratios. IFN-γ produced by T cells was measured. A representative of three separate experiments conducted by using 3 different PBMCs-donors is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t*-test.

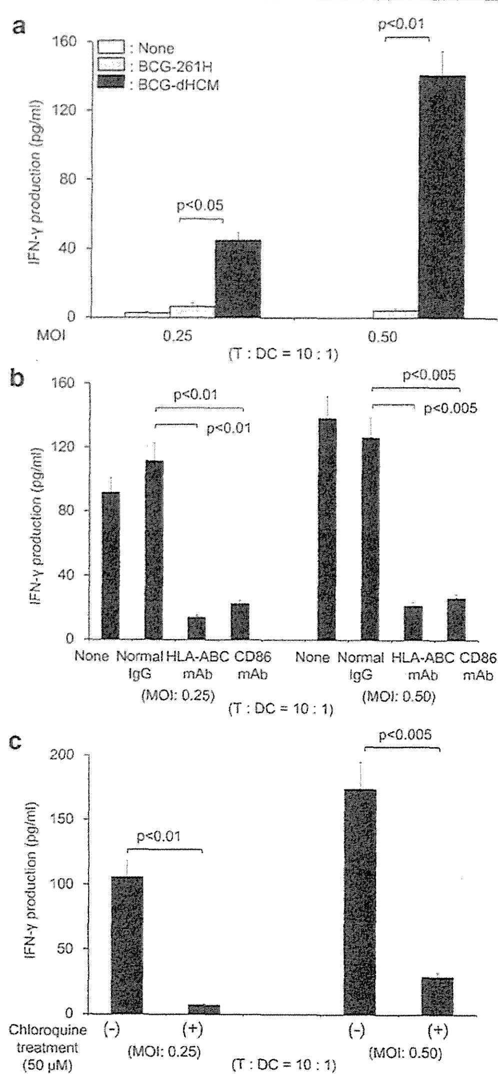

Figure 5 Activation of naive CD8^{+} T cells by BCG-dHCM through activation of DC. a. IFN-γ production from naïve CD8^{+} T cells by stimulation with BCG-dHCM. DC were infected with either BCG-261H or BCG-dHCM at the indicated MOI, and were used as a stimulator. 10^5 responder T cells were stimulated for 4 days with rBCG-infected DC at T: DC = 10: 1. **b.** Inhibition of T cell activation by the treatment of BCG-dHCM-infected DC with mAb. DC were infected with BCG-dHCM at the indicated MOI, and subsequently treated with 10 μg/ml of the mAb or normal murine IgG. These APCs were used as the stimulator of the indicated responder T cells (1 x 10^5/well) at the indicated T/DC ratios for 4 days. IFN-γ produced by T cells was measured. **c.** Effect of chloroquine treatment of DC on the activation of T cells. Immature DC were treated with chloroquine (50 μM, 2 h) or untreated, and subsequently infected with BCG-dHCM at the indicated MOI. These DC were used as the stimulator of the indicated responder T cells at the indicated responder/stimulator ratios. IFN-γ produced by T cells was measured. A representative of three separate experiments conducted by using 3 different PBMCs-donors is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titers were statistically compared using Student's *t*-test.

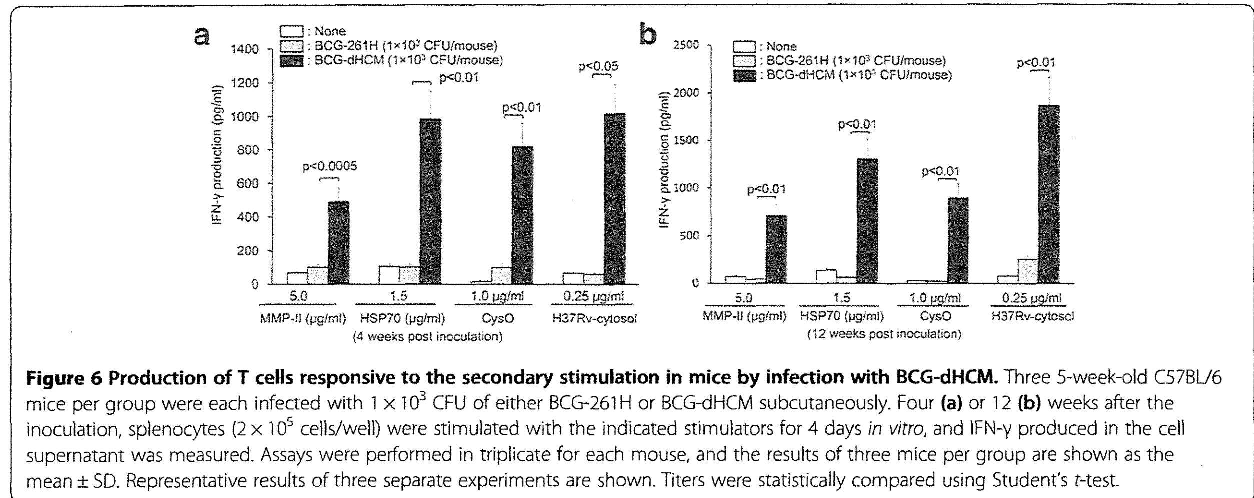

Figure 6 Production of T cells responsive to the secondary stimulation in mice by infection with BCG-dHCM. Three 5-week-old C57BL/6 mice per group were each infected with 1×10^3 CFU of either BCG-261H or BCG-dHCM subcutaneously. Four **(a)** or 12 **(b)** weeks after the inoculation, splenocytes (2×10^5 cells/well) were stimulated with the indicated stimulators for 4 days *in vitro*, and IFN-γ produced in the cell supernatant was measured. Assays were performed in triplicate for each mouse, and the results of three mice per group are shown as the mean ± SD. Representative results of three separate experiments are shown. Titers were statistically compared using Student's *t*-test.

Production of T cells responsive to the secondary stimulation by BCG-dHCM *in vivo*

The ability of BCG-dHCM to produce T cells highly responsive to the secondary *in vitro* stimulation was examined by *in vivo* functional studies (Figure 6). C57BL/6 mice were subcutaneously inoculated with 1×10^3 CFU/mouse of either BCG-261H or BCG-dHCM 4 weeks before stimulation *in vitro* (Figure 6a). Not only MMP-II, HSP70 and CysO of which encoding genes were introduced into BCG-dHCM, but also H37Rv-derived cytosolic protein were used as a secondary *in vitro* stimulator. The T cells from mice inoculated with BCG-dHCM respond more vigorously to all stimulators and produced higher concentration of IFN-γ (Figure 6a) and IL-2 (not shown) than T cells from mice uninfected or infected with BCG-261H. To examine the long-term effect of the inoculation of BCG-dHCM on the production of responsive T cells, C57BL/6 mice were subcutaneously inoculated with 1×10^3 CFU/mouse of either BCG-261H or BCG-dHCM 12 weeks before restimulation *in vitro* (Figure 6b). Again, a significantly higher concentration of IFN-γ was produced from splenic T cells obtained from mice inoculated with BCG-dHCM by all stimulators than those from mice uninfected or infected with BCG-261H.

Effect of BCG-dHCM vaccination on the multiplication of aerosol challenged *M. tuberculosis*

C57BL/6 mice vaccinated with either BCG-261H or BCG-dHCM (1×10^3 CFU/mouse) for 6 weeks were challenged with 100 CFU per lungs of H37Rv by aerosol infection. Six weeks later, the MTB recovered from both lungs and spleen was enumerated (Figure 7). While the mice vaccinated with BCG-261H minimally inhibit the multiplication of MTB, the vaccination with BCG-dHCM significantly inhibited the growth of H37Rv in lungs (Figure 7a). Similarly, the multiplication of MTB in spleen is significantly inhibited by the vaccination with BCG-dHCM (Figure 7b).

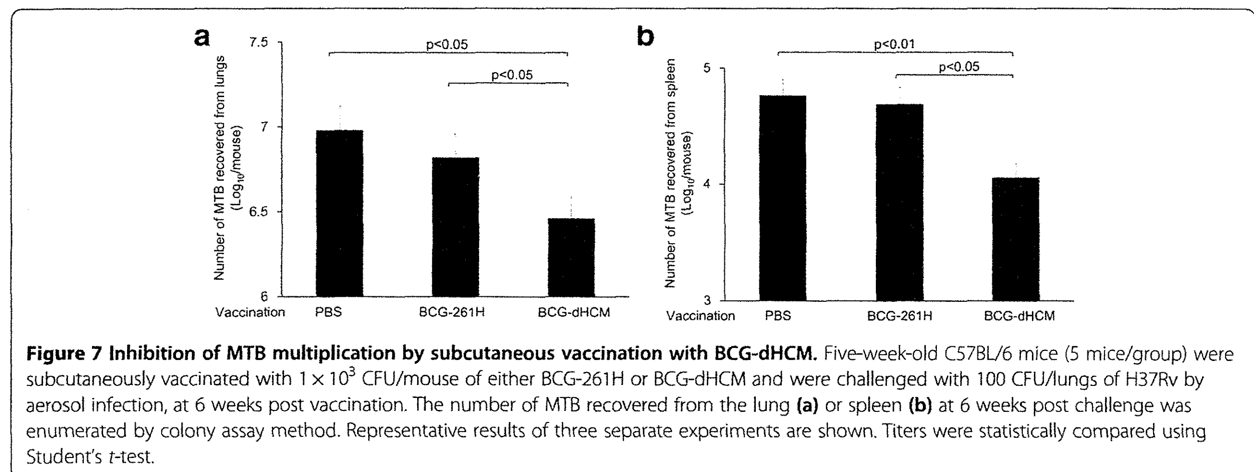

Figure 7 Inhibition of MTB multiplication by subcutaneous vaccination with BCG-dHCM. Five-week-old C57BL/6 mice (5 mice/group) were subcutaneously vaccinated with 1×10^3 CFU/mouse of either BCG-261H or BCG-dHCM and were challenged with 100 CFU/lungs of H37Rv by aerosol infection, at 6 weeks post vaccination. The number of MTB recovered from the lung **(a)** or spleen **(b)** at 6 weeks post challenge was enumerated by colony assay method. Representative results of three separate experiments are shown. Titers were statistically compared using Student's *t*-test.

