LipC (Rv0220) Is an Immunogenic Cell Surface Esterase of *Mycobacterium tuberculosis*

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We have reported previously the identification of novel proteins of *Mycobacterium tuberculosis* by the immunoscreening of an expression library of *M. tuberculosis* genomic DNA with sera obtained from *M. tuberculosis*-infected rabbits at 5 weeks postinfection. In this study, we report the further characterization of one of these antigens, LipC (Rv0220). LipC is annotated as a member of the Lip family based on the presence of the consensus motif “GXSG” characteristic of esterases. Although predicted to be a cytoplasmic enzyme, we provide evidence that LipC is a cell surface protein that is present in both the cell wall and the capsule of *M. tuberculosis*. Consistent with this localization, LipC elicits strong humoral immune responses in both HIV-negative (HIV⁻) and HIV-positive (HIV⁺) tuberculosis (TB) patients. The absence of anti-LipC antibodies in sera from purified protein derivative-positive (PPD⁺) healthy subjects confirms its expression only during active *M. tuberculosis* infection. Epitope mapping of LipC identified 6 immunodominant epitopes, 5 of which map to the exposed surface of the modeled LipC protein. The recombinant LipC (rLipC) protein also elicits proinflammatory cytokine and chemokine responses from macrophages and pulmonary epithelial cells. rLipC can hydrolyze short-chain esters with the carbon chain containing 2 to 10 carbon atoms. Together, these studies demonstrate that LipC is a novel cell surface-associated esterase of *M. tuberculosis* that is highly immunogenic and elicits both antibodies and cytokines/chemokines.

There are an estimated 9×10^6 new cases of tuberculosis (TB) and 2×10^6 TB-related deaths every year (55). Infection with *Mycobacterium tuberculosis* is initiated with the inhalation of a droplet bearing bacteria, and it takes months or years to progress to clinical TB. During this progression from initial infection to clinical disease, the *in vivo* bacteria adapt to continuously changing environments by altering their gene expressions (31, 34, 48, 50). Previous studies to delineate culture filtrate (CF) proteins of *M. tuberculosis* that are recognized by antibodies during the natural course of disease progression demonstrated that the repertoire of antigens enlarges with the progression of infection (34–36, 41). Interestingly, several of the *M. tuberculosis* antigens (malate synthase, MPT51, and ESAT6, for example) that elicit immune responses during the early stages of active infection have also been demonstrated to play important roles in the host-pathogen interaction (19–21, 57).

To identify additional antigenic proteins of *M. tuberculosis* that are expressed during the early stages of active infection, we used sera obtained from *M. tuberculosis* aerosol-infected rabbits that were bled at 4 to 5 weeks postinfection to screen an expression library of *M. tuberculosis* genomic DNA (44). Antibodies in these sera identified several proteins known to contribute to the infection and survival of *M. tuberculosis* (exported repetitive protein [ERP], KatG, and MtrA) as well as novel proteins (proline threonine repetitive protein [PTRP], PE-PGRS51, and LipC [Rv0220]) (3, 26, 44, 59). Interestingly, ERP, KatG, and PTRP are cell wall proteins of *M. tuberculosis* (3, 43, 58), and while the precise localization of MtrA in *M. tuberculosis* has not been reported, the *Mycobacterium leprae* homolog is also a cell wall protein (27). The current studies are focused on LipC.

LipC is annotated as a member of the Lip family based on the presence of the consensus motif “GXSG” characteristic of es-

terases and members of the hydrolase fold family (40). The Lip family is comprised of 24 putative carboxyl ester hydrolases. Of these, studies of 3 members, LipF (Rv3487c), LipH (Rv1399c), and LipY (Rv3097c), have been reported so far (5, 10, 60). The current studies demonstrate that LipC is a cell surface protein that is present in both the cell wall and the capsule of *M. tuberculosis*. Consistent with this localization, LipC is a strong inducer of antibodies in patients with active TB, and immunodominant epitopes of the protein have been mapped. LipC also elicits cytokine responses from both macrophage-like THP-1 and pulmonary epithelial A549 cells. Moreover, a recombinant and enzymatically active *M. tuberculosis* LipC was produced and purified from *M. smegmatis*, and the biochemical characterization revealed that LipC hydrolyzes short-chain esters. Together, these results suggest that LipC participates in the progression of active TB both by contributing to the utilization of lipid substrates for bacterial growth and replication and by modulating immune responses.

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MATERIALS AND METHODS

Bioinformatics analysis. Nucleic and protein BLAST searches with the current databases were performed at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>). Predictions of the theoretical molecular weights (ProtParam), transmembrane helices (TMHMM), and signal peptides (SignalP) were carried out with the respective software at the ExPASy Proteomics server (<http://ca.expasy.org/>). The NetOGlyc program was used to predict potential glycosylation sites, and MeMo (<http://www.bioinfo.tsinghua.edu.cn/~tigerchen/memo/links.html/>) was used to predict potential arginine or lysine sites that may be methylated. A model of the three-dimensional (3-D) structure of LipC was generated from the automatic protein structure homology modeling server using the I-TASSER server (33). The Rv0220 sequence was submitted against the nonredundant sequence database structural classification of proteins and the Protein Data Bank (PDB). The molecular graphics software program PyMol (<http://www.pymol.org/>) was used to display and draw the structure from the PDB files generated by the I-TASSER server.

Bacterial strains, plasmids, and growth media. Mycobacterial strains were grown in Middlebrook 7H9 broth (Becton Dickinson and Company [BD], Sparks, MD) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-saline (ADS) (0.5% bovine serum albumin, fraction V [Sigma, St. Louis, MO]; 0.2% dextrose; and 0.085% NaCl) at 37°C under constant shaking at 110 rpm. *Escherichia coli* strains were grown in LB broth (Difco Laboratories) at 37°C with shaking (220 rpm) in the presence of either hygromycin (200 µg/ml), kanamycin (50 µg/ml), ampicillin (100 µg/ml), or chloramphenicol (34 µg/ml), as required.

Immunoscreening of the λ gt11 library. The λ gt11 expression library of *M. tuberculosis* H37Rv DNA from the World Health Organization (WHO) was screened with pooled sera obtained from *M. tuberculosis* aerosol-infected rabbits at 5 weeks after aerosol infection as described previously (44). The reactive λ gt11 clones were purified, and the inserts were sequenced to identify the gene encoding the immunoreactive protein (44).

