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Delineating the physiological roles of the PE and catalytic domain of LipY in lipid consumption in mycobacteria-infected foamy macrophages

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Within tuberculous granulomas, a sub-population of *Mycobacterium tuberculosis* resides inside foamy macrophages (FM) that contain abundant cytoplasmic lipid bodies (LB) filled with triacylglycerol (TAG). Upon fusion of LB with *M. tuberculosis*-containing phagosomes, TAG is hydrolyzed and reprocessed by the bacteria into their own lipids, which accumulate as intracytosolic lipid inclusions (ILI). This phenomenon is driven by many mycobacterial lipases, among which LipY participates in the hydrolysis of host and bacterial TAG. However, the functional contribution of LipY’s PE domain in TAG hydrolysis remains unclear. Herein, enzymatic studies were performed to compare the lipolytic activity of recombinant LipY and its truncated variant lacking the N-terminal PE domain, LipY(ΔPE). Complementarily, an FM model was used where BMDM were infected with *M. bovis* BCG strains either overexpressing LipY or LipY(ΔPE) or carrying a *lipY*-deletion mutation prior to being exposed to TAG-rich VLDL. Results indicate that truncation of the PE domain correlates with increased TAG hydrolase activity. Quantitative electron microscopy analyses showed that (i) in the presence of lipase inhibitors, large ILI were not formed either because of an absence of LB due to inhibition of VLDL-TAG hydrolysis or inhibition of LB-neutral lipid hydrolysis by mycobacterial lipases; (ii) large ILI$^{+3}$ in the strain overexpressing LipY(ΔPE) were reduced and, (iii) the number of ILI$^{+3}$ profiles in the ΔlipY mutant was reduced by 50%. Overall, these results delineate the role of LipY and its PE domain in host and mycobacterial lipid consumption and show that additional mycobacterial lipases take part in these processes.
INTRODUCTION

With more than 10 million new cases and 1.7 million deaths in 2016, tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains one of the most devastating diseases worldwide (1). This serious illness is not fully treatable with current medication. Prognosis of the disease depends on the host’s ability to contain the bacilli at the site of infection within granulomas. Tuberculous granulomas are complex and dynamic immunological structures, which generate a wide range of microenvironments (2, 3) that are assumed to drastically affect the tubercle bacilli physiological properties (3, 4). Inside the high diversity of TB pulmonary lesions found during active disease or latent infection, a distinct bacterial sub-population appears associated with a specific cell type consisting of foamy macrophages (FM) characterized by the presence of numerous triacylglycerol (TAG)-filled lipid bodies (LB) in their cytosol (2, 5, 6). To date, the exact role of FM in TB pathogenesis remains elusive, however these specialized lipid-rich cells have been described in both experimentally-infected animals and patients suggesting that they might play an essential role in granuloma formation and maturation processes. Several independent studies have demonstrated that pathogenic mycobacteria during *in-vitro* FM infection, are able to survive in a slow or non-replicating state (5, 7). These experimental observations prompted the authors to propose that FM may also promote *M. tb* intracellular survival for extensive periods *in vivo*, although this remains to be demonstrated. A better understanding of how mycobacteria interact with and persist within FM is important to decipher this complex host-pathogen crosstalk that may sustain latent TB and, eventually, to identify new pharmacological approaches to control both active and latent TB. It is well-known that storage of large amounts of neutral lipids in the form of intracytosolic lipid inclusions (ILI) is one of the main characteristics of persistent mycobacteria (8). ILI are mainly composed of triacylglycerols (TAG) resynthesized by
mycobacterial TAG synthases from free fatty acids coming from the hydrolysis of host
TAG (9-12). Host lipid transfer to the phagosomal lumen occurs via mycobacterium-
induced fusion between LB and phagosomes (7). Lipids within ILI seem to serve as a
source of carbon and energy prior to reactivation of dormant bacilli (11, 13).

Although the origin of the ILI-containing lipids has become the focus of intense
research (5, 10, 11, 14, 15), the catalytic steps involved in the formation of mycobacterial
ILI are not completely understood (16, 17). It is obvious, however, that hydrolysis of host
TAG released into the phagosome involves specific mycobacterial lipolytic enzymes,
either secreted by the bacilli into the phagosomal lumen or associated with the outermost
layer of the mycobacterial cell wall (16, 18-20) whereas lipolytic enzymes involved in
hydrolysis of ILI-contained TAG must be located in the mycobacterial cytosol (11, 21, 22).
Among the many mycobacterial lipases found in \textit{M. tuberculosis}, LipY (Rv3097c) appears
as a major player in the degradation of TAG within ILI under \textit{in vitro} growth conditions
mimicking dormancy (11, 21, 22). LipY belongs to the hormone-sensitive lipase family
and displays an N-terminal PE domain that is specific to pathogenic mycobacterial
species (21-23). Relevant studies demonstrated that PE proteins are exported to the cell
wall via the type VII secretion system ESX-5 after recognition of the specific amino-acid
sequence YxxxD/E contained in the N-terminal PE domain (18, 24-26). Among the
enzymes of the PE family, only two members, PE-PGRS11 (or Rv0754), a functional
phosphoglycerate mutase (27) and LipY, have been biochemically characterized. LipY
was proposed to be a major contributor to the breakdown of stored TAG (21, 22). In
addition, a recombinant \textit{Mycobacterium smegmatis} strain overexpressing the full-length
LipY, exhibited a reduced TAG hydrolytic activity as compared to a LipY variant shortened
by its N-terminal PE domain, LipY(ΔPE), suggesting that the PE domain may negatively
modulate the activity of LipY (22, 28).
In the present study, the experimental model system of VLDL-driven FM (7) was used to delineate the physiological role of the PE domain of LipY in the hydrolysis of both host-derived TAG and mycobacterial TAG within ILI. Reasons for choosing this model system have been described in detail elsewhere (7, 12, 14, 29). After internalization of VLDL by receptor-mediated endocytosis, the neutral lipids of this lipoprotein will undergo hydrolysis in lysosomes. This will provide fatty acids for the subsequent biosynthesis of neutral lipids, including TAG, between the two leaflets of the endoplasmic reticulum, where LB, consisting of a phospholipid monolayer encapsulating neutral lipids, bud of. This model system generates defined conditions for inducing the formation or removal of LB, which triggers ILI formation or consumption, and helps delineating the role of host cell or mycobacterial lipases in these processes.