Discussion

IFN-γ produced by activated type 1 $CD4^+$ T cells and $CD8^+$ T cells has an essential function in both antimicrobial activity against MTB and limitation of lung inflammation associated with massive accumulation of neutrophils which are recruited by Th17 cells [7-9,46]. Therefore, one of the important aims of vaccination is to produce memory type $CD4^+$ T cells as well as memory type $CD8^+$ T cells capable of producing abundant dose of IFN-γ by responding rapidly to MTB-infected DC and macrophages. From the studies using T cell receptor-transgenic animal model, it is known that it needs several weeks for host T cells to start inhibiting the multiplication of aerosol infected MTB in primary mice [5,6]. It is unknown whether alveolar APCs could be primed by vaccination, so that time required for APC to move to the regional LN after phagocytosing MTB in lung could be reduced. However, it could certainly reduce the time necessary for T cells to be activated by the interaction with MTB-infected APCs in the LN by differentiating naïve T cells into memory subset by vaccination. BCG has been used widely as a vaccine against tuberculosis, but its effect is quite limited. BCG can only prevent the development of miliary tuberculosis and tuberculosis meningitis in child, but is not effective for prevention of adult lung tuberculosis [15]. However, BCG has many antigenic molecules common to that present in MTB, and moreover, the safety of BCG is well established [16,17]. Thus, we are of the opinion that the improvement of BCG by producing rBCGs would be the fastest route to produce more reliable single injection vaccine against tuberculosis. In this respect, rBCG should have some antigenic molecules which are present in MTB, and also should highly activate not only naïve T cells of both CD4 and CD8 subsets, but also APCs including DC and macrophages. Since both DC and macrophages express MMP-II-related peptide on their surface upon an infection with both H37Ra and H37Rv [31], and also MMP-II can ligate TLR2 and consequently activates NF-κB pathway of APCs [28-31], MMP-II is considered to be good target that could be used as an active vaccinating agent. In this study, we produced new rBCG termed BCG-dHCM, that is a urease-deficient rBCG that secrete the fusion protein composed of HSP70, CysO and MMP-II. In the production of BCG-dHCM, we used MMP-II as a central component, HSP70 as an adjuvant, and CysO as an element necessary to release Ag in APCs. BCG-dHCM secreted the fusion protein in both lysosome in which abundant enzyme is available and in phagosomes of BCG-susceptible cells. Thus BCG-dHCM strongly activated both subsets of naïve T cells and APCs. The efficient activation of APCs by BCG-dHCM is revealed through up-regulation of APC-associated molecules and by enhanced production of various proinflammatory cytokines including IL-12, IL-1β and TNFα from DC and macrophages. The efficient activation of these APCs can be assumed to be directly linked with the effective activation of adaptive immunities. Actually macrophages-infected with BCG-dHCM activated memory type $CD4^+$ T cells. Previous rBCG termed BCG-70M that is BCG introduced with HSP70-MMP-II fusion gene failed to activate memory type T cells through macrophages [32], thus, the high immunostimulatory function of BCG-dHCM seems to be owing to the high antigenic load on the surface of BCG-dHCM-infected macrophages, that is due to efficient translocation of BCG-dHCM into lysosome. The effective memory T cells are ones capable of responding to the molecules expressed on the surface of MTB-infected APCs, and are produced by activating naïve T cells in an Ag-specific manner [27,31]. In this respect, the activation of both naïve $CD4^+$ T cells and naïve $CD8^+$ T cells by BCG-dHCM was dependent on MHC and CD86 molecules expressed on APCs. Thus, BCG-dHCM seems to be stimulating naïve T cells in an Ag-specific fashion. Presumably, APCs infected MTB express various epitopes on their surface, indicating that the presence of clonal diversity of T cells, might provide better control of MTB. Subcutaneous inoculation of BCG-dHCM into C57BL/6 mice produced long lasting T cells responsive to *in vitro* secondary stimulation. Upon stimulation with not only MMP-II, CysO and HSP70, but also H37Rv-derived cytosolic (Figure 6) and membrane (not shown) protein, T cells obtained from mice inoculated with BCG-dHCM were efficiently reactivated and produced high concentration of IFN-γ. Although the exact reason why these T cells were capable of responding to MTB components is not clear, it may be reasonable to speculate that the translocation of BCG-dHCM into lysosome induced the degradation of BCG that leads to production of epitope derived from BCG itself. CysO is engaged in alternative cysteine biosynthesis pathway, which plays an essential role for the MTB survival in the oxidative condition [33,34]. Also, CysO is categorized in ubiquitin superfamily and is possible to direct protein towards proteosome degradation pathway [37]. In fact, in this study, we found that rCysO, produced by using *M. smegmatis* in a LPS-free condition, activated both innate and adaptive cellular responses, since it induced some cytokine production from APCs and phenotypic changes in DC, and also stimulated IFN-γ production from both subsets of T cells. When we introduced *CysO* gene into urease-deficient rBCG accompanying with HSP70-MMP-II fusion gene, CysO was produced as a part of fusion protein, in fact, the immunogenic function of CysO may be up-regulated because of the chaperone activity of HSP70, that may lead to production of CysO-specific memory T cells *in vivo*, although we could not elucidate the detailed mechanisms of the immunological function of CysO. Actually, mice inoculated with BCG-dHCM produced T cells that

respond to CysO vigorously. These results indicate that CysO protein was certainly secreted from rBCG in APC and was used for T cell activation. CysO expression in MTB is inhibited in hypoxic condition and is induced by reaeration [34], thus, CysO may be highly expressed in MTB in active phase, and, also, is involved in the survival of MTB in macrophages. Therefore, production of CysO reactive T cells may be advantageous for the induction of host defense reaction against MTB which is re-activated in macrophages. In this study, we used small dose of BCG, one fifth of usual dose [47], and by which the parent BCG cannot inhibit MTB growth in lungs in our hands (not shown), for vaccination of mice in order to elucidate the difference between BCG-261H and BCG-dHCM. As expected BCG-261H did not inhibit the MTB growth, but even a small dose of BCG-dHCM at least partially inhibits multiplication of MTB in both lungs and spleen. In this respect, BCG-dHCM seems to be superior to BCG-261H. The reason why BCG-261H did not inhibit the multiplication of MTB may be because we tested bacterial burden in lung and spleen at 6 wks, but not 4 wks which is a frequently used time point [47], after MTB challenge, because report suggests that 4 ~ 5 wks are necessary to reach the stable level of pulmonary bacterial burden even in naïve mice [6]. Also, due to BCG strain differences, there may be differences in protective effect in mice experiments. However, more detailed studies, for example, the difference between BCG-DHTM and BCG-dHCM, and the relation between the presence of CysO-reactive T cells and MTB replication in macrophages, are absolutely required, to prove the usefulness of CysO involvement for the production of vaccine against tuberculosis.

Conclusions

These results indicate that the secretion of polycomponent antigenic molecules can efficiently produce polyclonal Ag-specific T cells responsive to secondary stimulation *in vivo*, and may provide one possible tool for the development of better vaccine against tuberculosis.

Abbreviations

BCG: *Mycobacterium bovis* BCG; *M*: *Mycobacterium*; r: Recombinant; MTB: *M. tuberculosis*; HSP: Heat shock protein; MMP: Major membrane protein; IFN-γ: Interferon-gamma; DC: Dendritic cells; Ag: Antigen; MHC: Major histcompatibility complex; APC: Antigen-presenting cell; TLR: Toll-like receptor; PBMC: Peripheral blood mononuclear cell; mAb: Monoclonal antibody; MOI: Multiplicity of infection; CFU: Colony forming unit.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

YT and MM participated in the design of the study and carried out the cell culture experiments, YM and TT carried out animal studies, YT and TM conducted the construction of recombinant BCG. YT, YM and MM were involved in the preparation of the manuscript. All authors have read and approved the final manuscript.

Acknowledgements

We thank Ms. M. Kujiraoka for her technical support, and the Japanese Red Cross Society for kindly providing PBMCs from healthy donors. This work was supported in part by a Grant-in-Aid for "Research on Emerging and Re-emerging Infectious Diseases" (Grant no. H24-Shinko-Ippan-009 and H25-Shinko-Ippan-005 to M.M) from the Ministry of Health, Labour, and Welfare of Japan.

Received: 25 October 2013 Accepted: 21 March 2014
Published: 2 April 2014

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doi:10.1186/1471-2334-14-179
Cite this article as: Tsukamoto *et al.*: Polyclonal activation of naïve T cells by urease deficient-recombinant BCG that produced protein complex composed of heat shock protein 70, CysO and major membrane protein-II. *BMC Infectious Diseases* 2014 14:179.

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Diagnostic Microbiology and Infectious Disease 80 (2014) 62–65

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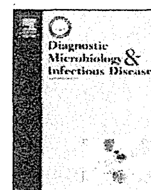

Mycobacteriology

Evaluation of major membrane protein-I as a serodiagnostic tool of pauci-bacillary leprosy

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ARTICLE INFO

Article history:
Received 16 January 2014
Received in revised form 21 April 2014
Accepted 11 June 2014
Available online 19 June 2014

Keywords:
Serodiagnosis
Leprosy
Major membrane protein-I

ABSTRACT

We have previously shown that the serodiagnosis using major membrane protein-II (MMP-II) is quite efficient in diagnosing leprosy. However, the detection rate of pauci-bacillary (PB) leprosy patients is still low. In this study, we examined the usefulness of major membrane protein-I (MMP-I) from *Mycobacterium leprae*. The MMP-I–based serodiagnosis did not show significantly high detection rate. However, when the mixture of MMP-I and MMP-II antigens was used, we detected 94.4% of multi-bacillary leprosy and 39.7% of PB patients. There were little correlation between the titers of anti–MMP-I antibodies (Abs) and that of anti-MMP-II Abs in PB patients' sera. Ten out of 46 MMP-II–negative PB leprosy patients were MMP-I positive, so that the detection rate of PB leprosy patient increased from 39.7% to 53.8% by taking either test positive strategy. We concluded that MMP-I can complement the MMP-II–based serodiagnosis of leprosy.

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1. Introduction

Leprosy is chronic infectious disease caused by an infection with *Mycobacterium leprae* (*M. leprae*), and a significant number of new cases are still detected in 2011; for instance, 219,075 new cases were reported (WHO, 2012). Leprosy usually leads to peripheral nerve injury and systemic deformity (Job, 1989; Stoner, 1979), and the development of the deformity might be preventable, if the sufficient chemotherapy is initiated at an early stage of infection. Thus, early detection of leprosy is quite essential. As leprosy is mainly endemic in developing countries, easy and inexpensive diagnosis is strongly desired.

The diagnosis of leprosy is conducted based on microscopic detection of acid-fast bacilli in skin smears or biopsies, along with clinical and histopathological evaluation of suspected lesions. However, these methods have low sensitivity because *M. leprae bacilli* cannot be detected easily (Shepard and McRae, 1968). Although PCR-based molecular methods have been developed (Donoghue et al., 2001; Martinez et al., 2006; Phetsuksiri et al., 2006), it is not practical to perform PCR in resource-poor settings area. In this respect, serodiagnosis is a reasonable method to diagnose leprosy. Phenolic glycolipid-I (PGL-I), which is supposed to be *M. leprae* specific, was discovered in 1981 (Hunter and Brennan, 1981). The PGL-I is currently accepted as the standard target antigen (Ag) for serodiagnosis of leprosy (Meeker et al., 1986; Schuring et al., 2006; Sekar et al., 1993). However, the method using PGL-I may be useful for the detection of multi-bacillary (MB) leprosy but is not sensitive enough for the detection of pauci-bacillary (PB) leprosy at least in some countries (Kai et al., 2008; Soebono and Klatser, 1991). In the previous study, we have focused on major membrane protein-II (MMP-II) from *M. leprae* (Maeda et al., 2007; Kai et al., 2008). MMP-II is one of the major proteins in the membrane fraction of *M. leprae*, and it induces immune response of host cells during infection (Maeda et al., 2005; Makino et al., 2005). We applied MMP-II as a serodiagnostic tool and found that the MMP-II–based serodiagnosis can increase the detection rate of PB leprosy patient. However, detection rate was still low at 39% (Maeda et al., 2007); thus, it is desirable to improve the sensitivity of the diagnostic tool.

In this study, we focused on major membrane protein-I (MMP-I) from *M. leprae*. MMP-I is 35-kDa major membrane protein expressed in *M. leprae*, which is identified as one of the most dominant Ags of *M. leprae* (Winter et al., 1995).

Although the function of MMP-I is still unknown, MMP-I may induce cell-mediated immune responses (unpublished observation) but has no homology with MMP-II. Therefore, MMP-I could be recognized by the different population of immune cells of leprosy patients and might be worth applying as a serodiagnostic Ag for the improvement of serodiagnosis. We purified recombinant MMP-I Ag using *Mycobacterium smegmatis* and evaluated its usefulness in the detection of both PB and MB leprosy patients.

2. Materials and methods

2.1. Study population

Sera were obtained with informed consent from healthy volunteers and leprosy patients in Japan. Frozen sera samples were used for the study. The samples studied comprised of MB ($n = 72$) and PB ($n = 78$) leprosy patients, either treated or untreated, from the National Sanatorium Oshimaseishoen. Classification of leprosy was performed by using the clinical criteria but was re-classified according to WHO

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http://dx.doi.org/10.1016/j.diagmicrobio.2014.06.004
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recommendations (http://www.who.int/lep/classification/en/index.html) for study purposes. In Japan, children are obligated to get vaccination with *Mycobacterium bovis* bacillus Calmette–Guerin (BCG); therefore, all healthy volunteers (n = 78) are likely to be BCG-vaccinated. Sera from healthy volunteers were used as negative controls in the enzyme-linked immunosorbent assay (ELISA) to determine the cut-off value for the positivity. This study is approved by the ethics committee of the National Institute of Infectious Diseases, Tokyo, Japan.

