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# Mammalian lipid droplets are innate immune hubs integrating cell metabolism and host defense

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**Key words:** Lipid droplets, mitochondria, innate immunity, immunometabolism, proteomics, antibiotics, cathelicidin.

## **Abstract**

Lipid droplets (LDs) are the major lipid storage organelles of eukaryotic cells and a source of nutrients for intracellular pathogens. Here, we demonstrate that mammalian LDs are endowed with a protein-mediated antimicrobial capacity, which is upregulated by danger signals. In response to lipopolysaccharide (LPS), multiple host defense proteins, including interferon-inducible GTPases and the antimicrobial cathelicidin, assemble into complex clusters on LDs. LPS additionally promotes the physical and functional uncoupling of LDs from mitochondria, reducing fatty acid metabolism while increasing LD–bacterial contacts. Thus, LDs actively participate in mammalian innate immunity at two levels: they are both cell-autonomous organelles that organize and utilize immune proteins to kill intracellular pathogens as well as central players in the local and systemic metabolic adaptation to infection.

Lipid droplets (LDs) are the major lipid storage organelles of eukaryotic cells (1). Common parasites (e.g. trypanosomes and *Plasmodium falciparum*), bacteria (e.g. mycobacteria and *Chlamydia*), and viruses (e.g. hepatitis C (HCV) and dengue (DENV)) induce and target LDs during their life cycles (2). The current view is that LDs support infection, providing invaders with substrates for survival and/or growth (3). However, successful innate defense is critical for survival and host immune responses have co-evolved with pathogens developing a plethora of defense mechanisms. Indeed, there is some limited evidence that LDs actively participate in innate defense (4, 5). For example, three innate immune system-related proteins localize to the LDs of infected cells: (i) viperin, which is active against two viruses assembled on LDs (HCV and DENV) (6); (ii) IFN- $\gamma$ -inducible GTPase (IGTP), which is required for resistance to *Toxoplasma gondii* (7); and (iii) histones on LDs, which increase the survival of bacterially challenged *Drosophila* embryos (8). Here, we analyzed whether mammalian LDs have a direct or regulated role in immune defense. Since all eukaryotic cells accumulate LDs, this innate defense mechanism may be ubiquitous and therefore serve as a suitable target for therapeutic intervention.

## Results

### **Mammalian LDs display regulated protein-mediated antibacterial activity**

We selected hepatic LDs as a proof of concept that mammalian LDs participate in innate immunity. The liver modulates the systemic immune response and hepatic LDs are targeted by LD-related pathogens (9). We tested the antibacterial capacity of hepatic LD proteins in a bacterial killing assay of *Escherichia coli*, an abundant component of the intestinal microbiota and cause of serious clinical infections. First, we injected mice with lipopolysaccharide (LPS), an activator of innate immunity (10). Since LPS-treated animals

(LPS-mice) reduce food intake, LPS-mice were additionally fasted and compared to mice injected with saline buffer and identically fasted (CTL-mice). Both treatments promoted similar hepatic triglyceride levels (Fig. 1, A and B; and Fig. 1C), although morphological differences between LDs were evident by transmission electron microscopy (TEM). The number of LDs in LPS-treated livers (LPS-LDs) was higher than in fasted animals (CTL-LDs) although LPS-LDs were smaller (Fig. 1, D and E). CTL- and LPS-LDs were purified (Fig. 1F; and fig. S1A) and LD proteins incubated with *E. coli*. Bacterial viability was estimated from the resulting colony-forming units (CFU). LD proteins reduced bacterial growth and LPS-LD proteins demonstrated enhanced antibacterial capacity (Fig. 1G). This enhancement was confirmed in suspension cultures (fig. S1C) and using LD proteins from fed mice (fig. S1, D and E). To determine LD antibacterial activity during an actual infection, mouse liver LDs were obtained after cecal ligation and puncture (CLP), a model of polymicrobial sepsis. CLP-LD proteins exhibited enhanced antibacterial capacity when compared to CTL-LDs (fig. S1, B and F). LPS- and CLP-LD proteins reduced bacterial growth even after a shorter incubation time (fig. S1, G and H). Bacterial growth was unaffected by oleic acid (OA), the major fatty acid component of hepatic LDs, or by cytosolic proteins from CTL- and LPS-livers (fig. S1, I and J). Thus, mammalian LDs have a protein-mediated antibacterial capacity, which is regulated by infection.

Next, we analyzed whether LDs reduce bacterial growth in human monocyte-derived macrophages (HMDMs) from healthy donors. In HMDMs, LD accumulation was promoted by incubation with OA, a fatty acid efficiently esterified into LDs (11). Untreated and LD-loaded HMDMs were infected with either non-pathogenic *E. coli* or the professional intramacrophage pathogen *Salmonella enterica* serovar Typhimurium (*Salm*). HMDMs responded to infection by increasing LD numbers (Fig. 1H). *E. coli* survival (Fig. 1I), but not

phagocytic capacity (Fig. 1K), was reduced in LD-loaded HMDMs. By contrast, LDs did not reduce *Salm* survival (Fig. 1J), in keeping with this pathogen's ability to avoid antimicrobial responses (12). In *E. coli*-infected macrophages, LDs were often in the proximity of bacteria (Fig. 1, M to R). Comparative analyses demonstrated that LDs were closer to and more frequently established longer contacts with *E. coli* than with *Salm* (Fig. 1L; and fig. S2, A and B). These LD-*E. coli* contact sites increased in loaded HMDMs (fig. S2, C and D). TEM analysis revealed that in LD-*E. coli* contact sites, the LD monolayer (containing LD proteins) produced an apparent discontinuity in the bacterial vacuolar membrane and probably interacted with the bacterial periplasm (Fig. 1, O to R; and fig. S2, E and F). Thus, LD-loaded macrophages display enhanced antibacterial capacity, which suggests the existence of docking mechanisms that enable or facilitate the engagement of antibacterial LD proteins with bacteria.

### **Quantitative mass spectrometry analysis of LPS-LDs**

To characterize the enhanced LPS-LD antibacterial capacity, we performed comparative mass spectrometry profiling of proteins differentially associated with LPS- or CTL-LDs (13). CTL- and LPS-livers were analyzed in parallel. Stringent analysis (FDR<1) of LPS-livers identified 8563 proteins of which 1136 (cut-off  $|\Delta Zq| \geq 1.8$ ) were differentially expressed (553 enriched/583 reduced) (Fig. 2A; table S1; and table S2). In LPS-LDs, 3392 proteins were identified (table S3), of which 689 were differentially distributed (317 enriched/372 reduced) (table S4; and table S5). Only 8% of the enriched and 0.8% of the downregulated proteins in LPS-LDs followed an equivalent profile in LPS-livers (Fig. 2A; Fig. 2B; and fig S3A), indicating autonomous changes in LPS-LDs. Functional annotation enrichment analysis revealed the upregulation of proteins related to the acute phase and inflammatory

responses and significant reduction of mitochondrial proteins co-fractionating with LDs (Fig. 2B; and fig S2A).

Published proteomic analyses show that approximately 7-10% of proteins in LD fractions are bona fide LD resident proteins (14, 15), reflecting the tight interaction of LDs with other organelles. Of 3392 identified proteins in LPS-LDs, 238 (7%) were annotated as LD-resident proteins by the Ingenuity Pathway Analysis (IPA) platform or by at least one of the above proteomic analyses (Fig. 2C; and table S6). Seventy-two of these LD proteins were LPS-regulated (59 enriched/13 reduced) (table S7). Thus, 30% of the identified LD proteome, including the five perilipins (PLINs), was LPS-sensitive. PLIN2 ( $\Delta Zq=6.47$ ) and RAB18 ( $\Delta Zq=7.10$ ) were highly enriched and PLIN5 was the only downregulated PLIN ( $\Delta Zq=\text{minus } 4.13$ ) (table S7). Two immune proteins previously described on LDs, viperin (RSAD2,  $\Delta Zq=8.12$ ) and IGTP (IRGM3,  $\Delta Zq=6.7$ ), were identified on LPS-LDs, validating our proteomic strategy (table S4). IPA analysis of these LD resident proteins demonstrated enrichment of innate immunity-related components and reduction of metabolism-related LD resident proteins (fig. S3B).