We first show that addition of lipase inhibitors, during exposure of infected cells to VLDL, inhibits the accumulation of lipids in the form of ILI by affecting either the macrophage lysosomal TAG hydrolase and, hence, LB formation, or mycobacterial lipases involved in the hydrolysis of host TAG delivered to phagosomes. Next, we demonstrate the modulatory role of its N-terminal PE domain on LipY function by, as a first approach, comparing the catalytic activity of purified LipY and LipY(ΔPE) variants using a range of lipids with various chain lengths. As a second approach, we used quantitative electron microscopy (EM) methods to examine differences in mycobacterial ILI formation in bone marrow-derived mouse macrophages (BMDM) exposed to VLDL to become foamy after they were infected with *M. bovis* BCG strains overexpressing different LipY variants or in which the *lipY* gene was deleted. This study indicates that other lipases, in addition to LipY, are likely to be involved in ILI formation. Removal of VLDL normally leads to the loss of TAG-containing ILI (7). When lipase inhibitors were
added after exposure to VLDL no loss of ILI occurred, a result that emphasizes the participation of several lipolytic enzymes in the hydrolysis of bacterial cytosolic TAG.
MATERIALS AND METHODS

Reagents. DMEM and glutaraldehyde grade I (EM grade) were purchased from Sigma (St Louis, MO, USA), FBS was from Biowest (Nuaillé, France), PBS was from GIBCO (distributed by Invitrogen, Villebon sur Yvette, France), commercial Very Low Density Lipoprotein (VLDL) was from Calbiochem-Merck (Darmstadt, Germany), osmium tetroxide and Spurr resin were from Electron Microscopy Sciences (distributed by Euromedex, Mundolsheim, France). All bacterial culture media were purchased from Life technologies (USA).

Bacterial strains and growth conditions. Escherichia coli DH10B cells (Invitrogen) used in cloning experiments were grown at 37°C in Luria Bertani (LB) broth (Invitrogen) or on LB agar plates. Culture media were supplemented with 200 µg/mL hygromycin B.

The M. smegmatis mc²155 groEL1ΔC strain (30) used for expression experiments was routinely grown at 37°C with shaking (220 rpm) in Middlebrook 7H9 medium (Difco) supplemented with 0.05% Tween 80 (v/v), 0.2% glycerol (v/v), 0.5% bovine serum albumin (BSA) (w/v) and 0.2% glucose (w/v) or on Middlebrook 7H11 (Difco) agar plates.

The M. bovis BCG Pasteur 1173P2 strain used for overexpression experiments was grown at 37°C with shaking (160 rpm) in Middlebrook 7H9 medium supplemented with 0.05% Tween 80 (v/v), 0.2% glycerol (v/v) and 10% Albumin Dextrose Catalase (v/v) (ADC). Preparation of M. smegmatis and M. bovis BCG electrocompetent cells and electroporation procedures were performed as described previously (31). Transformants were selected on Middlebrook 7H11 agar supplemented with 10% ADC and either 50 µg/mL hygromycin or 50 µg/mL kanamycin. Plates were incubated at 37°C for 3-5 days for M. smegmatis and for three weeks for M. bovis BCG.
Complementation of *M. bovis* BCG ΔlipY strain. A *M. bovis* BCG mutant lacking the lipY gene (ΔlipY) was grown in the presence of 50 µg/mL hygromycin (32). Complementation was performed by introducing pMV261::lipY (22) prior to selection on 7H11 Middlebrook agar medium supplemented with 10% ADC and kanamycin. Presence of the plasmid was checked by PCR and expression of LipY confirmed by immunoblotting. Briefly, bacterial lysates from the WT, ΔlipY and complemented strain (ΔlipY::Comp) were normalized for total protein content, electrophoretically separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were then saturated with 1% bovine serum albumin (BSA) in PBS, 0.1% Tween 20 and probed for 1 hour with mouse antiserum raised against LipY at a 1/2,500 dilution. After extensive washing, the membrane was incubated with horseradish peroxidase-conjugated IgG anti-mouse antibodies (Sigma-Aldrich). The GroEL2 protein was used as protein loading control and was revealed using the HisProbe™ HRP conjugate (Thermo-Scientific) which recognizes the natural poly-Histidine sequence present in its C-terminal domain (33, 34). Detection was achieved using the Pierce™ ECL Western Blotting substrate solution (Thermo-Scientific) and visualized using the ChemiDoc™ MP Imaging System (Bio-Rad).

Cloning, expression and purification of LipY and LipY(ΔPE). The lipY and lipY(ΔPE) genes were amplified by PCR using *M. tuberculosis* H37Rv genomic DNA and cloned into pSD26 under the control of the acetamide inducible promoter and carrying a hygromycin resistance cassette (35) or into pMV261 (36) downstream of the hsp60 promoter and containing a kanamycin resistance cassette, as reported previously (22). DNA sequence analysis of each insert was performed by GATC Biotech (Germany).
Expression and purification of recombinant LipY or LipY(ΔPE) were performed as previously reported (37) with some minor modifications. Briefly, *M. smegmatis* mc²155 groEL1ΔC strains carrying pSD26- lipY and pSD26- lipY(ΔPE), were used to inoculate 10 mL of 7H9 medium containing 50 μg/mL hygromycin. After three days of incubation at 37°C with shaking, 10 mL of the preparation were used to inoculate 400 mL of culture medium for a large-scale production. Cultures were grown at 37°C with shaking (220 rpm) until an OD<sub>600nm</sub> value between 2.5 and 3 was reached. Expression of recombinant proteins was induced by adding acetamide to a final concentration of 0.2% (w/v) for 16 h.

Bacteria were harvested, resuspended in ice-cold buffer A (10 mM Tris/HCl pH 8.0, 150 mM NaCl) (30 mL) containing 1% N-lauroylsarcosine and lysed using a French Press set 1100 psi. After centrifugation, the supernatant (S1) was recovered while the resulting pellet was resuspended in buffer A (30 mL) and sonicated twice for 30 s with 30 s breaks between each cycle and stirred overnight at 4°C. After centrifugation, the new supernatant (S2) was pooled with S1 supernatant and the mixture was loaded onto a Ni²⁺-NTA resin equilibrated with buffer A. The column was subsequently washed with buffer A without detergent prior to elution with increasing concentrations of imidazole. The eluted fractions were analysed by performing 12% SDS-PAGE as described previously (38). Fractions containing pure proteins were pooled, dialysed overnight against buffer A and concentrated by ultrafiltration to a final concentration of 0.6 mg/mL and stored at -80°C.

Theoretical physical properties (molecular mass, extinction coefficient at 280 nm and isoelectric point) of both proteins containing the His<sub>6</sub>-tag at the C-Terminal position were obtained from the ProtParam tool (http://ca.expasy.org/tools/protparam.html).

**Enzyme activity measurements using the pH-stat technique.** Enzymatic hydrolysis of emulsions of mono-, di- and triacylglycerol namely monobutylin (MC4), monolein (MC18),
diolein (DC18), tributyrin (TC4) and trioctanoin (TC8) were monitored titrimetrically for at least 5 min at 37°C using a pH-stat (Metrohm 718 STAT Titrino; Metrohm Ltd., Herisau, Switzerland). Assays were performed in 2.5 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl and 3 mM NaTDC (23, 39). Free fatty acids released were automatically titrated with 0.1 M NaOH to maintain a fixed end-point pH value of 7.5. The specific activity of both enzymes was expressed in units per mg of pure enzyme. One unit corresponds to the release of 1 µmole of fatty acid per minute.

