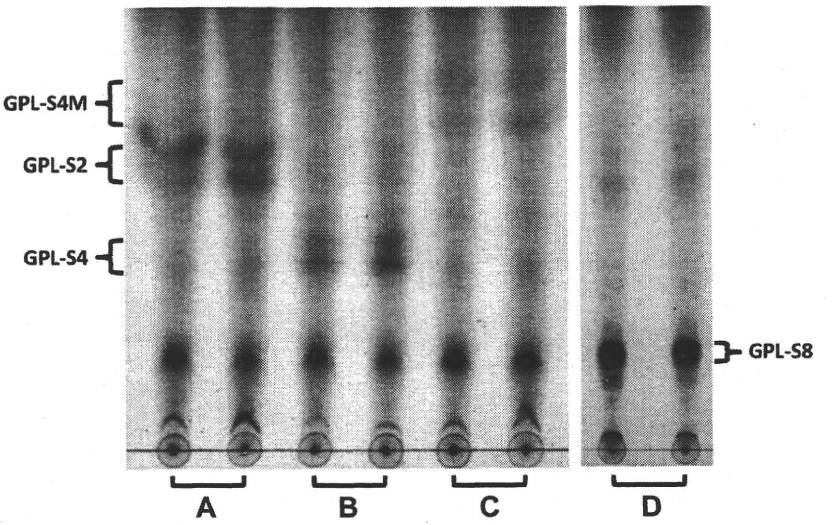

FIG. 2. TLC analysis of crude GPL extracts from recombinant *M. smegmatis* strains MS-S2/pYM301a (A), MS-S2/pYM-hlpA (B), MS-S2/pYM-hlpA-orf2 (C), and MS-S2/pYM-orf3-orf4-orf5 (D). GPL extracts were prepared from the total lipid fraction after a mild alkaline hydrolysis step. Each recombinant strain was tested by two samples derived from independent colonies. Samples were spotted and developed in $CHCl_3$-CH_3OH-H_2O (30:8:1 [vol/vol/vol]).

presence or absence of the methyl group in the fatty acid portion as described above. Thus, the results revealed that the mass unit difference between GPL-S2 (*m/z* 1,465.80, 1,479.82) and GPL-S4 (*m/z* 1,611.84, 1,625.85) was 146 and that between GPL-S2 and GPL-S4M (*m/z* 1,625.89, 1,639.90) was 160, demonstrating that the Rha and 4-*O*-Me-Rha residues were further added to the GPL-S2 to yield GPL-S4 and GPL-S4M, respectively.

The results from TLC, GC-MS, and MALDI-TOF MS analyses strongly suggested that *hlpA* and ORF2 are involved in the formation of 4-*O*-Me Rha. However, it is not clear whether the *hlpA* gene product functions as a glycosyltransferase that transfers a Rha via 1→4 linkage to a Fuc residue, which is observed only for serovar 4-specific GPL. To elucidate the function of *hlpA*, we determined the linkage of sugar moieties of GPL-S4 produced by recombinant strain MS-S2/pYM-hlpA (Fig. 2, lane B). The purified GPL-S4 was subjected to perdeuteriomethylation followed by GC-MS and gave four peaks corresponding to Fuc, 6-d-Tal, Rha, and 2,3,4-tri-*O*-Me-Rha (data not shown). The spectra of Rha and 6-d-Tal demonstrated that the linkage position between these two sugar residues is commonly observed in the oligosaccharide of all ssGPLs, and position C-3 of Rha is linked to the next one, which is consistent with the data previously reported (Fig. 5B and C) (25, 26). In addition, the detection of fragment ions at *m/z* 121, 168, and 206 in spectra of Fuc indicated that its positions C-2 and C-3 were deuteriomethylated (Fig. 5D), meaning that position C-1 of Fuc is linked to position C-3 of Rha and position C-4 of Fuc is linked to the next one. These observations were supported by the fact that GPL-S4 was structurally based on the oligosaccharide of serovar 2-specific GPL. The peak of 2,3,4-tri-*O*-Me-Rha was found to include mixed fragment ions (Fig. 5A). A group of fragment ions corresponding to the spectra of 2,3,4-tri-*O*-Me-Rha linked to alaninol of tetrapeptide was observed. The remaining fragment ions at *m/z* 121, 134, 168, and 181 indicate the presence of deuteriomethyl groups at positions C-2, C-3, and C-4 of the other Rha that is linked at the terminus of oligosaccharide in GPL-S4. These results indicate that position C-1 of terminal Rha is linked to position C-4 of Fuc. Accordingly, the oligosaccharide structures of GPL-S4 were determined to have Rha-(1→4)-Fuc-(1→3)-Rha-(1→2)-6-d-Tal at D-*allo*-Thr, demonstrating that *hlpA* encodes a rhamnosyltransferase that transfers a Rha residue via 1→4 linkage to a Fuc residue of serovar 2-specific GPL (Fig. 6).

DISCUSSION

It is known that serovar 4 is the most prevalent type, and serovar 4-specific GPL, particularly its oligosaccharide portion, plays a role in exhibiting the specific properties that belong to pathogenic factors. However, to date, the biosynthesis of its oligosaccharide portion has not been clarified. In this study, structural determination of three recombinant products, GPL-S2, GPL-S4, and GPL-S4M, revealed that *hlpA* and its downstream gene (ORF2) in the GPL biosynthetic gene cluster are involved in the formation of 4-*O*-methyl Rha, which is unique to serovar 4-specific GPL (Fig. 6). Previously, it was reported that the GPL biosynthetic gene cluster of MAC serovar 2 strains contained one gene whose amino acid sequences are similar to that of *hlpA* with 69% identity (14). This has been regarded as the gene not associated with GPL biosynthesis, because its amino acid sequences are similar to those of hemolytic proteins distributed in some species of bacteria (4). Thus, as shown in Fig. 6, it was surprising that *hlpA* from serovar 4 was found to encode a rhamnosyltransferase that plays a critical role in the pathway leading from serovar 2-specific GPL to serovar 4-specific GPL. For mycobacterium species, a BLAST analysis of HlpA revealed that its homologues are seen only in MAC serovar 2 and not in other species, including *Mycobacterium tuberculosis*. When we tested the

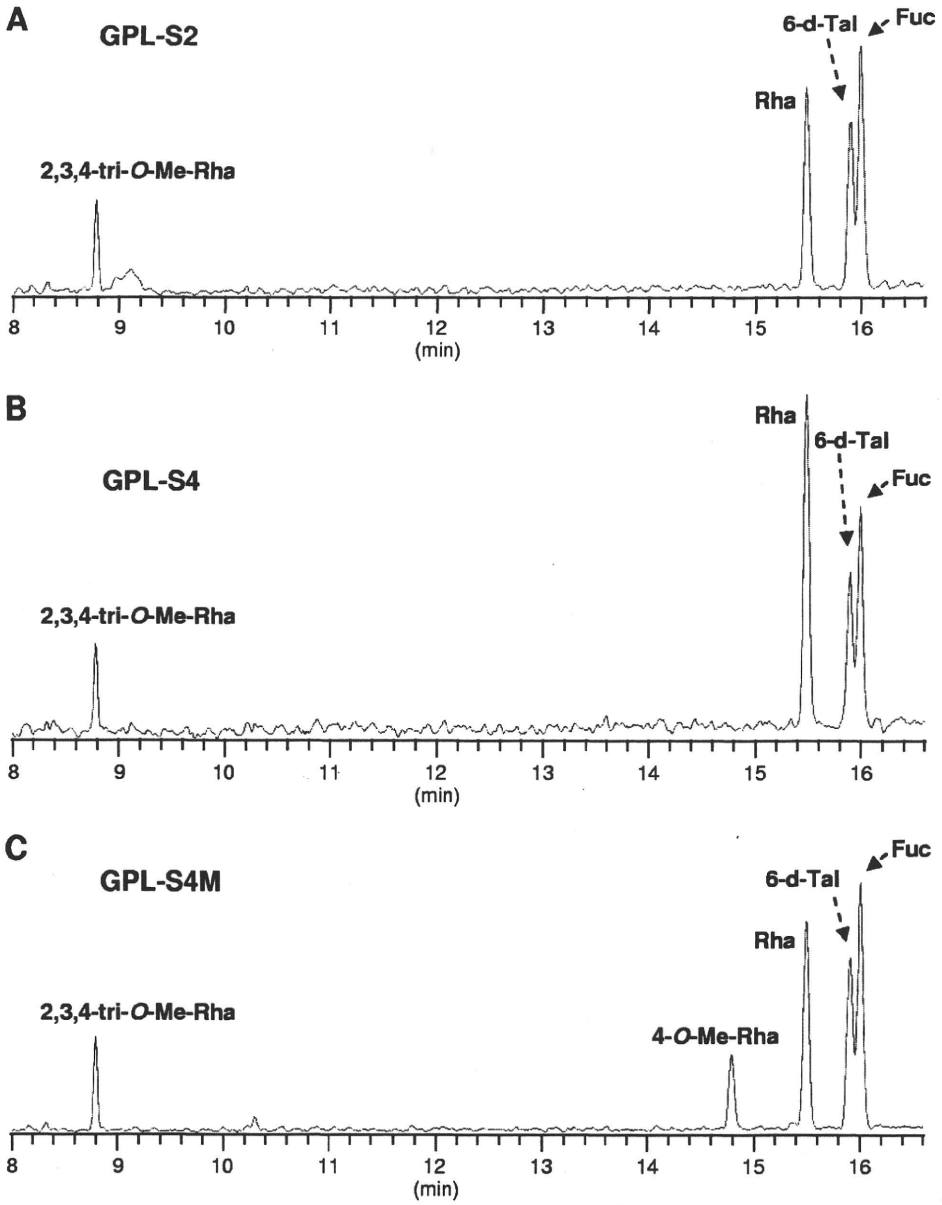

FIG. 3. GC-MS of alditol acetate derivatives from GPL-S2 (A), GPL-S4 (B), and GPL-S4M (C), which were purified from GPL extracts of recombinant *M. smegmatis* strains MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively.

function of HlpA from serovar 2, it did not serve as a glycosyltransferase like HlpA from serovar 4 (data not shown). At present, the function of HlpA from serovar 2 is still unclear, because the biosynthesis of the oligosaccharide portion in serovar 2-specific GPL has been fully elucidated (14, 26). The oligosaccharide structure of serovar 2-specific GPL is basic for several ssGPLs, including serovar 4-specific GPL. In the biosynthetic gene cluster of serovar 2-specific GPL, several insertion sequence (IS) elements are observed, raising the possibility that the HlpA from serovar 2 is retained through genomic alterations that induce biosynthetic changes from other ssGPLs to serovar 2-specific GPL. Therefore, HlpA in the serovar 2 strain originally might function as a glycosyltransferase in the biosynthesis of oligosaccharides of other serovars.

Most HlpA homologues are putatively categorized as hemolytic proteins because they are similar to one protein from *Prevotella intermedia*, which is actually proved to have hemolytic activity (4). Since the amino acid sequences of HlpA show 38% identity and 54% similarity to the above protein of *P. intermedia*, we predicted that HlpA also possesses hemolytic activity as an additional function. However, none was detected when *hlpA* was expressed in *M. smegmatis* and *E. coli* by plate assay using a sheep blood agar plate (data not shown). A BLAST analysis of HlpA homologues showed that they also

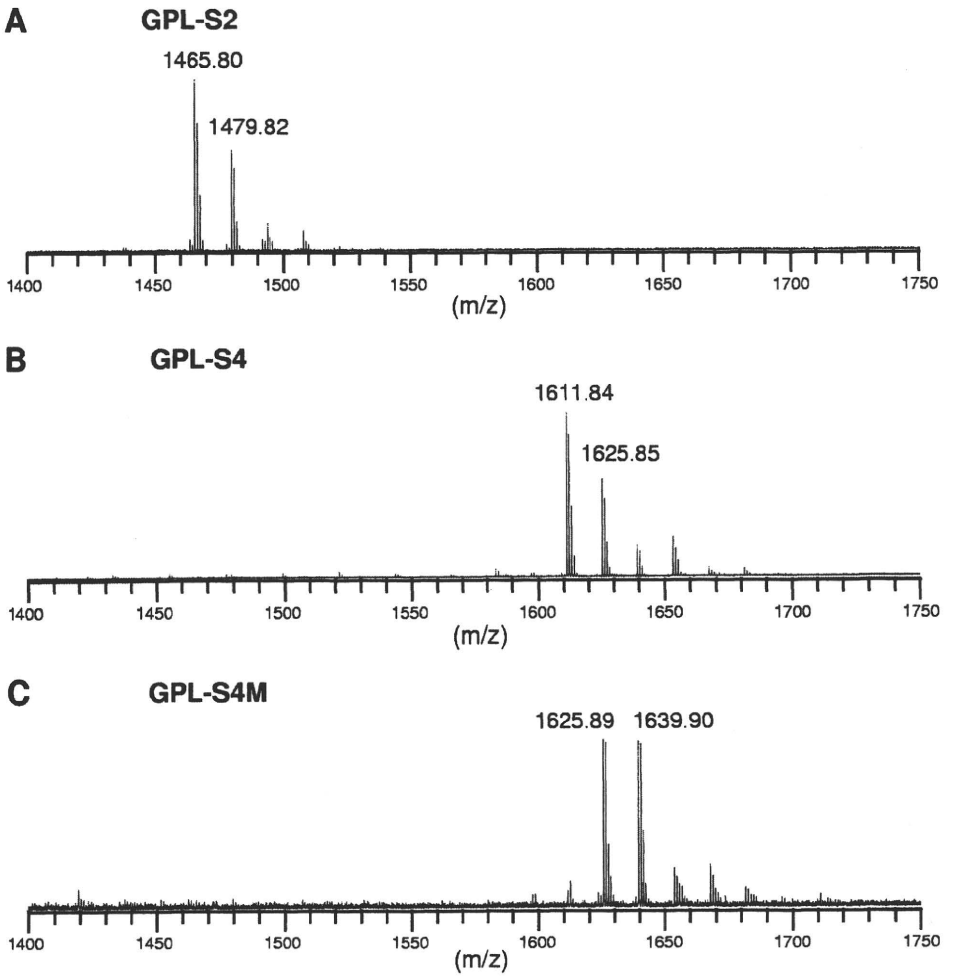

FIG. 4. MALDI-TOF MS of GPL-S2 (A), GPL-S4 (B), and GPL-S4M (C), which were purified from GPL extracts of recombinant *M. smegmatis* strains MS-S2/pYM301a, MS-S2/pYM-hlpA, and MS-S2/pYM-hlpA-orf2, respectively.

contained a partial motif of some glycosyltransferases and methyltransferases. Therefore, it is envisaged that the evolutionary ancestor of HlpA might have lost hemolytic activity in MAC or, conversely, have been altered to retain it in *P. intermedia* in the course of phylogenetic evolution between these bacterial species.

