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INVITED REVIEW

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Defensive-lipid droplets: Cellular organelles designed for antimicrobial immunity

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Summary

Microbes have developed many strategies to subvert host organisms, which, in turn, evolved several innate immune responses. As major lipid storage organelles of eukaryotes, lipid droplets (LDs) are an attractive source of nutrients for invaders. Intracellular viruses, bacteria, and protozoan parasites induce and physically interact with LDs, and the current view is that they "hijack" LDs to draw on substrates for host colonization. This dogma has been challenged by the recent demonstration that LDs are endowed with a protein-mediated antibiotic activity, which is upregulated in response to danger signals and sepsis. Dependence on host nutrients could be a generic "Achilles' heel" of intracellular pathogens and LDs a suitable chokepoint harnessed by innate immunity to organize a front-line defense. Here, we will provide a brief overview of the state of the conflict and discuss potential mechanisms driving the formation of the 'defensive-LDs' functioning as hubs of innate immunity.

KEYWORDS
bacteria, innate immunity, interferon, lipid droplets, parasites, virus

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1 | INTRODUCTION

To date, approximately 1400 species of human pathogens have been identified, including viruses, bacteria, fungi, protozoa, and helminths. As the *Earth Microbiome Project* advances, this number is expected to raise dramatically.[1] These pathogens cause 16 million deaths each year, and this situation is predicted to worsen in the future.[2] Biodiversity reduction and microbe genome plasticity will enable the estimated trillions of viruses and bacteria living on earth to colonize new host species. Furthermore, by 2050, an estimated 10 million people will die each year from antimicrobial resistant bacteria.[2] It is therefore imperative that countermeasures to face this global threat are identified and implemented as soon as possible.

A premise of our research is that countermeasures could be learnt from the sophisticated defense mechanisms that eukaryotes have developed over millions of years to cope with the pervasive presence of microbes. In eukaryotic cells, front-line defense is organized around rapid and generic responses collectively defined as "innate immunity". Being mechanisms generated to face a never-ending variety of microbes, innate immunity first detects common vulnerable aspects of pathogens and then organizes generic responses to confront invaders.[3]

Intracellular pathogens are largely or completely dependent on host nutrients. In eukaryotes, nutrients are stored in the form of lipids and managed by atypical organelles called lipid droplets (LDs).[4] Since Rudolf Virchow's description in 1863 that biopsies of *Mycobacterium leprae*-infected patients accumulate fat-laden cells,[5] the list of pathogens known to induce accumulation of host LDs has grown exponentially[3] (Table 1). Although mechanistic details remain largely unknown, LDs accrue in cells infected with some of the most medically relevant viruses (e.g., Hepatitis C, Zika, Dengue, and SARS-CoV), bacteria (e.g., *Mycobacterium*, *Chlamydia*, and *Salmonella*), and parasites (e.g., *Plasmodium*, *Trypanosoma*, and *Leishmania*). The most accepted explanation for these lipid-laden cells is that host LDs are first induced and then manipulated by virulence factors produced by pathogens to obtain substrates needed for growth, chronic persistence, or to evade immunity.[6–8]

In this context, our work and that of others has revealed that the LD–pathogen dynamic is far more complex than initially considered. Dependence on host lipids could be a generic weakness of microbes identified by innate immunity and thus the LD a strategic chokepoint for organizing a first defensive line.[3] A pioneering indication that LDs are active innate immunity players is found in studies describing that in virally infected cells, viperin (RSAD2), an interferon (IFN)-inducible broad-spectrum antiviral protein, resides on LDs to simultaneously participate in killing and transduction of type I IFN signaling.[9,10] This challenging concept was extended with the demonstration that histones on LDs protect *Drosophila* embryos when infected with Gram-negative and Gram-positive bacteria.[11]

More recently, we demonstrated that LDs have a complex antibacterial activity, which is upregulated in mice treated with the lipopolysaccharide (LPS), a potent activator of innate immunity, or subjected to a polymicrobial sepsis.[12] Quantitative profiling of the proteome of hepatic LDs purified from mice treated with LPS proved that viperin and histones are just components of a complex and multifaceted defensive strategy organized on/around LDs. A stringent analysis identified 689 proteins differentially regulated by LPS in purified LD fractions (317 enriched/372 reduced), a major share potentially related to immunity for the first time. Functional annotation analysis predicted that in LPS-activated cells, LDs accrue proteins (i) with anti-pathogenic activity, (ii) involved in immune signaling, (iii) mediating inflammation, and (iv) regulating immunometabolism. Among cellular organelles, LDs display a unique flexibility in terms of formation, protein/lipid composition, and interaction with other organelles and thus have the capacity to rapidly assume all these defensive mechanisms urgently activated by infected cells.[13]

Here, we will provide a summary of the cell biology of LDs and examine representative LD-pathogen dynamics that exemplify the state of the conflict. Further, we will discuss, from computational predictions and current annotations, mechanisms potentially driving the profound transformation of LDs into hubs of innate immunity. To define these newly formed LDs, we will use the term "defensive-LDs" (d-LDs) that, in our opinion, emphasizes the profound compositional and functional differences existing between the LDs assembled in infected cells and other well-characterized LDs that, activated by energy sensors, provide metabolic flexibility to healthy cells (Box 1).

2 | LIPID DROPLETS MANAGE THE NUTRIENTS OF EUKARYOTIC CELLS

The capacity to store nutrients in rich environments to be used during scarcity periods provides cells with the metabolic flexibility needed to survive when facing environmental fluctuations.[4] Triacylglycerol (TAG), a highly hydrophobic and reduced ester formed by glycerol and three fatty acids, is the preferred molecule for eukaryotes to store energy and nutrients. LDs are the specialized organelles evolved to gather, administrate and supply TAGs.[14] TAG-enriched LDs are ancient organelles present in the simplest unicellular organisms, such as green algae or yeast, to the most specialized mammalian cells, such as hepatocytes, cardiomyocytes, or macrophages.[4,15,16]

The cellular content of LDs is remarkably flexible; LDs can be promptly formed or efficiently consumed depending on the cellular status. In the presence of nutrients, LDs are rapidly formed by accumulating the fatty acids generated de novo, from glucose or amino acids (lipogenesis), and the lipids imported by cells from the extracellular medium (Figure 1A). Fatty acids are esterified into TAG by the sequential action of enzymes residing in the endoplasmic reticulum (ER)[14] (Figure 1B). Assisted by structural proteins, such as seipin, fat storage-inducing transmembrane protein 2 (FIT2), or LD assembly factor 1 (LDAF1),[16–18] TAGs are gradually accrued into lipid lens that phase separate within the ER bilayer to form a nascent LD.[19] As additional lipid arrives and esterifies, the lens progressively grows into the cytosol to generate a spherical organelle, encircled by a single monolayer of phospholipids, and with a hydrophobic core of TAGs,

TABLE 1 Pathogens inducing LDs in host cells and their proposed mechanism of action.

Pathogen	Host cell	Mechanism	References
Bacteria			
Mycobacterium tuberculosis (Mtb)	Human macrophages Mice	1. Restriction of bacterial growth 2. Inflammatory responses 3. Production of host defensive eicosanoid	[79,83,156,240]
Chlamydia trachomatis	Human Hela Mouse embryonic fibroblasts (MEF)	1. Association with reticulate bodies for lipid delivery 2. Remodeling of the LD proteome for bacterial growth	[58,63]
Chlamydia pneumoniae	Murine adipocytes	Mobilization of free fatty acids for bacterial growth	[161]
Salmonella Typhimurium	macrophages	LD accumulation for bacterial proliferation and secretion of PGE2	[162]
Viruses			
Dengue (DENV)	Hamster CHO Human HepG2/Hela/A549/Hek293T/ hepatocytes Monkey Vero E6 Mosquito Aag2	1. Scaffold for nucleocapsid formation 2. Virus replication	[45,166]
Sindbis	Mosquito Aag2	1. LD accumulation 2. Activation of Toll and IMD pathways	[241]
Hepatitis C (HCV)	Human HuH7/hepatocytes	Virus production	[168,169,242]
Zika (ZIKV)	Human primary astrocytes Human HuH7/HEK293T/Hela Murine primary MEF Monkey Vero	1. Increase the motility of LDs 2. Enhanced INFI/III production 3. Control early viral replication	[42,175]
Poliovirus (PV)	Human HeLa S3/HuH7	1. RC biogenesis 2. PV replication	[48]
Herpes simplex 1 (HSV-1)	Human primary astrocytes Human HuH7/HEK293T/Hela Murine primary MEF Monkey Vero	1. Enhanced INFI/III production 2. Control early viral replication	[42]
Rotavirus	Human MA104/Caco-2/BSC-1/Cos-7/ MA104	Generation of infectious progeny virus	[171]
Reovirus	Hamster CHO Monkey CV-1	Induction of apoptosis	[172]
SARS-Cov2	Human primary monocytes/A549 Monkey Vero-E6	1. Assembly viral platform 2. Increase pro-inflammatory mediators	[174]
Rabies	Human N2a/BSRSK-N-SH Mice C57BL/6	Facilitate viral budding	[173]
Parasites			
Plasmodium derivates	Mice hepatocytes Erythrocytes	1. LD increases associated with liver dysfunction 2. Accumulation or degradation of LDs in stage development specific manner of parasite	[243,244]
Candida albicans derivates	*Candida albicans* *Candida parapsilosis* *Candida tropicalis*	LD accumulation in response to squalene synthase inhibitor	[245]
Trypanosoma cruzi	Rat macrophages C57BL/6 mice macrophages	1. Increase in LD number and PGE2 production 2. Formation of inflammatory macrophages	[65,182]
Toxoplasma gondi	Human primary skeletal muscle	1. Lipids delivery to PV 2. Increase of inflammatory indicators PGE2 and COX-2	[180]
Leishmania amazonensis	Mouse myeloid dendritic leucocytes	LD accumulation and contact with PV	[181]

BOX 1 Defining defensive-LDs, implications in immunity and disease.

Eukaryotic cells rapidly accumulate LDs after infection by microbes or when activated by danger signals[3] (Figure 2A). When compared to LDs activated by energy sensors and assembled to produce metabolic energy during fasting ("fasting-LDs", f-LDs), the LDs of infected cells exhibit unique compositional and functional traits.[13] Comparative proteomics of hepatic LDs purified from fasted or from LPS-treated mice determined that at least 689 proteins are differentially regulated on/around LPS-activated LDs (317 enriched/372 reduced).[12] The Ingenuity Pathway Analysis concluded that these LDs have the potential to be innate immunity hubs functioning in several defensive fronts including killing, signaling, inflammation, and immunometabolism.[12] Our work and that of others is beginning to indicate that these could be generic roles assumed by the LDs of infected cells.[12,42,79,83,149] Therefore, to refer to the distinct LDs formed by activation of innate immunity programs, we propose the name of 'defensive-LDs' (d-LDs).

