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Microreview

Lipid droplet hijacking by intracellular pathogens

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Running title: Lipid droplet hijacking

Summary

Lipid droplets were long considered to be simple storage structures, but they have recently been shown to be dynamic organelles involved in diverse biological processes, including emerging roles in innate immunity. Various intracellular pathogens, including viruses, bacteria and parasites, specifically target host lipid droplets during their life cycle. Viruses such as hepatitis C virus, dengue virus and rotaviruses use lipid droplets as platforms for assembly. Bacteria, such as mycobacteria and chlamydia, and parasites, such as trypanosomes, use host lipid droplets for nutritional purposes. The possible use of lipid droplets by intracellular pathogens, as part of an anti-immunity strategy, is an intriguing question meriting further investigation in the near future.

Introduction

Lipid droplets (LDs) are intracellular structures that store neutral lipids. Until recently, they were classified in the same category as glycogen granules, as simple inert storage sites for energy, increasing and decreasing as a function of metabolic energy requirements. However, considerable interest has recently focused on LDs as dynamic organelles at the hub of lipid exchanges between intracellular compartments and energy metabolism (Pol *et al.*, 2014; Welte 2015). LDs interact dynamically with other organelles, including the endoplasmic reticulum (ER), mitochondria, endosomes, peroxisomes and the plasma membrane, exchanging both lipids and proteins (Gao *et al.*, 2015). However, a growing number of intriguing and unexpected functions have also recently been attributed to LDs, such as the constitution of a reservoir for various proteins, including transcription factors and chromatin components (Welte 2015). LDs are emerging as important new organelles for the life cycles of various intracellular pathogens.

LD biology

LDs are best known for their role in lipid storage in mammalian adipocytes in adipose tissue, but they are present in almost all eukaryotic cells, including yeasts, and have even been found in prokaryotes. Unlike membrane-bound organelles, the aqueous contents of which are enclosed by a phospholipid bilayer, LDs consist of a central core of hydrophobic (neutral) lipids, surrounded by a single layer of amphipathic lipids (reminiscent of half a membrane bilayer), associated with proteins. Several proteins are involved in the generation, maturation and degradation of LDs. These proteins include the perilipin (PLIN) family of proteins (previously known as the PAT family): PLIN1, PLIN2 (adipocyte differentiation-related protein - ADRP) and PLIN3 (tail-interacting protein 47 - TIP47), the founding members of the growing family of LD-associated proteins (Bickel *et al.*, 2009, Kimmel *et al.*, 2010). The triglycerides in LD hydrophobic core are generated by an elaborate biosynthetic pathway, the final step of which is catalyzed by the diacylglycerol (DAG) acyltransferases DGAT1 and DGAT2, converting DAG and fatty acids into triglycerides (Pol *et al.*, 2014). Both enzymes are located in the ER, where triglycerides accumulate at sites between the two layers of the ER membrane to form nascent LDs. According to the classical model of LD biogenesis, these organelles are generated by the continuous growth of these structures, which eventually

separate from the ER via a mechanism resembling budding (Thiam *et al.,* 2013). DGAT2 is inserted exclusively into the external leaflet of the ER membrane and can therefore diffuse onto the LD surface, promoting triglyceride synthesis and the continuation of LD growth (Wilfing *et al.,* 2013). Depending on cell type and conditions, the hydrophobic core may also contain proteins and cholesterol esters, the synthesis of which is catalyzed by cholesterol acyltransferases. LD turnover is rapid, with these structures continually being produced at the ER and degraded by the action of lipases bound to their surface, such as adipocyte triglyceride lipase (ATGL), or by lipophagy, an autophagic process (Zechner *et al.,* 2012). It has also been suggested that LDs act as general protein sequestration sites. This sequestration may modulate the interactions of these proteins with their binding partners, facilitate the storage of damaged or unfold proteins, or allow the movement of droplets for the delivery of proteins to specific compartments (Hodges *et al.,* 2010). LDs are thought to store a pool of histone proteins in the cytoplasm for use in DNA replication or repair outside of S phase (Welte 2015).