Expression and purification of rLipC. For the expression of recombinant LipC (rLipC) in *E. coli*, a 1,235-bp DNA fragment containing the entire *lipC* open reading frame was amplified from *M. tuberculosis* H37Rv genomic DNA using primers F1 5'-CCCATATGAACCAGCGACGCG-3' and R1 5'-CCCTCGAGTTGGCCGGCGTTTAGATG-3' (underlined sequences indicate NdeI and XhoI sites, respectively) and cloned into the pCR-Blunt cloning vector (Invitrogen, Carlsbad, CA). This intermediate plasmid (pCR-Blunt-*lipC*) was digested with NdeI and XhoI, and the resulting *lipC* gene was cloned into the pET14b expression vector (Novagen, EMD Biosciences, Inc., San Diego, CA) at NdeI and XhoI sites to produce an in-frame fusion with the His tag at the N-terminal position. The open reading frame of the recombinant plasmid (pET14b-*lipC*) was verified by DNA sequencing. The recombinant plasmid (pET14b-*lipC*) was transformed into *E. coli* BL21(DE3)(pLysS) cells (Invitrogen). After isopropyl- β -D-thiogalactopyranoside (IPTG) induction, the recombinant protein was expressed in inclusion bodies, and standard procedures with urea were used to obtain the purified His-tagged rLipC protein by affinity chromatography on a Ni-nitrilotriacetic acid (NTA) agarose column (Qiagen, Chatsworth, CA). Endotoxins were removed by washing the protein-loaded affinity column with 10 mM Tris-HCl in 6 M urea, followed by 0.5% amidosulfobetaine 14 (ASB-14) in 6 M urea. rLipC was eluted (20 mM Tris-HCl [pH 7.9], 1 M imidazole, 6 M urea), and fractions containing purified rLipC were pooled and dialyzed against 10 mM ammonium bicarbonate (pH 8.0) with stepwise-decreased concentrations of urea. The purified rLipC protein formed aggregates that were readily solubilized in 0.1% SDS. The *Limulus* amoebocyte lysate (LAL) assay was used to determine the endotoxin content in the purified LipC preparation according to the manufacturer's instructions (Bio Whittaker, Walkersville, MD). Proteomic analysis of rLipC was performed by quadrupole time-of-flight (Q-TOF) mass spectrometry at the New York University

(NYU) protein analysis facility. All studies except for the assessment of enzymatic activity were performed with this rLipC protein.

Since the rLipC protein obtained from *E. coli* was enzymatically inactive, *lipC* was also expressed in *M. smegmatis* to determine enzymatic activity and substrate specificity. Briefly, the *lipC* gene was amplified from *M. tuberculosis* H37Rv genomic DNA with primers F2 (5'-GCATCCATG GTACAGCGACGCGCCGCGGGTC-3') and R2 (5'-CTTATGAAGCT TAGATGACCTCTTTCGCGAACTG-3'), containing NcoI and HindIII restriction sites (underlined), respectively. The amplified *lipC* fragment was double digested (with NcoI and HindIII), and after purification, the fragment was cloned into vector pMyNt (a shuttle vector containing a tobacco etch virus (TEV)-cleavable N-terminal 6His tag, an acetamide-inducible promoter, and a hygromycin resistance gene; provided by EMBL-Hamburg). The resulting recombinant plasmid (pMyNt-*lipC*) was verified by DNA sequencing and electroporated into *M. smegmatis* mc²155. The bacteria expressing rLipC were cultured until an optical density at 600 nm (OD₆₀₀) value of 3 was reached, induced for 16 h by the addition of 0.2% (wt/vol) acetamide, and harvested by centrifugation. The bacterial pellet was suspended in 10 mM Tris buffer (pH 8.0) containing 300 mM NaCl and 1% N-laurylsarcosine (Sarkosyl) and broken with a French press. The His-tagged rLipC protein was purified by Ni-NTA agarose column-based affinity chromatography and eluted with 10 mM Tris buffer (pH 8.0) containing 300 mM NaCl and 100 mM imidazole. The fractions containing rLipC were pooled and further purified with a Superdex 200 column equilibrated with 10 mM Tris buffer (pH 8.0) containing 300 mM NaCl.

Localization of LipC. Purified rLipC from *E. coli* suspended in incomplete Freund's adjuvant (IFA; Sigma) was used to immunize a New Zealand White rabbit to obtain polyclonal antibodies (16). Immunoglobulin G (IgG) from normal rabbit serum or serum from the immunized rabbit was purified by protein A-Sepharose 4B columns (Amersham Biosciences). *M. tuberculosis* H37Rv subcellular protein fractions (cytoplasm [Cyt], total cell wall [TCW], SDS-extracted cell wall [SDS-CW], and culture filtrate [CF]) (NIH/NIAID TB Research Materials contract, CSU) and *M. tuberculosis* capsular material (kindly provided by Mary Jackson, Colorado State University) were separated by SDS-PAGE (5.0 µg/lane), and the Western blots were probed with rabbit anti-LipC IgG (1:1,000) or normal rabbit IgG, followed by alkaline phosphatase (AP)-conjugated anti-rabbit IgG (1:2,000) and BCIP (5-bromo-4-chloro-3-indolylphosphate)-Nitro Blue Tetrazolium (NBT) substrate (KPL, Inc., Gaithersburg, MD). Previous studies have demonstrated that *M. tuberculosis* malate synthase is present in all the subcellular fractions of *M. tuberculosis* (19). Identical Western blots of all subcellular fractions were probed with anti-malate synthase IgG (1:1,000) to ensure the integrity of the preparations used. The localization of LipC on intact bacterial cells was confirmed by immunoelectron microscopy. Briefly, *M. tuberculosis* clinical isolate CDC1551 and a transposon mutant with a *lipC* mutation in CDC1551 (CDC1551 *lipC*::Tn) were obtained from TARGET (Tuberculosis Animal Research and Gene Evaluation Taskforce, Johns Hopkins University [<http://webhost.nts.jhu.edu/target/>]). Bacteria from mid-log-phase cultures of *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551, and *M. tuberculosis* CDC1551 *lipC*::Tn were fixed and processed as described previously (43). The sections were examined under a Philips CM 10 TEM electron microscope at the NYU Image Core Facility.

