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

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**Short title:** ILI in mycobacterial lifecycle

**Keywords:** Metabolic adaptation, mycobacterial lipids, persistence, triacylglycerol, tuberculosis.

**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

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

35 **Abstract:**

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

54

55

## 56 **Introduction**

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

73 It is now clearly established that host-derived lipids such as triacylglycerols (TAG),  
74 cholesterol, phospholipids and free fatty acids (FFA) are being used for *Mtb* survival *in vivo*,  
75 by providing energy inside granulomatous lesions, therefore emphasizing the essential role  
76 of central lipid metabolism in mycobacterial long-term survival and persistence (Bloch and  
77 Segal 1956, Lee, et al. 2013, McKinney, et al. 2000, Nazarova, et al. 2017). During this  
78 specific phase, designated latent TB infection (LTBI), *Mtb* may harbour a wide range of  
79 phenotypes which is often dependent on the host-local immune state. However, very few  
80 bacilli are able to actively replicate and most of them are assumed to be in a slow/non-  
81 replicating state. This phenotypic behaviour is characterized by major and systematic  
82 physiological features such as low metabolic activity, bacterial division arrest, loss of acid  
83 fastness, increased antibiotics tolerance and presence of large amounts of TAG stored  
84 under the form of intracytosolic / intrabacterial lipid inclusions (ILI) (Caire-Brandli, et al.  
85 2014, Daniel, et al. 2016, Daniel, et al. 2011, Garton, et al. 2002, Garton, et al. 2008,  
86 Kapoor, et al. 2013, Santucci, et al. 2019a, Vilcheze and Kremer 2017).

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

90 through the lipolysis and the  $\beta$ -oxidation pathways. Numerous organisms (including some  
91 bacteria, parasites, plants and mammals) are able to synthesize TAG and to store them  
92 under the form of cytoplasmic granules (Murphy 2001, Murphy 2012). Over the years, these  
93 structures have been designated as lipid droplets (LD), lipid bodies, lipid inclusions,  
94 adiposomes, fat bodies or oil bodies (Daniel, et al. 2004, Walther and Farese 2009). In a  
95 homeostatic view, these organelles have been ascribed to regulate the storage of neutral  
96 lipids, remove toxic lipids, involved in cellular communication including stress response  
97 mechanisms as well as inflammatory processes (Monson, et al. 2021). In this review, the  
98 term LD refers to as the eukaryotic TAG inclusions while ILI designates the bacterial TAG  
99 inclusions. With respect to their chemical composition, TAG granules are essentially  
100 composed of neutral lipids (*i.e.*, TAG or sterol esters), surrounded by a phospholipid  
101 monolayer in which specific proteins, called lipid droplet-associated proteins, are inserted.  
102 To date, adipocytes, whose functions are mainly dedicated to the storage of neutral lipids in  
103 the form of LD within human body, are probably the best characterized cell type harboring  
104 TAG inclusions. In bacteria, species belonging to the *Rhodococcus* and *Mycobacterium*  
105 genus have been utilized as model systems to study TAG biosynthesis, ILI formation,  
106 storage and hydrolysis (Alvarez 2016, Santucci, et al. 2016, Wältermann and Steinbüchel  
107 2005).  
108 Nevertheless, with respect to *Mtb*, how the bacilli specifically realign their metabolism *in*  
109 *vivo*, use and store host-derived lipids under the form of ILI to persist in a non-replicating  
110 state and hydrolyse these lipids during reactivation, still remains poorly understood. The  
111 presence of such ILI has also been highlighted in several mycobacterial species including  
112 non-tuberculous mycobacteria (NTM), suggesting a conserved function of these lipid  
113 structures in the lifestyle of non-pathogenic, opportunistic and strict pathogens (Barisch, et  
114 al. 2015, Barisch and Soldati 2017, Santucci, et al. 2019a, Viljoen, et al. 2016) (**Figure 1**).  
115 In this review, we summarize the current knowledge and understanding of ILI composition  
116 and metabolic actors involved in their formation (synthases) and hydrolysis (lipases) with a  
117 special emphasis on mycobacterial species (**Figure 2**). We report how ILI have been  
118 discovered and how specific microenvironments govern their biosynthesis/mobilization. A  
119 non-exhaustive description of some of the recent approaches developed to study the  
120 dynamics of these processes in pathogenic mycobacteria is also presented. These include  
121 the development of experimental models, the use of specific classes of pharmacological  
122 inhibitors and biochemical and genetic strategies to delineate ILI biology. Finally, the recent

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

125

### 126 ***Historical discovery of TAG storage and intracytoplasmic bacterial lipid inclusions***

127 In prokaryotes, although ILI have been observed in both Gram<sup>+</sup> and Gram<sup>-</sup> bacteria, they  
128 remain a common characteristic of the *Actinobacteria* phylum (Alvarez and Steinbüchel  
129 2002). Indeed, many species belonging to the *Streptomyces*, *Nocardia*, *Dietzia*, *Gordonia*,  
130 *Rhodococcus* or *Mycobacterium* genus possess the capacity to synthesize and store TAG  
131 in the form of ILI (Alvarez 2016, Alvarez and Steinbüchel 2002). In mycobacterial species,  
132 ILI were first observed during the 1940s with the pioneering work of Burdon, Sheehan and  
133 Whitwell (Burdon 1946, Sheehan and Whitwell 1949). Using Sudan Black B, a non-  
134 fluorescent dye specific for neutral lipids (Hartman 1940), these authors demonstrated the  
135 presence of ILI within *in vitro* mycobacterial cultures, in samples isolates from infected  
136 guinea pigs, and also within sputum from TB patients (Burdon 1946, Sheehan and Whitwell  
137 1949). In the 1950s, the use of electron microscopy enabled, for the first time, imaging  
138 single bacterial cell at high-resolution, thereby revealing the presence of globular  
139 cytoplasmic structures in *Mycobacterium avium* (*Mav*) as well as in *Mycobacterium leprae*  
140 (Brieger and Glauert 1956a, Brieger and Glauert 1956b, Knaysi, et al. 1950). Both studies  
141 highlighted the heterogeneity within the population in term of bacterial size, shape and  
142 granule content. Interestingly, the authors stated that this type of globules were visible only  
143 after long incubation period in a rich culture medium, suggesting a co-relation between  
144 bacterial fitness and metabolism with the appearance of the granular structures. The first  
145 metabolic studies were carried out later on and demonstrated that supplementation of lipid  
146 substrates such as oleic acid (OA) or Tween-80 in the culture medium favoured the  
147 appearance of ILI in mycobacterial cells (Schaefer and Lewis 1965). At that time, the exact  
148 composition of the accumulated lipids was not established, and there was no mention  
149 regarding the exact phase of the bacterial cell cycle wherein these structures were visible  
150 (Schaefer and Lewis 1965). In the 1970s, the use of radiolabelled substrates, such as  
151 palmitic acid or OA, in *Mav* or *Mycobacterium smegmatis* (*Msmeg*) cultures provided  
152 evidence that the FFA incorporated within bacterial cells were mainly stored as TAG rather  
153 than simple FFA or phospholipids (Barksdale and Kim 1977, McCarthy 1971, Nakagawa, et  
154 al. 1976, Weir, et al. 1972). Using gas chromatography to analyse radiolabelled OA  
155 incorporated into TAG, two independent research groups demonstrated that modifications

156 in chain lengths had occurred (Nakagawa, et al. 1976, Weir, et al. 1972). Indeed, most of  
157 the stored TAG were composed of labelled C14:0, C16:0, C16:1, C18:1, C20:0 and C24:0  
158 long-chain fatty acids, suggesting that the lipid substrates can be either shortened or  
159 elongated. Moreover, upon OA treatment, the authors noticed that bacterial TAG  
160 accumulation was closely correlated with an increase in triacylglycerol synthase (TGS) (also  
161 known as diacylglycerol-acyltransferase; DGAT) activity, and that this phenomenon was  
162 exclusively restricted to the post-exponential or stationary growth phases (Nakagawa, et al.  
163 1976). Altogether, these early results favoured the emergence of new concepts regarding  
164 ILI biosynthesis and led to the assumption that the production of ILI might depend on  
165 various factors, in particular the composition of the culture medium as well as the  
166 physiological stage of the bacterial cell cycle.

