Chapter 3

Lipid Inclusions in Mycobacterial Infections

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Additional information is available at the end of the chapter

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1. Introduction

M. tuberculosis and *M. leprae* are intracellular pathogens. *M. tuberculosis* can survive up to decades in a phenotypically non-replicating dormant state, primarily in hypoxic granulomas in the lung [1]. The otherwise drug-susceptible dormant mycobacteria show the remarkable property to develop drug resistance within the granulomas of the host. These nonreplicative drug-resistant bacteria within the host´s tissues are called persisters [2].

Mycobacteria have outstanding mechanisms to escape from elimination and have a high degree of intrinsic resistance to most antibiotics, chemotherapeutic agents and immune eradication [3,4]. One major obstacle for host defence mechanisms and therapeutic intervention is the robust, mycolic acid-rich cell wall, which is unique among prokaryotes [3,5]. In the last years it has become apparent that mycobacteria induce the accumulation of lipids in the host cells and use them as energy and carbon source. This strategy is regarded as another crucial factor for the long term-survival of *M. tuberculosis* and *M. leprae* in the host. Most mycobacteria have the ability to synthesize lipid bodies as reservoirs for fatty acids. The lipid droplet- containing macrophages are called "foamy macrophages" and are the hallmark of *M. tuberculosis* and *M. leprae* infection.

M. leprae is the causative agent of leprosy. Leprosy is a chronic infectious disease caused by the obligate intracellular bacterium *Mycobacterium leprae* and is a major source of morbidity in developing countries [6,7]. Leprosy patients show two major manifestations of the disease, known as as lepromatous leprosy (LL), and tuberculoid leprosy (TT) [6]. TT is observed in patients with good T-cell mediated (Th1) immunity and is characterized by granuloma formation and death of Schwann cells (Scs) leading to myelin degradation and nerve destruction [8,9]. Patients with poor T-cell mediated immunity show the lepromatous type leprosy (LL), which leads to a high bacterial load inside host cells specially in Schwann cells and macrophages [8,10-12]. For both forms of leprosy damage of the nerves is observed [12].

Lepromatous leprosy lesions of the skin, eyes, nerves, and lymph nodes are characterized by tumor-like accumulations of foamy macrophages. The foamy macrophages are fully packed with lipid droplets (LDs) and contain high numbers of leprosy bacilli. These aggregations of foamy macrophages expand slowly and disfigure the body of the host [13].

The finding that *M. leprae* has insufficient fatty acid synthetase activity to support growth lead to the hypothesis that *M. leprae* scavenges lipids from the host cell [14]. Over the last years it has become evident that survival and persistence of *M. tuberculosis* is critically dependent on lipid body formation. Furthermore lipid body formation seems to be the prerequisite for transition of *M. tuberculosis* to the dormant state. The formation of foamy macrophages is a process which appears to be a key event in both sustaining persistent bacteria and release of infectious bacilli [15]. This goes along with the important observation that sputum from tuberculosis patients contains lipid body-laden bacilli [16,17].

In the dormant state lipids from lipid bodies appear to be the primary carbon source for *M. tuberculosis* in vivo. For *M. tuberculosis* several bacterial genes are upregulated during the dormant state and have been reported to be involved in lipid metabolism such as diacylglycerol acyltransferase (tgs1), lipase (lipY), and isocitrate lyase (icl) [18,19].

M. leprae has a small genome (3.2 Mb). The obligate intracellular organism shows a moderate genome degradation and several genes are absent when compared with other mycobacterial species. Due to the gene loss *M. leprae* is strongly dependent on the host for basic metabolic functions [8,20]. Macrophages infected with *M. leprae* contain oxidized host lipids and it has been observed that *M. leprae* upregulates 13 host lipid metabolism genes in T-lep lesions and 26 in L-lep lesions. The oxidized lipids inhibit innate immune responses and thus seem to be an important virulence factor for the organism [21].

This review highlights the importance of the LDs as one of the most unique determinant for persistence and virulence of *M. tuberculosis* and *M. leprae*. The formation of LDs in *M. tuberculosis and M. leprae* in infected host cells shall be compared and the lipid metabolism of both organisms will be discussed.

In this review we will use the term “lipid droplets” for lipid-rich inclusions in the host and “lipid bodies” for lipid-rich inclusions in the pathogen.

2. Biogenesis of lipid inclusions in bacteria and eukaryotes

The current models of lipid droplet biogenesis are still hypothetical and have been reviewed extensively by Murphy in 1999 and Ohsaki in 2009 [22,23]. The most common model supposes that the membrane protein diacyltransferase DGAT1 synthesizes triacylglycerols (TAG), which accumulate between the two membrane leaflets of the endoplasmic reticulum (ER) to be finally released by budding. The lipids are covered by a phospholipid monolayer from the ER membrane.

The formation of lipid bodies in bacteria has been even less characterized. Wältermann et al. suggested in 2005 that a bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltrans-

ferase, (WS/DGAT) synthesizes TAG for lipid body formation. WS/DGAT is an integral membrane protein and synthesizes a growing globule around the cytoplasmic portion of the enzyme. Finally the lipid body is released to the cytoplasm. The origin of the surface phospholipid monolayer is not known [22,24].

2.1. Lipid droplets in the host

The accumulation of lipid droplets occurs also in several infectious, and inflammatory conditions, including in atherosclerosis [25], bacterial sepsis [26], viral infections [27], and in mycobacterial infections [15,28,29]. *M. tuberculosis* infected macrophages store mostly neutral lipids, while cells infected with *M. leprae* seem to accumulate next to TAG a high degree of cholesterol and cholesterol esters [10,30].

LDs are observed in various cells of the immune system including macrophages, neutrophils, and eosinophils. The structure and composition of LDs is highly conserved. They contain a core of neutral lipid esters typically TAG, but also sterols and sterol esters [31-36]. The surface is covered by a phospholipid monolayer, which is composed at least in some cells by unique fatty acids [37].

