Intrabacterial lipid inclusions in mycobacteria: unexpected key players in survival and pathogenesis?

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Intrabacterial lipid inclusions in mycobacteria: unexpected key players in survival and pathogenesis?

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Abbreviations:

Acylglycerol-3-phosphate acyltransferase (AGPAT); bone marrow-derived macrophage (BMDM); diacylglycerol (DAG); diacylglycerol-acyltransferase (DGAT); dormancy survival regulator (Dos); fatty acid transport proteins (FATP); foamy macrophage (FM); free fatty acid (FFA); glycerol-3-phosphate (G3P); glycerol-3-phosphate acyltransferase (GPAT); heparin-binding hemagglutinin adhesin (HBHA); intrabacterial lipid inclusion/Intracytosolic lipid inclusion (ILI); latent TB infection (LTBI); lipid droplet (LD); lysophosphatidic acid (LPA); monoacylglycerol (MAG); *Mycobacterium abscessus* (*Mabs*); *Mycobacterium avium* (*Mav*); *Mycobacterium bovis* BCG (*Mbv* BCG); *Mycobacterium marinum* (*Mmar*); *Mycobacterium smegmatis* (*Msmeg*); *Mycobacterium tuberculosis* (*Mtb*); nitric oxide (NO); non-tuberculous mycobacteria (NTM); oleic acid (OA); peripheral blood mononuclear cells (PBMCs); phosphatidic acid (PA); phosphatidic acid phosphatase (PAP); tetrahydrolipstatin (THL); thin layer chromatography (TLC); triacylglycerol (TAG); triacylglycerol synthase

(TGS); tricarboxylic acid cycle (TCA);tuberculosis (TB); very low density lipoprotein (VLDL); wax ester (WE); wild-type (WT).

Abstract:

Mycobacterial species, including *Mycobacterium tuberculosis*, rely on lipids to survive and chronically persist within their hosts. Upon infection, opportunistic and strict pathogenic mycobacteria exploit metabolic pathways to import and process host-derived free fatty acids, subsequently stored as triacylglycerols under the form of intrabacterial lipid inclusions (ILI). Under nutrient-limiting conditions, ILI constitute a critical source of energy that fuels the carbon requirements and maintain redox homeostasis, promoting bacterial survival for extensive periods of time. In addition to their basic metabolic functions, these organelles display multiple other biological properties, emphasizing their central role in the mycobacterial lifecycle. However, despite of their importance, the dynamics of ILI metabolism and their contribution to mycobacterial adaptation/survival in the context of infection has not been thoroughly documented. Herein, we provide an overview of the historical ILI discoveries, their characterization, and current knowledge regarding the micro-environmental stimuli conveying ILI formation, storage and degradation. We also review new biological systems to monitor the dynamics of ILI metabolism in extra- and intracellular mycobacteria and describe major molecular actors in triacylglycerol biosynthesis, maintenance and breakdown. Finally, emerging concepts regarding to the role of ILI in mycobacterial survival, persistence, reactivation, antibiotic susceptibility and inter-individual transmission are also discuss.

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), remains a global health issue and one of the deadliest disease caused by a single infectious agent (WHO 2020). Upon primary infection by *Mtb*, granulomas constrain the infection by limiting *Mtb* replication and dissemination within the lungs. If for some individuals these dynamic and complex immunological structures may confer a protective effect by clearing the infection, some of them however are not able to fully eradicate the pathogen. Indeed, *Mtb* possesses the remarkable ability to persist within its host for extensive period of time without generating any clinical symptoms, before eventual reactivation that causes the active form of the disease (Ramakrishnan 2012, Russell 2001, Russell 2007). Recent advances have suggested that the current and commonly accepted estimation that 25-30% of the world's population are infected with TB and at risk of developing TB disease might be overestimated highlighting that more evidences are required to better define host clearance and bacterial persistence in the context of TB pathogenesis (Behr, et al. 2019). Therefore, understanding the cellular and molecular bases for *Mtb* clearance, survival, persistence and reactivation within granulomas remains a fundamental challenge, with the ultimate goal of developing new tools and approaches to better control TB.

It is now clearly established that host-derived lipids such as triacylglycerols (TAG), cholesterol, phospholipids and free fatty acids (FFA) are being used for *Mtb* survival *in vivo*, by providing energy inside granulomatous lesions, therefore emphasizing the essential role of central lipid metabolism in mycobacterial long-term survival and persistence (Bloch and Segal 1956, Lee, et al. 2013, McKinney, et al. 2000, Nazarova, et al. 2017). During this specific phase, designated latent TB infection (LTBI), *Mtb* may harbour a wide range of phenotypes which is often dependent on the host-local immune state. However, very few bacilli are able to actively replicate and most of them are assumed to be in a slow/non-replicating state. This phenotypic behaviour is characterized by major and systematic physiological features such as low metabolic activity, bacterial division arrest, loss of acid fastness, increased antibiotics tolerance and presence of large amounts of TAG stored under the form of intracytosolic / intrabacterial lipid inclusions (ILI) (Caire-Brandli, et al. 2014, Daniel, et al. 2016, Daniel, et al. 2011, Garton, et al. 2002, Garton, et al. 2008, Kapoor, et al. 2013, Santucci, et al. 2019a, Vilcheze and Kremer 2017).

TAG are neutral lipids, insoluble in water, comprising of three fatty acids esterified onto a glycerol backbone. They represent prominent energy storage molecules in many living organisms (eukaryotes and prokaryotes) and provide high amounts of ATP when mobilized

through the lipolysis and the β-oxidation pathways. Numerous organisms (including some bacteria, parasites, plants and mammals) are able to synthesize TAG and to store them under the form of cytoplasmic granules (Murphy 2001, Murphy 2012). Over the years, these structures have been designated as lipid droplets (LD), lipid bodies, lipid inclusions, adiposomes, fat bodies or oil bodies (Daniel, et al. 2004, Walther and Farese 2009). In a homeostatic view, these organelles have been ascribed to regulate the storage of neutral lipids, remove toxic lipids, involved in cellular communication including stress response mechanisms as well as inflammatory processes (Monson, et al. 2021). In this review, the term LD refers to as the eukaryotic TAG inclusions while ILI designates the bacterial TAG inclusions. With respect to their chemical composition, TAG granules are essentially composed of neutral lipids (*i.e.*, TAG or sterol esters), surrounded by a phospholipid monolayer in which specific proteins, called lipid droplet-associated proteins, are inserted.

To date, adipocytes, whose functions are mainly dedicated to the storage of neutral lipids in the form of LD within human body, are probably the best characterized cell type harboring TAG inclusions. In bacteria, species belonging to the *Rhodococcus* and *Mycobacterium* genus have been utilized as model systems to study TAG biosynthesis, ILI formation, storage and hydrolysis (Alvarez 2016, Santucci, et al. 2016, Wältermann and Steinbüchel 2005).

Nevertheless, with respect to *Mtb*, how the bacilli specifically realign their metabolism *in vivo*, use and store host-derived lipids under the form of ILI to persist in a non-replicating state and hydrolyse these lipids during reactivation, still remains poorly understood. The presence of such ILI has also been highlighted in several mycobacterial species including non-tuberculous mycobacteria (NTM), suggesting a conserved function of these lipid structures in the lifestyle of non-pathogenic, opportunistic and strict pathogens (Barisch, et al. 2015, Barisch and Soldati 2017, Santucci, et al. 2019a, Viljoen, et al. 2016) (**Figure 1**).

In this review, we summarize the current knowledge and understanding of ILI composition and metabolic actors involved in their formation (synthases) and hydrolysis (lipases) with a special emphasis on mycobacterial species (**Figure 2**). We report how ILI have been discovered and how specific microenvironments govern their biosynthesis/mobilization. A non-exhaustive description of some of the recent approaches developed to study the dynamics of these processes in pathogenic mycobacteria is also presented. These include the development of experimental models, the use of specific classes of pharmacological inhibitors and biochemical and genetic strategies to delineate ILI biology. Finally, the recent

discoveries regarding the physiological roles and impacts of these conserved organelles with respect to mycobacterial survival and pathogenicity are discussed.

Historical discovery of TAG storage and intracytoplasmic bacterial lipid inclusions

In prokaryotes, although ILI have been observed in both Gram^{+} and Gram^{-} bacteria, they remain a common characteristic of the *Actinobacteria* phylum (Alvarez and Steinbüchel 2002). Indeed, many species belonging to the *Streptomyces*, *Nocardia*, *Dietzia*, *Gordonia*, *Rhodococcus* or *Mycobacterium* genus possess the capacity to synthesize and store TAG in the form of ILI (Alvarez 2016, Alvarez and Steinbüchel 2002). In mycobacterial species, ILI were first observed during the 1940s with the pioneering work of Burdon, Sheenan and Whitwell (Burdon 1946, Sheehan and Whitwell 1949). Using Sudan Black B, a non-fluorescent dye specific for neutral lipids (Hartman 1940), these authors demonstrated the presence of ILI within *in vitro* mycobacterial cultures, in samples isolates from infected guinea pigs, and also within sputum from TB patients (Burdon 1946, Sheehan and Whitwell 1949). In the 1950s, the use of electron microscopy enabled, for the first time, imaging single bacterial cell at high-resolution, thereby revealing the presence of globular cytoplasmic structures in *Mycobacterium avium* (*Mav*) as well as in *Mycobacterium leprae* (Brieger and Glauert 1956a, Brieger and Glauert 1956b, Knaysi, et al. 1950). Both studies highlighted the heterogeneity within the population in term of bacterial size, shape and granule content. Interestingly, the authors stated that this type of globules were visible only after long incubation period in a rich culture medium, suggesting a co-relation between bacterial fitness and metabolism with the appearance of the granular structures. The first metabolic studies were carried out later on and demonstrated that supplementation of lipid substrates such as oleic acid (OA) or Tween-80 in the culture medium favoured the appearance of ILI in mycobacterial cells (Schaefer and Lewis 1965). At that time, the exact composition of the accumulated lipids was not established, and there was no mention regarding the exact phase of the bacterial cell cycle wherein these structures were visible (Schaefer and Lewis 1965). In the 1970s, the use of radiolabelled substrates, such as palmitic acid or OA, in *Mav* or *Mycobacterium smegmatis* (*Msmeg*) cultures provided evidence that the FFA incorporated within bacterial cells were mainly stored as TAG rather than simple FFA or phospholipids (Barksdale and Kim 1977, McCarthy 1971, Nakagawa, et al. 1976, Weir, et al. 1972). Using gas chromatography to analyse radiolabelled OA incorporated into TAG, two independent research groups demonstrated that modifications

in chain lengths had occurred (Nakagawa, et al. 1976, Weir, et al. 1972). Indeed, most of the stored TAG were composed of labelled C14:0, C16:0, C16:1, C18:1, C20:0 and C24:0 long-chain fatty acids, suggesting that the lipid substrates can be either shortened or elongated. Moreover, upon OA treatment, the authors noticed that bacterial TAG accumulation was closely correlated with an increase in triacylglycerol synthase (TGS) (also known as diacylglycerol-acyltransferase; DGAT) activity, and that this phenomenon was exclusively restricted to the post-exponential or stationary growth phases (Nakagawa, et al. 1976). Altogether, these early results favoured the emergence of new concepts regarding ILI biosynthesis and led to the assumption that the production of ILI might depend on various factors, in particular the composition of the culture medium as well as the physiological stage of the bacterial cell cycle.

