JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2011, p. 613–617 Vol. 49, No. 2
0095-1137/11/$12.00 doi:10.1128/JCM.00817-10

Multiple Cases of Cutaneous *Mycobacterium massiliense* Infection in a "Hot Spa" in Japan▽

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Received 23 April 2010/Returned for modification 2 August 2010/Accepted 7 December 2010

Seven body polishers working in the same "hot spa" presented with multiple red nodules and papules on their hands and forearms. A causative agent was successfully isolated from two of the subjects and from a swab sample collected from the underside of a bed cover in the body-polishing facility. The two cutaneous isolates and the environmental isolate were rapidly growing mycobacteria that formed nonphotochromogenic smooth or smooth/rough colonies on Ogawa egg slants. They were identified as *Mycobacterium massiliense* by multigenotypic analysis using the 16S rRNA, *hsp65*, and *rpoB* genes and the 16S–23S rRNA internal transcribed spacer (ITS) region. However, the use of the 16S rRNA gene sequence and/or DNA-DNA hybridization (DDH Mycobacteria Kit) alone would not distinguish *M. massiliense* from mycobacteria in the *M. chelonae-M. abscessus* group. The three isolates were significantly more susceptible to clarithromycin, doxycycline, and minocycline than the *M. abscessus* and *M. bolletii* reference strains. One cutaneous isolate and the environmental isolate were in a related cluster by randomly amplified polymorphic DNA PCR (RAPD-PCR). Of the several mycobacterial species found in the day spa, only *M. massiliense* was isolated from biopsy specimens of the skin lesions, suggesting that this bacterium is a human skin pathogen. This is the first known report of cutaneous *M. massiliense* infections that could not be attributed to a prior invasive procedure. This is also the first report of *M. massiliense* infection in Japan.

Mycobacterium massiliense was initially isolated from the sputum of a patient with pneumonia in France in 2004 (1). Epidemiologically, *M. massiliense* has been recognized as an emerging pathogen in the United States (16, 24) and Brazil, where outbreaks have been associated with postsurgical and cosmetic procedures (2, 4, 22). In Korea, an outbreak was linked to intramuscular injections of an antimicrobial agent (9). This bacterium was also the source of a lethal case of sepsis in Italy and has been found in cystic fibrosis patients in France (15, 20). Among pulmonary *M. abscessus* group isolates, almost half of the isolates in Korea and 30% of those in the Netherlands are *M. massiliense* (8, 21). It has been suggested that *M. massiliense* should be reclassified taxonomically as a subspecies of *M. abscessus* (11). The clinical significance of differentiating these two species has also been explored (7). However, *M. massiliense* has not been fully characterized. Although mycobacteria are a frequent source of dermal infection, *M. massiliense* has never been reported as an etiological agent. This report describes the first case of an *M. massiliense* dermal infection in Japan.

Case Reports

In November 2007, a 49-year-old female who worked as a body polisher in a hot spa developed multiple red nodules and papules on her hands and forearms. The number of lesions gradually increased over several months, precipitating a visit to a local hospital in June 2008 (case 1). A skin biopsy specimen of a nodule stained with hematoxylin and eosin (H&E) revealed that the lesion was a structured form of granuloma that contained giant cells and infiltrating lymphocytes with necrosis. Acid-fast bacilli were identified by Ziehl-Neelsen staining.

In October 2008, multiple red nodules and papules appeared on the hands and forearms of a 26-year-old female who worked in the same body-polishing facility as the individual with case 1. She visited the same local hospital in December 2008 (case 2) and received similar biopsy results: acid-fast bacilli and granuloma formation with giant cells and infiltrating lymphocytes.

In addition to cases 1 and 2, in the same spa during the same period, there were five more puzzling cases of body polishers with similar symptoms. Three of these patients (with cases 3 to 5) visited the hospital. However, the presence of acid-fast bacilli was not confirmed, even after the observation of granulomas in the skin biopsy specimen of case 3. In April 2009, environmental sampling was conducted at this hot spa in order to discover the causative agent(s).

MATERIALS AND METHODS

Identification and characterization of isolates. Skin samples were decontaminated with *N*-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) (13). Briefly, an equal volume of NALC-NaOH solution (2% NaOH, 1.45% sodium citrate, 0.5% NALC) was added to as much as 10 ml of a skin specimen homogenized in normal saline. The mixture was vortexed and allowed to stand for 15 to 20 min before neutralization with sterile 0.067 M phosphate buffer (pH 6.8), to a final volume of 50 ml, and centrifugation at 3,000 rpm for 20 min. The supernatant was discarded, and the sediment was resuspended in 2 ml of phosphate-buffered saline. Half of the sediment was stored at −80°C, while the other half was used

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▽ Published ahead of print on 15 December 2010.

TABLE 1. Primers used in this study

Primer	Sequence (positions)	Target and/or purpose (amplified fragment size)	Reference
8F16S	5′-AGAGTTTGATCCTGGCTCAG-3′ (8–27)[a]	16S rRNA gene, PCR (ca. 1,500 bp), sequencing	17
1047R16S	5′-TGCACACAGGCCACAAGGGA-3′ (1047–1028)[a]		
830F16S	5′-GTGTGGGTTTCCTTCCTTGG-3′ (830–849)[a]		
1542R16S	5′-AAGGAGGTGATCCAGCCGCA-3′ (1542–1523)[a]		
TB11	5′-ACCAACGATGGTGTGTCCAT-3′	*hsp65*, PCR (441 bp), sequencing	19
TB12	5′-CTTGTCGAACCGCATACCCT-3′		
MabrpoF	5′-GAGGGTCAGACCACGATGAC-3′ (2112–2131)[b]	*rpoB*, PCR (449 bp), sequencing	This study
MabrpoR	5′-AGCCGATCAGACCGATGTT-3′ (2559–2541)[b]		
ITSF	5′-TTGTACACACCGCCCGTC-3′	16S–23S ITS region, PCR (ca. 340 bp), sequencing	14
ITSR	5′-TCTCGATGCCAAGGCATCCACC-3′		
OPA2	5′-TGCCGAGCTG-3′	RAPD-PCR	25
OPA18	5′-AGGTGACCGT-3′		
INS-2	5′-GCGTAGTGCGTCGGTGACAAA-3′		

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

for acid-fast staining and inoculation into a 2% Ogawa egg slant (case 1) or Middlebrook 7H9 broth enriched with 10% oleic acid-albumin-dextrose-catalase (OADC; Nippon Becton Dickinson, Fukushima, Japan) (7H9 broth) (case 2). Mycobacterial isolates were subcultured on Middlebrook 7H11 agar plates enriched with 10% OADC (Nippon Becton Dickinson) for more than 3 days at 36.5°C.

A total of 15 environmental samples were collected from the body-polishing facility in sterile containers or bags. There were four water samples from different bathtubs, eight swab samples, and three scurf scrub equipment samples (two gloves and one brush). All samples were centrifuged at 3,000 rpm for 20 min to concentrate any organisms; the swab and equipment samples were stirred in sterile normal saline before centrifugation. Following centrifugation, precipitated samples were resuspended in normal saline and were added to 1.5 volume of 1 N hydrogen chloride. After incubation for 20 min, the samples were neutralized with 1 N NaOH. The mixture was centrifuged at 3,000 rpm for 20 min, and the sediment was resuspended in 1 ml of phosphate-buffered saline (5). Suspensions were inoculated onto 2% Ogawa egg slants or into 7H9 broth and were incubated at 36.5°C. Mycobacterial isolates were subcultured on Middlebrook 7H11 agar for more than 3 days at 36.5°C. The characteristics of the cultured isolates were determined as described previously (3).

DNA-DNA hybridization. DNA-DNA hybridization was performed with a DDH Mycobacteria Kit (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) to identify mycobacterial species (10). In brief, one-half loopful of a mycobacterial colony was used for the test. Biotin-labeled denatured DNA was extracted from a colony and was distributed into the wells of a microdilution plate where the single stranded DNA from 18 reference strains had been immobilized. After a 2-h hybridization at 55°C, hybridized DNA was detected with peroxidase-conjugated streptavidin and the substrate tetramethylbenzidine. The optical density at 630 nm was measured for each well within 30 min. The labeled strain was identified as one of the 18 species when the maximum color intensity was 1.9 times higher than the intensity of the negative control and the second strongest color intensity was lower than 70% of the maximum color intensity.

DNA extraction. One loopful of a mycobacterial colony on solid medium was suspended in 400 μl sterilized phosphate-buffered saline supplemented with 0.05% Tween 80 and was stored at −80°C until DNA was extracted. A frozen mycobacterial sample was crushed in a bead-beating instrument (Magnalizer; Roche Diagnostics) at 3,000 rpm for 90 s with zirconia beads (diameter, 2 mm). Total genomic DNA was purified from the crushed suspension using the High Pure PCR template preparation kit according to the manufacturer's instructions (Roche Diagnostics) and was stored at −20°C.

Sequence and phylogenetic analysis. Sequences of clinical and environmental isolates, which had been preliminarily identified as *M. abscessus* by the DDH Mycobacteria Kit, were compared to those of the reference strains *M. massiliense* JCM 15300^T, *M. chelonae* JCM 6388^T, *M. abscessus* JCM 13569^T, and *M. bolletii* JCM 15297^T, obtained from the Japan Collection of Microorganisms of the Riken BioResource Center (BRC-JCM; Saitama, Japan). The majority of the 16S rRNA gene, the partial *hsp65* and *rpoB* genes, and the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes were amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) with the primers listed in Table 1. Both strands were sequenced with the BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems), and were run on the ABI Prism 310 genetic analyzer (Applied Biosystems) (13). Analyses were performed after removal of the primers from the sequences.

Similarity searches were performed in the DNA Data Bank of Japan (DDBJ) (6). Phylogenetic analyses were performed using the MEGA software package, version 4.0.2 (Build no. 4028) (18). The tree was constructed using the neighbor-joining method with Kimura's two-parameter distance correction model with 1,000 bootstrap replications.

RAPD-PCR. Randomly amplified polymorphic DNA PCR (RAPD-PCR) (25) was performed with three random primers in order to compare clinical and environmental isolates with the *M. massiliense* JCM 15300^T reference strain (Table 1). In brief, 50 μl of a mixture containing 60 mM Tris-HCl (pH 9.0), 2.5 mM $MgCl_2$, 15 mM $(NH_4)_2SO_4$, 250 μM each deoxynucleoside triphosphate (dNTP), 50 pmol of the primer, 1 U of *Taq* DNA polymerase (Takara Bio Inc., Japan), and 100 ng of total genomic DNA, which was freshly extracted or stored for as long as 30 days at −20°C, was used for the PCR. Amplification was performed in the Takara PCR thermal cycler SP using 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. The PCR products were separated in the same run by 2% agarose gel electrophoresis and ethidium bromide staining. Strains were assigned to the same cluster when the same band patterns were observed with the three primers or one major band difference was observed in only one of the three primers.

Drug susceptibility assays. Drug susceptibility assays were performed with 7H9 broth microdilutions according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (23), with a modification in drug choice for rapidly growing mycobacteria. Amikacin (AMK), azithromycin (AZM), ciprofloxacin (CIP), clofazimine (CLF), clarithromycin (CLR), doxycycline (DOX), meropenem (MEM), minocycline (MIN), and panipenem (PAPM) were tested against the clinical and environmental isolates and the *M. abscessus*, *M. massiliense*, and *M. bolletii* reference strains. AZM was provided by Pfizer Japan Inc.; MEM and PAPM were provided by Dainippon Sumitomo Pharma Co. Ltd. and Daiichi Sankyo Co. Ltd., respectively; and the other drugs were purchased from Sigma-Aldrich Co. MIC testing was carried out in triplicate on different days, with two of three matching MICs used as the criteria for MIC determination. Susceptibility was evaluated according to the CLSI breakpoint recommendations.