Serovar 4 strains, including ATCC 35767, have been recognized as strains producing the serovar 4-specific GPL but not the serovar 8-specific GPL (24, 35). However, as shown in Fig. 1, we found that the GPL biosynthetic gene cluster contains three known genes (the ORF3, ORF4, and ORF5 genes) previously identified as biosynthetic genes responsible for the formation of 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl glucose residue in the oligosaccharide of serovar 8-specific GPL (25). TLC analysis showed that overexpression of three ORFs potentially produces the serovar 8-specific GPL, including the 4,6-*O*-(1-carboxyethylidene)-3-*O*-methyl glucose residue (Fig. 2, lane D), demonstrating that in the serovar 4 strain, there is inefficient expression of three genes, which might be caused by genomic alterations affecting their transcription, resulting in the loss of serovar 8-specific GPL. Moreover, HlpA homologues are often found in several species of cyanobacteria but not in other bacterial groups and mycobacterium species, implying the occurrence of a certain kind of "horizontal gene transfer" between these environmental bacteria. Thus, MAC seemed to incorporate foreign genes or realign preexisting genes to modify the oligosaccharide structures of GPLs for their survival in a varied environment. In terms of sugar composition and linkage affecting the properties of ssGPLs, the functional aspects of the 4-*O*-methyl-Rha residue, which influence the interactions with the host cell, are still unclear. In addition, the sugar linkage Rha-(1→4)-Fuc is seen only in serovar 4-specific GPL and not in other ssGPLs, suggesting that it might generate unique properties that differ notably from those generated by other sugar linkages. Also, the rarity of this sugar linkage could be one of the factors that define the specificity of MAC serovar 4, which would be resolved by further studies, including the generation of an *hlpA* knockout mutant. For functional characterization of *hlpA* and ORF2, we have adopted the gene expression experiment

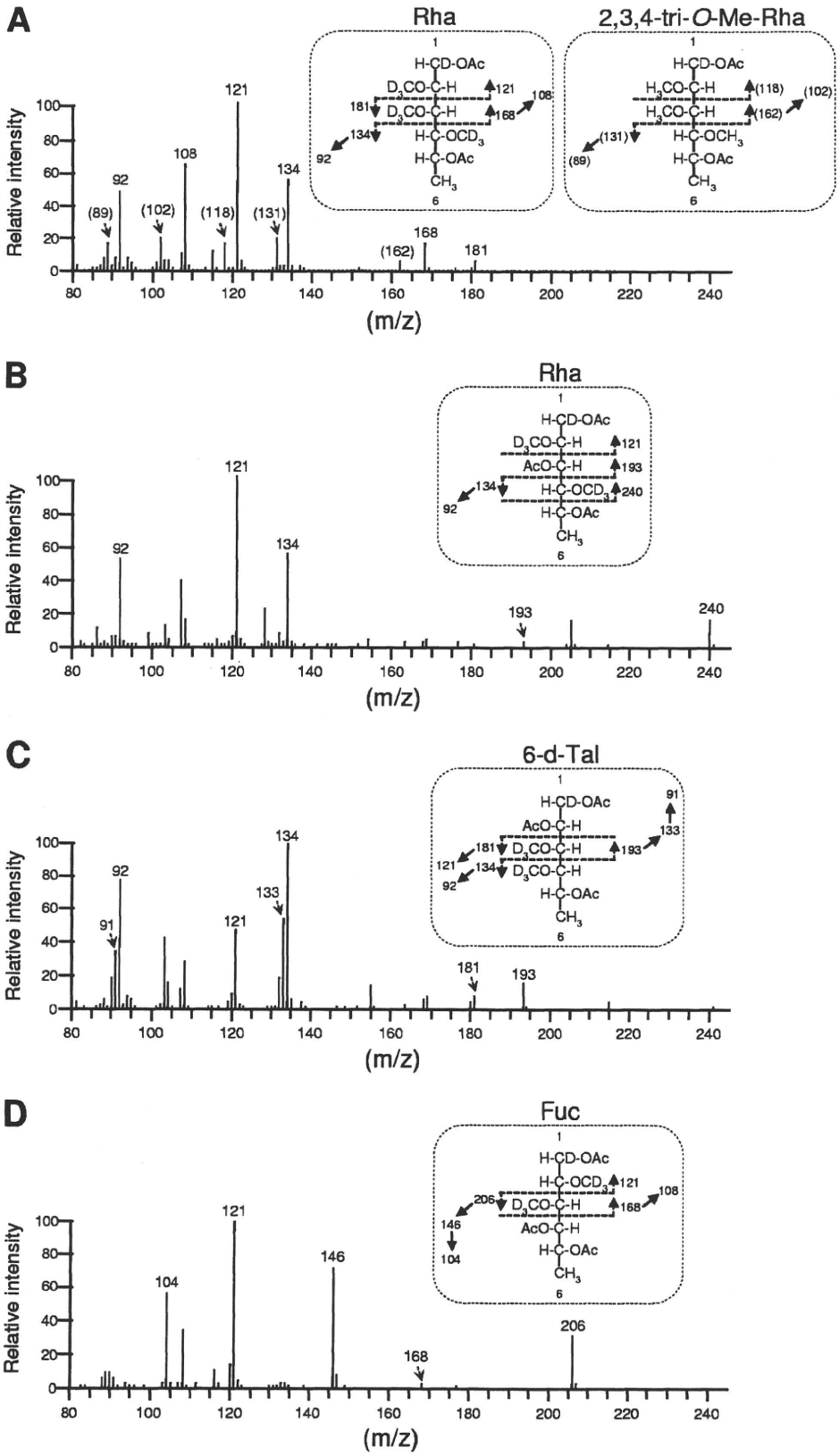

FIG. 5. GC-MS spectra and fragment ion assignments of 2,3,4-tri-*O*-Me-Rha (A), Rha (A and B), 6-*d*-Tal (C), and Fuc (D), which are derived from alditol acetates of sugars released from deuteriomethylated GPL-S4. Ac, acetate; D, deuterium.

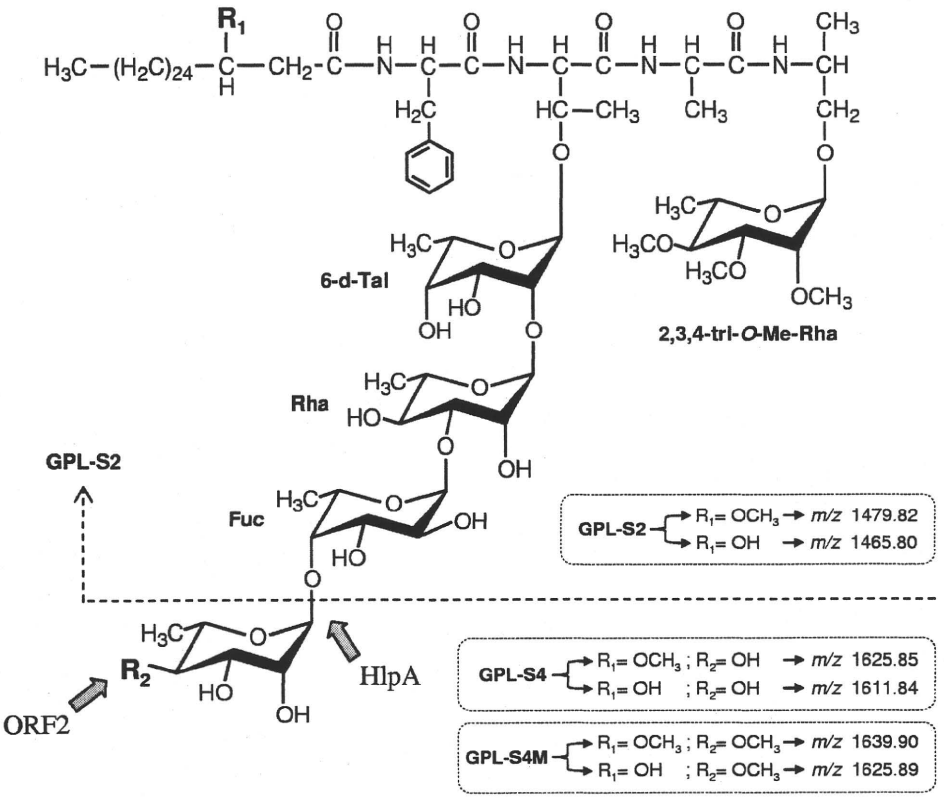

FIG. 6. Proposed structures and biosynthetic pathways for GPL-S2, GPL-S4, and GPL-S4M. Parentheses indicate structural differences between three compounds, which are deduced from MALDI-TOF MS analyses {pseudomolecular ions ($[M + Na]^+$)}.

using the *M. smegmatis* strain. Further enzymatic analyses, such as *in vitro* testing with recombinant proteins, would confirm our results. Taken together, these findings may contribute to understanding the mechanism for generation of structural and functional diversity of GPLs as well as their biological role in MAC.

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REFERENCES

1. **Askgaard, D. S., S. B. Giese, S. Thybo, A. Lerche, and J. Bennedsen.** 1994. Serovars of *Mycobacterium avium* complex isolated from patients in Denmark. J. Clin. Microbiol. **32:**2880–2882.
2. **Aspinall, G. O., D. Chatterjee, and P. J. Brennan.** 1995. The variable surface glycolipids of mycobacteria: structures, synthesis of epitopes, and biological properties. Adv. Carbohydr. Chem. Biochem. **51:**169–242.
3. **Barrow, W. W., T. L. Davis, E. L. Wright, V. Labrousse, M. Bachelet, and N. Rastogi.** 1995. Immunomodulatory spectrum of lipids associated with *Mycobacterium avium* serovar 8. Infect. Immun. **63:**126–133.
4. **Beem, J. E., W. E. Nesbitt, and K. P. Leung.** 1999. Cloning of *Prevotella intermedia* loci demonstrating multiple hemolytic domains. Oral Microbiol. Immunol. **14:**143–152.
5. **Belisle, J. T., K. Klaczkiewicz, P. J. Brennan, W. R. Jacobs, Jr., and J. M. Inamine.** 1993. Rough morphological variants of *Mycobacterium avium*: characterization of genomic deletions resulting in the loss of glycopeptidolipid expression. J. Biol. Chem. **268:**10517–10523.
6. **Birkness, K. A., W. E. Swords, P. H. Huang, E. H. White, C. S. Dezzutti, R. B. Lal, and F. D. Quinn.** 1999. Observed differences in virulence-associated phenotypes between a human clinical isolate and a veterinary isolate of *Mycobacterium avium*. Infect. Immun. **67:**4895–4901.
7. **Bjorndal, H., C. G. Hellerqvist, B. Lindberg, and S. Svensson.** 1970. Gas-liquid chromatography and mass spectrometry in methylation analysis of polysaccharides. Angew. Chem. Internat. Ed. Engl. **9:**610–619.
8. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
9. **Camphausen, R. T., R. L. Jones, and P. J. Brennan.** 1986. Structure and relevance of the oligosaccharide hapten of *Mycobacterium avium* serotype 2. J. Bacteriol. **168:**660–667.
10. **Chatterjee, D., and K. H. Khoo.** 2001. The surface glycopeptidolipids of mycobacteria: structures and biological properties. Cell. Mol. Life Sci. **58:** 2018–2042.
11. **Ciucanu, I., and F. Kerek.** 1984. A simple and rapid method for the permethylation of carbohydrates. Carbohydr. Res. **131:**209–217.
12. **Daffe, M., and P. Draper.** 1998. The envelope layers of mycobacteria with reference to their pathogenicity. Adv. Microb. Physiol. **39:**131–203.
13. **Daffe, M., M. A. Laneelle, and G. Puzo.** 1983. Structural elucidation by field desorption and electron-impact mass spectrometry of the C-mycosides isolated from *Mycobacterium smegmatis*. Biochim. Biophys. Acta **751:**439–443.
14. **Eckstein, T. M., J. T. Belisle, and J. M. Inamine.** 2003. Proposed pathway for the biosynthesis of serovar-specific glycopeptidolipids in *Mycobacterium avium* serovar 2. Microbiology **149:**2797–2807.
15. **Eckstein, T. M., F. S. Silbaq, D. Chatterjee, N. J. Kelly, P. J. Brennan, and J. T. Belisle.** 1998. Identification and recombinant expression of a *Mycobacterium avium* rhamnosyltransferase gene (*rtfA*) involved in glycopeptidolipid biosynthesis. J. Bacteriol. **180:**5567–5573.
16. **Fujiwara, N., N. Nakata, S. Maeda, T. Naka, M. Doe, I. Yano, and K. Kobayashi.** 2007. Structural characterization of a specific glycopeptidolipid containing a novel *N-acyl*-deoxy sugar from *Mycobacterium intracellulare* serotype 7 and genetic analysis of its glycosylation pathway. J. Bacteriol. **189:**1099–1108.
17. **Fujiwara, N., N. Nakata, T. Naka, I. Yano, M. Doe, D. Chatterjee, M. McNeil, P. J. Brennan, K. Kobayashi, M. Makino, S. Matsumoto, H. Ogura, and S. Maeda.** 2008. Structural analysis and biosynthesis gene cluster of an antigenic glycopeptidolipid from *Mycobacterium intracellulare*. J. Bacteriol. **190:**3613–3621.
18. **Horgen, L., E. L. Barrow, W. W. Barrow, and N. Rastogi.** 2000. Exposure of human peripheral blood mononuclear cells to total lipids and serovar-spe-

cific glycopeptidolipids from *Mycobacterium avium* serovars 4 and 8 results in inhibition of TH1-type responses. Microb. Pathog. **29:**9–16.
19. **Jeevarajah, D., J. H. Patterson, M. J. McConville, and H. Billman-Jacobe.** 2002. Modification of glycopeptidolipids by an *O*-methyltransferase of *Mycobacterium smegmatis*. Microbiology **148:**3079–3087.
20. **Julander, I., S. Hoffner, B. Petrini, and L. Ostlund.** 1996. Multiple serovars of *Mycobacterium avium* complex in patients with AIDS. APMIS **104:**318–320.
21. **Kano, H., T. Doi, Y. Fujita, H. Takimoto, I. Yano, and Y. Kumazawa.** 2005. Serotype-specific modulation of human monocyte functions by glycopeptidolipid (GPL) isolated from *Mycobacterium avium* complex. Biol. Pharm. Bull. **28:**335–339.
22. **Krzywinska, E., and J. S. Schorey.** 2003. Characterization of genetic differences between *Mycobacterium avium* subsp. *avium* strains of diverse virulence with a focus on the glycopeptidolipid biosynthesis cluster. Vet. Microbiol. **91:**249–264.
23. **Maekura, R., Y. Okuda, A. Hirotani, S. Kitada, T. Hiraga, K. Yoshimura, I. Yano, K. Kobayashi, and M. Ito.** 2005. Clinical and prognostic importance of serotyping *Mycobacterium avium-Mycobacterium intracellulare* complex isolates in human immunodeficiency virus-negative patients. J. Clin. Microbiol. **43:**3150–3158.
24. **McNeil, M., A. Y. Tsang, and P. J. Brennan.** 1987. Structure and antigenicity of the specific oligosaccharide hapten from the glycopeptidolipid antigen of *Mycobacterium avium* serotype 4, the dominant mycobacterium isolated from patients with acquired immune deficiency syndrome. J. Biol. Chem. **262:**2630–2635.
25. **Miyamoto, Y., T. Mukai, Y. Maeda, M. Kai, T. Naka, I. Yano, and M. Makino.** 2008. The *Mycobacterium avium* complex *gtfTB* gene encodes a glucosyltransferase required for the biosynthesis of serovar 8-specific glycopeptidolipid. J. Bacteriol. **190:**7918–7924.
26. **Miyamoto, Y., T. Mukai, Y. Maeda, N. Nakata, M. Kai, T. Naka, I. Yano, and M. Makino.** 2007. Characterization of the fucosylation pathway in the biosynthesis of glycopeptidolipids from *Mycobacterium avium* complex. J. Bacteriol. **189:**5515–5522.
27. **Miyamoto, Y., T. Mukai, F. Takeshita, N. Nakata, Y. Maeda, M. Kai, and M. Makino.** 2004. Aggregation of mycobacteria caused by disruption of fibronectin-attachment protein-encoding gene. FEMS Microbiol. Lett. **236:**227–234.
28. **Patterson, J. H., M. J. McConville, R. E. Haites, R. L. Coppel, and H. Billman-Jacobe.** 2000. Identification of a methyltransferase from *Mycobacterium smegmatis* involved in glycopeptidolipid synthesis. J. Biol. Chem. **275:**24900–24906.
29. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4:**1911–1919.
30. **Sweet, L., and J. S. Schorey.** 2006. Glycopeptidolipids from *Mycobacterium avium* promote macrophage activation in a TLR2- and MyD88-dependent manner. J. Leukoc. Biol. **80:**415–423.
31. **Sweet, L., W. Zhang, H. Torres-Fewell, A. Serianni, W. Boggess, and J. Schorey.** 2008. *Mycobacterium avium* glycopeptidolipids require specific acetylation and methylation patterns for signaling through toll-like receptor 2. J. Biol. Chem. **283:**33221–33231.
32. **Tassell, S. K., M. Pourshafie, E. L. Wright, M. G. Richmond, and W. W. Barrow.** 1992. Modified lymphocyte response to mitogens induced by the lipopeptide fragment derived from *Mycobacterium avium* serovar-specific glycopeptidolipids. Infect. Immun. **60:**706–711.
33. **Tsang, A. Y., J. C. Denner, P. J. Brennan, and J. K. McClatchy.** 1992. Clinical and epidemiological importance of typing of *Mycobacterium avium* complex isolates. J. Clin. Microbiol. **30:**479–484.
34. **Vergne, I., and M. Daffe.** 1998. Interaction of mycobacterial glycolipids with host cells. Front. Biosci. **3:**d865–876.
35. **Wayne, L. G., R. C. Good, A. Tsang, R. Butler, D. Dawson, D. Groothuis, W. Gross, J. Hawkins, J. Kilburn, M. Kubin, K. H. Schroder, V. A. Silcox, C. Smith, M. F. Thorel, C. Woodley, and M. A. Yakrus.** 1993. Serovar determination and molecular taxonomic correlation in *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*: a cooperative study of the International Working Group on Mycobacterial Taxonomy. Int. J. Syst. Bacteriol. **43:**482–489.
36. **Yakrus, M. A., and R. C. Good.** 1990. Geographic distribution, frequency, and specimen source of *Mycobacterium avium* complex serotypes isolated from patients with acquired immunodeficiency syndrome. J. Clin. Microbiol. **28:**926–929.