Viruses

Hepatitis C virus (HCV) is one of the most widely studied infectious agents in terms of interactions with LDs. The life cycle of this virus is closely tied to lipid metabolism, as viral particles circulating in the blood of infected patients are bound to lipoproteins, forming lipoviroparticles (Boyer *et al.,* 2014). In HCV-infected cells, the nucleocapsid (core) protein and a nonstructural protein of the viral replication complex, NS5A, are localized to LDs (Myanari *et al.,* 2007). The visualization of virus assembly in HCV-infected cells has proved challenging, but, by overproducing the structural proteins (the core and the two envelope proteins), virus-like particles can be observed budding at the ER membranes in close association with LDs (Hourioux *et al.,* 2007a; Roingeard *et al.,* 2008) (Figure 1A). It is currently thought that HCV formation involves the use of LDs as assembly platforms for the virus, with the core protein playing a key role in this mechanism. The core protein is the first protein translated from the viral RNA and is released from the single viral polyprotein encoded by the viral genome, through two consecutive cleavage events: the first, mediated by the signal peptidase and the second by signal peptide peptidase. These events generate a mature protein that diffuses laterally in the ER membranes towards the surface of the LDs (McLauchlan *et al.,* 2002). The HCV core protein interacts physically with DGAT1 in the ER membrane, and this interaction, coupled to active DGAT1 triglyceride synthesis, is required for the localization of core to LDs (Herker *et al.,* 2010). Unlike core, which is found almost

entirely on LDs, NS5A is found on both LDs and ER membranes. DGAT1 also interacts with NS5A, probably functioning as a molecular bridge between core and NS5A to ensure their targeting to the same LD (Camus *et al.,* 2013). Two other cell factors, TIP47 and the Ras-related protein Rab18 which are associated with LDs in hepatocytes, interact with NS5A and contribute to HCV formation (Vogt *et al.,* 2013; Salloum *et al.,* 2013). Rab 18 may promote the physical association of NS5A with other components of the viral replication machinery and LDs (Salloum *et al.,* 2013). HCV assembly probably involves the close apposition of LDs against viral replication sites located in specialized regions of ER membranes and generated by the nonstructural proteins (Ferraris *et al.,* 2013). NS5A, which has RNA-binding properties, may transport viral RNA from the replication sites to LDs for interaction with core, leading to the encapsidation of the newly synthesized viral RNA and the formation of virions. Very low-density lipoproteins (VLDL) are assembled in the luminal compartment of the ER and most of the lipids used for their production are derived from LDs. The nascent HCV particles, therefore, probably follows the VLDL assembly pathway, to generate virions with incorporated apolipoproteins. HCV/LD interaction is not restricted to viral morphogenesis, as chronic HCV infection is linked to LD accumulation, or steatosis, in the liver of patients with chronic HCV infection (Roingeard and Hourioux, 2008). This steatosis can affect the natural course of the infection, aggravating the progression of hepatic fibrosis. Levels of LD accumulation in HCV-infected cells have been shown *in vitro* to be directly linked to polymorphisms of the core protein sequence (Hourioux *et al.,* 2007b), although host genetic factors are the principal factors controlling the severity of liver steatosis *in vivo* (Roingeard, 2013).

GB virus B (GBV-B), which is closely related to HCV and causes acute hepatitis in experimentally infected tamarins, encodes a core protein that colocalizes with LDs, due to a region similar to the HCV core protein (Hourioux *et al.,* 2007a). Interaction with LDs is not unique to HCV and related viruses from the *Flaviviridae* family, as the core protein of dengue virus (DENV) has also been shown to localize with LDs, although these viruses infect different host cells (hepatocytes for HCV, mosquito and human monocytes and macrophages for DENV) (Samsa *et al.,* 2009). A nonstructural protein from DENV, NS3, cooperates with Rab18 to recruit the host fatty acid synthetase to sites of viral replication (Heaton *et al.,* 2010 ; Tang *et al.,* 2014). Interestingly, the DENV core protein has recently been shown to interact specifically with VLDL, suggesting that DENV may also form lipoviroparticles (Faustino *et al.,* 2014). Although much work remains to be done in the DENV model, core protein/LD association appears to be crucial for virus production in both cases.