167

### 168 ***Biophysicochemical factors impacting TAG anabolism and storage in vitro***

169 During their lifecycle, most bacterial species encounter unfavourable environments and are  
170 not only facing drastic modifications of surrounding physical and chemical factors but also  
171 important fluctuations in nutrient availability. To adapt and survive under such drastic  
172 conditions, bacteria have evolved metabolic strategies which include numerous biological  
173 pathways and specific molecular factors. In the context of TB, formation of pulmonary  
174 granulomatous lesions is one of the major hallmarks of *Mtb* infection. Within granulomas,  
175 the bacilli are exposed to various stresses, where the most significant ones are probably  
176 nutrient limitation (*i.e.*, carbon, nitrogen, iron starvation etc.), exposure to reactive  
177 oxygen/nitrogen intermediates including nitric oxide (NO) and oxygen depletion (Barry, et al.  
178 2009, Deb, et al. 2009, Honer zu Bentrup and Russell 2001, Timm, et al. 2003). These  
179 harsh conditions, alone or in combination, significantly alter the metabolic state and the  
180 fitness of the tubercle bacilli and, consequently, impact on TAG biosynthesis and  
181 accumulation (**Figure 3**), which are critical for *Mtb* survival during lung infection and anti-TB  
182 chemotherapy (Baek, et al. 2011, Garton, et al. 2008, Hammond, et al. 2015).

183

#### 184 *1) Specificity and bioavailability of carbon source*

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



189 MAG; diacylglycerol, DAG; TAG or steroids, *i.e.* cholesterol esters derivatives) can be  
190 converted into long or short chain FFA and then further imported and reprocessed by the  
191 tubercle bacilli (Bloch and Segal 1956, Kim, et al. 2010, Lee, et al. 2013, McKinney, et al.  
192 2000, Nazarova, et al. 2017). Interestingly, when cultured in a well-defined medium  
193 containing long-chain FFA or dextrose as the sole carbon source, ILI formation monitored  
194 by electron microscopy approaches revealed that TAG accumulation was visible only under  
195 lipid-rich conditions when *Mtb* H37Rv reaches stationary phase (Rodriguez, et al. 2014).  
196 This important result suggests that exogenous FFA, but not glucose, would be the stimulus  
197 responsible for TAG accumulation in *Mtb* and that the physiological mechanisms governing  
198 ILI formation in mycobacteria might be well conserved and dependent on the entry into the  
199 stationary phase. Importantly, conversion and storage of FFA into TAG appears as a  
200 fundamental process, which is conserved from bacteria to human. In particular, it is now  
201 well established that lipid droplet storage in eukaryotic cells is a dedicated biological  
202 mechanism that prevents lipotoxicity through excessive reactive oxygen species generation  
203 during FFA diet (Listenberger, et al. 2003).

204 Alternatively, when mycobacterial species such as *Msmeg* were exposed to a high  
205 concentration of glycerol and in absence of FFA as a lipid source, ILI accumulation was  
206 observed during the stationary phase and monitored by thin layer chromatography (TLC)  
207 and microscopic approaches (Armstrong, et al. 2016, Armstrong, et al. 2018, Garton, et al.  
208 2002, Santucci, et al. 2019a). The significant accumulation of ILI led the authors to propose  
209 that the FFA incorporated within TAG-forming inclusions was directly derived from *de novo*  
210 synthesis. This is of particular interest since it implies that the two distinct experimental  
211 designs mentioned above (*i.e.*, excess of exogenous FFA or high glycerol diet) are perfectly  
212 complementary highlighting the potential of easily applying these stimuli to modulate and  
213 characterize the pathways involved in ILI biosynthesis.

214

## 215 II) Nitrogen deprivation

216 Limitation of nitrogen has been well documented as another major cause triggering TAG  
217 biosynthesis and ILI formation (Alvarez, et al. 2000, Alvarez, et al. 1996, Garton, et al.  
218 2002, Santucci, et al. 2019a). First evidences were observed in *Streptomyces* sp. where the  
219 authors noticed an increase in the TAG content upon cultivation under nitrogen-deprived  
220 conditions (Olukoshi and Packter 1994). This was further confirmed with the *Rhodococcus*  
221 *opacus* strain PD630, capable to produce insoluble inclusions when grown in presence of

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

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

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

239 In fact, the biological pathways and specific role of putative conserved molecular factors  
240 involved in actinobacterial adaptation to nitrogen starvation have started to be investigated  
241 over the last decade (Amon, et al. 2008, Davila Costa, et al. 2017, Jessberger, et al. 2013,  
242 Williams, et al. 2015). Among them, the *nlpR* gene, which encodes a master regulator of  
243 lipogenesis upon nitrogen starvation in *Rhodococcus jostii* RHA1, has been recently  
244 identified as a key player in TAG metabolism (Hernandez, et al. 2017). This gene which is  
245 conserved in mycobacterial species such as *Msmeg* and *Mtb* (*MSMEG\_0432* and *Rv0260c*  
246 genes, respectively) has been demonstrated to be crucial in nitrogen assimilation processes  
247 (Jenkins, et al. 2013, Williams, et al. 2015). However, its direct implication in TAG  
248 biosynthesis/storage remains elusive. To date, only the mycobacterial PrrAB two-  
249 component system has been identified as a negative regulator directly linking nitrogen  
250 limitation and TAG accumulation in mycobacteria (Maarsingh and Haydel 2018).  
251 Interestingly, deletion of *prrAB* gene in *Msmeg* led to a significant overexpression of the  
252 genes encoding enzymes from the Kennedy pathway by which mammalian cells synthesize  
253 phospholipids and TAG for incorporation into membranes or lipid-derived signalling  
254 molecules, thus favouring TAG accumulation and storage (Maarsingh and Haydel 2018)  
255 **(Figure 4).**

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

261

### 262 III) Hypoxia

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

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

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

299

#### 300 IV) NO exposure

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

309 This NO stress experimental system has been used recently with the vaccine strain *Mbv*  
310 BCG to investigate the contribution of the heparin-binding hemagglutinin adhesin (HBHA) in  
311 ILI formation (Raze, et al. 2018). Analysis of the lipid content of *Mbv* BCG WT and  $\Delta hbhA$   
312 strains by fluorescence and electron microscopy, revealed that deletion of *hbhA* impairs ILI  
313 formation upon NO stress (Raze, et al. 2018), while re-introduction of *hbhA* in the mutant  
314 strain restored ILI accumulation to levels comparable to those found in the parental strain.  
315 This simple induction system, which mimics O<sub>2</sub> depletion, confirms that inhibition of aerobic  
316 respiration trigger FFA import and esterification onto TAG molecules inside mycobacteria.

317

#### 318 V) Iron starvation and oxidative stress

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

322 mechanism. It has been observed that low level of iron *in vivo* works as a stimulus for the  
323 expression of virulence genes in numerous microbial pathogens. In addition, several genes  
324 involved in lipid biosynthesis and accumulation are overexpressed in *Mtb* under iron  
325 starvation (Rodriguez, et al. 2002). In *Mtb* H37Rv, *in vitro* chemostat cultures under iron-  
326 poor conditions led to the accumulation of TAG and the production of an unidentified novel  
327 wax ester (WE) (Bacon, et al. 2007, Pal, et al. 2019). Iron depletion could also be achieved  
328 with deferoxamine mesylate salt, a powerful iron chelator. Using this approach, the authors  
329 demonstrated that *Mtb* H37Rv and several clinical strains were able to produce large  
330 amount of ILI (Vijay, et al. 2017). Since these specific starvation conditions are also known  
331 to induce an important oxidative stress in cells by increasing the production of radical  
332 oxygen species, when *Mtb* H37Rv and several clinical isolates were submitted to increasing  
333 concentration of H<sub>2</sub>O<sub>2</sub>, all strains accumulated TAG under the form of ILI. No additional  
334 information regarding this *in vitro* experimental design is available regarding NTM.