M. leprae infects preferentially macrophages and Schwann cells [11]. A typical feature of lepromatous leprosy is the survival and replication of. *M. leprae* within the lipid droplets stored in the enlarged phagosome of histiocytes. Lipid droplets are thought to be an important nutrient source for the bacillus. A major concern in leprosy is peripheral neuropathy. The damage to nerves of the peripheral nervous system is caused by the the infection of Schwann cells (SCs) by *M. leprae*. In LL nerve biopsies, highly infected SCs also contain lipid droplets and show a foamy appearance, such as Virchow cells found in dermal lesions [38]. The biology of the these foamy cells has been characterized poorly until now. Neither the origin or nature of the lipids has been elucidated yet. Only recently it *in vitro* studies by Mattos could show that ML induces the formation of lipid droplets in human SCs [10]. Moreover, the group found that LDs are promptly recruited to bacterial phagosomes. In SCs LD recruiting by bacterial phagosomes depends on cytoskeletal reorganization and PI3K signaling, but is independent of TLR2 bacterial sensing [10].

Important markers for the lipid accumulation in adipocytes or macrophages are lipid-droplet-associated proteins such as adipose differentiation-related protein ADRP and perilipin, which play essential roles in lipid-droplet formation [39]. After phagocytosis of live *M. leprae* ADRP expression is constantly upregulated in human monocytes. ADRP and perilipin are localized at the phagosomal membrane (Figure 4) [39].

2.2. Lipid bodies in the pathogen

Prokaryotes do not generally produce lipid bodies containing TAG. Accumulation of TAG in intracellular lipid-bodies is mostly restricted to bacteria belonging to the actinomycetes group [40].

Most mycobacterial species accumulate considerable amounts of TAG during infection [24,41-44]. The intracellular pathogen *M. tuberculosis* can survive up to decades in a pheno-

typically non-replicating dormant state, primarily in hypoxic granulomas in the lung [1]. The otherwise drug-susceptible dormant bacteria develop drug resistance within the granulomas of the host. These nonreplicative drug-resistant bacteria within the host´s tissues are called persisters [2].

It has been observed that persisters store large amounts of intracellular triacylglycerol lipid bodies (LBs) [15,17,28,45,46]. *M. tuberculosis* uses TAG from the lipid bodies as energy and carbon source under conditions such as starvation [47], oxygen depletion [48], and pathogen reactivation [49]. The observation that sputum from tuberculosis patients contains lipid body-laden bacilli, proves the importance of lipids for the survival of the bacterium in the host [17].

3. *M. tuberculosis* induces foamy macrophages in the host

M. tuberculosis infects primarily alveolar macrophages, which reside within alveoli. The infected macrophage leaves the alveoli and migrates then towards the next lung draining lymph node. *M. tuberculosis* inhibits the generation of the phagolysosome and the bacteria begin to multiply within the macrophage [50]. The host's immune response seems to be unable to clear the bacillus from the infected macrophages. Infected macrophages secrete TNF-α and chemokines, which recruit systemic monocytes. The macrophages start to enlarge and accumulate TAG in lipid droplets. These lipid-filled foamy macrophages (FM) are surrounded by an outer layer of lymphocytes. Within the foamy macrophages the bacteria resist in phagosomes, packed with lipid droplets.

Over the last years it has become evident that survival and persistence of *M. tuberculosis* is critically dependent on lipid body formation, and induction of foamy macrophages appears to be a key event in both sustaining persistent bacteria and and release of infectious bacilli [15].

M. tuberculosis-infected phagosomes engulf cellular lipid droplets and finally the bacteria are completely enclosed by cellular lipid droplets. Only enclosed by lipid droplets the bacteria form lipid bodies and cell replication comes to a halt and finally the bacteria enter the state of dormancy and induced drug resistance [19,28]. In the nonreplicative state *M. tuberculosis* induces several bacterial genes involved in lipid metabolism such as diacylglycerol acyltransferase (tgs1), such as diacylglycerol acyltransferase (*tgs1*), lipase (*lipY*), and isocitrate lyase (*icl*) are upregulated [19,46]. In conclusion lipid body formation seems to be absolutely necessary for transition of *M. tuberculosis* to the dormant state. This goes along with the important observation that sputum from tuberculosis patients contains lipid body-laden bacilli [17].

The final granuloma consists of a core of infected, lipid-laden macrophages, which are surrounded by an outer layer of additional differentiated macrophages. The outer shell consists of T lymphocytes, B lymphocytes, dendritic cells, neutrophils, fibroblasts and an extracellular matrix [29,51-53].

The development and composition of a human tuberculosis granuloma is depicted in Figure 1.

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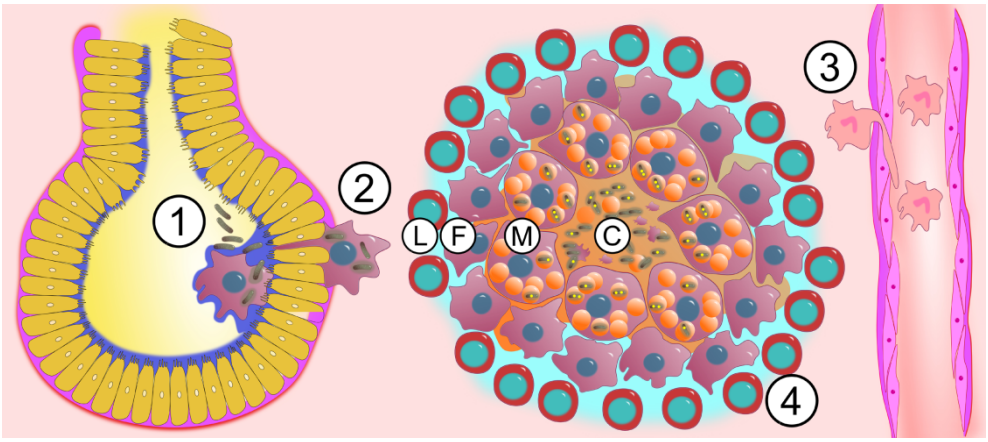

Figure 1. Development and structure of the human tuberculosis granuloma. 1, Uptake of *M. tuberculosis* by alveolar macrophages. 2, Migration of the infected macrophage towards the next lung draining lymph node. 3, Recruitment of systemic monocytes. 4, Granuloma formation. L, Lymphocytes at the periphery of the granuloma outside the fibrous outer layer. F, Fibrous capsule. Contains fibroblasts, collagen and other extracellular matrix proteins. M, Macrophage region with foamy macrophages. C, Caseum. Contains debris and lipids from necrotic macrophages. Orange, Lipid droplets of the macrophage. Yellow dots, Lipid bodies.