Biophysicochemical factors impacting TAG anabolism and storage in vitro

During their lifecycle, most bacterial species encounter unfavourable environments and are not only facing drastic modifications of surrounding physical and chemical factors but also important fluctuations in nutrient availability. To adapt and survive under such drastic conditions, bacteria have evolved metabolic strategies which include numerous biological pathways and specific molecular factors. In the context of TB, formation of pulmonary granulomatous lesions is one of the major hallmarks of *Mtb* infection. Within granulomas, the bacilli are exposed to various stresses, where the most significant ones are probably nutrient limitation (*i.e.*, carbon, nitrogen, iron starvation etc.), exposure to reactive oxygen/nitrogen intermediates including nitric oxide (NO) and oxygen depletion (Barry, et al. 2009, Deb, et al. 2009, Honer zu Bentrup and Russell 2001, Timm, et al. 2003). These harsh conditions, alone or in combination, significantly alter the metabolic state and the fitness of the tubercle bacilli and, consequently, impact on TAG biosynthesis and accumulation (**Figure 3**), which are critical for *Mtb* survival during lung infection and anti-TB chemotherapy (Baek, et al. 2011, Garton, et al. 2008, Hammond, et al. 2015).

I) Specificity and bioavailability of carbon source

In infected tissues, several types of lipids derived from both living and dead host cells can be used by the bacilli as a source of carbon and energy to further survive, persist or replicate. Among them, saturated or unsaturated FFA are probably the most abundant lipids, whereas more complex lipids such as phospholipids, glycerides (monoacylglycerol,

MAG; diacylglycerol, DAG; TAG or steroids, *i.e.* cholesterol esters derivatives) can be converted into long or short chain FFA and then further imported and reprocessed by the tubercle bacilli (Bloch and Segal 1956, Kim, et al. 2010, Lee, et al. 2013, McKinney, et al. 2000, Nazarova, et al. 2017). Interestingly, when cultured in a well-defined medium containing long-chain FFA or dextrose as the sole carbon source, ILI formation monitored by electron microscopy approaches revealed that TAG accumulation was visible only under lipid-rich conditions when *Mtb* H37Rv reaches stationary phase (Rodriguez, et al. 2014). This important result suggests that exogenous FFA, but not glucose, would be the stimulus responsible for TAG accumulation in *Mtb* and that the physiological mechanisms governing ILI formation in mycobacteria might be well conserved and dependent on the entry into the stationary phase. Importantly, conversion and storage of FFA into TAG appears as a fundamental process, which is conserved from bacteria to human. In particular, it is now well established that lipid droplet storage in eukaryotic cells is a dedicated biological mechanism that prevents lipotoxicity through excessive reactive oxygen species generation during FFA diet (Listenberger, et al. 2003).

Alternatively, when mycobacterial species such as *Msmeg* were exposed to a high concentration of glycerol and in absence of FFA as a lipid source, ILI accumulation was observed during the stationary phase and monitored by thin layer chromatography (TLC) and microscopic approaches (Armstrong, et al. 2016, Armstrong, et al. 2018, Garton, et al. 2002, Santucci, et al. 2019a). The significant accumulation of ILI led the authors to propose that the FFA incorporated within TAG-forming inclusions was directly derived from *de novo* synthesis. This is of particular interest since it implies that the two distinct experimental designs mentioned above (*i.e.*, excess of exogenous FFA or high glycerol diet) are perfectly complementary highlighting the potential of easily applying these stimuli to modulate and characterize the pathways involved in ILI biosynthesis.

II) Nitrogen deprivation

Limitation of nitrogen has been well documented as another major cause triggering TAG biosynthesis and ILI formation (Alvarez, et al. 2000, Alvarez, et al. 1996, Garton, et al. 2002, Santucci, et al. 2019a). First evidences were observed in *Streptomyces* sp. where the authors noticed an increase in the TAG content upon cultivation under nitrogen-deprived conditions (Olukoshi and Packter 1994). This was further confirmed with the *Rhodococcus opacus* strain PD630, capable to produce insoluble inclusions when grown in presence of

different hydrocarbon substrates under low nitrogen conditions (Alvarez, et al. 1996). Finally, the fact that *Rhodococcus* cells, grown in a medium with a fixed concentration of carbon source, accumulate increasing amounts of TAG in stationary phase and that this process is inversely proportional to the concentration of ammonium available in the broth medium, clearly supports the assertion that ILI formation *in vitro* is directly correlated with ammonium deprivation (Alvarez, et al. 2000).

In *Msmeg*, two-dimensional TLC analysis and fluorescence microscopy also demonstrated that TAG accumulation occurred after several days of growth under nitrogen-limiting conditions (Garton, et al. 2002). Moreover, gas chromatography coupled to mass spectrometry (GC-MS) analysis of the fatty acid profiles of TAG component stored following nitrogen starvation, revealed the presence of an increased proportion of already synthesized saturated FFA resulting from *de novo* synthesis (Garton, et al. 2002).

Recently, using defined-minimal media, our group confirmed that carbon excess and nitrogen limitation promote *in vitro* TAG accumulation under the form of ILI in both *Msmeg* and *Mycobacterium abscessus* (*Mabs*) (Santucci, et al. 2019a). Based these findings, it was proposed that the stress responses during nitrogen deprivation may be evolutionarily conserved among different mycobacterial species (Santucci, et al. 2019a).

In fact, the biological pathways and specific role of putative conserved molecular factors involved in actinobacterial adaptation to nitrogen starvation have started to be investigated over the last decade (Amon, et al. 2008, Davila Costa, et al. 2017, Jessberger, et al. 2013, Williams, et al. 2015). Among them, the *nlpR* gene, which encodes a master regulator of lipogenesis upon nitrogen starvation in *Rhodococcus jostii* RHA1, has been recently identified as a key player in TAG metabolism (Hernandez, et al. 2017). This gene which is conserved in mycobacterial species such as *Msmeg* and *Mtb* (*MSMEG_0432* and *Rv0260c* genes, respectively) has been demonstrated to be crucial in nitrogen assimilation processes (Jenkins, et al. 2013, Williams, et al. 2015). However, its direct implication in TAG biosynthesis/storage remains elusive. To date, only the mycobacterial PrrAB two-component system has been identified as a negative regulator directly linking nitrogen limitation and TAG accumulation in mycobacteria (Maarsingh and Haydel 2018). Interestingly, deletion of *prrAB* gene in *Msmeg* led to a significant overexpression of the genes encoding enzymes from the Kennedy pathway by which mammalian cells synthesize phospholipids and TAG for incorporation into membranes or lipid-derived signalling molecules, thus favouring TAG accumulation and storage (Maarsingh and Haydel 2018) (**Figure 4**).

Altogether, these results underscore conserved pathways involved in adaptation and regulation of lipid metabolism during nitrogen starvation in *Actinobacteria*, and this easy-to-use *in vitro* experimental system combined with molecular genetics and biochemical approaches should further contribute to increase our understanding of these biological pathways.

III) Hypoxia

Mycobacterial species require constant levels of oxygen for growth. However, many studies suggested that the tubercle bacilli encounter hypoxic microenvironments in both active disease as well as during LTBI (Via, et al. 2008, Wayne and Sohaskey 2001). To survive under these specific conditions, *Mtb* uses several adaptive pathways relying on the well-known *dormancy survival regulator* (Dos) regulon, which comprises of approximately 50 genes and controlled by the DosRST three-component system (Boon and Dick 2002, Park, et al. 2003, Sherman, et al. 2001). During low oxygen tension, several TGS-encoding genes are upregulated by *Mtb*, among which the *tgs1* (*i.e.*, *rv3130c*) gene being part of the Dos regulon is strongly was overexpressed (Daniel, et al. 2004). Genetic disruption of *tgs1* drastically reduces TAG accumulation during gradual depletion of oxygen while *trans*-complementation almost fully restored the WT phenotype, suggesting that *tgs1* is a critical factor in TAG synthesis and accumulation in *Mtb* H37Rv (Sirakova, et al. 2006). As a result, hypoxia-induced TAG accumulation has been acknowledged as a relevant *in vitro* model, termed as the “Wayne” hypoxia model, widely used to dissect the molecular mechanisms involved in ILI formation in *Mtb* and also in other species composing the *Mtb* complex (Levillain, et al. 2017, Low, et al. 2009, Low, et al. 2010). Similar experimental stimuli were successfully applied to the vaccine strain *Mycobacterium bovis* BCG (*Mbv* BCG), showing that *Mbv* BCG was capable to accumulate TAG under the form of ILI during hypoxia but not in aerated culture conditions (Low, et al. 2009). By combining the use of fluorescent FFA, confocal microscopy and high-performance liquid chromatography coupled with mass spectrometry, the authors also demonstrated that low oxygen levels promote esterification of exogenous FFA within TAG (Low, et al. 2009). By performing transposon mutagenesis screening on *Mtb* growth under hypoxia, Baek and Sassetti identified 34 genes involved in mycobacterial growth regulation and lipid metabolism during low oxygen tension (Baek, et al. 2011). Among them, *dosR* and *tgs1* mutants were over-represented, confirming their major contribution in the establishment of *Mtb* persitent-like state. Moreover, they clearly

demonstrated that growth reduction upon hypoxia was mediated by Tgs1 and TAG formation by down-regulating the tricarboxylic acid cycle (TCA).

More recently, transcriptional studies raised new concepts regarding the role of hypoxia in persistent-like state and dormancy establishment. Indeed, RNA-sequencing results suggested that both the nature of the carbon source (*i.e.*, dextrose or FFA) and oxygen depletion are involved in a successful adaptation leading to TAG accumulation inside ILI (Del Portillo, et al. 2018, Rodriguez, et al. 2014). These observations strongly suggest that hypoxia occurring in damaged tissue during infection may be directly linked to FFA uptake and metabolism within specific microenvironments surrounding the bacilli, and that all act together to promote a lipid-rich phenotype.

IV) NO exposure

In addition to oxygen deprivation, large amounts of NO have been shown to inhibit aerobic respiration and induce non-replicating persistent state. Several studies demonstrated that upon NO treatment, genes from the Dos regulon, including *tgs1,* were significantly upregulated in *Mtb* cultures (Ohno, et al. 2003, Voskuil, et al. 2003). This overexpression was not only associated with an important increase in TAG accumulation, but importantly, NO treatment was also responsible for a greater incorporation of radiolabelled acetate and exogenous FFA into TAG, resulting molecules mainly formed by saturated C16:0 to C28:0 acyl chains (Daniel, et al. 2004).

This NO stress experimental system has been used recently with the vaccine strain *Mbv* BCG to investigate the contribution of the heparin-binding hemagglutinin adhesin (HBHA) in ILI formation (Raze, et al. 2018). Analysis of the lipid content of *Mbv* BCG WT and Δ*hbhA* strains by fluorescence and electron microscopy, revealed that deletion of *hbhA* impairs ILI formation upon NO stress (Raze, et al. 2018), while re-introduction of *hbhA* in the mutant strain restored ILI accumulation to levels comparable to those found in the parental strain. This simple induction system, which mimics O_2 depletion, confirms that inhibition of aerobic respiration trigger FFA import and esterification onto TAG molecules inside mycobacteria.

V) Iron starvation and oxidative stress

Iron acquisition and storage has also been reported to play an essential role in *Mtb* survival and virulence (Bacon, et al. 2007). Within the host, making iron unavailable for the pathogens through sequestration or deprivation, represents an important defence

mechanism. It has been observed that low level of iron *in vivo* works as a stimulus for the expression of virulence genes in numerous microbial pathogens. In addition, several genes involved in lipid biosynthesis and accumulation are overexpressed in *Mtb* under iron starvation (Rodriguez, et al. 2002). In *Mtb* H37Rv, *in vitro* chemostat cultures under iron-poor conditions led to the accumulation of TAG and the production of an unidentified novel wax ester (WE) (Bacon, et al. 2007, Pal, et al. 2019). Iron depletion could also be achieved with deferoxamine mesylate salt, a powerful iron chelator. Using this approach, the authors demonstrated that *Mtb* H37Rv and several clinical strains were able to produce large amount of ILI (Vijay, et al. 2017). Since these specific starvation conditions are also known to induce an important oxidative stress in cells by increasing the production of radical oxygen species, when *Mtb* H37Rv and several clinical isolates were submitted to increasing concentration of H_2O_2, all strains accumulated TAG under the form of ILI. No additional information regarding this *in vitro* experimental design is available regarding NTM.