To identify relevant candidates on LPS-LDs, we initially performed hierarchical clustering of proteins with similar variation profiles across each individual replicate, likely reflecting co-regulation (Fig. 2D). Gene interaction analysis of correlated proteins revealed the existence of several functionally connected **protein networks** such as clusters of RAB GTPases, a cluster containing PLIN1 and histones, and a network of metabolism regulators including PLIN3, PNPLA2 (ATGL), and ACSL4 (fig. S4A). Notably, the cluster containing proteins ranking highest for enrichment ( $\Delta Zq>3.14$ ) nucleated around PLIN2 and included viperin, IGTP, and several immune GTPases (GVIN, IFGGA1, IFGGB55, IFI47, and IFI35) (Fig. 2D). These

functionally related proteins may also physically interact. We confirmed that PLIN2 interacts with IGTP (7) and detected a weak interaction with cathelicidin (fig. S4B). Finally, we performed a gene interaction analysis across the whole LPS-sensitive LD proteome ( $\Delta Zq > 1.8$ ). This analysis retrieved complex protein networks (Fig. 2E) suggesting that LDs are innate immune hubs integrating major intra- and extracellular responses.

We validated the proteomic data by immunoblotting and confirmed enrichment of PLIN2 and PLIN3 on LPS-LDs in contrast with the unregulated lipase HSL ( $\Delta Zq = 0.04$ ) (Fig. 3A). PLIN2 expression was further confirmed in mouse liver sections (fig. S5A). PLIN2 in LPS- and CLP-livers was predominantly expressed in hepatocytes around periportal regions where cells receive blood and regulatory inflammatory mediators. Direct transcriptional regulation of LD proteins by inflammatory stimuli (fig. S5 B) was assessed in human hepatic HuH7 cells treated with LPS, TNF, or IFN- $\gamma$ . *PLIN2* and *PLIN5* expression was differentially regulated by individual cytokines (fig. S5C). Thus, LPS likely regulates LD protein composition directly and in conjunction with paracrine signaling networks.

### **Physical and functional uncoupling of LPS-LDs and mitochondria**

Mitochondria are key organelles for innate immunity (16). During nutrient starvation, LDs contact mitochondria to supply fatty acids fueling oxidative phosphorylation (OXPHOS) (17). By contrast, challenged innate immune cells increase aerobic glycolysis and reduce OXPHOS (16). Therefore, uncoupling LPS-LDs and mitochondria (Fig. 2B) may contribute to a reduction of OXPHOS in infected cells. Reduced interaction between LPS-LDs and mitochondria was confirmed by decreased co-fractionation of ATP5D (a subunit of ATP synthase, an OXPHOS enzyme) when compared to CTL-LDs (Fig. 3, A and B). Functional annotation of reduced mitochondrial proteins co-fractionating with LPS-LDs matched with



the whole mitochondrial proteome (MitoCarta 2.0) (Fig. 3, C). This does not reflect a reduced mitochondrial content of LPS-livers **as determined by hepatic citrate synthase activity, and liver cytochrome oxidase (COI) gene copy number** (Fig. 3, D and E). The reduced number of contacts between LPS-LDs and mitochondria was then confirmed by TEM (Fig. 3F; and fig. S6). In these images, ER membranes often separated LPS-LDs and mitochondria (fig. S6C). Finally, we confirmed two functional consequences of uncoupling: (i) reduced mitochondrial beta-oxidation of lipids supplied by LDs in LPS-primary hepatocytes (Fig. 3, G and H); and (ii) lower levels of circulating ketones in LPS-mice serum (Fig. 3I). These results extend and mechanistically explain early observations showing reduced beta-oxidation and ketogenesis in rats infected with *Streptococcus pneumoniae*, *Francisella tularensis*, and *S. Typhimurium* (18).

PLIN5 tethers LDs and mitochondria (17). Interestingly, PLIN5 is the only PLIN downregulated in LPS-LDs (fig. S3B; table S5; and table S7). During fasting, to facilitate LD–mitochondria contacts, PLIN5 levels increase on hepatic LDs (Fig. 3J). However, PLIN5 levels on LDs were reduced when fasted mice were treated with LPS (Fig. 3, A and J). Further, human *PLIN5* expression promoted co-clustering of LDs and mitochondria in HuH7 cells (Fig. 3K). To explore the role of PLIN5 **during infection**, *PLIN5* was transfected in LPS-responsive HEK293-TLR4<sup>+</sup> cells (fig. S7, A to C) and the LD–mitochondria contacts quantified. *PLIN5* expression increased the number and length of these contacts (Fig. 3L; and fig. S7, D to F). In LPS-treated HEK293-TLR4<sup>+</sup> cells, the overall length of the contacts was reduced in CTL- but not in *PLIN5*-expressing cells (Fig. 3L). In *PLIN5*-expressing cells, LPS only modestly reduced the total number of contacts (fig. S7E) and increased the average length of remaining contacts (fig. S7F). Thus, LPS directly regulates dynamics of LD–

mitochondria contacts. Furthermore, PLIN5 downregulation appears to be involved in the LPS-induced metabolic reprogramming.

We next evaluated the role of PLIN5 in other aspects of immune defense. *PLIN5*-overexpressing HEK293 cells exhibited a significantly reduced capacity to clear *E. coli* by comparison to *PLIN3*-overexpressing control cells (Fig. 3, M and N). Furthermore, THP-1 cells lentivirally transduced with *PLIN5* and subsequently infected with *E. coli* exhibited increased numbers of LD–mitochondria contacts (fig. S7, G to I), reduced LD–bacteria interactions (fig. S7J), and impaired antimicrobial capacity (Fig. 3, O and P). Thus, LPS-mediated PLIN5 downregulation reduces LD–mitochondria tethering, enabling an effective antimicrobial response.

### **LDs accumulate and utilize innate immune proteins**

Our proteomic analyses predicted complex immune protein networks on LDs (Fig. 2, D and E; and fig. S4A). Given that many known antipathogenic proteins were associated with the PLIN2 cluster (Fig. 2D), we next assessed components of this cluster for LD association. The antiparasitic protein IGTP and the antiviral protein viperin as well as three GTPases (IIGP1, TGTP1, and IFI47) all associated with LDs (Fig. 4, A to C; fig. S8; and fig. S9). Thus, multiple proteins associated with responses to different classes of pathogens localize to LDs.

The PLIN2 cluster also includes cathelicidin (CAMP,  $\Delta Zq=7.25$ ), a broad-spectrum antimicrobial peptide with chemotactic and immunomodulatory properties (19). Cathelicidins are synthesized as proproteins which, after cleaving a N-terminal signal peptide, follow the exocytic pathway (Fig. S10A). We confirmed the accumulation of CAMP on LPS-LDs (Fig. 4A) and the distribution of a human-tagged CAMP between the ER and LDs of HuH7 cells

(Fig. 4B; and fig. S10, B to E). CAMP on LDs had a higher molecular weight than CAMP in the ER (Fig. 4C; and fig. S10E), suggesting that the CAMP hydrophobic domain functions as both a signal peptide cleaved for secretion via the ER as well as an as an uncleaved LD-targeting signal. An equivalent dual distribution occurs for other LD proteins containing signal peptides, such as apolipoproteins (20). Indeed, the low-molecular-weight (20 kDa) CAMP species corresponded to the protein with a cleaved signal peptide following the secretory pathway (fig. S10, F to H). Distribution of overexpressed CAMP, as well as other immune LD proteins, was not directly affected by LPS–TLR4 signaling (fig. S11). Thus, LPS does not directly regulate the intracellular trafficking of these proteins.

We next investigated the role of CAMP in HMDMs. Silencing of *CAMP* (Fig. 4D) impaired the antibacterial response of the macrophages against *E. coli* (Fig. 4E). Furthermore, although LD loading significantly reduced bacterial survival, this treatment regime was unable to do so in *CAMP*-silenced HMDMs. Thus, the antibacterial activity of LDs in HMDMs appears to require *CAMP*. To further explore this possibility, a LD-resident *CAMP* was engineered by substitution of the *CAMP* signal peptide with the ALDI LD-targeting motif (fig. S12; A and B) (21). Modified *CAMP* (LD-*CAMP*) accumulated on LDs of HuH7 cells (fig. S12, C to F) and showed a single electrophoretic mobility pattern, matching the higher molecular weight *CAMP* that localized to LDs (Fig. 4C; and fig. S12C). Next, HEK293 cells were transfected with LD-*CAMP* and protein distribution on LDs confirmed with anti-*CAMP* antibodies (Fig. 4F), demonstrating a native conformation. The antimicrobial capacity of LD-*CAMP* was then assessed. Bacterial loads of *E. coli*, *Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus* (MRSA) were significantly reduced in LD-*CAMP* expressing cells when compared to those expressing the *PLIN3* control (Fig. 4G). By contrast, *Pseudomonas aeruginosa* loads were not affected by LD-*CAMP*, suggesting that this pathogen subverts this

innate defense response. The impact of LD-*CAMP* overexpression on bacterial survival was dependent on LD formation (Fig. 4, H to J). The tagged LD-*CAMP* demonstrated a similar antibacterial activity to wild-type *CAMP* and a slightly augmented stability when compared to an untagged LD-*CAMP* (fig. S12, G to J). Thus, LDs act as a molecular switch in innate immunity, responding to danger signals by both reprogramming cell metabolism and eliciting protein-mediated antimicrobial defense.

