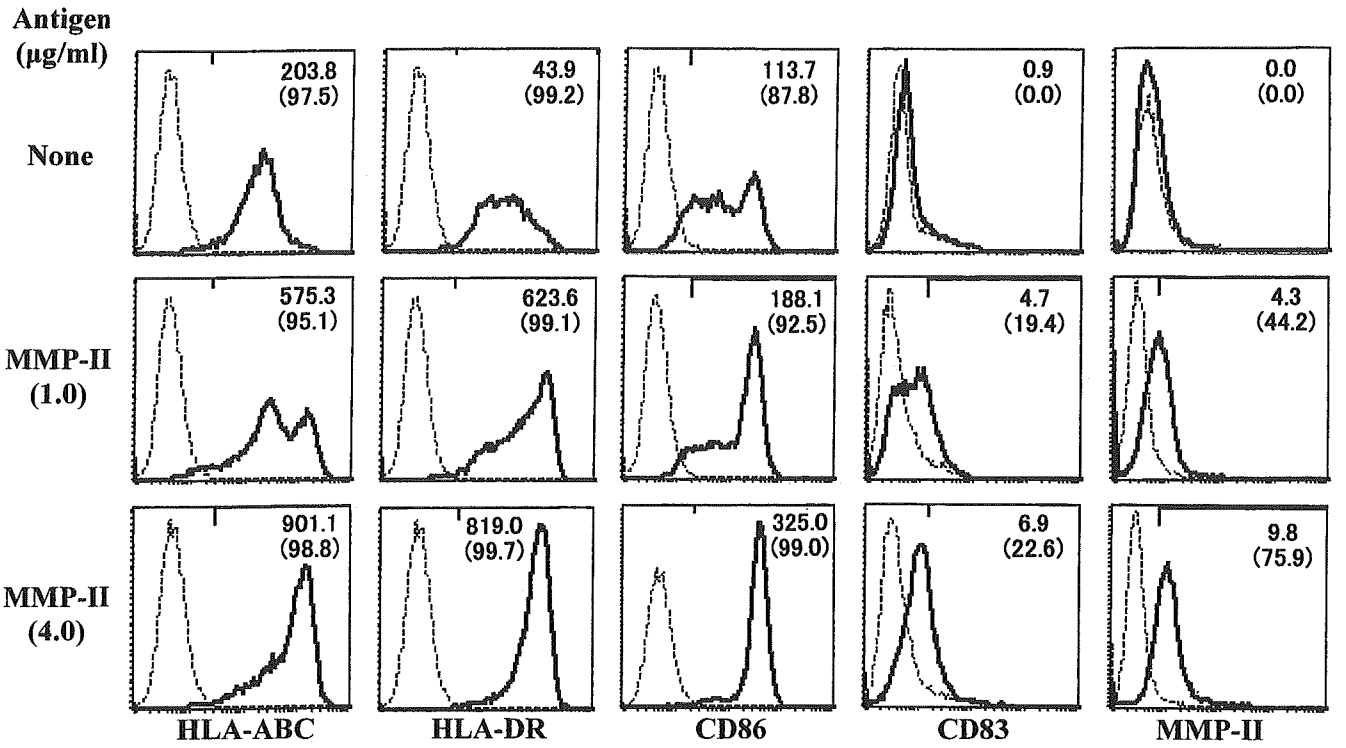

FIG. 4. Expression of various molecules on DCs pulsed with MMP-II. Monocyte-derived DCs from healthy individuals (PPD positive) were pulsed with the indicated dose of MMP-II. DCs were gated and analyzed. Solid curves, isotype-matched control IgG; broken curves, the indicated MAb. The number in the top right corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test MAb. The number in parentheses is the percentage of positive cells. Results of one experiment representative of three separate experiments are shown.

Assessment of APC function of DCs pulsed with cell membrane fractions. The ability of DCs pulsed with various fractions of the *M. leprae* cell membrane to stimulate autologous T cells was assessed using an autologous stimulator–T-cell mixed reaction as previously described (10, 24). The Ag-pulsed DCs were treated with 50 μg/ml of mitomycin C, washed extensively to remove extracellular Ags, and used as a stimulator. T cells were prepared as follows: freshly thawed PBMCs were depleted of major histocompatibility complex (MHC) class II^+ cells by using magnetic beads coated with a MAb to MHC class II Ag (Dynabeads 450; Dynal) and were further treated with beads coated with either a CD4 or a CD8 MAb to select T cells negatively as previously reported (10). The purity of $CD4^+$ T cells or $CD8^+$ T cells was more than 98%. The supernatant of the stimulator–T-cell mixture was collected on day 4 of coculture, and the level of gamma interferon (IFN-γ) produced was measured by an Opt EIA Human ELISA Set (BD PharMingen International).

Assessment of cytokine production. Levels of the following cytokines were measured: tumor necrosis factor alpha (TNF-α), IL-10, and IL-12 p70 produced from either macrophages or DCs by stimulation with MMP-II for 24 h in the presence or absence of a soluble form of CD40L (Pepro Tech). The murine MAb against TLR-2 (clone 2392; IgG1) with neutralizing activity was obtained from Genentech (San Francisco, CA). The optimal concentration of the anti–Toll-like receptor 2 (anti–TLR-2) Ab was determined in advance. The concentrations of IL-12 p70, IL-10, and TNF-α were quantified using the Opt EIA Human ELISA Set enzyme assay kits, available from BD PharMingen International.

Cell transfection and luciferase assay. Human embryonic kidney cells (HEK293) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% FCS, 50 mg/ml penicillin/streptomycin, and nonessential amino acids (Invitrogen, Carlsbad, CA) at 37°C in a humidified incubator with 5% CO_2. The cDNA of human TLR-2 was PCR amplified using a human spleen cDNA library (BD Biosciences, San Jose, CA) and inserted into pCIneo (Promega, Madison, WI). HEK293 cells (2×10^4) were transiently transfected with a mixture of plasmids—200 ng of pCIneo hTLR2, 25 ng of p5×NF-κB-luc

TABLE 1. Cytokine production from DCs stimulated with MMP-II[a]

DC stimulation (dose)	Concn (pg/ml) of the following cytokine:					
	IL-12 p70		TNF-α		IL-10	
	CD40L (−)	CD40L (+)	CD40L (−)	CD40L (+)	CD40L (−)	CD40L (+)
None	2.6 ± 0.2*,†	17.7 ± 0.4‡,§	2.6 ± 0.4¶,‖	15.4 ± 3.8**,††	2.0 ± 0.1	3.4 ± 0.1
MMP-II (1 μg/ml)	51.2 ± 0.5*	782.0 ± 8.7‡	345.4 ± 9.9¶	345.7 ± 19.3**	2.5 ± 0.3	1.7 ± 0.1
MMP-II (4 μg/ml)	404.0 ± 9.8†	1624.0 ± 11.0§	773.8 ± 11.1‖	747.3 ± 18.7††	2.2 ± 0.1	2.8 ± 0.3
LPS (0.3 pg/ml)	2.8 ± 0.3	18.7 ± 0.6	5.0 ± 1.3	36.0 ± 9.2	2.0 ± 0.3	3.0 ± 0.2

[a] Monocyte-derived DCs (10^5/well) were stimulated for 24 h with the indicated dose of MMP-II in the absence [CD40L (−)] or presence [CD40L (+)] of a soluble form of CD40L (1.0 μg/ml). The DCs were also stimulated for 24 h with 0.3 pg/ml of LPS, which is estimated to be present in 4 μg/ml of MMP-II. Results representative of more than three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± standard deviations. Titers with the same symbols are statistically compared by Student's *t* test, as follows: *, $P < 0.001$; ‡, $P < 0.001$; ¶, $P < 0.0005$; **, $P < 0.001$; †, $P < 0.001$; §, $P < 0.001$; ‖, $P < 0.0001$; ††, $P < 0.0005$.

TABLE 2. Cytokine production from macrophages stimulated with MMP-II[a]

Macrophage stimulation (dose)	Concn (pg/ml) of the following cytokine:		
	IL-12 p70	TNF-α	IL-10
None	2.0 ± 0.2	19.3 ± 1.8*,†	45.3 ± 8.8‡,§
MMP-II (1.0 μg/ml)	2.1 ± 0.3	122.2 ± 6.8*	149.9 ± 20.3‡
MMP-II (4.0 μg/ml)	1.9 ± 0.2	568.6 ± 12.4†	561.2 ± 31.9§
LPS (0.3 pg/ml)	1.8 ± 0.1	10.0 ± 2.0	15.6 ± 3.2

[a] Monocyte-derived macrophages (10^5/well) were stimulated for 24 h in the presence of a soluble form of CD40L (1.0 μg/ml) with the indicated dose of MMP-II. Results representative of more than three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± standard deviations. Titers with the same symbols are statistically compared by Student's *t* test, as follows: *, $P < 0.001$; ‡, $P < 0.005$; †, $P < 0.0005$; §, $P < 0.001$.

(Stratagene, La Jolla, CA), and 10 ng of pRL-TK-*Renilla* luciferase plasmid (Promega)—using the FuGENE 6 reagent (Roche Molecular Biochemicals, Indianapolis, IN), as previously described (38). Thirty-six hours after transfection, cells were treated with various amounts of glutathione *S*-transferase (GST), MMP-II, or peptidoglycan (PGN) as a positive control (for TLR-2-dependent luciferase activity) for a further 6 h. The cells were lysed in 70 μl of 1× passive lysis buffer (Promega), and luciferase activity in 10 μl of the cell lysate was measured using the Promega Dual-Luciferase Reporter Assay System according to the protocol provided by the manufacturer. Data were expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency.

Statistical analysis. Student's *t* test was applied to demonstrate statistically significant differences.

RESULTS

Identification of *M. leprae*-derived antigenic molecules. The cell membrane fraction from *M. leprae* was found to be the most T-cell stimulating (10, 22), although it may also contain some inhibitory molecules (10). The *M. leprae*-derived cell membrane fraction was solubilized and further fractionated using a gel filtration column to search for the antigenic molecules. Figure 1 shows the silver staining of each fraction, which revealed several proteins. Then we pulsed healthy donor-derived DCs with each of these fractions individually and examined the antigenicity of each fraction by monitoring IFN-γ production by DC-stimulated $CD4^+$ and $CD8^+$ T cells derived from PPD-positive healthy individuals (Fig. 2). Among the eight fractions, fractions 4 through 6 seemed to be efficient at stimulating cytokine production by $CD4^+$ T cells, and fractions 4 and 5 appeared to be involved in the activation of both $CD4^+$ and $CD8^+$ T cells. Thus, using these two fractions of cell membrane, we identified one of the antigenic molecules. The pooled PB leprosy sera, preadsorbed with *M. leprae* cytosol fractions, were used for Western blot analysis of the fractions (not shown). N-terminal sequencing of the serum-reactive bands common to fractions 4 and 5 identified MMP-II as one of the candidates. The presence of MMP-II in fractions 4 and 5 was further confirmed by Western blotting using a MAb against MMP-II (Fig. 3). For the purification of the protein, the MMP-II gene was amplified by PCR from the genomic DNA of *M. leprae*, and MMP-II protein was subsequently expressed in *E. coli* by using the T7 expression system (pET-28). The expressed protein was confirmed to be MMP-II by Western blot analysis (not shown), by comparison to purified MMP-II, used as a positive control (donated by P. J. Brennan, Colorado University).