Enzymatic pathways involved in TAG biosynthesis

If multiple environmental conditions are able to promote ILI formation in actinobacteria, they essentially rely on highly conserved pathways and enzymatic actors to synthesize TAG molecules. From a molecular perspective, these enzymatic reactions can be subdivided into three major steps, which are (i) the production and/or importation of fatty acyl-compounds; (ii) the formation of glycerol intermediates and (iii) the sequential esterification of the glycerol moiety with fatty acyl-residues (Alvarez and Steinbüchel 2002) (**Figure 4**).

The initial step consists in generating fatty acid moieties under the form of acyl-CoA molecules which will be subsequently condensed with glycerol-3-phosphate (G3P) substrates. FFA required for TAG biosynthesis can be either synthesized *de novo* through the action of the type I fatty acid synthetase (FAS-I) or directly imported from the environment *via* multiple transporters. Then, the G3P plays a pivotal role in the biosynthesis of glycerol intermediates, which are required for both membrane glycerophospholipid and TAG metabolism. The intrabacterial pool of G3P is generated by the complementary action of the glycerol kinase, which converts free glycerol into G3P and the G3P dehydrogenase that catalyzes the reduction of dihydroxyacetone phosphate into G3P by using the NAD(P)H cofactor as a reductant. The final step is mediated by the enzymes from the Kennedy pathway which condenses FFA and G3P into lysophosphatidic acid (LPA), and subsequently in phosphatidic acid (PA), DAG and TAG respectively. Despite their physiological and clinical relevance, very few information is available regarding these three major pathways in *Mtb*.

Free fatty acids uptake and biogenesis

De novo synthesis of FFA in mycobacteria is mediated by the multi-enzymatic complex, FAS-I, which uses acetyl-CoA and malonyl-CoA substrates to elongate fatty acids beyond C16 and up to C24-C26 (Bazet Lyonnet, et al. 2017, Gago, et al. 2019). Thus, FAS-I provides essential FFA precursors that will be used to produce mycolic acids, complex polyketides or TAG (Pawelczyk and Kremer 2014). In addition to *de novo* biosynthesis, it is now commonly acknowledged that the tubercle bacilli are able to acquire host-derived fatty acids and cholesterol which are critical carbon sources required for growth, chronic persistence and reactivation processes (Lovewell, et al. 2016). However, how these highly hydrophobic substrates were able to cross the thick and impermeable mycobacterial cell-wall was unclear until recently. Initial studies reported that mammalian transmembrane proteins involved in lipid acquisition also designated fatty acid transport proteins (FATP)

were highly conserved and found in a wide panel of organisms, including *Caenorhabditis elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae* and *Mtb* (Hirsch, et al. 1998). Ectopic overexpression of FACL6/Rv1206 in COS cells revealed that this mycobacterial FATP was able to facilitate FFA uptake, suggesting that this molecular actor might play a key role in lipid import (Hirsch, et al. 1998). Another independent study showed that the purified protein displayed acyl-CoA synthetase activity *in vitro* and heterologous expression in *E. coli* promoted fatty acid uptake/utilization (Daniel, et al. 2014). Moreover, genetic disruption of the gene encoding FACL6 in *Mtb*, showed that this protein is required for optimal incorporation of radiolabeled exogenous fatty acids and the production of intracellular TAG during *in vitro* dormancy (Daniel, et al. 2014). However, these findings failed to directly demonstrate that FACL6 acts as fatty acid transmembrane transporter *stricto sensu*.

In their seminal work, Pandey and Sassetti clearly demonstrated that *Mtb* imports cholesterol across the cell envelope by using the multi-subunit Mce transporter, Mce4 (Pandey and Sassetti 2008). Interestingly, *Mtb* genome contains four unlinked *mce* loci (*mce1-mce4*), which encode numerous proteins of unknown functions, nevertheless, the similarities among the 4 loci indicate that all of them might be transporters required to import hydrophobic molecules (Cole, et al. 1998, Ekiert, et al. 2017, Pandey and Sassetti 2008). Recently, Nazarova and colleagues identified Mce1 as *Mtb*'s main machinery involved in fatty acid import *in vitro* and *in cellulo* (Nazarova, et al. 2017). They also showed that LucA/Rv3723, plays a central role in exogenous carbon acquisition by facilitating both fatty acid and cholesterol uptake across the cell envelope by stabilizing protein subunits of the Mce1 and Mce4 transporters, respectively (Nazarova, et al. 2017). As expected, deletion of *lucA* led to important growth defects within mouse and human macrophages, fitness reduction in infected mice and decrease in lung inflammation, highlighting the importance of this molecular determinant in *Mtb* pathogenesis.

Biosynthesis of LPA, PA and DAG precursors

Modulation of the environmental conditions triggers bacterial metabolism reprogramming at transcriptional, translational and post-translational levels. In this context, the synthesis of TAG requires the upregulation of genes encoding key enzymes involved in the production of lipid precursors, such as proteins from the Kennedy pathway (Amara, et al. 2016, Chen, et al. 2014, Juarez, et al. 2017). This pathway is composed of four enzymatic reactions that act sequentially to generate TAG molecules from G3P and FFA substrates. (**Figure 4**)

Firstly, the enzyme glycerol-3-phosphate acyltransferase (GPAT) condenses FFA onto G3P. In bacteria, this reaction is performed by the highly conserved PlsB protein which is an essential enzyme involved in phospholipid biogenesis (Feng and Cronan 2011, Noga, et al. 2020). *Mtb* possesses two *plsB* genes encoding putative GPAT proteins named PlsB1/Rv1551 and PlsB2/Rv2482c respectively (Cole, et al. 1998) and still little is known about the function of these two proteins. However, heterologous expression of *plsB1*/*Rv1551* in *E. coli*, resulted in higher level of glycerophospholipids and cardiolipin when stimulated with exogenous fatty acids suggesting that Rv1551 is a functional GPAT (Law and Daniel 2017).

Secondly, the LPA must be converted into PA by an acylglycerol-3-phosphate acyltransferase (AGPAT). In *Mtb* genome, one gene named *Rv2182c* has been annotated as encoding a putative AGPAT (Cole, et al. 1998). Interestingly, several whole-genome mutagenesis experiments revealed that this gene is essential for *Mtb* growth *in vitro* (DeJesus, et al. 2017, Griffin, et al. 2011, Sassetti, et al. 2003) and bioinformatic analysis showed that it possesses numerous orthologs in a wide range of mycobacterial species emphasizing that such molecular determinant might play an important role in mycobacterial physiology. Alternatively, another putative AGPAT was identified associated to the ILI fraction obtained from *Mbov* BCG ILI upon growth in oxygen-limiting conditions. Indeed, the protein BCG1489c (which shares 100% identity with *Mtb* Rv1428c protein) has been described for containing a PlsC-like domain which might be responsible for AGPAT activity. Deletion of the gene *BCG1489c* resulted in a noticeable decrease in TAG accumulation under hypoxia when analysed by TLC, suggesting that this gene is required for optimal TAG synthesis (Low, et al. 2010).

Thirdly, the phosphate group from PA has to be cleaved from the substrate backbone by the action of a phosphatidic acid phosphatase (PAP) to generate DAG. This enzymatic reaction is mainly performed by protein belonging to the PAP2 superfamily. Indeed, two proteins from *Streptomyces coelicolor* named Lppα (SCO1102) and Lppβ (SCO1753) were identified as PAP enzymes involved in TAG accumulation (Comba, et al. 2013). Surprisingly, bioinformatic analysis revealed that only one of these two proteins is conserved in *Mtb*. The protein Rv0308 which is annotated as a putative conserved integral membrane protein, possesses C-terminus region similar to C-terminus of other integral membrane proteins or phosphatases (Cole, et al. 1998). Therefore, this protein might play an important role in ILI formation and other metabolic processes, however no experimental data are available regarding its physiological function.

TAG biosynthesis and ILI formation

The final and limiting step governing TAG synthesis is mediated by the key enzymes DGAT also known as TGS. Since the identification of the first DGAT (named AtfA) in *Acinetobacter calcoaceticus ADP1* (Kalscheuer and Steinbuchel 2003), homologs from different microorganisms have been identified in multiple genome databases. However, the homology is mainly restricted to certain groups of bacteria, especially actinomycetes, like *Mycobacterium, Rhodococcus* and *Streptomycetes*, correlating with previous reports on bacterial TAG and WE accumulation processes (Alvarez and Steinbüchel 2002, Wältermann and Steinbüchel 2005). The laboratory strain *Mtb* H37Rv possesses 15 AtfA homologous proteins (Daniel, et al. 2004, Kalscheuer and Steinbuchel 2003, Sirakova, et al. 2006). *In silico* analysis showed that among these 15 proteins, 11 contain the HHxxxDG active-site motif, required for the acyl-CoA acyltransferase activity involved in TAG synthesis, whereas the 4 remaining have modified active-site motifs without any typical consensus sequence. Comprehensive investigation by heterologous production in *E. coli* and determination of total lysate-associated TGS activity showed that all TGS displayed activity *in vitro*. The authors reported that 4 proteins exhibited higher specific activities, these enzymes were then renamed Tgs1-Tgs4 (Daniel, et al. 2004). In addition to these biochemical studies, genetic disruption coupled phenotypic assays allowed to determine that Tgs1 is the main contributor to TAG synthesis and the most active enzyme in *Mtb* in multiple *in vitro* models (Daniel, et al. 2004, Daniel, et al. 2011, Sirakova, et al. 2006).

Similarly, in *Mabs* seven Tgs-encoding genes were identified based on the presence of the HHxxxDG motif, among which Tgs1 was also found to be most active protein (Viljoen, et al. 2016). Site directed mutagenesis revealed that His144 and Gln145 are essential for enzymatic activity of Tgs1 in this species. Tgs1 could use a range of acyl-CoAs as fatty acyl donors however it is more active in presence of palmitoyl-CoA (Viljoen, et al. 2016). In an independent study, it was shown the heterologous overproduction of *Mtb* mycolytransferase Ag85A in *Msmeg* led to altered cell morphology, impaired division and the formation of ILI suggesting a possible role of Ag85A in TAG metabolism and ILI biosynthesis (Elamin, et al. 2011).

In lipid-poor conditions, these enzymes are mainly localized in the membrane fraction (Elamin, et al. 2011, Hayashi, et al. 2016, Viljoen, et al. 2016) supporting previous studies suggesting that TAG biosynthesis is initiated in the inner leaflet of the cytoplasmic membrane before being released into the bacterial cytosol (Waltermann, et al. 2005).

Indeed, lipid inclusions synthesis analysis *in vitro* and in *Rhodococcus opacus* PD630 demonstrated that TAG is first stored in the form of small lipid inclusions, which remain associated to the cytoplasmic membrane. Then, these small inclusions conglomerated to newly form pre-lipid inclusions which are then released into the cytoplasm as mature ILI with a large amount of associated proteins (Waltermann, et al. 2005) (**Figure 4B**)

ILI-associated proteomes, structural proteins and anchoring properties

The first isolation of ILI in Actinobacteria was achieved by phase separation following centrifugation in density gradients (Alvarez, et al. 1996), a simple and efficient protocol which had been previously developed to purify polyester(s) granules from *Pseudomonas oleovorans* (Preusting, et al. 1993). After isolation, samples were analysed by polyacrylamide gel electrophoresis, and the presence of numerous ILI-associated proteins was observed suggesting that ILI are not only constituted by lipid material (Alvarez, et al. 1996).