335

336 **Enzymatic pathways involved in TAG biosynthesis**

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

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

357

358 **Free fatty acids uptake and biogenesis**

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

370 were highly conserved and found in a wide panel of organisms, including *Caenorhabditis*  
371 *elegans*, *Drosophila melanogaster*, *Saccharomyces cerevisiae* and *Mtb* (Hirsch, et al.  
372 1998). Ectopic overexpression of FACL6/Rv1206 in COS cells revealed that this  
373 mycobacterial FATP was able to facilitate FFA uptake, suggesting that this molecular actor  
374 might play a key role in lipid import (Hirsch, et al. 1998). Another independent study showed  
375 that the purified protein displayed acyl-CoA synthetase activity *in vitro* and heterologous  
376 expression in *E. coli* promoted fatty acid uptake/utilization (Daniel, et al. 2014). Moreover,  
377 genetic disruption of the gene encoding FACL6 in *Mtb*, showed that this protein is required  
378 for optimal incorporation of radiolabeled exogenous fatty acids and the production of  
379 intracellular TAG during *in vitro* dormancy (Daniel, et al. 2014). However, these findings  
380 failed to directly demonstrate that FACL6 acts as fatty acid transmembrane transporter  
381 *stricto sensu*.

382 In their seminal work, Pandey and Sassetti clearly demonstrated that *Mtb* imports  
383 cholesterol across the cell envelope by using the multi-subunit Mce transporter, Mce4  
384 (Pandey and Sassetti 2008). Interestingly, *Mtb* genome contains four unlinked *mce* loci  
385 (*mce1-mce4*), which encode numerous proteins of unknown functions, nevertheless, the  
386 similarities among the 4 loci indicate that all of them might be transporters required to  
387 import hydrophobic molecules (Cole, et al. 1998, Ekiert, et al. 2017, Pandey and Sassetti  
388 2008). Recently, Nazarova and colleagues identified Mce1 as *Mtb*'s main machinery  
389 involved in fatty acid import *in vitro* and *in cellulo* (Nazarova, et al. 2017). They also showed  
390 that LucA/Rv3723, plays a central role in exogenous carbon acquisition by facilitating both  
391 fatty acid and cholesterol uptake across the cell envelope by stabilizing protein subunits of  
392 the Mce1 and Mce4 transporters, respectively (Nazarova, et al. 2017). As expected,  
393 deletion of *lucA* led to important growth defects within mouse and human macrophages,  
394 fitness reduction in infected mice and decrease in lung inflammation, highlighting the  
395 importance of this molecular determinant in *Mtb* pathogenesis.

396

### 397 ***Biosynthesis of LPA, PA and DAG precursors***

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

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

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

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



**439 TAG biosynthesis and ILI formation**

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

459 Similarly, in *Mabs* seven Tgs-encoding genes were identified based on the presence of the  
460 HHxxxDG motif, among which Tgs1 was also found to be most active protein (Viljoen, et al.  
461 2016). Site directed mutagenesis revealed that His144 and Gln145 are essential for  
462 enzymatic activity of Tgs1 in this species. Tgs1 could use a range of acyl-CoAs as fatty acyl  
463 donors however it is more active in presence of palmitoyl-CoA (Viljoen, et al. 2016). In an  
464 independent study, it was shown the heterologous overproduction of *Mtb* mycolyltransferase  
465 Ag85A in *Msmeg* led to altered cell morphology, impaired division and the formation of ILI  
466 suggesting a possible role of Ag85A in TAG metabolism and ILI biosynthesis (Elamin, et al.  
467 2011).

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

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

477

#### 478 ***ILI-associated proteomes, structural proteins and anchoring properties***

479 The first isolation of ILI in Actinobacteria was achieved by phase separation following  
480 centrifugation in density gradients (Alvarez, et al. 1996), a simple and efficient protocol  
481 which had been previously developed to purify polyester(s) granules from *Pseudomonas*  
482 *oleovorans* (Preusting, et al. 1993). After isolation, samples were analysed by  
483 polyacrylamide gel electrophoresis, and the presence of numerous ILI-associated proteins  
484 was observed suggesting that ILI are not only constituted by lipid material (Alvarez, et al.  
485 1996).

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

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

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

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

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

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

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

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

548 Upon characterisation of the ILI-associated proteome in *Rhodococcus*, Ding et al., identified  
549 another molecular actor that was overrepresented at the surface of ILI (Ding, et al. 2012).  
550 This protein named PspA is homolog to the phage shock protein A of *E. coli* which is  
551 involved the plasma membrane maintenance and homeostasis under stress (Joly, et al.  
552 2010). Surprisingly, deletion of *pspA* resulted in the formation of supersized ILI and an  
553 opposite phenotype was reported upon inactivation of its mycobacterial ortholog  
554 *pspA/MSMEG\_2695* in *Msmeg* (Armstrong, et al. 2016, Ding, et al. 2012). Despite these  
555 conflicting observations, this clearly demonstrates that PspA is an important protein in ILI  
556 homeostasis (Armstrong, et al. 2016, Ding, et al. 2012).

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

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

588

589

590 ***TAG breakdown and pharmacological inhibition of lipolysis***

591 The presence of ILI in mycobacterial cells, and more particularly *Mtb*, has been defined as  
592 one of the hallmarks of non-replicating persistent bacilli *in vitro* and *in vivo* (Daniel, et al.  
593 2011, Garton, et al. 2002, Garton, et al. 2008). These organelles are considered as one of  
594 the main strategies employed by mycobacteria to adapt and survive within drastic  
595 environmental conditions by providing a constant and sufficient amount of energy over long  
596 period of time.

597 Utilization of the energy stored in TAG requires hydrolysis by hydrolases and lipases to  
598 release free fatty acids which in turn are catabolized by  $\beta$ -oxidation (Menendez-Bravo, et al.  
599 2017). Depending on their nature and substrate specificity, lipolytic enzymes are commonly  
600 divided in four classes such as i) carboxylesterases acting on small and partially water-  
601 soluble carboxylester substrates; ii) true lipases hydrolyzing water-insoluble substrates such  
602 as MAG, DAG and TAG molecules; iii) phospholipases, acting on phospholipid substrates  
603 and iv) cutinase-like proteins consisting of a much more versatile family of enzymes able to  
604 degrade a wide range of substrate including carboxylesters, TAG, phospholipids, as well as  
605 cutin (Delorme, et al. 2012, Masaki, et al. 2005).

606 In order to constantly provide energy upon long-term persistence or during reactivation,  
607 degradation of mycobacterial TAG must be initiated by specific proteins displaying TAG-  
608 lipase activity. Pioneer work by Deb *et al.*, reported the presence of the *Mtb* Pro-Glu (PE)  
609 protein, encoded by *Rv3097c* and named LipY, which has a C-terminal domain with  
610 homology to the hormone-sensitive lipase family and containing the conserved GDSAG  
611 motif (Deb, et al. 2006). Biochemical characterization demonstrated that LipY displays TAG  
612 hydrolase activity *in vitro* (Deb, et al. 2006). Generation of a *lipY* deficient mutant, showed  
613 that TAG utilization upon carbon starvation was significantly decreased, indicating that LipY  
614 plays an important role for the hydrolysis of stored TAG. This was further confirmed by  
615 heterologous expression in *Msmeg*. However, since this latter species does not encode the  
616 ESX-5 secretion system, that allow the secretion of LipY, these results have to be  
617 considered with caution (Mishra, et al. 2008, Santucci, et al. 2019b). Interestingly, multiple  
618 comparison of full-length LipY with a recombinant variant shortened by its N-terminal PE  
619 domain (LipY( $\Delta$ PE)), revealed that full-length LipY exhibited reduced TAG-hydrolase  
620 activity, suggesting a negative modulation of the activity of LipY by its N-terminal PE  
621 domain (Garrett, et al. 2015, Mishra, et al. 2008, Santucci, et al. 2018, Santucci, et al.  
622 2019b). Enzymatic characterization of LipY showed that this protein also displays DAG-  
623 hydrolase activity, implying that this enzyme may also be involved in another essential step

