DISSERTATION

IFIDENTIFICATION AND CHARACTERIZATION OF LEPROSY T CELL ANTIGENS IN THE CONTEXT OF EARLY DIAGNOSIS AND CD1a RESTRICTED T CELL ACTIVATION

Submitted by

Hee Jin Kim

Department of Microbiology, Immunology and Pathology

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Doctoral Committee:

Advisor: Patrick J. Brennan

Noreen E. Reist
Alan R. Schenkel
Varalakshmi D. Vissa

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF LEPROSY ANTIGENS
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AND CD1a RESTRICTED T CELL ACTIVATION

Some of the work conducted, specifically the production of certain *M. leprae* recombinant proteins, has been published as follows: Spencer JS, Kim HJ, Wheat WH, Chatterjee D, Balagon MV, Cellona RV, Tan EV, Gelber R, Saunderson P, Duthie MS, Reece ST, Burman W, Belknap R, Mac Kenzie WR, Geluk A, Oskam L, Dockrell HM, Brennan PJ; on behalf of the IDEAL Consortium. Analysis of Antibody Responses to *Mycobacterium leprae* Phenolic Glycolipid I, Lipoarabinomannan, and Recombinant Proteins To Define Disease Subtype-Specific Antigenic Profiles in Leprosy. Clin Vaccine Immunol. 2011. 18:260-267.

The progression of leprosy disease highly correlates with predominant type 1/type 2 cytokines and proliferation of Th1/Th2 cells in response to *M. leprae*. Th1 immunity is thought to be protective to *M. leprae* infection but Th2 immunity is pathogenic. The primary objective of this dissertation was to identify and characterize a panel of new protein based T cell antigens, particularly those that induce Th1 immunity, in order to understand host-*M. leprae* interaction and to develop diagnostic tests for leprosy.

Cellular mediated immunity (CMI) based *in vitro* interferon-γ released assay (IGRA) to *M. leprae* specific antigens is known as the most promising diagnostic test to

detect those individuals in the early stages of *M. leprae* infection. Here, we identified two classes of antigens using advanced genomic and proteomic approaches.

Comparative bioinformatic analysis among mycobacterial genomes identified potential *M. leprae* unique proteins called "hypothetical unknowns" from the *M. leprae* genome. We performed cDNA based quantitative real time PCR to investigate the expression status of 136 putative open reading frames (ORFs) encoding hypothetical unknowns. Twenty six of *M.leprae* specific antigen candidates showed significant levels of gene expression compared to that of ESAT-6 (ML0049) which is an important T cell antigen of low abundance. Fifteen out of 26 selected antigen candidates were expressed and purified in *E. coli*. Serological analysis using the sera obtained from lepromatous leprosy patients and cavitary tuberculosis patients clearly indicated that 9 out of 26 selected proteins elicited *M .leprae* specific immune responses.

M. leprae membrane antigen (MLMA) induces a strong Th1 immune response. However, the large amount of lipomannans/lipoarabinomannans (LMs/LAMs) content hindered the further processing of this fraction and the optimal use of this fraction as diagnostic reagents in a CMI based assay. A simple sodium carbonate treatment successfully enriched membrane proteins into MLMA-SP (alkali soluble proteins of MLMA) and excluded the most hydrophobic lipids. Subsequent IFN-γ release assay (IGRA) of peripheral blood mononuclear Cells (PBMCs) from 16 mycobacterial laboratory/leprosy clinic workers revealed that MLMA-SP induced higher levels of IFN-γ secretion in the group exposed to leprosy patients, as compared to the group with environmental/professional mycobacterial exposure. Therefore, MLMA-SP and 9 of the

hypothetical unknowns may be good diagnostic reagents to improve both sensitivity and specificity in detection of individuals with asymptomatic leprosy.

Bacterial lipoproteins, containing an N-acyl di-O-acylglyceryl-cysteine unit, have been known to induce strong CMI response to bacterial infections through Toll like receptor (TLR-2) signaling. CD1-restricted T cells in response to mycobacterial lipid antigens efficiently and rapidly induce cytolytic T cell response to kill intracellular pathogens. In this work, we developed simple proteomic and chemical methodologies to enrich lipoproteins of low abundance from MLMA. T cell reactivity to these lipoprotein enriched fractions combined with proteolysis provided the first evidence that bacterial lipoproteins are a new class of CD1a restricted antigens recognized by a CD1a restricted T cell line derived from skin lesions of a leprosy patient. Further proteomic and genomic analyses identified lipoproteins, ML1086, ML2095 and ML2446, as CD1a antigen candidates. However, these three lipoproteins expressed in recombinant form in *M. smegmatis* were unable to activate a CD1a restricted T cell line (LCD4.15) though the acylation of all recombinant proteins was biochemically verified. The possible reasons for this result are: 1) that the antigen recognized by LCD4.15 would be another lipoprotein of the least abundance making it difficult to identify using current proteomic analysis; 2) that the bacterial lipoproteins of *in vitro* grown *M. smegmatis* could present different acylation patterns from those of *in vivo* grown *M. leprae*.

The presence of acylated cysteine at the N terminus of lipoprotein is primarily predicted by bioinformatic analysis but rarely verified by biochemical analysis. Lipopeptides of mycobacterial lipoproteins were prepared by proteolysis and organic extraction, and analyzed by gas chromatography-mass spectrometry (GC-MS) and

quadrapole time of flight liquid chromatography/mass spectrometry (Q-TOF LC/MS). GC-MS results revealed that the major fatty acids of the glyceryl portion of the lipoproteins are tuberculostearic acid, stearic acid, oleic acid and palmitic acid, which are also the dominant fatty acids of mycobacterial phospholipids. The N-linked fatty acid of the cysteine residue is predominantly palmitic acid. Interestingly, Q-TOF LC/MS data showed that mycobacterial lipoproteins appeared as di- and tri-acylated mature forms, but also had an array of fatty acids in various combinations. Di-acylated lipopeptides appeared more frequently than tri-acylated lipopeptides. The present study demonstrated a distinct fatty acid composition of the lipid portion of mycobacterial lipoproteins compared to other bacterial lipoproteins. Moreover, we defined the full spectrum of acylation pattern in tri- or di-acyled mycobacterial lipoproteins, the impact of which in host-bacteria responses is subject to further immunological investigation.

Here, we have identified and characterized a panel of new *M. leprae* derived protein antigens by developing novel biochemical and proteomic techniques. The knowledge and methologies presented in this dissertation will allow for further understanding of the physiology and pathogenicity of *M. leprae* in human hosts.

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I really appreciate my family for their support. I thank my parents for their endless love. I am profoundly indebted to my husband, Dr. Sebabrata Mahapatra, for his encouragement, understanding, sacrifice, patience and support during these difficult times. I also want to give my special thanks to my child, Ananya, for being a happy kid in spite of Mom's absence.

DEDICATION

This dissertation is dedicated to my husband, Sebabrata Mahapatra, and my child, Ananya Mahapatra, who have given their love and strength to accomplish my desire. I hope to be with Ananya one day, as she faces her own challenges and difficulties to pursue her dream.

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List of Abbreviations

1,25D3	1,25-dihydroxyvitamin D_3 or 1,25-dihydroxycholecalciferol
2D	Two-Dimensional
19 kDa/LpqH	19 kDa *M. tuberculosis* Lipoprotein Antigen
33 kDa/LpK	33 kDa *M. leprae* Lipoprotein Antigen
38 kDa/PhoS1	38 kDa *M. tuberculosis* Lipoprotein Antigen
45/47-kDa/MPT32	45/47-kDa *M. tuberculosis* Glycoprotein Antigen
AA	Amino Acid
Ala, A	Alanine
AraLAM	Uncapped Lipoarabinomannan
Arg, R	Arginine
Asp, D	Asparagine
BB	Mid Borderline Leprosy
BCG	*M. bovis* Bacillus Calmette-Guérim; a vaccine for tuberculosis
BL	Borderline Lepromatous Leprosy
BSA	Bovine Serum Albumin
BT	Borderline Tuberculoid Leprosy
$C_{\#}$	Carbon $_{\text{Number}}$
CD^{+}	Cluster of Differentiation Restricted
CFP-10	10 kDa *M. tuberculosis* Culture Filtrated Proteins, ML0050/Rv3874
CMI	Cell-Mediated Immunity
C-terminus	Carboxyl Terminus
CYP27b1	25-Hydroxy Vitamin D3-1α-Hydroxylase
Cys	Cysteine
DC-SIGN	Dendritic Cell-Specific Intracellular Adhesion Molecule-Grabbing Nonintegrin
ELISA	Enzyme-Linked Immunosorbent Assay
ERK1/2	Extracellular Signal-Regulated Kinase (ERK),
ESAT-6	Early Secretory Antigenic Target of 6 kDa, ML0049/Rv3875
GC	Gas Chromatography
Gly, G	Glycine
Gln, Q	Glutamine
Glu, E	Glutamate
HHC	Household Contacts
HLA	Human Leukocyte Antigen
IEF	Isoelectric Focusing
Ile, I	Isoleucine
IFN-γ	Interferon-γ
IGRA	IFN-γ Release Assay
IRAK-1 or 4	IL-1 Receptor-Associated Kinase-1 or 4
Leu, L	Leucine

LAM	Lipoarabinomannan
LepLAM	LAM of *M. leprae*
Lgt	Diacylglyceryl Transferase
LID-1	Recombinant Fusion Protein containing ML0405 and ML2331
LL	Lepromatous Leprosy
LM	Lipomannan
Lnt	N-Acyl Transferase
Lpr1086	Recombinant Hybrid Protein of ML1086 and Signal Sequence of LprG Expressed in M. smegmatis (Signal sequence of LprG was used to generate acylated form of ML1086 but removed during lipid modification)
LprG	26kDa Mycobacterial Lipoprotein, ML0557/Rv1411c
LspA	Signal Peptidase II
Lys, K	Lysine
Met, M	Methionine
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight
ManLAM	Mannose-Capped LAM
MAPK	Mitogen –Activated Protein Kinase
MARCO	Macrophage Receptor with Collageneous Structure
MB	Multibacillary Leprosy
MCP	Major Cytosolic Protein
MHC	Major Histocompatibility Complex
ML####	Sanger Identification Number of *M. leprae* Protein
ML Flow test	*M. leprae* Lateral Flow Test
MLCwA	*M. leprae* Cell Wall Antigens Excluding LAMs
MLMA	*M. leprae* Membrane Antigen or Membrane Fractions of *M. leprae*
MLMA-InsP	Alkali Insoluble (Sodium Carbonate Soluble) Proteins of of MLMA
MLMA-P	Trion X-114 Insoluble Fraction of MLMA-InsP
MLMA-SP	Alkali Soluble (Sodium Carbonate Soluble) Proteins of MLMA; Peripheral Membrane Protein of MLMA
MLSA-LAM	*M. leprae* Soluble (Cytosol) Antigens Excluding LAMs
MMP	Major Membrane Protein
MS	Mass Spectrometry
MyD88	Myeloid Differentiation Factor 88
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
NOD	Nucleotide-binding Oligomerization Domain Containing Protein
OM	Outer Membrane
ORF	Open Reading Frame
PAMP	Pathogen Associated Molecular Pattern
PB	Paucibacillary Leprosy
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction

PDIM	Phthiocerol Dimycocerosates
PGL	Phenolic Glycolipids
Phe, F	Phenylalanine
PIMs	Phosphatidylinositol Mannosides
PPD	Purified Protein Derivative of *M. tuberculosis*
Pup	Prokaryotic Ubiquitin-Like Protein
Pupylation	Prokaryotic Ubiquitin-Like Protein Modification
qRT-PCR	Quantitative Real Time PCR
QTOF	Quadrapole Time of Flight
Rv####	Sanger Identification Number of *M. tuberculosis* Protein
SE	Size Exclusion
Sec	Secretion
Ser, S	Serine
Sod	Superoxide Dismutase
TAT	Twin-Arginine Translocation
TB	Tuberculosis
TDM	Trehalose Dimycolate
TGF-β	Transforming Growth Factor- β
THP-1	Human Acute Monocytic Leukemia Cell Line
Thr, T	Threonine
TICAM1	Toll /Interleukin -1 Receptor -Domain-Containing Molecule 1
TIRAP/MAL	TIR Associated Protein /MyD88 Adaptor Like
TLR	Toll Like Receptor
TMM	Trehalose Monomycolate
TNF	Tumor Necrosis Factor
TRAF-6	TNF Receptor Associated Factor-6
TRAM	TRIF Related Adaptor Molecule (TRAM)
TRIF	TIR-Domain Containing Adaptor Protein-Inducing IFN-β
Trp, W	Tryptophan
TT	Tuberculoid Leprosy
Tyr, Y	Tyrosine
Val, V	Valine
VDR	Vitamin D Receptor
WHO	World Health Organization

CHAPTER 1 LITERATURE REVIEW: LEPROSY HISTORY, TREATMENT, EPIDERMIOLOGY, THE BACTERIUM, IMMUNE RESPONSE AND DIAGNOSIS

The primary objective of this dissertation is to identify and characterize a panel of new protein antigens that induce Th1 immunity. *Mycobacterium leprae*, the causative agents of leprosy, persist in the human host for long periods without presenting obvious clinical symptoms. Th1/Th2 immune response to *M. leprae* plays an important role in determining to develop leprosy in affected individuals. Most individuals exposed to the bacterium do not develop disease, but instead manifest cellular mediated immune (CMI) response to the bacterium. We hypothesized that a set of *M. leprae* antigens trigger protective Th1 immunity in host-pathogen interactions and also allow for the improved specificity and sensitivity of diagnosis to detect individuals in the early stages of *M. leprae* infection. To test these hypotheses, we embarked on the following two specific aims. *In aim one,* advanced bioinformatic and proteomic approaches enhanced the optimal selection of *M. leprae* antigens that can be used to detect CMI responses of *M. leprae* infected individuals, but not those of individuals with other mycobacterial infection or exposure. *In aim two, M. leprae* lipoproteins that induce protective CD1a restricted T cell immunity in leprosy skin lesions were identified and characterized by biochemical and genomic approaches combined with a T cell reactive assay.

Post-translational modification of mycobacterial proteins diversifies biological roles of proteins in host-pathogen interactions. Additionally, this dissertation hypothesized that mycobacterial lipoproteins contain the distinct mycobacterial fatty acid (tuberculostearic acid) and tri- or di-acyl glyceryl-cysteine unit at their N-terminus. To test this hypothesis, we developed the biochemical methodologies and defined the chemical structure of the lipid portion of the mycobacterial lipoproteins

1.1 History

Leprosy, caused by *Mycobacterium leprae*, predominantly appears in skin, peripheral nerves and body parts that are cooler than normal body temperature such as ear lobes, and other anterior parts of the body (127). Disease progression leads to disfiguring skin lesions, nerve damage and permanent disability (112,262). Leprosy is an ancient disease described in many archaic writings (148). The first description of this disease appears in the Ebers Papyrus, an ancient Egyptian medical document written around 1550 B.C. (148). However, the description of leprosy manifestations in these ancient medical texts is still controversial because the descriptions match other skin diseases (dermatitis, gas gangrene and fungal infections) (148). Later, in the 6th century B.C. (63,156), *Sushruta samhita* and Kautilya's *Arthashastra* in India and *Nei Ching* in the Chou Dynasty of China described the more typical clinical leprosy symptoms, which included sensory changes and deformities. In the West, leprosy first appeared in the records of ancient Greece after the army of Alexander the Great came back from India, and then in Rome in 62 B.C (168). Recent paleontological studies from ancient human skeletal

remains revealed that lepromatous leprosy had existed from 2000 B.C. in India (168,237,244).

There are two hypotheses about the origin and transmission of human leprosy. One hypothesis, proposed from paleontological analysis, is that leprosy extending from India was the origin of Western or Eastern Asian and North African leprosy (244); the other hypothesis supported from recent phylogeographical analysis of single nucleotide polymorphisms is that leprosy originated in Eastern Africa and then disseminated through Europe to the remainder of the world with continuous human migration (191).

Given the incurable deformity, unknown etiological features, misunderstandings, and abhorrence of the disease, led people to think that leprosy was a curse or punishment from the gods (38,112). Consequently, leprosy was left to be treated by priests or holy men, not physicians. Since the disease often appeared in family members, some people thought it was hereditary (112). Therefore, leprosy patients and their contacts were restricted to live in isolated leprosaria.

In 1873, the Norwegian scientist, Dr. Gerhard Henrik Armauer Hansen, discovered rod shaped bacteria, *M. leprae*, from leprous nodules under a microscope (38,112). He proved that leprosy is an infectious disease, not hereditary or the result of a divine curse (112,145,303). By using aniline dyes, Neisser was able to stain *M. leprae* in leprosy patients' tissue samples provided by Dr. Hansen and proved Dr. Hansen's theory (38,303). Therefore, leprosy is also known as Hansen's disease to honor Dr. Hansen's role in this significant discovery. In addition, leprosy is often called "Hansen's disease" by many clinicians in an attempt to protect patients from the stigmas associated with the term leprosy (38).

1.2 The disease

1.2.1 Clinical features

Leprosy presents as two contrasting spectra correlating with the type of host immune response against the pathogen and resulting in bacterial and disease progression (185). At one end of the disease spectrum, patients with tuberculoid leprosy (TT) or paucibacillary leprosy (PB) exhibit a strong CMI which is responsible for the protective immunity to *M. leprae* (194,262,272). Affected skin lesions contain few bacilli and well defined granulomas consisting of Langerhans cells, $CD4^+$ and $CD8^+$ T-cells (262,272).

At the opposite end of the spectrum, patients with lepromatous leprosy (LL) or multibacillary leprosy (MB) exhibit a strong humoral immune response and a high number of bacilli (175,262). Patients present numerous skin and nodular lesions on all parts of the body. The biopsies of LL patients contain a number of foamy lipid-laden macrophages with diffused abundant bacilli. According to the Ridley-Jopling classification (241), the disease spectrum is subdivided into five parts: TT, borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL) to LL forms (Figure 1.1).

Immunohistochemistry and gene expression profile analysis by using RT-PCR demonstrated that the Th1 cytokines such as IL-2, IL-12 and IFN-γ, were predominantly expressed in the skin lesions of TT patients. In contrast, Th2 cytokines including IL-4, IL-5, and IL-10 predominated in the skin lesions of LL patients (186,272) (Figure 1.1; further discussed in section 1.8).

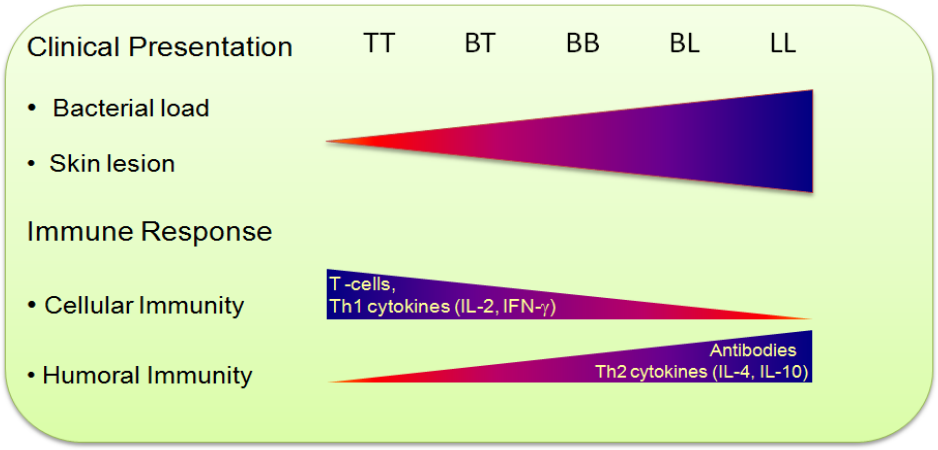

Figure 1.1 Disease spectrum of leprosy (adapted from Misch *et al.* (183) with permission). Clinical manifestation of leprosy is classified as TT, BT, BB, BL and LL (see above for definitions). The humoral immunity of host and bacterial load highly correlates.

1.2.2 Nerve damage

One hallmark of leprosy is damage to the peripheral nerves, causing sensory and motor impairment, with characteristic limb deformities and disability. *M. leprae*, as the causative agent of leprosy, preferentially invade and grow in the Schwann cells surrounding the axons of peripheral nerve cells. The tropism of *M. leprae* to Schwann cell is associated with the tissue specific expression of laminin-2 on the membrane of Schwann cell (183). By using a rat Schwann cell axon coculture system, Rambukkana *et al.* (234) demonstrated that the trisaccharide unit of phenolic glycolipid-I (PGL-I) on *M. leprae* binds specifically to the domain of the α2 chain of native laminin-2 in the basal lamina of the Schwann cell. In addition, *M. leprae* laminin binding protein 21 which is heavily methylated (see section 1.7.4) also mediates the intracellular entry into Schwann cells (183). Collectively, according to Rambukkana *et al.* (233), PGL-I and the laminin binding protein 21 are the key determinants in the tropism of *M. leprae* for peripheral

nerve invasion and induce demyelination which results in neurodegenerative disease (Figure 1.2).

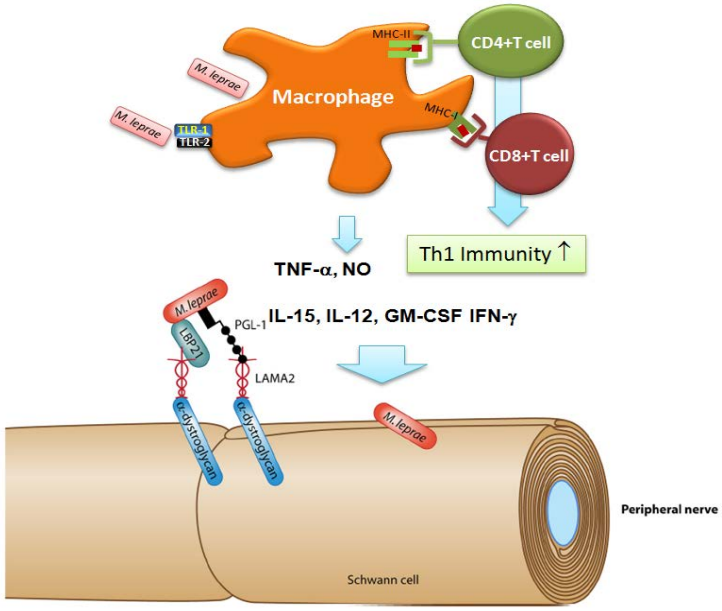

Figure 1.2 Proposed model of infection of peripheral nerve by *M. leprae* (adapted from Misch *et al.* (183) with permission). In non-immune-mediated nerve injury, laminin binding protein 21 (LBP21) and PGL-I in the *M. leprae* cell wall bind to the α2 chain of laminin-2 (LAMA2) and α-dystroglycan on the Schwann cell membrane permitting entry and subsequent damage to the peripheral nerve. In immune mediated mechanism, the activated macrophages induce proinflammatory cytokines, NO, influx of immune cells and Th1 cellular immunity leading to nerve damage.

Cell surface molecules of *M. leprae* usually activate monocytes or macrophage to induce proinflammatory cytokines such as TNF, IL-12, IL-6, IL-1, IL-15 and nitric oxide (NO). Nerve biopsies from leprosy patients reveal perineural and intraneural inflammation (183). The spontaneous leprosy reactions are acute inflammatory complications considered as medical emergencies during the course of treated or untreated leprosy (262). It may cause irreversible tissue damage and nerve destruction in leprosy patients if not treated timely. Two major clinical types of leprosy reactions are known: type 1 "reversal reactions" and type 2 “erythema nodosum leprosum” (262). Type 1 reactions frequently occur in BL, BB and BT patients and are due to the sudden

increases of CMI response to *M. leprae* antigens (183). However, type 2 reactions often exhibit in MB patients with poor CMI against *M. leprae* (183). These patients often experience neuritis with sensory and motor neuropathy (262). However, the triggering factors and mechanism of leprosy reactions are still unknown.

The influx of immune cells and edema via inflammatory response to *M. leprae* could cause the immune-mediated nerve injury (183). By using *in vitro* stimulation assays, this hypothesis indirectly supported that 19 kDa *M. tuberculosis* lipoprotein, which is representing mycobacterial toll like receptor-2 (TLR-2) ligand, recognized TLR-2/1 on the cultured Schwann cells and induced innate immune response (214) (Figure 1.2).

1.3 Diagnosis

Current standard diagnosis of leprosy by the World Health Organization (WHO) is based on the presence of one or more of the clinical signs as follows: 1) hypopigmented or reddish skin lesion consistent with leprosy and with definite sensory loss; and 2) thickened peripheral nerves (311). For treatment purposes, histopathological classification in leprosy diagnosis is important to determine the type and course of chemotherapy (94,312).

Major problems to leprosy diagnosis are that:1) there is no measure to detect individuals with asymptomatic *M. leprae* infection who could be healthy household contacts (HHCs) of MB patients but mostly have no known history of close contact with leprosy patients (81-83,277); 2) clinical symptoms-based diagnosis and a microscopic diagnosis by acid-fast bacilli staining is insensitive to greater than 70% of infected

patients, especially PB/TT patients (30); and 3) acid-fast bacilli staining on skin slit smear or biopsy specimen is rarely applied, nowadays, in routine diagnosis. Moreover, a microscopic diagnosis and classification by counting acid-fast bacilli in skin slit smear or biopsy is highly dependent on well-trained leprologists or laboratory technicians. Therefore, these problems often result in delayed and missed diagnosis, permitting disease progression and increasing potential disease transmission. Since multi-drug therapy (described in section 1.4.2) is capable to sterilize *M. leprae* infected individuals, early diagnosis and correct classification of leprosy are major requirements to control leprosy and eventually to prevent irreversible disability of leprosy patients.

PCR based assays targeting either *M. leprae* antigen coding sequences (18kDa/, 65 kDa/ GroEL antigens or 36kDa/Proline-rich antigen) or repeated DNA sequences, improves both sensitivity and specificity of leprosy diagnostics by detecting 95% of MB/LL patients and 55% of PB/TT patients (30,94,251). However, this advanced molecular technique cannot be used to detect preclinical leprosy due to poor resources in endemic regions. During the last decade, research towards immune based diagnostic assays has made rapid progress in early diagnosis of leprosy. Immune based diagnosis of leprosy is described in section 1.10 following a discussion of the major antigens of *M. leprae.*

1.4 Treatment

1.4.1 Early form of chemotherapy

Along with description of symptoms, the treatment of leprosy with Chaulmoogra oil was mentioned in a number of ancient medical documents (63,202). In the late 19th

century, a British surgeon serving in India introduced this plant into the West (63,202). It was extensively used on leprosy patients until the 1940s. Chaulmoogra oil consists of hydnocarpic and chaulmoogric acids. Early studies hypothesized that these compounds might inhibit the biosynthesis of bacterial cell envelope and/ or enhance the host defense mechanism (126). Jacobsen and Levy (125) showed that the growth of *M. intracellulare* was susceptible to 2 μg of hydnocarpic acid. Subsequently, the presence of biotin in culture media attenuated this action of hydnocarpic acid suggesting that the action mechanism of hydnocarpic acid might be related with biotin metabolism of bacteria (125). However, Levy (150) demonstrated that the multiplication of *M. leprae* in mouse food pad infection model was highly susceptible to chaulmoogric acids but not to hydnocarpic acids. The action mechanism of Chaulmoogra oil in the treatment of *M. leprae* infection is still obscure.

Later in 1937, a sulfone derivative (sodium salt of p,p′-diamninodiphenyl-sulfone-N-didextrose sulfate) was synthesized and put on the market under the name Promin by Parke, Davis and Co.. This drug was investigated for the treatment of various acid-fast bacilliary infections (327). A clinical study on leprosy patients reported that Promin inhibited the progress of leprosy in a significant number of cases. This resulted in its approval as standard chemotherapy for the treatment of human leprosy (154,155,327). However, long-term dapsone monotherapy with poor compliance resulted in the failure of the treatment and consequently dapsone-resistant leprosy emerged (262,264). Additional anti-microbial agents such as rifampicin and clofazimine were then introduced to treat dapsone-resistant leprosy (262,265).

1.4.2 Multidrug Therapy

In order to resolve the emergence of drug-resistant leprosy, the WHO recommended multidrug therapy as the new standard regimen for leprosy control in 1981 (265). The initial regimen consisted of a combined daily dose of dapsone and clofazimine and a monthly dose of rifampicin and clofazimine for two years or until skin smears became negative (262). In order to reduce the economical burden in developing countries, the multidrug therapy regimen was continuously modified and the period of treatment was shortened (30). The current regimen for MB patients consists of receiving 600 mg of rifampicin once a month, 100mg of dapsone daily, 300 mg of clofazimine once a month as well as 50 mg daily for 12 months. PB patients are treated with a combination of 600 mg monthly and 100 mg of dapsone daily for six months. The patients with a single lesion are treated with single dose of rifampicin (600 mg), ofloxacin (400 mg) a minocycline (100mg) (312).

1.5 Epidemiology

The 12th, 13th and 14th centuries were believed to be the times of the highest prevalence of leprosy (112). By the end of the 18th century, quarantining measures mentioned earlier in this section successfully decreased disease incidence (38,112). Currently, leprosy has become less prevalent, but continues to be problematic (112).

The route of transmission in the human host is still unknown, but it is suspected that nasal carriage or invagination through skin wounds, is the major ports of entry for *M. leprae* (302). The natural reservoirs of *M. leprae* are mostly humans and, perhaps, nine-banded armadillos, *Dasypus novemcinctus* (141). Armadillos with low body temperature

(34°C) are currently used as a host for the multiplication of large quantities of *M. leprae* for research purposes (262).

The WHO in 1976 estimated the total number of leprosy cases worldwide to be over 12 million (308). The WHO-directed MDT program introduced in 1982 has resulted in a dramatic reduction of global leprosy prevalence (265). The prevalence continued to be reduced from 5,351,408 in 122 countries in 1985 to 2,361,407 in 1991 in the top 32 endemic countries (209,309) (Figure 1.3). Given the success of the multidrug therapy program, the World Health Assembly in 1991 ambitiously projected the "elimination of leprosy as a public health problem by the year 2000" to achieve a level of prevalence of less than one case per 10,000 populations (265). Since then, effective chemotherapy treatments and efforts from governmental and non-governmental organizations have made it feasible to achieve the global goal of the leprosy elimination campaign (152). In a recent report from WHO, the global registered prevalence from 141 countries/territories at the beginning of 2010 was 211,903 and the number of new cases detected during 2009 was 244,796, which was significantly reduced from 620,638 in 2002 (309,310) (Figure 1.3). These remaining numbers of cases are predominantly in India and Brazil, but also in Indonesia, the Democratic Republic of the Congo, Central African Republic, Nepal, Nigeria, Mozambique, and Tanzania (310). Although the elimination campaign with multidrug therapy efficiently sterilized the patients and removed the biological reservoir of *M. leprae*, the number of new cases was sustained until now indicating that quiescent leprosy is continuously transmitted in endemic regions.

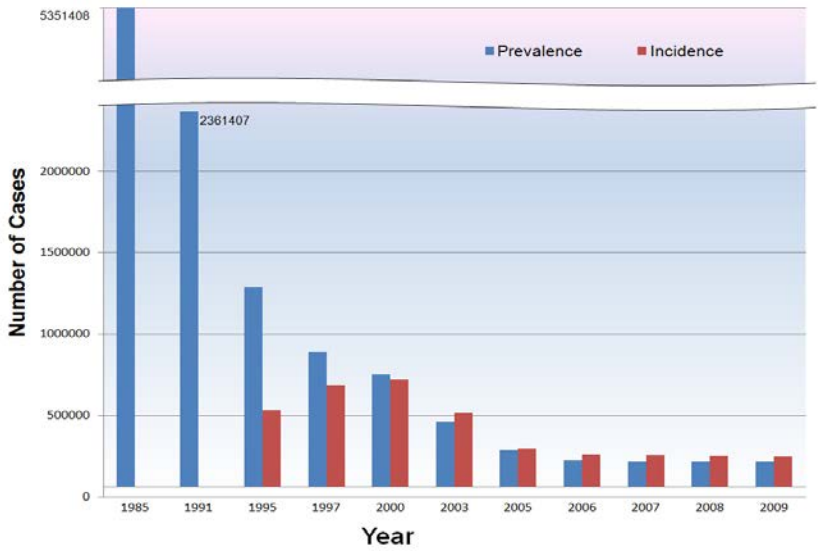

Figure 1.3 Global prevalence and incidence of leprosy, 1985-2009. The prevalence of leprosy is the total number of registered cases of leprosy at December 31 of a given year. The incidence is the number of new cases from January 1 to December 31 at a given year.

1.6 The bacterium: *Mycobacterium leprae*

M. leprae is an aerobic, rod-shaped and non-spore forming bacillus in the *Mycobacterium* spp., belonging to the family *Mycobacteriaceae* of the order *Actinomycetales* (262,302). Due to the possession of mycolic acids in a unique cell wall structure, *Mycobacterium* spp. are positive to the acid fast stain (16). *Mycobacterium* spp. are categorized into two subgroups: fast-growing (one to two hours doubling time) and slow growing (12 to 24 hours doubling time) (55). While the fast growers, such as *M. smegmatis* and *M. phlei*, are prevalent in the environment and are mostly nonpathogenic, the slow growing species including *M. tuberculosis*, *M. bovis*, *M. avium* and *M. avium paratuberculosis*, are highly adapted to survive in a host intracellular niche and are generally considered as the pathogens of mammalian diseases (55). Unlike other slow growing mycobacterial pathogens, *M. leprae* is a noncultivable intracellular bacterium with approximately 12-14 day generation time even in immunologically

defective mice (nu-/-) (47,151). Therefore, it takes an average of three to five years after infection for presentation of clinical symptoms in humans (302). Different from other mycobacteria, *M. leprae* preferentially invade the Schwann cell of the peripheral nerve and result in irreversible neurological injury causing sensory and sensorimotor loss in leprosy patients (233). The bacilli chronically infect and propagate inside of both macrophages and Schwann cells, contributing to pathogenesis of leprosy (194,233,262) (described in section 1.2.2).

1.6.1 Genome

Cole *et al.* (47) originally sequenced the entire genome of the *M. leprae* TN strain which had been purified from skin lesions of a multi-bacillary leprosy patient from Tamil Nadu, India and was subsequently propagated in a nine-banded armadillo. The genome of *M. leprae* is 3.27 megabase (Mb) in size and encodes 1614 coding sequences. Comparison with the genome to *M. tuberculosis* (4.41 Mb) with a high coding capacity (3,959 coding sequences), indicates that *M. leprae* contains an exceptionally large number of 1133 pseudogenes which are functionally active in *M. tuberculosis* (46,47) (Table 1.1).

Table 1.1 Comparison of genome features of *M. leprae* TN and *M. tuberculosis* (adapted from Scollard *et al.* (262) with permission)

Feature	*M. leprae*	*M. tuberculosis*
Genome size (bp)	3,268,203	4,411,532
G+C (%)	57.8	65.6
Protein coding (%)	49.5	91.2
Unknown genes	142	606
Protein-coding genes	1,604	3,993
Pseudogenes	1,133	6
Gene density (bp per gene)	2,024	1,106
Average gene length (bp)	1,007	1,008

This massive decay of genes results in the inactivation of important metabolic pathways, more frequently in catabolic pathways than in anabolic pathways, such as regulation, detoxification, DNA repair and transport or efflux of metabolites such as amino acids (AAs), peptides, cations and anions (47,301). The functional gene redundancy which is commonly found in *M. tuberculosis* does not occur in *M. leprae*; *M. leprae* has fewer genes in almost all functional categories (47,301). Genes involved in anabolic pathways and β-oxidation of fatty acids are conserved in *M. leprae.* Similarly, intracellular survival of *M. tuberculosis* was proven to depend on host lipid degradation and the glyoxylate shunt for energy production (47,246,262). In agreement with chemical analysis of cell wall structure (see section 1.6.3), the genes encoding for the biosynthesis pathway of the cell wall in *M. leprae* are considerably conserved (301). Comparative genomic analyses across *Mycobacterium* spp. indicate that the genome of *M. leprae* shares higher homology with *Mycobacterium* spp. (47). Therefore, this sequence similarity of genes encoding important immunogenic proteins results in considerable cross-reactivity in distinguishing individuals infected by *M. leprae* from those exposed to *M. tuberculosis* or other mycobacteria (277). In order to overcome this

problem, researches have vigorously evaluated the antigenic potential of hypothetical unknown proteins in functional gene class VI that have no/ low homology in any current mycobacterial database (80,82,277) (Table 1.2; see section 1.10.2).

Table 1.2 Functional classification of genes in *M. leprae* (http://www.pasteur.fr/recherche/unites/Lgmb/NATURE_DATA/ML_gene_list)

Class	Function
I	Small-molecules metabolism (sugar, carbon compound, fatty acids and amino acids)
II	Macromolecules metabolism (polysaccharide, lipopolysaccharide, cell envelope, RNA, DNA and proteins etc)
III	Cell process (Transport/binding proteins, cell devision, adaptation)
IV	Others (virulence, inserted elements, antibiotic production and resistance etc)
V	Conserved hypotheticals
VI	Hypothetical unknowns

By using conventional methods (6× coverage) and advanced next generation sequencing platforms (38× or 46× coverage), Monot *et al.* (192) recently completed the whole genome sequences of three different *M. leprae* strains including Br4923 which was isolated from a Brazilian patient, Thai53 from a Thai patient and NHDP 63 from a North American (192). This study revealed that all new three strains and the TN strain share 99.995% of sequence identity despite geographical distance. It clearly indicated that the genomes of *M. leprae* strains are highly conserved and lack polymorphisms in coding sequences (192). Additionally, 16 single nucleotide polymorphism sites which were newly found in 5 pseudogenes, verified the previous transmission patterns of early human leprosy by genotyping both extant and extinct strains of *M. leprae* worldwide (described in the section 1.1).

1.6.2 Proteome

Due to massive gene decay, *M. leprae* is incapable of independent growth, being restricted to human and certain animal models where it manages to persistently multiply in the host. Therefore, proteomic analysis of *M. leprae* has focused on understanding leprosy pathogenesis and developing tools for leprosy diagnosis.

In the early stage of proteomic analysis, the WHO Scientific Working Groups on the Immunology of Leprosy identified distinct protein antigens from extracts of *M. leprae* by using monoclonal antibodies (137). These were further screened as recombinant proteins from the early λgt11 library and identified as heat-shock proteins (70 kDa=DNAK; 65 kDa=GroEL; 12-14 kDa=10 kDa=GroES) (3,4). Further biochemical and immunological studies on large quantities of armadillo-derived *M. leprae* allowed subcellular fractionation, isolation and purification of the major native proteins including the major cytosolic protein (MCP)-I (GroES/ML0380), MCP-II (18 kDa/heat shock protein), MCP-III (Sod A/ML0072c), major membrane protein (MMP)-I (35 kDa/ML0841) and MMP-II (ML2038/bacterioferritin (BfrA)/22 kDa) (117). MMP-II in *M. leprae* was found to be a multimer with a molecular weight of 380 kDa. Analysis of the native form by using the absorption spectrum demonstrated that the 380 kDa multimer contained iron. This result indicates that MMP-II may play a crucial role in the intracellular survival of *M. leprae* by acquisition of iron (224).

However, much of this early proteomic approach highly depended on the immunogenicity of proteins, limiting identification to only immunodominant proteins. Later, the addition of 2-D gel electrophoresis technology and emerging genome sequence data allowed further identification of *M. leprae* proteins such as alkyl

hydroxide (AhpC), sulfate sufurtransferase (CysA), L7/L12 ribosomal binding protein (RplL), transcription activator in two component system (MtrA) and protein elongation factor (EF-Tu) (170).

By using advanced modern mass spectrometry technology, two consecutive studies indentified 382 proteins which were mostly enzymes for lipid degradation and biosynthesis, biosynthesis of cell envelope, lipid carriers, etc. (171,172). It suggested that mycobacterial cell wall and lipid metabolism play a major role in bacterial pathogenesis. Recently, Souza *et al.* (61) indentified 1046 proteins out of 1614 coding sequences proteins by using a high throughput proteomic approach combined with bioinformatic analysis by using a new ORF annotation algorithm. In this study, five new proteins encoded by pseudogenes were identified. However, it is not clear whether these proteins are functionally expressed in the *M. leprae* proteome.

1.6.3 Unique cell envelope

The mycobacterial cellular envelope constitutes 40% of the dry weight of the bacterium and, as for all members of the *Mycobacterium* genus, contains highly unusual, but mycobacteria typical, lipids, glycolipids, proteins and polymers (26). The unique cell wall of *Mycobacterium* spp., *M. tuberculosis* and *M. leprae,* has been intensely investigated and proven to be a major pathogenic contributor in the context of the survival of mycobacterial pathogens within phagocytic cells (55,124,246). The structures of its components have been well defined and share common characteristics with all *Mycobacterium* spp. (25,26). These structures are introduced in the following sections. Knowledge of some (e.g. the phosphatidylinositol mannosides (PIMs), lipomannans

(LMs), lipoarabinomannans (LAMs) is important in the context of the experimental work (e.g. Chapter 3); other structures are discussed more in the context of an overall perspective of the complex nature in the tubercle bacillus. This section will briefly review the basic features of the mycobacterial envelope related to pathogenic aspects and some of the typical features of *M. leprae.*

Originally, it was thought that the mycolyl arabinogalactan-peptidoglycan complex of *M. leprae* should be simpler than that of other mycobacteria due to the large scale of gene reduction or gene decay (301). However, detailed biochemical analysis does not support this concept.

The mycobacterial cell envelope consists of the plasma membrane, a phospholipid bi-layer but unique to mycobacteria and related bacteria in possessing esterified tuberculostearic acid and PIMs. Extending outward from the plasma membrane, the cell-wall core consists of peptidoglycan covalently attached to arabinogalactan, which in turn is attached to the mycolic acids (25). The outer membrane (OM) is loosely associated or intercalated by the mycolic acid layer through hydrophobic interaction in the outermost part of the cell (26) but serves to form a true bilayers (Figure 1.4).

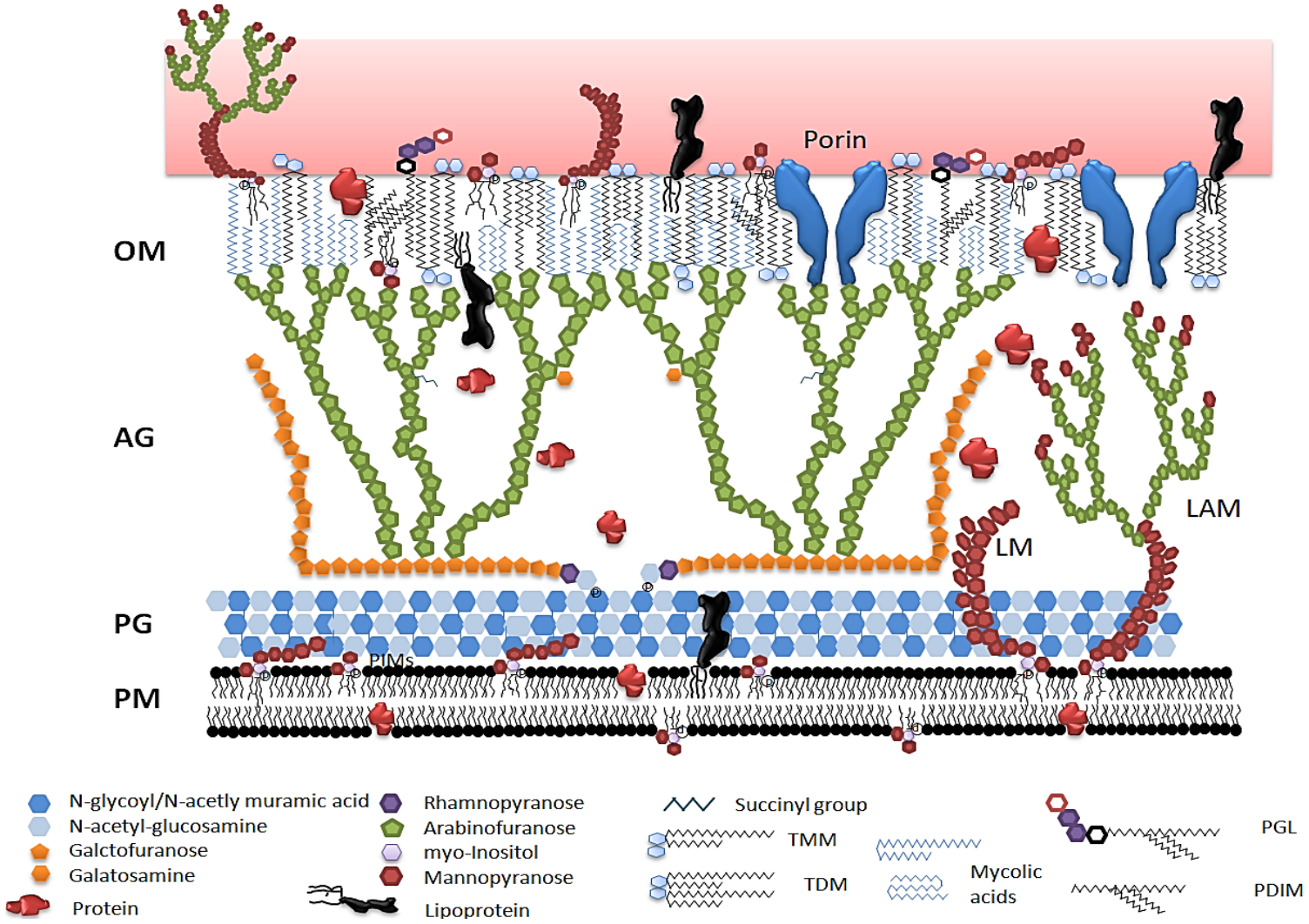

Figure 1.4 Schematic representation of the cell envelope of *Mycobacterium spp.* (adapted from Bhamidi *et al*. (15) and Niederweis *et al.* (207) with permissions). AG, arabinogalactan; PG, peptidoglycan; PM, plasma membrane.

1.6.3.1 Peptidoglycan

The peptidoglycan, a prokaryotic skeleton, maintains cell structure and resists osmotic pressure arising from the cytoplasm or external sources. It consists of the repetitive units of *N*-acetyl-α-D-glucosamine and modified muramic acid as a backbone (25). In the C3 position, the muramic acid is substituted with a tetrapeptide which consists of L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine (161) (Figure 1.5). Compared to *Escherichia coli*, there is extensive cross-linking between each peptide in the *Mycobacterium* spp. and provides the rigid structural integrity (25). However, the peptidoglycan of the *Mycobacterium* spp. is a unique mixture of *N* -glycolylated and *N* -acetylated muramic acids and the carboxylic acids of the peptide that are often amidated (164). Recent studies revealed that the *N*-glycoly muramic dipeptides of *M. tuberculosis* are more powerful than *N*-acetyl muramyl dipeptides in activation of proinflammatory cytokine secretion through the RIP-2 and NF-κB (Nuclear Factor Kappa-Light-Chain-enhancer of Activated B cells) intracellular networks (50). Also, the genetically mutated *namH* gene of *M. smegmatis* is responsible for the conversion of the N-acetyl muramic acid to *N*-glycolyl-muramic acid. The absence of the *N*-glycolyl function in NamH mutants substantially decreased NOD2 (nucleotide-binding oligomerization domain containing 2)-mediated TNF (tumor necrosis factors) secretion by infected macrophages compared to those of wild type *M. smegmatis* (50). However, *M. leprae* encodes *namH* as a pseudogene and is believed to be devoid of *N*-glycolyl muramic acids in its mature peptidoglycan. Recently, an advanced mass spectrometry based approach finally provided the biochemical evidence for this concept by analyzing the peptidoglycan of *in vivo* derived *M. leprae* (163) (Figure 1.5). Another unique feature of *M. leprae*

peptidoglycan is the replacement of L-alanine (L-Ala) in the first position of the tetrapeptide side chain with glycine due to its *in vivo* growth conditions (70,162) (Figure 1.5). The substitution of L-Ala with D-Ala in the peptidoglycan of *Staphylococcus aureus* and *Bacillus subtilis* was demonstrated to contribute to evading the recognition by NOD2 in host cells (121). Taken together, the loss of glycolyl function in muramic acids and the substitution of L-Ala with Gly in peptidoglycan may favor *M. leprae* in host-pathogen interaction, a question yet to be addressed.