This experimental approach was subsequently optimized to identify proteins associated to the TAG-containing fractions by LC-MS and bioinformatic analysis (Chen, et al. 2014, Ding, et al. 2012). This led to the identification of 228 and 177 proteins *Rhodococcus jostii* RHA1 and *Rhodococcus opacus* PD630 ILI proteomes, respectively. *In silico* analyses revealed that, among many proteins belonged to distinct families involved in various processes, including enzymatic, transport, cell-division, transcription/translation processes and proteins with unknown functions (Chen, et al. 2014, Ding, et al. 2012).

With respect mycobacterial, only two independent investigations have been carried out in order to identify possible ILI-associated proteins (Armstrong, et al. 2018, Low, et al. 2010). The original study performed by Low and colleagues identified six novel proteins bound to an ILI-enriched fraction from the vaccine strain *Mbv* BCG cultivated under oxygen limiting conditions (Low, et al. 2010). Among them, the authors found the well-characterised Tgs1 (BCG3153c/Rv3130c) and Tgs2 (BCG3794c/Rv3734c) proteins, the heat shock protein hspX (BCG2050c/Rv2031c), a putative acylglycerol-phosphate acyltransferase (BCG1489c/Rv1428c), a putative bifunctional long chain acyl-CoA synthase-lipase (BCG1721/Rv1683) as well as a protein with unknown function (BCG1169c/Rv1109c). Heterologous expression of these genes in yeast showed that the corresponding proteins were indeed targeted to ILI, and overexpression/inactivation of the corresponding genes,

followed by TLC analysis, confirmed that five of these genes were required for optimal TAG accumulation during hypoxia-induced persistence (Low, et al. 2010).

Lately, a complementary study was performed in the saprophytic species *Msmeg* and the enrichment in ILI-associated proteins was analysed upon growth in glycerol-rich Sauton's medium. In this experimental setting, the authors found more than 400 proteins associated to the purified lipid fraction (Armstrong, et al. 2018).

While these results obtained in *Msmeg* were similar to those from *Rhodococcus*, it is still not well understood why there are so many differences regarding protein abundance between these two studies. Multiple factors including the experimental growth conditions, but also species-specific features could be responsible for such striking differences. Unfortunately, the biological reason(s) responsible for these discrepancies remain unknown. Moreover, one obvious limitation in these biological settings is the requirement of bacterial cell-lysis which can lead to massive *trans*-contamination of the lipid fraction with highly hydrophobic or membrane proteins, thus generating a large number of false positives. In that context, the development of next-generation, non-invasive approaches such as proximity labelling proteomics will be of great interest for a spatio-temporal description of the protein composition of ILI in mycobacteria. It is now well acknowledged that ILI are constituted by structural proteins that play a critical role in TAG homeostasis by modulating ILI size, shape but also fusion/fission events. To date, three major proteins have been extensively characterised as key structural factor involved in ILI formation/maintenance in mycobacteria.

Initially discovered by performing an elegant genetic screening for TAG deficient mutants, *tadA* was identified as an important factor requested for TAG accumulation in *R. opacus* PD630. This highly conserved actinobacterial gene encodes a protein which contains a C-terminal domain belonging to the heparin-binding hemagglutinin family and has been described for regulating ILI size/shape (Ding, et al. 2012, MacEachran, et al. 2010). Indeed, deletion of this gene, triggered an important reduction of ILI size which can be restored by *trans*-complementation (MacEachran, et al. 2010). Interestingly, complementation with a truncated version devoid of the C-terminal domain was unable to phenocopy the WT strain suggesting a key role of this lysine-rich heparin-binding domain in ILI storage (MacEachran, et al. 2010). Similar results were recently generated with its mycobacterial ortholog, HbhA, highlighting a potential conserved structural function (Raze, et al. 2018).

Within eukaryotic cells, major structural proteins involved in LD biosynthesis, maintenance and degradation are perilipins also known as PLIN. This family is constituted of proteins that

associate with the surface of LD and regulate numerous metabolic processes, impacting the intracellular LD number, shape and size (Kimmel and Sztalryd 2016). Daniel and colleagues, identified a *Mtb* protein (PPE15) which was overproduced under *in vitro* multiple-stress conditions inducing non-replicating persistence and displaying sequence similarity with the mammalian perilipin-1 protein (PLIN1) (Daniel, et al. 2016). Genetic inactivation and TLC analysis showed that *ppe15/rv1039c* is required for TAG accumulation (Daniel, et al. 2016). In addition, fluorescence microscopy revealed that the *ppe15* mutant failed to form Nile-Red positive granules within the well-established *in vitro* multiple-stress model and *in vitro* human granulomas, thus emphasising a critical role in ILI storage in numerous experimental model (Daniel, et al. 2016).

Upon characterisation of the ILI-associated proteome in *Rhodococcus*, Ding et al., identified another molecular actor that was overrepresented at the surface of ILI (Ding, et al. 2012). This protein named PspA is homolog to the phage shock protein A of *E. coli* which is involved the plasma membrane maintenance and homeostasis under stress (Joly, et al. 2010). Surprisingly, deletion of *pspA* resulted in the formation of supersized ILI and an opposite phenotype was reported upon inactivation of its mycobacterial ortholog *pspA*/*MSMEG_2695* in *Msmeg* (Armstrong, et al. 2016, Ding, et al. 2012). Despite these conflicting observations, this clearly demonstrates that PspA is an important protein in ILI homeostasis (Armstrong, et al. 2016, Ding, et al. 2012).

These three mycobacterial proteins which exert different effects on ILI number, size and shape, however share some key interesting features regarding their subcellular localisation. PPE15, HbhA and PspA proteins have been described for being either membrane-associated (Kobayashi, et al. 2007), exported (Raze, et al. 2018), or secreted *via* macromolecular machineries (Chen, et al. 2017). Strikingly, these proteins are also located at the ILI surface in *Msmeg* (Armstrong, et al. 2018). Where these proteins are subcellularly produced, how they dynamically alternate between intra- and extra-cellular localisation and what the physiological role(s) of these dual localisations are, remain to be fully addressed. Recently, it has been proposed that a small conserved domain might be responsible for the targeting of these determinants at the surface of ILI (Armstrong, et al. 2018). Indeed, the presence of a specific domain either in N-terminal or C-terminal seems critically required for facilitating ILI localisation. Regarding HbhA, it has been shown that its C-terminal domain, which shares also similarities with the apolipoprotein A1/A4/E protein, was responsible for ILI anchoring and aggregation *in vitro* (Ding, et al. 2012, MacEachran, et al. 2010).

Based on *in silico* analysis, it was hypothesized that some conserved domains of the PPE15 protein, which are conserved in multiple PLIN, would potentially be responsible for its interaction with ILI, notably *via* its C-terminal hydrophobic region. However, no experimental validation supporting this issue has been performed yet (Daniel, et al. 2016). More recently, an in-depth characterisation of the PspA binding properties allowed to define a putative conserved biochemical pattern that would facilitate proteins interaction with ILI (Armstrong, et al. 2018). Among the five alpha-helix domains (H1 to H5) of PspA, it was reported that the first helix (H1) was necessary and sufficient for subcellular localization of PspA at the ILI surface. The generation and expression of a recombinant GFP variant fused to one or three N-terminus repeated H1 tag(s) allowed to localise the GFP protein onto ILI (Armstrong, et al. 2018). By combining their proteomic approach, with these observations and bioinformatic analysis, the authors were able to identify and validate that specific amphipathic helices are responsible for binding to the ILI surface inside mycobacteria (Armstrong, et al. 2018). However, to date, there is still no information regarding the spatiotemporal dynamics of this association process at the ILI surface. Such investigation will be of great interest in the coming years to finely decipher how, where and when these proteins interact with ILI and govern their homeostasis.

TAG breakdown and pharmacological inhibition of lipolysis

The presence of ILI in mycobacterial cells, and more particularly *Mtb*, has been defined as one of the hallmarks of non-replicating persistent bacilli *in vitro* and *in vivo* (Daniel, et al. 2011, Garton, et al. 2002, Garton, et al. 2008). These organelles are considered as one of the main strategies employed by mycobacteria to adapt and survive within drastic environmental conditions by providing a constant and sufficient amount of energy over long period of time.

Utilization of the energy stored in TAG requires hydrolysis by hydrolases and lipases to release free fatty acids which in turn are catabolized by β-oxidation (Menendez-Bravo, et al. 2017). Depending on their nature and substrate specificity, lipolytic enzymes are commonly divided in four classes such as i) carboxylesterases acting on small and partially water-soluble carboxylester substrates; ii) true lipases hydrolyzing water-insoluble substrates such as MAG, DAG and TAG molecules; iii) phospholipases, acting on phospholipid substrates and iv) cutinase-like proteins consisting of a much more versatile family of enzymes able to degrade a wide range of substrate including carboxylesters, TAG, phospholipids, as well as cutin (Delorme, et al. 2012, Masaki, et al. 2005).

In order to constantly provide energy upon long-term persistence or during reactivation, degradation of mycobacterial TAG must be initiated by specific proteins displaying TAG-lipase activity. Pioneer work by Deb *et al*., reported the presence of the *Mtb* Pro-Glu (PE) protein, encoded by *Rv3097c* and named LipY, which has a C-terminal domain with homology to the hormone-sensitive lipase family and containing the conserved GDSAG motif (Deb, et al. 2006). Biochemical characterization demonstrated that LipY displays TAG hydrolase activity *in vitro* (Deb, et al. 2006). Generation of a *lipY* deficient mutant, showed that TAG utilization upon carbon starvation was significantly decreased, indicating that LipY plays an important role for the hydrolysis of stored TAG. This was further confirmed by heterologous expression in *Msmeg*. However, since this latter species does not encode the ESX-5 secretion system, that allow the secretion of LipY, these results have to be considered with caution (Mishra, et al. 2008, Santucci, et al. 2019b). Interestingly, multiple comparison of full-length LipY with a recombinant variant shortened by its N-terminal PE domain (LipY(ΔPE)), revealed that full-length LipY exhibited reduced TAG-hydrolase activity, suggesting a negative modulation of the activity of LipY by its N-terminal PE domain (Garrett, et al. 2015, Mishra, et al. 2008, Santucci, et al. 2018, Santucci, et al. 2019b). Enzymatic characterization of LipY showed that this protein also displays DAG-hydrolase activity, implying that this enzyme may also be involved in another essential step

upon TAG breakdown (Santucci, et al. 2019b). In addition to LipY, other *Mtb* enzymes have been reported to display true lipase activity. Among them Rv1984c/Cfp21, which belongs to the cutinase-like family proteins, is able to hydrolyze different substrate including medium-chain carboxylic esters, MAG and TAG (Dedieu, et al. 2013, Schué, et al. 2010). Interestingly, the putative bifunctional long chain acyl-CoA synthase-lipase (BCG1721/Rv1683) has been showed to be involved in TAG degradation (Low, et al. 2010). This enzyme possesses a putative N-terminal lipase and a C-terminal ACSL domain, expressing a TAG-lipase activity when overproduced in *Mbv* BCG or in yeast, and this activity was abolished when S150A a point mutation was introduced in the predicted lipase catalytic site (Low, et al. 2010). A previously uncharacterized membrane-associated protein, annotated as Rv2672/Msh1, was reported to express both lipase and protease activities. This protein, which was mainly conserved among actinomycetes, is required for host-TAG hydrolysis, albeit no investigation regarding intrabacterial lipid consumption was done (Singh, et al. 2017). *Mtb* also encodes one monoacylglycerol lipase named Rv0183 which converts monoacylglycerol into free glycerol and fatty acid, which can be further used as a source of energy (Côtes, et al. 2007). Genetic knock-out of *MSMEG_0220*, the ortholog of *Rv0183* in *Msmeg*, triggers drastic changes in morphology and resulted in important antibiotic susceptibility modifications (Dhouib, et al. 2010) but no information is available regarding ILI metabolism. Altogether, this shows that *Mtb* possesses numerous enzymes required for TAG breakdown and generation of FFA but more investigation is required to comprehensively decipher their respective involvement. Two main experimental strategies have been used *in vitro* to better apprehend the process of lipid consumption in mycobacteria. The first one, is based on a simple series of events and mimics fluctuations from a nutrient-limiting to a nutrient-replete condition. Indeed, once the level of any limiting chemical element, metabolites and/or energetic substrates raises again, bacterial cells initiate a global metabolic reprograming and rapidly start consuming stored TAG in order to fuel their regrowth. Such an experimental model was used by Low and colleagues to investigate the contribution of ILI hydrolysis during regrowth from hypoxia-induced dormancy (Low, et al. 2009).