2.2. Purification of MMP-I and MMP-II

The MMP-I gene (ML0841) was cloned from the genome DNA of *M. leprae*, using primers: 5′-GAGGATCCACGTCGGCTCAGAATGAGTC-3′ and 5′-ATACTAGTTCACTTGTACTCATGGAACT-3′. The amplified gene was expressed in *M. smegmatis* using pMV261 expression vector. The recombinant protein was His6-tagged and purified with Cu^{2+} resin (ABT Agarose Bead Technologies, Tampa, FL, USA). The protein obtained was electrophoresed on sodium dodecyl sulfate–polyacrylamide gels, then the gel was stained with Instant Blue (Expedeon protein solution, San Diego, CA, USA), and a single band of MMP-I protein was observed. The MMP-II gene (ML2038c) was expressed and purified as previously described (Maeda et al., 2007).

2.3. ELISA

The ELISA for the detection of anti-MMP-I antibodies (Abs) was performed as described previously with several modifications (Maeda et al., 2007). Briefly, 96-well plates (Nunc Maxisorp, Thermo Fischer Scientific Inc., Waltham, MA, USA) were coated overnight with MMP-I Ag at a concentration of 1 μg/mL, MMP-II Ag at 2 μg/mL, or the mixture of MMP-I (1 μg/mL) and MMP-II (2 μg/mL) Ags. All Ags were diluted in 0.1 mol/L carbonate buffer (pH 9.5). After blocking with 10% fetal bovine serum (FBS)-containing phosphate-buffered saline (PBS), the plates were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). The optimal concentration of both Ags was determined in advance. Human sera diluted 100-fold were added and incubated at room temperature for 2 hours. After washing with PBS-T, biotinylated anti-human IgG Ab (Vector Laboratories, Burlingame, CA, USA) was added at a concentration of 0.5 μg/mL and incubated for 1 hour. Then, the plates were incubated with reagents from a Vecstain ABC kit (Vector Laboratories) for 30 min. These reagents include avidin and biotinylated horse-radish peroxidase, and this enzyme binds to biotinylated anti-human IgG Ab via avidin. After further washing with PBS-T, a substrate solution consisting of 0.2 mg/mL of 2,2′-Azino-bis (3-ethylbenzothiazolone-6-sulfonic acid) and 0.02% H_2O_2 in 0.1 mol/L citrate buffer was added until a blue color developed, and the reaction was stopped by adding 2 N H_2SO_4. Optical density (OD) was measured at 405 nm using a spectrophotometer. Plate-to-plate variations in OD readings were controlled using a common standard serum with an OD value of 0.360. The volume of all solutions used in the 96-well plate was 50 μL/well.

2.4. Statisitical analyses

The data were analyzed using MEDCALC software (MedCalc, Ostend, Belgium). A receiver operating characteristic (ROC) curve was drawn to calculate the cut-off levels using the OD values of MB leprosy patients' sera and healthy controls. The McNemar test was applied to determine the P value. When the number of inconsistent pairs was less than or equal to 25, the calculation of 2-sided P value was done based on the cumulative binomial distribution. The P value of <0.05 was considered to be statistically significant. The κ value was calculated to determine the agreement between the 2 tests.

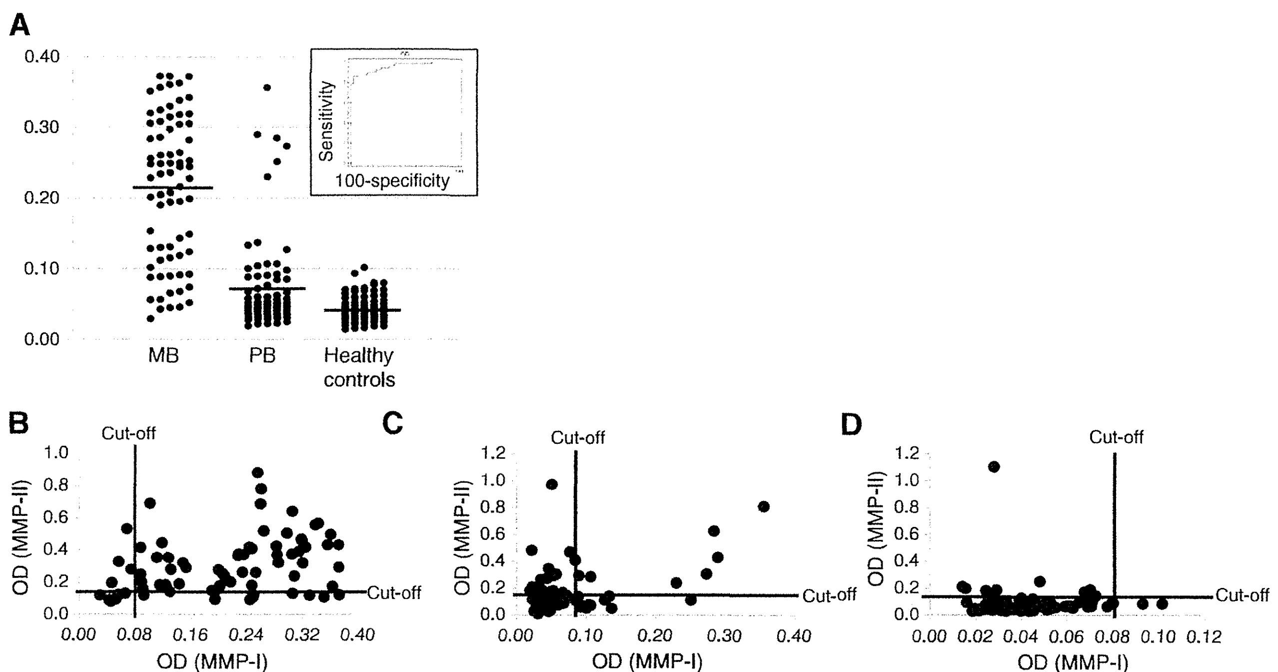

Fig. 1. OD values of each sample were determined by MMP-I–based serodiagnosis and MMP-II–based serodiagnosis. (A) Sample sera from MB leprosy patients (left), PB leprosy patients (middle), and healthy controls (right) were subjected to MMP-I–based ELISA. OD value (wave length: 405 nm) of each sample was plotted. The thick horizontal lines show the average of OD in each group. (Inset) ROC curve analysis of MMP-I–based ELISA. The cut-off value was determined as 0.080, and the area under the ROC curve was 0.952. (B–D) The results of MMP-I–based ELISA and MMP-II–based ELISA were plotted. Sample sera from MB leprosy patients (B), PB leprosy patients (C), and healthy controls (D) were subjected to MMP-I–based or MMP-II–based ELISAs. The x-axis shows the OD value of MMP-I–based ELISA, and the y-axis shows that of MMP-II–based ELISA. Thick lines show the cut-off value of each analysis (MMP-I, 0.080; MMP-II, 0.13).

Table 1
Positivity rates of MMP-I– and MMP-II–based serodiagnosis in various groups of subjects by ELISA.

	MMP-I				MMP-II				McNemar test	Inter-rater agreement
	Tested	Positive	%	95% CI	Tested	Positive	%	95% CI	MMP-I versus MMP-II	κ
MB leprosy	72	62	86.1	75.9–93.1	72	58	80.6	69.5–88.9	$P = 0.3877$	0.403
PB leprosy	78	20	25.6	16.4–36.8	78	32	41.0	30.0–52.8	$P = 0.0518$	0.101
Healthy subjects	78	2	2.6	0.3–8.9	78	9	11.5	5.4–20.8	$P = 0.0654$	−0.044

The data in Fig. 1 were summarized. CI = confidence interval.

3. Results and discussion

3.1. Detection of leprosy patient by the MMP-I-based serodiagnosis

We purified recombinant MMP-I and MMP-II Ags and measured the anti-MMP-I IgG Ab levels in the leprosy patients' sera. We also compared the levels of anti–MMP-I IgG Ab with those of anti-MMP-II IgG Ab. The study population consisted of MB leprosy, PB leprosy patients, and normal healthy BCG-vaccinated individuals from Japan. Some of the patients were already under treatment, so that not all patients were active leprosy patients. The OD value of each sample is plotted in Fig. 1A for MMP-I–based ELISA. The cut-off value was calculated by ROC curve analysis, using the OD values of MB leprosy patients' sera and healthy controls (Fig. 1A, inset). The cut-off value was 0.080 for MMP-I–based ELISA, and the area under the ROC curve was 0.952. The cut-off value for MMP-II–based ELISA was 0.13. From the data thus obtained, we determined the positivity rates of each group and summarized in Table 1. As shown in Table 1, 86.1% of MB ($n = 72$, 62/72), 25.6% of PB leprosy patients ($n = 78$, 20/78), and 2.6% of healthy subjects ($n = 78$, 2/78) had positive anti–MMP-I IgG Ab levels in their sera. We then compared those results with anti–MMP-II IgG Ab levels in their sera. As shown in Table 1, the percent positivities of anti–MMP-II Ab levels were 80.6% in MB leprosy patients (58/72), 41.0% in PB leprosy patients (32/78), and 11.5% in healthy subjects (9/78). The percent positivities of anti–MMP-I Ab in sera of both MB and PB leprosy patients were not significantly high in comparison to anti–MMP-II Ab ($P = 0.3877$ and $P = 0.0518$, by McNemar test). To represent as a dotplot, MMP-I OD values were plotted on X axis and that of MMP-II on Y axis in Fig. 1B (MB leprosy), 1C (PB leprosy), and 1D (healthy controls).

3.2. Usefulness of combination of MMP-I and MMP-II for sensitive detection of leprosy

We analyzed the effect of the use of the mixture of MMP-I and MMP-II Ags for the improvement of the detection rate of leprosy.

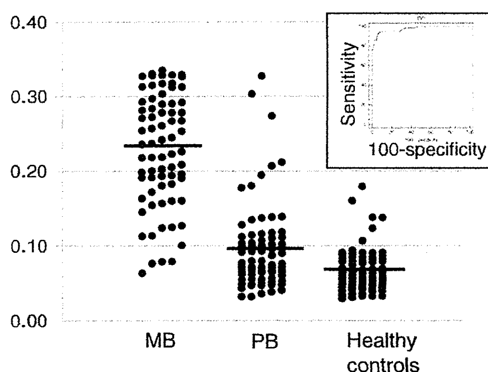

Fig. 2. OD values of each sample were determined by the combinational use of MMP-I and MMP-II Ags for ELISA. Sample sera from MB leprosy patients (left), PB leprosy patients (middle), and healthy controls (right) were subjected to MMP-I– and MMP-II–based ELISA. OD value of each sample was plotted. The thick horizontal lines show the average OD value in each group. The cut-off value was calculated by ROC curve shown in the inset. The cut-off value was determined as 0.094, and the area under the ROC curve was 0.974.

MMP-I Ag and MMP-II Ag were mixed and were used as the Ag for ELISA. All sera used in Table 1 were analyzed. The OD value of each sample is plotted in Fig. 2. The cut-off value was calculated by ROC curve analysis, using the OD value of MB leprosy patients' sera and healthy controls (Fig. 2, inset). The cut-off value was 0.094, and the area under the ROC curve was 0.974. From those data, we determined the positivity rates of each group and summarized in Table 2. The percent positivities against Ag-mixture were 94.4% (68/72) for MB, 39.7% (31/78) in PB leprosy patients, and 9.0% (7/78) for healthy subjects. The detection rate of MB leprosy patients was significantly higher than that using MMP-II Ag alone ($P = 0.002$, by McNemar test). Since both MMP-I and MMP-II Ags are immunogenic (Maeda et al., 2005, 2007; Winter et al., 1995, and our unpublished observations), it would be speculated that most MB leprosy patients produce IgG Abs against either MMP-I or MMP-II. In contrast, the detection rate of PB leprosy patients remained at the same level as MMP-II–based serodiagnosis.