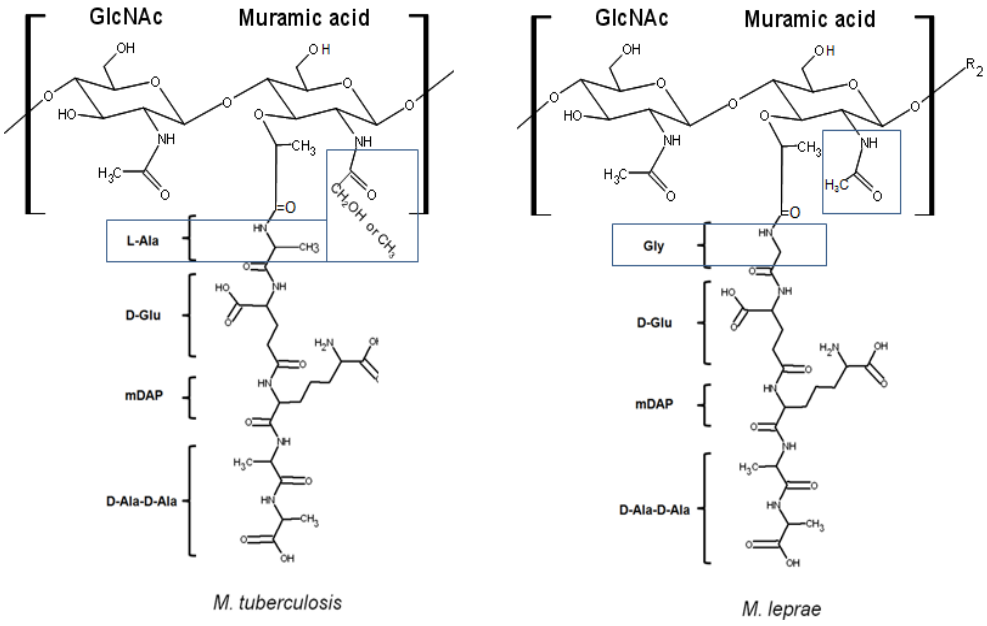

Figure 1.5 Structure of the repeating unit of mycobacterial peptidoglycan (adapted from Mahapatra *et al.* (161) with permission). Boxes indicate the differences between *M. leprae* and *M. tuberculosis.* GlcNAc, *N*-acetyl-α-D-glucosamine.

1.6.3.2 Arabinogalactan

The fundamental features of arabinogalactan are similar among all mycobacteria including *M. leprae* (70). However, early structural analyses indicated a simpler, less extended structure in the case of *M. leprae* (301). Arabinogalactan is a tripartite structure consisting of the linker unit, an alternating β-(1→5) and β-(1→6) linked D-galactofuran

(30 units long), and a branched D-arabinofuran, a typical mycobacterial glycopolymer (25) (Figure 1.6).

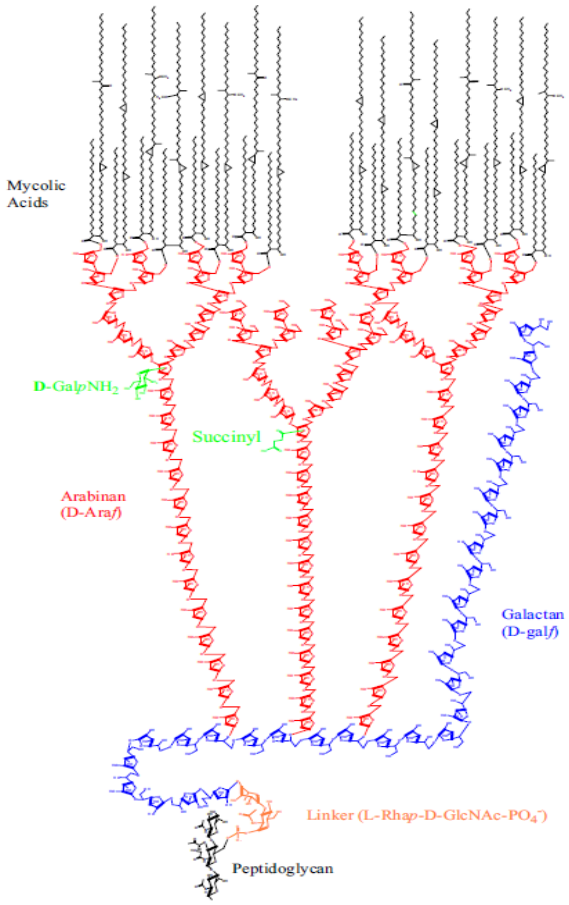

Figure 1.6 Structure of mycolyl arabinogalactan and its attachment to peptidoglycan (from Bhamidi *et al.* (15) with permission). Two-thirds of 31-unit long arabinans (red) attached to mycolyl residues. Three chains are present per galactan (blue) chain. The $GalNH_2$ or the succinyl residue (green) is attached to an internal branching point of the arabinan.

The linker unit, α-rhamnopyranosyl-(1→3)-α-*N*-acetyl-α-D-glucosamine-(1→phosphate) of arabinogalactan is covalently attached at the C6 position of muramic acid residues in peptidoglycan through a phophodiester bond (Figure 1.6). Bhamidi *et al* (15) analyzed the arabinogalactan of *M. tuberculosis*, and revealed that three arabinan chains are present per galactan chain and two-thirds of the 31-unit long arabinan are attached to

mycolyl residues. Either succinyl or glucosaminosyl groups are individually linked at an internal branching point of two-thirds of the arabinan (Figure 1.6).

1.6.3.3 Mycolic Acids

Mycolic Acids are α-alkyl, β-hydroxyl fatty acids, 70 to 90 carbons in length (Figure 1.7), forming a lipophilic protective barrier and contributing to inherent drug resistance to some drug and solute uptake. The majority of mycolic acids exists as bound esters of arabinogalactan, but is also present as extractable lipids, such as trehalose monomycolate, trehalose dimycolate in the outer membrane (OM) (see section 1.6.3.5) (Figures 1.4 and 1.10). The structure of the mycolic acids consists of C_{20} to C_{25} α-branches and C_{50} to C_{70} β- meromycolate chains containing one or two functional groups (keto, methoxy, epoxy, wax ester) and varying within *Mycobacterium* spp. (26).

In *M. leprae*, α-branch mycolic acids are shorter than that of other mycobacteria (C_{20} vs $C_{24\text{-}26}$) and the β-chains do not contain the methoxy function (69). The complete loss of mycolic acids is lethal for mycobacteria but results in viable *Corynebacterium glutamicum* which otherwise shares mycobacterial cell wall features (173). This *C. glutamicum* mutant is more susceptible to hydrophilic antibiotics (173,207). In addition, genetic mutation of either modification or length of mycolic acids in mycobacteria alters the permeability of hydrophilic materials. Taken together, mycolic acids serve as a significant permeability barrier responsible for some of the special physiological feature of mycobacteria such as impermeability to uptake nutrients and drugs and slow growth (207).

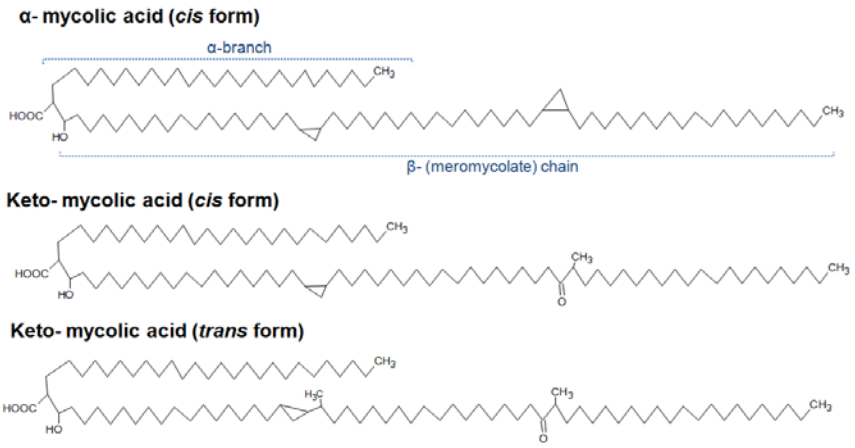

Figure 1.7 Structures of mycolic acids (adapted from Takayama *et al.* (288) with permission). Three major forms of mycolic acids are found in *M. leprae*, *M. tuberculosis*, *M. bovis* and *M. bovis* BCG.

1.6.3.4 Phostphatidylinositol mannosides (PIMs), lipomannans (LMs), and lipoarabinomannans (LAMs)

The PIMs, LMs and LAMs are among the most abundant and highly conserved components in all mycobacteria. They are found in the plasma membrane or/and the OM of all *Mycobacterium* spp. and exist via non-covalent hydrophobic interactions. Their insertion into the membraneous bilayers is derived from their acylated phosphatidyl portion (26,292) (Figure 1.8.A). The phosphatidyl inositol (*sn*-glycerol-3-phospho-1-D-*myo*-inositol) unit of PIMs (PIM_1 to PIM_6) is modified with one to six mannose residues (208). The inositol group can additionally be acylated at the C3-position and contain a mannose residue at the C2-position (Figure 1.8.A). This mannose also can be acylated at the C6-position. Current research on their essentiality suggests that these molecules play an important role to maintain cell viability in all *Mycobacterium* spp. (123). LMs consist of PIM_4 with a long α (1→6)-linked mannan branched with alternative α (1→2)-linked mannose residues. LAMs are derived from LMs by attaching an arabinan chain at the

mannan backbone (Figure 1.8.B). The arabinan segment of LAMs consists of α (1→5)-D-arabinofuranose polymers branching at internal residue to 3,5-α-D-arabinofuranose residues (208). The terminal non-reducing arabinose residues may be capped with mannosyl residues (ManLAM) or devoid of these residues (AraLAM) (208). ManLAMs are mostly found in pathogenic slow growing mycobacteria, such as *M. tuberculosis, M. bovis, M. avium, M. leprae;* the mannose caps consist of a single mannose, or di- or tri-mannosides containing α(1→2) linkages (39,208) (Figure 1.8.B). LAMs are more abundant in *M. leprae* and have less mannose capping and simpler arabinans in comparison to LAMs of *M. tuberculosis* (208,292).

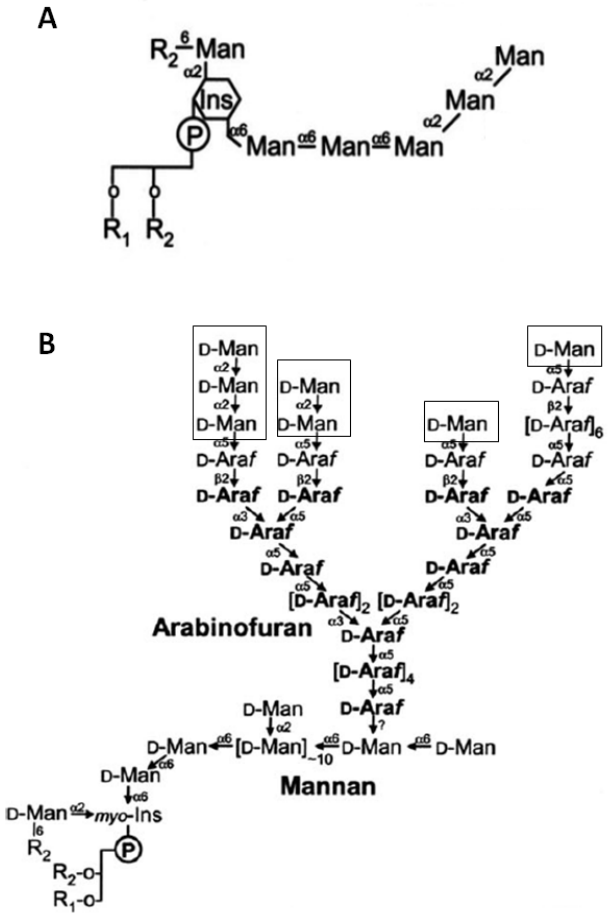

Figure 1.8 Schematic structures of mycobacterial PIM_6 and ManLAMs. (from Berg *et al.* (14) with permission). A) PIM_6, B) ManLAMs; Boxes indicate mannose capping: R1, palmitic acid; R2, tuberculostearic acid.

The presence of mannose caps on LAMs has been demonstrated to contribute to the successful pathogenesis of virulent mycobacteria (157,256,259,260). In addition, the number, nature, and position of the acyl residues are important in the interaction of LAM with receptors such as CD14, and TLR-4 (130). The immunomodulatory role of these components, particularly LMs and LAMs, has been extensively investigated in terms of pathogenesis (see section 1.8.2).

1.6.3.5 Glycolipids: Phenolic glycolipids (PGLs) and phthiocerol dimycocerosates (PDIM); Trehalose containing glycolipids (TDMs, TMMs)

Similar to LAM and PIMs, components of the OM including PDIM, PGL, trehalose dimycolate (TDM, also known as cord factor), trehalose monomycolate (TMM) may associate or even intercalate with the mycolic acids via hydrophobic interactions (26,54) (Figure 1.4). The PGLs, polyketide derived virulence factors, are dominantly produced in the slow growing mycobacteria including *M. leprae, M. kansasii, M. bovis, M. microti* and a few of the clinically isolated *M. tuberculosis* strains particularly, apparently, in the case of MDR tuberculosis (26,55). The PDIM, the lipid base of the PGLs is composed of a mixture C_{33}-C_{41} diols with two polymethylated branched fatty acids (C_{27}-C_{33}), mycocerosic and phthioceranic acids (26,124) (Figure 1.9). PDIM itself also contributes to the pathogenesis of mycobacterial disease by protecting from antimicrobial activity of nitric oxide and suppressing the secretion of proinflammatory cytokines by activated macrophages (124). The PGL structure is generally composed of a PDIM core, a phenol and a considerably hydrophobic oligosaccharide part containing one to four deoxysugars modified with multiple O-methyl groups (55). The PGL-I, II

and III in *M. leprae* are major antigenic glycolipids. PGL-I contains the *M. leprae* specific saccharide of 3-O-methyl (Me)-rhamnose, 2,3-di-O-Me-rhamnose, and 3,6-di-O-Me-glucose attached to the phenol of the PDIM via a glycosidic bond (116) (Figure 1.9). This uniquely modified sugar induces the species specific humoral response (41) and also plays an important role in host pathogen interaction (see sections 1.2.2 and 1.10.4) (35,233,258).

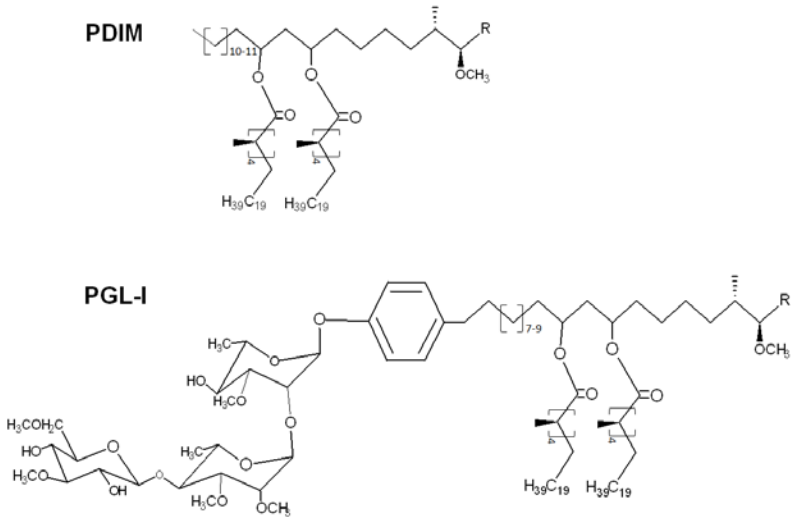

Figure 1.9 Structures of phthiocerol dimycocerosates (PDIM) and Phenolic glycolipids (PGLs) from *M. leprae* (adapted from Jackson *et al.* (124) with permission). Two of the polymethylated mycocerosic acids attach at long-chain β-diols (phthiocerol moiety) of PDIM via ester bonds. The PGL-I from *M. leprae* consists of phenolphthiocerol esterified by two polymethylated mycocerosic acids and a trisaccharide unit consisting 3-O-methyl (Me)-rhamnose, 2,3-di-O-Me-rhamnose, and 3,6-di-O-Me-glucose at the phenol groups (R=-CH_2-CH_3 or –CH_3) (116). The structure of the oligosaccharide unit of the PGL from *M. tuberculosis* is different (2,3,4-tri-O-Me-fucose-(1-3)-rhamnose-(1-3)-2-O-Me-rhamnose) (124). The oligosaccharide structures of glycolipids from environmental mycobacteria are different as well.

Trehalose containing lipids present in OM of *M. tuberculosis* including trehalose 6,6′-O-dimycolate (TDM), trehalose 6-O-monomycolate (TMM) and sulfolipids which consist of trehalose 2′-sulfate acylated with hydroxyphthioceranic, phthioceranic and saturated fatty acids (palmitic or stearic acid) (26) (Figures 1.10 and 1.22). All of these

lipids are found predominantly in the cultures of virulent *M. tuberculosis* (55) and play an important role in the pathogenesis of tuberculosis including pro-inflammatory cytokines (primarily TNF-α) production and granuloma formation of macrophages (19,124). TDM, also known as a cord factor, induces the potent inflammatory responses of macrophage via TLR-2 signaling (19). TDM and TMM were not found in *M. leprae,* though a significant numbers of leprosy patients presented seroreactivity to TDM and its precursor, TMM, derived from *M. tuberculosis* (132). Recently, Kai *et al*. (132) isolated both *M. leprae*-derived TDM and TMM from armadillo tissue infected with the bacteria. Subsequent MALDI-TOF mass analysis revealed that these trehalose based lipids of *M. leprae* possessed both α-mycolates (C_{78}) and keto- mycolates (C_{81} or C_{83}). However, sulfolipids are absent from *M. leprae* due to deletion of a series of genes which are responsible for biosynthesis of their acyl chains (47).

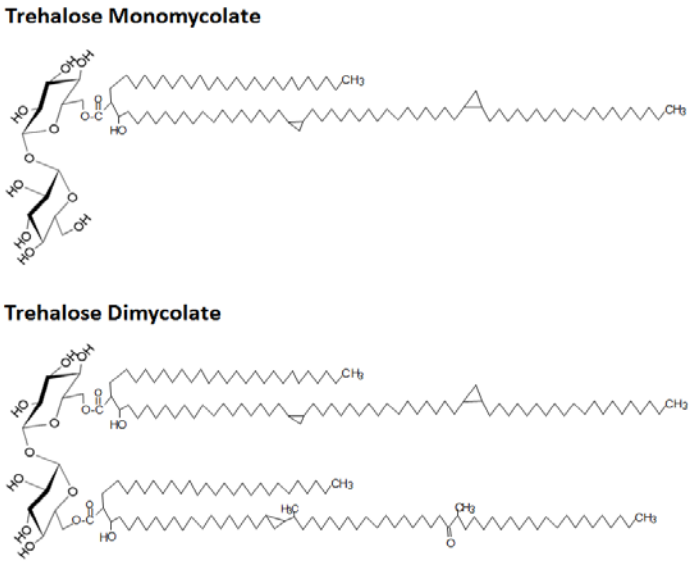

Figure 1.10 Structures of trehalose monomycolate and trehalose dimycolate (adapted from Takayama *et al.* (288) with permission). In *M. leprae*, α- or/and keto-mycolates are estrified to the trehalose head groups of both TMM and TDM.

1.6.3.6 Outer membrane (OM)

The OM of *Mycobacterim* spp. consists of an asymmetric bilayer containing pore forming proteins (porins), lipoproteins and free lipids including the phthiocerol-containing lipids, PIMs, LMs, LAM, TDM, TMM, and the diacyl- and polyacyl-trehaloses (25) and, above all, the arabinogalactan-bound mycolates (Figure 1.4). Minnikin proposed the existence of a pseudo OM akin to that of Gram negative bacteria (54). OM has since then been considered as an asymmetrical lipid bilayer consisting of covalently linked mycolic acid solely comprising the inner leaflet and the extractable lipids restricted to the outer leaflet (26,54).

Recently, Hoffmann *et al.* (110) and Zuber *et al.* (328), by using cryo-electron tomography and vitreous sections, provided a new perspective on the spatial organization of the cell envelope, an altered version of the original Minnikin's model. One major change is the existence of extractable lipids in both the inner and outer leaflets of the OM leading to symmetrical lipid bilayers. The other difference is that the OM is thinner than conventional models supporting the functional accommodation of porins (110). The thickness of the OM of *Mycobacterium* spp. is 8nm (110). However, the theoretical length (around 9nm) of the meromycolate of mycolic acid in an extended conformation is too long to fit this new model (110). New findings raise the question of whether meromycolates in the hydrophobic matrix of the OM have an elongated or a folded conformation (207).

Zuber *et al*. (328) proposed a new model with a folded meromycolates in a symmetric OM, unlike that previously depicted as the Figure 1.11. Since there is no direct experimental evidence of the conformation of mycolic acids, both extended and

folded conformations of mycolic acids are equally acceptable in Figure 1.11 model of the organization of the OM of the mycobacterial envelope (207).

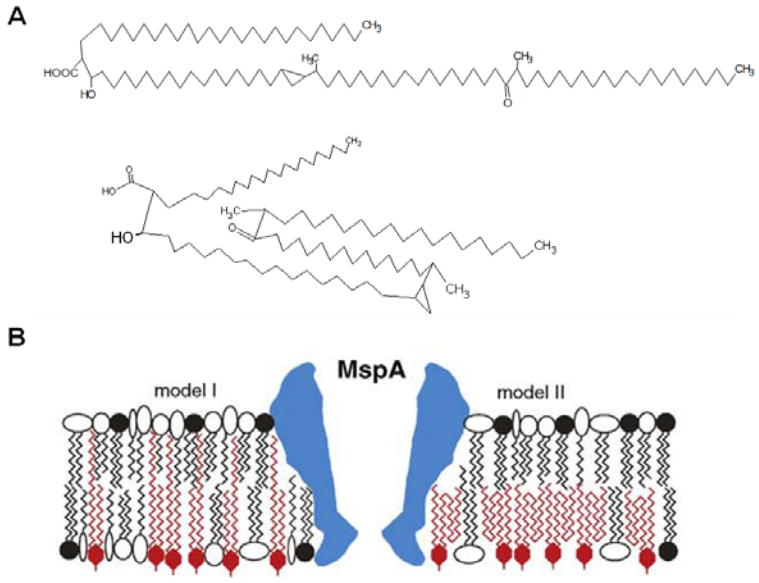

Figure 1.11 Models of the mycobacterial outer membrane (OM) (adapted from Niederweis *et al*. (207) with permission). A) Schematic structure of mycolic acids in the elongated and folded conformation; B) Models of the mycobacterial OM. The mycolic acids covalently attached to the arabinogalactan polymer are drawn in red and inserted either in the elongated conformation (model I, left side) or the folded conformation (model II, right side). A porin, MspA from *M. smegmatis* , is represented in blue. Open symbols of lipid headgroups indicate apolar groups; filled symbols represent polar groups. These free lipids could occur in both leaflets of the outer membrane.

1.6.3.7 Biosynthesis and genetics of cell wall components

In previous sections, we described the chemical definition of the mycobacterial cell envelope mostly in the context of the pathogenesis of mycobacterial diseases. The chemical structures of its components are very similar among all *Mycobacterium* spp. but are different from those of other bacteria and such structures are absent in host eukaryotes. Therefore, the biosynthesis of cell wall components has been long investigated in order to develop new drugs against *M. tuberculosis*. The availability of genome sequences of *Mycobacterium* spp. combined with recent advances in both genetic manipulation and

analytical techniques allow for an understanding of the basic genetics and biosynthesis of cell wall components of *M. tuberculosis* (25). Genome analyses of *M. leprae* revealed that the important genes responsible for the biosynthetic pathways of cell wall components are conserved and share high degrees of homology with orthologues of *M. tuberculosis* (27,47,301). Therefore, the genetics and biosynthesis of mycolyl arabinogalactan-peptidoglycan complex, PDIMs and LMs/LAMs/PIMs in *M. leprae* probably resemble those of *M. tuberculosis*. These topics have been reviewed by Crick *et al.* (52), Guilhot *et al.* (97), Kaur *et al.* (136), among others.

1.7 Post-translational modification of mycobacterial proteins

Post-translational protein modifications such as protein acylation, glycosylation, methylation, phosphorylation and ubiquitination diversify the biochemical structure of proteins. These chemical groups on mature proteins ultimately influence protein localization, stability, regulation and biological functions which cannot be predicted by the genomic DNA sequences (33)

The primary structure of certain mycobacterial proteins can be modified by acylation and phosphorylation (240,305). In addition, growing evidence suggests that mycobacteria possess protein modification machineries for glycosylation (253,299), methylation (227) and ubiquitination (33), which are typically found in eukaryotes. The exact biochemical structures of post-translationally modified proteins are largely unknown as well as their physiological or pathogenic roles. This section reviews post-translationally modified mycobacterial proteins, primarily on lipoproteins, with emphasis on the biosynthesis, structure and pathogenic roles.

1.7.1 Lipoproteins

Bacterial lipoproteins were initially identified in *E. coli* (20,21). This archetypical bacterial lipoprotein, Braun's lipoprotein, is secreted across the plasma membrane structures and attaches to the peptidoglycan (285). Lipoproteins are acylated at the thiol group of the N-terminal cysteine (Cys) residue through a thioester linked diacylglycerol unit. An additional acyl group is linked through an amide bond to the N terminus of Cys (134) (Figure 1.12). Chemically identical lipoproteins have been found in a variety of bacteria primarily on the basis of sequence analysis (9). However, the chemical structure of few lipoproteins has been experimentally verified to date.

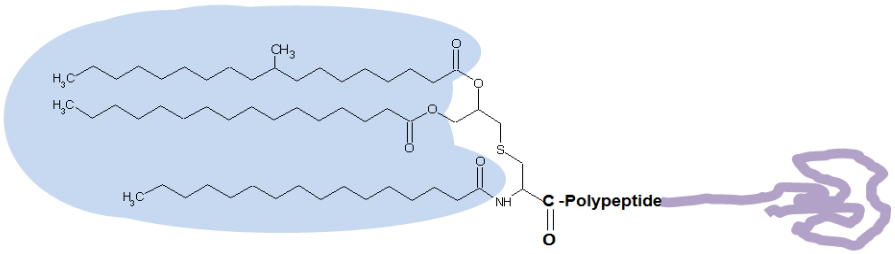

Figure 1.12 General structure of a mycobacterial lipoprotein (adapted from Tschumi *et. al.* (295) with permission). The shaded fatty acids reflect on the composition of phopholipids.

Bacterial lipoproteins have been long studied as a Toll like receptor-2 (TLR-2) ligand that contains conserved pathogen associated molecular patterns (PAMPs) and initiates an inflammatory response (289). The N-terminal acylated region of lipoproteins interacts with TLR-2 on the antigen presenting cells as well as various nonprofessional immune cells, and induces innate and adaptive immune responses required for host defense against microbial pathogens (2,255). In 1999, it was demonstrated that the mycobacterial lipoprotein LpqH/19 kDa strongly stimulated IL-12 induction in Th1

immunity through TLR-2 in human macrophage cell lines (28). Since then, not only LpqH/19 kDa (45,79,205,210,219), but LprG (78,114,115), LprA (222), PhoS1/38 kDa (131,236) from *M. tuberculosis* and 33 kDa (158,319) from *M. leprae* have been studied in terms of Th1 immunity and mycobacterial pathogenesis.

1.7.1.1 Identification

Identification of bacterial lipoproteins has been conducted largely by three approaches: 1) metabolically with radiolabeled fatty acids, those mostly found in phospholipids on accounting of their precursor role in lipoprotein biosynthesis; 2) disturbing lipid modification processes by mutating the signal peptide sequence of prolipoproteins which direct to the lipid modification machinery and result in a mature lipoprotein; and 3) identification of the conserved consensus sequence within the prolipoprotein signal peptides (240).

Biosynthesis of bacterial lipoproteins depends on the presence of a Type II signal peptide containing the consensus sequence $[LVI]_{-3}\,[ASTVI]_{-2}\,[GAS]_{-1}\,C_{+1}$ for lipid modification, commonly known as the "lipobox" (9,304) (Figure 1.13). The signal sequence of secretory proteins, including lipoproteins, consists of three parts: 1) an N-terminal region containing 3-15 amino acids (AAs) with at least two positively charged AAs; 2) a central hydrophobic region which contains 7-22 AA with predominantly hydrophobic and non-charged residues; 3) a cleavage region which distinguishes the Type II signal peptide of lipoproteins from the Type I signal peptide of non-acylated proteins (especially glycoproteins). The cleavage region of the Type II signal peptide possesses the lipobox at -3 to +1 at Cys but a Type I signal peptide contains the Ala-X-

Ala motif as the cleavage site (120,285) (Figure 1.13). These features of signal peptides have facilitated the identification of putative lipoproteins via bioinformatic analysis (9,285) (http://www.mrc-lmb.cam.ac.uk/genomes/dolop/).

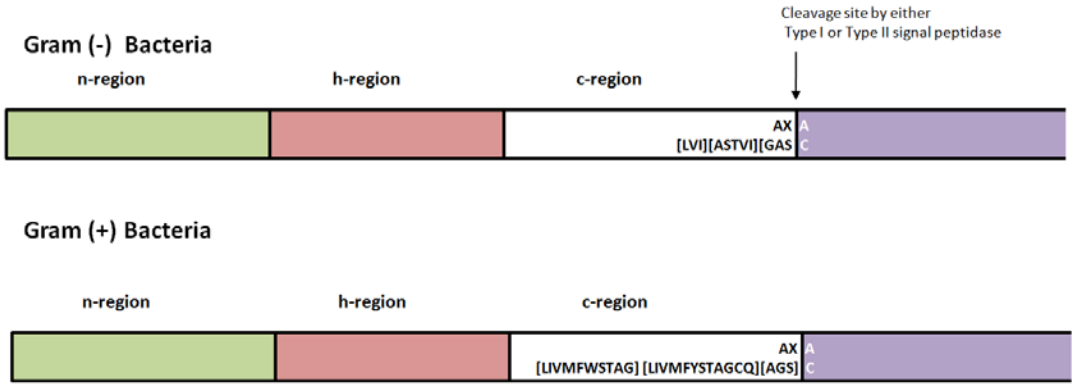

Figure 1.13 The tripartite region of signal peptides recognized by either Type I or Type II signal peptidase in Gram-positive or Gram-negative bacteria (adapted from Hutchings *et al.* (120) with permission). Type I signal peptidase recognizes the AXA motif at the cleavage region (c-region). Type II signal peptidase recognizes the lipobox. The N-terminal region (n-region) containing 3-15 amino acids (AAs) with at least two of positively charged AAs and the hydrophobic region (h-region) consisting of 7-22 AA of hydrophobic and non-charged residues. In the case of Gram-positive bacteria, the consensus sequence of the lipobox is more variable than in Gram-negative bacteria.

More than 2,000 bacterial lipoproteins have been identified and account for 1% to 3% of coding genes in each bacterial genome (9). The signal sequences of 90 lipoproteins from Gram-positive organisms have been extensively studied and established as a consensus Prosite profile PS51257 ([DERK]$_{-6}$ [LIVMFWSTAG]$_{-3}$[LIVMFYSTAGCQ]$_{-2}$[AGS]$_{-1}$C$_{+1}$) (120) (Figure 1.13). This *in silco* analysis has indentified 99 and 31 putative lipoproteins in *M. tuberculosis* and *M. leprae* genome respectively (143,149,286,296) (Table 1.3).

Few mycobacterial lipoproteins have been experimentally characterized in terms of physiological function (68,142,284,295,321). Evidence of acylation in putative mycobacterial lipoproteins has mostly been provided by their appearance in the detergent

phase generated from Triton X-114 phase separation (18). However, this method could not fully characterize protein acylation because lipoproteins colocalized with integral membrane proteins in the resultant detergent phase. Young *et al.* (321) metabolically labeled *M. tuberculosis* lipoproteins by using radiolabeled fatty acids. Two-dimensional (2-D) gel electrophoresis and autoradiography of the resultant proteins revealed that several proteins were acylated including 19 kDa and 38 kDa. In *M. leprae*, 33 kDa/LpK/ML0603 (149) expressed in *E coli* was studied by metabolic labeling with [^{14}C] glycerol and globomycin treatment **(**a specific inhibitor of signal peptidase II, see section 1.7.1.2), leading to the accumulation of the precursor form of LpK (158).

1.7.1.2 Biosynthesis

The biosynthetic machinery of bacterial lipoproteins was identified and fully characterized in Gram-negative bacteria, especially *E. coli* (105,240,249,250,317). In Gram-positive bacteria, recombinant Braun's lipoproteins expressed in both *Bacillus subtilis* and *Staphylococcus aureus* were structurally equivalent to the three-acylated forms produced in *E. coli* (287). Therefore, it is believed that bacterial lipoproteins from Gram-positive and Gram-negative organisms are structurally similar and share similar biosynthetic machinery.

The lipoprotein biosynthetic machinery consists of three enzymes. The first enzyme is diacylglyceryl transferase (Lgt) which covalently links the diacylglycerol residue at the thiol group of Cys in the lipobox (120,249) (Figure 1.14). The orthologues of this enzyme have been identified as Rv1614 in *M. tuberculosis* and ML1274 in *M. leprae,* respectively (149,296). The second enzyme is a signal peptidase II (LspA)

(240,318). LspA/Rv1539/ ML1199 (149,296) cleaves the signal peptide resulting in a new free N-terminus of di-acylated Cys (240,318) (Figure 1.14).

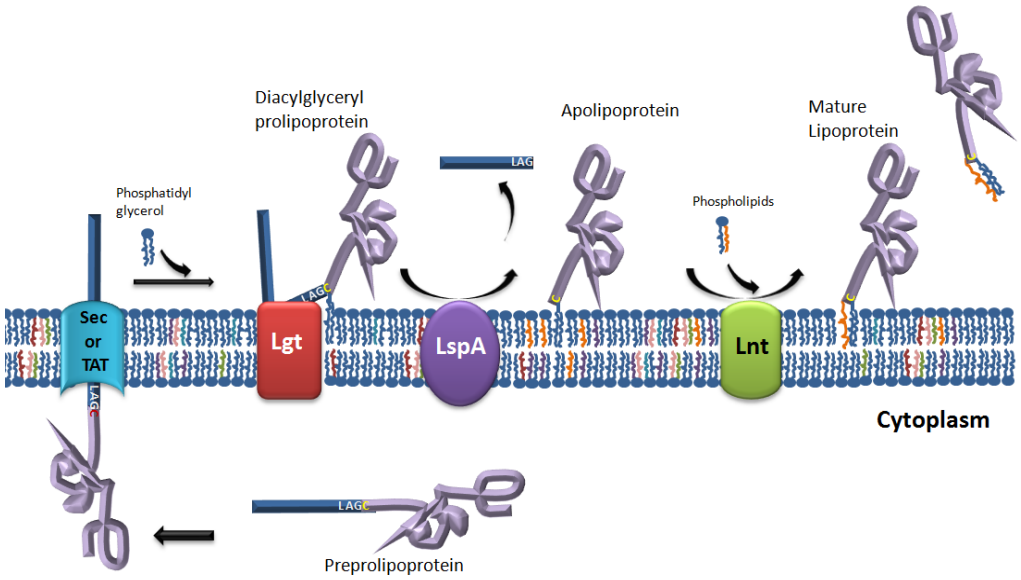

Figure 1.14 Biosynthesis of bacterial lipoproteins (adapted from Hutchings *et al.* (120) with permission). The precursor lipoprotein synthesized in the cytoplasm translocates across the plasma membrane by either the Sec or Tat protein export system (detailed in section 1.7.1.3). First, diacylglyceryl transferase (Lgt) covalently links the diacylglycerol residue to the thiol group of Cys in the lipobox. Second, signal peptidase (LspA) cleaves the signal peptide of the diacylglyceryl prolipoprotein and N-acyl transferase (Lnt) subsequently attaches the third fatty acid to the amide group of Cys. Mature lipoproteins are either retained within the plasma membrane or transported to the outer membrane of the cell wall in Gram-negative bacteria.

The *M. tuberculosis* LspA/Rv1539 enzyme has been partially characterized (248). The growth rate of the *lspA*/*rv1537* deletion *M. tuberculosis* mutant in both mice and macrophage cell lines was decreased but was unaffected in *in vitro* cultured media (248). This suggests that although LspA/Rv1539 is not essential for growth and is not a good drug target, its activity might contribute to the pathogenesis of mycobacterial diseases (240,248).

The N-acyl transferase (Lnt), as a final processing enzyme (120), adds an amide-linked fatty acid (Figure 1.14). In the process of protein acylation, the acyl groups are

donated from bacterial membrane phospholipids such as phosphatidylglycerol, phosphatidylethanolamine and cardiolipin (13,98). Lnt/Rv2051/ML1441 (149,296) is conserved in all mycobacteria and contains 2 domains: an Lnt domain at the N-terminus and a polyprenol monophosphomannose synthase domain at the C-terminus (240). The Rv2051 expressed in either *M. smegmatis* or *E. coli* has been demonstrated to attach *in vitro* guanosine diphosphate mannose onto endogenous mycobacterial polyprenol monophosphates acceptors, which are responsible for biosynthesis of LMs and LAMs (101,240). Recently, the Lnt function of Rv2051 was characterized by using an *lnt* deletion mutant of *M. smegmatis* (295). The subsequent MALDI-TOF analysis showed that this *lnt* deletion mutant failed to acylated the recombinant LppX but the complementation of *lnt/rv2051* in an *lnt* deletion mutant of *M. smegmatis* restored the N-acylation of LppX. Additionally, this study provided the first direct evidence that the mycobacterial lipoproteins contain the species specific tuberculostearic acid derived from phospholipids as the acyl donor (295).

1.7.1.3 Transport

Lipoproteins are exported from the cytoplasm, the site of their synthesis, across both the inner membrane and hydrophobic cell wall. In general, there are conserved protein export systems for the majority of proteins: the classic secretion (Sec) pathway and the twin-arginine translocation (TAT) pathway (204). In some bacteria, there are also specialized protein export systems that translocate specific subsets of proteins (204,242). Lipoproteins are modified and simultaneously transported through the interaction of their Type II signal peptide and protein export systems (Figures 1.13 and

1.14). The positively charged AAs in the n-region of the signal sequence attach to the negatively charged inner surface of the cytoplasmic membrane. The hydrophobic region of the signal peptide is inserted into the cytoplasmic membrane through the Sec or TAT machinery (120,177,304) (Figure 1.14).

The Sec-dependent pathway is a conserved protein export system in all procaryotes (22,204). The Sec-dependent system is composed of several proteins: SecA (ATPase) as an energy dependent motor in the cytoplasm; Sec B as a chaperone in the cytoplasm; SecYEG as a translocase complex in the membrane; and YajC. During the translational process, the signal peptide of a secretory protein binds with the signal recognition particle and forms a ternary complex with the ribosome. This complex targets a precursor protein to the Sec machinery through FtsY (204) (Figure 1.15.A). SecB maintains a preprotein in an unfolded state that is capable to translocate (Figure 1.15.B). SecA accepts a precursor protein from either chaperone, Sec B or signal recognition particle/ribosome/protein complex (200,204). SecA and the target protein associate with the SecYEG complex at the cytoplasmic site. Subsequent conformational changes of the Sec dependent machinery translocate the protein across the plasma membrane and attach the lipid onto the protein (22,204) (Figure 1.15). YajC increases the efficiency of this type of protein export (200,204) (Figure 1.15). Unlike the Sec dependent pathway, the TAT system exports the folded state of preproteins across the plasma membrane (Figure 1.15.C). The N-terminal signal sequence of proteins destined for Tat-dependent translocation typically contains a two arginine motif at the n-region and is longer than in Sec dependent translocation (204).

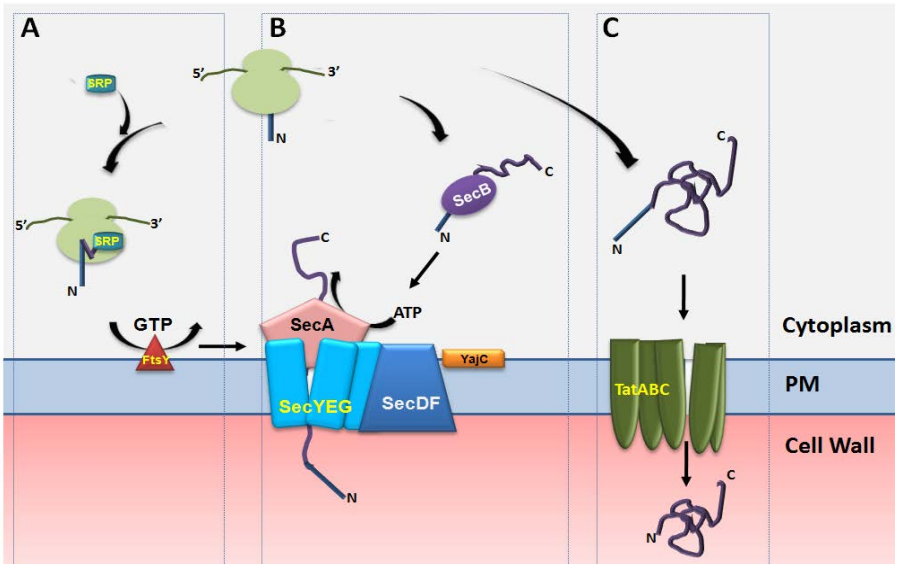

Figure 1.15 Schematic overview of the secretion (Sec) and twin-arginine translocation (TAT) pathways (adapted from Natale *et al.* (204) with permission). Sec dependent translocation of unfolded precursor protein, A) during and B) after translation. C) Translocation of folded precursor proteins by the Tat translocase (see above description). SRP, signal recognition particle.

Mycobacteria have two homologs of SecA: SecA1/Rv3240/ML0779 and SecA2/Rv1821/ ML2082c (22,149,242,296). As in other bacteria, SecA1 is essential and similar to well characterized SecA proteins (242). SecA2 is present in *Streptococcus gordonii*, *Streptococcus parasanguinis*, *M. tuberculosis* and *Listeria monocytogenes* which are capable to survive in hostile intracellular niches (242). SecA2 is assumed to be responsible for secretion of the following virulence factors: SodA (superoxide dismutase, Rv3846); KatG (catalase-peroxidase-peroxynitritase T, Rv1908); Acr (alpha-crystalline, Rv2031) found in *M. tuberculosis* (22,23). However, it still remains to be addressed how to distinguish SecA1 and SecA2 dependent protein export. Based on genomic analysis, *M. tuberculosis* has a functional TAT pathway consisting of TatA/Rv2094c/ ML1331, TatB/Rv1224/ ML1079, tatC/Rv2093c/ ML1332 (149,177,296). Bioinformatics analysis predicted 10-15% of putative lipoproteins as the substrates of TAT in *M. tuberculosis* (120). Currently, β-lactamase and phopholipase C were experimentally verified as

substrates of TAT by using genetic mutants of the Tat components (177,238,240). In *M. leprae*, bioinformatics analysis predicted seven putative lipoproteins as TAT substrates (149).

1.7.1.4 Localization

Proteins on the surface of bacteria are major contributors to host-pathogen interaction including nutrient acquisition, adhesion, invasion and efflux of drugs (242,248). In Gram-negative bacteria, lipoproteins are exported to the outer membrane (OM) or retained in the outer leaflet of the plasma membrane (213). Some lipoproteins contain Asp_{+2} as a sorting signal, allowing it to attach to the plasma membrane (291). In the periplasm, lipoproteins are transported to the OM by the LolABCDE complex consisting of the ATP binding cassette transporters and chaperones (203,213). The lipid moiety of lipoproteins serves as an anchor to attach to the inner membrane or OM in Gram-negative or Gram-positive bacteria cell wall (203,230). Therefore, lipoproteins of Gram-positive bacteria are believed to be functional equivalents of periplamic proteins in Gram-negative bacteria (120). Localization of mycobacterial lipoproteins across this layer has rarely been defined. Proteomic analysis on subcellular fractionation of *M. tuberculosis* revealed that the majority of mycobacterial lipoproteins are found in the cell wall and membrane fractions (89,176). Current findings about mycobacterial OM (mentioned in section 1.6.3.6) suggest that mycobacterial lipoproteins may either associate with the OM or exist in the periplasm between OM and plasma membrane. Transport mechanisms across this layer have yet to be defined, but may be similar to the Lol transport system in *E. coli* (213,291).

1.7.1.5 Physiological Function

Mycobacterial lipoproteins are believed primarily to play an important role in maintaining cell wall integrity and are classified into 5 categories according to their physiological role (286) (Table 1.3). However, this classification heavily relies on comparative bioinformatic analysis of other bacterial genomes. Physiological functions of a few lipoproteins in *Mycobacterium* spp. have been experimentally characterized by using genetic mutants of either mycobacterial lipoproteins or the biosynthetic machinery proteins, LspA and Lnt. In the following, the physiological roles of mycobacterial lipoproteins will be briefly reviewed, focusing on those that have been experimentally verified.

Table 1.3 Functional categories of *M. leprae* and *M. tuberculosis* putative lipoproteins (adapted from Sutcliffe and Harrington (286) with permission)

Functional category	Sub-category	*M. leprae* ORF /*M.tuberculosis* ORF	*M.tuberculosis* ORF without *M. leprae* orthologue
Transporters			
- SBPs in ATP binding cassette transporter	Iron	ML1729/Rv3044(FecB)	Rv0265c (FecB2)
	Molybdenum and Phosphate	ML2095/Rv0928(PstS3)	Rv0932c(PstS2), Rv0934 (PstS1), Rv1857 (ModA)
	Peptides	ML0303/Rv0411c(GlnH) ML0489/Rv2585c ML1093/Rv1244 (LpqZ) ML1121/Rv1280c (OppA)	Rv3666c, Rv3759c
	Sugars	ML1086/Rv1235 (LpqY) ML1427Rv2041c	Rv2833c
	Sulfates	ML0615/Rv2400c (SubI)	
- Lipid Carrier	PIMs DIM PIMs, LMs, LAMs	ML0136/Rv2945 (LppX) ML0557/Rv1411c (LprG) ML1497/Rv1166 (LpqW)	
Enzymes			
- Cell wall metabolism		ML1923/Rv3593 (LpqF)	Rv0399c (LpqK), Rv0838 (LpqR), Rv2905 (LppW) Rv1922, Rv2864c (pbpB)
- Degradative process	Esterases	ML0715/Rv3298c (LpqC)	Rv0671 (LpqP)
	Glycosyl hydrolase		Rv0237 (LpqI)
	Phosphorylase		Rv2293c
	Proteinase/ peptidases	ML1339/Rv2672 ML1633/Rv2224c	Rv0418 , Rv0419
- Metabolic activities	Copper oxidase		Rv0846c
	FAD-linked oxidase		Rv2251
	γ-Glutamyl transferase		Rv2394 (ggtB)
	Oxidoreductases and thioredoxins	ML2412 /Rv0526 (dsbF)	Rv0132c (Fgd2), Rv1677 Rv3006 (LppZ)
	Phosphoglycerate mutase		Rv3390

Functional category	**Sub-category**	***M. leprae* ORF /*M.tuberculosis* ORF**	***M.tuberculosis* ORF without *M. leprae* ortholog**
Adhesion and cell invasion		ML2593(LprK)/Rv0173(Mce1E)	Rv2873 (MPT83), Rv0593 (Mce2E), Rv1970 (Mce3E), Rv3495c (Mce4E)
Signaling and sensory functions		ML0775/Rv3244c (LpqB)	Rv1009 (RpfB), Rv1270c, Rv1368 (LprF), Rv1690 (LprJ), Rv1911c, Rv2403c, Rv3576
Unknown	Inter-related Lpp of unknown function	ML0246/Rv1016c (LpqT) ML0426/Rv2518c (LppS) ML2446/ Rv0483 (LprQ)	Rv0583c (LpqN), Rv0604 (LpqO), Rv2999 (LppY), Rv1228 (LpqX), Rv2341 (LppQ)
	The LppA paralog family		Rv2543 (LppA), Rv2544 (LppB), Rv2796c (LppV)
	Others	ML0319/Rv3584 (LpqE) ML0603/ Rv2413c (LpK) ML0902/Rv2171 (LppM) ML1099/ Rv1252c (LprE) ML1115 /Rv1274 (LprB) ML1116 /Rv1275 (LprC) ML1315/ Rv2116 (LppK) ML1560/Rv2843 ML1966/ Rv3763 (LpqH)	Rv0179c(LprO), Rv0344c(LpqJ), Rv0381c, Rv0460, Rv0679c, Rv0847(LpqS), Rv0962c(LpqP), Rv1064c (LpqV), Rv1270c(LprA), Rv1418(LprH), Rv1541c(LprI), Rv1799 (LppT), Rv1881c(LppE), Rv1921c(LppF), Rv2046 (LppI), Rv2080(LppJ), Rv2138(LppL), Rv2270(LppN), Rv2290(LppO), Rv2330c(LppP), Rv2784c(LppU), Rv3016(LpqA), Rv3623(LpqG)

All lipoproteins categorized by their functional motif. The function of ORF in shade has been experimentally verified. SBP, solute binding proteins.