When compared with f-LDs, the d-LDs formed during a polymicrobial sepsis or in response to LPS demonstrated a significantly enhanced protein-mediated antibiotic activity in classical bacterial killing assays.[12] In contrast to activated f-LDs, activated d-LDs are depleted of proteins related to mitochondrial and phospholipid metabolism but enriched in proteins involved in immunity (Figure 2B). Such a different proteome is, at least in oxidative cells, potentially determined by the relative composition of PLINs, with d-LDs enriched in PLIN2 but depleted of PLIN5.[12] Forced PLIN5 expression in infected fibroblasts and macrophages diminishes the amplitude of the defensive response against bacteria.[12] The low PLIN5 levels on d-LDs likely reduces crowding to favor recruitment of PLIN2 and of newly synthesized defensive proteins, with some of them physically interacting with PLIN2.[12,40] Furthermore, the low PLIN5 levels on d-LDs lessen their interaction with mitochondria and the signaling that, driven by PLIN5 during fasting, potentiates oxidative metabolism in cells.[4] Therefore, by cancellation of key traits of f-LDs, d-LDs likely contribute to generate a particular metabolic environment conducive for defense.

Among the upregulated proteins, the d-LDs assembled in host cells recruit viperin, CAMP, IRGs (IGTP, IFI47, IIGP1, and TGTP) (12), histones,[11] GBPs (GBP2 and GBP6), and Rab GTPases (Rab7 and Rab18) (Bosch et al, unpublished) (Figure 2C). Other studies have described that the LDs of infected cells accrue RNF213/ISG15 (134), HIG-2,[79] STING/TBK1,[97] and COX-2/PGE_2 synthase,[84] some of them also identified in the proteomic characterization of d-LDs[12] (Figure 2B,C).

In contrast to f-LDs interacting with mitochondria, d-LDs interact with the phagolysosomal membranes containing invaders.[43,48,49,63,71,89,160] These contact sites could be driven by IRGMs, GBPs, and Rab GTPases.[87–89,115] Electron microscopy, designed for membrane preservation, revealed that d-LDs generate a discontinuity in the phagolysosomal membrane possibly allowing physical interaction of antimicrobial proteins with the bacterial outer membrane[3,12] (Figure 2D). Proteins on dLDs, such as CAMP and histones, form functional connected complexes and cooperate in different steps of the killing.[105] In support to the existence of these trafficking mechanisms and contact sites, d-LDs efficiently accumulate and deliver hydrophobic antibiotics into bacterial inclusions to reduce microbes' viability.[90,91]

In silico analysis predicts that intricated signaling networks, involving transcriptional programs and posttranslational modifications, converge to generate d-LDs. Upstream regulation inference analysis anticipates that a major share of the proteome programming upon activation of d-LDs is mediated by IFNs, TLR2 and TLR4/NF-kB, and SRF/SMADs axes[12] (Figure 3A,B). The stability and function of d-LD proteome is regulated by PTMs. The d-LD proteome is enriched in substrates of cytokine-activated kinases, including AKT, GSK3, and IKK[12] (Figure 4B). The activity of the ubiquitin-proteasomal system on d-LD proteins likely regulates the extend and duration of the defensive response.[144–146] Furthermore, the RNF213-driven ISGylation of LD proteins (see details in section 5), is involved in the switching from f- to d-LDs[133,149] (Figure 4C). Additional signals contributing to the programming of d-LDs have been attributed to receptor tyrosine kinases, such as the EGFR in virally infected cells,[42] and additional transcription factors, such as HIF-1 in *Mtb* infected cells.[79]

The new concept of the d-LD could have important implications for understanding progression of diseases in which cells display an abnormal accumulation of LDs, including obesity, cancer, and aging.[157,158] These pathological processes are all characterized by chronic inflammation and cellular damage. For example, after a stroke microglia accumulates LDs resembling to d-LDs and containing proteins such as viperin, ISG15, RNF213, IFI47, TGTP1, IIGP1, and GBP6. The accumulation of d-LDs is exacerbated in the microglia of old individuals driving an exaggerated type I IFN immune response and worsening the neurological outcome.[159] Hence, the equilibrium between f- and d-LDs could be somehow disrupted in unhealthy cells and contribute to pathogenesis.

cholesterol esters, and other hydrophobic molecules such as vitamins or pigments (Figure 1C). Although requiring the coordination of many different proteins and formation/transformation of many lipid species,[14] the biogenesis of LDs is an extremely rapid metabolic reaction occurring a few minutes after the arrival/formation of lipids.[19] Complexes of functionally connected enzymes at the sites of

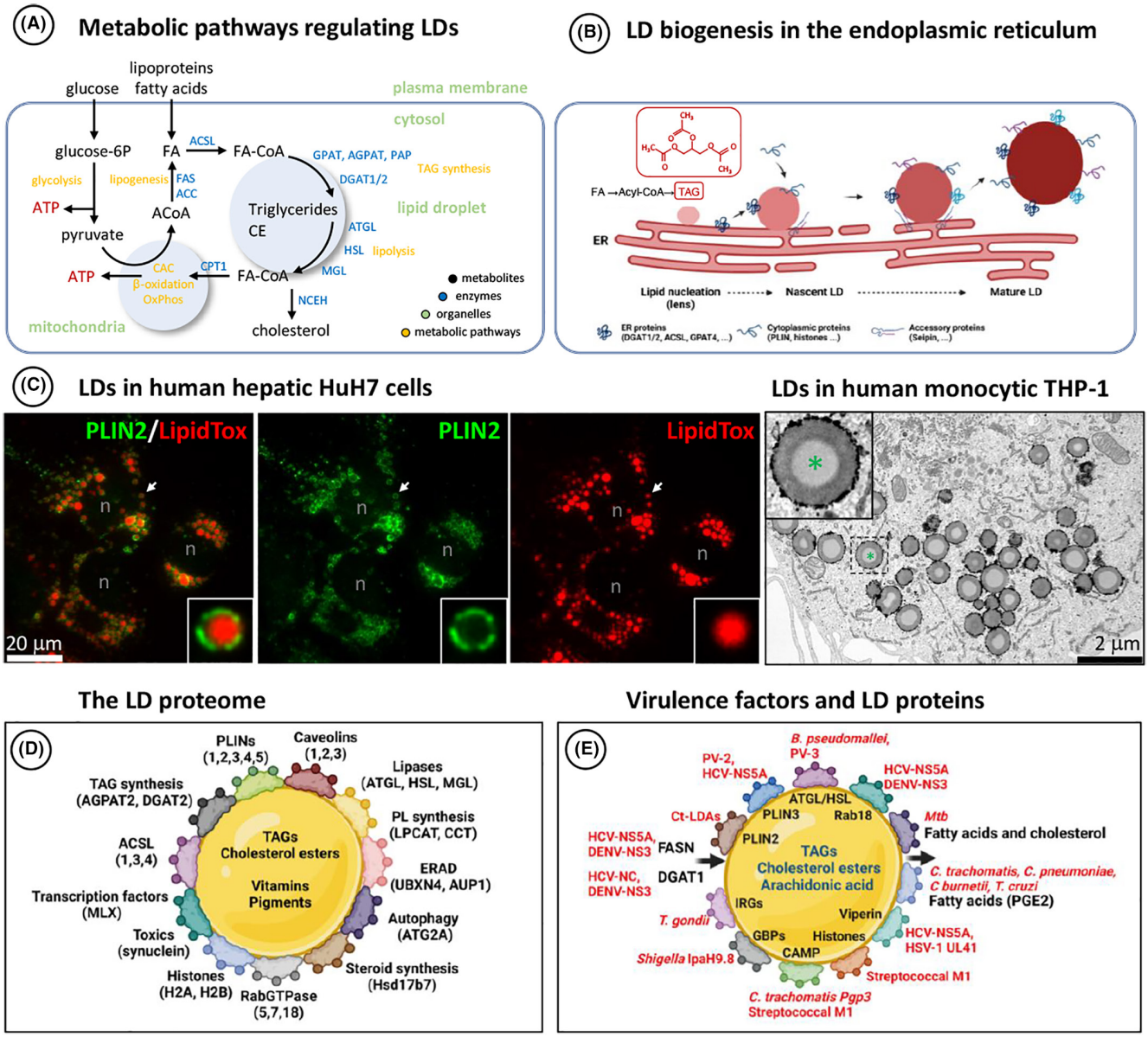


FIGURE 1 Lipid droplets: metabolism, morphology, and composition. (A) Simplified scheme of the main metabolic pathways and intermediate metabolites involved in the biogenesis and consumption of LDs. See the text for additional details. FA, fatty acid; FA-CoA, acyl-CoA; CPT1, carnitine palmitoyltransferase I; CAC, citric acid cycle; FASn, fatty acid synthase; OxPhos, oxidative phosphorylation; ACC, acetyl-CoA carboxylase; GPAT, glycerol-3-phosphate acyltransferase; AGPAT, 1-acyl-sn-glycerol-3-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diglyceride acyltransferase-1 and -2; ACSL, acyl-CoA synthetases; ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase; MAGL, monoacylglycerol lipase; NCEH, neutral cholesterol ester hydrolase. (B) Schematic representation of LD biogenesis occurring in the endoplasmic reticulum (ER). After esterification, neutral lipids accumulate within the ER bilayer forming a lens structure that undergoes a phase separation within the ER bilayer and grows into the cytosol forming a nascent LD. Cytoplasmic and ER proteins are recruited to the surface of LDs, facilitating their growth, and budding into mature LDs. Accessory proteins cooperate during the process. The esterification of fatty acids (FA) into triacylglycerol (TAG) is illustrated in the upper panel (red: the chemical structure of TAG). (C). Hepatic HuH7 cells were treated with oleic acid to induce LD formation for 16 hours (left panel). PLIN2 (green) was localized with specific antibodies and neutral lipid stained with LipidTox. (N) indicates the nucleus of the cells. The arrow marks the LD in the high magnification inset. THP-1 cells were processed for TEM analysis (right panel). Lipid droplets are distinguished by their spherical morphology, by being relatively low electron dense, and by being delimited by a single monolayer of phospholipids. (D) Simplified scheme representing major proteins on LDs. (E) The scheme contains a few examples of LD proteins (black) manipulated by virulence factor secreted by pathogens in host cells (red) (see the text for details).

biogenesis and on LDs provide a high efficiency to the process.[14] These LDs are relatively static organelles and accumulate in the cell center waiting for the lipids and proteins they store to be required.[20]

The surface of LDs is a crowded domain (Figure 1D). Dynamic competition between LD proteins allows rapid remodeling of the organelle's proteome to efficiently respond to fluctuations in

nutritional, metabolic, and stress conditions.[21] To accurately administer nutrients, the surface of LDs accommodates at steady state an estimated 150 different proteins.[22,23] The LD proteome includes its own family of regulatory proteins, the perilipins (PLINs).[24] The main function of PLINs (−1 to −5) is to modulate the activity of the lipases residing on the LD surface.