**Lipase activity assays on TAG from Pomegranate oil.** Corning UV 96-well microplates were coated as recently described (40) using TAG from Pomegranate oil, containing up to 80% punicic acid (C18:3) equally present at the 3 positions of the glycerol backbone. The lipase activity was measured at 37°C in 10 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 6 mM CaCl₂, 1 mM EDTA, 0.001% (w/v) 3,5-di-tert-4-butylhydroxytoluene (BHT) and 3 mg/mL β-Cyclodextrine (β-CD). The formation of the β-CD/free punicic acid complex was continuously monitored at 275 nm for 60 min.

**In silico Protein Modeling.** Three-dimensional structural models of LipY and LipY(ΔPE) proteins were generated with the automatic protein structure homology modeling server using the I-Tasser software program (41, 42). The alignment of the LipY and LipYΔPE sequences were performed using Multalin multiple sequence alignment (43) and the result was displayed with ESPript (44). The structural overlay and figures were drawn using the PyMOL Molecular Graphics System (version 1.8.6.0, Schrödinger, LLC).

**Infection of bone marrow-derived mouse macrophages (BMDM) with recombinant** *M. bovis BCG strains.* Bone marrow cells were isolated from the femurs of 6- to 8-week-
old C57BL/6 female mice and seeded onto tissue culture dishes (Falcon; Becton Dickinson Labware, Meylan, France) 35 mm in diameter (4x10^5 cells per dish). The culture medium was DMEM with high glucose (1 g liter⁻¹) and high carbonate (3.7 g liter⁻¹) concentrations supplemented with 10% heat-inactivated FBS, 10% L-cell conditioned medium (a source of colony-stimulating factor 1, CSF-1), and 2 mM L-glutamine. Five days after seeding, the adherent cells were washed twice with DMEM and refed with complete medium. Medium was then renewed on day 6. No antibiotics were added. On day 7, the cells were infected for 4 h at 37°C with wild type or different recombinant M. bovis BCG strains at a multiplicity of infection (MOI) of 5 for EM studies, washed in 4 changes of ice-cold PBS to eliminate non-ingested bacteria, and further incubated in complete medium devoid of antibiotics. For long-term cultures, the medium was changed twice a week.

**Treatment with VLDL and chase after treatment.** After active replication of M. bovis BCG for 6 days, infected macrophages were exposed to Very Low Density Lipoprotein (VLDL), for 24 h. The volume of VLDL was adjusted so as to expose cells (1 x 10^6 per dish) to 180 µg TAG per mL of medium. In some instances, infected cells were exposed to VLDL simultaneously with either tetrahydrolipstatin (THL also named Orlistat, 60 µg/mL in ethanol) or MmPPOX (15 µg/mL in ethanol) for 24 h (23). Cells were also exposed to VLDL for 24 h, washed and incubated in a VLDL-free fresh medium with or without THL or MmPPOX for 24 h.

**Processing for conventional electron microscopy (EM).** Cells were fixed for 1 h at room temperature with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 0.1 M sucrose, 5 mM CaCl₂, and 5 mM MgCl₂, washed with complete
cacodylate buffer, and post-fixed for 1 h at room temperature with 1% osmium tetroxide in the same buffer devoid of sucrose. They were washed with buffer, scraped off the dishes, concentrated in 2% agar in cacodylate buffer, and treated for 1 h at room temperature with 1% uranyl acetate in 30% methanol. Samples were dehydrated in a graded series of ethanol solutions and embedded in Spurr resin. Thin sections (70 nm thick) were stained with 1% uranyl acetate in distilled water and then with lead citrate.

Quantification and statistical analysis. At the time points indicated in the figures, 150 to 300 intraphagosomal mycobacteria per sample were examined under an EM to score the percentage of each category of *M. bovis* BCG ILI profiles. The cells were examined at random, and care was taken to avoid serial sections. Histograms represent the mean ± standard deviation of at least three independent counts. Statistical analyses were performed using GraphPad Prism 4.03 and differences were considered statistically significant when *P*-values were ≤ 0.05 using two tailed Student’s *t*-tests.
RESULTS

ILI formation depends on lipid processing by macrophage and mycobacterial lipases. Previously, we have shown the dependence of ILI formation on the presence of LB in the host, confirmed the finding that host TAG provided the bulk of lipid for ILI formation and demonstrated that VLDL-derived TAG must first be processed by host lysosomal lipases (7). The requirement of host lipases for the release of fatty acids had been tested by exposing *M. avium*-infected macrophages for 24 h to VLDL in the presence of the anti-obesity drug, tetrahydrolipstatin (THL or Orlistat®). THL inhibits human digestive lipases within the gastro-intestinal tract (45) as well as a variety of other serine-hydrolases (46, 47), including mycobacterial LipY, a major enzyme responsible for hydrolysis of ILI-contained TAG (21).

In the present work, we applied the above procedure to cells infected with wild-type *M. bovis* BCG. As described in our previous study (7), endocytosis of VLDL and transfer to lysosomes was not affected by THL but TAG and derivatives in VLDL were not degraded as usual. These lipids were easily recognized by their electron-transparency and were seen to fill the entire lumen of most lysosomes, leaving only small spaces with the usual electron-dense lysosomal contents (Fig. 1A). This was not observed when infected cells were exposed to VLDL only, in which case lysosomes keep their normal electron-dense appearance (Fig. 1B). In the presence of THL, very few, if any, LB were observed with an average value of approximately 0.32 LB per cell (Supplemental Material, Fig. S1). As expected, with this extremely low level of LB (approximately 23 times less than the untreated cells), mycobacterial profiles with large ILI filling most of the mycobacterial cytoplasm (defined as ILI+3 in (7)) were infrequent (Fig. 1C) as opposed to when LB were formed in the presence of VLDL only (Fig 1D). A quantitative evaluation (Fig. 1G) showed that the amount of ILI+3 and ILI+2 profiles remained low, less than 1%
and 23% ± 1.1, respectively, in presence of THL vs 25% ± 7.4 and 45% ± 2, respectively, in the absence of this lipase inhibitor. Consequently, the amount of ILI(+/−) profiles was 2.5 fold higher in presence of THL (with: 78% ± 9.6; without: 31% ± 0.5).

These results suggest that mycobacterial lipases were unable to process TAG in the VLDL core directly and that VLDL-derived TAG first had to be processed by lysosomal lipases in order to hydrolyze TAG into diacylglycerides (DAG) and monoacylglycerides (MAG) and FFA for subsequent re-processing into TAG and accumulation in LB. Because no LB were formed, it was not possible to gain information on the role of mycobacterial lipases on host TAG hydrolysis. However, THL has also been reported as a non-specific inhibitor of a wide range of serine-hydrolases from both mammals and bacterial species (13, 19, 48, 49) and, therefore one cannot exclude that mycobacterial enzymes are also inhibited by THL, thereby impacting ILI formation.