Enhanced Activation of T Lymphocytes by Urease-Deficient Recombinant Bacillus Calmette-Guérin Producing Heat Shock Protein 70-Major Membrane Protein-II Fusion Protein

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

To activate naive T cells convincingly using *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), recombinant BCG (BCG-D70M) that was deficient in urease, expressed with gene encoding the fusion of BCG-derived heat shock protein (HSP) 70 and *Mycobacterium leprae*-derived major membrane protein (MMP)-II, one of the immunodominant Ags of *M. leprae*, was newly constructed. BCG-D70M was more potent in activation of both $CD4^+$ and $CD8^+$ subsets of naive T cells than recombinant BCGs including urease-deficient BCG and BCG-70M secreting HSP70–MMP-II fusion protein. BCG-D70M efficiently activated dendritic cells (DCs) to induce cytokine production and phenotypic changes and activated $CD4^+$ T cells even when macrophages were used as APCs. The activation of both subsets of T cells was MHC and CD86 dependent. Pretreatment of DCs with chloroquine inhibited both surface expression of MMP-II on DCs and the activation of T cells by BCG-D70M–infected APCs. The naive $CD8^+$ T cell activation was inhibited by treatment of DCs with brefeldin A and lactacystin so that the T cell was activated by TAP- and proteosome-dependent cytosolic cross-priming pathway. From naive $CD8^+$ T cells, effector T cells producing perforin and memory T cells having migration markers were produced by BCG-D70M stimulation. BCG-D70M primary infection in C57BL/6 mice produced T cells responsive to in vitro secondary stimulation with MMP-II and HSP70 and more efficiently inhibited the multiplication of subsequently challenged *M. leprae* than vector control BCG. These results indicate that the triple combination of HSP70, MMP-II, and urease depletion may provide a useful tool for inducing better activation of naive T cells. *The Journal of Immunology*, 2010, 185: 6234–6243.

Mycobacterium leprae is a causative bacterium of leprosy (1, 2). Leprosy is clinically divided into two major categories, paucibacillary and multibacillary leprosy (2). In the lesion of paucibacillary leprosy, $CD1a^+$ dendritic cells (DCs) are involved, and the substantially activated T cells are observed (3, 4). These observations indicate that host defense activity against *M. leprae* is chiefly conducted by adaptive immunities, and both IFN-γ–producing type 1 $CD4^+$ T cells and $CD8^+$ T cells act to inhibit the active multiplication of *M. leprae*. Thus, few numbers of bacilli are observed in the lesion of paucibacillary leprosy. The activation of T cells is induced by DCs loaded with bacilli or its component, which display various antigenic molecules on their surface, including the immunodominant Ags (5, 6). Previously, we identified major membrane protein (MMP)-II (gene name bfrA or ML 2058) as one of the immunodominant Ags of *M. leprae* (7). MMP-II ligates TLR2 and consequently activates the NF-κB pathway (7). DCs pulsed with MMP-II protein activate both naive and memory type $CD4^+$ and $CD8^+$ T cells to produce IFN-γ in an Ag-specific fashion (7, 8). Further, the MMP-II is supposed to be recognized by T cells in vivo of *M. leprae*-infected individuals, including paucibacillary leprosy patients (8).

Multidrug therapy introduced by the World Health Organization has been effective to reduce the number of leprosy patients registered. However, the drug therapy seems ineffective to reduce the number of newly developed leprosy patients; thus, the useful vaccine is essential to control the number of new patients. So far, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is used as a vaccine against leprosy, although not broadly (9–11). However, nowadays, BCG is not recognized as a reliable vaccine, because an overall efficacy of BCG against leprosy is reported to be 26%, which is calculated by meta-analyses enrolling several studies and observations (12). However, BCG intrinsically possesses the ability to activate type 1 $CD4^+$ T cells, although not convincingly, and may share some antigenic molecules with *M. leprae* (9, 10). These observations suggest that the improvement of BCG may be one of the critical ways to develop new effective vaccines against leprosy. However, BCG also has its intrinsic defect, an activity to inhibit the fusion of BCG-infected phagosomes with lysosomes (13–15). This defect seems to be a major factor associated with unconvincing activation of naive T cells. Therefore, we tried to upregulate the T cell-stimulating activity of BCG by overcoming the intrinsic defect of the bacteria. First of all, we produced recombinant BCG (rBCG) (BCG-SM) that secretes MMP-II in the infected cells (16). As expected, BCG-SM substantially activated both naive $CD4^+$ and $CD8^+$ T cells and consequently inhibited the growth of *M. leprae* to some extent, but not completely, in the

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Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; DC, dendritic cell; HSP, heat shock protein; MMP, major membrane protein; MOI, multiplicity of infection; rBCG, recombinant BCG.

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footpad of C57BL/6 mice (17). It is known that parent BCG partially activates naive $CD4^+$ T cells but is not efficient in stimulating naive $CD8^+$ T cells quickly to produce IFN-γ (14, 16). In this respect, the fact that BCG-SM can activate DCs to produce IL-12p70 and both subsets of naive T cells to produce IFN-γ indicates that secretion of MMP-II of *M. leprae* presumably in phagosomes of APCs of host cells is a useful strategy to activate both APCs and T cells (16). Usefulness of the enhancement of secretion of vaccinated BCG-derived Ags is revealed in the other intracellular infection system such as *Mycobacterium tuberculosis*, in which the active secretion of Ag85 complex was effective in inhibiting the replication of subsequently challenged *M. tuberculosis* (18).

Then, we undertook two other strategies to further enhance the T cell-stimulating activity of BCG. One of them was aimed at potentiating the activation of naive $CD4^+$ T cells. BCG possesses urease, which produces ammonia and inhibited the acidication of BCG-infected phagosomes to avoid the fusion with lysosomes (19, 20). To inhibit the ammonia production, we produced urease-deficient BCG (BCG-ΔUT-11-3) (15). BCG-ΔUT-11-3 was feasibly translocated into lysosomes and activated both DCs and naive $CD4^+$ T cells of human (15). Further, BCG-ΔUT-11-3 efficiently produces memory type $CD4^+$ T cells in mice that can recognize *M. leprae*-derived proteins (15). Thus, the disruption of the *UreC* gene of BCG is useful tool to enhance the $CD4^+$ T cell-activating activity of BCG. The second strategy for potentiation of BCG activity is aimed to provide BCG the ability of activating IFN-γ–producing $CD8^+$ T cells quickly and strongly. To this end, we used heat shock protein 70 (HSP70) (21–24). The gene encoding HSP70 was directly connected with that of MMP-II and was extrachromosomally transformed into BCG (production of BCG-70M). BCG-70M secreted HSP70–MMP-II fusion protein and activated not only Ag-specific naive $CD8^+$ T cells polyclonally, but also naive $CD4^+$ T cells and DCs (25). Thus, the production and secretion of HSP70 in phagosomes accompanied by MMP-II seems an effective strategy to activate human naive $CD8^+$ T cells using BCG.

Because we employed two independent strategies to overcome the intrinsic defect of BCG (inhibition of phagosome-lysosome fusion), in this study, we combined the two strategies and produced another rBCG (BCG-D70M) that is deficient in urease activity but is introduced with the gene encoding HSP70–MMP-II fusion protein and evaluated its immunostimulatory activities. The BCG-D70M showed the strongest activities in terms of activation of naive $CD4^+$ and $CD8^+$ T cells among the rBCGs produced by us so far.

Materials and Methods

Preparation of cells and Ags

Peripheral blood was obtained from healthy purified protein derivative-positive individuals under informed consent. In Japan, BCG vaccination is compulsory for children (0–4 y old). PBMCs were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (26). The viability of T cells obtained from cryopreserved PBMCs was >90%, and no selection in terms of functionality was induced in both monocytes and T cells by the cryopreservation of PBMCs. For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 mAb (Dynabeads 450, Dynal Biotech, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on collagen-coated plates, and the nonplastic adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (27). Monocyte-derived DCs were differentiated as described previously (26, 28). Briefly, monocytes were cultured in the presence of 50 ng rGM-CSF (PeproTech EC, London, U.K.) and 10 ng/ml rIL-4 (PeproTech) (28). On day 4 of culture, immature DCs were infected with rBCG at an indicated multiplicity of infection (MOI), and, on day 6 of culture, DCs were used for further analyses of surface Ag and mixed lymphocyte assays. Macrophages were differentiated as described previously (29, 30). In brief, monocytes were cultured in the presence of 10 ng/ml rM-CSF (R&D Systems, Minneapolis, MN). On day 5 of culture, macrophages were infected with rBCG at an indicated MOI, and, on day 7 of culture, they were used for further analyses of mixed lymphocyte assay. The rMMP-II protein was produced as described previously (7, 31), and the rHSP70 Ag was purchased (HyTest, Turku, Finland).

Vector construction and preparation of rBCG

The genomic DNAs from BCG substrain Tokyo and from *M. leprae* Thai-53 strain were extracted by using phenol-chloroform. The oligonucleotide primers for the *hsp70* gene were FMb70Bal (5′-aaaTGGCCAtggctcgtgcggtcggg-3′; capital letters indicate a BalI site) and RMb70Eco (5′-aaaGAATTCcttggcctcccggccg-3′; capital letters indicate an EcoRI site). The primers for MMP-II sequence from *M. leprae* genomic DNA was amplified with FMMP Eco4 (5′-aaaGAATTCcaaggtgatccggatgt-3′; capital letters indicate an EcoRI site) and RMMP Sal (5′-tgaGTCGACttaactcggcggccggga-3′; capital letters indicate a SalI site). The amplified products were digested with appropriate restriction enzymes and cloned into BalI-SalI–digested parental pMV261 plasmid. For replacing kanamycin resistance gene to hygromycin resistance cassette, the XbaI-NheI fragment from pYUB854 (32) was cloned into SpeI-NheI fragment of each plasmid (32). The rBCG that lacks *ure*C gene (BCG-ΔUT-11) was produced as described previously (15). The hygromycin cassette was removed by using a plasmid encoding γδ-resolvase (γδ-*tnp*R) encoded in pTYUB870 (32). The unmarked BCG having the hygromycin gene was named BCG-ΔUT-11-3. The HSP70–MMP-II fusion protein expression vector was introduced into BCG-ΔUT-11-3 by electroporation method. BCG-70M was produced as described previously (25). BCG-ΔUT-11-3 containing pMV-HSP70-MMP-II as an extrachromosomal plasmid is referred to as BCG-D70M and that containing pMV-261-hygromycin is referred to as BCG-261H (BCG vector control). rBCGs were grown to log phase and stored at 10^8 CFU/ml at −80°C. Preinfection to DCs and macrophages, BCGs were counted by colony assay method. There is no significant difference in the in vitro culture growth between BCG-261H and BCG-D70M.

Western blot analyses of the fusion protein HSP70–MMP-II extracted from rBCGs

To verify the expression of MMP-II and HSP70 in rBCGs, we prepared cell lysates from BCG-70M and BCG-D70M as described previously (16). Briefly, the protein fraction of the rBCGs was prepared as follows: harvested cells were washed with PBS and sonicated. Disrupted cells were centrifuged at 10,000 × *g* at 4°C, and the supernatant was taken as the cell lysate. SDS-PAGE and electroblotting were carried out using standard methods. Western blotting was performed as follows: a polyvinylidene difluoride membrane having the transferred protein was blocked in 5% skim milk and then incubated with anti–MMP-II mAb 202-3 (IgG2a) or anti-mycobacterial HSP70 mAb (HyTest), which is not cross-reactive to mammalian HSP70 homologs. Anti-Ag85B rabbit polyclonal Ab was used as an internal control. An alkaline phosphatase-conjugated anti-mouse IgG Ab (Biosource International, Camarillo, CA) or alkaline phosphatase-conjugated anti-rabbit IgG Ab (Tago, Burlingame, CA) was used as the secondary Ab. Color development was performed using NBT/BCIP detection reagent (Calbiochem, San Diego, CA).

Analysis of cell surface Ag

The expression of cell surface Ag on DCs and lymphocytes was analyzed using FACSCalibur (BD Biosciences). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich, St. Louis, MO), and 1×10^4 live cells were analyzed. For the analysis of the cell surface Ag, the following mAbs were used: FITC-conjugated mAb against HLA-ABC (G46-2.6, BD Biosciences, San Jose, CA), HLA-DR (L243, BD Biosciences), CD86 (FUN-1, BD Biosciences), CD83 (HB15a, Immunotech, Marseille, France), CD1a (NA1/34, DakoCytomation, Glostrup, Denmark), CD62L (Dreg 56, BD Biosciences), CCR7 (clone 150503, R&D Systems), CD27 (M-T271, BD Bioscience), and PE-conjugated mAb to CD162 (TB5, Exbio, Praha, Czech), CD8 (RPA-T8, BD Biosciences), and CD4 (RPA-T4, BD Biosciences).

The expression of MMP-II on rBCG-infected DCs was determined using the mAb against MMP-II (M270-13, IgM, κ), which probably detects MMP-II complexed with MHC molecules on the surface of DC (8), followed by FITC-conjugated anti-mouse Igs Ab (Tago-immunologicals,

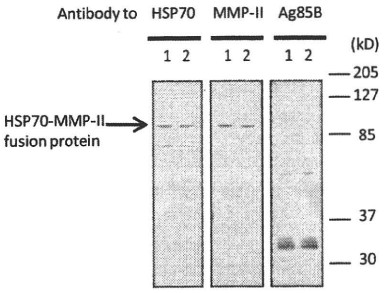

FIGURE 1. Western blotting analysis of protein fraction extracted from BCG-70M and BCG-D70M. An mAb to either MMP-II or HSP70 was used to detect HSP70–MMP-II fusion protein. An anti-Ag85B rabbit polyclonal Ab was also used to detect Ag85B (an internal control). *Lane 1*, Cell lysate of BCG-70M; *lane 2*, cell lysate of BCG-D70M.

Camarillo, CA). For the inhibition of the intracellular processing of phagocytosed bacteria, DCs were treated with 50 μM chloroquine (Sigma-Aldrich) for 2 h, washed, and subsequently infected with rBCG and subjected to analyses of MMP-II surface expression. The intracellular production of perforin was assessed as follows: naive $CD8^+$ T cells were stimulated with rBCG-infected DCs for 5 d in the presence of naive $CD4^+$ T cells, and $CD8^+$ T cells were surface stained with PE-labeled mAb to CD8 and were fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using Permeabilizing solution (BD Biosciences) and stained with FITC-conjugated mAb to perforin (δG9, BD Biosciences) or FITC-labeled isotype control.