For improvement in the detection rate of PB leprosy, we focused on the inter-rater agreement between the MMP-I–based and the MMP-II–based serodiagnosis. As shown in Table 1, the κ value was low for PB leprosy (κ value: 0.10). This indicates that there is little correlation between the Ab titers against MMP-I and MMP-II in this population of leprosy patients (Fig. 1C). This speculation directed us to conduct more detailed analysis of the titers of anti–MMP-I and anti–MMP-II Abs in those sample sera. We classified the sample sera into 4 groups, "MMP-I negative and MMP-II negative", "MMP-I negative and MMP-II positive", "MMP-I positive and MMP-II negative", and "MMP-I positive and MMP-II positive" from the results of ELISA obtained in Table 1 (Table 3A). In PB leprosy sera, the proportions of the sera classified into the second group and the third group was higher than expected (28.2% and 12.8%, respectively). Thus, we reclassified the sera into 2 groups, "Consistent" and "Not consistent" (Table 3B). "Consistent" group includes "MMP-I negative and MMP-II negative" and "MMP-I positive and MMP-II positive" groups, those have agreement in the results of ELISA using MMP-I Ag and MMP-II Ag, while "Not consistent" group includes "MMP-I negative and MMP-II positive" and "MMP-I positive and MMP-II negative" sera. Interestingly, only 59.0% of PB leprosy sera showed consistency between the results of anti–MMP-I Ab-based ELISA and anti–MMP-II Ab-based ELISA (Table 3B). The results are in contrary to the results of MB leprosy sera, as 83.3% of sera have consistency (Table 3B). These results suggested the possibility that the MMP-I Ag

Table 2
Positivity rates of MMP-I and MMP-II-mixed serodiagnosis in various groups of subjects by ELISA.

	MMP-I + MMP-II				McNemar test versus MMP-II
	Tested	Positive	%	95% CI	
MB leprosy	72	68	94.4	86.4–98.5	$P = 0.002$
PB leprosy	78	31	39.7	28.8–51.5	$P = 1$
Healthy subjects	78	7	9.0	3.7–17.6	$P = 0.753$

The data in Fig. 2 were summarized.

Table 3
(A) Sample sera were classified by the positivity of anti–MMP-I Ab and anti–MMP-II Ab. The figures in brackets show the rate positivity in each group. (B) Sample sera were reclassified by the consistency of the result of the ELISA. The row "Consistent" shows the number of sera that have been classified in the group "MMP-I negative and MMP-II negative" or "MMP-I positive and MMP-II positive". The row "Not consistent" shows the number of sera that have been classified in the group "MMP-I negative and MMP-II positive" or "MMP-I positive and MMP-II negative".

	MB (n = 72)	PB (n = 78)
(A)		
MMP-I negative and MMP-II negative	6 (7.7%)	36 (46.1%)
MMP-I negative and MMP-II positive	4 (5.6%)	22 (28.2%)
MMP-I positive and MMP-II negative	8 (11.1%)	10 (12.8%)
MMP-I positive and MMP-II positive	54 (75.0%)	10 (12.8%)
(B)		
Consistent	60 (83.3%)	46 (59.0%)
Not consistent	12 (16.6%)	32 (41.0%)

can rescue the people who are diagnosed as negative in MMP-II serodiagnostic test, which may successfully reduce false-negative results. When MMP-II Ag–based serodiagnosis was followed by MMP-I Ag–based analysis, 53.8% of PB leprosy patients (42/78) were Ab positive, and 91.7% of MB leprosy patients (66/72) were Ab positive, although relatively high percentages of healthy subjects (14.1%, 11/78) were Ab positive (Table 4, data of Table 1 were re-analyzed), and the exact reason for this percentage remains to be evaluated. Therefore, both MMP-I Ag and MMP-II Ag may be quite useful diagnostic tools for both MB and PB leprosy.

There is the discrepancy between the results obtained by mixing MMP-I and MMP-II Ags in the same well and those by individual ELISA in the detection rate of PB leprosy. We found that the individual serological tests performed using the Ag mixture are not always successful in detecting all PB leprosy patients in separate ELISA assays. The theoretical calculation indicates that MaxiSorp ELISA plate surface has the capacity to adsorb about 650 ng/cm^2 of globular protein such as IgG; it is likely that both MMP-I and MMP-II Ags can be adsorbed (150 ng/well). The probable explanation for the low detection rate of PB leprosy may be the hydrophobic characteristics of both MMP-I and MMP-II Ags. Such characteristics may interfere with the adsorption of those Ags on ELISA plate. However, as far as we tested other ELISA plates including AGC technoglass Co. Ltd. and the other Ag-coating buffers, we could not improve the detection rate of PB leprosy using mixed Ags. Another way to modify the assay such as using specific Abs to MMP-I and MMP-II Ags for coating the plates and then layering the Ag may also help to detect more Abs, but presently, such specific Abs are not available. On the other hand, some of MB leprosy patients have polyclonally activated B cells, which might lead to produce IgG Abs with high affinity; therefore, the use of mixture of MMP-I and MMP-II Ags could be applicable.

Previous study has shown that the detection rate of serodiagnosis using MMP-II is similar for leprosy patients in Vietnam to those in Japan. In contrast, the detection rate of PGL-I–based serodiagnosis is significantly lower for Vietnamese patients than Japanese (Kai et al., 2008). The reason why the sensitivity of PGL-I–based serodiagnosis is different between Vietnamese and Japanese is still unclear. It could be speculated that the major pathogenic and non-pathogenic mycobacterial species found in each region differs and the inhabitants in such region could be influenced by those mycobacteria that would lead to production of region-specific Abs. Therefore, the detection rate of leprosy may differ when MMP-I–based serodiagnosis is applied to leprosy patients of endemic countries.

The sera used in this study were obtained from both treated and untreated leprosy patients. However, because of the lack of records on the treatment history, we could not perform comparative studies between untreated and treated patients. The detailed cohort studies are absolutely required to reveal the utility of MMP-I Ag in primary diagnosis, especially in endemic areas. Also, we need the testing of cross-reactivity of MMP-I and MMP-II Ags with other mycobacterial diseases, especially because homologs of MMP-I are identified in several other mycobacteria and possible infection of those bacteria may have impact on the detection of leprosy. So far, cell-mediated immune responses using MMP-I and MMP-II Ags seem not helpful for the diagnosis of PB leprosy, so that serological tool may be advantageous for the PB diagnosis. Through the establishment of simple tool for the serodiagnosis using both MMP-I and MMP-II Ags, we may be able to achieve easy and inexpensive diagnosis of leprosy of both MB and PB type.

Table 4
Positivity rates of MMP-I or MMP-II ELISAs in various groups of subjects.

	MMP-I or MMP-II		
	Tested	Positive	%
MB leprosy	72	66	91.7
PB leprosy	78	42	53.8
Healthy subjects	78	11	14.1

Acknowledgments

This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour, and Welfare of Japan (H22-Shinko-Ippan-007 and H24-Shinko-Ippan-009).

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Protection against *Mycobacterium leprae* Infection by the ID83/GLA-SE and ID93/GLA-SE Vaccines Developed for Tuberculosis

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Despite the dramatic reduction in the number of leprosy cases worldwide in the 1990s, transmission of the causative agent, *Mycobacterium leprae*, is still occurring, and new cases continue to appear. New strategies are required in the pursuit of leprosy elimination. The cross-application of vaccines in development for tuberculosis may lead to tools applicable to elimination of leprosy. In this report, we demonstrate that the chimeric fusion proteins ID83 and ID93, developed as antigens for tuberculosis (TB) vaccine candidates, elicited gamma interferon (IFN-γ) responses from both TB and paucibacillary (PB) leprosy patients and from healthy household contacts of multibacillary (MB) patients (HHC) but not from nonexposed healthy controls. Immunization of mice with either protein formulated with a Toll-like receptor 4 ligand (TLR4L)-containing adjuvant (glucopyranosyl lipid adjuvant in a stable emulsion [GLA-SE]) stimulated antigen-specific IFN-γ secretion from pluripotent Th1 cells. When immunized mice were experimentally infected with *M. leprae*, both cellular infiltration into the local lymph node and bacterial growth at the site were reduced relative to those of unimmunized mice. Thus, the use of the *Mycobacterium tuberculosis* candidate vaccines ID83/GLA-SE and ID93/GLA-SE may confer cross-protection against *M. leprae* infection. Our data suggest these vaccines could potentially be used as an additional control measure for leprosy.

Prevalence rates for leprosy have sharply declined over the last 20 years, with the major breakthrough being attributed to the provision of free-of-charge treatment to all diagnosed leprosy patients. The stalled decreases in both global prevalence and new case detection rates of leprosy over the last decade indicate that additional measures are likely required. The relative success of leprosy control, however, has prompted the integration of leprosy-specific programs into general health facilities and has also reduced the resources available to research (most notably specialized investigators and funds) (1, 2). During the same period, the World Health Organization (WHO) has declared tuberculosis (TB) a global public health emergency. Indeed, over 2 billion people are now believed to be infected with *Mycobacterium tuberculosis*, and multi- and extremely drug-resistant strains are rapidly emerging (3, 4). Numerous groups are actively engaged in developing replacement or supplementary vaccines as an alternative or additional control strategy for the TB epidemic (5). Defined antigens, delivered as plasmid DNA, vectored DNA, or as recombinant proteins in adjuvant, have proven effective in animal models, and at least nine subunit TB vaccines have entered clinical trials (6–11).

The *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccines represent an important component within TB control programs, since they provide at least partial protection against tuberculosis (12–14) and leprosy (15). In several countries, both TB and leprosy are endemic, and the contribution of mass BCG vaccination is often overlooked as a factor within leprosy control (16). The application of emerging TB vaccines to leprosy control programs could be logistically and economically beneficial as a public health intervention, but the capacity of such vaccine candidates to elicit protective responses against infection with *Mycobacterium leprae* (the causative agent of leprosy) has not been investigated.

As chimeric fusion proteins comprised of three and four *M. tuberculosis* proteins, the TB vaccine candidate antigens ID83 and ID93 each present *M. tuberculosis* proteins selected from various categories (17). When combined with the synthetic Toll-like receptor 4 ligand (TLR4L) glucopyranosyl lipid adjuvant (GLA) in a stable emulsion (SE), ID93 boosts the effects of BCG, protecting mice and guinea pigs against infection with *M. tuberculosis* (7, 18). The current study was designed to examine the potential of the ID83/GLA-SE and ID93/GLA-SE vaccines to protect against *M. leprae* infection.

MATERIALS AND METHODS

Subjects and samples. Recently diagnosed and previously untreated leprosy patients and controls from an area to which leprosy is endemic (EC) were recruited at Centro de Referencia em Diagnostico e Terapeutica and Hospital Anuar Auad, Goiânia, Goiás State, Brazil. Leprosy patients were categorized as paucibacillary (PB) by clinical, bacilloscopic, and histological observations (bacterial index, skin lesions, nerve involvement, and histopathology) carried out by qualified personnel. Blood was obtained from tuberculosis patients (*M. tuberculosis* sputum-positive, HIV-negative individuals with clinically confirmed pulmonary tuberculosis) who were undergoing treatment. EC were healthy individuals who had never had tuberculosis, had no history of leprosy in the family, and were living in the area to which leprosy is endemic. All had previously been immunized with BCG, and all blood samples were obtained after informed consent and after local ethics committee approval.

Received 6 June 2014 Returned for modification 26 June 2014
Accepted 5 July 2014
Published ahead of print 14 July 2014
Editor: J. L. Flynn
Address correspondence to Malcolm S. Duthie, mduthie@idri.org.
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doi:10.1128/IAI.02145-14

TABLE 1 Homology of ID83 and ID93 components between mycobacteria

Vaccine(s)	Component (*M. tuberculosis* protein)[a]	Homolog (% identity) from: *M. leprae*[b]	*M. bovis*
ID83 and ID93	Rv1813	Not found	Mb1843c (100)
	Rv2608	Not found	Mb2640 (99.8)
	Rv3620	ML1055 (57.9 [in 95-aa overlap])	
ID93 only	Rv3619	ML1056 (64.15 [in 92-aa overlap])	

[a] *M. tuberculosis* H37Rv strain; homologs were searched for using Tuberculist.
[b] *M. leprae* TN strain. aa, amino acid.

