tially clarified by the GPL. We propose that the lipid components in the cell envelope are important for MAIC infection and that the structure modification must be taken into account. These findings shed light on the better understanding of the structure-function relationships of GPLs and may open a new avenue for the prevention of MAIC infections.

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Apoptosis-Inducing Activity of Clofazimine in Macrophages[▽]

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Clofazimine is a riminophenazine compound which has been used for the treatment of leprosy since the 1960s. Although the drug is effective in the management of leprosy reactions because of its anti-inflammatory activity, the mechanism leading to the cessation of inflammation is not well understood. In the present study, it was shown that clofazimine exhibits apoptosis-inducing activity in macrophages. When human monocyte-derived macrophages were cultured *in vitro* in the presence of clofazimine, the cells exhibited a marked decrease in metabolic activity and showed shrinkage in cell size, indicating cell death. Nuclear condensation and fragmentation were also observed by Giemsa and Hoechst 33248 stains. The endonuclease inhibitor $ZnCl_2$ inhibited the clofazimine-induced cell death. Significant enhancement of caspase-3 activity was observed in clofazimine-treated macrophages and THP-1 cells. Collectively, these results suggest the apoptosis-inducing activity of clofazimine in macrophages, which may also be responsible for the antibacterial properties of clofazimine.

Clofazimine (B663) is a phenazine iminoquinone derivative, specifically, a riminophenazine dye with the empirical formula $C_{27}H_{22}C_{12}$. In the 1950s, Barry et al. synthesized a large number of compounds by progressive chemical alteration of the anilinoaposafranine molecule, several of which showed antituberculous activity both *in vitro* and in experimental animals (1). Of these compounds, clofazimine (or Lamprene or B663) was found to be highly active against mycobacteria with the least toxicity. Chang (4) observed the antibacterial activity of clofazimine against *Mycobacterium lepraemurium* at about the same time as its anti-*M. leprae* activity was reported by Browne (2) and Browne and Hogenzeil (3). Later, after the introduction of the mouse footpad method of Shepard and Chang (22), its antibacterial activity against *M. leprae* was demonstrated (18).

Clofazimine has bifunctional activity: antibacterial and anti-inflammatory. It was used in the treatment of leprosy for its antibacterial action against *M. leprae*. Later, it was also found to possess an anti-inflammatory action which makes it a very useful drug in the treatment of acute reactions, including erythema nodosum leprosum (ENL), neuritis, iritis, etc., although its mechanism of action is unknown (2). *In vitro* studies on the effect of clofazimine on immune cells have been conducted. Clofazimine increases superoxide anion production and degranulation by stimulated neutrophils. and tumor necrosis factor alpha (TNF-α) potentiates this enhancement (15). The mechanism underlying this pro-oxidative effect seems to involve stimulation of phospholipase A2 (PLA2) activity with subsequent accumulation of arachidonic acid and lysophospholipids, which act as second messengers to activate oxidase (10). In addition, a number of reports have demonstrated the effects of clofazimine that might predict increased immune activity. Lysosomal enzyme activity of cultured macrophages was upregulated by clofazimine (21). Peripheral blood monocytes from healthy volunteers have been demonstrated to exhibit increased major histocompatibility complex class II expression following incubation with clofazimine (25). Increased oxygen uptake during phagocytosis was observed in neutrophils derived from patients with pyoderma gangrenosum during clofazimine therapy (5). Suppressor T-cell activity was decreased in mycobacteria-infected mice during clofazimine treatment (26). However, the mechanisms underlying the anti-inflammatory action of clofazimine are still unclear.

In the present study, we examined the effect of clofazimine on macrophages and found that the drug possessed apoptosis-inducing activity.

MATERIALS AND METHODS

Drug and chemicals. Clofazimine (Sigma-Aldrich Co., St. Louis, MO), rifampin (catalog no. R3501; Sigma-Aldrich Co.), and dapsone (DDS; Biomol Research Inc., Butler Pike Plymouth Meeting, PA) were dissolved in dimethyl sulfoxide (DMSO) and stored at −30°C until use. Ampicillin was obtained from Sigma-Aldrich Co.

Culture of human macrophages and isolation of bacilli. Human peripheral blood was obtained under informed consent from healthy individuals. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) gradient centrifugation (12). The cells were suspended in AIM-V medium (Gibco BRL, Invitrogen Corp., Carlsbad, CA), and 1×10^6 PBMCs were cultured in a well of a 24-well tissue culture plate (Falcon; Becton Dickinson Labware, Becton Dickinson and Company, Franklin Lakes, NJ) containing 13-mm round coverslips (Nunc Thermanox coverslips; Nalge Nunc, Thermo Scientific, Rochester, NY) at 37°C in a 5% CO_2 incubator for adherence of monocytes. After 1 h incubation, the coverslips were washed with Hanks' balanced salt solution (HBSS; Sigma-Aldrich Co.) to remove nonadherent cells. The monocytes on the coverslips were cultured in a new 24-well plate containing RPMI 1640 medium (Sigma-Aldrich Co.) supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS; Bio Whittaker Co., Walkersville, MD), 2 mM L-glutamine, and 100 μg/ml ampicillin (RPMI-10F) in the presence of 40 ng/ml of granulocyte-macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN). After 10 days, the cells were differentiated into macrophages and used for experiments. In some experiments, PBMCs were cultured in 35-mm cell culture dishes (Corning Inc., Corning, NY) for adherence, and adherent monocytes were cultured for 10 days. Human monocytic leukemia cell line THP-1 was maintained in RPMI 1640 medium containing 15% fetal bovine serum.

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M. leprae (Thai-53 strain) was isolated from the footpads of BALB/c *nu/nu* mice that had been inoculated with *M. leprae* 8 months prior to isolation, and the bacillary number was enumerated according to the method of Shepard and Chang (22).

Light and phase-contrast microscopy. Macrophages on the coverslip were fixed with absolute methanol, followed by performing Giemsa stain (Wako Co., Japan). After they were mounted on a glass slide, the cells were observed under a light microscope (Optiphot-2; Nikon Co., Tokyo, Japan). Photographs were taken with a digital camera (Nikon F70s). Macrophages in 35-mm dishes were incubated in the presence of clofazimine and observed under a phase-contrast microscope (Olympus CKX41 with ×10- and ×20-objective lenses). Photographs were taken with an Olympus DP50 system. Image acquisition and data processing were done using the DP controller software.

Fluorescence microscopy. Fluorescence staining for DNA was employed. Macrophages were cultured in an 8-well chamber slide (Lab-Tek II chamber slide system; Nalge Nunc). The cells were incubated in the presence of clofazimine and subsequently fixed with 2.5% glutaraldehyde in phosphate-buffered saline (PBS). Hoechst 33342 dye (Sigma-Aldrich Co.) in PBS was added to the wells at a final concentration of 10 μM, and the slide was incubated for 1 h at 37°C. The cells on the slide were observed under a fluorescence microscope (Olympus BX60 with a ×40-objective lens) equipped with an Olympus DP50 system. The digital images were processed with DP controller software.

Determination of cell death. Cell viability was determined using the colorimetric method (Cell Titer 96 aqueous nonradioactive cell proliferation assay; Promega Corp., Madison, WI). Briefly, cells in a 24-well plate were incubated in the presence of clofazimine in phenol red-free RPMI 1640 medium containing 10% FBS, followed by addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt/phenazine methosulfate solution (formazan reagent). After 1 h incubation at 37°C, an aliquot of medium was transferred into a well of a 96-well plate, and the developed color was measured by a microplate reader at 490 nm. In addition, the activity of lactate dehydrogenase (LDH), released from dead cells into culture supernatants, was measured by a colorimetric assay (Cytotox 96 nonradioactive cytotoxicity assay; Promega Corp.). The color that developed in a sample incubated with LDH substrate was measured by a microplate reader at 490 nm (Vmax; Molecular Devices Corp., Sunnyvale, CA).