624 upon TAG breakdown (Santucci, et al. 2019b). In addition to LipY, other *Mtb* enzymes have  
625 been reported to display true lipase activity. Among them Rv1984c/Cfp21, which belongs to  
626 the cutinase-like family proteins, is able to hydrolyze different substrate including medium-  
627 chain carboxylic esters, MAG and TAG (Dedieu, et al. 2013, Schué, et al. 2010).  
628 Interestingly, the putative bifunctional long chain acyl-CoA synthase-lipase  
629 (BCG1721/Rv1683) has been showed to be involved in TAG degradation (Low, et al. 2010).  
630 This enzyme possesses a putative N-terminal lipase and a C-terminal ACSL domain,  
631 expressing a TAG-lipase activity when overproduced in *Mbv* BCG or in yeast, and this  
632 activity was abolished when S150A a point mutation was introduced in the predicted lipase  
633 catalytic site (Low, et al. 2010). A previously uncharacterized membrane-associated protein,  
634 annotated as Rv2672/Msh1, was reported to express both lipase and protease activities.  
635 This protein, which was mainly conserved among actinomycetes, is required for host-TAG  
636 hydrolysis, albeit no investigation regarding intrabacterial lipid consumption was done  
637 (Singh, et al. 2017). *Mtb* also encodes one monoacylglycerol lipase named Rv0183 which  
638 converts monoacylglycerol into free glycerol and fatty acid, which can be further used as a  
639 source of energy (Côtés, et al. 2007). Genetic knock-out of *MSMEG\_0220*, the ortholog of  
640 *Rv0183* in *Msmeg*, triggers drastic changes in morphology and resulted in important  
641 antibiotic susceptibility modifications (Dhouib, et al. 2010) but no information is available  
642 regarding ILI metabolism. Altogether, this shows that *Mtb* possesses numerous enzymes  
643 required for TAG breakdown and generation of FFA but more investigation is required to  
644 comprehensively decipher their respective involvement. Two main experimental strategies  
645 have been used *in vitro* to better apprehend the process of lipid consumption in  
646 mycobacteria. The first one, is based on a simple series of events and mimics fluctuations  
647 from a nutrient-limiting to a nutrient-replete condition. Indeed, once the level of any limiting  
648 chemical element, metabolites and/or energetic substrates raises again, bacterial cells  
649 initiate a global metabolic reprogramming and rapidly start consuming stored TAG in order to  
650 fuel their regrowth. Such an experimental model was used by Low and colleagues to  
651 investigate the contribution of ILI hydrolysis during regrowth from hypoxia-induced  
652 dormancy (Low, et al. 2009).  
653 By monitoring bacterial regrowth, the authors demonstrated that ILI mobilization was  
654 required to support *Mbv* BCG regrowth during the transition from hypoxia to normoxia (Low,  
655 et al. 2009). Interestingly, Orlistat (Tetrahydrolipstatin; THL), a serine-hydrolase inhibitor  
656 known for covalently binding and inhibiting mammalian and bacterial lipolytic enzymes as  
657 well as fatty acid synthases, was able to prevent the degradation of TAG and to impair *Mbv*

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

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

676 The second experimental model mainly relies on carbon starvation, which allows to  
677 artificially trigger TAG consumption. This approach has been widely used to investigate the  
678 mechanisms and dynamics of ILI hydrolysis by first developing an image-based quantitative  
679 method to assess lipolysis and ILI dynamics by time-lapse fluorescence microscopy at the  
680 single-bacterial cell level. This specific system combined with a microfluidic disposal  
681 allowed to monitor intracellular bacterial lipid levels over time with a high temporal  
682 resolution (Dhouib, et al. 2011). In this experimental system, upon carbon deprivation using  
683 PBS-induced starvation, *Msmeg* rapidly hydrolyses the stored TAG, which results in a rapid  
684 consumption of ILI; and this lipolytic activity was abolished in the presence of THL, as  
685 observed by fluorescence microscopy and TLC analysis (Dhouib, et al. 2011). More  
686 recently, using an *in vitro* model based on nitrogen starvation followed by carbon  
687 deprivation, the relative rate of ILI consumption in *Msmeg* and *Mabs* species was  
688 determined (Santucci, et al. 2019a). Moreover, heterologous overexpression of the well-  
689 characterized *Mtb* lipase LipY under these conditions led to a significant decrease in TAG  
690 content in *Msmeg* (Daleke, et al. 2011, Deb, et al. 2006, Mishra, et al. 2008, Santucci, et al.  
691 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 MmPPOX 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  $\beta$ -oxidation, releasing acetyl-CoA and the reduced co-factors NADH and  
715 FADH<sub>2</sub> 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 through 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

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

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

### 730 **Biological systems to study ILI metabolism within host cells**

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

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

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

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

767 The first investigations in human immune cells were performed by using an *in vitro* model of  
768 mycobacterial granulomas (Peyron, et al. 2008, Puissegur, et al. 2004). In this biological  
769 system, peripheral blood mononuclear cells (PBMCs) isolated from the blood of healthy  
770 donors were infected with *Mtb* for 3 or 11 days and further processed for conventional  
771 electron microscopy (Peyron, et al. 2008). This approach showed how *Mtb* promotes LD  
772 formation in *in vitro* human granulomas, but also allowed to describe the fusion events  
773 between host-LD and the mycobacterium-containing vacuole. Such observations were also  
774 correlated with the presence of ILI, suggesting that ILI formation in *Mtb* might derived from  
775 the acquisition of lipids contained within host-LD (Peyron, et al. 2008). Complementary  
776 experiments within PBMC-derived macrophages showed that, over time, the bacterial  
777 burden within FM was significantly lower in comparison to the one in lipid-poor cells.  
778 Transcriptomic analysis revealed that *dosR*, *icl* and *tgs1* genes were massively upregulated  
779 during infection of FM, suggesting that foamy PBMCs may provide a niche for *Mtb* long-  
780 term persistence in a lipid-rich non-replicating state (Peyron, et al. 2008). Interestingly,  
781 these observations were further confirmed in both human monocytic cell line THP-1 and  
782 PBMC-derived macrophages exposed to 1% of O<sub>2</sub> (Daniel, et al. 2011). In such a hypoxia-  
783 based cellular model, the authors used exogenous isotopically labelled FFA followed by  
784 TLC analysis to report that *Mtb* assimilated host-derived TAG as a lipid source which was  
785 subsequently used to build up ILI in a *tgs1*-dependent manner (Daniel, et al. 2011).  
786 However, one cannot exclude that some of the exogenous lipids were directly transferred to  
787 the bacteria and might impact ILI formation in this experimental model.

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

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

794 To date, this close relationship between loss of acid fastness, low metabolic activity, lipid  
795 accumulation and antibiotic susceptibility is still not well understood (Vilcheze and Kremer  
796 2017). Although the key players related to acid fast property of mycobacteria remain largely  
797 unexplored, the association of mycolic acids and other cell wall-associated (glyco)lipids with  
798 acid fast property of *Mtb* was reported, essentially based on genetically defined mutants  
799 found to be attenuated in mice. The approach of this study will not only help improving  
800 detection of dormant bacilli but also beneficial for future vaccine developments (Vilcheze  
801 and Kremer 2017). In addition to Tgs1, the  $\beta$ -ketoacyl-acyl carrier protein synthase KasB,  
802 involved in mycolic acid synthesis, could be one of the major actors involved in loss of acid  
803 fastness and long-term persistence (Bhatt, et al. 2007, Vilcheze and Kremer 2017,  
804 Vilcheze, et al. 2014). Recently, long-term live-cell imaging of *Mtb*-infected human  
805 monocytes-derived macrophages allowed to monitor LD content at the single-cell level, and  
806 confirmed that *Mtb* replication correlated a decrease in intracellular TAG, suggesting that  
807 LD-contained lipids serve as an important carbon source during infection (Greenwood, et al.  
808 2019). Unexpectedly, by combining light, electron and ion microscopy imaging, the authors  
809 not only demonstrated that the anti-tubercular antibiotic bedaquiline accumulates within  
810 host LD, but also that host-lipid consumption is a dynamic process that enhanced the  
811 delivery of bedaquiline within *Mtb* (Greenwood, et al. 2019). The cumulative results in  
812 human primary cells clearly strengthen the fact that the intricate interplay between  
813 mycobacterial and host lipid metabolism plays a crucial role in the mycobacterial lifecycle,  
814 antibiotic susceptibility and treatment outcomes (Agarwal, et al. 2020).