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Determining reactivity by 24-h WBA. Whole-blood assays (WBA) were performed with venous undiluted heparinized whole blood (Greiner). Within 2 h of collection, blood was added to each well of a 24-well plate (450 μl/well; Sigma, St. Louis, MO) and incubated with antigens at 37°C, 5% CO_2. For each assay, stimulations were conducted with 10 μg/ml of recombinant protein, 10 μg/ml *M. leprae* cell sonicate (provided by John Spencer, Colorado State University, Fort Collins, CO, under NIH contract N01 AI-25469), or 1 μg/ml phytohemagglutinin (PHA) (Sigma). After 24 h, plasma was collected and stored at −20°C. Gamma interferon (IFN-γ) content within the plasma was determined by enzyme-linked immunosorbent assay (ELISA), used according to the manufacturer's instructions (QuantiFERON CMI; Cellestis, Carnegie, Australia). The detection limit of the test was 0.05 IU/ml. For data interpretation, we assigned as a positive result a concentration above an arbitrary cutoff point of 0.5 IU/ml.

Mice and immunizations. Wild-type C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA). Mice were immunized with recombinant protein formulated with saline, SE, or GLA-SE to provide a final protein concentration of 100 μg/ml antigen and 200 μg/ml GLA-SE (19). Mice were immunized up to 3 times by subcutaneous (s.c.) injection of 0.1 ml vaccine at the base of the tail at 2-week intervals. Mice were maintained under specific-pathogen-free conditions, and all procedures were approved by the appropriate institutional animal care and use committees.

Antibody responses. Mouse sera were prepared by collection of retroorbital blood into Microtainer serum collection tubes (VWR International, West Chester, PA), followed by centrifugation at 1,200 rpm for 5 min. Each serum sample was then analyzed by antibody capture ELISA. Briefly, ELISA plates (Nunc, Rochester, NY) were coated with 1 μg/ml recombinant antigen in 0.1 M bicarbonate buffer and blocked with 1% bovine serum albumin (BSA)-phosphate-buffered saline (PBS). Then, in consecutive order and following washes in PBS-Tween 20, serially diluted serum samples, anti-mouse IgG, IgG1, or IgG2c-horseradish peroxidase (HRP) (all from Southern Biotech, Birmingham, AL), and 2,2′-azino-bis(3-ethylbenzthiazolinesulfonic acid) (ABTS)-H_2O_2 (Kirkegaard & Perry Laboratories, Gaithersburg, MD) were added to the plates. Plates were analyzed at 405 nm (EL_x808; Bio-Tek Instruments Inc., Winooski, VT). The endpoint titer was determined as the last dilution to render a positive response, determined as 2 times the mean optical density of the replicates derived from sera from unimmunized mice in the Prism software program (GraphPad Software, La Jolla, CA).

Antigen stimulation and cytokine responses. Single-cell suspensions were prepared by disrupting spleens between sterilized frosted slides. Red blood cells were removed by lysis in 1.66% NH_4Cl solution, and then mononuclear cells were enumerated by ViaCount assay with a PCA system (Guava Technologies, Hayward, CA). Single-cell suspensions were cultured at 2×10^5 cells per well in duplicate in a 96-well plate (Corning Incorporated, Corning, NY) in RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS) and 50,000 units penicillin-streptomycin (Invitrogen). Cells were cultured in the presence of 10 μg/ml antigen for 96 h, after which culture supernatants were collected and cytokine content was assessed. Cytokine concentrations within culture supernatants were determined by ELISA. ELISA kits for determination of mouse IFN-γ, interleukin 5 (IL-5), IL-13 and tumor necrosis factor alpha (TNF-α) were performed according to the manufacturer's instructions (eBioscience, San Diego, CA), and optical density was determined using an ELx808 plate reader (Bio-Tek Instruments Inc., Winooski, VT).

Alternatively, for the elucidation of intracellular cytokine expression, cells were cultured at 37°C for 16 h in the presence of 1 μg/ml phorbol myristate acetate (PMA)-ionomycin (Sigma, St. Louis, MO) or 10 μg/ml recombinant antigen and Golgi Stop (BD Biosciences, San Diego, CA). Cells were fixed and permeabilized in Cytofix/Cytoperm (BD Biosciences, San Diego, CA). To stain, cells were first incubated with the anti-FcγII/IIIR antibody 2.4G2 to block nonspecific binding, before addition of a cocktail of fluorescently conjugated antibodies to identify cytokine-producing antigen-experienced T helper cells (anti-CD4, anti-CD3ε, anti-CD44, anti-IL-2, anti-IFN-γ and anti-TNF [all from eBioscience]). Flow cytometry was performed using an LSR Vantage instrument (BD Biosciences), and the data were analyzed using the FlowJo software program (Treestar, Ashland, OR).

***M. leprae*-induced inflammation.** To assess *M. leprae*-induced inflammation, live *M. leprae* bacilli (Thai-53 strain) were purified from the footpads of *nu/nu* mice at National Hansen's Disease Programs and shipped overnight on ice to the Infectious Disease Research Institute (IDRI) for inoculations (20). Heat-killed *M. leprae* (HKML) bacteria were obtained by heating bacilli at 70°C for 1 h and then quenching on ice. Mice were inoculated with 1×10^6 bacilli in a volume of 10 μl by intradermal (i.d.) injection into the ear pinnae. Twelve weeks later, single-cell suspensions were prepared from the (auricular) draining lymph nodes (DLN), and cell numbers were determined by ViaCount assay with a PCA system (Guava Technologies).

Determination of bacterial burden. To assess *M. leprae* growth, live *M. leprae* bacilli (Thai-53 strain) were purified from the footpads of *nu/nu* mice at National Institute of Infectious Diseases. Six C57BL/6 mice per group were s.c. vaccinated with a total of 5 μg/mouse of either the ID83 or ID93 fusion protein or GLA-SE (10 μg/mouse) as a negative control 3 times with an interval of 3 weeks between inoculations and a month later were challenged with 5×10^3 *M. leprae* bacilli by subcutaneous (s.c.) injection into each footpad. Footpads were harvested 7 months later, and the bacilli were enumerated by direct microscopic counting of acid-fast bacilli according to the method of Shepard and McRae, with a limit of detection of 3,700 bacilli (21). Animal studies were reviewed and approved by the Animal Research Committee of Experimental Animals of the National Institute of Infectious Diseases (Tokyo, Japan) and were conducted according to their guidelines.

Statistics. For human data, the nonparametric Kruskal-Wallis analysis-of-variance test was used to compare the IFN-γ levels among all of the groups, and the Mann Whitney U test was applied for comparison between two groups. The P values for mouse studies were determined using the Student t test. Statistics were generated using the software program MS Excel (Microsoft Corporation, Redmond, WA) or Prism (GraphPad Software, Inc., La Jolla, CA). Statistical significance was considered when the P values were <0.05.

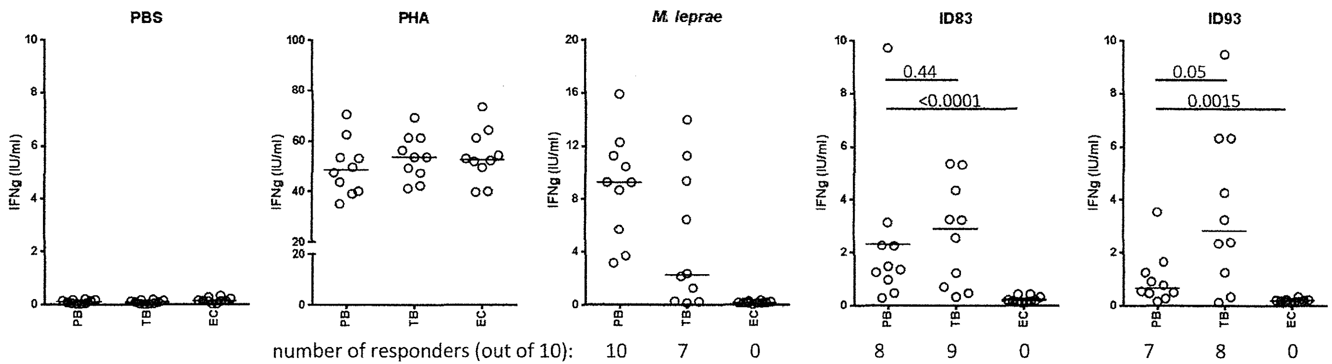

FIG 1 The ID83 and ID93 fusion proteins are recognized by PB leprosy patients. Whole blood from the PB, TB, and EC groups was cultured for 24 h in the presence of antigen, and IFN-γ content in the plasma was measured by ELISA. Data from each individual is represented by a point, and the black bar indicates the median IFN-γ value. *P* values are indicated.

RESULTS

Recognition of ID83 and ID93 by leprosy patients. Replication and dissemination of *M. leprae* is limited in PB leprosy patients, suggesting that their potent cellular immune response is associated with limited or localized disease. Antigens that are recognized by immune cells of PB patients may therefore be the key to identifying an effective subunit vaccine against leprosy. We assessed the abilities of two TB vaccine candidate antigens we have developed, ID83 (a fusion of three *M. tuberculosis* proteins, Rv1813, Rv2608, and Rv3620) and ID93 (a fusion of four *M. tuberculosis* proteins, Rv1813, Rv2608, Rv3619, and Rv3620) (Table 1), to stimulate patient-specific IFN-γ recall responses in WBA. While none of the EC samples exhibited IFN-γ levels above 0.5 IU/ml, blood from TB patients reacted strongly to these proteins and secreted significant levels of IFN-γ (Fig. 1). IFN-γ levels were observed above this threshold in 8 of 10 TB patient samples stimulated with either protein. Although not as potently recognized as by blood from TB patients, ID83 and ID93 were also well recognized by PB leprosy patient blood (Fig. 1). IFN-γ levels above 0.5 IU/ml were observed in 8 of 10 PB patient samples stimulated with ID83 and 6 of 10 samples stimulated with ID93. Taken together, these data indicate that the TB vaccine candidate antigens ID83 and ID93 are also applicable to leprosy.

Impact of vaccine formulation on immune response. To examine this vaccine potential, mice were immunized with either ID83/SE or ID83/GLA-SE. Immunization with either formulation induced an antigen-specific IgG response (Fig. 2). However, although antigen-specific IgG1 responses were similar, GLA-SE induced a significantly greater anti-ID83 IgG2c response than SE (Fig. 2). These data indicate that immunization with the GLA-SE,

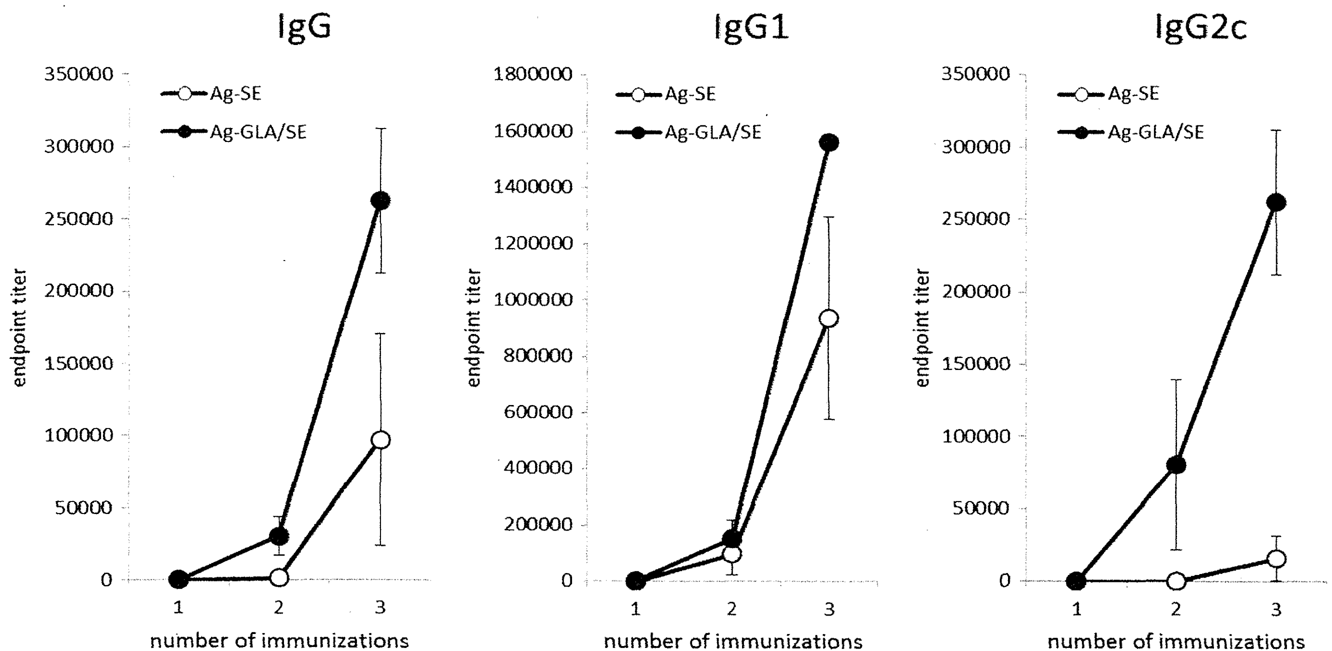

FIG 2 Immunization with ID83/GLA-SE but not ID83/SE promotes strong antigen-specific IgG2c responses. Mice were injected s.c. with ID83/SE and ID83/GLA-SE at biweekly intervals for a total of 3 immunizations. Serum was collected before each immunization and 1 month after the final immunization. Antigen-specific serum IgG, IgG1, and IgG2c endpoint titers were determined by antibody-capture ELISA.