Rotaviruses replicate in enterocytes and have been shown to highjack LDs for their own purposes (Figure 1B). Early stages of viral assembly and replication take place in virus-induced cytoplasmic inclusion bodies called viroplasms, from which double-layered particles (DLPs) are released. These particles acquire an outer layer from the rough ER, to form triple-layered particles (TPLs) (Trask *et al.,* 2012). These TPLs contain four major capsid proteins (VP2, VP4, VP6 and VP7) and two minor proteins (VP1 and VP3). Mature virions, which are non-enveloped viruses, are thought to be released through the exocytosis pathway, after removal of the ER membrane. Release from the infected cell exposes the virion to gastrointestinal tract proteases, resulting in the cleavage of VP4 into VP5 and VP8, for which the production of fully infectious virions (Trask *et al.,* 2012). The viroplasm, which contains an active RNA replication complex and is essentially formed by two nonstructural proteins NSP2 and NSP5, colocalizes with LDs in infected cells (Cheung *et al.,* 2010). LDs recruitment close to the viroplasm begins soon after initial infection, and the number of viroplasm-LD complexes increases during the replication cycle (Cheung *et al.,* 2010). Lipidome analysis has shown that the total lipid content of the cell increases during rotavirus infection, consistent with an increase in the interaction of LDs with viroplasms (Gaunt *et al.,* 2013a). Chemical compounds blocking fatty-acid synthesis or interfering with LD homeostasis, such as triacsin C, have been shown to decrease the number and size of viroplasms and the number of infectious viruses produced (Gaunt *et al.,* 2013b).

Finally, different viral proteins from various viral models, including the μ1 outer capsid protein of reoviruses (Coffey *et al.,* 2006) and the agnoprotein of the polyomavirus BK (Untersatb *et al.,* 2010), have been shown to interact with LDs. The biological relevance of these associations remains to be determined in these viral models, but these findings suggest that various viruses have evolved mechanisms for interacting with LDs and, possibly, for subverting the function of these organelles for thier to use as a platform for viral particles assembly.

Bacteria

The formation of “foamy”, LD-filled macrophages is one of the key characteristics of mycobacterial infection. *Mycobacterium tuberculosis* (Russel *et al.,* 2009), *Mycobacterium bovis* (D’Avila *et al.,* 2006) and *Mycobacterium leprae* (Mattos *et al.,* 2011) infections lead to the accumulation of LDs within host cells. In foam cells, mycobacteria are present within phagosomes tightly apposed to LDs (Peyron *et al.,* 2008 ; Mattos *et al.,* 2011). In infected

cells, LDs are redistributed to the vicinity of vacuoles containing mycobacteria, very soon after bacterial engulfment, and neutral lipids subsequently accumulate within the bacterium-containing phagosome (Barisch C *et al.,* 2015). It has been suggested that the fatty acids released from the host triglycerides stored in LDs are the major source of energy and carbon for *Mycobacterium tuberculosis* during chronic infection. *Mycobacterium tuberculosis* uses triglycerides synthesized by its own triacylglycerol synthetase as its principal source of energy. Host-derived fatty acids are imported and used for the synthesis of triglycerides, which are then deposited in bacterial LDs (Daniel *et al.,* 2011). *Mycobacterium tuberculosis* encodes its own lipase, which may release stored fatty acids in times of energy shortage but can also degrade host triglycerides for use by the pathogen (Deb *et al.,* 2006). Studies in the *Mycobacterium leprae* model demonstrated that impairment of the initial recruitment of host cell LDs to the phagosome by interfering with cytoskeletal function decreases bacterial survival in infected cells (Mattos *et al.,* 2011).

The accumulation of LDs within infected cells has been observed for another intracellular bacterium, *Chlamydia trachomatis*, which infects epithelial mucosal cells. This bacterium interacts with LDs by inducing the translocation of an entire LD from the host cytoplasm into the lumen of the parasitophorous vacuole containing the bacterium, in a process resembling endocytosis (Cocchiaro *et al.,* 2008). It has been suggested that the chlamydial protein Lda3 plays a major role in this organelle sequestration by replacing ADRP at the surface of the LD and favoring the formation of links between the LD and the vacuole membrane (Cocchiaro *et al.,* 2008). Host LDs appear to be crucial for the capture of triglycerides and cholesterol esters by *Chlamydia trachomatis* for bacterial growth during active propagation, as bacterial inclusions are small and bacterial growth is inhibited in triacsin C-treated cells (Kumar *et al.,* 2006). The incorporation of LDs into the bacterium-containing vacuole has also been observed *in vivo*, in a mouse model of infection with a related bacterium, *Chlamydia muridarum* (Rank *et al.,* 2011).