## Discussion

Pathogens require host-derived lipids to support their life cycles, with LDs providing a source of these lipids (22). As a result, LDs also have the potential to deliver effective host defenses against intracellular pathogens. We show that at least 30% of the LD proteome is LPS-sensitive, suggesting that innate immunity has developed a host defense program that includes extensive LD remodeling. Our analyses demonstrate that complex clusters of immunity-related proteins organize on LDs of infected cells. In addition to previously described LD-resident immune proteins, such as viperin and IGTP, we have identified IIGP1, TGTP1, and IFI47. Our analysis also identified *CAMP* as a professional antibacterial protein efficiently functioning on LDs. These proteins may act individually, in a coordinated manner, and/or synergistically to kill pathogens.

Mechanisms of LD trafficking and docking with phagocytic and parasitophorous membranes, observed here and described for several pathogens (23-26), may facilitate the delivery of immune proteins located on the LD surface. Accumulation on LDs may provide stability to these proteins and may restrict these potentially cytotoxic peptides to LDs preventing indiscriminate cellular damage (27). In this respect, we have shown that LPS triggers

physical separation of LDs and mitochondria, at least partly due to reduced PLIN5 levels on LPS-LDs (28). Uncoupling likely reflects both a self-protection program (to avoid mitochondrial damage, in view of their prokaryotic evolutionary origin), and a means to maximize or increase the number of LDs available to interact with bacteria. Simultaneously, the reduced LD–mitochondria interaction may lead to distinctive immunometabolic features: (i) the accumulation of host LDs resulting from reduced mitochondria-mediated LD consumption; (ii) reduced OXPHOS displayed by infected cells due to decreased fatty acid oxidation; and (iii) the low rates of ketogenesis displayed by infected animals.

In conclusion, these studies highlight the fact that mammalian LDs constitute an intracellular first line of defense. LDs actively participate in at least two levels of the innate immune response, accumulating and utilizing antibacterial proteins, as well as regulating immune cell metabolism. Since widespread resistance to current antibiotics is common among pathogens, understanding the cellular mechanisms eliciting LD-mediated defense may inform future strategies for the development of anti-infective therapies (29, 30).

## **Materials and methods**

### **Plasmids**

pCMV6-*IGTP*-myc-FLAG (MR224617), pCMV6-*CAMP*-myc-FLAG (RC208872), pCMV6-*IIGP1*-myc-FLAG (MR206520), pCMV6-*TGTP1*-myc-FLAG (MR206553), and pCMV6-*IFI47*-myc-FLAG (MR206684) were purchased from OriGene Technologies (Rockville, MD, US). pcDNA3.1-*VIPERIN*-FLAG (OHu13432) was from Genscript (Piscataway, NJ, US). pcDNA3.1-*PLIN5*-FLAG (OHu04126) from GenScript was subcloned into pCMV6-myc-FLAG vector using primers containing EcoRI and XmaI sites. The LD-*CAMP* construct was derived from the plasmid pCMV6-*CAMP*-myc-FLAG: an equivalent EcoRI/BspEI sequence of pCMV6-*CAMP*-myc-FLAG was designed replacing the *CAMP* signal peptide

(MKTQRDGHSLGRWSLVLLLLGLVMPLAII) with the hydrophobic domain of *ALDI* (MDALVFLQLLVLLLLTLPLHLLALLGC) acquired from GeneScript, cloned in a PUC57 plasmid. Both fragments were swapped after an EcoRI/BspEI digestion. *CAMP*  $\Delta$ N mutant, results from deletion of the amino acids 1-32. The cDNAs were acquired from GenScript and subcloned into pCMV6-myc-FLAG vector following the same strategy. pCMV6-*CAMP*-untagged was generated by PCR using primers containing EcoRI and XmaI sites. The plasmid pCMV6-*PLIN2*-myc-FLAG tagged was derived from the plasmid pGFP-*PLIN2*, provided by Dr John McLauchlan (Institute of Virology, Glasgow) and subcloned into pCMV6 by PCR using primers containing EcoRI and XhoI sites. The plasmid pCMV6-*PLIN3*-myc-FLAG tagged was derived from the plasmid pCDNA 3 $\times$ myc-tagged *PLIN3* provided by S. Pfeffer (Stanford University School of Medicine, Stanford, CA), and subcloned into pCMV6 by PCR using primers containing EcoRI and XhoI sites. The lentiviral system utilizing pFTRE3G-PGK-puro (kindly provided by James Murphy, Walter and Elizabeth Hall Institute of Medical Research) for doxycycline-inducible gene expression has previously been described (31,32). The plasmid pFTRE3G-*PLIN5* was obtained by subcloning *PLIN5* into pFTRE3G by PCR using primers containing BamHI sites.

## **Mouse studies**

### ***Animals and models of infection***

C57BL/6J male mice (8-10 weeks old) were purchased from Charles River Laboratories (Wilmington, MA, US). Animals were kept under a controlled humidity and lighting schedule with a 12 hours dark period. Food and water were available ad libitum. All animals received humane care in compliance with institutional guidelines regulated by the European Community. The experimental protocols were approved by the Animal Care Committee of the University of Barcelona. The day before the experiment, animals were fasted overnight (16 hours) and in some cases intraperitoneally injected with 200  $\mu$ L of saline buffer (CTL) or 6 mg/kg LPS (final dose) (L2639, Sigma-Aldrich, St Louis, MO, US). In some experiments (fed condition) food was available ad libitum. To induce sepsis by cecal ligation and puncture (CLP), mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine (Richter Pharma AG, Wels) and 10 mg/kg xylazine (Rompun, Bayer, Leverkusen, Germany) and a 1 cm incision was made on the abdomen. The cecum was exposed and ligated below the ileocecal junction. A double puncture was made using a 22G needle, to induce severe sepsis. Sham-operated animals (CTL) underwent an identical laparotomy but without CLP.

All mice received 1 mL of sterile saline subcutaneously as fluid resuscitation and antibiotic therapy by subcutaneous injection of 10 mg/kg meropenem (Merck Research Laboratory, Whitehouse Station, NJ, US) 6 hours after surgery.

### ***Histological analysis***

Liver sections were prepared and processed for hematoxylin-eosin staining as previously described (33). For immunohistochemistry, liver sections were prepared and processed as described previously (34). The slides were blocked by incubation in 5% normal goat serum in PBS for 1 hour at room temperature followed by incubation with anti-PLIN2 antibody (1:200; ab78920, Abcam, Cambridge, UK) overnight at 4°C. Sections were then washed three times in PBS and incubated with secondary goat anti-rabbit IgG Alexa Fluor 647 (1:250; A21244, ThermoFisher Scientific, Waltham, MA, US) for 45 min at RT. After washing three times in PBS, slides were mounted with Dako Fluorescence Mounting Medium (Agilent Dako, #S3023).

### ***Liver fractionation, and hepatic LD purification***

After liver perfusion with 0.9% NaCl and 0.1% EDTA solution, the liver was placed on a Petri dish, chopped with a scalpel for two minutes and transferred into a Dounce tissue grinder at a ratio of 1 g of tissue to 3 mL of homogenization buffer (25 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM EDTA, and 5 mM EGTA). After three up-and-down strokes of each loose- and tight-pestle, the liver homogenate was centrifuged at 500 g for 10 minutes at 4°C. 2.5 mL of the resulting post-nuclei supernatant (PNS) were mixed with an equal volume of 2.5 M sucrose and placed at the bottom of a sucrose step gradient of 25%, 15%, 10%, and 5% (w/v) sucrose in homogenization buffer, with an additional top layer of 25 mM Tris-HCl, pH 7.5, 1 mM EDTA and 5 mM EGTA, and centrifuged at 12,000 g for 1 hour at 4°C (SW-41Ti rotor, Beckman Coulter, Pasadena, CA, US). Six or seven fractions were collected from the top. Equal volumes of each fraction were used for immunoblotting. To purify LDs, the LD fraction on the top of the gradient was recovered and concentrated by re-floating LDs at 16,000 g for 10 minutes at 4°C. The lower phase containing the excess buffer was removed by aspiration with a syringe and four volumes of ice-cold acetone were added to precipitate proteins and kept 48 hours at -20°C. The samples were centrifuged at 16,000 g for 10 minutes at 4°C, the pellet washed with cold acetone 3 times, air-dried and reconstituted with 10 mM Tris-HCl, pH 7.5. After sonication, protein concentration was quantified by CBQCA protein quantitation kit (ThermoFisher Scientific). To purify cytosol extracts, 200 µL of PNS plus

600  $\mu\text{L}$  of homogenization buffer were centrifuged at maximal speed for 1 hour (1,000,000 x g in S140-AT Fixed Angle Rotor, ThermoFisher Scientific). A syringe (23G needle) was inserted below the floating LDs to remove 200  $\mu\text{L}$  of cytoplasm and proteins were precipitated as described previously.

