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Sepsis-induced lipid droplet accumulation enhances antibacterial innate immunity through mechanisms dependent on DGAT-1 and interferon-beta

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ABSTRACT

Lipid droplets (LDs) are lipid-rich organelles recognized as central players in lipid homeostasis, signaling, and inflammation. While their functions in inflammation are well-documented, the mechanisms of LDs in antibacterial immunity and infection resistance remain less understood. Our results show that *E. coli*-infection trigger immunometabolic reprogramming and LD accumulation in macrophages. Moreover, purified LDs from LPS-stimulated and *E. coli*-infected macrophages exhibited direct *E. coli* anti-bacterial activity. Pharmacological inhibition or genetic knockdown of DGAT1, a key enzyme in triglyceride synthesis, reduced LD formation, bacterial clearance, and pro-inflammatory responses (nitric oxide, PGE_2, CCL2, IL-6). Notably, DGAT1 inhibition impaired the expression of IFN-β and several interferon-stimulated genes (ISGs), including viperin, iNOS, cathelicidin and IGTP, in *E. coli*-infected macrophages. In a cecal-ligation and puncture model of sepsis in C57BL/6 mice, DGAT1 inhibition reduced sepsis-induced LD accumulation in peritoneal cells and decreased levels of IFN-β, CCL2, nitric oxide, and lipid mediators (PGE_2, LTB_4, and RvD1). Furthermore, DGAT1 inhibition accelerated sepsis-related mortality, coinciding with elevated bacterial loads in the peritoneum and bloodstream at 6- and 24-hour post-sepsis. Our results demonstrate that LDs are critical regulators of innate immunity infection resistance, contributing to both bacterial clearance and the coordination of a protective proinflammatory response during sepsis through mechanisms dependent on DGAT-1 and Type I IFN.

1. INTRODUCTION

Lipid droplets (LDs) are dynamic lipid-rich organelles that play essential intracellular roles, including regulating cell lipid homeostasis, intracellular signaling, and inflammatory processes [1,2]. LD accumulation is heavily influenced by the flux of cellular lipid metabolism, including both the uptake and synthesis of neutral lipids, as well as lipid-consuming processes like lipolysis and β-oxidation [3]. Accumulate evidence place LDs in leukocytes and other cells of innate immunity as major organelles in inflammation and critical regulators of different inflammatory diseases, acting as markers of leukocyte activation [4] and platforms for a broad-range synthesis of eicosanoids and cytokine signaling [4,5].

Numerous studies on pathogen-leukocyte interactions have demonstrated a strong link between LDs and bacterial infections [6–8]. Activation of Toll-Like Receptors (TLRs) plays a central role in inducing LD accumulation in leukocytes [9–12]. Additionally, bacterial virulence factors have been shown to drive LD formation during infections by pathogens such as *Salmonella enterica* Typhimurium, *Listeria monocytogenes*, and *Mycobacterium tuberculosis* [13–15]. Intracellular bacteria may exploit LDs as an energy source and as a mechanism to evade the immune system [6]. In pathogenic bacteria, LDs serve as platforms for prostaglandin E_2 (PGE_2) synthesis, which has been linked to increased IL-10 production and suppression of Th1 cytokines, promoting bacterial survival and/or proliferation [16–18].

The perception of LDs solely as pro-pathogenic factors in infectious and inflammatory contexts has recently been challenged [19–21]. Emerging evidence suggests that LDs play a pivotal role in interferon (IFN) signaling pathways and the effectiveness of early innate responses [22–24]. Recent data show that LDs are a central hub in innate immunity, responding to danger signals by reprogramming cellular metabolism and

serving as autonomous platforms for innate immune responses [25]. In this context, the presence of antibacterial peptide cathelicidin (CAMP) in LDs confers greater protection against bacterial infection in human macrophages [25]. These findings position LDs as key regulators of metabolic reprogramming and facilitators of antimicrobial mechanisms [25]. Furthermore, the involvement of LDs in the antibacterial response is evolutionarily conserved, being first reported in *Drosophila* [26].

Sepsis is a complex life-threatening syndrome caused by dysregulated inflammatory and metabolic host response to infection [27]. The balance between infection resistance and tissue tolerance may be central to understanding sepsis at the molecular level [28,29]. Alterations in lipid metabolism and increased LDs are observed during sepsis [12,30], however although LD functions in inflammation are well-documented, the role of LDs in antibacterial immunity and infection resistance and their roles in sepsis survival remain less understood. During sepsis, LDs are critical for amplifying the proinflammatory response but also contribute to the disruption of tissue tolerance mechanisms leading to increased organ dysfunction [30]. Notably, the pathways driving the breakdown of tissue tolerance may play a key role in infection resistance [28,29]. In this study, we investigated the formation of LDs and the triglyceride synthesis in antibacterial innate immunity during sepsis. Our results demonstrate that LDs are critical regulators of resistance to infection, contributing to both bacterial clearance and the coordination of a protective proinflammatory response during sepsis.

2. Materials and Methods

Reagents

Recombinant Murine interferon γ (IFNγ) was obtained from PeproTech. *E. coli* (K-12 strain) Texas Red™ conjugate Bioparticles (Cat# E2863), Lipopolysaccharides from *Escherichia coli* (serotype O111:B4, Cat# L4931), A922500 (Cat# A1737), PF-04620110 (Cat# PZ0207), CI976 (Cat# C3743), oleic acid (Cat# O1008), saponin from *Quillaja* bark (Cat# S7900), and Oil Red O (Cat# O0625) were obtained from Sigma-Aldrich/Merck (St. Louis, MO). The Luria-Bertani broth (Cat# K25-1551), and Tryptic Soy Agar (Cat# K25-610052) was obtained from Kasvi (São José do Pinhais, PR, Brazil). RPMI-1640 (cat# 22400-089), penicillin-streptomycin (Cat# 15140148), gentamicin (Cat# 15750060), and L-glutamine (Cat# 25030081) were obtained from Gibco (Grand Island, NY, USA). DAPI (Cat# D1306) and Fluoromount-G Mounting Medium (Cat# 00-4958-02) were purchased from ThermoFisher Scientific (Waltham, MA, USA).

Mice

Adult male C57BL/6J mice (25-30 g, 10-12 weeks old) were supplied by the Institute of Science and Technology in Biomodels from Oswaldo Cruz Foundation. Mice were maintained with standard rodent diet (AIN-93 M) and water available *ad libitum* with 12/12 h light/dark cycle (7:00 AM-7:00 PM) under controlled temperature (23 ± 1 °C) and humidity (60 ± 5%). The Institutional Animal Welfare Committee approved all animal experiments in agreement with the Brazilian National guidelines supported by CONCEA (Conselho Nacional de Controle em Experimentação Animal) under license number L025/15 and L005/20 (CEUA/FIOCRUZ).

127

128 **Macrophages cell culture**

129 To obtain bone marrow-derived macrophages (BMDM), bone-marrow cells
130 isolated from femur and tibia of mice were cultured for 7 days in RPMI-1640 medium
131 supplemented with 18% (vol/vol) L929-derived M-CSF conditioned medium, 20%
132 (vol/vol) heat-inactivated fetal bovine serum, 1% L-glutamine (vol/vol), and 1%
133 penicillin-streptomycin (vol/vol) as previously described by Assunção et al., (2017).
134 Differentiation was performed at 37 °C in a humidified 5% CO_2 incubator. After seven
135 days, adherent macrophages were harvested and seeded for assays. Differentiated
136 macrophages were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal
137 bovine serum (FBS, vol/vol), 1% L-glutamine (vol/vol), and 1% penicillin/streptomycin
138 (vol/vol). BMDM cells culture was performed at a density of $0.1x10^6$ – $1x10^6$ cells/ml,
139 and the cell culture were performed at 37°C in 5% CO_2.

140

141 **Bacterial strains and growth conditions**

142 *Escherichia coli strain Seattle 1946* (ATCC 25922) used in this study were
143 obtained from the Enterobacteria Collection (CENT) of the Oswaldo Cruz Foundation.
144 *E. coli* strain Seattle 1946 is a whole-genome sequenced quality control strain that does
145 not produce verotoxin and that does not present antibiotic resistance mechanisms. The
146 bacteria were cultured in Luria-Bertani broth (LB) for 16–18 h at 37°C to obtain
147 stationary growth phase cultures. Before infection, the bacteria were washed three times
148 with Phosphate-Buffered Saline (PBS), centrifuged (1,000 × g) for 10 min at 4°C. The
149 bacteria were resuspended in PBS and diluted in RPMI with FBS to an appropriate
150 multiplicity of infection (MOI) according to the optical density (OD) at 600 nm. *E.*

coli (K-12 strain) Texas Red™ conjugate Bioparticles were also washed three times with PBS, centrifuge (1,000 × g) for 10 min at 4°C, and resuspended in PBS.