Nucleotide sequence accession numbers. The DNA sequences of the 16S rRNA (1,468 bp), *hsp65* (401 bp), *rpoB* (409 bp), and ITS (298 bp) fragments from the reference strains (*M. massiliense* JCM 15300^T, *M. chelonae* JCM 6388^T, *M. abscessus* JCM 13569^T, and *M. bolletii* JCM 15297^T) and the clinical and environmental isolates have been deposited in the International Nucleotide Sequence Databases (INSD) through the DDBJ under accession numbers AB548592 to AB548611.

TABLE 2. Similarities of nucleotide sequences between case isolates and reference strains of closely related mycobacterial species

Isolate	Species for comparison[a]	% Identity			
		16S rRNA (1,468 bp)	*hsp65* (401 bp)	*rpoB* (409 bp)	ITS (298 bp)
Isolate 1	*M. abscessus*	99.9	98.8	97.6	99.0
	M. massiliense	99.9	100	100	100
	M. bolletii	99.9	99.3	98.3	99.0
	M. chelonae	99.8	92.5	96.1	89.9
Isolate 2	*M. abscessus*	99.9	98.8	97.6	99.0
	M. massiliense	99.9	100	100	100
	M. bolletii	99.9	99.3	98.3	99.0
	M. chelonae	99.8	92.5	96.1	89.9
Environmental isolate	*M. abscessus*	99.9	98.8	97.6	99.0
	M. massiliense	99.9	100	100	100
	M. bolletii	99.9	99.3	98.3	99.0
	M. chelonae	99.8	92.5	96.1	89.9

[a] Reference strains used for comparison were *M. abscessus* JCM 13569^T, *M. massiliense* JCM 15300^T, *M. bolletii* JCM 15297^T, and *M. chelonae* JCM 6388^T.

RESULTS

Isolation from skin and environmental samples. Bacteria isolated from the skin biopsy specimens of cases 1 and 2 were provisionally identified as *M. abscessus* by the DDH Mycobacteria Kit. None of the four environmental samples from the bathtubs yielded mycobacteria. However, mycobacteria grew from four swabs and two gloves used for the scurf scrub. The swab isolate from the underside of the bed cover in the body-polishing room was tentatively identified as *M. abscessus* by the DDH Mycobacteria Kit. The five remaining mycobacterial isolates included *M. nonchromogenicum*, from the stone wall of the body-polishing room; *M. terrae*, from a glove; and three *M. fortuitum* isolates (one from the spring spout, one from the wood wall of the body-polishing room, and one from a glove).

The clinical and environmental (bed cover) isolates were rapidly growing mycobacteria that formed nonphotochromogenic colonies at 25 to 37°C on 2% Ogawa egg slants and 7H11 agar plates but did not grow at 42°C. The isolates were negative for niacin, nitrate reduction, and Tween 80 hydrolysis and were positive for 5% NaCl tolerance, arylsulfatase (3 days), catalase, and urease. However, differences in colony morphology were observed: isolate 1 and the environmental isolate formed smooth colonies, while isolate 2 produced rough colonies.

Genotypic analysis. Nucleotide sequence analysis was performed with the three isolates and four reference strains (*M. abscessus*, *M. massiliense*, *M. bolletii*, and *M. chelonae*). The sequences of the 1,468-bp fragment of the 16S rRNA gene from the three isolates were identical. Only single or triple mismatches with *M. abscessus*, *M. massiliense*, and *M. bolletii*, or with *M. chelonae*, respectively, were found at nucleotide positions 1008 or 999, 1039, and 1265. The sequences of *hsp65*, *rpoB*, and the ITS region were also identical among the three isolates, showed complete identity with those of *M. massiliense*, and were 89.9 to 99.3% similar to those of *M. abscessus*, *M. bolletii*, and *M. chelonae* (Table 2). Phylogenetic trees, developed using sequences from the *hsp65* and *rpoB* genes, clustered the isolates with *M. massiliense* (Fig. 1), although the clustering was not as clear with trees developed using sequences from the 16S rRNA gene and the 16S-23S rRNA ITS region (data not shown). Confirmation of these three isolates as *M. massiliense* led to the supposition that *M. massiliense* might be the underlying cause of the cutaneous lesions and that the environment of the day spa led to the acquisition of the infections.

Randomly amplified polymorphic DNA PCR. Strain typing was performed by RAPD-PCR with three random primers to clarify the relatedness of the clinical and environmental *M. massiliense* isolates. A comparison of the OPA2 band patterns (Fig. 2, lanes 1 to 4) revealed distinct differences in the amplification patterns of the clinical isolates versus the *M. massiliense* reference strain. The patterns of isolate 1 and the environmental isolate differed by a minor band. The OPA18 and INS-2 band patterns of isolate 1 and the environmental isolate were identical or differed by only one minor band, though these band patterns were clearly different between the clinical isolates and the reference strain (Fig. 2, lanes 5 to 8 and 9 to

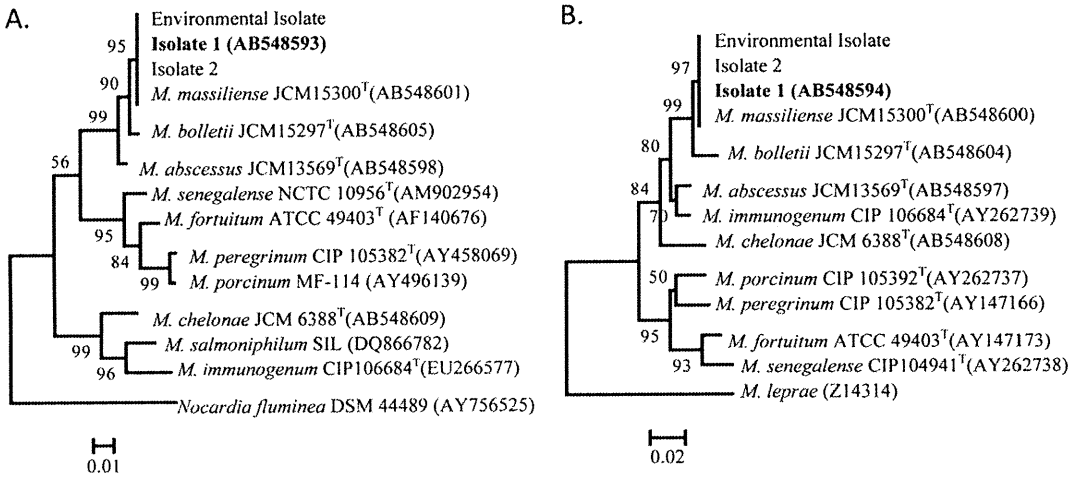

FIG. 1. Phylogenetic analysis based on the *hsp65* (A) and *rpoB* (B) genes of isolate 1 (boldface) and other rapidly growing mycobacteria. The numbers at the nodes are the percentages of bootstrap levels supported by 1,000 resampled data sets. Bootstrap values of <50% are not shown. *Nocardia fluminea* (A) and *M. leprae* (B) were used as outgroups.

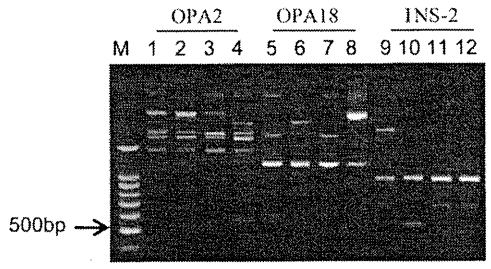

FIG. 2. Comparison of the RAPD-PCR patterns of the clinical isolates (isolates 1 and 2), the environmental isolate, and a reference strain (*M. massiliense* JCM 15300^T) with three different primers. Lanes 1, 5, and 9, DNA from isolate 1; lanes 2, 6, and 10, DNA from isolate 2; lanes 3, 7, and 11, DNA from the environmental isolate; lanes 4, 8, and 12, DNA from the *M. massiliense* reference strain; lane M, DNA size marker (100-bp ladder). RAPD-PCR patterns produced with primers OPA2 (lanes 1 to 4), OPA18 (lanes 5 to 8), and INS-2 (lanes 9 to 12) are shown.

12). Therefore, isolate 1 and the environmental isolate were assigned to the same cluster by RAPD-PCR analysis but were different from isolate 2.

Assays for susceptibility to antimicrobial agents. The results of tests of the susceptibilities of the clinical and environmental isolates to antimicrobial agents are shown in Table 3. All three isolates exhibited susceptibility patterns similar to that of the *M. massiliense* reference strain (1, 11, 16), such as susceptibility to clarithromycin, minocycline, doxycycline, and amikacin and resistance to ciprofloxacin. The strains were also tested against azithromycin, clofazimine, meropenem, and panipenem, though these were not on the list of CLSI-recommended drugs (23). Notably, the MICs of azithromycin for the three isolates and the *M. massiliense* reference strain were lower than those for the *M. abscessus* and *M. bolletii* reference strains. No differences in the MIC were observed with clofazimine, meropenem, and panipenem.

DISCUSSION

In 2004, *M. massiliense* was proposed as a new species in the *M. chelonae-M. abscessus* group (1). Its 16S rRNA gene had complete identity with that of *M. abscessus* and more than 99.6% similarity with the *M. chelonae* and *M. immunogenum* genes. Therefore, genotypic analysis using single-target sequencing of the 16S rRNA gene would not distinguish *M. massiliense* from other mycobacteria in the *M. chelonae-M. abscessus* group. Two independent groups have reported on the inaccuracy of single-target sequencing for the diagnosis of *M. massiliense* (11, 12). Similarly, the DDH Mycobacteria Kit could not distinguish *M. massiliense* from *M. abscessus*, because the objective species of this kit were limited to 18 mycobacterial species: *M. tuberculosis*, *M. kansasii*, *M. marinum*, *M. simiae*, *M. scrofulaceum*, *M. gordonae*, *M. szulgai*, *M. avium*, *M. intracellulare*, *M. gastri*, *M. xenopi*, *M. nonchromogenicum*, *M. terrae*, *M. triviale*, *M. fortuitum*, *M. chelonae*, *M. abscessus*, and *M. peregrinum*. However, with this kit, the one isolate provisionally identified as *M. abscessus* was easily distinguished from several environmental surveillance mycobacterial isolates.

The appearance of skin lesions among day spa workers led to the collection and analysis of workplace environmental samples. Although environmental surveillance was performed several months after case 1 first presented with symptoms, RAPD-PCR showed that isolate 1 and the environmental isolate were part of the same cluster (Fig. 2). In contrast, the same analysis revealed that isolate 2 belonged to a different cluster. The relationship between isolate 1 and the environmental isolate suggests that the unhygienic conditions in the day spa led to the acquisition of the infections, but the cause and effect could not be resolved, because the origin of isolate 2 was not specified. RAPD-PCR typing also showed that the *M. massiliense* reference strain isolated in France had a different amplification pattern, which was indicative of the geographical distinction between the Japanese and French isolates.