DNA electrophoresis. THP-1 cells or macrophages were harvested from the culture, and DNA was purified by a spin column method (E.Z.N.A. tissue DNA kits; Omega Bio-Tek, Norcross, GA). Briefly, 5×10^6 cells incubated in the presence of clofazimine were harvested, centrifuged at 2,000 rpm for 5 min, washed once with PBS, and resuspended in PBS. Protease was added, the mixture was heated at 65°C for 5 min, and buffer BL was added. After the mixture was heated at 70°C for 10 min, ethanol was further added. The mixture was applied to a HiBind spin column and centrifuged. DNA bound to the column was finally eluted, and the DNA preparation was subjected to electrophoresis in a 1% agarose gel, followed by ethidium bromide staining, and DNA was visualized by UV transillumination.

Western blotting. THP-1 cells or macrophages incubated with clofazimine were washed once with PBS(−) and lysed in lysis buffer (CelLytic-M; Sigma-Aldrich Co.) containing 2 protease inhibitor cocktails (phosphatase inhibitor cocktail 1 and phosphatase inhibitor cocktail 2; Sigma-Aldrich Co.). In the case of clofazimine-treated adherent macrophages, the cells were scraped off the dishes with a rubber policeman. The lysates were incubated for 10 min on ice and centrifuged at 13,000 rpm for 5 min. The protein concentration was determined. Ten micrograms of total protein was loaded onto an SDS-PAGE gel. After running the electrophoresis, the proteins in the gel were transferred onto an Immobilon PSQ membrane (Millipore Corporation, Billerica, MA). After washing with Tris-buffered saline (2.42 g Tris base and 8 g NaCl per 1 liter, pH 7.6) containing 0.05% Tween 20 (TBS-T), the membrane was blocked with 5% skim milk (Amersham ECL Plus Western blotting reagent pack; GE Healthcare Life Sciences, Amersham Place, Buckinghamshire, United Kingdom) for 1 h at room temperature. The membrane was washed 3 times with TBS-T and incubated overnight with 1:3,000-diluted primary anticleaved caspase-3 antibody (cleaved caspase antibody sampler kit; Cell Signaling Technology Inc., Danvers, MA). The membrane was then incubated with 1:10,000-diluted horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. Finally, proteins were detected by incubating the membrane with HRP substrate (Immobilon Western chemiluminescent HRP substrate; Millipore Corporation), and the membrane was exposed to X-ray film (Amersham Hyperfilm ECL; GE Healthcare). For reprobing of the membrane, the membrane was washed with TBS-T and incubated with stripping buffer (Restore Plus Western blot stripping buffer; Pierce, IL). After the membrane was blocked, it was used again for probing different antibodies, such as cleaved caspase-9 and poly(ADP-ribose) polymerase (PARP) antibodies (cleaved caspase antibody sampler kit; Cell Signaling Technology Inc.) and beta-actin antibody (Cell Signaling Technology Inc.).

Colorimetric caspase assay. Colorimetric substrates for caspases were used to determine caspase-3 activity (colorimetric caspase assay kits; Biovision Research Products, CA) in lysates of cells incubated in the presence of clofazimine. Briefly, 5×10^6 cells were pelleted and lysed with chilled lysis buffer. After centrifugation, the supernatant was transferred to a new tube, and reaction buffer and a substrate for caspase-3, Asp-Glu-Val-Asp-*p*-nitroanilide, were added to the tube. After incubation for 2 h at 37°C, the samples were transferred into a well of a 384-well plate and read by a plate reader at 405 nm (Infinite F200; Tecan Systems Inc., San Jose, CA). The background reading was obtained by subtracting the reading for the reaction buffer from the reading for the lysate samples.

PGE_2 assay. The amount of prostaglandin E_2 (PGE_2) in the culture supernatants was measured by enzyme-linked immunosorbent assay (catalog no. 514010; Cayman Chemical Co., MI).

RESULTS

Morphological changes observed after treatment with clofazimine. Macrophages differentiated from human monocytes were incubated in the presence of 10 μg/ml of clofazimine for 20 h. The change in cell morphology was observed under a phase-contrast microscope. As shown in Fig. 1B, in the presence of clofazimine, the cells exhibited shrinkage in cell size and membrane blebbing. The death of more than 80% of cells was observed (Fig. 1B). As a control, Fig. 1A shows the normal morphology of macrophages. By Giemsa stain, too, these clofazimine-treated cells exhibited shrinkage in cell size, accompanied by the appearance of fragmented smaller nuclei (arrow in Fig. 1D), suggesting the apoptotic nature of the cells. Nontreated macrophages showed intact nuclei (arrow in Fig. 1C). Again, the change of nuclear structure was confirmed by Hoechst dye staining. Under a fluorescence microscope, nuclear condensation and membrane blebbing were observed in the clofazimine-treated cells (Fig. 1F and G), in contrast to normal cells, which showed intact nuclei (Fig. 1E). Similar fragmentation or condensation of chromatin was observed in THP-1 cells (data not shown). Such morphological changes were not observed in THP-1 cells treated with rifampin or dapsone at a concentration up to 50 μg/ml. Also, DMSO, which was used as a solvent for clofazimine at a concentration of 0.2%, had no effect on cell morphology or cell functions (negative control).

Cell death-inducing activity of clofazimine determined by colorimetric assay. Cell death was determined by a biochemical analysis using a colorimetric method. The conversion of the tetrazolium compound into soluble formazan is accomplished by metabolically active cells. When higher concentrations up to 10 μg/ml of clofazimine were employed in macrophage cultures, decreased color intensity of soluble formazan was observed, indicating cell death (Fig. 2A). Cell death was also observed in THP-1 cells (Fig. 2B). Hansen's disease is caused by infection of macrophages with *M. leprae*; therefore, we are curious to know whether *M. leprae* infection affects the cell death-inducing activity of clofazimine. When we infected macrophages with *M. leprae* at a multiplicity of infection (MOI) of 10 or 30, we found no significant difference in the induction of cell death in the presence of 10 μg/ml clofazimine, indicating that the bacilli did not inhibit or enhance clofazimine-induced cell death (Fig. 2C). Another method of determining cell death is by measurement of LDH release from