1.7.1.5.a Transporter; Solute binding proteins of the ATP Binding Cassette transport systems and lipid carriers

The majority of lipoproteins are classified as solute binding proteins of the ATP binding cassette transport system. They import the hydrophilic substrates (phosphate, peptides, iron and sugar) into cells via membrane associated permeases in an energy dependent manner (286) (Table 1.3). A gene deletion study in a mouse infection model showed that several of the mycobacterial solute binding proteins play an important role in intracellular survival in the early stages of infection (34,254). All phosphate binding proteins, 38 kDa Rv0934/PstS-1, Rv0932/PstS-2 and Rv0928/Pst-3/ML2095 (149,296), share the conserved phosphate binding domain as *E. coli* phosphate binding proteins (36,165). In particular, 38 kDa (Rv0934/PstS-1, PhoS1, PhoS/ML0746) (149,296), a lipoglycoprotein, is abundant on the cell surface or in the cultured supernatant of *in vitro* grown *M. tuberculosis* (36). The 38kDa Rv0934/PstS-1 was experimentally shown to have phosphate binding capacity by using immunoblot analysis of *M. tuberculosis* which was cultured in phosphate starvation condition (75).

Comparative genome analysis indicated that 50% of putative mycobacterial lipoproteins are not conserved in other bacteria, but are very similar among *Mycobacterium* spp. particularly between *M. tuberculosis* and *M. leprae* (240). The mycobacterial cell envelope is highly enriched with unusual lipids. The function of these unknown lipoproteins has been speculated to be as lipid carriers for a hydrophobic precursor of those lipids on the cell envelope of mycobacteria. Several recent studies support this hypothesis, demonstrating that LppX/Rv2945/ML0136, LpqW/Rv1166/ML1497, and LprG/Rv1411c /ML0557 (149,296) transport lipid

precursors across the mycobacterial cell envelope (68,142,284). All three lipid carriers were also found in *M. leprae* proteomic analysis implying the importance of their physiological role (172). The *lpqW* disruption mutant and its complementation with *lpqW* have indicated that this lipoprotein acts as a lipid carrier of PIM_4 in the biosynthesis of polar PIMs and LAMs (142,169).

The *lppX*/*rv2945c* is located in a gene cluster which is involved in the synthesis and transport of the PDIM complex to the cell envelope. Disruption of this gene cluster by transposon mutagenesis attenuated *in vivo* growth of *M. tuberculosis*, but complementation with *lppX* was able to recover the growth rate of this transposon mutant. Lipid analysis of both mutants revealed that LppX is responsible for translocating PDIMs across the plasma membrane and releasing into the culture medium (284).

LprG/Rv1411c has high sequence similarity to LppX and is associated with *M. tuberculosis* virulence (17). It has mostly been investigated as a TLR-2 ligand to elicit protective immunity (67,78,270). Drage *et al.* (68) demonstrated that LprG plays an important role in the *in vivo* growth of *M. tuberculosis* by using a *lprG* knockout mutant. In this study, recombinant LprG/Rv1411c from *M. smegmatis* showed high binding affinity with the tri-acylated glycolipid portion of LMs, LAMs and PIMs. The crystal structure of LprG/Rv1411c with PIMs revealed that the hydrophobic pocket of LprG accommodated three acyl chains (68). The results indicate that LprG serves as a lipid carrier to assemble and to transport acylated glycolipids in cell wall biogenesis. Additionally, LMs, LAMs and PIMs bound to LprG enhanced TLR-2 sensing compared to LprG alone (68).

1.7.1.5.b Enzymes

Lipoproteins enzymes can be divided into three subcategories; cell wall metabolic enzymes, degradative enzymes and others enzymes (Table 1.3). However, the exact functions of these enzymes are still unclear (286). SodC/Rv0432/ML1925c (149,296) contribute to the survival of *M. tuberculosis* in activated macrophages, suggesting that it plays an important role in the defense against the oxidative burst produced *in vivo* (229). SodC/Rv0432 was originally assumed to be a lipoglycoprotein localized in the cellular envelope, but was later found to be only a glycoprotein lacking acylation in the culture of *in vitro* grown *M. tuberculosis* (252). However, it is unclear how SodC/Rv0432 avoids or misses the lipid modification step during protein export.

1.7.1.5.c Adhesion and cell invasion

MPB83 and Mce lipoproteins are primarily categorized in the roles of adhesion and cell invasion. Several studies, predominantly using gene knockout mutants, attempted to address the pathogenic roles of MPB83 and Mce lipoproteins in a mouse model. However, conclusion from the results are still unclear (286) (Table 1.3).

1.7.1.5.d Signaling and sensory function

LpqB/Rv3244c/ML0775 (149,296) is located downstream of the two-component signal transduction system MtrAB (see section 1.7.3) (113,286). The *M. smegmatis lpqB* defective mutant revealed that LpqB/Rv3244c interacted with the extracellular domain of MtrB and enhanced MtrA phosphorylation. This mutant also exhibited a large enhancement of susceptibility to multiple antibiotics with diversified action mechanisms.

Therefore, the LpqB in combination with MtrAB possibly affects cell division and cell homeostasis in response to environmental stress (206).

In both *M. tuberculosis* and *M. smegmatis*, LprF /Rv1368 and LprJ/Rv1690 interact and form a tertiary structure with the histidine kinase KdpD which is involved in the signal transduction pathway (296). RpfB/Rv1009 (296) is the only lipoprotein among the five resuscitation-promoting factor (Rpf) proteins of *M. tuberculosis* that stimulate dormant cells to divide (240). The inactivation of *rpfB/rv1009* significantly delayed the growth rate of *M. tuberculosis* in the persistently infected mice model, suggesting a role of RpfB/Rv1009 as a resuscitation-promoting factor (297) (Table 1.3).

1.7.1.5.e Unknown function

The physiological/pathogenic roles of most of the mycobacterial lipoproteins are unknown. These include the 19 kDa/LpqH/ Rv3763/ML1966, LprA/Rv1270c, LppA and LppB, ML0603/33 kDa (45,78,79,115,131,149,158,210,219,222,275,296) (Table 1.3). However, the immunological role of these lipoproteins (described in section 1.8.1) has been intensively investigated in the context of developing a vaccine and elucidating host-pathogen interactions.

1.7.2 Glycoproteins

1.7.2.1 Identification and structure

Glycosylation of proteins was thought to be a unique phenomenon of higher eukaryotes, an event that is either absent or very scarce in prokaryotes (153). The 38 kDa/PhoS1/Rv0934 was first characterized as a glycoprotein associated with, or

covalently linked to, sugars similar to those in lipo/arabinomannan, i.e. mostly mannose (56). By using immunoblot analysis of Concanavalin A, putative mycobacterial glycoproteins were identified including the 38 kDa/Pst1/Rv0934 and the 45 kDa/MPT32/Rv1860 (74). The concept of protein glycosylation in *M. smegmatis* was further enhanced by using the mutated LpqH/Rv3763 which substituted Thr-Thr, the presumed glycosylation motif, to Val-Val. This mutated LpqH/Rv3763 expressed in *M. smegmatis,* abolished the Concanavalin A binding affinity but was unaffected in wild type (109). Eleven different *M. tuberculosis* lipoproteins were screened by a neural network (NetOglyc) analysis and expressed in *M. smegmatis* by using the 19kDa lipoprotein signal peptide (108). Concanavalin A immunoblot detected the glycosylations of eight recombinant lipoproteins including 19 kDa/LpqH/Rv3763, 38 kDa/PhoS1/Rv0934, MPT83/Rv2873, GlnG/Rv0411c, LppN/Rv2270, LppQRv2431, LprI/Rv1541c and SodC (108,296). Recombinant LprA/Rv1270c (222) and LprG/Rv1411c (270) were recently shown to be Concanavalin A-reactive as mycobacterial glycoproteins.

Direct biochemical evidence of protein glycosylation has been demonstrated for only three proteins, 45/47-kDa/MPT32/Rv1860 (64,65), SodC/Rv0432 from *M. tuberculosis* (252) and MPB83 from *M. bovis* (181). By mass spectrometry and N-terminal AA sequencing, two studies (64,65) showed that four of the Thr residues of 45/47-kDa/MPT32/Rv1860 were attached to α-D-mannose, α-D-mannobiose or α-D-mannotiose solely by α(1→2) linkages. Michell *et al*. (181) identified an O-glycosylated Thr-Thr doublet at the N-terminus of secreted MPB83 in *M. bovis* by site directed mutagenesis, Concanavalin A immunoblot and ESI mass spectrometry. MPB83 in *M.*

bovis predominantly contained a mannose and mannobiose via an $\alpha(1\rightarrow3)$ linkage (181). With the same approaches, Sartain *et al.* (252) showed that SodC in *M. tuberculosis* contained six O-glycosylated residues within a 13-amino-acid region near the N-terminus of the protein.

1.7.2.2 Biosynthesis and function

Mycobacterial protein glycosylation resembles eukarytotic protein O-mannosylation and mostly occurs in the Thr/Ser rich region of proteins (252). The eukaryotic O-mannosyl transferases contain an Asp-Glu motif which plays a crucial role in the catalysis of glycosylation (153). The biosynthetic mechanism of mycobacterial glycoproteins is largely unknown. Recently, VanderVen *et al* (299) provided evidence that the Rv1002c/ML0192 which also contained the conserved Asp-Glu motif, was involved in the first step of protein glycosylation. In this study, the Asp-Glu motif of Rv1002c was substituted to either Ala or Asp and Ala-Asp. The *in vitro* enzyme catalytic assay revealed that the wild type Rv1002c efficiently transferred radiolabeled [^{14}C] mannose from guanosine diphosphate mannose to synthetic peptide compared to the substituted mutants (299). By using mass spectrometry, the glycosylation pattern analyses of recombinant hybrid secretory proteins, with/or without Type I signal peptide, showed that the Sec-dependent protein export system was required for protein O-mannosylation in *M. tuberculosis* (299). However it is still not clear whether other glycosyltrasferases are involved in the elongation step of protein mannosylation.

Protein glycosylation plays numerous roles in eukaryotes, including protein stability, protein localization, protein folding, receptor recognition and enzymatic activity

(153). The functional roles of prokaryotic protein glycosylation are still obscure but may contribute to maintenance of bacterial shape, protection against proteolytic digestion, enzyme-substrate binding and direct interaction with the host immune system (298). Additionally, glycosylation of both 45/47-kDa/MPT32/Rv1860 and LprG/Rv1411c is required for T-cell recognition of these proteins in order to serve as Major Histocompatibility Complex-II (MHC-II) restricted epitopes (111,245,270). Glycosylated MPT32 as an adhesion molecule was capable of capturing pulmonary C-type lectin surfactant protein, which is often facilitated in immune suppression by pathogens, from crude human bronchoalveolar lavage fluid (232). The Thr to Val substitutions at the glycosylation site of 19kDa lipoglycoprotein resulted in proteolytic cleavage suggesting glycosylation may serve to protect against proteolysis (109).

1.7.3 Phosphoproteins

In bacteria, protein phosphorylation is a major signal transduction system to sense and adapt to environmental conditions by two conserved components, a histidine protein kinase and a response regulator protein (282). The coupled phosphorylation and dephosphorylation conveys intracellular signals to regulate cellular responses. In response of environmental stimuli, histidine protein kinase is autophosphorylated at a histidine residue of the cytoplasmic sensor domain by its conformational change. This phosphate group of histidine protein kinase is transferred to an aspartate residue of histidine protein kinase. Subsequent phosphorylation of response regulator elicits the regulation of gene expression and intracellular responses (282) (Figure 1.16.A). The *M. tuberculosis* genome encodes 11 pairs of histidine protein kinase- response regulator

known as two-component systems and a few isolated histidine protein kinases and response regulators (46) but only three histidine protein kinase-response regulator s are found in *M. leprae* (Table 1.4).

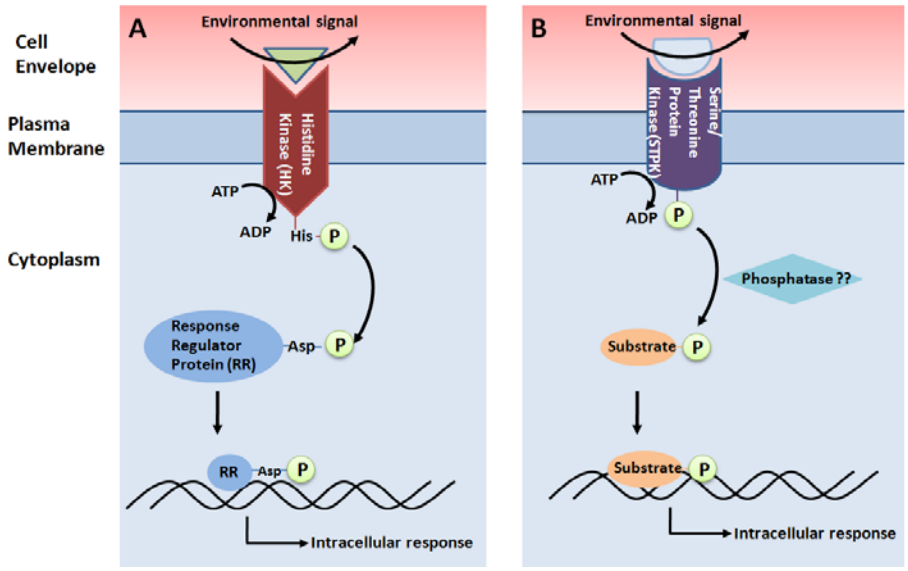

Figure 1.16 Schematic overview of phosphorylation and signal transduction of bacterial proteins. A) Two component system B) Phosphorylation system of serine/threonine protein kinases; the existence of a partner phosphatase is still unclear.

Eukaryotic-like serine/threonine protein kinases have two domains: the kinase domain at the N-terminus transmits from the environmental signal to the C-terminus region, which induces intracellular responses through a transmembrane segment, and phosphorylated substrates (46) (Figure 1.16.B). A partner phosphatase in eukaryotes detaches a phosphate group from Ser, Thr or Tyr of serine/threonine protein kinases as the primary signal transduction pathway but it is still unclear if the process occurred in prokaryotes. Compared to other bacterial genomes, mycobacterial genomes contain a relatively small number of histidine protein kinase-response regulator systems (305). Eleven of the annotated serine/threonine protein kinases in the *M. tuberculosis* genome are assumed to compensate for the paucity of histidine protein kinase-response regulator

(46,305) but only four putative serine/threonine protein kinases are found in *M. leprae* (8). Recent studies revealed that serine/threonine protein kinases regulate intracellular metabolic processes including transcription, cell division, virulence and chemotaxis (96) (Table 1.4).

Table 1.4 Mycobacterial phosphoproteins (adapted from Av-Gay and Everett (8) with permission).

	Gene Name	ORF from *M. tuberculosis*	ORF from *M. leprae*	Signal/ Substrate	Function
Two component system (HK/RR)	MprA/ MprB [325]	Rv0982/Rv0981	ML0174/ ML0174	?	Maintenance of persistent infections
	TcrS/ TcrR [106]	Rv1032/Rv1033	ML0260/ML0261	?	?
	RegX3/ SenX3 [92]	Rv0490/Rv0491	ML2440c/ML2439	Phosphate starvation	?
	DevS/ DevR [122,247]	Rv3132/Rv3133	-/-	Hypoxia, NO	Induction of dormacy
	MtrB/MtrA [324]	Rv3245/Rv3248	ML0774/ ML0773	?	Cell wall Integrity/ cell division
STPKs	PknA [135,300]	Rv0015	ML0017	?/ EmbR	Cell elongation /division
				?/mtFabH	Regulation of mycolic acid biosynthesis
	PknB[135,305]	Rv0014	ML0016	?/ EmbR	Cell elongation /division
	PknE [188]	Rv1743	ML0992*	?	?
	PknF [8,190,300,305]	Rv1746	ML1266	Cell growth / Rv1747 ? /mtFabH (Rv0533)	Membrane transport/ regulation of mycolic acid biosynthesis
	PknG [8,51]	Rv0410	ML0304	Glutamine level	Amino-acid uptake, stationary-phase metabolism
	PknH [8,189]	Rv1266	ML0992*	Stress response/ EmbR, Rv0681, Rv3330	Arabinan metabolism

All genes in *M. tuberculosis* have been demonstrated to autophosphorylate their recombinant forms and their function has been evaluated mostly with gene deletion mutants. Underlined ORF in *M. leprae* indicates a pseudogene. HK, histidine protein kinase; RR, response regulator; STPKs, serine/threonine protein kinases, NO, nitric oxide. *: ML0992c is similar to both PknE/Rv1743 and PknH/Rv1266.

1.7.4 Methylated Proteins

The heparin-binding-hemagglutinin has been shown to bind sulfated or glycoconjugated surface proteins of nonphagocytic cells postulated to be important for extrapulmonary dissemination (179,180,225). Recombinant heparin-binding-hemagglutinin/Rv0485/ ML2454c (149,296) produced in *M. smegmatis* was found to be multiple methylated on 13 lysine residues but the *E. coli*-expressed proteins heparin-binding-hemagglutinin/Rv0485/ML2454c was not observed to be methylated (227,228). Methylated heparin-binding-hemagglutinin was well recognized by sera of tuberculosis and leprosy patients (60,266,276,326) and also induced strong T-cells responses from *M. tuberculosis* infected individuals (290).

M. leprae surface-exposed laminin-binding protein/ML1683c (149) plays an important role in invasion of Schwann cells. Soares *et al*. (276) demonstrated that the recombinant *M. leprae* laminin-binding protein interacts with the extracellular matrix and host cell surface. In this study, the C-terminus region of recombinant *M. leprae* laminin-binding protein was shown to be methylated by immunoblot analysis.

However, the mechanism and function of protein methylation in *Mycobacterium* spp. are still unknown. It has been proven that methylated heparin-binding-hemagglutinin enhanced binding affinity to epithelial glycosaminoglycans by using an anti-methyllysine antibody blocking assay (226). *E. coli* expressed recombinant heparin-binding-hemagglutinin and laminin-binding protein are susceptible to proteolytic digestion. However, the chemical methylation of these recombinant heparin-binding-hemagglutinin and laminin-binding protein by using $NaBH_4$ and formalin, restores the resistance to proteolysis (227).

1.7.5. Pupylated Proteins (Pupylation: prokaryotic ubiquitin-like protein modification)

Pupylation was recently characterized in *M. tuberculosis* (58). In eukaryotes, almost all proteins destined for proteasomal degradation are covalently attached to ubiquitin at lysine (Lys) residues of the C-terminus. Ubiquitin is a long immature polypeptide which is processed by ubiquitin-specific proteases and ubiquitin carboxy-terminal hydrolases. The resultant 76 AAs ubiquitin is first adenylated and activated by an E1 enzyme and ATP. The activated ubiquitin is subsequently transferred to a ubiquitin-conjugating enzyme (E2) and a protein ligase (E3), conjugating at a Lys residue of the target substrate through iso-peptide bonds (33) (Figure 1.17.A).

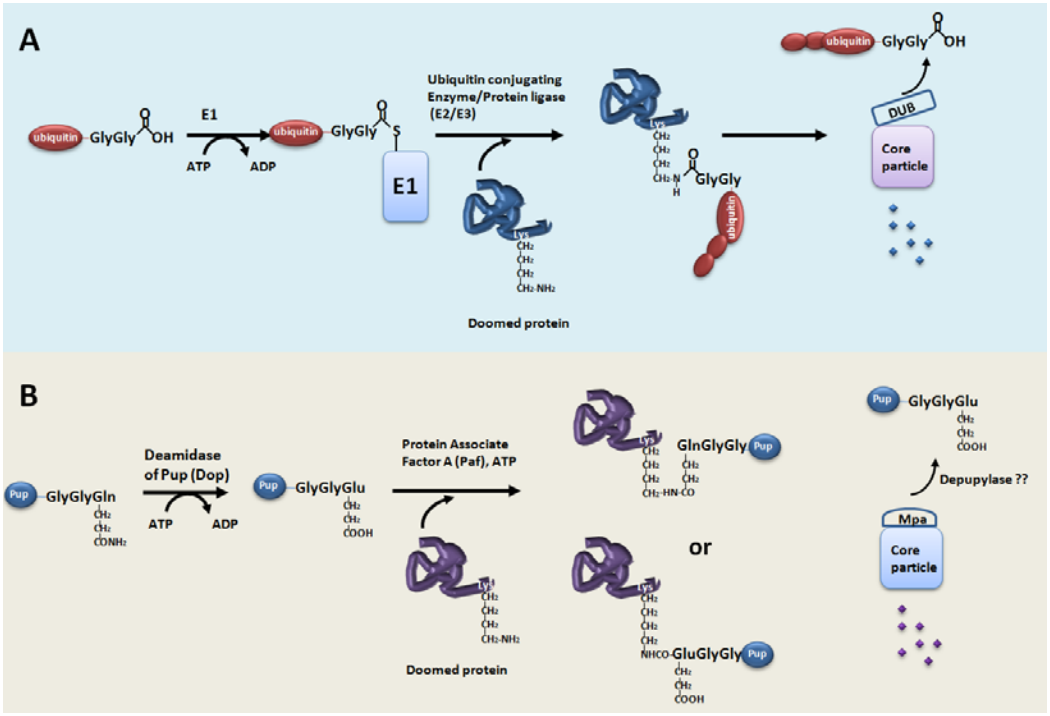

Figure 1.17 Comparison of the ubiquitin and prokaryotic ubiquitin-like protein (Pup) proteasome pathways (adapted from Burns and Darwin (33) with permission). A) Ubiquitin is adenylated at the C-terminal di-glycine, followed by a series of thioesterification reactions and finally conjugated to a substrate protein. Ubiquitin is removed by deubiquitinases (DUBs) and the substrate protein is unfolded and delivered into the proteasome complex consisting core particles. B) Pup is first deamidated at the C-terminal glutamine and then conjugated to substrate proteins. Pup targets proteins to *Mycobacterium* proteasome associated ATPase (Mpa) and the proteasome complex.

E3 ligases contain various substrate binding activities that provide specificity to the ubiquitin–proteasome system. In general, polyubiquitin chains can be recognized by the regulatory complex of proteasomes, then removed and recycled by deubiqutinase for further ubiquitination (57)

Bacterial proteasomes and pupylation have been identified exclusively in Actinomycetes, including *Mycobacterium, Streptomyces, Rhodococcus, Frankia,* and experimentally verified in *Methanococcus jannaschii* by degrading model peptide substrates (57). Similar to ubiquitin, Pup (prokaryotic ubiquitin-like protein/Rv2111/ ML1321) (149,296) is attached at Lys residues of protein substrates via its C-terminus. Pup has a terminal GlyGly motif at the C-terminus followed by either glutamate (Glu) or glutamine (Gln) (Figure 1.17.B). Prior to conjugation to the Lys residue of target proteins, the Gln of Pup is deamidated to Glu via catalysis of Pup deamidase/ Rv2112/ML1320 (149,283,296). Proteasome-associated factor A/Rv2097/ML1328 (149,283,296) catalyses conjugation of Pup to proteasome-substrates complex in the presence of ATP and Pup deamidase (33,57). The mechanism and functions of proteasome in prokaryotic physiology are largely unknown.

Nitric oxide produced by activated macrophage efficiently kills invading pathogenic bacteria; however, *M. tuberculosis* can persist even in the presence of nitric oxide (57,58). The *mpa* (*Mycobacterium proteasome associated ATPase/rv2115/ml1316*) and proteasome-associated factor (*rv2097*) mutants increased *in vitro* nitric oxide sensitivity and attenuated *M. tuberculosis* virulence in infected mouse model compared to the wild type (58). Additionally, *mpa* mutants have been proven to alter the expression levels of Rv2111c, FabD and PanA compared to the wild-type strain, implicating

Mpa/Rv2115 in a protein degradation pathway (221). Genome analysis revealed that *M. leprae* contain Pup and all proteasome related components, including proteasome-associated facto, *mpa* and the protease core genes *prcB* and *prcA* (149) (Figure 1.18). The Pup-proteasome system is conserved in all *Mycobacterium* spp. and might be crucial to intracellular survival of *M. tuberculosis* and *M. leprae* in the presence of the immune response and nitric oxide. However, the mechanism of proteasome-associated nitric oxide tolerance remains unclear in terms of host-pathogen interaction.

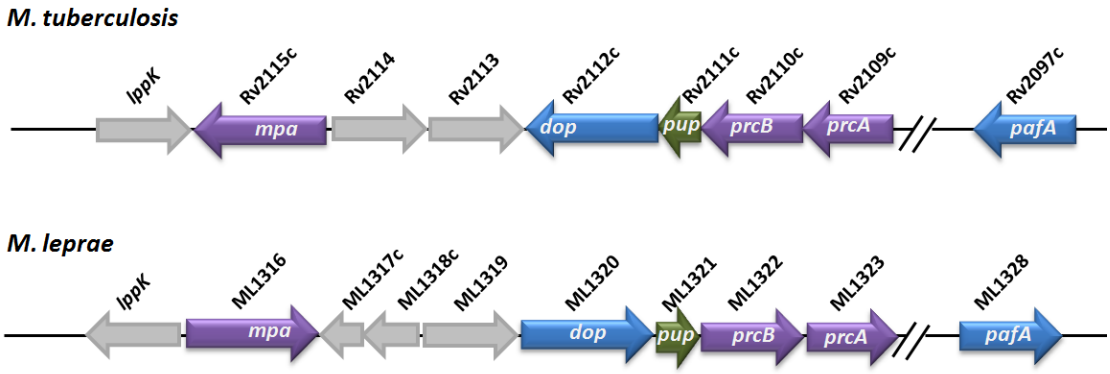

Figure 1.18 Gene arrangement of Pup and proteasome in both *M. tuberculosis* and *M. leprae* (adapted from Darwin *et al*. (57) with permission). Genes of the bacterial proteasome core consist of a β-subunit gene (*prcB*) and an α-subunit (*prcA*). Dop, deamidase of Pup; pafA, proteasome-associated factor.

1.8 Immune response

The immune response to *M. leprae*, like other pathogens, is divided into the innate and adaptive immune response. Adaptive immune response to *M. leprae* infection is thought to be mediated by $CD4^+$ T-cells, which play a critical role in both protective and pathogenic immune response (185). Differentiation of $CD4^+$ T cells is initiated by the innate immune system, which provides costimulatory molecules to allow for activation and proliferation of T cells in response to specific antigens (53). Subsequently, activated

$CD4^+$ T cells produce the polarized cytokines (Th1 or Th2) in response to antigens presented by Major Histocompatibility Class II (MHC-II) complexes on antigen presenting cells. Th1 cells secrete IL-2 and IFN-γ which activate cellular mediated immunity and cytotoxicity T cells (53). In contrast, Th2 cells produce IL-4 and IL-5 mostly activating a humoral response.

More than 90-95% of *M. leprae*-exposed individuals are resistant to disease, while developing a strong immune response to the bacteria (3,81). As previously mentioned (see section 1.2.1), tuberculoid leprosy (TT) patients exhibit strong cellular mediated immunity (CMI) to *M. leprae* antigens and can localize the infection to a few affected lesions. On the other hand, lepromatous leprosy (LL) patients present a weak CMI and higher antibody levels to *M. leprae* antigens, having higher bacterial loads in diffused skin lesions. Therefore, the CMI response is thought to represent protective immunity to *M. leprae* infection, while humoral immune response is considered to favor the multiplication of bacteria in human hosts (summarized in Figures 1.19 and 1.21). Since there is no available facile whole animal model to study *M. leprae* infection in the laboratory, the role of Th1/Th2 immunity in *M. leprae* infection has been indirectly evaluated by *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) or T cell clones derived from leprosy patients, and comparative immunohistology between TT and LL skin lesions.

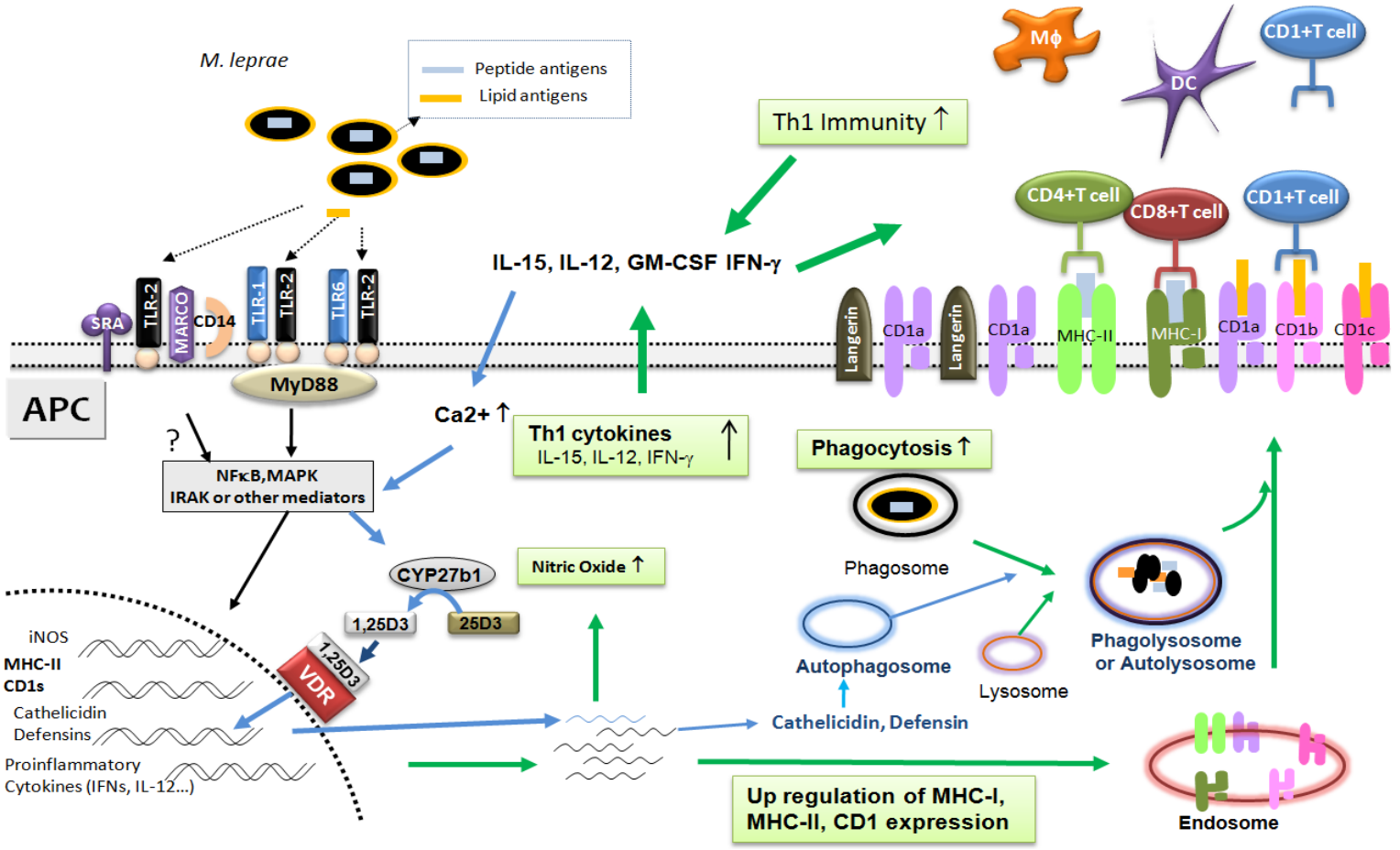

Figure 1.19 Immune response in tuberculoid leprosy (TT) lesions. In TT lesions, several pattern recognition receptors on phagocytes recognize *M. leprae* and initiate innate immunity via a MyD88 dependent signaling pathway or unknown mechanisms (mostly shown in the left side of the figure). Particularly, VitD dependent antimicrobial activity via TLR signaling induces autophagy to control intracellular *M. leprae* (illustrated in blue and described in section 1.7.2). In response to *M. leprae*, activated antigen presenting cells (APCs) produce high levels of proinflammatory cytokines and upregulate the surface expression of MHC-I, II, CD1s instructing adaptive Th1 immunity (illustrated at the right upper corner). Th1 cytokines (IL-2 and IFN-γ) produced by T cells elicit the recruitment of the cellular immune components and enhance the antimicrobial activity and antigen presentation of APCs (shown in green). VDR, vitamin D receptor; GM-CSF, granulocyte-macrophage colony stimulating factor..

Th1 cytokines, IL-2 and IFN-γ, are predominantly found in skin lesions of TT patients. Early immunohistochemical analysis of T-cell phenotype showed that the ratio of $CD4^+$ versus $CD8^+$ T cells in TT lesions was 2:1. $CD4^+$ T helper cells and $CD8^+$ T-cells were also more abundant in skin lesions of TT patients than in those of LL patients (186). MHC class II-restricted $CD4^+$ T-cell clones from TT patients were shown to secrete high levels of IFN-γ (216). Additionally, granulysin-expressing $CD4^+$ T cells frequently appeared in TT skin lesions (211). Later, immunohistochemistry study combined with RT-PCR analysis showed that the expression of IL-12 in TT lesions was 10-fold greater than LL lesions (272). Subsequently, recombinant IL-12 (rIL-12) successfully induced the proliferation of type 1 $CD4^+$ T cell clones from TT patients but failed to stimulate that of type 2 $CD8^+$ T cell clones from LL patients (274).

In contrast, Th2 cytokines, IL-4 and IL-5, and IL-10 highly expressed in LL lesions and $CD8^+$ T cells were more frequently observed than $CD4^+$ T cells by immunohistochemical analysis of these lesions (186). The phenotypes of both T cell subsets were found to be equivalent to those of a naïve subset or a suppressor subset of T cells. $CD8^+$ T cell clones from these patients secreted high levels of IL-4 (186). Sieling *et al.* (274) showed that the presence of IL-4 and IL-10 inhibited IL-12 secretion by monocytes upon *in vitro* stimulation with *M. leprae*. This result suggested that Th2 cytokines representing Th2 immunity in LL lesions could play an important role in T cell anergy, favoring *M. leprae* dissemination in LL lesions. Several hypotheses exist to explain the T cell anergy in LL lesions including the genetic predisposition of human host and the immune modulation of *M. leprae* (see sections 1.8.2 and 1.9). The efficient innate immunity observed in TT skin lesions successfully controls *M. leprae* infection. It

suggests that the failure of innate immunity allows *M. leprae* survival in phagocytes and subsequently delays the initiation of adaptive immunity, contributing to the pathogenesis of leprosy.

Collectively, the progression of leprosy disease highly correlates with the polarized Th1/Th2 cytokines and T cell proliferation in response to *M. leprae*. In conjunction with the clinical features of leprosy (see section 1.2.1), growing evidence indicates that human genetic polymorphisms involved in the innate or adaptive immune response strongly influence the disease outcome (see section 1.9).

1.8.1 Innate immunity

Innate response is initiated through interaction between the pathogen associated molecular patterns (PAMPs) on the surface of pathogens and pattern recognition receptors such as TLRs, C type lectin receptors, NOD and NOD like receptors on or within host phagocytes (289). Among several pattern recognition receptors, TLR-2 plays an important role in immune recognition of *M. leprae* as well as other mycobacteria (128). The heterodimer TLR-2/1 is triggered by tri-acylated lipoproteins while heterodimer TLR-2/6 is activated by di-acylated lipoproteins (289) (Figure 1.20).

Bacterial lipoproteins activate pro-inflammatory signals via TLR-2 and induce Th1 immunity (2,255). TLRs, as integral membrane glycoproteins, share a conserved intracellular Toll /interleukin-1 receptor domain in the cytoplasmic region. The extracellular leucine rich repeats domain of TLRs recognizes PAMPs and is responsible for the signal transduction pathway in innate immune system (289) (Figure 1.20). The leucine rich repeats domain of TLRs is sensitized by a heterodimer with either TLR-1 for

the tri-acylated form or TLR-6 for the di-acylated form (2) (Figure 1.20). X-ray crystallography of TLR-2 and TLR-1/ or -6 revealed that extracellular M-shaped structures of the TLR-2/1 and the TLR-2/6 heterodimer interacted with lipid portion of their ligands (289).

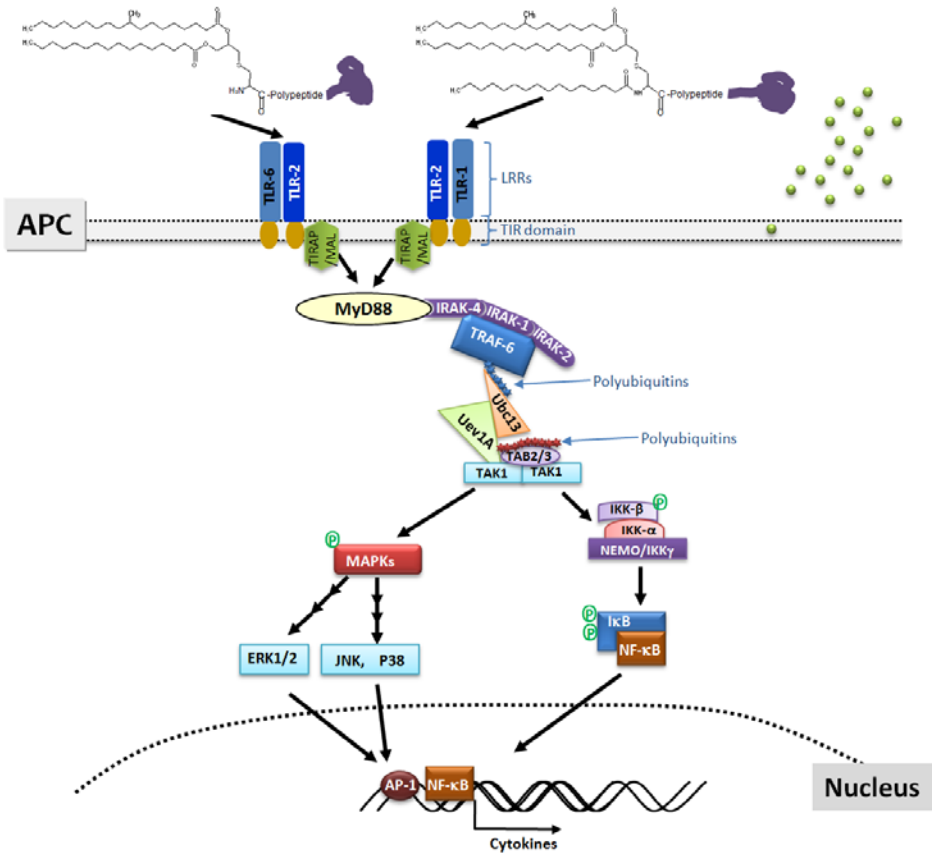

Figure 1.20 Myeloid differentiation factor 88 (MyD88) dependent toll like receptor-2 (TLR-2) signaling pathway (adapted from Takeuchi and Akira (289) with permission). Either TLR-2/1 or TLR-2/6 recognizes the bacterial lipoproteins and recruits MyD88 by bridging with TIRAP/MAL. MyD88 interacts with IRAK-4. IRAK-4 is activated and hyperphosphorylates IRAK-1/ IRAK-2. IRAKs dissociate from MyD88 and interact with TRAF-6 (=E3 ubiquitin protein ligase). TRAF-6 catalyzes Lys 63-linked polyubiquitin on itself and generates an unconjugated polyubiquitin chain activating Ubc13, Uev1A and TGF-β-activated kinase-1 (TAK-1) complex. Subsequent TAK-1 binding protein-2 (TAB-2)-TAB-3 complex induce the phosphorylation of the IKK (IκB kinase subunit)-γ/NF-κB essential modulator (NEMO) and MAPKs. The activation of the IKK complex (IKK-α, IKK-β and IKKγ) leads to the translocation NF-KB to the nucleus by phosphorylation and degradation of IκB. Mitogen–activated protein kinase (MAPKs) including ERK-1/2, p38, and JNK (c-Jun N-terminal kinase) activate activator protein-1 (AP-1) to induce the production of proinflammatory cytokines and antimicrobial activity.

TLR signaling pathways are intracellularly mediated through five Toll /interleukin-1 receptor domain domains containing adaptors, including myeloid differentiation factor 88 (MyD88), Toll /interleukin-1 receptor domain associated protein /MyD88 adaptor like (TIRAP/MAL), Toll /interleukin-1 receptor domain containing adaptor protein-inducing IFN-β /or Toll /interleukin-1 receptor domain-containing molecule 1, TRIF related adaptor molecule and sterile-α and armadillo motif-containing protein. While TLR-4 signaling is mediated by both MyD88 dependent and independent pathways, TLR-2 signaling relies solely on the MyD88 dependent pathway (2). MyD88 by association with IL-1 receptor-associated kinase -1 (IRAK-1) and IRAK-4 triggers mitogen–activated protein kinase, leading to the activation of NF-κB and activator protein-1as transcription factors (220,289).

As TLR-2 ligands, mycobacterial lipoproteins such as *M. tuberculosis* 19 kDa/LpqH/Rv3793 (45,79,205,210,219), LprG (78,114,115), LprA (222), PhoS1 (131,236) and *M. leprae* 33 kDa/LpK/ML0603 (158,319) can mostly activate three different types of cellular mediated immunity (CMI) on antigen-presenting cells such as macrophages and dendritic cells: 1) increase cytokine production (IL-1, IL-12, IL-18, IFN-γ and TNF-α) (143,144); 2) enhance the antimicrobial activity produced reactive nitrogen intermediates, reactive oxygen intermediates and antimicrobial peptides (28,193,320); and 3) induce the expression of MHC-I, II and CD1 on the cell surface and then enhance adaptive immunity (102,104,195) (Figure 1.19). The N-terminal acylated moiety of mycobacterial lipoproteins is mostly responsible for activation of innate immunity and has been demonstrated using signal peptide deletion mutants of

LprG/Rv1411c and LprA/Rv1270 (114,222) or synthetic lipopeptides derived from 19 kDa/LpqH/Rv3793 or 33 kDa/LpK/ML0603 (143).

Additionally, several mycobacterial lipoproteins were investigated as vaccine candidates to induce efficient adaptive immunity, particularly in *M. tuberculosis* infections (131,235,236,270). *M. tuberculosis* 19 kDa/LpqH and 38 kDa/PhoS1 induce antibody production and strong memory $CD4^+$ T cells and $CD8^+$ cytotoxic T cells from human and mice (131,235). Recently, Sieling *et al.* (270) demonstrated that both recombinant LprG/Rv1411c and LprG/ML0557 were recognized by $CD4^+$ MHC-II restricted T cell clones from a leprosy lesion and patient blood.

Pathogenic mycobacteria actively inhibit, subvert or modulate the antigen presentation by suppressing the expression of MHC-I, II and CD1 molecules delaying adaptive immunity (88,102,119,128,139,140,210,219). Differential TLR-2 signaling modulates downstream MAPK signaling pathways contributing to the pathogenesis of mycobacterial disease by the regulation of antimycobacterial activities including apoptosis and MHC-II antigen presentation (128). Prolonged *in vitro* exposure of murine and human macrophages to LpqH/Rv3763, LprG/1411c and LprA/Rv1270c, inhibited the expression of MHC class II molecule (78,131,210,222). Pennini *et al.* (223) demonstrated that LpqH/Rv3763 inhibited the expression of MHC-II transcription activator or the IFN-γ dependent histone acetylation, which control MHC-II transcription activator promoter, in murine macrophage RAW264.7 cell line but not in TLR-2 -/- mice. Immunoblot analysis of both LpqH/Rv3763-stimulated macrophages by using anti-phospho-38 and extracellular signal-regulated kinase (ERK) antibodies showed that the subsequent regulation of MHC-II was mediated by the p38 or ERK pathways (223).

However, this finding needs to be carefully investigated because the MAPKs cascade is redundantly shared with other pattern recognition receptors particularly C-type lectin receptors. Several biochemical studies demonstrated the glycosylation of 19kDa/LpqH/Rv3763 (109), 38kDa/PhoS1/Rv0934(74), LprA/Rv1270c (222) and LprG/1411c (270). The glycosylated MPT32, as an adhesion molecule, was capable to bind the C-type lectin on phagocytic cells (see section 1.7.2.2) (232) implying that glycosylation of mycobacterial lipoproteins could synergistically contribute to this type of immune evasion response. Additionally, glycolipids copurified with recombinant lipoproteins can modulate the immune response which was recently demonstrated using recombinant LprG (68). Since mycobacterial lipoproteins used in previous immunological studies have never been structurally characterized, further studies need to clarify the immune mechanisms in mycobacterial infection by using structurally characterized mycobacterial lipoproteins.

In *M. leprae* infection, immunohistochemical analysis of leprosy lesions showed that TLR-2 and TLR-1 were more strongly expressed in TT lesions than LL lesions (143,194) suggesting that immune recognition via TLR-2 signaling plays an important role in the development of protective immunity against *M. leprae* infection.

In *M. tuberculosis* infection, LMs (130), LAMs (107,130,313), trehalose dimycolate (19), and the 19 kDa lipoprotein (28,147) activated TLR-2 on macrophages with or without the other cell surface receptors, TLR-1, CD14, scavenger receptor A, dectin-1 and macrophage receptor with collageneous structure, to produce the subsequent proinflammatory cytokines (TNF-α, IFN-γ, IL-12) and antimicrobial activity (Table 1.5) (Figure 1.19). As previously described, the structures of these mycobacterial PAMPs are

similar to those of *M. leprae* with some structural modifications. It remains to be addressed whether the structural differences of PAMPs between *M. leprae* and other pathogenic mycobacteria affect the TLR-2 signaling pathway in terms of the pathogenesis of leprosy.

Table 1.5 TLR recognition of mycobacterial components (adapted from Jo *et al.* (128) with permission)

Mycobacterial components	TLR	Species
33 kDa lipoprotein[143,319]	TLR-2	*M. leprae*
MMP-II[166]	TLR-2	*M. leprae*
19 kDa lipoprotein/LpqH[28,210]	TLR-2	*M. tuberculosis, M. bovis*
38kDa lipoprotein/PhoS1[131]	TLR-2, TLR-4	*M. tuberculosis*
LprA[222]	TLR-2	*M. tuberculosis, M. bovis*
LprG[78]	TLR-2	*M. tuberculosis, M. bovis*
LMs[231]	TLR-2	*M. tuberculosis, M. bovis*
AraLAMs[313]	TLR-2	*M. smegmatis*
PIMs[90]	TLR-2	*M. tuberculosis, M. bovis, M. smegmatis*
Trehalose dimycolate[19]	TLR-2	*M. tuberculosis*

A recent major research advance in understanding mycobacterial innate immunity was that the vitamin D pathway plays a key role in antimicrobial mechanisms. Vitamin D receptor is intracellularly activated by 1,25D3, an active form of VitD. The 25-hydroxy vitamin D3-1α-hydroxylase (CYP27b1) is responsible for catalyzing 25D (an inactive form of vitamin D) into 1,25D3 (an active form of vitamin D) (323) (Figures 1.12; shown in blue). Analysis of gene expression profiles showed that Vitamin D receptor, CYP27b1 and cathelicidin were highly induced in skin lesions of TT patients (194). In response to *M. tuberculosis*, upregulated 1,25D3 and Vitamin D receptor highly induced the expression of both cathelicidin and defensin and subsequently activated autophagy, which is destructive to intracellular pathogens (323). Recently, Shin *et al.* (267) revealed that the 19 kDa/LpqH from *M. tuberculosis* causes the upregulation of

autophagy, NO, and antimicrobial peptides. Therefore, VitD antimicrobial activity via TLR-2 signaling plays an important role in the control of mycobacterial infections.