Parasites

The intracellular protozoan *Trypanosoma cruzi*, the causal agent of Chagas disease, has been shown to induce the accumulation of large LDs in infected macrophages (Melo *et al.,* 2003; 2006) (Figure 1C). Similar events have been described for other protozoan parasites, including *Leishmania amazonensis* (Lecoeur *et al.,* 2013), *Leishmania major* (Rabhi *et al.,* 2012) and *Toxoplasma gondii* (Gomes *et al.,* 2014). LDs recruited close to the parasite-

containing vacuole have been shown to be sites of enhanced prostaglandin E2 (PGE2) synthesis favoring parasite growth (D'Avila *et al.,* 2011). Both cells containing internalized and non-parasitized cells were found to have larger numbers of LDs compared to control, non-infected cells, suggesting a bystander amplification of the response. However, parasitized cells had significantly more LDs than non-parasitized cells, consistent with the direct induction of LD biogenesis by parasite uptake (D'Avila *et al.,* 2011). As observed with *Chlamydia* and other pathogens, LDs are frequently seen around an within the parasitophorous vacuole (Melo e*t al.,* 2006; Melo & Dvorak, 2012; Toledo *et al.,* 2016). Overall, it is thought that host lipids acquired from LDs play a key role in sustaining successful parasite replication within the parasitophorous vacuole (Toledo *et al.,* 2016). However, it remains unclear whether this LD translocation is a stage of phagosome maturation favoring parasite survival or a host cell defense mechanism (Melo & Dvorak, 2012).

LD as an innate immune signaling platform

Accumulation of LDs in host cells infected with different pathogens may modulate the production of an innate immune response. Increased LD formation observed during infections depend not only on direct host-pathogen interaction but also on a bystander amplification system through pathogen components, such as lipopolysaccharide (LPS), present in all Gram-negative bacteria, and the mycobacterial cell wall component lipoarabinomannan (LAM), a virulence factor of *M. tuberculosis,* that are potential toll-like receptors (TLRs) ligands (Pacheco *et al.,* 2002; D'Avila *et al.,* 2006; Cao *et al.,* 2007). Infection with parasites such as *Trypanosoma cruzi* also leads to increased LD genesis through mechanisms that involve pattern recognition receptors (D'Avila et al., 2011).

LDs formed during pathogen infections function as platforms for synthesis of inflammatory mediators. LDs are recognized sites of arachidonic acid (AA), which is stored sterified mainly in phospholipids in these organelles (Melo & Weller, 2016). Following stimulation with pathogens and/or their components, AA is released as free fatty acid within newly formed LDs and enters the cyclooxygenase (COX) or lipoxygenase (LOX) pathways to generate eicosanoids such as PGE2 or leukotriene B4 (LTB4), respectively (Bozza *et al.,* 2007; Melo & Weller, 2016) (Fig. 2). These eicosanoids are signaling molecules with a wide range a biological functions including inflammation and immunity modulation (Bozza *et al.,* 2007). Moreover, PGE2, which potentially inhibits Th1 responses, may contribute to a

permissive environment for pathogen proliferation (Toledo *et al.,* 2016). LDs may be involved in other functions during the host response to pathogen infections. The proinflammatory cytokine tumor necrosis factor–α (TNF-α) is detected in LDs of mast cells, eosinophils, and macrophages in Crohn's disease (Beil *et al.* 1995) and after *in vivo* LPS stimulation of neutrophils and monocytes from septic patients (Pacheco *et al.* 2002). However, the association of LDs with cytokines remains to be understood.

