Oxylipin metabolism is controlled by mitochondrial β-oxidation during bacterial inflammation

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Oxylipins are potent biological mediators requiring strict control, but how they are removed en masse during infection and inflammation is unknown. Here we show that lipopolysaccharide (LPS) dynamically enhances oxylipin removal via mitochondrial β-oxidation. Specifically, genetic or pharmacological targeting of carnitine palmitoyl transferase 1 (CPT1), a mitochondrial importer of fatty acids, reveal that many oxylipins are removed by this protein during inflammation in vitro and in vivo. Using stable isotope-tracing lipidomics, we find secretion-reuptake recycling for 12-HETE and its intermediate metabolites. Meanwhile, oxylipin β-oxidation is uncoupled from oxidative phosphorylation, thus not contributing to energy generation. Testing for genetic control checkpoints, transcriptional interrogation of human neonatal sepsis finds upregulation of many genes involved in mitochondrial removal of long-chain fatty acyls, such as ACSL1,3,4, ACADVL, CPT1B, CPT2 and HADHB. Also, ACSL1/Acs1 upregulation is consistently observed following the treatment of human/murine macrophages with LPS and IFN-γ. Last, dampening oxylipin levels by β-oxidation is suggested to impact on their regulation of leukocyte functions. In summary, we propose mitochondrial β-oxidation as a regulatory metabolic checkpoint for oxylipins during inflammation.
Oxygenated polyunsaturated fatty acids (PUFA) (oxylipins) are essential bioactive lipid mediators generated during inflammation/infection. They are generated by cyclooxygenases (COX), lipooxygenases (LOX), or cytochrome P450s (CYP), expressed in a variety of cells and tissues. They signal through activation of G protein-coupled receptors (GPCRs) at sub nM concentrations. Oxylipin signaling requires deactivation; however, our understanding of how this happens during infection is poor. Systemic pathways for individual oxylipins, including thromboxane, prostacyclin, and hydroxyeicosatetraenoic acids (HETEs) were uncovered in healthy humans decades ago. There, infusion of exogenous labeled lipids enabled determination of half-life and metabolites, some of which appear immediately, slowly disappearing over several minutes. Urinary metabolite analysis became the gold standard for whole body oxylipin analysis. Separately, peroxisomal β-oxidation of individual oxylipins was explored using liver microsomes. There, partial β-oxidation revealed truncated products termed dinors (minus 2 carbons) and tetrans (minus 4 carbons). These were identified as stable intermediates in tissue, plasma and urine.

Aside from peroxisomes, mitochondria contain fully competent β-oxidation machinery, used for the first steps of FA-dependent energy metabolism, FA oxidation (FAO). Here, FA are converted to acetyl-CoA which enters the tricarboxylic cycle (TCA) providing substrates for oxidative phosphorylation (OxPhos). Although mediated by distinct enzymes to peroxisomes, mitochondrial β-oxidation also involves sequential removal of 2-carbon fragments from the carboxyl terminus. More recently, there has been significant interest in how mitochondrial FAO supports innate and adaptive immunity in T cells and macrophages. Saturated FA such as palmitate or stearate are considered the main β-oxidation substrates; however, oxylipins generated abundantly during inflammation have not been considered. We recently showed that endogenously generated platelet oxylipins are removed by carnitine palmitoyltransferase-1 (CPT1), the mitochondrial import protein for FA. However, it is not known whether mitochondria also remove oxylipins in other leukocytes, nor how this might be regulated during inflammation/infection, if this impacts lipid signaling, and if it supplies acetyl-CoA to OxPhos. These are important questions since large amounts of diverse oxylipins are generated during inflammation/infection, with downstream autocrine and paracrine signaling being a major contributor to the overall inflammatory response. Currently, there is significant interest in how mitochondria contribute to inflammation, and their role in immunometabolism.

To address these questions, we examine the role of mitochondria in removing oxylipins in macrophages during inflammatory activation in vitro and in vivo, and how modulation of physiological oxylipin levels impacts leukocyte signaling relevant to an acute inflammatory challenge (macrophages, neutrophils, T cells). Genetic and lipidomic approaches characterize a metabolic β-oxidation network for oxylipins and their metabolites, during inflammation. The study identifies a mechanism for dampening oxylipin signaling that could be more broadly relevant in inflammation and infection.