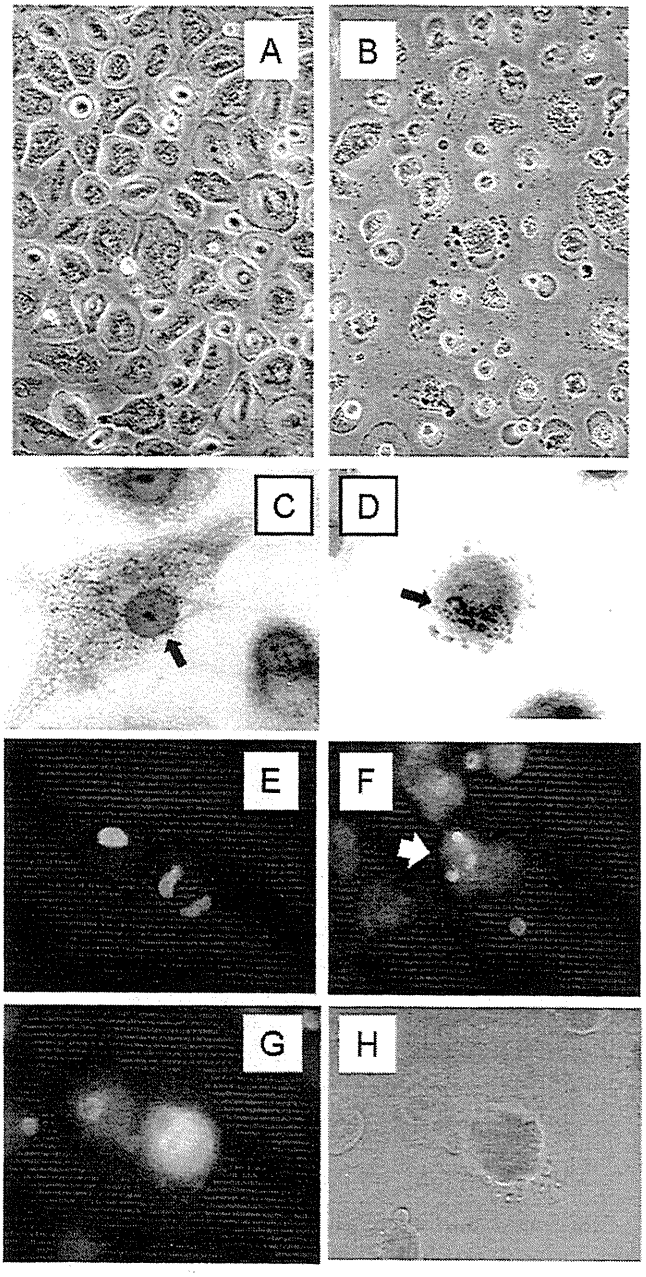

FIG. 1. Cell death induced in macrophages by clofazimine. Photographs were taken under a phase-contrast microscope (A and B) with a ×20-objective lens. (B) Human monocyte-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine for 20 h. (A) Cells cultured in the absence of clofazimine showed normal morphology. Giemsa stain of clofazimine-treated macrophages was also performed (C and D). Human monocyte-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine (D) or in the absence of clofazimine (C) for 24 h. Photographs were taken under a light microscope with a ×100-objective lens. Fragmentation of the nucleus was significant in the clofazimine-treated cells (arrow in panel D), in contrast to the intact morphology of the nucleus in normal cells (arrow in panel C). Nuclear condensation and fragmentation of clofazimine-treated macrophages were also confirmed under a fluorescence microscope (E to G). Macrophages were incubated in the presence of 10 μg/ml of clofazimine, followed by fixation and stained with a nucleus-staining dye, Hoechst 33342. The cells were observed under a fluorescence microscope (×40-objective lens). Cells cultured without clofazimine (E), clofazimine-treated cells (F and G), and a phase-contrast image of panel G (H) are shown.

dead cells. As shown in Fig. 3, more LDH release was observed in the manner dependent on the concentration of clofazimine.

Clofazimine treatment induces DNA ladder formation in macrophages. We examined the condition of DNA in clofazimine-treated THP-1 cells. Agarose gel electrophoresis showed fragmentation of DNA into integer multiples of 180 bp, a so-called DNA ladder (Fig. 4A), suggesting that DNA endonuclease was activated by clofazimine treatment. Therefore, we examined the effect of one of the apoptosis inhibitors, $ZnCl_2$, which is known to possess suppressing activity for endonuclease, and found that clofazimine-induced DNA fragmentation in THP-1 cells was completely blocked by $ZnCl_2$ treatment even at a low concentration of 0.25 mM $ZnCl_2$ (Fig. 4B), although it is still not clear whether $ZnCl_2$ can directly block the activity of clofazimine. Moreover, it was evident that neither cell death nor DNA fragmentation was induced by other antileprosy drugs, such as DDS or rifampin (Fig. 4C).

Clofazimine-induced cell death is mediated by activation of caspase-3. Caspases are known to be central regulators of apoptotic cell death, and caspase-3, which locates downstream of the caspase pathway, is one of the key executioners of apoptosis. Upon apoptotic stimulation, caspases are cleaved into active fragments. Figure 5 shows a Western blot analysis of extracts from THP-1 cells and macrophages cultured in the presence of clofazimine. Enhanced expression of cleaved caspase-3 was detected in cells (Fig. 5A and B). In addition, caspase-9 was also cleaved. A DNA-repairing enzyme, PARP, which is cleaved by caspase-3, was significantly activated in clofazimine-treated THP-1 cells (Fig. 5A). We next measured the caspase activity by colorimetric assay (Fig. 5C). The induction of caspase-3 by clofazimine was significantly high in macrophages as well as THP-1 cells.

Clofazimine enhanced PGE_2 production in *M. leprae*-infected macrophages. Monocyte-derived macrophages were preincubated in the presence of clofazimine for 4 h, followed by replenishment with *M. leprae*-containing medium for 20 h. The culture supernatants were collected, and the PGE_2 concentration was measured. As shown in Fig. 6, clofazimine clearly enhanced PGE_2 production in macrophages.

DISCUSSION

Riminophenazines are structurally phenazine compounds which were derived from lichens historically and were targeted for treatment of tuberculosis. The first clinically developed phenazine compound was clofazimine, whose activity has been extended to other mycobacterial diseases (1, 17). In test animals, the drug was found to inhibit the growth of mycobacteria *in vivo*, as well as *in vitro* (22), but the molecular mechanism of clofazimine in inducing anti-*M. leprae* activity is still not yet clear.

In the present study, it was found that both human monocyte-derived macrophages and THP-1 cells exhibited marked decreases in their metabolic activity in the presence of 10 μg/ml clofazimine. Under a phase-contrast microscope, 80% of the cells showed irregular morphology with shrinkage in cell size, and by a precise time course study, it was revealed that the morphological changes were evident from 6 h incubation with clofazimine. From this early time point, the cell body began to shrink, accompanied by membrane blebbing, which was also