E. coli infection of macrophages and treatments

An *in vitro* gentamicin protection assay was used to measure the phagocytosis and intracellular survival of *E. coli* based on the method described by Lissner et al. (1983), with modifications of Souza et al. (2021). The macrophages were seeded at 1 x 10^5 and 5 x 10^5 cells per well in 24- and 12-well places (flat-bottom, tissue-culture-treated plates; Costar), respectively, and were incubated for 12 h. Meanwhile, *E. coli* cells were cultured overnight at 37°C with agitation. Macrophages were infected with *E. coli* at a MOI of 100 for 1 h. Then, the culture medium was discarded, and the cells were washed with PBS with 100 μg/mL gentamicin three times. RPMI-1640 supplemented with 100 μg/mL gentamicin was added to each well to kill non-phagocyted bacteria, and incubation was continued for another 1 h. After incubation, media with gentamicin were removed, and fresh media was added for the remainder of the time. A similar protocol was used for *E. coli* bioparticles stimulation at MOI of 100. Alternatively, BMDMs were stimulated with LPS serotype 0111:B4 (500 ng/mL) plus murine IFNγ recombinant (10 ng/mL) for 24h. All experiments were done in triplicate.

To enhance LD accumulation, 40 μM oleic acid was added to the cell culture and incubated for 16 h prior to *E. coli* infection. To inhibit LD biogenesis, the DGAT1 inhibitors A922500 or PF-04620110, or the ACAT1 inhibitor CI976, were added 30 min before infection and maintained throughout the infection period. All inhibitors were dissolved in dimethyl sulfoxide (DMSO; Sigma).

For Colony-forming unit (CFU) analysis, BMDM cells were washed three times with PBS and lysed with 10% Triton X-100 solution at the indicated time points. The CFU of bacteria was counted by plating the appropriate dilution in TSA plates.

Lipid droplets staining and quantification

BMDM were fixed with 3.7% formaldehyde for 10 min, and LDs were stained with 0.3% Oil Red O' (diluted in isopropanol 60%) as previously described [33]. Preparations were analyzed with a 60× objective in FluoView FV10i Olympus confocal microscope (Tokyo, Japan). Images were acquired, colored, and merged using Olympus FV10-ASW and open-source ImageJ software (https://imagej.nih.gov/ij/). The Oil red O-stained LDs were measured using the open-source ImageJ software.

Measurement of Lactate

Lactate levels in cell-free culture supernatant were measured using an enzymatic lactate kit (Labtest, cat. # 138-1/50) performed as per manufacturer's instruction.

Nitric Oxide Assay

The nitric oxide (NO) levels in culture supernatants and peritoneal lavage supernatants were measured by the colorimetric Griess assay. Briefly, 25µL of the sample was mixed with an equal part of Griess reagent (1% sulfanilamide / 0,1% N-(1-naphtyl) -ethylenediamine dihydrochloride in 2.5% H_3PO_4) in a 96-well plate. The color development was assessed at 450 nm with a microplate reader (Spectramax, Molecular Devices, Inc., USA). As a standard curve, a solution of sodium nitrite ($NaNO_2$) was used at an initial concentration of 200µM, diluted in fresh culture medium.

199

200 **Quantitative PCR (qPCR)**

201 RNA was extracted with QIAamp Viral RNA (Qiagen®) from cells seeded (10^5
202 cells/well) in 24-well plates. Quantitative RT-PCR was performed using a dye-based
203 GoTaq 1-Step RT-qPCR System (Promega, Fitchburg, WI, USA) in a StepOne™ Real-
204 Time PCR System (ThermoFisher, Carlsbad, CA, USA). Amplifications were carried out
205 in 15 µL reaction mixtures containing 2× reaction mix buffers, 1x of probe-based oligos
206 from predesigned TaqMan Gene Expression Assays (ThermoFisher, Carlsbad, CA, USA),
207 and 5 µL of RNA template. The program for probe-based amplifications was 10 min at
208 95 ◦C followed by 50 cycles of 15 s at 95◦C and 1 min at 60 ◦C. The relative mRNA
209 expression was calculated by the 2^{-} ΔΔCt method. The *β-actin* (*actb*) expression was
210 used as a reference gene. The probe-based oligos were all Predesigned Taqman Gene
211 Expression Assays: *fasn* (ref: Mm00662322, g1, FAM), *dgat1* (ref: Mm00515643_m1,
212 FAM), *dgat2* (ref: Mm00499536_m1, FAM), *acat1* (ref: Mm00507463_m1, FAM), *plin2*
213 (ref: Mm00475794_m1, FAM), *plin3* (ref: Mm04208646_g1, FAM), *pnpla2* (ref:
214 Mm00503040_m1, FAM), *camp* (ref: Mm01241632_g1, FAM), *cd36* (ref:
215 Mm01135198_m1, FAM), *abca1* (Ref: Mm00442646_m1, FAM), *il-1β* (ref:
216 Mm00434228_m1, FAM), *il-10* (ref: Mm00439614_m1, FAM), *ptgs2* (ref:
217 Mm00478374_m1, FAM), *5-lo* (ref: Mm01182747_m1, FAM), *15-lo* (Cat#
218 Mm00507789_m1) *nox2* (ref: Mm00440502_m1, FAM), *arg1* (ref: Mm00475988_m1,
219 FAM), *chi3l3* (ref: Mm00657889_m1, FAM), *mr1* (ref: Mm00468487_m1, FAM), and
220 *actb* (ref: Mm02619580_g1, FAM).

221

222 **Immunofluorescence staining**

BMDMs were seeded on coverslips and fixed using 3.7% formaldehyde after 48 h of infection for 20 min at room temperature. Cells were rinsed three times with PBS containing 0.1 M $CaCl_2$ and 1 M $MgCl_2$ (PBS/CM) and then permeabilized with 0.1% Triton X-100 plus 0.2% BSA in PBS/CM for 10 min (PBS/CM/TB). Cox-2 was labeled by the rabbit polyclonal antibody anti-COX-2 (cat. sc-1745, Santa Cruz Biotechnology) at a 1:500 dilution overnight, followed by a mouse anti-IgG-DyLight 550 at a 1:1,000 dilution for 1 h with 0.2 μg/ml BODIPY493/503 dye for 5 min for LD staining. Slides were mounted using antifade mounting medium (VECTASHIELD). Nuclear recognition was based on DAPI staining (1 μg/ml) for 5 min. Fluorescence microscopy was analyzed with a 100x objective lens (Olympus).

Flow cytometry

Macrophages ($5x10^5$) were incubated with the appropriate amount of purified rabbit polyclonal antibody anti-GLUT1 (Cat# 21829-1-AP, Proteintech, USA), Alexa Fluor® 488 rat anti-mouse CD206 (MMR) Antibody (Cat# 141710, Biolegend, USA), APC mouse anti-mouse CD36 (Cat# 562744, BD Biosciences, USA), APC hamster anti-mouse CD80 (Cat#560016), and/or PE rat anti-mouse F4/80 Antibody (Cat#123109, Biolegend, USA) for 30 min at 4°C, after incubation with 10% of FBS in PBS/0.1% Sodium Azide to block non-specific binding. For GLUT1 staining, Alexa546 goat anti-rabbit IgG was added to all wells and incubated for 30 min. As negative control cells receive only secondary antibodies. At least 10^4 cells were acquired per sample in FACSCalibur flow cytometer and CellQuest™ software (Becton Dickinson, San Jose, CA, USA). All data were displayed on a log scale of increasing fluorescence intensity and presented as histograms. Analysis was performed by using FlowJo™ Software (Becton Dickinson, San Jose, CA, USA).

248

249 **Sepsis Induction and Treatment**

250 Sepsis was induced by Cecal Ligation and Puncture (CLP), according to Reis et
251 al. (2017), with modifications of Teixeira et al. (2023). Briefly, C57BL/6 mice were
252 anesthetized with intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10
253 mg/kg) and a 1 cm incision was made on the abdomen. The cecum was exposed and
254 linked below the ileocecal junction. Four perforations were created using a 22-gauge
255 needle (severe sepsis model), allowing a small amount of fecal material to be expelled
256 into the peritoneal cavity, and the cecum was gently relocated. The area was sutured with
257 nylon 3-0 (Shalon) in two layers. Sham-operated animals (control) with identical
258 laparotomy but without ligation and punctures.