When lipids are needed, LDs are promptly but progressively activated to meet the cellular demands. PLINs are phosphorylated by energy sensors, such as the protein kinase A (PKA), to function as scaffolds that reorganize the LD surface by releasing, excluding, or attracting other proteins such as acyl-CoA synthetases (ACSL), accessory proteins (e.g., comparative gene identification-58, CGI58), and lipases (adipose triglyceride lipase, ATGL; hormone sensitive lipase, HSL; and monoacylglycerol lipase, MAGL).[4] Especially during periods of prolonged starvation, TAG-LDs are also metabolized by acid lipases in a process conducted by different types of autophagy; lipophagy involving small parts of LDs, macroautophagy involving the whole organelle, and chaperone-mediated autophagy involving specific LD proteins.[4,20,25–27] These LDs activated in nutrient poor environments will be defined from here as "fasting-LDs" (f-LDs).

Activated LDs are highly dynamic organelles forming contact sites with most cellular organelles.[28] Up to 26 Rab GTPases, regulators of membrane trafficking, reside on LDs.[22] The presence of these GTPases reflects the potential of LDs for delivering lipids and proteins where and when necessary, although the mechanisms involved are still largely unresolved.[29,30] For example, in a process activated by the energy sensor 5′ AMP-activated protein kinase (AMPK) when sensing low ATP levels, f-LDs move on microtubules to physically interact with mitochondria and, by forming a "metabolic synapse", locally deliver the fatty acids that will be oxidized to produce metabolic energy.[20] The enormous potential of LDs in supplying cells with energetic substrates is reflected in the fact that these organelles provide the liver with the necessary metabolic energy to regenerate after a 70% hepatectomy, a compensatory hyperplasia accomplished in just 7 days.[31]

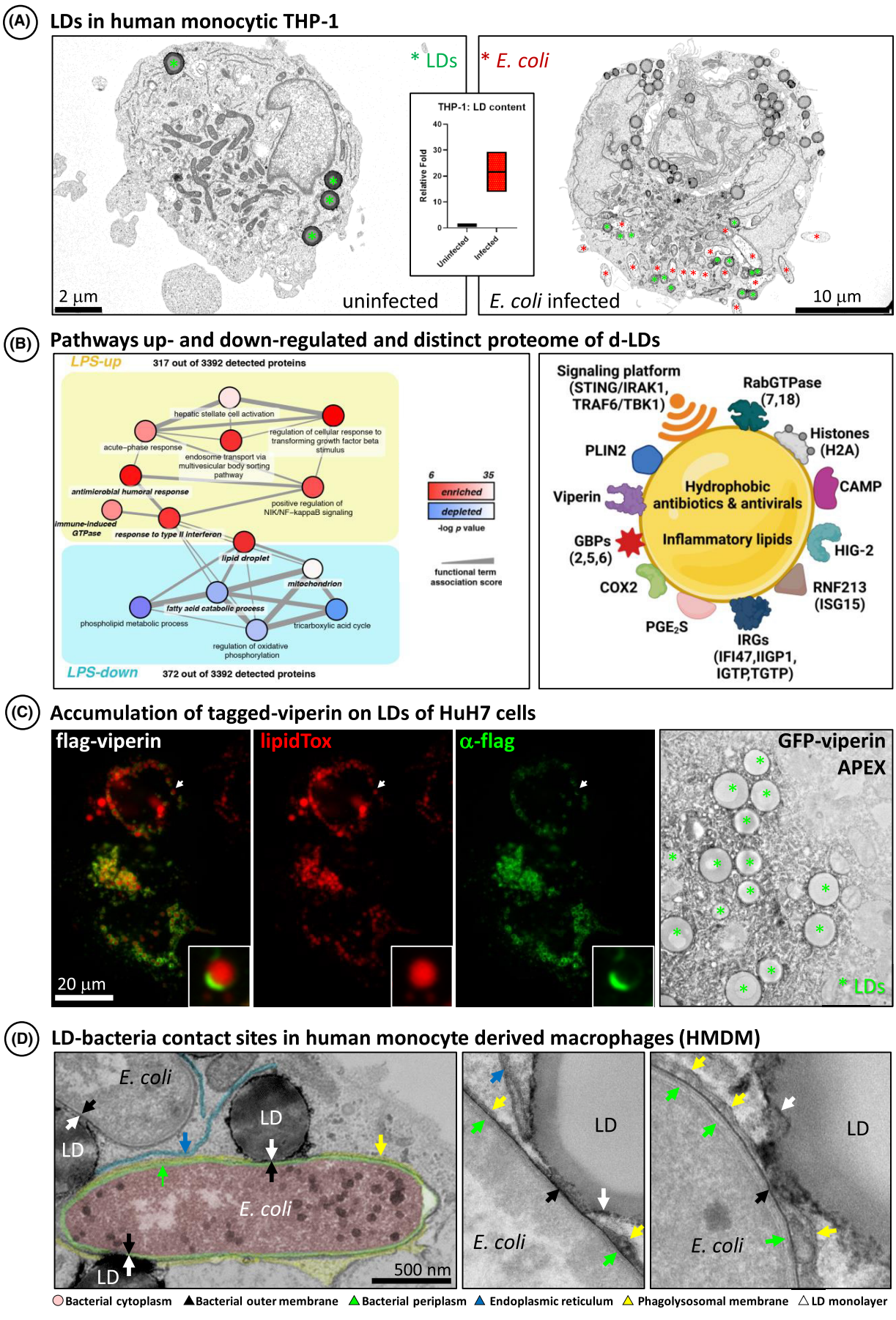
Furthermore, reflecting additional roles beyond lipid administration, the LD monolayer accommodates proteins not obviously related to lipids, such as histones,[32] toxic proteins,[33] caveolins,[34,35] transcription factors,[36] proteins of the ubiquitin system,[37] components of the ER associated degradation of proteins,[38,39] and immune-related proteins.[40] The role of LDs in maintaining cellular homeostasis by functioning as generic stress buffers has been proposed.[21,29] A major share of the knowledge behind this paradigm shift about the functions of LDs has been enabled by the use of systematic molecular profiling approaches, capable of revealing and describing non-intuitive systems-level relationships.[41]

3 | LIPID DROPLETS, AN ATTRACTIVE SOURCE OF NUTRIENTS FOR INTRACELLULAR PATHOGENS

Accumulation of host LDs is observable during the first hour following infection of protozoan parasites, bacteria, and viruses[42,43] (Figure 2A) (Box 2, 3, and 4). This is well characterized in the case of positive-strand RNA viruses, the largest group infecting eukaryotes, that parasitize host LDs in all steps of their lifecycle.[44] These pathogens exploit LDs to generate replication compartments, for virion assembly, to form lipoviroparticles for egression, and by forcing host metabolism to produce the energy needed to fuel the aforementioned processes.[45–51] For example, Poliovirus (PV) produces PV-2 and PV-3 that displace PLIN-3 and activate ATGL and HSL to release the fatty acids that, converted into phospholipids, generate the replication compartments in the ER[48] (Figure 1E). Hepatitis C and Dengue viruses produce HCV-NS5a and DENV-NS3 to activate fatty acid synthase (FASN, key lipogenic enzyme) and diacylglycerol O-acyltransferase 1 (DGAT1, key fatty acid esterifying enzyme) to generate the LDs that will be used for viral packaging.[51–54] Viral components are concentrated on the LD monolayer by physically interacting with abundant LD resident proteins such as PLINs and Rab GTPases.[51,52,54–57]

Similarly, intracellular Gram-positive and -negative bacteria induce and target host LDs.[3] When infecting fibroblasts or macrophages, *Chlamydia trachomatis* rapidly induces formation of host LD.[58,59] To obtain fatty acids and cholesterol, *C. trachomatis* manipulates host LDs by secreting Ct-LDAs (LD-associated proteins).[59–62] For example, Ct-LDA3 displaces PLIN-2 and promotes the apparent translocation of host LDs into the bacterial inclusion.[63] Further, *C. trachomatis*, *Chlamydia pneumoniae*, and *Coxiella burnetii* use the fatty acids from host LDs for the synthesis of prostaglandin E2 (PGE2), an immune suppressor employed also by *Trypanosoma cruzi*

FIGURE 2 Defensive-lipid droplets: formation, composition, and interaction with bacteria. (a) THP-1 cells (left panel) were infected with *Escherichia coli* for 1 hour (central panel) or 8 hours (right panel) and subsequently processed for flow cytometry or TEM analysis. Infected THP-1 rapidly accumulated LDs as shown in the graph (arbitrary fluorescence units corresponding to LipidTox quantification) and in the image (green asterisks). Bacteria are indicated with red asterisks. (b) The left panel shows relevant functional annotation terms among the proteins enriched (red) or depleted (blue) in d-LDs. Functions are displayed as a network based on their relationship and hit overlap through the REVIGO open resource.[238] The right panel shows a schematic representation of d-LDs and includes some proteins discussed in the text. The presence of GBP and RNF213 was detected by proteomics.[12] (c) Tagged (flag- and GFP-) human forms of viperin were transfected in human hepatic HuH7 cells. Cells were treated with OA to induce LD formation. Cells were fixed after 16 hours and stained with anti-flag antibodies (green) and LipidTox staining (LD, red). In the right panel, GFP-viperin distribution was analyzed using an APEX genetic tag together with stable nanobodies against GFP and analyzed by TEM. (d) Human monocyte derived macrophages (HMDMs) were infected with *E. coli* for 4 hours, fixed, and processed for TEM analysis. The bacteria and the ER have been pseudocolored (blue: putative ER; red: *E. coli* interior [excluding periplasm]; green: periplasm with bounding membranes; yellow: vacuolar membrane). White arrows indicate the LD monolayer and black arrows the bacterial outer membrane.



BOX 2 Lipid droplets and bacteria.

In this box, we provide an overview of what is known regarding the mechanisms underlying the induction and utilization of LDs by several bacterial pathogens (Table 1).

Intracellular bacteria cannot survive inside host cells without continuous nutritional fatty acid support. Cytosolic bacteria or vacuole-containing bacteria secrete virulence factors that will hijack LDs to complete their life cycle and sustain their optimal growth and persistence. Bacteria will benefit from the contact with LDs to activate TAG lipolysis and subsequently fulfill their replication needs from fatty acids.

