

TABLE 6. Stability of polymorphic microsatellite loci detected among strains subjected to serial passage in nude mice

Generation	No. of copies at locus:							
	(AC)8a	(AC)8b	(AC)9	(TA)10	(AT)15	(AT)17	(TA)18	(GTA)9
Thai53 4th	11	7	7	11	13	13	20	11
Thai53 7th	11	7	7	11	13	13	20	11
Thai53 11th	11	7	7	11	13	13	20	11
Kyoto-1 3rd	7	8	7	12	15	12	16	15
Kyoto-1 5th	7	8	7	12	15	12	16	15
Kyoto-1 7th	7	8	7	12	15	12	16	15
Kyoto-1 8th	7	8	7	12	15	12	16	15
Zensho-4 ^a	8	8	7	10	20	13	15	13
Zensho-4 1st	8	8	7	10	20	13	15	13
Zensho-4 2nd	8	8	7	10	20	13	15	13
Zensho-4 3rd	8	8	7	10	20	13	15	13
Zensho-4 4th	8	8	7	10	20	13	15	13
Korea3-2 ^a	7	8	7	15	17	14	15	14
Korea3-2 1st	7	8	7	15	17	14	15	14
Korea3-2 2nd	7	8	7	15	17	14	15	14
Korea3-2 3rd	7	8	7	15	17	14	15	14
Korea3-2 4th	7	8	7	15	17	14	15	14

^a Biopsy specimen.

no polymorphism which may have little or no long-term epidemiological value. In bacteria, loci containing a tandem repeat from the microsatellite class have been called simple sequence contingency loci. Altered number of repeats allows reversible on and off states of expression for the corresponding gene. In such an extreme situation, the microsatellite is not suitable for strain identification, epidemiological, or phylogenetic studies (7). The slight allele shift found in a few loci, (CA)6, 23-3, 27-5, and 12-5, was on one hand supposed to be a result of replication slippage, which can lead to an increase or a decrease in the copy number of the repeat element during cell division and inclined to be attributed to the locus with no polymorphism, and on the other hand, there might be different subpopulations of *M. leprae*.

As far as we are concerned, the nine combined microsatellite

loci with strong discriminatory capacity which have been also tested for its stability and reproducibility in this study were the most likely and reliable sites for genetic diversity for *M. leprae*. Our previous study showed the 10-copy TTC repeat was the predominant genotype in a leprosy-endemic village (11). Nonetheless, by TTC alone, no conclusion could be drawn that bacteria were from the same population. Meanwhile, the multibase household which was examined the identical genotype for TTC alone does not mean an identical *M. leprae* isolate at all. As a matter of fact, when eight polymorphic loci rather than TTC, (AC)8a, (AC)8b, (AC)9, (TA)10, (AT)15, (AT)17, (TA)18, and (GTA)9, were added to the genotype, *M. leprae* could be further subdivided into two populations. Moreover, all 27 strains could also be identified individually by these combined nine polymorphic loci. Hence, this group of locus is

TABLE 7. Application of potential polymorphic loci for household leprosy patients

Patients ^a	Sample no.	No. of copies at polymorphic locus ^b :								
		TTC or (AGA)20	(AC)8a	(AC)8b	(AC)9	(TA)10	(AT)15	(AT)17	(TA)18	(GTA)9
Son*	1	13	12	7	10	10	13	15	14	9
Father	2	13	12	7	10	10	13	15	14	9
Sister	3	13	12	7	10	10	13	15	14	9
Brother	4	13	12	7	10	10	13	15	14	9
Brother	5	13	12	7	10	10	13	15	14	9
Son*	6 (left earlobe)	12	10	7	9	10	13	13	11	11
	7 (right earlobe)	12	10	7	9	10	13	13	11	11
Brother	8 (earlobe)	12	10	7	9	10	13	13	11	11
	9 (back)	12	10	7	9	10	13	13	11	11
Son*	10	8	10	7	9	8	16	9	15	11
Brother	11	8	10	7	9	8	16	10	15	12

^a *, supposed index case.

^b STRs with variation in the sane TTC repeat strains were indicated by boldface type.

anticipated to a wide application for clinical samples as a preliminary molecular typing system in leprosy epidemiology.