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

825 2018). In this biological system, the use of exogenous FA combined with the fluorescent  
826 lipid BODIPY-palmitate demonstrated that exogenous lipids can be acquired by the bacteria  
827 during macrophage infection (Podinovskaia, et al. 2013). The Mce1/LucA machinery was  
828 identified as a key factor in lipid acquisition (Nazarova, et al. 2017). In the presence of the  
829 fluorescent lipid BODIPY-palmitate, flow cytometry combined with fluorescence and  
830 electron microscopy approaches demonstrated that a *Mtb*  $\Delta$ lucA::hygR mutant was unable  
831 to utilize FFA and cholesterol during BMDM infection, and consequently to accumulate ILI  
832 (Nazarova, et al. 2017). The authors further infected BMDM with a mCherry fluorescent  
833 transposon mutant library, then pulse-labelled the infected cells with the latter fluorescent  
834 substrate and measured bacterial incorporation of BODIPY-palmitate by flow cytometry to  
835 identify which alternative *Mtb* genes are involved in FFA uptake and ILI formation  
836 (Nazarova, et al. 2019). Intracellular bacteria with BODIPY-low level were isolated and  
837 plated on selective agar. Sequencing of the transposon insertion sites confirmed the  
838 important role for the Mce1/LucA transporter but also allowed identifying new genes  
839 involved in FFA import/metabolism such as *rv0966c*, *rv0200/omamB*, *rv3484/cpsA* or  
840 *rv2583c/reIA* (Nazarova, et al. 2019). Such findings clearly support the idea that ILI  
841 formation derives from the dynamic acquisition of host-lipids. It is however important to  
842 specify that direct acquisition of labelled lipids by *Mtb* cannot be ruled out and might have  
843 also occurred in that biological system.

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

859 sharply decreased, suggesting an increase in lipolysis that was correlated with a  
860 simultaneous resumption of mycobacterial division (Caire-Brandli, et al. 2014). This *in vitro*  
861 FM model was subsequently used to investigate how *Mbv* BCG acquires host lipids in the  
862 phagosomal lumen (Santucci, et al. 2018). Deletion and overexpression of the *lipY* gene,  
863 encoding the well-characterized mycobacterial TAG-lipase, revealed that, while LipY plays  
864 an important role in extra-bacterial lipid consumption, this lipase is not essential for ILI  
865 formation within FM (Santucci, et al. 2018). In addition to slow- growing mycobacteria, this  
866 model has also been used to investigate the intracellular behaviour of the emerging  
867 pathogen *Mabs* within FM (Viljoen, et al. 2016). The authors demonstrated that *Mabs* is  
868 able to interact with host-LD and synthesize ILI in a Tgs1-dependent manner (Viljoen, et al.  
869 2016).

870 Alternatively, the dynamics of host-lipid consumption and accumulation under the form of ILI  
871 was studied during the infection of the amoeba *Dictyostelium discoideum* with the fish  
872 pathogen *Mycobacterium marinum* (*Mmar*) (Barisch, et al. 2015, Barisch and Soldati 2017).  
873 This system, based on the exogenous addition of FFA to promote lipid-rich phenotype and  
874 initially used to identify new proteins involved in LD synthesis within the amoeba (Du, et al.  
875 2013) allowed to monitor in real-time the interaction of host LD with the mycobacterium-  
876 containing vacuole by using fluorescence microscopy techniques (Barisch, et al. 2015).  
877 Following this approach, the authors showed that perilipin proteins were able to coat  
878 cytosolic bacteria in a RD1-dependent manner regardless of the presence or the absence  
879 of exogenous FFA (Barisch, et al. 2015). Similar results were observed with the eukaryotic  
880 TAG synthase Dgat2, but not Dgat1 (Barisch and Soldati 2017). Upon FFA treatment, TAG  
881 production was totally abolished in a *Dictyostelium* mutant lacking both *dgat1* and *dgat2*,  
882 but this loss was accompanied with an significant increase in endoplasmic reticulum (ER)  
883 membrane proliferation (Barisch and Soldati 2017). Surprisingly, the authors showed that,  
884 under these specific conditions, *Mmar* was still able to synthesize its own ILI by depleting  
885 the ER-like structures and using host phospholipids as main substrates (Barisch and Soldati  
886 2017). Finally, they demonstrated that *Mmar* remained metabolically active and acid-fast  
887 positive in that biological system. This suggests that some mechanisms linking lipid  
888 accumulation, cell-wall alterations and persistence might be different among mycobacterial  
889 species and require to be further investigated.

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

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

899  
900

### 901 ***Beneficial role of ILI metabolism in the mycobacterial lifecycle?***

#### 902 *1) ILI, carbon source and mycobacterial metabolism*

903 Although LD and ILI have been considered as “just fat” organelles for decades, it is now  
904 well acknowledged that the biological function of these complex structures goes beyond  
905 that simplistic view (Cabruja, et al. 2017, Deb, et al. 2009, Henne 2019, Martinot, et al.  
906 2016). In the context of chronic mycobacterial infection, accumulation of TAG within ILI has  
907 been proposed to serve as a major source of carbon and energy in non-replicating bacteria  
908 (Daniel, et al. 2011, Garton, et al. 2002, Garton, et al. 2008, Peyron, et al. 2008) Indeed,  
909 slow and long-term TAG breakdown in the absence of exogenous FFA or *de novo* synthesis  
910 provide an important acetyl-CoA pool *via*  $\beta$ -oxidation, which can be rerouted to other  
911 metabolic pathways or to sustain ATP production and plasma membrane and cell wall  
912 integrity (Cabruja, et al. 2017, Deb, et al. 2009, Martinot, et al. 2016). However, the use and  
913 combination of several experimental models have recently shed new insights into additional  
914 roles of these dynamic organelles in bacteria. Among these alternative functions, ILI  
915 formation has appeared essential to limit the reductive stress and lipotoxicity generated by  
916 excess of endogenous FFA (Rodriguez, et al. 2014). During infection, the biosynthesis of  
917 TAG and phthiocerol dimycocerosates have been shown to be essential for limiting  
918 metabolic stress (Lee, et al. 2013). Similar results have been found in primary fibroblast  
919 cells and yeasts where TAG biosynthesis is essential to prevent lipotoxicity and cell death  
920 (Garbarino, et al. 2009, Garbarino and Sturley 2009, Listenberger, et al. 2003, Petschnigg,  
921 et al. 2009). This implies that, like the eukaryotic LD, ILI might also act as conserved  
922 organelles involved in the storage of toxic fatty acids, which must be sequestered during  
923 specific conditions to prevent the irreversible damages for the cell (Wältermann and  
924 Steinbüchel 2005). Alternatively, storage of evaporation-resistant lipids has been proposed  
925 as a bacterial strategy to overcome water deficiency under dehydration, where oxidation  
926 would be one of the main mechanisms to produce water molecules (Alvarez, et al. 2004).

927 Recently, it has been demonstrated that ILI contained within the saprophytic strain  
928 *Rhodococcus jostii* RHA1 are able to bind to genomic DNA through the action of one ILI-  
929 associated protein (Zhang, et al. 2017). Indeed, the authors showed that the protein  
930 RHA1\_Ro02105, homolog of the mycobacterial HBHA, known for its role in ILI formation  
931 when localized intracellularly (Raze, et al. 2018), was able to bind and stabilize DNA, thus  
932 enhancing bacterial survival under genotoxic stresses (Zhang, et al. 2017). Interestingly,  
933 interactions of LD with nucleic acid or histone proteins have also been reported in  
934 mammalian cells (Layerenza, et al. 2013, Uzbekov and Roingeard 2013). Despite the fact  
935 that the biological significance of these interactions remains obscure, one can hypothesize  
936 that such processes could be highly conserved and directly involved in DNA maintenance  
937 and/or gene expression modulation.