259 After 6h and 24h post-surgery, Sham and CLP mice were orally treated with
260 A922500 at a dose of 3 mg/kg or vehicle. All mice received 500 µL of sterile saline with
261 meropenem (10 mg/kg; Merck) subcutaneously as fluid resuscitation and antibiotic
262 therapy at 6h and 24h hours after surgery. Animals were monitored for 48h for survival
263 and clinical score analysis. The clinical score was determined by the observation of
264 following signs: piloerection, curved trunk, alterations in gait, seizures, limb paralysis,
265 coma, respiratory rate, skin color alterations, heart rate, lacrimation, palpebral closure,
266 decreased grip strength, limb, abdominal and body tone and body temperature alterations.
267 The clinical evaluation was based on a multifactorial SHIRPA protocol, with the
268 modifications of Reis et al. (2017). Blood glucose levels were measured using a glucose
269 meter (Gtech) at 6h and 24 post-surgeries. A small incision was made at the tip of the
270 mouse's tail, and a drop of blood was placed on the test strip inserted into the device.

271 Six or twenty-four hours after sepsis induction, the animals were euthanized,
272 blood was collected via cardiac puncture, and the peritoneal cavity was opened and

washed with 3 ml of sterile saline. The peritoneal lavage was collected for total and differential cell count, CFU evaluation, LDs, cytokines, chemokines, lipid mediators, and NO quantification. For CFU evaluation, peritoneal lavage and serum from each animal was diluted and plated on tryptic soy agar (TSA) plates. After 24 hours of incubation at 37°C, the bacterial colonies were enumerated manually. Myeloperoxidase activity was measured spectrophotometrically, following the method of by Bradley et al., (1982) with minor modifications. Briefly, liver tissue samples were homogenized in 0.5% hexadecyltrimethylammonium bromide (HTAB; Sigma Chemical Co., St. Louis, MO) prepared in 50 mmol/L potassium phosphate buffer (pH 6.0), using 100 µL of buffer per 50 mg of tissue. The homogenates were centrifuged at 40,000 × *g* for 30 minutes at 4°C. Supernatants were collected for MPO activity analysis. In 96-well plates, 50 µL of the supernatant was mixed with 50 µL of HTAB buffer and 50 µL of orthodianisidine dihydrochloride (0.167 mg/mL; Sigma), followed by incubation at 37°C for 30 minutes. Subsequently, 50 µL of hydrogen peroxide (0.0005%, Sigma) was added to initiate the reaction. After 10 minutes, absorbance was measured at 460 nm using a spectrophotometer. MPO activity was normalized to tissue weight (mg).

In each experiment, 7 to 15 cages, each containing 5 mice, were used. After weighing, cages were randomly assigned to experimental groups. Within each cage, all mice were numbered from 1 to 5 using tail markings. Each experiment included 5 mice from the sham group, 10–15 from the CLP group, and 10–15 from the CLP+iDGAT1 group. The experimental n was determined based on the 50% mortality rate associated with severe sepsis, as previously documented in the literature[36]. All surviving mice from each experiment were included in all subsequent analyses. A total of 5 independent experiments were conducted, involving 175 animals, including those used for bone marrow collection for macrophage differentiation.

298

299 **Leukocyte Count and Lipid Droplet Staining**

300 Peritoneal lavage samples were diluted in Turk's solution (2% acetic acid), and the
301 total cell counts were performed with optical microscopy in the Neubauer
302 Hemocytometry chamber. For differential cell count and LD Staining, the samples were
303 cytocentrifuged in a microscope slide and fixed with 3.7% formaldehyde for 10 min. Cells
304 were washed three times in PBS, slides were stained with 0.3% Oil Red O' (diluted in
305 isopropanol 60%) for 2 minutes at room temperature. Cells were washed once in 30%
306 isopropanol and three times in distilled water. Slides were rinsed and counterstained with
307 Mayer's hematoxylin for 3 minutes. After incubation, the slides were washed three times
308 in distilled water. Mounting solutions and coverslips were added. LDs were enumerated
309 by optical microscopy in 50 consecutive leukocytes.

310

311 **Measurements of Cytokines and Chemokines**

312 CXCL1 / KC, CCL2 / MCP-1, IL-1β, IL-6, IL-10, IL-12p40, TNF-α, IFN-β and
313 IFN-γ in cell-free culture supernatants and peritoneal lavage were measured using mouse
314 Duoset ELISA kit (R&D Systems, USA) according to manufacturer's instructions.
315 Cathelicidin-related antimicrobial peptide (CAMP) in peritoneal lavage was measured
316 using mouse Sandwich ELISA kit (Cat# CSB-E15061m, CUSABIO, USA). The level of
317 IFN-alpha in cell-free culture supernatants and peritoneal lavage was measured using
318 mouse IFN-alpha All Subtype ELISA Kit (Catalog #MFNAS0, R&D Systems, USA)

319

320 **Measurements of Lipid mediators of inflammation**

321 According to the manufacturer's instructions, levels of Prostaglandin E2
322 (PGE_2), Leukotriene B4 (LTB_4) and Resolvin D1 (RvD1) in macrophages supernatant
323 and peritoneal lavage were measured using EIA kits (Cayman Chemical, USA).
324
325 **Lipid droplets purification and Bacterial Plate Assay**
326 BMDMs were seeded at a density of 25×10^7 cells/ flask (175 cm^2). The next day,
327 cells were stimulated with LPS serotype 0111: B4 (500 ng/mL) plus murine IFN-γ
328 recombinant protein (10 ng/mL), oleic acid (40 μM) or *E. coli* (MOI 100) for 24 h, at
329 37°C in 5% CO_2. LDs were isolated from 4 culture flasks for each condition as previously
330 described by Samsa *et al*, 2009 [37]. Cells were gently scraped into the media and
331 centrifuged at 200g for 15 minutes at 4°C using a swing-out bucket rotor.. The cell pellet
332 was washed twice in PBS and then resuspended in 2.5 mL of lysis buffer (20 mM Tris, 1
333 mM EDTA, 1 mM EGTA, 100 mM KCl buffer (pH 7.4), followed by incubation on ice
334 for 5 min. Using a Cell Disruption Vessel, we lysed cells by applying 750 psi of nitrogen
335 gas for 5 minutes in an ice bath. The cell lysate was collected in 15 mL conical tubes. The
336 total cell lysate was centrifuged at 900*g* for 15 min at 4°C. The resulting post nuclear
337 supernatant was collected and applied to a sucrose gradient (1.08 M, 0.27 M, and 0.135
338 M) and subjected to ultracentrifugation (150,000 x g for 70 min at 4°C). After
339 ultracentrifugation, the first fraction was rich in isolated LDs. TEE-KCl buffer was added
340 to the first fraction, and the LDs were isolated a second time by centrifugation at 14,000
341 × g for 15 min. As a control for proper cell fractionation, the activity of the cytoplasmic
342 enzyme lactate dehydrogenase (LDH; Promega, G1780) was assayed.
343 Colony formation assays were conducted to assess the antibacterial activity of
344 LDs as described by Bosch *et al* (2020). Exponentially growing *E. coli* cultures in LB
345 broth at 37 °C were harvested at an OD_{600} of 1. The cells were then centrifuged, washed,

and resuspended in 10 mM Tris-HCl buffer (pH 7.4) supplemented with 1% (v/v) LB medium. Aliquots of 100 µL of this bacterial suspension were incubated with 100 µL of intact LDs prepared using a sucrose gradient. Following incubation, the samples underwent 6–8 serial 1:10 dilutions in 10 mM Tris-HCl (pH 7.4) and were plated in triplicate on LB agar plates. CFU were quantified after 16 hours of incubation at 37 °C.

DGAT1 Knocking down assays.

BMDMs were plated (2,5 x 10^5 cells/well) in 24-well culture plates and incubated for 12 h at 37 °C and 5% CO_2. Cells were transfected with 100 pmol/µL siRNA targeting DGAT1 (Cat# s64951, ThermoFish Scientific) or scramble RNA (Cat# 4390844, ThermoFish Scientific) in Opti-MEM (Gibco, USA), using Lipofectamine RNAiMax (ThermoFish Scientific). Alternatively, macrophages were also transfected with 5µM self-deliverable AUM*silence* oligos for DGAT1 (Cat# AUM-SIL-A-100-Dgat1-1) or scramble RNA (AUM-SCR-A-100) according to the manufacturers' instructions (AUM BioTech, LLC, USA). After 48 hours of recovery, BMDMs were then infected with *E. coli*. The efficiency of *knocking down assays* was measured 48-72 h post-transfection through quantitative RT-PCR performed using dye-based GoTaq 1-Step RT-qPCR System.