APC functions of DCs

The ability of BCG-infected DCs and macrophages to stimulate T cells was assessed using an autologous APC–T cell coculture as previously described (6, 28). Purification of $CD4^+$ and $CD8^+$ T cells was conducted by using negative-isolation kits (Dynabeads 450, Dynal Biotech) (28). The purity of the $CD4^+$ and $CD8^+$ T cells was >95% when assessed using an FACS-Calibur (BD Biosciences). Naive $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with mAb to CD45RO, which were followed by beads coated with mAb to goat anti-mouse IgGs (Dynal Biotech). The purity of both subsets of naive T cells was >97%. However, there was no contamination of memory type T cells in the naive T cell preparations. More than 98% of $CD45RA^+$ T cells were positive in the expression of CCR7 molecule. Memory type T cells were similarly produced by the treatment of cells with mAb to CD45RA Ag. The purified responder cells (1×10^5/well) were plated in 96-well round-bottom tissue-culture plates, and DCs or macrophages infected with rBCG were added to give the indicated APC/T cell ratio. Supernatants of APC–T cell cocultures were collected on day 4, and the cytokine levels were determined. In some cases, rBCG-infected DCs and macrophages were treated with mAb to HLA-ABC (W6/32, mouse IgG2a, κ), HLA-DR (L243, mouse IgG2a, κ), CD86 (IT2.2, mouse IgG2b, κ, BD Biosciences), MMP-II (M270-13), or normal mouse IgG. The optimal concentration was determined in advance. Also, in some cases, immature DCs and macrophages were treated with the indicated dose of chloroquine, brefeldin A (Sigma-Aldrich), or lactacystin (Sigma-Aldrich) and subsequently infected with BCG-D70M. The optimal dose of these reagents was determined in advance.

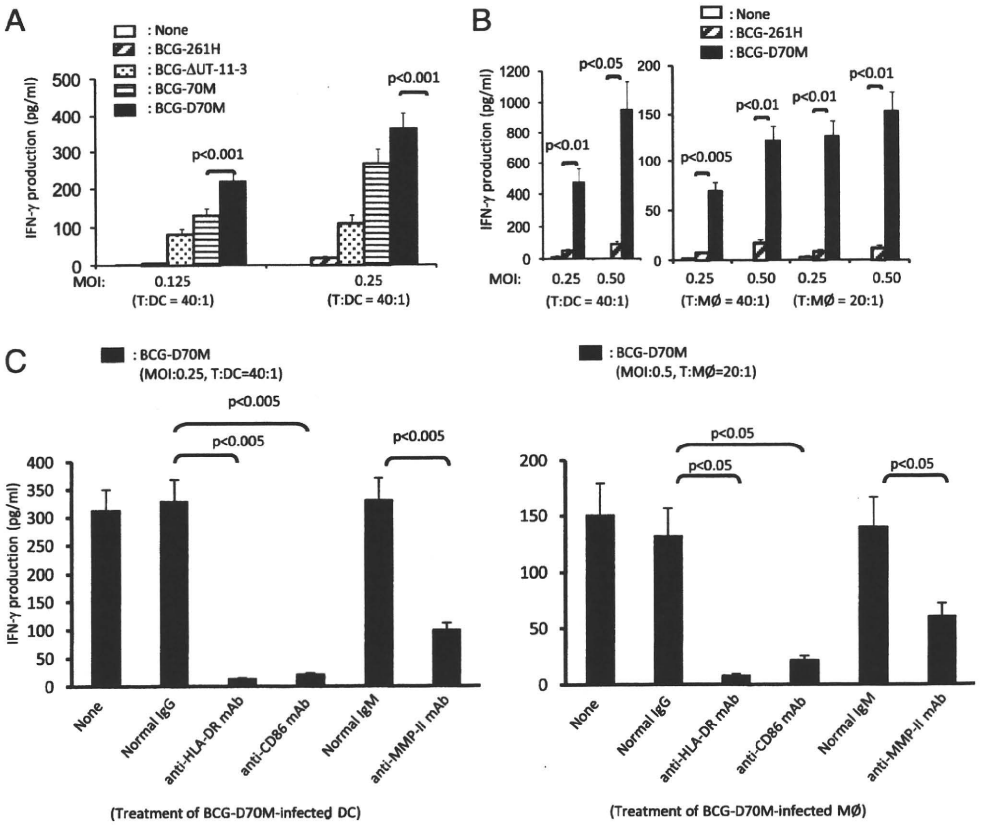

FIGURE 2. *A*, IFN-γ production from naive $CD4^+$ T cells by stimulation with rBCG. Monocyte-derived DCs were infected with BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M at the indicated MOI and were used as a stimulator of naive $CD4^+$ T cells in a 4-d culture. Total of 10^5 responder T cells were stimulated with the BCG-infected DCs at T/DC ratio of 40:1. *B*, IFN-γ production from unseparated $CD4^+$ T cells by stimulation with rBCG. Monocyte-derived DCs or macrophages were infected with either BCG-261H or BCG-D70M at the indicated MOI and were used as a stimulator of unseparated $CD4^+$ T cells in a 4-d culture. Total of 10^5 responder T cells were stimulated with the indicated dose of BCG-infected DCs or macrophages. *C*, Inhibition of naive or unseparated $CD4^+$ T cell activation by the treatment of BCG-D70M–infected DCs or BCG-D70M–infected macrophages, respectively, with mAb. Monocyte-derived DCs or macrophages were infected with BCG-D70M at the indicated MOI and subsequently treated with 10 μg/ml mAb to HLA-DR, CD86, or MMP-II Ags or normal murine IgG or IgM. These APCs were used as the stimulator of responder $CD4^+$ T cells (1×10^5/well) at the indicated T/APC ratio. IFN-γ produced by T cells was measured. A representative of four separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student *t* test.

Measurement of cytokine production

Levels of the following cytokines were measured: IFN-γ produced by $CD4^+$ and $CD8^+$ T cells, and IL-12p70, TNF-α, and IL-1β produced by DCs or macrophages stimulated for 24 or 48 h with rBCGs. The concentrations of these cytokines were quantified using the enzyme assay kit Opt EIA Human ELISA Set (BD Biosciences).

Animal studies

For inoculation into mice, rBCGs were cultured in Middlebrook 7H9 medium to log phase and stored at 10^8 CFU/ml at −80°C. Before the aliquots were used for inoculation, the concentration of viable bacilli was determined by plating on Middlebrook 7H10 agar plate. Three 5-wk-old C57BL/6J mice (Clea Japan, Tokyo, Japan) per group were inoculated s.c. with 0.1 ml PBS or PBS containing 1×10^3 rBCGs. The animals were kept in specific pathogen-free conditions and supplied with sterilized food and water. Four or 12 wk postinoculation, the spleens were removed, and the splenocytes were suspended at a concentration of 2×10^6 cells/ml in culture medium. The splenocytes were stimulated with an indicated concentration of rMMP-II or rHSP70 (HyTest) in triplicate in 96-well round-bottom microplates (15, 16). The individual culture supernatants were collected 3 to 4 d poststimulation, and IFN-γ was measured using the Opt EIA Mouse ELISA Set (BD Biosciences). Five C57BL/6 mice per group were vaccinated with 1×10^3 CFU/mouse either BCG-261H or BCG-D70M for 4 wk and were challenged with 5×10^3/mouse of *M. leprae* in footpad. Thirty-two wk later, the footpad was removed. The number of *M. leprae* grown in the footpad was enumerated by Shepard method (33). Animal studies were reviewed and approved by the Animal Research Committee of Experimental Animals of the National Institute of Infectious Diseases and were conducted according to their guidelines.

Statistical analysis

Student *t* test was applied to determine the statistical differences.

Results

Activation of naive T lymphocytes by BCG-D70M

Because BCG-D70M possess two modified characteristics, 1) a lack of urease activity and 2) an expression of HSP70 and MMP-II fusion protein, we assessed the expression level of HSP70 and MMP-II proteins in BCG strains: BCG-70M and BCG-D70M. Both BCG-70M and BCG-D70M equivalently expressed both HSP70 and MMP-II molecules as examined by Western blot analyses using Ag85B as an internal control (Fig. 1). Further, the T cell activation activity of BCG-D70M was evaluated by using not only vector control BCG (BCG-261H), but also rBCGs that lacks urease activity (urease-deficient BCG-ΔUT-11-3 and BCG-70M that secretes HSP70–MMP-II fusion protein) as control BCG (Fig. 2*A*). When these rBCGs were infected to DCs to use as a stimulator of naive $CD4^+$ T cells, both BCG-ΔUT-11-3 and BCG-70M showed higher T cell-stimulating activity than vector control BCG. However, BCG-D70M showed the highest T cell-stimulating activity among these rBCGs at both MOIs: 0.125 and 0.25. More than 350 pg/ml IFN-γ was produced by stimulation with BCG-D70M (MOI: 0.25; T/DC ratio: 40:1). Also, at different T/DC ratios, BCG-D70M exhibited the highest activity (not shown). On addition to IFN-γ, TNF-α was also efficiently produced by BCG-D70M stimulation (not shown). So far, when macrophages were used as APCs, it was difficult to activate $CD4^+$ T cells to produce IFN-γ significantly. In contrast to the activity of rBCGs, such as BCG-261H, BCG-ΔUT-11-3, and BCG-70M (15, 25), newly constructed BCG-D70M efficiently stimulated $CD4^+$ T cells to produce the cytokine through macrophages at various conditions, although the T cell-stimulating activity of macrophages was much less efficient comparing to that of DCs (Fig. 2*B*). More than 100 pg/ml IFN-γ was produced from responder $CD4^+$ T cells; however, BCG-D70M–infected macrophages failed to induce the production of significant dose of IFN-γ from naive $CD4^+$ T cells (not shown). Also, BCG-D70M did not induce IFN-γ production from DCs or macrophages (not shown). Although normal murine IgG did not affect the T cell-stimulating activity of both BCG-D70M–infected DC and the BCG-D70M–infected macrophages, the treatment of these APCs with either anti–HLA-DR mAb, anti-CD86 mAb, or anti–MMP-II mAb significantly inhibited the activation of naive $CD4^+$ T cells and $CD4^+$ T cells, respectively (Fig. 2*C*). More than 90% of IFN-γ production was inhibited by the treatment of APCs when mAb to HLA-DR or CD86 was used. Furthermore, when naive $CD8^+$ T cells were stimulated with DC infected with various rBCGs, BCG-D70M induced the strongest activation of naive $CD8^+$ T cells (Fig. 3*A*). Both BCG-70M and BCG-D70M induced significant IFN-γ production, but BCG-D70M activated the T cells more strongly than BCG-70M. More than 400 pg/ml IFN-γ can be produced from naive $CD8^+$ T cells. These phenomena were observed consistently at various conditions including the different MOIs and T cell/DC ratios, although high doses of BCG-D70M and high doses of BCG-D70M–infected DCs were required to induce the production of abundant IFN-γ from naive $CD8^+$ T cells compared with the dose required to stimulate naive $CD4^+$ T cells. Again, when BCG-D70M–infected DCs were treated with mAb to either HLA-ABC or CD86, the IFN-γ production from naive $CD8^+$ T cells was significantly inhibited, whereas normal murine IgG did not affect the activation of the responder T cells (Fig. 3*B*).

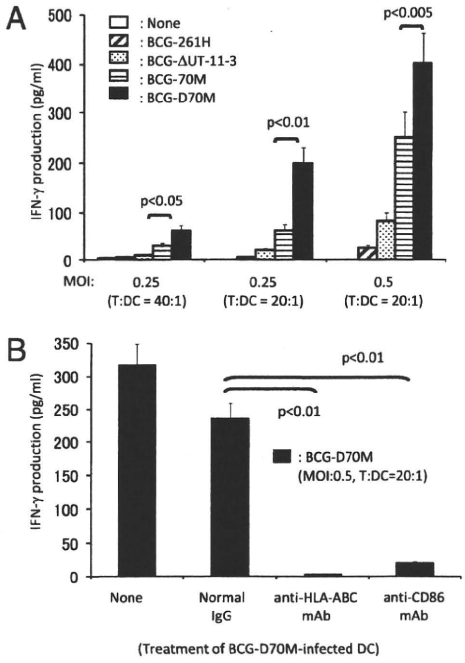

FIGURE 3. *A*, IFN-γ production from naive $CD8^+$ T cells by stimulation with BCG. Monocyte-derived DCs were infected with BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M at the indicated MOI and were used as a stimulator of responder naive $CD8^+$ T cells in a 4-d culture. Total of 10^5 responder T cells were stimulated with the BCG-infected DCs at the indicated T/DC ratio. *B*, Inhibition of naive $CD8^+$ T cell activation by the treatment of BCG-D70M–infected DCs with mAb. Monocyte-derived DCs were infected with BCG-D70M at an MOI of 0.5 and subsequently treated with 10 μg/ml mAb to HLA-ABC or CD86 Ags or normal murine IgG. These DCs were used as the stimulator of naive $CD8^+$ T cells (1×10^5/well) at T/DC ratio of 20:1. IFN-γ produced by T cells was measured. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student *t* test.

Effect of BCG-D70M on DCs

To stimulate responder T cells efficiently, APCs susceptible to BCG infection should be adequately activated. We assessed the activation of DCs from the aspects of cytokine production and phenotypic changes (Fig. 4). When DCs were stimulated with various rBCGs including BCG-261H, BCG-ΔUT-11-3, BCG-70M, and BCG-D70M, BCG-D70M stimulated DCs to produce IL-12p70 most efficiently at both MOIs: 0.25 and 0.5 (Fig. 4*A*). Further, BCG-D70M induced significantly higher dose of IL-1β and TNF-α production from DCs and also induced a higher dose of TNF-α from macrophages compared with BCG-261H (Fig. 4*B*). To assess the phenotypic changes induced by BCG-D70M infection, we assessed the expression of MHC, CD86, CD83, and CD1a molecules on DCs (Fig. 4*C*). Infection with BCG-D70M induced significantly higher level of expression of HLA-ABC, HLA-DR, CD86, and CD83 Ags compared with BCG-261H infection. The expression of CD1a was significantly downregulated. We used various dose of rBCGs for the assessment, and the similar changes were observed (not shown). These results indicated that BCG-D70M more efficiently activated DCs than BCG-261H.