Chlamydia trachomatis is an obligate intracellular bacterium that causes genital and ocular diseases. During infection, LDs are translocated from the host cytoplasm to the lumen of the bacteria-containing parasitophorous vacuole at IncA-enriched subdomains, enabling the replication of the bacteria. The docking of LDs to the vacuole is promoted through the removal of PLIN2 from the surface of LDs by the chlamydial protein Lda3 which could initiate lipolysis.[63] LDs isolated from cells infected with *C. trachomatis* are enriched in proteins related to lipid metabolism and LD biogenesis.[58] Furthermore, treatment with triacsin C (an inhibitor of LD biogenesis) negatively impacted *C. trachomatis* replication, further indicating the role of LD in bacterial growth.[59] A second example of *Chlamydia* species, *Chlamydia pneumoniae* proliferates inside host cells by activating FABP4/HSL-mediated lipolysis which releases fatty acids from LDs needed for ATP synthesis, a vital molecule for bacterial replication.[161]

Salmonella enterica is a facultative intracellular pathogen that causes salmonellosis. Following *S. enterica typhimurium* macrophages infection, TLR2 signaling is activated leading to LD accumulation via SPI1-related T3SS activity. The pharmacologic inhibition of DGAT1 and cytosolic phospholipase A2 lowered bacterial proliferation and abrogated the synthesis of PGE2.[162]

Mycobacterium tuberculosis (*Mtb*), the causal agent of tuberculosis, is a facultative intracellular pathogen that primarily targets lung macrophages. Activation of TLR2/4 by *Mtb* components leads to an important reprogramming of energy and lipid metabolism, with inhibition of lipolysis and fatty acid oxidation and concomitant increase in lipid uptake, mobilization and de novo synthesis leading to LD accumulation.[68,69] Intracellular *Mtb* can import fatty acids deriving from host LDs, suggesting that the pathogen uses them as a lipid source. However, maintenance of LDs in infected macrophages appears to require IFN-γ-driven induction of HIF-1α, which inhibits lipolysis and blocks *Mtb*'s acquisition of LD-derived fatty acids.[79] In *Mtb*-infected macrophages LD formation also boosts the production of host protective eicosanoids such as LXB4 and PGE2.[79] In support of host LDs having antimycobacterial functions, chemically or genetically inhibiting fatty acid oxidation in macrophages increased their ability to control *Mtb* growth.[163,164]

to evade immunity.[64,65] *Burkholderia pseudomallei* activates the host nuclear receptor NR1D2 that downregulates ATGL and prevents the autophagy-dependent suppression of infection.[66]

One of the most medically relevant examples of bacteria interacting with LDs is *Mycobacterium tuberculosis* (*Mtb*), the leading cause of death from a single infectious agent.[67] By extensively rewiring host cell metabolism, *Mtb* infection causes the accumulation of LDs and the formation of foamy macrophages.[68,69] Because *Mtb* persists in a dormant state within such foamy macrophages in vitro and in the lung granulomas of tuberculous patients, it is commonly accepted that host LDs benefit the pathogen by providing a nutrient source.[70–72] *Mtb* expresses coordinated fatty acid and cholesterol import systems that support bacterial survival in vivo.[73] Remarkably, *Mtb* synthesizes its own LDs, a process involving TAG from host LDs and requiring the interaction of host LDs with the bacteria-containing phagosomes.[70,71,74]

4 | ARE LIPID DROPLETS ACTIVE DEFENSIVE ORGANELLES?

In this context, few studies have begun to illuminate a more complex relationship between invaders and LDs, in which the accumulation of host LDs could reflect a defensive response.[3] For example, host LDs rapidly accumulate even in cells infected with killed pathogens.[43,75,76] The formation of host LDs is triggered by common pathogen-associated molecular patterns (PAMPs), such as LPS or lipoarabinomannan, and danger-associated molecular patterns (DAMPs), such as synthetic nucleic acids.[42,75,77] Furthermore, LDs also accumulate in uninfected cells in the vicinity of infected cells.[43,78] Thus, driven by signals produced and emitted by infected cells when sensing danger, formation of host LDs seems to be a defensive innate immunity response when identifying common microbial molecular patterns.[79,80]

The LDs assembled by host cells could actively participate in the defense. Hepatic cells with reduced LD content had a concomitant reduction in Type I and III IFN production when infected with Sendai, Herpes simplex 1, and Zika viruses.[42,81] Similarly, inhibitors of LD formation markedly attenuated expression of anti-viral genes driven by Type II IFN in pancreatic beta cells.[82] In the case of bacteria, LDs of *Mtb*-infected macrophages critically mediate the production of protective eicosanoids,[79,83] a process at least partially conducted by host LD enzymes and lipids.[84] Hence, infection-induced LDs participate in innate immune signal transduction and production of inflammatory mediators.[3,68,85]

Despite the growing possibility that LDs have assumed defensive roles, until recently only three immunity-related proteins had

BOX 3 Lipid droplets and viruses.

In this box, we will provide a few examples of viruses to briefly uncover the role of LDs in viral immunity (Table 1).

The classical role of LDs during viral infection is particularly highlighted in the positive single strand RNA (+ssRNA) viruses from the *Flaviviridae* family[45,165] (Table 1). These viruses induce a rearrangement of host membranes to provide a specialized replication compartment (RC) within host cells for the synthesis of viral genome. The ER membrane is the preferable site for +ssRNA viruses, where the RC is employed for genome packaging, viral replication, translation, assembly, and immune evasion. Since the ER is the formation site of LDs, it is not surprising that a strong connection was described between LDs and +ssRNA virus during their life cycle, where many viral proteins are trafficked to or interacting with LDs.[165]

For instance, Dengue virus capsid proteins strongly bind to LDs to have a scaffold for nucleocapsid formation during encapsulation[166,167] and activate LDs consumption to release fatty acids for virus replication.[45] Another well-studied example of the *Flaviviridae* virus family is the Hepatitis C virus, where the core protein is targeted to LDs for efficient viral replication and assembly through the formation of a RC.[168,169]

Poliovirus, belonging to the enterovirus's family, utilizes its 2C protein to generate a contact site between LDs and the RC. Consequently, viral proteins activate the lipolysis machinery by interacting with HSL and ATGL (lipases on the surface of LD) to enable the generation of fatty acids from LD and thereby provide lipids for RC biogenesis and Poliovirus replication.[48]

Rotavirus early-stage assembly in infected cells arises in viroplasms which are shown to be associated with LDs proteins[170] such as PLIN2 through the rotaviral protein NSP5.[171] Intriguingly, the inhibition of LD formation using chemical compounds like isoproterenol plus isobutylmethylxanthine and triacsin C lowered the number of viroplasms and inhibited the production of progeny viruses.[171] On the other hand, the reovirus capsid protein micro1 induces apoptosis in infected cells when co-localized with LD, ER, and mitochondria.[172]

Rabies viral infection upregulates N-myc downstream regulated gene-1 (NDRG1), which increases the expression of DGAT1 and DGAT2, responsible for the TAG biogenesis in LDs and thus promotes LDs formation. Then, rabies virus employs LDs carriers to facilitate pudding process for virus production.[173]

During SARS-CoV-2 infection, viral proteins and double stranded-RNA were found in close proximity to LDs in host cells. The pharmacological inhibition of LD formation using A922500 (DGAT1 inhibitor) impeded not only the replication of the virus, but also the release of pro-inflammatory mediators and cell death.[174]

Following Zika virus infection, the motility of LDs was significantly increased, while their number remains unaffected. In fact, LD displacement (peaked at 48 h post-infection) and mean speed (peaked at 8 h post-infection) were enhanced in a time-dependent manner in infected cells.[175]

Influenza A virus triggers autophagy in infected cells, which contributes to the accumulation of LDs in infected cells. As LDs can enhance the viral replication, treatment with atorvastatin (an HMG-CoA reductase inhibitor) reduced Influenza A virus reproduction with partial suppression of ER stress and Reactive oxygen species (ROS).[176]

Beyond the conventional role of LDs as supporters of viral infection, a paradigm shift in the field points to an antiviral role of LDs as part of immune responses.[85] Infected cells secrete antiviral cytokines, mainly IFNs, which trigger the expression of ISGs. Some of these ISGs are localized on LDs (e.g. viperin and immunity-related GTPase), which confer to them the capability of counteracting viral propagation. In fact, when Herpes simplex 1 and Zika viruses infect cells, LDs are produced at early time points (2 h post-infection) to facilitate the magnitude of the early antiviral immune response via the enhanced secretion of type I and III IFNs.[42] In addition, the localization of viperin on the surface of LDs enables its interaction with the HCV-NS5A protein which causes the inhibition of Hepatitis C virus replication.[177]

been localized to LDs: (i) viperin, which is active against viruses assembled on LDs such as Hepatitis C and Dengue viruses[10]; (ii) IFN-γ-inducible GTPase (IGTP), related with antigen cross-presentation and required for resistance to *Toxoplasma gondii*[40]; and (iii) histones that on LDs increase survival of bacterially infected *Drosophila* embryos.[11] This picture must be radically revised with the recent demonstration that the protein complements of LDs changes dramatically upon infection, with immense implications for understanding the role of LDs in defense.[12] Proteomic analysis of LDs purified from the liver of mice treated with LPS, identified 689 regulated proteins (317 enriched/372 reduced), including changes in ~30% of the annotated "core" LD proteome.[12] The Ingenuity Pathway Analysis suggested that these LDs could be involved in different aspects of the innate immune defense, including killing, signaling, and inflammation, and thus, from hereafter will be referred as "defensive-LDs" (d-LDs) (Box 1).

BOX 4 Lipid droplets and parasites.

LDs constitute a nutrient-fueling organelle for the replication of parasites[178] (Table 1). Some parasites survive inside their parasitophorous vacuole, while others are living in the cytoplasm of host cells. Protozoan parasite infection with *Trypanosoma cruzi*, *Leishmania amazonensis*, *Leishmania major*, and *Toxoplasma gondii* induces an accumulation of large LDs in host cells.[179–182] Although both populations of parasitized and non-parasitized cells accumulate LDs, the number of LDs is higher in infected cells containing parasites, consistent with the fact that LD biogenesis is triggered by the parasite uptake.[183] During parasite infections (e.g., *Trypanosoma cruzi*, *Toxoplasma*) LDs are recruited in direct contact with the parasitophorous vacuole, where they constitute a site for lipid delivery and PGE2 synthesis to favor parasite growth.[65,183]

Several parasites (e.g., *Plasmodium falciparum* and *Toxoplasma gondii*) can synthesize neutral lipids and store them in their own LDs, specifically in lipid-enriched conditions.[89] Parasite LDs of *Schistosoma mansoni* are associated with hemozoin (heme toxic metabolite), which could suggest a heme detoxification role of LDs during parasite blood feeding cycle.[184]

The crosstalk between protozoan parasites and mammalian host in disease pathogenesis is continuing to be unveiled. Parasites have evolved ways to attract LDs to[182] engulf them, and to steal their lipid components. Therefore, LDs act as a bystander amplification of the host response to parasite infection, which could constitute an interesting therapeutic target.

We experimentally confirmed that d-LDs are simultaneously enriched in viperin, IGTP, histones, and other immunity-related GTPases (IRG) such as IIGP1, TGTP1, and IFI47 (Figure 2B,C). Furthermore, d-LDs also recruit cathelicidin (CAMP), a broad-spectrum antimicrobial peptide with chemotactic properties (see details in following sections). Demonstrating a direct participation of d-LDs in intracellular defense, cells expressing a genetically engineered LD-associated CAMP were more resistant to the infection of *Escherichia coli*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Listeria monocytogenes*. Intriguingly, *Pseudomonas aeruginosa* was not affected by LD-CAMP, suggesting that some bacteria may have evolved mechanisms of resistance.