Based on published reports, this is the first presentation of cutaneous *M. massiliense* infections that were not preceded by an invasive procedure. *M. massiliense* may be more pathogenic to human skin than other species, since only *M. massiliense* was isolated from the skin biopsy specimens, though several species of mycobacteria were isolated from the day spa facility. The antimicrobial susceptibility profile of *M. massiliense* is shown in Table 3. Further studies are required to determine if the profile differs from those of other members of the *M. chelonae-M. abscessus* group and if any differences can be used as a typing tool. Interestingly, the MICs of azithromycin, clarithromycin,

TABLE 3. Results of drug susceptibility tests

Antimycobacterial drug[a]	MIC (μg/ml) for:					
	Isolate 1	Isolate 2	Environmental isolate	*M. massiliense* JCM 15300^T	*M. abscessus* JCM 13569^T	*M. bolletii* JCM 15297^T
AMK	16	16	16	16	16	16
AZM	16	32	16	16	64	128
CIP	8	16	8	8	8	8
CLF	1	2	1	2	1	2
CLR	0.25	0.25	0.25	0.25	4	4
DOX	2	8	1	1	64	64
MEM	8	8	16	8	16	8
MIN	1	2	0.5	0.5	16	8
PAPM	64	32	64	64	64	64

[a] AMK, amikacin; AZM, azithromycin; CIP, ciprofloxacin; CLF, clofazimine; CLR, clarithromycin; DOX, doxycycline; MEM, meropenem; MIN, minocycline; PAPM, panipenem.

doxycycline, and minocycline for both clinical isolates, the environmental isolate, and the *M. massiliense* reference strain were much lower than those for the *M. abscessus* and *M. bolletii* reference strains. Reinvestigation of the genotypic and drug susceptibility characteristics of the *M. chelonae-M. abscessus* group is needed. However, some differences in drug susceptibilities have been described that may allow clinicians to differentiate *M. massiliense* from other mycobacteria in the *M. chelonae-M. abscessus* group and to design specific therapies targeting the organism (1, 11, 16). Further study is needed to document the clinical features of, and treatment options for, cutaneous *M. massiliense* infection.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor, and Welfare of Japan (for Y.H., M.M., and N.I.) and by a Grant-in-Aid for Scientific Research (C) from The Ministry of Education, Culture, Sport, Science, and Technology of Japan (for Y.H.).

We thank Pfizer Japan Inc., Dainippon Sumitomo Pharma Co. Ltd., and Daiichi Sankyo Co. Ltd. for the kind gifts of AZM, MEM, and PAPM, respectively.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2011, Article ID 347594, 8 pages
doi:10.1155/2011/347594

Review Article

Innate Immune Effectors in Mycobacterial Infection

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Received 4 October 2010; Revised 13 December 2010; Accepted 22 December 2010

Academic Editor: Carl Feng

Tuberculosis, which is caused by infection with *Mycobacterium tuberculosis* (Mtb), remains one of the major bacterial infections worldwide. Host defense against Mtb is mediated by a combination of innate and adaptive immune responses. In the last 15 years, the mechanisms for activation of innate immunity have been elucidated. Toll-like receptors (TLRs) have been revealed to be critical for the recognition of pathogenic microorganisms including mycobacteria. Subsequent studies further revealed that NOD-like receptors and C-type lectin receptors are responsible for the TLR-independent recognition of mycobacteria. Several molecules, such as active vitamin D_3, secretary leukocyte protease inhibitor, and lipocalin 2, all of which are induced by TLR stimulation, have been shown to direct innate immune responses to mycobacteria. In addition, Irgm1-dependent autophagy has recently been demonstrated to eliminate intracellular mycobacteria. Thus, our understanding of the mechanisms for the innate immune response to mycobacteria is developing.

1. Introduction

In humans, tuberculosis is one of deadly infectious diseases. Indeed, approximately 2 million tuberculosis patients die every year. The risk of disease is also increased by emergence of acquired immune deficiency syndrome and development of multidrug-resistant mycobacteria [1]. Therefore, it is important to understand the host defense mechanisms against mycobacteria. Inhalation of aerosols containing *Mycobacterium tuberculosis* (Mtb) causes tuberculosis. After inhalation, Mtb invades alveolar macrophages to enter into the host and establish the infection. The host, in turn, ignites defense responses through sequential activation of immunity, a combination of innate and adaptive immune systems. In the adaptive phase of immune responses, the importance of Th1/IFN-γ-mediated responses in mycobacterial infection has been well established [2]. In contrast, although macrophages are the major target of invasion by Mtb, how the innate arm of immunity mediates host defense against mycobacteria had long remained unknown. However, the mechanisms behind innate immune responses have been revealed in the past 15 years following the identification and characterization of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) [3]. Furthermore, it has been elucidated that TLR-dependent activation of innate immunity controls the development of adaptive immune responses [4]. The involvement of PRRs other than TLRs in the recognition of mycobacteria has also been revealed. In addition to the induction of adaptive immune responses, the PRR recognition of mycobacteria induces expression of several effector molecules participating in the innate host responses. The role of these innate effector molecules in mycobacterial infection is being elucidated. PRR-independent mechanisms for mycobacterial killing, such as autophagy, have also been revealed. In this paper, we will describe recent advances in our understanding of effectors that mediate innate immune responses against mycobacteria.

2. Toll-Like Receptors in Mycobacterial Infection

Innate immune responses after mycobacterial infection are initiated by recognition of mycobacterial components by PRRs, with mycobacterial components activating several

TLRs (Figure 1). Genomic DNA from a *Mycobacterium bovis* strain, bacillus Calmette–Guérin (BCG), have an ability to augment NK cell activity and induce type I IFNs from murine spleen cells and human peripheral blood lymphocytes. The immunostimulatory activity of mycobacterial DNA was ascribed to the presence of palindromic sequences including the 5′-CG-3′ motif, now called CpG motif [5], and now known to activate TLR9 [6]. The mycobacterial cell wall consists of several glycolipids. Among these, lipoarabinomannan (LAM) lacking mannose end capping, lipomannan (LM), and phosphatidyl-*myo*-inositol mannoside (PIM) are recognized by TLR2 [7, 8]. The 19-kDa lipoprotein of Mtb also activates macrophages via TLR2 [9, 10]. TLR4 is also presumed to recognize mycobacterial components.

The *in vivo* importance of the TLR-mediated signal in host defense to Mtb was highlighted in studies using mice lacking MyD88, a critical component of TLR signaling. MyD88-deficient mice are highly susceptible to airborne infection with Mtb [11–13]. In contrast to mice lacking MyD88, mice lacking individual TLRs are not dramatically susceptible to Mtb infection. Susceptibility of TLR2-deficient mice to Mtb infection varies between different studies [14, 15], while TLR4-deficient mice do not show high susceptibility to Mtb infection [16, 17]. A report demonstrates that TLR9-deficient mice are susceptible to Mtb infection and mice lacking both TLR2 and TLR9 are more susceptible [18]. These findings indicate that multiple TLRs might be involved in mycobacterial recognition. However, a recent report using mice lacking TLR2/TLR4/TLR9 indicated that these triple KO mice show a milder phenotype than MyD88-deficient mice [12]. Therefore, more intensive examination is required to reveal whether TLRs or molecules other than TLRs activating MyD88 mediate innate immune responses to mycobacterial infection. This study also demonstrated that Th1-like adaptive immune responses are induced even in Mtb-infected MyD88-deficient mice [12]. Therefore, the TLR/MyD88-independent component of innate immunity is involved in the induction of adaptive immune responses during mycobacterial infection. The TLR/MyD88-independent response might be induced by other PRRs described below.

3. Non-TLRs in Mycobacterial Infection

Several recent findings have indicated that PRRs other than TLRs evoke innate immune responses [19]. These include RIG-I-like receptors, NOD-like receptors (NLRs), and C-type lectin receptors. Among these PRRs, NOD-like receptors and C-type lectin receptors have been implicated in the innate recognition of mycobacteria (Figure 2).

NOD2 is a member of NLRs that recognize muramyl dipeptide (MDP), a core component of bacterial peptidoglycan, in the cytoplasmic compartment. Macrophages from NOD2-deficient mice show a defective cytokine production after Mtb infection [20]. Similarly, mononuclear cells of individuals homozygous for the *3020insC NOD2* mutation show a defective cytokine response after stimulation with Mtb [7]. Activation of the NOD2-mediated pathway is induced by stimulation with live Mtb, but not by heat-killed Mtb [8]. Live Mtb, which is localized in the phagosomal compartment within macrophages, stimulates the cytosolic NOD2 pathway by inducing phagosomal membrane damage [21]. The NOD2 ligand MDP is N-acetylated in most bacteria. However, MDP is N-glycolylated by N-acetyl muramic acid hydroxylase (NamH) in mycobacteria. Analyses using *M. smegmatis* namH mutant and NOD2-deficient mice showed that N-glycolyl MDP is recognized by NOD2. In addition, N-glycolyl MDP is the more potent NOD2 activator than N-acetyl MDP [22]. Thus, NOD2 contributed to the recognition of mycobacteria.

Several members of the NLR family, such as NLRP1, NLRP3, and IPAF, induce assembly of the inflammasome, which leads to caspase-1-dependent secretion of IL-1β and IL-18 [23]. The involvement of IL-1β and IL-18 in mycobacterial infection was demonstrated in studies using knockout mice [24–27]. A recent study demonstrated that mycobacteria inhibit the inflammasome-dependent casapase-1 activation leading to defective IL-1β production [28]. The inhibition of caspase-1 activation has further been shown to be mediated by an Mtb gene, *zmp1*, which encodes a putative Zn^{2+} metalloprotease. Thus, Mtb has a strategy that evades the inflammasome-mediated innate immune responses.

C-type lectin receptors, such as mannose receptor, were originally reported to mediate phagocytosis of mycobacteria [29]. Another C-type lectin receptor, DC-SIGN, has been shown to recognize mycobacteria, and thereby modulate the function of dendritic cells [30–32]. Recognition of mycobacteria by dectin-1 has been shown to induce gene expression such as TNF-α, IL-6, and IL-12 [33, 34]. In addition, macrophage inducible C-type lectin (Mincle) has recently been shown to recognize trehalose-6,6′-dimycolate (TDM: also called cord factor), a mycobacterial cell wall glycolipid that is the most studied immunostimulatory component of Mtb [35, 36], thereafter modulating macrophage activation. Thus, several C-type lectin receptors are involved in the recognition of mycobacteria.

CARD9 is involved in the signaling pathways of several PRRs including TLRs, NOD-like receptors, and FcRγ-associated C-type lectin receptors through association with Bcl-10 and MALT. Therefore, it is not surprising that CARD9-deficient mice are highly susceptible to Mtb infection. However, interestingly the high susceptibility of CARD9-deficient mice to the infection has been shown to be excessive inflammatory responses due to defective production of the immunosuppressive cytokine IL-10 [37]. Mincle is a member of C-type lectin receptors associated with FcRγ [38]. Accordingly, TDM-induced immune responses are mediated by the signaling pathway activating CARD9 [36, 39].

TLRs and C-type lectin receptors are expressed on the plasma membrane or the endosomal/phagosomal membrane, whereas NOD-like receptors are expressed within the cytoplasm. Indeed, distinct patterns of TLR- and NOD-like receptor-mediated gene expression profiles have been demonstrated in infection with intracellular bacteria [40]. Thus, several PRRs recognize mycobacteria in distinct sites within the host cells (macrophages) to synergistically induce effective host defense responses.