Characteristics of BCG-D70M

Previously, we reported that BCG-70M, which was genetically manipulated to produce HSP70–MMP-II fusion protein, induced expression of MMP-II on the surface of BCG-70M–infected DCs (25). We then analyzed the BCG-D70M–infected DCs in terms of MMP-II expression (Fig. 5*A*). Whereas DCs uninfected or infected with BCG-261H did not express MMP-II significantly, BCG-D70M induced significant expression of MMP-II. Further, when immature DCs were treated with chloroquine, an inhibitor of phagosomal acidification, in advance to the infection with BCG-D70M, the MMP-II expression was significantly inhibited. In addition to the MMP-II expression, the chloroquine treatment on DCs affects the activation of responder T cell by BCG-D70M (Fig. 5*B*). IFN-γ production from naive $CD4^+$ T cells by stimulation with BCG-D70M (MOI: 0.125 and 0.25) was significantly

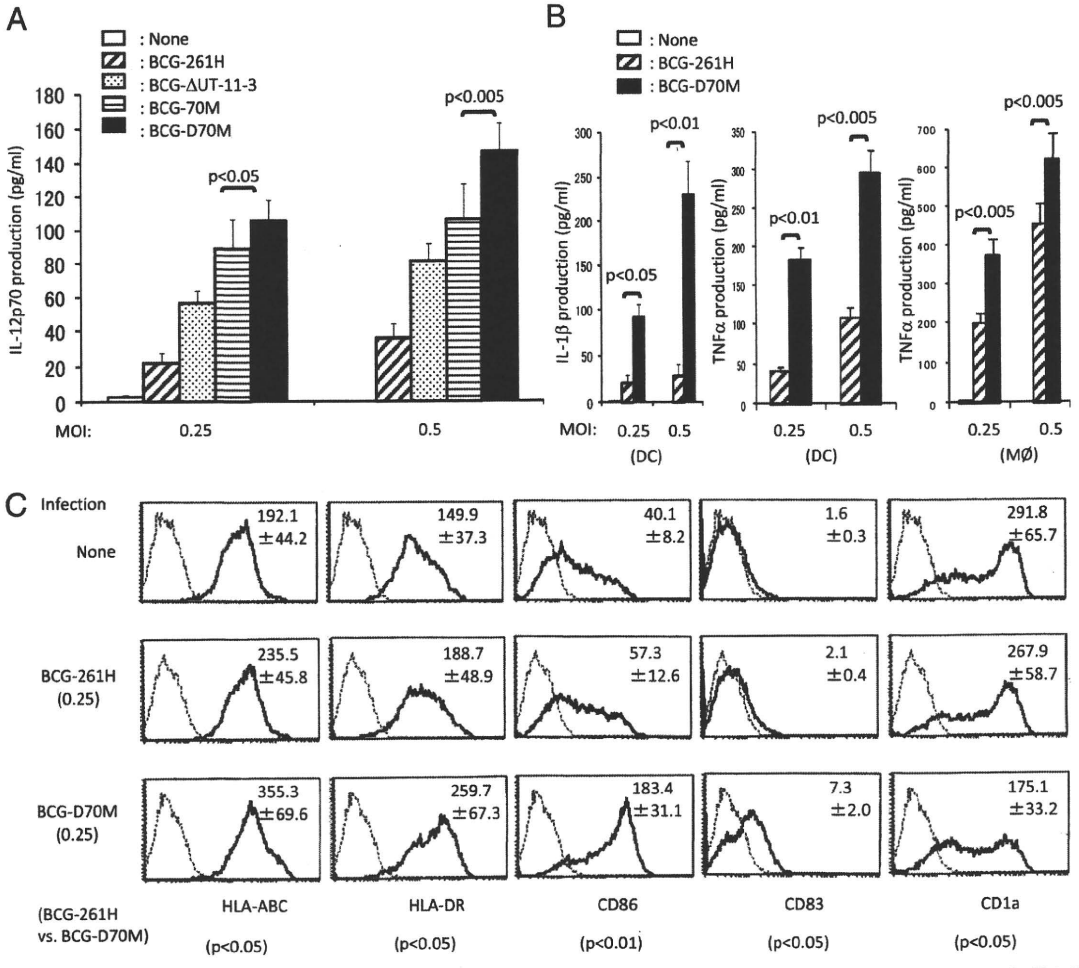

FIGURE 4. *A*, IL-12p70 production from DCs stimulated with rBCG. Monocyte-derived DCs from 5 d of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated dose of BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M for 48 h. The concentration of IL-12p70 was determined by the ELISA method. *B*, Cytokine production from DCs or macrophages stimulated with rBCG. DCs produced using rGM-CSF and rIL-4 or macrophages from 5 d of culture in the presence of rM-CSF were stimulated with either BCG-261H or BCG-D70M for 24 h. The concentration of the indicated cytokine was determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student *t* test. *C*, Upregulation of APC-associated molecules and activation marker on DCs by BCG-D70M infection. Monocyte-derived immature DCs were infected with either BCG-261H or BCG-D70M at an MOI of 0.25 and cultured for another 2 d in the presence of rGM-CSF and rIL-4. The DCs from day 5 of culture were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. Representative results of three separate experiments are shown. The number in the top right corner of each panel represents the mean ± SD for three independent experiments of the difference in the fluorescence intensity between the control IgG and test mAb. Titers of BCG-261H–infected DCs and BCG-D70M–infected DCs were statistically compared using Student *t* test.

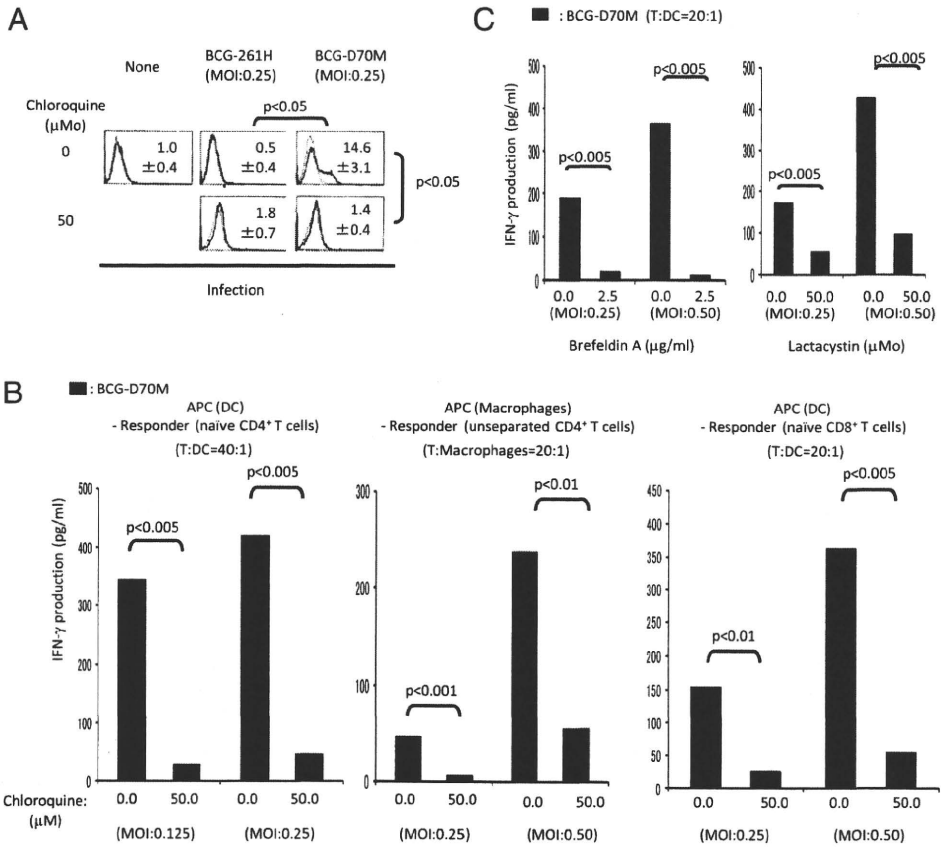

FIGURE 5. *A*, Expression of MMP-II on DCs. Immature DCs were either treated with 50 μM chloroquine for 2 h or untreated and subsequently infected with either BCG-261H or BCG-D70M at an MOI of 0.25. After 2 d culture in the presence of rGM-CSF and rIL-4, DCs were gated and analyzed. Dotted lines, control normal IgM; solid lines, anti–MMP-II mAb (IgM). The number in the top right corner of each panel represents the mean ± SD for six independent experiments of the difference in mean fluorescence intensity between the control IgM and the test mAb. Representative results of six separate experiments are shown. *B*, Effect of chloroquine treatment of DCs and macrophages on the activation of T cells. Immature DCs from 4 d of culture were treated with chloroquine (50 μM, 2 h) or untreated and subsequently infected with BCG-D70M at the indicated MOI. These DCs were used as the stimulator of responder autologous naive $CD4^+$ T cells or naive $CD8^+$ T cells (1×10^5/well) at T/DC ratio of 40:1 or 20:1, as indicated. Macrophages from 5 d of culture in the presence of M-CSF were treated with chloroquine as above and subsequently infected with BCG-D70M at the indicated MOI. These macrophages were used as the stimulator of responder autologous unseparated $CD4^+$ T cells (1×10^5/well) at T/DC ratio of 20:1. IFN-γ produced by T cells was measured. *C*, Effect of treatment of immature DCs with brefeldin A or lactacystin on the activation of naive $CD8^+$ T cells. Immature DCs from 4 d of culture were treated with the indicated dose of brefeldin A or lactacystin or untreated and subsequently infected with BCG-D70M at the indicated MOI. These DCs were used as the stimulator of responder autologous naive $CD8^+$ T cells (1×10^5/well) at T/DC ratio of 20:1. IFN-γ produced by T cells was measured. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student *t* test.

inhibited by chloroquine. Also, on treatment of macrophages with chloroquine, ~90% of IFN-γ produced from $CD4^+$ T cells by BCG-D70M stimulation (MOI: 0.25 and 0.50) was inhibited. Similarly, the production of IFN-γ from naive $CD8^+$ T cells was significantly inhibited by the chloroquine treatment of DCs, which were subsequently infected with BCG-D70M (MOI: 0.25 and 0.50). We further confirmed that BCG-D70M secreted 92 kDa protein (molecular mass: MMP-II 22 kDa and HSP70 70 kDa) in vitro (not shown). These results suggest the possibility that the secreted HSP70–MMP-II fusion protein is one of the responsible elements for the activation of both $CD4^+$ T cells and $CD8^+$ T cells as observed in BCG-70M. Further, we previously reported that BCG-70M stimulated naive $CD8^+$ T cells through TAP- and proteosome-dependent cytosolic cross-presentation pathway, because the T cell activation was inhibited by the pretreatment of DCs with brefeldin A and lactacystin (25). In this study, we assessed whether BCG-D70M activates naive $CD8^+$ T cells by using the similar cross-presentation pathway (Fig. 5*C*). When immature DCs were treated with either brefeldin A or lactacystin and were subsequently infected with BCG-D70M at MOI 0.25 or 0.50, the IFN-γ production from naive $CD8^+$ T cells was significantly inhibited.

Effect of $CD4^+$ T cells on the activation of naive $CD8^+$ T cells

Because BCG-D70M activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells, we assessed the influence of the presence of $CD4^+$ T cells on the activation of naive $CD8^+$ T cells (Fig. 6*A*). The naive unseparated T cell population was stimulated with DCs infected with BCG-261H or BCG-D70M, and $CD8^+$ T cells were gated and analyzed by FACS. Compared to $CD8^+$ T cells stimulated with BCG-261H, the BCG-D70M–stimulated naive $CD8^+$ T cells produced significantly higher number of perforin-producing $CD8^+$ T cells and $CD62L^{low}$ $CD8^+$ T cells. Further, $CCR7^{low}$ $CD8^+$ T cells and $CD27^{low}$ $CD8^+$ T cells were more efficiently produced by BCG-D70M stimulation (Fig. 6*A*). The efficient production of these $CD8^+$ T cells was observed with different doses of BCG; however, in the absence of naive $CD4^+$ T cells, these changes were not induced (not shown). Also, $CD4^+$ T cells producing intracellular perforin was not produced from naive $CD4^+$ T cells by the stimulation with BCG-D70M–infected

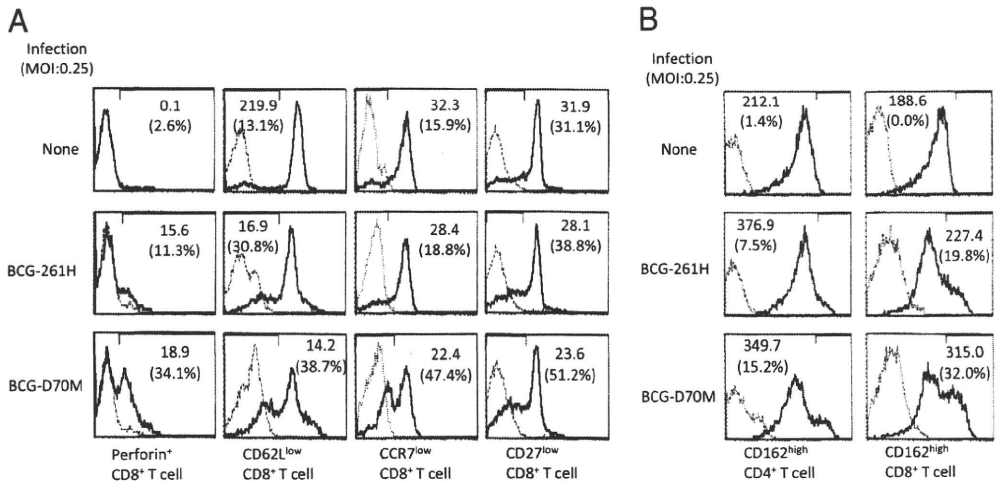

FIGURE 6. *A*, Perforin production and expression of activation or memory marker on naive $CD8^+$ T cells stimulated with DCs infected with BCG-D70M. Monocyte-derived DCs were infected with either BCG-261H or BCG-D70M at an MOI of 0.25 and were cocultured with naive T cells (T/DC 20:1) for 5 d. The stimulated $CD8^+$ T cells were gated and analyzed for perforin production and expression of the indicated molecules. The number in the top right corner of each panel represents the difference in the fluorescence intensity between the control IgG and the test mAb. The number in the parenthesis indicated either percent perforin-positive $CD8^+$ T cells or $CD8^+$ T cells expressing the test Ags among $CD8^+$ T cell population. *B*, Expression of migration marker on naive T cells. Monocyte-derived DCs were infected with either BCG-261H or BCG-D70M at an MOI of 0.25 and were cocultured with naive T cells (T/DC 20:1) for 5 d. The stimulated T cells of either $CD4^+$ or $CD8^+$ subsets were gated and analyzed for expression of CD162 molecules. The number in the top right or left corner of each panel represents the difference in the fluorescence intensity between the control IgG and the test mAb. The number in the parentheses indicated percent $CD162^{high}$ T cells among $CD4^+$ or $CD8^+$ T cell population. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as the mean ± SD. Titers were statistically compared using Student *t* test.

DCs. These results indicate that BCG-D70M may produce effector $CD8^+$ T cells having killing activity and memory type $CD8^+$ T cells efficiently from naive population. When BCG is used as a vaccine, it is required to produce memory T cells having a high migratory function (34). To examine this point, we assessed the expression of CD162 molecules on both $CD4^+$ T cells and $CD8^+$ T cells, which were stimulated with DCs infected with BCG-D70M (Fig. 6*B*). On BCG-D70M stimulation, both $CD4^+$ T cells and $CD8^+$ T cells that express high levels of CD162 Ag were produced more efficiently than by the stimulation with BCG-261H. A similar difference between BCG-261H and BCG-D70M was induced when different doses of BCG were used (not shown).

Production of T cells responsive to the secondary stimulation by BCG-D70M in vivo

The ability of BCG-D70M to produce T cells highly responsive to the secondary in vitro stimulation was examined by in vivo functional studies (Fig. 7). C57BL/6 mice were s.c. inoculated with 1×10^3/mouse of rBCGs 4 wk prerestimulation in vitro. Both MMP-II and HSP70 proteins were used as a restimulator. These proteins induced IFN-γ production from T cells in all infected or uninfected mice because they have high immunogenicity, and BCG-Tokyo, a parental strain of all rBCGs, has the gene encoding BCG-derived MMP-II. However, splenic T cells from BCG-D70M–infected mice respond most vigorously to the stimulators and produced higher doses of IFN-γ (Fig. 7*A*) and IL-2 (not shown) than those from mice uninfected or infected with control rBCGs including BCG-261H, BCG-ΔUT-11-3, and BCG-70M. To examine the long-term effect of the inoculation of rBCGs on the production of such responsive T cells, C57BL/6 mice were s.c. inoculated with 1×10^3/mouse of rBCGs 12 wk before the restimulation. Again, a significantly higher dose of IFN-γ (Fig. 7*B*) was produced from splenic T cells obtained from mice inoculated with BCG-D70M by the stimulation with MMP-II and HSP70 than those from mice uninfected or infected with control rBCGs.

Effect of BCG-D70M vaccination on the multiplication of M. leprae *in vivo*

C57BL/6 mice vaccinated with either BCG-261H or BCG-D70M (1×10^3 CFU/mouse) for 4 wk were challenged with 5×10^3 *M. leprae* in the footpad. Thirty-two weeks later, the footpad was removed, and the *M. leprae* recovered from the footpad was enumerated (Fig. 8). A total of 2×10^5 *M. leprae* were recovered from mice inoculated with PBS and challenged with *M. leprae*. Although the mice vaccinated with BCG-261H inhibited the multiplication of *M. leprae* significantly, the BCG-D70M vaccination significantly and more efficiently inhibited the *M. leprae* multiplication than BCG-261H. A similar difference was observed when 1×10^2 CFU/mouse rBCG was inoculated for the inhibition of *M. leprae*.