Electron microscopy of human macrophages infected with *E. coli* confirmed the commonly observed interaction of d-LDs with phagocytic and parasitophorous membranes.[12] Although the nature of this junction and the mechanism of any protein, peptide, or lipid transfer from d-LDs to bacteria is unknown, our imaging methods preserving lipid membranes showed that the d-LD monolayer produced an apparent discontinuity in the phagolysosomal membrane, possibly allowing interaction of the antibacterial proteins on d-LDs with the bacterial outer membrane[3,12] (Figure 2D). These studies demonstrate the existence of sensing, trafficking, and docking mechanisms to facilitate the engagement of d-LD proteins with intracellular pathogens. Relocation of LDs to interact with the lysophagosome containing *Mycobacterium marinum* was observed only 10 minutes after phagocytosis,[86] being likely mediated by Rab GTPases such Rab7 or Rab18.[87–89] In silico analysis demonstrated that twenty-three different Rab GTPases are enriched on d-LDs, including Rab7 and Rab18,[12] suggesting candidate interactors among other organelles (Table 2).

In support of the existence of mechanisms to deliver toxic molecules from d-LDs into invaders, recent studies have described that LDs are efficient antibiotic reservoirs and suppliers. Bedaquiline is a highly lipophilic antibiotic reducing *Mtb* viability by interacting with ATP synthase. In infected macrophages, host LDs operate as intracellular bedaquiline reservoirs and suppliers enhancing its antimycobacterial activity.[90] Similarly, the broad-spectrum antiviral compound ST-669 accumulates within LDs to restrict chlamydial inclusion development and *Coxiella burnetii* growth.[91] Thus, the dynamic interaction of d-LDs with bacteria could be therapeutically exploited.

Functional annotation analysis of the proteins upregulated on d-LDs suggested that these organelles may simultaneously participate in (i) killing by using bactericidal proteins and lipids, increasing antimicrobial autophagy, and generating toxic nucleotides; (ii) immune responses by acting as intracellular signaling platforms; (iii) controlling production of pro- and anti-inflammatory lipids in different phases of the response; and (iv) dynamic regulation of immunometabolism by regulating lipid distribution within host cells (Box 5). Indeed, characterizing the protein and lipid composition of d-LDs has the potential to illuminate completely novel mechanistic aspects of the host-pathogen battlefront.

5 | THE BIOLOGICAL ADVANTAGES OF LIPID DROPLETS AS HUBS OF INNATE IMMUNITY

In addition to organelles attracting intracellular pathogens, d-LDs present several assets for antimicrobial defense. The rapid formation and consumption of LDs make them a suitable site for the assembly of urgently formed but transient signaling platforms. Collecting immune proteins at the surface of d-LDs could facilitate proximity between functionally connected enzymes and proteins sequentially contributing to defensive reactions. Further, because most antibiotic and antiviral compounds are cytotoxic, restriction of these molecules on d-LDs assures safety for the rest of cellular organelles. Indeed, d-LDs simultaneously handle a bunch of potentially toxic proteins that, as viperin, CAMP, histones, IRGs, and guanylate binding proteins (GBPs), participate in complementary, sequential, or synergistic defensive mechanisms (Figure 2B).

TABLE 2 List of Rab-GTPases upregulated in hepatic LDs of mice treated with LPS and their potential roles in innate immunity.

Rab-GTPase	*ΔZq*	Roles in innate immunity	References
1	3.10	Phagocytosis and phagosome maturation	[246]
1b	3.41	Antiviral innate immunity	[247]
2	3.99	Phagocytosis and phagosome maturation	[246]
2b	2.93	Phagocytosis and phagosome maturation?	[246]
3d	2.68	Autophagy	[246]
5a	2.50	Phagocytosis and phagosome maturation?/ degranulation and secretion?	[246]
5c	2.56	Phagocytosis and phagosome maturation?/ degranulation and secretion?	[246]
7 L1	1.65	Phagocytosis and phagosome maturation?/ degranulation and secretion?	[246]
7a	3.43	Phagocytosis and phagosome maturation/ Antigen presentation?	[246]
8b	1.65	Degranulation and secretion?	[246]
8a	2.57	Degranulation and secretion?	[246]
10	1.82	Phagocytosis and phagosome maturation	[246]
12	2.58	Autophagy	[246]
13	2.22	ND	–
14	2.80	Phagocytosis and phagosome maturation	[246]
17	2.25	Phagocytosis and recycling endosomes	[248]
18	7.10	Trafficking of proteins to facilitate viral replication	[54]
20	5.47	Phagocytosis and phagosome maturation	[246]
21	2.19	TLR4 endosomal traffic	[249]
22a	2.10	Phagocytosis and phagosome maturation	[246]
27a	3.00	Autophagy	[246]
30	3.89	Degranulation and secretion	[246]
32	2.36	Degranulation and secretion	[246]
33b	1.69	Degranulation and secretion?	[246]
35	2.34	Phagocytosis	[250,251]

Note: Light blue color represents the speculated role of Rabs in innate immunity based on the same family member. *ΔZq* indicated the relative enrichment of the Rab GTPase on d-LDs when compared with f-LDs.

Abbreviation: ND, not determined.

Viperin (RSDA2) is an ancient core factor of the IFN mediated innate immunity in vertebrates.[92] Viperin is a broad-spectrum antiviral protein and a key transducer of the type I IFN-mediated response in many cell types, including macrophages and hepatocytes.[92] After synthesis in response to different PAMPs, viperin is targeted to the ER and LDs[93] (Figure 2C). By using a variety of antiviral mechanisms, viperin inhibits replication of a large number of viruses such as Hepatitis C, Dengue, Zika, West Nile, and Influenza A viruses.[92] Viperin is a radical S-adenosyl-L-methionine (SAM) enzyme that catalyzes the conversion of cytidine triphosphate (CTP) into 3′-deoxy-3′,4′-didehydro-CTP (ddhCTP), a ribonucleotide that functions as a chain terminator of viral RNA synthesis.[94] Viperin restricts replication of Zika and Tick-borne encephalitis viruses by targeting NS3 to proteasomal degradation, a process involving an unknown E3 ubiquitin ligase.[95] Furthermore, viperin nucleates signaling platforms that, like the STING/IRAK1 and TRAF6/TBK1 axes, activate IRF3 and IRF7 to regulate the IFN-mediated immune response[10,96,97] (Figure 4). HCV NS5A deactivates viperin to catalyze the conversion reaction of CTP to ddhCTP.[98] The ribonuclease UL41 produced by the Herpes simplex virus 1 (HSV-1) can degrade the mRNA of viperin to restrain its antiviral function.[99]

Cathelicidin (CAMP) is a broad-spectrum antimicrobial peptide with chemotactic and immunomodulatory properties.[100] Antimicrobial effects of CAMP have been observed against fungal, bacterial, and viral pathogens. For example, CAMP is secreted by adipocytes to protect the skin during *Staphylococcus aureus* infection.[101] The C-terminal domain of CAMP is proteolytically cleaved to produce LL37, the active peptide that folding as an alpha-helix causes damage in pathogen's membranes. A pool of CAMP is intracellularly retained and accumulated on d-LDs, at least in macrophages and hepatocytes.[12] Cells expressing a genetically engineered LD-associated CAMP were more resistant to different bacterial

BOX 5 Lipid droplets in the regulation of immunometabolism.

With the popularity and substantial development of the immunometabolism concept in the past years, LDs emerged as a central bridge integrating cell metabolism and immunity. Despite the increased knowledge of metabolic pathways governing cellular fates, the understanding of immunometabolism is still in its infancy. Immune cells are in constant transition between rested and activated phenotypes, which entails reattribution of nutrients into different metabolic pathways to sustain functional changes.[185–187] As an accepted metabolic fact, immune cells highly rely on aerobic glycolysis when mediating inflammatory processes; while they exploit fatty acid oxidation for energy production when switching to their immune modulatory and reparative roles.[188]

Being an energetic reservoir, LDs orchestrate host immunity through the release of lipids that could (i) act directly as signaling molecules or (ii) be converted into inflammatory mediators.[189] The release of free fatty acids from the breakdown of TAG or other esterified lipids activates several signaling pathways in immune cells through their binding to cell surface receptors such as TLRs, G protein-coupled receptors (GPCRs), intracellular transport proteins and nuclear receptors. Sterol regulatory element-binding proteins (SREBPs), PPARs, and NF-κB represent some examples of nuclear receptors activated by the release of fatty acids.[190,191]

In addition, LDs stockpile polyunsaturated fatty acids (PUFAs) which are precursors of lipid mediators such as eicosanoids and specialized pro-resolving mediators (SPMs). These lipid intermediates are massively and instantaneously released from activated immune cells to alter inflammatory and immune responses in their microenvironment by binding to GPCRs on target cells.[192,193] The initiation of cellular responses does not follow a general trend, but rather different fatty acids, eicosanoids, SPMs and related oxygenated fatty acids species have distinct abilities to trigger pathways in a context dependent manner, according to the immune cell type and the presence of specific receptors on target cells.[194,195] This amalgam of complex and dynamic lipid mediators controls transcriptional programs integrating immunity, metabolism, and inflammation that results in disparate physiological outcomes.[190]

One of the illustrations of the metabolic adaptation to activating signaling is the polarization phenotypes of macrophages known as pro-inflammatory M1 and pro-resolving M2.[196,197] Upon exposure to TLR, IFN-γ, or TNF-α ligands, macrophages employ aerobic glycolysis (upregulation of glucose uptake through GLUT-1) and the pentose phosphate pathway to meet their energetic needs through the TLR/NF-κB,[198] HIF1α,[199] and/or AKT/mTOR[200] signaling. Emerging macrophages, called M1, are characterized by an increase in lipogenesis (fatty acids and cholesterol) to support their inflammatory and phagocytic roles.[201] Consequently, the fatty acid pool will rise in the cytoplasm which triggers the formation of LDs through TLR-mediated upregulation of LD-associated proteins, induction of ER stress and inhibition of ATGL (increase of its endogenous inhibitor HILPDA).[202,203] The formation and accumulation of LDs are typically described in the pro-inflammatory status of macrophages, which fuel immune responses by storing bioactive molecules.[204]

When shifting to the M2 phenotype, macrophages rely on fatty acid oxidation and OXPHOS. Following TGF-β, IL-4/10/13 stimuli, an upregulation of STAT6, GATA3, PCG-1α, and PPARγ[205,206] is provoked leading to a reduced glycolytic rate and an increased expression of CD36 and lysosomal lipolysis genes.[207,208] Thus, the breakdown of lipids and cholesterol efflux are prompted by the LXR transcription factor to prevent the accumulation of LDs.