We then resorted to the oxadiazolone inhibitor, MmPPOX, known to inhibit a variety of mycobacterial serine-hydrolases including those of the hormone-sensitive lipase (HSL) family (50), and more specifically LipY (18, 23). After exposure of M. bovis BCG-infected cells to VLDL in the presence of MmPPOX, host lysosomes were not affected in the same way as during THL treatment. Here, most lysosomes retained their usual electron-dense appearance. Less than 25% of the lysosomes contained small lipid droplets (Fig. 1E) and only 10% were filled with TAG and derivatives as in the case of macrophages exposed to VLDL with THL. Notably, with MmPPOX the relative number of LB per cell was 6 to 7 times higher than with a THL treatment (Supplemental Material, Fig. S1), with an average value of 2.1 LB per cell (thus leading to a 3.5-fold reduction in LB content, in comparison with an untreated sample). These data suggest that host lysosomal lipases were poorly affected by MmPPOX. As a result, VLDL-derived TAG
could be broken down into di- and mono-glycerides and fatty acids for re-processing into TAG and accumulation in LB.

Strikingly, during MmPPOX treatment mycobacterial profiles with large ILI were not observed as demonstrated (Fig. 1F). A quantitative evaluation of mycobacterial profiles containing the different categories of ILI showed that those with large (ILI+3) and medium-sized (ILI+2) ILI reached less than 5% and 20%, respectively, of the amount generated by cell exposure to VLDL in absence of MmPPOX (Fig. 1G). These data suggest that the cell-surface exposed mycobacterial lipases involved in hydrolysis of host TAG delivered to wild-type M. bovis BCG-containing phagosomes are involved in ILI formation and that MmPPOX appears to inhibit the mycobacterial lipases more efficiently than THL. By comparing the LB/ILI relative ratio from the two treated samples, a clear difference in the effects of the two inhibitors was observed where LB/ILI+3 and LB/ILI+2 ratios were 9.8 and 12.4 times higher, respectively, in MmPPOX than in THL treated samples (Supplemental Material, Fig. S1).

Altogether, these data further support that in our system, VLDL has to be processed by host lipases in order to newly synthesize host LB, and that these neutral lipid-rich organelles have to be delivered within mycobacteria-containing phagosomes for subsequent hydrolysis into free fatty acids, which will allow ILI formation. However, these experiments do not allow to identify specific mycobacterial lipase(s) involved in this process, although the LipY form associated with the mycobacterial surface remains the most likely candidate.

**Biochemical characterization of rLipY and rLipY(ΔPE).** The LipY hydrolase from M. tuberculosis has a dual location, firstly in the mycobacterial cytosol where it has direct access to ILI for subsequent lipid hydrolysis (21, 22) and, second, at the mycobacterial
cell surface in a truncated/mature form following removal of its N-terminal PE domain (18, 21, 22, 51). This dual localization has been explained by Daleke et al. (18) who demonstrated that the PE domain of LipY is essential for secretion by ESX-5 (18, 25, 26).

It is not known, however, whether this secretion also requires additional partners such as PPE proteins, which are known to form pairs with PE proteins. Mishra et al. proposed that the PE domain not only plays a role in the recognition by ESX-5 for secretion of LipY but also contributes to the enzymatic activity of the protein (22). Overexpression of LipY(ΔPE), a LipY version lacking its N-terminal PE domain, in M. smegmatis was associated with increased activity as compared to the strain overexpressing the full-length protein (22). Nevertheless, whether lack of the PE domain directly affects LipY activity or whether the increased activity of LipY(ΔPE) results from an indirect effect, presumably due to the absence of a possible partner, remains unknown. To address this issue, we compared the enzymatic activity of purified LipY and LipY(ΔPE) recombinant proteins.

Because the E. coli expression system failed to express large amounts of LipY in an active form, we opted for M. smegmatis mc²155 groEL1ΔC as a surrogate host which allowed producing around 30 mg of pure and active recombinant LipY from a 400 mL culture volume (Fig. 2A). Regarding rLipY(ΔPE), 9 mg of pure (Fig. 2A) and active enzyme were obtained from equivalent cultures, thus indicating that the PE domain is not needed for proper folding of the protein. This was confirmed by further biochemical analyses and circular dichroism spectra determination of both recombinant enzymes (data not shown). The lower rLipY(ΔPE) yield could rely on the absence of the PE domain that exposes the lipid binding site of LipY, promoting a higher degree of interactions between molecules and excessive protein aggregation.

The specific activity of rLipY and rLipY(ΔPE) was determined using a range of lipid substrates differing in their chain lengths and by combining titrimetric and
spectrophotometric techniques. Regardless of the lipid substrates used, rLipY(ΔPE) exhibited higher specific activities (1.2 to 2.5 times depending on the substrate) than rLipY (Table 1). Both proteins were preferentially active on short-chain emulsified MAG and TAG, i.e., monobutyrin (61.0 ± 2.0 and 92.0 ± 5.0 U/mg, respectively) and tributyrin (129.0 ± 6.0 and 267.0 ± 24.0 U/mg, respectively) and displayed the same chain length specificity since their respective specific activities decreased gradually as the substrate chain length increased (Table 1). These results unambiguously show that the lipase activity of LipY is significantly enhanced in the absence of its PE domain, consistent with previous findings (22). Moreover, LipY has a nonspecific lipase activity able to hydrolyse DAG and MAG as well as TAG. Therefore, LipY has the potential to degrade total host TAG converting them into glycerol and free fatty acids, products that can subsequently be directly absorbed by the bacteria.

The N-terminus contains 4 α-helices (α1 to α4), all lacking in LipY(ΔPE) (Fig. 2B). Molecular modelling was performed allowing us to propose the overall structures of both the full-length and the truncated LipY proteins (Fig. 2C) and to address whether these secondary structures may alter either the optimal recognition and/or binding of the substrates to LipY. The α1-α4 helices of the PE domain are connected to a linker unit composed of three helices (α5-α7), presumably providing a high degree of flexibility to the PE and core (C-terminal) domains. The latter, comprising the active site of LipY belongs to the α/β hydrolase fold family and is composed of a central β-sheet (β1-β8 strands) surrounded by nine α-helices. Similar to most lipases, the catalytic serine (Ser309) is located in a nucleophile elbow between the β5 strand and the α13 helix. The presence of the PE domain (Fig. 2C) clearly reduces the accessibility of the active site to the substrate. Removal of the PE domain allows the linker portion (green) to move to the side of the catalytic domain, thus opening access to the active site. This large opening
presumably allows a better accommodation of the lipid substrates into the active site, thereby contributing to the increased enzymatic activity of LipY(ΔPE).