938

#### 939 II) *ILI and disease progression*

940 Another beneficial role of ILI is provided by the observation of lipid-rich bacilli in sputum  
941 from TB-positive individuals originated from Gambia and the United-Kingdom, which raised  
942 the hypothesis that these structures might play a key role in the transmission of the tubercle  
943 bacilli (Garton, et al. 2008). Thus, ILI may favour bacterial survival during the transit from an  
944 individual to another and may also help the bacilli to more easily adapt to the environmental  
945 pressure encountered in a new host (Garton, et al. 2008). These speculations are also in  
946 line with findings regarding the Beijing family of *Mtb* strains (sub-branch of lineage 2 also  
947 known as the East Asian lineage), which has been associated with increased epidemic  
948 spread and reported to constructively produce high amounts of TAG (Domenech, et al.  
949 2017, Reed, et al. 2007, Tong, et al. 2020). To investigate whether ILI accumulation may  
950 confer a significant advantage in the establishment and outcomes of infection, zebrafish  
951 embryos were infected with lipid-rich and lipid-poor *Mabs* (Santucci, et al. 2019a).  
952 Comparison of the bacterial loads, inflammatory response and larval survival indicated that  
953 the lipid-rich persistent-like phenotype favoured bacterial replication and significantly  
954 increased the number of granulomatous lesions per fish which is in agreement with a  
955 previous study obtained with non-replicating *Mtb* in CB6F1 mice (Dietrich, et al. 2015,  
956 Dietrich, et al. 2016, Santucci, et al. 2019a). This was also correlated with an earlier larval  
957 mortality, suggesting that pre-existing ILI might confer a specific advantage when colonizing  
958 a new host. However, more investigations are required to better understand these important  
959 physiopathological aspects. Several studies emphasized that both host and bacterial TAG  
960 metabolism are a critical factor influencing and determining the outcome of the infection



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

967

### 968 *III) ILI and antibiotic efficacy*

969 The presence of ILI in mycobacteria has been also reported to drastically impact antibiotic  
970 susceptibility which is crucial in the context of bacterial infection. Indeed, non-replicating  
971 bacteria with reduced metabolic activity are more tolerant to antibiotic treatments (Baek, et  
972 al. 2011, Deb, et al. 2009, Hammond, et al. 2015, Kapoor, et al. 2013, Santucci, et al.  
973 2019a). By using an *in vitro* multiple-stress dormancy model, Deb and colleagues showed  
974 that specific microenvironments drive the appearance of lipid-loaded, rifampicin and  
975 isoniazid-tolerant phenotypes (Deb, et al. 2009). Another complementary study  
976 demonstrated that rerouting the carbon flux and TAG synthesis influence both growth rate  
977 and antibiotic susceptibility *in vitro* and *in vivo* (Baek, et al. 2011). This was further  
978 confirmed by Hammond and colleagues who clearly established a link between the  
979 intrabacterial lipid content and drug tolerance in species belonging to the *Mtb* complex as  
980 well as in NTM (Hammond, et al. 2015). By discriminating old/young and lipid-rich/poor  
981 bacteria, this study raised an interesting concept suggesting that the age of the culture or  
982 the growth rate are not always linked to increased tolerance (Hammond, et al. 2015).

983

### 984 **Concluding remarks and perspectives**

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

993

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1002

1003 **Authors contribution**

1004 PS and SC proposed and conceptualized this work. IM, PS and SC wrote the draft of the  
1005 manuscript. LK and JFC completed and revised the entire manuscript. IP, VP and LK  
1006 provided light and electron microscopy micrographs and helped IM and SC in figures  
1007 editing. All authors listed have made a substantial, direct and intellectual contribution to the  
1008 work which was supervised by SC. All authors approved the manuscript for publication.

1009

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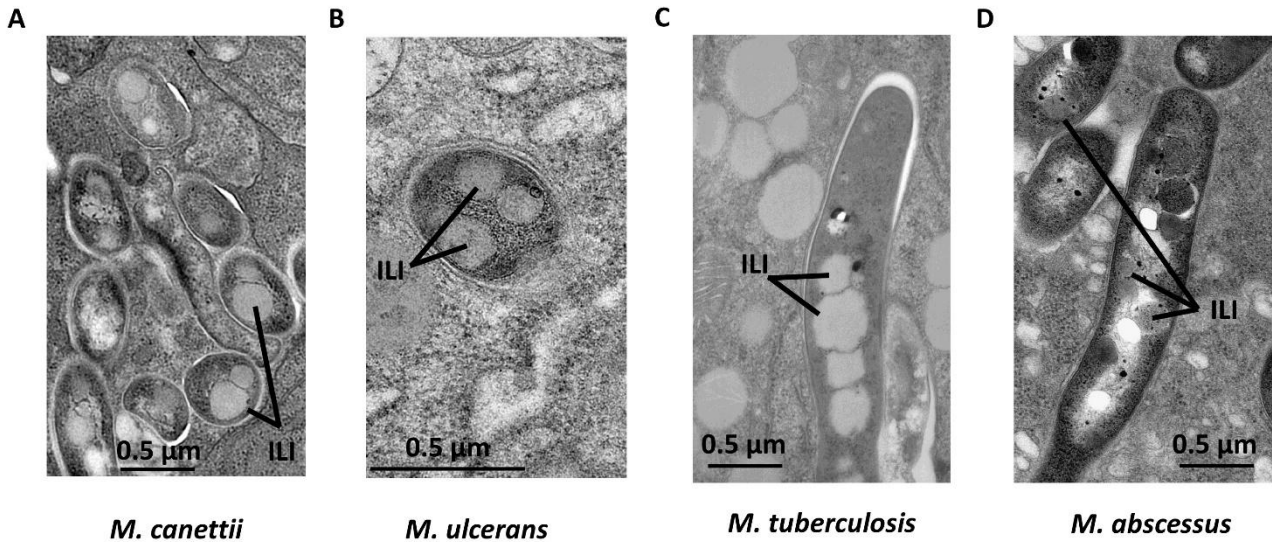
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1382 **Figures and Figure legends**