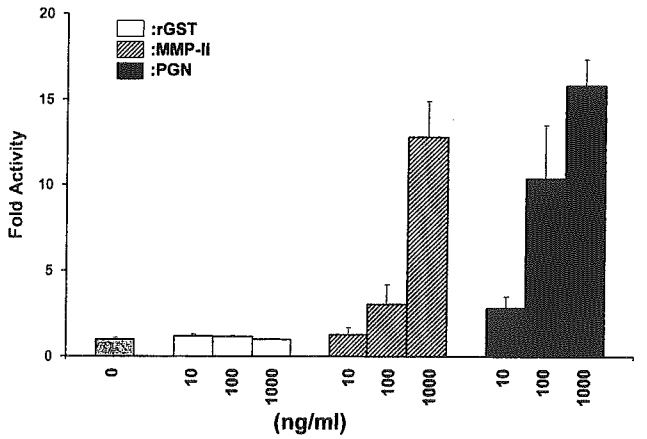

FIG. 5. NF-κB-dependent reporter gene activity of the TLR2 transfectant was measured after stimulation with or without 10, 100, or 1,000 ng/ml of rGST, MMP-II, or PGN, as described in Materials and Methods. Data are expressed as fold induction relative to the activity of *Renilla* luciferase, which is an internal control for transfection efficiency in the dual-luciferase reporter assay. Results of one experiment representative of two separate experiments are shown. Assays were done in triplicate, and the results are expressed as means ± standard deviations.

Antigenicity of *M. leprae*-derived MMP-II. The ability of MMP-II to evoke cellular immunity was assessed using DCs and macrophages as APCs. Previously we demonstrated that the cytosol fraction from *M. leprae* was less efficient at the induction of DC maturation and that the whole cell membrane fraction partially induced DC maturation (22). In contrast, when immature DCs were pulsed with MMP-II, they up-regulated the expression levels of HLA-ABC, HLA-DR, CD86, and CD83 Ags on the surfaces of DCs in an Ag dose-dependent manner, and the percentage of $CD83^+$ cells was found to increase significantly (Fig. 4). The expression of MMP-II on the surfaces of MMP-II-pulsed DCs was revealed using a MAb to MMP-II (Fig. 4). The functional aspects of MMP-II in terms of APC activation were assessed by measuring production of cytokines, such as IL-12 p70, IL-10, and TNF-α, by APCs (Tables 1 and 2). The bioactive form of IL-12 was released from DCs by pulsing MMP-II in the absence of CD40L, and the cytokine production level was enhanced by copulsing DCs with CD40L and MMP-II (Table 1). Obviously IL-12 was not produced from DCs by stimulation with the amount of LPS estimated to be present in 4 μg/ml of MMP-II. Furthermore, DCs produced TNF-α in the presence or absence of CD40L, but they did not produce any significant amount of IL-10 due to MMP-II stimulation. These results suggested that MMP-II could activate DCs and induce their maturation. Macrophages derived from monocytes did not produce IL-12 p70 by stimulation with MMP-II, but they produced TNF-α and IL-10 (Table 2), which are found predominantly in granulomatous mycobacterium-infected lesions. These results indicate that MMP-II also activated macrophages, but macrophages and DCs seem to have distinct functional roles. All cytokines were produced in an Ag dose-dependent fashion.

Involvement of TLR-2 in activation of DCs. In order to elucidate the mechanism by which MMP-II activates DCs, we examined the relationship of MMP-II and TLR-2, because

TABLE 3. Effect of the TLR-2-antagonistic Ab on IL-12 p70 production by DCs[a]

DC stimulation (dose)	IL-12 p70 production (pg/ml) in the presence of:				
	TLR-2-antagonistic Ab at the following concn (μg/ml):			Control IgG at the following concn (μg/ml):	
	0	5.0	10.0	5.0	10.0
MMP-II (1.0 μg/ml)	603.1 ± 11.0*†	491.2 ± 10.2*	178.1 ± 8.8†	658.2 ± 11.3	675.9 ± 10.7
MMP-II (4.0 μg/ml)	1,210.2 ± 20.0‡,§	949.0 ± 9.3‡	805.3 ± 7.9§	1,290.3 ± 12.4	1,403.8 ± 31.5

[a] Monocyte-derived immature DCs (10^6/ml) were treated with the indicated dose of a TLR-2-antagonistic MAb or an isotype-matched control IgG and were subsequently stimulated for 24 h with MMP-II in the presence of CD40L (1.0 μg/ml). Results representative of more than three separate experiments are shown. Assays were done in triplicate, and results are expressed as means ± standard deviations. Titers with the same symbols were statistically compared by Student's *t* test, as follows: *, $P < 0.0001$; †, $P < 0.0005$; ‡, $P < 0.0001$; §, $P < 0.0001$.

TLR-2 is reported to be highly associated with induction of innate immunity against mycobacterial infection (1, 4, 30). When HEK293 cells that had been cotransfected with pCIneo TLR-2, p5×NF-κB-luc, and pRL-Tk-*Renilla* luciferase were pulsed with MMP-II, significant levels of luciferase activity were induced in an Ag dose-dependent manner, levels comparable to those induced by PGN, a well-defined TLR-2-associated bacterial Ag (Fig. 5). Similar results were also obtained using *M. leprae*-derived MMP-II. Such changes were not induced by rGST, a negative-control protein. Furthermore, when the surface TLR-2 Ag on DCs was masked by an antagonistic Ab to TLR-2, IL-12 p70 production by DCs stimulated with MMP-II was significantly, though partially, suppressed (Table 3). The isotype-matched control IgG did not affect IL-12 p70 production by MMP-II-stimulated DCs. As expected, the TLR-2-antagonistic Ab did not suppress IL-12 p70 production by DCs stimulated with LPS (a ligand for TLR-4) (not shown). These results indicate that MMP-II might use TLR-2 as its ligand on APCs, resulting in stimulation of DCs.

DISCUSSION

Leprosy is a broad-spectrum disease (34). One representative manifestation is PB leprosy. Studies on clinical specimens of the skin lesions indicate that the infection is localized and the spread of *M. leprae* is suppressed as a consequence of activation of cellular immune responses (20, 31, 36). On the other hand, MB leprosy usually manifests widespread infection due to the lack of an efficient response to *M. leprae* components. The mechanisms leading to the broad spectrum are not fully understood yet, but these observations suggest the presence of an Ag with immunomodulating activities that modify the immune responses in vivo. So far, however, such Ags have not been identified. Previously we evaluated the APC function of professional APCs and found that DCs were superior to macrophages in activating T cells (10). When we examined the DC-mediated antigenicity of subcellular components of *M. leprae* for identification of immunomodulating molecules, we found that the cell membrane fraction was more suitable than other fractions (22). Therefore, the *M. leprae* membrane fraction was size fractionated, and each fraction was examined for its T-cell-stimulating ability by using DCs as APCs. Two of the fractions with high activity were examined by reaction to PB leprosy sera, and subsequently the N terminus of the reactive protein was sequenced. As a result, MMP-II was identified as one of the antigenic cell membrane proteins, and the result was confirmed by Western blotting of the various fractions using an anti-MMP-II antibody (Fig. 3).

MMP-II was originally identified from *M. leprae* as a major native protein in 1990 (13) and was recognized as being identical to mycobacterial bacterioferritin (32). Purification of MMP-II by reverse-phase chromatography revealed a large molecular mass of 380 kDa and a ferroxidase center residue. MMP-II contains 1,000 to 4,000 atoms of iron per molecule of protein (32). A homology search on the mycobacterial nucleotide database revealed that MMP-II is conserved among *M. leprae*, *M. tuberculosis*, and *M. avium*. The percent homology at the amino acid level is about 86% among these species. The previous studies reported that MMP-II was recognized in vivo by B and T cells. Sera from patients were reported to have higher IgG titers to MMP-II, regardless of the clinical type of leprosy, than sera from healthy individuals (7). Also, T cells from leprosy of both the PB and the MB type were stimulated by MMP-II to proliferate and to produce both IFN-γ and IL-5 (29). However, tuberculosis patients or individuals who have had contact with leprosy patients have not been examined yet. Also, the influence of MMP-II on the innate immune response has not yet been clarified.

MMP-II stimulated DCs to produce TNF-α and a bioactive form of IL-12 (IL-12 p70) (Table 1) and induced their maturation, as observed by their phenotypic changes (Fig. 4). Further, MMP-II also stimulated macrophages to produce TNF-α and IL-10 (Table 1). These cytokines were produced by stimulation with either MMP-II derived from *M. leprae* (not shown) or MMP-II overexpressed in *E. coli* (Table 1). DCs and macrophages play distinct roles in the host defense against mycobacterial infection (9). DCs are central to the initiation of Ag-specific T-cell responses (6, 27, 36), and in our preliminary experiments, DCs pulsed with purified MMP-II stimulated both $CD4^+$ and $CD8^+$ T cells from PPD-positive healthy individuals to produce IFN-γ (not shown). The activated form of macrophage is involved in the formation of tuberculoid granulomatous lesions (5, 9). These results indicate that MMP-II might contribute to the immune regulation of host cells against mycobacteria. Then we investigated what could be the MMP-II ligand that is expressed on APCs. TLR-2 is associated mainly with innate immunity and has been shown to recognize the molecular pattern of pathogens (4, 11, 18, 26, 33). In mycobacterial infection, it has been reported that a 19-kDa lipoprotein isolated from *M. tuberculosis* ligated TLR-2 (4, 19), and the *M. leprae* 33-kDa lipoprotein could be another candidate participating in the TLR-2-associated innate immune system

(19). In our study using the TLR-2 reporter assay with HEK293 cells, we found that TLR-2 is likely to be involved in the recognition of MMP-II in spite of the fact that MMP-II lacks the triacylated region. This finding surprised us, but a similar ligation of protein to TLR-2 has also been reported for neisserial porins HSP60 and HSP70, which have no posttranslational modification of acylation (2, 25, 39). IL-12 production by MMP-II-stimulated DCs was partially inhibited by a TLR-2-antagonistic Ab, which indicates that other receptors are also involved in signals leading to IL-12 production.

The data in this report, taken together, indicate that MMP-II has an immunomodulating activity and contributes to the activation of innate immunity. Further study should be pursued to evaluate its host defense-associated activity against leprosy and other mycobacterial infections that pose a worldwide threat.

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Immunostimulatory activity of major membrane protein-II from *Mycobacterium leprae*

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Abstract

We examined the antigenicity of an immunomodulatory protein, major membrane protein (MMP)-II, from *Mycobacterium leprae*, since host defense against *M. leprae* largely depends on adaptive immunity. Both unprimed and memory T cells from healthy individuals were stimulated by autologous MMP-II-pulsed monocyte-derived dendritic cells (DCs) to produce IFN-γ. The DC-mediated IFN-γ production was dependent on the expression of MHC, CD86, and MMP-II antigens. Memory T cells from paucibacillary (PB) leprosy more extensively responded to MMP-II-pulsed DCs than T cells from healthy individuals, while comparable IFN-γ was produced by unprimed T cells. Memory T cells from multibacillary leprosy, which are normally believed to be anergic, were activated similarly to those from healthy individuals by MMP-II-pulsed DCs. These results suggest that memory T cells from PB leprosy are primed with MMP-II prior to the manifestation of the disease, and MMP-II is highly antigenic in terms of activation of adaptive immunity.