By monitoring bacterial regrowth, the authors demonstrated that ILI mobilization was required to support *Mbv* BCG regrowth during the transition from hypoxia to normoxia (Low, et al. 2009). Interestingly, Orlistat (Tetrahydrolipstatin; THL), a serine-hydrolase inhibitor known for covalently binding and inhibiting mammalian and bacterial lipolytic enzymes as well as fatty acid synthases, was able to prevent the degradation of TAG and to impair *Mbv*

BCG growth resumption (Low, et al. 2009). The same group further employed a THL analogue in an activity-based protein profiling assay to identify the target proteins responsible for TAG hydrolysis upon reactivation of *Mbv* BCG (Ravindran, et al. 2014). This approach unravelled many lipolytic enzymes as potential targets needed for lipid metabolism and re-growth. These include carboxylesterases belonging to the Hormone-Sensitive Lipase Lip-family members (*i.e*., LipD, LipG, LipH, LipI, LipM, LipN, LipO, LipV, LipW, LipY) and the thioesterase TesA (Deb, et al. 2006, Mishra, et al. 2008, Ravindran, et al. 2014).

Nevertheless, it is important to mention that these experiments where performed with the vaccine strain *Mbv* BCG and that the role of TAG hydrolysis during outgrowth following hypoxia is still largely unknown in *Mtb*. Investigating whether such biological process is conserved within both *Mtb* laboratory and clinical strains will be of great interest. It should also be pointed out that such powerful approach, mainly based onto pharmacological inhibition, presents some limitations. The low selectivity of THL and its propensity to block a wide range of mycobacterial enzymes may impact numerous biological processes specially when assessing bacterial growth. In this context, confirmation of the observed phenotypes with a complementary approach such as gene overexpression or inactivation is often required (Low, et al. 2009).

The second experimental model mainly relies on carbon starvation, which allows to artificially trigger TAG consumption. This approach has been widely used to investigate the mechanisms and dynamics of ILI hydrolysis by first developing an image-based quantitative method to assess lipolysis and ILI dynamics by time-lapse fluorescence microscopy at the single-bacterial cell level. This specific system combined with a microfluidic disposal allowed to monitor intracellular bacterial lipid levels over time with a high temporal resolution (Dhouib, et al. 2011). In this experimental system, upon carbon deprivation using PBS-induced starvation, *Msmeg* rapidly hydrolyses the stored TAG, which results in a rapid consumption of ILI; and this lipolytic activity was abolished in the presence of THL, as observed by fluorescence microscopy and TLC analysis (Dhouib, et al. 2011). More recently, using an *in vitro* model based on nitrogen starvation followed by carbon deprivation, the relative rate of ILI consumption in *Msmeg* and *Mabs* species was determined (Santucci, et al. 2019a). Moreover, heterologous overexpression of the well-characterized *Mtb* lipase LipY under these conditions led to a significant decrease in TAG content in *Msmeg* (Daleke, et al. 2011, Deb, et al. 2006, Mishra, et al. 2008, Santucci, et al. 2019b). Importantly, using a model of infected foamy macrophages (FM), it has been

692 demonstrated that specific lipid deprivation within host-cells triggers rapid LD consumption
693 and subsequently ILI breakdown (Caire-Brandli, et al. 2014, Santucci, et al. 2018). In all
694 cases, addition of the serine-hydrolase inhibitor THL or the oxadiazolone-core compound
695 M*m*PPOX targeting more specifically *Mtb* lipolytic enzymes belonging to the Lip-family
696 members (Delorme, et al. 2012, Nguyen, et al. 2018, Santucci, et al. 2019b), showed that
697 ILI hydrolysis was dependent on mycobacterial lipolytic enzyme activities (Santucci, et al.
698 2018, Santucci, et al. 2019a). These results support the view that lipolytic enzymes are
699 essential for TAG hydrolysis and can be modulated by pharmacological inhibition (**Figure**
700 **5**). They are also consistent with previous findings reporting the importance of lipolytic
701 enzymes during ILI mobilization, suggesting that these processes are well conserved
702 across fast- and slow-growing mycobacteria (Dhouib, et al. 2011, Low, et al. 2009,
703 Santucci, et al. 2019a).
704 Collectively, the results gained from various experimental systems have led to the common
705 consensus, that mycobacterial species have evolved specific complex biological pathways
706 to detect and protect themselves against a wide range of exogenous stresses, mainly by
707 modulating their metabolic profiles (Kumar, et al. 2011). It is clear that the availability of
708 such *in vitro* experimental models will greatly facilitate our understanding of mycobacterial
709 adaptation, lipid metabolism and pathogenesis in a near future.
710
711 ***TAG-derived FFA and TAG export***
712 Upon ILI degradation, TAG-derived molecules such as DAG, MAG and FFA can be
713 redirected in many biological pathways. To date, it is commonly accepted that FFA are
714 mainly used for β-oxidation, releasing acetyl-CoA and the reduced co-factors NADH and
715 $FADH_2$ providing energy *via* the TCA cycle and the respiratory chain. However, TAG-
716 derived products may also serve as precursor for membrane phospholipids, mycolic acids
717 or complex lipids such as dimycocerosate esters, thus playing a central role in
718 mycobacterial physiology (Aschauer, et al. 2018, Cabruja, et al. 2017, Lee, et al. 2013,
719 Menendez-Bravo, et al. 2017). Indeed, acyl-CoA molecules react with malonyl-ACP to
720 generate very long chain mero-mycolyl-ACP though the type II fatty acid synthase,
721 ultimately leading to the production of mycolic acids (Cabruja, et al. 2017). FFA degradation
722 also lead to the generation of propionyl-CoA, which can generate methyl-malonyl-CoA
723 through the methyl-malonyl pathway, subsequently utilized to produce methyl-branched

lipid for the building blocks of the bacterial cell wall (Lee, et al. 2013, Minnikin, et al. 2002, Russell, et al. 2010).

Alternatively, TAG can also be directly exported *via* the LprG-Rv1410 machinery (Martinot, et al. 2016). If the contribution of such machinery in the context of hypoxia-induced persistence has not been investigated yet, one can speculate that such transport system might be required for optimal regrowth.

Biological systems to study ILI metabolism within host cells

Lipid metabolism is an extremely complex process, particularly during mycobacterial infection which is modulated by numerous factors. Inside granulomas, mycobacteria encounter different extracellular environments and interact with host immune cells. Among the cells, the FM or lipid-loaded macrophages, represent an atypical cell type that has been the subject of intensive research over the last decades (Russell, et al. 2009). Although they might play a critical role in persistence and reactivation of the tubercle bacillus, leading to the spread of the disease, the exact role of these specific cells in mycobacterial pathogenesis remains unclear. Multiple cellular models have been developed to dissect the mechanism by which mycobacteria acquire lipids from the host and use them for synthesizing their own ILI, and to understand how this process influences the outcome of the infection (Barisch and Soldati 2017, Santucci, et al. 2016).

Intracellular acquisition and accumulation of lipids from the host was first described in non-immune cells where *Mtb* was able to accumulate ILI and survive in a non-replicating state within adipocytes (Neyrolles, et al. 2006). These results were recently confirmed with *Mycobacterium canettii*, another member of the *Mtb* complex (Bouzid, et al. 2017).

This infection model using adipose cells further allowed to investigate the expression of specific genes required for host-derived lipids consumption and biosynthesis of ILI. Transcriptional studies of *Mtb*-infected adipocytes revealed that the *dosR* gene encoding the master regulator of the dormancy regulon and the *icl* gene encoding the main isocitrate lyase were both highly upregulated (Rastogi, et al. 2016). Moreover, *tgs1* and *tgs2* as well as several genes encoding lipolytic enzymes belonging to the Lip (*lipF*, *lipH*, *lipN*, *lipX* and *lipY*) and cutinase-like (*culp5*, *culp7* and *culp6*) families were also strongly upregulated, thus suggesting an important role of these enzymes in host-derived lipid degradation and accumulation (Rastogi, et al. 2016). In an independent study, transcriptome profiling by RNA-sequencing performed on bacilli from infected adipocytes revealed that genes involved in *de novo* FA synthesis, such as *fas* or *acpA*, were downregulated, whereas those predicted to be involved in TAG biosynthesis were significantly overexpressed. Surprisingly

tgs1 was not listed among them (Nandy, et al. 2018). In addition, no significant differences were observed regarding the expression of lipolytic enzymes at 10 days post-infection, suggesting that temporal changes are an important factor that needs to be considered in this biological system. Importantly, the authors reported the suppression of the transcription factor IdeR, suggesting decreased iron uptake by *Mtb* in the adipocyte model not in preadipocyte infection, in order to prevent oxidative stress (Nandy, et al. 2018). Finally, using an oxidative-sensitive stress on *Mtb* Δ*IdeR* mutant, they showed that incorporation of OA triggers a reduction of the tubercle bacilli cytoplasm and further promote its survival in lipid-rich environment (Nandy, et al. 2018).

The first investigations in human immune cells were performed by using an *in vitro* model of mycobacterial granulomas (Peyron, et al. 2008, Puissegur, et al. 2004). In this biological system, peripheral blood mononuclear cells (PBMCs) isolated from the blood of healthy donors were infected with *Mtb* for 3 or 11 days and further processed for conventional electron microscopy (Peyron, et al. 2008). This approach showed how *Mtb* promotes LD formation in *in vitro* human granulomas, but also allowed to describe the fusion events between host-LD and the mycobacterium-containing vacuole. Such observations were also correlated with the presence of ILI, suggesting that ILI formation in *Mtb* might derived from the acquisition of lipids contained within host-LD (Peyron, et al. 2008). Complementary experiments within PBMC-derived macrophages showed that, over time, the bacterial burden within FM was significantly lower in comparison to the one in lipid-poor cells. Transcriptomic analysis revealed that *dosR*, *icl* and *tgs1* genes were massively upregulated during infection of FM, suggesting that foamy PBMCs may provide a niche for *Mtb* long-term persistence in a lipid-rich non-replicating state (Peyron, et al. 2008). Interestingly, these observations were further confirmed in both human monocytic cell line THP-1 and PBMC-derived macrophages exposed to 1% of O_2 (Daniel, et al. 2011). In such a hypoxia-based cellular model, the authors used exogenous isotopically labelled FFA followed by TLC analysis to report that *Mtb* assimilated host-derived TAG as a lipid source which was subsequently used to build up ILI in a *tgs1*-dependent manner (Daniel, et al. 2011). However, one cannot exclude that some of the exogenous lipids were directly transferred to the bacteria and might impact ILI formation in this experimental model.