1.8.2 Immune modulation

Mycobacteria enter into phagocytes through receptor mediated phagocytosis. These receptors responsible for entry are the complement receptors and C-type lectin receptors, including mannose receptor (CD206), DC-SIGN (CD209, dendritic cell-specific intracellular adhesion molecule-grabbing nonintegrin) and scavenger receptors. The receptors recognize the carbohydrate containing molecules in mycobaterial envelopes, such as ManLAMs, PGL-I, glycoproteins (262). These molecules can modulate the intracellular signaling cascades of the host and suppress CMI, favoring the intracellular survival of mycobacteria (10,102,246). This immune modulation is thought to be an important mechanism of T cell anergy which appears in LL skin lesions (Figure 1.21).

M. leprae enter macrophages through interaction of complement (C3), complement receptors on phagocytes and PGL-I of *M. leprae* (257,258) suppressing the IL-2 dependent pathway of Th1 cell activation (37,178,258). ManLAMs interact with mannose receptors and DC-SIGNs on macrophages, dampening antimicrobial activity by modulating the production of cytokines and nitric oxide (130,262). Although different from macrophages, DC-SIGN on dendritic cells binds ManLAMs to uptake mycobacteria and suppresse dendritic cells maturation (208). The virulent mycobacteria in phagosomes actively produce ManLAMs which inhibit the Ca^{2+} dependent recruitment of type 3 phospatidyl inositol 3- kinase into the phagosomes (130).

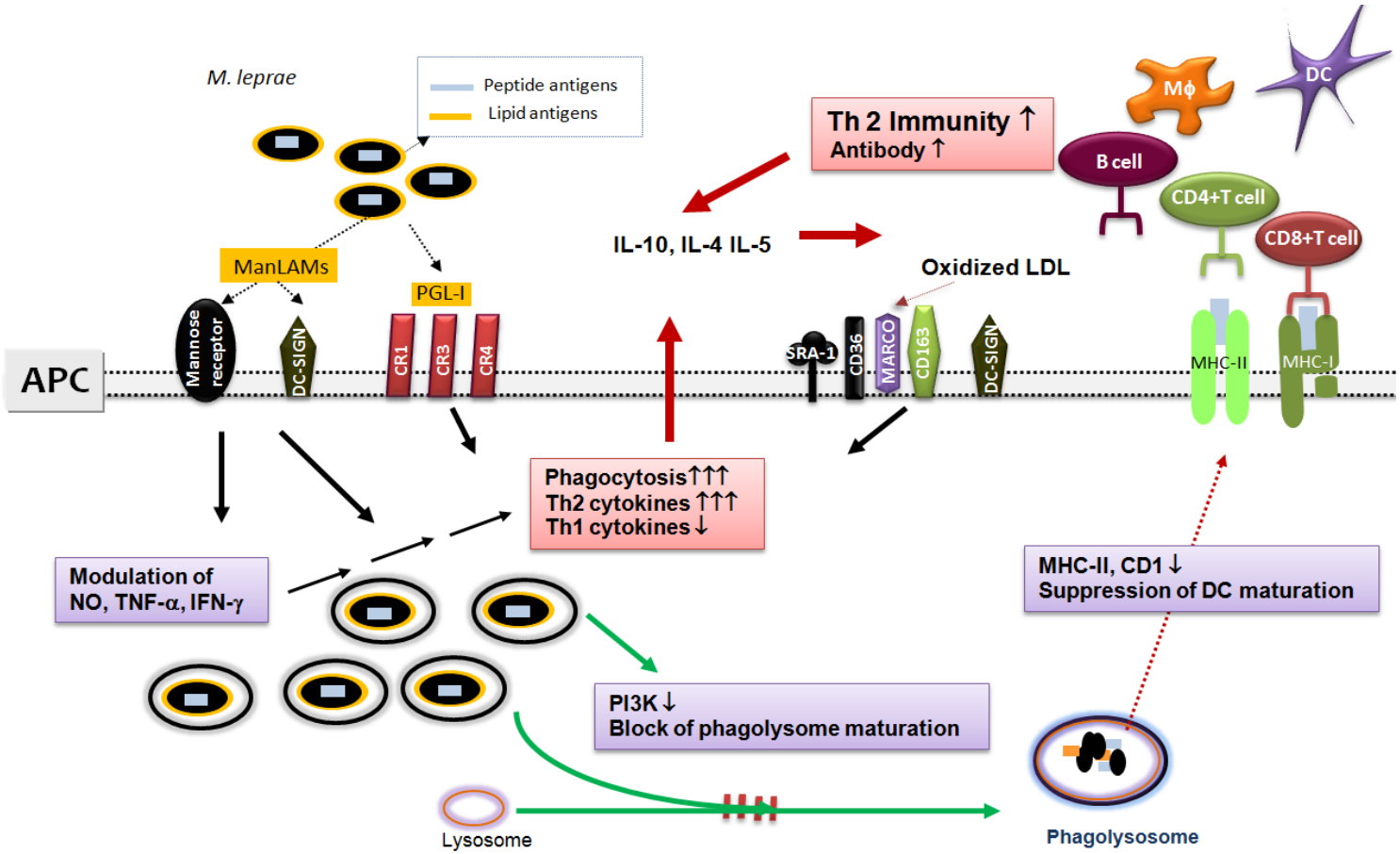

Figure 1.21 Immune response in lepromatous leprosy (LL) lesions. Some PAMPs of *M. leprae*, particularly carbohydrate-containing cell surface molecules, enhance phagocytosis of *M. leprae* by interacting with host pattern recognition receptors, primarily complement receptors (CRs) and C-type lectin receptors (CLRs) Purple boxes indicate the immune modulation of ManLAMs as major virulence factors. *M. leprae* in the phagosome of antigen presenting cell (APCs) actively produce ManLAMs which inhibit phagolysosome maturation, resulting in the suppression of dendritic cells maturation. This event allows escaping *M. leprae* antigens to be presented and to induce Th 2 immunity in LL lesions (illustrated in red). The resulting Th 2 cytokines suppress the antimicrobial activity of APCs but also deposit host oxidized low-density lipoproteins (LDLs) in *M. leprae* infected phagocytes, favoring *M. leprae*.

This action depletes phosphatidyl inositol 3-phosphate in the membrane of phagosomes and inhibits normal phagolysosomal maturation (130) (Figure 1.21). Subsequently, it allows mycobacteria to avoid killing and the presentation of mycobacterial antigens to T cells.

1.8.3 CD1 restricted T cells

The CD1 family of MHC-class-I-like glycoprotein (CD1a, CD1b, CD1c, CD1d and CD1e) presents both foreign and self lipids to T cells (11,29,199). In antigen presentation by CD1, the alkyl chain(s) of an amphipathic lipid antigen is (are) inserted into the hydrophobic and invariant antigen binding pocket of CD1 but the hydrophilic head of the lipid antigen is exposed out of this binding pocket (11). Nonpolymorphic CD1 molecules present many mycobacterial nonpeptide antigens including LM, PIM, LAM (59,268) mycolic acids (146,196), didehydroxy-mycobactin (198), mannosyl β-1-phosphomycoketides (174,197) and sulfolipid Ac_2SGL (91) (Figures 1.7, 1.8 and 1.22) and activate clonal $CD1^+$ T cells. Importantly, the immune response to these nonpeptide antigens has been more frequently observed in patients infected by *M. tuberculosis* or *M. leprae* compared to healthy individuals with positive tuberculosis skin tests (59,91).

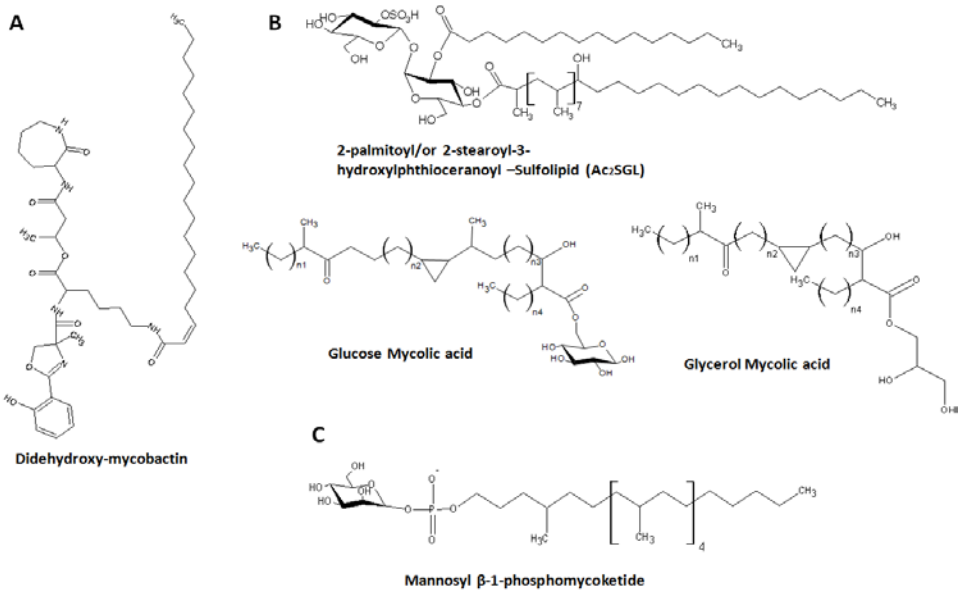

Figure 1.22 Chemical structures of CD1 restricted mycobacterial antigens (adapted from Barral and Brenner (11) with permission) A) CD1a antigens. B) CD1b antigens. C) CD1c antigens. n1=17, n2=12, n3=14, n4=24.

CD1 restricted T-cells activated by mycobacterial antigens contribute to developing protective immunity in mycobacterial infection by releasing high levels of IFN-γ (269), but also by proliferation of cytolytic T cells (12,194). These cytolytic T cells efficiently kill cells infected by live virulent *M. tuberculosis* (281). The cytolytic activity of $CD1^+$ T cells in mycobacterial infections is categorized into two types: 1) $CD4^-CD8^-$ CD1 restricted T cells kill the mycobacteria-infected cells by the induction of apoptosis, but do not kill intracellular *M. tuberculosis.* LAMs and glucose monomycolate are presented to $CD1b^+$ T cell lines derived from leprosy patients (196,268); 2) $CD8^+$ CD1 restricted T cells lyse the infected cells via the expression of granulysin and kill approximately 50% of intracellular bacteria (280,281). Most CD1 antigens are recognized by these types of T cell lines. Recently, a $CD4^+$ CD1 restricted T

cell clone was found in *M. tuberculosis* infection and recognized glycerol monomycolates but its cytolytic activity was not characterized yet (146).

Higher levels of $CD1^+$dendritic cells are found in the skin lesions of TT patients compared to those in LL skin lesions (144,271,273). Langerins, as a C-type lectin receptor, are highly expressed on the surface of these $CD1^+$dendritic cells which are known as Langerhans cells (273). Langerhans cells in skin lesions of TT patients coexpressed CD1a and langerin, and efficiently presented non-peptide antigens which were assumed to be the mycolic acids and arabinogalactan complex (273). However, the structure of antigens presented by CD1a has not been defined.

After human blood monocytes were infected with *M. tuberculosis*, flow cytometric analysis of these cells showed that lipid antigen presentation by CD1b restricted dendritic cells was detected within 24 hr. However, peptide antigen presentation of MHC-II restricted dendritic cells was found to be delayed or absent after four days though the dendritic cells were fully mature (104). Therefore, CD1 restricted T cell immunity plays an important role in controlling pathogenic mycobacteria in the early stages of infection.

1.9 Host genetic influences on human leprosy

Several human genetic studies have provided evidence of linkage between genes of the immune response on chromosome 6, 9, 11, 12, 13, 15, 16, 17 and 20, and leprosy susceptibility (3,95,262). Based on results of complex segregation and linkage analysis, genetic susceptibility was found to influence the immune response in leprosy as a two stage model (3,262). At stage 1, the group1 genes (Parkin and Parkin co regulated gene

(PARK2/PACRG), lymphotoxin A and natural resistance-associated macrophage protein 1) contribute to establish innate immunity eventually leading to leprosy resistance in 90-95% of *M. leprae* exposed individuals (Figure 1.23). At stage 2, 5-10% of susceptible individuals developed TT (CMI response), BT, BL and LL (humoral immune response) depending on group 2 genes such as those for TNF-α, TLR-1/2, IFN-γ, IFN-γ receptor, IL-10, MHC-I, MHC-II, transporter associated with antigen processing (TAP), IL-12 and its receptor (IL12RB1) and Vitamin D receptor (VDR) (3,262) (Figure 1.23).

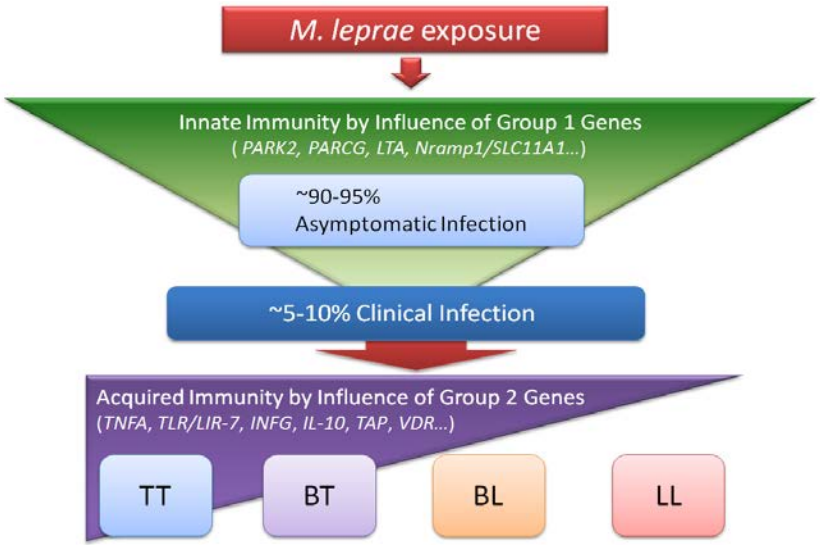

Figure 1.23 Two-stage model of genetic susceptibility on human leprosy. Upon *M. leprae* exposure, group 1 genes result in either susceptibility or resistance by establishing innate immunity. Group 2 genes in 5-10% of infected individuals determine the clinical presentation of leprosy by affecting acquired immunity.

One of the most important finding in leprosy research is that loci within *PARK2* and a coregulated gene, *PACRG*, located on chromosome 6 q25-q27 are correlated with the development of human leprosy, despite the fact that leprosy is clearly an infectious disease (182). Both genes are liked to susceptibility to Parkinson disease. PARK2 codes for E3 ubiquitin–protein ligase that is involved in the delivery of polyubiquitinated proteins to the proteasome complex in other neurodegenerative diseases (95) but the

function of PACRG is unknown. Both genes are strongly expressed in Schwann cells and monocyte derived macrophages (182). Single nucleotide polymorphisms in the 5′ regulatory region shared by both *PARK2* and *PACRG*s, were found to be associated with susceptibility to general leprosy by genome scans of both a Vietnamese patient population and a Brazilian population. Additionally, two of the single nucleotide polymorphisms (PARK2_e01, position 697; PARK2_e01, position-2599) associated with leprosy susceptibility (182). However, these single nucleotide polymorphisms in an Indian population revealed different effects in the regulation of genetic susceptibility to leprosy (95,261); the role of these genes in *M. leprae* infection is still not clear.

Several studies showed that TLR-1 and 2 genes contribute to the development of leprosy. Recent genetic polymorphism studies support that TLR-1 T to G nucleotide substitution at 1,805 single nucleotide polymorphism of TLR-1 (I602S), is associated with a decreased incidence of leprosy among white Europeans (129,316). Significantly, this study provided the first experimental evidence to directly evaluate the biological function of mutated TLR-1 in conjunction with gene susceptibility to leprosy. A common single nucleotide polymorphism, I602S, within TLR-1, leads to aberrant trafficking of TLR-1 to the cell surface and diminishes responses of blood monocytes to bacterial lipopeptides (129).

In general, genetic polymorphisms could potentially affect clinical outcomes of leprosy. However, the great degree of genetic diversity within and among populations and complexity of molecular interactions in individuals interfere in efforts to analyze the potential risk of such human genetic factors for leprosy (95).

1.10 Immune based diagnosis

As previously discussed in section 1.2.1, all leprosy patients characteristically exhibit an immune response to *M. leprae* antigens; tuberculoid leprosy (TT) or paucibacillary leprosy (PB) patients predominantly present CMI to bacterial antigens but lepromatous leprosy (LL) or multibacillary leprosy (MB) patients exhibit humoral immune response. Therefore, a substantial amount of research focused on developing immune based diagnostic tests for the diagnosis of leprosy (6,7,24,32,41,66,71-73,80-82,84-87,187,277,306).

House hold contacts (HHCs) with close contact to MB patients, develop strong CMI and delayed type hypersensitivity to *M. leprae* antigens but do not present clinical symptoms of leprosy (85). Therefore, CMI based diagnostic tests have been extensively investigated to detect *M. leprae* infection in the early stages and also to distinguish it from infections due to other mycobacterial exposure/infection.

As previously described, most individuals exposed to *M. leprae* do not develop the disease, but raise an effective CMI response to *M. leprae* (3,81,85). A CMI based assay to *M. leprae* specific antigens is thought to be the most promising diagnostic tool to detect asymptomatic leprosy in the early stages of *M. leprae* infection (24,84,85). This immune response to *M. leprae* antigens has proven to be predominantly dependent on the presence of memory T-cells secreting IFN-γ and IL-2 as the "inducer" of CMI (218). Therefore, CMI based diagnosis has focused on evaluating the ability of memory T cells to produce IFN-γ in response to a panel of *M. leprae* specific antigens as a consequence of mycobaterial infection and exposure (49,201,217). In order to be used routinely in clinical practice, this type of diagnostic assay has been developed to measure either local

delayed type hypersensitivity *in vivo* or the IFN-γ level released *in vitro* to *M. leprae* specific antigens (24,84).

1.10.1 Skin test

delayed type hypersensitivity is manifested by infiltration of T cells and monocytes into the affected tissues (217). It occurs 24-72 hr after an immunized or infected individual is challenged by microbial antigens (218). In 1890, the tuberculin skin test was developed for measuring local delayed type hypersensitivity reflecting past or recent exposure to *M. tuberculosis* using crude *M. tuberculosis* antigens (purified protein derivatives of *M. tuberculosis*; PPD) (218).

In 1919, Mitsuda revealed that volunteers with or without leprosy developed a local granulomatous induration to injected Lepromin-H 3-4 weeks later. This Lepromin-H had been prepared by autoclaving suspensions of macerated nodules from untreated LL patients and evoked the reaction known as the Mitsuda reaction (184). Later, it was observed that PB/TT patients and HHCs showed an early response to Lepromin-H between 48-72 hr, after injection (76). This Fernandez reaction represents a delayed type hypersensitivity response to *M. leprae* soluble antigens similar to PPD of *M. tuberculosis* (24). However, a substantial amount of healthy individuals in endemic region produced a false positive response to the Lepromin H (24,262). Thus, the lepromin test is only used for classification and prognosis of leprosy (24,262,277).

Two more recent skin test reagents, Convits' and Rees' antigens, were prepared by fractionation of armadillo derived *M. leprae* to produce a new type of the leprosy skin test antigen (24). Efficacy of both antigens on human subjects was extensively studied in

different geographical endemic countries. It was proven that both antigens induced positive reactions in BT and TT patients but were negative in LL patients (48,99). However, both Convits' and Rees' antigens induced variable skin test responses in HHCs and endemic healthy population (100).

A major advantage of the skin test is that it costs little to prepare and does not require any laboratory infrastructure to perform the diagnostic test in poor resource settings where leprosy is most prevalent. (217,218). In order to improve the specificity of the leprosy skin test, fractionation of native whole cell lysate of *M. leprae* generated two subcellular fractions, cell wall antigens without LAMs (MLCwA) and soluble antigens devoid of LAMs (MLSA-LAM); Both MLCwA and MLSA-LAM had been subjected to Triton X-114 phase separation to deplete the large amount of LAMs responsible for T-cell cross-reactivity and suppression of their immune response (24). An *in vitro* stimulation study on peripheral blood mononuclear cells (PBMCs) showed that both MLCwA and MLSA-LAM induced strong CMI response in leprosy patients and HHCs but little cross-reactivity in PPD-positive healthy individuals in leprosy endemic regions (167,306). These products had completed Phase II trials in Nepal in 2010. Data analysis now on the way will reveal the diagnostic potential of these antigens.

1.10.2 Antigen identification

The major focus of research in the development of leprosy diagnosis was on studies to identify *M. leprae* specific antigens, those stimulating both B- and T-cell responses. Identification of *M. leprae* antigens, particularly protein based antigens, has been accomplished using two different approaches: 1) immunological and biochemical

analysis of *M. leprae* derived molecules and; 2) *in silico* analysis of mycobacterial genomes (a postgenomic approach). Table 1.6 summarizes all antigens which have been evaluated by both CMI and serological assay formats.

The first immunological and biochemical approach to antigen identification focused on the characterization of the dominant *M. leprae* protein antigens, those which are abundantly expressed in *M. leprae.* They include GroES (243), MCP-II (322), Sod A (322), major membrane proteins-I (MMP-I) (294,315) and MMP-II (224) as well as the glycolipid antigens, LAMs, PIMs and PGL-I (117) (see section 1.6.2). However, the challenge encountered with these abundant *M. leprae* protein antigens is that many of these antigens share over 80% of amino acid sequence identity/similarity with orthologues in other mycobacteria resulting in low specificity of the corresponding diagnostic assays (66). Although *M. avium* contains an orthologue of MMP-I (294,315), MMP-I elicited strong T-cell proliferation from peripheral blood mononuclear cells (PBMCs) of TT patients (263,294). MMP-II shares higher homology with orthologues among mycobacteria but this protein is low abundance in other mycobacteria and elicited strong seropositivity in leprosy patients but negligible reactivity in *tuberculosis* (TB) patients (62,160,166,212,263).

Completion of whole genome sequencing of *Mycobacterium* spp. was anticipated to resolve the low specificity of both TB and leprosy diagnostic tests (6,7,66,73,81,278,279). A post genomic approach was utilized to identify TB antigens. An early antigenic target was the 6 kDa (ESAT-6) secretory protein. It appears in culture filtrated proteins of *M. tuberculosis* and is encoded by genes in the region of deletion-1 which is deleted from all BCG vaccine strains (4,5,307).

Table 1.6 *M. leprae* antigens and their potential use in diagnostics (adapted from Goulart and Goulart (94) with permission)

***M. leprae* antigens**	**Homology with other mycobacteria**	**Immune responses**	**Types of test to be used**	**Observation**
MLSA-LAM	high	CMI	Skin test	Highly associated with abundant immunogens (7, 65, 45, 35, 28, 18 and 10kDa), *M. leprae* lysates and PPD.[167,306]
MLCwA	high	CMI	Skin test	Highly associated with abundant immunogens (7, 65, 45, 35, 28, 18 and 10kDa), *M. leprae* lysates and PPD.[167,306]
GroES	89.9% with *M. tuberculosis*	Humoral immunity	ELISA	High titers of IgG1 in all disease spectra.[118]
p25-40 derived from *M. leprae* GroES	86.6% with *M. tuberculosis*	CMI	IGRA	Specifically detected 5-80% of PB and HHCs.[42,43,138]
MMP-I/ 35 kDa/ML0841	92.2% with *M. avium*	Humoral immunity	ELISA/ IGRA	Specifically detected 97.5% of MB.[293] *M. smegmatis* -derived 35-kDa induced strong T cell immunity in the majority of PB and HHCs but not in MB.[294]
p60-76, p132-151, p206-224 and p267-286 derived from MMP-I	92.2%, 84%, 94% and 89% with *M. avium*, respectively	Humoral immunity	ELISA	Specifically detected *M. leprae* exposure 30% for PB, 48% for MB, 22% for HHCs.[44]
p 206-2242 derived from MMP-I	94% with *M. avium*	CMI	IGRA	Specifically detected *M. leprae* exposure 63% for PB, 66% for HHCs.[314]
MMP-II	90.1% with *M. tuberculosis*	Humoral immunity	ELISA (IgG)	Elicited humoral responses predominantly in leprosy patients but negligible responses in TB patients.[112,114] Detected 39-48% of PB and 85% of MB. Antibody titer decreased 50% to 18% after chemotherapy.[103,133,159]
ESAT-6	38% with *M. tuberculosis*	CMI /humoral immunity	IGRA	Induced a strong CMI from PBMCs of leprosy patients as well as *tuberculosis* patients and TB HHCs.[86,279]
CFP-10	40% with *M. tuberculosis*	CMI /humoral immunity	IGRA	Cross-reactive with *M. tuberculosis.*[87,278]

M. leprae antigens	Homology with other mycobacteria	Immune responses	Types of test to be used	Observation
ML0308, ML2498	No	CMI/ Humoral	ELISA IGRA	Detected TT and LL patients.[7]
Ep1-0308 derived from ML0308	No	CMI	IGRA	Proposed a simple robust whole blood test for early diagnosis of leprosy.[6]
ML0678, ML0757, ML2177, ML2244, ML2498	No [a]	Humoral immunity	IGRA	Strongly recognized circulating antibodies in MB patients.[7]
ML0008, ML0126, ML1057, ML2567	No	CMI	IGRA	Showed IFN-γ responses only in paucibacillary patients.[277]
Peptides:p67, p68 and p69 from ML1419; p75 from ML2177; p91 from ML2347; and p92 from ML2452	No	CMI	IGRA	Detected 60-100% of PB patients and 50-100% of HHCs.[277]
ML0576, ML1989, ML1990, ML2283 and ML2567	No		IGRA	Significantly inducted IFN-γ and detected 94% of negative PGL-1 patients. ML2283 is most specific antigen to detect *M. leprae* exposure/infection.[82,83]
Peptides (ML1989 p11, ML1990 p7, ML2283 p5, and ML2567 p3)	No	CMI	IGRA	Detected all PB patients (n=6) and 13 out of 14 HHCs.[83]
LAMs PGL-1 ND-O-BSA NT-P-BSA	No	Humoral immunity	ELISA/ ML flow test	Detected 74–100% of MB and 13.6% of PB patients. [31,32,93,215] Used for operational classification.[40,41]
ML0405, ML2331	38%& 82% with *M. tuberculosis*	Humoral immunity	ELISA (IgG)	Specifically identified LL/borderline lepromatous (BL) patients Detect 84-88% of MB and 16-20% of PB.[239] (71,73)
LID-1, fusion construct of ML405 and ML2331	N/A	Humoral immunity	ELISA (IgG)	Detected 92% of MB and 48% of PB patients.[71,73]

a, ML0757 showed 77% with *M. tuberculosis* CDC at 2010 search; NA, not shown in literature. ML flow test, *M. leprae* lateral flow test; IGRA, *in vitro* interferon-γ release assay; ELISA, enzyme-linked immunosorbent assay:

In vitro IFN-γ release assay (IGRA) of PBMCs to ESAT-6 specifically differentiates TB patients from healthy individuals who were immunized with the BCG vaccine or exposed to environmental mycobacteria (218,307).

M. leprae ESAT-6/ML0049 and CFP-10/ML0050 show only 38% and 40% of AA sequence identity with orthologues of *M. tuberculosis* (149,296). Consequently, these antigens generated no cross-reactive CMI or humoral responses in mice immunized with either *M. leprae* or *M. tuberculosis* (278,279). However, both *M. leprae* ESAT-6 and CFP-10 induced a strong CMI from PBMCs of leprosy patients as well as TB patients and HHCs of TB patients in endemic regions (86,87).

Comparative bioinformatic analysis of the genomes of *Mycobacterium* spp. allowed the identification of *M. leprae* unique proteins into a group called as "hypothetical unknowns" (functional class VI) (47,149,277). Several hypothetical unknowns containing T-cell epitopes restricted to major HLA (human leukocyte antigen)-DR alleles were identified as potential antigen candidates and were evaluated in leprosy and control populations of various endemic regions by using IGRA (6,7,80,81,85,277). Geluk *et al.* (80) revealed that the selected hypothetical unknowns proteins, ML0576, ML1989, and ML1990, ML2283 and ML2567 induced significant levels of IFN-γ secretion from PBMCs of PB patients and HHCs, but not from most endemic healthy individuals and TB patients. However, a few of endemic controls and TB patients responded to these antigens due to previous exposure to *M. tuberculosis* or environmental mycobacteria in endemic settings.

In order to enhance the specificity of IGRA, Geluk *et al* (81) and Spencer *et al.* (277) generated several peptide antigens derived from hypothetical unknowns.

Subsequently, IGRA of PBMCs from PB patients and HHCs revealed that these peptide antigens can detect 60%-100% of the PB patients and 50-100% of the leprosy HHC group. Overall, these peptides enhanced the specificity of IGRAs which could distinguish leprosy patients or HHCs from individuals exposed to other mycobacteria but the inherent low sensitivity of these tests made it difficult to detect individuals with preclinical leprosy from healthy populations.

Additionally, ML0405 and ML2331 were identified from the *M. leprae* genomic expression library using sera of untreated LL patients (239). ML0405, ML2331 and LID-1, a fusion protein of both proteins, were capable of detecting antibodies in sera of LL and BL patients in various geographical regions (48,50).

1.10.3 In vitro *Interferon-γ Release Assays (IGRAs)*

In mycobacterial infections, IFN-γ is a potent pro-inflammatory mediator to induce both memory $CD4^+$ T cells and $CD8^+$ T cells (201,217). Since IFN-γ is a stable cytokine and robustly induced upon stimulation, the level of IFN-γ secretion by PBMCs is facilitated to measure the induction levels of CMI to mycobacterial antigens as an outcome of mycobaterial infection (49,201,217).

As previously mentioned, abundant protein antigens of *M. leprae* as well as *M. tuberculosis* share a great deal of sequence similarity with orthologues among other mycobacteria. The antigens with this type of sequence similarity when applied to IGRAs generated high sensitivity but poor specificity particularly in endemic regions where most individuals are exposed to environmental mycobacteria and are vaccinated with BCG (82,218,263). A new type of IGRA emerged in the case of TB diagnosis which has been

developed commercially and is available as the QuantiFERON-TB or QuantiFERON-CMI tests. This test achieved impressive specificity for TB diagnosis by using overlapping peptide antigens derived from *M. tuberculosis* ESAT-6 (77,201,218).

Quanti-FERON types IGRAs have been adapted to develop CMI-based assays for early diagnosis of preclinical leprosy (6,7,80,82,85,277). As previously described, IGRAs based on hypothetical unknown proteins or their peptides, can detect individuals exposed to or infected by *M. leprae*, especially HHCs with MB or PB patients (6,7,80,82,85,277). Recent studies in five different geographical regions (Brazil, Nepal, Bangladesh, Pakistan and Ethiopia) revealed that IGRA of the peptides derived from hypothetical unknowns (see section 1.10.2) specifically discriminated *M. leprae*-exposed healthy individuals from endemic healthy populations but showed low sensitivity to detect the *M. leprae* exposure (82,84). Therefore, further searches for *M. leprae* specific antigens should continue to enhance both sensitivity and specificity of leprosy diagnosis in endemic regions.

1.10.4 Serodiagnosis

Leprosy serodiagnosis is capable of detecting 75-100% MB patients but show very poor performance in the case of HHCs and PB patients where only 15-40% are positive (1,73,263). Antibody levels in leprosy serodiagnosis are often used as a surrogate marker to measure the bacterial load in skin lesions of leprosy patients. Serological assays to *M. leprae* specific PGL-I and MMP-I detected more than 95% and 89% of MB patients and 33% and 40% of PB patients respectively, and showed no response in the case of TB patients or endemic healthy volunteers (31,32,41).

Seroreactivity of leprosy patients to MMP-I decreased after effective chemotherapy (314). Serodiagnosis of leprosy has been used to classify leprosy patients in order to assess the efficacy of chemotherapy and to predict relapse (263).

Serology based antibody detection is the most feasible diagnostic format to apply in leprosy endemic regions (49,72). Currently, the only available field friendly test for diagnosis of leprosy is the detection of circulating anti-PGL-I antibodies in patents' sera (72,73,83). The terminal trisaccharide unit of PGL-I, which is responsible for *M. leprae* specificity in serodiagnosis, is conjugated to bovine serum albumin (BSA) and human serum albumin (HSA) via either an octyl linker (NDO-BSA/HSA) or a phenyl linker (NT-P-BSA) (32,215). The resulting semi-synthetic glycoconjugates are incorporated into user friendly lateral flow tests (known as ML flow test) (32).

Recently, several studies revealed the potential of leprosy serodiagnosis to identify individuals with the risk of developing leprosy (72,93,103,133). A five-year monitoring study of 1,396 of HHCs using simple ML flow test identified 10.4% of HHCs who are test positive and subsequently developed leprosy symptoms. The disease development of HHCs with positive ML flow tests occurred six times higher than that of individuals with negative ML flow tests (93). However, this ML flow test often exhibits a higher background response making it difficult to distinguish the true antibody response from nonspecific antibody binding by detecting anti-PGL-I IgM antibody.

In order to overcome this problem, Maeda *et al* (159) demonstrated that seroreactivity of recombinant MMP-II in patients' sera was able to detect 39-48 % PB patients as well as 85% MB patients in non-endemic (Japan) and endemic regions (Indonesia and Vietnam). After chemotherapy, anti-MMP-II antibody titers in sera of

leprosy patients decrease by 18-50%. Subsequently, seropositivity of recombinant MMP-II could predict disease progression of leprosy patients and HHCs similar to that of PGL-I (103,133). The fusion construct of ML0405 and ML2331, LID-1, was also proven to detect individuals who were in preclinical leprosy but presented clinical symptom, 6-8 month later (73).

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CHAPTER 2 GENE EXPRESSION PROFILE AND IMMUNOLOGICAL EVALUATION OF UNIQUE HYPOTHETICAL UNKNOWN PROTEINS OF *MYCOBACTERIUM LEPRAE* BY USING QUANTITATIVE REAL TIME-PCR

From a manuscript in preparation by **Hee Jin Kim,** Kalyani Prithiviraj, Nathan Groathouse, John S. Spencer and Patrick J. Brennan. Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado.

2.1 Abstract

Cellular mediated immunity (CMI) based *in vitro* interferon-γ released assay (IGRA) to *M. leprae* specific antigens is known as the most promising diagnostic test to detect those individuals in the early stages of *M. leprae* infection. However, diagnosis of leprosy is a major obstacle toward disease control, and has been compromised in the past by the lack of specific markers. Comparative bioinformatic analysis among mycobacterial genomes identified potential *M. leprae* unique proteins called "hypothetical unknowns" from the *M. leprae* genome. Due to the massive gene decay and the prevalence of pseudogenes, it is unclear whether any of these proteins are expressed or are immunologically relevant. Here, we performed cDNA based quantitative real time PCR to investigate the expression status of 136 putative open reading frames (ORFs) encoding hypothetical unknowns. Twenty six *M. leprae* specific antigen candidates showed significant levels of gene expression compared to that of ESAT-6 (ML0049) which is an important T cell antigen of low abundance in *M. leprae*.

Fifteen out of 26 selected antigen candidates were expressed in *E. coli* and purified. The seroreactivity to these recombinant proteins using the pooled sera of lepromatous leprosy patients and cavitary tuberculosis patients revealed that 10 out of 15 recombinant hypothetical unknowns elicited *M. leprae* specific immune responses. Therefore, 9 of the recombinant hypothetical unknowns may be good diagnostic reagents to improve both sensitivity and specificity in the detection of individuals with asymptomatic leprosy.

2.2 Introduction

The diagnosis of leprosy is solely based on clinical symptoms, requiring the presence of a neurologic deficit and skin lesions (4,39), but is only specific to patients with progressive disease. More than 70% of infected patients are acid-fast bacilli staining negative and do not present the analgesic skin lesions, especially paucibacillary/ tuberculoid leprosy (PB/TT) patients (4) (see section 1.3). Since the presence of skin lesions in these patients is variable, their clinical symptoms are not sufficient to specifically diagnose leprosy (4). These problems are accentuated in the diagnosis of individuals with subclinical *M. leprae* infection including leprosy house hold contacts (HHCs) who have close contact with leprosy patients and may also serve as important sources of ongoing leprosy transmission (4,9,29,39).

Several *M. leprae* antigens were identified and evaluated for their diagnostic potential by serological or CMI based tests (1-3,5,6,8-11,13-19,30,31,33,37) (see section 1.10.2). Until now, serological tests by a single *M. leprae* specific antigen, phenolic glycolipid-I (PGL-I), successfully detects circulating antibody in sera of multibacillary/lepromatous leprosy (MB/LL) patients. However, this test fails to detect PB/TT patients and HHCs who usually present low seroreactivity but strong CMI

response to mycobacterial antigens (11,30). Both *in vitro* IFN-γ release assays (IGRAs) and a simple delayed type hypersensitivity skin test have been developed to detect individuals in the early stages of leprosy using *M. leprae* protein based antigenic fractions such as *M. leprae* cell wall antigens without LAMs (MLCwA), *M. leprae* soluble antigen without LAMs (MLSA-LAM) and the major individual immunogenic proteins (3,3,13,26,29,38). However, a major obstacle of the application of the IGRA to the major *M. leprae* protein fractions or antigens is that most of these share a great deal of homology among other mycobacteria causing undesirable cross-reactivity from individuals who were vaccinated with BCG and/or exposed to *M tuberculosis* and/or non-tuberculosis mycobacteria.

Comparative genomic analysis between *M. leprae* and other mycobacteria has identified up to 142 hypothetical unknowns as *M. leprae* specific proteins (7,14,30) (described in section 1.10.2). During the last decade, either recombinant proteins or synthetic peptides originating from these ORFs, and which contain T cell epitopes restricted via major HLA (human leukocyte antigen)-DR alleles, have been studied as *M. leprae* specific antigens (1,2,13-16,30). CMI based IGRA to *M. leprae*-specific antigens can distinguish individuals exposed to/infected by *M. leprae* from endemic healthy controls in Brazilian population (13,14,16,30). However, the levels of IFN-γ secretion to these antigens, particularly their peptides, were found to be too low to distinguish some individuals exposed to *M. leprae* from healthy individuals in different geographical regions (15,30), suggesting that the low response to these peptide antigens could misdiagnose individuals with preclinical leprosy. Low sensitivity of current IGRA raises a question of whether any of the hypothetical unknowns are expressed, because

approximately 50% of genes encoding for functional proteins, which were found in other mycobacteria, are deleted or inactivated in the *M. leprae* genome (7,36).

The aim of this study was to identify *M. leprae*-specific proteins that could induce high levels of IFN-γ secretion by peripheral blood monocyte cells (PBMCs) from individuals with asymptomatic *M. leprae* infection/ exposure, as well as TT/PB patients. In order to achieve this goal, we performed cDNA based quantitative real time PCR (qRT-PCR) to investigate the expression status of 136 *M. leprae* specific ORFs (hypothetical unknowns) and selected twenty six of the promising antigen candidates which showed detectable gene expression levels for recombinant protein production. Subsequent serological analysis using sera of LL patients and cavitary tuberculosis (TB) patients evaluated the immunological potentials of all new recombinant antigen candidates.

2.3 Materials and methods

2.3.1 Isolation of M. leprae *RNA*

M. leprae Thai-53 (10.2 mg, 2×10^{10} cells) provided by the Leprosy Contract at Colorado State University (CSU) were washed with 10 mM Tris-HCl (pH 8.0), 1mM EDTA buffer and pelleted by centrifugation at 600 × g for 10 min. The bacteria were subjected to a single tube homogenization/RNA extraction protocol according to Williams *et al*. (40). Briefly, bacteria were resuspended with 1 ml of TRIzol (Invitrogen Life Technologies, Calsbad , CA) and were mechanically lysed in a vial containing lysing matrix B (MP Biomedical LLC, Solon, OH) using a Fast Prep-24 (MP biomedical LLC, Solon, OH). The resultant homogenate solution was added with 200 µl of chloroform-

isoamyl alcohol (24:1, v/v) and subjected to centrifugation at 27,000 × g for 20 min. Nucleic acids in the aqueous phase were precipitated by adding 100 μl of 3 M Na-acetate (pH 5.2) and 500μl of isopropanol and followed by incubation at -20 °C for 1 hr. Total RNA was recovered by centrifugation at 27,000 × g for 30 min at 4 °C. According to the manufacturer's instructions, the Turbo DNA free kit (Ambion, Austin, TX) was used to remove the DNA contaminants in the RNA solution prior to cDNA synthesis.

2.3.2 Primer design for quantitative real time PCR (qRT-PCR) analysis

All DNA sequences of 136 hypothetical unknown ORFs in functional class VI (7) (http://www.pasteur.fr/recherche/unites/Lgmb/NATURE_DATA/ML_gene_list) were obtained from the *M. leprae* genome database, Leproma (22). The OLIGO6 Primer Analysis Software (Molecular Biology Insights Inc, Cascade, CO) was used to design the specific primer set to each target gene of hypothetical unknown ORFs. ML2244, ML2249, ML2567, ML2151, ML0567 and ML0678 were excluded. The genes encoding ML2249 and ML2151 were too small to design proper primers. Gene expression levels of ML2567, ML2244 ML0678 and ML0567 were already studied by us or collaborators (13,30). In order to enhance the efficiency of qRT-PCR, the primer set to each target gene was designed to produce a PCR product of 200-400 bp in size. The specificity of each primers set to template was analyzed by comparing the genomes analyses of *M. tuberculosis* (35), *M. avium*, *M. bovis* BCG and *M. smegmatis* TIGR Microbial Database (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi) through a BLAST search.

2.3.3 Synthesis of complementary DNA (cDNA) and qRT-PCR assay

Total RNA transcripts of *M. leprae* Thai-53 were converted to cDNA using the SuperScript III First Strand Synthesis System kit (Invitrogen Life Technologies, Calsbad, CA) with random hexamers according to the manufacturer's instructions. Total 2.5 µg of cDNA were obtained. The qRT-PCR assay of each gene was performed with *M. leprae* cDNA (10ng of cDNA, equivalent to 8×10^7 cells) and a dilution series of quantified *M. leprae* genomic DNAs (10 fg, 50 fg, 100 fg, 500 fg, 1 pg, 10 pg, 100 pg and 1 ng per microliter) for relative quantification of cDNA by using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen Life Technologies, Calsbad, CA) on the iCycler Real Time PCR Machine (BioRad Laboratories, Hercules, CA). All PCRs with a volume of 25 µl were performed in 96-well optical-grade PCR plates (BioRad Laboratories, Hercules, CA) in triplicate. The primer sets of ML0380, ML2038 and ESAT-6, which were previously proven to be expressed in *M. leprae* (30,42), were used to optimize the PCR conditions. An initial DNA denaturation step at 95 °C for 5 min was followed by 30 cycles of denaturation at 95 °C for 30 sec, primer annealing at 64 °C for 20 sec and primer extension at 72 °C for 45 sec, with a final extension step at 72 °C for 5 min. The iCycler iQ Software (BioRad Laboratories, Hercules, CA) was used to analyze the qRT-PCR assays with a correlation co-efficiency (≥ 0.95) and PCR efficiency (85%-105%).

2.3.4 Cloning of hypothetical unknowns ORFs from **M. leprae**

To express 24 of the selected novel antigen candidates in *E. coli*, genes encoding these hypothetical unknowns were PCR amplified from *M. leprae* Thai 53 genomic DNA

using the *rTth* DNA polymerase XL (Applied Biosystems, Carlsbad, CA). PCR amplification was performed using primer sets which include the *NdeI* and *HindIII* sites, respectively (underlined), specific to the upstream and downstream of the open reading frame as listed in Table 2.1). Each of the target genes was amplified using a Touchdown PCR. This method had a high initial annealing temperature at 64 °C that decreased by an additional 1 °C in each of the first 7 cycles, followed by 25 cycles at 58 °C. PCR products were directly digested with restriction enzymes and cloned into the expression vector pET 29a (+) (Novagen, Madison, WI), which contained the coding sequence for a 6-Histidine tag at the C-terminus of expressed proteins to facilitate the purification of recombinant proteins. The DNA sequences of all recombinant clones were confirmed by automated nucleotide sequencing at the Proteomics and Metabolomics Facilities (PMF), Colorado State University.

Table 2.1 List of primers used in cloning of 24 selected hypothetical unknown ORFs.

Name	Sequence	Name	Sequence
ML0023 forward	AACATATGCCGACCAACCCCATTTC	ML0953 forward	AACATATGGCGATTTCGGCCGTTATGGCTG
ML0023 reverse	AAAAGCTTAGTCCCCCTTCGCCCACT	ML0953 reverse	AAAAGCTTGCGTGCGTCGTCGCAGTGAT
ML0070 forward	AACATATGCTGACCGGGGTGACGAAC	ML0959 forward	AACATATGGCTCGCACTCTGCGATGTT
ML0070 reverse	AAAAGCTTCTCCCTGTGGGGACTGTT	ML0959 reverse	TAAAGCTTAGAAGACGCACTCGGGCTG
ML0121 forward	AACATATGGTTTGGCACATGACACATTCG	ML1010 forward	ACCATATGCGCGGCAGTCAACACAAGG
ML0121 reverse	AAAAGCTTGGAGGAGTCCAGAATGTCTTT	ML1010 reverse	AAAGCTTCAGCCTAGCGACGTTGCTG
ML0141 forward	AACATATGCGTCTCAAAGTCATTGACAGC	ML1384 forward	AACATATGACGGACGGCATCCAGCAGT
ML0141 reverse	AAAAGCTTGCCAACGTCTATATGCCGCT	ML1384 reverse	AAAAGCTTGTCGCCCGGACCAACAC
ML0188 forward	AACATATGGATCCGATCAATTCGCCCA	ML1575 forward	AACATATGATGCCCTGGGGATCGCCTG
ML0188 reverse	AAAAGCTTCGCAATATATGTCCGCAA	ML1575 reverse	AAAAGCTTCAACCCAGCAGGTCCACCA
ML0217 forward	AACATATGGGTGTCGGTCGCCCATGTG	ML1949 forward	AACATATGTTCTGGGTTGGGGTACGCG
ML0217 reverse	AAAAGCTTAGCACTTAGCTCATCAATG	ML1949 reverse	AAAAGCTTCTACTGGCGTCCTGGAATTT
ML0448 forward	AACATATGCAGGCCTGGTACGTATXTAG	ML2044 forward	AACATATGGAAACGATCGTGCTCCCCGA
ML0448 reverse	CCAAGCTTCTATCTCATAATTACGTGTT	ML2044 reverse	AAAAGCTTCGACACCACCGACCACTACA
ML0527 forward	TACATATGGTTTACCTGGAAAACCATGG	ML2307 forward	AACATATGGCAAAAACACGACCGGCC
ML0527 reverse	TCAAGCTTTGTCTTGCCCGCCGCTTCG	ML2307 reverse	AAAAGCTTGCTAACATTGCGGTGCTTGC
ML0588 forward	AGCATATGGTGATTGGCAGCCTTGACATT	ML2313 forward	AACATATGGCCAGTGCTTCTCAAACACA
ML0588 reverse	ACAAGCTTCCAGTGTCCCGGCAGCAT	ML2313 reverse	ATCTCGAGGTTCTCGCCGAGGATGC
ML0614 forward	AACATATGTGGAAGGCACGGACAGCATT	ML2651 forward	AACATATGATCTTGAGCTCGATATGGCT
ML0614 reverse	AAAACGTTCCCGCGCCGAATCA	ML2651 reverse	AAAAGCTTGCGCCAGATAGGCAGGATTT
ML0755 forward	AACATATGGCACTGCGCGAGGCCACCAA	ML2630 forward	AAACATATGCAGCATCAACCCCACCAC
ML0755 reverse	AAAAGCTTTTAGCGTGACCTCCTCGCAC	ML2630 reverse	TTAAGCTTATGCGTCGCTGCTGCTTT
ML0920 forward	ACCATATGAGCACACTGCACAAGGTCAA	ML2666 forward	AACATATGGCGTGGCTGGAGCAGTGGAA
ML0920 Lower	AAAAGCTTTTGGTAGGCGTAAAAGCCGGTT	ML2666 reverse	AAAAGCTTGCCAACGTCTATATGCCGC

The sequences recognized by restriction enzymes are underlined; CATATG is specific for *Nde*I and AAGCTT for *Hind*III

2.3.5 Purification of recombinant **M. leprae** *proteins*

The plasmids containing the novel antigen candidates were introduced into the *Escherichia coli* expression host BL21 Star (DE3) pLysS (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's instruction. The transformants were grown to the log phase (optical density (O.D.) at 600 nm of 0.5) at 37 °C in Luria-Bertani (LB) broth with 50 μg/ml kanamycin. Expression of recombinant proteins was induced by adding 0.2-0.5 mM IPTG. The cells were cultured at 25 °C for overnight. The cultured cells were harvested by centrifugation at 4°C and frozen at -70 °C. The cells were resuspended in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl) containing 10 μg/ml of DNase and 10 μg/ml of RNase, with a protease inhibitor cocktail (P8340, Sigma, St. Louis, MO) and 20 μM PMSF (phenylmethanesulfonyl fluorid, Sigma, St. Louis, MO), and were disrupted by intermittent probe sonication with a Soniprep 150 sonicator (Sanyo MSE, London, UK) for 10 min. The lysates were centrifuged at 5,000 × g for 5 min to remove unbroken cells and the supernatants were centrifuged for 30 min at 27,000 × g at 4 °C. Supernatants were subjected to a Ni-NTA agarose column chromatography (Qiagen, Valencia, CA). The column was washed with 20 × column volumes of the same buffer. Recombinant proteins were eluted with 1 × column volume of lysis buffer containing stepwise increments of imidazole (5, 10, 20, 50,100,150 and 200 mM) (31). All recombinant hypothetical unknowns were found in the 50-200 mM imidazole fractions. Only 15 out of the 24 novel candidates were expressed and purified for further serological analyses.