Patients and control subjects. Sera from 19 purified protein derivative-negative (PPD⁻) and 29 PPD-positive (PPD⁺) healthy subjects; 70 HIV-negative (HIV⁻), acid-fast-bacillus (AFB) sputum smear-positive TB patients (HIV⁻ TB⁺); and 45 HIV-positive (HIV⁺) AFB smear-positive TB patients (HIV⁺ TB⁺) from India, a country where TB is endemic, were included in these studies. As per the DOTS (directly observed therapy, short term) programs guided by the WHO, the diagnosis of TB in countries where the disease is endemic is based on the microscopic examination of sputum smears for AFB, since this test is highly specific (54). Cultures are too expensive and complex for TB diagnosis in high-burden settings; for this reason, the bacteriological confirmation of

TABLE 1 Human subjects included in the study^a

Infection status	Origin	No. of subjects	AFB sputum smear status	Treatment status
HIV ⁻ TB ⁺	India	70	Positive	Untreated
HIV ⁺ TB ⁺	India	45	Positive	Untreated
HIV ⁺ TB ⁻	United States	46	ND	ART
PPD ⁺	United States	7	ND	NA
	India	11	ND	NA
	China	7	ND	NA
	Cameroon	2	ND	NA
	Japan	1	ND	NA
	Colombia	1	ND	NA
PPD ⁻	United States	6	ND	NA
	India	10	ND	NA
	China	3	ND	NA

^a AFB, acid-fast bacilli; ART, antiretroviral therapy; ND, not done; NA, not applicable.

TB in patients included in these studies was based on microscopy. Sera from 46 HIV⁺ asymptomatic patients on antiretroviral therapy were also included (HIV⁺ TB⁻). All the TB patients were bled prior to the initiation of TB therapy (Table 1). All individuals were bled, with informed consent, after permissions from the appropriate ethical committees had been obtained.

In vivo expression of LipC during active TB. The presence of antibodies to LipC in serum specimens from TB patients and PPD⁺ subjects was tested to determine the *in vivo* expression of LipC during active and latent TB. Western blots of rLipC (75 ng per lane) were blocked with 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) overnight, washed with PBST (2% Tween 80 in PBS), and incubated with sera (1:50) from 6 PPD⁻ and 6 HIV⁺ TB⁻ subjects as well as from 6 PPD⁺, 6 HIV⁻ TB⁺, and 6 HIV⁺ TB⁺ patients at 4°C overnight. After extensive washing, the blots were probed with a mixture of protein A-AP (1:2,000; Sigma) and anti-human IgA-AP (1:1,000; Sigma) followed by BCIP-NBT substrate (KPL, Inc., Gaithersburg, MD). To confirm the results obtained in the above-described experiments, sera from 20 additional subjects (10 PPD⁺ subjects and 10 HIV⁻ TB⁺ patients) were evaluated similarly. Semiquantitative analysis of the reactivity on Western blots was performed by using an Epson Perfection 4990 Professional scanner (Epson), and the relative intensity values were determined with Image J software (<http://rsbweb.nih.gov/ij/>).

Identification of immunodominant regions of LipC. Having confirmed the *in vivo* expression of LipC in humans during active TB, epitope mapping of LipC was performed to identify the immunodominant regions. Overlapping N-terminal biotin-labeled peptides (20-amino-acid [aa] length with a 10-aa overlap; total, 40) covering the entire LipC sequence (PEPscreen; Sigma) were captured (2.5 µg/ml; 50 µl/well) in wells of commercial streptavidin-coated enzyme-linked immunosorbent assay (ELISA) plates (Roche Diagnostic, Indianapolis, IN). Each peptide was probed with 50 µl of individual sera (final concentration, 1:40) from 13 PPD⁻ and 23 PPD⁺ subjects and 60 HIV⁻ TB⁺ patients (39, 43). After washing, the wells were probed with a mixture of protein A-AP (1:2,000; Sigma) and anti-human IgA-AP (1:1,000; Sigma). *p*-Nitrophenyl phosphate (pNpp) was used to detect the peptide-bound antibodies. Mean ODs of the sera from the 36 healthy subjects (13 PPD⁻ and 23 PPD⁺) plus 3 standard deviations (SD) were used as a cutoff to determine positive responses in TB patients. Twelve peptides that were recognized by antibodies in sera from >50% of the 60 HIV⁻ TB⁺ patients in the initial screening were retested twice, and positive reactivity in 3/3 or 2/3 ELISAs was considered positive. The reactivity of the 6 peptides that were consistently recognized by >50% of the HIV⁻ TB⁺ patients was evaluated with sera from 46 HIV⁺ TB⁻ and 45 HIV⁺ TB⁺ patients.

Cell viability assay. Since rLipC remains soluble only in the presence of 0.1% SDS, experiments were performed to confirm that the SDS concentration being used does not affect cell viability in the cytokine studies.

The LipC stock (400 µg/ml) was diluted to 1 µg/ml in medium (final concentration of SDS of 0.00025% [~0.01 mM]), and 200 µl of solution was added to ~4 × 10⁴ A549 cells/well or 2 × 10⁵ phorbol myristate acetate (PMA)-differentiated THP-1 cells in triplicate for 24 h. At the end of the incubation, the viability of cells was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions (Invitrogen).

Confirmation of the lack of endotoxin contamination. The LAL assay showed that the concentration of endotoxin in the purified rLipC preparation was <10 ng/ml. For the biological confirmation of the absence of contamination with endotoxin, 2 × 10⁵ PMA-differentiated THP-1 cells were exposed to 200 µl medium containing 1 ng/ml lipopolysaccharide (LPS), 1 ng/ml LPS that was preincubated with 10 ng/ml polymyxin B for 1 h, 1 µg/ml LipC, and 1 µg/ml LipC that was preincubated with 10 ng/ml polymyxin B in triplicate. Culture supernatants were collected 24 h later and used for cytokine assessments with commercially procured ELISA kits according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego, CA).

Cytokine responses to rLipC. A total of 2 × 10⁵ THP-1 or 4 × 10⁴ A549 cells/well (in 200 µl of medium) were exposed to different concentrations of the rLipC protein for 24 h, and the supernatants were harvested and assayed for interleukin-12 (IL-12), tumor necrosis factor alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1), and IL-8 using commercial sandwich ELISA kits (BD Biosciences Pharmingen), as described above.