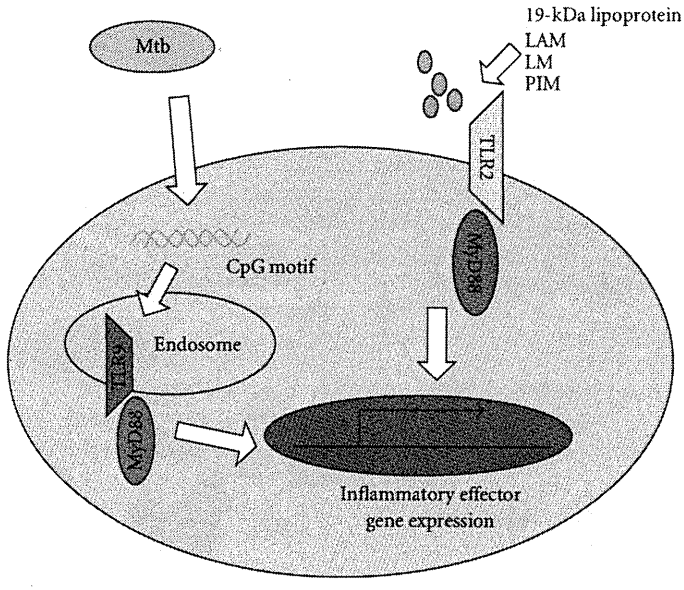

FIGURE 1: Recognition of mycobacteria by Toll-like receptors. TLR2 recognizes several mycobacterial-derived components. TLR9 recognizes mycobacterial DNA including the CpG motif within endosomal compartments. TLR-dependent recognition of mycobacteria induces activation of signaling pathways via the adaptor molecule MyD88, leading to activation of gene expression.

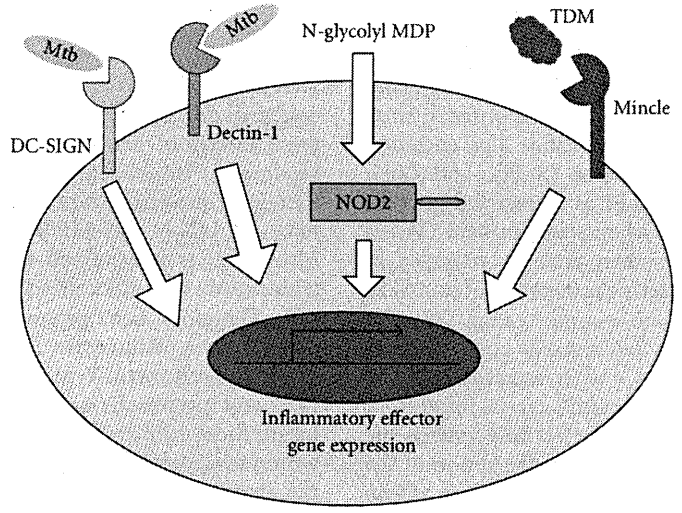

FIGURE 2: Recognition of mycobacteria by pattern recognition receptors. Several pattern recognition receptors, such as NOD-like receptors and C-type lectin receptors, mediate the TLR-independent recognition of mycobacteria. NOD2, a member of NOD-like receptors, recognizes mycobacterial N-glycolyl MDP within the cytoplasm. DC-SIGN and dectin-1 are members of C-type lectin receptors, which are implicated in the recognition of mycobacteria. In addition, Mincle has been shown to recognize TDM (a mycobacterial cell wall glycolipid).

4. Effectors for Mycobacterial Killing

The recognition of mycobacteria by several PRRs induces the expression of several genes that mediate host defense (Figure 3). Among these gene products, vitamin D receptor (VDR) and Cyp27b1, a 25-hydroxyvitamin D_3 1-α-hydroxylase that catalyzes inactive provitamin D into the bioactive form of vitamin D (1, 25 $(OH)_2D_3$), have been shown to be induced by TLR2 ligands in human macrophages [41]. Stimulation of macrophages with 1, 25 $(OH)_2D_3$ induces the expression of the antimicrobial peptide cathelicidin, and thereby enhances the antimycobacterial killing activity [42]. In addition to cathelicidin, the small cationic antimicrobial peptide defensin mediates innate immune responses to Mtb [43, 44]. Experimental infection of the lung epithelial cell line A549 with Mtb strongly induces production of human β-defensin HBD-2, which leads to Mtb killing [43]. HBD-2 expression has also been shown to be induced by TLR2 [45].

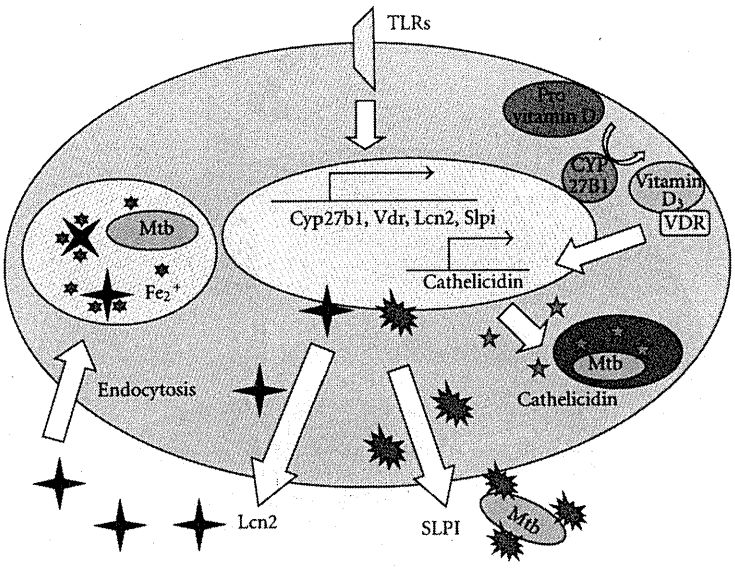

FIGURE 3: TLR-dependent innate response to mycobacteria. Several TLR-dependent gene products mediate innate immune responses to mycobacteria. Mycobacterial stimulation of TLR2 induces expression of Cyp27b1 and vitamin D receptor (VDR), both of which are involved in vitamin D_3-dependent induction of cathelicidin which directly kills mycobacteria. TLR-dependent induction of SLPI mediates disruption of the mycobacterial cell wall. Lcn2, which is also induced by TLR stimulation, is internalized into the alveolar epithelial cells and inhibits mycobacterial growth by sequestering iron uptake.

Gene expression analyses of the lung of mycobacteria-infected mice have identified several TLR-dependent genes that are involved in innate immune responses during mycobacterial infection. These genes include *Slpi*, encoding secretory leukocyte protease inhibitor (SLPI), and *Lcn2*, encoding lipocalin 2 (Lcn2). SLPI is a secreted protein composed of two cysteine-rich whey acidic protein (WAP) domains [46–48]. SLPI was named after its presence in secretions and its function as a serine protease inhibitor. SLPI was originally shown to mediate wound healing [49, 50]. SLPI is produced by bronchial and alveolar epithelial cells as well as alveolar macrophages and is secreted into the alveolar space at the early phase of mycobacterial respiratory infections. Recombinant mouse SLPI effectively inhibits the *in vitro* growth of BCG and Mtb through disruption of the mycobacterial cell wall structure. Cationic residues within the WAP domains of SLPI are essential for the disruption of mycobacterial cell walls. Moreover, SLPI-deficient mice are highly susceptible to mycobacterial infection [51]. The mechanism by which SLPI attaches to the membrane of mycobacteria has been elucidated. SLPI recognizes mannan-capped lipoarabinomannans and phosphatidylinositol mannoside, which are conserved in mycobacteria. Thus, SLPI might act as a PRR in order to bind to the mycobacterial membrane [52].

Lcn2 (also known as neutrophil gelatinase-associated lipocalin, 24p3, or siderocalin) was originally identified in the granules of human neutrophils. Lcn2 is a member of the lipocalin protein family and able to bind to small hydrophobic molecules, siderophore. It is a bacterial molecule made in iron-limited environment and facilitates iron uptake by bacteria [53–58]. The expression of Lcn2 is increased in macrophages of LPS-treated mice [59]. In addition, it is secreted into the alveolar space by alveolar macrophages and epithelial cells during the early phase of respiratory mycobacterial infection. Lcn2 inhibits *in vitro* growth of Mtb by binding the mycobacterial siderophore carboxymycobactin, thereby sequestering iron uptake. Moreover, Lcn2-deficient mice are highly susceptible to intratracheal infection with Mtb. Lcn2 is internalized into alveolar epithelial cells by endocytosis and colocalized with mycobacteria within the cells. Therefore, Lcn2 presumably sequesters iron uptake of mycobacteria within epithelial cells and thereby inhibits their intracellular growth. Within macrophages, the endocytosed Lcn2 and mycobacteria show distinct patterns of subcellular localization, which might allow growth of mycobacteria within macrophages [60]. Thus, Lcn2, which is secreted into the alveolar space during the early phase of mycobacterial infection, is endocytosed into alveolar epithelial cells, thereby inhibiting mycobacterial growth [61].

5. Autophagy in Mycobacterial Infection

Phagocytosis of myobacteria and PRR-dependent recognition of mycobacteria activate several effector functions in macrophages (Figure 4). Maturation of phagosomes is a crucial step in the elimination of intracellular bacteria. The natural-resistance-associated macrophage protein (Nramp1), which is encoded by Slc11a1, is thought to mediate transportation of divalent cations in the phagosomal membrane and thereby sequesters iron (Fe^{2+}) from mycobacteria to enhance bacterial killing by macrophages [62]. Polymorphisms of the *SLC11A1* gene have been associated with susceptibility to several infectious diseases,

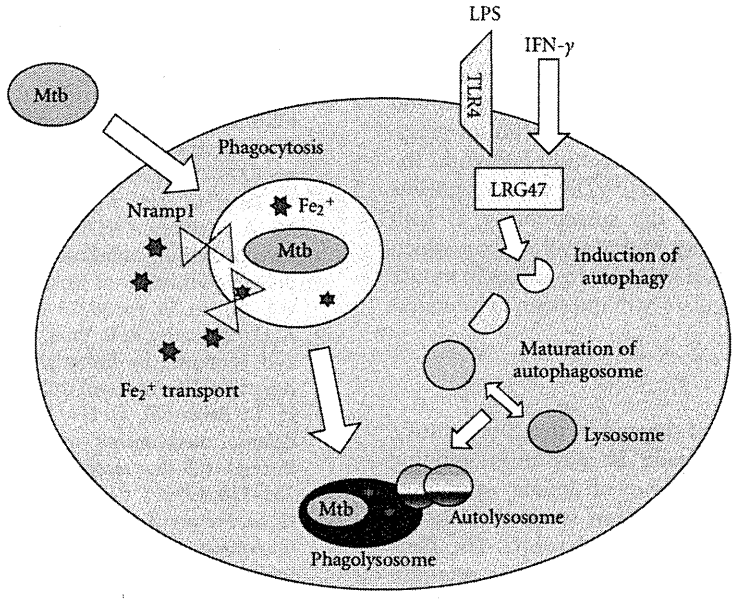

FIGURE 4: Effectors that mediate mycobacterial killing in macrophages. Macrophages eliminate invading mycobacteria by activating several effector functions, such as phagosomes and autophagy. Nramp1 is expressed in the phagosomal membrane and presumably mediates mycobacterial killing by sequestering iron uptake. IFN-γ and the TLR4 ligand induce expression of LRG47, which in turn stimulates autophagy in macrophages. Autophagy is responsible for mycobacterial killing by promoting fusion of mycobacterial phagosomes to lysosomes.