### ***Bacterial killing assay (BKA)***

*Escherichia coli* (ATCC 25922) were grown to an O.D. at 600 nm of 1 and diluted 1:100 (1.5 x 10<sup>5</sup> colony-forming units (CFU/mL)). 100  $\mu\text{L}$  of bacterial culture were mixed with 15 or 25  $\mu\text{g}$  of LD-proteins and incubation buffer (33 mM KH<sub>2</sub>PO<sub>4</sub>, 60 mM K<sub>2</sub>HPO<sub>4</sub>, 10 mM Na<sub>2</sub>SO<sub>4</sub>, 1.7 mM sodium citrate, 10 mM MgSO<sub>4</sub>) was added up to 200  $\mu\text{L}$ . Cultures were incubated for the indicated times at 37°C in a shaking incubator. Serial dilutions were plated in triplicate on LB-agar plates and surviving bacteria were quantitated as CFU/mL after overnight incubation at 37 °C. Alternatively, overnight incubations were centrifuged at 11,000 g for 30 seconds and the bacterial pellet was resuspended in PBS and measured the absorbance by optical density at 600 nm with a Modulus Microplate Multimode Reader (Promega, Madison, WI, US). For monitoring bacterial growth in the presence or absence of OA (175  $\mu\text{g}/\text{mL}$ ) or gentamicin (200  $\mu\text{g}/\text{mL}$ ), *E. coli* cultures were diluted to an O.D. at 600 nm of 0.1 in 96-well flat-bottom plates and incubated at 37°C shaking. O.D. at 600 nm readings were taken every 20 minutes and monitored using a POLARstar Omega reader (BMG Labtech, Germany).

### ***Serum parameters, hepatic triacylglycerol quantification, and mitochondrial content***

Blood was extracted by cardiac puncture and sera obtained after centrifugation of blood samples at 6,000 g for 15 minutes at 4°C in serum heparin separator tubes (Becton Dickinson, Franklin Lakes, NJ, US). Ketone bodies in serum were measured using a Ketone Body Assay Kit (MAK134; Sigma-Aldrich) according to the manufacturer's instructions. Triacylglycerol content of the liver was determined using the Triglyceride Detection Kit following manufacturer's instructions (BioSystems, Barcelona, Spain). Citrate synthase activity was measured as a reliable marker of mitochondrial content as previously described (36).

### ***Fatty acid beta-oxidation***

Primary hepatocytes were isolated as previously (37). To accumulate radiolabeled fatty acids in LDs, cells were treated for 4 hours with 1  $\mu\text{Ci}/\text{mL}$  of [<sup>14</sup>C]-OA 175  $\mu\text{g}/\text{mL}$  (NEC317050C,



PerkinElmer, Waltham, MA, US); next the media was replaced with fresh media at 175  $\mu\text{g}/\text{mL}$  of OA for an additional 4 hours followed by an overnight incubation with DMEM 0% FCS, low glucose (0.75 g/L) with or without LPS (100  $\mu\text{g}/\text{mL}$ ) and sealed. Oxidation measurements were performed by trapping the released [ $^{14}\text{C}$ ] carbon dioxide in a parafilm-sealed system on filter paper soaked in 1 M potassium hydroxide and measured using a Wallac 1409 Liquid Scintillation Counter. The rate of beta-oxidation was calculated as the amount of trapped [ $^{14}\text{C}$ ] carbon dioxide in relative units produced per  $0.5 \times 10^6$  cells. Results are expressed as the beta-oxidation rate relative to the untreated condition. Lipid soluble intermediates include those incompletely oxidized acid-soluble metabolites containing  $^{14}\text{C}$  and were obtained after precipitation with perchloric acid and measured using a liquid scintillation counter.

## **Human macrophages studies**

### ***Cell culture***

Human monocyte-derived macrophages (HMDMs) were obtained by differentiating CD14<sup>+</sup> monocytes as previously described (35). The human monocytic THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD, US). Cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI, Gibco, ThermoFisher Scientific) containing 10% heat inactivated FBS (Bovogen Biologicals, Melbourne, VI, Australia), 5mM sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 50 U/mL penicillin (Invitrogen, Carlsbad, US) and 50  $\mu\text{g}/\text{ml}$  streptomycin (Invitrogen). Infection media are similar to complete media but without penicillin/streptomycin.

### ***Bacterial strains and infection assays***

For HMDMs infection, the following bacterial strains were used: *Salmonella Typhimurium* SL1344 and *Escherichia coli* K-12 MG1655. THP-1 cells were infected with *E. coli* K-12 MG1655. To induce LD formation, cells were treated with OA (178  $\mu\text{g}/\text{mL}$  final) 18 hours prior infection. Bacterial infections were performed as previously described (35), with a multiplicity of infection (MOI) of 10 for *S. Typhimurium* and 100 for *E. coli*.

### ***Flow cytometry***

HMDMs were seeded at  $0.5 \times 10^6$  cells/mL and treated with or without OA (178 ng/mL) for 16 hours. The next day, heat-killed pHrodo™ Green *E. coli* BioParticles™ Conjugate

(#P35366, ThermoFisher Scientific) was added to the well (50 µg/well) for 90 minutes. Cytochalasin D (BioMol, Plymouth Meeting, PA) (10 µM, 30 minutes pre-treatment) was used as positive control to block phagocytosis (ThermoFisher Scientific). Cells were then harvested in ice-cold PBS containing 0.1% sodium azide and 25 mM EDTA. Flow cytometric analysis was performed using a GALLIOS Flow Cytometer (Beckman Coulter) and data were analyzed using Kaluza Analysis 1.3. software.

### ***Fluorescence and quantitation of LD-bacteria proximity***

HMDMs, plated on coverslips, were treated with OA (175 µg/mL) for 16 hours, then infected with *E. coli* strain MG1655 (MOI 10) or *Salmonella* SL1344 strain (MOI 10), both expressing mCherry constitutively. At 4 hours post-infection, cells were stained with BODIPY 647 (10 µg/mL; Molecular Probes Eugene, OR, US) for 30 minutes, before being washed with PBS and fixed with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA, US) for 10 minutes. Cells were then stained with DAPI (20 ng/mL) and mounted on slides.

### ***HMDM siRNA experiments***

Day 6 HMDMs were harvested and resuspended in IMDM complete media containing 10 mM HEPES buffer (pH 7.2-7.5, Gibco). Combined sets of CAMP siRNA (GGAAGCUGUGCUUCGUGCUAUAGAU, AUCUAUAGCACGAAG CACAGCUUCC, GACAUCAGUUGUGAUAAAGGAUAACA, UGUUAUCCUUAUCAC AACUGAUGUC, GCUUCACAGUGAAAGAGACAGUGUG, CACACUGUCUCCUUC ACUGUGAAGC) or scramble siRNA were used as previously described (38). After 24 hours recovery, cells were treated with OA (37.5 µg/mL) for another 18 hours. HMDMs were then infected with *E. coli*.

### ***Gene overexpression in THP-1 by lentiviral transduction***

Lentiviral transduction was used for gene overexpression of *PLIN5* in THP-1 cells as previously described (38).

## **Cell culture studies**

### ***Cell culture and treatments***

HuH7 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Cromwell, CT, US) 10% v/v fetal bovine serum (Biological Industries)

supplemented with 4 mM L-glutamine, 1 mM pyruvate (Sigma-Aldrich) and with 50 U/mL penicillin, 50 µg/ml streptomycin and non-essential amino acids (Biological Industries). HEK293 cells stably expressing human Toll-like receptor (HEK293-TLR4<sup>+</sup>) have been characterized previously (39). OA treatments were performed using OA (O1008, Sigma-Aldrich) conjugated to fatty acid-free BSA (A8806, Sigma-Aldrich) at a molar ratio of 6:1. Cells were treated with recombinant human TNF $\alpha$  (20 ng/mL; 300-01A, Preprotech, Rocky Hill, NJ, US), and IFN $\gamma$  (10ng/mL; 300-02, Preprotech) and LPS (500 ng/mL) for 16 hours. Cells expressing CAMP- $\Delta$ N mutant were treated with MG132 (5 µM; 474790, Merck) for 24 hours.

### ***Transfection***

300,000 HuH7 cells or 400,000 HEK293 or HEK293-TLR4<sup>+</sup> cells were seeded in 6-well plates. 24 hours after plating, cells were transfected using GENEJET PLUS (SignaGen, Rockville, MD, US), following the manufacturer's instructions. 6 hours after transfection cells were treated with OA 175 µg/mL for 16 hours.