It is thought that tandem repeats located within the regulatory region of a gene can constitute an on/off switch of gene expression at the transcriptional level (7). In spite of this, the intergenic or interspseudogenic location of the microsatellite loci makes it unlikely that a difference in repeat copy number would itself confer any biological advantage, but it is possible that it acts as a marker for some biologically distinct subpopulation of bacteria and that it is carried along during selective expansion of this subpopulation (28). On the basis of this, the repeat unit which was differed by one copy number at (AT)₁₇ and (GTA)₉ loci when multibase household were subjected to the nine combined polymorphic loci indicated the presence of two subpopulations of *M. leprae* which have different dominant genotypes and presumably have propagated independently of each other. With regard to the fully matching polymorphism profile in each sample from the same dwelling at each polymorphic microsatellite loci, it was possible that the patients were infected by the same strain of *M. leprae* that survived in some common infectious sources. Taking these into consideration, there might be certain publicly shared infectious sources other than multibacillary cases played a role in the transmission of leprosy.

The unique function of *rhoT* was precisely evaluated in tracking the possible worldwide spread of leprosy and its contribution to the study of anthropology and archaeology (10, 13). We analyzed the other two loci with characteristics common to locus 6-3a in *rhoT* and found no regularity and significance such as *rhoT* had.

Young et al. (28) compared microsatellite profiles for samples taken from different anatomic sites from the same individual and observed that while skin, blood, and nasal cavity samples consistently generated matching profiles, frequent mismatches were found when bacteria in skin and nerves were compared. Mostly, we obtained identical repeats from different skin lesions on the same individual such as samples 6 (left earlobe), 7 (right earlobe) and 8 (earlobe) and 9 (back). Rarely, different skin lesions from the same patient exhibited mismatch profiles at the TTC locus (data was not shown). This probably suggested different bacterial populations coexisted instead of resulting from replication slippage. Nevertheless, the underlying mechanisms are awaiting to be elucidated.

Molecular typing systems are undergoing rapid technical improvements. Advance in the understanding of the biological basis of microbial biodiversity at subspecies levels will improve the conceptual framework required for proper epidemiologic interpretation of disease transmission. Wider application of these systems will surely contribute to the epidemiology of leprosy transmission and, therefore, allow for more effective control and prevention strategies to reach a world free of leprosy.

ACKNOWLEDGMENTS

This study was supported by a Health Research Grant of Emerging and Re-emerging Infectious Diseases, Ministry of Health, Labor and Welfare, Government of Japan, a grant of the Postdoctoral Fellowship Program, Japan Society for the Promotion of Science, a grant of the Foundation for Hansen's diseases in Asia, and a grant of the U.S.-Japan Cooperative Medical Science Program.

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Identification of an Immunomodulating Agent from *Mycobacterium leprae*

Yumi Maeda,¹ Tetsu Mukai,¹ John Spencer,² and Masahiko Makino^{1*}

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan,¹ and Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523-1677²

Received 22 October 2004/Returned for modification 1 December 2004/Accepted 7 January 2005

A search for an immunomodulating agent from mycobacteria was carried out using *Mycobacterium leprae*. The antigenicity of each fraction of the bacterial membrane, which contains the most antigenic components of *M. leprae*, was assessed by using sera from paucibacillary leprosy. N-terminal sequencing of the serum-reactive protein and functional assessment of the membrane fractions using monocyte-derived dendritic cells (DCs) identified major membrane protein II (MMP-II) as one of the efficient T-cell-activating candidates. Purified MMP-II stimulated DCs from healthy individuals to produce interleukin-12 p70 and up-regulated the surface expression of major histocompatibility complex class I and II, CD86, and CD83 molecules. Also, there was an increase in the percentage of CD83⁺ cells in the DC population. Furthermore, MMP-II-pulsed DCs expressed their derivatives on their surfaces. Using Toll-like receptor 2 (TLR-2)-dependent receptor constructs, we found that TLR-2 signaling was involved in DC maturation induced by MMP-II. Taken together, MMP-II can be recognized as an immunomodulating protein in terms of activation of antigen-presenting cells and innate immunity.