Western blotting

BMDM cells were harvested using ice-cold lysis buffer pH 8.0 (1% Triton X-100, 2% SDS, 150 mM NaCl, 10 mM HEPES, 2 mM EDTA containing protease inhibitor cocktail - Roche). Cell lysates were heated at 100°C for 5 min in the presence of Laemmli buffer pH 6.8 (20% β-mercaptoethanol; 370 mM Tris base; 160 µM bromophenol blue; 6% glycerol; 16% SDS). Twenty µg of protein/sample were resolved by electrophoresis

on SDS-containing 10% polyacrylamide gel (SDS-PAGE). After electrophoresis, the separated proteins were transferred to nitrocellulose membranes and incubated in blocking buffer (5% nonfat milk, 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). Membranes were incubated overnight with a 1:1000 dilution of the following primary antibodies: anti-Cathelicidin (#ab180760, Abcam), anti-IRGM3 (#14979S, Cell Signaling), anti-Viperin (#13996S, Cell Signaling), anti-iNOS (#ab15323, Abcam), anti-PLIN-2 (#15294-1-AP, ProteinTech), anti-β-tubulin (66240-1-Ig, ProteinTech), and anti-β-actin (#66009-1-Ig, ProteinTech). After the washing steps, they were incubated with IRDye—LICOR or HRP-conjugated secondary antibodies. All antibodies were diluted in blocking buffer. The detections were performed by Supersignal Chemiluminescence (GE Healthcare) or by fluorescence imaging using the Odyssey system. Densitometric analyses were performed using Image Studio Lite version 5.2. Protein expression levels were first normalized to β-actin or β-tub expression and then further normalized to the uninfected group.

Statistical analysis

Data obtained in this study were presented as mean ± SEM of three to six independent experiments. The unpaired two-tailed t-test was used to evaluate the significance of the two groups. Multiple comparisons among three or more groups were performed by one-way ANOVA followed by Tukey's multiple comparison test. The significance of the survival curves was evaluated using the log-rank (Mantel−Cox) test. For all analyses, a p-value of ≤ 0.05 was considered statistically significant. The statistical analysis was conducted using GraphPad Prism v.8 software (GraphPad, La Jolla, CA, USA).

3. RESULTS

Escherichia coli induced LD accumulation is part of pro-inflammatory reprogramming of murine macrophages.

LD accumulation in leukocytes is a well-documented phenomenon both in experimental models of sepsis as well as in septic patients [12,38]. However, the role of LD during infections caused by extracellular bacteria has not been fully clarified. Our first step was to investigate the participation of LD in the antibacterial response of macrophages. For it, we infected primary macrophages with *Escherichia coli*, a classical extracellular bacterium. The *E coli* infection led to a significant LD accumulation in macrophages at 24 hours post-infection (hpi) **(Figure 1A-B and S1A)**. To deepen this finding, we evaluated the expression of the main genes involved in lipid metabolism and LD formation in BMDMs at 12 hpi. Differently from the classical LPS + IFN-γ model **(Figure S1B)**, the *E. coli* infection induced a discrete change in gene expression of lipid metabolism-associated genes at 12 hpi **(Figure 1C)**. The *E. coli* infection led to a slight increase of lipid metabolism-associated genes *plin2*, *dgat1* and *cd36*, and a decrease in the expression of lipolytic gene *pnpla2/atgl* **(Figure 1C)**. In comparison, stimulation with LPS + IFN-γ led to a more expressive remodeling of the expression of all genes involved in lipid metabolism **(Figure S1B)**. These findings indicate that *E. coli* infection triggers a lipid metabolic profile distinct from that induced by the classical LPS + IFN-γ.

Our next step was to evaluate whether the accumulation of neutral lipids could be associated with *E. coli* intracellular viability. Unlike pathogenic bacteria [14,39], *E. coli* viability decreased sharply between 1 hour and 24 hours **(Figure 1D)**, suggesting that LD accumulation does not contribute to the intracellular survival of bacterium. We investigated the potential association between LD accumulation and a pro-inflammatory

response during *E. coli* infection. First, we evaluated the expression of the key enzymes involved in lipid mediator synthesis (*cox-2*, *5-lo*, and *15-lo*). The *E. coli* infection increases the expression of *cox-2* and decreases the expression of *5-lo* and *15-lo* **(Figure 1E).** In addition, COX-2 expression was elevated and found near LDs **(Figure 1F)**. Reinforcing this finding, the infection led to an increase in the synthesis of PGE_2, but not LTB_4 or RvD1 **(Figure 1G)**. Moreover, *E. coli* infection induces classical glycolytic shift **(Figure S1C and 1H)** and pro-inflammatory reprogramming in macrophages. This was evidenced by elevated nitric oxide production **(Figure 1I),** increased expression of *inos/nox2* and *il-1b* mRNA **(Figure 1J)**, and higher levels of pro-inflammatory chemokines (CCL2 and CXCL1) and cytokines (IL-1β, IL-6, IL-12p40, and TNF) in the supernatant **(Figure 1K)**. There was no increase in the gene expression of M2 biomarkers (*il-10*, *arg-1*, *ch3l3*) **(Figure 1J)**, nor in the production of IL-10 **(Figure 1K)**. In host-pathogen interaction, several works have reported that LDs are essential for enhancing the synthesis of IFN in infected cells, playing a critical role in the early innate response to viral infection[40]. Our next step was to evaluate whether *E. coli* infection could trigger IFN production in macrophages. Interestingly, the *E. coli* infection induces the release of IFN-β but not IFN-α or IFN-γ by macrophages **(Figure 1L)**, suggesting a selective IFN response within this extracellular bacterial context.

LDs exhibit anti-bacterial functions in murine macrophages.

Our previous data in human macrophages indicated that the interaction between LDs and *E. coli* was associated with a decrease in bacterial viability [25]. In murine macrophages, LDs interact with *E. coli* **(Figure 2A)**, but these interactions are relatively infrequent, occurring in only about 19% of cases. Bacterial interactions with LDs can promote bacterial survival or inhibit their viability depending on the bacteria and the host

cell and stimulatory conditions [6]. Using bioparticles of *E. coli*, we observed that this interaction occurred independently of bacterial viability **(Figure 2B)**. As a proof-of-principle of the protective role of LDs for bacterial infection, we purified LDs (**Figure 2C)** from LPS+IFN-stimulated macrophages, and we conducted a BPKA assay as reported by Bosh *et al*. (2020). LDs from LPS+IFN-γ-stimulated macrophages demonstrated enhanced bactericidal activity than the control (**Figure 2D-G**). We also investigated whether LDs from *E. coli*-infected BMDMs exhibit bactericidal activity. As shown in **Figure 2H-J,** LDs from *E. coli*-infected BMDMs displayed enhanced bactericidal activity, comparable to that induced by LPS+IFN and greater than the control. Notably, LDs from OA-stimulated BMDMs did not exhibit bactericidal activity. (**Figure 2H-I**).

Our next step was to investigate the contribution of these findings to control bacterial loads into the macrophages. Our first experimental strategy was to stimulate LD biogenesis by pretreating macrophages with 40 μM oleic acid (OA) for 16 h before bacterial infection. Pretreatment with OA promoted LD biogenesis, increasing LDs size and numbers at 1 hpi and 24 hpi **(Figure 2K-L).** OA reduced the number of viable intracellular bacteria at 24 hpi **(Figure 2M and Figure S2A).** However, the reduction in bacterial numbers at 1 hpi following OA pretreatment suggests it may also be linked to decreased bacterial uptake, indicating that OA may modulate bacterial load through an LD-independent pathway.

Our next strategy was to impair LD accumulation by inhibiting the main pathway associated with neutral lipid synthesis **(Figure 2N)**. Treatment with a DGAT1 inhibitor (iDGAT1) significantly reduced *E. coli*-induced LD accumulation **(Figure 2O)**, while the ACAT1 inhibitor showed minimal effects on LD biogenesis. We then examined whether DGAT1 or ACAT1 inhibition could alter macrophage intracellular bacterial loads.

DGAT1 inhibition increased intracellular bacterial counts to 24 hpi **(Figure 2P)**, a phenomenon not associated with altered bacterial uptake **(Figure S2B)**. In contrast, ACAT1 inhibition did not affect macrophage bactericidal activity. We further evaluated whether iDGAT1 could influence *E. coli* proliferation. The iDGAT1 treatment showed no effect on *E. coli* growth in LB medium **(Figure S2C).** Taken together, these results suggest that changes in the lipid metabolism of macrophages affect the killing capacity of macrophages, with LDs exerting anti-bacterial functions.

Inhibition of LD accumulation reduced the inflammatory response of macrophages and impaired the IFN production and function.