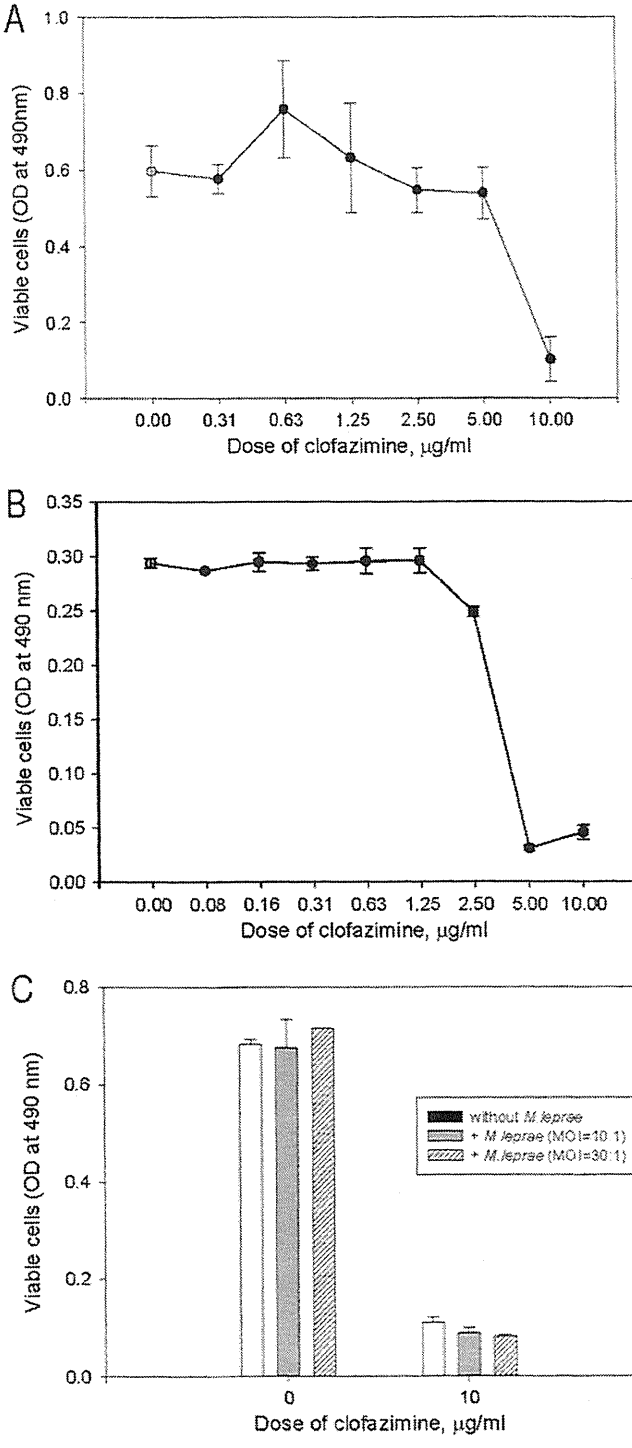

FIG. 2. Clofazimine-induced cell death in macrophages and THP-1 cells. Human monocyte-derived macrophages (A) and THP-1 cells (B) were incubated with various concentrations of clofazimine for 24 h, followed by determination of viable cells by the Cell Titer 96 cell proliferation assay. The results are representative of three independent cell culture tests. The cell death-inducing effect of clofazimine in the presence of *M. leprae* was also examined. Monocyte-derived macrophages were infected with *M. leprae* at an MOI of 10 or 30 per cell for 24 h. The infected cells were further incubated with 10 µg/ml clofazimine for another 24 h, followed by determination of viable cells by Cell Titer 96 cell proliferation assay (C). The results are representative of three independent cell culture tests. OD, optical density.

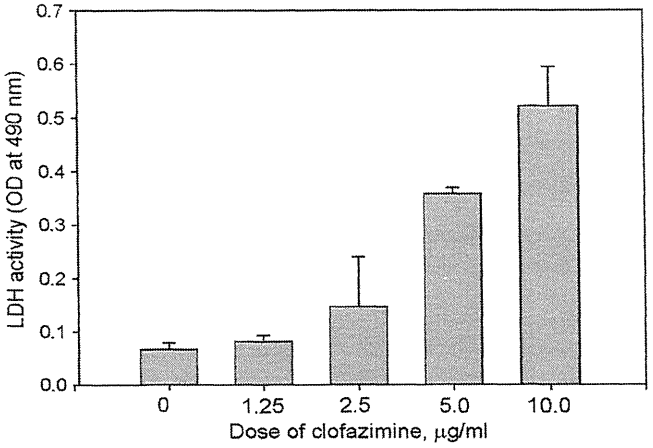

FIG. 3. LDH release from clofazimine-treated macrophages. Human monocyte-derived macrophages were incubated in the presence of the indicated concentrations of clofazimine for 24 h, and the LDH activity was measured. The results were obtained from triplicate cultures and are representative of three independent cell culture tests. OD, optical density.

evident from Giemsa stain and Hoechst staining of the nuclei (Fig. 1). Interestingly, the dose of clofazimine (10 µg/ml) required to cause cell death was equivalent to the dose required to exhibit anti-*M. leprae* activity *in vitro* by radiorespirometry (data not shown), the dose of which is in concordance with the dose required to kill *M. leprae* reported by Franzblau and O'Sullivan (7). Moreover, in our study, at 5-µg/ml concentrations of clofazimine, *M. leprae* viability was lowered in *in vitro* experiments with *M. leprae*-infected macrophages, and with this dose, *M. leprae* was found not to inhibit clofazimine-induced cell death. Therefore, clofazimine might inhibit mycobacterial growth through an alternative way by inducing apoptosis of host cells. Although the concentration of clofazimine in sera of patients taking regular doses of the drug is as low as 1 to 2 µg/ml, fat-soluble clofazimine readily accumulates in cells. In one patient, 7 months treatment with clofazimine (200 mg/day) resulted in accumulation of needle-shaped crystal inclusions in his alveolar macrophages (20). In another report, clofazimine-induced crystal-storing histiocytosis was observed in a leprosy patient (23). So, we are of the opinion that in some cells, the concentration of clofazimine is higher (10 to 20 µg/ml) than in others, so we have used a concentration of 10 µg/ml for our experiments.

Normally, cells undergo distinct morphological changes when they progress through either necrosis or apoptosis. Necrosis occurs when cells are exposed to an extreme variance from physiological conditions, resulting in damage to the plasma membrane. As such, necrosis is characterized by cell swelling and disruption of cellular organelles, with little change in the chromatin initially. In contrast, apoptotic cells shrink in size, undergo membrane blebbing, and exhibit marked alterations in their chromatin structure at an early stage under normal physiological conditions. As mentioned earlier, treatment with clofazimine resulted in highly condensed chromatin within the nucleus and membrane blebbing, indicating macrophages undergoing apoptosis. To confirm this, DNA from clofazimine-treated THP-1 cells was examined. Fragmented DNA was

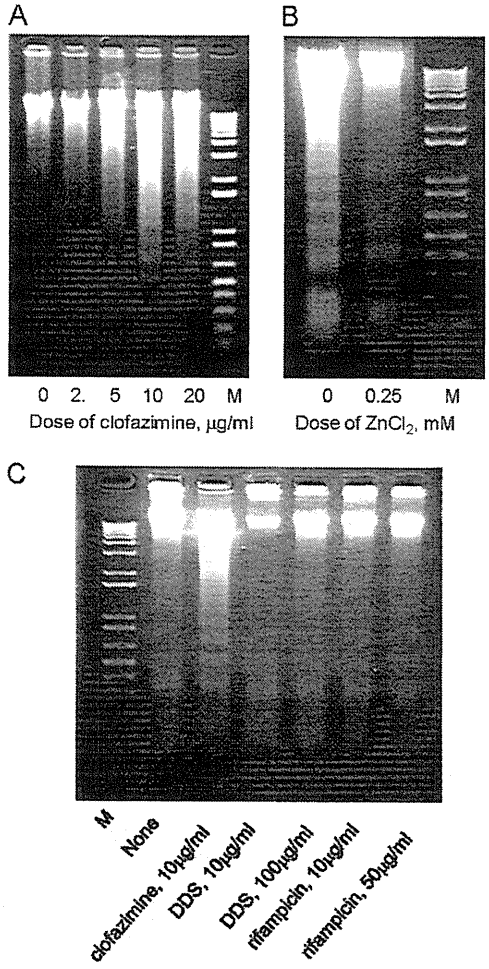

FIG. 4. DNA ladder formation in clofazimine-treated THP-1 cells and effects of other antileprosy drugs on DNA ladder formation. (A) THP-1 cells were incubated in the presence of the indicated concentrations of clofazimine for 4 h, followed by purification of DNA for agarose gel electrophoresis. An ethidium bromide-stained agarose gel is shown. (B) An endonuclease inhibitor, $ZnCl_2$, was examined for its effect on clofazimine-induced ladder formation. THP-1 cells were incubated in the presence of 10 μg/ml clofazimine and $ZnCl_2$ for 4 h. DNA was purified for electrophoresis. (C) THP-1 cells were incubated in the presence of clofazimine, DDS, and rifampin for 4 h, followed by purification of DNA. An ethidium bromide-stained agarose gel is shown. Lanes M, molecular weight marker.