Mycobacterial infection is a major public health risk worldwide, and around one-third of the world's population is estimated to be latently infected. *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, accounts for 8 to 10 million new active cases and 2 million deaths annually (12, 41). Nontuberculous mycobacterial infections of immunocompromised individuals, such as human immunodeficiency virus type 1 (HIV-1)-infected patients (8, 28), evoke serious concern, and *Mycobacterium leprae* induces a chronic progressive peripheral nerve injury which leads to systemic deformity (16, 37). The sole immunomodulating agent currently available for human use against mycobacterial diseases is *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). However, its protective effect against mycobacterial infection is unconvincing, especially to elderly people, and thus, its use is limited (3). Various efforts are currently being made to develop immunomodulating agents, but convincing protection against mycobacterium-induced diseases has not been achieved to date. The development of other useful molecules as immunomodulating agents is greatly desired. In this study, we attempted to find such antigenic molecules in *M. leprae* subfractions.

M. leprae is the causative agent of human leprosy, for which a broad disease spectrum is clinically observed (34). Most individuals infected with *M. leprae* do not manifest leprosy, but a few manifest the disease, depending on their immunological status. The representative spectra are the tuberculoid, or paucibacillary (PB), leprosy and the lepromatous, or multibacillary (MB), leprosy. In the former disease spectrum, localized skin and nerve lesions are observed, and T cells act chiefly to lo-

calize bacterial spread and, thus, disease lesions (20, 31, 36). In contrast, in the latter disease spectrum, such cell-mediated immune responses are not efficiently evoked; rather, T cells show *M. leprae* antigen (Ag)-specific anergic responses. In both types of leprosy, the protective effect of antibody (Ab) is not observed. These observations indicate that the bacterial component Ags capable of modulating immune responses should be identified. Previously it has been demonstrated that monocyte-derived dendritic cells (DCs), which are the most potent antigen-presenting cells (APCs), are capable of stimulating both memory and naïve CD4⁺ and CD8⁺ T cells (14, 15, 21). Also, we reported that DCs played a central role in stimulating T cells (10, 18, 22); however, macrophages stimulated T cells less efficiently. Furthermore, we showed that among subcellular components of *M. leprae*, the cell membrane fraction was quite antigenic and contained molecules which stimulated DCs to produce interleukin-12 (IL-12) p70 (22). However, the molecules associated with DC activation have not been elucidated. For identification of an APC-associated immunomodulator, the following issues should be addressed: (i) the ability of the immunogen to activate APCs, including both DCs and macrophages; (ii) the ability of the immunogen to be processed and presented on the surfaces of these APCs, because mycobacteria are intracellular parasitic pathogens. In this context, we fractionated the *M. leprae*-derived cell membrane fraction, screened the fractions for such a protein by using DCs as APCs, and subsequently evaluated the newly identified molecule, major membrane protein II (MMP-II), in terms of innate immunity.

MATERIALS AND METHODS

Preparation of cells and bacteria. Peripheral blood was obtained with informed consent from healthy individuals who were positive for purified protein derivative (PPD) due to *M. bovis* BCG vaccination. We are aware that PPD-negative individuals would help to provide full information for these experi-

* Corresponding author. Mailing address: Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan. Phone: 81-42-391-8059. Fax: 81-42-391-8212. E-mail: mmaki@nih.go.jp.

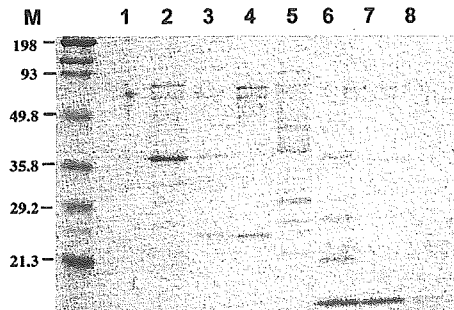


FIG. 1. *M. leprae* membrane fractions were separated into eight fractions by gel filtration as described in Materials and Methods. Then 3 μ g of each fraction was run on a 12% SDS-polyacrylamide gel, and silver staining of the gel was performed.