### ***Bacterial strains and infection assays***

The bacterial strains used were: *E. coli* (ATCC 25922), MRSA (Methicillin-resistant *Staphylococcus aureus*, strain 162057-900), *Pseudomonas aeruginosa* (ATCC 27853) and *Listeria monocytogenes* (strain 10403S). 400,000 HEK293 or HEK293-TLR4<sup>+</sup> cells were seeded in 6-well plates and transfected the next day. 6 hours after transfection, culture media was replaced for antibiotic-free cell culture medium in presence of OA (175 µg/mL) and left overnight. Bacteria were grown overnight to stationary phase. Next day, bacteria were diluted 1/10 and grown to an O.D. at 600 nm of 0.54-0.56, washed twice and resuspended in antibiotic-free cell culture medium and used at a multiplicity of infection (MOI) of 0.5. Each infection was performed in triplicate wells. After 1 hour, extracellular bacteria were removed by incubation with 200 µg/mL gentamycin-containing medium (G1914, Sigma-Aldrich) for 1 hour, followed by incubation with 20 µg/mL gentamycin-containing medium for 4 hours. To determine intracellular bacterial loads cell were lysed with 0.1% Triton X-100 (T8787, Sigma-Aldrich) for 5 minutes and plated onto LB medium supplemented with 1.5% (w/v) agar.

### ***Cell fractionation***

HuH7 cells were seeded at  $1.5 \times 10^6$  cells/plate, transfected the next day and loaded with 175  $\mu\text{g}/\text{mL}$  OA overnight. Three 100 mm culture plates were used per each condition. Sucrose density gradient was performed as previously described (40).

### ***Protein purification and co-immunoprecipitation***

For purification of myc-tagged proteins, HuH7 cells were plated in 100 mm culture plates at  $10 \times 10^6$  cells/plate, transfected the next day and loaded with 175  $\mu\text{g}/\text{mL}$  OA overnight. Myc-tagged proteins from the cellular extract and secreted into the media were purified using a c-myc protein purification kit (MBL, Nagoya, Japan) according to the manufacturer's instructions. Samples were processed by SDS-PAGE and analyzed by immunoblotting. For immunoprecipitation, transfected HuH7 cells (five 100 mm culture plates per condition), were collected and lysed in buffer containing 50mM Tris-HCl, at pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 supplemented with proteases and phosphatases inhibitors. Cell lysates were homogenized with a 23G needle syringe 10 times, centrifuged 20 minutes at 16,000 g, at 4 °C, and the supernatant was incubated with 1  $\mu\text{g}$  of anti- FLAG antibody for 2 hours at 4°C, followed by addition of protein G Sepharose beads (P3296, Sigma-Aldrich) for 1 hour at 4 °C. Immunoprecipitated proteins were washed three times with lysis buffer, suspended in 2 $\times$  Laemmli buffer and analyzed by immunoblotting.

### ***Gene expression by quantitative PCR (qPCR)***

For quantitative RT-PCR, total RNA was isolated from liver homogenates, HuH7 or HEK293-TLR4<sup>+</sup> cells using the RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. 1  $\mu\text{g}$  of total RNA was used for cDNA synthesis, using the High Capacity cDNA Reverse Transcription Kit (Applied Bioscience, ThermoFisher Scientific) according to the manufacturer's instructions. qRT-PCR was performed using the Brilliant SYBR Green qPCR Master Mix (# 600548, Agilent Technologies, Santa Clara, CA, US) and detected by the Mx3000P QPCR System (Agilent Technologies).

The primers used for real-time PCR were:

*GAPDH*: forward, 5'- CGACTTCAACAGCAACTCCCACTCTTCC-3' and reverse 5'- TGGGTGGTCCAGGGTTTCTTACTCCTT-3'. *Cytochrome C oxidase subunit I (COI)*: forward, 5'- GCCCCAGATATAGCATTCCC-3' and reverse 5'-

GTTCATCCTGTTCTGCTCC-3'. *PLIN2*: forward 5'-ACACCCTCCTGTCCAACATC-3' and reverse 5'-AAGGGACCTACCAGCCAGTT-3'. *PLIN5*: forward 5'-GCGGTCTGCGATGTTTACAG-3' and reverse 5'-CTCCGAAGGTTGCTGGAGAA-3'. *Rab18*: forward 5'-GACGTGCTAACCACCCTGAA-3' and reverse 5'-AACACCCTGTGCACCTCTAT-3'. *HSL*: forward 5'-CACCAGCCAACACTCAGCTA-3' and reverse 5'-GTGTGAGGAGGGTCATCGTT-3'. *HPRT*: forward 5'-GCAGTACAGCCCCAAAATGG-3' and reverse 5'-AACAAAGTCTGGCCTGTATCCAA-3'. *CAMP*: forward 5'-CTGTCCCCATACACCGCTTC-3' and reverse 5'-GACACAGTGTGCCCCAGGAC-3'. *TNF $\alpha$* : forward 5'-CCATGTTGTAGCAAACCCTCAA-3' and reverse 5'-GCTGGTTATCTCTCAGCTCCA-3'. *IL8*: forward 5'-AGACAGCAGAGCACACAAGC-3' and reverse 5'-ATGGTTCCTTCCGGTGGT-3'. *18 S*: forward 5'-CGGCTACCACATCCAAGGAA-3' and reverse 5'-GCTGGAATTACCGCGGCT-3'. The relative expression of each mRNA normalized to the internal reference GAPDH (liver), 18S (cultured cells), or hypoxanthine phosphoribosyl transferase (HPRT; macrophages).

#### ***TLR4-mediated Interleukin 8 release Assay***

HEK293-TLR4<sup>+</sup> cells were seeded at 400,000 cells /plate in 48-well plates in the presence or absence of LPS (250 ng/mL) for 18 hours. 100  $\mu$ L of culture supernatant were used to measure Interleukin 8 (IL-8) levels using the Human ELISA IL-8 Set assay ( 555244; BD OptEIA™ , BD Biosciences) according to the manufacturer's protocol and detected by Epoch Multiplate Spectrophotometer (BioTek, Winooski, VT, US).

#### ***Immunofluorescence***

HuH7 cells were grown in 10mm glass coverslips. For HEK293 and HEK293-TLR4<sup>+</sup> cells, glass coverslips were coated with 50  $\mu$ g/mL of fibronectin (Sigma-Aldrich) for 30 minutes at room temperature and rinsed twice with PBS before seeding cells. Cells were fixed for 60 minutes in 4% paraformaldehyde, permeabilized in 0.15% Triton X-100 for 10 minutes, followed by blocking with 1% BSA (A7906, Sigma-Aldrich), 0.1% Tween in PBS for 15 minutes. Labelling was achieved by incubating cells for 1 hour at room temperature with primary antibodies diluted in blocking solution: rabbit polyclonal anti-PLIN2 (1:500;

ab108323, Abcam), rabbit polyclonal anti-CAMP (1:200; ab 180760, Abcam), rabbit polyclonal anti-TOM20 (1:500; ab186734, Abcam), mouse monoclonal anti-FLAG (1:500; F1804; Sigma). Primary antibodies were detected with donkey anti-mouse IgG Alexa Fluor 488 (A21202), donkey anti-mouse IgG Alexa Fluor 555 (A31570), donkey anti-rabbit IgG Alexa Fluor 555 (A31572), and chicken anti-mouse IgG Alexa Fluor 647 (A31572) from ThermoFisher Scientific, diluted 1:250 in blocking solution. Finally, cells were labelled with DAPI (1:4000; ThermoFisher) and LDs were stained with BODIPY 493/503 (1:1000; Molecular Probes) for 10 minutes at room temperature, washed twice with PBS and coverslips were mounted with Mowiol (475904; Calbiochem, Merck). Alternatively, LDs were labelled with LipidTOX Deep Red (H34477; Molecular Probes) at 1:100 dilution in mounting media.