The role of LDs in dictating the fate of myeloid and lymphoid lineage is poorly investigated due to the complexity of their metabolic regulation and the tissue-dependent microenvironment. A study conducted by Ecker et al,[209] distinguished the metabolic behavior of different subsets of T-cells in the presence and absence of glucose. When glucose is abundant in the medium, both naïve and effector memory T-cells accumulate LDs and survive on glycolysis. However, during glucose deprivation, only effector T-cells fail to upregulate fatty acid synthesis, OXPHOS, and to reduce glutaminolysis, which allow them to maintain high levels of IFN-γ and preserve their T-cell function in nutrient-depleted microenvironments.[209] A second study exemplified the implication of unsaturated fatty acids in the regulation of the Myeloid suppressor cells (MSC) phenotype.[210] Treatment with sodium oleate (C18:1) and linoleate (C18:2), but not stearate (C18:0), confers a functional phenotype to MSC and suppresses T-cell activation through the formation of LDs, facilitating tumor escape from the immune system.[210] In agreement with this study, Wu H et al demonstrated that Tumor-associated macrophages (TAMs) enriched in LDs endured an in vitro polarization and promoted tumor growth in vivo, uncovering a new therapeutic strategy to restore immune surveillance by inhibiting LDs in TAMs.[211] In neutrophils, LDs lipolysis and autophagy-mediated lipid degradation are essential to supply the mitochondria with free fatty acids for a correct neutrophil differentiation in the bone marrow.[212] During chronic airway inflammation, innate lymphoid cells (ILC) increase their external glucose and lipid uptake.[213] The free fatty acids accumulating in the cytoplasm are stored in LDs and converted into phospholipids to sustain the proliferation of the tissue-resident type 2 ILCs via mTOR signaling. When mice are fed with a ketogenic diet, ILC2-mediated airway inflammation is resolved through an impairment of lipid metabolism and LDs formation.[213]

species including *E. coli*, MRSA, and *Listeria monocytogenes*. *C. trachomatis* produces Pgp3, a virulence factor, that neutralizes the antichlamydial activity of CAMP.[102]

Histones participate in several aspects of immunity including the extracellular-mediated antimicrobial and inflammatory responses.[103] Histones accumulate on LDs of *Drosophila* embryos[104] and mammalian cells.[11,12,22] In the presence of LPS or lipoteichoic acid, LD-histones could be released to kill both Gram-negative and Gram-positive bacteria.[104] Interestingly, Histone H2A and CAMP constitute a synergistic antibiotic mechanism, with CAMP forming pores to allow the entry of H2A into bacteria to bind bacterial DNA and to inhibit transcription of *E. coli* and *Staphylococcus aureus*.[105,106] Hence, by bringing together histones and CAMP, the d-LDs might optimize bacterial killing. Strains of the group A *Streptococcus*, belonging to the hypervirulent M1T1 serogroup, have developed a virulence factor, the Streptococcal M1 Protein, which binds and neutralizes both histones and CAMP.[107,108]

The IFN-inducible immune GTPases orchestrate anti-microbial activities against a diverse range of pathogens such as bacteria, protozoan, and viruses.[109] The d-LDs are enriched in IRGs such as IFI47, IIGP1, or TGTP1[12] and, as suggested by proteomic studies,[12] in GBPs such as GBP2, GBP5, or GBP6.[12,110] GBPs and IRGs specifically target phagolysosomal membranes enclosing invaders and restrict intracellular vacuolar pathogen replication by disrupting the vacuolar compartment.[111] By transporting antimicrobial cargo to the pathogen-containing vacuole, GBPs participate in the resistance to bacteria such as *Listeria monocytogenes* or *Mycobacterium bovis BCG*, and parasites such *Toxoplasma gondii*.[112,113] In addition, GBPs mediate in the activation of the inflammasome to regulate pyroptosis, cytokine production, and defensive autophagy.[111,114,115] *Shigella* secretes IpaH9.8, an E3 ubiquitin ligase, that targets GBPs to degradation and promotes the spread of bacteria and death of infected mice.[116,117] Virulent *Toxoplasma gondii* secretes ROP18, a kinase, which phosphorylates IRGs to inhibit their relocation and functioning on the parasitophorous vacuole.[118]

Although important during infection, histones and CAMP can be quite harmful for the host and promote cell damage and inflammation.[119,120] Thus, compartmentalization of toxic antibacterial proteins on d-LDs is likely avoiding indiscriminate cellular damage. Due to its prokaryotic origin, this mechanism could be especially relevant for mitochondria. Among the five PLINs, PLIN5 was the only one downregulated on d-LDs.[12] An important role of PLIN5 is to function as a tether between LDs and mitochondria.[121] Thus, low PLIN5 levels on d-LDs promote physical and functional disconnection from mitochondria and a concomitant reduction of oxidative metabolism and ketogenesis, characteristic hallmarks of immunometabolic response to infection.[122,123] Forcing PLIN5 expression in infected cells increased the number of contacts d-LD with mitochondria, reducing the number of interactions d-LD with bacteria, and compromising the antimicrobial capacity of cells,[12] as described in more detail in the following sections.

6 | PROGRAMMING DEFENSE-LIPID DROPLETS TO FUNCTION AS INNATE IMMUNITY HUBS

One of the most intriguing questions arising from these studies is how innate immunity drives the formation of the d-LDs that finally become defensive hubs. When compared with f-LDs, such a transformation involves the downregulation of 372 proteins.[12] The d-LDs purified from the liver of LPS-treated mice liver were depleted of proteins involved in mitochondrial metabolism, such as PLIN5 and VPS13D,[121,124] and proteins related to phospholipid metabolism, such PCYT1A, PLPP3, and ABCB4 (Figure 2B). Forced PLIN5 expression in fibroblasts and macrophages reduces the amplitude of the defensive response when infected with *E. coli*.[12] Hence, acquisition of the defensive d-LD capacity seems to require switching off the metabolic activity of f-LDs that, largely mediated by PLIN5 especially in oxidative cells, is probably interfering in the defense.

The impaired immune response of cells expressing high levels of PLIN5 could be caused by several traits that make this PLIN a unique member of the family. First, PLIN5 stimulates interaction of LDs with mitochondria[121,125] and thus, reduces the number of d-LDs available to interact with bacteria.[12] Second, because PLIN5 and PLIN2 compete for mutually excluding subdomains on LDs,[126] the low levels of PLIN5, and its mitochondrial tethering, probably reduces molecular crowding to allow the documented enrichment of PLIN2 on d-LDs.[12] By physically interacting with immune proteins, such as CAMP or IGTP,[12,40] PLIN2 could organize on d-LDs clusters of functionally connected defensive proteins.[12] Finally, because PLIN5 activates the key oxidative SIRT-1/PPARα/PGC-1α axis,[127] the absence of PLIN5 on d-LDs and concomitant reduction of oxidative metabolism are probably generating a metabolic environment conducive for defense. Indeed, many examples illustrate that the activity of the PKA/SIRT-1/PPARα/PGC-1α axis must be low for an optimal immune response.[128-133]

Among the 317 proteins enriched on d-LDs, we inferred with high confidence the upstream regulation of 140 proteins, predicting a strong control of d-LDs by the Toll like receptors TLR2 and TLR4/NF-κB and the SRF/SMADs signaling axes (Figure 3A). Remarkably, 79 of these annotated proteins are direct targets of IFNs, indicating that these cytokines are fundamental signals transcriptionally driving the remodeling of d-LDs (Figure 3A,B).

Molecular underpinnings, physiopathological roles, and evolutionary conservation of the IFN networks have been the subject of intense research[134] (Box 6). A plethora of PAMPs and DAMPs trigger the initiation of signaling pathways activating transcription factors that, as NF-κB, AP-1, IRF-3, and IRF-7, finally converge to stimulate the expression of IFNs. These IFNs function as autocrine and paracrine cytokines activating the Signal transducer and activator of transcription (STAT) stimulating expression of IFN-stimulated genes (ISGs) and triggering a plethora of defensive programs[3] (Figure 4A). Among the hundreds of ISGs, in addition to viperin, d-LDs accumulate IRGs and GBPs[12] (Figure 3B). Beyond their intrinsic antimicrobial activity (previous section), these GTPases may function as scaffolds

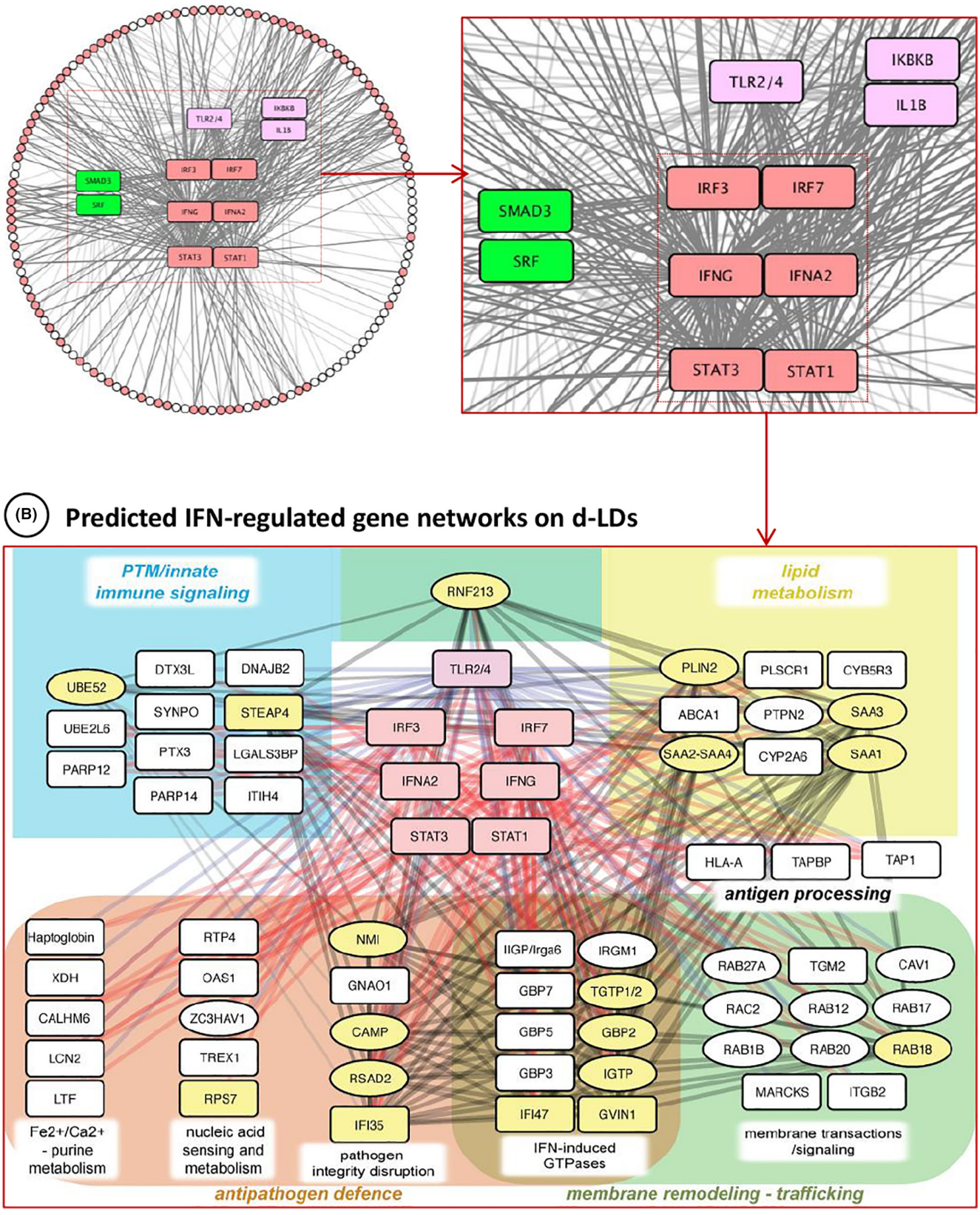


FIGURE 3 Upstream regulators of the defensive-lipid droplet proteome. (A) Analysis for main upstream regulators of LPS-induced LD proteins using the IPA™ resource identifies 140 polypeptides, of which 79 are annotated as directly regulated by the interferon network (red hue nodes in the circular layout; stronger stroke edges; not including relationships with other upstream regulators). Extensive crosstalk is also predicted with two other major upstream regulatory modules (TLR2/4-NF-kB; SRF/SMADs). (B) A detailed depiction of gene networks inferred from those genes predicted as directly regulated by the interferon network. Functional grouping is highlighted.