3.1. Lipid body formation in *M. tuberculosis* is critically dependent on lipid droplets from the host

Host lipids from lipid droplets are used by the pathogen as substantial nutrient source. Middlebrook already demonstrated in the late 1940s that mycobacterial growth *in vitro* was enhanced by supplementation with oleic acid [54]. Over the last years several groups have reported that *M. tuberculosis* within foamy macrophages produces lipid bodies, suggesting that they are able to accumulate host cell lipids [19,55]. Mycobacterial growth inside adipocytes is strictly dependent upon TAG provided by lipid droplets in host cells [55], and it has been shown that *M. tuberculosis* incorporates intact host TAG into bacterial TAG [46].

The utilization of host lipids in vivo does not only promote survival but may also increases virulence and modulate the immune response to infection. Growth of *M. tuberculosis* on fatty acids such as such propionate or valerate during infection leads to increased production of the surface-exposed lipid virulence factors, phthiocerol dimycocerosate (PDIM) and sulfolipid-1 (SL-1) [56].

Cholesterol utilization was also identified to be required for mycobacterial persistence [57]. In 2008 Pandey and Sassetti found that *M. tuberculosis* can grow using cholesterol as a primary carbon source and that the mce4 transporter is required for cholesterol uptake. *M. tuberculosis* contains four homologous mce operons, mce1–mce4, which are thought to encode lipid transporters [57,58].

Especially *M. leprae* infected macrophages show an increased accumulation of cholesterol and cholesterol [10,30]. But in contrast to *M. tuberculosis* the *M. leprae* genome encodes only one

operon for cholesterol uptake (mce1). All *M. leprae* five *mce* genes were overexpressed during intracellular growth in mouse and human biopsies [59,60]. This observation suggests, that the intracellular bacilli population induces cholersterol uptake of the infected cell and subsequently uses the stored cholesterol as carbon and energy source.

Cholesterol is also essential for uptake of *M. tuberculosis* and *M. leprae* in macrophages. Cholesterol accumulates at the site of mycobacterial entry in macrophages and promotes mycobacterial uptake. Cholesterol mediates the recruitment of TACO from the plasma membrane to the phagosome [61]. TACO, also termed as CORO1A, is a coat protein that prevents phagosome-lysosome fusion and thus degradation of mycobacteria in phagolysosomes (Figure 4) [61,62]. This mechanism for the formation of TACO-coated phagosomes promotes intracellular survival [62,63].

3.2. Lipid body formation in *M. tuberculosis* is critically dependent on lipid droplets

Host lipids from lipid droplets are used by the pathogen as substantial nutrient source. Middlebrook already demonstrated in the late 1940s that mycobacterial growth *in vitro* was enhanced by supplementation with oleic acid [54]. Host lipids play an important role during infection. They appear to be the primary carbon source for *M. tuberculosis* in vivo. Over the last years several groups have reported that *M. tuberculosis* within foamy macrophages produces lipid bodies, suggesting that they are able to accumulate host cell lipids [19,55]. Neyrolles et al. showed that mycobacterial growth inside adipocytes occurs only after the formation of lipid droplets in the host cell. This result emphasizes that *M. tuberculosis* is dependent upon TAG provided by lipid droplets in host cells [55]. In 2011 Daniel et al. finally demonstrated that *M. tuberculosis* inside foamy macrophages imports fatty acids derived from host TAG and incorporates them intact into bacterial TAG. Moreover the group proved the accumulation of TAG in lipid bodies [46].

The utilization of host lipids in vivo does not only promote survival but may also increases virulence and modulate the immune response to infection. Growth of *M. tuberculosis* on fatty acids such as such propionate or valerate during infection leads to increased production of the surface-exposed lipid virulence factors, phthiocerol dimycocerosate (PDIM) and sulfolipid-1 (SL-1) [56].

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3.3. Biosynthesis of TAG and formation of lipid bodies in *M. tuberculosis*

Biosynthesis of TAG consists of the sequential esterification of the glycerol moiety with fatty acyl-residues by various acyltransferases. Fatty acid biosynthesis consists of the stepwise addition of acetyl groups, which are provided by acetyl-CoA. The initial step is the transfer of an acetyl group from acetyl-CoA to a small protein, called acyl carrier protein (ACP). In the

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following two-carbon fragments are added sequentially to yield fatty acids of the desired length. *M. tuberculosis* uses both type I and type II FAS systems for fatty acid elongation. The multifunctional FAS I enzyme (*Rv2524c*) catalyzes the de novo synthesis of C_{16}- and C_{18}-S-ACP. These fatty acids are converted to the CoA derivative and used primarily for the synthesis of membrane phospholipids. By continuous elongation of these fatty acids FAS I produces specifically the C20- and C26-S-ACP products, and these fatty acids are released as the CoA derivatives. The C20 fatty acid is transferred to the FAS II system for the synthesis of the very-long-chain mero segment of α-, methoxy-, and ketomycolic acids [64]. The transfer from the FAS I to the FAS II system occurs by a key condensing enzyme, the ketoacyl ACP synthase III (FabH). FabH catalyzes the decarboxylative condensation of malonyl-ACP with the acyl-CoA products of the FAS I system (Figure 2). Two distinct cyclopropane synthases, MmaA2 and PcaA introduce cyclopropane rings into the the growing acyl chain [64-66].