Discussion

M. leprae is well-known as a representative slow-growing *Mycobacterium*. Usually, *M. leprae* needs 12–14 d for one division and at least 2–5 y for the manifestation of the disease. In vivo studies using the immunodeficient nude mouse indicate that adaptive immunities play an important role in inhibiting the multiplication of *M. leprae*, and the activation of both $CD4^+$ T cells and $CD8^+$ T cells is an essential element for controlling *M. leprae* infection (5, 6, 35). Although $CD4^+$ T cells chiefly act at the initial phase of infection, the contribution of $CD8^+$ T cells in terms of IFN-γ production and killing of mycobacteria-infected host cells is necessary in the chronic phase of the infection (36). BCG was used so far as vaccine against leprosy; however, its efficacy is nowadays considered not as convincing as expected (12). The reason for why BCG cannot prevent the leprosy manifestation convincingly may be due to its inadequate ability to stimulate T cells. The poor T cell-stimulating activity seems to be based on the intrinsic defect of BCG not being able to enter the lysosome feasibly. Also, poor stimulation of T cells would result in the meager production of competent memory T cells, including both $CD4^+$ and $CD8^+$ subsets, capable of convincingly responding

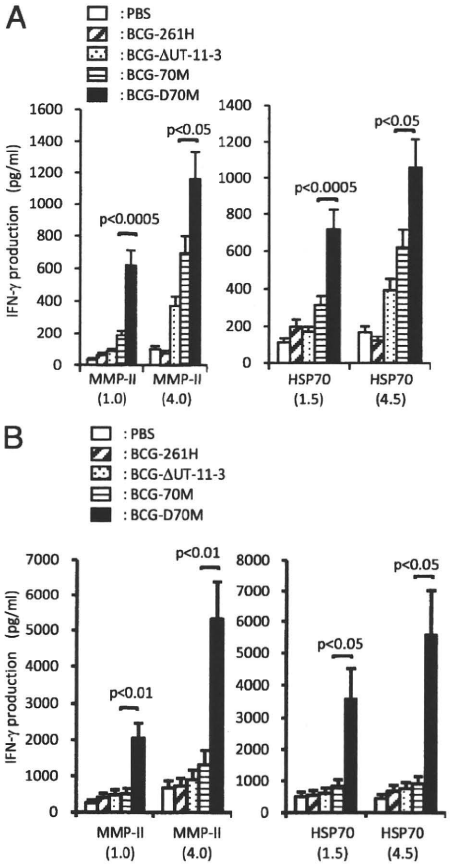

FIGURE 7. *A*, Production of T cells responsive to the secondary in vitro stimulation in C57BL/6 mice by infection with rBCG. Three 5-wk-old C57BL/6 mice per group were infected with 1×10^3 CFU/mouse BCG-261H, BCG-ΔUT-11-3, BCG-70M, or BCG-D70M s.c. Four weeks postinoculation, splenocytes (2×10^5 cells/well) were stimulated with the indicated dose of either MMP-II or HSP70 for 4 d in vitro, and IFN-γ produced in the cell supernatant was measured. *B*, Production of T cells capable of responding to the secondary stimulation in C57BL/6 mice a long time postinfection with rBCG. Three 5-wk-old C57BL/6 mice per group were infected with 1×10^3 CFU/mouse rBCGs s.c. Twelve weeks postinoculation, splenocytes (2×10^5 cells/well) were stimulated with the indicated dose of either MMP-II or HSP70 for 3 d 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 *t* test.

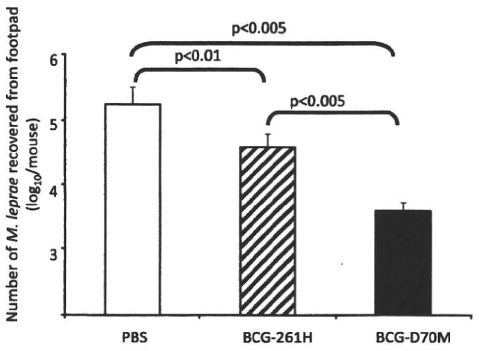

FIGURE 8. Inhibition of *M. leprae* multiplication by s.c. vaccination with BCG-D70M. Five-week-old C57BL/6 mice (5 mice/group) were vaccinated with 1×10^3 CFU/mouse either BCG-261H or BCG-D70M s.c. and were challenged with 5×10^3 bacilli/mouse *M. leprae* in footpad 4 wk postvaccination. The number of *M. leprae* recovered from the footpad at 32 wk postchallenge was enumerated by Shepard's method (33). Representative results of three separate experiments are shown. Titers were statistically compared using Student *t* test.

to mycobacterial Ags. Especially, BCG cannot activate naive $CD8^+$ T cells adequately in the absence of $CD4^+$ T cell-derived help (14), so that BCG may poorly control the disease in the chronic phase or in the inhibition of disease manifestation for a long time postinfection (14). This fact is important when the growth rate of *M. leprae* is taken into account.

However, BCG has also intrinsic benefit, because it activates human naive $CD4^+$ T cells to produce IFN-γ to some extent. Therefore, we tried to improve the potency of BCG, especially with regard to immunostimulatory activities. We chiefly focused on overcoming the defect of BCG—that is, the ability to avoid the fusion of BCG-infected phagosomes with lysosomes. One of the approaches we carried out previously is the production of *UreC* gene-deficient rBCG (BCG-ΔUT-11-3), which successfully produces acidic phagosomes and facilitates them to fuse with lysosomes (15). In fact, BCG-ΔUT-11-3 efficiently colocalizes with lysosomes and preferentially and effectively stimulates human naive $CD4^+$ T cells (15). Therefore, the disruption of the *UreC* gene of BCG seems to be a useful strategy to translocate BCG to lysosomes. However, unfortunately, BCG-ΔUT-11-3 did not activate naive $CD8^+$ T cells effectively. Then, the second approach for overcoming the lack of phagosome–lysosome fusion was carried out—that is, to induce the secretion of immunodominant Ag into phagosome. In this study, we used MMP-II as the immunodominant Ag of *M. leprae* (7). In one case, sole MMP-II protein (production of BCG-SM) and in the other case HSP70–MMP-II fusion protein (production of BCG-70M) secreting BCG was constructed (16, 25). Both BCGs were quite efficient in the induction of activation of not only naive $CD8^+$ T cells, but also naive $CD4^+$ T cells. However, BCG-70M was superior to BCG-SM in activating both subsets of T cells, especially naive $CD8^+$ T cells (not shown). The activation of naive $CD8^+$ T cells by BCG-70M is highly dependent on the secretion of HSP70–MMP-II fusion protein, because the activation seems to be induced by TAP- and proteosome-dependent cross-presentation of the secreted protein (24, 25, 37). Therefore, the secretion of MMP-II in the combination with HSP70 seems to be an efficient strategy to overcome the intrinsic defect of BCG.

Because the two independent strategies for overcoming the intrinsic defect of BCG were useful, we tried to combine both strategies and produced new rBCG (BCG-D70M), in which BCG-ΔUT-11-3 was integrated with gene encoding HSP70–MMP-II fusion protein. As previously reported (25), BCG-70M secreted 92 kDa HSP70–MMP-II fusion protein after being phagocytozed by APCs, and the secreted protein was transported to functional lysosomes. In the phagolysosomes, some portions of HSP70–MMP-II fusion protein could be degraded, but rest of the protein may be sequestrated into the cytosol, where they could be degraded and used for cross-priming $CD8^+$ T cells. In this respect, when immature DCs were pretreated with chloroquine and subsequently infected with newly produced BCG-D70M, both the expression of MMP-II and the activation of naive $CD4^+$ and $CD8^+$ T cells by the rBCG were inhibited. Thus, protein secreted from BCG-D70M seems to be responsible for the activation of naive T cells. Further, the activation of naive $CD8^+$ T cells by BCG-D70M was also abolished by pretreatment of immature DCs with lactacystin, a proteosomal protein degradation blocker and brefeldin A that is an inhibitor of antegrade Golgi transportation and of TAP-dependent transportation. Therefore, it is highly likely that the 92-kDa fusion protein secreted from BCG-D70M could be sequestrated into cytosol from lysosome, degraded in proteosome, and used for loading on MHC class I molecules through the TAP-

dependent pathway. Thus, similar to BCG-70M, BCG-D70M also used the cytosolic pathway, which is known as the most effective cross-presenting pathway (38), to cross-prime $CD8^+$ T cells. In this respect, it is known that HSP plays an important role in the induction of the cytosolic cross-presentation pathway (39, 40). HSP70 secreted as a part of the fusion protein seems to be closely associated with the cross-priming $CD8^+$ T cells. The activation of both naive $CD8^+$ T cells and naive $CD4^+$ T cells by BCG-D70M was induced in an Ag-specific fashion, because treatment of BCG-D70M–infected DCs with mAb to MHC molecules or CD86 Ag inhibited the IFN-γ production from naive T cells. However, the naive $CD4^+$ T cells seemed to be polyclonally activated by the stimulation, because the treatment of DCs with mAb to MMP-II partially, but significantly, inhibited the activation (Fig. 2*C*). In C57BL/6 mice, a single injection of BCG-D70M produced T cells capable of responding to both MMP-II and HSP70 several weeks postinoculation. Therefore, the HSP70–MMP-II fusion protein activated both APCs and T cells by the similar mechanisms as observed in in vitro experiments and was probably used as antigenic molecules in vivo. Because *M. leprae*-infected DCs expressed MMP-II–derived antigenic determinants on their surface (7, 16), the production of T cells responsive to MMP-II in vivo may be useful to prevent the disease manifestation. This speculation seems to be supported by the present observation that the vaccination with BCG-D70M more efficiently inhibited the multiplication of *M. leprae* in vivo than that with vector control BCG.

The activities stimulating both subsets of naive T cells of BCG-D70M were strongest among the all rBCGs produced so far including BCG-70M. Although all of the rBCGs showed the dose-dependent effect in the T cell activation, BCG-D70M showed the strongest activity in terms of the T cell activation, even if an MOI 1.0 of BCG was used. Further, BCG-D70M most strongly activated DCs as revealed by IL-12p70 production from DCs. Because BCG-70M activated DCs through the binding of HSP70–MMP-II fusion protein with TLR2 (25), BCG-D70M seems to activate DCs with a similar mechanism, at least partially. However, it did not induce an apoptotic cell death of target APCs including DCs and macrophages, the in vitro growth rate of BCG-D70M was almost identical with that of BCG-261H, and further, the infectivity of these rBCGs to host cells in both in vitro and in vivo was identical (not shown). It is likely that the stronger DC- and T cell-activating ability of BCG-D70M than BCG-70M might be due to the absence of ammonia, products of *UreC* gene-encoding urease, in the phagosome. The urease depletion may facilitate the translocation of HSP70–MMP-II fusion protein secreted in phagosomes from BCG-D70M into lysosomes. However, another explanation could be that the absence of ammonia may facilitate the translocation of BCG-D70M itself to lysosomes, because it has previously reported that BCG-ΔUT-11-3 more efficiently entered lysosomes than parent BCGs, which possess the *UreC* gene (15, 19). BCG-D70M translocated into lysosomes or phagolysosomes secreted HSP70–MMP-II fusion protein. Therefore, it can be speculated that a larger dose of secreted protein that could be efficiently processed would be available in lysosomes, so that much or many types of antigenic peptides could be loaded on the MHC molecules. This speculation is important because it has recently been reported that quick activation of $CD8^+$ T cells by BCG requires the high antigenic load on MHC class I molecules (41). These results indicate that the deletion of urease from BCG and integration of gene encoding fusion protein into BCG may act synergistically, although further detailed analyses is required.

The strong ability of BCG-D70M to stimulate T cells enables macrophages to activate $CD4^+$ T cells. The $CD4^+$ T cells stimulated by BCG-D70M through macrophages seemed to be activated in an Ag-specific manner, because the IFN-γ production from the T cells was largely blocked by the treatment of BCG-D70M–infected macrophages with mAbs to MHC class II and CD86 Ags. So far, rBCG including BCG-ΔUT-11-3 and BCG-70M did not activate $CD4^+$ T cells efficiently through macrophages in the absence of costimulators such as CD40L and IFN-γ (15, 25). The definite reason for why BCG-D70M, but not BCG-70M, could activate $CD4^+$ T cells through macrophages remains unanswered. However, the secreted fusion protein either in the phagosome or phagolysosome could be associated with $CD4^+$ T cell activation through macrophages, because pretreatment of macrophages with chloroquine abolished their T cell-stimulating activities. BCG infects not only DCs, but also macrophages, which are highly active in phagocytosis of bacteria; thus, the successful activation of $CD4^+$ T cells by macrophages upon an infection with BCG-D70M would provide many chances to heterogeneous $CD4^+$ T cells to receive antigenic stimuli. The $CD4^+$ T cell activation by macrophages should contribute to the efficient production of high doses of IFN-γ and to the production and maintenance of abundant memory T cells. In addition, in the presence of the help of $CD4^+$ T cells, naive $CD8^+$ T cells were differentiated into $CCR7^{low}CD8^+$ and $CD27^{low}CD8^+$ memory type T cells by the stimulation with BCG-D70M. Also, they produced phenotypically activated $CD62L^{low}CD8^+$ T cells as well as perforin-producing effector $CD8^+$ T cells. Therefore, the efficient activation of naive and memory type $CD4^+$ T cells may contribute to the efficient production of effector and memory $CD8^+$ T cells. In our hands, we could not confirm the possibility that the functional perforin-producing $CD8^+$ T cells, which are produced from naive T cells, can be further differentiated into memory subsets. If this were the case, effector $CD8^+$ T cells having killing activity can be immediately and efficiently produced from such memory T cells upon an infection with *M. leprae* in vivo.

It has been reported that to prevent the disease manifestation induced by infection with mycobacteria, such as *M. tuberculosis*, by vaccination, the vaccinating agents should be able to produce memory T cells that have a high potency to migrate into the infection site (34). Thus, we evaluated whether BCG-D70M can produce T cells with a migration activity by monitoring the surface expression of CD162 molecules. The stimulation of naive T cells with BCG-D70M–infected DCs induced the expression of CD162 on both $CD4^+$ T cells and $CD8^+$ T cells. Therefore, it could be assumed that BCG-D70M may be a convincing stimulator of naive T cells.

Taken together, in this study, we newly constructed an rBCG that is deficient in production of urease, but instead produced HSP70–MMP-II fusion protein and is capable of effectively and strongly activating both naive $CD4^+$ and $CD8^+$ T cells, thus overcoming the intrinsic defect of BCG. Using the triple combination of expressing HSP70 and MMP-II protein in BCG and depletion of urease may result in sufficient production of memory T cells by activating both subsets of naive T cells in human.

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Disclosures

The authors have no financial conflicts of interest.

References

1. WHO Expert Committee on Leprosy. 1982. *Sixth Report of WHO Technical Report Series 675*. World Health Organization, Geneva, Switzerland.