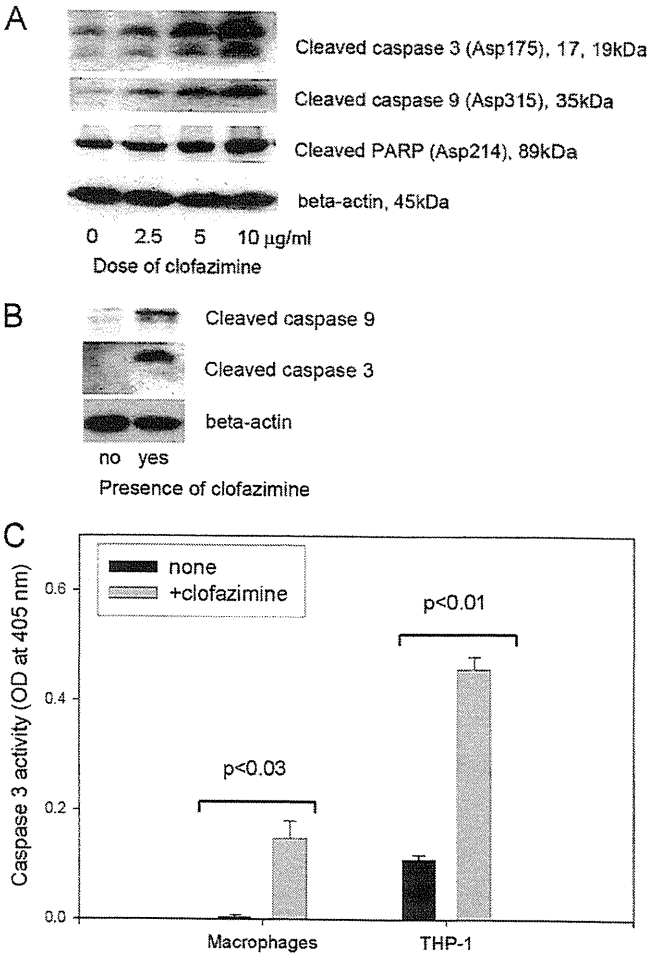

FIG. 5. Expression of caspase in clofazimine-treated THP-1 cells and macrophages. THP-1 cells were incubated in the presence of the indicated concentrations of clofazimine for 6 h, and cell lysates were processed for detection of cleaved caspase-3, caspase-9, and PARP by Western blotting (A). Similarly, monocyte-derived macrophages were incubated in the presence of 10 μg/ml of clofazimine, and the cell lysates were examined for cleaved caspase-3 and caspase-9 expression (B). The caspase activity in clofazimine-treated macrophages and THP-1 cells was analyzed. Macrophages were incubated in the presence of 10 μg/ml of clofazimine for 6 h, and the caspase-3 activity in the cell lysates was determined by colorimetric assay (C). The results are representative of three independent cell culture tests.

demonstrated, suggesting that DNA endonuclease was activated causing apoptosis.

We observed that *M. leprae* by itself does not induce apoptosis of human cells. Similarly, infection of mouse macrophages with viable *M. leprae* was shown not to induce apoptosis (11). Although apoptosis is induced when macrophages infected with *M. leprae* are treated with clofazimine, the host cell viability does not change significantly in the presence of *M. leprae*. Nevertheless, the viability of *M. leprae* in macrophages was significantly lower in clofazimine-treated cells than infected cells not treated with clofazimine (data not shown). Therefore, we can speculate that clofazimine induces apoptosis of *M. leprae*-infected macrophages, which in turn inhibits *M. leprae* growth.

Consequently, we investigated the pathways involved in the execution of macrophage apoptosis (6, 14). We observed enhanced expression of cleaved caspase-3, caspase-9, and PARP following clofazimine treatment in THP-1 cells (Fig. 5A). Colorimetric assay also indicated enhanced caspase-3 activity in both macrophages and THP-1 cells treated with clofazimine (Fig. 5C), suggesting the involvement of caspases in clofazimine-induced apoptosis.

Apoptosis has been shown to be effective in therapy of chronic inflammatory diseases (16). An immunomodulatory drug, thalidomide, is used for treatment of ENL in leprosy patients, and its anti-inflammatory activity is believed to be through the downregulation of production of the proinflammatory cytokine TNF-α (19). Gockel et al. showed that thalidomide induces apoptosis in human monocytes (8). Clofazimine

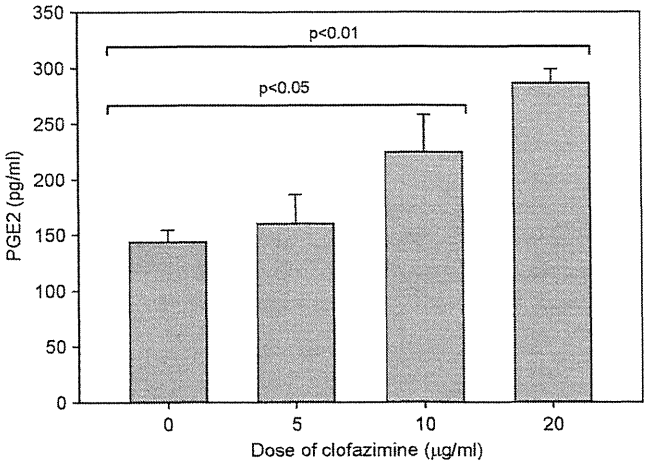

FIG. 6. Enhancement of PGE_2 production by clofazimine in *M. leprae*-infected macrophages. Macrophages were preincubated in the presence of the indicated doses of clofazimine for 4 h, followed by infection with *M. leprae* (5×10^6/well), and the culture was continued for another 20 h. The amount of PGE_2 in the culture supernatants was measured. The results are representative of three independent cell culture tests.

is known to have a favorable influence on the reversal reaction in human leprosy (2). Browne and Hogenzeil found that clofazimine controlled persistent exacerbations in patients who were corticosteroid dependent for controlling the passing reactions, including ENL, and suggested that the drug may exert a suppressive effect on the development of acute exacerbation in lepromatous leprosy (3). These observations have been followed by those of later workers, and one of the special indications for use of clofazimine is the presence of acute reactions or a chronic recurrent reaction in lepromatous leprosy. These clinical data suggest that the mechanisms underlying the action of the drug in these leprosy patients mainly seem to be anti-inflammatory, although there is no direct evidence. Macrophages are capable of elaborating a series of biochemical products with potent immunomodulatory activities. We have observed enhancement of the production of PGE_2, when macrophages were pretreated with clofazimine. PGE_2 is released from arachidonic acid by PLA2. The enzyme is reported to be stimulated in clofazimine-treated neutrophils (10). With respect to B-cell function, prostaglandins of the E series (PGE) inhibit both B-cell proliferation and the generation of antibody-forming cells, and also, B-cell tolerance is induced by PGE_2 (9, 24). T-cell proliferation is also suppressed by PGE_2 (13). The reaction to leprosy involves antibody (ENL caused by immune complex) and cells (delayed-type hypersensitivity mediated mainly by T cells). The mechanisms underlying the immunomodulatory role of clofazimine are still not clear, but the present study clarifies certain aspects. Apoptosis induced in macrophages might explain the anti-inflammatory activities of clofazimine *in vivo*.