Keywords: *M. leprae*; Leprosy; Major membrane protein-II; T cell; Dendritic cell; IFN-γ; Memory T cell; Paucibacillary leprosy; Host defense

1. Introduction

Mycobacterium leprae is the causative agent of human leprosy, in which a chronic progressive peripheral nerve injury leading to systemic deformity is induced [1,2]. Most individuals infected with *M. leprae* do not manifest leprosy, but a few manifest the disease depending on their immunological status. Leprosy exhibits a wide range of clinical features and therefore, a broad disease spectrum is observed [3]. The representative spectra are the paucibacillary (PB) leprosy and multibacillary (MB)[1] leprosy. In the former disease spectrum, the localized skin and nerve lesions are observed and both $CD4^+$ and $CD8^+$ T cells chiefly act to localize the bacterial spread and, thus, disease lesion [4–6]. In contrast, in the latter disease spectrum, such cell-mediated immune responses are not efficiently evoked, but, rather, T cells show *M. leprae* Ag-specific anergic response [3]. The

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[1] *Abbreviation used:* MMP-II, major membrane protein-II; *M.*, *Mycobacterium*; DC, dendritic cell; MB, multibacillary; IFN, interferon; MHC, major histocompatibility complex; PB, paucibacillary; BCG, *M. bovis* bacillus Calmette-Guérin; Ab, antibody; Ag, antigen; APC, Ag-presenting cell; MDT, multi-drug therapy; TLR, Toll-like receptor; PBMCs, peripheral blood mononuclear cells; m, monoclonal; L, ligand; MLM, *M. leprae*-derived cell membrane; LPS, lipopolysaccharide; LAM, lipoarabinomannan.

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vaccine currently examined for human use against the mycobacterial diseases is *M. bovis* bacillus Calmette-Guérin (BCG). However, its protective effect on *M. leprae* infection is not so convincing. Various efforts have currently been done for the development of new immunostimulatory agents, however, we still do not have an effective anti-leprosy vaccine. Also, in both forms of leprosy, the protective effects of antibody (Ab) in blood could not be observed. Therefore, the identification of the useful bacterial component antigens (Ags) which have immunomodulatory and immunostimulatory activities are desired.

Previously we demonstrated that dendritic cells (DCs), which are the most potent Ag-presenting cell (APC) capable of stimulating both memory and unprimed $CD4^+$ and $CD8^+$ T cell subsets [7–9], played a central role in stimulating T cells of both healthy individuals and PB leprosy patients [10,11]. However, macrophages less efficiently stimulated T cells [12]. Using DCs as APCs, we reported that cell membrane fraction of *M. leprae* was the most T cell stimulatory fraction [11], and therefore we identified major membrane protein-II (MMP-II) from this fraction as an immunomodulatory molecule.

MMP-II was originally identified from *M. leprae* as a major native protein in 1990 and was recognized to be identical to *M. paratuberculosis* bacterioferritin. Purification of MMP-II by reverse-phase chromatography, revealed a large molecular mass of 380 kDa, which has a ferroxidase-center residue. MMP-II contained 1000–4000 atoms of iron per molecule of protein. In the previous study, we showed that purified MMP-II stimulated DCs to produce IL-12 p70, and TNFα through the ligation to toll-like receptor (TLR)-2 [13]. In this study, we evaluated the immunostimulatory activity of purified MMP-II using DCs as APCs, since type 1 T cells response is most closely associated with host defense against *M. leprae* [1,2,14]. Furthermore, we assessed if MMP-II is associated with the activation of T cells in PB leprosy patients.

2. Materials and methods

2.1. Preparation of cells and bacteria

Peripheral blood was obtained under informed consent from healthy volunteers who were PPD-positive due to *M. bovis* BCG vaccination at childhood, and from five cases each of PB and MB leprosy patients. The status of patients used in this study are as follows: PB leprosy: 2 female and 3 male, age range (31–56), and MB leprosy: 1 female and 4 male, age range (21–53). All patients were under multi-drug therapy (MDT) for less than 7 months. We are aware that PPD-negative individuals would help to provide full information for these experiments because *M. leprae* and *M. bovis* BCG share some common Ags. However, in Japan, such individuals are not available for study, because *M. bovis* BCG vaccination was compulsory for children (0–4 year-old) until some years ago. PPD-negative individuals in Japanese population are the ones who do not respond to BCG vaccination; and therefore, it is likely that they may suffer from unknown human disease or immuno-insufficiency. Therefore these individuals cannot be used for our experiments. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [15]. For preparation of the monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal (m) Ab (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ fraction of the PBMCs were plated on collagen-coated plates and cultured for 60 min at 37 °C. The non-plastic-adherent cells were then removed by extensive washing and the remaining adherent cells were used as monocytes and precursors of DCs [14]. Monocyte-derived DCs were differentiated from the plastic-adherent cells as described [15,16]. Briefly, the plastic-adherent cells were cultured in 3 ml of RPMI 1640 medium containing 10% FCS for 5 days in the presence of 50 ng of rGM-CSF (Pepro Tech EC, London, England) and 10 ng of rIL-4 (Pepro Tech) per milliliter. rGM-CSF and rIL-4 were supplied every 2 days and 400 μl of medium was replaced as described previously [16]. In some cases, DCs unpulsed or pulsed with Ags were further treated with a soluble form of CD40 ligand (L) (Pepro Tech) to obtain fully matured DCs capable of efficiently activating T cells. The purity of DCs obtained was 90.5% as judged by the expression of CD1a.

2.2. Purification of whole cell membrane fraction of M. leprae and MMP-II

The whole cell membrane fraction (MLM) was obtained according to previous report [13]. Briefly, the mycobacterial suspension was mixed with Zirconium beads in the presence of protease inhibitors at a ratio of approximately 1:1 (v/v) and homogenized using Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo), at 1500 rpm for 90 s 3–4 times. The beads were separated and the suspension was centrifuged at 10,000*g* for 30 min. The supernatant was then further ultra-centrifuged at 100,000*g* for 1 h. The resulting pellet was suspended in PBS, washed 2 times and taken as the membrane fraction. The *MMP-II* gene was PCR amplified from *M. leprae* chromosomal DNA and cloned into *Escherichia coli* expression vector as described previously [13]. Briefly, the *MMP-II* gene was inserted into the expression plasmid pET28 (Novagen, Madison, WI) and transformed into *E. coli* strain ER2566 (New England

BioLabs, Beverly, MA). The expressed protein was eluted using Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA) and used for all the experiments conducted in this paper. The amount of lipopolysaccharide (LPS) in the purified MMP-II protein was determined by using Limulus amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be less than 70 pg/mg of MMP-II, of which level did not affect the T cell stimulating activity of DCs as described previously [13].

2.3. Assessment of an antigenicity of MMP-II

The immunostimulatory activity of MMP-II was assessed by using DCs as APCs and autologous T cells as responder population as previously described [10,16]. The Ag-pulsed DCs were treated with 50 μg/ml mitomycin C, washed extensively to remove extracellular Ags, and were used as a stimulator. Freshly thawed PBMCs were depleted of MHC class II^+ cells by using magnetic beads coated with mAb to MHC class II Ag (Dynabeads 450; Dynal) and further treated with beads coated with either CD4 or CD8 mAb to select T cells negatively as previously reported [10]. The purity of $CD4^+$ T cells or $CD8^+$ T cells was more than 98%. Unprimed and memory population of responder T cells was purified from these $CD4^+$ and $CD8^+$ T cells by depleting $CD45RO^+$ memory cells and $CD45RA^+$ naïve cells, respectively. For depletion, mAb to CD45RO or CD45RA (Dako, Glostrup, Denmark) were used, and were followed by immunomagnetic beads coated with mAb to mouse IgG (Dynal). The purified responder cells (1×10^5/well) were plated in 96-well round-bottomed tissue culture plates and DCs were added to give a DC responder $CD4^+$ T cell ratio of 1:10, 20, 40 or 80 and a DC responder $CD8^+$ T cell ratio of 1:5, 10 or 20. The supernatant of stimulator-T cell mixture was collected on day 3 or 4 of co-cultures for assessment of cytokine concentration. To identify molecules restricting T cell activation, the following purified mAbs were used: anti-HLA-ABC Ab (W6/32), anti-HLA-DR (L243), and anti-CD86 Ab (IT2.2, BD PharMingen International). Also, the purified mAb to MMP-II (IgM, k), which were raised in mice [13], was used to mask the MMP-II Ag expressed on the surface of DCs. The optimal concentrations of mAbs were determined in advance. Lipoarabinomannan (LAM) and mAb to LAM were donated by Dr. P.J. Brennan (Colorado State University).

2.4. Assessment of cytokine production

Levels of the following cytokines were measured; IFN-γ and IL-2 produced by $CD4^+$ and $CD8^+$ T cells stimulated for 3 or 4 days with Ag-pulsed DCs. The concentrations of IL-2 and IFN-γ were quantified using the enzyme assay kits Opt EIA Human ELISA Set available from BD PharMingen International.

2.5. Statistical analysis

Student's t test was applied to demonstrate statistically significant differences.

3. Results

3.1. Antigenicity of M. leprae-derived MMP-II

The ability of MMP-II to evoke cellular immunity was assessed, since MMP-II stimulated DCs to produce IL-12p70 and MMP-II-pulsed DCs expressed its derivatives on the surface [13]. We examined whether MMP-II-pulsed DCs activated both unprimed and memory T cells by using IL-2 and IFN-γ as a reporter cytokine. When $CD45RO^-$ unprimed $CD4^+$ and $CD8^+$ T cells obtained from healthy PPD-positive individuals were stimulated with autologous MMP-II-pulsed DCs, they produced both IL-2 (Table 1) and IFN-γ (Table 2). While 4 μg/ml of MMP-II was required to stimulate DCs for production of significant dose of IL-2 from $CD4^+$ and $CD8^+$ T cells (Table 1), 1 μg/ml of the Ag was sufficient for significant IFN-γ production from both T cells (Table 2). Then, $CD45RA^-$ memory $CD4^+$ and $CD8^+$ T cells, obtained from healthy donors, were stimulated with autologous MMP-II-pulsed DCs (Table 3). In contrast to unprimed T cells, both memory T cell subsets efficiently produced IFN-γ even when they were stimulated with DCs which were pulsed with MMP-II in the absence of maturation factor such as CD40L. MMP-II was found to be more potent than whole membrane fraction (positive control) or lipoarabinomannan (negative control) in the stimulation of both $CD4^+$ and $CD8^+$ T cells. However, no IL-4 was produced from these T cells (not shown). CD40L further up-regulated the IFN-γ production from both T cell subsets. In both cases, $CD4^+$ T cell predominantly produced IFN-γ. The IFN-γ production was induced in a manner dependent on both MMP-II dose (Table 3) and CD40L concentration (not shown). Both unprimed and memory T cells were not stimulated by DCs which were treated with the amount of LPS estimated to be present in 4 μg/ml of MMP-II (not shown).

The role of MHC and co-stimulatory molecules of MMP-II-activated DCs on T cell activation was determined by using mAbs towards these molecules. The IFN-γ production from T cells was suppressed by the treatment of these DCs with mAbs towards HLA-ABC, HLA-DR or CD86 Ags (Table 4). More than 85% of IFN-γ production from $CD4^+$ T cells was suppressed by mAb to HLA-DR and more than 80% of the cytokine production was suppressed by mAb to CD86. However, the cytokine production was not suppressed by mAb to HLA-ABC Ags. On the other hand, the IFN-γ production from $CD8^+$ T cells was suppressed by the DC treatment with mAb to HLA-ABC (85–90% suppression) or

Table 1
IL-2 production from unprimed T cells stimulated with MMP-II-pulsed DCs[a]

MMP-II (μg/ml)	Unprimed $CD4^+$ T cells stimulation by DCs (IL-2, pg/ml)		Unprimed $CD8^+$ T cells stimulation by DCs (IL-2, pg/ml)	
T/DC:	10	20	5	10
0.0	$4.9 \pm 0.8^{b,c}$	$4.4 \pm 1.1^{d,e}$	$1.0 \pm 0.0^{f,g}$	$0.5 \pm 0.0^{h,i}$
1.0	9.3 ± 2.1^{b}	8.7 ± 1.3^{d}	4.1 ± 0.8^{f}	2.9 ± 0.2^{h}
4.0	23.7 ± 3.1^{c}	18.4 ± 1.1^{e}	17.6 ± 1.4^{g}	7.8 ± 1.1^{i}

[a] The responder $CD45RO^-$ unprimed $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from healthy individuals, were stimulated for 3 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC). Immature DCs were pulsed with MMP-II, treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate and results are expressed as means ± SD. The titers having the same alphabet were statistically compared by Student's *t* test.
[b] $p < 0.05$.
[c] $p < 0.005$.
[d] $p < 0.001$.
[e] $p < 0.0001$.
[f] $p < 0.05$.
[g] $p < 0.005$.
[h] $p < 0.005$.
[i] $p < 0.01$.