Recent studies have suggested that LDs may also act as reservoirs of antiviral and antibacterial proteins. Viperin (virus inhibitory protein, ER-associated, interferon-inducible) is targeted to the cytoplasmic face of the ER and is also particularly abundant on the surface of LDs (Hinson and Cresswell 2009). Viperin can interact with the HCV NS5A protein at the surface of the LD, inhibiting HCV replication, and its antiviral activity is dependent on its presence on the surface of the LDs (Helbig *et al.,* 2011). Viperin has also been shown to inhibit the replication of DENV (Helbig *et al.,* 2013) and chikungunya virus (Teng *et al.,* 2012), although its localization to the LD is not specifically required in this case. Histones at the surface of the LD have recently been shown to be associated with an unexpected intracellular antibacterial defense system (Anand *et al.,* 2012). In experiments performed *in vitro* with LDs purified from *Drosophila* embryos, LD-bound histones were released and killed bacteria following activation by the LPS or lipoteichoic acid produced by the bacteria. This effect was also observed *in vivo* following the injection of various bacterial species into wild-type *Drosophila* embryos, as shown by comparison with embryos that had been genetically modified to eliminate the LD-bound histones. When mice were challenged with LPS, to mimic bacterial infection, the levels of LD-bound histones increased in the liver, suggesting that the loading of LDs with histones to kill bacterial invaders is a conserved immune mechanism (Anand *et al.,* 2012). Irgm3 is an immunity-related GTPase from the large family of interferon-induced proteins; it specifically localizes to the LD surface in interferon-γ-stimulated mouse dendritic cells (Bougneres *et al.,* 2009). Interestingly, Irgm3 is required for resistance to *Toxoplasma gondii* (Hunn *et al.,* 2011) and *Leishmania donovani* (Murray *et al.,* 2015) infections in mice.

Final remarks and future directions

Various intracellular pathogens hijack host LDs to complete their own life cycle, as shown in the model in Figure 2. Several viruses use host LDs as a platform for assembly, and some intracellular bacteria and parasites usurp host LDs for nutritional purposes. LDs are also

increasingly recognized as important players in innate immunity, involved in the synthesis of inflammatory mediators (Fig. 2). It remains unclear whether the intracellular pathogens targeting LDs also do so as a means of interfering with innate immunity thus favoring pathogen proliferation. Conversely, the location of innate immunity proteins on the surface of LDs may reflect an optimal host-cell strategy for fighting intracellular pathogens trying to hijack LDs. The multiple functions performed by LDs in cells are only just being elucidated, and further investigations in various host cell/pathogen models should help us to decipher the full range of LD functions.