ments, however, in Japan, such individuals are not available for study, because *M. bovis* BCG vaccination is compulsory for children (0 to 4 years old). Moreover, PPD-negative individuals in the Japanese population are those who do not respond to BCG vaccination, and therefore, it is likely that they suffer from an unknown human disease or immune insufficiency. Therefore, these individuals cannot be used as controls 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 (23). Macrophages were differentiated by culturing plastic-adherent CD14⁺ monocytes with RPMI 1640 medium containing 20% of fetal calf serum (FCS) and 1% penicillin G (Katayama Chemical, Osaka, Japan) in the presence of 5 ng/ml of macrophage colony-stimulating factor (R & D Systems, Abingdon, United Kingdom), as previously described (40). For preparation of the monocytes, CD3⁺ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMCs using immunomagnetic beads coated with an anti-CD3 monoclonal antibody (MAb) (Dynabeads 450; Dynal, Oslo, Norway). The CD3⁻ fraction of the PBMCs was 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 macrophages and DCs (23). Monocyte-derived DCs were differentiated from the plastic-adherent cells as described previously (23, 24). 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 recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (Pepro Tech EC Ltd., London, England) and 10 ng of rIL-4 (Pepro Tech) per ml. rGM-CSF and rIL-4 were supplied every 2 days, and 400 μ l of medium was replaced as described previously (24). In some cases, DCs unpulsed or pulsed with Ags were further treated with a soluble form of CD40 ligand (CD40L) (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. Since *M. leprae* cannot be cultivated or grown in vitro, *M. leprae* (Thai-53) was obtained from an armadillo liver that

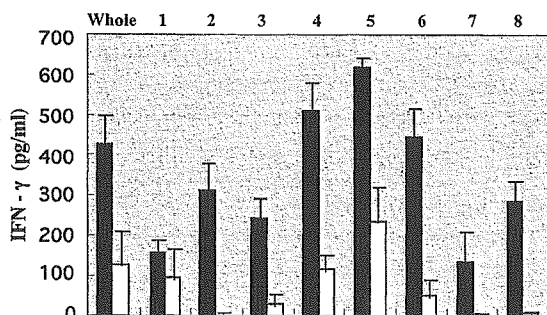


FIG. 2. IFN- γ production by T cells stimulated with membrane fraction-pulsed DCs. The responder CD4⁺ and CD8⁺ T cells (10^5 /well) were stimulated for 4 days with autologous DCs which had been previously stimulated with 10 μ g/ml of various membrane fractions of *M. leprae* obtained by gel filtration. Solid bars, IFN- γ production from CD4⁺ T cells; open bars, IFN- γ production from CD8⁺ T cells.

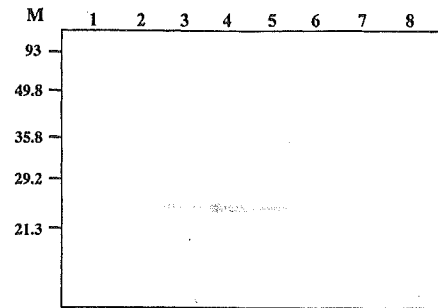


FIG. 3. Western blot of *M. leprae* membrane fractions. Five micrograms of various membrane fractions of *M. leprae* was run on a 12% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane, which was further probed with an anti-MMP-II MAb. An alkaline phosphatase-conjugated anti-mouse IgG Ab was used as the secondary Ab, and the protein was detected with NBT/BCIP reagent.

had been previously infected with *M. leprae*. The isolated bacteria were counted by Shepard's method (35) and were frozen at -80°C until use. The viability of *M. leprae* was assessed by using a fluorescent diacetate/ethidium bromide test (17).

Fractionation of *M. leprae* protein and N-terminal sequencing. The fractionation of the mycobacterial proteins into cell wall, membrane, and cytosolic fractions was carried out according to previous reports (13, 18, 22). Briefly, the mycobacterial suspension was mixed with zirconium beads in the presence of protease inhibitors at a ratio of approximately 1:1 (vol/vol) and homogenized using Beads Homogenizer, model BC-20 (Central Scientific Commerce, Tokyo, Japan), at 1,500 rpm for 90 s three to four times. The beads were separated, and the suspension was centrifuged at $10,000 \times g$ for 30 min. The supernatant was then further ultracentrifuged at $100,000 \times g$ for 1 h. The resulting pellet was suspended in phosphate-buffered saline, washed twice, and taken as the membrane fraction. For the identification of *M. leprae* antigenic molecules, the membrane fraction was further fractionated using a fast protein liquid chromatography system (Amersham Bioscience, New Jersey). Four hundred micrograms of protein was run on a Superose 12 column (Amersham Bioscience) in 50 mM Tris-HCl, 0.5 M NaCl, and 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) at a flow rate of 0.5 ml/min. Fractions were collected, concentrated, buffer exchanged to 50 mM Tris-HCl using Microcon centrifuge filter units YM-3 (Millipore, Bedford, MA), and run on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The gel was stained with "Daiichi" silver stain (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Western blotting was performed using PB patients' pooled sera, at a dilution of 1:25, which had been preadsorbed with the *M. leprae* cytosolic fraction. Alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) (Biosource, Camarillo, CA) was used as the secondary Ab, and detection was performed by using the nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) reagent (Calbiochem, San Diego, CA). N-terminal peptide sequencing of the protein which reacted to the sera was performed at the Center for Instrumental Analysis, Hokkaido University.