Table 2
IFN-γ production from unprimed T cells stimulated with MMP-II-pulsed DCs[a]

MMP-II (μg/ml)	Unprimed $CD4^+$ T cells stimulation by DCs (IFN-γ, pg/ml)		Unprimed $CD8^+$ T cells stimulation by DCs (IFN-γ, pg/ml)	
T/DC:	10	20	5	10
0.0	$18.5 \pm 2.9^{b,c}$	$14.0 \pm 3.1^{d,e}$	$1.4 \pm 0.2^{f,g}$	$0.9 \pm 0.1^{h,i}$
1.0	66.2 ± 8.7^{b}	44.1 ± 9.3^{d}	10.0 ± 1.8^{f}	7.9 ± 0.9^{h}
4.0	134.3 ± 11.0^{c}	115.9 ± 13.9^{e}	27.7 ± 2.9^{g}	14.3 ± 3.9^{i}

[a] The responder $CD45RO^-$ unprimed $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from healthy individuals, were stimulated for 4 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC). Immature DCs were pulsed with MMP-II, treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate and results are expressed as means ± SD. The titers having the same alphabet were statistically compared by Student's *t* test.
[b] $p < 0.005$.
[c] $p < 0.005$.
[d] $p < 0.05$.
[e] $p < 0.005$.
[f] $p < 0.05$.
[g] $p < 0.005$.
[h] $p < 0.005$.
[i] $p < 0.05$.

mAb to CD86 Ag (more than 85% suppression). Furthermore, the IFN-γ production was also suppressed by the treatment of MMP-II-pulsed DCs with mAb to MMP-II Ag (Table 4). The percent suppression was ~65% in $CD4^+$ T cells and ~65% in $CD8^+$ T cells. mAb to LAM, used as a negative control Ab, did not suppress the IFN-γ production. No significant suppression was observed on the cytokine production from $CD4^+$ and $CD8^+$ T cells stimulated with DCs unpulsed with any Ags (not shown).

3.2. Efficiency of MMP-II-pulsed DCs in the activation of leprosy T cells

It is known that T cells of PB leprosy have conserved an ability to respond to *M. leprae* infection, but those of MB leprosy are unresponsive towards *M. leprae* Ags [3]. Using T cells from five patients of each disease type, we evaluated the T cells responses to MMP-II-pulsed DCs. First, $CD45RO^-$ unprimed $CD4^+$ and $CD8^+$ T cells obtained from PB leprosy were stimulated with autologous MMP-II-pulsed DCs (Table 5). Both subsets of T cells produced a significant dose of IFN-γ and 1 μg/ml of MMP-II was enough to produce the cytokine. However, the amounts of cytokine produced from these T cells were comparable to that produced from T cells obtained from healthy individuals (Table 2). Next, we evaluated the memory $CD4^+$ T cells and $CD8^+$ T cells. The CD45RA-negative T cells were obtained from healthy individuals, PB and MB leprosy patients, and were stimulated with autologous DCs which were pulsed with MMP-II in the absence or presence of CD40L (Fig. 1). Again, T cells from healthy individuals responded to MMP-II-pulsed DCs, but, both $CD4^+$ and $CD8^+$ T cells from PB leprosy produced significantly higher level of IFN-γ than T cells from healthy individuals when stimulated with autologous CD40L-stimulated, MMP-II (0.5 μg/ml)-pulsed DCs. In addition, DCs which were not

Table 3
IFN-γ production from memory T cells stimulated with MMP-II-pulsed DCs[a]

Antigen (μg/ml)	CD40L stimulation of DCs	Memory $CD4^+$ T cells stimulation by DCs (IFN-γ, pg/ml)		Memory $CD8^+$ T cells stimulation by DCs (IFN-γ, pg/ml)	
	T/DC:	40	80	10	20
None	(−)	$8.9 \pm 0.2^{b,c}$	9.6 ± 1.9^{d}	$1.1 \pm 0.1^{e,f}$	0.0 ± 0.0
MMP-II 1.0	(−)	48.7 ± 5.3^{b}	3.3 ± 0.9	7.1 ± 0.9^{e}	2.0 ± 0.0^{g}
MMP-II 4.0	(−)	144.5 ± 4.4^{c}	48.8 ± 2.3^{d}	24.1 ± 3.1^{f}	5.0 ± 0.2^{g}
None	(+)	36.4 ± 1.9	1.5 ± 0.1	6.9 ± 1.3	0.3 ± 0.0
MMP-II 1.0	(+)	117.9 ± 3.6	43.8 ± 1.7	56.6 ± 3.1	13.3 ± 1.0
MMP-II 4.0	(+)	$308.6 \pm 11.3^{h,i}$	$172.8 \pm 4.0^{j,k}$	$153.3 \pm 7.9^{l,m}$	$29.8 \pm 3.4^{n,o}$
MLM 4.0	(+)	79.2 ± 11.3^{h}	39.0 ± 2.1^{j}	35.9 ± 2.8^{l}	12.4 ± 4.8^{n}
LAM 4.0	(+)	32.9 ± 9.8^{i}	2.0 ± 1.2^{k}	10.2 ± 2.0^{m}	2.1 ± 1.0^{o}

[a] The responder $CD45RA^-$ memory type $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from healthy individuals, were stimulated for 4 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC). Monocyte-derived immature DCs were pulsed with various dose of MMP-II, whole *M. leprae*-derived membrane fraction (MLM) or LAM, untreated or treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate and results are expressed as means ± SD. The titers having the same alphabet were statistically compared by Student's *t* test.

[b] $p < 0.01$.
[c] $p < 0.0005$.
[d] $p < 0.00005$.
[e] $p < 0.01$.
[f] $p < 0.01$.
[g] $p < 0.005$.
[h] $p < 0.00001$.
[i] $p < 0.00001$.
[j] $p < 0.0001$.
[k] $p < 0.0001$.
[l] $p < 0.001$.
[m] $p < 0.001$.
[n] $p < 0.005$.
[o] $p < 0.005$.

conditioned by CD40L also induced T cell activations, but lesser dose of the cytokine was produced from both T cell subsets. In contrast to PB patient, both subsets of T cells from MB leprosy produced comparable level of cytokine to healthy individuals when they were stimulated with the CD40L-stimulated, MMP-II-pulsed DCs. Little IL-4 was produced from patients' T cells stimulated with MMP-II-pulsed DCs.

4. Discussion

Leprosy represents broad spectrum disease [3]. One representative manifestation is PB leprosy. Studies on the clinical specimens of the skin lesions indicate that the infection is localized, and the spread of *M. leprae* is suppressed by a consequence of activation of both $CD4^+$ and $CD8^+$ T cell subsets [4–6]. On the other hand, MB leprosy usually manifests widespread infection due to the lack of efficient cellular immune response against *M. leprae* components. The mechanisms leading to the broad spectrum are not fully understood yet, but these observations suggest the presence of some host defense associated Ags that trigger the immune responses. However, so far such Ags have not been identified. Previously we evaluated the T cell stimulating function of professional APC and found that DC was superior to macrophage in activating T cells [10,12]. When we examined the antigenicity of subcellular components of *M. leprae* for identification of DC-mediated antigenic molecules, we found that the cell membrane fraction was more adept than other fractions in terms of T cell stimulating ability [11]. Based on these observations, we identified MMP-II from *M. leprae* cell membrane fraction as one of the components, capable of acting as an immunomodulatory agent [13]. In our previous study, we found that purified MMP-II stimulated DCs to produce bioactive IL-12 and TNFα through ligation to TLR-2 on the surface of DCs. However, these DCs did not produce detectable level of IL-10 [13]. As is widely accepted, IL-12 is an important APC-mediated cytokine capable of driving Th1 T cell responses [17], and TLR-2 serves as a bridge to link innate and adaptive immune responses [18,19]. Therefore, in this study, we examined the capability of MMP-II to evoke adaptive immunity, especially in respect to type 1 T cell responses. Consequent to the induction of adaptive immunity, both unprimed and memory T cells are activated to produce type-1 cytokine, such as IFN-γ, which is an extremely important cytokine involved in the host defense, since it can activate macrophages and consequently kill the bacteria [4,14]. When MMP-II-pulsed DCs were evaluated as an inducer of

Table 4
Suppression of IFN-γ production from T cells by treatment of DCs with mAb[a]

Responder	T/DC	IFN-γ production (pg/ml)					
	mAb treatment:	Control	MHC class I	MHC class II	CD86	MMP-II	LAM
$CD4^+$ T cells	40	$392.1 \pm 22.2^{b,c,d}$	360.4 ± 23.1 (8.1%)	51.4 ± 8.8^{b} (86.9%)	74.1 ± 12.7^{c} (81.1%)	101.3 ± 20.1^{d} (74.2%)	401.9 ± 31.4 (0.0%)
	80	$240.9 \pm 20.2^{e,f,g}$	219.3 ± 11.8 (9.0%)	17.8 ± 8.1^{e} (92.7%)	19.1 ± 9.8^{f} (92.1%)	81.3 ± 9.0^{g} (66.3%)	253.2 ± 21.9 (0.0%)
$CD8^+$ T cells	10	$230.8 \pm 21.3^{h,i,j}$	24.1 ± 11.0^{h} (89.6%)	202.4 ± 30.1 (12.3%)	33.7 ± 6.9^{i} (85.4%)	29.5 ± 3.9^{j} (87.2%)	229.8 ± 30.8 (0.4%)
	20	$48.9 \pm 9.6^{k,l,m}$	6.1 ± 2.1^{k} (87.5%)	51.4 ± 3.9 (0.0%)	6.0 ± 1.8^{l} (87.7%)	17.3 ± 4.2^{m} (64.6%)	50.3 ± 5.6 (0.0%)

[a] The responder CD45RA$^-$ memory type $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from healthy individuals, were stimulated for 4 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC), in the presence of various mAb. mAb to LAM was used as negative control Ab. Immature DCs were pulsed with MMP-II, treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD. Parenthesis indicates percent suppression by mAb. The optimal concentration of mAb was determined in advance. The titers having the same alphabet were statistically compared by Student's t test.
[b] $p < 0.001$.
[c] $p < 0.0005$.
[d] $p < 0.00005$.
[e] $p < 0.001$.
[f] $p < 0.001$.
[g] $p < 0.005$.
[h] $p < 0.001$.
[i] $p < 0.005$.
[j] $p < 0.005$.
[k] $p < 0.05$.
[l] $p < 0.05$.
[m] $p < 0.01$.