Next, we evaluated whether the inhibition of LD accumulation by A922500 treatment could influence the pro-inflammatory response induced by *E. coli* infection in macrophages. Inhibiting LD accumulation was associated with a significant decrease in the inflammatory lipid mediator PGE_2 but not LTB_4 in this model **(Figure 3A)**. Additionally, DGAT1 inhibition reduced the levels of lactate production **(Figure 3B)**. These results prompted us to analyze the activation of macrophages treated with iDGAT1. We found that DGAT1 inhibition did not affect the expression levels of F4/80 **(Figure S3A)**, CD80 **(Figure S3B)**, and GLUT1 **(Figure S3C)**. Likewise, the expression of M2-marker CD206 remained unchanged when compared to *E. coli* infection in untreated group **(Figure S3D)**. Interestingly, *E. coli* infection-induced CD36 expression was enhanced by iDGAT1 treatment **(Figure S3E)**, suggesting a potential compensatory mechanism.

Next, we evaluated the impact of iDGAT1 treatment on inflammatory cytokines and chemokines production. The inhibition of LD accumulation reduced the production of CCL2, IL-1β and IL-6 induced by *E. coli* infection **(Figure 3C),** but not the lytic cell

death induced by bacterial infection measured by LDH activity (**Figure 3D)**. However, no alteration was observed in *E. coli* -induced CXCL1, IL-10, IL-12p40, TNF or IFN-γ in the iDGAT1-treated group **(Figure S3F)**. These results suggest that DGAT1 pathway plays a selective role in modulating inflammatory mediators without fully deactivating the macrophage response.

Furthermore, preventing LD accumulation with iDGAT1 inhibits *E. coli*-induced IFN-β **(Figure 3E)**. In addition, we investigated whether iDGAT1 treatment affects the expression of interferon-stimulated genes (ISGs). iDGAT1 reduced *E. coli*-induced expression of *camp* and *nox2* at the mRNA level, but not of Plin2 **(Figure 3F)**. To confirm these findings at the protein level, we assessed the expression of CAMP and iNOS. Inhibition of DGAT1 in macrophages decreased CAMP and iNOS protein levels **(Figure 3G-H)** and impaired nitric oxide production, consistent with the reduced iNOS expression **(Figure 3I)**. Moreover, treatment with iDGAT1 partially reduced Plin2 expression in *E. coli*-infected macrophages **(Figure 3G-H)**. We also evaluated the expression of Viperin, and IRGM3/IGTP, which have previously been linked to LDs presence in host cells [25,41,42]. As shown in **Figure 3J-K,** treatment with the DGAT1 inhibitor reduced the expression of Viperin and IRGM3 in *E. coli*-infected macrophages.

To confirm these findings, we tested whether another DGAT1 inhibitor could replicate the effects observed with A922500 during *E. coli* infection. For this purpose, we used PF-04620110, a structural unrelated high selective DGAT1 inhibitor [43]. We found that DGAT1 inhibition by PF-04620110 also reduced LD accumulation **(Fig. S4A-C)**, which was followed by decreased PGE_2 synthesis **(Fig. S4D)**. Furthermore, DGAT1 inhibition with PF-04620110 diminished the levels of IFN-β, IL-1β, IL-6, CCL2 and TNF **(Fig. S4E-I)**. Moreover, the inhibition of DGAT1 also reduced lactate and nitric oxide (NO) production **(Fig. S4J-K)**. Notably, DGAT1 blockade led to an increased bacterial

load at 24 hours post-infection (hpi) **(Fig. S4L)**. Together, these findings suggest a significant role of DGAT1 and LD accumulation in the host signaling response critical for resistance to bacterial infection.

The loss of DGAT1 expression decreases LD accumulation and inflammatory function in bacterial infection.

To further confirm the role of DGAT1 in LD accumulation and inflammatory response during *E. coli* infection, we knocked down *dgat1* expression in murine macrophages. Initially, we tested two methods. The first approach utilized siRNA with lipofectamine, which reduced *dgat1* expression by approximately 50% **(Figure S5A)**. The second approach employed self-delivery siRNA technology, which achieved around 75% knockdown efficiency in primary macrophages **(Figure 4A and Figure S5B-C)**. Due to its higher effectiveness, we proceeded with the self-delivery siRNA method. Knockdown of *dgat1* reduced *E. coli*-induced LD accumulation **(Figure 4B-C)** and increased bacterial persistence but did not affect bacterial uptake **(Figure 4D and S5D)**. In alignment with the pharmacological inhibition findings, reducing DGAT1 expression decreased the production of PGE_2 **(Figure 4E),** and the levels of IL-6, CCL2, and IFN-β **(Figure 4F-H)**. We also evaluated the expression of *nox-2* and *camp*, critical genes involved in control of bacterial infection. We found that DGAT1 knockdown corresponded with reduced *camp and nox2* at the mRNA level **(Figure 4I-J)** and decreased NO production **(Figure 4K)** at 24hpi. Furthermore, DGAT1 knockdown by siRNA also attenuated the *E. coli*-induced upregulation of *Ptgs2* expression at the mRNA level **(Figure 4L)**.

Mammals possess two diacylglycerol acyltransferase isoforms (DGAT1 and DGAT2) that catalyze the final and committed step in triglyceride synthesis [44]. While

previous studies demonstrated that TLR agonists can induce DGAT2 expression in macrophages[45], we sought to evaluate this effect in our experimental system. Both *E. coli* infection and LPS+IFN-γ treatment significantly upregulated *dgat2* expression by 12 hours post-infection (hpi) **(Figure 4M).** To determine whether DGAT1 siRNA knockdown might exert off-target effects or trigger compensatory DGAT2 upregulation, we assessed *dgat2* expression following DGAT1 silencing. Notably, DGAT1 siRNA treatment did not alter *dgat2* expression in either uninfected or *E. coli*-infected cells **(Figure 4M)**. Unlike pharmacological DGAT1 inhibition, DGAT1 knockdown did not impact lactate production **(Figure 4O)** or LDH release **(Figure 4P)**. Collectively, these results confirm the central role of DGAT1 in LD accumulation and in regulating bacterial activity in murine macrophages.

DGAT1 inhibition prevents sepsis-induced LD accumulation and downregulates the protective inflammatory response.

We next investigated whether the DGAT1 inhibitor A922500 could prevent sepsis-induced LD accumulation in peritoneal leukocytes during the early stages of sepsis **(Figure 5A)**. In 6h and 24h post-sepsis, the DGAT1 inhibition reduced sepsis-induced LD accumulation in peritoneal leukocytes **(Figure 5B-C).** During sepsis, LDs have been shown to serve as a platform for the synthesis of lipid inflammatory mediators in leukocytes [46]. The inhibition of LD accumulation significantly reduced the production of lipid inflammatory mediators, including PGE_2, LTB_4 and RvD1 **(Figure 5D)**. The prevention of LD accumulation also impaired sepsis-induced IL-6 and CCL2 in 6h and 24h post-sepsis, respectively **(Figure 5E-F)**. No significant differences were observed between untreated and treated sepsis groups in IL-1β, IL-12p40, IL-10, CXCL1 and TNF production **(Figure S6A-E)**. Due to reduced CCL2 levels, we also investigated whether

inhibiting DGAT1 could impair the activation of peritoneal macrophages. However, our results showed that the treatment did not reduce the pro-inflammatory activation macrophages marker (F4/80) **(Figure S6F)**. There was an increase in CD80 and GLUT1 expressions in cells derived from the CLP-iDGAT1 treated group, but no changes in CD206 expressions were observed between the groups **(Figure S6F-I).**

Our previous work showed that LPS-induced LDs in the liver are associated with upregulation of several interferon-stimulated genes (ISGs), including Viperin and CAMP [25]. Here we show that Viperin, HMGB1 and CAMP are upregulated in the hepatic sepsis-induced LDs **(Figure S7A-B)**, suggesting elevated systemic interferon levels during sepsis. In this context, we investigated whether sepsis induces ISG upregulation in peritoneal macrophages. We observed sepsis-induced expression of IRGM, followed by increased Plin2 expression in these cells. **(Figure 5G)**. We also investigated the interferon production in the peritoneal lavage induced by sepsis. The polymicrobial sepsis did not affect the production of IFN-α **(Figure 5H)** but increased the production of IFN-β at 24h **(Figure 5I)**, accompanied by a modest and transient elevation in IFN-γ levels at 6h post-sepsis **(Figure 5J)**. The downregulation of LD accumulation induced by the IDGAT1 inhibition during sepsis was followed by a significant decrease in IFN-β levels 24h post-sepsis **(Figure 5I)**. We then evaluated whether DGAT1 inhibition could impair the antibacterial mechanisms during infection. We found that preventing LD accumulation reduced sepsis-induced increase of NO **(Figure 5K)** and impaired secretion of antibacterial peptide CAMP **(Figure 5L)**. Altogether, these data support the role of DGAT and lipid accumulation in leukocyte inflammatory response during sepsis.