**Distribution of ILI profiles in *M. bovis* BCG strains over-expressing various LipY variants in VLDL-driven FM.** To investigate the functional contribution of the PE domain of LipY in host TAG hydrolysis and consequently ILI formation, macrophages were infected with various *M. bovis* BCG strains for 6 days, before being exposed to VLDL for 24 h to induce FM formation (7). The strains used in this experiment were the wild-type BCG strain (WT), which is characterized by the native expression of LipY and was used as reference, and three other recombinant BCG strains harboring distinct pMV261-derived constructs, leading to the overproduction of either LipY, LipY(ΔPE) or LipY(ΔPE) in which Ser309 was replaced by an Ala residue (LipY(ΔPE)$^{S309A}$) yielding a catalytically-inactive protein (22). Cells were then fixed and processed for EM and profiles of intracellular mycobacteria were examined for the presence and extent of ILI formation in both VLDL-treated and untreated macrophages. As before (7), intracellular mycobacteria were divided into 4 categories according to their ILI size (Figs. 3, A-D). ILI$^1$ profiles with no ILI (Fig. 3A), ILI$^{+1}$ for profiles displaying a few small ILI 0.1 µm in width at most (Fig. 3B), ILI$^{+2}$ for profiles displaying several ILI approximately 0.2 to 0.3 µm in width (Fig. 3C), and ILI$^{+3}$ for profiles displaying several ILI approximately 0.4 to 0.5 µm in width and occupying most of the mycobacterial cytoplasm (Fig. 3D). The relative abundance of each type of ILI profile was scored on approximately 200 different mycobacterial profiles per sample (Fig. 3E). As expected, in the absence of exposure to VLDL, 95% of the *M. bovis* BCG profiles were ILI$^{(+1/-)}$, regardless of the strain (data not shown). In contrast, after a 24 h exposure to VLDL, ILI$^{+2}$ and ILI$^{+3}$ were predominantly present among the different *M. bovis* BCG strains (Fig. 3E) as already found with *M. avium* (7). Further examination of
the quantitative data showed that both the WT and the LipY-overexpressing strain displayed similar amounts of ILI profiles of each category with ILI[^3]: 27% ± 4.8 and 25% ± 4.1; ILI[^2]: 43% ± 3 and 40% ± 4.1; ILI[^+1/-]: 29% ± 3.9 and 35% ± 6.8, respectively.

Likewise, and as expected, the relative abundance of the 4 types of ILI profiles in the strain over-expressing the catalytically-inactive form LipY(ΔPE)[S309A] was similar to the ones scored in the WT strain and the strain overexpressing LipY (Fig. 3E). In sharp contrast with these strains, overexpression of LipY(ΔPE) resulted in a substantial drop in the percentage of ILI[^3] (15% ± 4.4 vs. 27% ± 4.8) and a concomitant increase in ILI[^+1/-] profiles (50% ± 7.5 vs. 29% ± 3.9), as compared to the WT strain. Determination of the ILI[^3]/ILI[^+1/-] ratio profiles showed that overexpression of LipY(ΔPE) triggers important changes in the intracellular pool of ILI in comparison with the full-length LipY or the inactive form LipY(ΔPE)[S309A] (Fig. 3E). These data are fully consistent with the increased *in vitro* activity of rLipY(ΔPE) over rLipY (Table 1).

**Additional mycobacterial lipases contribute to host TAG hydrolysis and ILI formation.** Despite the primary role of LipY in host lipid hydrolysis, it is very likely that additional mycobacterial lipases participate in this process, a hypothesis emphasized by the fact that the genome of *M. tuberculosis* encodes for at least 24 putative lipases (19, 20, 23, 32, 52). To test this hypothesis, macrophages were infected with a *lipY*-deleted *M. bovis* BCG mutant before being exposed to VLDL for 24 h. The cells were then fixed and processed for EM and profiles of intracellular mycobacteria were examined for the presence and extent of ILI formation in both the WT and mutant strains. The profiles of the mutant clearly displayed smaller ILI than those of the WT strain (Fig. 4A vs 4C). The relative abundance of each category of ILI was then scored on approximately 150 different mycobacterial profiles per sample (Fig. 4E). Although all categories of ILI were
found in the profiles of the ΔlipY mutant, the percentage of ILI+3 profiles was reduced by approximately 50% (16% ± 5.8 vs. 35% ± 2.9) with a concomitant increase in the percentage of ILI+1/ (46% ± 10.9 vs. 27% ± 5.5) with respect to the WT strain. Functional complementation of the ΔlipY mutant was performed with pMV261::lipY, which allows constitutive expression of the full-length lipY leading to the strain ΔlipY::Comp. Production of the protein was confirmed by immunoblotting using polyclonal antibody directed against the M. tuberculosis LipY protein (Fig. 4D). As mentioned earlier (18, 22), LipY is not produced by wild-type M. bovis BCG in vitro under standard growth conditions explaining the lack of a reactive band in the corresponding crude lysate. In contrast, a specific immunoreactive band was detected in ΔlipY::Comp, indicating that LipY is constitutively produced in this strain. Thus, ΔlipY::Comp was used to infect macrophages for six days, before being exposed to VLDL for 24 h. Analysis of the ILI profile indicates that complementation restores the WT phenotype, characterized by 39% ± 5.3 and 23% ± 4.2 for ILI3+ and ILI+1/3, respectively (Fig. 4B and 4E). Likewise, the ILI3+/ILI+1/3 ratio was severely impacted in ΔlipY and was restored upon functional complementation of the mutant, clearly confirming the impact of the lipY deletion on intrabacterial lipid accumulation (Fig 4F).

Taken collectively, these results reveal the major contribution of LipY in the breakdown of host-derived TAG within the phagosomal lumen and highlight the role of additional mycobacterial lipases in hydrolysis of host TAG and accumulation of TAG in the form of large ILI.

Consumption of TAG within ILI correlates with the reduction of LB and the activity of cytosolic mycobacterial lipases. To dissect the physiological link between LB in FM and ILI in mycobacteria, we had previously investigated whether the formation of large
ILI (ILI\(^{+3}\)) could be reversed by removal of VLDL (7). After exposure of infected macrophages to VLDL for 24 h, the infected cells had been washed and re-incubated in fresh medium without lipoprotein. Within 24 h after removal of VLDL, infected cells had lost their LB and ILI\(^{+3}\) were no longer visible in the mycobacterial profiles (7), thus demonstrating the direct link between the presence/absence of LB and ILI formation/consumption. The same method applied to *M. bovis* BCG-infected cells and the present work yielded comparable results (Fig. 5D).