Table 5
IFN-γ production from PB leprosy unprimed T cells stimulated with MMP-II-pulsed DCs [a]

MMP-II (μg/ml)	Unprimed $CD4^+$ T cells stimulation by DCs (IFN-γ, pg/ml)		Unprimed $CD8^+$ T cells stimulation by DCs (IFN-γ, pg/ml)	
T/DC:	10	20	5	10
0.0	$20.9 \pm 3.9^{b,c}$	15.3 ± 4.1^{d}	$2.1 \pm 0.4^{e,f}$	$1.9 \pm 0.6^{g,h}$
1.0	73.2 ± 9.2^{b}	22.4 ± 10.3	14.6 ± 2.8^{e}	10.9 ± 1.3^{g}
4.0	173.2 ± 13.0^{c}	60.6 ± 9.9^{d}	33.8 ± 3.1^{f}	20.9 ± 2.7^{h}

[a] The responder CD45RO$^-$ unprimed $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from PB leprosy, were stimulated for 4 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC). Immature DCs were pulsed with MMP-II, treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate and results are expressed as means ± SD. The titers having the same alphabet were statistically compared by Student's t test.
[b] $p < 0.005$.
[c] $p < 0.005$.
[d] $p < 0.01$.
[e] $p < 0.05$.
[f] $p < 0.005$.
[g] $p < 0.005$.
[h] $p < 0.005$.

adaptive immunity, both $CD4^+$ and $CD8^+$ T cells produced IFN-γ. MMP-II was more potent than whole membrane protein (Table 3) in the stimulation of both $CD4^+$ and $CD8^+$ T cells. Although the detailed process of $CD8^+$ T cell activation by soluble protein like MMP-II is not fully covered, cross priming may be largely involved in activating $CD8^+$ T cells. The cytokine production was restricted by MHC molecules (Table 4), in concordance with the previous report that T cells from PB and MB leprosy were restricted by HLA-DR molecules [20]. Also the IFN-γ production from T cells was suppressed by mAb to CD86, which indicates that the activation of both $CD4^+$ T cells and $CD8^+$ T cells was largely dependent on the expression of CD86 Ag on DCs. Furthermore, the cytokine production was Ag-specific, since more than 60% of IFN-γ production was suppressed by the treatment of the DCs with MMP-II mAb (Table 4). The fact that the activation of both $CD4^+$ and

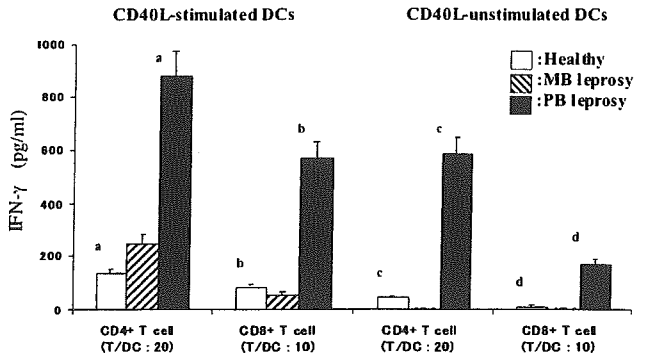

Fig. 1. IFN-γ production of T cells from healthy individuals (PPD-positive), PB and MB leprosy. The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous MMP-II (0.5 μg/ml)-pulsed DCs at an indicated T cell:DC ratio. The Ag-pulsed DCs were untreated or treated with CD40L (1 μg/ml) for 24 h before T cell stimulation. Representative result obtained from five different patients and healthy individuals are shown. Assays were done in triplicate and results are expressed as mean ± SD of the representative donor. $^a p < 0.001$, $^b p < 0.005$, $^c p < 0.005$, and $^d p < 0.005$.

$CD8^+$ T cells were suppressed by the MMP-II mAb may depend on the immunological feature of the mAb, which should be clarified. In addition to IFN-γ, the MMP-II-pulsed DCs stimulated unprimed $CD4^+$ T cells to produce IL-2 (Table 1). Therefore, it may be deduced that MMP-II is efficient in the activation of DC-mediated adaptive immunity, although other characteristics of MMP-II protein need to be further evaluated.

We further evaluated the potential of MMP-II to activate T cells from leprosy patients. Although DCs from the patients expressed MHC molecules, CD86 and CD80 Ags comparable to the level expressed by healthy individuals (not shown), memory $CD4^+$ and $CD8^+$ T cells from PB patients produced significantly higher dose of IFN-γ than T cells from healthy individuals by stimulation with autologous MMP-II-pulsed DCs (Fig. 1). In addition to CD40L-stimulated MMP-II-pulsed DCs, less matured DCs, which were pulsed with MMP-II in the absence of any additional maturation factor including CD40L, were also quite efficient in the IFN-γ production from both subsets of memory T cells from PB leprosy (Fig. 1). However, in contrast to memory T cells, the IFN-γ production from unprimed T cells obtained from PB leprosy was comparable to that from healthy individuals. We may deduce from these results that peripheral blood T cells of PB leprosy are primed with Ags of *M. leprae* through professional APCs prior to the clinical manifestation of the disease, and one of the Ags responsible for retaining the specific memory T cells could be MMP-II. Therefore, MMP-II can be considered as one of the candidates involved in T cell activation of PB leprosy.

In contrast to PB patient, T cells from MB leprosy responded to MMP-II only when it was pulsed to DCs in the presence of CD40L. Although, the exact reason for the difference between PB and MB leprosy in the requisite form of DCs is not fully covered. However, one possible explanation for the poor cellular immune response in MB leprosy might be associated with recent observation that some MB leprosy has TLR-2 polymorphism [21,22]. We have not examined whether our patients had such polymorphism, but, if so, it might lead to less efficient ligation of MMP-II to the receptor. Another explanation is that T cells cannot be efficiently primed with *M. leprae* derived antigenic components in MB leprosy patients for reasons that are still uncovered. This possibility is more likely, because T cells from MB leprosy produced equivalent dose of IFN-γ by MMP-II stimulation to that produced by healthy individuals T cells.

Taken together, these observations indicate that MMP-II is highly potent in terms of immune stimulation, and is an antigenic element in T cell activation for the control of the growth of the bacilli. Further study should be pursued to evaluate its ability as host defense associated molecule against leprosy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellimm.2005.04.001.

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Identification and Characterization of the Genes Involved in Glycosylation Pathways of Mycobacterial Glycopeptidolipid Biosynthesis

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Glycopeptidolipids (GPLs) are major components present on the outer layers of the cell walls of several nontuberculous mycobacteria. GPLs are antigenic molecules and have variant oligosaccharides in mycobacteria such as *Mycobacterium avium*. In this study, we identified four genes (*gtf1*, *gtf2*, *gtf3*, and *gtf4*) in the genome of *Mycobacterium smegmatis*. These genes were independently inactivated by homologous recombination in *M. smegmatis*, and the structures of GPLs from each gene disruptant were analyzed. Thin-layer chromatography, gas chromatography–mass spectrometry, and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry analyses revealed that the mutants Δgtf1 and Δgtf2 accumulated the fatty acyl-tetrapeptide core having *O*-methyl-rhamnose and 6-deoxy-talose as sugar residues, respectively. The mutant Δgtf4 possessed the same GPLs as the wild type, whereas the mutant Δgtf3 lacked two minor GPLs, consisting of 3-*O*-methyl-rhamnose attached to *O*-methyl-rhamnose of the fatty acyl-tetrapeptide core. These results indicate that the *gtf1* and *gtf2* genes are responsible for the early glycosylation steps of GPL biosynthesis and the *gtf3* gene is involved in transferring a rhamnose residue not to 6-deoxy-talose but to an *O*-methyl-rhamnose residue. Moreover, a complementation experiment showed that *M. avium gtfA* and *gtfB*, which are deduced glycosyltransferase genes of GPL biosynthesis, restore complete GPL production in the mutants Δgtf1 and Δgtf2, respectively. Our findings propose that both *M. smegmatis* and *M. avium* have the common glycosylation pathway in the early steps of GPL biosynthesis but differ at the later stages.

The mycobacterial cell envelope has a unique structure that contains a complex of covalently linked peptidoglycan, arabinogalactan, and mycolic acids (7, 11). The outer layer of the cell envelope is composed of several types of glycolipids that affect the surface properties of mycobacterial cells (7, 11). Glycopeptidolipids (GPLs) are a major class of glycolipid present on the outer layer of several species of nontuberculous mycobacteria, such as *Mycobacterium avium* complex, *M. scrofulaceum*, *M. chelonae*, *M. fortuitum*, and *M. smegmatis* (31). GPLs have a common fatty acyl-tetrapeptide core consisting of tetrapeptide amino alcohol (D-Phe-D-*allo*-Thr-D-Ala-L-alaninol) and amide-linked long-chain fatty acid (C_{26-34}). The fatty acyl-tetrapeptide core is glycosylated with 6-deoxy-talose (6-d-Tal) and variable *O*-methyl-rhamnose (*O*-Me-Rha) residues, termed non-serovar-specific GPLs (nsGPLs), which are also the main products of *M. smegmatis* GPLs (1, 4, 10). The GPLs of *M. avium* have a more complicated structure, in which an additional Rha residue is added to 6-d-Tal of nsGPLs to be extended with various haptenic oligosaccharides, which are important surface antigens, resulting in serovar-specific GPLs (ssGPLs) (1, 4, 31).

There are some evidences that GPLs may be responsible for pathogenicity. It has been shown that the some of the ssGPLs are immunosuppressive and are able to induce a variety of cytokines, which affect host responses to infection (3, 15, 18, 29). Also, ssGPLs are identified as the factors modulating the phagocytosis and phagosome-lysosome fusion (17, 21). The altered GPL structure is also known to affect the colony morphology relevant to variable virulence (14, 30).

The biosyntheses of GPLs, particularly nsGPLs, have been characterized for *M. smegmatis*. Several biosynthetic genes encoding enzymes such as *O*-methyltransferase, acetyltransferase, and peptide synthetase have been identified (5, 16, 25, 26), but less is known about the genes involved in the glycosylation steps of the GPLs. The only glycosyltransferase gene that has been characterized is *rtfA* from *M. avium*, which is responsible for transferring the Rha residue to 6-d-Tal of nsGPLs to form ssGPLs (12). However, the initial glycosylation steps for the formation of nsGPLs remain unknown. Recently, it was shown that GPLs from *M. smegmatis* has a unique structure in which nsGPLs are further glycosylated, unlike ssGPLs (23, 24, 32), but these unique GPLs are produced in a carbon-starved situation, which is not a normal growth condition.

In this study, to clarify the glycosylation step leading to the formation of nsGPLs and its further products, we focused on four of the *M. smegmatis* genes having high similarity to *M. avium rtfA*, whose functions remain uncharacterized. Here, we have undertaken the gene disruption approach for generating each mutant in *M. smegmatis*, characterized their biochemical phenotypes, and finally hypothesized new biosynthetic pathways associated with glycosylation of GPLs.

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TABLE 1. Bacterial strains and vectors used in this study

Strain or vector	Characteristic(s)	Source or reference
Bacteria		
E. coli		
DH5α	Cloning host	
STBL2	Cloning host	
M. smegmatis		
mc^2155	Wild type	27
Δgtf1	*gtf1* disruptant	This study
Δgtf2	*gtf2* disruptant	This study
Δgtf3	*gtf3* disruptant	This study
Δgtf4	*gtf4* disruptant	This study
M. avium		
JATA51-01 (ATCC 25291)	Source of *gtfA* and *gtfB*	
Vectors		
pYUB854	Cosmid vector	2
phAE87	Phasmid vector carrying full-length DNA of mycobacteriophage PH101	2
pMV261	*E. coli-Mycobacterium* shuttle vector carrying *hsp60* promoter cassette	28
pYUBgtf1	pYUB854 with *gtf1*-disrupted sequences for generating recombinant mycobacteriophage	This study
pYUBgtf2	pYUB854 with *gtf2*-disrupted sequences for generating recombinant mycobacteriophage	This study
pYUBgtf3	pYUB854 with *gtf3*-disrupted sequences for generating recombinant mycobacteriophage	This study
pYUBgtf4	pYUB854 with *gtf4*-disrupted sequences for generating recombinant mycobacteriophage	This study
pMVgtf1	pMV261 with *gtf1*	This study
pMVgtf2	pMV261 with *gtf2*	This study
pMVgtf3	pMV261 with *gtf3*	This study
pMVgtf4	pMV261 with *gtf4*	This study
pMVgtfA	pMV261 with *gtfA*	This study
pMVgtfB	pMV261 with *gtfB*	This study

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA manipulation. Bacterial strains and vectors used and constructed are listed in Table 1. Mycobacterial strains for DNA manipulation were grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween 80 or Middlebrook 7H10 agar (Difco) with 0.5% glycerol, and each was supplemented with 10% albumin-dextrose-catalase enrichment (Difco). *M. smegmatis* strains for GPL production were cultured in Luria-Bertani (LB) broth with 0.05% Tween 80. DNA manipulation including isolation of DNA, transformation, and PCR was carried out as described previously (22). *E. coli* strain DH5α was used for routine manipulation and propagation of plasmid DNA. *E. coli* strain STBL2 was used for construction of phasmid vectors derived from phAE87. Antibiotics was added as required: kanamycin, 50 μg/ml for *E. coli* and 25 μg/ml for *M. smegmatis*; hygromycin B, 150 μg/ml for *E. coli* and 75 μg/ml for *M. smegmatis*.