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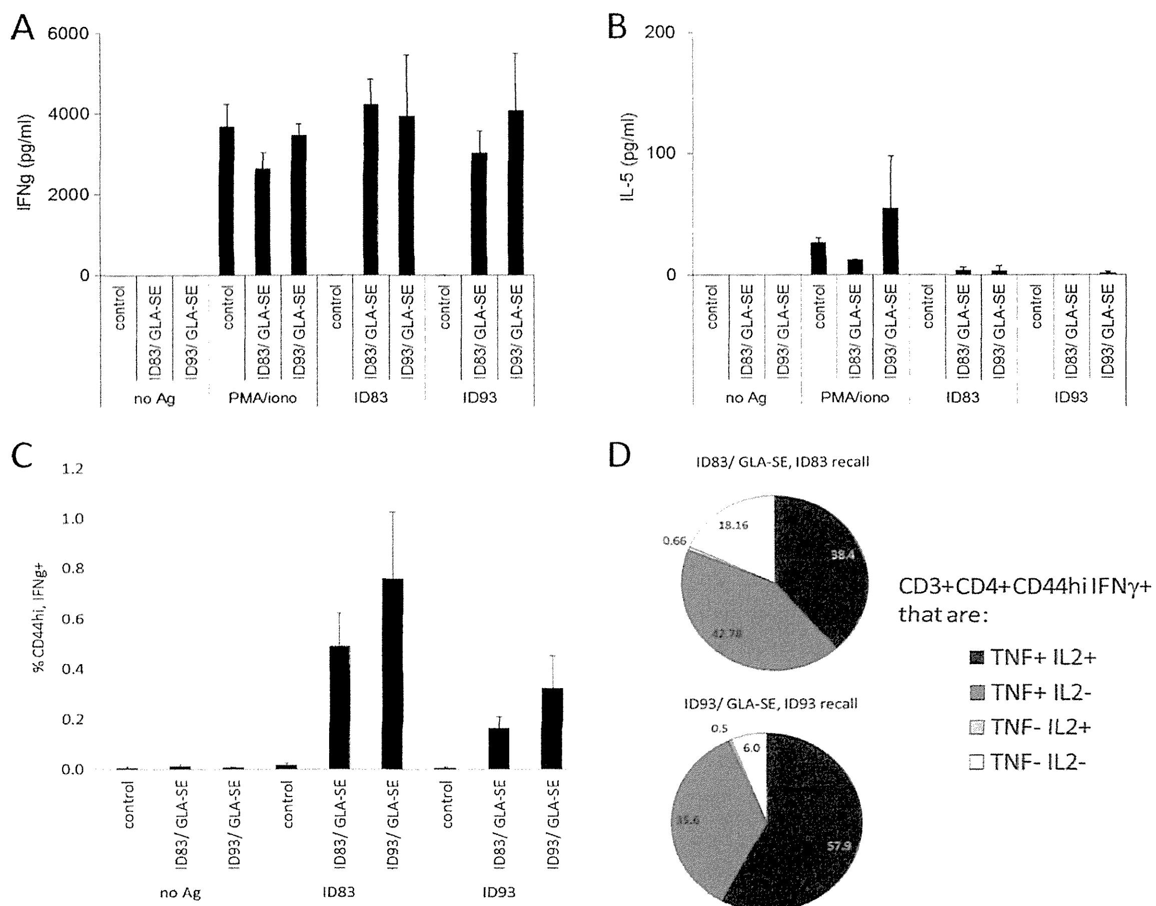

FIG 3 Immunization with either ID83/GLA-SE or ID93/GLA-SE stimulates pluripotent Th1 antigen (Ag)-specific responses. Mice were injected s.c. with ID83/GLA-SE and ID93/GLA-SE at biweekly intervals for a total of 3 immunizations. Single-cell suspensions of spleen cells were prepared 1 month after the final immunization and cultured with 10 μg/ml protein. Culture supernatants were collected after 4 days, and IFN-γ (A) or IL-5 (B) content was determined by ELISA. Results are shown as means and SE; $n = 3$ per group. iono, ionomycin. Alternatively, cells were cultured with antigen and BD Golgi Stop for 16 h and then fixed and stained to determine the percentage (C) of $CD3^+$ $CD4^+$ $CD44^{hi}$ IFN-γ^+ cells by flow cytometry. Results are shown as means and SE; $n = 3$ per group. The phenotype of each $CD3^+$ $CD4^+$ $CD44^{hi}$ IFN-γ^+ cell was further delineated by costaining for IL-2 and/or TNF. In panel D, results are shown as percent $CD3^+$ $CD4^+$ $CD44^{hi}$ IFN-γ^+ cells exhibiting each phenotype. Data are representative of at least 3 independent experiments.

but not the SE, formulation promotes a Th1-like response that could provide cross-protection against *M. leprae* infection.

Elicitation of pluripotent Th1 responses by TB vaccines. To further characterize this vaccine potential, mice were immunized with either ID83/GLA-SE or ID93/GLA-SE. Spleen cells from immunized mice responded to antigen stimulation by secreting large amounts of IFN-γ but very little IL-5, indicating the generation of a strong Th1 response (Fig. 3A and B). Furthermore, many of the antigen-specific IFN-γ-secreting cells also secreted both IL-2 and TNF, and the vast majority secreted at least one of these additional cytokines (Fig. 3C).These data indicate that immunization with either ID83/GLA-SE or ID93/GLA-SE promotes a Th1-like response that could be protective against *M. leprae* infection.

***M. leprae*-induced inflammation is reduced in mice immunized with TB vaccines.** We previously demonstrated that *M. leprae* infection of the ear causes local inflammation that can be interrupted by drug treatment (22, 23). We hypothesized that appropriate vaccination with ID83 and ID93 would limit development of local inflammation. Mice were immunized by s.c. injection at the base of the tail and then infected in the ears with *M. leprae*. In agreement with our hypothesis, 12 weeks after infection, fewer cells were recovered from DLN of ID83/GLA-SE- or ID93/GLA-SE-immunized mice than from DLN of mock immunized mice (Fig. 4A). The DLN cell numbers of infected, immunized mice were similar to those of HKML-inoculated, infected mice (Fig. 4A). Taken together, these data indicate that the local inflammation observed following *M. leprae* ear infection can be limited by immunization with the candidate TB vaccines.

Immunization with TB vaccines reduces *M. leprae* burden. To investigate if the vaccines could limit growth of *M. leprae*, immunized mice were infected with *M. leprae* in the footpad and bacilli numbers were assessed 7 months later. Immunization with either vaccine decreased bacterial numbers compared with those of mice injected with adjuvant alone (Fig. 4B). Taken together, our experimental data indicate that defined subunit vaccines intended for TB could also be useful for leprosy.

DISCUSSION

Proteins that elicit IFN-γ responses from PB leprosy patients and healthy household contacts of multibacillary (MB) patients

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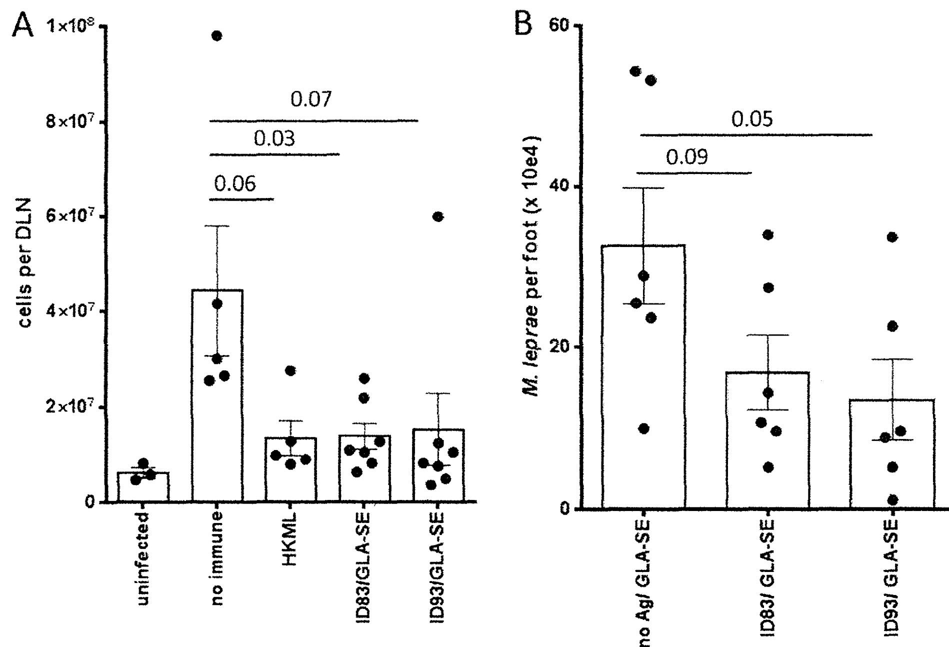

FIG 4 Immunization with TB vaccines reduces lymphadenopathy induced by *M. leprae* infection and reduces *M. leprae* burden. Mice were injected s.c. with ID83/GLA-SE and ID93/GLA-SE at biweekly intervals, for a total of 3 immunizations. In panel A, 1 month after the last immunization, mice were infected with 1×10^6 *M. leprae* bacilli in each ear, and DLN cell numbers were determined 12 weeks later. Results are shown as means and SE ($n = 5$ per group), and data are representative of three independent experiments. Student's t test was used to calculate P values between each group. In panel B, 1 month after the last immunization, mice were infected with 5×10^3 *M. leprae* bacilli in each foot, and bacillus numbers were determined 7 months later. The Mann-Whitney test was used to calculate P values between each group, and results are shown as individual points for each animal, group mean, and SE ($n = 6$ per group).

(HHC) are potential targets of the immune response that naturally limits *M. leprae* infection. Utilizing such proteins within subunit vaccines thus has the potential to disrupt disease development and bacterial dissemination. In this report, we demonstrate that the chimeric fusion proteins ID83 and ID93, each developed as antigen candidates within TB vaccines, are recognized by blood from PB leprosy patients. When either antigen was formulated with a TLR4L-containing adjuvant (GLA-SE), immunization stimulated strong, pluripotent Th1 responses and inhibited *M. leprae*-induced inflammation and bacterial growth in mice. Thus, the use of the *M. tuberculosis* candidate vaccines ID83/GLA-SE and ID93/GLA-SE may confer cross-protection against *M. leprae* infection and could potentially be used as a control measure for leprosy.