Enzymatic characterization of rLipC. The activity of rLipC expressed in *M. smegmatis* was measured by using *p*-nitrophenyl (pNP) esters (Sigma) with carbon chain lengths ranging from C₂ to C₁₆. The release of pNP was monitored at 410 nm by using a 96-well plate spectrophotometer and quantified by using a calibration curve of pNP [$\epsilon_{\lambda=410\text{ nm}} = 30\text{ mM}^{-1}$]. Enzymatic reactions were performed with 2.5 mM Tris buffer (pH 8.0) containing 300 mM NaCl (with 0, 1, and 4 mM sodium taurodeoxycholate [NaTDC]) at 37°C over a period of 15 min in a final volume of 300 µl containing various amounts of enzyme and 1 mM substrate. Results are expressed as specific activities in international units/mg, corresponding to 1 µmol pNP released per minute and per mg of enzyme. rLipC activity was also investigated by using pH-stat (Metrohm AG, Switzerland), fluorescent, and spectrophotometric assays for various lipids as well as phospholipids to detect lipase or phospholipase activity, respectively.

Statistical analysis. Comparisons between the reactivities with sera from PPD⁻ and PPD⁺ healthy controls as well as TB patients were performed by calculating *P* values with a nonparametric Mann-Whitney test using GraphPad Prism, version 5, software (GraphPad Software, Inc., San Diego, CA). A *P* value of <0.01 was considered statistically significant.

RESULTS

Identification and characteristics of LipC. The *lgt11* clone identified by the serum pool from *M. tuberculosis*-infected rabbits expressed the C-terminal half (aa 231 to 403) of the *lipC* (Rv0220) gene product (data not shown). Bioinformatics analysis predicts LipC to be a 44-kDa (403-aa) protein with a pI of 10.4. Three amino acids, Ala, Arg, and Val, are overrepresented (12.3, 11.1, and 10.5%, respectively) in the LipC amino acid sequence. SignalP analysis predicted no signal sequence in the LipC protein. No transmembrane helices were identified by TMHMM software. At the Tuberculist data website (<http://tuberculist.epfl.ch/>), LipC is annotated as an esterase based on the presence of the carboxylesterase type B serine active site (GXSG) and the hydrolase fold that is characteristic of lipases/esterases. The amino acid sequence of lipC shows <40% identity with the other 23 members of the Lip family. BLAST-P identified homologous proteins (~100% identity) in *M. tuberculosis* H37Ra, *M. bovis* AF2122/97, *M. bovis* BCG strain Pasteur 1173P2, *M. bovis* BCG strain Tokyo 172, and all *M.*

FIG 1 3-D model of LipC. (A) Ribbon representation. (B) Molecular surface of the 3-D structure of LipC. (C and D) Rotation of 180° of the structures depicted in panels A (C) and B (D). The catalytic triad (Ser237, Asp334, and His367) is represented by cyan sticks in panels A and C. The localization of the immunodominant peptides of LipC identified by epitope mapping, LipC3 (red), LipC6 (orange), LipC24 (blue), LipC26 (green), LipC34 (yellow), and LipC39 (magenta), on the 3-D model is also depicted.

tuberculosis clinical isolates sequenced so far. The protein is absent in *M. leprae* TN and *M. leprae* Br4923. Proteins showing 70 to 80% identity are present in the nontuberculous mycobacteria (NTM) *M. avium* paratuberculosis K-10, *M. avium* 104, *M. avium* ATCC 2529, *M. ulcerans* Agy99, *M. kansasii* ATCC 12478, *M. parascrofulaceum* ATCC BAA-614, *M. intracellulare* ATCC 13950, *M. marinum*, and *M. smegmatis* mc²155. In *M. abscessus*, a protein showing only 23% identity was found. No glycosylation sites but 3 potential Arg residues that may be methylated were predicted.

The LipC protein model was generated from 10 templates available in the PDB that are structurally closest to proteins like the brefeldin A esterase (PDB accession number 1JKM) or the carboxylesterase from *Archaeoglobus fulgidus* (accession number 1JJI). This model reveals that LipC is a globular protein similar to other α/β -hydrolase serine enzymes and consists of 11 α -helices and a central β -sheet core containing six parallel β -strands and one antiparallel β -strand (Fig. 1). The catalytic triad is composed of Ser237 (nucleophilic residue), Asp334 (charge relay network), and His367 (proton carrier) residues (Fig. 1). Ser237 is located within the nucleophilic elbow connecting strand β 4 and helix α 8 at the center of the protein.

Expression and purification of rLipC. When expressed in *E. coli*, rLipC formed inclusion bodies and was purified under denaturing conditions, but the removal of urea at the final stages of purification led to the aggregation and precipitation of the protein in several buffers (ammonium bicarbonate, PBS, or Tris) that

FIG 2 Expression and purification of rLipC. Lane 1, molecular mass markers; lane 2, rLipC purified from *E. coli* separated on 10% SDS-PAGE gels and stained with Coomassie blue; lanes 3 to 5, Western blots of rLipC (from *E. coli*) probed with anti-His (lane 3), rabbit anti-LipC IgG (lane 4), and normal rabbit IgG (lane 5); lane 6, rLipC purified from *M. smegmatis* separated on a 10% SDS-PAGE gel and stained with Coomassie blue; lanes 7 to 9, Western blots of rLipC (from *M. smegmatis*) probed with anti-His (lane 7), rabbit anti-LipC IgG (lane 8), and normal rabbit IgG (lane 9).

were used for refolding. The rLipC protein could be kept soluble in the presence of low concentrations (0.1%) of SDS. The purified recombinant protein appeared as a single ~45-kDa band on SDS-PAGE gels and was recognized by commercial anti-His antibody and anti-LipC antibodies (Fig. 2). The identification of rLipC was confirmed by protein sequencing (Table 2). Since the rLipC protein expressed in *E. coli* was enzymatically inactive, the protein was also expressed in *M. smegmatis*. rLipC expressed in *M. smegmatis* was also recognized by anti-His antibody and anti-LipC antibodies (Fig. 2). The identity of LipC expressed in *M. smegmatis* was also confirmed by protein sequencing (data not shown).