Generation of the gene disruptants. The targeted genes (*gtf1*, *gtf2*, *gtf3*, and *gtf4*) were selected by BLAST analysis of unfinished *M. smegmatis* genome sequences deposited in the database of The Institute for Genomic Research (TIGR) (http://www.tigr.org) with the *rfA* gene of *M. avium* as the query nucleotide sequence. Each gene was inactivated by inserting a hygromycin-resistant cassette (*hyg*) using the specialized transducing phage system (2). To construct the disrupted sequences, around 1.0-kb fragments both upstream and downstream of each gene were amplified from *M. smegmatis* mc^2155 genomic DNA using the following two pairs of primers: US1 and UA1 for upstream of *gtf1* and DS1 and DA1 for downstream of *gtf1*; US2 and UA2 for upstream of *gtf2* and DS2 and DA2 for downstream of *gtf2*; US3 and UA3 for upstream of *gtf3* and DS3 and DA3 for downstream of *gtf3*; US4 and UA4 for upstream of *gtf4* and DS4 and DA4 for downstream of *gtf4*. The PCR products were digested with each restriction enzyme and cloned into the corresponding sites flanking *hyg* of pYUB854 to give pYUBgtf1 (*gtf1*), pYUBgtf1 (*gtf2*), pYUBgtf3 (*gtf3*), and pYUBgtf4 (*gtf4*). These plasmids were used for packaging into the phasmid vector phAE87 to construct a specialized transducing mycobacteriophage for gene disruption as described previously (2). The *M. smegmatis* mc^2155 strain infected with the above mycobacteriophage at a multiplicity of infection of 10 was incubated at 37°C for 3 h in 7H9 broth without Tween 80. Harvested bacterial cells were then plated and cultured on 7H10 agar containing 75 μg/ml hygromycin B for 1 week. The hygromycin B-resistant colonies were selected, and their genomic DNA was subjected to PCR analysis to confirm the disruption of each gene using the following primers: U1 and D1 for *gtf1*; U2 and D2 for *gtf2*; U3 and D3 for *gtf3*; and U4 and D4 for *gtf4* (Fig. 1A to D).

Construction of the *gtf* expression vectors. The *gtf* genes of *M. smegmatis* and *M. avium* were amplified from each genomic DNA using the following primers: GTF1S and GTF1A for *gtf1*, GTF2S and GTF2A for *gtf2*, GTF3S and GTF3A for *gtf3*, GTF4S and GTF4A for *gtf4*, GTFAS and GTFAA for *gtfA*, and GTFBS and GTFBA for *gtfB*. The PCR products were digested with each restriction enzyme and cloned into the corresponding site of pMV261 to give pMVgtf1 (for the *gtf1* gene), pMVgtf2 (for the *gtf2* gene), pMVgtf3 (for the *gtf3* gene), pMVgtf4 (for the *gtf4* gene), pMVgtfA (for the *gtfA* gene), and pMVgtfB (for the *gtfB* gene). These vectors were used for complementation and overexpression experiment.

Isolation and purification of GPLs. The total lipids were extracted from harvested bacterial cells with $CHCl_3/CH_3OH$ (2:1, vol/vol) for several hours at room temperature. The extracts from the organic phase were separated from the aqueous phase and evaporated to dryness. For isolation of crude deacylated GPLs, total lipid fractions were subjected to mild alkaline hydrolysis as previously described (22, 25). For analytical thin-layer chromatography (TLC), the total lipid fraction after mild alkaline hydrolysis was spotted on silica gel 60 plates (Merck) and developed in $CHCl_3$-CH_3OH (9:1 [vol/vol]). Deacylated GPLs and other compounds were visualized by spraying with 10% H_2SO_4 and charring. Each total lipid fraction was extracted from an equal weight of harvested cells. Purified deacylated GPLs were separated from the total lipid fraction after mild alkaline hydrolysis by preparative TLC on the same plates and extracted from the bands corresponding to each GPLs. β-Elimination and per-deuteriomethylation treatment for determination of the linkage positions of sugar moieties were carried out as described previously (6, 9, 12).

GC/MS analysis. For monosaccharide analysis, purified deacylated GPLs or total lipid fraction after mild alkaline hydrolysis was hydrolyzed in 2 M trifluoroacetic acid (2 h, 120°C), and released sugars from deacylated GPLs were reduced with $NaBD_4$ (sodium borodeuteride) and then acetylated with pyridine-acetic anhydride (1:1 [vol/vol]) at room temperature overnight. Each total lipid fraction was extracted from an equal weight of harvested cells. The resulting

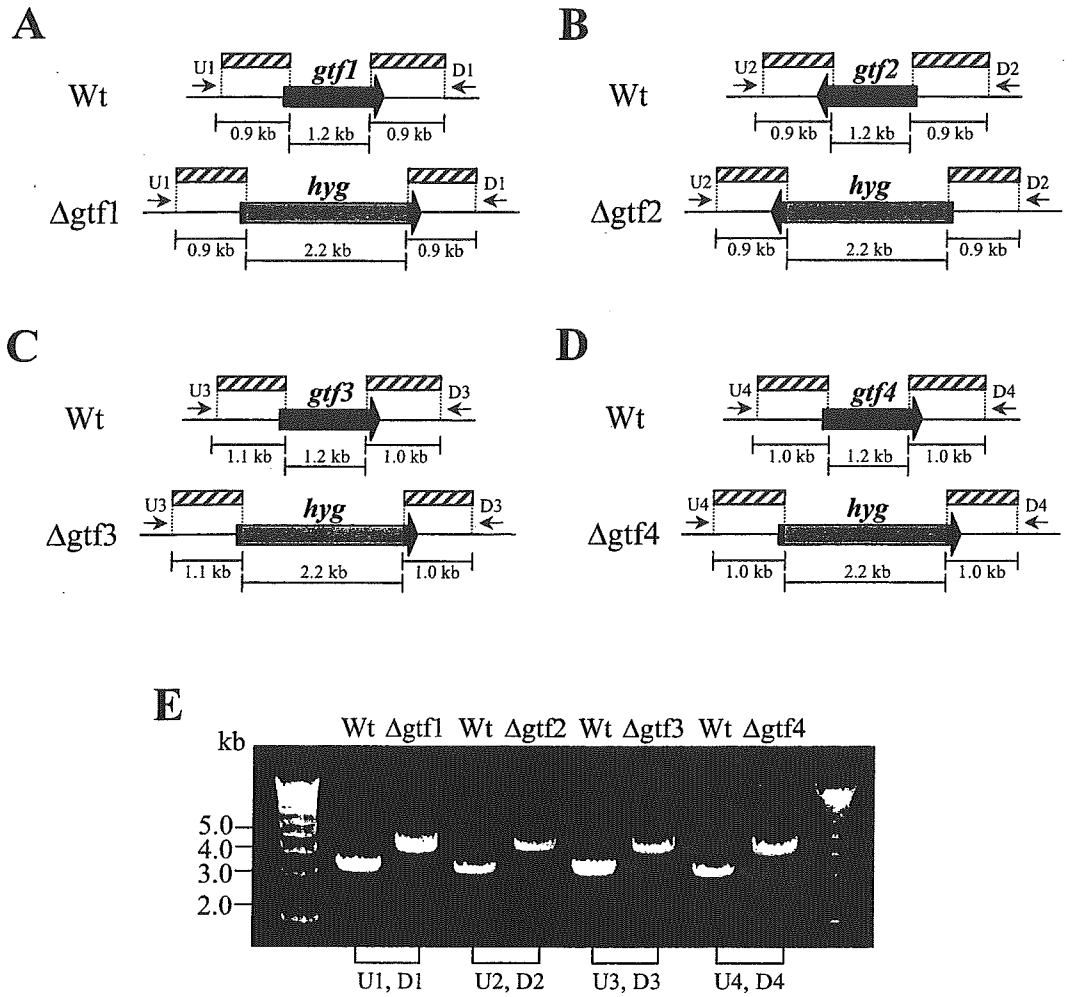

FIG. 1. Generation of *gtf* gene disruptants. (A to D) Schematic diagram of each *gtf* region on the chromosome of the wild-type *M. smegmatis* mc^{2}155 strain (Wt) and its gene disruptants Δgtf1, Δgtf2, Δgtf3, and Δgtf4. The shaded boxes indicate the regions included in recombinant phage for gene disruption. The black arrows represent the coding region of each *gtf* gene. The gray boxes represent the hygromycin resistance cassette (*hyg*). The primers used for PCR analysis are indicated by small arrows. (E) PCR analyses of the wild type and each disruptant using the primers indicated above.

alditol acetates were separated and analyzed by gas chromatography–mass spectrometry (GC/MS) on TRACE DSQ (Thermo electron) instrument equipped with an SP-2380 column (SUPELCO) using helium gas. The temperature program was from 52 to 172°C at 40°C/min and then 172 to 250°C at 3°C/min.

MALDI-TOF/MS analysis. To determine the total mass of the purified deacylated GPLs, matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectra (in the positive mode) were acquired on a QSTAR XL (Applied Biosystems) with a pulse laser emitting at 337 nm. Samples mixed with 2,5-dihydroxybenzoic acid as the matrix were analyzed in the reflectron mode with an accelerating voltage operating in positive ion mode of 20 kV.

RESULTS

Disruption of *gtf1*, *gtf2*, *gtf3*, and *gtf4* by allelic exchange. Four genes showing high similarity to the *rtfA* gene, involved in GPL biosynthesis of *M. avium*, were identified for the *M. smegmatis* mc^{2}155 strain (12). The homologies of their corresponding amino acid sequences with that of RtfA were around 60%. Three genes were found in the GPL biosynthetic gene cluster, namely, *gtf1*, *gtf2*, and *gtf3* (GenBank accession no. AY138899.1) (16), whereas one gene, designated *gtf4* (TIGR database no. 4839918 to 4841162), was located far from the other three genes. To examine whether these genes are responsible for GPL biosynthesis, we generated four gene disruptants, designated Δgtf1, Δgtf2, Δgtf3, and Δgtf4, using the specialized transducing mycobacteriophage containing the entire open reading frame, replacing with the hygromycin resistance cassette (2). For confirmation of the gene disruption, PCR analysis was performed on chromosomal DNA from each disruptant. To avoid the amplification of disrupted sequences derived from residual mycobacteriophage, we designed and used the primers located outside the sequences included in each mycobacteriophage as shown in Fig. 1A to D. As expected, around 3.0-kb fragments were amplified from mc^{2}155 (wild type), whereas around 4.0-kb fragments were amplified from each disruptant, because most of the *gtf* coding region (1.2 kb) was replaced by the hygromycin resistance cassette (2.2 kb) (Fig. 1E). These results demonstrated that allelic exchanges involving replacement of the *gtf* genes with the disrupted constructs have been successful.