Analysis of cell surface Ags. The expression of cell surface Ags on DCs was analyzed using FACScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO), and 10^4 live cells were analyzed. For analysis of cell surface Ags, fluorescein isothiocyanate-conjugated MAbs against HLA-ABC (G46-2.6; PharMingen, San Diego, CA), HLA-DR (L243; PharMingen), CD86 (FUN-1; PharMingen), and CD83 (HB15a; Immunotech, Marseille, France) were used. A murine MAb against MMP-II was raised by immunizing a mouse with purified MMP-II. The optimal concentrations of MAbs were determined in advance.

Identification and purification of MMP-II. The MMP-II gene was PCR amplified from *M. leprae* chromosomal DNA and cloned into an *Escherichia coli* expression vector. Briefly, the MMP-II gene was inserted into the expression plasmid pET28 (Novagen Inc., Madison, WI) and transformed into *E. coli* strain ER2566 (New England Biolabs Inc., Beverly, MA). The expressed protein was eluted using Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA) and used for all experiments. The amount of lipopolysaccharide (LPS) in the purified MMP-II protein was determined by using a *Limulus* amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be less than 70 pg per mg of MMP-II, a level that did not affect the maturation of DCs.

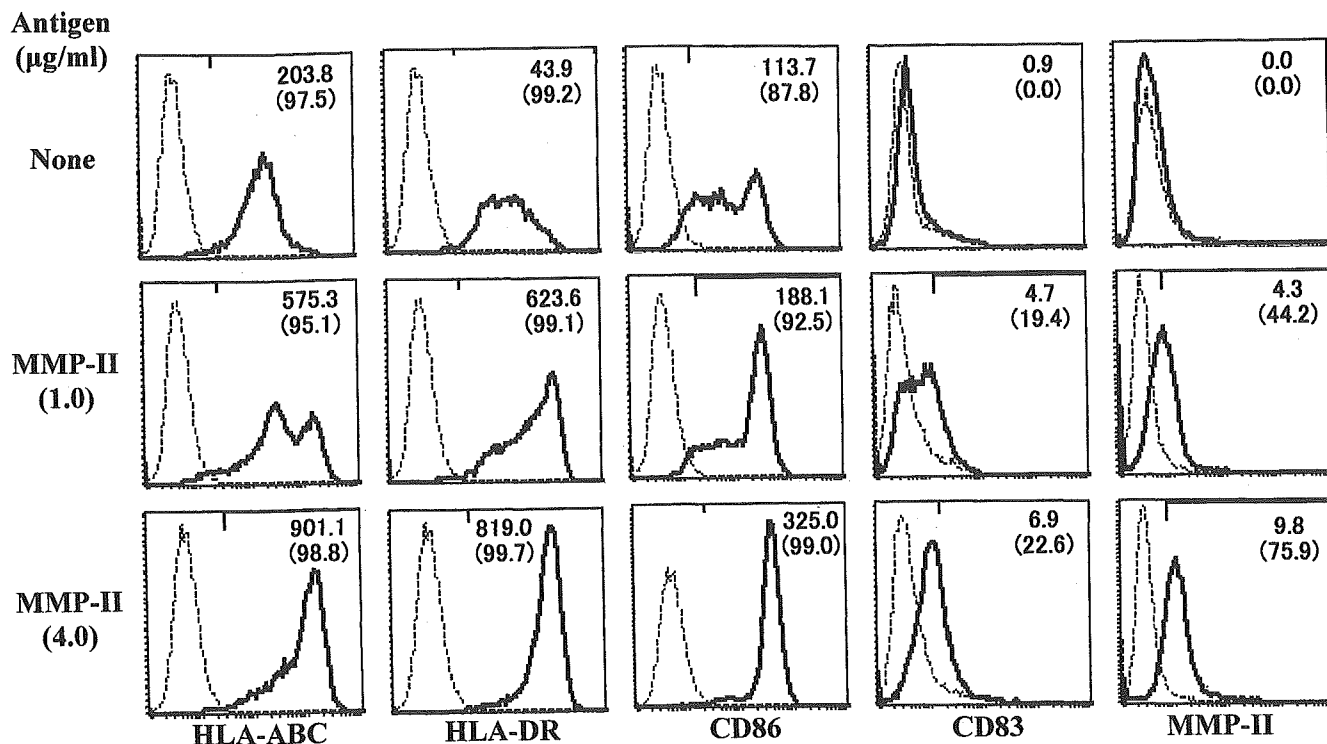


FIG. 4. Expression of various molecules on DCs pulsed with MMP-II. Monocyte-derived DCs from healthy individuals (PPD positive) were pulsed with the indicated dose of MMP-II. DCs were gated and analyzed. Solid curves, isotype-matched control IgG; broken curves, the indicated MAb. The number in the top 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% CO₂. 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: *, $P < 0.001$; †, $P < 0.001$; ‡, $P < 0.001$; §, $P < 0.001$; ¶, $P < 0.0005$; ||, $P < 0.0005$; **, $P < 0.001$; ††, $P < 0.001$; ‡‡, $P < 0.0005$.

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 \pm 0.2	19.3 \pm 1.8 ^{*,†}	45.3 \pm 8.8 ^{‡,§}