While many groups are attempting to develop vaccines for other neglected tropical diseases, difficulties inherent in leprosy research (e.g., our inability to culture *M. leprae in vitro* and the very long duration of experimental infections) have severely restricted the investigation and advancement of leprosy vaccines. There is an enormous effort, however, to develop vaccines against *M. tuberculosis*, and at least nine subunit vaccines have entered clinical trials (11). Taking advantage of TB research efforts could provide an efficient way to codevelop a vaccine for both leprosy and TB. As chimeric fusion proteins comprised of three and four *M. tuberculosis* proteins, ID83 and ID93 each present *M. tuberculosis* proteins selected from various categories (the PE/PPE family of proteins and the EsX family of virulence factors, associated with latent growth of *M. tuberculosis* and expressed during hypoxia, respectively). In this study, our original intent was to examine the T cell responsiveness of Brazilian TB patients to the ID83 and ID93 vaccine antigen candidates, using leprosy patients as a known mycobacterium-infected control group to determine specificity of the responses. Indeed, TB patients did respond strongly through the secretion of IFN-γ. *In silico* predictions revealed extremely low homology between the amino acid sequences of the individual proteins contained in ID83 and ID93 and the published *M. leprae* genome (24) (Table 1). It was therefore surprising that PB leprosy patients responded well to antigen stimulation. The response of leprosy patients was most likely due to *M. leprae* infection and, because EC did not respond, not due to previous BCG immunization or other factors, such as possible latent *M. tuberculosis* infection or exposure to other environmental mycobacteria. These data provide validation for the potential use of these vaccine candidates for the prevention of leprosy. It has previously been demonstrated that even though the identities between *M. tuberculosis* and *M. leprae* ESAT-6 and CFP-10 (36% and 40%, respectively) were very low and the heterologous proteins were not cross-reactive in terms of serum antibody responses in leprosy patients, there was a strong cross-reactive response at the T cell level in both TB and leprosy patients (25). Thus, the cell-mediated responses for low-homology proteins appear to be more promiscuous and are likely contained within similar T cell epitopes within the heterologous proteins.

When *M. leprae* infection manifests disease, leprosy can present across a diverse bacteriologic, clinical, immunologic, and pathological spectrum. The hallmark neuropathy associated with leprosy arises not only from the direct infection of peripheral nerves by *M. leprae*, a unique trait among bacteria, but also from the inflammatory and immunologic responses to the infection. Indeed, immune-inflammatory episodes known as leprosy reac-

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tions are the main cause of irreversible nerve damage and can be severe enough to require hospitalization. Thus, the promotion of strong immune responses could theoretically precipitate immune pathology, and it is therefore of paramount importance to ensure the safety of a vaccine for leprosy. ID83 subunit vaccines containing synthetic TLR4 or TLR9 agonists generated a Th1 immune response and protected mice against challenge with *M. tuberculosis* (19). Experimental infection of mice with *M. leprae* does not precipitate the nerve damage that is a common feature in leprosy patients (21), but it is noteworthy that vaccination with the TB vaccines reduced the *M. leprae*-induced lymphadenopathy. This observation implies that the vaccines, at least at the DLN level, do not elicit strong infection-site inflammation that causes immune pathology. Further exploration in armadillos that do develop neuropathy following *M. leprae* infection appears prudent (26–28).

Currently the Leprosy Control Program in Brazil recommends BCG vaccination for all intradomiciliary contacts of both MB and PB leprosy that already have a BCG scar or the ones that haven't been previously vaccinated. In this regard, a new TB vaccine that could also protect against leprosy could also provide additional protection for exposed contacts of leprosy patients. The majority of research directed toward a vaccine for leprosy dates back a few decades and has focused on the use of related whole mycobacteria or purification of protein fractions from *M. leprae*. Purified and/or recombinant 10-kDa, 25-kDa, and 65-kDa proteins provided protection when administered with Freund's adjuvant (29). Modern vaccine standards, regulations, and safety concerns suggest that more refined products should be developed. The development, production, and advancement of vaccines through the necessary regulatory processes is not trivial or inexpensive, however, and it is therefore noteworthy that at least nine subunit TB vaccines have already passed such scrutiny and have entered clinical trials (11). Our strongest indication that any of these TB vaccines could also contribute toward leprosy control is the demonstration of reduced *M. leprae* burdens in ID83- and ID93-immunized mice following experimental infection.

In summary, despite the positive impact that the widespread provision of multidrug therapy has had on the global prevalence of leprosy, there are indications that further effort and additional strategies are required in the push for elimination. It is our strong opinion that this effort should include an effective vaccine and that perhaps the smoothest path toward this would be the use of TB vaccines that also protect against leprosy. Our data suggest that new TB vaccine initiatives that are advancing the ID83- and ID93-based vaccine candidates could also be highly beneficial for the sustained control of leprosy.

ACKNOWLEDGMENTS

We are extremely grateful to the patients and staff of the Centro de Referência em Diagnóstico e Terapêutica, Goiânia/Goiás for their cooperation and support.

This work was conducted with support from American Leprosy Missions, UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) grant no. A20509, The Heiser Program for Research in Leprosy and Tuberculosis of The New York Community Trust, National Institutes of Health grant AI-078054 and contract HHSN272200800045C, and the Bill and Melinda Gates Foundation (grant 42387). M.M.A.S. is a recipient of a fellowship from National Counsel of Technological and Scientific Development (CNPq/Brazil) (grant no. 304869/2008-2), and R.M.O. was supported by a scholarship awarded by the Brazilian Federal Agency for the Support and Evaluation of Graduate Education (CAPES). This work was also partly supported by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases (grant no. H21-Shinko-Ippan-007, to M. Makino and Y. Maeda) from the Ministry of Health, Labor and Welfare of Japan.

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Discrimination of *Mycobacterium abscessus* subsp. *massiliense* from *Mycobacterium abscessus* subsp. *abscessus* in Clinical Isolates by Multiplex PCR

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The rapidly growing mycobacterium *M. abscessus sensu lato* is the causative agent of emerging pulmonary and skin diseases and of infections following cosmetic surgery and postsurgical procedures. *M. abscessus sensu lato* can be divided into at least three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii*. Clinical isolates of rapidly growing mycobacteria were previously identified as *M. abscessus* by DNA-DNA hybridization. More than 30% of these 117 clinical isolates were differentiated as *M. abscessus* subsp. *massiliense* using combinations of multilocus genotyping analyses. A much more cost-effective technique to distinguish *M. abscessus* subsp. *massiliense* from *M. abscessus* subsp. *abscessus*, a multiplex PCR assay, was developed using the whole-genome sequence of *M. abscessus* subsp. *massiliense* JCM15300 as a reference. Several primer sets were designed for single PCR to discriminate between the strains based on amplicons of different sizes. Two of these single-PCR target sites were chosen for development of the multiplex PCR assay. Multiplex PCR was successful in distinguishing clinical isolates of *M. abscessus* subsp. *massiliense* from samples previously identified as *M. abscessus*. This approach, which spans whole-genome sequencing and clinical diagnosis, will facilitate the acquisition of more-precise information about bacterial genomes, aid in the choice of more relevant therapies, and promote the advancement of novel discrimination and differential diagnostic assays.

Members of the *Mycobacterium chelonae-M. abscessus* group of rapidly growing mycobacteria (RGM), *M. abscessus sensu lato*, have been identified not only as sources of pulmonary infections but also as emerging pathogens of nosocomial infections following cosmetic surgery and postsurgical procedures (1–4). The taxonomic status of *M. abscessus sensu lato* has not been resolved; however, *M. massiliense* and *M. bolletii* were characterized as new species distinct from *M. abscessus* (5, 6). Although the clinical significance of *M. massiliense* has been emphasized (7, 8), it was proposed by Leao et al. in 2011 that *M. massiliense* and *M. bolletii* should be reclassified as a united subspecies of *M. abscessus*, *M. abscessus* subsp. *bolletii*, and that a new subspecies, *M. abscessus* subsp. *abscessus*, should be designated (9). However, a recent whole-genome study strongly supported the hypothesis that the species can be divided into at least three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* (10). *M. abscessus* subsp. *massiliense* was initially isolated from the sputum of a patient with pneumonia in France in 2004 (5). In 2005, an outbreak of *M. abscessus* subsp. *massiliense* infection was linked to intramuscular injections of an antimicrobial agent in South Korea (11). This bacterium was also the source of a lethal case of sepsis in Italy (12) and has been found in cystic fibrosis patients in France (13). Several cases of bacteremia and cutaneous pulmonary infections have also been reported in Japan (14–17).

A novel approach is required to differentiate *M. abscessus* subsp. *massiliense* from *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* because conventional methods such as biochemical assays and 16S rRNA genotyping cannot make the discrimination. Moreover, the clinical profile of *M. abscessus* subsp. *massiliense* is different from those of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*. In particular, antibiotic treatment with clarithromycin is more effective against *M. abscessus* subsp. *massiliense* lung infections, with resistance developing more readily in cases of *M. abscessus* subsp. *abscessus* lung disease. Therefore, differentiating *M. abscessus* subsp. *massiliense* from *M. abscessus* subsp. *abscessus* is critical in the clinical environment (7). A significant difference between *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *abscessus-M. abscessus* subsp. *bolletii* in susceptibility to various antimycobacterial drugs has also been observed in our laboratory (17). A significant difference between *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* in treatment response was also noted (18). However, the incidence of *M. abscessus* subsp. *bolletii* infection is very rare, making it difficult to separate its clinical profile from that of an *M. abscessus* subsp. *abscessus* infection.

In Japanese hospitals, a commercially available DNA-DNA hybridization (DDH) assay is frequently used for the clinical identi-

Received 24 May 2013 Returned for modification 23 June 2013
Accepted 1 November 2013
Published ahead of print 6 November 2013
Editor: K. C. Carroll

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.01327-13.

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doi:10.1128/JCM.01327-13

TABLE 1 Primers used in this study

Primer	Sequence	Target and/or purpose (amplified fragment size)	Reference
8F16S	5′-AGAGTTTGATCCTGGCTCAG-3′ (positions 8 to 27)[a]	Mycobacterial 16S rRNA gene, PCR (ca. 1,500 bp), sequencing	21
1047R16S	5′-TGCACACAGGCCACAAGGGA-3′ (positions 1047 to 1028)[a]		
830F16S	5′-GTGTGGGTTTCCTTCCTTGG-3′ (positions 830 to 849)[a]		
1542R16S	5′-AAGGAGGTGATCCAGCCGCA-3′ (positions 1542 to 1523)[a]		
TB11	5′-ACCAACGATGGTGTGTCCAT-3′	Mycobacterial *hsp65* gene, PCR (441 bp), sequencing	22
TB12	5′-CTTGTCGAACCGCATACCCT-3′		
MabrpoF	5′-GAGGGTCAGACCACGATGAC-3′ (positions 2112–2131)[b]	Mycobacterial *rpoB* gene, PCR (449 bp), sequencing	17
MabrpoR	5′-AGCCGATCAGACCGATGTT-3′ (positions 2559–2541)[b]		
ITSF	5′-TTGTACACACCGCCCGTC-3′	Mycobacterial 16S-23S ITS region, PCR (ca. 340 bp), sequencing	23
ITSR	5′-TCTCGATGCCAAGGCATCCACC-3′		

[a] Nucleotide positions were assigned using the *Escherichia coli* 16S rRNA gene sequence as a reference.
[b] Primer design and nucleotide positions were based on the *M. tuberculosis rpoB* gene sequence (GenBank/EMBL/DDBJ accession no. L27989).

fication of mycobacterial strains. However, the reference panel for the DDH Mycobacteria kit consists of only the 18 most common strains of mycobacteria. Although the DDH test is able to clearly differentiate *M. chelonae* from the rest of the *M. chelonae-M. abscessus* group, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* are not included in the panel (19). In fact, isolates from different subpopulations of patients were all identified as *M. abscessus* by the DDH assay. However, these isolates appeared to have different responses to several antimycobacterial drugs (20). These observations led us to develop a simple genotyping test to discriminate *M. abscessus* subsp. *massiliense* from *M. abscessus* using the whole-genome data of *M. abscessus* subsp. *massiliense* as a reference sequence.