They also demonstrated that upon FM infection, *Mtb* lost acid fastness and became Nile-Red positive, two well-acknowledged features of non-replicating persistent bacilli (Daniel, et al. 2011). This transition from acid fast-positive to acid fast-negative and Nile-red positive has been shown to be *tgs1*-dependent in an *in vitro* model of granuloma. Additionally, such

phenotype was associated to an increase tolerance to the front-line drug rifampicin (Kapoor, et al. 2013).

To date, this close relationship between loss of acid fastness, low metabolic activity, lipid accumulation and antibiotic susceptibility is still not well understood (Vilcheze and Kremer 2017). Although the key players related to acid fast property of mycobacteria remain largely unexplored, the association of mycolic acids and other cell wall-associated (glyco)lipids with acid fast property of *Mtb* was reported, essentially based on genetically defined mutants found to be attenuated in mice. The approach of this study will not only help improving detection of dormant bacilli but also beneficial for future vaccine developments (Vilcheze and Kremer 2017). In addition to Tgs1, the β-ketoacyl-acyl carrier protein synthase KasB, involved in mycolic acid synthesis, could be one of the major actors involved in loss of acid fastness and long-term persistence (Bhatt, et al. 2007, Vilcheze and Kremer 2017, Vilcheze, et al. 2014). Recently, long-term live-cell imaging of *Mtb*-infected human monocytes-derived macrophages allowed to monitor LD content at the single-cell level, and confirmed that *Mtb* replication correlated a decrease in intracellular TAG, suggesting that LD-contained lipids serve as an important carbon source during infection (Greenwood, et al. 2019). Unexpectedly, by combining light, electron and ion microscopy imaging, the authors not only demonstrated that the anti-tubercular antibiotic bedaquiline accumulates within host LD, but also that host-lipid consumption is a dynamic process that enhanced the delivery of bedaquiline within *Mtb* (Greenwood, et al. 2019). The cumulative results in human primary cells clearly strengthen the fact that the intricate interplay between mycobacterial and host lipid metabolism plays a crucial role in the mycobacterial lifecycle, antibiotic susceptibility and treatment outcomes (Agarwal, et al. 2020).

The use of mouse bone marrow-derived macrophages (BMDM) further emerged as a good alternative to human macrophages by-passing the donor-to-donor variability occurring when working with human blood samples. In addition, BMDM represent a valuable tool to easily investigate/monitor lipid accumulation in cells derived from specific mouse-models with distinct genetic background (Knight, et al. 2018, Podinovskaia, et al. 2013). Thanks to fluorescence microscopy on infected BMDM exposed to OA, *Mtb* was found to promote significantly the retention of the foamy phenotype (Podinovskaia, et al. 2013). Recent results obtained in *Mtb*-infected BMDM suggest that such accumulation of LD would be dependent on the host macrophage activation profile and essentially mediated by the transcription factor hypoxia-inducible factor 1α (Hif1-α) (Genoula, et al. 2020, Knight, et al.

2018). In this biological system, the use of exogenous FA combined with the fluorescent lipid BODIPY-palmitate demonstrated that exogenous lipids can be acquired by the bacteria during macrophage infection (Podinovskaia, et al. 2013). The Mce1/LucA machinery was identified as a key factor in lipid acquisition (Nazarova, et al. 2017). In the presence of the fluorescent lipid BODIPY-palmitate, flow cytometry combined with fluorescence and electron microscopy approaches demonstrated that a *Mtb* Δ*lucA*::hygR mutant was unable to utilize FFA and cholesterol during BMDM infection, and consequently to accumulate ILI (Nazarova, et al. 2017). The authors further infected BMDM with a mCherry fluorescent transposon mutant library, then pulse-labelled the infected cells with the latter fluorescent substrate and measured bacterial incorporation of BODIPY-palmitate by flow cytometry to identify which alternative *Mtb* genes are involved in FFA uptake and ILI formation (Nazarova, et al. 2019). Intracellular bacteria with BODIPY-low level were isolated and plated on selective agar. Sequencing of the transposon insertion sites confirmed the important role for the Mce1/LucA transporter but also allowed identifying new genes involved in FFA import/metabolism such as *rv0966c*, *rv0200*/*omamB*, *rv3484*/*cpsA* or *rv2583c*/*relA* (Nazarova, et al. 2019). Such findings clearly support the idea that ILI formation derives from the dynamic acquisition of host-lipids. It is however important to specify that direct acquisition of labelled lipids by *Mtb* cannot be ruled out and might have also occurred in that biological system.

Another experimental system using BMDM as model was developed to investigate how mycobacteria acquire lipids from their hosts. Here, infection with pathogenic mycobacteria is first performed allowing active replication of the bacilli inside infected cells, then infected BMDM are further exposed for 24h to TAG-rich very low density lipoprotein (VLDL) as a lipid source (Caire-Brandli, et al. 2014). Such alternative exogenous lipid source was selected since it requires CD36-mediated internalisation and endolysosomal processing before promoting the appearance of LD in the infected cells. This model was developed in order to limit the exposure of intracellular mycobacteria to exogenous FFA which rapidly diffuse across membranes and the endolysosomal network (Caire-Brandli, et al. 2014). By combining this model with quantitative analysis of EM observations, it was shown that LD rapidly fuse with the *Mav*-containing phagosome, which was associated with an increase in ILI levels (Caire-Brandli, et al. 2014). Upon VLDL treatment intracellular mycobacteria displayed a thinned cell wall, became elongated and the quantification of bacterial septum in addition to CFU scoring demonstrated that mycobacterial division was arrested (Caire-Brandli, et al. 2014). Conversely, upon VLDL removal, both host LD and ILI abundance

sharply decreased, suggesting an increase in lipolysis that was correlated with a simultaneous resumption of mycobacterial division (Caire-Brandli, et al. 2014). This *in vitro* FM model was subsequently used to investigate how *Mbv* BCG acquires host lipids in the phagosomal lumen (Santucci, et al. 2018). Deletion and overexpression of the *lipY* gene, encoding the well-characterized mycobacterial TAG-lipase, revealed that, while LipY plays an important role in extra-bacterial lipid consumption, this lipase is not essential for ILI formation within FM (Santucci, et al. 2018). In addition to slow- growing mycobacteria, this model has also been used to investigate the intracellular behaviour of the emerging pathogen *Mabs* within FM (Viljoen, et al. 2016). The authors demonstrated that *Mabs* is able to interact with host-LD and synthesize ILI in a Tgs1-dependent manner (Viljoen, et al. 2016).

Alternatively, the dynamics of host-lipid consumption and accumulation under the form of ILI was studied during the infection of the amoeba *Dictyostelium discoideum* with the fish pathogen *Mycobacterium marinum* (*Mmar)* (Barisch, et al. 2015, Barisch and Soldati 2017). This system, based on the exogenous addition of FFA to promote lipid-rich phenotype and initially used to identify new proteins involved in LD synthesis within the amoeba (Du, et al. 2013) allowed to monitor in real-time the interaction of host LD with the mycobacterium-containing vacuole by using fluorescence microscopy techniques (Barisch, et al. 2015). Following this approach, the authors showed that perilipin proteins were able to coat cytosolic bacteria in a RD1-dependent manner regardless of the presence or the absence of exogenous FFA (Barisch, et al. 2015). Similar results were observed with the eukaryotic TAG synthase Dgat2, but not Dgat1 (Barisch and Soldati 2017). Upon FFA treatment, TAG production was totally abolished in a *Dictyostelium* mutant lacking both *dgat1* and *dgat2*, but this loss was accompanied with an significant increase in endoplasmic reticulum (ER) membrane proliferation (Barisch and Soldati 2017). Surprisingly, the authors showed that, under these specific conditions, *Mmar* was still able to synthesize its own ILI by depleting the ER-like structures and using host phospholipids as main substrates (Barisch and Soldati 2017). Finally, they demonstrated that *Mmar* remained metabolically active and acid-fast positive in that biological system. This suggests that some mechanisms linking lipid accumulation, cell-wall alterations and persistence might be different among mycobacterial species and require to be further investigated.

Altogether, these findings highlight the complexity of mycobacterial lipid metabolism within host cells. Observations from the last two decades have raised numerous questions and emerging concepts regarding the acquisition of host-derived lipids by mycobacteria. Still

very little is known about the physiological roles and dynamics of LD in the context of bacterial infection. Indeed, how membrane-bound or cytosolic bacteria interact with LD in order to exploit their content, remains to be further explored. In that context, investigating the potential occurrence and contribution of LD-derived lipids acquisition *in vivo* and further deciphering to which extent this consumption process influence *Mtb* pathogenesis and disease progression is of great interest.

Beneficial role of ILI metabolism in the mycobacterial lifecycle?

I) ILI, carbon source and mycobacterial metabolism

Although LD and ILI have been considered as "just fat" organelles for decades, it is now well acknowledged that the biological function of these complex structures goes beyond that simplistic view (Cabruja, et al. 2017, Deb, et al. 2009, Henne 2019, Martinot, et al. 2016). In the context of chronic mycobacterial infection, accumulation of TAG within ILI has been proposed to serve as a major source of carbon and energy in non-replicating bacteria (Daniel, et al. 2011, Garton, et al. 2002, Garton, et al. 2008, Peyron, et al. 2008) Indeed, slow and long-term TAG breakdown in the absence of exogenous FFA or *de novo* synthesis provide an important acetyl-CoA pool *via* β-oxidation, which can be rerouted to other metabolic pathways or to sustain ATP production and plasma membrane and cell wall integrity (Cabruja, et al. 2017, Deb, et al. 2009, Martinot, et al. 2016). However, the use and combination of several experimental models have recently shed new insights into additional roles of these dynamic organelles in bacteria. Among these alternative functions, ILI formation has appeared essential to limit the reductive stress and lipotoxicity generated by excess of endogenous FFA (Rodriguez, et al. 2014). During infection, the biosynthesis of TAG and phthiocerol dimycocerosates have been shown to be essential for limiting metabolic stress (Lee, et al. 2013). Similar results have been found in primary fibroblast cells and yeasts where TAG biosynthesis is essential to prevent lipotoxicity and cell death (Garbarino, et al. 2009, Garbarino and Sturley 2009, Listenberger, et al. 2003, Petschnigg, et al. 2009). This implies that, like the eukaryotic LD, ILI might also act as conserved organelles involved in the storage of toxic fatty acids, which must be sequestered during specific conditions to prevent the irreversible damages for the cell (Wältermann and Steinbüchel 2005). Alternatively, storage of evaporation-resistant lipids has been proposed as a bacterial strategy to overcome water deficiency under dehydration, where oxidation would be one of the main mechanisms to produce water molecules (Alvarez, et al. 2004).

Recently, it has been demonstrated that ILI contained within the saprophytic strain *Rhodococcus jostii* RHA1 are able to bind to genomic DNA through the action of one ILI-associated protein (Zhang, et al. 2017). Indeed, the authors showed that the protein RHA1_Ro02105, homolog of the mycobacterial HBHA, known for its role in ILI formation when localized intracellularly (Raze, et al. 2018), was able to bind and stabilize DNA, thus enhancing bacterial survival under genotoxic stresses (Zhang, et al. 2017). Interestingly, interactions of LD with nucleic acid or histone proteins have also been reported in mammalian cells (Layerenza, et al. 2013, Uzbekov and Roingeard 2013). Despite the fact that the biological significance of these interactions remains obscure, one can hypothesize that such processes could be highly conserved and directly involved in DNA maintenance and/or gene expression modulation.