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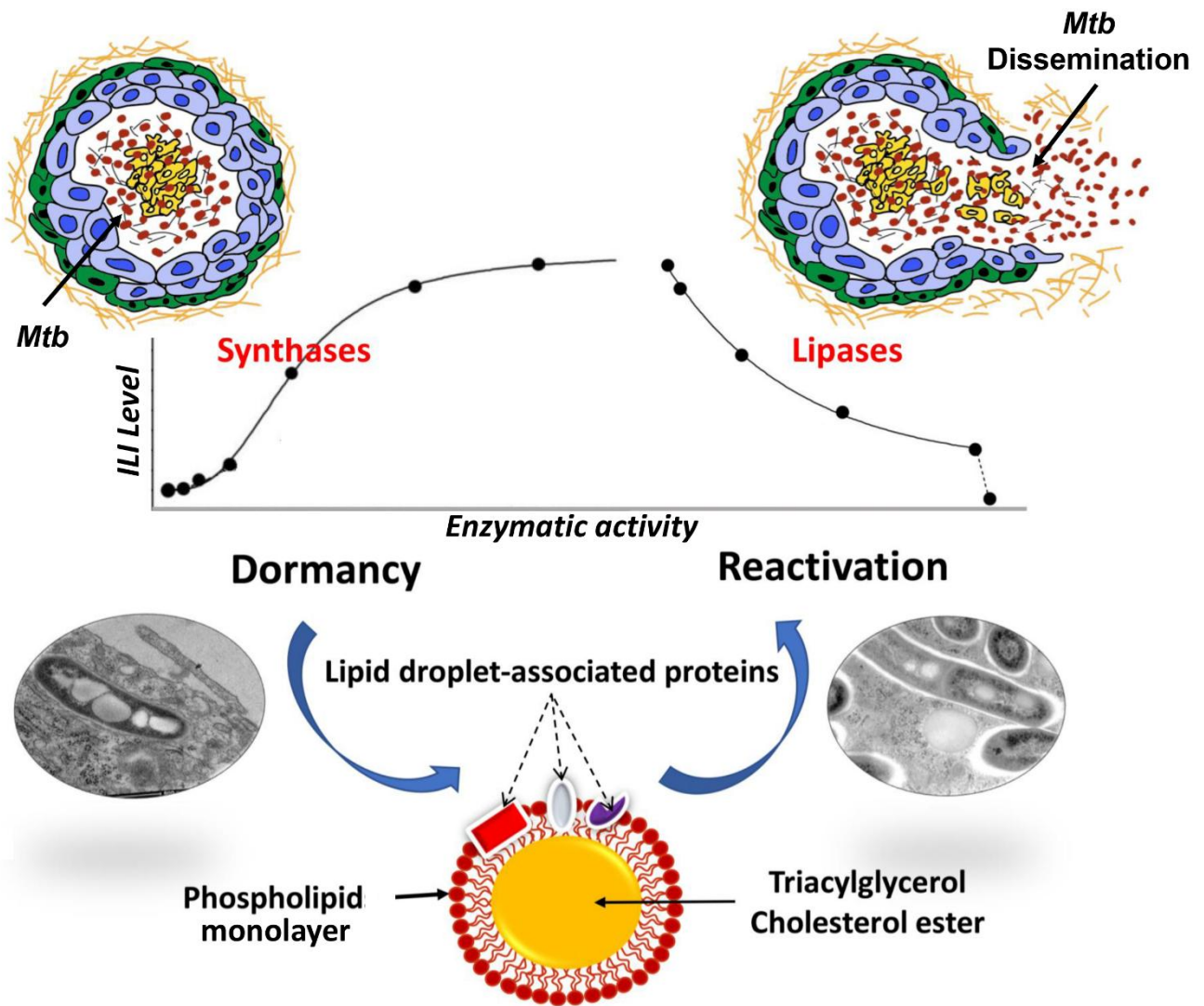
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1387 **Figure 1:** Electron microscopy images of ILI in various intracellular pathogenic  
1388 mycobacteria inside different hosts. **(A)** 3T3-L1 mature adipocytes infected with *M. canettii*.  
1389 **(B)** Mouse tail tissue infected with *M. ulcerans*. **(C)** *Mtb*-infected cell from a sputum sample  
1390 from a TB-positive patient. **(D)** *M. abscessus*-containing macrophage in a zebrafish embryo.  
1391 ILI are indicated with black lines and scale bars represent 0.5 μm. All electron microscopy  
1392 micrographs depicted are from the authors' personal collections.  
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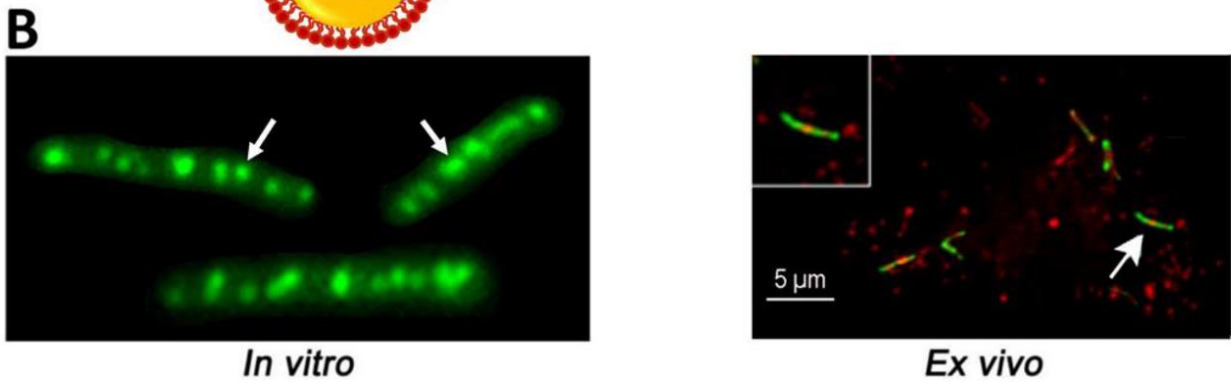
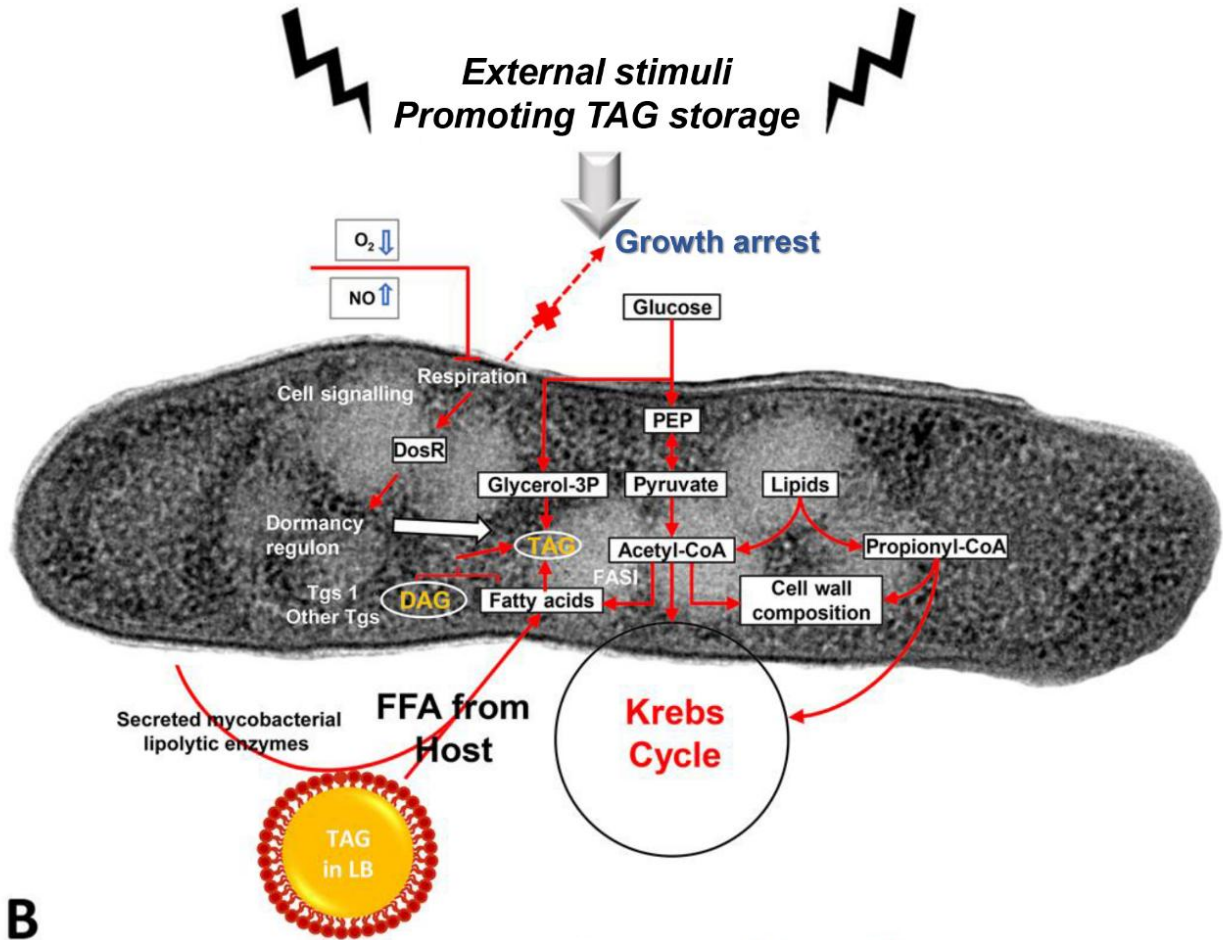