## **Microscopy**

### ***Optical and Fluorescence Microscopy***

Imaging of hematoxylin-eosin staining was performed with a Leica DMRB optical microscope (Leica, Wetzlar, Germany) equipped with a Leica DFC450 digital camera, using the 63x oil immersion objective lens. For immunohistochemistry and immunofluorescence, images were collected using a Leica AF600 motorized microscopy system (Leica Microsystems, Mannheim, Germany) equipped with a DMI6000 microscope, a Leica PL APO 63x Numerical aperture 1.4 oil immersion, a high-resolution monochrome ORCA-spark CMOS Digital Camera and a mercury metal halide bulb Leica EL6000 as light source. DAPI was acquired with a band pass excitation filter 340-380 nm, dichromatic mirror (400 nm) and a long pass emission filter (425 nm). A488 was acquired with a band excitation filter 480/40 nm, dichromatic mirror 505 nm and a band pass emission filter (527/30 nm). A555 was acquired with a band pass excitation filter 531/40 nm, dichromatic mirror reflection 499-555 and transmission 659-730 nm and a band pass emission filter (593/40 nm). A647 was acquired with excitation band pass filter 628/40 nm, dichromatic mirror reflection 549-651 nm and transmission 699-726 nm and a band pass emission filter (692/40 nm). Images were collected using the LAS X Navigator software. High-resolution images of liver areas were captured using the Tile Scan acquisition mode. For quantitation of LD-bacteria proximity, images were taken with 63x objective lens using a Zeiss Axiovert 200 Upright Microscope Stand with LSM 710 Meta Confocal Scanner, with spectral detection and Airyscan super resolution detector. 2-photon imaging with a fully tunable Mai Tai eHP DeepSee 760-

1040nm laser (Zeiss, Oberkochen, Germany). Images were analyzed using the Adobe Photoshop CS3 software (Adobe Systems Inc. San Jose, CA, US) and ImageJ (NIH).

### ***Electron microscopy and morphological measurements***

Liver samples, HMDMs or THP-1 cells in 3 cm dishes were processed for transmission electron microscopy (TEM) as described previously (42). For TEM, ultrathin sections (60 nm) were cut using an ultramicrotome (EM U26, Leica, Germany) and collected on copper mesh grids. Imaging was conducted on a Hitachi 7700 (Tokyo, Japan) at 80kV. For serial blockface scanning electron microscopy (SBF-SEM) the stub was transferred to a Zeiss Sigma scanning electron microscope fitted with a Gatan 3view. Sectioning and imaging were conducted at 50 nm intervals with a voxel size of 11.5 x 11.5 nm, allowing for a field of view of 46 x 46  $\mu$ m. Data obtained from SBF- SEM were analyzed using Imod software (43). Image stacks were aligned manually using the Midas command. Structures of interest were then segmented using the manual drawing tool aided by an automated interpolator tool. A mesh was placed on the objects allowing them to be viewed in 3D.

### **Image Analysis**

Image Analysis was performed using FIJI-Image J (Wayne Rasband, NIH, USA (44, 45). Custom-made macros were programmed with instructions for the automated image analysis pipelines.

### ***LD-mitochondria contacts***

Confocal images from fluorescently labelled HEK293 cells, Mitochondria (TOM20), LD (BODIPY), PLIN5 and nuclei (DAPI), were acquired to analyze contacts between mitochondria and LD under LPS and PLIN5 expression (Fig. S7). Briefly, cells were segmented, individualized, and stored as Regions of Interest (ROI). LD segmentation was achieved through a Trainable Weka Segmentation classifier (46) on LD (BODIPY) channel image and mitochondria were segmented by intensity thresholding (autothreshold method “Otsu”). Contact regions between mitochondria and LD were first obtained by using the Colocalization Highlighter plugin (Pierre Bourdoncle, Institut Jacques Monod, Service Imagerie, Paris) and converted to a contour line section by skeletonization. Contact length and contact counts were quantified from each cell and stored in the results table. Mean PLIN5

intensity was quantified from each cell to differentiate expressing PLIN5 cells. Computer code available at <https://zenodo.org/badge/latestdoi/280189667>.

### ***Distribution of selected tagged human LD-proteins***

To analyze distribution of selected tagged human LD-proteins in HuH7 cells, confocal z sections from cells labelled with DAPI, anti-FLAG antibodies, anti-PLIN2 antibodies and LipidTox were acquired. Briefly, cells defined manually and LD intensity thresholded and converted to binary image were stored in ROI Manager. The sum of intensities from anti-FLAG or anti-PLIN2 from LD ROIs was divided by the sum of intensities of anti-FLAG or anti-PLIN2 from each cell, multiplied by 100 and expressed as percentage of anti-FLAG or anti-PLIN2 protein on LDs respectively. LDs that contained at least one pixel of anti-FLAG or anti-PLIN2 labelling were counted as positive LDs for that labelling. The total counts of positive LDs for anti-FLAG or anti-PLIN2 was divided by the total amount of LDs and expressed as a percentage. Computer code available at <https://zenodo.org/badge/latestdoi/280200243>.

### **Immunoblotting**

Cells were washed twice with cold PBS before being scraped into ice-cold 10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA 0.1% Triton X-100 and a mixture of protease and phosphatase inhibitors. Cells were homogenized by sonication at 4 °C. Protein was quantified with the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, US). Immunoblotting of cells was performed as described previously (41). The blots were incubated with primary antibodies 1 hour at room temperature. Primary antibodies: rabbit polyclonal anti-GFP (1:5000; ab290, Abcam), rabbit polyclonal anti-PLIN2 (1:5000; ab78920, Abcam), rabbit polyclonal anti-PLIN5 (1:1000; ab222811, Abcam), rabbit polyclonal anti-EEA1 (1:200; ab2900, Abcam), rabbit polyclonal anti-VAP-A (1:5000; ab181067, Abcam), mouse monoclonal anti-viperin (1:1000; ab107359, Abcam), rabbit polyclonal anti-CAMP (1:1000; ab180760, Abcam), guinea pig polyclonal anti-PLIN 3 (1:500; GP32, Progen, Heidelberg, Germany), guinea pig polyclonal PLIN5 (1:1000; GP31, Progen), guinea pig polyclonal PLIN2 (1:2000; GP41, Progen), mouse monoclonal anti-GM130 (1:2000; Labs 810822, BD-Biosciences San Jose, CA, US), mouse monoclonal anti-Na/K ATPase (1:1000; 05-369 Upstate-Millipore, Darmstadt, Germany) rabbit polyclonal anti-HSL (1:1000; 4107, Cell Signaling, Leiden, The Netherlands), mouse monoclonal anti-IGTP (1:200; sc-136317, Santa Cruz Biotechnology, Dallas, TX, US), rabbit polyclonal anti-CAMP (1:500; TA306515,



OriGene), mouse monoclonal anti-FLAG (1:1000; F1804, Sigma-Aldrich), mouse monoclonal anti-ATP synthase (1:500; 7H10BD4F9, ThermoFisher Scientific), and goat polyclonal anti-GAPDH (1:5000; A00191, GenScript). After incubation with primary antibodies, membranes were washed and incubated with peroxidase-conjugated secondary antibodies (1:3000): goat anti-rabbit IgG (H + L)-HRP conjugate (1706515, BioRad), goat anti-mouse IgG (H + L)-HRP conjugate (1706516, BioRad) and peroxidase affinitypure donkey anti-goat IgG (H+L) (705-035-147, Jackson ImmunoResearch, Ely, UK). HRP-conjugated secondary antibodies were detected with ECL (Biological Industries) and visualized using ImageQuant LAS4000 (GE Healthcare, Chicago, IL, US). Immunoblotting quantification was performed using the Fiji-ImageJ software (NIH).

### **Quantitative proteomics and functional annotation analyses**

Proteins were precipitated from either isolated lipid droplets (four and five independent replicates for CTL- and LPS-treated, respectively) or liver homogenates (three independent replicates per condition) with ice-cold acetone and solubilized in lysis buffer (50 mM Tris-HCl, pH 7.5, 2% SDS and 10 mM TCEP (Tris-(2-carboxyethyl)-phosphine hydrochloride). Protein concentration in spun supernatants was determined by infrared spectrometry. ~100 µg of total protein per sample were digested using standard FASP procedures. After alkylation, proteins were digested overnight at 37°C with modified trypsin (Promega) in 50 mM ammonium bicarbonate at 30:1 protein:trypsin (w/w) ratio. Resulting peptides were eluted in 50 mM ammonium bicarbonate and 0.5 M sodium chloride, and trifluoroacetic acid (TFA) was added to a final concentration of 1%. Eluates were desalted through C18 Oasis-HLB cartridges (Waters corporation, Milford, MA US), dried and resuspended in 100 mM Triethylammonium bicarbonate (TEAB) buffer. Equal amounts of each peptide sample were labelled using the 10-plex TMT Reagents (ThermoFisher Scientific) according to manufacturer's protocol. For increased proteome coverage, TMT-labelled samples were fractionated by high-pH reverse phase chromatography (Pierce High pH Reversed-Phase Peptide Fractionation Kit, # 84868; ThermoFisher Scientific). Labelled peptides were chromatographed through a C-18 reversed phase nano-column (75 µm I.D. x 50 cm, 2 µm particle size, Acclaim PepMap RSLC, 100 C18; ThermoFisher Scientific) in a continuous acetonitrile gradient consisting of 0-30% B in 360 minutes, 50-90% B in 3 minutes (A= 0.1% formic acid; B=90% acetonitrile, 0.1% formic acid; flow rate of 200 nL/minutes) for analysis in an Orbitrap Fusion mass spectrometer (ThermoFisher Scientific). MS/MS spectra from the