**Results**

**Inhibition of CPT1 prevents secretion of diverse peritoneal oxylipins in vivo.** Mitochondrial β-oxidation of native long-chain FA requires uptake of their FA-CoA derivatives via the outer membrane transporter, CPT1, of which there are three isoforms, a,b,c. However, whether oxylipin species can be imported via CPT1 in macrophages is unknown. To test this in vivo, the pan CPT1 inhibitor etomoxir was injected intraperitoneally (i.p.) into wild-type C57BL/6 mice alone or with the bacterial lipid LPS (1 μg), then peritoneal lavage harvested after 6 h, and analyzed using LC/MS/MS. A large number of oxylipins were detected in cell-free supernatant with several showing expected elevations in response to LPS (Fig. 1a, b and Supplementary Fig. 1a). These are broadly categorized into lipids generated by 12/15-LOX, COX-1/2, or CYP450, although some are from more than one pathway (Supplementary Data File 1). Many specialist pro-resolving mediators (SPM) including resolvins or protectins were not conclusively detected. For maresin (Mar1, 7R,14S-dHDOHE), very small peaks were seen that fell below our limit of quantitation (LOQ) for the assay. When injecting larger amounts of sample and broadening the retention time window, two peaks were seen with one having the same retention time as the authentic standard (Supplementary Fig. 1b). It was not possible to generate a convincing MS/MS spectrum from tissues that matched the standard due to the low levels present. The detection of two isomers suggests diastereomers of 7,14-di-HDOHE, and the peaks may also contain co-eluting stereoisomers. This is suggestive of non-enzymatic origin and this peak is more appropriately named 7,14-diHDOHE.

Etomoxir stimulated small increases in several oxylipins, however when included with LPS, a far stronger impact was seen, and levels of many lipids were effectively doubled (Fig. 1a, b). This suggests that CPT1 in peritoneal mitochondria actively transports bioactive oxylipins during health, but in infection, its role is significantly enhanced.

**CPT1 regulates the metabolism of endogenously generated oxylipins by peritoneal macrophages.** In vivo, peritoneal oxylipins could be metabolized by mitochondria in resident macrophages, B cells and/or peritoneal membrane (mesothelium). Naïve resident macrophages, which express COX-1 and 12/15-LOX (Alox15), are likely to be the primary basal source. Thus, naïve peritoneal macrophages from wild-type mice were assessed for mitochondrial removal of endogenous oxylipins. We focused on extracellular forms since most are secreted to activate GPCRs extracellularly. Serum-free media was used to prevent contamination with blood-derived oxylipins. LPS (24 h) stimulated prostaglandin (PG) secretion by naïve resident peritoneal macrophages, consistent with COX-2 induction (Fig. 1c and Supplementary Fig. 1c). Although 12/15-LOX is highly expressed in naïve peritoneal macrophages, its loss in culture leads to the relatively low generation of monohydroxy FA (12-HETE and 12/15-HEPEs) (Fig. 1d and Supplementary Fig. 1d). Overall, oxylipin species generated by peritoneal macrophages were similar to those in lavage, although the total number/diversity was less with fewer CYP-derived species and no SPMs detected (Supplementary Fig. 1c).

Etomoxir significantly increased detected levels of several monohydroxy lipids and COX-2 products (Fig. 1c, d). This indicates that both their formation and removal is increased by inflammation, similar to in vivo (Fig. 1a, b). 12-HETE, 12-HEPE and 15-HEPE increased with etomoxir but were suppressed by LPS, likely due to inflammation-associated loss of 12/15-LOX (Fig. 1d and Supplementary Fig. 1d). Importantly, in vivo and in vitro, COX-2 and 12/15-LOX products were consistently increased by CPT1 inhibition.

Several mono- and di-hydroxy oxylipins were only detected when CPT1 was blocked, indicating that their mitochondrial metabolism exceeds their generation (Fig. 1d and Supplementary Fig. 1d). Thus, CPT1 prevents their secretion. In contrast, during
LPS challenge, the eicosapentaenoic acid product 17,18-diHETE, generated by soluble epoxide hydrolase oxidation of CYP-derived 17,18-EET, was suppressed by etomoxir (Supplementary Fig. 1e). Overall, these data indicate that peritoneal macrophage oxylipin secretion (12/15-LOX or COX-2 derived) is counterbalanced by CPT1-mediated uptake into mitochondria. However, CYP-derived lipids show the opposite, where CPT1 inhibition dampens levels.

Bone marrow-derived macrophages (BMDM) consume diverse oxylipins from serum. Macrophages are exposed to oxylipins...
from other immune or stromal cells during inflammation. Serum contains numerous oxylipins from 12-LOX and COX-1, primarily monohydroxy isofoms and thromboxane, generated by white cells and platelets, but few PGs. Serum forms during innate immune responses, thus oxylipin metabolism may occur in local or systemic sites. To examine this, we tested primary murine bone marrow-derived cells differentiated to macrophages, then treated with MCSF, alone or along with LPS/IFN-γ or IL-4 (henceforth referred to as M0, M1, and M2) respectively as in vitro of models for macrophage inflammation. All macrophage phenotypes consumed significant amounts of exogenous monohydroxy oxylipins from 10% serum-containing medium, including 12-HETE/12-HEPE (12-LOX, generated by platelets), 5-HETE/5-HEPE (5-LOX, generated by neutrophils), and 9-13-HODEs (Supplementary Fig. 2a). 11-HETE/11-HEPE and 15-HETE/15-HEPE were removed by M0(MCSF) and M2(IL-4) cells; however, they appeared higher or unchanged for M1(LPS/IFN-γ) since they were simultaneously generated by COX-2, induced by LPS/IFN-γ (Supplementary Fig. 2a). Furthermore, M1(LPS/IFN-γ) cells secreted large amounts of PGs and thromboxane due to COX-2, causing a net increase in their extracellular levels. (Supplementary Fig. 2a). Thus, the overall pattern was consumption of serum oxylipins by all macrophage populations, coupled with simultaneous generation of COX-2 PGs by M1(LPS/IFN-γ). Last, low dose etomoxir (25 μM) significantly increased PGs released by M1(LPS/IFN-γ) cells (Supplementary Fig. 2b). Overall, this demonstrates that mitochondrial uptake of PGs suppresses their secretion from classic inflammatory BMDM cells (M1(LPS/IFN-γ)), in the same manner as seen with naïve peritoneal macrophages and in vivo with LPS peritonitis, while all BMDM cell types consume significant amounts of HETEs and HEPEs.