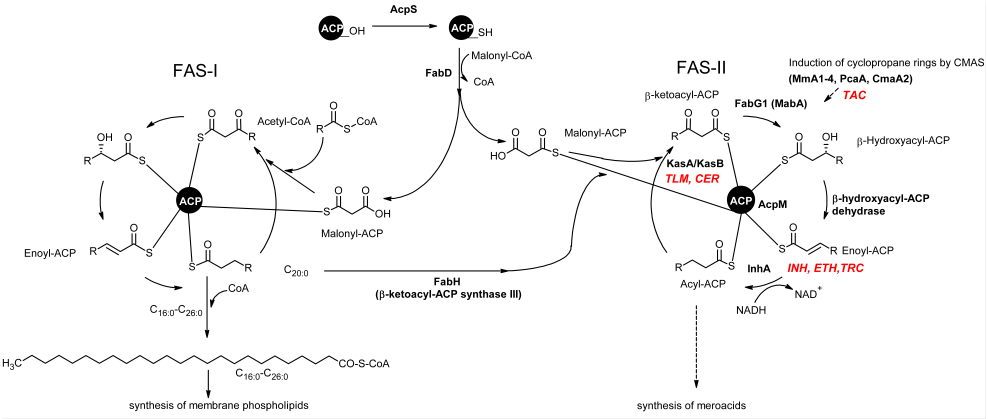

Figure 2. Fatty acid biosynthesis in *Mycobacterium tuberculosis*. The FAS-II elongation module uses the substrates R-CO-S-ACP and malonyl-S-ACP derived from malonyl-S-CoA, generated by FabD. FabH condenses both substrates R, long-chain alkyl group. Enzymes involved in these reactions are as follows: FabG1, a β-ketoacyl-ACP reductase catalyzes the reduction of beta-ketoacyl-ACP substrates to beta-hydroxyacyl-ACP. β-hydroxyacyl-ACP dehydrase. 2-trans-enoyl-ACP reductase (InhA). The β-ketoacyl-ACP synthase (KasA/KasB) catalyzes the addition of of two carbons from malonyl-ACP to R-CO-S-ACP (See text for details). R, long-chain alkyl group. ACP, acyl carrier protein. Enzymes are in bold letters. Selected inhibitors are depicted in red bold letters. TLM, thiolactomycin. CER, cerulenin. ETH, ethionamide. INH, isoniazid. TRC, triclosan. TAC, thiacetazone.

Esterification of fatty acids with glycerol-3-phosphate occurs via sequential acylation of the sn-1,2 and 3 positions of glycerol-3-phosphate, and removal of the phosphate group before the last acylation step. The terminal reaction is the esterification of diacylglycerol (DAG) with acyl-CoA by an diacylglycerol acyltransferase [40]. Animals and plants use diacylglycerol acyl-transferases (DGAT) for the terminal esterification. DGATs catalyze exclusively the esterification of acyl-CoA with diacylglycerol. Bacteria do not contain

DGATs but only bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferases (WS/DGAT). WS/DGATs, mediate next to TAG formation the synthesis of waxes by esterification of acyl-CoA with alcohol [67]. The genome of *M. tuberculosis* codes for 15 genes which contain the highly conserved putative active site motif of WS/DGATs (HHxxxDG). These genes were designated as "tgs", triacylglycerol synthases, but have only a weak sequence similarity to other WS/DGAT sequences. All 15 expressed mycobacterial Tgs proteins show diacylglycerol acyltransferase activity and Tgs1 has the highest activity of all enzymes [48]. Gene disruption of *tgs1* results in a drastic reduction of major C26 long-chain fatty acid in *M. tuberculosis* grown under hypoxic conditions. Thus Tgs1 appears to be a major contributor to TAG synthesis in *M. tuberculosis* so far [48,68]. And moreover two homologous proteins to Tgs1 and Tgs2 (BCG3153c and BCG3794c) and another poorly characterized acyltransferase (BCG1489c) were found to be exclusively associated to lipid bodies. The disruption of *BCG3153c, BCG3794c*, and *BCG1489c* reduces TAG accumulation during the hypoxia-induced nonreplicating state, revealing that the enzymes are involved in TAG synthesis during latency and pathogenicity [69].

Ten of the 15 tgs genes in *M. tuberculosis* are located adjacent or proximal to 11 lip genes that are annotated as probable phospholipases or lipases-esterases-carboxylesterases. Some tgs genes may be cotranscribed with neighboring lip genes and may synthesize triacylglycerols from the released fatty acids from the host [18]. Lip gene products may be important for utilization of TAGs during dormancy and upon reactivation after dormancy. The tgs gene Rv0221 is located near lipC (*Rv0220*), lipW (*Rv0217c*), acyl-CoA synthetase (*Rv0214*), acyl-CoA dehydrogenase (*Rv0215c*), and an integral membrane acyltransferase (*Rv0228*). This clustering of genes of the fatty acid metabolism suggests that these genes may be cotranscribed and may release fatty acid from host TAG, carry out the transport of fatty acids and finally catalyze the re-synthesis of TAGs in the pathogen. Rv0221 and LipC have to be shown to be catalytical active. [18,70].

In summary Tgs enzymes play a major role in TAG synthesis, lipid body formation and maintenance.

Ag85A, a mycoltransferase, that is known to catalyze the formation of the cord factor was recently found to have additional DGAT activity [71]. The kinetic parameters are quite similar to those reported for the *M. tuberculosis* Tgs1-4, but the primary sequence of Ag85A does not contain the active site motif of WS/DGATs or TGS enzymes (HHxxxDG) [48,68,71]. Ag85A belongs to the α/β hydrolase fold family and contains the consensus GXSXG sequence. The enzyme is a carboxylesterase with an additional acyltransferase activity. Overexpression of Ag85A induces lipid body formation in *M. smegmatis*. The enzyme is located in the mycobacterial cell wall, suggesting that it may be involved in the maintenance of lipid droplets in the host cell [71].

The genome of *M.leprae* contains also mycolytransferase 85 complex genes (A, B and C). Transcripts of these genes are upregulated either in infected nude mouse or human skin lesions [59].