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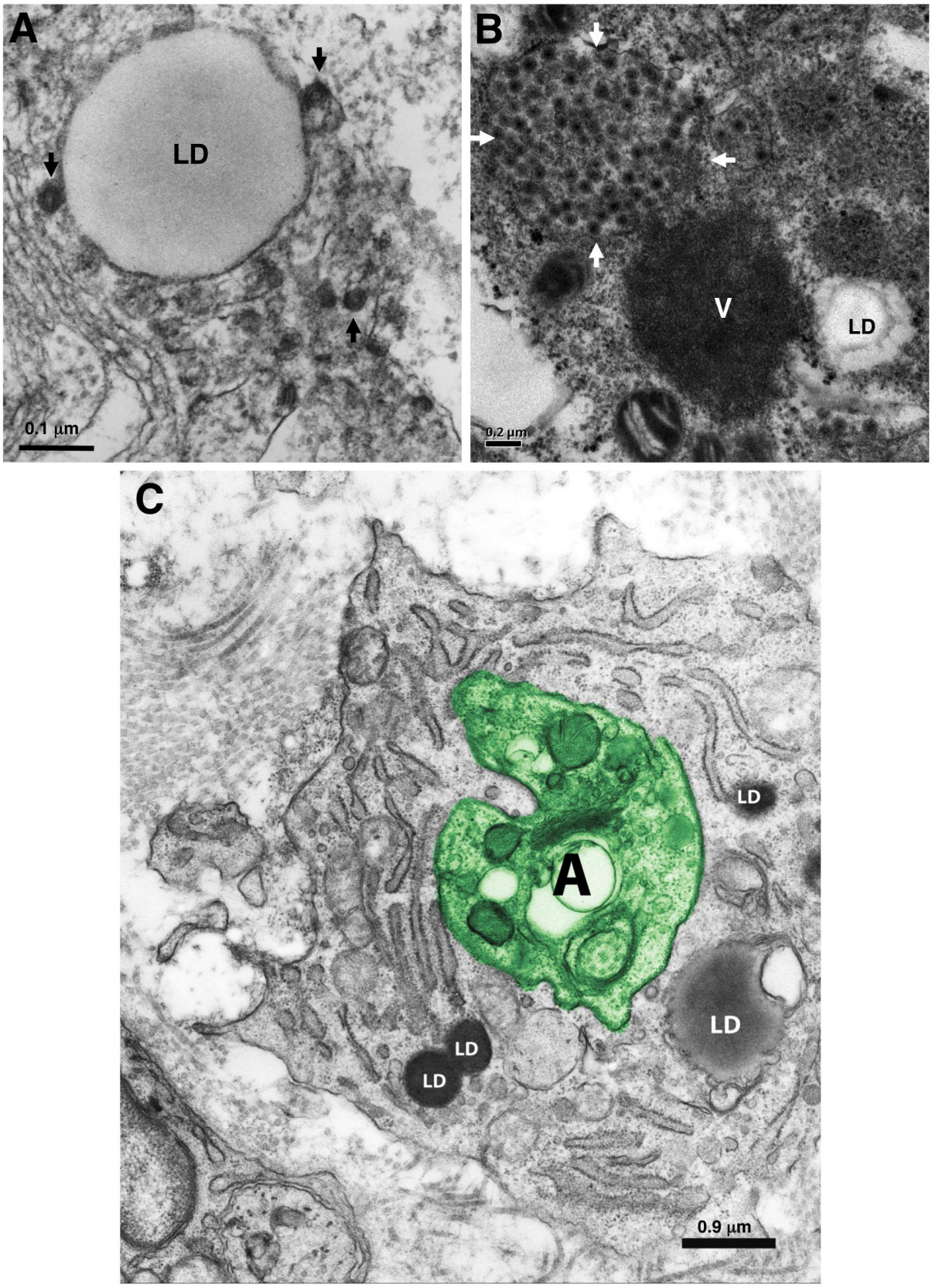


Fig.1. Lipid droplets as platforms for viral assembly and sources of nutrients for intracellular parasites.

A. Hepatitis C virus (HCV) assembly probably occurs at endoplasmic reticulum (ER) membranes connected to lipid droplets (LD), as HCV structural proteins self-assemble into HCV-like particles (arrows) that bud at ER membranes closely associated with LDs.

B. Rotaviruses are formed in ER-derived vacuoles (delimited by the white arrows) closely associated with cytoplasmic inclusion bodies called viroplasms (V), which are located close to LDs and serve as the site of viral replication.

C. LDs with different sizes and electron densities accumulate around an amastigote in the cytoplasm of an inflammatory macrophage from the heart (A, highlighted in green). The amastigote is the intracellular form of *Trypanosoma cruzi*.