To determine whether mycobacterial lipases were involved in ILI consumption, we applied the same chase strategy, but in the absence or presence of lipase inhibitors. After exposure of WT *M. bovis* BCG-infected cells to VLDL for 24 h to allow ILI formation, the cells were washed and re-incubated in fresh medium without lipoprotein but in the absence or presence of either THL or *MmpPPOX* for an additional 24 h. The morphological appearance of the *M. bovis* BCG profiles was observed under the EM (Fig 5). In the absence of a lipase inhibitor, ILI\(^{+3}\) profiles were seldom encountered (Fig. 5A). In contrast, many ILI\(^{+3}\) profiles were encountered during a chase in presence of either THL (Fig. 5B) or *MmpPPOX* (Fig. 5C). Scoring of the relative amount of each category of ILI profiles in each condition showed, as before (7), that the percentage of ILI\(^{+3}\) profiles was reduced by 90% when cells were chased in medium without inhibitors (Fig. 5D). In contrast, when the chase medium contained either THL or *MmpPPOX* approximately 80 to 90% of the ILI\(^{+3}\) profiles were retained (Fig. 5D). The fact that at 24 h the relative abundance of ILI\(^{+3}\) was 5 times higher in the presence of THL or *MmpPPOX* (21% ± 0.6 and 23% ± 0.8, respectively) than in the absence of lipase inhibitors (3.6% ± 0.8) indicates that ILI consumption is indeed dependent on mycobacterial lipase activity. These results are in agreement with previous studies showing that hydrolysis of mycobacterial ILI is
associated with the decrease of LB in the host (7) and the presence of a (several) mycobacterial lipase(s) (13, 32), and more particularly LipY (11, 21, 22).
DISCUSSION

In previous work, VLDL was used as a source of lipids to generate FM (7) since it is well known that such cells play a major role in the persistence and reactivation phases of TB within granulomas (5, 6, 11, 53). Quantitative analyses of detailed EM observations performed after exposure of *M. avium*-infected cells to VLDL had shown that macrophages became foamy and mycobacteria formed large ILI for which host TAG was essential. Lipid transfer occurred *via* mycobacterium-induced fusion between LB and phagosomes. This experimental tool clearly constitutes a well-defined cellular system in which to study changed metabolic states of intracellular mycobacteria that may relate to persistence and reactivation of TB. As a follow-up to this proof of concept, this FM model was used here to demonstrate the extensive accumulation of ILI inside *M. bovis* BCG and to investigate in detail the role of host lipases in LB formation, without which there can be no transfer of host TAG to mycobacterium-containing phagosomes, as well as the physiological role of the mycobacterial lipase, LipY, in the degradation of TAG inside mycobacterium-containing phagosomes and mycobacterial ILI. By a variety of approaches, we analyzed the contribution of macrophage and bacterial lipases in LB and ILI formation/degradation, and propose the following model depicted in Fig. 6.

After its internalization by receptor-mediated endocytosis, VLDL is transferred to lysosomes where host lysosomal lipases hydrolyze TAG into its derivatives and free fatty acid that serves to form LB. In a first set of experiments, we used two different families of lipase inhibitors, THL and *MmpPPOX*, which, surprisingly, act by targeting different types of lipases within cells. On the one hand, by exposing infected cells to VLDL in the presence of THL, this lipase inhibitor shows preferences for host lipases, since TAG hydrolysis in lysosomes was blocked. Macrophages were unable to become foamy and, as a result, *M. bovis* BCG was unable to form ILI. On the other hand, exposure of cells to
VLDL in presence of MmPPOX, blocked weakly the host lipases in the lysosomes and consequently did not profoundly affect the formation of LB, but mycobacteria still remained unable to form ILI. Our results suggest that the effect of this inhibitor is more specifically directed against mycobacterial lipases. The fact that these two families of inhibitors target lipases located at different sites could be directly related to their chemical structures and biophysical properties. In contrast to MmPPOX, THL is indeed a highly lipophilic molecule with physico-chemical properties similar to those of a diacylglycerol molecule which makes it soluble in VLDL. By using these two different families of lipase inhibitors, we can study either the host lipases or the mycobacterial lipases. We, therefore, provide strong evidence that ILI formation i) involves both host lysosomal and mycobacterial lipases, and ii) is strictly dependent on host LB in our experimental model. In Dictyostelium discoideum, Mycobacterium marinum, was found to produce ILI without using TAG as the major carbon source (54, 55). Indeed, the use of a Dictyostelium knockout mutant for both Dgat enzymes (dgat1 and dgat2 genes), which is unable to produce LB, allowed the authors to demonstrate that a resulting excess of free fatty acids is predominantly incorporated into phospholipids triggering a massive ER-membrane proliferation. Fluorescent and EM approaches coupled with TLC lipid analyses suggested that M. marinum uses phospholipids to build-up ILI. This observation leads to a new hypothesis that intracellular mycobacteria have access to a wide range of host lipids, and one cannot exclude the possibility that bacteria in FM may have direct access to free fatty acids under conditions other than the experimental conditions of our FM model. Although this hypothesis has to be fully addressed experimentally in future studies, the current knowledge about ILI formation in mycobacteria within FM seems to predominantly rely on host-TAG degradation.
Among the large number of lipolytic enzymes encoded by the *M. tuberculosis* genome, LipY remains the only one carrying an N-terminal PE domain, which was subsequently found to be a signal of recognition by the ESX-5 secretion system (18, 26). Herein, we investigated whether the PE domain may directly participate in the enzyme’s TAG activity. Our results are in agreement with a previous study demonstrating that LipY is the enzyme with the highest potential for hydrolyzing TAG stored inside *M. tuberculosis* (21). The authors extend our earlier report suggesting that the PE domain plays a role in modulating the catalytic activity of LipY (22). Comparison of the catalytic activities of rLipY and rLipY(ΔPE) clearly indicates that LipY(ΔPE) is more active than LipY, regardless of the substrate used. Similar results came from another group who used only one synthetic non physiological substrate (28). Our data, therefore, add new insights into the functional role of the PE domain, which is shared by a large number of proteins in pathogenic mycobacteria. Interestingly, *Mycobacterium marinum* contains a protein homologous to LipY, termed LipY\textsubscript{mar}, in which the PE domain is substituted by a PPE domain (22). As for LipY, overexpression of LipY\textsubscript{mar} in *M. smegmatis* significantly reduced the TAG pool, and this was further pronounced when the PPE domain of LipY\textsubscript{mar} was removed, suggesting that PE and PPE domains can share a similar functional role. Therefore, given the analogy between LipY and LipY\textsubscript{mar}, it is conceivable to speculate that the PPE domain, like the PE domain, directly modulates the activity of LipY\textsubscript{mar} and that the lower catalytic activity of LipY\textsubscript{mar} may result from steric hindrance of the PPE domain, altering the recognition/accommodation of TAG within the lipid-binding site. Since, like *M. tuberculosis* or *M. bovis* BCG, *M. marinum* also produces ILI during infection (54) (and unpublished data), it is tempting to speculate that LipY\textsubscript{mar} plays a crucial role in ILI formation during *M. marinum* infection.
To further delineate the role of the PE domain in modulating the activity of LipY in an infectious context, experiments were carried out by extending our original experimental model of FM (7). BMDM were infected with *M. bovis* BCG strains overexpressing either LipY or LipY(ΔPE) and exposed to VLDL as a lipid source. Quantitative analyses of EM observations allowed to perform a detailed investigation on the role of the mycobacterial lipase, LipY, in the degradation of host TAG transferred to the mycobacterium-containing phagosomes. Importantly, the difference in *in vitro* activity of LipY and LipY(ΔPE) could be reconciled in this model of infection where a significant reduction of the ILI+3 category was found with the BCG strain overexpressing LipY(ΔPE). This is consistent with the fact that, in contrast to LipY which is secreted, LipY(ΔPE) fails to be transported by ESX-5 and, as a consequence, its accumulation inside mycobacteria boosts ILI degradation (22, 25).