MATERIALS AND METHODS

Bacterial strains. An environmental isolate (strain LRC AbsB-1) and 117 clinical isolates were obtained for differential diagnosis from hospitals in Japan (see the Appendix for the list of the hospitals). Of these, 109 strains were isolated from sputum samples and 8 were obtained from skin lesions (see Table S1 in the supplemental material). All of the isolates had been classified as *M. abscessus* based on the results of DDH analysis (DDH Mycobacteria kit; Kyokuto Pharmaceutical Industrial, Tokyo, Japan). This kit contains 18 strains of mycobacteria on the reference panel, which includes *M. abscessus* but not *M. abscessus* subsp. *massiliense* or *M. abscessus* subsp. *bolletii* (19).

Reference strains of the rapidly growing mycobacteria *M. abscessus* subsp. *massiliense* JCM 15300^T, *M. chelonae* JCM 6388^T, *M. abscessus* subsp. *bolletii* JCM 15297^T, and *M. abscessus* subsp. *abscessus* JCM 13569^T (ATCC 19977) were obtained from the Japan Collection of Microorganisms of the Riken Bio-Resource Center (BRC-JCM; Ibaraki, Japan). All bacterial strains were subcultured on 2% Ogawa egg slants or 7H11 agar plates.

Following development of the multiplex PCR assay, several laboratory and clinical isolates that had been classified using DDH assays and/or sequencing were applied to this assay. They included isolates of *M. avium* complex, *M. fortuitum*, *M. gordonae*, *M. kansasii*, *M. lentiflavum*, *M. peregrinum*, *M. shimoidei*, *M. szulgai*, *M. triplex*, *M. tuberculosis*, and *M. xenopi*.

DNA extraction. DNA extraction was performed as described previously (17). In brief, a loopful of bacilli was suspended in 400 μl sterilized phosphate-buffered saline supplemented with 0.05% Tween 80 and stored at −80°C until the extraction was performed. A frozen sample was crushed with zirconia beads (2 mm in diameter) in a bead-beating instrument. Total genomic DNA was purified from the crushed suspension using a High Pure PCR template preparation kit according to the manufacturer's instructions (Roche Diagnostics) and stored at −20°C.

Sequence analysis. The sequences of the clinical and environmental isolates, which had been preliminarily identified as *M. abscessus* with the DDH kit, were compared to those of the *M. abscessus* subsp. *massiliense* JCM 15300^T, *M. abscessus* subsp. *bolletii* JCM 15297^T, *M. chelonae* JCM 6388^T, and *M. abscessus* subsp. *abscessus* JCM 13569^T reference strains. The sequences of the majority of the 16S rRNA gene, partial *hsp65* and *rpoB* genes, and the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes were amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) with the primers listed in Table 1. Both strands were sequenced with BigDye Terminator cycle sequencing kit ver. 3.1 (Applied Biosystems) and run on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Analyses were performed after removing the primers from the sequences (24).

Short-read DNA sequencing using Illumina Genome Analyzer II. A cDNA library of *M. abscessus* subsp. *massiliense* JCM 15300^T, containing fragments of approximately 500 bp in length, was prepared using a genomic DNA Sample Prep kit (Illumina, San Diego, CA). DNA clusters were generated on a slide using a Cluster Generation kit (ver. 2) on an Illumina Cluster Station (Illumina), according to the manufacturer's instructions. All sequencing runs for 83-mers were performed using Illumina Genome Analyzer II (GA II) and an Illumina sequencing kit (ver. 3). Fluorescent images were analyzed using Illumina base-calling pipeline 1.4.0 to obtain FASTQ-formatted sequence data.

***De novo* assembly of short DNA reads.** Prior to *de novo* assembly, reads were divided into 40-, 50-, 60-, or 70-mers from the 5′ end of 83-mer reads followed by nucleotide trimming based on the *phred* quality value (cutoff of 14) using the Euler-SR *qualitytrimmer* command (25). These trimmed sequences were then assembled using Euler-SR v1.0 (25) with the default parameters (vertex size, 25).

Genome scaffold analysis using reference sequences. Reference sequence-assisted gap closing was performed with OSLay v1.0 software (26) using the *Mycobacterium abscessus* ATCC 19977 chromosome DNA sequence as a reference genome (GenBank accession no. NC_010397). Homologous regions between the *de novo* assembly of short reads and ATCC 19977 chromosome DNA were identified by BLASTN searches with 1E-10 as a cutoff value (setting parameters, -m 8 -e 1E-10). Predicted supercontigs (an ordered and oriented set of contigs that contained gaps) were visualized by OSLay (26). Tentative scaffolds of *M. abscessus* subsp. *massiliense* chromosomal DNA sequence were obtained in the same manner as the supercontigs. Pairwise alignment between those genome sequences was performed using a BLASTN homology search (27) followed by visualization of the aligned images with the Artemis Comparison Tool (ACT) (28).

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TABLE 2 List of DNA sequence accession numbers (AB548592 to AB548611)

Strain	16S rRNA (1,468 bp)	*hsp65* (401 bp)	*rpoB* (409 bp)	ITS (298 bp)
M. abscessus subsp. *massiliense* JCM 15300^T	AB548602	AB548601	AB548600	AB548603
M. abscessus subsp. *bolletii* JCM 15297^T	AB548606	AB548605	AB548604	AB548607
M. abscessus subsp. *abscessus* JCM 13569^T	AB548599	AB548598	AB548597	AB548596
M. abscessus subsp. *massiliense* strain A1	AB548592	AB548593	AB548594	AB548595
M. chelonae JCM 6388^T	AB548610	AB548609	AB548608	AB548611

PCR assays. Single-PCR and multiplex PCR assays differentiating *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* were conducted with the listed primers (see Table 4). In brief, 50 μl of a mixture containing 50% AmpliTaq Gold 360 Master Mix (Applied Biosystems), 2% GC enhancer, 0.5 μM (each) primer, and 0.1 μg template DNA was used for PCR with a single set of primers. Amplification was performed in the Mastercycler gradient (Eppendorf) using 95°C for 10 min; 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide.

Nucleotide sequence accession numbers. The DNA sequences of the 16S rRNA (1,468-bp), *hsp65* (401-bp), *rpoB* (409-bp), and ITS (298-bp) fragments from the reference strains (type strains of *M. abscessus* subsp. *massiliense* JCM 15300^T, *M. chelonae* JCM 6388^T, *M. abscessus* subsp. *bolletii* JCM 15297^T, *M. abscessus* subsp. *abscessus* JCM 13569^T, and *M. abscessus* subsp. *massiliense* cutaneous isolate strain A1) were deposited into the International Nucleotide Sequence Databases (INSD) through the DNA Databank of Japan (DDBJ) under accession numbers AB548592 to AB548611 (see Table 2). The draft genome sequences of *M. abscessus* subsp. *massiliense* were deposited under accession numbers BAOM01000001 to BAOM01000060.

RESULTS

Multilocus sequence analysis. Nucleotide sequence analysis was performed with the isolates and reference strains (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, *M. abscessus* subsp. *bolletii*, and *M. chelonae*). The sequences of the 1,468-bp fragment of the 16S rRNA genes of the 118 isolates and the reference strains were almost identical, with only 1-bp mismatches or no mismatches with *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* found at nucleotide position 1007 or 1008 or 1407 or 1408 and additional 3-bp mismatches with *M. chelonae* at nucleotide positions 999, 1039, and 1265 (17). However, differences were observed with the sequences of *hsp65*, *rpoB*, and the ITS region (Table 3). There were two distinct groups. The strains in the first group either had the same *hsp65*/*rpoB*/ITS sequence as the *M. abscessus* subsp. *abscessus* type strain (type 1) or had a 1- or 2-bp difference in *hsp65* and/or the ITS region (type 1a or type 1b). In this group, 58.5% of the strains were classified as *M. abscessus* subsp. *abscessus*, in accordance with the DDH results. The strains in the second group had the same *hsp65*/*rpoB*/ITS sequence as the *M. abscessus* subsp. *massiliense* type strain, with the exception of one base in the ITS region (type 2) or one or two or no base pair differences in the ITS region and/or *rpoB* (type 2a, type 2b, type 2c, and type 2d). In this group, 36.4% of the strains classified as *M. abscessus* by DDH were actually *M. abscessus* subsp. *massiliense*. The strains in the third group, with only 3 isolates included, had the same *hsp65*/*rpoB*/ITS sequence as the *M. abscessus* subsp. *bolletii* type strain, with the exception of 1-bp differences in *rpoB* (type 3a) and/or the ITS region and/or *hsp65* (type 3b and type 3c). In this group, 2.5% of the strains classified as *M. abscessus* by DDH were actually *M. abscessus* subsp. *bolletii*. The remaining three clinical isolates could not be identified by the sequences, because they had discordant sequencing results. The sequences of the *hsp65* genes and ITS regions of the two isolates were identical to those of *M. abscessus* subsp. *abscessus*; however, they carried the *rpoB* sequence of *M. abscessus* subsp. *bolletii* with the 1-bp mismatch (DS type 4). The third isolate had the *M. abscessus* subsp. *massiliense hsp65* and ITS region sequences and the *rpoB* sequence of *M. abscessus* subsp. *abscessus* (DS type 5). An examination of the data from combinations of multilocus sequence analyses can be used to clearly discriminate *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* with 97.5% accuracy.

Whole-genome sequence analysis and primer design. The whole-genome sequence of the *M. abscessus* subsp. *massiliense* type strain was compared with that of the *M. abscessus* subsp. *abscessus* type strain (GenBank accession no. NC_010397) using the ACT to visualize pairwise alignments between the sequences. At least eight notable regions, containing 50- to 800-bp differences, were identified as candidates for PCR targets (i.e., 50- to 800-bp insertions or deletions in *M. abscessus* subsp. *massiliense* compared to *M. abscessus* subsp. *abscessus*). Figure S1 in the supplemental material shows a representative region, which had a 494-bp insertion in *M. abscessus* subsp. *massiliense* at position 3694233 of *M. abscessus* subsp. *abscessus*. The eight regions were labeled MAB_0022c, MAB_0104c, MAB_0357c, MAB_1112c, MAB_1176c, MAB_2847c, MAB_3644, and MAB_4614 with reference to the locations of the open reading frames (ORFs). Regions around MAB_0022c, MAB_0104c, MAB_3644, and MAB_4614 were associated with coding sequences, whereas MAB_0357, MAB_1112c, MAB_1176, and MAB_2847 were associated with noncoding sequences. Eight primer sets were designed using locations in the borders of regions that would sharply differentiate *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* based on the sizes of their PCR amplicons (Table 4). Single-PCR tests were performed to cull the two best primer sets. The following check points were used as selection criteria. (i) Were the isolates amplified? (ii) Were the amplicons clear single bands? (iii) Were the amplicons the correct size? (iv) Were the PCR results discriminating *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense* consistent with the multilocus sequence analysis? All clinical isolates were used in the single-PCR tests; however, tests were terminated when check point 1, 2, or 3 was negative, with the first 20 strains selected. Figure S2 in the supplemental material shows single-PCR results targeting MAB_1176c as representative. As shown in that figure, PCR of all *M. abscessus* subsp. *abscessus* strains produced an amplicon of the expected size, ca. 210 bp. Four clinical *M. abscessus* subsp. *massiliense* strains and the *M. abscessus* subsp. *massiliense* type strain produced an amplicon of ca. 860 bp. However, 8 clinical strains produced an unexpected size of ca. 400 bp. These results suggested that the MAB 1176c primer sets were not suitable for PCR, since the insertion sequence in the *M. abscessus* subsp. *massiliense* clinical strains was not conserved as a uniformly sized sequence of ca. 860 bp. Single-PCR results are shown in Table S2 in the supplemental material. Amplification using MAB 3644, MAB 0022c, and MAB 1112c primer sets produced amplicons of

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