In conclusion, our findings suggest that clofazimine induced apoptosis of macrophages through the activation of caspases. The data indicate that the action of clofazimine in leprosy patients may be at least partially mediated by apoptosis.

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

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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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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

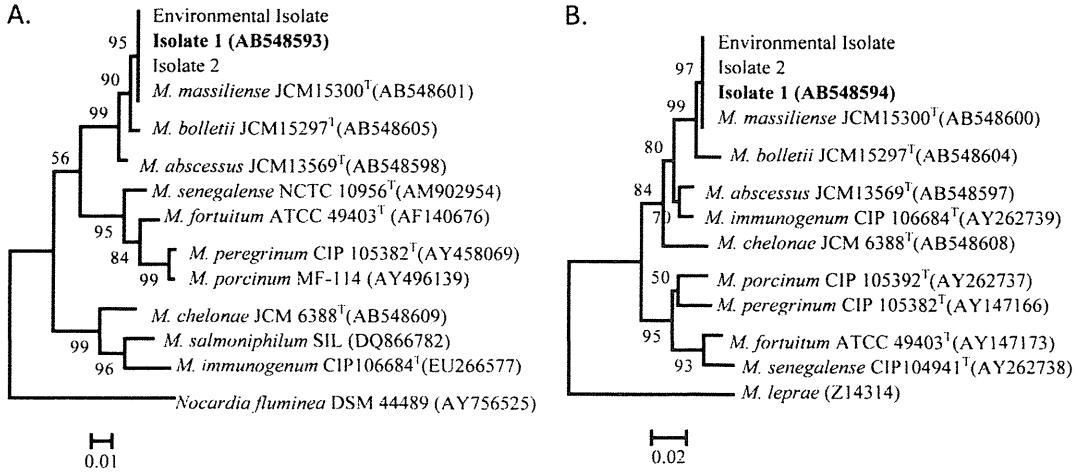

FIG. 1. Phylogenetic analysis based on the *hsp65* (A) and *rpoB* (B) genes of isolate 1 (boldface) and other rapidly growing mycobacteria. The numbers at the nodes are the percentages of bootstrap levels supported by 1,000 resampled data sets. Bootstrap values of <50% are not shown. *Nocardia fluminea*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

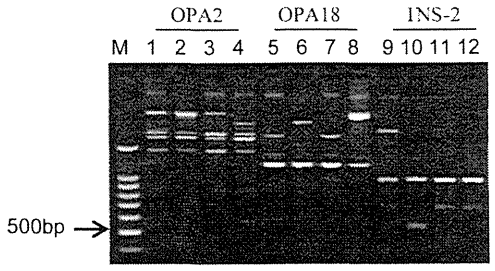

FIG. 2. Comparison of the RAPD-PCR patterns of the clinical isolates (isolates 1 and 2), the environmental isolate, and a reference strain (*M. massiliense* JCM 15300^T) with three different primers. Lanes 1, 5, and 9, DNA from isolate 1; lanes 2, 6, and 10, DNA from isolate 2; lanes 3, 7, and 11, DNA 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.

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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.

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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

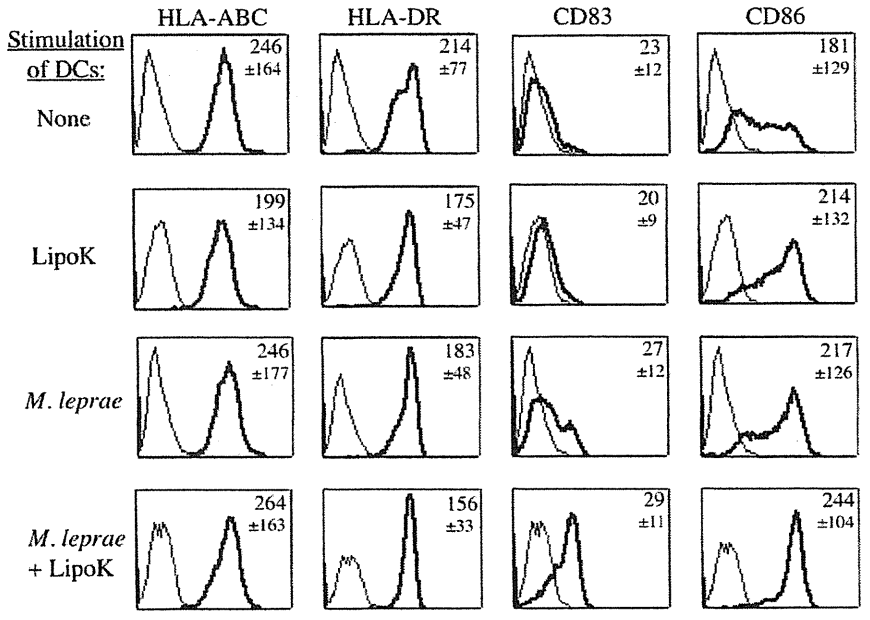

Figure 1. Expression of the surface markers on DCs after stimulation of *M. leprae* infected DCs with LipoK. The expression of cell surface markers on DCs, was analyzed using FACSCalibur. Dead cells were eliminated from the analysis by staining with 7-amino actinomycin D (7-AAD) stain. LipoK was used 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.
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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.

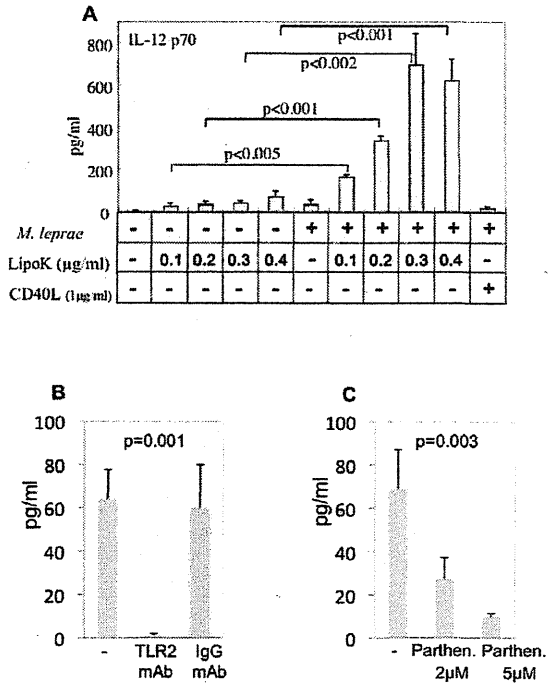

Figure 2. Production of IL-12 p70 from DCs. (**A**) Enhanced induction of IL-12 p70 from DCs by stimulation with LipoK and *M. leprae*. DCs were stimulated with the antigens on day 4 after the start of culture from monocytes. After 24 hours, IL-12p70 concentration in the culture supernatant was measured 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.