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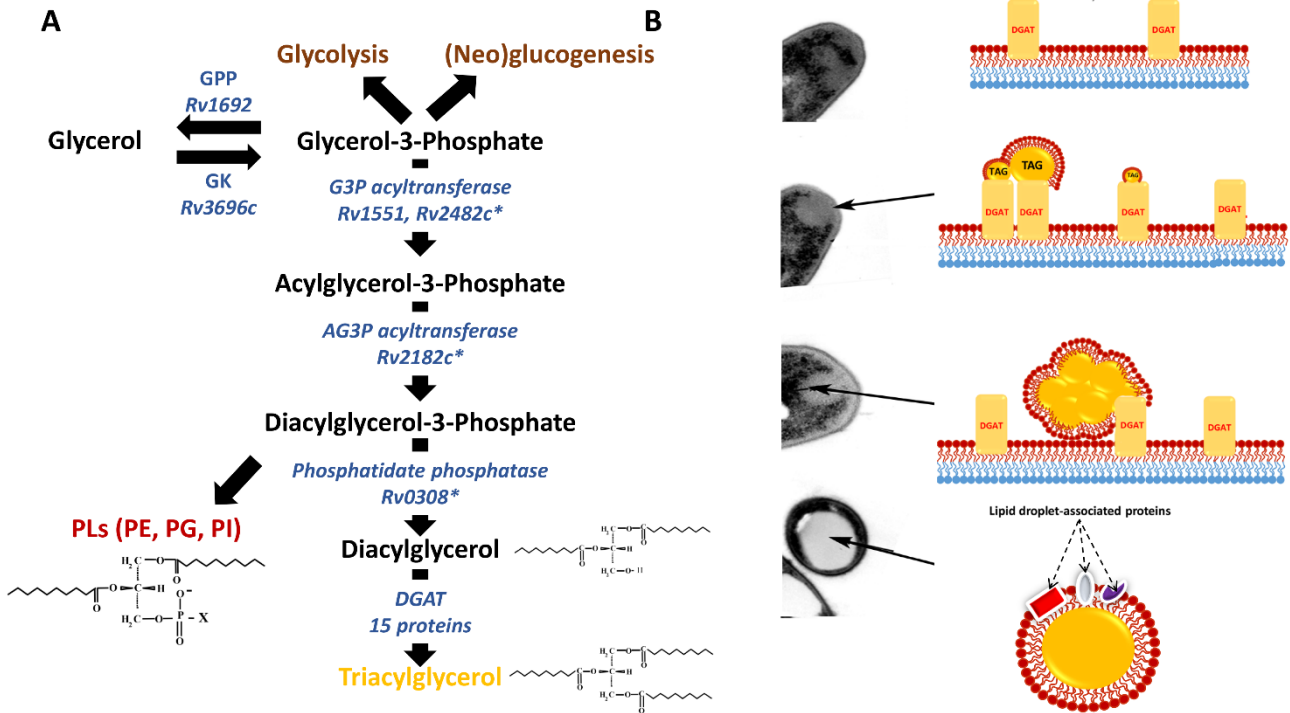
**Figure 2:** Scheme showing the role of synthases during ILI formation stage and the engagement of lipases during the ILI consumption stage, which are related to dormancy and reactivation, respectively. As observed in the electron microscopy picture on the right, total ILI consumption is not required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

# A Environment Infection

Osmolarity, Temperature, Altered pH, Hypoxia, Iron/zinc/nitrogen limitation, ROS/PNS



1402  
 1403 **Figure 3:** Different stimuli promoting ILI formation in mycobacteria are indicated by black  
 1404 arrows. **(A)** The metabolic pathway inducing TAG synthesis and their accumulation to form  
 1405 ILI is indicated inside bacteria. From the literature host FFA providing from TAG contained  
 1406 in LD are mainly due to the action of secreted mycobacterial lipolytic enzymes (Santucci, et  
 1407 al. 2018), **(B)** Confocal microscopic imaging of mycobacteria under ILI-promoting conditions  
 1408 using *in vitro* (left panel) and *ex vivo* (right panel) models. For the latter, BMDM were  
 1409 infected with the GFP-expressing *Mbv* BCG strain. Six days later, cells were exposed to  
 1410 VLDL for 24 hr. Cells were then treated with BODIPY (red label), which stains neutral lipids  
 1411 prior fixation and observation under the confocal microscope. After a 24 hr exposure to  
 1412 VLDL, both the macrophage and *Mbv* BCG (arrow and insert) contained BODIPY-stained  
 1413 granules (orange). All electron and confocal microscopy pictures were from the authors'  
 1414 personal collections.



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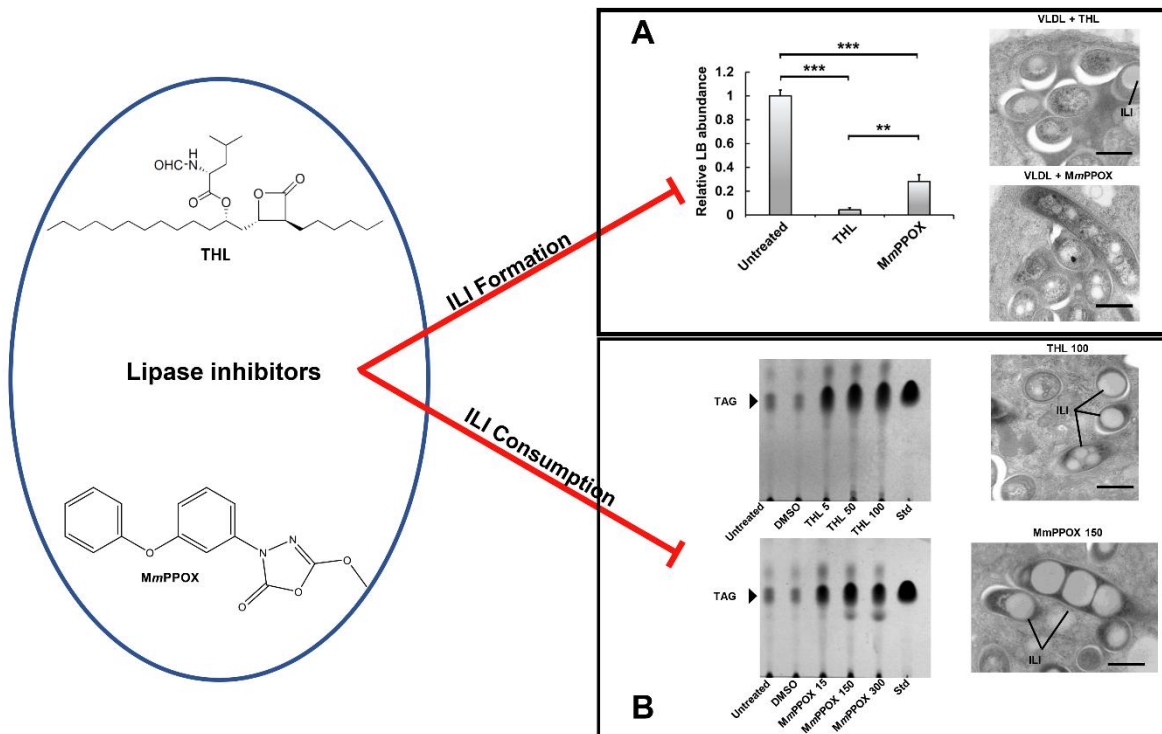
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**Figure 4:** Synthesis of TAG into Actinobacteria. **(A)** Enzymes and substrates involved in TAG synthesis (Kennedy pathway), homolog in *M. tuberculosis* are indicated. **(B)** Scheme of lipid droplet formation based on the electron microscopy observation 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.



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1427 **Figure 5:** Pharmacological inhibition targeting lipolytic enzymes modulates ILI formation  
 1428 and consumption. Chemical structures of lipases inhibitors THL and MmPPOX are  
 1429 displayed on the left panel. On the right panel, a schematic representation displays their  
 1430 effects onto **(A)** ILI formation through enzymatic inhibition of host-cell proteins involved in  
 1431 LD biogenesis within the VLDL-induced FM model (Adapted from (Santucci, et al. 2018))  
 1432 and **(B)** ILI breakdown *in vitro* and during macrophages infection. Electron micrographs and  
 1433 thin layer chromatography (adapted from (Santucci, et al. 2019a)) show the absence of  
 1434 TAG degradation in presence of THL or MmPPOX compounds. Scale bars represent 0.5  
 1435  $\mu\text{m}$ .

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