including tuberculosis [63, 64]. However, *in vivo* studies have shown that Nramp1-deficient mice are not more susceptible than wild-type mice to infection with virulent Mtb [65]. Thus, the role of Nramp1 in mycobacterial infection is still controversial. This might be due to the presence of other killing mechanisms for mycobacteria in macrophages. Indeed, autophagy has recently been shown to be involved in host defense against several intracellular pathogens that reside within phagosomes [66]. Autophagy was originally identified as a homeostatic mechanism for the catabolic reaction of cellular constitutes [67, 68]. It has been demonstrated that autophagy mediates innate immune responses against mycobacteria by promoting phagolysosomal maturation within macrophages [69, 70]. Autophagy is induced by IFN-γ-dependent induction of a member of the immunity-related p47 guanosine triphosphatases (IRG) family, LRG47 (also known as Irgm1) in murine macrophages [69]. The importance of LRG47 in resistance to Mtb infection was demonstrated in LRG47-deficient mice, which show high susceptibility to infection [71]. A subsequent study demonstrated that stimulation of macrophages with the TLR4 ligand LPS leads to the MyD88-independent induction of autophagy, which enhances mycobacterial colocalization with the autophagosomes. Since LPS stimulation induces expression of LRG47, the TLR signaling establishes a close relationship between innate immunity and autophagy in mycobacterial infection [72]. In humans, the most equivalent gene to murine Irgm1 is IRGM. IRGM has also been implicated in the induction of autophagy in mycobacteria-infected human macrophages [73]. Irgm1 has been shown to associate with the mycobacterial phagosome by interacting with phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P(2)) and PtdIns(3,4,5)P(3) [74]. The connection of the IRG family of proteins with autophagy has been further demonstrated in an alternative intracellular infection model. In this study, Irgm3 (also known as IGTP) has been implicated in autophagy induction in macrophages infected with *Toxoplasma gondii* [75].

p62 (also called A170 or SQSTM1) directly binds to cytosolic polyubiquitinated proteins and thereby induces their autophagic clearance [76, 77]. It has also been shown that p62 targets intracellular *Salmonella typhimurium* decorated by ubiquitinated proteins to induce autophagy [78]. In the case of mycobacteria residing in the phagosome, p62 delivers cytosolic ubiquitinated proteins to autophagolysosomes where they are proteolytically processed to products that are able to kill mycobacteria [79]. In accordance with this finding, it has been shown that mycobacterial killing by ubiquitin-derived peptides is enhanced by autophagy [80].

As described above, 1, 25 $(OH)_2D_3$ mediates antimycobacterial activity via induction of cathelicidin. A recent report demonstrated that 1, 25 $(OH)_2D_3$-mediated expression of cathelicidin induces autophagy [81]. Thus, several innate immune effectors are closely interacted.

6. Human Genetics in Tuberculosis

In addition to the intensive studies using murine models, considerable advances have been made in our understanding of the susceptibility to Mtb infection in humans through the identification of mutations and polymorphisms of

innate immunity-related genes in tuberculosis patients. As described above, polymorphisms of the *SLC11A1* gene are associated with tuberculosis. Subsequent studies identified a significant distinction between tuberculosis patients and healthy controls in *TLR2* Arg753Gln polymorphism genotype, indicating that the *TLR2* polymorphism influences the susceptibility of Mtb infection [82]. *VDR* polymorphisms have also been implicated in the susceptibility of Mtb infection [83]. These studies suggest that several genes, which have been revealed to be critical in innate responses in mouse models of Mtb infection, regulate Mtb infection in humans.

7. Conclusion

Since the discovery of TLRs at the end of the 20th century, rapid advances have been made in our understanding of the mechanisms for activation of innate immunity. Accordingly, innate immunity has been revealed to have a pivotal role in host defense against mycobacteria. The TLR-independent mechanisms for the innate immune response to mycobacteria have also been elucidated. The emergence of multidrug-resistant Mtb is now a major public health problem all over the world. In this context, it is highly critical to develop a new strategy for the treatment of Mtb-infected patients that supplements the conventional antimycobacterial chemotherapeutic drugs. More precise understanding of the innate immune response to Mtb will pave the way for the development of an effective drug that targets the host innate immunity for the treatment of tuberculosis.

Acknowledgments

The authors thank C. Hidaka for secretarial assistance. This paper is supported by the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labour and Welfare and the Osaka Foundation for the Promotion of Clinical Immunology.

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PLoS NEGLECTED TROPICAL DISEASES

A Lipopeptide Facilitate Induction of *Mycobacterium leprae* Killing in Host Cells

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Abstract

Little is known of the direct microbicidal activity of T cells in leprosy, so a lipopeptide consisting of the N-terminal 13 amino acids lipopeptide (LipoK) of a 33-kD lipoprotein of *Mycobacterium leprae*, was synthesized. LipoK activated *M. leprae* infected human dendritic cells (DCs) to induce the production of IL-12. These activated DCs stimulated autologous $CD4^+$ or $CD8^+$ T cells towards type 1 immune response by inducing interferon-gamma secretion. T cell proliferation was also evident from the CFSE labeling of target $CD4^+$ or $CD8^+$ T cells. The direct microbicidal activity of T cells in the control of *M. leprae* multiplication is not well understood. The present study showed significant production of granulysin, granzyme B and perforin from these activated $CD4^+$ and $CD8^+$ T cells when stimulated with LipoK activated, *M. leprae* infected DCs. Assessment of the viability of *M. leprae* in DCs indicated LipoK mediated T cell-dependent killing of *M. leprae*. Remarkably, granulysin as well as granzyme B could directly kill *M. leprae in vitro*. Our results provide evidence that LipoK could facilitate *M. leprae* killing through the production of effector molecules granulysin and granzyme B in T cells.

Citation: Maeda Y, Tamura T, Fukutomi Y, Mukai T, Kai M, et al. (2011) A Lipopeptide Facilitate Induction of *Mycobacterium leprae* Killing in Host Cells. PLoS Negl Trop Dis 5(11): e1401. doi:10.1371/journal.pntd.0001401

Editor: Kenji Hirayama, Institute of Tropical Medicine (NEKKEN), Japan

Received May 16, 2011; **Accepted** October 6, 2011; **Published** November 22, 2011

Funding: This work was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan for "Research on Emerging and Re-emerging Infectious Diseases" (Grant no. H21-Shinko-Ippan-007 to T.M.), and a grant from Ohyama Health Foundation (to Y.M.), Tokyo. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The introduction of multidrug therapy in 1982 and the WHO campaign for the 'elimination of leprosy as a public health problem', have contributed greatly to the decrease in the prevalence rate over the past three decades. But leprosy still remains to be a public health problem in some countries, and the number of new cases detected during the last three years, remain steady [1]. The disease presents as a clinical spectrum that correlates with the level of the immune response to the pathogen [2]. Patients with lepromatous form of the disease have poor cellular immunity, resulting in extensive intracellular proliferation of *Mycobacterium leprae* bacilli in the skin and nerves. On the other hand, patients with the tuberculoid form of the disease are relatively resistant to the bacilli, so that few, if any, demonstrable bacilli are seen in the lesions [2,3]. For patients with abundant bacilli, whose lesions are characterized by type-2 cytokines, there is a need to up-regulate the T-cell mediated type 1 immune responses, by immunotherapeutic means to kill the bacilli.

We have previously identified a lipoprotein of *M. leprae*, a 33-kD lipoprotein (ML0603) [4]. Truncated protein, having the N-terminal 60 amino acids of 33-kD lipoprotein, had cytokine inducing ability in human monocytes, in contrast to the C-terminal 192 amino acids having no such ability [5]. In this study, we synthesized the lipopeptide (LipoK) having the N-terminal 13 amino acids of the 33-kD *M. leprae* lipoprotein linked to tri-palmitoylated portion of a lipid. Since GC mass spectrometry of mycobacterial lipoproteins provided evidence for the presence of three fatty acids (either palmitic, stearic or tuberculostearic acid), we assumed that tri-palmitoylated peptide would represent the natural lipoprotein of *M. leprae* [6,7]. Further, N-acyl transferase (Lnt) activity was identified in mycobacteria, which transfers the amide-linked acyl group to the N-terminal cysteine residue [6]. This presence of Lnt activity would indicate the presence of triacylated lipoproteins in mycobacteria, although the exact lipid structure of *M. leprae* lipoprotein is still to be determined. Previously, it was observed that hexameric peptides with tri-palmitoyl modification, corresponding to 19-kD and 33-kD lipoproteins of *M. leprae*, partially activates cells through TLR2-TLR1 heterodimers [8,9]. Since dendritic cells (DCs) are the most potent antigen presenting cells capable of bacilli uptake, antigen presentation and initiating acquired immune responses, DCs were used as target antigen presenting cells, in the present study [10,11]. As expected, it was found that LipoK, delivered signals through TLR2, and activated *M. leprae* infected DCs to produce abundant IL-12, although, LipoK does not produce IL-12, in non-infected DCs. Several mechanisms are known to be involved in the clearance of intracellular bacteria, including interferon gamma (IFN-γ) release, apoptosis induction of the host cells and anti-microbial activity of $CD8^+$ cytotoxic T lymphocytes (CTL) [12–15]. CTL mediated killing of mycobacteria, was demonstrated in tuberculosis by Thoma-Uszynski *et al.* They showed that $CD8^+$ CTL-mediated killing of *M. tuberculosis* was dependent on granule exocytosis [16].

In the present study, we analyzed whether *M. leprae* infected DCs, activated through LipoK could undergo functional changes and subsequently induce type 1 T cell activation to kill the bacilli. We observed that LipoK is a potent inducer of T cells equipped

Author Summary

We observed that LipoK (*Mycobacterium leprae* lipopeptide with 13 amino acids) is capable of inducing a good immune response in *M. leprae* infected human dendritic cells (DCs). These activated DCs had up-regulated expression of costimulatory molecule CD86 as well as CD83 (well known maturation marker) on their surface, and secreted IL-12, which is an important cytokine involved in the host defense against pathogens. Importantly, these mature DCs were capable of further driving type 1 responses by stimulating $CD4^+$ T cells and $CD8^+$ T cells for proliferation and interferon-gamma production. Further, both subsets of T cells were capable of producing cytotoxic granules: granulysin and granzyme B. *In vitro* experiments proved that these molecules are capable of killing *M. leprae* directly. It is the first report of the type, which proves that granulysin as well as granzyme B could partially kill *M. leprae*. LipoK would facilitate in inducing the immune responses in patients' harboring *M. leprae*.

with cytolytic function, which can largely contribute to the killing of *M. leprae* in host cells.