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The *M. leprae* genome shows only one predicted gene product which has has a significant degree of identity to any the Tgs enzymes from *M. tuberculosis* [18]. The tgs gene product ML1244 shows 72% identity to Rv2484c from *M. tuberculosis*. *Rv2448* is located next to a carboxylesterase lipQ, (*Rv2485c*), a probable glycerol-3-phosphate acyltransferase, (*Rv2482c*), a lysophosphatidic acid acyltransferase-like protein (*Rv2483c*), and a probable enoyl-CoA hydratase (*Rv2486*). The gene cluster of lipid metabolism genes suggests a possible involvement of the gene products in the synthesis of TAG [18]. A few tgs genes (*Rv3234c*, *Rv3233c*, *Rv2285*, and *Rv1425*) are located proximal to lipoproteins, which may serve as donors or acceptors of fatty acids [48]

3.4. Activation of TAG – Lipases and esterases of *M. tuberculosis*

Neutral lipids in the core of the lipid body are hydrolyzed by lipases or esterases, yielding fatty acids for energy generation and anabolism of membrane phospholipids.

In the genome of *M. tuberculosis* H37Rv twenty-one genes are termed as putative lipases (*lip* A to W, except K and S) [72]. The annotation was only based on the presence of the consensus sequence GXSXG, which is characteristic for the large group of the α/β hydrolase fold protein family, which includes lipases as well as esterases, proteases, peroxidases, epoxide hydrolases and dehalogenases [72]. Thus the members of the lip group have only a very low level of sequence identity of ~20% and might have another function apart from lipid hydrolysis. Only the gene product of *Rv3097c* (LipY)) shows reasonable hydrolase activity for long-chain TAG with chain lengths ranging from C4 to C18. Overexpression of LipY induces extensive TAG hydrolysis. Disruption of *lipY* markedly reduces but does not completely inactivate TAG hydrolase activity, which suggests the presence of other lipases in *M. tuberculosis* [47,49].

Overexpression of *LipY* in *M. bovis* Bacillus Calmette-Guérin reduces protection against infection in mice, indicating that lipY plays a central role in TAG hydrolysis and virulence [47,73,74]. LipY contains a PE (Pro-Glu) domain, that is involved in modulation of LipY activity [73]. The PE domain contains a signal sequence for secretion of LipY by the ESX-5 system. It has been implicated that the secreted LipY is loosely associated with the bacterial surface where it may hydrolyze host's TAG [75].

Several other esterases, next to the members of the Lip group have been identified and biochemically characterized. They all belong also to the α/β hydrolase fold family and showing the minimal GXSXG motif. In 2007 Côtes et al. characterized a novel lipase Rv0183. The enzyme is only found in the cell wall and culture medium. This observation suggests that Rv0183 is involved in the degradation of the host cell lipids e.g. when M. tuberculosis infects adipocytes [55,76]. Another probably cell wall-associated carboxylesterase is encoded by Rv2224c. The esterase Rv2224c was found to be required for bacterial survival in mice [77]. The substrate spectrum of Rv2224c is poorly characterized and until now it is unknown whether the enzyme uses TAG as substrate [77]. Furthermore the three-dimensional structures of the esterases Rv0045c (PDB 3P2M) [78], Rv1847 (PDB 3S4K), and LipW (3QH4) from *M. tuberculosis* have been determined, but unfortunately it is not known whether these enzymes are involved in TAG hydrolysis.

3.5. Lipase genes of *M. leprae*

In the *M. leprae* genome only 2 lipase genes (*lipG*, *lipU*) were found. But *M. tuberculosis* has also only six expressed Lip enzymes, showing reasonable hydrolase activity for long-chain triacylglycerols. (LipY, LipC, LipL, LipX, LipK, LipG). LipG and LipU from *M. leprae* are homologous with LipG and LipU from *M. tuberculosis* and show sequence identities of 72 and 79%, respectively. The lipases LipG and LipU from *M. tuberculosis* show very low and no activity with long chain triacylglycerols as substrates [47]. *M. tuberculosis* LipY is suspected to be a major functional lipase, which utilizes stored triacylglycerols (TAG) during dormancy and reactivation of the pathogen [47,49]. LipY shows only a weak similarity with *M. leprae* LipU (23 % identity). In summary it appears that *M. leprae* uses different lipases for the hydrolysis of fatty acids than *M. tuberculosis*.

3.6. Enzymes of the β-oxidation and glyoxylate cycle

M. tuberculosis can grow on fatty acids as sole carbon source and it has been demonstrated that fatty acid oxidation is important for survival of the pathogen in the lungs of mice [79,80]. Fatty acids are oxidized via the β-oxidation cycle and the glyoxylate shunt, to replenish TCA cycle intermediates during growth [81]. The β-oxidation cycle consists of five biochemical reactions, where one molecule acetyl-CoA of the fatty acid is split off per cycle. The genome of *M. tuberculosis* encodes around 100 genes, designated as fad genes (fatty acid degradation) with putative roles in the β-oxidation of fatty acids. While *E. coli* has only one enzyme for each step of the β-oxidation cycle, *M. tuberculosis* seems to have several backup enzymes for each reaction [82]. The initial step of β-oxidation is the formation of acyl-CoA from free fatty acids and Coenzyme A and is catalyzed by acyl-CoA synthase. In *M. bovis* BCG one Acyl-CoA synthase (BCG1721) (Rv1683) has been identified to be exclusively bound to lipid bodies. Nonreplicating mycobacteria, which overexpress a BCG1721 construct with an inactive lipase domain displayed a phenotype of attenuated TAG breakdown and regrowth upon resuscitation. These results indicate that the gene might be essential for TAG hydrolysis and growth [69].

Together with malate synthase, isocitrate lyase (ICL) is the key enzyme of the glyoxylate cycle that catalyzes the cleavage of isocitrate to glyoxylate and succinate [81,83]. The *M. tuberculosis* genome codes for two isocitrate lyases, icl and icl2, which are essential for the fatty acid metabolism and jointly required for in vivo growth and virulence. Disruption of icl has only little effect on survival in macrophages and bacterial loads in lungs of infected mice. Only disruption of both lyase genes results in a fast elimination of bacteria from lungs of infected mice and infected macrophages [79,80]. These results strongly suggest that both icl genes are required for mycobacterial persistence.