MMP-II (1.0 μ g/ml)	2.1 \pm 0.3	122.2 \pm 6.8 [*]	149.9 \pm 20.3 [‡]
MMP-II (4.0 μ g/ml)	1.9 \pm 0.2	568.6 \pm 12.4 [†]	561.2 \pm 31.9 [§]
LPS (0.3 pg/ml)	1.8 \pm 0.1	10.0 \pm 2.0	15.6 \pm 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 μ g/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 \pm 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 μ l of 1 \times passive lysis buffer (Promega), and luciferase activity in 10 μ l 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 mole-

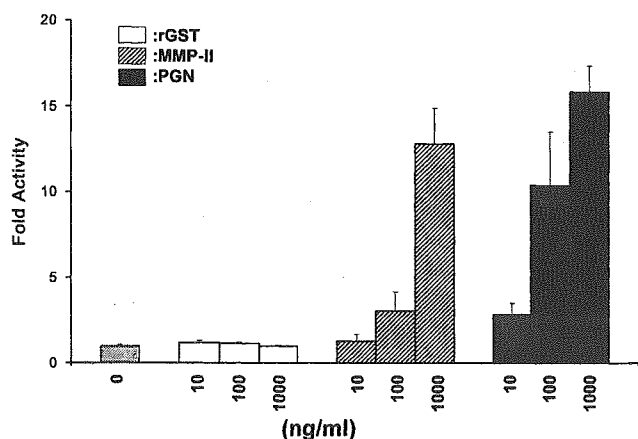


FIG. 5. NF- κ B-dependent reporter gene activity of the TLR2 transfectant was measured after stimulation with or without 10, 100, or 1,000 ng/ml of rGST, MMP-II, or PGN, as described in Materials and Methods. Data are expressed as fold induction relative to the activity of *Renilla* luciferase, which 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 \pm standard deviations.

cules. 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).

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 μ g/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 (10⁶/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.

ACKNOWLEDGMENTS

We appreciate the valuable comments of S. H. E. Kaufmann on the manuscript. We are grateful to N. Makino for the preparation of the manuscript. We acknowledge K. Takahashi, Hokkaido University, for N-terminal sequencing of the protein. We also thank the Japanese Red Cross Society for kindly providing PBMCs from healthy donors. Purified MMP-II was provided by P. J. Brennan (Colorado State University) through NIH contract 1-AI-25468, "Leprosy Research Support."

This work was supported in part by funds from the health science research grants Research on Emerging and Re-emerging Infectious Diseases and Grant-in-Aid for Research on HIV/AIDS from the Ministry of Health, Labour and Welfare of Japan.

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Editor: W. A. Petri, Jr.