Materials and Methods

Ethics statement, cell culture and preparation of the bacteria

Peripheral blood was obtained from healthy Japanese individuals under informed consent. But no information of the donor (exposure to bacilli) was provided. In Japan, BCG vaccination is compulsory for children (0~4 years old). Monocyte-derived DCs were differentiated from monocytes using GM-CSF and IL-4 as described earlier [17,18]. Animal studies were carried out in strict accordance with the recommendations from Japan's Animal Protection Law. The protocol was approved by the Experimental Animal Committee, of the National Institute of Infectious Diseases, Tokyo (Permit Number: 210001). *M. leprae* (Thai-53 strain) is passaged in athymic *nu/nu* mice (Clea Co, Tokyo) [19]. At 8 to 9 months post-infection, the footpads were processed to recover *M. leprae* [20]. For all experiments, *M. leprae* was freshly prepared. The multiplicity of infection (MOI) was determined based on the assumption that DCs were equally susceptible to infection with *M. leprae* [21], and immature DCs were infected with *M. leprae* at MOI 50 in all experiments. Human cells without the bacilli was cultured at 37°C, but when infected with the bacilli, the cells were cultured at 35°C, which is the minimal temperature at which the cells can survive in *in-vitro* experiments. LipoK having the structure Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy) -propyl)-Leu-Pro-Asp-Trp-Leu-Ser-Gly-Phe-Leu-Thr-Gly-Gly-OH, was synthesized by Bachem (Bubendorf, Switzerland). Using LAL assay (QCL-1000, Lonza), endotoxin was undetectable in original LipoK preparation (50 μg/ml). Therefore, any contaminating LPS in the synthesized product could be ruled out. Monoclonal Ab to TLR2 was kindly provided by Genentech, and mAb to mannose receptor and DC-SIGN were obtained from BD Biosciences. Parthenolide obtained from Santa-Cruz was used at a concentration of 2 and 5 μM. CD40L (Pepro Tech) was used at the concentration of 1 μg/ml, whenever needed.

Analysis of cell surface Ags on DCs and measurement of IL-12 production

Immature DCs were stimulated with *M. leprae* and/or LipoK for 48 hours. The expression of cell surface antigens on DCs, were analyzed using FACSCalibur flow cytometer (BD Biosciences). Dead cells were eliminated from the analysis by staining with 7-amino actinomycin D stain. For analysis of cell surface Ag, the following mAb were used: FITC-conjugated mAb against HLA-ABC (G46-2.6), HLA-DR (L243) and CD86 (FUN-1), purchased from PharMingen, and CD83 (HB15a, Immunotech). The ability of DCs to produce IL-12 on stimulation with either LipoK and/or *M. leprae*, was assessed. DCs were stimulated with the Ags on day 4 after the start of culture from monocytes. After 24 hours, OptEIA Human IL-12 (p70) ELISA Set (BD Biosciences) was used to determine the concentration of IL-12 p70 in the culture supernatant.

DC-T cell co-cultures

The ability of *M. leprae*-infected DCs to stimulate T cells was assessed using an autologous DC-T cell co-culture. $CD4^+$ T cells and $CD8^+$ T cells were purified using respective T cell enrichment Set (BD IMag) from freshly thawed PBMCs. The purity of $CD4^+$/$CD8^+$T cells was determined to be more than 95%. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates, and mitomycin C-treated DCs which were pulsed with Ag, were added to give the indicated DC: $CD4^+$ or $CD8^+$ T cell ratio. Supernatants of DC-T cell co-cultures were collected on day 4, and IFN-γ production was measured by ELISA, using Opt EIA Human IFN-γ ELISA Set (BD Biosciences). In other experiments, Ag-pulsed DCs were treated with mAb to HLA-ABC (W6/32), HLA-DR (L243), CD86 (IT2.2) or normal mouse IgG. For obtaining naïve T cells, anti-CD45RO mAb (Dako) and anti-mouse IgG Ab Dynabeads M-450 (Invitrogen) were used to negatively select the cells. Since BCG is compulsory for children in Japan, it is likely that naïve T cells respond to *M. leprae* antigens, some of which are cross reactive to *M. bovis* BCG.

Measurement of T cell proliferation by CFSE labeling

DCs stimulated with Ags were co-cultured with the CFSE labeled total T cells. CFSE (Molecular Probes) was added at the concentration of 1 μM and incubated at 37°C for 10 min and stabilized according to the manufacturers' protocol. A total of 1×10^6 cells/well were seeded in a 24-well plate at a DC:T cell ratio of 1:6. After 8 days co-culture, cells were co-stained with PE conjugated anti-CD4 mAb and APC conjugated anti-CD8 mAb (BD Biosciences). CFSE signal of gated T cells were analysed.

Confocal microscopy

Imaging of cells was performed using laser scanning microscope LSM5-Exciter (Carl Zeiss). DCs grown on a 13-mm coverglass in a 24-well plate, were infected with *M. leprae* and/or stimulated with LipoK for 48 hours. T cell from the same donor was purified using the Dynal T cell isolation kit, and co-cultured with DCs for additional 3 days, after washing out extracellular bacilli. Cells were fixed in 2% paraformaldehyde, and the bacilli stained with 0.01% auramine O as described [22]. Anti-*M. leprae* membrane (minus LAM) polyclonal antibody was kindly provided by Dr. John S. Spencer through the NIH/NIAID Leprosy Research Support (N01 A1-25469). Fixed cells were blocked with normal human IgG (10 μg/ml), and stained with the above polyclonal antibody (1 μg/ml) for 30 min in PBS containing 0.1% saponin and 0.5% BSA, and the secondary antibody used was Alexa Fluor 633-conjugated goat anti-rabbit IgG (Molecular Probes), and images were recorded on fluorescent confocal microscope using a 63× oil objective, 488-nm and 633-nm lasers. Data was processed using the LSM software ZEN 2007. All bacilli observed were not surface attached as observed by section scanning (Z-stack Navigation).

Determination of intracellular levels of perforin, granzyme B and granulysin

After 7 days co-culture of purified T cells with DC pulsed with *M. leprae* and/or LipoK, intracellular detection of cytolytic effector molecules was performed. Briefly, GolgiStop (BD Biosciences) was added to the media for the last 12 hours of culture. Cells were first surface stained, fixed, permeabilized, and finally stained with FITC conjugated anti-perforin mAb or anti-granzyme B mAb or isotype control IgG2a (BD Biosciences). For the determination of intracellular levels of granulysin, the procedure was followed as for the intracellular stain of perforin, except that the surface stain used was FITC conjugated-CD4 and APC conjugated anti-CD8 mAb (BD Biosciences), and subsequently PE conjugated granulysin (eBioscience, GmbH, Germany) was used to determine the percentage of granulysin producing cells.

Determination of *M. leprae* viability in DCs

Since *M. leprae* cannot be cultured in vitro, we measured the viability of the bacilli, by the measurement of radioactive CO_2 production from oxidation of palmitic acid as described previously [23]. DCs were infected with *M. leprae* with or without LipoK, and co-cultured with T cells in some cases. Six days later, cells were harvested and washed 3 times in PBS, and centrifuged, so that *M. leprae* that might have escaped from the DCs into the media could be eliminated from our assay. Cell lysates were prepared as follows: 0.1 N NaOH solution was added to the cells for few minutes and then neutralized with the equal volumes of 0.1 N HCl solution. Subsequently, equal volume of 2 times concentrated Middlebrook 7H9 broth supplemented with ADC was added. ^{14}C labeled palmitic acid was added to the lysates of DCs and cultured at 33°C. Seven days later, the amount of $^{14}CO_2$ evolved and trapped on the filter paper was measured using a Packard 1500 TRI-CARB liquid scintillation analyzer. In a likewise manner, direct effect of *M. leprae* killing was observed by incubation of the bacilli with 3 µg/ml of granulysin (R&D systems) or granzyme B (Calbiochem) for a period of 3 days at 33°C, and then ^{14}C labeled palmitic acid was added to determine the viability as described above.

Statistical analysis

The unpaired student's t test was used to find the significance of the two sets of data. Differences were considered as statistically significant if $p<0.05$. All experiments were performed at least 3 times with different blood donors, unless otherwise stated, and the reproducibility of the experiment was evaluated. In some cases, ANOVA was used for probability calculation.

Results

LipoK activated human dendritic cells

We investigated the effect of LipoK stimulation on human monocyte derived DCs. All DCs were CD1a positive and CD14 negative [21]. When LipoK was used as a stimulant for immature DCs, maturation of DCs was observed as shown in Fig. 1. Up-regulation in the expression of CD83 (maturation marker of DCs) and CD86 (co-stimulatory molecule) was observed in LipoK stimulated DCs, the level of which, was similar to that of *M. leprae* infected DCs. *M. leprae* was used at the multiplicity of infection (MOI): 50 in all the experiments. The expression of the CD83 and CD86 molecules was more pronounced when LipoK was used to stimulate *M. leprae* infected DCs. The expression of HLA-ABC and HLA-DR molecules was not significantly different in LipoK stimulated *M. leprae* infected DCs from non-infected DCs, after 48 hours. Although, at earlier time points (18 hours after stimulation with antigen), a higher expression of HLA-ABC and

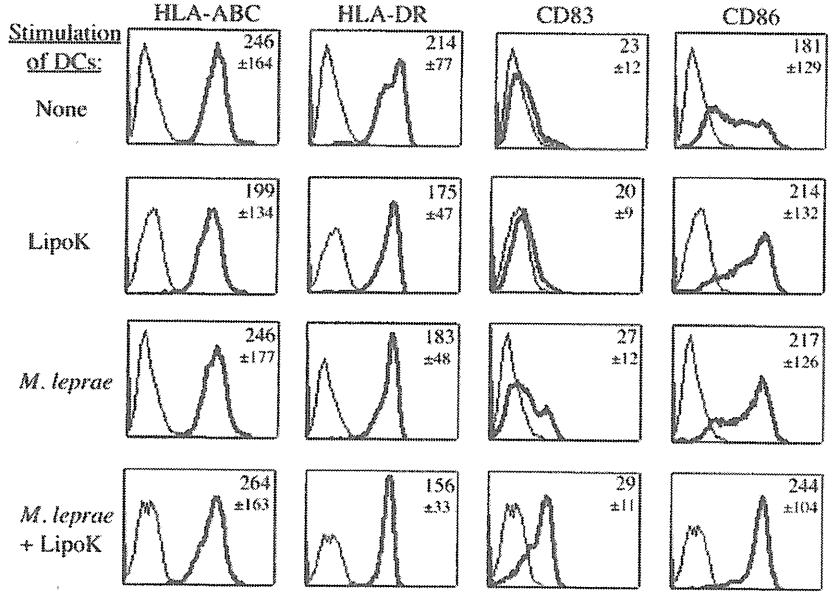

Figure 1. Expression of the surface markers on DCs after stimulation of *M. leprae* infected DCs with LipoK. The expression of cell surface markers on DCs, was analyzed using FACSCalibur. Dead cells were eliminated from the analysis by staining with 7-amino actinomycin D (7-AAD) stain. LipoK was used at a concentration of 0.3 µg/ml. The following mAb were used: FITC-conjugated mAb against HLA-ABC, HLA-DR, CD83 and CD86. Black light lines, isotype-matched control IgG. Black solid lines show the fluorescence intensity of the respective surface markers of DCs. Numbers indicate the mean fluorescence intensity with SD of the respective surface markers. Representative data of three separate experiments with different donors is shown.
doi:10.1371/journal.pntd.0001401.g001

HLA-DR is observed in LipoK stimulated *M. leprae* infected DCs compared to non-stimulated.

Alternatively, when the IL-12 p70 secreted by DCs was measured, increasing dose of LipoK on *M. leprae* infected DCs produced the cytokine, with maximal cytokine production at LipoK concentration of 0.3 μg/ml (Fig. 2A). LipoK alone did not produce statistically significant amounts of IL-12 at the concentration of 0.3 μg/ml compared to the non-stimulated DCs. Another TLR-2 agonist, peptidoglycan could produce IL-12 (data not shown), probably due to the heterogeneous nature of the peptidoglycan which contains long peptide linkages. LipoK probably need other protein/peptide molecules to activate IL-12 production in DCs. Also, *M. leprae* infection alone did not produce IL-12 in DCs. When CD40 ligand (CD40L) was used to stimulate *M. leprae* infected DCs, IL-12 production was negligible.