LipC is a cell surface protein of *M. tuberculosis*. As reported previously, anti-malate synthase antibodies showed strong reactivity with an ~81-kDa protein in the Cyt preparation of *M. tuberculosis* (19). Smaller amounts of malate synthase were present in the CW and SDS-CW preparations, while strong protein bands were present both in the capsule and CF preparations. Anti-malate synthase IgG did not react with the rLipC protein (Fig. 3A, top). In contrast, anti-LipC IgG showed strong reactivity with the ~45-kDa rLipC protein band. The same antibody showed weak reactivity with a doublet of proteins at ~45 and ~47 kDa in the Cyt preparation of *M. tuberculosis*. Stronger reactivity with the doublet was observed for the TCW and the SDS-CW protein preparations. Only the ~47-kDa protein was identified in the capsular preparation, while no protein in the CF reacted with these antibodies (Fig. 3A, middle). Purified IgG from normal rabbit serum showed no reactivity with any protein in any subcellular fraction

TABLE 2 Sequencing of rLipC by Q-TOF mass spectrometry

LipC peptide fragment mapped	No. of identified peptides	LipC aa positions
AAGSTGVAYIR	1	6–16
HASDFLSATAK	2	60–70
DLTLPGINEVR	3	71–81
GIVSPDDLAVEWPAPER	12	98–114
VLYGDDPAQLLDVWR	22	123–147
AIQGYAVLSR	1	173–182
FVDFLER	2	290–296
TGPTAHAIALFLNQVHR	1	377–393

FIG 3 Localization of LipC protein. (A) *M. tuberculosis* H37Rv subcellular protein fractions (5 μ g/lane) and rLipC (75 ng/lane) were separated on 10% SDS-PAGE gels and transferred onto a nitrocellulose membrane. The blots were probed with anti-malate synthase (MS) IgG (top), anti-LipC IgG (middle), and normal rabbit IgG (bottom). Lanes: 1, rLipC; 2, cytoplasm; 3, total cell wall; 4, SDS-extracted cell wall; 5, capsule; 6, culture filtrate. (B) Immunoelectron microscopy of ultrathin sections of *M. tuberculosis* H37Rv (a and b), the *M. tuberculosis* CDC1551 *lipC*::Tn mutant (c), and *M. tuberculosis* CDC1551 (d). The bacterial sections were probed with either rabbit normal IgG (a) or rabbit anti-LipC IgG (b, c, and d).

(Fig. 3A, bottom). The surface localization of LipC was confirmed by the presence of gold particles on *M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551 bacterial cells (Fig. 3Bb and d) and their paucity on the cell surface of CDC1551 *lipC*::Tn bacterial cells (Fig. 3Bc) when examined by immunoelectron microscopy. No LipC was detected on the bacterial cells when IgG from normal rabbit serum was used instead of anti-LipC antibodies (Fig. 3Ba).

Expression of LipC in vivo. To determine if LipC is expressed during *M. tuberculosis* infection in humans, the reactivity of rLipC with sera from PPD⁻, HIV⁺ TB⁻, as well as PPD⁺ healthy subjects and TB patients was examined by Western blotting. Visual examination showed that while sera from non-TB subjects showed background reactivity with the rLipC protein, there was no difference between the reactivities of sera from PPD⁻ and HIV⁺ TB⁻ subjects and those from the PPD⁺ subjects. In contrast, sera from most HIV⁻ TB⁺ and HIV⁺ TB⁺ patients showed stronger reactivity (Fig. 4A). To confirm these results, sera from additional randomly selected PPD⁺ subjects and TB patients were tested, and similar results were obtained (Fig. 4B). When the intensity of the rLipC protein probed with anti-His antibodies was considered 100 units, the relative intensity obtained with individual sera from PPD⁻, HIV⁺ TB⁻, and PPD⁺ subjects ranged from 1 to 14 units, and there was no difference among sera from the 3 subgroups of subjects ($P > 0.01$). In contrast, reactivity with the 6 HIV⁻ TB⁺ sera ranged from 7 to 60 units (median reactivity = 28.5 units), and for the 6 HIV⁺ TB⁺ sera, it ranged from 33 to 75 units (median reactivity = 62.5 units). The difference in intensi-

ties between the non-TB subjects and the TB patients was statistically significant ($P < 0.01$). Using mean intensities of PPD⁻, PPD⁺, and HIV⁺ TB⁻ sera plus 3 SD as a cutoff, sera from 11/12 TB patients in the first experiment (Fig. 4A and C) and from 8/10 TB patients in the second experiment (Fig. 4B and D) were positive for the presence of anti-LipC antibodies. Thus, sera from ~85% of the TB patients had anti-LipC antibodies. The reactivity of a subset of the control and TB sera was evaluated with rLipC expressed in *M. smegmatis*; the reactivity was similar to that observed with rLipC expressed in *E. coli* (data not shown).

Epitope mapping of LipC. When the reactivities of the 40 overlapping peptides of LipC were tested with sera from 13 PPD⁻ and 23 PPD⁺ subjects, there was no difference in the reactivities of sera from these two types of subjects with any of the peptides. In contrast, sera from a vast majority (57/60; 95%) of the HIV⁻ TB⁺ patients had antibodies against at least one LipC peptide (Fig. 5A); most sera (56/60; 93%) had antibodies to >1 peptide. As expected, there was a wide variation in the recognition of individual peptides with the HIV⁻ TB⁺ sera, in which different peptides were recognized by 0 to 70% of the patients (Fig. 5A). Twelve peptides were recognized by $>50\%$ of the HIV⁻ TB⁺ sera (52 to 70%). These peptides were retested twice for reactivity with the same sera. For 6 peptides (LipC3, LipC6, LipC24, LipC26, LipC34, and LipC39) (Fig. 5B and Table 3), the reactivity was maintained (52 to 65%). In contrast, the level of reactivity with the remaining 6 peptides was lower during the subsequent assays (32 to 43%). For the 6 peptides that were consistently recognized by $>50\%$ of the HIV⁻ TB⁺ sera, there was no difference between the reactivities with sera from PPD⁺ and PPD⁻ subjects ($P = 0.29$ to 0.74), while the level of reactivity of all 6 peptides was significantly higher ($P < 0.001$) with sera from patients. The combined level of reactivity with the 6 peptides was ~83% for HIV⁻ TB⁺ patients. These 6 peptides were also reproducibly recognized individually with $>50\%$ of the HIV⁺ TB⁺ sera (Fig. 5C), and the combined level of reactivity with the peptides was ~82%, while the vast majority of HIV⁺ TB⁻ patients lacked antibodies to any of these peptides (Fig. 5C). Mapping of the 6 immunodominant peptides onto the model of the 3-D structure of LipC showed that 5 peptides (LipC3, LipC6, LipC26, LipC34, and LipC39) were totally exposed to the solvent, while LipC24 was buried in the folded conformation of the protein (Fig. 1).