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

Masahiko Makino^{a,*}, Yumi Maeda^a, Norihisa Ishii^b

^a Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan

^b Department of Bioregulation, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan

Received 21 January 2005; accepted 2 April 2005

Available online 10 May 2005

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.

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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

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.

* Corresponding author. Fax: +81 42 391 8212.
E-mail address: mmaki@nih.go.jp (M. Makino).

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 TNF α 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 com-

mon 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 μ l 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 10,000g for 30 min. The supernatant was then further ultra-centrifuged at 100,000g 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 amoebocyte 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 ± 0.8 ^{b,c}	4.4 ± 1.1 ^{d,e}	1.0 ± 0.0 ^{f,g}	0.5 ± 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 ± 2.9 ^{b,c}	14.0 ± 3.1 ^{d,e}	1.4 ± 0.2 ^{f,g}	0.9 ± 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 ($\mu\text{g/ml}$)	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 \pm 1.9 ^d	1.1 \pm 0.1 ^{e,f}	0.0 \pm 0.0
MMP-II 1.0	(-)		48.7 \pm 5.3 ^b	3.3 \pm 0.9	7.1 \pm 0.9 ^c	2.0 \pm 0.0 ^g
MMP-II 4.0	(-)		144.5 \pm 4.4 ^c	48.8 \pm 2.3 ^d	24.1 \pm 3.1 ^f	5.0 \pm 0.2 ^g
None	(+)		36.4 \pm 1.9	1.5 \pm 0.1	6.9 \pm 1.3	0.3 \pm 0.0
MMP-II 1.0	(+)		117.9 \pm 3.6	43.8 \pm 1.7	56.6 \pm 3.1	13.3 \pm 1.0
MMP-II 4.0	(+)		308.6 \pm 11.3 ^{h,i}	172.8 \pm 4.0 ^{h,k}	153.3 \pm 7.9 ^{l,m}	29.8 \pm 3.4 ^{n,o}
MLM 4.0	(+)		79.2 \pm 11.3 ^h	39.0 \pm 2.1 ^j	35.9 \pm 2.8 ⁱ	12.4 \pm 4.8 ⁿ
LAM 4.0	(+)		32.9 \pm 9.8 ⁱ	2.0 \pm 1.2 ^k	10.2 \pm 2.0 ^m	2.1 \pm 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 $\mu\text{g/ml}$), 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 \pm 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 profes-

sional 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 TNF α 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 \pm 23.1 (8.1%)	51.4 \pm 8.8 ^b (86.9%)	74.1 \pm 12.7 ^c (81.1%)	101.3 \pm 20.1 ^d (74.2%)	401.9 \pm 31.4 (0.0%)
	80		240.9 \pm 20.2 ^{e,f,g}	219.3 \pm 11.8 (9.0%)	17.8 \pm 8.1 ^e (92.7%)	19.1 \pm 9.8 ^f (92.1%)	81.3 \pm 9.0 ^g (66.3%)	253.2 \pm 21.9 (0.0%)
CD8 ⁺ T cells	10		230.8 \pm 21.3 ^{h,i,j}	24.1 \pm 11.0 ^h (89.6%)	202.4 \pm 30.1 (12.3%)	33.7 \pm 6.9 ⁱ (85.4%)	29.5 \pm 3.9 ^j (87.2%)	229.8 \pm 30.8 (0.4%)
	20		48.9 \pm 9.6 ^{k,l,m}	6.1 \pm 2.1 ^k (87.5%)	51.4 \pm 3.9 (0.0%)	6.0 \pm 1.8 ^l (87.7%)	17.3 \pm 4.2 ^m (64.6%)	50.3 \pm 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 μ g/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 \pm 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 (μ g/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 \pm 4.1 ^d	2.1 \pm 0.4 ^{e,f}	1.9 \pm 0.6 ^{g,h}
1.0		73.2 \pm 9.2 ^b	22.4 \pm 10.3	14.6 \pm 2.8 ^e	10.9 \pm 1.3 ^g
4.0		173.2 \pm 13.0 ^c	60.6 \pm 9.9 ^d	33.8 \pm 3.1 ^f	20.9 \pm 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 μ g/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 \pm 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