In a previous study (7), we had shown that removal of VLDL, induced a rapid decline of both LB and ILI. Here, we show that exposure of infected cells to lipase inhibitors, added during the chase period following an exposure to VLDL, strongly affects TAG hydrolysis within ILI, which remain abundant and large. These results provide evidence that cytosolic LipY is also involved in ILI consumption.

Our data also indicate that the dual localization of LipY impacts on its activity. Since the PE-containing domain is cleaved off by the ESX-5 secretion system (18), our results suggest that the mycobacterial surface-anchored form of LipY is more active than the intracytosolic full-length protein. From these results, it can be inferred that LipY is more active against host lipids targeted to phagosomes via LB-phagosome fusion than towards TAG stored within ILI. Indeed, as illustrated in (Fig. 6), while a fraction of LipY is found in the cytosol, consistent with its role in the catabolism of intracellular TAG in ILI, a significant fraction of mature LipY lacking its PE domain is also localized at the outer
of the mycobacterial cell envelope, where it catalyzes the breakdown of exogenously available TAG, such as those found within the LB of the FM. This scenario is consistent with the idea that *M. tuberculosis* depends on fatty acids as a preferred energy source during infection (56) where LipY represents a critical enzyme for the utilization of host lipids. However, the ILI+3 profile in the mutant ΔlipY, clearly indicates the active participation of other mycobacterial lipases in this process, a finding consistent with the presence of a large array of lipolytic-encoding genes found in pathogenic mycobacteria (57). Among these, Rv0183 has been shown to exhibit a preference for MAG or DAG and to be localized to the cell wall (19, 58), whereas Cfp21, a secreted cutinase-like enzyme also expresses TAG lipase activity (20, 52). These two proteins may represent putative candidates which, in addition to LipY, may participate in the breakdown of host TAG transferred to phagosomes, and the release of free fatty acids which may be utilized by multiple TAG synthases (59) to carry out the synthesis of TAG in the mycobacterial cytosol (Fig. 6). Utilization of these TAG in ILI is essential for the regrowth of mycobacteria during their exit from hypoxic non-replicating conditions (32) and reactivation of latent infection to cause an active disease.
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**FIGURE LEGENDS**

**Figure 1:** Addition of lipase inhibitors during exposure to VLDL affects host LB formation and accumulation of mycobacterial lipids in the form of ILI. At day 6 p.i. with WT *M. bovis* BCG, BMDM were exposed for 24 h to VLDL in the absence or presence of lipase inhibitors. The cells were then processed for EM and analyzed for host LB and mycobacterial ILI formation. *(A, B, E)* Morphological appearance of host LB and lysosomes (Ly): *(A)* exposure to VLDL and THL: LB are scarce and Ly are filled with electron-translucent lipids (Ly⁺); *(B)* exposure to VLDL only: cells contain many LB and Ly retain their normal appearance, with dense contents only; *(E)* exposure to VLDL and *MmPPOX*: LB are present and most Ly either retain their normal appearance or contain low amounts of lipids in the form of lipid droplets (Ly⁺/−). *(C, D, F)* Morphological appearance of ILI within mycobacterial profiles: *(C)* exposure to VLDL and THL: no ILI⁺³ mycobacterial profiles; *(D)* Exposure to VLDL only: many mycobacterial profiles are ILI⁺³; *(F)* Exposure to VLDL and *MmPPOX*: ILI remained small and no ILI⁺³ mycobacterial profiles were observed. Bars in panels A to F, 0.5 µm. *(G)* Dependence of steady-state levels of ILI⁺³, ILI⁺² and ILI⁺¹/² profiles on the absence or presence of lipase inhibitors. Error bars indicate the standard deviations (SD) based on the results of 2 to 4 independent experiments. For each experiment, 150 to 300 profiles were examined for each treatment. Statistical analysis was performed by using two tailed Student’s *t*-test. Results were compared to an untreated sample and ** correspond to a *P* value <0.01 and *** correspond to a *P* value <0.001.

**Figure 2:** Purification and structural modelling of LipY and LipY(ΔPE). *(A)* Protein purity assessed on SDS-PAGE. Six µg of protein were loaded onto a 12% polyacrylamide
gel and stained with Instant Blue solution. Lane 1, rLipY; lane 2, rLipY(ΔPE). MW, Molecular Weight standards (10 µg, Euromedex). (B) Alignment of the LipY and LipY(ΔPE) amino acid sequences. The secondary structures identified from the corresponding three-dimensional models are indicated above the sequences using the same color codes as those used in the structural models depicted in C: PE domain in blue, linker unit in green and catalytic domain in pink. Black stars below the sequence indicate the catalytic triad. (C) Overall view of the structural models of LipY and LipY(ΔPE). On the left part, the LipY and LipY(ΔPE) 3D-models were generated by I-TASSER software. The PE domain is represented in blue and in both cases, the catalytic triad composed of serine (Ser309), aspartic acid (Asp383) and histidine (His414) residues are also indicated. The green part represents the polypeptide linking the catalytic domain to the PE domain. The black arrow shows the displacement of the linker after deletion of the PE domain, leading to a large opening of the active site and allowing a better accommodation of the substrates.

Figure 3: Distribution of ILI profiles in *M. bovis* BCG strains over-expressing various LipY variants in VLDL-driven FM. BMDM were infected with BCG strains over-expressing different LipY variants. After exposure to VLDL for 24 h, cells were fixed, and processed for EM. Bacterial profiles were divided into 4 different categories in terms of the presence and size of ILI (ILI−, ILI+1, ILI+2, ILI+3). (A) ILI− is no ILI. (B) ILI+1 is less than 5 small ILI up to 0.1 µm in width. (C) ILI+2 is several ILI 0.2 to 0.3 µm in width. (D) ILI+3 is several ILI 0.4 to 0.5 µm in width and extending across the full width of the *M. bovis* BCG cytosol. Bars in panels A to D, 0.5 µm. (E) Comparative analysis of the percentage of each category of ILI profiles in wild-type (WT) vs. *M. bovis* BCG strains over-expressing different LipY variants (LipY, LipY(ΔPE), LipY(ΔPE)S309A). Error bars indicate the standard deviations based on the results of 2 to 4 independent experiments. For each experiment,
100 to 250 profiles were examined for each strain. Statistical analysis was performed by using two tailed Student’s t-test. Results were compared to the WT strain and * correspond to a P value <0.05 and ** correspond to a P value <0.01. **(F) Comparative analysis of ILI$^{3+/+}$/ILI$^{1+/1-}$ ratios in WT and LipY overexpressing strains. Ratios were calculated by dividing the percentage of ILI$^{3+}$ with the percentage of ILI$^{1+/1-}$ bacteria collected between 2 to 4 independent experiments. Error bars indicate the standard deviations and statistical analysis was performed by using two tailed Student’s t-test where * correspond to a P value <0.05 and ** correspond to a P value <0.01.