II) ILI and disease progression

Another beneficial role of ILI is provided by the observation of lipid-rich bacilli in sputum from TB-positive individuals originated from Gambia and the United-Kingdom, which raised the hypothesis that these structures might play a key role in the transmission of the tubercle bacilli (Garton, et al. 2008). Thus, ILI may favour bacterial survival during the transit from an individual to another and may also help the bacilli to more easily adapt to the environmental pressure encountered in a new host (Garton, et al. 2008). These speculations are also in line with findings regarding the Beijing family of *Mtb* strains (sub-branch of lineage 2 also known as the East Asian lineage), which has been associated with increased epidemic spread and reported to constructively produce high amounts of TAG (Domenech, et al. 2017, Reed, et al. 2007, Tong, et al. 2020). To investigate whether ILI accumulation may confer a significant advantage in the establishment and outcomes of infection, zebrafish embryos were infected with lipid-rich and lipid-poor *Mabs* (Santucci, et al. 2019a). Comparison of the bacterial loads, inflammatory response and larval survival indicated that the lipid-rich persistent-like phenotype favoured bacterial replication and significantly increased the number of granulomatous lesions per fish which is in agreement with a previous study obtained with non-replicating *Mtb* in CB6F1 mice (Dietrich, et al. 2015, Dietrich, et al. 2016, Santucci, et al. 2019a). This was also correlated with an earlier larval mortality, suggesting that pre-existing ILI might confer a specific advantage when colonizing a new host. However, more investigations are required to better understand these important physiopathological aspects. Several studies emphasized that both host and bacterial TAG metabolism are a critical factor influencing and determining the outcome of the infection

(Garton, et al. 2008, Jaisinghani, et al. 2018, Kim, et al. 2010, Russell, et al. 2009). A concept of bacterial immunostat lipid-regulated that control host immunopathologic response has been recently proposed (Queiroz and Riley 2017), whereby the synthesis of many different acylated forms of lipid, directly linked to TAG metabolism (Lee, et al. 2013, Singh, et al. 2009) represents a specific strategy used by the tubercle bacilli to decrease the host response and further establish a long-term infection (Queiroz and Riley 2017).

III) ILI and antibiotic efficacy

The presence of ILI in mycobacteria has been also reported to drastically impact antibiotic susceptibility which is crucial in the context of bacterial infection. Indeed, non-replicating bacteria with reduced metabolic activity are more tolerant to antibiotic treatments (Baek, et al. 2011, Deb, et al. 2009, Hammond, et al. 2015, Kapoor, et al. 2013, Santucci, et al. 2019a). By using an *in vitro* multiple-stress dormancy model, Deb and colleagues showed that specific microenvironments drive the appearance of lipid-loaded, rifampicin and isoniazid-tolerant phenotypes (Deb, et al. 2009). Another complementary study demonstrated that rerouting the carbon flux and TAG synthesis influence both growth rate and antibiotic susceptibility *in vitro* and *in vivo* (Baek, et al. 2011). This was further confirmed by Hammond and colleagues who clearly established a link between the intrabacterial lipid content and drug tolerance in species belonging to the *Mtb* complex as well as in NTM (Hammond, et al. 2015). By discriminating old/young and lipid-rich/poor bacteria, this study raised an interesting concept suggesting that the age of the culture or the growth rate are not always linked to increased tolerance (Hammond, et al. 2015).

Concluding remarks and perspectives

It is clear that mycobacterial lipid metabolism, particularly ILI formation and breakdown, are key events that are directly linked to survival, persistence, pathogenicity and drug tolerance. Understanding how the bacilli acquire host lipids, how they metabolise them in order to persist and reactivate remain a fascinating challenge. The combination of genetic, biochemical or pharmacological approaches along with the development of easy-to-use *in vitro* biological stimuli and new cellular/animal models is crucial to gain further insights into these physiological processes, inspiring new ways for feeding the discovery of new therapeutic strategies against mycobacterial diseases.

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Authors contribution

PS and SC proposed and conceptualized this work. IM, PS and SC wrote the draft of the manuscript. LK and JFC completed and revised the entire manuscript. IP, VP and LK provided light and electron microscopy micrographs and helped IM and SC in figures editing. All authors listed have made a substantial, direct and intellectual contribution to the work which was supervised by SC. All authors approved the manuscript for publication.

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Figures and Figure legends

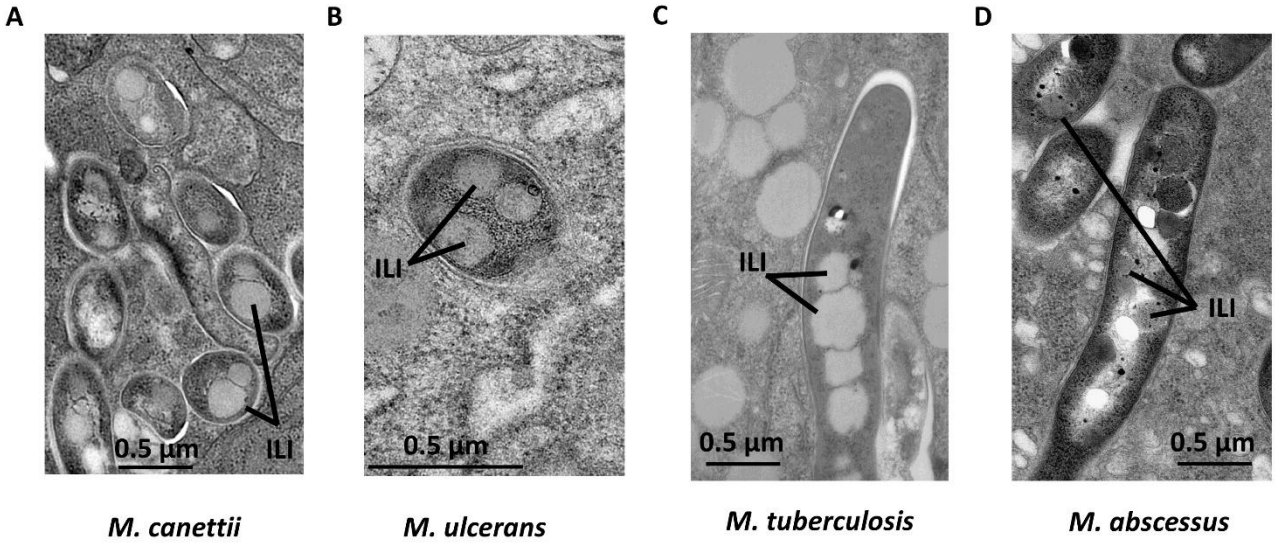

Figure 1: Electron microscopy images of ILI in various intracellular pathogenic mycobacteria inside different hosts. **(A)** 3T3-L1 mature adipocytes infected with *M. canettii*. **(B)** Mouse tail tissue infected with *M. ulcerans.* **(C)** *Mtb*-infected cell from a sputum sample from a TB-positive patient. **(D)** *M. abscessus*-containing macrophage in a zebrafish embryo. ILI are indicated with black lines and scale bars represent 0.5 µm. All electron microscopy micrographs depicted are from the authors' personal collections.

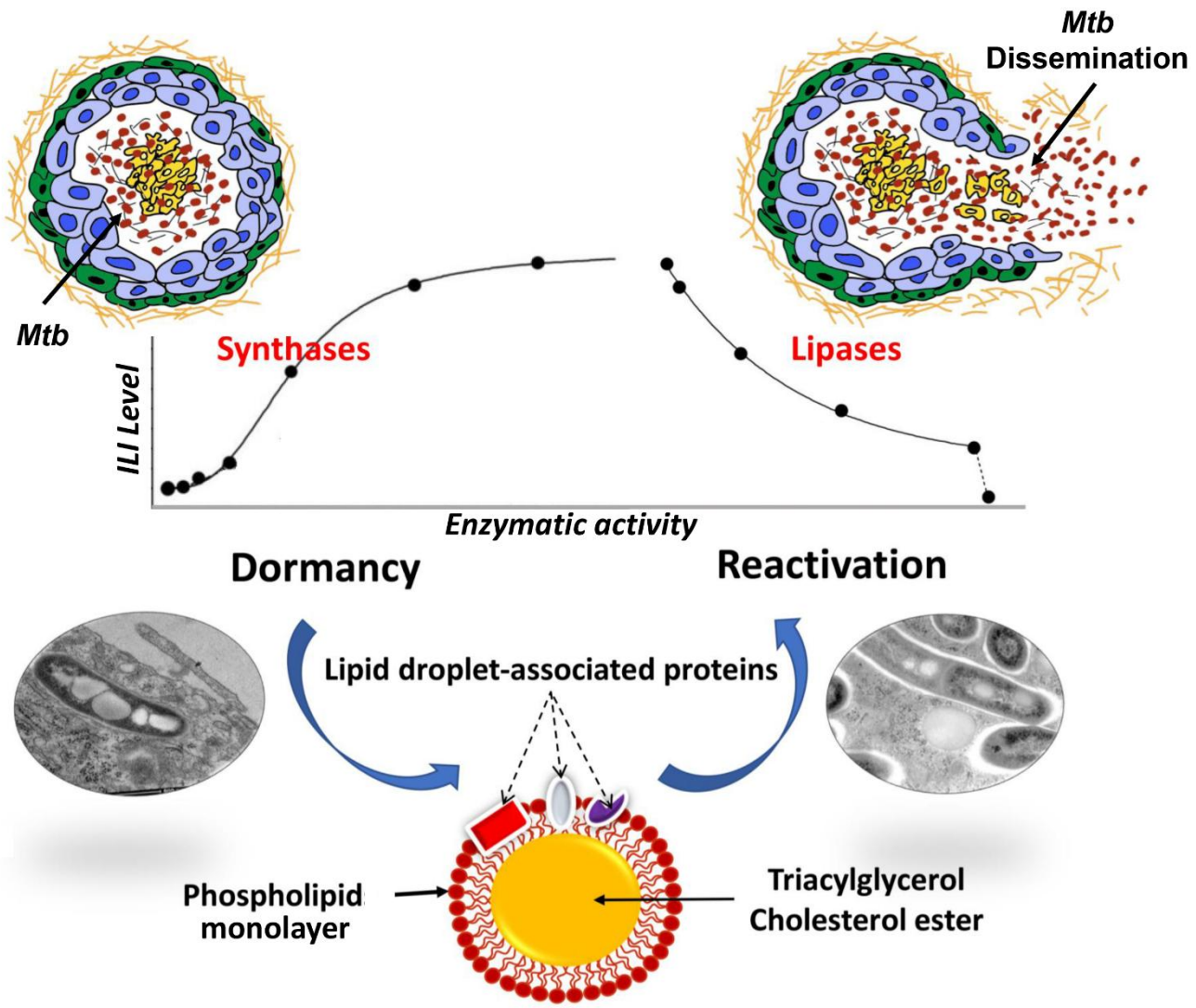

Figure 2: Scheme showing the role of synthases during ILI formation stage and the engagement of lipases during the ILI consumption stage, which are related to dormancy and reactivation, respectively. As observed in the electron microscopy picture on the right, total ILI consumption is not required for regrowth/reactivation/transmission. All electron and confocal microscopy pictures were from the authors' personal collections.