The inhibition of DGAT1 led to the loss of control of the bacterial load in sepsis

The impairment of resistance mechanism compromises the host's ability to control infections [28,47]. Furthermore, the increased bacterial burden in the peritoneal cavity is directly linked to the impaired bactericidal capacity of peritoneal leukocytes[48]. In this context, we assessed the bacterial load in both the peritoneal cavity and the bloodstream during sepsis. Bacterial load was significantly higher in the peritoneum of septic mice treated with iDGAT1 compared to untreated mice at both 6h and 24h post-sepsis **(Figure 6A)**. To determine whether the loss of bacterial control was associated with impaired leukocyte recruitment, we also evaluated the cell number in the peritoneal cavity. However, no significant differences were observed in the total or differential leukocyte counts between untreated and treated sepsis groups at either 6 hours **(Figure 6B)** or 24 hours post-sepsis **(Figure 6C)**. Moreover, the bacterial load was also significantly elevated in the serum of iDGAT1-treated mice at both times analyzed **(Figure 6D)**, indicating increased bacterial dissemination and a loss of control over the infection. During systemic infection, the liver plays a critical role in clearing bacteria from the bloodstream, thereby preventing their dissemination throughout the body [49]. To investigate whether DGAT1 inhibition affects hepatic leukocyte recruitment, we measured myeloperoxidase activity. iDGAT1-treated mice exhibited significantly reduced hepatic myeloperoxidase activity both at 6h **(Figure 6E)** and 24 h **(Figure 6F)**, indicating decreased leukocyte infiltration. This impaired recruitment may contribute to the observed increase in bacteremia. Collectively, our findings demonstrate that LD accumulation enhances both the antibacterial capacity of macrophages and their inflammatory response during sepsis.

We further assessed whether DGAT1 inhibition could modulate systemic metabolism, which might ultimately influence LD biogenesis in leukocytes. iDGAT1 treatment did not affect the acute hyperglycemia associated with ketamine/xylazine

620 anesthesia in mice [50,51] at 6h post-surgery **(Figure S6C)**. However, iDGAT1 promoted
621 recovery from sepsis-induced hypoglycemia at 24 h post-surgery **(Figure S6D)**. Notably,
622 DGAT1 inhibition had no effect on sepsis-induced hypertriglyceridemia at either point
623 analyzed (Figure S6E-F). We next investigated whether pretreatment with the DGAT1
624 inhibitor in combination with the antibiotic meropenem would affect clinical outcomes in
625 severe sepsis models. iDGAT1 pretreatment worsened clinical scores at 6 hours post-
626 sepsis, with scores remaining elevated throughout all the observation period **(Figure 6F).**
627 Furthermore, DGAT1 inhibition, even in the presence of meropenem, accelerated
628 mortality in severe sepsis, shifting the mortality peak to the first 24 hours **(Figure 6G)**.
629 These findings suggest that triglyceride synthesis plays an essential protective role in
630 sepsis survival.

4. DISCUSSION

LDs are evolutionary conserved organelles with major functions in metabolism and immune response [1-4]. In leukocytes and other cells of innate immunity LDs are signaling induced organelles acting as platforms for amplified synthesis of eicosanoids and cytokine signaling [4,5]. LDs have been reported in infectious disease across all pathogen classes, from viruses [52,53] and protozoa [54,55], to bacteria [11,16] and fungi [56]. Host LDs are often exploited by specialized pathogens to evade the immune system and/or serve as an energy source for their survival and/or replication [19–21]. However, recent findings highlight a drastic transformation in the participation of LDs within infectious and inflammatory contexts. Emerging research suggests that LDs support host defense [19–21,57], acting as regulators of immunometabolism [25] and essential platforms for producing protective eicosanoids, cytokines and antibacterial peptides [46,57]. Additionally, neutral lipid synthesis and LD accumulation significantly enhance the pro-inflammatory profile of macrophages [58,59]

LD biogenesis is strongly associated with PAMP recognition by TLRs in leukocytes [9,12], with different TLRs being engaged depending on the specific pathogen [9,11]. Similar to the classic LPS model, *E. coli* infection induces LD accumulation in macrophages, which is associated with glycolytic reprogramming [59–61]. This *E. coli*-induced LD accumulation is a multifaceted process primarily linked to increased lipid uptake (via CD36), reduced lipolysis (via ATGL), and elevated expression of LD structural proteins. These findings align with those of Feingold *et al.* (2012), who showed that TLR activation promotes neutral lipid accumulation in macrophages through multiple pathways [60]. However, *E. coli*-induced LD accumulation involves minimal changes in the

655 expression of lipid synthesis enzymes. In fact, *de novo* lipid synthesis is not required for
656 the LD formation of classical activated macrophages [62].

657 This work demonstrates that LDs play a central role in the response to bacterial
658 infections, serving both as hubs integrating the immunometabolic response of
659 macrophages and as direct players in antibacterial activity. Importantly, the direct
660 antibacterial function of LDs is observed in models that involve activation of the innate
661 immune response. This interpretation is reinforced by the lack of antibacterial activity in
662 LDs from OA stimulation. Beyond LD direct antimicrobial role, LDs contribute to pro-
663 host defense through additional mechanisms. Inhibition of LDs through the genetic and
664 pharmacological inhibition of DGAT1, decreased the expression of key proteins in the
665 control of the antibacterial response, in particular iNOS and ISGs as well as modulate
666 eicosanoid synthesis. In vitro, this disruption increased bacterial load, and in vivo, it led
667 to the loss of infection control and systemic bacterial dissemination.

668 Despite catalyzing the same reaction, DGAT1 and DGAT2 play distinct roles in
669 triglyceride synthesis, DGAT1 primarily mediates lipid remodeling, whereas DGAT2
670 drives de novo synthesis pathways [63]. Although DGAT2 is more highly expressed in
671 activated macrophages [45], in LPS-stimulated macrophages rely more critically on DGAT1
672 for triglycerides synthesis and LD accumulation, while DGAT2 may contribute to
673 triglyceride synthesis in a supportive capacity [59]. Our findings highlight DGAT1 central
674 role in both LD accumulation and the antibacterial response of macrophages, consistent
675 with this phenomenon. Notably, modulation of DGAT1 expression did not affect DGAT2
676 levels, suggesting independent regulatory mechanisms for these enzymes. On the other
677 hand, recent findings demonstrate that DGAT2 affects glucose uptake and oxidation,
678 presumably by facilitating the synthesis of triglycerides from endogenous fatty acids [64,65].
679 Additionally, some DGAT1 inhibitors, at concentrations typically used to block lipid

droplet formation, may also partially affect DGAT2 activity [43,66,67]. This cross-reactivity could help explain some of the differences seen between siRNA-mediated DGAT1 knockdown and pharmacological inhibition, especially in lactate and nitric oxide production. However, other potential off-target effects of these inhibitors cannot be excluded.

In the context of immunometabolism, triglyceride synthesis has been identified as a critical component of the inflammatory response in various experimental models [30,58,68]. Castoldi et al. (2020) reported that triglyceride synthesis and LD accumulation significantly enhance the pro-inflammatory profile of LPS-stimulated macrophages, primarily by serving as a platform for PGE_2 synthesis [59]. Pharmacological or genetic inhibition of DGAT1 attenuated the proinflammatory response, characterized by reduced IL-1β and IL-6 production. This impaired cytokine production in DGAT1-deficient macrophages was restored upon PGE_2 supplementation [59]. These results agree with what we observed in *E. coli* infection, where blocking LD accumulation resulted in decreased PGE_2 synthesis, reduced IL-6 and IL-1β production, and increased bacterial load. In sepsis, DGAT1 inhibition reduced the eicosanoids and IL-6 synthesis but failed to inhibit IL-1β synthesis. These differences between *in vitro* and *in vivo* findings may be attributed to the considerable heterogeneity in function and metabolic programming among macrophage subsets [69–71]. Peritoneal macrophages have a heightened oxidative phosphorylation capacity under homeostatic conditions, driven by glutamine and fatty acid oxidation, when compared to BMDMs or alveolar macrophages. This distinctive metabolic phenotype may explain the increased CD80 expression observed in peritoneal macrophages from iDGAT1-treated animals, suggesting enhanced proinflammatory activation, a response that was absent in BMDMs. In contrast, iDGAT1 did not affect the upregulation of GLUT-1, a well-established marker of proinflammatory activation [71, 59].

Notably, DGAT1 overexpression has been shown to reduce fatty acid-induced inflammatory responses in macrophages from diet-induced insulin-resistant mice [72].