2.3.6 Subjects and samples

Leprosy patients diagnosed at the Leonard Wood Memorial Center for Leprosy Research, Cebu, the Philippines were included in this study. Leprosy patients (n=70) were classified according to the Ridley-Jopling classification system (28) based on bacterial index, histological and clinical observations carried out by experienced leprologists and a leprosy pathologist. All leprosy patient sera were collected at the time of initial diagnosis prior to beginning multidrug therapy. Serum samples from cavitary TB patients (n=30) were from a cohort of newly diagnosed TB patients from the Tuberculosis Trials Consortium Study Group 22. Eleven of the patients were sputum smear negative, while nineteen were smear positive (ranging from 1+ to 4+, too many to count). The sera were provided by Dr. William Mac Kenzie from a serum bank repository from the Centers for Disease Control in Atlanta, Georgia. Serum samples from all sources, made anonymous and coded to protect donor identities, were obtained with informed consent and/or with permission from the local ethics committee or institutional review boards of the relevant countries and institutions involved (32). Sera were pooled from eight randomly selected lepromatous patients whose bacterial index was 6. Five sera of cavitary TB patients were pooled to investigate their cross-reactivity to *M. leprae* recombinant antigens.

2.3.7 Western blot analysis

Protein quantities of all subcellular fractions were measured by the bicinchoninic acid assay reagent (Pierce, Rockford, IL). Each novel recombinant antigen candidate (0.5 μg/lane) was subjected to electrophoresis in 15% SDS-PAGE gel and transferred onto

nitrocellulose membrane. Blots were blocked with blocking buffer (3% BSA in PBS/0.05% Tween80) for 2 hr and were then probed with both diluted sera (1:5,000 dilutions for the leprosy sera and 1:1000 for TB sera). Blots were performed by probing with secondary anti-human IgG alkaline phosphatase (Sigma, St. Louis, MO) and developed by using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tretrazolium (Sigma, St. Louis, MO) (33).

2.4 Results

2.4.1 Establishment of the gene expression profile of **M. leprae** *specific hypothetical unknown ORFs and selection of novel antigen candidates*

Previously, the global analysis of *M. leprae* transcripts using microarray revealed that a few of the hypothetical unknown genes were expressed during infection (42). Although this global analysis has provided valuable information in bacterial pathogenesis studies, it is not sufficient to evaluate the functional status of hypothetical unknowns that were usually expressed at low levels.

In order to verify the gene expression level of all hypothetical unknowns (class VI), we applied real time PCR assays using a set of target-specific primers and SYBR green I as fluorescent probes to determine the relative quantification of hypothetical unknown ORFs in cDNAs synthesized from total *M. leprae* RNAs. SYBR green I binds to dsDNA generated with each progressive cycle of the PCR and emits a fluorescent signal; fluorescence generated is quantitatively measured to track the amplification of DNA in PCR. There is a quantitative relationship between the amount of starting template and the amount of the PCR product at an exponential phase of the PCR (25).

We established the gene expression profile of 136 hypothetical unknown ORFs which had not been previously studied in our laboratory. This target based gene expression analysis revealed that a majority of the hypothetical unknown ORFs (60%) expressed less than 100fg of mRNA (Figure 2.1 and Table 2.2).

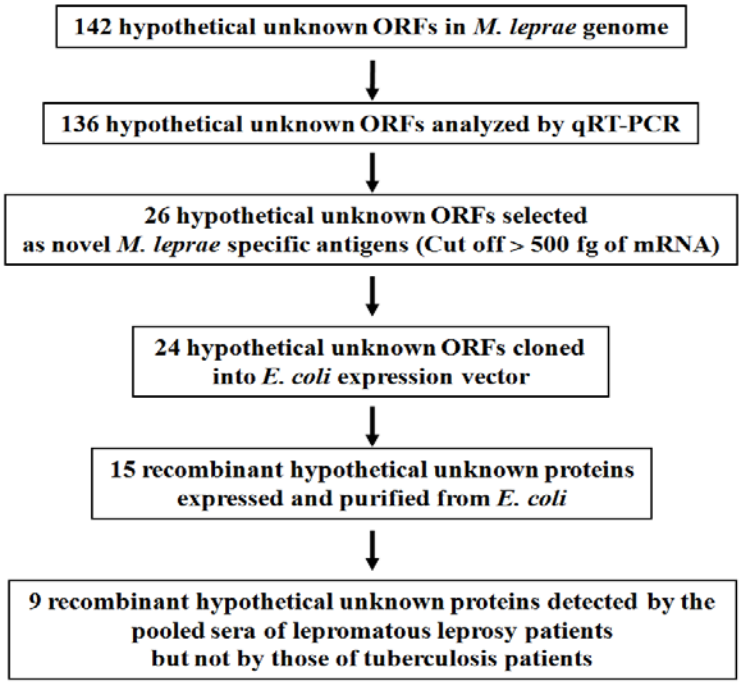

Figure 2.1 Summary of the hypothetical unknowns investigated in the present study.

The transcript of EAST-6/ML0049, which has proved to be an important T-cell antigen (30), was expressed at low level in both nude-mouse derived *M. leprae* and *M. leprae* recovered from MB patients (42). The gene expression levels of the screened 26 hypothetical unknown genes were found to be in the range of 1 pg~500 fg, which is similar to that of ESAT-6 (Table 2.3). Of 26 hypothetical unknowns, the immunogenicity of ML0573 and ML0574 was already evaluated in endemic regions (13).

Therefore, 24 hypothetical unknowns were cloned in order to express in *E. coli* (Figure 2.1).

The class VI hypothetical unknown proteins consisting of 142 theoretical ORFs had no orthologues among any mycobacterial genome databases (7,30) at the time that this work was started. Subsequent advances in next generation DNA sequencing technology has rapidly completed a great number of microbial genome sequences and the advanced bioinformatics analysis methodology has further refined the annotation of *M. leprae* ORFs on the previous genome sequences (41). In a reflection of this trend, the current list of functional class VI (http://www.pasteur.fr/recherche/unites/Lgmb/NATURE_DATA/ML_gene_list) has been adjusted continuously but has retained the majority of genes from the previous list of genes. In order to evaluate whether these class VI hypothetical unknown proteins have homology with other genes expressed in other human pathogens, the sequence similarity of 26 *M. leprae*-specific proteins were examined by BLASTP (http://www.ncbi.nlm.nih.gov/BLAST). A few genes contained orthologues among other pathogens (data not shown) but appeared as hypothetical genes.

Based on e-values, 26 new antigen candidates have been categorized into 4 subclasses. In BLASTP analysis, the similar protein sequence to these candidates (21 of 26) are less than 100 AAs with high gap score and its identities was less than 25%, indicating that the selected antigen candidates cannot have orthologues in other pathogen and are considered to be *M. leprae* specific (12,27).

Table 2.2 Analysis of qRT-PCR of 136 hypothetical unknown ORFs.

	Ct value of genomic DNA								Ct value	Level of gene
ORF	1ng	100g	10pg	1pg	500fg	100fg	50fg	10fg	of cDNA	expression
ML0009		no	signal							
ML0840		no	signal							
ML0939		no	signal							
ML2045		no	signal							
ML2669		no	signal							
ML0464		22.3	25.8	29.5	32	33.5	34	36.4	36.7	<10fg
ML0926		22.1		30	32	35.9	36.6	41	N/A	<10fg
ML1148	18.6	22.1	25.2	28.9	29.4	32.8	33.2	35.4	35.2	10fg
ML0473		21.4	24.4	28.4	30.7	33.4	34	36.4	34.8	10fg< <50fg
ML0218		33.2	36.7	36.3	38.1	38.7	39	42.7	40.1	10fg< <50fg
ML0291		21.4	24.6	28.4	29.8	31.2	33	34.5	34.3	10fg< <50fg
ML0293		21.2	24.8	28.8	30.4	31.1	32.7	36.8	34	10fg< <50fg
ML0938		21.9		28.9	30	31.3	32.2	34	33.4	10fg< <50fg
ML1001		23.4	27.3	32.6	34.5		36	38.7	36.5	10fg< < 50fg
ML0325		25.6	27	32	33.1	34.7		37.9	35.4	10fg< <100fg
ML0927		26.4	30.8	33.9	34.2	37.5		41	38.3	10fg< <100fg
ML0152		22.1	26	29.5	30.4	32.5	34.1		35.6	< 50fg
ML0162		23.7	27.9	32.4	40.6	35	36.1		37	<50fg
ML0470	17.4	21	24.6	28.7	29.7	31.7	32.1		36.4	<50fg
ML0659		29	34	39.5		41.6	42.8		N/A	<50fg
ML1057							38.9		39.6	<50fg
ML1210	19.5	23.5	27.4	32	32.5	35.8	38.8		N/A	<50fg
ML1605	18.6	23.3	26.3	30.3	31.4	34			N/A	<50fg
ML1915	21.1	24.9	29	32.4	33.6	35.7	36.6		37	<50fg
ML1976	20.4	24.2	27.9	31.9	32.5	34.5	35.6		N/A	<50fg
ML1979	19.4	24	27.6	32.5	33	34.9	35.3		36.9	<50fg
ML1989	19.6	23.6	27.7	31.8	34	36			36.5	<50fg
ML2013	19.7	23.8	27.9	31.1	33.2	35			N/A	<50fg
ML2346				33.5	34.1	36.1	36.5	N/A	38	<50fg
ML2476	18.8	23.8	26.7	30.2	31.2	33.6	33.7		34.2	<50fg
ML2478	18.3	23.8	26.9	31.4	33.1	35.7	35		N/A	<50fg
ML2491	24	29.5	33.5	37.9	39.5	N/A	41.5		41.9	<50fg
ML2562	20.5	24.7	29.3	32.1	33.2	35	36.1		37.1	<50fg
ML2603	23	30	35.6	39	40.5	41.9	42.6		N/A	<50fg
ML2629	18.7	24.6	28.7	33.5	33.1	37	36.9		N/A	<50fg
ML0024		24.6	28.3	31.6	n/a	37.6	39	n/a	38.1	50fg
ML0664		22.4	26.1	30.4		34.1	34.3		34.5	50fg
ML0777		22.1	26	29.5	30.8	32.8	35		35.5	50fg
ML1188	18.5	22.5	26.1	30	30.5	32.9	34.3		34.5	50fg
ML1189		23.7	27.9	32.1	33	36.1	37		36.8	50fg
ML1292	17.7	21.4	25.1	29	30.2	32	33		33.6	50fg
ML1420	19	22.9	26.5	30.1	31.9	34.1	34.7		39.8	50fg
ML1761	18.1		25.8	29.7	30.1	31.9	32.9		32.6	50fg
ML2091	19	23.4	27	31.2	31.8	32	34.2		34.2	50fg
ML2170	23.5	28.5	31.9	36.1	36.8	38.5	39.3	41.2	39.4	50fg
ML2172	22	26.4	31.7	33.7	36	38.5	39	43	39.6	50fg
ML2497	20.9	26.7	30.3	34.8	36	39	40.5		40.4	50fg
ML0025			35	37.1	38	39.4	42.5		41.1.	50fg< <100fg
ML0126				32.7	33	34.6	36.8	n/a	35.6	50fg< <100fg
ML0292		20.8	24.4	27.7	28.7	31.9	32.6	34.6	31	50fg< <100fg
ML0369		23.3	26.1	30.6	32.1	33.4	36.4		34.5	50fg< <100fg
ML0863		22.7	26	29	31	32	34		33.2	50fg< <100fg
ML0957		23	25.9	29.7	32	32.8	35.3		34.7	50fg< <100fg

	Ct value of genomic DNA								Ct value	Level of gene
ORF	1ng	100pg	10pg	1pg	500fg	100fg	50fg	10fg	of cDNA	expression
ML1018	18.7	22.4	25.7	27.9	30.2	31.6	34		32.5	50fg< <100fg
ML1243	23.9	29	33.2	38.5	39.1	40.8	42.5		42.3	50fg< <100fg
ML1275	18.8	22.9	26.7	31.3	31.7	33.4	36		34.1	50fg< <100fg
ML1523	18.6	22.4	26.7	29.8	30.8	33.4	34.7		34.5	50fg< <100fg
ML1602	18	21.9	25.4	29.2	30.2	31.5	34.3	36.5	33.2	50fg< <100fg
ML1763	18.9		26.9	30.5	31	33.5	36		34	50fg< <100fg
ML1788	18.8	22.5	26	30.2	31.2	33.2	34.8	39	34	50fg< <100fg
ML1829	17.8	22.5	26.3	29.8	30.5	31	32.7		32.5	50fg< <100fg
ML2201	18.9	23.3	27.1	31	31.2	32.3	32.8		32.6	50fg< <100fg
ML2452	19	24	27.9	32	32.6	34.6			35.2	<100fg
ML2265	17.6	21.6	25.1	29	29.4	N/A	N/A		31.3	<100fg
ML2178	21.4	25.9	31.2	34.6	35.9	37			N/A	<100fg
ML1119	20.3	24.3	29.2	33.2	33.2	36			N/A	< 100fg
ML1517	21.4	25.9	30.9	34.2		36.4			38.8	<100fg
ML1604	17.9	22	25.5	29.6	30.2	32.8			34.2	<100fg
ML1717	18.8		26.2	30.6	31.1	33.3			35	<100fg
ML1821	18	22.4	25.7	29.7	33.6	34.4			40.7	<100fg
ML1011	18	21.8	25	28.7	30.2	32.4	32.8	35	32	100fg
ML1106	19	22.8	26.4	29.9	31.3	33.6		38.8	33.3	100fg
ML1344c	17.5	21.4	24.7	28.5	29.3	31.8	N/A		34.3	100fg
ML2158	18.9	24.1	27.6	31.5	32.7	33.2	35		33.6	100fg
ML0394				34.8	n/a	38.1	n/a	39.4	38.5	100fg
ML0663		23.2	26.2	31	31.9	33			32.9	100fg
ML0949		22.6	31	32.1	35	36.1			36.8	100fg
ML2264	18.8	23.6	27.3	32	33.3	35.4	36.2		35.5	100fg
ML2468	18	22.1	25.2	29.5	30	32.5	34.4		32.1	100fg
ML0950		23.8	28	31.1	32	33.6			35.7	<100fg
ML0964		25.3	29.5	32.8	33.4	35			36.6	< 100fg
ML0950		23.8	28	31.1	32	33.6			35.7	<100fg
ML0472		21.3	25.3	28.2	31.9	32.8		34.4	32	100fg< <500fg
ML0656		22.4	25.8	30	30.8	34.3	35		33.6	100fg< <500fg
ML0679	18.5	22.4	25.9	29.5	30.5	33.4	33.8		31	100fg< <500fg
ML0947		21.9	29.1	30.7	31.8	32.5	33.5	39	32	100fg< <500fg
ML1186	18.5	22.3	25.7	29.3	31	35.1			34.5	100fg< <500fg
ML1294	17.2	21.4	24.6	28.1	29.3	31.7	33.2		31	100fg< <500fg
ML1572		21.5	25.8	29.7	30.4	32.6	33.5		31.7	100fg< <500fg
ML1601	18	22.2	25.3	29.2	31	34.3			32.1	100fg< <500fg
ML1603	18.1	22	25.7	28.7	29.4	32.1			31	100fg< <500fg
ML1793	18.7	21.9	26.5	30.8	31	32.6	34.5		31.8	100fg< <500fg
ML1796	18.3	22.8	26	30.4	32.2	32.8	33.6		32.5	100fg< <500fg
ML1928	17.5	21.8	25	28.4	29.1	31.8	32.7		30.9	100fg< <500fg
ML1972	19.3	23.4	26.6	30.6	32.1	34.4	35.5		33.3	100fg< <500fg
ML2035	22.5	26.3	29.6	33.3	33.8	36			35	100fg< <500fg
ML2176	19.2	23.9	28.4	31.5	32.5	34.2	38.7	39.8	32.5	100fg< <500fg
ML2252	19.9	24.3	27.2	32	32.8	36	37.9	41.1	35.5	100fg< <500fg
ML2288	19.4	24	27	31.8	32.7	36.2			34.5	100fg< <500fg
ML2379	18.8	24.3	27.7	32	32.4	36.7	37		33.7	100fg< <500fg
ML2621	19.6	25.3	29.5	33.7	36	37.9	39		36.8	100fg< <500fg
ML0963		23.4	29.8	32.7	35				N/A	<500fg
ML2283	17.7	22.7	25.6	29.7	31.1	34.9			31	500fg
ML2284	19.2	24	31.6	32	34.3	35.2			34.2	500fg
ML0023		24.6	26.9	38.9	37.1	37.9	38.6	n/a	37.4	500fg
ML0121		23.4	26.9	31.8	32.9	33.5	36.2		31.8	500fg

	Ct value of genomic DNA								Ct value	Level of gene
ORF	1ng	100pg	10pg	1pg	500fg	100fg	50fg	10fg	of cDNA	expression
ML0127		25.3	29.9	32.7	33.2	34.3	n/a	n/a	33.5	500fg
ML0265		29.3	32.1		35	39.1	42.7		35.4	500fg
ML0757		23	26.7	30	31.8	33.1	35.4		31.7	500fg
ML0928		28.8	34	39	40	42			40.3	500fg
ML1445	19	22.7	26.3	30	32	34	36		32.4	500fg
ML0448		21.7	25.2	29.1	33.3	34.2			31.4	500fg< <1pg
ML0573		21.6	24.7	28.8	33.4	34.8	34	41	32	500fg< <1pg
ML0755		23.2	26.7	31	31.8	33.1	35.4		31.7	500fg< <1pg
ML0920		23.8	27.4	31.1	32.5	35			31.5	500fg< <1pg
ML0953		27	29.5	33.8	35.1			39.2	34.1	500fg< <1pg
ML1949	19.6	23.7	27.1	31.4	32.9	34.1			32	500fg< <1pg
ML2630	17.3	22.8	26.5	30.9	31.6	32.9	N/A		31.3	500fg< <1pg
ML0141			36	37.6	39.2	40.6	42.7	n/a	36.5	1pg
ML0188		23.9	29.1		35.4	36.4	36.8	n/a	34.6	1pg
ML0217		23.8	28.4	33.9	36.9		37.1	40.4	32	1pg
ML0070		25.2	30.7	33.6	33.5	34	34.2	35.4	33.6	1pg
ML0574		23.1	27	30.5	31	32	33.2		30.9	1pg
ML0588		22.8	26	30.5	31.8	32.7	34.8		30.1	1pg
ML0614	20.8	25	29.1	32.4	36	37.5			32	1pg
ML0959		24	29	32.5	33.4	36.2			32.6	1pg
ML1010	18.4	22.4	25.2	28.7	30.2	32.2	32.9		28.5	1pg
ML1384	18.8	22.7	25.2	30.4	31	34.8	35.2		30.7	1pg
ML1575	26.4	31.1	34.4	37.8	38.9	41.4			37.9	1pg
ML2044	21	25.7	30	33.7	34.7	37	39.1		33.9	1pg
ML2307	19.9	24.6	27.2	31.8	33.2	34.8	35.2		31.5	1pg
ML2313	20.9	26	30.5	34.3	35.8	38.7			32.3	1pg
ML2651	19.3	23.1	27.3	30.9	32	35.7	N/A		31.1	1pg
ML2666	19.5	25.1	28	32	32.3	34.7	35.6		32.1	1pg
ML0527		22.4	25.4	29.2	30.2	32.4	33.2		28.9	> 1pg
Positive control for qRT-PCR										
ESAT-6		22	26	29.4	31.7	32.4	35.2		29	≈1pg
ML0380		28	31.5	36.7					32.1	1pg< <10pg
ML2038		20.2	24	27.5	31	32.9	34		26.2	1pg< <10pg

The Ct (cycle threshold) is the number of cycles at which the fluorescent signal generated in PCR just exceeds the background fluorescent level (threshold) where it reaches at an exponential phase of the PCR (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocument/cms_042502.pdf). The expression level of each gene was determined by comparing to results of qRT-PCR assay generated using a series of genomic DNA standard and were from 8×10^7 of *M. leprae*.

Table 2.3 Analysis of qRT-PCR of the 26 selected hypothetical unknown ORFs.

	Ct value of genomic DNA and cDNA							Ct value	Level of
ORF	100pg	10pg	1pg	500fg	100fg	50fg	10fg	of cDNA	gene expression
ML0023	24.6	26.9	38.9	37.1	37.9	38.6	n/a	37.4	500fg
ML0070	25.2	30.7	33.6	33.5	34	34.2	35.4	33.6	1pg
ML0121	23.4	26.9	31.8	32.9	33.5	36.2		31.8	500fg
ML0448	21.7	25.2	29.1	33.3	34.2			31.4	500fg< <1pg
ML0573	21.6	24.7	28.8	33.4	34.8	34	41	32	500fg< <1pg
ML0755	23.2	26.7	31	31.8	33.1	35.4		31.7	500fg< <1pg
ML0920	23.8	27.4	31.1	32.5	35			31.5	500fg< <1pg
ML0953	27	29.5	33.8	35.1			39.2	34.1	500fg< <1pg
ML1949	23.7	27.1	31.4	32.9	34.1			32	500fg< <1pg
ML2630	22.8	26.5	30.9	31.6	32.9	n/a		31.3	500fg< <1pg
ML0141		36	37.6	39.2	40.6	42.7	n/a	36.5	1pg
ML0188	23.9	29.1		35.4	36.4	36.8	n/a	34.6	1pg
ML0217	23.8	28.4	33.9	36.9		37.1	40.4	32	1pg
ML0574	23.1	27	30.5	31	32	33.2		30.9	1pg
ML0588	22.8	26	30.5	31.8	32.7	34.8		30.1	1pg
ML0614	25	29.1	32.4	36	37.5			32	1pg
ML0959	24	29	32.5	33.4	36.2			32.6	1pg
ML1010	22.4	25.2	28.7	30.2	32.2	32.9		28.5	1pg
ML1384	22.7	25.2	30.4	31	34.8	35.2		30.7	1pg
ML1575	31.1	34.4	37.8	38.9	41.4			37.9	1pg
ML2044	25.7	30	33.7	34.7	37	39.1		33.9	1pg
ML2307	24.6	27.2	31.8	33.2	34.8	35.2		31.5	1pg
ML2313	26	30.5	34.3	35.8	38.7			32.3	1pg
ML2666	25.1	28	32	32.3	34.7	35.6		32.1	1pg
ML0527	22.4	25.4	29.2	30.2	32.4	33.2		28.9	> 1pg

The transcription levels of 26 hypothetical unknown ORFs were similar to that of ML0049, an immunologically important antigen expressed at fairly low levels.

However, five selected antigens which are ML0614 in subclass VI.c and all of subclass VI.d appeared to contain orthologues in other *Mycobacterium* spp. by only sequence analysis but their physiological role and expression status are not experimentally verified yet (22). Therefore, these five candidates and are currently not considered as the hypothetical unknowns (22).

Table 2.4 Amino acids (AAs) sequence similarity of the 26 selected *M. leprae*-specific proteins.

Subclass	ORF	Molecular Mass (kDa)	Sequence Similarity	Protein with mximal similarity[1] (Identities (%), Ratio of coverage[2], maximal stretch[3] or gap in sequence alignment[4])
VI.a	ML0070	9.1	none	N/A[4]
	ML0141	9.3	none	N/A
	ML0448	10	none	N/A
	ML0527	8.7	none	N/A
	ML0574	11.4	none	N/A
	ML0959	13.6	none	N/A
	ML1010	8.4	none	N/A
	ML1384	12.2	none	N/A
	ML1575	11.1	none	N/A
	ML2044	7.9	none	N/A
	ML2651	11.6	none	N/A
	ML2666	8.8	none	N/A
VI.b	ML0121	9.6	3e-08	Hypothetical proteins of *M. paratuberculosis (*59%, 34/153, 8)
	ML0023	11.7	3e-06	Transposage for IS3514B of *Propionbacterium freudenreichii* (31%, 36/401, 7)
	ML0188	9.2	3e-08	Conserved hypothetical unknown of M. paratuberculosis (46%, 36/ 230, 7)
	ML0217	8.4	1e-06	Transposage of *Rhodococcus jostii* (98%, 80/346, 80)
	ML0573	9.5	1e-05	Hypothetical protein of *M. kansasii* (41%, 30/282, 4AAs)
	ML0588	8.5	3e-04	PPE protein of *M. tuberculosis* (55%, 21/539, 4)
	ML0953	8.6	1e-06	Putative nucleic acid binding protein of *M. kansasii* (85%, 54/131, 13)
VI.c	ML0614*	10.2	3e-17	Hypothetical proteins of *M. tuberculosis* T46 (66%, 43/67, 4AAs)
	ML2630	13	7e-10	conserved membrane protein of *M. tuberculosis* T92, (54%, 36/309, 5)
	ML1949	12.5	5e-17	Hypothetical proteins of *M. kansasii* (54%, 41/483, 8)
VI.d	ML0755*	9.6	1e-22	Hydrolase of *M. kansasii* (69%, 50/183, 12AAs)
	ML0920*	6.3	1e-102	Hypothetical protein of *M. tuberculosis* T17 (83%, 182/218, 8 gaps)
	ML2307*	9.3	4e-52	Transcription factor WhiB4 of *M. paratuberculosis* (92%, 101/116, 5 gaps)
	ML2313*	21.8	3e-71	Transcriptional regulator (PadR) of *M. avium 104* (67%, 163/242, 50 gaps)

All ORFs in subclass VI.a appear no homology. *M. leprae* proteins with BLASTP values between $1x10^{-4}$ to $1x10^{-8}$ are considered to have low homology (Subclass VI.b). Proteins with e-values between $1x10^{-8}$ and $1x10^{-20}$ have moderate homology (Subclass VI.c) and less than $1x10^{-20}$have high homology (Subclass VI.c). 1; Most similar one of several proteins found in BLASTP. 2; Number of matched AAs of the *M. leprae* hypothetical unknown to number AAs of most similar in sequence alignment. 3: Number of AAs found in longest aligned sequence. 4: Number of gap AAs indicate different sequence in sequence alignment. *: ORFs are currently found to have orthologues and not considered as hypothetical unknown.

2.4.2 Purification and serological reactivity of the recombinant M. leprae specific antigens

Fifteen of the 24 new candidates were successfully expressed as 6 Histidine tag fusion proteins in the *E. coli*-T7 promoter driven vector system and purified by immobilized metal affinity chromatography (Figure 2.2.A). Since ML0573 and ML0574 were already studied by other group (13), we exclude them in the present study. In order to evaluate the immunogenicity of these novel antigens, we performed Western blot analysis using sera of both LL and cavitary TB patients (Figure 2.2.B and C).

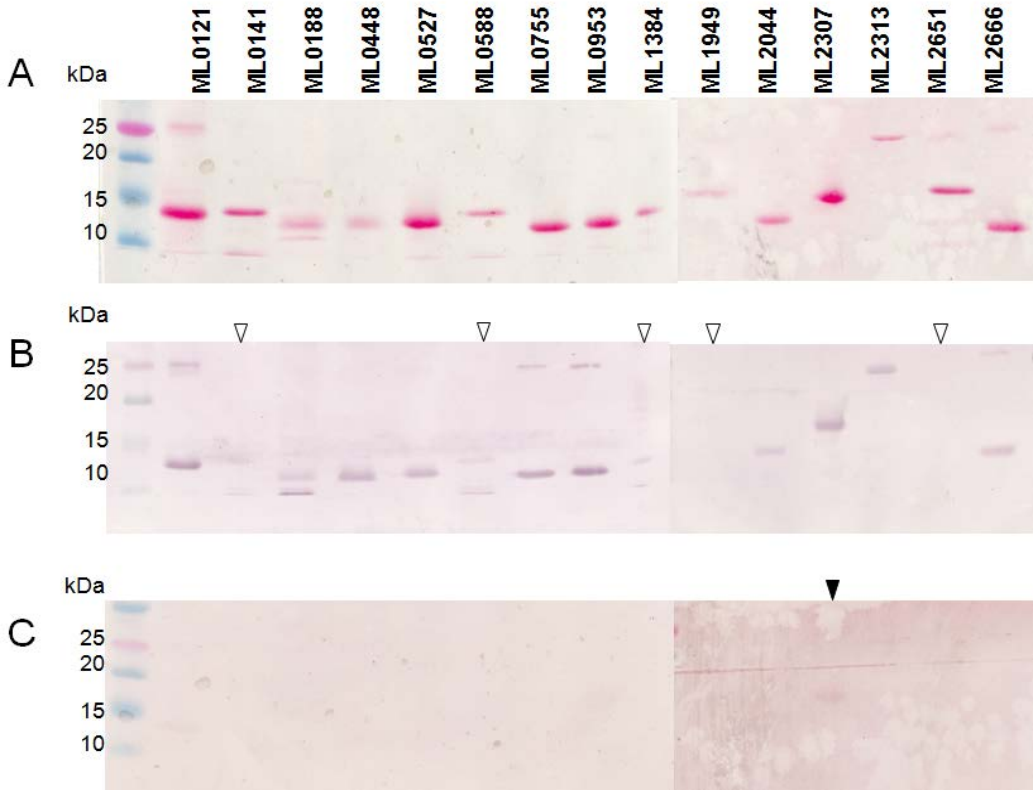

Figure 2.2 Western blot analysis of the recombinant forms of selected hypothetical unknown proteins. A) Transferred blot with Ponceu-S staining after running SDS-PAGE of the recombinant candidates. B) Western blot hybridized with pooled sera of LL patients (1:5000 dilution) whose bacterial index is around 6. C) Western blot from identical gel hybridized with pooled cavitary TB patients. The open arrow head indicates the candidates which did not react with LL patients' sera and the filled arrow head shows the reactivity to TB patients' sera.

The seroreactivity to all recombinant hypothetical unknowns revealed that 10 of the 15 recombinant antigens were recognized by circulating antibodies in the pooled sera from LL patients (Figure 2.2.B). Western blot analysis using pooled sera of 5 cavitary TB patients detected a single recombinant antigen out of the 15 proteins, ML2307, which had high homology with orthologues of other bacteria (Table 2.4 and Figure 2.2.C). Although all ORFs of subclass VI.d showed higher homology with the orthologues of *M. tuberculosis* than other candidate antigens (Table 2.4), sera of cavitary TB patients did not react to ML0755, ML0920, and ML2313 in this analysis (Figure 2.2.C). ML0141, ML0588, ML1384, ML2651 in subclass VI.a and ML1949 in subclass VI.c did not induce the seropositivity of LL or TB patients (Figure 2.2.B and C). Therefore, ML0121, ML0188, ML0448, ML0527, ML0755, ML0953, ML1384, ML2044, ML2313 and ML2666 induced *M. leprae* specific immune response in LL patients.

2.5 Discussion

The overall objective of this work was to identify the *M. leprae* specific antigens that can improve the sensitivity and specificity of CMI-based *in vitro* IFN-γ release assays (IGRAs), and to detect individuals exposed to/ infected by *M. leprae*.

Previously, 31 hypothetical unknown ORFs and hundreds of their peptides were identified as novel antigens from the 142 hypothetical unknown ORFs and their antigenic potentials were evaluated in order that the antigens are used for early diagnosis of leprosy by using the IGRA format (1,2,13,15,30). These *M. leprae* specific antigens were shown to be valuable diagnostic reagents in identifying those infected by *M. leprae* specifically, occupational contacts or HHCs of MB or PB patients (13,14,16,30). However, all

proteins and peptides were recognized by a significant number of endemic controls, but also these *M. leprae*-derived peptides showed considerable variation in reactivity to racially/ geographically different study populations (15,16). Most of the problems with these IGRAs originated from the low levels of CMI to hypothetical unknown proteins and hypothetical-unknown-derived peptides.

The genome of *M. leprae* contains exceptionally large number of pseudogenes (7) but also many hypothetical unknowns. These hypothetical unknowns (class VI; see Table 1.2) are low molecular weight proteins (13,30). Therefore, it was speculated that ORFs of hypothetical unknowns are not genuine ORFs or are barely expressed and available to T cell recognition causing the low sensitivity of IGRAs. Here, we established the functional status of 136 *M. leprae* unique proteins (class VI) to enhance the screening process of novel antigens by using target based qRT-PCR assays (Table 2.2). Of the class VI genes, twenty six of the hypothetical unknown ORFs were selected as being actively expressed with high positive PCR signals (>500 fg of cDNA) (Table 2.3). Only two of the 26 selected ORFs have already been studied or are being evaluated as T cell antigens according to the literature (ML0573 and ML0574) (1,2,13,16,30).

Two initial studies by Dr. Geluk and colleagues (13,16) showed that five of the recombinant hypothetical unknowns (ML0126, ML1420, ML1989, ML2283 and ML2346) and twenty two of their peptides induced the *M. leprae* specific T cell response from PB patients or HHCs in Brazilian population. In order to apply for the clinical use, IGRA validated whether five of the most promising recombinant hypothetical unknowns and their peptides can induce IFN-γ secretion from PB patients or HHCs in various leprosy endemic areas in Brazil, Bangladesh, Nepal, Pakistan and Ethiopia (15). The

ML2283 recombinant protein and ML2283-derived peptides were recognized by 20% and 25% of PB patients and HHCs but only detected by 6 % of TB patients (n=50) suggesting that ML2283 and ML2283-derived peptide induced the most *M. leprae* specific T cell response (15). However, the levels of IFN-γ production to other antigens were mostly found in the range of 100 pg/ml to 200 pg/ml, which are close to cutoff (100 pg/ml),and detect considerable numbers of endemic controls (30 %) and TB patients (20 % to 40 %) due to the low sensitivity of IGRA. All Brazilian subjects exhibited no T cell response to any of peptide antigens and the ML1420-derived peptide induced *M. leprae* specific T cell response only in the Pakistan population (15) suggesting that low sensitivity of IGRA to peptide antigens caused the variable reactivity in racially/geographically different study populations (15).

In the present study, the gene expression level of ML2283 was found to be 500fg which was relatively high compared to the other hypothetical unknowns. The ORFs of ML0126, ML1420, ML1989 and ML2346 which induced variable responses among three populations, were expressed in the lower amount of 100 fg (Table 2.2). The literature and our study showed that the low sensitivity of IGRA to current peptide antigens may be attributable to low antigen availability and poor T cell recognition of the parent proteins by the host immune system.

A number of well known *M. leprae* abundant antigens (major membrane protein-I (MMP-I), MMP-II, GroES, ESAT-6/ML0049 and CFP10/ML0050) induce strong cellular mediated and humoral immune responses in PB and MB patients respectively (20,23,24,31,33,34). Araoz *et al* (1,2) showed that a few of the recombinant hypothetical unknown proteins can be recognized by circulating antibodies in a few sera of MB

patients but also induced the *M. leprae* specific T cell response in PB and HHCs. Surprisingly, our study showed that the majority of the selected antigen candidates were detected by circulating antibodies in sera of LL patients suggesting that these antigens can be processed and accommodated on the Major Histocompatibility Complex (MHC) molecule to present to T-helper cells (Figure 2.2.B). Although all cavitary TB patients presented high bacterial loads, the seroreacivities of these patients were undetectable to most of the antigen candidates by Western blot analysis (Figure 2.2.C).

Recently, several serological studies to abundant antigens (PGL-I, LID-1 and MMP-II) showed that 9-10 % of HHCs exhibited the seropositivities to these antigens and 10 % of individuals with seropositivity developed the clinical leprosy within 6-8 months (5,9,21). Due to low antibody titers, 15-30% of PB patients can be detected by ELISA to the abundant antigens but the level of ELISA (close to cutoff) in these patients causes the high false positivity rates (≥ 10%) (29). Therefore, it is still questionable that a stand-alone serological test is used to detect individuals with asymptomatic leprosy. Recent studies in our laboratory (32) examined the reactivity patterns of patient sera to PGL-I, lipoarabinomann (LAM), and six recombinant proteins of abundant protein antigens (ML1877, ML0841, ML2028, ML2038, ML0380, and ML0050) by Western blot analysis and ELISA. This analysis showed that antibody response patterns highly correlate with a particular disease state (32), suggesting that these serological test lead to better patient management. Since 9 selected antigens induced the *M. leprae* specific antigens in LL patients, it would be useful to determine the disease states and allow for a more critical assessment of the form of disease within the leprosy spectrum and for understanding of leprosy pathogenesis.

Therefore, these novel recombinant antigens have considerable potential to improve the sensitivity and specificity of CMI-based diagnosis. The CMI response to all of these purified recombinant antigens is now being evaluated against PBMCs of leprosy patients and HHCs in endemic regions. In the future, the identification of *M. leprae*-specific peptides from these novel candidates that are restricted to all major HLA-DR types and specifically activate the maximal IFN-γ in PBMCs from PB patients and HHCs will allow improvements in the sensitivity and specificity of IGRA for early diagnosis of leprosy.

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CHAPTER 3 EVALUATION OF NOVEL ANTIGENS FROM *MYCOBACTERIUM LEPRAE* CYTOSOLIC MEMBRANE FOR THE EARLY DIAGNOSIS OF LEPROSY

From a manuscript form in preparation by **Hee Jin Kim**, Chaman Ranjit, Alan R. Schenkel and Patrick J. Brennan. Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado.

3.1 Abstract

The great needs towards successful control of leprosy are tools to detect *Mycobacterium leprae* infected individuals. The assessment of cellular mediated immune (CMI) responses to *M. leprae* specific antigens is thought to be the most promising diagnostic platform for the early detection of leprosy. Until now, two classes of novel antigens have been applied to CMI based diagnosis of leprosy: *M. leprae* subcellular fractions, MLSA-LAM and MLCwA, generated by proteomic approach; and protein/peptide antigens, selected by comparative genomic and bioinformatic analysis. However, CMI tests based on protein antigens are still problematic in detection of *M. leprae* infection/exposure in endemic regions due to low sensitivity to individual recombinant protein/peptide antigens and low specificity to *M. leprae* native protein antigens.

M. leprae membrane antigen (MLMA) induces a strong CMI response in tuberculoid leprosy patients. However, the large amount of lipomannans (LMs) and

lipoarabinomannans (LAMs) hinders the further processing of this fraction and the optimal use of this fraction as a diagnostic reagent in CMI based assays. Here, we developed a simple sodium carbonate treatment and successfully enriched membrane proteins into an alkali soluble membrane protein fraction (MLMA-SP) excluding the most hydrophobic lipids. Subsequent *in vitro* IFN-γ release assays (IGRA) of peripheral blood monocyte cells (PBMCs) from 16 mycobacterial laboratory/leprosy clinic workers revealed that MLMA-SP induced higher levels of IFN-γ secretion in the group exposed to leprosy patients, as compared to the group with environmental/occupational mycobacterial exposure. Therefore, MLMA-SP may contain immunogens to allow improvements in both sensitivity and specificity for the early diagnosis of leprosy.

3.2 Introduction

Since the 1980's, our laboratories have examined the native *M. leprae* proteins from armadillo derived *M. leprae* to encourage antigen discovery for diagnosis of leprosy (27,34,35,43-45). Several major antigens had been identified from subcellular compartments and their immunological potentials were evaluated toward the development of immune based diagnosis, particularly CMI-based diagnostic tests (see section 1.10). Two promising *M. leprae*-native-protein-rich antigenic fractions had been translated into clinical products, the so called "bench to bedside research". MLSA-LAM and MLCwA (5) have just completed Phase II clinical trials as delayed type hypersensitivity skin test antigens to be used for diagnosis of human leprosy in routine clinical practice.

However, major obstacles remain in the identification of *M. leprae* native antigens which are: 1) the inability to cultivate *M. leprae in vitro* makes it extremely difficult to produce sufficient quantities of refined reagents; and 2) the sharing of *M. leprae* antigens among other mycobacteria. These problems have been overcome using postgenomic approaches to generate either recombinant proteins or peptides derived from *M. leprae* specific hypothetical unknowns (previously described in section 1.10.2). *In vitro* IFN-γ release assays (IGRAs) to these new antigens can diagnose individuals exposed to/or infected by *M. leprae* (2,3,17-19,21,51). However, the low level of IFN-γ production to peptide antigens make difficult to detect the *M. leprae* infected individuals in geographically/racially different endemic populations (20). Therefore, it is necessary to continue the identification of *M. leprae* specific antigens to greatly enhance sensitivity and specificity of IGRA for early diagnosis.

Either whole *M. leprae* or *M. leprae* whole cell lysates in infected dendritic cells suppressed surface expression of the antigen presenting molecules (MHC-I and II) and co-stimulatory molecules (CD80, CD83) as well as IL-12 and TNF-α (29,39). However, MLMA induced the expression of MHC-II, CD86 and CD83 on dendritic cells and stimulated IL-12 p70 production from dendritic cells (29,30). The MLMA also induced IFN-γ production from naïve and memory $CD4^+$ T cells and $CD8^+$ T cells by stimulating dendritic cells (29). These data suggest that MLMA contains highly immunogenic molecules which could enhance the sensitivity of current IGRA formatted diagnosis by efficiently activating antigen presenting cells and being presented to T cells.

Major concerns in studying MLMA as diagnostic reagents are that most *M. leprae* proteins share high sequence similarity with orthologues in other mycobacteria which can

raise undesirable cross-reactivity due to infection with other mycobacteria (8,9,36,50). However, the massive gene decay and reduction of *M. leprae* could create a distinct native membraneous protein profile which could achieve different antigenic profiles from those of other mycobacteria. Earlier studies in our laboratories also showed that the proteome of MLMA by gel and MS analyses appeared to be unique (25,27,35). MLMA was predominated by two major membrane protein I (MMP-I) and MMP-II (27). Since MMP-I and MMP-II were absent or of low abundance in other mycobacteria, these two antigens had already been shown to induce *M. leprae*-specific T cell response in paucibacillary/tuberculoid leprosy (PB/TT) patients and humoral response in multibacillary/lepromatous leprosy (MB/LL) patients (11,30,32,41,47,52). However, MLMA also harbors large quantities of lipomannans (LMs) and lipoarabinomannans (LAMs) which cause immune modulation and cross-reactivity with other mycobacteria (5,29). CMI assays based on the proteins from MLMA have never been fully explored.

The aim of our work was to develop and evaluate the immunogenic proteins from MLMA which could improve the sensitivity and specificity of CMI based assay to detect individuals with asymptomatic leprosy in endemic regions. In this study, we developed the procedure to enrich the membrane associated proteins from MLMA and evaluated the resultant MLMA-SP by IGRA in individuals professionally exposed to leprosy patients.

3.3 Materials and methods

3.3.1 Isolation of M. leprae and preparation of M. leprae membrane antigen (MLMA)

M. leprae Thai-53 was isolated from armadillo spleens and livers by the 'Draper protocol' (48) with modifications. Briefly, the *M. leprae* infected armadillo tissues (25g),

invariably spleen or liver, provided by Dr. Truman from Louisiana State University, were homogenized in a sterilized chamber with 75 ml of homogenization buffer (150 mM EDTA, pH7.2) at 4 °C for 3 min by using the Omni-mixer (Omni International, Kennesaw, GA). The homogenate was then centrifuged at 5,000 × g for 5 min at 4 °C to remove residual non-homogenized tissue. The pellet was rehomogenized with 50 ml of homogenization buffer for 3 min. The resulting supernatants from both homogenization steps were combined and then centrifuged at 10,000 × g for 10 min at 4 °C. The pellet was resuspended with 200 ml of an extraction buffer (0.1M NaOH, 10 mM EDTA) to extract out residual tissue pigments, incubated with stirring for 2 hr at 4°C and centrifuged at 10,000 × g for 30 min at 4 °C. The bacteria-tissue pellet was washed, resuspended with 150 ml of buffered water (0.1 M Tris-HCl, pH 7.2, 0.1% Tween80) and digested for 3 hr in collagenase (20 mg per gram of infected tissue; Sigma, St. Louis, MO) at 37 °C. Subsequent digestion was performed with trypsin and alpha-chymotrypsin (20mg per gram of infected tissue; Sigma, St. Louis, MO) at 37 °C for 2 hr. The bacterial pellet was collected by centrifugation at 10,000 × g for 30 min at 4 °C, resuspended in PEG/DEX buffer (6% polyethylene glycol (PEG), 8% dextran, 10 mM KH_2PO_4, 10mM NaCl) and mixed by vortexing. The resulting PEG/DEX-bacteria mixture was then placed into a 500-ml separatory funnel and subjected to 2-phase partition over approximately 1 hr at 25 °C. The residual tissue-pigments were in the lower dextran phase, while the *M. leprae* bacilli were in the upper PEG phase. The resulting PEG-bacteria solution was then centrifuged at 27,000 × g for 30 min at 4°C. The bacteria pellet was washed and resuspended with buffered water. The quantity of *M. leprae* was evaluated by reading an O.D. at 540nm corresponding to 0.365 mg/ml of bacteria (49).

The bacteria (200 mg, obtained from approximately 116 g of *M. leprae* infected armadillo tissues) were washed twice with 5 ml of lysis buffer (50 mM Tris- buffer pH 7.4, 10 mM magnesium chloride) by centrifugation at 3,000 × g for 10 min. The cells were resuspended in the lysis buffer with a protease inhibitor cocktail (P8340; Sigma, St. Louis, MO), then disrupted by intermittent probe sonication (MSE Sonic prep150, MSE-Sanyo, Palisades Park, NJ) for 30 cycles (60 sec bursts/90 sec of cooling on ice). The whole cell sonicate was digested with 10 µg/ml of DNase and 10 µg/ml of RNase for 30 min at 4 °C and centrifuged at 27,000 × g for 30 min. The supernatant was recentrifuged at 100,000 × g for 3 hr to provide the insoluble pellet fraction, the MLMA (34).

3.3.2 Protein enrichment of the MLMA

The MLMA was resuspended to a protein concentration of 1mg/ml with ice-cold 100 mM Na_2CO_3, pH 11.4 (16). The resulting suspension was stirred for 30 min at 4 ºC and then centrifuged at 100,000 × g (3 hr, 4 ºC). The released proteins, which are mostly basic peripheral membrane proteins (16), were found in the supernatant (MLMA-SP). The alkali insoluble proteins of MLMA (MLMA-InsP) in the pellet were resuspended with lysis buffer. Both MLMA-SP and MLMA-InsP were dialyzed against 10 mM ammonium bicarbonate and lyophilized and kept at -70 °C prior to use for T cell simulation assays. The LPS content of all antigens as measured by the Limulus amoebocyte lysate test (BioWhittaker, Walkersville, MD) was less than 2 ng/mg of protein. For protein analysis, the MLMA-InsP was subjected to ice-cold 1% Triton X-114 solubilization and then to ultracentrifugation (100,000 × g, for 3 hr). The pellet (P) fraction called MLMA-P was utilized for the identification of CD1a restricted antigens

(see Chapter 4). The resulting supernatant was applied to phase separation and generated the aqueous phase and detergent phase; the detergent phase contained the integral plasma membrane proteins and lipoproteins (4). Figure 3.1 summarizes the experimental procedure.