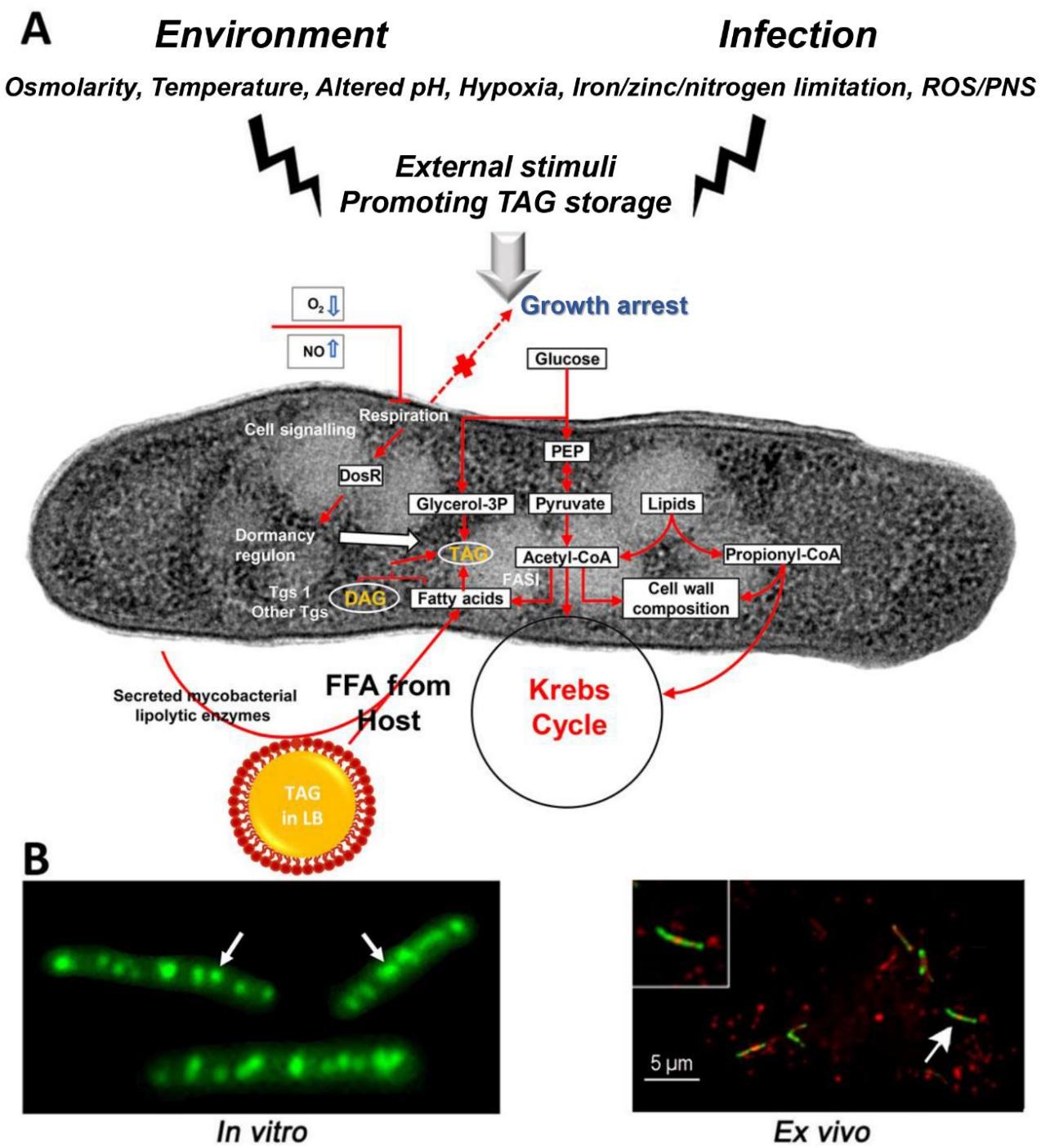

Figure 3: Different stimuli promoting ILI formation in mycobacteria are indicated by black arrows. **(A)** The metabolic pathway inducing TAG synthesis and their accumulation to form ILI is indicated inside bacteria. From the literature host FFA providing from TAG contained in LD are mainly due to the action of secreted mycobacterial lipolytic enzymes (Santucci, et al. 2018), **(B)** Confocal microscopic imaging of mycobacteria under ILI-promoting conditions using *in vitro* (left panel) and *ex vivo* (right panel) models. For the latter, BMDM were infected with the GFP-expressing *Mbv* BCG strain. Six days later, cells were exposed to VLDL for 24 hr. Cells were then treated with BODIPY (red label), which stains neutral lipids prior fixation and observation under the confocal microscope. After a 24 hr exposure to VLDL, both the macrophage and *Mbv* BCG (arrow and insert) contained BODIPY-stained granules (orange). All electron and confocal microscopy pictures were from the authors' personal collections.

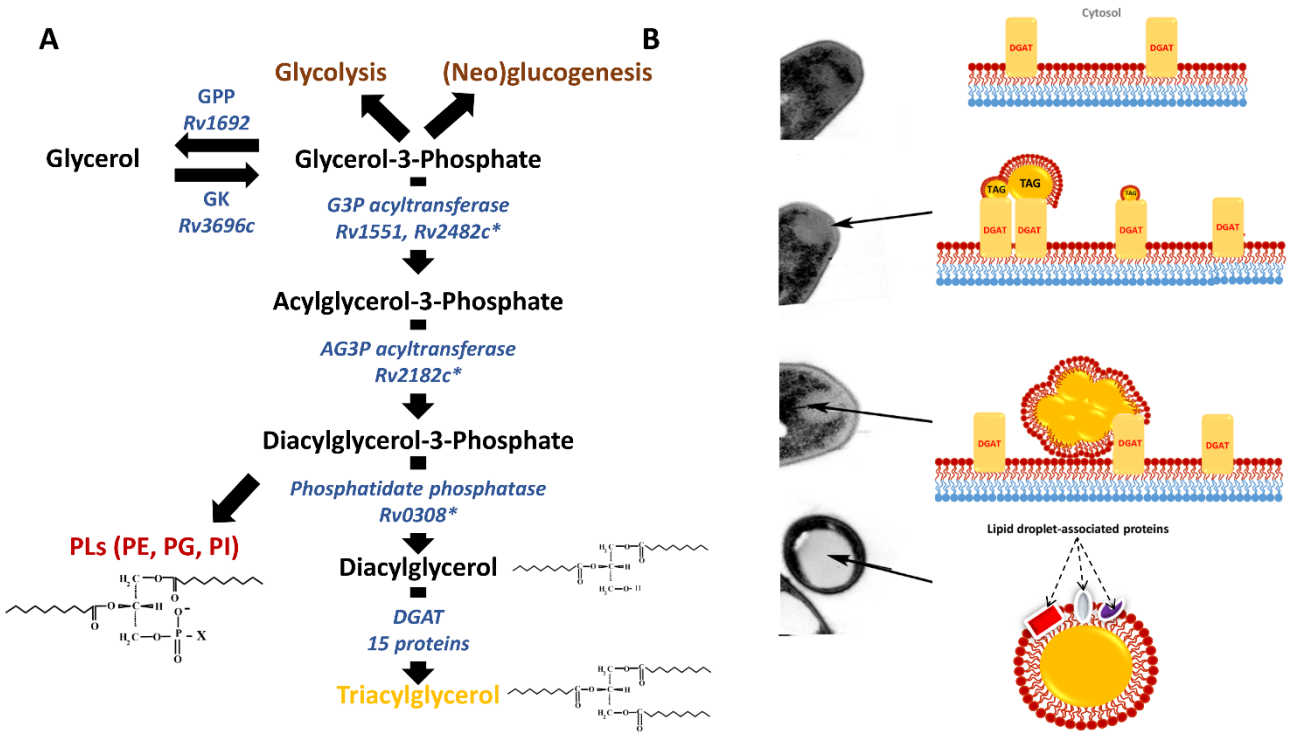

Figure 4: Synthesis of TAG into Actinobacteria. **(A)** Enzymes and substrates involved in TAG synthesis (Kennedy pathway), homolog in *M. tuberculosis* are indicated. **(B)** Scheme of lipid droplet formation based on the electron microscopy observation in *R. opacus* PD630 (adapted from (Wältermann and Steinbüchel 2005)). GPP: glycerol phosphate phosphatase (*Rv1692* (Larrouy-Maumus, et al. 2013)), GK: Glycerol kinase (*Rv3696c* (Maarsingh and Haydel 2018)), GPAT: Glycerol 3 Phosphate acyltransferase (*Rv1551* (Law and Daniel 2017), *Rv2482c*), PAP, Phosphatidate phosphatase (Rv0308*),* DGAT: Diacylglycerol acetyl transferase, PL: Phospholipids, X: Ethanolamine or Choline or Inositol, TAG Triacylglycerol. Star means putative.

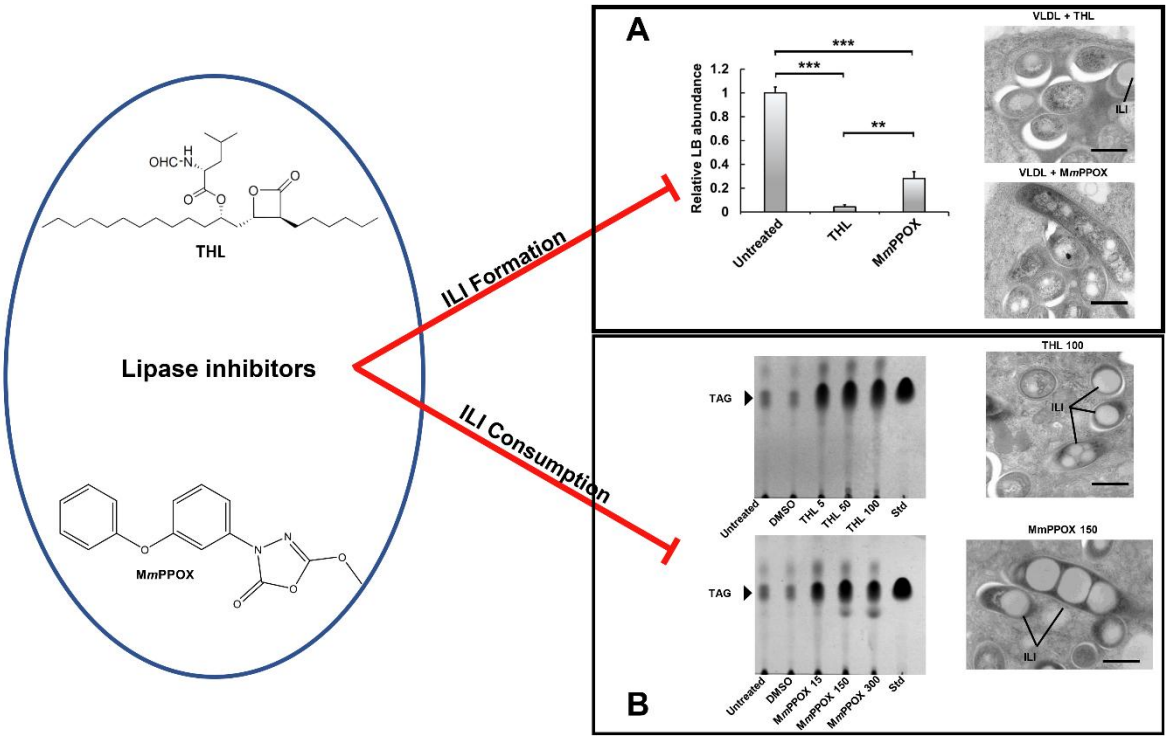

Figure 5: Pharmacological inhibition targeting lipolytic enzymes modulates ILI formation and consumption. Chemical structures of lipases inhibitors THL and M*m*PPOX are displayed on the left panel. On the right panel, a schematic representation displays their effects onto **(A)** ILI formation through enzymatic inhibition of host-cell proteins involved in LD biogenesis within the VLDL-induced FM model (Adapted from (Santucci, et al. 2018)) and **(B)** ILI breakdown *in vitro* and during macrophages infection. Electron micrographs and thin layer chromatography (adapted from (Santucci, et al. 2019a)) show the absence of TAG degradation in presence of THL or M*m*PPOX compounds. Scale bars represent 0.5 µm.