LDs serve as reservoirs of arachidonic acid and upon stimulation compartmentalize eicosanoid synthesis enzymes, which fuels the heightened synthesis of eicosanoids - key inflammatory lipid mediators [4]. Therefore, blocking LD formation has a similar effect to inhibiting the mobilization or utilization of these lipid stores [4]. In this context, key enzymes such as ATGL and HSL, which release arachidonic acid from triglycerides, and cPLA2, which act on phospholipids, play essential roles in eicosanoid synthesis. Their coordinated activity ensures the availability of free arachidonic acid required to produce eicosanoids. The inhibition of $cPLA_2$ reduces the synthesis of PGE_2 and consequently inflammatory responses induced by pathogenic bacteria, including *Samonella* Thyphimurium [14], *L. monocytogenes* [15] and hypervirulent *Klebsiella pneumoniae* [73]. Similarly, inhibiting ATGL-mediated triglyceride breakdown in BMDMs [74] and microglia [75,76] impaired eicosanoid synthesis and dampened the pro-inflammatory response. The finding that blocking LD accumulation attenuates inflammatory responses parallels studies showing that inhibition of LD lipolysis similarly reduces LPS-induced inflammation [74–76].

Our data highlights further the complexity of PGE_2's role in inflammation and infection both *in vitro* and *in vivo*. Notably, PGE_2 can be either pro-inflammatory or anti-inflammatory depending on the pathogen, the infection site and stage, and the concentration of this mediator at the infection site [77–81]. During *S. typhimurium* infection, PGE_2 was also identified as an inducer of glycolytic reprogramming and the pro-inflammatory response in macrophages [80]. On the other hand, lactate also stimulates PGE_2 synthesis [82], which can inhibit β-oxidation and lead to LD accumulation [83]. Additionally, PGE_2 regulates iNOS in macrophages, acting as either an activator or repressor,

depending on its concentration [84]. Moreover, non-primed BMDMs have been shown to preferentially produce higher levels of PGE_2 over LTB_4 [85], whereas peritoneal macrophages have been reported to secrete these eicosanoids in relatively similar amounts but in a stimulus-dependent way [48,86,87].

In this pro-host context, the protective role of LDs in infection has been associated with IFN response [24,40,88]. However, the role of type I IFNs in bacterial infections remains less understood compared to their role in viral infections [89,90]. Our results demonstrate that preventing LD accumulation through DGAT1 inhibition impairs IFN-β release, reducing the expression of key antibacterial proteins, including iNOS and CAMP, which leads to an increase in bacterial load and sepsis-induced mortality. It is important to emphasize that the increase in bacterial load observed in vivo is not attributed to sepsis-induced impairment of leukocyte migration to the infection site. The results are consistent with Mancuso et al. (2006), who reported that IFN-α/β signaling is essential for host defense against various bacteria, including *E. coli* [91]. In the absence of IFN-α/β signaling, a marked reduction in macrophage production of NO, and TNF-α was observed after stimulation with live bacteria or with purified LPS [91]. Moreover, IFN-β also mediates time-dependent increases in the mRNA levels of microsomal PGE synthase-1 and COX-2, and induced PGE_2 production [92]. In the polymicrobial sepsis model, mice deficient in IFN-α/β receptor (IFNAR) display persistently elevated peritoneal bacterial counts compared with wild-type mice [93]. Furthermore, Bosch et al. (2020) reported that LPS triggered remodeling of the LD proteome, leading to an increase in several innate immune proteins, many of which belonged to the ISG family, including Viperin, IGTP and CAMP. Interestingly, we observed that inhibiting LD accumulation not only reduced IFN-β release but also suppressed ISG expression. Our findings align with recent evidence demonstrating that type I IFN production and signaling is strongly dependent on cellular

metabolism [94,95]. Further studies will be required to better characterize the signaling and the interplay of LDs in type I interferon biology and functions. One limitation of this study was the difficulty in isolating enough LDs from primary macrophages for thorough proteomic analysis. While we had enough LDs to show they had antibacterial activity, the quantity was insufficient for the detailed proteomic analysis needed to identify specific proteins such as CAMP and their exact location within the LDs. To address this, protein from sepsis-induced liver LDs, which contain a higher protein content, was analyzed, and CAMP was detected. This finding provides supporting evidence for the association of CAMP with LDs in the context of inflammation.

The role of LDs as enhancers of inflammatory response also can be a two-edged sword. In sepsis, tissue damage results from a maladaptive inflammatory and metabolic response mounted to resist infection; this is associated with inadequate mechanisms of tissue tolerance [96]. Recently, our group demonstrated that inhibiting hepatic LD accumulation by targeting the enzyme DGAT1 reduces levels of inflammatory mediators and lipid peroxidation while improving liver function in sepsis [30]. The exacerbation of mechanisms of resistance to infection has been associated with tissue damage, which ultimately can lead to multiple organ failure [28,29]. However, these same mechanisms involved in organ failure are essential to control bacterial loading, such as LTB_4, NO, and CCL2 [48,97]. The same phenomenon has been reported to IFN-β, its effects during bacterial infections can be either protective or detrimental, depending on the specific bacterium and host status [98,99].

In summary, our data suggests that LD biogenesis in *E. coli*-infected macrophages plays a critical role in controlling bacterial load and enhancing the innate immune response. In sepsis, preventing LD accumulation through DGAT1 inhibition disrupts the production of inflammatory mediators and antibacterial factors, leading to increased

780 bacterial load and higher sepsis-associated mortality. These findings highlight LD
781 accumulation as a component of cellular metabolic reprogramming and a molecular
782 switch in innate immunity. Given the growing resistance to current antibiotics, this study
783 provides insights into the molecular mechanisms underlying antimicrobial defense, which
784 could inform the development of novel anti-bacterial strategies.
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Declaration of Competing Interest

The authors declare no competing interests.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding authors upon reasonable request.

Author Contributions:

FP-D and PB conceptualized the study. FP-D was responsible for design and conducting the experiments, literature review, prepared the figures and tables. The manuscript was written by FP-D and PB and edited by all authors. JCS, EKS, RVS, HGG, HE-S, TSS, TC-F, FFM, LP, MMC, DMO, VCS and LS-M contributed to the *in vitro* data collection and analysis. EKS, GI and MAJ contributed to microscopy data collection and analysis. GI and MFSC contributed to cytometry data collection and analysis. FP-D, LT, EFTS, TSS, AFC, PMAS, TC-F, TI, and PAR performed *in vivo* treatment and infection. LT, LS-M, MFSC, PAR, and PB contributed to study design and critically revised the article. All authors approved the submitted version.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT4/OpenAI, Deepseek, Gemini/Google and Copilot/Microsoft in order to improve readability and language of the work. After using these tools/services, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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FIGURE LEGENDS

Figure 1: *E. coli* infection induced LD biogenesis and proinflammatory reprogramming in murine macrophages. BMDMs were infected with *E. coli* (MOI 100) for 1 hour, followed by gentamicin treatment to remove extracellular bacteria. (A) Confocal images showing Oil Red O-labeled LDs (red) in infected BMDMs. Nuclei were stained with DAPI (blue). Scale bar: 10 μm.(B) Quantification of LDs at 1 and 24 h post-infection (hpi). (C) Relative mRNA expression of lipid metabolism genes (*plin2, plin3, fasn, dgat1, acat1, cd36, pnpla2/atgl, abca1*) at 12 hpi via RT-qPCR. Data normalized to *actb* (D) Intracellular *E. coli* CFUs at 1 and 24 hpi. (E) mRNA expression of *ptgs-2/cox-2, 5-lo,* and *15-lo* in noninfected (NI) and infected BMDMs at 6 hpi. (F) Immunofluorescence analyses of *E. coli*-infected BMDMs at 24 hpi. Confocal images were stained for COX-2 (red) and Bodipy 493/503 -labeled LDs (green). Nuclei were stained with DAPI (blue). Scale bar: 10 μm. (G) Levels of PGE_2, LTB_4, and RvD1 in supernatants at 24 hpi (EIA assay). (H) Lactate levels in supernatants at 24 hpi (enzymatic assay). (I) Nitrite (NO) levels in supernatants at 24 hpi (Griess method). (J) Expression of pro-inflammatory (*il-1b, inos*) and anti-inflammatory (*il-10, ch3l3, arg1*) genes at 6 hpi via RT-qPCR. (K) Levels of cytokines (CCL2, CXCL1, IL-10, IL-1β, IL-6, IL-12p40, TNF) at 24 hpi (ELISA). (L) Levels of pan-IFN-α, IFN-β, and IFN-γ at 24 hpi (ELISA). Data represent mean ± SEM from three independent experiments. Significant difference ($p < 0.05$) compared to NI group.