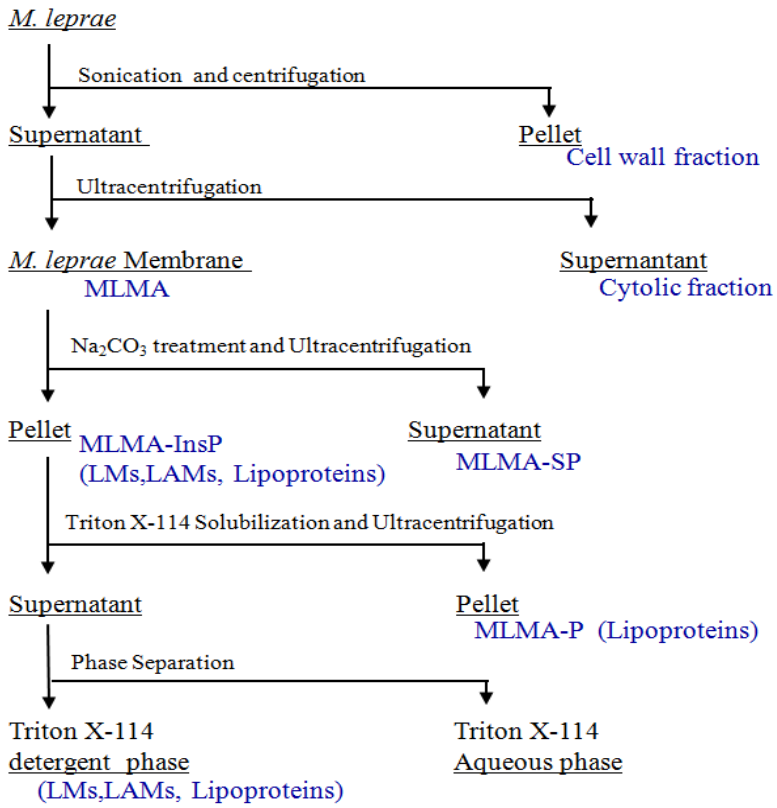

Figure 3.1 Experimental scheme for the fractionation of *M. leprae* membrane antigens (MLMA).

Both resulting aqueous and detergent phases, generated from Triton X-114 phase separation, were subjected to cold acetone precipitation to remove the detergents for gel analysis. Protein quantities of all subcellular fractions were measured by the bicinchoninic acid assay reagent. All fractions were analyzed by SDS-PAGE. A periodic acid oxidation and silver staining step visualized PIMs, LMs and LAMs, and the protein components, respectively (27). Identical gels were transferred onto nitrocellulose

membranes for Western blot analyses by the procedure previously described in section 4.2.7. Briefly, blots were blocked and then probed with monoclonal antibody CS38 to detect MMP-I and mouse polyclonal anti-MMP-II antibody to detect MMP-II. Both antibodies were provided by the NIH Leprosy Contract, CSU. Blots were probed by secondary anti-mouse IgG alkaline phosphatase and developed by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tretrazolium (Sigma, St. Louis, MO).

3.3.3 T cell stimulation assay

3.3.3.1 Study subjects

Sixteen healthy volunteers were recruited at the Mycobacterial Research Laboratories in CSU. All volunteers were employees or collaborators in the laboratories and many of these individuals had worked extensively with mycobacteria and/or cared for leprosy patients. A questionnaire was used to assess the exposure level of all volunteers to nonviable laboratory strain of mycobacteria (*M. smegmatis*, *M. bovis* BCG, *M. tuberculosis* H37Rv) or *M. leprae*. Informed consent forms had been authorized by all volunteers prior to blood donation. All tests and procedure were approved by the Institutional Review Board Committee at CSU.

3.3.3.2 T cell stimulation assay with MLMA derived antigens and IFN-γ Enzyme-Linked ImmunoSorbent Assay (ELISA).

Twenty milliliter of blood from each subject was drawn by venipuncture, heparinized and PBMCs were isolated by Ficoll (GE Healthcare, Pittsburgh, PA) density centrifugation according to Spencer *et al.* (51). PBMCs were resuspended in AIM-V

medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA). PBMCs were plated at 2×10^6 cells/ml in 96 well round bottom plates (Costar Corporation, Cambridge, MA) in triplicate and stimulated with each of the membrane subfractions (2 µg/ml such as MLMA, MLMA-SP, MLMA-InsP), or mitogen PHA (0.5 µg/ml; Sigma, St. Louis, MO), or PPD (1 µg/ml; Mycos Research, Fort Collins, CO), or media alone as negative controls. After five days of culture at 37ºC in a humidified 5% CO_2 atmosphere, supernatants were collected and stored immediately at -70 ºC until further analysis. The supernatants were tested for IFN-γ levels by using a IFN-γ ELISA Kit (UCytech, Utrecht, the Netherlands). All values of released IFN-γ on stimulation were subtracted by the value of IFN-γ released by media alone negative control.

3.3.3.3 PGL-I rapid lateral flow test

The PGL-I Rapid Antibody Test kits (Standard Diagnostics Company, Kyonggi-do, Korea) based on synthetic ND-O-BSA were provided by the Leprosy Contract, CSU (courtesy of Dr. Sang Nae Cho, Yonsei University, Seoul, Korea) and were utilized to detect circulating anti-PGL-I antibody in the sera of all participants. After the isolation of PBMCs, 5 µl of plasma were diluted with 45 µl of the running buffer provided in the kit and a total 50 µl was applied onto the window of the kit. The test was read after 10 min. Positivity was determined by appearance of both test line and control line while negativity was the appearance of the single control line (7).

3.3.3.4 Statistical analysis

Differences in IFN-γ levels between antigens tested and test groups were analyzed with the one-tailed Turkey-Kramer test by JMP 8.0 Statistical Discovery Software (SAS Institute Inc., Cary, NC).

3.4 Results

3.4.1 Subfractionation of MLMA by sodium carbonate treatment

In the identification of native protein antigens, chromatographic approaches have been used to prefractionate the native protein pools and to enrich certain proteins in isolated fractions (15). However, this proteomic approach as applied to membrane fractions is difficult due to complications with both extraction and solubilization of amalgamated hydrophobic proteins (15) and it becomes uneconomical when using *M. leprae* membrane proteins due to the paucity of membrane fraction, i.e. MLMA versus MLCwA and MLSA. In the case of *M. leprae*, the membrane fractions also contain large amounts of LAMs and LMs rendering this task particularly difficult. Therefore this study focused on a simple biochemical approach accompanied by a cellular based assay to indentify immunodominant proteins in *M. leprae* membranes.

The sodium carbonate treatment converts the closed vesicles of membrane lipids to open membrane sheets, and peripheral proteins are released in soluble form (16). This simple method combined with ultracentrifugation successfully released the alkali soluble peripheral membrane proteins with a great reduction of LAMs, LMs and PIMs from MLMA (Figure 3.2.A and B).

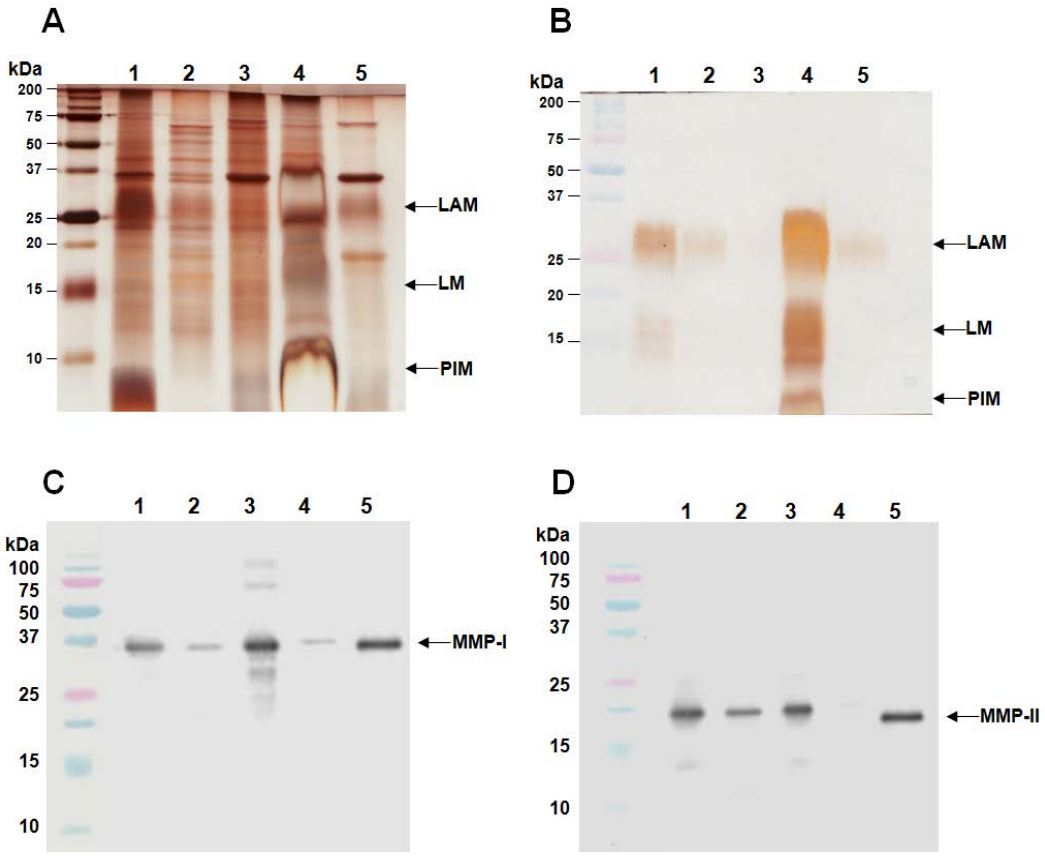

Figure 3.2 SDS-PAGE and Western blot analysis of subfractionated proteins of MLMA. A) Protein pattern of MLMA derived fractions was visualized by silver staining. B) LMs, LAMs, PIMs in identical gels were revealed as diffuse bands with average masses of 30, 20 and 8 kDa respectively by sliver staining combined with sodium periodate. C) Western blot pattern obtained with CS-38 (anti-MMP-I monoclonal antibody). D) Western blot pattern obtained with anti MMP-II polyclonal antibody; 5 µg of each protein fraction was run in each lane. Lane 1 is MLMA; lane 2, MLMA-SP; lane 3, MLMA-P; lane 4, the TritonX-114 detergent phase proteins from MLMA-InsP; lane 5, all of the Triton X-114 aqueous phase proteins from MLMA-InsP.

After dialysis of the alkali soluble protein in the supernatant, the MLMA-SP contained most of the membrane proteins (Figure 3.2). The two dominant major membrane proteins, MMP-I (ML0841, 35 kDa) and MMP-II (ML2038, bacterioferritin, 18kDa), were found in MLMA-SP by gel and Western blot analyses. Subsequently, Triton X-114 separation of the alkali insoluble proteins (MLMA-InsP) showed that the integral plasma membrane proteins were enriched into the Triton X-114 detergent phase along with most of the LAM, LM, and PIMs in membrane subcellular fractions (Figure

3.2.A and B). The integral membrane proteins from MLMA made up approximately 4% of the total MLMA but were masked by the enormous amount of lipoglycans (LMs and LAMs). The Triton X-114 aqueous phase was devoid of proteins according to protein quantification assay (bicinchoninic acid protein assay). Since MMP-I and MMP-II were proven to be peripheral proteins, Western blot analysis showed that Triton X-114 detergent phase contained a minimal amount of MMP-I and no MMP-II (Figure 3.2.C and D).

3.4.2 Classification of the study groups

In order to evaluate the immunogenicity of the new *M. leprae* subfractions, MLMA-SP and MLMA-InsP were evaluated by measuring IFN-γ production of PBMCs from a group of healthy laboratory workers some of whom may have or have had a history of exposure to leprosy/tuberculosis patients or the respective bacteria. This experiment is a presage for future applications to populations in leprosy endemic regions and the only type of experiment achieved within our own laboratory setting. Also the levels of exposure to *M. leprae* or other mycobacteria was determined as a result of the PGL-I rapid lateral flow test (also known as ML flow test; see section 1.10.4) and previous TB skin test or BCG vaccination and questionnaires (Table 3.1) regarding periods of working experience with leprosy patients.

Table 3.1 Characteristics of participating study population.

	NonExp group (4)	labMycoExp group (7)	LepExp group (5)
Duration of Exposure to Leprosy Patient	N/A[a]	N/A	one-15years[b]
Duration of Exposure To Native Mycobacterial Products[c]	Less than 4 months	3 years- 40 years	N/A
Positivity of TB skin test	0/4	6/7	2/5
No. BCG Vaccination	0	one or 3[d]	one
Endemic Site born/lived	0/4	6/7	5/5
Positivity to PGL-I lateral flow test	0/4	0/7	5/5

a; Not applicable. b; All individuals had direct contact with leprosy patients and were involved in sampling the blood/or skin slit smears. Two individuals physically examined leprosy patients on a regular basis. Average contact was one or two active leprosy cases per week. c; The mycobacterial products were native antigens from *M. tuberculosis* H37Rv, *M. bovis* BCG. Two of seven individuals had additionally worked with *M. leprae*. d; Most of the individuals had received a single dose of the BCG vaccine but one individual had 3 doses of the BCG vaccine at different times.

The participants in this study were categorized into 3 groups (Table 3.1): 1) Nonexposed group (NonExp group, n= 4), i.e. those who were born in the U.S.A., were both TB skin test and PGL-I test negative, and had worked for less than 4 months in a mycobacterial laboratory; 2) Laboratory nonviable mycobacteria exposed group (labMycoExp group, n= 7), i.e. those who were born in leprosy/tuberculosis endemic countries and/or had experience in handling nonviable laboratory mycobacteria or *M. leprae* in mycobacterial laboratories for 3-40 years. Six of seven individuals in this group were TB skin test positive but PGL-I test negative; and 3) Leprosy patient exposed group (LepExp group, n=5), i.e. those who were PGL-I test positive, born in leprosy endemic countries/regions and had extensively worked with leprosy patients but not significantly with TB patients. However, three of individuals in this group were TB skin

test negative (Table 3.1). Seroreactivity of those individuals to the PGL-I test appeared to be a good correlation with exposure time to leprosy patients.

3.4.3 Production of IFN-γ by PBMCs in response to MLMA derived antigens

PBMCs from the NonExp group did not produce IFN-γ in response to any of the membrane derived antigens (MLMA, MLMA-SP and MLMA-InsP) or PPD, but responded well to the positive mitogen control (PHA) (Figures 3.3 and 3.4). In the NonExp group, MLMA-SP induced a low level of IFN-γ production (mean=112.1 pg/ml), whereas MLMA-InsP induced the highest level (mean=358.2 pg/ml) among the four test antigens. The low level of IFN-γ production in non-exposed individuals tends to confirm that both MLMA-SP and MLMA show little or no response to an immunocompetent population selected for minimal mycobacterial exposure (Figure 3.4).

The MLMA and the two subfractions showed differences in their ability to induce IFN-γ secretion between the labMycoExp and LepExp groups. The LepExp group showed the highest responses to MLMA and MLMA-SP (Figures 3.3 and 3.4). Individuals in the labMycExp group born in endemic regions exhibited higher CMI responses to all tested antigens as compared to the NonExp group due perhaps to low but continuous exposure to other mycobacterial antigens. One individual in the labMycoExp group out of 7 individuals received three doses of BCG vaccine. This individual consistently had higher levels of IFN-γ production to all antigens (PPD, MLMA and MLMA-P) except MLMA-SP.

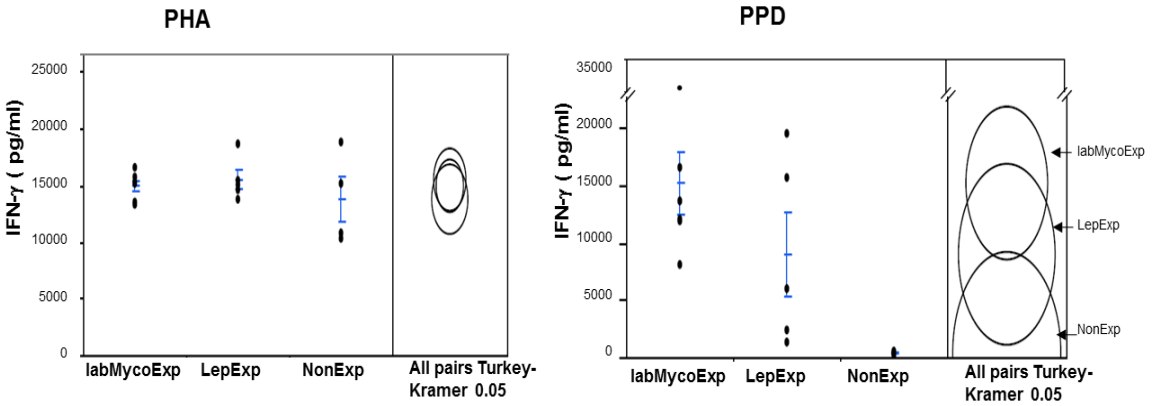

Figure 3.3 IFN-γ production by PBMCs induced in response to PHA and PPD. PPD (1μg/ml) and PHA (0.5 μg/ml) stimulated the PBMCs from all experimental groups which were to NonExp group (n=4), the labMycoExp group (n=7) and the LepExp group (n=5). The levels of released IFN-γ in cultured supernants of PBMCs were measured by IFN-γ ELISA. All values were corrected by subtraction of mean negative control values. The vertical bars indicate 0.95 confidence intervals. One way Turkey-Kramer tests were assessed for significance at p=0.05 (JMP 8.0 Statistical Discovery Software). The right side of graphical presentation in each graph shows the statistical difference between groups. The less overlap between circles indicates significant differences.

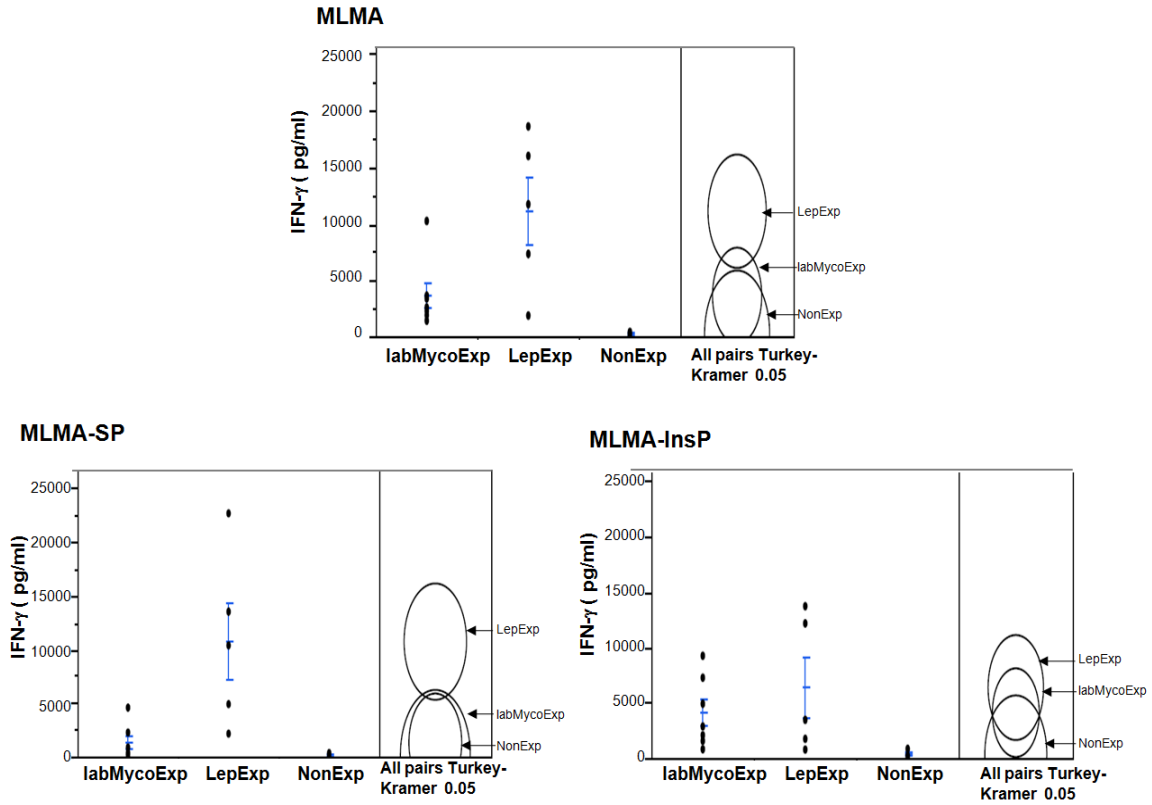

Figure 3.4 IFN-γ production by PBMCs induced in response to three of the MLMA derived subfractions. MLMA, MLMA-SP and MLMA-InsP (2μg/ml) were used to stimulate the PBMCs from the same three test groups for Figure 3.2. Analysis and evaluation are described in Figure 3.3.

Variance in MLMA-SP responses was in part attributed to the reduced immune response of three individuals, all of whom were exposed to leprosy patients with positivity of PGL-I lateral flow test but negativity of TB skin test. Also these three individuals showed relatively low levels of IFN-γ production in response to PPD which induced high level of IFN-γ production from all individuals in the labMycoExp group and other members of the LepExp group. However, MLMA-SP compared to either MLMA-InsP or PPD was capable of inducing relatively higher levels of IFN-γ production from three of these low responders (Figures 3.3 and 3.4).

3.5 Discussion

The aims of this work were: 1) to enrich the immunodominant proteins from the native cytosolic membrane fraction (MLMA) of *M. leprae* to be used eventually, presumably as recombinant proteins, for CMI based assays; and 2) to evaluate this fraction of *M. leprae* to detect individuals exposed to/ infected by *M. leprae* prior to development of clinical symptoms of leprosy, as a prelude to application for CMI based test for *M. leprae* infection in leprosy endemic regions.

Mycobacterial membrane fractions contain substantial amounts of LMs and LAMs which are exceptionally abundant in the membrane fractions of *M. leprae*, as well as the more conventional phospholipids (6,26,34). LMs and LAMs, immune modulators, induce proinflammatory cytokines and eventually promote the differentiation of naïve $CD4^+$ T cells into Th1 IFN-γ producing cells via TLR-2 or/and TLR-4 signaling (28,40). Additionally, ManLAMs from LAMs suppress immune response to mycobacteria (40). These heterogeneous glycolipids, LMs LAMs and PIMs, are structurally very similar

across *Mycobacterium* spp. (40). In mycobacterial infections, IFN-γ is a potent proinflammatory mediator often induced in the course of the CMI response (18,31). IFN-γ secretion of PBMCs has been utilized as a readout of CMI based assays to mycobacterial antigens as a consequence of mycobaterial infection or exposure (10,38,42). Therefore, LM and LAMs are primarily responsible for cross-reactivity with related mycobacteria when applied in immune based diagnostic assay formats.

In an earlier study of our laboratory, a single Triton X-114 phase separation of the *M. leprae* subcellular membrane fraction dominantly enriched two major membrane proteins (MMP-I and MMP-II) and partially excluded LMs, LAMs and PIMs (27). However, the resulting membrane proteins in the aqueous phase were recovered with a great loss of protein quantities due to the precipitation of most proteins and subsequent mandatory detergent removal procedures (27). Here, we showed for the first time that simple treatment with an alkaline sodium carbonate solution successfully excluded most of the LM, LAM, and PIM content from the highly hydrophobic integral and peripheral protein antigens in MLMA without use of detergent and enriched a majority of membrane proteins typically obscured by enormous amount of LMs and LAMs (Figure 3.2). The protein recovery rate of the MLMA-SP was sufficient to pursue further immunological studies using a CMI based assay format.

Previously, *M. leprae* cell wall and soluble cytosolic fractions, MLSA-LAM and MLCwA, were shown to induce slightly higher levels of IFN-γ production from PBMCs of house hold contacts (HHCs) and TT patients but less cross-reactivity to PPD-positive healthy individuals, as compared to *M. leprae* crude lysates (12,33,53). The health-care workers and HHCs developed strong Th1 responses to the *M. leprae* derived antigens

presumably by continuous contacts with MB and PB patients (17,21,47,51). Levels of IFN-γ production to the mixed proteins antigen, MLSA or MLCwA, were quite varied from one IU/ ml to near 500 IU/ml in HHCs though they could discriminate well between leprosy patients and endemic healthy controls (33,53). The MLMA-SP, similar to these mixed protein antigens, produced relatively higher amounts of IFN-γ from the PBMCs of individuals in the LepExp group than MLMA, MLMA-InsP or PPD (Figures 3.3 and 3.4) while MLMA-InsP generated similar levels of IFN-γ from the PBMCs of individuals in both labMycoExp and LepExp groups. Therefore, the results suggest that the enhanced reduction of LMs and LAMs in MLMA-SP can improve the specificity of IGRA to detect exposure of leprosy.

Recently, several studies revealed that a few of HHCs were positive by the PGL-I rapid lateral flow test (13,22,23). In this present study, all individuals in the LepExp group were seropositive in the PGL-I rapid lateral flow test depending on their exposure time to leprosy patients (Table 3.1). Three individuals in this group showed low levels of IFN-γ response to MLMA-SP and even PPD (MLMA-InsP and PPD). Some individuals exposed to/infected by *M. leprae* induce no/low CMI response to crude mycobacterial antigens (PPD) or *M. leprae* antigens (33). Recent studies showed that host genetic polymorphisms in different components of the type 1 cytokine pathway failed to induce CMI responses and affect human resistance to mycobacterial diseases including leprosy (1,24,46) (described in section 1.9). It is possible that the low Th1 immunity of the three individuals in the LepExp group to mycobacterial antigens (PPD and MLMA-InsP) is associated with genetic susceptibility to leprosy (Figures 3.3 and 3.4). However, MLMA-SP caused relatively higher amounts of IFN-γ production than either MLMA-

InsP or PPD even in these same low T cell responders, implying that the MLMA-SP might contain the immunogenic molecules necessary to develop Th1 immune response.

Taken together, a single sodium carbonate treatment successfully enriched most of the membrane associated proteins from crude MLMA and simultaneously excluded most of the LAMs and LMs (Figure 3.2). The immune response to the resultant MLMA-SP seemingly discriminated individuals with leprosy exposure from those with other forms of mycobacterial exposure, implying that the MLMA-SP subfraction contains more *M. leprae* specific immunogenic antigens than MLMA or MLMA-InsP. Although, this initial work is promising, further evaluation of the MLMA-SP in TT patients and HHCs of MB patients is required prior to application in leprosy endemic clinical settings.

In the development of CMI based diagnostic tests using *M. leprae* specific antigens, the ultimate goal is to detect *M.leprae* infected individuals with asymptomatic leprosy as the biological reservoirs and to support the decision for early treatment of those affected individuals. CMI based IGRAs in combination with highly selected proteins or peptides were shown to identify the occupational contacts (LepExp group in the present study) or HHCs, who are exposed to/infected by *M. leprae* (5,20,21) but do not exhibit the obvious clinical symptoms as well as TT/PB patients. Prospective cohort studies on leprosy patients in chemoprophylaxis and their families revealed that the HHCs of PB and MB patients have a high risk of developing disease (14,37). The majority of these HHCs with asymptomatic infection and appreciable CMI reactivity are protected by maintaining an effective CMI response to continuous exposure (21), but a few of these individuals may be carriers of contagious *M. leprae* with/without developing leprosy (14,37). Therefore, follow-up studies should be performed to address the question of whether the HHCs

screened by IGRA using *M. leprae* specific antigens are resistant/susceptible to the development of clinical leprosy. Since leprosy is a slow onset disease with low incidence rate, such studies should be conducted with a large number of contacts over long periods of time in order to obtain conclusive results. The extensive literature shows a distinct cytokine profile between TT and LL patients. Therefore, additional analysis of other cytokine profiles (i.e. IL-2, TNF-, TGF-ß, IL-4, and IL-10) during these clinical follow-up studies will allow for the discovery of new biomarkers in T cell assays of *M. leprae* antigens which can be used to identify protection against leprosy/disease progress. This type of prospective research will allow for the further understanding of leprosy pathogenesis and provide the evident indicator to initiate chemotherapy of individuals with true subclinical leprosy, preventing disease progression and transmission of *M. leprae* to uninfected individuals.

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CHAPTER 4 IDENTIFICATION AND BIOCHEMICAL DEFINITION OF NEW *MYCOBACTERIUM LEPRAE* ANTIGENS PRESENTED BY CD1a OF LANGERHANS CELLS

This work has been done in the collaboration with Dr. Peter Sieling and Dr. Robert Modlin, in University of California, Los Angeles. Protein production/fractionation at Colorado State University by Hee Jin Kim. T cell assays at UCLA by Dr. Peter Sieling

4.1 Introduction

CD1 proteins are expressed on the surface of B cells, myeloid dendritic cells, Langerhans cells and other antigen presenting cells (37). They are structurally related to the Major Histocompatibility Complex-I (MHC-I), a peptide antigen presenting molecule (4). A CD1 molecule consists of two chains: β 2 microglobulin (β 2m) and a heavy chain containing three extracellular domains (α1-α3) (Figure 4.1). On the basis of sequence comparisons, the molecules can be divided into two groups: Group 1 consists of CD1a, CD1b, CD1c, and CD1e, which are involved in antigen presentation of microbial lipids to clonal T cells; Group 2 contains only CD1d, presenting lipid antigens to natural killer T cells (2,4,32).

In antigen presentation, CD1 holds the alkyl chains of amphipathic lipids into a deep hydrophobic groove of CD1 heavy chain formed between the α1 and α2 helices (2,32) (Figure 4.1). The hydrophilic parts of these antigens are exposed on the surface of CD1 molecules and presented to T cell receptors (2,32) (Figure 4.1). Many of the unique

mycobacterial lipids found in the cell envelope can be presented by CD1a, CD1b and CD1c to activate a variety of T cell clones. These include mycolates (21,29), lipoglycans (8,41), sulfoglycolipid (14), mycobactin (31) and mannosyl β-1-phospho-mycoketides (27,30) (see Figures 1.7, 1.8 and 1.22).

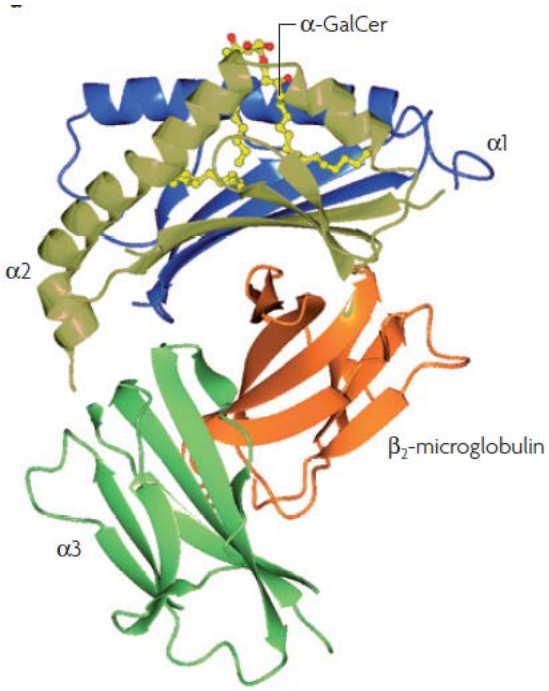

Figure 4.1 Crystal structure of murine CD1d loaded with a synthetic α-galactosylceramide (adapted from Barral and Brenner (2) with permission). The acyl chains of α-galactosylceramide (α-GalCer) (yellow) are inserted into the hydrophobic binding groove which is formed between α1 (blue) and α2 (light green) helices of the heavy chain of CD1. The hydrophilic oxygenated (red) sugar of this CD1d antigen is exposed to T cell receptors of a $CD1^+$ T cell.

CD1-restricted T-cells ($CD1^+$ T cells) induce protective Th1 immunity to intracellular pathogens such as mycobacteria (see section 1.8.3). Particularly, the mycobacteria specific $CD1^+$ T cells secrete potent cytolytic molecules such as perforin, granulysin and efficiently kill cells that are intracellularly infected by live virulent *M. tuberculosis* (3,47,48). Importantly, $CD1^+$ T cells appear frequently in patients infected by *M. tuberculosis* or *M. leprae* (8,14).

In *M. leprae* infection, Toll like receptor-1 (TLR-1), TLR-2 and CD1s are more strongly expressed in skin lesions of tuberculoid leprosy (TT) patients, as compared to those of lepromatous leprosy (LL) patients (20,43). TLR-2 signaling by synthetic lipopeptides (derived from mycobacterial lipoproteins) efficiently mediates differentiation of $CD1^+$ dendritic cells from peripheral blood mononuclear cells (PBMCs) of TT patients (20). Langerhans cells, as abundant dendritic cells in TT skin lesions, coexpress high levels of CD1a and langerin (CD207), as one of the C-type lectin receptors (52). Langerin can mediate phagocytosis and efficient innate immunity in skin lesions via interaction with carbohydrate containing cell surface molecules such as mannose, N-acetyl glucosamine and fucose (44). The $CD1a^+$Langerhans cells efficiently present mycobacterial lipids to $CD1a^+$ T cells which can produce high levels of IFN-γ and granulysin (34,44) suggesting that bacterial lipoprotein may serve as CD1a restricted antigens or could be involved in CD1a antigen presentation via Langerhans cells.

In this study, we hypothesized that Langerhans cells present a unique set of microbial antigens, bacterial lipoproteins, to CD1a restricted T cells. Therefore, the aim of this study was to identify and to biochemically characterize lipoprotein(s) as novel *M. leprae* specific antigens recognized by CD1a-restricted T cell clones from leprosy skin lesions.

4.2 Materials and methods

4.2.1 T cell lines, proliferation and IFN-γ released assays

T cell lines were derived from skin lesions of borderline tuberculoid leprosy (BT) patients according to Sieling *et al*. (44). Briefly, lymphocytes were obtained from patients' skin lesions with a tissue sieve and isolated by Ficoll-Hypaque (GE healthcare,

Pittsburgh, PA) gradient centrifugation. T cell lines were activated by adding recombinant IL-2 (rIL-2; 1nM; Chiron Diagnostics, Emeryville, CA) and irradiated PBMCs from the same donors. Subsequently, T cell lines were cultured with $CD1^+$ antigen presenting cells which were stimulated by *M. leprae* lysates. The established T cell lines were maintained in rIL-2 containing media and propagated by adding heterologous PBMCs and PHA (Sigma, St. Louis, MO). In antigen specific T cell proliferation assays, T cells (1×10^4) and $CD1^+$ antigen presenting cells (usually 1×10^4) were cultured with or without of bacterial antigens in microtiter wells (in triplicate) at 37 °C in a 7% CO_2 incubator. After 3 days, cells were pulsed with [^{3}H] thymidine (1 μCi/well; ICN Biomedicals, Irvine, CA), harvested 4–6 hr later and subjected to liquid scintillation counting. For *in vitro* IFN-γ release assays, T cells (1×10^4) were stimulated in the same manner as the proliferation assay but incubated only for 24 hr. The supernatants were collected for measuring IFN-γ levels by an ELISA assay kit (BD Pharmingen, San Jose, CA). All of these biological experiments were conducted by Dr. Sieling in UCLA.

4.2.2 Proteinase K treatment of M. leprae membrane antigen (MLMA)

The 50 μg of MLMA was treated with or without 2 unit/ml of proteinase K (Sigma, St. Louis, MO) in PBS. For reaction controls, proteinase K was heat-inactivated and added into MLMA. All reactions were incubated at 37 °C overnight and inactivated for T cell assays by heating at 100°C for 10 min.

4.2.3 Sub-fractionation of MLMA

The hydrophobic MLMA-P (Trion X-114 insoluble fraction of MLMA-InsP) was generated from MLMA by the methods described in section 3.3.2. Figure 4.2 summarized the experimental procedure.

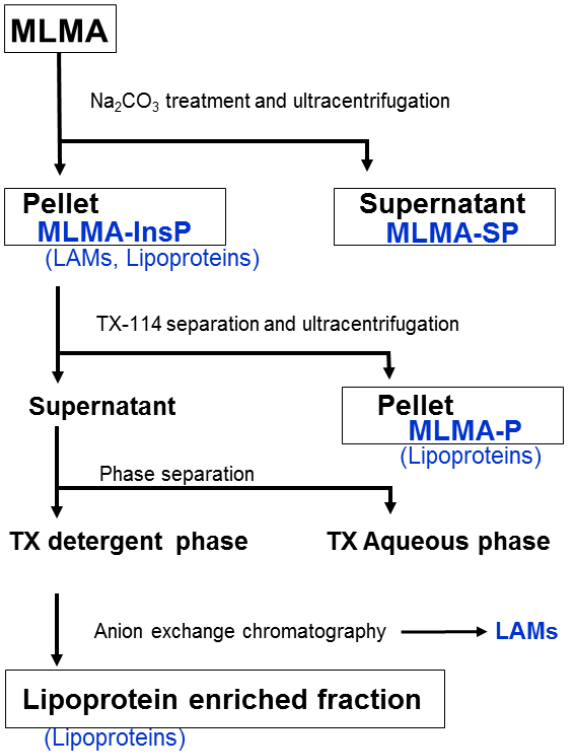

Figure 4.2 Experimental scheme for the sub-fractionation of MLMA (modified from Figure 3.1)

Briefly, MLMA-InsP was subjected to 1% Triton X-114 solubilization and ultracentrifugation (100,000 × g, 3 hr). The resultant Triton X-114 insoluble fraction (MLMA-P) in the pellets was subjected to cold acetone precipitation to remove residual detergent. The supernatant was subjected to phase separation which allowed enriching lipoproteins into the detergent phase. This detergent phase fraction was applied to cold acetone precipitation.

4.2.3.1 Anion exchange chromatography of MLMA-P for preparation of a lipoprotein enriched fraction

The protein pellet of the Triton X-114 (200 μg) detergent phase fraction was subjected to solubilization with 1 ml of denaturing buffer (8M urea, 50 mM Tris (pH 11.0), 20 mM dithiothreitol) for 3 hr at 4 °C and was centrifuged to remove the insoluble materials for 30min at 27,000 × g at 4 °C. The supernatants were applied onto an anion exchange column (Q Sepharose; GE healthcare, Pittsburgh, PA) to remove LAMs (lipoarabinomannan), LMs (lipomannan) and PIMs (phosphatidylinositol mannosides), and washed with 5 × column volumes of denaturing buffer. The resulting lipoprotein enriched fraction (40 μg) was recovered by adding 500 μl of elution buffer (8M urea, 50 mM Tris-HCl (pH 4.0), 20 mM dithiothreitol) and was dialyzed against 10 mM ammonium bicarbonate prior to T cell simulation assays.

4.2.3.2 Size exclusion (SE) fractionation of MLMA-P

The MLMA-P was further fractionated on the basis of molecular mass of its constituents using the Whole Gel Eluter (Bio-Rad Life Science, Hercules, CA) according to the manufacturer's instructions. Briefly, the MLMA-P (1 mg) was dissolved in 1 ml of NuPAGE LDS sample buffer (Invitrogen Life Technologies, Carlsbad, CA) containing 50 mM dithiothreitol and was electrophoretically separated in a precast 4-12% NuPAGE Novex Bis-Tris mini preparative gel (Invitrogen Life Technologies, Carlsbad, CA) with MES Running Buffer (Invitrogen Life Technologies, Carlsbad, CA). The gel was placed in a Mini Whole Gel Eluter (Bio-Rad Life Science, Hercules, CA). Subsequently, an

electric current (75 mA, 30 min) was applied to recover the separated proteins into 14 fractions in an elution buffer (60 mM Tris 40 mM CAPS, pH 9.3).

4.2.3.3 Preparative isoelectric focusing (IEF) fractionation of MLMA

The MLMA (2 mg) was dialyzed against 10mM ammonium bicarbonate, lyophilized and resuspended with 2 ml of IEF buffer (7 M urea, 2 M thiourea (Invitrogen Life Technologies, Carlsbad, CA), 0.5% octyl β-D-glucopyranoside (Sigma, ST. Louis, MO), 0.5% ASB-14 (Sigma, ST. Louis, MO), 4% ampholyte (pH range 4 to 7; Invitrogen Life Technologies, Carlsbad, CA), 1% ampholyte (pH range 3 to 10; Invitrogen Life Technologies, Carlsbad, CA), 10 mM DTT). The protein solution was separated into six fractions by liquid-phase isoelectric focusing with a Mini-Rotofor apparatus (Bio-Rad Life Science, Hercules, CA) under constant power of 15 watt for 3 hr, according to the manufacturer's instructions. Each fraction was dialyzed and subjected to cold acetone precipitation.

4.2.4 Limulus amoebocyte lysate test, bicinchoninic acid assay and SDS-PAGE analysis of protein fractions

Protein quantities of all protein fractions and recombinant proteins were determined by the bicinchoninic acid assay. Individual fractions were analyzed by electrophoresis in custom made 15% SDS-PAGE or commercial 4-12% NuPAGE gels (Invitrogen Life Technologies, Carlsbad, CA). The gels were visualized using a periodic acid oxidation step for LMs, LAMs and PIMs, and silver staining for the proteins. All resultant protein fractions were dialyzed against 10 mM ammonium bicarbonate and

lyophilized until use. LPS contents of all protein fractions were determined by the Limulus amoebocyte lysate test and were less than 2 ng/mg of protein.

4.2.5 Butanol extraction of MLMA and MLMA-P

Both MLMA and MLMA-P (1mg per protein) were resuspended with 1ml of PBS and added to 1ml of PBS saturated butanol. Subsequently, the preparations were incubated on a rocker at 25 °C for 30 min and centrifuged at 27,000 × g for 10 min at 25 °C. The samples were separated into three phases. Delipidated proteins (devoid of noncovalently linked proteins) were located at both the interphase and the lower water phase while lipids were in the upper organic phase. The interphase and lower water phase were combined and were dialyzed against 10 mM ammonium bicarbonate. The lipids in upper phase were dried under N_2 gas. Fractions were analyzed by thin layer chromatography in $CHCl_3$:CH_3OH:H_2O (65:35:5) developed with 0.2% sulfuric acid spray, to assess the lipid content, and by SDS-PAGE to analyze the protein content.

4.2.6 In gel tryptic digestion and electrospray ionization-MS analysis

Protein fractions which induced IFN-γ production of LCD4.15, were applied to electrophoresis (4-12% NuPAGE gel; Invitrogen Life Technologies, Carlsbad, CA) and proteins stained using Coomassie Brilliant Blue R-250 (Invitrogen Life Technologies, Calsbad, CA). Coomassie stained proteins spots were excised from the gel and subjected to in gel digestion with trypsin (Roche Applied Sciences, Indianapolis, IN), according to Marques *et al*. (25). Briefly, protein gel pieces were destained three times with 25 mM ammonium bicarbonate (pH 8.0) in 50% acetonitrile and dried in a speed-vacuum

concentrator (ThermoFisher Scientific, Hampton, NH). The gel pieces were reconstituted in 15 µl of 200 mM ammonium bicarbonate (pH8.0) containing 20 ng/µl of trypsin and were incubated overnight at 37 °C. The resulting peptides were recovered by twice extracting with 100 µl of 50% acetonitrile containing 5% trifluoroacetic acid with vortexing for 30 min and dried in a speed-vacuum concentrator. Dried peptides were resuspended with 20 µl of 5% acetonitrile, 0.1% acetic acid and subjected to electrospray ionization-MS analysis at the PMF (CSU).

Peptides were purified and concentrated using an on-line enrichment Agilent Zorbax (Agilent, Santa Clara, CA) C_{18} column 5µm (5 × 0.3mm). Subsequent chromatographic separation was performed on a reverse phase nanospray column (Agilent 1100 nanoHPLC Zorbax C_{18}, 5µm, 75 µm ID × 150mm column; Agilent, Santa Clara, CA) by using a 42 min linear gradient from 25%-55% buffer B (90% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min. Peptides were eluted directly into the mass spectrometer (Thermo Scientific LTQ Linear Ion Trap, ThermoFinnigan, San Jose, CA) and spectra were collected over a *m/z* range of 200-2000 Da using a dynamic exclusion limit of 2 MS/MS spectra of a given peptide mass. Compound lists of the resulting spectra were generated by using Bioworks 3.0 software (ThermoFinnigan, San Jose, CA) with an intensity threshold of 5,000 and 1 scan/group. MS/MS spectra were searched against the NCBI nonredundant protein database and the *M. leprae* database (1614 entries) (22) using the Mascot database search engine (version 2.3). The scaffold software 3.0 (Proteome Software, Portland, OR) was applied to verify peptide identifications by the use of MASCOT 2.2 and SEQUEST search engine probabilistically validating the peptide and protein identifications to all MS/MS sequencing results (25).

4.2.7 Analysis of lipoproteins by using a bacterial lipoprotein database and BLASTP algorithm

AA sequences of putative *M. leprae* lipoproteins were analyzed by a bacterial lipoprotein database (DOLOP, http://www.mrc-mb.cam.ac.uk/genomes/dolop/) (see section 1.7.1.1). The AA sequences of the N terminal regions (Cys at +1 to +17) from selective lipoproteins were compared to orthologues of *M. tuberculosis* (51) by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome)

4.2.8 Cloning of M. leprae lipoproteins into **M.** *smegmatis expression vector pVV16*

Eight genes encoding putative lipoproteins were amplified from genomic DNA of *M. leprae* Thai-53 using the Touchdown PCR method (see section 2.3.4). The primers (IDT Technology, Denver, CO) contained the recognition sequences of unique restriction enzymes, *Nde*I, or *Bam*HI and *Hind*III respectively, and were specific to the upstream and the downstream of the ORFs (Table 4.1). All PCR products were directly digested with restriction enzymes and cloned into the *E. coli-M. smegmatis* shuttle expression vector pVV16, provided by Dr. Vissa (CSU), which allowed to produce the 6-Histidine tag at the C-terminus of the target protein. The DNA sequences of all recombinant clones were confirmed by automated nucleotide sequencing at the PMF, CSU. Each of recombinant clones was transformed into the expression host, *M. smegmatis* mc^2155.

Table 4.1 List of primers used in cloning of putative lipoprotein ORFs

Name	Sequence	Name	Sequence
ML1086 forward	AACATATGGTGGTGAGTCGGCGC	ML0489 forward	AACATATGCGGTCCGGCTTATTCG
ML1086 reverse	AAAAGCTTCGGCAACAAGCCCTTC	ML0489 reverse	AAAAGCTTTCGTACTAACGCCCAT
ML0706 forward	AACATATGAAGGCTGCCGTGACAC	ML0303 forward	TGGATCCGATGACGCGCCAGTT
ML0706 reverse	AAAAGCTTCCACTGCTGCACCAAG	ML0303 reverse	AAAAGCTTATCCACATACCTCGGTGTG
ML2095 forward	AACATATGAAGCTCAACCAGTTTGGT	ML2593 forward	AACATATGAGGCGTGCAATGAGTG
ML2095 reverse	AAAAGCTTAGCGATGGCATTAGCC	ML2593 reverse	AAAAGCTTGCATCGCCTATCGG
ML2446 forward	AACATATGATCGCATCCACTCCGC	ML1699 forward	AAACATATGCGGATAGGGCGGTC
ML2446 reverse	AAAAGCTTACCACCGGGTCTAGCC	ML1699 reverse	AAGCTTGGTTTTGTCGTCGTTG

Recognition sequence of the restriction enzyme is shaded. AAGCTT is the recognition sequence for *HindIII*, CATATG for *Nde*I and GGATCC for *Bam*HI

4.2.9 Subcellular fractionation of M. *smegmatis and detection of the recombinant lipoproteins*

Each of *M. smegmatis* recombinant clones containing lipoprotein gene constructs, pVV16:*lpr1086*, pVV16:*ml2095* or pVV16:*ml2446*, was cultured in 2 L of Luria-Bertani (LB) broth or M7H9 broth (supplemented with 0.2% glycerol) with 50 μg/ml of kanamycin and 25 μg/ml of hygromycin to mid-log phase at 37 °C (42), centrifuged at 4 °C and frozen at -70°C. The cells (10 g, wet weight) were resuspended with lysis buffer (50mM Tris-HCl (pH8.0), 150mM NaCl) containing 10 μg/ml of DNase, 10 μg/ml of RNase and protease inhibitor cocktail (P8340, Sigma, St. Louis, MO), and disrupted by intermittent probe sonication with a Soniprep 150 sonicator (MSE Sonic Prep150, MSE-Sanyo, Palisades Park, NJ) for 10 cycles (60 sec bursts/90 sec of cooling on ice). The lysates were centrifuged at 5000 × g for 5 min to remove unbroken cells and the supernatants were centrifuged at 27,000 × g for 30 min at 4 °C. The pellets contained insoluble cell walls. The supernatants were applied to ultracentrifugation at 100,000 × g for 3 hr at 4 °C and were separated into cytosol fraction in the supernatant and membrane fraction in the pellet. In order to verify the localization of recombinant proteins, all

fractions were analyzed by 15% SDS-PAGE and Western blot using anti-6-Histidine tag polyclonal antibody (1:3000 dilutions; Sigma, St. Louis, MO).