Cytokine responses to LipC. The cytokine responses elicited from THP-1 and A549 cells by rLipC were also evaluated. The viabilities of both cell types were unaffected by the low concentration of SDS (0.00025% SDS) present in the antigen preparation used to stimulate them (Fig. 6A). THP-1 cells exposed to 1 ng/ml LPS produced ~2,400 pg/ml of IL-12 and ~2,500 pg/ml of TNF- α . The preincubation of LPS with 10 ng/ml of polymyxin B completely abrogated the production of both cytokines (Fig. 6B). Cells exposed to 1 μ g/ml rLipC expressed ~2,800 pg/ml and ~2,700 pg/ml IL-12 and TNF- α , respectively. The preincubation of rLipC with polymyxin B had no effect on cytokine production, confirming that the observed cytokine responses were not due to a contaminating endotoxin (Fig. 6B).

THP-1 cells exposed to different concentrations of rLipC showed a dose-dependent production of IL-12, TNF- α , IL-8, and MCP-1, and in most cases, the peak response was attained at 0.1 μ g/ml (Fig. 6C). MCP-1 was the most highly induced cytokine (~7,000 pg/ml), followed by IL-8 (~3,500 pg/ml), IL-12 (~3,000 pg/ml), and TNF- α (~2,500 pg/ml) (Fig. 6C). Minimal or no

FIG 4 Reactivity of rLipC with sera from TB patients and control subjects. (A) Western blots of rLipC (75 ng/lane) were probed with sera (1:50) from 6 PPD⁻ (lanes 2 to 7), 6 PPD⁺ (lanes 8 to 13), 6 HIV⁺ TB⁻ (lanes 14 to 19), 6 HIV⁺ TB⁺ (lanes 20 to 25), and 6 HIV⁻ TB⁺ (lanes 26 to 31) subjects. (B) Probing of rLipC (75 ng/lane) blots with sera from 10 additional PPD⁺ healthy subjects (lanes 2 to 11) and 10 additional HIV⁻ TB⁺ patients (lanes 12 to 21). Lanes 1 in both panels A and B show the reactivity of rLipC with anti-His antibody. (C and D) Reactivity of rLipC with sera from each subject was measured by determining the intensity of the rLipC band in panels A and B by use of Image J software. The relative intensities of rLipC bands in panels A and B calculated by considering anti-His antibody reactivity as 100 units are plotted in panels C and D, respectively.

cytokine levels (<400 pg/ml) were detected in supernatants from unstimulated cells (Fig. 6C).

A549 cells stimulated with rLipC expressed IL-8 and MCP-1 in a dose-dependent manner, and as was observed with THP-1 cells, the peak level of MCP-1 induction was higher (~5,500 pg/ml) than the peak level of induction of IL-8 (~1,600 pg/ml) (Fig. 6D).

Enzymatic characterization of LipC. To evaluate the substrate specificity of LipC, the enzymatic activity of proteins expressed and purified from *M. smegmatis* against *p*-nitrophenyl esters was investigated by using substrates with acyl chain lengths ranging from C₂ to C₁₆ in presence or absence of NaTDC (1 or 4 mM) (Fig. 7). The presence of NaTDC enhanced slightly the specific activity for substrates ranging from C₂ to C₁₀ compared to the buffer alone. The activity of rLipC increased with the chain length of the substrate, reaching maximum specific activities of 160 mU/mg ($K_m = 2.89$ mM; $V_{max} = 0.48$ μ M/min) and 110 mU/mg ($K_m = 2$ mM; $V_m = 0.69$ μ M/min), with and without NaTDC, respectively.

No activity was detected for substrates with carbon chain lengths greater than C₁₀ under any of the conditions used. Using the pH-stat method (12), fluorescent spectrometric assays (R. Verger, C. Serveau-Avesque, and H. Chahinian, 17 August 2006, PCT patent application WO2006/85009), and radioactive assays (37), no activity was detected on mono-, di-, or triacylglycerol whatever the carbon chain length, indicating that this enzyme is a strict esterase and does not have any lipase activity (data not shown). Also, all attempts to detect phospholipase activity failed (data not shown).

DISCUSSION

The sequencing of the *M. tuberculosis* genome revealed that *M. tuberculosis* possesses ~150 genes that code for enzymes involved in fatty acid degradation (6), suggesting that *M. tuberculosis* utilizes host lipids during growth and replication *in vivo*. The analysis

of the genes by bioinformatics methods identified a family of 24 putative carboxyl hydrolases with the characteristic GX SXG motif. Twenty of the Lip family members are predicted to be present in the cytoplasm of the bacterium, and four of these family members are predicted to be present in the periplasmic space; LipC is predicted to be a cytoplasmic enzyme (40). However, the experimental approaches used in this study clearly demonstrate that native LipC is present primarily in the cell wall and the capsule of *M. tuberculosis*. This is reminiscent of the findings with 2 other Lip family members, LipF and LipY, both of which are also associated with the cell wall (28, 45). In confirmation of this localization *in vivo*, like LipF and LipY, LipC also participates in immune responses by eliciting antibodies. It is interesting that anti-LipC antibodies raised by immunization with rLipC (~45 kDa) recognize the appropriate protein band as well as an additional ~47-kDa protein in the native cell wall subcellular fractions but only the ~47-kDa band in the capsular fraction. This finding suggests that the native LipC present on the bacterial surface in the capsule may have posttranslational modifications, while the cytoplasm and cell wall have both the modified and the nonmodified LipC proteins. The nature of these posttranslational modifications remains to be determined.