2. Ridley, D. S., and W. H. Jopling. 1966. Classification of leprosy according to immunity. A five-group system. *Int. J. Lepr. Other Mycobact. Dis.* 34: 255–273.
3. Sieling, P. A., D. Jullien, M. Dahlem, T. F. Tedder, T. H. Rea, R. L. Modlin, and S. A. Porcelli. 1999. CD1 expression by dendritic cells in human leprosy lesions: correlation with effective host immunity. *J. Immunol.* 162: 1851–1858.
4. Sieling, P. A., M. T. Ochoa, D. Jullien, D. S. Leslie, S. Sabet, J. P. Rosat, A. E. Burdick, T. H. Rea, M. B. Brenner, S. A. Porcelli, and R. L. Modlin. 2000. Evidence for human $CD4^+$ T cells in the CD1-restricted repertoire: derivation of mycobacteria-reactive T cells from leprosy lesions. *J. Immunol.* 164: 4790–4796.
5. Modlin, R. L., J. Melancon-Kaplan, S. M. M. Young, C. Pirmez, H. Kino, J. Convit, T. H. Rea, and B. R. Bloom. 1988. Learning from lesions: patterns of tissue inflammation in leprosy. *Proc. Natl. Acad. Sci. USA* 85: 1213–1217.
6. Hashimoto, K., Y. Maeda, H. Kimura, K. Suzuki, A. Masuda, M. Matsuoka, and M. Makino. 2002. *Mycobacterium leprae* infection in monocyte derived dendritic cells and its influence on antigen-presenting function. *Infect. Immun.* 70: 5167–5176.
7. Maeda, Y., T. Mukai, J. Spencer, and M. Makino. 2005. Identification of an Immunomodulating Agent from Mycobacterium leprae. [Published erratum in 2005 *Infect. Immun.* 73: 4458.] *Infect. Immun.* 73: 2744–2750.
8. Makino, M., Y. Maeda, and N. Ishii. 2005. Immunostimulatory activity of major membrane protein-II from *Mycobacterium leprae. Cell. Immunol.* 233: 53–60.
9. Lombardi, C., E. S. Pedrazzani, J. C. Pedrazzani, P. F. Filho, and F. Zicker. 1996. Protective efficacy of BCG against leprosy in São Paulo. *Bull. Pan Am. Health Organ.* 30: 24–30.
10. Pönnighaus, J. M., P. E. Fine, J. A. Sterne, R. J. Wilson, E. Msosa, P. J. Gruer, P. A. Jenkins, S. B. Lucas, N. G. Liomba, and L. Bliss. 1992. Efficacy of BCG vaccine against leprosy and tuberculosis in northern Malawi. *Lancet* 339: 636–639.
11. Sharma, P., R. Mukherjee, G. P. Talwar, K. G. Sarathchandra, R. Walia, S. K. Parida, R. M. Pandey, R. Rani, H. Kar, A. Mukherjee, et al. 2005. Immunoprophylactic effects of the anti-leprosy Mw vaccine in household contacts of leprosy patients: clinical field trials with a follow up of 8-10 years. *Lepr. Rev.* 76: 127–143.
12. Setia, M. S., C. Steinmaus, C. S. Ho, and G. W. Rutherford. 2006. The role of BCG in prevention of leprosy: a meta-analysis. *Lancet Infect. Dis.* 6: 162–170.
13. Reyrat, J. M., F. X. Berthet, and B. Gicquel. 1995. The urease locus of *Mycobacterium tuberculosis* and its utilization for the demonstration of allelic exchange in *Mycobacterium bovis* bacillus Calmette-Guérin. *Proc. Natl. Acad. Sci. USA* 92: 8768–8772.
14. Grode, L., P. Seiler, S. Baumann, J. Hess, V. Brinkmann, A. Nasser Eddine, P. Mann, C. Goosmann, S. Bandermann, D. Smith, et al. 2005. Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guérin mutants that secrete listeriolysin. *J. Clin. Invest.* 115: 2472–2479.
15. Mukai, T., Y. Maeda, T. Tamura, Y. Miyamoto, and M. Makino. 2008. $CD4^+$ T-cell activation by antigen-presenting cells infected with urease-deficient recombinant *Mycobacterium bovis* bacillus Calmette-Guérin. *FEMS Immunol. Med. Microbiol.* 53: 96–106.
16. Makino, M., Y. Maeda, and K. Inagaki. 2006. Immunostimulatory activity of recombinant *Mycobacterium bovis* BCG that secretes major membrane protein II of *Mycobacterium leprae. Infect. Immun.* 74: 6264–6271.
17. Maeda, Y., T. Tamura, M. Matsuoka, and M. Makino. 2009. Inhibition of the multiplication of *Mycobacterium leprae* by vaccination with a recombinant *M. bovis* BCG strain that secretes major membrane protein II in mice. *Clin. Vaccine Immunol.* 16: 1399–1404.
18. Horwitz, M. A., B. W. Lee, B. J. Dillon, and G. Harth. 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA* 92: 1530–1534.
19. Schaible, U. E., S. Sturgill-Koszycki, P. H. Schlesinger, and D. G. Russell. 1998. Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. *J. Immunol.* 160: 1290–1296.
20. Höner zu Bentrup, K., and D. G. Russell. 2001. Mycobacterial persistence: adaptation to a changing environment. *Trends Microbiol.* 9: 597–605.
21. Binder, R. J., and P. K. Srivastava. 2005. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells. *Nat. Immunol.* 6: 593–599.
22. Flechtner, J. B., K. P. Cohane, S. Mehta, P. Slusarewicz, A. K. Leonard, B. H. Barber, D. L. Levey, and S. Andjelic. 2006. High-affinity interactions between peptides and heat shock protein 70 augment $CD8^+$ T lymphocyte immune responses. *J. Immunol.* 177: 1017–1027.
23. Tobian, A. A. R., D. H. Canaday, W. H. Boom, and C. V. Harding. 2004. Bacterial heat shock proteins promote CD91-dependent class I MHC cross-presentation of chaperoned peptide to $CD8^+$ T cells by cytosolic mechanisms in dendritic cells versus vacuolar mechanisms in macrophages. *J. Immunol.* 172: 5277–5286.
24. Tobian, A. A. R., C. V. Harding, and D. H. Canaday. 2005. *Mycobacterium tuberculosis* heat shock fusion protein enhances class I MHC cross-processing and -presentation by B lymphocytes. *J. Immunol.* 174: 5209–5214.
25. Mukai, T., Y. Maeda, T. Tamura, M. Matsuoka, Y. Tsukamoto, and M. Makino. 2009. Induction of cross-priming of naive $CD8^+$ T lymphocytes by recombinant bacillus Calmette-Guerin that secretes heat shock protein 70-major membrane protein-II fusion protein. *J. Immunol.* 183: 6561–6568.
26. Makino, M., and M. Baba. 1997. A cryopreservation method of human peripheral blood mononuclear cells for efficient production of dendritic cells. *Scand. J. Immunol.* 45: 618–622.
27. Wakamatsu, S., M. Makino, C. Tei, and M. Baba. 1999. Monocyte-driven activation-induced apoptotic cell death of human T-lymphotropic virus type I-infected T cells. *J. Immunol.* 163: 3914–3919.
28. Makino, M., S. Shimokubo, S. I. Wakamatsu, S. Izumo, and M. Baba. 1999. The role of human T-lymphotropic virus type 1 (HTLV-1)-infected dendritic cells in the development of HTLV-1-associated myelopathy/tropical spastic paraparesis. *J. Virol.* 73: 4575–4581.
29. Makino, M., Y. Maeda, Y. Fukutomi, and T. Mukai. 2007. Contribution of GM-CSF on the enhancement of the T cell-stimulating activity of macrophages. *Microbes Infect.* 9: 70–77.
30. Makino, M., Y. Maeda, M. Kai, T. Tamura, and T. Mukai. 2009. 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.
31. Maeda, Y., M. Gidoh, N. Ishii, C. Mukai, and M. Makino. 2003. Assessment of cell mediated immunogenicity of *Mycobacterium leprae*-derived antigens. *Cell. Immunol.* 222: 69–77.
32. Bardarov, S., S. Bardarov Jr, Jr., M. S. Pavelka Jr, Jr., V. Sambandamurthy, M. Larsen, J. Tufariello, J. Chan, G. Hatfull, and W. R. Jacobs Jr, Jr. 2002. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis, M. bovis* BCG and *M. smegmatis. Microbiology* 148: 3007–3017.
33. Shepard, C. C., and D. H. McRae. 1968. A method for counting acid-fast bacteria. *Int. J. Lepr. Other Mycobact. Dis.* 36: 78–82.
34. Mittrücker, H.-W., U. Steinhoff, A. Köhler, M. Krause, D. Lazar, P. Mex, D. Miekley, and S. H. E. Kaufmann. 2007. Poor correlation between BCG vaccination-induced T cell responses and protection against tuberculosis. *Proc. Natl. Acad. Sci. USA* 104: 12434–12439.
35. Ramasesh, N., L. B. Adams, S. G. Franzblau, and J. L. Krahenbuhl. 1991. Effects of activated macrophages on *Mycobacterium leprae. Infect. Immun.* 59: 2864–2869.
36. Kaufmann, S. H. E., and A. J. McMichael. 2005. Annulling a dangerous liaison: vaccination strategies against AIDS and tuberculosis. *Nat. Med.* 11(4 Suppl): S33–S44.
37. Schiavo, R., D. Baatar, P. Olkhanud, F. E. Indig, N. Restifo, D. Taub, and A. Biragyn. 2006. Chemokine receptor targeting efficiently directs antigens to MHC class I pathways and elicits antigen-specific $CD8^+$ T-cell responses. *Blood* 107: 4597–4605.
38. Burgdorf, S., and C. Kurts. 2008. Endocytosis mechanisms and the cell biology of antigen presentation. *Curr. Opin. Immunol.* 20: 89–95.
39. Martin, C. A., D. L. Kurkowski, A. M. Valentino, and F. Santiago-Schwarz. 2009. Increased intracellular, cell surface, and secreted inducible heat shock protein 70 responses are triggered during the monocyte to dendritic cell (DC) transition by cytokines independently of heat stress and infection and may positively regulate DC growth. *J. Immunol.* 183: 388–399.
40. Ziętara, N., M. Łyszkiewicz, N. Gekara, J. Puchałka, V. A. Dos Santos, C. R. Hunt, T. K. Pandita, S. Lienenklaus, and S. Weiss. 2009. Absence of IFN-β impairs antigen presentation capacity of splenic dendritic cells via down-regulation of heat shock protein 70. *J. Immunol.* 183: 1099–1109.
41. Ryan, A. A., J. K. Nambiar, T. M. Wozniak, B. Roediger, E. Shklovskaya, W. J. Britton, B. Fazekas de St Groth, and J. A. Triccas. 2009. Antigen load governs the differential priming of CD8 T cells in response to the bacille Calmette Guerin vaccine or Mycobacterium tuberculosis infection. *J. Immunol.* 182: 7172–7177.

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Suppressed induction of mycobacterial antigen-specific T_h1-type $CD4^+$ T cells in the lung after pulmonary mycobacterial infection

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Abstract

Although the importance of T_h1-type immune response in protection against mycobacterial infection is well recognized, its regulatory mechanism in the *Mycobacterium tuberculosis* (Mtb)-infected lung is not well characterized. To address this issue, we analyzed kinetics of induction of mycobacterial antigen-specific $CD4^+$ T_h1 T cells after mycobacterial infection in P25 TCR-transgenic (Tg) mice which express TCR α and β chains from a mycobacterial Ag85B-specific MHC class II A^b-restricted $CD4^+$ T-cell clone. To supply normal regulatory T-cell repertoire, we transferred normal spleen T cells into the P25 TCR-Tg mice before infection. High dose subcutaneous infection with Mtb or *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) induced P25 TCR-Tg $CD4^+$ T_h1 cells within a week. In contrast, high-dose Mtb or BCG infection into the lung failed to induce P25 TCR-Tg $CD4^+$ T_h1 cells at the early stage of the infection. Furthermore, low-dose Mtb infection into the lung induced P25 TCR-Tg $CD4^+$ T_h1 cells on day 21 in the mediastinal lymph node but not in the lung. IL-10 was partially involved in the suppression of T_h1 induction in the lung because pretreatment of mice with anti-IL-10 antibody resulted in increase of P25 TCR-Tg $CD4^+$ T_h1 cells in the Mtb-infected lung on day 21 of the infection, whereas neutralization of transforming growth factor-β, another important suppressive cytokine in the lung, showed no effects on the T_h1 induction. Our data suggest that induction of anti-mycobacterial $CD4^+$ T_h1 cells is suppressed in the mycobacteria-infected lung partially by IL-10.

Keywords: infection, lung, *Mycobacterium*, TCR transgenic mouse, T_h1

Introduction

Tuberculosis caused by *Mycobacterium tuberculosis* (Mtb) infection is a leading cause of morbidity and mortality responsible for 1.7 million deaths annually (1). Mycobacterial antigen-specific IFN-γ-producing $CD4^+$ (T_h1) and $CD8^+$ (Tc1) T cells have pivotal roles in the protective immune response against tuberculosis (2–4). The T cells also

contribute to the generation of granulomas in which aggregated macrophages engulfed Mtb are surrounded by a cuff of lymphocytes, including the $CD4^+$ and $CD8^+$ T cells (5, 6).

Mycobacterium bovis bacillus Calmette–Guérin (BCG) is an attenuated strain of *M. bovis* established by Calmette and Guérin for vaccination against Mtb. BCG vaccination to children has been reported to be efficacious against infant tuberculosis, especially miliary tuberculosis and tuberculous meningitis (7–9). However, prophylactic effect of BCG against adult pulmonary tuberculosis is variable in that the efficacy has ranged from 0 to 80% (10–12). Therefore, a new vaccine effective against adult pulmonary tuberculosis is required.

Since mucosal tissues of respiratory tract show unique immunoregulatory mechanism (13), a new vaccine strategy would be required to induce efficient protective immunity in the Mtb-infected lung. However, it is not well understood how induction of T_h1 and Tc1 responses is regulated in the lung after Mtb infection. In tissues such as skin and muscle that are usually used to inoculate vaccines, dendritic cells (DC) capture the inoculated pathogens or antigens and migrate into draining lymph node (LN) to present the antigen to T cells to initiate immune response. In contrast, pulmonary immune response was initiated not only in the lung-draining LN but also in bronchus-associated lymphoid tissue (14). Although it has been reported that pulmonary immune response is regulated by alveolar macrophages and cytokines such as IL-10 and transforming growth factor (TGF)-β (13), their involvement in regulation of anti-mycobacterial T_h1 cells in mycobacteria-infected lung is not clearly understood.

Mtb Ag85B is known as a major Mtb antigen that induces T_h1 response. A 15-mer peptide (Peptide-25, P25) covering amino acid residues 240–254 of the Ag85B has been reported to be recognized by $CD4^+$ T cells in MHC class II H-$2A^b$-restricted manner and induced T_h1 T cells producing IFN-γ and IL-2 (15–17). A transgenic (Tg) mouse line that expresses functional TCR specific for the P25 epitope of Ag85B in an A^b-restricted manner (P25 TCR-Tg mice) has been established (18). The P25 TCR-Tg $CD4^+$ T cells were activated to proliferate in the lung-draining mediastinal lymph node (MLN) from day 14 of pulmonary Mtb infection (19). Kinetics analysis of mycobacterial antigen-specific T cells using another Tg mice expressing TCR specific for Mtb-derived ESAT6 antigen showed that the T cells are activated between 7 and 10 days after pulmonary Mtb infection, whereas T_h1 response was demonstrated in the lung 15 days after the infection (20). For all the results, kinetics of T_h1 induction was not compared between vaccine strain BCG and virulent Mtb and between subcutaneous (s.c.) and pulmonary infection routs.

By using the P25 TCR-Tg mice, we analyzed kinetics of induction of mycobacterial antigen-specific $CD4^+$ T_h1 T cells after Mtb or BCG infection in the lung and compared the kinetics with that induced by s.c. infection. Furthermore, regulatory mechanism of the mycobacterial antigen-specific T_h1 T-cell induction in the lung was analyzed in the Tg T-cell system.

Methods

Animals

The P25 TCR-Tg mice express Ag85B P25 epitope-specific H-$2A^b$-restricted TCR from $CD4^+$ T-cell clone BP1 under C57BL/6 background (18). B6 Ly5.1 mice were a kind gift of Dr Yasunobu Yoshikai (Kyushu University, Fukuoka, Japan). Mice were used at 8–12 weeks of age. To supply normal T-cell repertoire to the P25 TCR-Tg mice, each P25 TCR-Tg mouse was transferred with 5×10^7 spleen cells from naive B6 Ly5.1 mice 1 day before infection (Normal T-cell repertoire-supplied (N)-P25 TCR-Tg mice). Experiments were conducted according to the Institutional Ethical Guidelines for Animal Experiments and the Safety Guideline for Living Modified Organism Experiments of the University of the Ryukyus under approval of the Animal Experiments Safety and Ethics Committee and the Living Modified Organism Experiments Safety Committee of the University of the Ryukyus, respectively.