All enzymes involved in lipid metabolism in lipid bodies are summarized in Table 1.

M. leprae has approximately one-third as many potential fad enzymes with probable roles in the β-oxidation. Even though *M. leprae* genome contains less necessary β-oxidation cycle genes than *M. tuberculosis*, transcript analysis revealed expression of acyl-CoA metabolic enzymes including *echA1* (ML0120, putative enoyl-CoA hydratase), *echA12* (ML1241,

possible enoyl-CoA hydratase), *fadA2* (ML2564, acetyl-CoA-acetyltransferase), *fadB2* (ML2461, 3-hydroxyacyl-CoA dehydrogenase), *fadD19* (ML0352, acyl-CoA synthase), *fadD26* (ML2358, fatty acid-CoA-ligase), *fadD29* (ML0132, probable fatty-acid-CoA synthetase), *fadD28* (ML0138, possible fatty-acid-CoA synthetase), *fadE25* (ML0737, probable acyl-CoA dehydrogenase) and *fadE5* (ML2563, acyl-CoA dehydrogenase) [59,60]. This gives strong evidence that host lipids provide the main carbon and energy sources for *M. leprae* during infection.

The *M. leprae* genome contains a gene, coding for an isocitrate lyase, *aceA*. The amino acid-sequence of AceA (ML1985c) shows 80 % identity with its homologue from *M. tuberculosis* ICL2 (Rv1915/1916). *AceA* is upregulated in both *M. leprae*-infected nude mouse and human lesions. [59]. A second icl gene, as observed in *M. tuberculosis*, is not present in the genome of *M. leprae*. This finding is of particular interest, because both lyases, *icl* and *icl2*, are jointly required for in vivo growth and virulence [79,80]. Deletion of *icl1* or *icl2* has little effect on bacterial growth in macrophages [80]. So far the *M. leprae* AceA might play a slightly different role in as the both isocitrate lyases in *M. tuberculosis.*

4. Lipid composition in *M. leprae* infected cells

In 1863, Virchow described foamy cells, which form droplets and surround *M. leprae* within the phagolysomes. [84,85]. This lipid capsule forms a characteristic electron-transparent zone. In contrast to *M. tuberculosis*, the presence of lipid bodies seem to be rather exceptional in *M. leprae* [85]. The lipid capsule contains mycoserosoic acids of phthiocerol dimycocerosates as well as phenolic glycolipids [86,87]. Brennan reported the full characterization of three phenol-phthiocerol triglycosides by *M. leprae* [84]. It has been postulated that many of these molecules together with phosphatidylinositol mannosides and phospholipids are released from the cell wall after synthesis, forming the capsule-like region [11]. The dominant lipid in the cell wall which gives *M. leprae* immunological specificity is phenolic glycolipid-1 (PGL-1). Phenolic glycolipid 1 has been isolated in relatively high concentrations from purified bacteria and from *M. leprae* infected tissues [88]. PGL-1 is thought to be a major component of the capsule in *M. leprae* and constitutes an important interface between bacteria and host [89]. It has been suggested that PGL-1 is involved in the interaction of *M. leprae* with the laminin of Schwann cells, thus PGL-1 hight play a role in peripheral nerve-bacillus interactions [90]. Moreover, phenolic glycolipids seem to be involved in the in the stimulation of suppressor T-cells in lepromatous leprosy [91]. Recently it was reported that also LDs from *M. leprae* infected SCs and macrophages accumulate mainly host derived lipids, such as oxidized phospholipids [92]. BODIPY stains infected SCs, indicating that LDs contain neutral lipids, such as triacylglycerols (TAG), but it seems as *M. leprae*-infected cells accumulate large amounts of cholesterol and choesterol esters [10].

Gene	Protein	DGAT activity *(in vitro)*	Lipid body associated	Influence on lipid bodies / TGA accumulation	Modulation of virulence	*M. leprae homologue*	Reference
Rv3130c (*tgs1*)	DGAT	+	+	Δ*tgs1* decreases TAG accumulation	NA	ML1244	[46,48,68]
Rv3734c (*tgs2*)	DGAT	+	+	NA	NA	ML1244	[48,68]
Rv3234c (*tgs3*)	DGAT	+	NA	NA	NA	ML1244	[48,68]
Rv3088 (*tgs4*)	DGAT	+	NA	NA	NA	ML1244	[48,68]
Rv1760	DGAT	+	NA	NA	NA	ML1244	[48,68]
Rv2285	DGAT	+	NA	NA	NA	ML1244	[48,68]
Rv3804c (*85A*)	DGAT	+	NA	Overexpression increases production of lipid bodies	NA	ML0097 (85A)	[71]
BCG1489c [*Rv1428c*]	DGAT	NA	+	Δ*BCG1489c* reduces TAG accumulation	NA	ML2427c	[69]
BCG3153c (*tgs1*) [*Rv3130c*]	DGAT	NA	+	Δ*BCG3153c* reduces TAG accumulation	NA	ML1244	[69]
BCG3794c (*tgs2*) [*Rv3734c*]	DGAT	NA	+	Δ*BCG3794c* reduces TAG accumulation	NA	ML1244	[69]
BCG1489c [*Rv1428c*]	acyltransferase	NA	+	Δ*Rv1428c* reduces TAG accumulation	NA		[69]

Gene	Protein	TGA-hydrolyzing activity (*in vitro*)	Lipid body associated	Influence on lipid bodies / TGA accumulation	Modulation of virulence	*M. leprae* homologue	Reference
Rv3097c (*lipY*)	Lipase/esterase	+	NA	Δ*lipY* reduces TAG hydrolysis. Overexpression increases TAG hydrolysis	Overexpression increases virulence in mice	ML0314c (*lipU*) ML1053 ML1183c	[47]
Rv1399c (*lipH*)	Lipase/esterase	+	NA	NA	NA	ML0314c (*lipU*)	[72]
BCG1721 [*Rv1683*]	Lipase/esterase	+ (in vivo*)	+	+ (*)	NA	ML1346	[69]
Rv0183	Lipase/esterase	Hydrolyzes only monoacylglycerides	NA	NA	NA	ML2603	[76]
Rv2224c	Lipase/esterase	NA	NA	NA	Gene disruption decreases virulence in mice	ML1633c	[77]