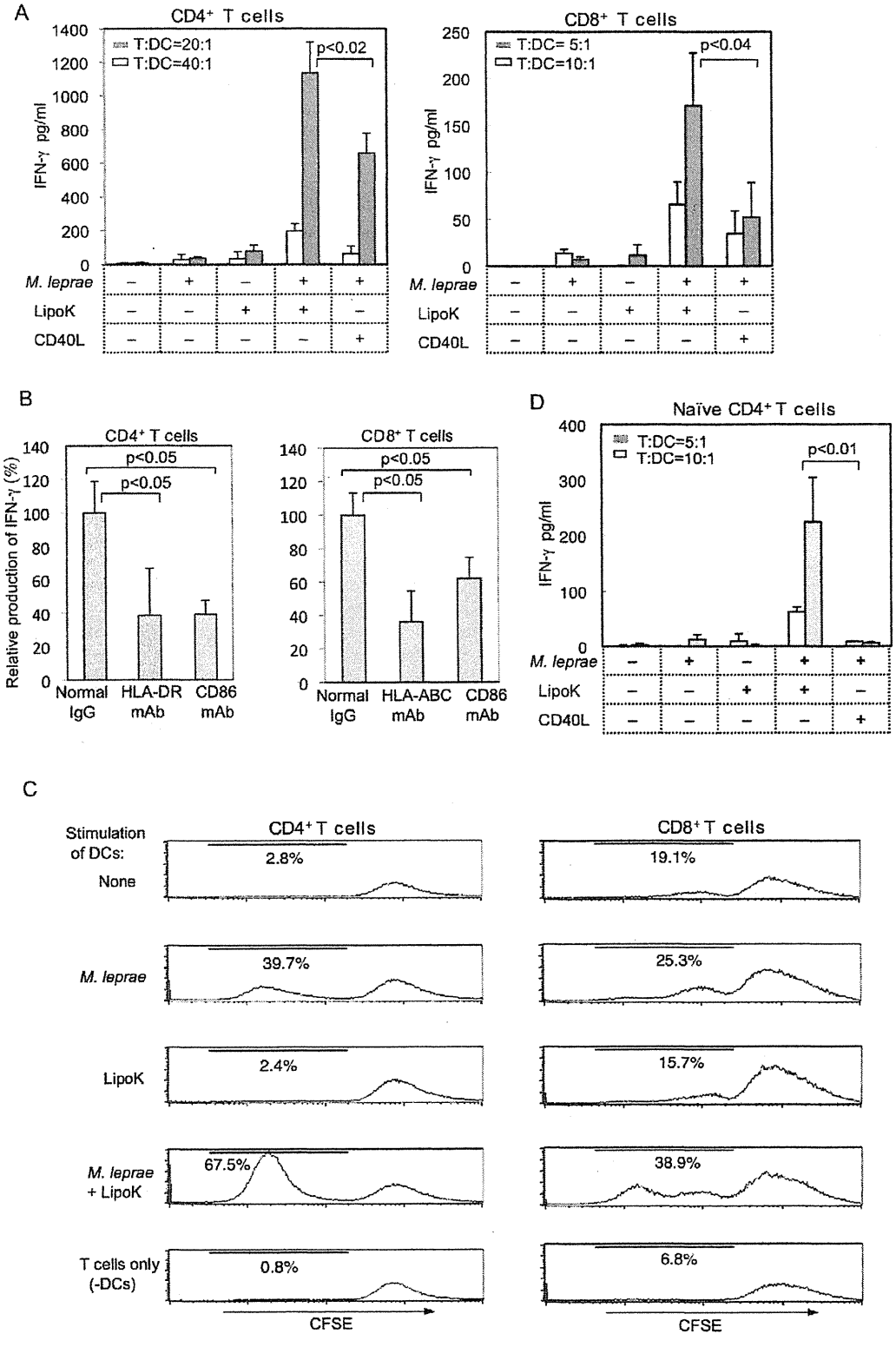

Figure 3. T cell activity as determined IFN-γ production and T cell proliferation. (**A**) The effect of LipoK on *M. leprae*-infected DCs to stimulate T cells was assessed using an autologous DC-CD4$^+$ or DC-CD8$^+$ T cell co-culture. IFN-γ production in the supernatant was measured by ELISA, after 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.
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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

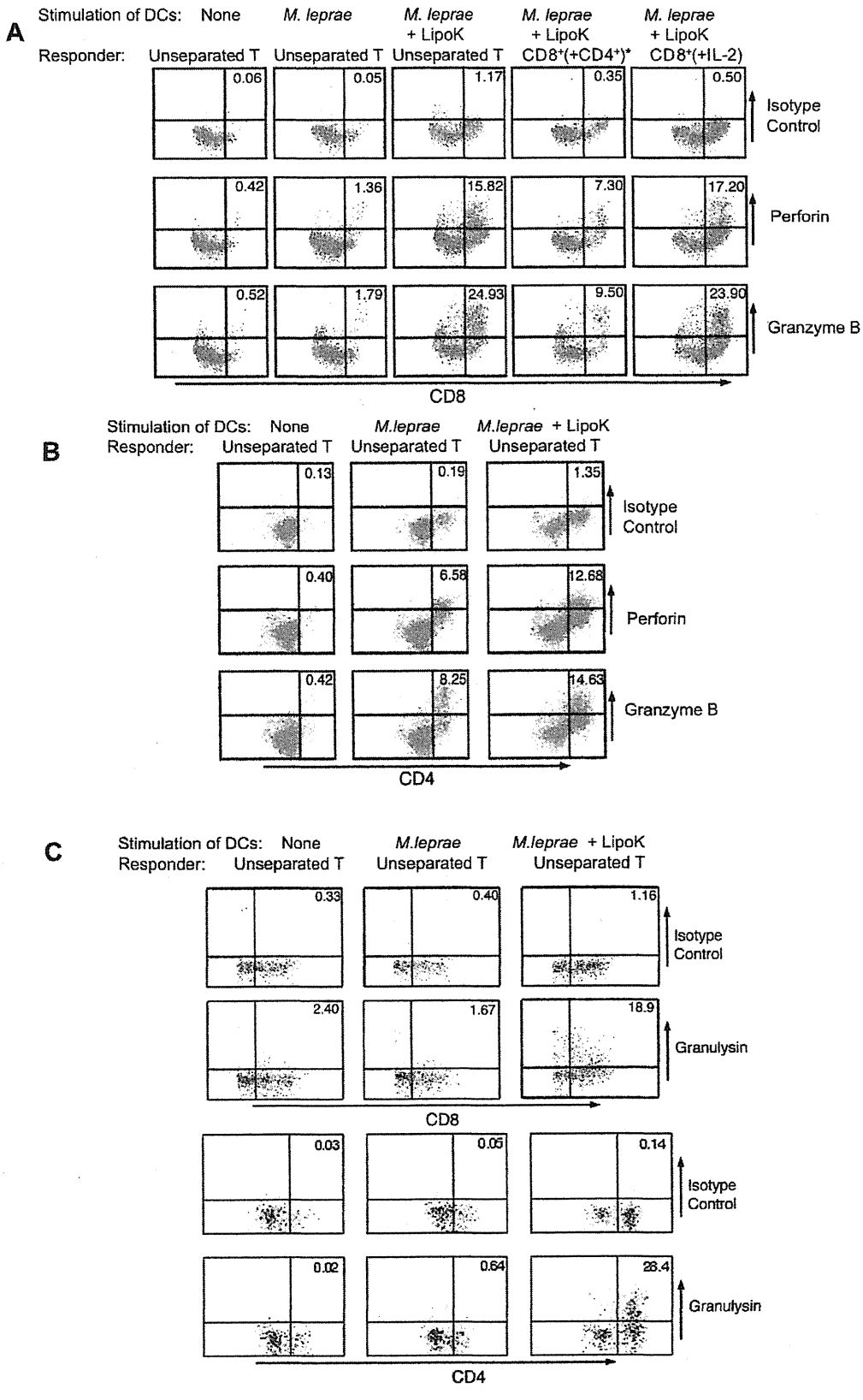

Figure 4. Production of perforin, granzyme B and granulysin in CD8$^+$ T cells as well as CD4$^+$ T cells. (**A**) Enhanced production of perforin and granzyme B from CD8$^+$ T cells cultured with LipoK stimulated *M. leprae* infected DCs. Intracellular staining of perforin and granzyme B was performed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