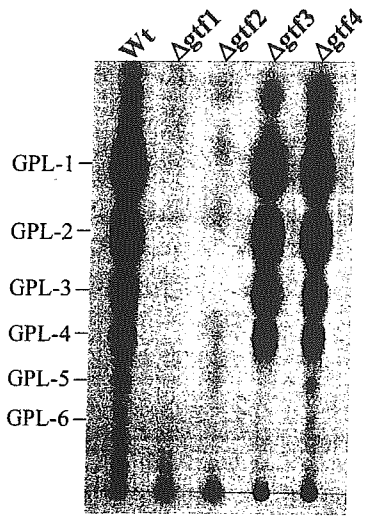

FIG. 2. TLC analyses of crude GPL extracts from the *M. smegmatis* mc^2155 strain (Wt) and its gene disruptants. The total lipid fraction after mild alkaline hydrolysis was spotted on plates and developed in $CHCl_3$-CH_3OH (9:1 [vol/vol]). GPLs were visualized by spraying with 10% H_2SO_4 and charring. Each total lipid fraction was extracted from an equal weight of harvested cells.

TLC analysis of gene disruptants. To investigate the effects of the mutation in each *gtf* gene, we examined GPL production of four gene disruptants. TLC analyses of total lipid fraction after mild alkaline hydrolysis revealed that wild-type mc^2155 mainly produced six components, designated GPL-1 to -6, whereas Δgtf1 and Δgtf2 lacked all six components and Δgtf3 lacked two minor ones (GPL-5 and GPL-6) found in the wild type (Fig. 2). In contrast, no differences in TLC profile were observed between Δgtf4 and the wild type (Fig. 2).

Characterization of Δgtf1 and Δgtf2. In Δgtf1 and Δgtf2, the TLC analyses showed that six GPL components contained in the wild type had disappeared. On the other hand, there is the possibility that both disruptants contained GPL derivatives which are structurally incomplete and hard to be detected by TLC analyses. To characterize the sugars included in GPL derivatives from both disruptants and to compare with the wild type, each total lipid fraction after mild alkaline hydrolysis was hydrolyzed, and the released monosaccharides as their alditol acetates were examined by GC/MS. Figure 3 shows that the profiles of the wild type gave three peaks corresponding to 2,3,4-tri-*O*-Me-Rha, 3,4-di-*O*-Me-Rha, and 6-d-Tal (Fig. 3A), whereas Δgtf1 lacked 6-d-Tal (Fig. 3B) and Δgtf2 lacked 3,4-di-*O*-Me-Rha and 2,3,4-tri-*O*-Me-Rha (Fig. 3C). Complementation of both disruptants with each respective gene restored the TLC profile of GPLs to that observed for the wild type (not shown). Therefore, the *gtf1* and *gtf2* genes are found to be responsible for transferring the 6-d-Tal and Rha residues, respectively.

Structural determination of GPL-5 and GPL-6 for characterization of Δgtf3. The TLC profile of Δgtf3 showed that two spots (GPL-5 and GPL-6) disappeared (Fig. 2). To reveal the biosynthetic role of the *gtf3* gene, GPL-5 and GPL-6 were purified from mc^2155 and their structures were determined. GC/MS analyses showed that GPL-5 and GPL-6 contained 6-d-Tal and 3,4-di-*O*-Me-Rha, which were identified as sugar

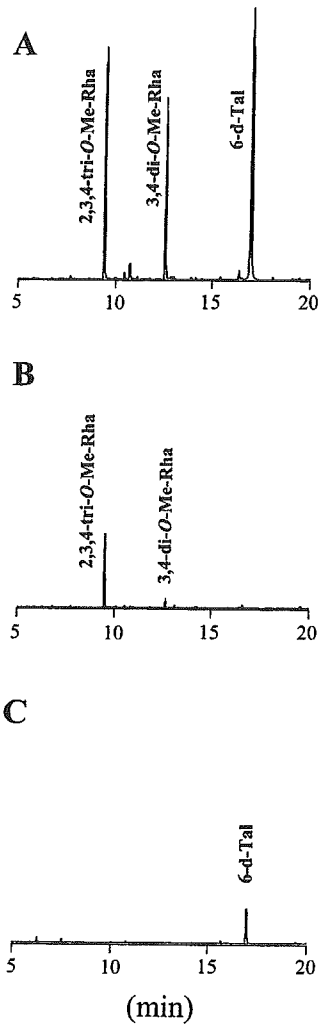

FIG. 3. GC/MS analyses of alditol acetates of sugars released from crude GPLs. GPLs were extracted from *M. smegmatis* strains: (A) mc^2155 strain, (B) Δgtf1, and (C) Δgtf2. Alditol acetate derivatives were prepared from the total lipid fraction after mild alkaline hydrolysis, which was extracted from an equal weight of harvested cells.

moieties of GPL-3 and GPL-4 (Fig. 4A). However, an extra sugar, 3-*O*-Me-Rha, was also detected (Fig. 4A). MALDI-TOF/MS analyses revealed that the main molecular ions of GPL-5 (*m/z* 1,333.8) and GPL-6 (*m/z* 1,319.8) were 160 mass units higher than those of GPL-3 (*m/z* 1,173.9) and GPL-4 (*m/z* 1,159.9), respectively (Fig. 4B). These results confirmed the presence of 3-*O*-Me-Rha in GPL-5 and GPL-6 and also suggested that 3-*O*-Me-Rha was further added to GPL-3 and GPL-4. Although GPL-5 and GPL-6 contained same three sugars, the spectra showed that the main molecular ion of GPL-5 (*m/z* 1,333.8) was 14 mass units higher than that of GPL-6 (*m/z* 1,319.8) (Fig. 4Ba and 4Bb). These differences in total mass may be due to O methylation of fatty acid as observed in structures of GPL-1 and GPL-3, suggesting that fatty acid of GPL-5 was O methylated like GPL-1 and GPL-3 (16). To investigate the sugar linked to D-*allo*-Thr of the fatty acyltetrapeptide core, GPL-5 and GPL-6 were subjected to β-elimination treatment. The main ion peaks of treated GPL-5 and

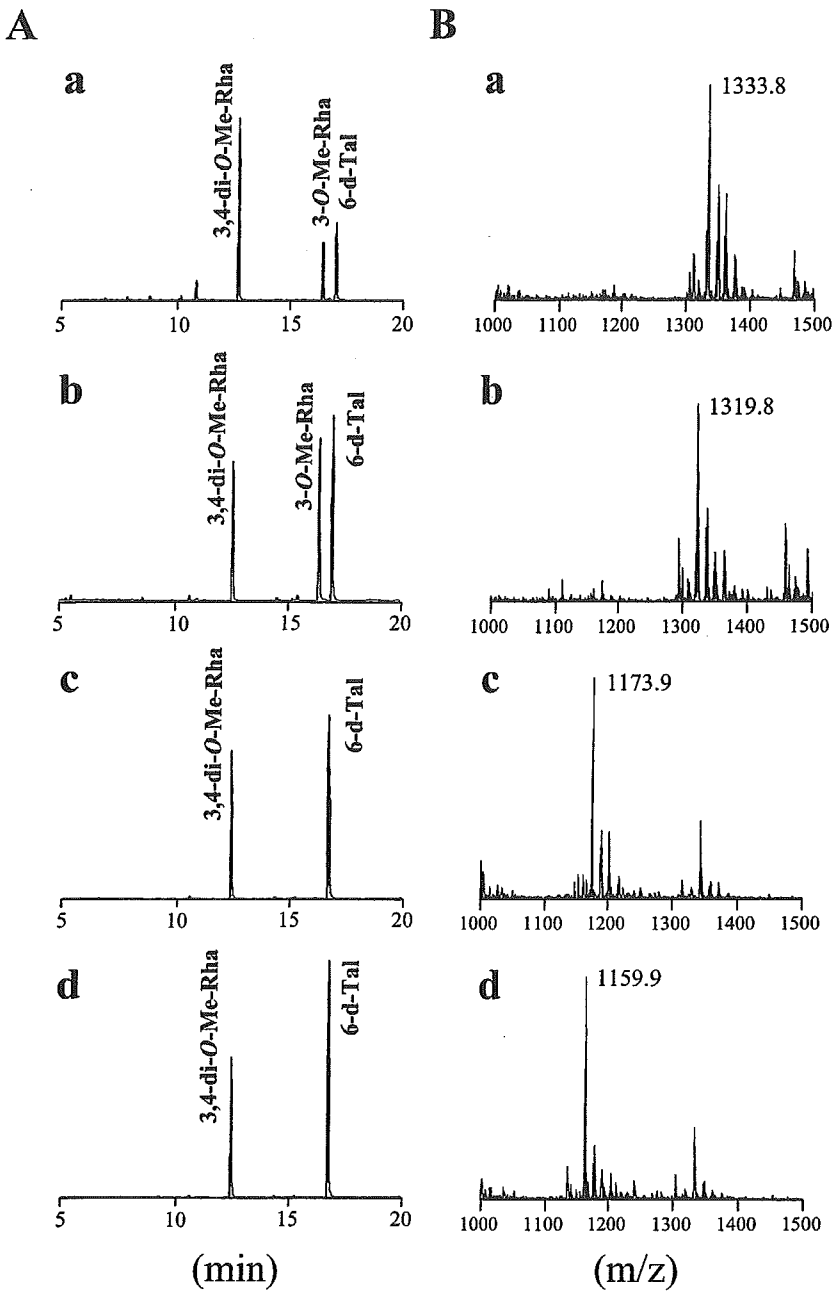

FIG. 4. Biochemical characterization of GPL-5 (a), GPL-6 (b), GPL-3 (c), and GPL-4 (d). (A) GC/MS analysis of alditol acetates of sugars released from each purified GPL. (B) MALDI-TOF/MS analysis of total molecular mass of each purified GPLs. (C) MALDI-TOF/MS analysis of total molecular mass of purified GPL-5 (a) and GPL-6 (b), which were subjected to β-elimination.

GPL-6 were *m/z* 1,171.7 and 1,157.7, respectively, which resulted in the loss of total mass of 162, suggesting that 6-d-Tal was linked to the position of D-*allo*-Thr (Fig. 4C). The linkage position of the sugars linked to the L-alaninol site of GPL-5 and GPL-6 was then determined by GC/MS analyses followed by perdeuteriomethylation. As shown in Fig. 5A, the GC profiles of alditol acetates from perdeuteriomethylated GPL-5 gave three peaks corresponding to 6-d-Tal, 3-*O*-Me-Rha, and 3,4-di-*O*-Me-Rha. The characteristic spectra of 3-*O*-Me-Rha and 3,4-di-*O*-Me-Rha, which are predicted to be linked to L-alaninol, are illustrated in Fig. 5B and C, respectively. The spectrum of 3-*O*-Me-Rha gave fragment ions at *m/z* 121, 134, and 165, which represent the presence of a deuteriomethyl group at positions C-2 and C-4. In contrast, no deuteriomethyl group was observed in 3,4-di-*O*-Me-Rha, whose C-2 position was acetylated, supported by the detection of fragment ions at *m/z* 131 and 190. The results from GC/MS analyses of perdeuteriomethylated GPL-6 were the same as those for GPL-5 (not shown). These observations demonstrated that GPL-5 and GPL-6 have the same sugar moieties, which are 6-d-Tal at

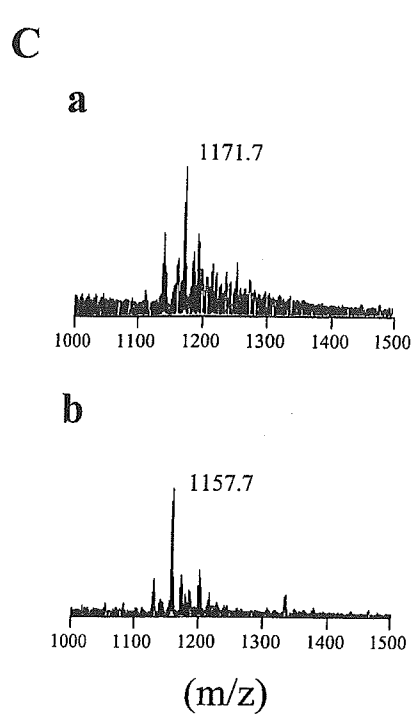

FIG. 4—*Continued.*

D-*allo*-Thr and 3-*O*-Me-rhamnosyl-(1→2)-3,4-di-*O*-Me-Rha at L-alaninol, indicating that 3-*O*-Me-Rha was linked to GPL-3 and GPL-4 (Fig. 6).