Nth most intense parent ions were analyzed along the chromatographic run. For peptide identification, all spectra were analyzed with Proteome Discoverer (v. 2.1.0.81) using SEQUEST-HT (ThermoFisher Scientific) and queried onto the Uniprot database with the following search parameters: 2 maximum missed tryptic sites; precursor and fragment mass tolerances of 2 Da and 0.02 Da, respectively; carbamidomethyl cysteine and TMT modifications at N-terminal and Lys residues as fixed modifications, and methionine oxidation as dynamic modification. Peptide identification was performed using the probability ratio method (47), and false discovery rate (FDR) was calculated using inverted databases. The relative abundance of each protein was estimated from ion intensities of peptides with an FDR  $\leq 1\%$  and expressed in units of standard deviation according to their estimated variances ( $Zq$  values), as previously described (48). Hierarchical clustering was computed across all individual replicates (averaged distance), and a 0.78 correlation cut-off was established for subsequent analysis. Functional protein analysis was performed using the system biology triangle (SBT) algorithm and Ingenuity Pathway Analysis (IPA, QIAGEN) (49). Upstream pathway analysis and network modeling of interested protein clusters were run using the IPA platform (QIAGEN), and network representation was layered out using Cytoscape 2.0. Analysis shown in Fig. 3C shows the relative proportion of proteins identified for each indicated functional annotation term (retrieved from DAVID resource 6.7, including all KEGG and GO terms), from either mitochondrial protein identified among LPS-LD downregulated proteins, or the MitoCarta 2.0 reference proteome. Mass spectrometry data have been deposited in Peptide Atlas ([ID: PASS01610](#)).

### **Statistical analysis**

All data shown in graphs are the mean + SD. Statistical significance was determined using paired *t*-test, one-way ANOVA multiple comparisons test, or two-sided students *z*-test on proportions, as specified in figure legends (not significant (ns), \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ ).

### **Figure preparation**

Figures were created using Microsoft PowerPoint (Microsoft 365 MSO). Images were edited with Adobe Photoshop CS3 software (Adobe Systems Inc.). GraphPad Prism 7 (GraphPad Software) was used to create graphs and calculate statistical significances.

## Supplementary Materials

Materials and Methods

Figs. S1 to S12

Tables S1 to S7

References (31-49)

## References and Notes

1. A. Pol, S. P. Gross, R. G. Parton, *J Cell Biol* **204**, 635 (Mar 03, 2014).
2. A. L. Vallochi, L. Teixeira, K. D. S. Oliveira, C. M. Maya-Monteiro, P. T. Bozza, *Front Immunol* **9**, 1022 (2018).
3. P. Roingeard, R. C. Melo, *Cell Microbiol* **19** (Jan, 2017).
4. M. Knight, J. Braverman, K. Asfaha, K. Gronert, S. Stanley, *PLoS Pathog* **14**, e1006874 (Jan, 2018).
5. R. C. Melo, A. M. Dvorak, *PLoS Pathog* **8**, e1002729 (2012).
6. E. R. Hinson, P. Cresswell, *Proc Natl Acad Sci U S A* **106**, 20452 (Dec 01, 2009).
7. L. Bougneres *et al.*, *Immunity* **31**, 232 (Aug 21, 2009).
8. P. Anand *et al.*, *Elife* **1**, e00003 (Nov 13, 2012).
9. U. Protzer, M. K. Maini, P. A. Knolle, *Nat Rev Immunol* **12**, 201 (Feb 24, 2012).
10. J. C. Kagan, *Trends Immunol* **38**, 696 (Oct, 2017).
11. A. Herms *et al.*, *Nat Commun* **6**, 7176 (May 27, 2015).
12. J. Behnsen, A. Perez-Lopez, S. P. Nuccio, M. Raffatellu, *Trends Immunol* **36**, 112 (Feb, 2015).
13. P. Navarro *et al.*, *J Proteome Res* **13**, 1234 (Mar 7, 2014).
14. N. Krahmer *et al.*, *Mol Cell Proteomics* **12**, 1115 (May, 2013).
15. K. Bersuker *et al.*, *Dev Cell* **44**, 97 (Jan 8, 2018).
16. E. L. Mills, B. Kelly, L. A. J. O'Neill, *Nat Immunol* **18**, 488 (Apr 18, 2017).
17. M. Bosch, R. G. Parton, A. Pol, *Semin Cell Dev Biol* (Mar 4, 2020).
18. H. A. Neufeld, J. A. Pace, F. E. White, *Metabolism* **25**, 877 (Aug, 1976).
19. A. Fabisiak, N. Murawska, J. Fichna, *Pharmacol Rep* **68**, 802 (Aug, 2016).
20. X. Shu, L. Nelbach, R. O. Ryan, T. M. Forte, *Biochim Biophys Acta* **1801**, 605 (May, 2010).
21. S. Turro *et al.*, *Traffic* **7**, 1254 (Sep, 2006).
22. C. L. Libbing, A. R. McDevitt, R. P. Azcueta, A. Ahila, M. Mulye, *Cells* **8** (Apr 15, 2019).
23. J. L. Cocchiari, Y. Kumar, E. R. Fischer, T. Hackstadt, R. H. Valdivia, *Proc Natl Acad Sci U S A* **105**, 9379 (Jul 8, 2008).
24. P. Peyron *et al.*, *PLoS Pathog* **4**, e1000204 (Nov, 2008).
25. K. A. Mattos *et al.*, *Cell Microbiol* **13**, 259 (Feb, 2011).
26. S. J. Nolan, J. D. Romano, I. Coppens, *PLoS Pathog* **13**, e1006362 (Jun, 2017).
27. J. S. Mader, N. Mookherjee, R. E. Hancock, R. C. Bleackley, *Mol Cancer Res* **7**, 689 (May, 2009).
28. S. N. Keenan *et al.*, *Diabetes* **68**, 543 (Mar, 2019).
29. D. J. Greenwood *et al.*, *Science* **364**, 1279 (Jun 28, 2019).

30. R. Dubey *et al.*, *Nat Chem Biol* **16**, 206 (Feb, 2020).
31. D. M. Moujalled *et al.*, *Cell Death Dis* **4**, e465 (Jan 17, 2013).
32. J. M. Murphy *et al.*, *Immunity* **39**, 443 (Sep 19, 2013).
33. M. Bosch *et al.*, *Am J Pathol* **186**, 517 (Mar, 2016).
34. A. Tutusaus *et al.*, *Cell Mol Gastroenterol Hepatol* **9**, 349 (2020).
35. R. Kapetanovic *et al.*, *FASEB J* **30**, 1901 (May, 2016).
36. A. Barrientos, *Methods* **26**, 307 (Apr, 2002).
37. M. Bosch *et al.*, *Curr Biol* **21**, 681 (Apr 26, 2011).
38. C. J. Stocks *et al.*, *J Leukoc Biol* (May 22, 2020).
39. S. Lauer, Y. A. Kunde, T. A. Apodaca, B. Goldstein, E. Hong-Geller, *Cell Immunol* **255**, 8 (2009).
40. A. Kassan *et al.*, *J Cell Biol* **203**, 985 (Dec 23, 2013).
41. A. Pol, D. Ortega, C. Enrich, *Biochem J* **323** ( Pt 2), 435 (Apr 15, 1997).
42. A. Herms *et al.*, *Curr Biol* **23**, 1489 (Aug 5, 2013).
43. J. R. Kremer, D. N. Mastronarde, J. R. McIntosh, *J Struct Biol* **116**, 71 (Jan-Feb, 1996).
44. J. Schindelin *et al.*, *Nat Methods* **9**, 676 (Jun 28, 2012).
45. C. A. Schneider, W. S. Rasband, K. W. Eliceiri, *Nat Methods* **9**, 671 (Jul, 2012).
46. I. Arganda-Carreras *et al.*, *Bioinformatics* **33**, 2424 (Aug 1, 2017).
47. S. Martinez-Bartolome *et al.*, *Mol Cell Proteomics* **7**, 1135 (Jun, 2008).
48. P. Navarro *et al.*, *J Proteome Res* **13**, 1234 (Mar 7, 2014).
49. F. Garcia-Marques *et al.*, *Mol Cell Proteomics* **15**, 1740 (May, 2016).