As could be expected, TLR-2 antagonistic Ab completely blocked IL-12 production, whereas mannose receptor Ab did not, suggesting that IL-12 production from LipoK stimulated *M. leprae* infected DCs was TLR-2 dependent (Fig. 2B). When DCs were pre-treated with parthenolide, which is known to inhibit NF-kB activity [24], it was found that both 2 μM and 5 μM could significantly inhibit the production of IL-12 in a dose-dependent manner (Fig. 2C), indicating that NF-kB is involved in the IL-12 production from these LipoK stimulated DCs.

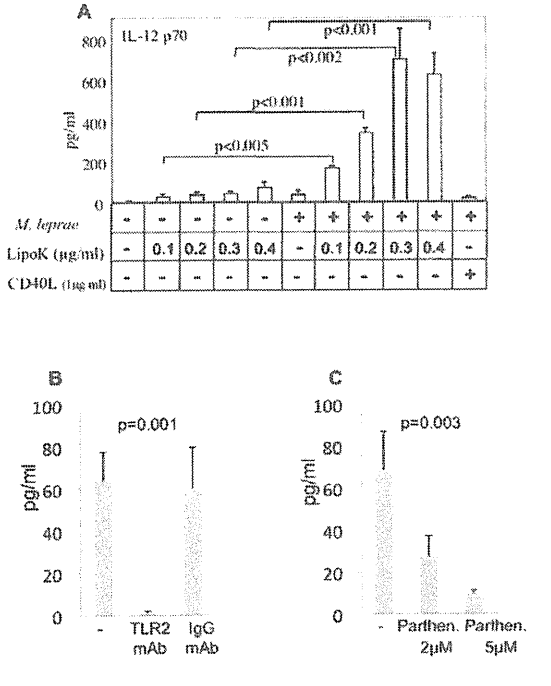

Figure 2. Production of IL-12 p70 from DCs. **(A)** Enhanced induction of IL-12 p70 from DCs by stimulation with LipoK and *M. leprae*. DCs were stimulated with the antigens on day 4 after the start of culture from monocytes. After 24 hours, IL-12p70 concentration in the culture supernatant was measured by the enzyme assay kit Opt EIA Human ELISA Set. The antigens used for the stimulation were: *M. leprae* and LipoK at different concentrations 0.1 μg/ml, 0.2 μg/ml, 0.3 μg/ml, and 0.4 μg/ml, CD40L was used at 1 μg/ml. **(B)** IL-12 p70 production from LipoK stimulated *M. leprae* infected DCs, is inhibited by antagonistic antibodies to TLR-2, and not by control normal IgG. **(C)** Effect of 2 μM and 5 μM of parthenolide (parthen.) on the IL-12 production was observed. Representative data of three separate experiments with different donors is shown. The probability by ANOVA was calculated to be 0.001 for (B) and 0.003 for (C).
doi:10.1371/journal.pntd.0001401.g002

LipoK pulsed human DCs activated human T cells *ex vivo*

To investigate the effect of LipoK on T cell responses, purified $CD4^+$ and $CD8^+$ T cells from autologous donors were cultured with activated DCs. IFN-γ release was measured as correlates of T cell activation. When the IFN-γ levels were compared, DCs activated with *M. leprae* and LipoK produced significantly higher dose of IFN-γ from $CD4^+$ T cells, when compared to that produced by DCs stimulated with *M. leprae* or LipoK alone, or *M. leprae* in presence of CD40L (Fig. 3A), at both high (T:DC = 20:1) and low (T:DC = 40:1) dose of DCs. Note that *M. leprae*-infection or LipoK-stimulation alone was not efficient in stimulating T cells. Similarly, secretion of IFN-γ was also observed from $CD8^+$ T cells but at lower level compared to that from $CD4^+$ T cells. Again there was significantly high production of IFN-γ from $CD8^+$ T cells co-cultured with LipoK stimulated *M. leprae*-infected DCs compared to that from CD40L stimulated *M. leprae*-infected DCs (Fig. 3A). Although the IL-12 p70 production differed in LipoK stimulated *M. leprae*-infected DCs and CD40L stimulated DCs, no IL-12 production was observed from these mitomycin treated DCs which were co-cultured with T cells. In addition, as shown in Fig. 3B, although normal murine IgG did not affect the T cell stimulating activity of both $CD4^+$ and $CD8^+$ T cells, mAbs to HLA-ABC and HLA-DR, inhibited $CD8^+$ T cells and $CD4^+$ T cell activation of LipoK-stimulated *M. leprae*-infected DCs' respectively. The results indicated that the activation of these T cells were MHC Class II- and Class I-dependent in $CD4^+$ T cell and $CD8^+$ T cells respectively. The inhibition was comparable to that of inhibition of IFN-γ production by mAb to co-stimulatory molecule CD86.

Proliferation of these LipoK activated $CD4^+$ and $CD8^+$ T cells, was confirmed by the CFSE labeling of T cells. The labeling experiment was preferable because it could measure proliferation of individual T cell subsets even in the presence of the other subsets. *M. leprae* stimulation of DCs resulted in proliferation of 39.7% of total $CD4^+$ T cells, but stimulation with both LipoK and *M. leprae* resulted in proliferation of 67.5% of total $CD4^+$ T cells. LipoK stimulation alone did not induce any significant proliferation of $CD4^+$ T cells (Fig. 3C). The profiles of flow cytometric analyses showed that 25.3% of $CD8^+$ T cells proliferated by stimulation with *M. leprae* alone, but higher number of cells proliferated (38.9%) in presence of LipoK stimulus.

Subsequently, we examined the response of naïve T cells to LipoK activated DCs. When naïve $CD4^+$ T cells were cultured with DCs activated with *M. leprae* and LipoK, significantly higher dose of IFN-γ was produced in comparison to those cultured with DCs stimulated with *M. leprae* alone or LipoK alone. Production of IFN-γ was low from those activated with *M. leprae* and CD40L (Fig. 3D). It was observed that the IFN-γ production from naïve $CD8^+$ T cells, co-cultured with DCs stimulated with *M. leprae* and LipoK was meager.

When *M. bovis BCG* was used for infecting DCs, the MOI of the bacilli had to be lowered to almost 1~10, because higher MOI (50) would kill the DCs in *in-vitro* culture. BCG when infected at MOI:1 produced 156 pg/ml of IFN-γ from CD8 T cells, but when LipoK was used to stimulate BCG infected DCs, the amount of IFN-γ increased to 380 pg/ml, indicating that LipoK could lead to further T cell activation of BCG infected DCs. It is also likely that LipoK stimulation could increase the production of perforin and granulysin in *M. tuberculosis* infected host cells.

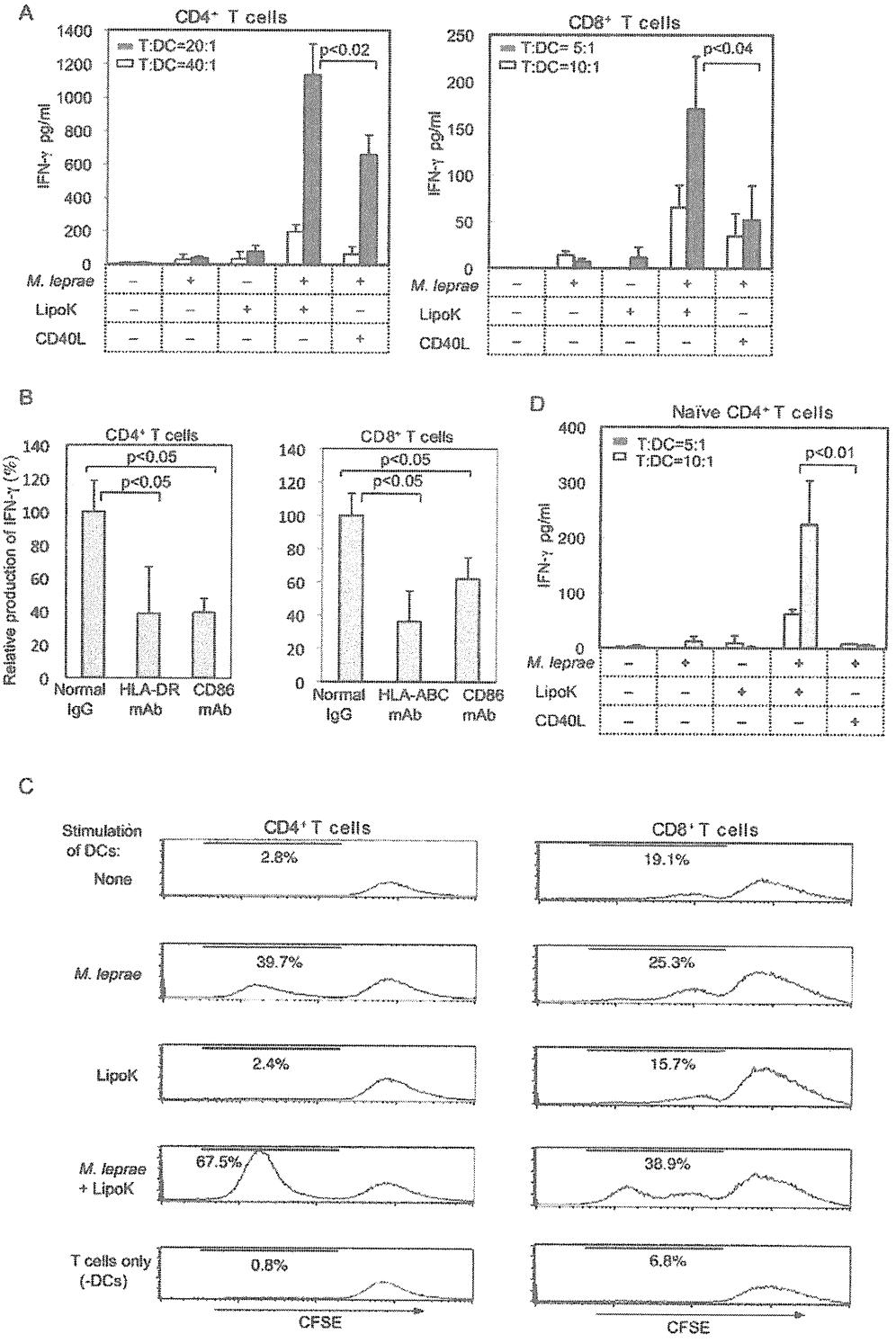

Figure 3. T cell activity as determined IFN-γ production and T cell proliferation. (A) The effect of LipoK on *M. leprae*-infected DCs to stimulate T cells was assessed using an autologous DC-$CD4^+$ or DC-$CD8^+$ T cell co-culture. IFN-γ production in the supernatant was measured by ELISA, after 4 days co-culture. (B) Effect of normal murine IgG or mAb to HLA-ABC/HLA-DR or CD86 on IFN-γ production from T cells co-cultured with *M. leprae* infected DCs simulated with LipoK. The production of IFN-γ from Ab non-treated T cells, cultured with LipoK and *M. leprae* stimulated DCs, is considered 100% and the actual value of IFN-γ produced from $CD4^+$ T cells is 250 pg/ml and that from $CD8^+$ T cells is 47 pg/ml at T cell:DC ratio of 10:1. (C) Proliferation of $CD4^+$ and $CD8^+$ T cells as assessed by CFSE labeling of T cells. DCs were mixed with autologous CFSE labeled T cells at a T cell:DC ratio of 10:1. Proliferating T cells were analysed by FACSCalibur on day 7 after co-culture. The percentage of proliferated cells is indicated. The

lowest histogram shows unstimulated T cells. (D) IFN-γ production from DC-naïve $CD4^+$ T cell co-culture. IFN-γ production was measured after 4 days co-culture with stimulated DCs. Representative data of four separate experiments with different donors is shown. Assays were performed in triplicate and the results are expressed as the mean +SD.
doi:10.1371/journal.pntd.0001401.g003