P25 TCR-Tg T cell-specific anti-idiotype mAb

B cells from rats immunized with BP1-derived TCR-expressing rat TG40 cells were fused with SP2/0 myeloma cells, and a B-cell hybridoma that react with $CD4^+$ T cells of the P25 TCR-Tg mice but not to other T cells was established as the producer of anti-idiotype mAb KN7.

Microorganisms

Mtb H37Rv strain was grown in Middlebrook 7H9 broth (BD, Sparks, MD, USA) with albumin-dextrose-catalase enrichment (BD) at 37°C for 3 weeks. Viable bacterial number was determined on 7H10 agar plates (BD) with oleic acid-albumin-dextrose-catalase (OADC) enrichment (BD). BCG Tokyo strain was purchased from Japan BCG (Kiyose, Japan). The bacteria were re-suspended in PBS before use. Mtb was infected s.c. into footpad or i.t. with 1×10^3 or 5×10^6 colony-forming unit (CFU) in 50 μl of PBS. BCG were infected s.c. into footpad or i.t. with 5×10^6 CFU in 50 μl of PBS. Bacterial number in infected organs was determined by plating serially diluted organ homogenates onto 7H10 agar plates with OADC enrichment.

Cell preparation

Single-cell suspensions were prepared from the lungs as described (21). Footpad-draining popliteal and inguinal lymph node (DLN), lung-draining MLN and spleens were suspended by passing through 30-mm stainless steel mesh. Adherent splenocytes of B6 Ly5.1 mice were collected after 90 min culture and used as antigen presenting cells (APC).

Bacterial burden and cytokine assay

Mice were infected with s.c. into footpad or i.t. with 5×10^6 CFU of Mtb or BCG, and the DLN, MLN and lung were removed 1, 2 and 4 weeks later, homogenized in distilled water and plated on 7H10 agar (BD) to determine bacterial number. The lung homogenates were also used to determine IFN-γ levels by ELISA (R&D systems, Minneapolis, MN, USA).

Cell culture

Cells were suspended in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% FBS (Equitech Bio, Kerrville, TX, USA), 100 U ml^{-1} of penicillin (Meiji,

Yokohama Japan) and 100 μg ml^{-1} of streptomycin (Meiji) at 5×10^6 cells per ml and cultured with 5 μg ml^{-1} purified protein derivative (PPD) of Mtb (Japan BCG), recombinant (r) Ag85B of BCG or P25 peptide (FQDAYNAAGGHNAVF) (Invitrogen, Carlsbad, CA, USA). rAg85B was produced from yeast *Pichia pastoris* as secretory protein by using pPIC9K expression vector (Invitrogen) containing the full-length Ag85B gene cloned from the genomic DNA of BCG Tokyo strain.

FACS analysis

To analyze surface molecules on freshly isolated cells, cells were stained with biotin-conjugated anti-P25 TCR idiotype (KN7), anti-CD4 (Caltag, Burlingame, CA, USA), anti-CD69 (Caltag), anti-CD3 (PharMingen, San Jose, CA, USA) and anti-TCRβ (PharMingen) mAb. Before surface staining, cells were treated with HBSS containing 5% of 2.4G2 anti-FcRγ hybridoma supernatant to block non-specific binding.

To detect IFN-γ^+ T cells, cells were cultured in the presence or absence of PPD for 24 h, at 37°C in 5% of CO_2 with breferdin A (Golgi Plug, BD) for the last 6 h. The cells were pretreated with anti-FcRγ mAb, surface stained with allophycocyanin-conjugated anti-CD4 and biotin-conjugated anti-P25 TCR idiotype KN7 mAb followed by streptavidin–PE, then permeabilized and fixed with Cytofix/Cytoperm Reagent (BD) according to the manufacturer's instructions. The fixed/permeabilized cells were stained with FITC-conjugated anti-IFN-γ mAb (eBioscience, San Diego, CA, USA).

To label proliferating cells *in vivo*, mice were given 5-bromo-2-deoxyuridine (BrdU; Sigma–Aldrich, St Louis, MI, USA) at 0.8 mg ml^{-1} in drinking water during the last 3 days before analysis (22, 23). To label proliferating cells *in vitro*, naive splenocytes from the P25 TCR-Tg mice were stimulated with antigen for 7 days at 37°C 5% CO_2, then re-stimulated for 48 h with the antigen and APC in the presence of 10 μM BrdU. The BrdU-labeled cells were detected using a BrdU Flow kit (BD) according to the manufacturer's instructions.

The stained cells were analyzed with the FACSCalibur flowcytometer (BD) and CellQuest software (BD).

Reverse transcription–PCR

Total RNA was extracted, reverse transcribed and amplified by PCR as described (21). The PCR product was electrophoresed on a 1.8% agarose gel and then was observed by Gel Documentation system (Bio-Rad, Hercules, CA, USA). To perform real-time PCR assay, the cDNA was amplified using the iCycler iQ and the amplification data were analyzed by the $2^{-\Delta\Delta CT}$ method using Real-Time PCR Optical System Software Version 3.0 (Bio-Rad) as described (21). The PCR primers used were as follows: *TGF-β* sense (5′-ATTCCTGGCGTTACCTTGG-3′), *Tgfβ* antisense (5′-CCTGTATTCCGTCTCCTTGG-3′); *Il10* sense (5′-AGGGAGATTATATTATATGATGGG-3′), *Il10* antisense (5′-TTTCTCACCTCTCTTAGG-3′); *Il4* sense (5′-CGAAGAACACCACAGAGACTGAGCT-3′), *Il4* antisense (5′-GACTCATTCATGGTGCAGCTTATCG-3′) and *Actβ* sense (5′-TGGAATCCTGTGGCATCCATGAAA-C-3′), *Actβ* antisense (5′-TAAAACGCAGCTCAGTAACAGTCCG-3′).

Neutralizing mAb

Anti-IL-10 (SXC-1) mAb and anti-TGF-β (1D11) mAb were purified from hybridoma culture supernatants and 400 μg of the mAb was i.v. injected as indicated.

Statistical analysis

To compare more than three groups, a Kruskal–Wallis test was used to analyze difference of mean ranks of the groups, followed by a post-hoc test when significant result was obtained using the Kruskal–Wallis test. All the statistical analyses were carried out using Statcel2 software (OMS, Tokorozawa, Japan). A P value of <0.05 was considered to indicate significant difference.

Results

Experimental design to detect mycobacterial antigen-*specific T_h1 T cells in the lung*

To confirm the specificity and detection sensitivity of the anti-idiotype mAb KN7 established against the P25 TCR-Tg T cells, splenocytes prepared from the P25 TCR-Tg mice were stained with the mAb KN7. The mAb stained ~90% of TCRβ$^+$ spleen T cells of the P25 TCR-Tg mice (Fig. 1A), whereas negligible fraction of spleen cells of the wild-type C57BL/6 mice was stained with the KN7 mAb (data not shown). In addition, the P25 TCR-Tg mice and wild-type C57BL/6 mice were found equally susceptible for Mtb infection and capable to produce IFN-γ in the lung 4 weeks after lung infection (Fig. 1B and C) or i.v. infection (data not shown) of Mtb, suggesting that the P25 TCR-Tg mice are not vulnerable to Mtb infection.

Although there is a report showing three amino acid substitutions within the P25 epitope of BCG Tokyo strain compared with that of Mtb H37Rv strain (24), BCG Pasteur strain (GenBank AM408590) is known to have the identical epitope sequence to that of Mtb H37Rv strain. Therefore, we decided to re-examine genomic DNA sequence of the Ag85B gene from BCG Tokyo strain, and we found that BCG Tokyo strain has identical P25 epitope sequence to that of Mtb H37Rv (data not shown). In *in vitro* culture, the naive P25 TCR-Tg CD4$^+$ T cells are activated and proliferated equally to the synthetic P25 peptide, BCG Tokyo strain-derived rAg85B, as well as to the PPD of Mtb that contains high amount of Ag85B (25) as determined by CD69 expression and BrdU incorporation (Fig. 1D). These data confirmed that the P25 TCR-Tg CD4$^+$ T cells can recognize P25 epitope of both Mtb and BCG origin. Therefore, we decided to use both Mtb and BCG Tokyo strain for *in vivo* infection experiments of the P25 TCR-Tg mice and evaluated *in vitro* T-cell response using PPD which contain Mtb-derived Ag85B to stimulate the P25 TCR-Tg CD4$^+$ T cells in the following experiments.

To determine kinetics of induction of mycobacterial antigen-specific T_h1-type CD4$^+$ T cells, we initially transferred naive P25 TCR-Tg T cells (Ly5.2$^+$) into wild-type C57BL/6 Ly5.1 mice and then i.t. infected the mice with Mtb. Although *in vivo* proliferation of the transferred P25 TCR-Tg CD4$^+$ T cells was demonstrated after pulmonary Mtb infection (19), we failed to stably detect IFN-γ production of the transferred

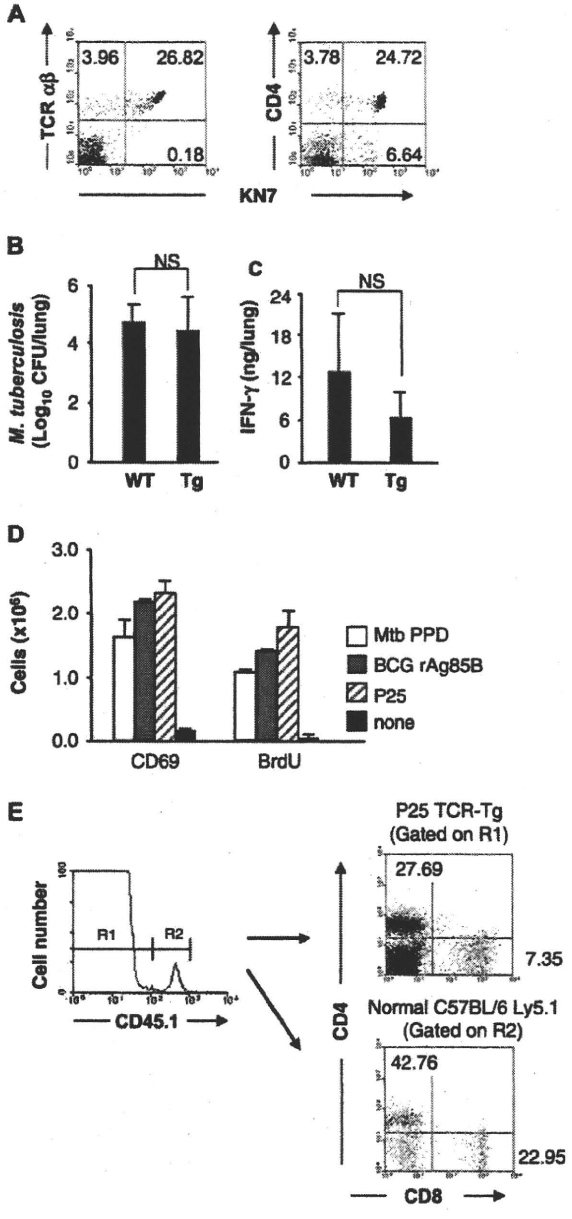

Fig. 1. Phenotype and anti-mycobacterial response of the P25 TCR-Tg mice. (A) Splenocytes from the naive P25 TCR-Tg mice were stained with anti-idiotypic mAb KN7, with anti-TCRβ or with anti-CD4 mAb. The cells were analyzed by FACS with analysis gate on lymphocytes. (B) Bacterial burden in the lungs of the wild-type or P25 TCR-Tg mice was analyzed 4 weeks after lung infection with 200 CFU of Mtb. The lungs were homogenized, plated on 7H10 agar and incubated to count the number of colonies. (C) IFN-γ levels in the lung homogenates were measured by ELISA 4 weeks after Mtb lung infection. (D) Activation and proliferation of the naive P25 TCR-Tg splenocytes were analyzed *in vitro*. Naive splenocytes from P25 TCR-Tg mice were cultured for 7 days with Mtb-derived PPD (open bars), BCG-derived rAg85B (gray bars), P25 peptide (striped bars) or without antigen (closed bars). Cells were re-stimulated for 48 h with relevant antigen, and the number of CD69-expressing and BrdU-incorporated P25 TCR-Tg CD4$^+$ T cells were measured by the FACS. The data shown are representatives of two independent experiments. (E) Detection of donor-derived CD45.1 cells in the lung of N-P25 TCR-Tg mice. Splenocytes of C57BL/6 Ly5.1 mice were intraperitoneal injection into P25 TCR-Tg mice. The next day, lymphocytes prepared from the lung were stained with anti-CD45.1 (Ly5.1), anti-CD4 and anti-CD8, and donor-derived CD45.1$^+$ T cells were detected by FACS. The data also demonstrate CD4 and CD8 expression of the donor-derived and the recipient P25 TCR-Tg mouse-derived T cells. The recipient-derived T cells are consisted of higher percentage of CD4$^+$ T cells which express Tg TCR, whereas donor-derived T cells showed normal CD4:CD8 ratio (~2:1).

P25 TCR-Tg CD4$^+$ T cells using KN7 mAb or anti-CD45.2 (Ly5.2) mAb in the mycobacteria-infected lung because the P25 TCR-Tg CD4$^+$ T cells are <1% of the total lung T cells even when 5×10^6 T cells were transferred, and IFN-γ$^+$ T cells represent <10% of the Tg CD4$^+$ T cells on day 28 after the infection (data not shown). Therefore, we concluded that a system which contains higher number of the P25 TCR-Tg CD4$^+$ T cells with normal T-cell regulatory system is required to analyze kinetics of mycobacterial antigen-specific T_h1-type T cells using the Tg mice. To overcome this problem, the P25 TCR-Tg mice were transferred with the normal T-cell repertoire of C57BL/6 Ly5.1 mice to construct the normal T cell-supplied (N)-P25 TCR-Tg mice. Twenty-four hours after the construction of the N-P25 TCR-Tg mice, the donor C57BL/6-Ly5.1-derived T cells were detectable with anti-CD45.1 mAb (Ly5.1) in the lung, (Fig. 1E). The N-P25 TCR-Tg mice were used to analyze the kinetics of activation and T_h1 differentiation of the P25 TCR-Tg CD4$^+$ T cells under normal immunoregulation after mycobacterial infection.

The Ag85B-specific P25 TCR-Tg CD4$^+$ T cells were activated and differentiated to T_h1 cells rapidly after BCG s.c. infection

Using the N-P25 TCR-Tg mice, we examined kinetics of bacterial number and activation, proliferation and T_h1 differentiation of the mycobacterial Ag85B-specific P25 TCR-Tg CD4$^+$ T cells after mycobacterial infection. We first analyzed the response of the P25 TCR-Tg CD4$^+$ T cells after s.c. infection of BCG into the footpads. After s.c. infection of 5×10^6 CFU of BCG, the bacteria was stably detected in the DLN until day 28 of the infection (Fig. 2A). The number of P25 TCR-Tg CD4$^+$ T cells in the DLN increased from day 3 and peaked on day 28 after the infection (Fig. 2B). The s.c. BCG infection induced expression of early T-cell activation marker CD69, proliferation detected by BrdU incorporation and IFN-γ expression of the P25 TCR-Tg CD4$^+$ T cells that are detected by FACS analysis (Fig. 2B). The IFN-γ expression was induced by antigen recognition of the P25 TCR-Tg CD4$^+$ T cells because IFN-γ production was not induced in the absence of *in vitro* antigen stimulation of the T cells (Fig. 2C). The number of the CD69$^+$ (Fig. 2D) and IFN-γ$^+$ (Fig. 2F) P25 TCR-Tg CD4$^+$ T cells peaked on day 3, and BrdU uptake of the T cells peaked on day 7 (Fig. 2E) after the s.c. BCG infection. Although the P25 TCR-Tg CD4$^+$ T cells showed the highest number on day 28 of the s.c. infection and IFN-γ$^+$ Tg CD4$^+$ T cell number also increased slightly on day 28, the increase was not statistically significant. Inoculation of low doses (1×10^3 to 1×10^4 CFU) of BCG failed to induce detectable activation and T_h1 induction