formaldehyde, permeabilized in 0.1% saponin, and stained with FITC conjugated anti-perforin mAb or anti-granzyme B mAb or isotype control IgG2a. Figure shows the dot plot of the gated $CD8^+$ T cells. The right hand quadrant shows $CD8^{high}$ T cells (activated $CD8^+$ T cells) and the number indicates the percentage of perforin or granzyme B positive T cells among gated $CD8^{high}$ T cells. *To determine whether direct interaction between $CD4^+$ and $CD8^+$ T cells for perforin and granzyme B production from $CD8^+$ T cells, is needed, $CD4^+$ T cells were cultured in inserts in a 24-well plate, and were not allowed to interact directly with $CD8^+$ T cells. As a control experiment, exogenous IL-2 (in the left hand dot plot) at a concentration of 50 U/ml was added to $CD8^+$ T cells. (**B**) Enhanced expression of perforin and granzyme B from $CD4^+$ T cells. The right hand quadrant shows $CD4^{high}$ T cells, and the number indicates percentage of $CD4^{high}$ T cells producing perforin and granzyme B. (**C**) Enhanced expression of granulysin from $CD8^+$ and $CD4^+$ T cells, co-cultured with LipoK and *M. leprae* stimulated DCs. The protocol was followed as per the staining of perforin, except that the surface stain used was FITC conjugated-CD4 and APC conjugated anti-CD8 mAb, and subsequently PE conjugated granulysin was used. Figure shows the dot plot of the gated $CD8^+$ and $CD4^+$ T cells. The right hand quadrant shows $CD8^{high}$ or $CD4^{high}$ T cells (activated T cells) and the number indicates the percentage of granulysin positive T cells among gated $CD8^{high}$ and $CD4^{high}$ T cells. Representative data of three separate experiments with different donors is shown.

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antibody could almost totally inhibit the IL-12 production from DCs, as well as the T cell activating function of DCs (not shown), probably through blocking of the classical NF-kB pathway. Indeed, parthenolide, one of the major sesquiterpene lactones, known to inhibit NF-kB activity [24], inhibited the IL-12 production from DCs stimulated with *M. leprae* and LipoK. Also, IL-12 was efficiently produced when *M. leprae* was viable and not dead. Thus, although the exact mechanisms remain to be elucidated, some cell surface molecules and secreted components of *M. leprae* are responsible for the production of IL-12, which further modulates type 1 T cell responses [32,33].

A number of mechanisms are known to be involved in the clearance of intracellular bacteria, such as IFN-γ release, apoptosis induction of the macrophages and anti-microbial activity of CTL [12,15]. Production of IFN-γ could boost the ability to kill pathogens in host cells. In fact, it was found that LipoK activated *M. leprae* infected DCs, highly stimulated both memory $CD4^+$ and $CD8^+$ T cells, as well as naïve $CD4^+$ to produce IFN-γ, and further assisted in the proliferation of both T cell subsets (Fig. 3).

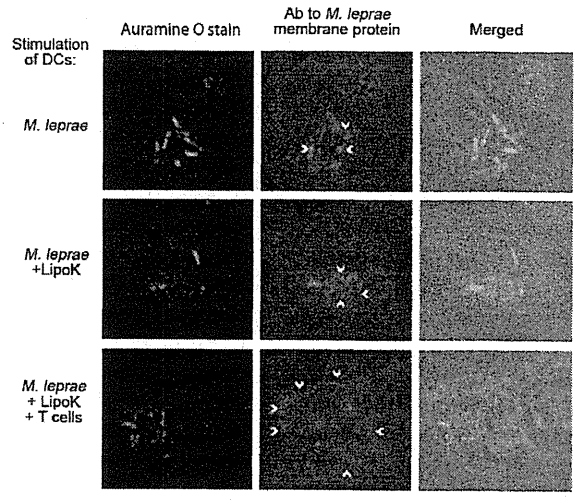

Figure 5. Localization of the membrane components of *M. leprae* at the periphery of the DCs. DCs were infected with either *M. leprae* alone or further stimulated with LipoK for 2 days and in some cases co-cultured with T cells. After 3 days co-culture with T cells, cover glass with attached DCs were fixed and observed under confocal microscopy-LSM5 Exciter. *M. leprae* was stained with Auramine O (shown in green) and *M. leprae* membrane components were stained with polyclonal rabbit antibody raised against the membrane fraction of *M. leprae* (depicted in red fluorescence). Alexa Fluor 633 conjugated anti-rabbit antibody (Molecular Probes) was used as the secondary antibody. Arrowheads indicate the positively stained region. Experiments were performed twice with different donors.

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Inhibition of MHC class I and class II molecules on DCs, 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. Further, proteolytic processing of *M. leprae* antigens was probably enhanced by LipoK treatment of DCs, since incubation with anti-*M. leprae* membrane Ab showed positive staining at the periphery of DCs, when co-cultured with T cells (Fig. 5). In addition, preliminary results showed that expression of MHC class I and II molecules on LipoK activated DCs, were elevated in those co-cultured with T cells. Thus, LipoK could probably assist in the processing and presentation of *M. leprae* antigens, and thereby, highly activate T cells.

The other important parameter, for the clearance of mycobacteria from the host cell, is their potential to activate antimicrobial effector mechanisms in human T cells. DCs have been shown to be involved in CTL induction following uptake of antigenic particles [25,34,35,36]. $CD8^+$ T cells co-cultured with LipoK

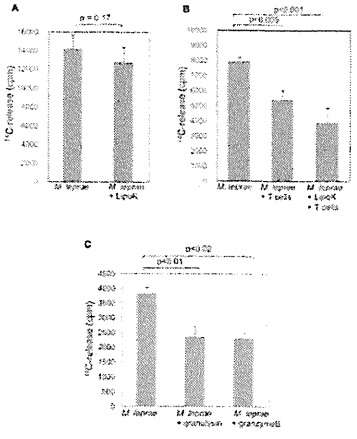

Figure 6. Reduction in the viability of *M. leprae* in DCs after co-culture with T cells and LipoK stimulation. (**A**) DCs were infected with *M. leprae* and stimulated with LipoK, 2 days later, cells were collected and the viability of *M. leprae* in DCs was measured by the radiorespirometric assay (metabolic CO_2 release) as described in Materials and Methods. In brief, ^{14}C labeled palmitic acid was added to the lysates of DCs and cultured at 33°C. After 7 days of culture, the amount of $^{14}CO_2$ evolved was measured using a Packard 1500 TRI-CARB liquid scintillation analyzer. (**B**) DCs were infected with *M. leprae* as in **A**, and co-cultured with T cells. Six days after the co-culture, DCs were lysed, and the viability of *M. leprae* was determined by the radiorespirometric assay. (**C**) *M. leprae* at a concentration of 1×10^7/well/200 µl in Middlebrook 7H9 media was incubated with granulysin or granzyme B for a period of 3 days at 33°C, and the viability determined as described in **A**. Unpaired Student's t test was used to find the statistical significance of the two sets of data. Representative data of three separate experiments is shown.

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