BOX 6 Interferons in innate immunity.

Interferons are a group of small cytokines that were first described as entities "interfering" with viral infection.[214] There are three families of IFNs: (i) Type I, consisting of IFN-α, IFN-β (most abundant IFNs), IFN-ω, IFN-κ, IFN-ε, IFN-ζ, IFN-δ, and IFN-τ subtypes,[215] (ii) Type II, including IFN-γ,[216] and (iii) Type III, encompassing IFN-λ1 (IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and IFN-λ4 subgroups.[217] In this section, we will briefly discuss the implication of type I and II IFNs in bacterial infections.

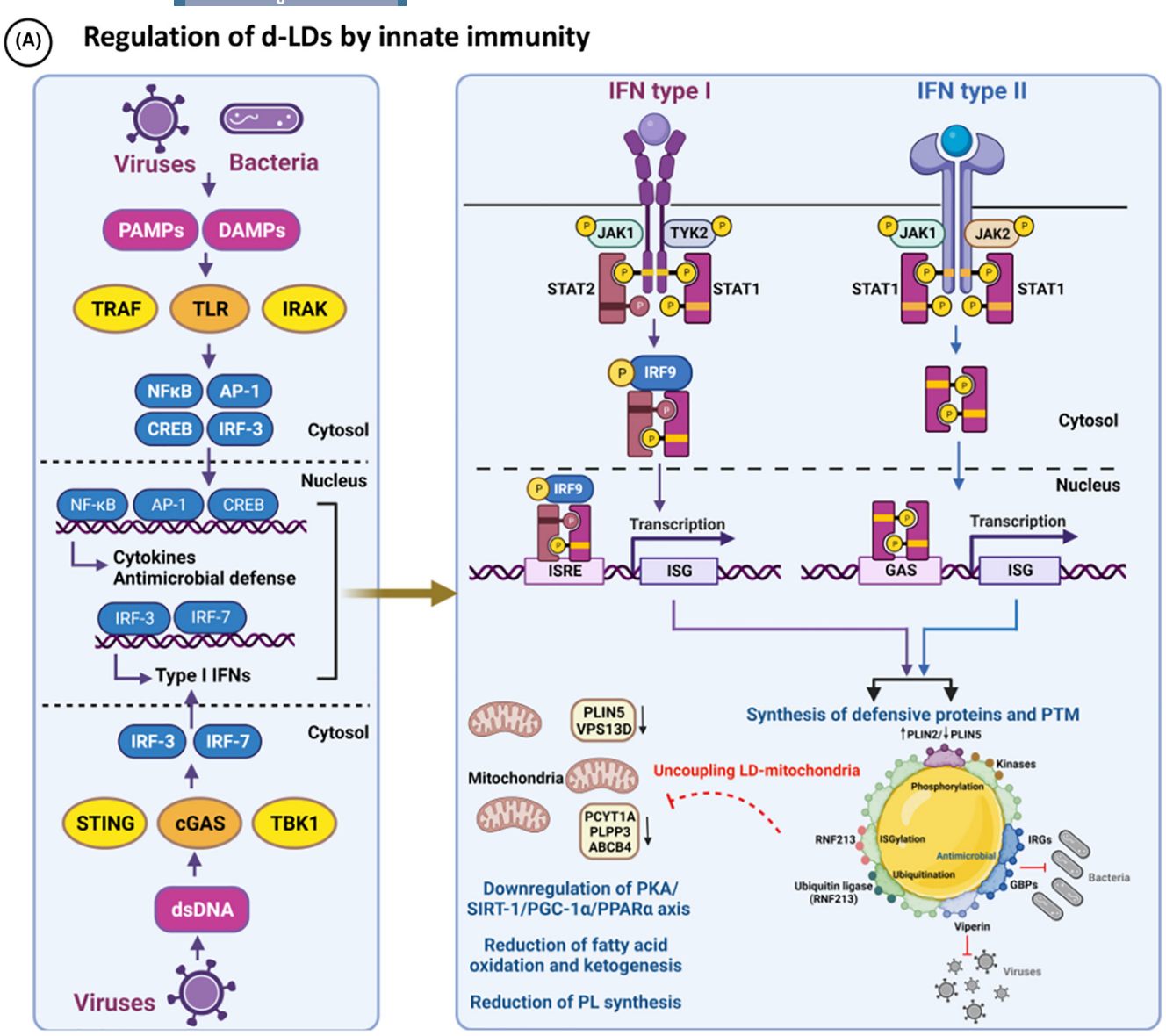
During infection, bacteria secrete PAMPs, which are detected by host cells through pattern recognition receptors (PRRs), enabling the production of type I and II IFN cytokines.[218] There are distinct PRRs, including TLRs, retinoic acid-inducible gene-I-like receptors (RLRs), nucleotide-binding oligomerization domain-like receptors (NLRs), and cyclic GMP-AMP synthase (cGAS). IFNs signal in an autocrine and paracrine manner upon recognition by their respective receptors, triggering the assembly of signaling complexes (JAK/STAT) and the activation of intracellular adaptor proteins (mitochondria-antiviral signaling protein [MAVS], STING, MyD88 and TRIF).[219] This cascade of signaling pathways will lead to the expression of interferon-stimulated genes (ISGs) which enhance pathogen detection, activate innate immune cells and promote adaptive immune responses[218] (Figure 4).

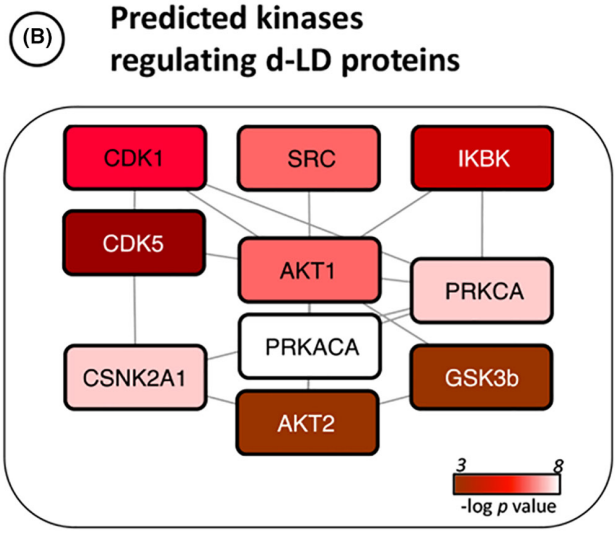
The role of IFNs in host defense against bacterial infections is enigmatic. They play diverse and context-dependent functions, varying according to the pathogen and the host. While it has been described that IFNs have mostly antibacterial properties in vitro, their functions are more complex to delineate in vivo because they can harbor beneficial or detrimental effects on pathogens. For instance, ISGs-mediated type I IFN impeded the replication of many intracellular bacteria in tissue culture models, such as *Mtb*,[220] *C. trachomatis*,[221] *Listeria monocytogenes*,[222] and *Shigella flexneri*.[223] In vivo, type I IFN mainly blocked the migration process of bacteria across endothelial and epithelial barriers, specifically in *E. coli*,[224] *Helicobacter pylori*,[225] and *Streptococcus pneumoniae*.[226] In contrast, IFN signaling can promote bacterial infection. For example, IFN-β exacerbated *Mtb* infection, where a correlation has been established between the presence of type I IFN-inducible transcripts and the disease's pathogenesis. In addition, some respiratory bacterial superinfection (*Streptococcus pneumoniae* or *Staphylococcus aureus*), could be triggered by IFN signaling secondary to viral infections.[227,228]

Considering the disparate role of IFNs in bacterial infection, the field will benefit from more comprehensive examination of IFN responses using different types of pathogens. In this context, the enrichment of Interferon-inducible GTPases on LDs is an interesting ground to explore in innate immunity. Type I and II IFNs are known to induce the expression of more than 2000 ISGs, including prominently the family of IFN-inducible GTPases.[218] Four subgroups of IFN-inducible GTPases exist, encompassing immunity-related GTPases (IRGs, 47 kDa), guanylate-binding proteins (GBPs, 65 kDa), Myxoma proteins (Mx, 72–82 kDa), and very large inducible GTPases (VLIGs or GVINs, 200–285 kDa).[115] Among these subgroups, IRGs and GBPs have gained much attention due to their capacity to destroy the pathogen's replication vacuole and to interfere with the spatial arrangement of bacteria.

IRGs confer resistance to host cells (in mice or in IFN-primed cells) by targeting the pathogen's vacuole through recruiting and loading Irgbs (Irgb6, Irgb10, Irgd), followed by vesiculation and blebbing, leading finally to the disruption of the vacuole's membrane and exposure of the pathogen to the cytoplasm.[110,229] Afterwards, host cells either undergo necrosis which will lead to the simultaneous death of the pathogen and the host cell[229] or Irgb-dependent autophagy process is activated as a potential mechanism for pathogen clearance.[230]

GBPs can destabilize the vacuole (*Mycobacterium bovis BCG*, *Salmonella typhimurium*)[112,231] similarly to IRGs, and also restrict directly the professional cytosolic pathogens (*Listeria monocytogenes* and *Francisella novicida*).[112,232] GBPs undergo homo-oligomerization and hetero-oligomerization processes, isoprenylation and ultimately targeting the endomembranes via the catalytic activity of the GTPase domain.[233,234] Moreover, GBPs are able to recruit in close vicinity to the bacteria's vacuole (i) NADPH oxidase components (gp91phox and p22phox) to promote the NADPH oxidase activity and (ii) autophagy factor p62 to trigger vacuole rupture.[112] GBPs function goes beyond cell-autonomous immunity, as they were shown to drive inflammasome complexes assembly through canonical and non-canonical pathways, leading to pyroptotic cells death.[114,235] For instance, during *Shigella flexneri* and *Salmonella typhimurium* infections, GBP1 binds to bacterial LPS through electrostatic interactions and recruits other GBPs to the signaling platform. This will enable the activation of caspase 4, triggering the assembly of the non-canonical inflammasome and subsequently bacteria clearance by pyroptosis.[236,237] Furthermore, a study conducted by Meunier et al showed that GBP2-mediated *Francisella novicida* killing leads to the release of bacterial DNA, which is a prerequisite for efficient AIM2 inflammasome activation.[232]