Gene	Protein	Isocitrate cleavage (*in vitro*)	Lipid body associated	Influence on lipid bodies / TGA accumulation	Modulation of virulence	*M. leprae* homologue	Reference
Rv0467 (*icl*), *Rv1915-1916* (*icl2*)	isocitrate lyase	+	NA	NA	The Δ*icl* , Δ*icl2* strain shows no intracellular replication	ML1985c (aceA)	[79,80,93-95]

Table 1. Enzymes involved in lipid body metabolism in *M. tuberculosis* and *M. bovis* BCG. Homologous genes in *M. tuberculosis* H37Rv are written in square brackets. NA, not applicable. *, expressed in yeast as recombinant protein. DGAT, diacylglycerol acyltransferase

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5. Induction of lipid droplet biogenesis

Since the biogenesis of lipid droplets in macrophages seems an absolute requirement for intracellular bacteria to establish infections, we will discuss mechanisms involved in foam cell formation and development of lipid droplets.

5.1. Scavenger receptor mediated lipid droplet biogenesis in *M. tuberculosis*

Upon infection with pathogenic bacteria macrophages generate reactive oxygen species (ROS). The release of ROS generates oxidative stress, and results not only in damage to cellular structures but also to oxidation of fatty acids, such as low density lipoproteins (OxLDL) in granulomas. The binding of OxLDL to type 1 scavenger receptors CD36 and LOX1 induces increased surface expression of both receptors, leading to uptake of OxLDL [96-98]. In addition, CD36 increases the uptake of *M. tuberculosis* by macrophages [99]. The increased rate of OxLDL uptake results in the accumulation of oxidized lipids, which finally leads to the formation of foamy macrophages [98]. *M. tuberculosis* and *M. leprae* benefit from the accumulated OxLDL in the infected macrophage. OxLDL-laden lung macrophages show enhanced replication of intracellular *M. tuberculosis* compared to macrophages loaded with non-oxidized LDL [98]. The presence of oxidized phospholipids in *M. leprae* infected macrophages down-regulates the innate immune response and contributes to pathogenesis [92]. Moreover, scavenger receptor-deficient phagocytes are characterized by a reduced intracellular bacterial survival and a lower cytokine response [100].

5.2. TLR mediated LD formation in *M. bovis* and *M. leprae*

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) and *M. leprae* are recognised by the Toll-like receptors (TLR) TLR6 and TLR2 [101,102]. Mycobacterium bovis Bacillus Calmette-Guérin induced lipid body formation is TLR2 mediated [103]. The mycobacterial surface molecule lipoarabinomannan (LAM) induces the formation of foamy macrophages by binding to TLR2 [104] (Figure 3).

M. leprae association to macrophages is mediated by binding of the bacteria to TLR2 and TLR6. Heterodimerization of TLR2 and TLR6 leads to downstream signalling and subsequent LD formation [102,105]. Macrophage association is not dependent on binding to TLR2 or TLR6. Neither a $TLR2^{-/-}$ or $TLR6^{-/-}$ knockout macrophage shows reduced binding to *M. leprae*. This suggests that both TLR2 and TLR6 can bind *M. leprae* alone, or/and the presence of other receptors, binding to *M. leprae*. The $TLR2^{-/-}$ or $TLR6^{-/-}$ knockout macrophages do also not completely abolish LD formation, but show only reduced LD formation [102]. This suggests the presence of additional signalling pathways for LD formation. In SCs TLR6, but not TLR2, is essential for *M. leprae*-induced LD biogenesis in [101]. In LL lesions, accumulated with LD enriched macropages the genes for ADRP and CD36 are up-regulated [30,92,102]. This suggests also an involvement of CD36 in LD formation of *M. leprae* (Figure 4) [99].

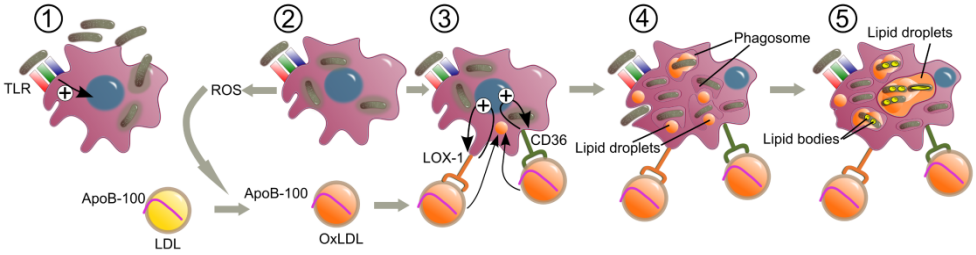

Figure 3. Induction of lipid droplet biogenesis in macrophages by *Mycobacterium tuberculosis*. 1) Recognition of bacteria by Toll-like receptors (TLR) trigger phagocytosis and subsequent formation of lipid droplets. 2) The infected macrophage produces reactive oxygen species (ROS), which oxidize LDL. 3) The binding of OxLDL to type 1 scavenger receptors CD36 and LOX1 induces increased surface expression of both receptors and increases uptake of host´s oxidized fatty acids. 4) Mycobacterium-laden phagosomes internalize lipid droplets. 5) Within the lipid droplets the bacteria form lipid bodies and finally enter the dormant state. ApoB-100, apolipoprotein B-100.