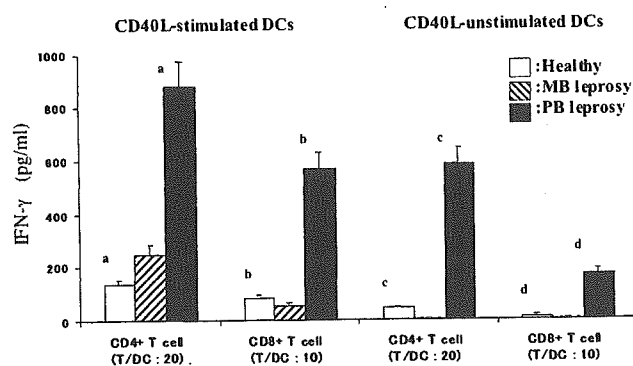


Fig. 1. IFN- γ production of T cells from healthy individuals (PPD-positive), PB and MB leprosy. The responder CD4⁺ and CD8⁺ T cells (1×10^5 /well) were stimulated for 4 days with autologous MMP-II (0.5 μ g/ml)-pulsed DCs at an indicated T cell:DC ratio. The Ag-pulsed DCs were untreated or treated with CD40L (1 μ g/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 \pm 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

We are grateful to Ms. N. Makino for the preparation of the manuscript. We also thank the Japanese Red Cross Society for kindly providing PBMCs from healthy donors. Purified LAM and mAb to LAM were provided by Dr. P.J. Brennan (Colorado State University) through NIH Contracts No.1-AI-25468 "Leprosy Research Support." This work was supported in part by funds from Health Sciences Research Grants-Research on Emerging and Re-emerging Infectious Diseases and from Grant-in-Aid for Research on HIV/AIDS from the Ministry of Health, Labour and Welfare of Japan.

Appendix A. Supplementary data

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

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

Yuji Miyamoto,¹ Tetsu Mukai,¹ Noboru Nakata,¹ Yumi Maeda,¹ Masanori Kai,¹
Takashi Naka,² Ikuya Yano,² and Masahiko Makino^{1,*}

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan,¹ and Japan BCG Central Laboratory, 3-1-5 Matsuyama, Kiyose, Tokyo 204-0022, Japan²

Received 2 August 2005/Accepted 10 October 2005

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 (*gfl1*, *gfl2*, *gfl3*, and *gfl4*) 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 $\Delta gfl1$ and $\Delta gfl2$ accumulated the fatty acyl-tetrapeptide core having *O*-methyl-rhamnose and 6-deoxy-talose as sugar residues, respectively. The mutant $\Delta gfl4$ possessed the same GPLs as the wild type, whereas the mutant $\Delta gfl3$ 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 *gfl1* and *gfl2* genes are responsible for the early glycosylation steps of GPL biosynthesis and the *gfl3* 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 gflA* and *gflB*, which are deduced glycosyltransferase genes of GPL biosynthesis, restore complete GPL production in the mutants $\Delta gfl1$ and $\Delta gfl2$, 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-deoxytalose (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.

* Corresponding author. Mailing address: Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan. Phone: 81-42-391-8059. Fax: 81-42-391-8212. E-mail: mmaki@nih.go.jp.

TABLE 1. Bacterial strains and vectors used in this study

Strain or vector	Characteristic(s)	Source or reference
Bacteria		
<i>E. coli</i>		
DH5 α	Cloning host	
STBL2	Cloning host	
<i>M. smegmatis</i>		
mc ² 155	Wild type	27
Δ gtf1	gtf1 disruptant	This study
Δ gtf2	gtf2 disruptant	This study
Δ gtf3	gtf3 disruptant	This study
Δ gtf4	gtf4 disruptant	This study
<i>M. avium</i>		
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	<i>E. coli</i> - <i>Mycobacterium</i> 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 DH5 α 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 μ g/ml for *E. coli* and 25 μ g/ml for *M. smegmatis*; hygromycin B, 150 μ g/ml for *E. coli* and 75 μ g/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 *rffA* 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* mc²155 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*), pYUBgtf2 (*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* mc²155 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 μ g/ml hygromy-

cin 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 CHCl₃/CH₃OH (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 CHCl₃-CH₃OH (9:1 [vol/vol]). Deacylated GPLs and other compounds were visualized by spraying with 10% H₂SO₄ 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 perdeuteriomethylation 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 NaBD₄ (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