Overexpression of *gtf1*, *gtf2*, *gtf3*, and *gtf4* in *M. smegmatis* mc²155. To investigate the effects of overexpression of each gene on GPL biosynthesis, we constructed four *gtf*-overexpressed strains in wild-type mc²155 and compared the profile of total GPLs by TLC analyses. The results showed that the profiles of Wt/pMVgtf1, Wt/pMVgtf2, and Wt/pMVgtf4 were the same as that of Wt/pMV261, whereas Wt/pMVgtf3 produced two major compounds whose biochemical data corresponded to those of GPL-5 and GPL-6 (Fig. 7).

Characterization of *M. avium gtfA* and *gtfB*. We showed that both *M. smegmatis gtf1* and *gtf2* were responsible for glycosylation of the fatty acyl-tetrapeptide core. Comparison of the genome sequences encompassing the GPL biosynthetic gene cluster among several species of *M. avium* have shown that *gtfA* and *gtfB* (GenBank accession no. AF125999.1) are very similar to *M. smegmatis gtf1* and *gtf2*, respectively, in the corresponding putative amino acid sequences and might contribute to the glycosylation of the fatty acyl-tetrapeptide core (13). However, the function of each gene has not been thoroughly analyzed (13). Therefore, to confirm the role of *gtfA* and *gtfB*, we complemented Δgtf1 and Δgtf2 with the *gtf* expression vectors carrying *gtfA* (pMVgtfA) and *gtfB* (pMVgtfB). As shown in Fig. 8, TLC analyses revealed that *gtfA* and *gtfB* restored the production of wild-type GPLs in Δgtf1 and Δgtf2, respectively, whereas transformants with reverse vectors (Δgtf1/pMVgtfB and Δgtf2/pMVgtfA) did not produce wild-type GPLs. These results suggested that the function of *M. avium gtfA* and *gtfB* is the same as that of *M. smegmatis gtf1* and *gtf2*, respectively.

DISCUSSION

It has been shown that the *rtfA* gene of *M. avium* encodes a rhamnosyltransferase which synthesizes ssGPLs, while other genes involved in the glycosylation of the fatty acyl-tetrapeptide core remain unknown (12). In this study, we focused on the four genes of *M. smegmatis*, which show high similarity to *rtfA*, and generated their disruptants to characterize the role in the GPL biosynthesis.

In the early glycosylation steps of the fatty acyl-tetrapeptide core, we observed that the disruption of *gtf1* abolished the whole GPLs and led to the accumulation of *O*-Me-Rha derivatives without 6-d-Tal in Δgtf1 (Fig. 3B). Thus, we propose that the *gtf1* gene product catalyzes the transfer of 6-d-Tal to fatty acyl-tetrapeptide core. It is reported that the *M. avium* 104Rg strain, which has a spontaneous deletion in the genome region including *gtfA*, also accumulated *O*-methylated and nonmethylated Rha without 6-d-Tal (13, 30). This property is directly supported by our result that the *gtfA* could complement Δgtf1 (Fig. 8). However, *M. avium* 104Rg mainly contained nonmethylated Rha, whereas Δgtf1 derived from *M. smegmatis* mc²155 contained only *O*-Me-Rha. These different observations may be due to differences in the substrate specificity of methyltransferase, because 2,3,4-tri-*O*-Me-Rha was present in *M. smegmatis* mc²155 but was not identified in *M. avium* species (8, 25).

When the *gtf2* gene was disrupted, we detected 6-d-Tal without Rha derivatives in GC/MS analysis, which demonstrates that the *gtf2* gene contributes to the transfer of Rha to the fatty acyl-tetrapeptide core (Fig. 3C). In addition, complementation revealed that the *gtfB* gene of *M. avium* had the same function as *gtf2* (Fig. 8). In the previous studies of GPL biosyntheses, the mutant accumulating 6-d-Tal-containing derivatives without the Rha residue have not been isolated from GPL-producing species so far. Our results directly indicated for the first time that 6-d-Tal-containing derivatives could be an intermediate for the biosynthetic pathways of GPLs.

As for the order of glycosylation steps regulated by *gtf1* and *gtf2*, we cannot determine which step takes place earlier, since both disruptants accumulated the intermediates having different component (Fig. 3B and C). For *M. avium* serovar 2, Eckstein et al. proposed a pathway in which the transfer of the Rha residue to the fatty acyl-tetrapeptide core occurred prior to that of 6-d-Tal, because a mutant strain, 104Rg, having the *gtfA* region deleted, accumulated the fatty acyl-tetrapeptide core with only the Rha residue (13). However, our results lead to the interesting possibility that there are two alternative glycosylation pathways for the formation of nsGPLs (Fig. 9). If the glycosylation should occur in a single pathway, we would expect the accumulation of a nonglycosylated intermediate in either of the disruptants, because one of the genes, *gtf1* or *gtf2*, would be responsible for the first step of glycosylation converting the fatty acyl-tetrapeptide core to a glycosylated intermediate. Thus, the detection of glycosylated intermediates from both Δgtf1 and Δgtf2 suggests that (i) the fatty acyl-tetrapeptide core could be the substrate for both Gtf1 and Gtf2 and (ii) the glycosylated intermediates could also be the substrates for both Gtf1 and Gtf2. We prove here that Gtf1 and Gtf2 have broad substrate specificity and propose that the fatty acyl-tetrapeptide core is glycosylated by Gtf1 and Gtf2 at the same

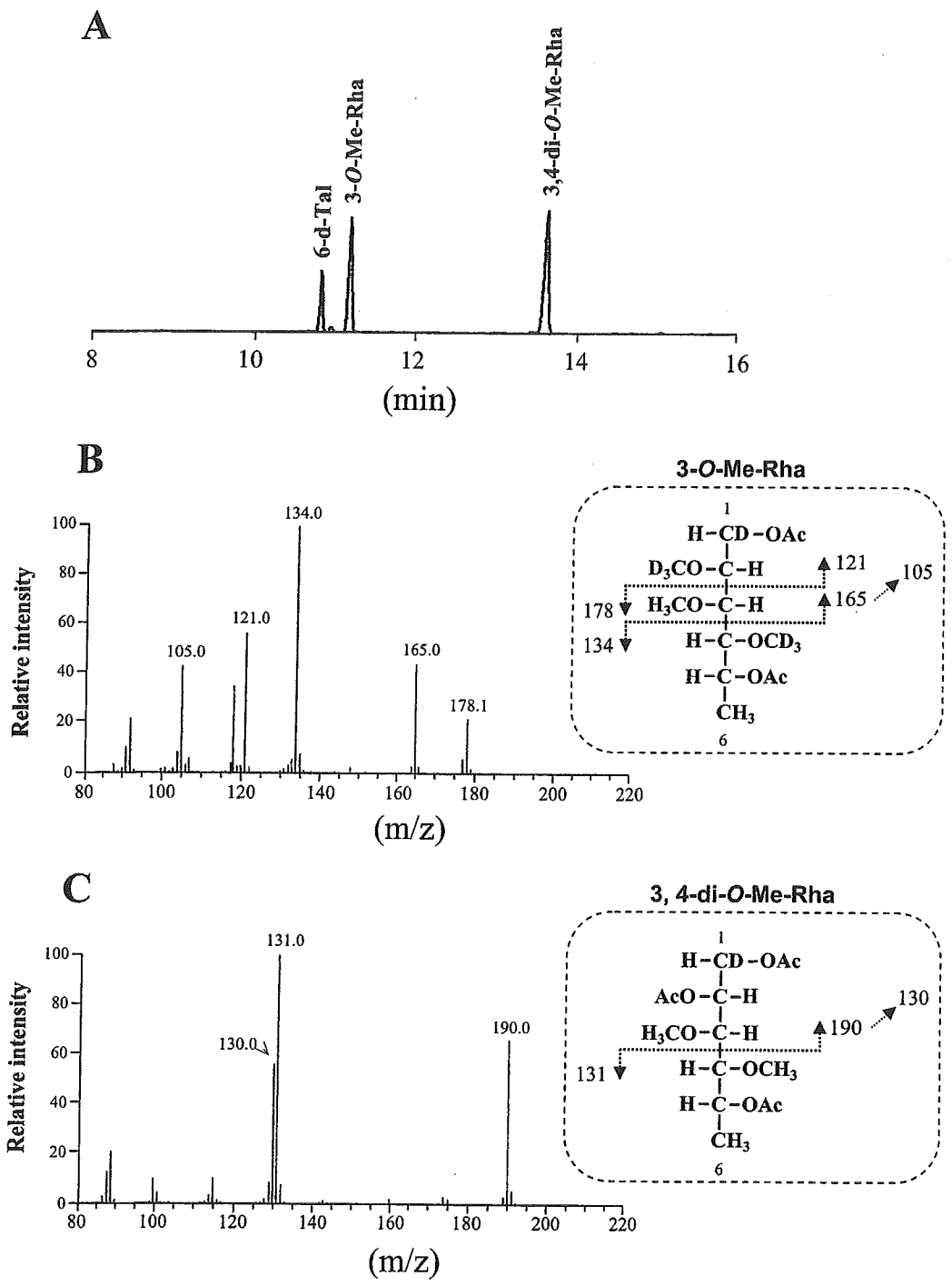

FIG. 5. GC/MS analysis of alditol acetates of sugars released from perdeuteriomethylated GPL-5. (A) GC profile. (B) Mass spectrum and fragment ion assignment corresponding to 3-*O*-Me-Rha. (C) Mass spectrum of fragment ion assignment corresponding to 3,4-di-*O*-Me-Rha.

time and then converted to the nsGPLs having both 6-d-Tal and *O*-Me-Rha via cross-glycosylations (Fig. 9).

Structural determination of GPL-5 and GPL-6 revealed that L-alaninol of the fatty acyl-tetrapeptide core was glycosylated with disaccharide (3-*O*-Me- and 3,4-di-*O*-Me-Rha), which was structurally different from GPLs including GPL-1 to -4 and ssGPLs (Fig. 6). However, it is reported that *M. fortuitum* complex produced GPLs which are glycosylated as in GPL-5 and GPL-6 as major components (19, 20). Therefore, these observations suggest that this type of glycosylation is not specific for *M. smegmatis*. GC/MS analyses of GPL-5 and GPL-6 indicated the presence of 3-*O*-Me-Rha in addition to 3,4-di-*O*-Me-Rha, and analyses of perdeuteriomethylated GPL-5 and GPL-6 showed that position C-1 of 3-*O*-Me-Rha is linked to position C-2 of 3,4-di-*O*-Me-Rha. Recent studies have shown that *M. smegmatis* mc^{2}155 newly produces two polar GPLs