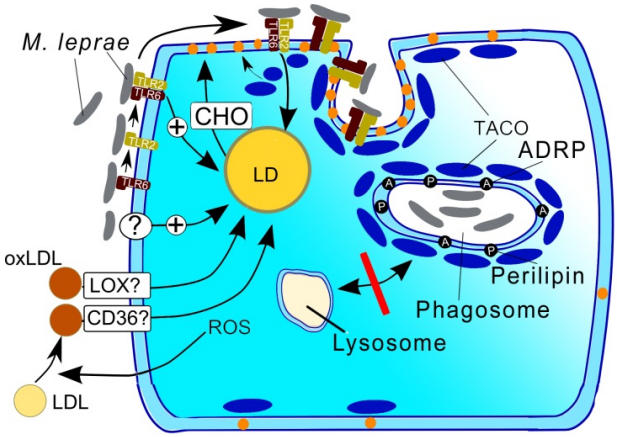

Figure 4. Basic mechanisms of lipid droplet induction in *M. leprae* infected macrophages. *M. leprae* attaches to TLR2 and TLR6. Heterodimerization of TLR2 and TLR6 induces downstream signalling and subsequent accumulation by LD formation. [102,105]. In SCs TLR6, but not TLR2, is essential for *M. leprae*-induced LD biogenesis [101]. Cholesterol from the LDs accumulates at the site of mycobacterial entry and promotes mycobacterial uptake. Cholesterol also recruits TACO from the plasma membrane to the phagosome [61]. TACO prevents phagosome-lysosome fusion and promotes intracellular survival [62,63]. Hypothetical uptake of oxidized lipids by scavenger receptors in *M. leprae*: Reactive oxygen species might oxidize low-density lipoprotein (LDL) to oxLDL, which is thought to be subsequently bound and taken up by scavenger receptors CD36 and LOX1. CHO, cholesterol. Unknown mechanisms for LD induction are indicated with a question mark.

5.3. Mycolic acids induce the formation of foamy macrophages

Mycolic acids and oxygenated mycolic acids are strong inducers of monocyte-derived macrophages differentiation into foamy macrophages [19,106]. Peyron et al. demonstrated

that that a set of oxygenated mycolic acids specifically produced by highly virulent mycobacteria species (*M. tuberculosis*, *M. avium*) were responsible for the formation of foamy macrophages [19].

6. Clinical implications

Several enzymes of the mycobacterial lipid-biosynthesis are regarded as targets for new antitubercular compounds. The research focused on enzymes, involved in the biosynthesis of lipid compounds of the mycobacterial cell wall [107]. Especially the biosynthesis of the highly toxic cord factor is an attractive target. The cord factor is synthesized by the antigen 85 complex [108,109]. It was recently shown that one member of the complex, antigen 85A is involved in the formation of intracellular lipid bodies [71]. Antigen 85 is an important virulence factor. It has been shown that *M. tuberculosis* requires the expression of Ag85A for growth in macrophages [110]. *M. tuberculosis* strain lacking Ag85C shows an decrease of 40% in the amount of cell wall linked mycolic acids [111,112]. The treatment by a trehalose analogue, 6-azido-6-deoxy-α,α'-trehalose (ADT) inhibits the activity of all members of Ag85 complex *in vitro* [108, 113]. Also ethambutol targets the synthesis of arabinogalactan, isoniazid and ethionamide inhibit biosynthesis of mycolic acids [107].

The most potent inhibitor for mycolic acid biosynthesis is isoniazid (INH). INH is a prodrug which is converted to the isonicotinoyl radical by KatG. INH forms a covalent adduct with NAD. This INH-NAD adduct inhibits FAS-II enoyl-ACP reductase InhA, which in consequence leads to inhibition of mycolic acid biosynthesis, and ultimately to cell death [114-117]. The inhibitors of fatty acid biosynthesis are summarized in Figure 2 and Table 2.

Synthesis step	Enzyme	Compound / class	References
FAS-I and FAS-II	KasA/KasB	Cerulenin (2R,3S-epoxy-4-oxo-7,10-trans,trans-dodecanoic acid amide	[118] [119]
FAS-II	KasA/KasB	TLM (Thiolactomycin)	[120-122]
FAS-II	KasA/KasB	Platensimycin	[123]
	InhA	INH (Isoniazid)	[124]
	InhA	ETH (Ethionamide)	[125]
	InhA	TRC (Triclosan)	[126]
	InhA	alkyl diphenyl ethers (Triclosan derivatives)	[127]
	InhA	2-(o-Tolyloxy)-5-hexylphenol (PT70)	[120]
Cyclopro-panation	CMASs (cmaA2, mmaA2 or pcaA)	TAC (Thiacetazone)	[128]
	MmaA4	TAC (Thiacetazone)	[128]

Table 2. Inhibitors of fatty acid biosynthesis

7. Conclusion

The formation of lipid inclusions during infection in the host as well as in the pathogen during intracellular infection with *M. tuberculosis* and *M. leprae* plays an important role in pathogenesis. A hallmark of intracellular infection is the formation of foamy macrophages. *M. tuberculosis* and *M. leprae* induce the formation of lipid droplets in the host cell. The accumulated lipids are used as energy and carbon source. In fact *M. tuberculosis* seems to switch completely to fatty acid catabolism at the transition from the acute to the chronic phase of infection. The central role of fatty acid metabolism during the dormant state of *M. tuberculosis* is underlined by the finding that both isocitrate lyase, icl and icl2, are essential for intracellular replication in the lung [79,80]. The TAG metabolism and the resulting formation of lipid inclusions of host and pathogen play a fundamental role in infection. Indeed TAG-derived fatty acids from the host cell are imported into *M. tuberculosis* and incorporated into bacterial TAG [46]. In conclusion the enzymes involved in lipid droplet metabolism are essential for survival of the pathogen in the lung and thus attractive targets for novel drugs. Especially enzymes with DGAT activity such as Tgs and Ag85A seem to be promising drug target candidates. Another promising targets seem to be the recently discovered cell wall associated and secreted esterases, which are involved in the utilization of host cell lipids such as Rv0183 and LipY [55, 75,76]. Future studies should also focus on the lipid metabolism of *M. leprae*, an organism which upregulates several genes of the host´s lipid metabolism during infection [92]. The regulation of lipid droplet formation in the host cell is another important topic. Recent sudies revealed that intracellular pathogens induce the expression of LDL receptor and scavenger receptors CD36 and LOX1 for the internalization of native and oxidized fatty acids. Especially the generation of oxidized lipids by macrophage-derived reactive oxygen species seems to be an important mechanism for the induction of scavenger receptors.

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