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## Figure Legends

**Fig. 1. Mammalian LDs display regulated protein-mediated antibacterial activity.**

(A and B) Hematoxylin-eosin-stained sections (A) and TEM images (B) of CTL- (top) or LPS-livers (bottom). Red arrows (insert in A) and asterisks (B) indicate LDs, and “n” shows

the selected hepatocyte nucleus. Images are representative of 5 (A) or 2 mice (B) per condition. Scale bars: 100  $\mu\text{m}$  (A); and 5  $\mu\text{m}$  (B). (C) Hepatic triacylglycerol levels (TG) in CTL- and LPS-mice (5 mice per condition). (D and E) Hepatic LD number (D) and mean LD area (E) measured in TEM images of CTL- or LPS-livers. For each condition, at least 13 random liver sections, obtained from two mice per condition, were quantified (see fig. S6). (F) CTL- and LPS-livers were fractionated in sucrose density gradients and LDs floated onto the top fraction (“LDs”), as assessed by anti-PLIN2 immunoblotting (see fig. S1A) (representative of 5 mice per condition). (G) *E. coli* were incubated for 16 hours in standard medium (gray) or medium supplemented with proteins from CTL- (black) or LPS-LDs (red bar). CFU measurements were normalized to the standard medium condition ( $n \geq 7$ ) (see fig. S1). (H) Unloaded (black) and OA-loaded HMDMs (red bars) were infected with *E. coli* or *Salm* for 4 hours. LD number per cell was quantified in TEM images. At least eight macrophages per group, obtained in three independent experiments, were analyzed. (I and J) Control (black) and OA-loaded HMDMs (red bars) were infected with *E. coli* (I) or *Salm* (J) and bacterial loads (CFU) determined 24 hours later ( $n = 5$ ). (K) Control (black) and OA-loaded HMDMs (red bars) were incubated with pHrodo *E. coli* and bacterial loads measured (fluorescence units) ( $n = 3$ ). Cyt D was used to inhibit phagocytosis. (L) Length of LD–bacteria contacts per cell was measured in TEM images of OA-loaded HMDMs infected with *E. coli* or *Salm* for 4 hours. At least 15 macrophages per group, obtained in three independent experiments, were analyzed (see fig. S2, A to D). (M to R) Control (M, O, and P) and OA-loaded HMDMs (N and R) were infected with *E. coli* for 4 hours and analyzed in TEM images. Representative images have been pseudocolored blue (ER), red (*E. coli* interior), green (periplasm), and yellow (vacuolar membrane) (see fig. S2, E and F) (representative of three independent experiments). Scale bars: 2  $\mu\text{m}$  (M and N) and 0.5  $\mu\text{m}$  (O and R). All graphs show means  $\pm$  SD; not significant (ns), \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$  in a paired *t* test (C to E, H to J, and L), and one-way ANOVA test (G and K).

## Fig. 2. Quantitative mass spectrometry analysis of LPS-LDs.

(A) The figure summarizes changes in the proteome of LPS-livers ( $n = 3$ ) and LPS-LDs ( $n = 5$ ) when compared to CTL-livers ( $n = 3$ ) or CTL-LDs ( $n = 4$ ), respectively. “Identified” (gray letters) indicates identified proteins and “Regulated” (black letters) proteins significantly modified by LPS. Among modified proteins, yellow and blue circles indicate up- and downregulated proteins, respectively (tables S1 to S5). (B) Functional annotation enrichment analysis of proteins increasing ( $|\Delta Zq| > 1.8$ ; yellow graphs) or decreasing ( $|\Delta Zq| < \text{minus} 1.8$ ;

blue graphs) on LPS-LDs when compared to CTL-LDs. Enrichment as compared to the mouse genome for each category is expressed as  $-\log(P\text{-value})$ . Analyses for CTL- and LPS-livers is shown in fig. S3A. (C) Pie charts summarizing LPS-induced changes in bona fide LD proteins. Protein details are in tables S6 and S7 and annotated interactions in fig. S3B. (D) Hierarchical clustering of  $Zq$  values across replicates identifies functionally coherent protein subsets similarly regulated by LPS (threshold for cluster analysis:  $r > 0.78$ ). The cluster nucleated around PLIN2 is included. Five additional clusters are detailed in fig. S4A. (E) Gene subnetwork from IPA analysis of all identified proteins upregulated in LPS-LDs.

### Fig. 3. Physical and functional uncoupling of LPS-LDs and mitochondria.

(A) Relative enrichment of selected proteins. Protein enrichment in LPS-LDs illustrated by a heatmap code (blue, depletion; yellow, enrichment). The  $\Delta Zq$ , UniProt ID, ranking (tables S3 to S5), and a representative immunoblot (representative of three mice per condition) are indicated. (B) Fed-, CTL-, and LPS-livers were fractionated in sucrose gradients and LD-mitochondria co-fractionation determined by immunoblotting of ATP5D (a subunit of ATP synthase) (representative of three mice per condition). (C) Functional categories of downregulated mitochondrial proteins cofractionating with LPS-LDs are compared with the whole mitochondrial proteome (MitoCarta 2.0). (D and E) The mitochondrial content of CTL- (black) and LPS-livers (red bars) was determined by citrate synthase activity (D) and DNA copy number of COI (E, relative to GAPDH) ( $n = 6$ ). (F) Percentage of LDs interacting with mitochondria in CTL- (black) and LPS-livers (red bars) was quantified in TEM images. At least 15 random sections, obtained from two mice per condition, were analyzed (see fig. S6). (G and H) Mitochondrial beta-oxidation (G) and formation of soluble intermediates (H, ketone bodies) of lipids stored in LDs were quantified for 16 hours in primary hepatocytes left untreated (black) or treated with LPS (red bars) (4 mice per condition). (I) Ketones in sera of CTL- (black) and LPS-mice (red bars) (4 mice per condition). (J) Fed-, CTL-, and LPS-livers were fractionated in density gradients and PLIN5 distribution analyzed by immunoblotting (representative of five mice per condition). (K) HuH7 cells were transfected with a tagged *PLIN5*, and labeled with anti-FLAG antibodies (PLIN5), anti-TOM20 antibodies (mitochondria), and LipidTox (LDs). Contours of a representative transfected and non-transfected cell are indicated. The insert (right panels) shows an additional transfected cell. The arrow indicates a mitochondrion completely enwrapping a LD (representative of three independent experiments). Scale bar: 20  $\mu\text{m}$ . (L) LPS sensitive HEK293-TLR4<sup>+</sup> cells transfected with a tagged *PLIN5* were loaded with OA (black) or with OA + LPS (red dots).

The length of LD–mitochondria contacts per cell was measured in confocal microscopy images (see example in fig. S7, D to F). **66 transfected cells and 470 non-transfected cells, obtained from three independent experiments, were analyzed.** (M and N) HEK293 cells were transfected with FLAG-tagged *PLIN3* or *PLIN5*, loaded with OA, and protein expression determined by immunoblotting (M). Cells were infected with *E. coli*, and bacterial loads quantified after 4 hours (N) (**n = 4**). (O and P) THP-1 cells were transduced with *PLIN5*-encoding or empty lentiviral vectors. *PLIN5* expression was confirmed by immunoblotting (O). Transduced cells were infected with *E. coli* and bacterial loads evaluated after 8 hours (P) (**n = 3**) (see fig. S7, G to J). All graphs show means  $\pm$  SD; not significant (ns), \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , in a paired *t* test (D, E, G to I, M and N), one-way ANOVA test (L), and two-sided Student's *z* test on proportions (F).

#### **Fig. 4. LDs accumulate and utilize innate immune proteins.**

(A to C) Relative enrichment of selected proteins. Protein enrichment in LPS-LDs was evaluated as in Fig. 3. Accumulation of transfected proteins on LDs was confirmed in HuH7 cells by immunofluorescence (B) and fractionation in density gradients (C) (see fig. S8; fig. S9; fig. S10; and fig. S12). Scale bar: 20  $\mu$ m. (D and E) HMDMs were transfected with a scrambled (Scr) or with a *CAMP* siRNA, and *CAMP* expression determined by qRT-PCR (D). Then, unloaded and OA-loaded HMDMs were infected with *E. coli* for 8 hours, and bacterial loads (CFU) were quantified (**n = 5**). (F) HEK293 cells were transfected with a tagged LD-*CAMP* (fig. S12) and loaded with OA. LD-*CAMP* was detected on LDs (LipidTox) with anti-FLAG and anti-*CAMP* antibodies. **The image is representative of three independent experiments.** Scale bar: 20  $\mu$ m. (G) HEK293 cells were transfected with LD-*CAMP* (red) or *PLIN3* (black bars), loaded with OA, and infected with the indicated bacteria for 4 hours. Bacterial loads were quantified and CFU values normalized to *PLIN3*-cells (**n  $\geq$  3**). (H to J) LD-*CAMP*-transfected HEK293 cells were incubated in control (black) or OA containing medium (red). Cellular LD-*CAMP* levels (H) and LD accumulation (I) were assessed by immunoblotting with anti-*CAMP* antibodies. These cells were then infected with *E. coli* for 4 hours and bacterial loads were quantified (J) (**n = 7**). All graphs show means  $\pm$  SD; not significant (ns), \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  in a paired *t* test.