4.2.10 Purification of recombinant lipoproteins

The harvested recombinant *M. smegmatis* (80-100 g) from 20 L of culture were subjected to subcellular fractionations. Both resulting cell wall and membrane fractions were resuspended with denaturing buffer (50 mM Tris- HCl (pH 8.0), 8M urea), incubated on the rocker at 4°C for 1 hr and centrifuged at 27000 × g for 30 min at 4°C. The supernatant was applied onto Ni-NTA agarose column (Qiagen, Valencia, CA) and the column was washed with 20 × column volumes of the same buffer. The recombinant proteins were eluted with 1 × column volume of denaturing buffer containing stepwise gradients of imidazole (5, 10, 20, 50,100,150 and 200 mM) (46). The recombinant lipoproteins (200 μg, obtained from approximately 100 g of each *M. smegmatis* clone) were dialyzed against 10mM ammonium bicarbonate, lyophilized and kept at -70 °C

4.2.11 Construction the hybrid Lpr1086 by using the signal sequence of M. tuberculosis LprG (Rv1411c)

The DNA sequence coding for the Rv1411c signal sequence was amplified from the pVV16 construct with *rv1411c* provided by Dr. Belisle (CSU) using the two step PCR (72 °C for annealing and extension steps, and 94 °C for the denaturing step). In this reaction, the forward primer (5'-TGGCAGCGAGGACAACTTGA 3') was specific to the 135 base pair upstream region from the translational initiation site of pVV16: *rv1411c*, and reverse primer

(5' GGTGGGAGCCTGCGTGCCCCCGCTGCAGCCGGCAACGACAGTGGCGGCGA

TGC 3') contained the nucleotide sequence encoding the signal peptide and Cys_{+1} of Rv1411c (shaded). This reverse primer also contained 24bps of nucleotide sequence encoding consecutive 6 AAs (Gly_{+2} to Gln_{+7}) of the ML1086 (underlined). The resulting PCR product encoding the Rv1411c signal sequence and 6 AAs (Gly_{+2} to Gln_{+7}) of ML1086, was then purified from agarose gel electrophoresis and used as a macroprimer in a second round of PCR. Subsequently, the hybrid *lpr1086* was generated using the macroprimer, ML1086 reverse primer (Table 4.1) and the pVV16 construct with *ml1086* as template (24). The PCR product, *lpr1086*, was cloned into the pVV16 vector. The DNA sequence of this recombinant clone was confirmed by automated nucleotide sequencing at the PMF (CSU). The pVV16: *lpr1086* was transformed into *M. smegmatis* mc^2155 to express and purify the recombinant lipoprotein.

4.3 Results

4.3.1 Characterization of the LCD4.15 T-cell clone

Dr. Sieling in UCLA had established CD1a-restricted T-cell lines from BT patients. These T cell lines were mostly activated by Langerhans cells' presentation of the antigens in several mycobacterial extracts including *M. tuberculosis*, *M. smegmatis* and *M. leprae*. None of these clones was activated by the didehydroxy-mycobactin, which was proven as a CD1a restricted antigen in *M. tuberculosis* infection, or several of the known CD1 antigens including glucose monomycolate, mycolic acid, ManLAM and mannosyl β-1-phosphomycoketid (see section 1.8.3; data not shown). However, one T cell clone, LCD4.15, recognized solely *M. leprae* whole cell lysates (Figure 4.3.A).

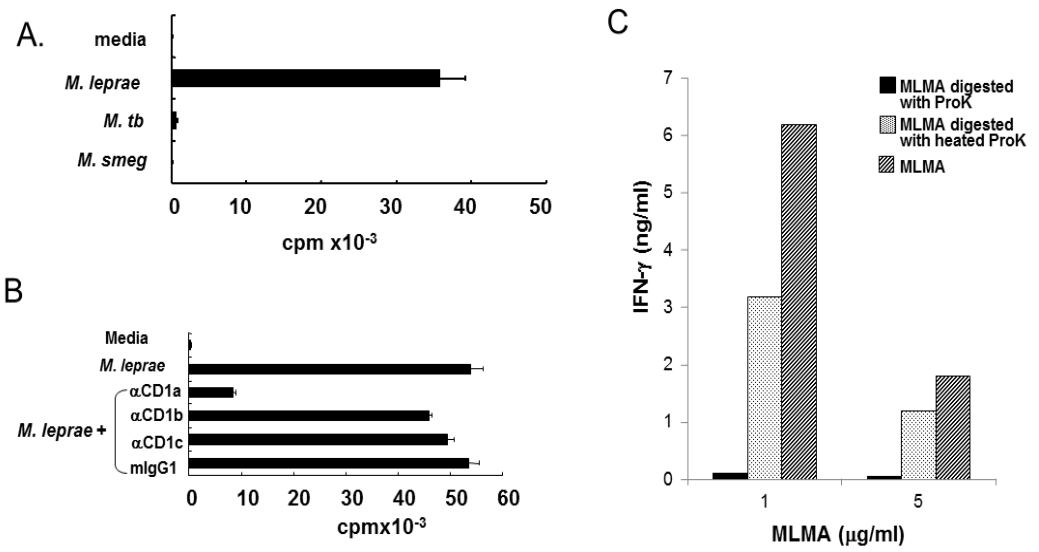

Figure 4.3 Proliferative response and IFN-γ production of LCD4.15. A) Proliferation assay of LCD4.15 in response to lysates of *M. tuberculosis* (*M. tb*), *M. smegmatis* (*M. smeg*) and *M. leprae*. B) CD1 blocking assay by using IgGs of CD1 isoforms. mIgG1 indicates mouse IgG. C) T cell reactivity after proteinase K (ProK) treatment of MLMA.

Blocking of CD1a by neutralizing IgG abolished the response to *M. leprae* whole cell lysates of this T cell clone (Figure 4.3.B). In contrast to other $CD1^+$ T cell clones recognized by mycobacterial glycolipids, this CD1a-restricted T cell line was unable to recognize proteinase K-treated *M. leprae* extracts (Figure 4.3.C). Since the *mbt* operon, which is responsible for the biosynthesis of mycobactin (nonribosomal lipopeptide), is absent in *M. leprae* (5), other biosynthetic intermediates related to mycobactin cannot be the antigen candidates recognized by LCD4.15. Collectively, the responses of LCD4.15 was restricted to CD1a antigen presentation by Langerhans cells and was protease-sensitive indicating that this line is capable of recognizing a *M. leprae* specific lipoprotein or a protein-lipid complex.

4.3.2 Activation of LCD4.15 in response to MLMA derived protein subfractions

Previous biochemical studies of CD1 antigens, supported by CD1crystallization studies, indicated that CD1 restricted antigens are generally amphipathic lipids (32). Therefore, we hypothesized that this new CD1a protein antigen would be covalently or noncovalently linked with lipids to retain the amphipathic feature of a CD1 antigen and would be enriched in hydrophobic protein pools, probably MLMA. Previously, as described in section 3.3.2, subfractionation of MLMA generated MLMA-SP (peripheral membrane protein of MLMA), MLMA-P (highly hydrophobic MLMA) and a lipoprotein enriched fraction (Figure 4.4.A and B).

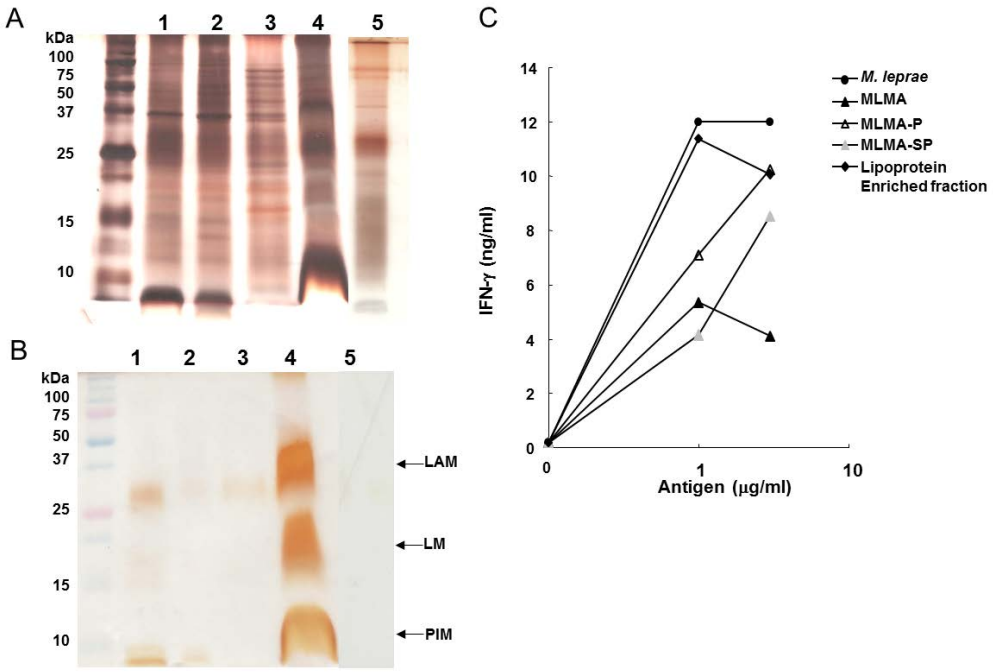

Figure 4.4 SDS-PAGE analysis of MLMA derived fractions and IFN-γ production by LCD4.15 induced in response to antigens.

A) MLMA fractions were stained with silver nitrate. B) Identical gel stained with silver staining in combination with sodium periodate for LMs, LAMs and PIMs. 5 μg of each protein fraction was run in individual lanes. Lane 1, MLMA; lane 2, MLMA-P; lane 3, MLMA-SP, lane 4, MLMA TX-114 detergent phase, and lane 5, lipoprotein enriched fraction. C) T cell reactivity to MLMA and each of subfractions derived from MLMA.

Overall, all MLMA derived fractions efficiently activated LCD4.15. T cell reactivity was especially strong on stimulation with both lipoprotein enriched fractions and MLMA-P which contained most of the lipoproteins and hydrophobic proteins but was devoid of LMs and LAMs (Figure 4.4.C). Thus, this novel antigen(s) might be a lipoprotein, or required a lipid/lipoprotein as carrier for lipidic antigen presentation.

In order to evaluate the role of lipoproteins in CD1a antigen presentation to LCD4.15, butanol extraction removed the noncovalently linked lipids from proteins proteins and possible nonribosomal lipopeptides (other than mycobactin) from MLMA-P and MLMA. Thin layer chromatography analysis showed that delipidated MLMA and MLMA-P proteins (devoid of noncovalently linked lipids) in the water phase did not contain free lipids, which were mostly extracted into the resulting organic phase (Figure 4.5.A).

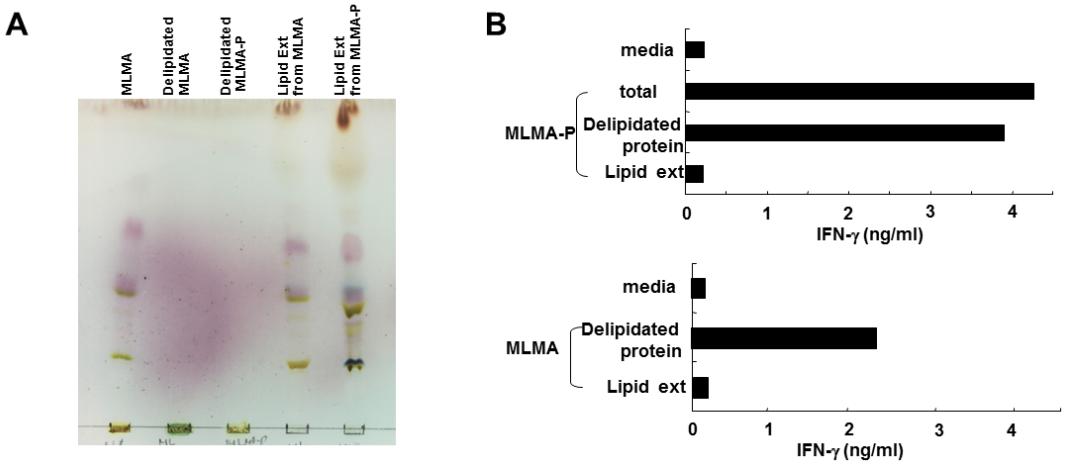

Figure 4.5 Thin layer chromatography analysis and T cell reactivity of MLMA and MLMA-P before/after delipidation. A) Thin layer chromatography analysis. MLMA and MLMA-P were delipidated with butanol and subjected to Thin layer chromatography in $CHCl_3$:CH_3OH:H_2O (65:35:5). The plates were developed with a sulfuric acid spray. B) IFN-γ production from LCD4.15 in response to MLMA, MLMA-P, delipidated version of the fractions (protein fractions without covalently linked lipids) and the lipid fractions. Lipid ext, lipid extracts from delipidation of MLMA or MLMA-P.

T cell reactivities to both delipidated fractions on LCD4.15 were equivalent to those of the parent MLMA-P and MLMA, but extracted lipids did not induce any T cell reactivity on stimulation of LCD4.15 (Figure 4.5.B). The results indicated that a lipoprotein, which is covalently acylated, was the likely new CD1a restricted *M. leprae* specific antigen recognized by LCD4.15. This was the first evidence that a bacterial lipoprotein is involved in CD1 restricted antigen presentation.

4.3.3 Identification of a CD1a restricted M. leprae specific antigen by using mimic Multi-Dimensional Protein Identification Technology

In order to identify the precise CD1a antigens, the sequential multi-dimensional protein identification technology (11) might not be feasible with the very limited supply of *M. leprae* available. Therefore, the MLMA-P and MLMA were subjected to the mimic sequential multi-dimensional protein identification technology which consisted of two different separation methods. The first was size exclusion (SE) fractionation by SDS-PAGE gel elution and the other was a preparative IEF fractionation, combined with T cell reactivity assays.

The first size exclusion (SE) fractionation generated 14 fractions from MLMA-P (Figure 4.6.A). T cell reactivities of all fractions revealed that the SE fraction 8 strongly induced IFN-γ production on stimulation of LCD4.15 indicating that this T cell clone recognizes a protein antigen in a range of molecular weight 37 to 55 kDa (Figure 4.6.B). In order to indentify this new CD1a antigen, three consecutive fractions (SE fraction 7 to 9) were subjected to in gel tryptic digestion and MS based peptide identification.

Bioinformatics analyses of MS/MS data from all three fractions identified more than 100 proteins where two or more unique peptides were identified.

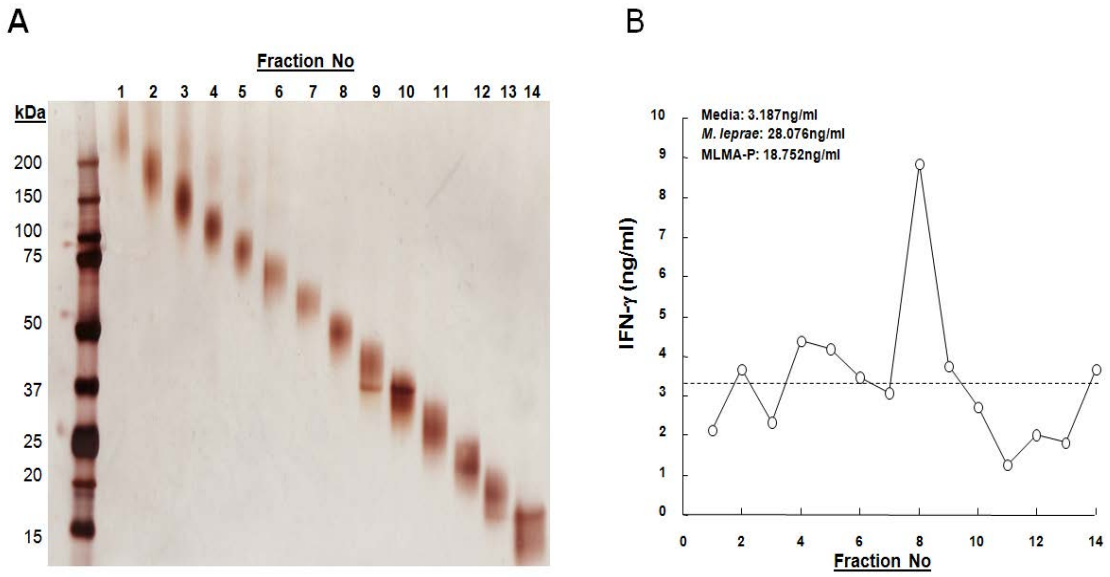

Figure 4.6 Size exclusion (SE) Fractionation of MLMA-P. A) SDS-PAGE analysis of fractions after size exclusion (SE) fractionation of MLMA-P; B) Production of IFN-γ in response to resultant fractions on LCD4.15.

Secondly, the preparative IEF fractionation generated six additional fractions. The T cell reactive assay of LCD4.15 revealed that a CD1a antigen was enriched in IEF fraction 4 which was in the range of pI 6 and 8 (Figure 4.7). This protein fraction was subjected to peptide identification by electrospray ionization-MS/MS analysis.

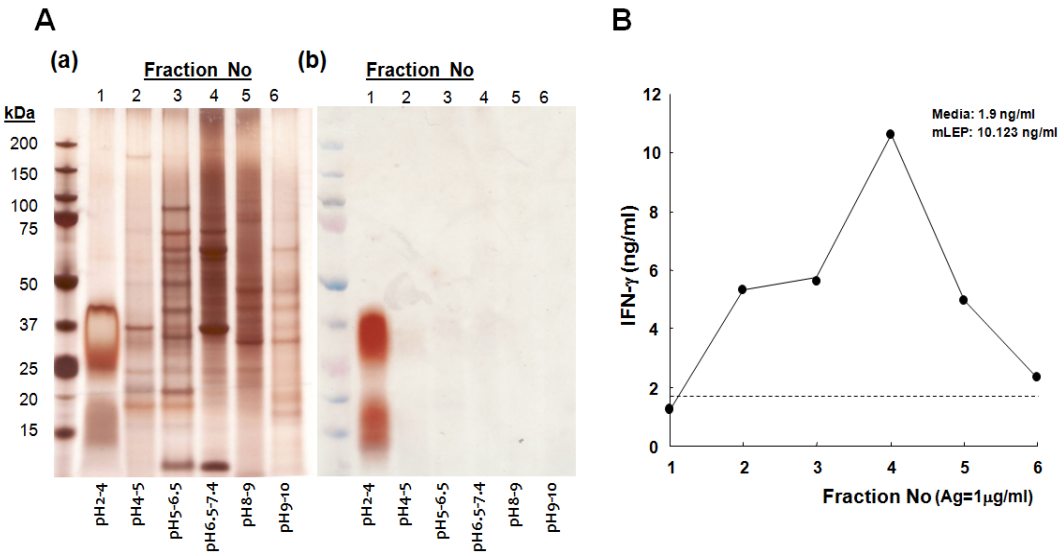

Figure 4.7 Preparative Isoelectric Focusing (IEF) fractionation of MLMA. A) SDS-PAGE analysis of fractions after IEF fractionation of MLMA-P; (a) silver stained gel for the proteins (b) sodium periodate stained gel for the carbohydrates: B) Production of IFN-γ in response to resultant fractions on LCD4.15.

A total of 24 proteins were identified by MS/MS analyses, common to both SE fraction 8 and IEF fraction 4, but were absent in SE fraction 7 and 9. Bioinformatic analysis screened five of the most plausible antigen candidates that could be covalently acylated. Among the five possible candidates (Table 4.2), two lipoproteins, ML1086 (a solute binding protein, LpqY) and ML2095 (a phosphate binding protein, Pst3), were identified as possible CD1a antigen candidates. In mass spectrometry analysis, the AA sequences extracted from unique peptides which allowed the identification of ML1086 or ML2095, corresponded to 8% or 33% of sequence coverage of the predicted AA sequence of ML1086 or ML2095 respectively (Table 4.2). Three other proteins identified in this manner were found to be involved in the fatty acid biosynthetic pathway and these proteins could be transiently acylated. Therefore, further study focused on the expression and evaluation of recombinant proteins, ML1086 and ML2095.

Table 4.2 List of CD1a antigen candidates indentified by proteomic analysis of both IEF fraction 4 and SE fraction 8.

ORF (Protein)	Function	MW (kDa)	pI	Peptide Sequence	Sequence Coverage (%)	Protein probability (%)
ML2565 (FabG4)	3-ketoacyl-(acyl-carrier-protein) reductase	47.325	6.172	LTEGLVGNGSISK VVVIGTTPDATTSTDER WTDSFGGLVFDATGITAPAELK LNSLLQGGLPVDVAETIAYFAIPASNAVTGNVIR	19%	95%
ML2639	Aldehyde dehydrogenase	54.090	9.642	IQPTVIVDPDPEGPLMTK LPFGGVGESGIGAYHGK ALQALMVENEGAIAQALADDLDRNPVEAFIVDIAATVSEAK	12%	95%
ML2563c (FadE3)	Acyl-CoA dehydrogenase	66.567	4.992	IQPTVIVDPDPEGPLMTK IDSLYEGTTAIQAQDFFFR ALTDIQGMEASLTGYLMAAQQDVTSLYK FITSGDSDDLFENIFHLVLARPEGAGPGTK ALQALMVENEGAIAQALADDLDRNPVEAFIVDIAATVSEAK	16%	95%
ML1086 (LpqY)	Solute-binding transport lipoprotein	50.402	5.322	TLGDMSYTDIGR TALESFVAATQQHVTGEVR LSAQDVVADFSDNLAEELNFR	8%	95%
ML2095 (Pst3)	Periplasmic phosphate-binding lipoprotein	37.714	5.418	FVNAFER YPDAQVGR NDESGTTDNFQR IATSADPEPIAISVDSVGK AFLQSTIGGGQNGLGDNGYVPIPDSFK IFNGSIASWNDPAIQALNTGVALPAEPIHVVFR	33%	95%

MS/MS data of the peptides were searched against the NCBI database using the Mascot or SEQUEST algorithms. The scaffold software 3.0 was used to validate MS/MS based peptide and protein identifications. Protein probabilities were assigned by the Protein Prophet algorithm.

4.3.4 Bioinformatics based identification of a novel CD1a restricted antigen

Since lipoproteins mostly exist as low abundant proteins in the mycobacterial native proteome, bioinformatics based analysis was applied to identify putative lipoproteins using the 'lipobox' which is the consensus sequence of lipoproteins (see section 1.7.1.1 ; Figure 1.13). T cell reactivities of SE fractions on LCD4.15 suggested that the new CD1a antigen could be a lipoprotein in the range of molecular mass 37 to 55 kDa (Figure 4.6). Besides ML1086 andML2095 which were identified by proteomics analysis, six putative lipoproteins in the molecular mass range were additionally identified as CD1a antigen candidate(s) according to their molecular mass (Table 4.3).

Table 4.3 List of eight lipoprotein candidates selected by bioinformatics and proteomics analyses.

Gene	Mass	Alignment of N-terminus with homology in *M. tuberculosis*		
ML0489	58.9	ML0489	LTAC	SGSAAGE I VYVVDGAL
		Rv2585c	LTAC	SGSAAAQ I DYVVDGAL
ML1699, LppZ	39.1	ML1699	LSSC	ARFNNTQSQPFTTKP
		Rv3006	SSGC	ARFNDAQSQPFTTEP
ML1086 , LpqY	50.4	ML1086	VLAC	GAGGDQLV ISFYTPA
		Rv1235	AAAC	GADSQGLVVSFYTPA
ML2446c, LprQ	46.9	ML2446c	FVAC	AGSA IKPSEKKTTPAP
		Rv0483	LVAC	AGKVTKLAEKRPPPAP
ML2593, LprK	42.3	ML2593	LSSC	GWRGISNVSI PGGPG
		Rv0173	LSSC	GWRGISNVAI PGGPG
ML0706, PmmB	56.9	ML0706	LAAC	DPDELAARFTRALRFG
		Rv3308	LAAC	GPDELKARFSRPLAF
ML2095, PstS3	37.7	ML2095	LSGC	GSDNN AAVGSARTGP
		Rv0928	LSAC	GNDDNVTGGGATTGQA
ML0303, GlnH	35.7	ML0303	LAAC	GHAELLT V ASMPTLPP
		Rv0411	LASC	GHSETLGV EAT PTLPL

Grey box shows the lipobox. Pink boxes shows difference between *M. leprae* and *M. tuberculosis* orthologues

4.3.5 Expression of putative lipoproteins

The literature shows that the fast growing avirulent strain, *M. smegmatis*, shares highly conserved phospholipids and the machinery of post translational modification with pathogenic mycobacteria including *M. tuberculosis* and *M. leprae* (13,16,17,36,39,50,54).

Therefore, all genes encoding the selected lipoproteins were cloned into a mycobacterial expression vector, pVV16, and every attempt was made to overexpress these mycobacterial recombinant lipoproteins. However most of these candidates were not expressed in *M. smegmatis* with the exception of recombinant lipoproteins ML2446 and ML2095. These recombinant proteins were expressed at low levels and solely enriched in the cell wall subcellular fraction of recombinant *M. smegmatis* (Figure 4.8).

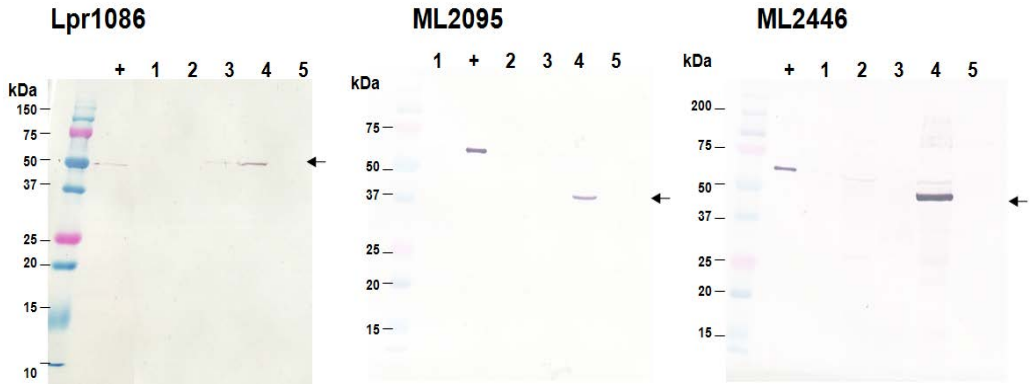

Figure 4.8 Subcellular localization of recombinant lipoproteins by using Western blot with anti-6-Histidine tag antibody. Lane 1 is whole lysate from *M. smegmatis* containing pVV16. Lane 2, 3, 4 and 5 are cytosolic, membrane, cell wall, and culture supernatant respectively of *M. smegmatis* containing each lipoprotein clone. + is a non-lipoprotein with a 6-Histidine tag as a positive control. Arrows indicate the recombinant lipoproteins. Lpr1086 is the recombinant fusion protein which contains the signal sequence of LprG/Rv1411c and ML1086 without its own signal sequence.

The purpose of this study was to identify and biochemically define the CD1a antigenic portion which would be the N-terminal part of acylated lipoprotein. Therefore, several genetic engineering technologies were applied to express the N-terminal part of lipoproteins in the present study. The first attempt was to express truncated forms of the recombinant lipoproteins which contained an acylated N-terminal part but their trans-membrane domains were excluded. Trans-membrane domains of lipoproteins or integral membrane proteins has been found to cause toxicity to the expression host when produced as recombinant protein (26). The second approach was to construct a fusion

gene containing the N-terminal portion of the gene of interest (signal sequence with +20 AAs from Cys_{+1}) and the C-terminal protein of *rv1411c* (without its signal sequence). Several previous studies showed that Rv1411c was successfully over-expressed in *M. smegmatis*; we expected that the C-terminal protein of *rv1411c* would help to express the N-terminal parts of target lipoproteins (10,12,15). However both approaches were unsuccessful to express the recombinant lipoproteins. Unexpectedly, we observed that a fusion construct (encoding for signal sequence ML1086 and Rv1411c) was expressed with substantially lower level compared to the intact *rv1411c* construct (data not shown) suggesting that the foreign signal sequence of ML1086 may affect the expression level of recombinant Rv1411c. Therefore, a final attempt was made to construct a fusion gene consisting of *ml1086* (without its signal sequence) and only the signal sequence of *rv1411c* into the pVV16 expression vector. The resulting *lpr1086* is a hybrid of *ml1086* and signal sequence of *rv1411c*. Lpr1086 was successfully expressed in *M. smegmatis* and enriched into both cell wall and membrane subcellular fractions (Figure 4.8). This method was applied to the remaining lipoproteins but it was not successful.

4.3.6 Purification, characterization and evaluation of T cell reactivity of recombinant Lpr1086, ML2095 and ML2446

The purified recombinant ML2095, ML2446 and Lpr1086 were analyzed by SDS-PAGE and Western blot with anti-6-Histidine tag antibodies (Figure 4.9.A and B). All three recombinant proteins were subjected to in gel tryptic digestion. Subsequent MS/MS data verified that all recombinant proteins were derived from the ORFs of ML1086, ML2095 and ML2446, respectively.

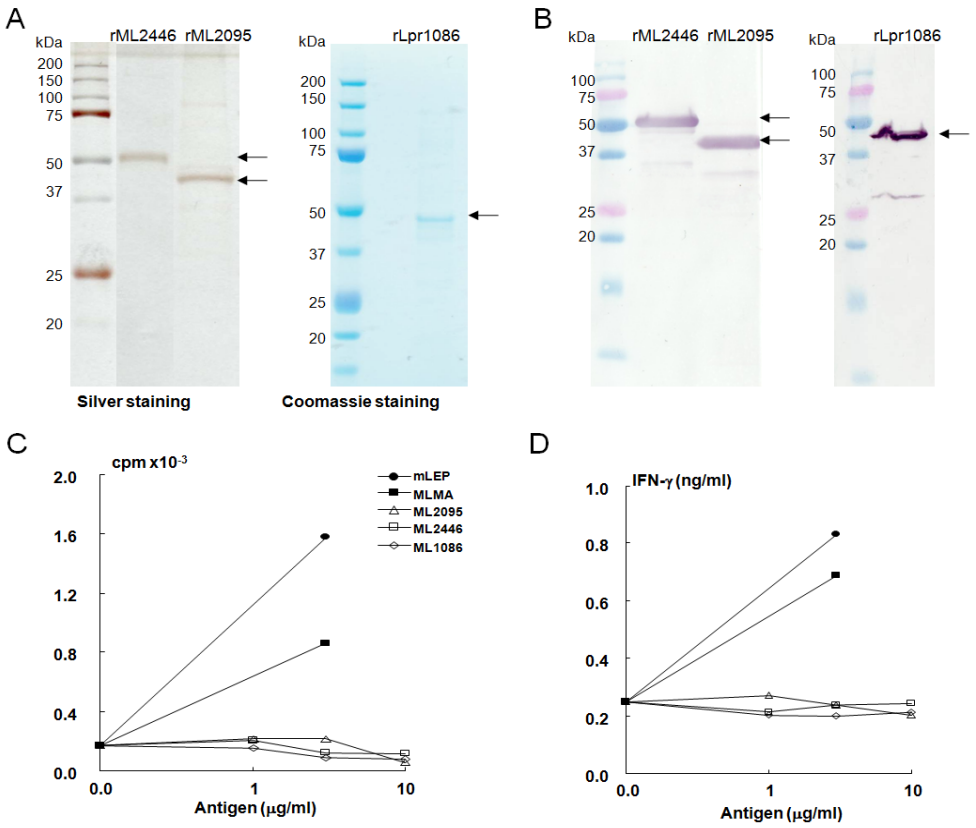

Figure 4.9 SDS-PAGE analysis and CD1a-restricted T cell response to recombinant ML2446, ML2095 and Lpr1086 from *M. smegmatis*. A) Silver or Coomassie staining of SDS-PAGE; B) Western blot hybridized with anti-6-Histidine tag polyclonal antibody (1:5000). C) Proliferative response**;** D) IFN-γ production of LCD4.15 to rLpr1086, rML2095 and rML2446 Arrow indicates the recombinant protein (see section 4.2.11).

In order to analyze protein acylation, all three recombinant lipoproteins were treated with proteinase K followed by $CHCl_3$: CH_3OH: H_2O (10:10:3) extraction. The resulting lipopeptides in the organic solution were analyzed by Q-TOF LC/MS (see Chapter 5). Q-TOF LC/MS results verified that Lpr1086, ML 2095 and ML2446 contain di- or tri-acylated glycerylcysteine residues consisting of two or three of fatty acids (Table 5.1). However, all recombinant Lpr1086, ML 2095 and ML2446 expressed in *M. smegmatis* failed to stimulate the CD1a restricted T cell clone, LCD 4.15 (Figure 4.9.C and D).

4.4 Discussion

The major aim of this study was to identify a lipoprotein as a new class of CD1a antigens. This antigen would eventually allow understanding of the immune relationship between TLR activation and CD1a antigen presentation in cutaneous infections.

According to the crystal structures, the dimension of the antigen binding groove is 1300 Å for CD1a representing the smallest binding groove, as compared to 2200 Å for CD1b which forms the largest antigen binding pocket (32,56). While CD1b can load long alkyl chains that are C_{16} to C_{80} in length (2,32), CD1a presents relatively shorter alkyl chain of lipidic antigen but also can accept two short fatty acyl chains. CD1a restricted T cells recognize several T cell antigens including didehydroxymycobactin, and sulfatides (32). In *M. tuberculosis* infection, didehydroxy-mycobactin, a nonribosomal lipopeptide related to mycobactin siderophores, is the only known antigen recognized by mycobacterial specific CD1a restricted T cells clone. The structure of didehydroxy-mycobactin consists of a short acyl chain (C_{20}) and an invariant 5mer peptide which are linked via ester, oxazaline ring and amide bonds (53) (Figure 1.22). This broad structural similarity provided the hypothesis that bacterial lipoproteins which contain the N-acylated cysteine could be potential antigens for CD1 restricted T cells (31). Recently, Van Rhijn *et al.* (53) synthesized an N-acyl glycine dodecamer peptide similar to myristosylated peptide found in virus and mammalian cells. This synthetic lipopeptide was recognized by CD1c restricted T cells suggesting that bacterial lipoproteins could serve as CD1 restricted antigens and induce powerful protective immune response. Sieling *et al.* (42) demonstrated that both recombinant LprG/Rv1411c and LprG/ML0557 were recognized by $CD4^+$ MHC-II restricted T cell clones from a leprosy lesion and patients' blood.

Therefore, it has been hypothesized that bacterial lipoproteins are the most powerful vaccine candidates which can induce strong innate immunity via TLR-2 signaling and directly cause potent adaptive immunity in response to intracellular pathogens by serving as both MHC-II and CD1 restricted T cell antigens (28,32).

In this dissertation, we found that a CD1a-restricted T-cell line, LCD4.15, from skin lesions of leprosy patients specifically recognized *M. leprae* lysates through antigen presentation of $CD1a^+$ Langerhans cells (Figure 4.3.A and B). Unlike other CD1 restricted T cells, the response of LCD4.15 to *M. leprae* lysate was protease sensitive indicating that a bacterial lipoprotein could be an antigen for this clone (Figure 4.3.C). We developed simple proteomic and chemical methods to enrich a minor population of lipoproteins but excluded most of the lipid components which were often found to be the CD1 restricted antigens (Figures 4.4 and 4.5). Subsequent T cell reactivities of delipidated protein fractions revealed that a lipoprotein was possibly a new class of $CD1a^+$ antigen recognized by LCD4.15. Here, we provided the tentative evidence that CD1a can present a lipopeptide derived from a novel bacterial lipoprotein in intracellular pathogens.

The three possible antigen candidates, ML1086, ML2095 and ML2446, were identified by proteomic and genomic analysis, were expressed in *M. smegmatis*. Q -TOF LC/MS analysis of all three recombinant proteins showed the presence of di- or tri-acylated glycerylcysteine residues at their N terminus (Table 5.1). However, recombinant ML1086, ML2095 and ML2446, at least expressed in *M. smegmatis* and presumably with the proper lipid function, failed to activate the CD1a restricted T cell clone, LCD4.15 (Figure 4.9). The possible reasons for this outcome are: 1) the antigen

recognized by LCD4.15 could be another lipoprotein, less abundant such that it could not be detected by current proteomic analysis; 2) the recombinant lipoproteins expressed in *in vitro* grown *M. smegmatis* could present different acylation patterns from those of *M. leprae,* and this foreign acylation pattern could disturb the presentation of this protein for CD1a; 3) a nonribosomal lipopeptide, which consists of a short acyl chain and a highly polar peptide such as to be water soluble and synthesized only in *M. leprae,* could be involved in the activation of LCD4.15 by Langerhans cells. The nonribosomal lipopeptides in procaryotes commonly contain D-amino acids rather than L-amino acids which are the building blocks of protein synthesis by ribosomes (40,49). These lipopeptides are mostly protease resistant and also extractable into organic solvent, suggesting that a nonribosomal lipopeptide may be the least possible CD1a restricted antigen candidates.

The knowledge that the activation of a single CD1a T cell clone in mycobacterial infection is restricted to both a dehydroxy unsaturated (C20:1) acyl chain and the peptide of didehydroxy-mycobactin (31), suggests the second hypothesis. The activation of CD1a restricted T cells may be uniquely specific to both lipid and peptide structures compared to that of other group 1 CD1s. *M. tuberculosis* in infected cells metabolize host lipid as a primary carbon source to generate energy via the glyoxylate shunt (18,38). Recently, Carvalho *et al.* (7) revealed that *M. tuberculosis* could simultaneously catabolize multiple carbon sources but other bacteria preferred one or two types of carbon sources to support their growth, providing the evidence that mycobacteria are highly capable of adapting to various environmental conditions, particularly in phagocytes. Depending on carbon sources, aerobic conditions, nitric oxide or acidic stress and iron

availability, *in vivo* grown mycobacteria contain different lipid profiles from *in vitro* grown mycobacteria (1,9,18,23,33,35,38,45). *M. tuberculosis* grown *in vivo* were found to possess uncommon structural modifications on several mycobacterial lipids particularly PDIM, sulfolipid-1 and possibly phenolic glycolipid all involved in the pathogenesis of mycobacterial disease (19). Although *M. leprae* has undergone massive gene decay (5,55), genes involved in anabolic pathways and β-oxidation of fatty acids are conserved to favor intracellular survival of *M. leprae* (6). In addition, mycobacteria exogenously uptake oleic acid (C18:1) derived from Tween added to growth media and incorporate it into triacylglycerol under hypoxic conditions that are thought to be similar to conditions in persistent mycobacterial infections (23). Our data (Table 5.1) also showed that bacterial lipoproteins contain several isoforms depending on acylation patterns such as di- or tri- acylated forms with various combinations of fatty acids even in *in vitro* grown *M. smegmatis* (see Chapter 5). The data suggest that *M. leprae*, an obligatory intracellular pathogen, could differently modify its lipoproteins in comparison to *in vitro* grown mycobacteria. Consequently, these distinct acylation patterns of *M. leprae* lipoproteins could result in the species-specific T cell reactivity of LCD4.15. Therefore, further chemical analysis of lipoproteins should be performed on recombinant forms expressed in mycobacteria that are grown under immune pressure which mimic conditions such as nitric oxide or iron stress, hypoxic conditions etc. A major obstacle to performance of this type of study is that Lpr1086, ML2095 and ML2446 were expressed at low levels in *M. smegmatis*. Although it is difficult to obtain sufficient quantities of recombinant lipoproteins for chemical analysis, such trials could provide the general acylation profile of lipoproteins which presumably appear in *in vivo* grown mycobacteria.

Based on the defined lipid profiles of *in vivo* grown mycobacterial lipoproteins, the subsequent synthetic lipopeptides derived from ML1086, ML2095 or ML2446 could identify the CD1a antigens as an intracellular lipid antigen.

Pathogenic mycobacteria continuously develop molecular mechanisms to favor their growth in phagosomes where they can escape from the host immune system but where they obtain nutrients to grow and multiply. Several mycobacterial CD1 antigens are found to be either biosynthetic intermediates or metabolites (21,27,29-31). The structural variants of CD1 antigens in the mycobacterial cell envelope could be major virulence factors (21,53), suggesting that these intracellular lipid CD1 antigens could be naturally selected to aid the mycobacterial survival in phagocytes. Therefore, $CD1^+$ T cell immune response to these mycobacterial lipids could be a key mechanism to eliminate intracellular pathogens. *M. leprae* uniquely evolves to survive in the human host implying that the structural modifications of pathogen associated molecular patterns (PAMPs) found in *M. leprae* (described in section 1.6.3) may play a unique biological role in host-*M. leprae* interaction which has never been fully examined yet. In this context, this novel *M. leprae* CD1a antigen may allow an understanding of the *M. leprae*-host interaction as well as in designing a novel vaccine against this intracellular pathogen.

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CHAPTER 5 STRUCTURAL ANALYSIS OF THE LIPID COMPONENTS OF MYCOBACTERIAL LIPOPROTEINS

From a manuscript form in preparation by Hee Jin Kim, John T. Belisle, Mark J. Sartain, Sebabrata Mahapatra and Patrick J. Brennan. Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado 80523-1677, USA;

5.1 Abstract

Bacterial lipoproteins, containing an N-acyl di-O-acylglyceryl-cysteine unit, have long been known to affect the immune system through macrophage stimulation, and, in general, the N-terminal triacylated region is important in the cell-mediated immune response to bacterial infections through Toll like receptor 2 (TLR-2). The presence of acylated cysteine at the N terminus of lipoprotein is primarily predicted by bioinformatic analysis but rarely verified by biochemical analysis. The aim of this study was to define the structure of the N-acylated mycobacterial lipoproteins. Lipopeptides derived from mycobacterial lipoproteins were prepared by proteolysis and organic extraction, and analyzed by gas chromatography-mass spectrometry (GC-MS) and quadrapole time of flight liquid chromatography/mass spectrometry (Q-TOF LC/MS). GC-MS results revealed that the major fatty acids of the glyceryl portion of the lipoproteins are tuberculostearic acid, stearic acid, oleic acid and palmitic acid, which are also the dominant fatty acids of mycobacterial phospholipids. The N-linked fatty acid of the cysteine residue is predominantly palmitic acid. Interestingly, Q-TOF LC/MS data showed that mycobacterial lipoproteins appeared as di- and tri-acylated mature forms, but

also had an array of fatty acids in various combinations. Di-acylated lipopeptides appeared more frequently than tri-acylated lipopeptides. The present study demonstrated a distinct fatty acid composition of the lipid portion of mycobacterial lipoproteins. Moreover, we defined the full spectrum of the acylation pattern in mycobacterial lipoproteins, the impact of which in host-bacteria responses is subject to further immunological investigation.

5.2 Introduction

Bacterial lipoproteins, those containing lipid covalently linked to an N-terminal cysteine residue, have been extensively studied in Gram-negative bacteria (4,15). The lipidated N-terminus is presumed to contribute several physiological features of bacteria such as cell wall integrity, transport systems, and signal transduction into the outer leaflet of the cytoplasmic membrane (16,32,38). Analysis of the signal sequence of the N-terminus of prolipoproteins has revealed the consensus sequence Leu-(Ala, Ser)-(Gly, Ala)-Cys at -3 to +1 positions (in relation of cysteine), known as the "lipobox". This motif representes the signal cleavage region of about three-fourths of all bacterial lipoproteins (15,37) (see section 1.7.1). The prolipoprotein undergoes sequential modification and processing catalyzed by diacylglyceryl transferase (Lgt), signal peptidase II (LspA), and N-acyl transferase (Lnt) to form mature lipoproteins, and the major glycerophospholipids such as phosphatidylglycerol, phosphatidylethanolamine and cardiolipin serve as fatty acyl donors for this reaction (15) (Figure 1.14). The cysteine (Cys) residue at the +1 site contains a sulfhydryl-linked diacylglycerol and an N-linked fatty acid (4,15) (Figure 1.13). The ester-linked fatty acids in lipoproteins are generally

similar in composition to those of bulk phospholipids, and 65% of the amide-linked fatty acids are palmitic acids (4).

About 99 putative lipoproteins with the consensus sequence for lipid modification have been identified in the *M. tuberculosis* genome and 31 in the case of *M. leprae* (21,24,38,42) (Table 1.3). The immunodominant antigens, 19kDa/LpqH and 38kDa/PhoS1 of *M. tuberculosis*, have been described as lipoprotein based on metabolic labeling with [^{35}S]-Met and [^{9,10}H]-palmitic acid) (48). Recently, MALDI-TOF analysis of in gel digested lipopeptides provided the evidence that the mycobacterial lipoproteins contain the species specific tuberculostearic acid derived from phospholipids as the acyl donor (41).

Several publications indicate that mycobacterial lipoproteins have affected the immune response to bacterial infections through macrophage activation and the release of certain cytokines, leading to the amplification of Th1 immunity (6,11,14,22,27). Although current research highlights the role of lipoproteins for the development of new immune therapies and vaccines, two major gaps in our knowledge of mycobacterial lipoproteins are the fact that the evidence for *M. leprae* protein acylation has largely relied on bioinformatic analysis; and that the chemical structures of *M. leprae* lipoproteins are presumed to be identical in all mycobacteria. Therefore, the goal of this present study was to define the full spectrum of acylation pattern in mycobacterial lipoproteins.

5.3 Materials and methods

5.3.1 Preparation of a hydrophobic protein fraction from Mycobacterium smegmatis *through Triton X-114 phase separation*

M. smegmatis was grown in a glycerol/alanine salts medium to mid-log phase at 37 °C, centrifuged at 4 °C (10 g, wet weight), washed with PBS, resuspended in the same buffer at 4 °C, and disrupted by intermittent probe sonication with a Soniprep 150 sonicator (MSE Sonic prep150, MSE-Sanyo, Palisades Park, NJ) for 30 min. The suspension of disrupted cells was diluted to a protein concentration of 1mg/ml with PBS followed by the addition of Triton X-114 (Sigma, St. Louis, MO) to a final concentration of 4%. The solution was stirred at 4 °C for 24 hr, and the cellular debris was removed by centrifugation at 27,000 × g for 20 min at 4°C (3). The supernatant was maintained at 25°C for 2 hr until clouding occurred, then centrifuged at 1500 × g for 10 min at 4 °C. The proteins in the resulting aqueous and detergent phases were recovered by precipitation with 5 volumes of chilled (-20 °C) acetone and centrifugation (3). The hydrophobic proteins arising from the detergent phase were extracted with $CHCl_3$:CH_3OH (2:1, v/v) to remove free fatty acids, and a phenol–water partition step was applied to remove lipomannan/lipoarabinomannan (LMs/LAMs) (27). The hydrophobic proteins at the interphase of the phenol partition were lyophilized and the concentration of the proteins was determined by bicinchoninic acid assay (Pierce, Rockford, IL). The hydrophobic and hydrophilic protein populations were analyzed by SDS-PAGE and 2-D IEF gel electrophoresis (26,27).

5.3.2 Proteolysis of **M. smegmatis** *hydrophobic proteins and release of the ester-linked fatty acids*

The hydrophobic protein fraction was suspended in 200 ml of PBS and incubated with 10 unit/ml proteinase K (Sigma, St. Louis, MO) overnight at 37°C. The reaction was stopped by the addition of 200 ml $CHCl_3$:CH_3OH (2:1, v/v) and the lipopeptides extracted into $CHCl_3$-CH_3OH (2:1, v/v). These were subjected to thin layer chromatography (TLC) in $CHCl_3$:CH_3OH:H_2O (65:35:5), and the plates developed with 0.2% ninhydrin in 95% C_2H_5OH. The ester-linked fatty acids of the lipopeptides were released by mild alkali treatment with $CHCl_3$:CH_3OH:0.8N NaOH (10:10:3, v/v/v) at 50 °C for 30 min (7). Free fatty acids were separated from the deacylated peptides by partitioning between hexane and water (6:1, v/v) (25). The deacylated peptides in the water phase were subjected to TLC in $CHCl_3$:CH_3OH:H_2O (65:35:5) and the plates developed with ninhydrin.