Up-regulation of perforin, granzyme B and granulysin production in $CD4^+$ and $CD8^+$ T cells

To determine whether cytotoxic effect could be induced in highly activated T cells, we analysed the intracellular production of perforin and granzyme B in DC co-culture system with unseparated T cells. As seen in Fig. 4A, 15.8% of activated $CD8^{high}$ T cells produced perforin and 24.9% produced granzyme B when stimulated with DCs activated with *M. leprae* and LipoK, in comparison to those co-cultured with DCs activated with *M. leprae*, showing 1.4% of perforin and 1.8% of granzyme B-producing T cells. Thus, prominent enhancement of both perforin and granzyme B producing $CD8^+$ T cells was observed. Recently, since $CD4^+$ T cells are also known to possess direct cytotoxic potential [25], we measured the percentage of $CD4^+$ T cells producing perforin and granzyme B. When LipoK and *M. leprae* stimulated DCs were co-cultured with T cells, 12.7% of $CD4^{high}$ T cells produced perforin and 14.6% of those cells produced granzyme B, whereas in presence of *M. leprae* stimulated DCs, 6.6% produced perforin and 8.3% produced granzyme B (Fig. 4B). These data indicated that in addition to $CD8^+$ T cells, $CD4^+$ T cells also had the capacity to produce significant amounts of perforin and granzyme B. Nevertheless, the percentage of $CD8^+$ T cells producing these cytolytic proteins was 1.2~1.7 fold higher than $CD4^+$ T cell. Then, we examined, whether $CD8^+$ T cells alone without the direct contact with $CD4^+$ could have the same capacity. When $CD4^+$ T cells were allowed to culture in inserts, so that there was no direct contact between $CD8^+$ and $CD4^+$ T cells, there was decreased production of both perforin (7.3% v/s 15.8%) and granzyme B (9.5% v/s 24.9%) producing $CD8^+$ T cells (Fig. 4A). So, a direct contact of $CD4^+$ and $CD8^+$ T cells was necessary for sufficient production of cytolytic proteins. When we examined whether exogenous IL-2 could substitute the action of $CD4^+$ T cells, we found that addition of 50 U/ml of IL-2 (excess amount) to $CD8^+$ T cells, could produce both perforin and granzyme B equivalent to that of $CD8^+$ T cells co-cultured with LipoK stimulated, *M. leprae* infected DCs in the presence of $CD4^+$ T cells. However such high levels of IL-2 cannot be produced from host cells, in our experimental setting.

The intracellular level of another cytolytic protein, granulysin, was then examined. Enhancement of granulysin producing $CD8^+$ T cells was observed when co-cultured with DCs activated with *M. leprae* and LipoK. As seen in Fig. 4C, 18.9% of activated $CD8^{high}$ T cells and 28.4% of activated $CD4^{high}$ T cells produced granulysin when co-cultured with DCs activated with *M. leprae* and LipoK, in comparison to those co-cultured with DCs activated with *M. leprae*, (1.7% of $CD8^{high}$ T cells and 0.6% of $CD4^{high}$ T cells).

Mycobacterium leprae components were observed at the periphery of the infected DCs stimulated with LipoK, and co-cultured with T cells

To examine the fate of *M. leprae* in activated DCs, the cells were stained with anti-*M. leprae* membrane polyclonal antibody. Confocal microscopy revealed rod shaped *M. leprae* as observed by auramine-O stain, and membrane components seem to be rather localized in the region where *M. leprae* are present (Fig. 5). Strickingly, those DCs stimulated with LipoK for 48 hours and co-cultured with T cells for additional 3 days showed membrane staining at the periphery of the DCs (Fig. 5 arrowheads shown), probably due to processing of the bacilli in activated DCs.

Killing of *M. leprae* in DCs, by the LipoK stimulation

We determined the viability of *M. leprae* in DCs after stimulation with LipoK in the presence of autologous $CD4^+$ and $CD8^+$ T cells. Since *M. leprae* is uncultivable *in vitro*, the viability of *M. leprae* in DCs, after co-culture with the T cells for a week, was determined by the radiorespirometric assay. The amount of radioactive CO_2 evolved which reflects the rate of ^{14}C-palmitic acid oxidized by *M. leprae*, was measured by the scintillation counter. No significant reduction in $^{14}CO_2$ production was observed, from DCs, not co-cultured with T cells, even in the presence of LipoK stimulation (Fig. 6A). But, when the bacilli were recovered from DCs stimulated with LipoK and co-cultured with T cells, $^{14}CO_2$ production were significantly lower ($p<0.001$) than those recovered from DCs not stimulated with LipoK or T cells. The result indicates that approximately 50% reduction in the viability of *M. leprae* was observed in LipoK activated DCs and co-cultured with T cells compared to those obtained from DCs not stimulated with LipoK (Fig. 6B), indicating that T cells were essential and LipoK stimulation to DCs, was necessary to kill *M. leprae* in DCs. To further determine whether the cytolytic granules namely, granulysin and granzyme B could directly kill *M. leprae*, the bacilli was incubated with human granulysin or granzyme B for a period of 3 days at 33°C. Statistically significant reduction of $^{14}CO_2$ was observed when the bacilli were incubated with granulysin as well as granzyme B (Fig. 6C).

Discussion

In the present study we investigated the role of *M. leprae*-derived synthetic lipopeptide (LipoK), which consists of N-terminal 13 amino acids of the 33-kD *M. leprae* lipoprotein (Accession no. ML0603) linked to Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl group in the induction of intracellular killing of *M. leprae* through immuno-activation. Previously, we observed that the 33-kD lipoprotein and the truncated form of the protein induced the production of IL-12 in human peripheral blood monocytes [4,5]. Although human DCs are potent inducers of acquired immune responses, when DCs were exposed to *M. leprae*, they are inefficient in activating T cells [21,26]. It is generally recognized that, stimulation of T cells by intracellular pathogens, such as mycobacteria, is achieved by the coordinated processing of the antigens in the phago-lysosome of APCs and the expression of the antigenic determinants on APCs. Furthermore, CD40-CD40L interaction on immature DCs, are known to contribute to cell mediated responses in leprosy [27,28]. In fact, when *M. leprae* infected DCs were stimulated with CD40L, up-regulation of CD83 and CD86 molecules was observed (not shown). However, we found that CD40L failed to induce the production of IL-12 p70 in *M. leprae* infected DCs. In contrast to CD40L stimulation, LipoK stimulation on *M. leprae* infected DCs induced significant production of IL-12. Further, the expression of CD40 on DCs was not enhanced by stimulating *M. leprae* infected DCs with LipoK. It was evident that IL-12 inducing ability of these matured DCs was mediated by TLR2, and not by other receptors such as mannose receptor or DC-SIGN, as observed in DCs exposed to *M. tuberculosis* or *M. bovis* BCG [29,30,31]. The TLR2 antagonistic

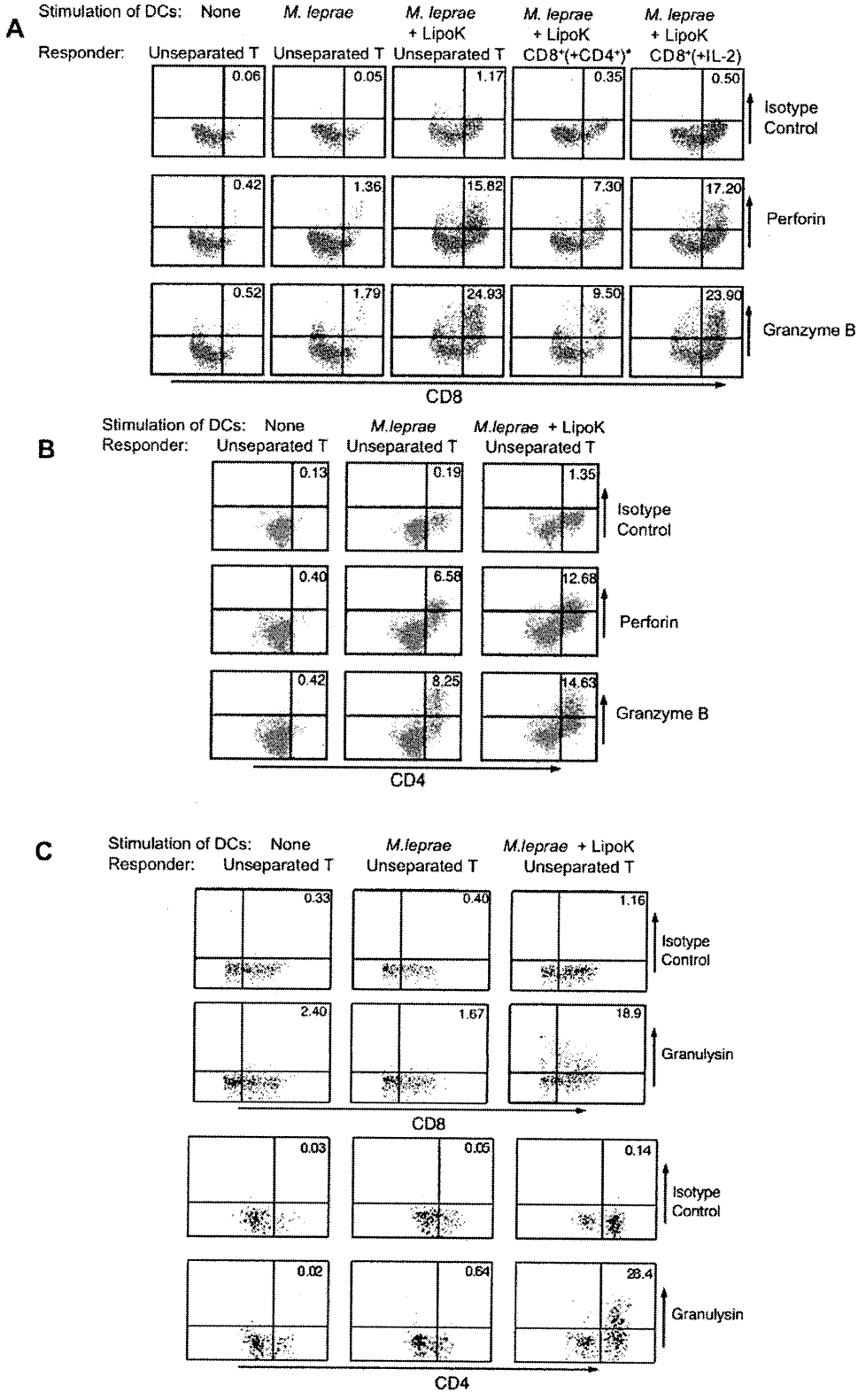

Figure 4. Production of perforin, granzyme B and granulysin in CD8$^+$ T cells as well as CD4$^+$ T cells. **(A)** Enhanced production of perforin and granzyme B from CD8$^+$ T cells cultured with LipoK stimulated *M. leprae* infected DCs. Intracellular staining of perforin and granzyme B was performed as follows: Cells were first stained with PE conjugated anti-CD4 or APC conjugated anti-CD8 mAb. Then, the cells were fixed in 2%