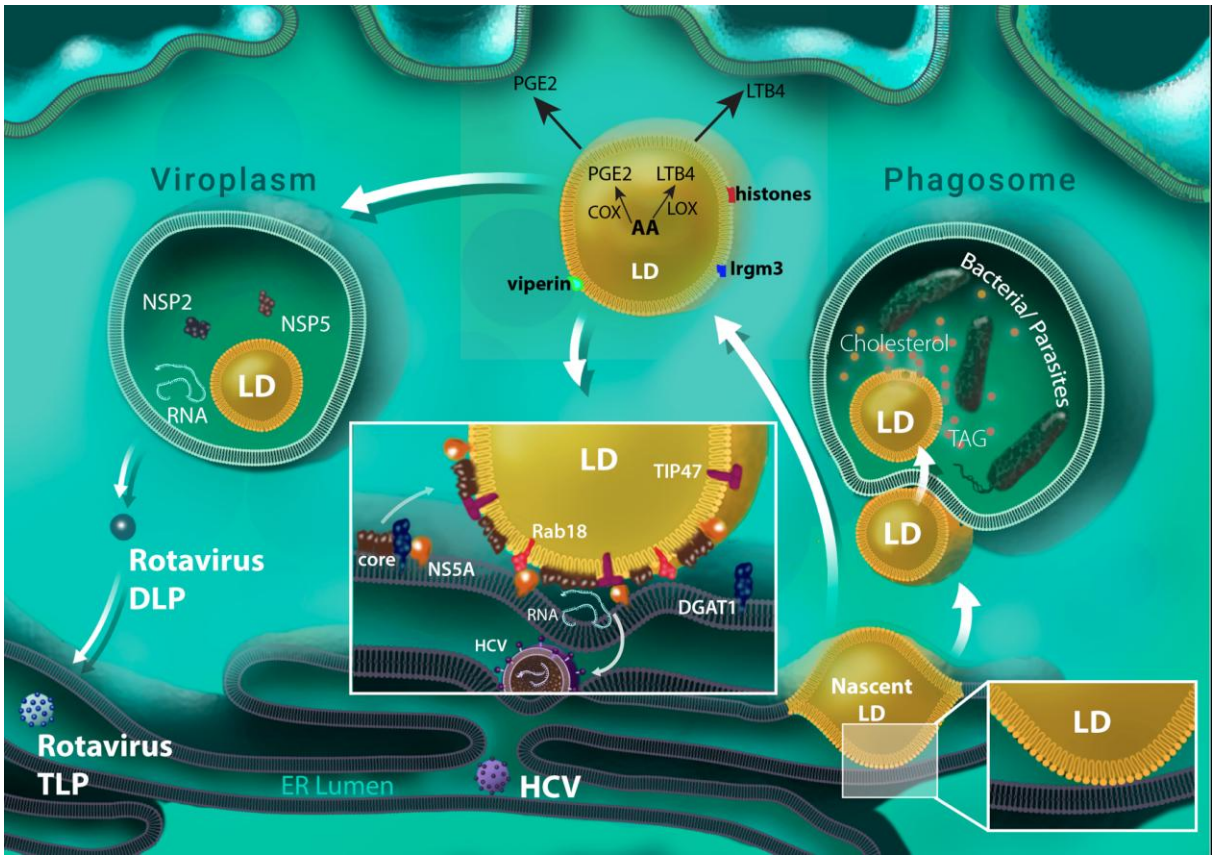


Fig.2. A model explaining the role of lipid droplets (LDs) during infections with different intracellular pathogens. In response to infections with bacteria, parasites and viruses, LDs are formed from the endoplasmic reticulum (ER) and accumulate in the cytoplasm. The interaction of LDs with phagosomes containing bacteria or parasites leads to the discharge of LD contents, such as cholesterol and triacylglycerol (TAG), which serve as source of lipids for pathogen growth. In viral infections, LDs are involved in different stages of viral replication. In hepatitis C virus (HCV) infection, diacylglycerol O-acyltransferase 1 (DGAT1), a protein located on the ER membrane, promotes interactions between the virus-encoded nucleocapsid protein (core) and the viral nonstructural protein 5A (NS5A) on the surface of the ER, ensuring that these proteins are targeted to the same LD. Core protein and NS5A complexes are then detected at the surface of LDs, together with the LD-associated proteins PLIN3/TIP47 and Rab18, which facilitate the interaction of LDs with specialized areas of the ER containing NS5A. Assembly starts when the core protein is transferred from the surface of LDs to the cytosolic ER membrane, together with viral RNA. In rotavirus infection, the viroplasm, wich contains viral RNA replication complexes and the viral nonstructural proteins NSP2 and NSP5, colocalizes with LDs. Double-layered particles (DLPs) released from viroplasms acquire an outer layer from the ER to form triple-layered particles (TPLs). LDs are also important players in innate immunity. Host-pathogen

interaction leads to the biosynthesis and secretion of inflammatory mediators such as prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) through arachidonic acid (AA)-derived pathways (cyclooxygenase - COX - and lipooxygenase - LOX -, respectively). PGE2 may potentially inhibit Th1 response thus favoring pathogen proliferation. In contrast to hijacking mechanisms to the pathogen benefit, innate immunity actors such as viperin (for virus inhibitory protein, ED associated, interferon inducible) and Irgm3 (for immunity-related GTPase family member m3), but also histones, are localized at the LD surface and may act as antiviral or antibacterial proteins.