5.3.3 Purification and analysis of lipopeptides from **M. smegmatis** *hydrophobic proteins*

The lipopeptides from preparative TLC plates were solubilized in 1 ml of 25 mM octyl glucopyranoside in 0.1 M ammonium acetate (pH 7.0) and 50 mM $CaCl_2$ (22). Insoluble materials were removed by centrifugation and the soluble lipopeptides were applied to a preparative (10 × 250 mm) Macherey and Nagel HPLC column with Nucleosil 120-7C_{18} as stationary phase and eluted at 40 °C with a water/2-propanol gradient (29,30). The amide-linked fatty acids were prepared by hydrolysis of the deacylated lipopeptides with 6 N HCl at 110 °C for 18 hr. The free fatty acids were

methyl esterified with 3M methanolic HCl (Supelco Inc, Bellefonte, PA) at 80 °C for 16 hr (1) recovered by partitioning between hexane and water and analyzed by gas chromatography on a BPX-70 column (Supelco Inc, Bellefonte, PA) in a model 5890 Gas Chromatogram (Hewlett-Packard) with flame ionization detector. For analysis of amino acids, the lipopeptides were hydrolyzed with 6 N HCl (Thermo Fisher Scientific, Rockford, IL) at 110 °C for 18 hr under N_2 gas and sequentially derivatized with 3N isobutanolic HCl and heptafluorobutyric anhydride, at 120 °C for 20 min and 150 °C for 5 min, respectively (25). The N-(O)-heptafluorobutyryl isobutyl amino acid esters of the lipopeptides were analyzed by gas chromatography/electron impact mass spectrometry (GC/EIMS) (Finnigan Polanis Ion Trap Mass Spectrometer). L-α-aminoadipic acid (10nmol) was added as internal standard.

5.3.4 Quadrapole time of flight (Q-TOF) LC/MS analysis of lipopeptides from recombinant lipoproteins Lpr1086, ML2095 and ML2446

The recombinant lipoproteins Lpr1086, ML2095 and ML2446 (see chapter 6) were suspended in 200 µl of water and incubated with 0.5 unit/µl of proteinase K (Sigma, St. Louis, MO) for overnight at 37 °C. A negative control reaction was included with the same treatment to exclude false value for MS data analysis. The reactions were stopped by heat inactivation (100 °C for 10 min). The resulting lipopeptides were extracted into $CHCl_3$-CH_3OH (10:10:3, v/v/v). The samples were clarified by centrifugation and subjected to liquid chromatography-mass spectrometry (LC-MS). Ten microliters of lipopeptides were applied either to a Waters X-Bridge (Waters, Milford, MA,) C_{18} column 3.5 µm (2.1 × 150 mm) that was connected to an Agilent 1200 series HPLC

system. The lipopeptides were eluted with 99% CH_3OH, 5 mM CH_3COONH_4 to 100% linear gradient of 79% n-propyl alcohol, 20% hexane, 5 mM CH_3COONH_4 at a flow rate of 320 µl/min. The eluant was introduced directly into either an Agilent 6220 Accurate-Mass TOF or Agilent 6250 Q-TOF mass spectrometer (Agilent, Santa Clara, CA,) quipped with an Agilent multimode source with simultaneous ESI and atmospheric pressure chemical ionization capability. The positive ion MS data were collected using Agilent Mass Hunter work station software (Agilent, Santa Clara, CA). MS data obtained from each sample were processed using the Molecular Feature Extractor of the Mass Hunter Qualitative Analysis software to generate a file of molecular mass features, their retention times, and their relative abundance. A preset minimum 100 counts as cut off was used to filter out extremely low abundance molecular features and were searched against a custom database containing a list of all possible lipopeptides generated from each lipoprotein. The resulting MS data were processed with Agilent MassHunter Qualitative Analysis software to extract molecular features; i.e. obtain a defined exact mass and retention times of compounds in each sample. The relative abundance of these filtered molecular features were compared with the negative reaction control and indentified true lipopeptides which are significantly higher (at least 5 fold) in relative abundance.

5.4 Results

5.4.1 Preparation and analysis of lipoproteins of M. smegmatis

Phase separation of the lysate of *M. smegmatis* in Triton X-114 allowed a clear differentiation of proteins into the hydrophilic population found exclusively in the

aqueous phase and the hydrophobic proteins in the detergent phase (3,27). The hydrophobic protein fraction (Figure 5.1.A) was subjected to proteolysis to generate the lipopeptides from the inherent lipoproteins extracted with $CHCl_3$:CH_3OH (2:1, v/v) and analyzed by TLC. Ninhydrin showed violet spots for the resolved lipopeptides, whereas the undigested proteins stayed at the origin (Figure 5.1.B). The lipopeptides were isolated by preparative TLC and subjected to mild alkali-treatment to remove the ester-linked fatty acids, which applied to TLC in the same solvent resulted in a marked change in polarity in that the now deacylated lipopeptides remained at the origin and the free fatty acids migrated in the plate (Figure 5.1.C).

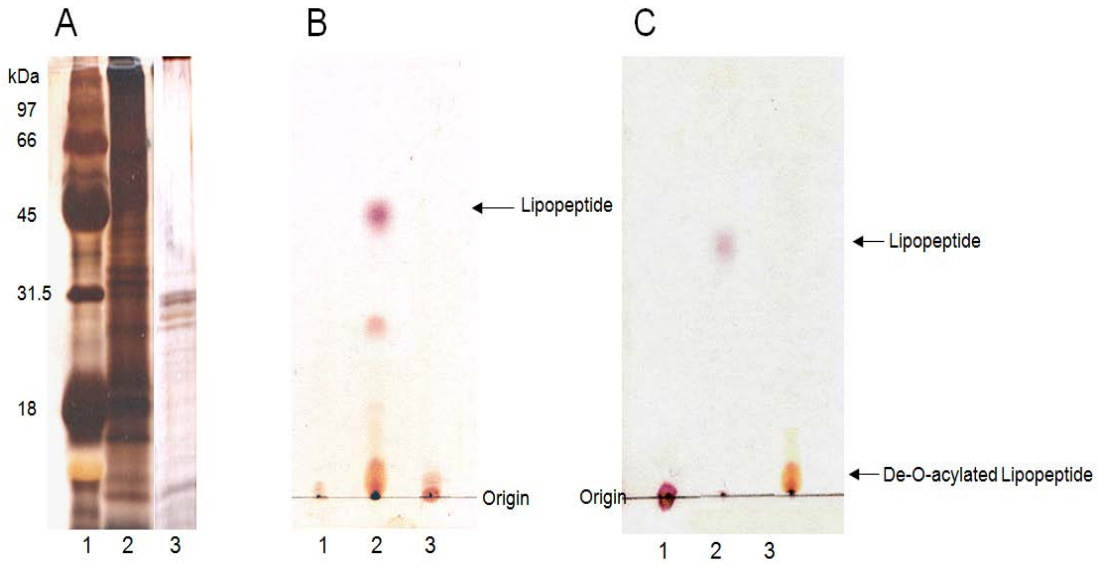

Figure 5.1 Proteolysis of lipoproteins and thin layer chromatography analysis of the alkali-treated lipopeptides. The lipoproteins from *M. smegmatis* were subjected to proteolysis with 10 unit/ml proteinase K for 2 hr at 37°C. The lipopeptides were extracted with $CHCl_3$:CH_3OH (2:1, v/v) and subjected to TLC in $CHCl_3$:CH_3OH:H_2O (65:35:5), and the plates were developed with 0.2% ninhydrin. A) SDS-PAGE, Lane 1, standard molecular weight maker; lane 2, lipoproteins; lane 3, lipoproteins after proteolysis: B) TLC of lipopeptides. Lane 1, lipoproteins; lane 2, lipopeptides in organic phase after extraction with organic solvent; lane 3, peptides in the water phase after extraction with organic solvent: C) TLC of deacylated lipopeptide. Lane1, lipoproteins; lane 2, lipopeptides; lane 3, deacylated peptides after mild alkali-treatment.

5.4.2 Purification and analysis of lipopeptides

Four products isolated by HPLC (Figure 5.2.A) were subjected to TLC analysis. Only P3 reacted strongly to both ninhydrin and charing reagents (Figure 5.2.B and C) but was phosphorus-negative (data not shown). To identify the fatty acids of the lipopeptides of the *M. smegmatis* lipoproteins, the HPLC-purified peak P3 was subjected to mild alkali treatment followed by acid hydrolysis with 6N HCl at 110°C for 18 hr and the alkali- and acid-released fatty acids were separately analyzed by GC-MS.

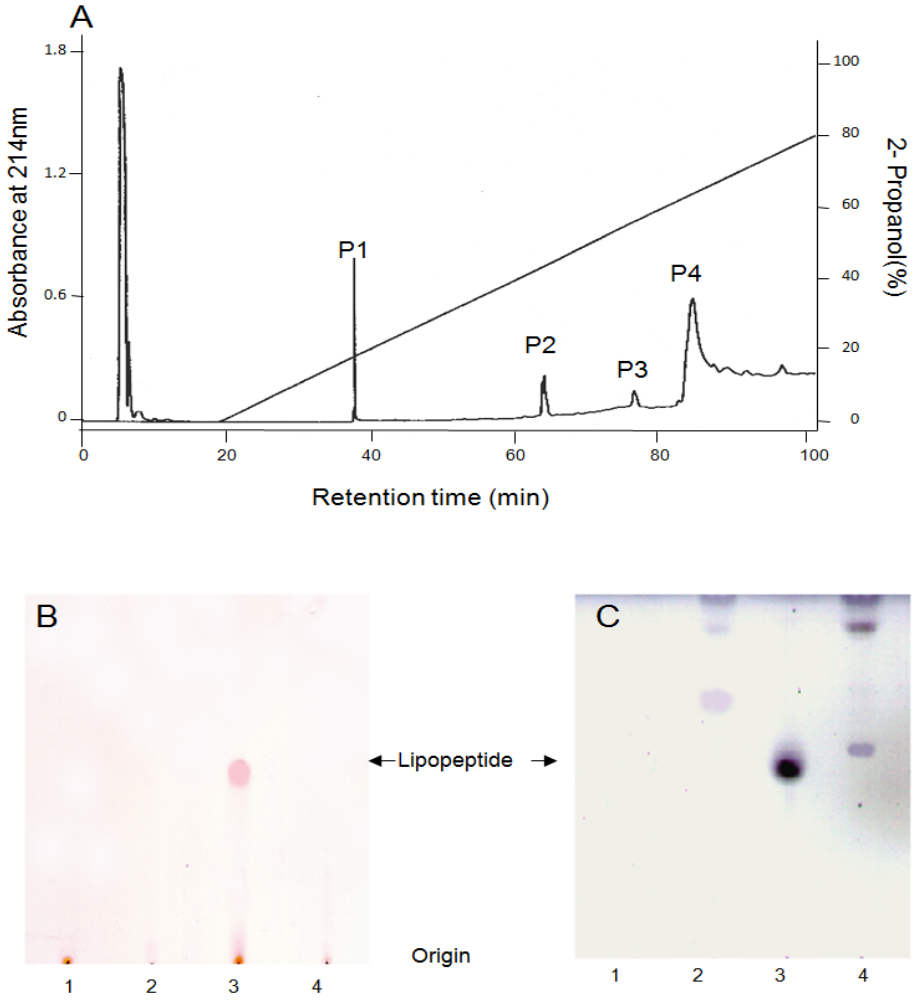

Figure 5.2 Purification of lipopeptides by reverse-phase HPLC. Lipopeptides were reconstituted with octyl glucopyranoside and applied to a 10 × 250mm reverse-phase C_{18} column. Elution was at 40ºC with a water/2-propanol gradient. A) HPLC profile of lipopeptide: B) TLC of HPLC purified peaks stained by ninhydrin ; Lane 1, P1; lane 2, P2; lane 3, P3; lane 4, P4: C) TLC of the HPLC purified peaks stained by 10% cupric sulfate.

The alkali-labile fatty acids from the diacylglycerol portion of the lipopeptides were identified as tuberculostearic acid (C19:0) (*m/z* 312), stearic acid (C18:0) (*m/z* 298) and palmitic acid (C16:0) (*m/z* 270) (Figure 5.3.A) (33), whereas the alkali-stable acid-labile fatty acids were all palmitic acid (C16:0) (Figure 5.3.B). In some other preparations of lipopeptides, small quantities of oleic acid were observed.

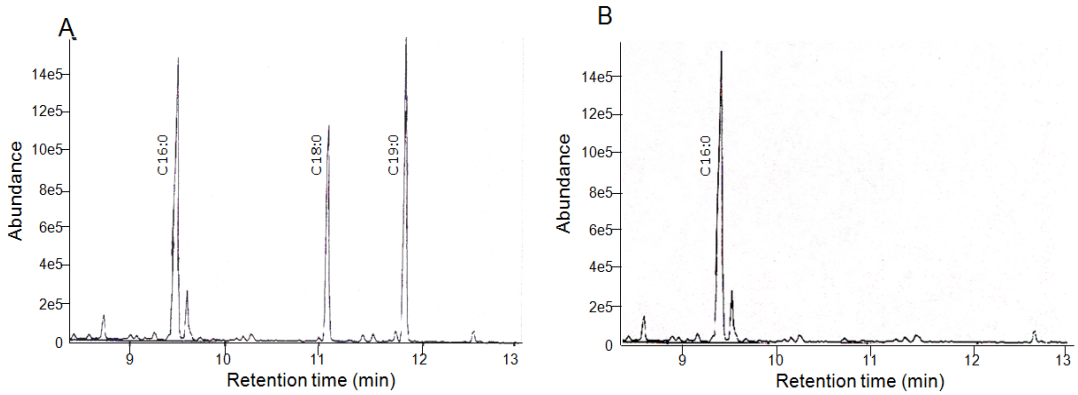

Figure 5.3 Analysis of the fatty acids of lipopeptides by GC-MS. The ester-linked fatty acids of the lipopeptides had been released by mild alkali treatment. The amide-liked fatty acids were prepared by hydrolysis of the deacylated lipopeptide with 6N HCl and hexane extraction. A) GC-MS profile of ester-linked fatty acids of the lipopeptide. B) GC-MS profile of amide-linked fatty acids of the lipopeptide.

After 6N HCl hydrolysis and derivatization of the lipopeptide to identify the amino acid composition, the N(O)-heptafluorobutyryl isobutyl amino acid esters were analyzed by GC-MS, which demonstrated the presence of lysine (*m/z* 280, 50%), arginine (*m/z* 504, 6%), leucine (*m/z* 240, 36.7%; *m/z* 282, 13.2%), threonine (*m/z* 252, 43.6%; *m/z* 253, 46%) (27). Lysine, arginine, leucine, and threonine were in the ratio of about 2:1:1:1 (Figure 5.4). The high content of basic amino acids explains the ninhydrin reactivity of the lipopeptides of the lipoproteins. In order to define further chemical structure, this lipopeptide was applied to subsequent MALDI-TOF MS analysis but no

identifiable fractions were obtained. The last step, unveiled for obligatory definition of the functionally crucial lipopeptdes of mycobacterial lipoproteins will be repeated.

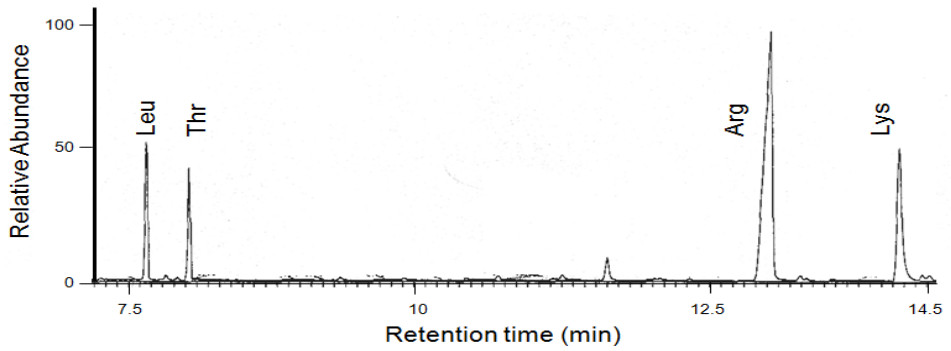

Figure 5.4 Amino acid analysis of lipopeptides by GC-MS. The lipopeptides were hydrolyzed with 6N HCl and dervatized into N(O)-heptafluorobutyryl amino acid esters. Amino acid identification was established by their retention time and typical ion mass fragmentation patterns.

5.4.3 Identification of lipopeptides from recombinant Lpr1086, ML2095 and ML2446 by Q-TOF LC/MS analysis

Purified recombinant Lpr1086, ML2095 and ML2446 (see Chapter 4) were treated with proteinase K and subjected to organic extraction. Subsequently, the extracted lipopeptides were subjected to Q-TOF LC/MS analysis. In order to experimentally identify lipopeptides, the custom lipopeptide databases of Lpr 1086, ML2095 and ML2446 were constructed by calculating exact mass of hypothetical lipopeptides in combination of fatty acids including palmitic acid (C16), pamitoleic acid (C16:1), stearic acid (C18), oleic acid (C18:1) and tuberculostearic acid (C19). Each database contained theoretical masses of all hypothetical lipopeptides containing di-and tri-acylated glycerylcysteines, to di- and tri-acylated 20mer peptide. Since some of lipoproteins were found to be glycosylated, the custom database also contained theoretical masses of mono- to penta-glycosylated units with di- and tri-acylated 20mer

peptide. The resulting MS data of each lipopeptide pool were searched against this custom database and were analyzed to evaluate the chemical structure based on the retention time and the exact mass of each compound. Lipopeptides experimentally found were summarized and compared with theoretical mass in Table 5.1. The amino acid sequences of these peptides are shown in Figure 5.5. Q-TOF LC/MS data analysis additionally identified 18, 25 and 12 possible lipopeptides from custom databases of ML2095, ML2446 and Lpr1086 respectively but was unable to create their molecular formula from experimental masses (data not shown); the masses of these unverified lipopeptides were greater than 1500 resulting in an inability to generate molecular formula. Present Q-TOF LC/MS analysis revealed that ML2095, ML2446 and Lpr1086 were found as both di-acylated and tri-acylated lipoproteins. Palmitic acid, oleic acid, stearic acid and tuberculostearic acid were heterogeneously found in glycerylcysteine of each lipopeptides but palmitoleic acid was not found. Interestingly, the di-acylated forms were found more frequently than tri-acylated forms of recombinant lipoproteins expressed in *M. smegmatis*.

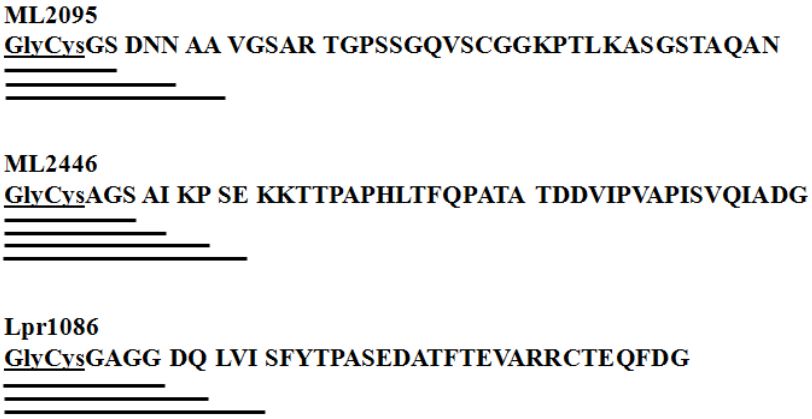

Figure 5.5 N- terminal amino acid sequence of mature ML2095, ML2446 and Lpr1086 and a map of lipopeptides observed by Q-TOF LC/MS analysis. GlyCys represents glycerylcysteine. Each line indicates peptide sequences that were found by Q-TOF LC/MS analysis.

Table 5.1 List of lipopeptides observed by Q-TOF LC/MS analysis.

Source of Lipopeptides	Observed Lipopeptides	Theoretical Mass	Experimental Mass	Retention Time (min)	Molecular Formula
All of three	C16,C16-GlyCys	671.5159	671.5176	1.88	$C_{38}H_{73}NO_6S$
recombinant	C16,C16,C19-GlyCys	951.7925	951.7909	12.16	$C_{57}H_{109}NO_7S$
lipoproteins	C16C,16,C18:1-GlyCys	935.7609	935.7607	11.49	$C_{56}H_{105}NO_7S$
ML2095	C18,C18-GlyCysGSDNN	1214.7447	1214.7476	3.61	$C_{59}H_{108}N_8O_{16}S$
	C19,C19-GlyCysGSDNN	1242.7760	1242.7822	4.5	$C_{61}H_{114}N_8O_{16}S$
			1342.8080	5.02	$C_{64}H_{116}N_{10}O_{18}S$
	C16,C19-GlyCysGSDNNAA	1342.8034			
			1368.8135	5.78	$C_{66}H_{120}N_{10}O_{18}S$
	C18,C19-GlyCysGSDNNAA	1368.814			
			1354.7998	5.85	$C_{65}H_{116}N_{10}O_{18}S$
	C18,C18:1-GlyCysGSDNNAA	1354.803			
			1827.0830	4.62	$C_{80}H_{144}N_{18}O_{25}S$
	C16,C16-GlyCysGSDNNAAVGSAR	1827.0792			
	C16,C18,C18-GlyCysGS	1109.8615	1109.8616	14.61	$C_{63}H_{119}N_3O_{11}S$
ML2446	C16,C18:1-GlyCysAGSAIKP	1250.8539	1250.8504	5.51	$C_{69}H_{127}N_9O_{14}S$
	C19,C18:1-GlyCysAGSAIKP	1292.9032	1292.9054	7.34	$C_{72}H_{133}N_9O_{14}S$
	C16, C18, C18-GlyCysAGS	1109.8616	1106.8592	14.37	$C_{66}H_{124}N_4O_{11}S$
	C16, C18, C19-GlyCysAGS	1123.8772	1123.8757	14.87	$C_{67}H_{126}N_4O_{11}S$
	C16,C18,C18:1-GlyCysAGSAI	1289.9501	1289.9468	12.29	$C_{76}H_{142}N_6O_{14}S$
			1775.2357	16.23	$C_{98}H_{179}N_{11}O_{20}S$
	C18,C18:1,C19-GlyCysAGSAIKPSE	1775.2364			
Lpr1086	C16,C18:1-GlyCysGAGG	939.6330	939.6307	3.42	$C_{49}H_{80}N_5O_{10}S$
	C18:1,C19-GlyCysGAGG	981.6799	981.6763	3.57	$C_{52}H_{95}N_5O_{10}S$
			1549.9987	4.83	$C_{78}H_{141}N_{11}O_{18}S$
	C18,C19-GlyCysGAGGDQLVI	1550.0020			
	C16,C16,C19-GlyCysGAGGDQ	1436.9795	1436.9811	14.26	$C_{75}H_{136}N_8O_{17}S$
	C16,C16,C19-GlyCysGAGGDQLVI		1762.2132	15.34	$C_{92}H_{167}N_{11}O_{19}S$
		1762.2160			

C16, C18, C18:1 and C19 represent palmitic acid, stearic acid, oleic acid and tuberculostearic acid, respectively.

5.5 Discussion

The aim of this work was to develop methods for the study of mycobacterial lipoproteins and, particularly, to identify the fatty acid composition of the N-terminal acylated region and to explain the biological roles of protein acylation in host pathogen interaction.

Although MALDI-TOF analysis of in gel digested lipopeptide provided the biochemical evidence of mycobacterial protein acylation (41), the existence of

lipoproteins in *Mycobacterium* is still supported predominantly by the presence of lipoprotein consensus sequences in the genome databases and the report of Young and Garbe (48) in demonstrating that metabolic labeling of *M. tuberculosis* with [^{3}H]-palmitic acid and L-[^{35}S]-cysteine followed by phase separation demonstrated that four major antigens, 19 kDa, 26 kDa, 27 kDa, and 38 kDa, were labeled (48).

In the present study, proteolysis of the hydrophobic protein fraction followed by lipid extraction was the single most important step in allowing identification of the fatty acid composition of mycobacterial lipoproteins. The mycobacterial lipoproteins are predominantly substituted with palmitic acid, stearic acid, and tuberculostearic acid in the glyceryl portion and palmitic acid in amide-linkage to the cysteine residue (Figure 5.3 and Table 5.1). The acylation pattern of other bacterial lipoproteins is usually dominated by palmitic acid (13), oleic acid (28,49), or hydroxystearic acid (44). Although chemical composition of the lipid portion of bacterial lipoproteins is rarely characterized, it is believed that the acylation profiles of bacterial lipoproteins are largely influenced by the distribution of fatty acids on the cellular lipids, particularly phosphoglycerides (1,12,15). The chemical composition of these phospholipids is varied depending on culture conditions (1,13). Our results (Figure 5.3) and those of Tschumi *et al.* (41) indicate that the mycobacterial lipoproteins have an array of fatty acids similar to those of mycobacterial phospholipids (5,19,41). The presence of tuberculostearic acid is noteworthy, since it is mostly associated with phosphatidyl inositol, the phosphatidyl inositol mannosides, LM and LAM (19), implying that turnover of these characteristic mycobacterial phospholipids may contribute to the acylation pattern of mycobacterial lipoprotein.

However, MALDI-TOF analysis of the lipopeptides derived from *M. smegmatis* native lipoproteins failed to elucidate the exact biochemical structure. This inability of MS based analysis may have been due to the long hydrocarbon chains of the lipid portion resulting in low recovery into the analytic instrument as well as low ionization efficiency during MS (43). This problem is accentuated when analytic materials are heterogeneous and low abundant similar to the lipopeptides derived from *M. smegmatis* native lipoproteins.

In order to define the chemical structure of mycobacterial lipoproteins, we identified eight ORFs coding for proteins containing the lipobox (Table 4.3) from the *M. leprae* genome and expressed in *M. smegmatis*. However, attempts to express many of the recombinant equivalents were mostly unsuccessful, since, presumably, the recombinant lipoproteins were highly toxic for the host cell and expressed at low level. The *advancement of analytical techniques has* made it possible to identify and quantify low abundant compounds from complex biological samples such as urine and serum (2,8,9). We developed a strategy based on an HPLC separation system with high mass accurate Q-TOF LC/MS detection of mycobacterial lipids. In order to overcome difficulties on MS based analysis of lipopeptide, we utilized this platform to directly further analyze low abundant lipopeptides.

The biosynthetic machinery of bacterial lipoproteins was first identified and fully characterized in Gram-negative bacteria (15,31,35,36,46). The lipoprotein biosynthetic machinery consists of three enzymes. The diacylglyceryl transferase (Lgt) covalently links the diacylglycerol residue at the thiol group of Cys in the lipobox (16,35). Subsequently, signal peptidase II (LspA) cleaves the signal peptide of diacylglyceryl

prolipoprotein (31,47) and the N-acyl transferase (Lnt) (16) adds an amide-linked fatty acid (Figure 1.14). Lgt and LspA are conserved in all bacteria but Lnt is predominantly found in Gram-negative bacteria (31). Chemical analysis showed that several lipoproteins exist as the tri-acylated form in Gram-negative bacteria (*E. coli*, *Borrelia burgdorferi*, *Neisseria gonorrhoeae* and *Porphyromonas gingivalis*) but those of mycoplasma were the di-acylated forms (39,40). Although *Bacillus subtilis* and *Staphlycoccus aureus* were reported to contain Lnt, there is little known of protein acylation in Gram-positive bacteria (39-41). The three essential enzymes, Lgt, LspA and Lnt (1,15,31), have been identified in the genomes of *M. smegmatis*, *M. tuberculosis* and *M. leprae* by homology alignment (31,32,34,41). The enzymatic activity of *M. tuberculosis* Lnt has been characterized by genetic deletion mutation and its complementation (41). Our results showed that mycobacterial lipoproteins have an array of fatty acids with different combinations. Table 5.1 revealed that di-acylated lipopeptides appeared more repeatedly than tri-acylated lipopeptides in *M. leprae* recombinant lipoproteins expressed in *M. smegmatis*. Recent findings showed that *S. aureus* containing *lnt* can produce both di- and tri-acylated forms of lipoprotein and the di-acylated forms of lipoproteins are more potent immune modulators (39,40). Despite the fact that mycobacteria possess functionally active Lnt, our data showed that mycobacterial lipoproteins exist as both di-and tri-acylated forms similar to the acylation pattern of lipoproteins in *S. aureus* (Table 5.1). Drage *et al.* (10) showed that rLprA, rLprG. rLpqH and rPhoS1 induce high levels of TNF-α production in TLR-2 signaling dependent manner on stimulation of TLR2-/-, TLR1-/- and TLR6-/- macrophages. In that study, the TLR response to rLprG or rLpqH was restricted to TLR-1 while that of LprA

or Phos1 was not required for either TLR-1 or TLR-6. TLR responses of all lipoproteins have varied depending on the involvement of CD14 and CD36 (10). Since all lipoproteins were not biochemically defined and expressed in *M. smegmatis* or *M. tuberculosis* (H37Ra; avirulent strain), it is still obscure how these mycobacterial lipoproteins influence TLR signaling in mycobacterial infection but implies that mycobacterial lipoproteins exist as several isoforms such as di-, tri or non-acylated forms.

Recently, Leng *et al*. (23) revealed that lipoproteins containing unsaturated fatty acid induces higher gene expression of the Th1/Th17-associated cytokines in bone marrow derived macrophages than synthetic lipopeptide containing saturated fatty acid via TLR-2 signaling mechanism, suggesting that different acylation pattern of lipoprotein can induce different immune responses. Interestingly, Q-TOF LC/MS analysis in the present study also showed that some lipopeptides contained the unsaturated fatty acid oleic acid (C18:1) (Table 5.1).

Overall, we concluded that mycobacterial lipoproteins (ML1086, ML2095 and ML2446) expressed in *M. smegmatis* are present as di- or tri-acylated isoforms and also contain a set of fatty acids (palmitic acids, stearic acids, oleic acids and tuberculostearic acids) at the N terminal cysteine. This new information raises two major questions. The first is whether the protein acylation pattern of the virulent mycobacteria, *M. leprae* and *M. tuberculosis*, in the host is identical to that of *M. smegmatis* characterized in the present study or whether one di-/tri acylated isoform is predominant in the *in vivo* grown virulent mycobacteria. The great challenge in addressing this question is that bacterial lipoproteins are generally of low abundance and hydrophobic in the native proteome, making it difficult to define their chemical structures even in the *in vitro* grown

mycobacteria. As previously mentioned in Chapter 4, the most plausible approach to resolve this problem would be the use of recombinant *M. smegmatis* expressing the lipoproteins of *M. tuberculosis* or *M. leprae*, to define the acylation pattern of mycobacterial lipoproteins generated under the conditions that mimic *in vivo* growth. The second question is whether two different acylated isoforms of lipoproteins with various combinations of fatty acids play different biological roles in the pathogenesis of mycobacterial disease by influencing TLR-2/1 and/or TLR-2/6 signaling. Recently, genetic polymorphisms studies in the TLR-2 gene and TLR-2 related genes showed that the tri-acylated lipoproteins may contribute to the pathogenesis of leprosy but di-acylated lipoproteins play an important role in development of tuberculosis by modulating TLR-2 responses (17,18,20,45). Therefore, it would be of interest to address whether the proportional difference between di- and tri-acylated mycobacterial lipoproteins influences the TLR-2 signaling by manipulating the involvement of TLR-1or TLR-6 and/or other costimulatory molecules and whether these differences result in different disease outcomes. Future work using synthetic di- or tri-acylated lipopeptides containing various combinations of fatty acids should be performed to understand the biological relevance of distinct mycobacterial lipopeptides in terms of the pathogenesis of mycobaterial infections. Indeed, the methodologies and knowledge presented in this dissertation will help to define the immunological role of lipoprotein in the mycobacterial pathogens, *M. tuberculosis* and *M. leprae*.

5.6 References

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CHAPTER 6 CONCLUSIONS AND FUTURE DIRECTIONS

The WHO-directed MDT strategy introduced in 1982 efficiently sterilized patients and removed much of the biological reservoir of *M. leprae*. However, the number of new cases has been sustained indicating that current diagnosis, the basis of the MDT intervention strategy, is not sufficient to detect *M. leprae* infected individuals. The early diagnosis of leprosy is a foremost priority for more effective control of leprosy and to interrupt *M. leprae* transmission in endemic regions.

M. leprae persist in the host for an extremely long time without presenting the obvious clinical symptoms. The disease progression of leprosy is highly governed by the host immune status and exhibits two contrasting disease spectra (strong CMI in the form of Th1 immunity to humoral Th2 immunity) in affected individuals. However, the physiology, pathogenicity and immunopathogenesis of *M. leprae* in human hosts is far from understood. These knowledge gaps obstruct the development of sensitive and specific diagnosis of leprosy that can distinguish *M. leprae* infection from other mycobacterial infections. Importantly, the long incubation time of *M. leprae* in most affected individuals makes it a challenge to detect asymptomatic leprosy particularly that which could be found in house hold contacts (HHCs) of multibacillary leprosy (MB) patients but mostly in individuals without a known history of close contact with leprosy patients (6-8,38). During the last decade, vigorous research efforts of our own and others

have agreed that CMI-based *in vitro* IFN-γ release assays (IGRA) to *M. leprae* specific antigens are the most promising and pragmatic diagnostic tool to detect individuals in the early stages of *M. leprae* infection.

In general, the identification of applicable antigens has been achieved through numerous chromatographic approaches in order to reduce the complexity of native protein pools and to enrich certain key proteins into isolated fractions. Due to the inability to culture *M. leprae* in the laboratory, this type of proteomic approach becomes impractical as applied to *M. leprae* derived proteins. *M. leprae* specific antigens have been identified from both native *M. leprae* fractions and recombinant proteins or synthetic peptides using simple proteomic approaches combined with *in silico* bioinformatic analyses of mycobacterial genomes. However, the physiological functions of these antigens during disease progression are rarely characterized. In this dissertation, we have identified and characterized a panel of new *M. leprae* derived protein antigens using advanced genomic techniques and developed simple and novel proteomic techniques that allow for the enrichment of antigens present in low abundance and to a very limited amount, applied these in diagnostic settings.

6.1 Leprosy diagnostic antigens

By using postgenomic approaches (see section 1.10), 31 ORFs and hundreds of peptides were identified from 142 “hypothetical unknowns” as *M. leprae* specific antigens; these hypothetical unknowns had not been annotated in the original genome definition. Some of hypothetical unknowns were shown to improve the specificity of IGRA for leprosy diagnosis (5,6,8,38). However, these recombinant hypothetical

unknown proteins and peptides were recognized by a significant number of endemic controls but the *M. leprae*-derived peptides showed considerable variation in reactivity to racially/geographically different study populations (7,8). Most of the problems with these IGRAs originated from low levels of IFN-γ response to hypothetical unknown proteins and particularly peptides derived from hypothetical unknowns.

For the first time, we established the expression status of 136 *M. leprae* unique proteins (hypothetical unknowns) to address correlation between antigen availability and immunogenicity and eventually to enhance the screening process of novel antigens using the target based qRT-PCR assays (Chapter 2). In our analysis, 60% of the hypothetical unknowns expressed less than 100pg of mRNA and thus were found to be transcriptionally inactive including hypothetical unknowns already studied in the literature (gene expression level was (Table 2.2). Our analysis showed that the low sensitivity of IGRA to previously studied hypothetical unknowns was attributable to low antigen availability, leading to poor T cell recognition of parent proteins by the host immune system. In this dissertation, twenty-six antigen candidates that showed a detectable level of gene expression were identified as the most promising antigens to improve the sensitivity and specificity of IGRA diagnostic tests (Table 2.3). Subsequent seroreactivity to these selective recombinant antigens showed that 9 of the selected antigen candidates were recognized by circulating antibodies in sera of lepromatous leprosy (LL) patients but not in sera of tuberculosis (TB) patients (Figure 2.2). This result suggests that these antigens might be processed and accommodated on the MHC molecule to present to T helper cells. Therefore, these novel recombinant antigens have great potential to improve the sensitivity and specificity of CMI-based diagnosis in the

detection of *M. leprae*-infected individuals with asymptomatic leprosy. All of these purified recombinant antigens are now being evaluated against PBMCs of leprosy patients and HHCs in endemic regions.

The *M. leprae* membrane antigen (MLMA) studied in Chapter 3 induced IFN-γ production of both naïve and memory $CD4^+$ T cells and $CD8^+$ T cells on stimulation of dendritic cells (23) suggesting that MLMA may contain strong immunogens to improve the sensitivity of IGRA for leprosy diagnosis. However, this MLMA also contains large amounts of lipomannans/lipoarabinomannan (LMs/LAMs) making it difficult to isolate low abundant membrane associated proteins and also causing cross-reactivity with other mycobacteria in CMI based assays. Therefore, the identification and characterization of T cell antigens associated with native membrane proteins had never been fully explored. In this dissertation, we developed simple and novel proteomic and biochemical techniques to create the first generation of membrane associated diagnostic protein antigens (MLMA-SP, alkali soluble proteins of MLMA) from which most of the LAMs/LMs were simultaneously excluded (Figure 3.2). Additionally, these techniques allowed the identification and characterization of lipoproteins as CD1a restricted antigens from this membrane fraction (Chapter 4). IGRA of 16 volunteers having worked in mycobacterial laboratories/leprosy clinics indicated that the MLMA-SP induced higher levels of IFN-γ production from peripheral blood mononuclear cells (PBMCs) of the group exposed to leprosy patients, as compared to those individuals with possible environmental mycobacterial exposure (Figure 3.4). This result indicated that this protein pool may contain the immunogenic molecules to mount Th1 immune response but obviously much more work is required. The massive gene decay and reduction in the

M. leprae genome creates a unique protein profile in MLMA. Earlier studies from our laboratory have provided evidence that the unique proteins appearing in MLMA can achieve a distinct antigenic profile different from that caused by other mycobacteria. Therefore, further identification of the individual antigens within MLMA-SP may improve the specificity as well as sensitivity of IGRA. Though this introductory work was seemingly promising, further evaluation of the MLMA-SP in tuberculoid leprosy (TT) patients and HHCs of MB patients is required prior to the application in leprosy endemic clinical settings.

Until now, we and others intensively explored *M. leprae* antigens to be utilized for the early diagnostics of leprosy by proteomic and bioinformatic approaches. In order to utilize CMI based diagnostic tests for routine clinical practice, future follow-up studies of large numbers of HHCs over long periods of time should address the question of whether the individuals indentified by IGRA using highly selected *M. leprae* proteins or peptides, are resistant/susceptible to the development of clinical leprosy. This type of longitudinal study will also allow for understanding the pathogenesis of leprosy and dictate decisions to introduce the chemotherapy to asymptomatic leprosy patients. Although two promising antigens, MLSA-LAM and MLCwA, had been translated into clinical products, major remaining challenges related with these CMI-based assays are to ensure affordability to patients and the easiness of performance combined with affordability in poor resource endemic settings where leprosy is most prevalent. Therefore, the future research to achieve “elimination of global leprosy” should be directed to development of a cost effective and user friendly IGRA applicable in leprosy endemic settings.

6.2 Identification and characterization of lipoproteins as a CD1a restricted antigen

Lipoproteins in bacterial cell surfaces contribute to a variety of physiological features (section 1.7.1) but appear to be low abundant proteins mostly within membranes. However, the N-terminal acylated moiety of lipoproteins is primarily responsible for the activation of innate immunity as well as the expression of MHC-I, II and CD1 on the cell surface, leading to adaptive immunity (9,18,19,24). Currently a few studies have revealed that mycobacterial lipoproteins are directly involved in adaptive immunity by serving as MHC-II restricted T cell antigens and enhancing memory T cell immunity (20,35). Therefore, mycobacterial lipoproteins containing an N-acyl di-O-acylglyceryl-cysteine unit have been long highlighted in terms of developing new vaccines or immunomodulatory therapies for controlling intracellular infectious diseases.

As previously discussed (Chapters 1 and 4), CD1-restricted T cells in response to mycobacterial lipids efficiently and rapidly induced Th1 immunity by secreting high levels of IFN-γ and proliferating cytolytic T cells to kill intracellular pathogens such as *M. tuberculosis* and *M. leprae* (1,10,26,39). Toll like receptor-1 (TLR-1), TLR-2 and CD1s in skin lesions of tuberculoid leprosy (TT) patients are more strongly expressed than in those of LL patients (19,36). TLR-2 signaling on stimulation with synthetic bacterial lipopeptides efficiently mediates to differentiate $CD1^+$dendritic cells from PBMCs (19). The CD1a restricted Langerhans cells, an abundant dendritic cell in TT skin lesions, efficiently present mycobacterial lipids to $CD1a^+$ T cells which can produce high levels of IFN-γ and granulysin (31,37), suggesting that bacterial lipoprotein may serve as CD1a restricted antigens or could be involved in CD1a antigen presentation via Langerhans cells.

Didehydroxymycobactin, a nonribosomal lipopeptide related to the mycobacterial mycobactin siderophores, is the sole known antigen presented by CD1a molecules in *M. tuberculosis* infection. Bacterial lipoproteins share structural similarity with didehydroxy-mycobactin. Therefore, bacterial lipoproteins containing lipids covalently linked to cysteine have been proposed as CD 1 restricted antigens and are believed to be important in the context of vaccines for controlling intracellular pathogens; however, this proposition has not been proven yet.

The proteomic approach to the identification of native proteins based on 2 D gel analysis or LC-MS/MS mainly estimates protein abundance based on peptide identification. However many hydrophobic proteins in low abundance are difficult to identify due to inability to enrich and/or resistance to common trypsin digestion. Therefore, these low abundant proteins have been neglected in terms of their functional role in host pathogen interactions. Growing evidence is showing that post-translationally modified mycobacterial proteins play an important role in the pathogenesis of mycobacterial disease as well as induction of protective Th1 immunity (4,14,30,32-34). However, many of these proteins, particularly lipoproteins, are or low abundance and are enriched in the hydrophobic membrane fractions.

This present work developed simple proteomic and chemical methodologies to enrich lipoproteins of low abundance from MLMA but excluding all lipid components that themselves were often found to be CD1 restricted antigens (Chapters 4 and 5). Subsequent T cell reactivity to delipidated *M. leprae* native proteins (devoid of noncovalently linked lipids) combined with proteolysis revealed that bacterial lipoprotein(s) were a new class of CD1a restricted antigens recognized by the CD1a

restricted T cell line, LCD4.15, derived from leprosy patients (Figures 4.3, 4.4 and 4.5). This was the first provisional evidence that CD1 could present a lipopeptide derived from natural lipoproteins in an intracellular bacteria. However, recombinant ML1086, ML2095 and ML2446 which were identified as CD1a restricted antigens by proteomic and genomic analyses, failed to activate LCD4.15 suggesting that these three lipoproteins expressed in *M. smegmatis* could not be the antigen to activate the LCD4.15 T cell clone via CD1a presentation (Figure 4.9). Since the identification of these lipoproteins was highly dependent on bioinformatic analysis, it raised the question of whether these lipoproteins are truly acylated in the mycobacterial proteome.

There are convincing data to suggest that polymorphisms in the TLR-2 gene and TLR-2 related genes may contribute to the pathogenesis of mycobacterial disease by modulating TLR-2 responses to di- or tri-acylated lipoproteins (13,15,17,45). Especially, the TLR-1 I602S SNP was associated with a decreased incidence of leprosy (13) but TIRAP S180L SNP related to TLR-2/6 signaling protected against pneumococcal disease, bacteremia, malaria and tuberculosis (17). The results indicated that different TLR-2/1 or TLR-2/6 responses by sensing di- or tri-acylated lipoproteins may differently affect the progression of mycobacterial diseases (4,12,14,18,30,32). Prolonged TLR-2 signaling by lipoproteins are known to inhibit the expression of MHC class II molecules (4,14,30,32) contributing to the pathogenesis of mycobacterial disease by the regulation of anti-mycobacterial activities including phagolysosomal maturation, apoptosis and antigen presentation (12). Several mycobacterial lipoproteins were found to be glycosylated (3,32,35). A glycosylated mycobacterial protein, MPT-32, was found to be capable of capturing pulmonary C-type lectin receptor, which is often used for immune

suppression by pathogens (34) (see section 1.7.2). This information implies that the glycosylated parts of mycobacterial lipoproteins also contribute to immune evasion mediated by prolonged TLR-2 response to their acylated functions. Since mycobacterial lipoproteins which were used in previous immunological studies have never been structurally characterized, the immunological roles of lipoproteins in mycobacterial infection are still unclear.

In order to encourage immune mechanistic study of TLR-2 responses to mycobacterial lipoproteins, we established several methodologies to define the chemical structure of N-terminal acylated portion of lipoproteins (Chapter 5). The major challenges of MS based analysis of lipoproteins are the low recovery and low ionization efficiency of lipopeptides due to the long hydrocarbon chains of lipoproteins (44). Here, we showed that proteolysis of the lipoproteins followed by lipid extraction allowed enhanced recovery of the resulting lipopeptides. The high mass accurate Q-TOF LC/MS detection made it possible to identify the full spectrum of lipopeptides derived from mycobacterial lipoproteins. Our study (Figure 5.3) and that of Tschumi *et al.* (43) revealed that mycobacterial lipoproteins have an array of fatty acids similar to those of mycobacterial phospholipids (2,16,43). The tuberculostearic acid is predominantly associated with phosphatidyl inositol, the phosphatidyl inositol mannosides and LM and LAM (16), suggesting that these characteristic mycobacterial phospholipids could be the source of acyl functions of mycobacterial lipoproteins. Interestingly, our results showed that mycobacterial lipoproteins appeared as di- and tri-acylated isoforms, and had an array of fatty acids including palmitic acid, stearic acid, oleic acid or tuberculosteraric acid (Table 5.1). The di-acylated lipopeptides appeared more frequently than tri-acylated

lipopeptides. Currently, some literature has revealed that the di-acylated forms of lipoprotein are more potent immune modulators as compared to tri-acylated forms (40,41). Therefore, it will be important to study whether the coapperance of di- and tri-acylated isoforms of lipoproteins with various proportions in *M. leprae* or *M. tuberculosis* could play different biological roles in the pathogenesis of mycobacterial disease by affecting TLR-2/1 and/or TLR-2/6 signaling.

The lipoproteins acylated with unsaturated fatty acids induced higher gene expression level of the Th1/Th17-associated cytokines compared to that of synthetic lipopeptides containing saturated fatty acids (22). This new information suggests that different acylation pattern of lipoproteins can induce different immune responses via TLR-2 signaling. Th17 cells particularly produce high levels of IL-17 to promote the recruitment of neutrophils and to result in tissue damage, but efficiently control *M. tuberculosis* in the early stages of infection (42). However, IL-17 in chronic infection mediates a shift of Th1 immunity to Th2 or T regulatory immunity which favors the pathogenesis of mycobacterial diseases. This knowledge suggests that the differential acylation profiles of mycobacterial lipoproteins, which we showed in this study, may differentially influence TLR-2 signaling of phagocytes and mediate protective Th1 immunity or pathogenic Th2 immunity to *M. leprae* infection. Therefore, future work should focus on understanding the biological relevance of these biochemically defined lipoproteins in the pathogenesis of leprosy and tuberculosis.

An unsolved question from our current studies is the nature of CD1a restricted antigens recognized by LCD 4.15. As discussed in Chapter 4, the activation of this T cell clone was highly specific to *M. leprae,* which is obligatorily capable of growing *in vivo*.

Recent mycobacterial lipidomics studies have shown that lipids of *in vivo* grown mycobacteria were differently modified from those of *in vitro* grown mycobacteria such as in PDIM, sulfolipid-1, and phenolic glycolipids (11), which are abundantly found in the cell envelope of mycobacteria but are also known as virulence factors. Since the chemical structures of mycobacterial CD1 antigens have rarely been defined or even found in the case of *in vitro* grown mycobacteria (21,25,27-29), we speculated that *M. leprae* solely grown under pressure of the host immune system may result in a unique acylation pattern of lipoproteins leading to *M. leprae* specific activation of LCD4.15. Therefore, further biochemical analysis of mycobacterial lipoproteins from *in vivo* versus *in vitro* grown mycobacteria could allow identifying the CD1a antigens as intracellular lipid antigens. Since many of the mycobacterial cell wall components are important contributors to mycobaterial pathogenesis, CD1a restricted antigens may further allow an understanding of host-*M. leprae* interaction. Finally, the biochemical, genetic and proteomic methodologies and knowledge presented in this dissertation will allow further understanding the biological roles of mycobacterial lipoproteins in the pathogenesis of mycobacterial disease such as tuberculosis and leprosy.

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