Figure 2: Lipid droplets contributed to antibacterial activity of macrophages. (A) Confocal images of Oil Red O-stained lipid droplets (LDs, red) in *E. coli*-infected BMDMs. Nuclei stained with DAPI (blue). Scale bar: 10 μm. (B) Quantification of the distance between LD and bacteria was performed using

Fiji/ImageJ software from confocal images. (C) Confocal images of Bodipy-labeled LDs (green) in BMDMs stimulated with Texa-red *E. coli* bioparticles (red). Nuclei stained with DAPI (blue). Scale bar: 10 μm. (D) Experimental design of bacterial plate killing assays (BPKA). (E-G) Macrophages stimulated with LPS (500 ng/mL) and IFN-γ (10 ng/mL) for 24 h. LDs purified by sucrose gradient were tested in BPKA. (F) BPKA results in using LDs from non-stimulated (NS) and LPS+IFN-γ-stimulated macrophages. (F) CFU quantification (n=9/group). (G) Relative bacterial viability compared to saline control (n=9/group). (H-J) Bacterial killing assay results using LDs from non-infected, *E. coli*-infected, and OA-stimulated macrophages. (I) CFU quantification (Buffer n = 6, NI n = 8, *E. coli* n = 8, OA n = 3). (J) Relative bacterial viability compared to saline control (n = 8/group). (K) Confocal images of BMDMs treated with oleic acid (OA, 40 μM) for 16 h after 1 hpi and 24 hpi. Scale bar: 10 μm. At least 100 cells across 10 fields analyzed per group. (L) LD quantification in infected BMDMs (≥100 cells per group across 10 fields/experiment). (M) Relative intracellular bacterial CFUs in macrophages with or without OA pretreatment after 24 hpi. (N-P) BMDMs infected with *E. coli* (MOI 100) with or without DGAT1 (A922500, 1.5 or 10 μM) or ACAT1 (CI976, 1 or 10 μM) inhibitors. (M) Experimental design (BioRender). (N) LD enumeration in infected and uninfected macrophages at 24 hpi (≥100 cells/10 fields/group). (O) Relative intracellular bacterial CFUs at 24 hpi (A922500:10 μM or CI976:10 μM). Data represent mean ± SEM from three experiments. *$p<0.05$ compared to non-infected controls.

Figure 3: DGAT1 inhibition disrupted proinflammatory reprogramming and interferon signaling induced by *E. coli* in macrophages. BMDMs were infected with *E. coli* (MOI 100) with or without 10 μM DGAT1 inhibitor (A922500) pretreatment. (A) PGE_2 and LTB_4 levels in cell-free supernatants at 24 hpi were measured by EIA. (B)

Lactate levels in supernatants at 24 hpi were assessed using an enzymatic assay. (C) CCL2, IL-1β, and IL-6 levels in supernatants at 24 hpi were determined by ELISA. (D) LDH release in supernatants indicated cellular viability. (E) IFN-β levels in supernatants at 24 hpi were measured by ELISA. (F) Relative mRNA expression of *camp*, *nox-2* and *plin2* genes in noninfected (NI) and infected BMDMs at 12 hpi, normalized to *actb* (mean $2^{-\Delta\Delta Ct}$ ± SEM; n = 3). (G) CAMP, iNOS, and Plin2 expression in cell lysates were analyzed by Western blotting, using β-tubulin as loading controls. (H) Graphs of band densitometry of CAMP, iNOS and Plin2 obtained after loading normalization and expressed as fold change over mock control. (I) Nitrite (NO) levels in supernatants at 24 hpi were quantified by the Griess method. (J) Viperin and IRGM3/IGTP expression in BMDM lysates were analyzed by Western blotting, using β-actin as loading controls. (K) Graphs of band densitometry of Viperin and IRGM3/IGTP obtained after loading normalization and expressed as fold change over mock control. Data represents the mean ± SEM of three independent experiments. (*) indicates a value significantly different ($p<0.05$) from that of the respective noninfected group.

Figure 4: RNAi-mediated DGAT1 knockdown impaired antibacterial activity, proinflammatory reprogramming, and interferon signaling in E. coli-infected macrophages. BMDMs were transfected with 5 μM self-deliverable AUMsilence oligos for DGAT1 or scramble RNA (SCR). After 48 h, BMDMs were infected with *E. coli* (MOI 100). (A) *dgat1* mRNA levels were assessed 48 h post-transfection by RT-qPCR and normalized to *actb* (mean $2^{-\Delta\Delta Ct}$ ± SEM; n = 3). (B) Oil Red O-stained LDs (red) in infected BMDMs at 24 hpi, with nuclei stained by DAPI (blue). Scale bar: 10 μm. (C) LD quantification in infected BMDMs (≥100 cells per group across 10 fields/experiment). (D) Relative intracellular bacterial CFU at 24 hpi. (E) PGE_2 levels in supernatants at 24

hpi by EIA. Levels of (F) IL-6, (G) CCL2, and (H) IFN-β in supernatants at 24 hpi by ELISA. (I-J) *camp* and *nox2* mRNA levels were assessed 48 h post-transfection by RT-qPCR and normalized to *actb* (mean $2^{-\Delta\Delta Ct} \pm$ SEM; n = 3). (K) Nitrite (NO) levels in supernatants at 24 hpi by the Griess method. (L) Relative mRNA expression of *cox-2* gene in noninfected (NI) and infected BMDMs at 6 hpi, normalized to *β-actin* (mean $2^{-\Delta\Delta Ct} \pm$ SEM; n = 3). (M-N) Relative mRNA expression of *dgat2* gene in noninfected (NI) and infected BMDMs at 6 hpi, normalized to *β-actin* (mean $2^{-\Delta\Delta Ct} \pm$ SEM; n = 3). (O) Lactate levels in supernatants at 24 hpi by enzymatic assay. (P) Cellular viability assessed by LDH release in supernatants. Data represent the mean ± SEM of three independent experiments. (*) indicates a significant difference ($p < 0.05$) compared to the respective noninfected group or SCR E. coli-infected group.

Figure 5: DGAT1 inhibition reduces proinflammatory responses in the peritoneum of septic mice. Sham or septic (CLP) mice were orally treated with A922500 (DGAT1 inhibitor, 3 mg/kg) or vehicle. At 6 h or 24 h post-surgery, peritoneal lavage was collected. (A) *In vivo* experimental design for DGAT1 inhibition. (B) Oil Red O-stained lipid droplets (red) in peritoneal leukocytes, counterstained with Mayer's hematoxylin. Scale bar: 20 μm. (C) Lipid droplet enumeration in peritoneal leukocytes (≥100 cells/group across 10 fields/experiment). (D) PGE_2, LTB_4, and RvD1 levels in peritoneal lavage at 24 h post-surgery by EIA. Levels of (E) IL-6, (F) CCL2) in peritoneal lavage at 6 h and 24 h post-surgery by ELISA. (G) IRGM3 and Plin2 expression in peritoneal leukocytes lysates were analyzed by Western blotting, using β-actin as loading controls. (H) pan-IFN-α, (I) IFN-β and (J) IFN-γ, (K) nitrite, and (L) cathelicidin (CAMP) in peritoneal lavage at 6 h and 24 h post-surgery by ELISA.. Data (4–6 mice/group) are presented as means ± SEM, analyzed by one-way ANOVA with Tukey's post hoc test. *$p < 0.05$

compared to sham; #p < 0.05 compared to untreated CLP. CLP: cecal ligation and puncture.

Figure 6: DGAT1 inhibition increases bacterial load and sepsis severity. Sham or septic (CLP) mice were orally treated with A922500 (DGAT1 inhibitor, 3 mg/kg) or vehicle. (A) Bacterial CFU quantification in peritoneal at 6 h and 24 h post-surgery. (C-D) Total and differential leukocyte counts in peritoneal lavage at (C) 6 h and (D) 24 h post-surgery, including total leukocytes, mononuclear cells (Mono), and polymorphonuclear leukocytes (PMN). (D) Bacterial CFU quantification in serum at 6 h and 24 h post-surgery. (E-F) MPO activity in liver tissues obtained by an enzymatic assay at (E) 6 h and (F) 24 h post-surgery. (G) Experimental design for survival and clinical score analysis. Mice were monitored for 48 h to assess (H) clinical sepsis severity scores and (I) survival. Data (4–8mice/group for A-F; 10 mice/group for H-I) are presented as means ± SEM, analyzed by one-way ANOVA with Tukey's post hoc test. *p < 0.05 compared to sham; #p < 0.05 compared to untreated CLP. CLP: cecal ligation and puncture.