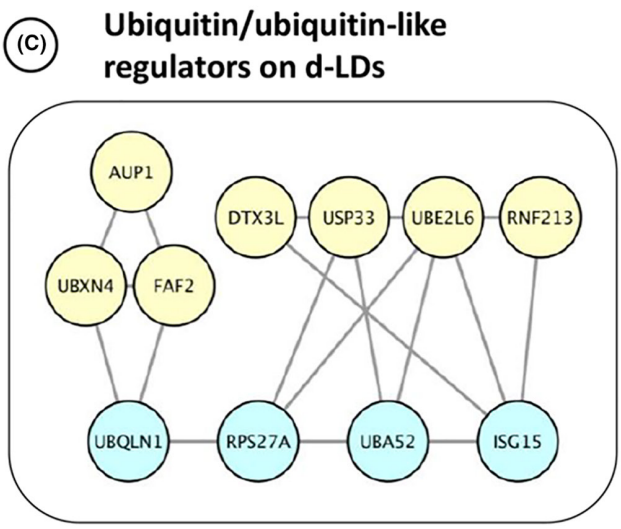


FIGURE 4 Programming defensive-lipid droplets by innate immunity. (A) Schematic representation of LD remodeling by innate immunity signaling and IFN. In the left blue box, when stimulated by PAMPs and DAMPs, TLRs and cGAS (orange) transduce the immune signaling by activating adaptor proteins (e.g., TRAF and STING) and kinases (e.g., IRAK and TBK1) (yellow). Kinases trigger activation and translocation of transcription factors (e.g., NF-KB, AP-1, CREB, and IRF-3, blue) into the nucleus to stimulate the expression of cytokines, antimicrobial defense genes and Type I IFN. In the right blue box, Type I and II IFNs signal through the phosphorylation of JAK/STAT proteins leading to the transcription of IFN-stimulated genes (ISGs). Subsequently, the relative composition of PLINs on LDs changes (high levels of PLIN2 but low levels of PLIN5) and expressed defensive proteins (e.g. viperin, GBPs, IRGs) accumulate on d-LDs. These proteins can be posttranslationally modified (PTMs, phosphorylation by kinases, ISGylation by RNF213, ubiquitination by ubiquitin ligase RNF213). In addition, d-LDs are physically and functionally disconnected from mitochondria (low PLIN5 and VPS13D) with a concomitant decrease in fatty acid oxidation and ketogenesis. In parallel, the low levels of PLIN5 on d-LDs reduces the activity of the PKA/SIRT-1/PPARα/PGC-1α axis. The contact of d-LDs with bacteria and viruses increases to facilitate killing and pathogen clearance. (B,C) Inference of two regulatory PTM layers that could modulate the recruitment and activity of the d-LD proteome. (B) Relevant kinases predicted to be overrepresented among phosphorylation acceptor sites of the LPS-LD proteome. Their enrichment estimation and their potential relationships, displayed as a network, was computed using the Kinase Substrate Enrichment open resource KSEA3[239] (C) LD core proteins found as responsive to LPS, predicted/reported to participate in ISGylation and ubiquitylation of protein substrates.

on d-LDs for recruiting signaling and inflammatory proteins and, because they are also involved in lipid and mitochondrial metabolism,[135–137] participate in the metabolic rewiring mediated by d-LDs in host cells.

In fact, the IFN signaling is highly sensitive to the metabolic status of cells and thus, it may in turn be positively or negatively regulated by LDs.[12] For example, the Stimulator of interferon genes (STING), a key inductor of type I IFN, is only active when the ER cholesterol levels are low.[138,139] In macrophages low cholesterol levels heighten the activation of the STING-TBK1-IRF3 axis to enhance type I IFN production and resistance to MHV-68, Influenza A, and Human immunodeficiency virus 1.[138] Conversely, replenishing cells with free cholesterol reduced the expression of type I IFN in virally infected cells.[138] Thus, because LDs are major cholesterol reservoirs and suppliers, the activity of d-LDs and the IFNs signaling could generate a loop to determine the intensity and duration of the defensive programs.

Following the example of f-LDs, we anticipate that the activity of the d-LD proteome is finely modulated by phosphorylation. During the transition from fed to fasting conditions, PLINs and other LD proteins are dynamically phosphorylated by energy sensors (such as PKA and AMPK) to function as scaffolds that reorganize the LD proteome and gradually activate the lipolytic, signalling, and trafficking machineries functioning on f-LDs.[140–142] Kinase-specific acceptor site prediction and substrate enrichment analysis, using combined machine learning approaches and public datasets, confirmed that the LD proteome is tightly regulated by PKA (PRKACA) (Figure 4B) but additionally predicted that d-LD proteins are phosphorylated by kinases involved in different aspects of immunity such as PKB (AKT), Glycogen synthase kinase-3 (GSK3), Casein Kinase II Subunit Alpha (CSNK2A), and the I-kappa B kinase (IKK) (Figure 4B).[12] Indeed, as occurring during fasting, protein phosphorylation could dynamically adjust the activity of the d-LD proteome and the intensity of the response to meet the cellular demands. These regulatory circuits can be exploited by pathogens: virulent *Toxoplasma gondii* secretes ROP18, a kinase that phosphorylates IRGs to inhibit their relocation to the parasitophorous vacuole.[118]

Beyond stimulating expression of defensive proteins and activation/inactivation of kinases, IFNs could regulate the d-LD proteome through a variety of posttranslational modifications (PTM). For example, extent and duration of the defensive IFN-mediated response is finely regulated by ubiquitination of the proteins conducting the signaling.[143] Attached to lysine residues, ubiquitin(s) determines protein function and proteasomal degradation of a wide range of proteins. Several LD proteins are locally regulated by ubiquitination and degraded by the ubiquitin-proteasomal system[144–146] (Figure 4C). Reflecting the importance of these systems on d-LDs, protein ubiquitination is both an efficient defensive mechanism and a virulence strategy exploited by hosts and invaders. Shigella secretes IpaH9.8, an E3 ubiquitin ligase that targets GBPs to degradation.[116] Viperin, through an unknown E3 ubiquitin ligase, restricts flaviviruses replication by stimulating ubiquitination and proteasomal degradation of NS3.[95]

One PTM getting increasing attention is mediated by the Interferon stimulated gene 15 (ISG15).[147] ISG15 is a peptide resembling ubiquitin, which is covalently attached to lysine residues of a wide range of substrates to modify their stability and function. Different E3 ubiquitin ligases mediate the incorporation of ISG15 to proteins, a process known as ISGylation. The E3 ubiquitin ligase RNF213 (mysterin) resides on LDs and mediates ISGylation of a variety of proteins that regulate LD dynamics.[133,148] RNF213 is responsive to IFNs and highly enriched on purified d-LDs (Figures 3B and 4c).[12] After conjugation with ISG15, RNF213 assembles on LDs forming oligomers that function as scaffolds for other ISGylated proteins to participate in the defense against *Listeria monocytogenes* and Herpes simplex virus type 1.[149] When infected with Vaccinia virus, macrophages lacking ISG15 exhibit impaired LD formation, an aberrant profile of lipid metabolism enzymes, and markedly shifted towards an increased fatty acid oxidation,[133] traits of f-LDs. Thus, ISGylation is an IFN-stimulated PTM that may regulate the biogenesis, defensive, and metabolic properties of d-LDs. Interestingly, RNF213 also mediates ubiquitylation of bacterial LPS to restrict proliferation of cytosolic *Salmonella* by inducing bacterial autophagy,[150] providing an additional weapon to the killing arsenal of d-LDs.

7 | CONCLUDING REMARKS, OPEN QUESTIONS, AND FUTURE DIRECTIONS

Long perceived as inert cytoplasmic fat inclusions, LDs have been traditionally described as ectopic sites of fat accumulation or simply as triglycerides.[151] Therefore, it is not surprising that the long-known accumulation of fat occurring in infected cells was attributed to nutritional strategies driven by pathogens.[6–8] Only three decades ago, pioneering studies identifying the first regulatory proteins on LDs, oleosins in maize and PLIN-1 in adipocytes,[152,153] started to change such a simplistic view.[151,154] Today, LDs are recognized as complex organelles and much is known about their key role in overseeing a variety of intracellular and environmental stresses, far beyond their main contribution to nutrient administration.[4,21,155] The use of novel systematic molecular profiling approaches, capable of revealing within the LD proteome non-intuitive systems-level relationships, decisively shifted the paradigm.[41] Although the presence of antiviral and antibacterial proteins on LDs was described long ago,[9,11] recent work from a number of different groups, including some high-throughput analyses, has illuminated the complex and bidirectional relationship between LDs and invaders.[12,42,79,149,156] The hypothesis that LDs are strategic chokepoints organizing a first defensive line of innate immunity has increasing support.[3,13,68,85]

Although there is a great deal of uncertainty surrounding this novel research field, the profound remodeling of the LD proteome in cells sensing danger signals suggests that LDs are armed with a plethora of agents required for immune defense. Indeed, our studies point to the possibility that innate immunity has developed a sophisticated defense program functioning around d-LDs and potentially involving regulation of hundreds of proteins.[12] Downregulated proteins likely switch-off the metabolic functions of LDs to generate the metabolic environment conducive for defense.[12] Upregulated proteins could mediate killing but also signaling and inflammation.[12,42,79] The increasing list of identified virulence factors specifically targeting LD proteins, including d-LD immune proteins (Figure 1E), illuminates an active conflict and indicates that d-LDs are opponents to be defeated by pathogens.

It is interesting to speculate about the fate of d-LDs after the resolution of infection. In the absence of danger signals, the metabolic signals regulating LD formation may take over, and LDs may return to their basic function, lipid reservoirs and suppliers. However, LDs accumulating in pathological conditions such as obesity, cancer, or aging are, like d-LDs, associated with inflammation and cell damage.[157,158] For example, we have recently observed that after brain ischemia LDs accumulate in microglia, a population of immune cells residing in the brain.[159] These LDs resemble d-LDs and contain ISGs such as viperin, ISG15, RNF213, IFI47, TGTP1, IIGP1, and GBP6. After a stroke in elderly patients, the accumulation of d-LDs is exacerbated with a concomitantly aggravated type I IFN immune response that worsened the neurological outcome.[159] Hence, the equilibrium between f- and d-LDs could be disrupted and promote disease.

In conclusion, many questions arise from the early discoveries summarized here. How are d-LDs directed to the vacuole-containing bacteria? What are the killing mechanisms adopted by d-LDs once they encounter bacteria? What lipids within d-LDs have a role in antimicrobial defense? How is the equilibrium between f-LDs and d-LDs regulated? How can bacteria avoid the d-LDs? Whether the findings detailed here, only tested for some pathogens in particular experimental conditions and particular cell types, could be generic branches of innate immunity deserve further investigation. We anticipate that the evolutionary race between hosts and microbes will lead to distinct scenarios, with LDs defeating the invader sometimes but pathogens surpassing the defenses in others. Whichever the case, characterizing the role of LDs in innate immunity by answering these questions will illuminate strategic chokepoints to be potentiated or corrected in future therapeutic interventions.

AUTHOR CONTRIBUTIONS

The authors jointly wrote the manuscript and designed figures.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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