Previous studies suggested that there may be interspecies differences in the profiles of antigens recognized by antibodies (17, 18). Since LipC was originally identified by antibodies in sera from *M. tuberculosis*-infected rabbits, it was important to confirm its expression during TB in humans. Thus, sera from subjects with latent or active *M. tuberculosis* infection were evaluated for the presence of anti-LipC antibodies. Previous studies also reported differential expressions of some *M. tuberculosis* antigens in HIV⁻ TB⁺ and HIV⁺ TB⁺ patients (34, 36). For this reason, sera from both HIV⁻ TB⁺ and HIV⁺ TB⁺ patients were included. Sera from healthy subjects with no evidence of *M. tuberculosis* infection

FIG 5 Epitope mapping of LipC protein. (A) Reactivities of overlapping peptides of LipC with sera from 60 HIV⁻ TB⁺ patients and 36 PPD⁻ or PPD⁺ healthy subjects tested by ELISA. The mean optical density (OD) at 405 nm plus 3 standard deviations with sera from PPD⁻ or PPD⁺ healthy subjects was used as a cutoff to determine positive reactivity. Percentages of sera from PPD⁻ and PPD⁺ healthy subjects (hollow bars) and HIV⁻ TB⁺ patients (black bars) showing positive reactivity with individual peptides are plotted. (B) Reactivities of 6 LipC immunodominant peptides with sera from 13 PPD⁻ (black circles) and 23 PPD⁺ (hollow circles) healthy subjects and HIV⁻ TB⁺ patients (black triangles). The OD values obtained with individual serum specimens in one representative experiment are plotted. (C) OD values obtained by the reactivity of individual sera from 36 PPD⁻ or PPD⁺ healthy subjects (black circles), 46 HIV⁺ TB⁻ asymptomatic subjects (hollow circles), and 45 HIV⁺ TB⁺ patients (black triangles) with 6 immunodominant peptides of LipC from one representative experiment. The dotted lines in panels B and C indicate the cutoff (mean OD + 3 SD with sera from PPD⁻ and PPD⁺ subjects) for determining positive reactivity with each peptide.

(PPD⁻) as well as HIV⁺ TB⁻ subjects (from the United States) were included as negative controls. Antibodies to LipC were present in sera from ~90% of the TB patients tested (Fig. 4), whether or not coinfecting with HIV, indicating the *in vivo* expression of the proteins during active infection in both types of patients. In contrast, sera from PPD⁺ subjects showed reactivity similar to that of sera from PPD⁻ subjects, demonstrating the absence of an *in vivo* expression of LipC during latent TB.

We have demonstrated that immunodominant epitopes of an-

tigenic proteins can replace and improve upon the detection of antibodies to the parent protein (39, 43). Antibodies to at least one epitope of LipC were present in ~95% of the HIV⁻ TB⁺ patients and absent in the vast majority of PPD⁺ subjects, confirming the results of the Western blot studies. Moreover, reactivity with just the 6 immunodominant peptides was sufficient to identify a vast majority (>80%) of the HIV⁻ TB⁺ patients. This level of reactivity is similar to that observed with immunodominant peptides of PTRP, which is also a cell wall protein that was identified by antibodies in rabbit sera obtained at 5 weeks postinfection and whose immunodominant epitopes were mapped with sera from the same cohorts as those that were used in this study (43). Moreover, as was observed for PTRP and its immunodominant peptides, there was no difference in the recognition of LipC and its peptides between HIV⁻ TB⁺ and HIV⁺ TB⁺ patients. Our earlier studies with culture filtrate proteins (CFP) of *M. tuberculosis* showed that some antigens (malate synthase and MPT51, for example) are well recognized by antibodies from both cavitary and noncavitary TB patients, demonstrating that these antigens are expressed *in vivo* in both types of patients (34, 53). In contrast, other antigens (38-kDa protein and MPT32, for example) appear to be expressed primarily during cavitary TB, since noncavitary TB patients lack antibodies to them (34). The similar immunogenicities of LipC and its peptides in sera from smear-positive HIV⁻ TB⁺ patients, most of

TABLE 3 Amino acid sequences of immunodominant LipC peptides

Peptide	Sequence
LipC3	ARPADYMLALS VAGGSLPVV ^a
LipC6	TAIGVWGARGHASFSLATAK ^b
LipC24	IAVAGCSAGGHLSALAGLTA ^c
LipC26	NDPQYQAE LPEGSDTSVDAV ^d
LipC34	GSRDCVIPVEQARSFVERLR ^e
LipC39	AHAIALFLNQVHRSRAQFAK ^f

^a Shown in red in Fig. 1.

^b Shown in orange in Fig. 1.

^c Shown in blue in Fig. 1.

^d Shown in green in Fig. 1.

^e Shown in yellow in Fig. 1.

^f Shown in magenta in Fig. 1.

FIG 6 Cytokine production by THP1 and A549 cells in response to rLipC. (A) Viability of A549 and THP-1 cells at 24 h with medium alone, medium containing 1 $\mu\text{g/ml}$ rLipC, and medium containing 0.00025% SDS. The percent viability calculated by considering cells exposed to medium alone as 100% is plotted. (B) Production of IL-12 and TNF- α by THP-1 cells exposed to LPS alone (1 ng/ml), LPS (1 ng/ml) preincubated with polymyxin B (10 ng/ml), rLipC (1 $\mu\text{g/ml}$), and rLipC (1 $\mu\text{g/ml}$) preincubated with polymyxin B (10 ng/ml). (C) Induction of IL-12, TNF- α , IL-8, and MCP-1 in THP-1 cells after incubation with various concentrations of rLipC and 1 $\mu\text{g/ml}$ of LPS. (D) Production of IL-8 and MCP-1 by A549 cells exposed to different concentrations of rLipC and 1 $\mu\text{g/ml}$ LPS.

FIG 7 Enzymatic activity of rLipC. The enzymatic activity of rLipC was measured by using *p*-nitrophenyl esters with carbon chain lengths ranging from C₂ to C₁₆ as substrates using various concentrations of NaTDC. The means $\pm$ SD of specific activities (mU/mg) obtained with 3 independent replicates are plotted.

whom had cavitary lesions, and HIV⁺ TB⁺ patients, who were mostly noncavitary, indicate that it is expressed by *M. tuberculosis* during replication in both noncavitary and cavitary environments *in vivo*.

Inexpensive and rapid point-of-care (POC) tests based on the detection of antibodies to antigens and/or epitopes selected by a careful analysis of their immunogenic potential in patients and controls have been devised for other diseases (1, 13). However, a recent WHO-sponsored analysis of the performances of currently available commercial serological tests for TB diagnosis showed that none of these tests provide accurate and reliable results for either pulmonary or extrapulmonary TB patients (46, 47, 56). These commercial tests are based primarily on crude mixtures of *M. tuberculosis* antigens or on antigens that elicited antibodies in immunized animals rather than on the careful analysis and selection of antigens relevant to human TB during the natural course of the disease. Our systematic studies of humoral responses in TB patients have identified a panel of potential candidates for devising a rapid POC test for TB, and immunodominant epitopes of some of these highly immunogenic *M. tuberculosis* antigens have been delineated (11, 34, 36, 39, 43, 44). Since combinations of multiple antigens provide an increased sensitivity of antibody detection (11, 15, 17, 42, 53), it is likely that combinations of immunodominant epitopes from multiple highly immunogenic proteins of *M. tuberculosis* will enable the development of a peptide-based rapid test for TB. One or more of the six epitopes of LipC defined in these studies could make significant contributions to such a diagnostic test.