**Figure 4:** LipY and additional mycobacterial lipases contribute to host TAG hydrolysis and ILI formation. At day 6 p.i. with either WT *M. bovis* BCG, ΔlipY or ΔlipY complemented (ΔlipY::Comp) strains, BMDM were exposed for 24 h to VLDL. The cells were then processed for EM and analysed for mycobacterial ILI formation. (A) BMDM infected with the ΔlipY strain: most of the ILI profiles were small, (B) BMDM infected with the ΔlipY::Comp strain: numerous large ILI were observed, (C) BMDM infected with WT *M. bovis* BCG: ILI$^{+3}$ profiles were abundant. Bars in panels A and C, 0.5 µm and B 1 µm, (D) Western blot analysis of WT, ΔlipY or ΔlipY::Comp strains. Equal amounts of lysates were immunoblotted and the full-length LipY was detected using a polyclonal antibody. GroEL2 was included a as loading control. (E) Comparative evaluation of the percentage of each category of ILI profiles formed in either the WT or the ΔlipY strain. Error bars indicate the standard deviations based on the results of 2 to 4 independent experiments. For each experiment, 150 to 300 profiles were examined for each treatment. Statistical analysis was performed with the two-tailed Student’s t-test. Results were compared to the WT strain and * correspond to a P value <0.05. **(F) Comparative analysis of ILI$^{3+/+}$/ILI$^{1+/1-}$ ratio of WT, ΔlipY or ΔlipY::Comp strains. Ratios were calculated by dividing the percentage of ILI$^{3+}$ with the percentage of ILI$^{1+/1-}$ bacteria collected between 2 to 4
independent experiments. Error bars indicate the standard deviations (SD) and statistical analysis was performed with the two tailed Student’s $t$-test where * correspond to a $P$ value <0.05.

**Figure 5: Consumption of TAG within ILI correlates with the activity of cytosolic mycobacterial lipases.** At day 6 p.i. with WT *M. bovis* BCG, BMDM were exposed to VLDL for 24 h and then re-incubated in VLDL-free culture medium without or with THL or *Mm*PPOX for 24 h. The cells were then processed for EM and the percentage of each category of ILI profiles was assessed. (A) VLDL for 24 h followed by a 24 h-chase in medium devoid of inhibitors. Cells contain few ILI$^+3$ profiles. (B) VLDL for 24 h followed by a 24 h-chase in medium with THL. Cells still contain ILI$^+3$ profiles. (C) VLDL for 24 h followed by a 24 h-chase in medium with *Mm*PPOX. Cells still contain ILI$^+3$ profiles. Bars in panels A and B, 0.5 µm and C 0.7 µm (D) Quantitative evaluation of the percentage of each category of ILI profiles immediately after a 24 h exposure to VLDL (no I, 0 h) or after a 24 h chase in culture medium without inhibitor (no I, 24 h), with THL (THL, 24 h) or with *Mm*PPOX (*Mm*PPOX, 24 h). Error bars indicate the standard deviations based on the results of 2 to 3 independent experiments. For each experiment, 150 to 300 profiles were examined for each treatment. Statistical analysis was performed by using two tailed Student’s $t$-test. Results without inhibitors were compared to the “No I 0h” condition where # correspond to a $P$ value <0.05 and ## correspond to a $P$ value <0.01. Whereas results with the inhibitors where compared to the “No I 24 h” condition where * correspond to a $P$ value <0.05 and ** correspond to a $P$ value <0.01

**Figure 6: Proposed model for LipY in vivo activity as a function of its localization.** The PE domain of LipY functions as a secretion signal recognized by the ESX-5 secretion
system. (1): Endocytic uptake of VLDL composed of phospholipids (P), triglycerides (T) and cholesterol (C) and transfer to lysosomes (Ly). (2): Hydrolysis of TAG in VLDL and formation of TAG-rich host LB after release of free fatty acids (FA) from Ly. (3): Fusion of LB with the membrane of mycobacterium-containing phagosomes. Host TAG is released into the phagosomal lumen during this process. (4): During its translocation across the mycobacterial cell envelope via ESX-5, the LipY N-terminal PE domain is cleaved off. After secretion into the phagosome, LipY(ΔPE) remains closely associated with the mycobacterial outermost surface, where it hydrolyses host TAG. At this stage, other mycobacterial lipases are likely to be involved in the degradation of the LipY end-product, releasing free fatty acid (FA), which are then transported to the mycobacterial cytosol where TAG are resynthesized by the TAG synthases (TGS). TAG then accumulate to form intracytosolic lipid inclusions (ILI). (5): Under specific conditions, TAG within ILI can be hydrolyzed by cytosolic LipY.
Table 1: Specific activities of rLipY and rLipY(ΔPE) on various lipid substrates\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>rLipY</th>
<th>rLipY(ΔPE)</th>
<th>rLipY(ΔPE)/rLipY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monobutyrin\textsuperscript{(b)} (MC4)</td>
<td>61.0 ± 2.0</td>
<td>92.0 ± 5.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Monolein\textsuperscript{(a)} (MC18)</td>
<td>6.6 ± 0.5</td>
<td>17.0 ± 1.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Diolein\textsuperscript{(a)} (DC18)</td>
<td>3.5 ± 0.2</td>
<td>5.6 ± 0.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Tributyrin\textsuperscript{(b)} (TC4)</td>
<td>129.0 ± 6.0</td>
<td>267.0 ± 24.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Trioctanoin\textsuperscript{(a)} (TC8)</td>
<td>20.0 ± 2.0</td>
<td>38.0 ± 1.0</td>
<td>1.9</td>
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<tr>
<td>Pomegranate oil TAG</td>
<td>4.0 ± 0.2</td>
<td>4.8 ± 0.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All substrates were assayed at concentrations above their solubility limits. Experiments were performed at 37°C in 15 mL of 2.5 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl and 1\textsuperscript{(a)} or 3\textsuperscript{(b)} mM NaTDC. * One unit corresponds to 1 µmole of fatty acid released per min. Values are means of 3 independent experiments ± SD.
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Figure 3

A. ILI⁻¹
B. ILI⁺¹
C. ILI⁺²
D. ILI⁺³

E. ILI profiles (%)
F. ILI⁻¹/ILI⁺¹ ratio

Graphs showing the distribution and ratio of ILI in different conditions.