The use of peptides is economically advantageous over recombinant purified proteins, which are expensive to produce and difficult to purify and pose considerable challenges for maintaining batch-to-batch consistency and long-term stability. Moreover, the use of peptides eliminates the nonimmunogenic and sometimes cross-reactive portions of the antigens. The use of biotin-conjugated peptides enables the equal binding of individual peptides to streptavidin-coated plates, thus eliminating differences between their binding capacities when being tested by ELISA. Despite equal binding, some peptides that are recognized by antibodies in a high proportion of TB patients during the first screen performed poorly when retested. This was also observed when

epitope mapping of other immunogenic proteins was performed (39, 43). In some cases, solubilized peptides were seen to gradually aggregate and precipitate during storage, suggesting that they undergo conformational changes in solution; for other peptides, it was unclear why the reactivity decreased during retesting. It is for this reason that the reactivity of each peptide needs to be evaluated multiple times so that only stable and highly reactive peptides are finally selected for test development.

Antibodies in sera from a few (<10%) of the HIV⁺ TB[−] subjects showed reactivity with the 6 LipC peptides. Since these HIV⁺ TB[−] patients were from the United States and were asymptomatic and on antiretroviral therapy (ART), it is unlikely that they were infected with any nontuberculous mycobacteria (NTM). The cross-reactivity observed with some of these sera is likely due to the hypergammaglobulinemia caused by HIV infection and may be eliminated during the optimization of conditions during the development of the rapid test.

As expected from B cell epitopes, 5 of the 6 immunodominant peptides mapped to the exposed surface of the modeled protein (Fig. 1). The presence of antibodies to LipC24, which is not surface exposed, indicates that *in vivo*, the shedding of the bacterial capsule may result in the release of LipC in a somewhat unfolded conformation, providing the B cells with access to this region.

While macrophages are well known to be the host cells for *M. tuberculosis*, there is increasing evidence for important roles for alveolar epithelial cells in TB (2, 19, 20, 23, 52). We therefore evaluated the ability of the purified LipC protein to elicit cytokines from both THP-1 and A549 cells. While MCP-1 was the most highly induced cytokine, IL-8 was also expressed by the two cell types (Fig. 6C and D). Both MCP-1 and IL-8 are involved in the recruitment of macrophages, neutrophils, dendritic cells, and T cells and play important roles in the formation of granulomas (51). Importantly, IL-8 and MCP-1 were also demonstrated to be present in the bronchoalveolar lavage fluid of TB patients (24). LipC also induced the expression of TNF- α and IL-12 from THP-1 cells. TNF- α is known to play a key role in granuloma formation and induce macrophage activation and has immunoregulatory properties (51). IL-12 is known to play a crucial role in the induction of gamma interferon (IFN- γ), which activates macrophages to kill the bacteria (7). The *in vivo* expression of LipC in rabbits at 4 to 5 weeks post-aerosol infection, when granuloma formation occurs, and the ability of LipC to stimulate proinflammatory cytokine and chemokine production suggest that the precise role of LipC in granuloma formation upon aerosol infection with *M. tuberculosis* warrants investigation in animal models. In this regard, previous studies showed that another carboxylesterase (Rv2224c), which was also anchored to the cell wall, modulates innate immune responses to *M. tuberculosis* in mice (25).

Previous studies have shown that *M. tuberculosis* stores energy in the form of triacylglycerol (TAG) as it goes into the latent state, which is utilized for survival during dormancy and reactivation when bacterial replication is resumed (9). TAG is also the common form of stored energy in hibernating animals and oil seeds when the level of metabolic activity is low (30). TAG is hydrolyzed via the glyoxylate cycle, which has been shown to be used by *M. tuberculosis* during intracellular survival (29). Interestingly, in *M. tuberculosis*, malate synthase, which participates in the glyoxylate cycle, is present not only in the cytoplasm but also on the cell wall and in the capsule (19), and antibodies to malate synthase are strongly associated with clinical TB but not latent TB, suggesting

enhanced expression when bacteria are replicating actively *in vivo*. Previous studies have also demonstrated that LipY hydrolyzes TAG (10), and LipF hydrolyzes both short-chain esters and phosphatidylcholine, which is the major constituent of biological membranes (45, 60). Both LipY and LipF also localize to the bacterial cell wall and elicit antibodies in patients with active TB (8, 10, 28, 45, 60). Together, these data suggest that LipY, LipF, and LipC may be used in tandem by *M. tuberculosis* for the utilization of host lipid substrates during active replication and growth *in vivo*.

There is increasing evidence that bacterial cell wall/surface-localized enzymes also multitask as antigenic proteins that play roles in the modulation of immune responses and/or in the invasion of host cells (4, 19, 32, 38, 49). For example, besides LipC, LipY, LipF, malate synthase, MPT51, Ag85B, Ag85C, and Rv2224c are all cell wall/cell surface-located enzymes of *M. tuberculosis* that elicit humoral, cellular, and/or inflammatory immune responses (10, 19, 21, 22, 25, 53, 57). Ag85B, malate synthase, and MPT51 were also reported previously to be adhesins of *M. tuberculosis* that bind to extracellular matrix proteins like fibronectin and laminin (14, 19, 21, 57). Interestingly, the GehD lipase of *Staphylococcus epidermidis* is a collagen-binding protein (4); the GbpD protein of *Streptococcus mutans* has both lipase and glucan-binding activities (38), and an extracellular lipase of *Pseudomonas aeruginosa* participates in the release of inflammatory mediators from granulocytes and monocytes (32). The current studies demonstrate that LipC is a cell surface-associated esterase of *M. tuberculosis* that is highly immunogenic and elicits both antibodies and cytokines/chemokines. We have identified immunodominant regions of LipC, which may be important constituents of a peptide-based serodiagnostic test for TB. The role of LipC in infection and disease progression in TB merits further investigation.

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