Dynamic control of oxylipins by CPT1 during the inflammatory challenge in RAW macrophages. Next, we used RAW cells, a model for macrophage inflammatory responses in vitro, amenable to genetic modification. These don’t express 12/15-LOX, therefore, to examine the impact of CPT1 on its products, cells stably overexpressing Alox15 (RAWAlox15) were generated. Basally, neither RAWmock nor RAWAlox15 cells secreted many oxylipins, with only 9-/13-HODE, two PGs, and three di-hydroxy products of CYP released in low amounts (Supplementary Fig. 3a). LPS (24 h, 100 ng/ml) stimulated robust secretion of many oxylipins, including several PGs (COX-2) 9/13-HODEs, and 11- and 15-HETEs, HETEs and HEPEs (likely from COX-2) (Fig. 2a and Supplementary Figs. 3a, b and 4a–c). The primary 12/15-LOX products 12-HETE and 14-HDOHE were only detected in RAWAlox15 cells, and their generation was increased by LPS (Fig. 2a). CPT1 inhibition had little impact on basal oxylipin secretion (Supplementary Fig. 3a). However, it significantly elevated the LPS-dependent generation of monohydroxy FA (HETEs, HDOHES, HETEs, and HODEs) from RAWAlox15 cells (Fig. 2a). These data indicate that secreted oxylipins normally represent only a fraction of the overall amounts made by 12/15-LOX. Thus, LPS increases CPT1-mediated removal of 12/15-LOX products, similar to in vivo and peritoneal macrophages. Many primary 12/15-LOX products (12-HETE, 14-HDOHE) were seen, while others (9-HOTrE, 9-HODE) may be byproducts of secondary propagation. In etomoxir/LPS-treated RAWAlox15 (but not RAW cells), a lipid suggestive of resolvinD5 (RvD5, 7S,17S-diHDOHE) was detected, however of the two peaks, only one matched the retention time of the authentic standard (Supplementary Fig. 4a, b). The lipid was very low in abundance, and it was not possible to generate a reliable MS/MS spectrum from cell supernatant. As there were two peaks, they likely represent diastereomers of 7,17-diHDOHE, and the individual peaks may also contain co-eluting stereoisomers. Thus, we labeled this 7,17-diHDOHE and suggest it arises from non-enzymatic oxidation. No SPMs were detected in any other samples from RAW or RAWAlox15 cells.

Several COX-2 derived PGs and 14,15-diHETE (both CYP) were significantly decreased by etomoxir in RAW cells (Supplementary Fig. 4c). This mirrored the impact of CPT1 inhibition on peritoneal macrophage 17,18-diHETE. In summary, while LOX products were consistently elevated by etomoxir in RAW or all primary macrophages, for RAW cells alone, both COX and CYP-derived lipids were suppressed.

Exogenous 12-HETE is metabolized to diene and triene tetranor metabolites via mitochondrial and non-mitochondrial β-oxidation in RAW cells. To determine the fate of oxylipins removed by CPT1, we examined the metabolism of exogenous 12-HETE by RAW macrophages. 12(S)-HETE was rapidly removed following LPS stimulation, coinciding with the formation and subsequent metabolism of two tetranor 12-HETEs (Supplementary Fig. 4d–f). These were confirmed by MS/MS as a triene, 8-hydroxy-4Z,6E,10Z-hexadecatetraenoic acid (comparison with an authentic standard), and a diene, proposed as 8-hydroxy-6,10-hexadecadienoic acid (through comparison with34) (Supplementary Fig. 5a–c). Both will be 8(S) since they originate from 12(S)-HETE. As confirmation, 12(S)-HETE-d8 was added to RAW cells (3 h), and a deuterated diene was detected (Supplementary Fig. 5d, e). The MS/MS fragmentation of these lipids is shown (Supplementary Figs. 12–14). For the tetranor diene, fragmentation matched the expected gas-phase chemistry of hydroxylated FAs, where cleavage occurs next to the hydroxyl group (Supplementary Schemes 13 and 14). For the triene, a daughter ion at m/z 165.1 was seen, with high-resolution MS/MS of the standard confirming this as C11H17O (Supplementary Fig. 6a and Supplementary Scheme 12). NMR for the triene is shown in Supplementary Fig. 7. Almost all triene and diene were detected extracellularly, and over 4–8 h, both disappeared from supernatant and cell pellets (Supplementary Fig. 4e, f). This suggests that macrophages generate and secrete primary and secondary metabolites of HETEs, but then re-internalize them for further metabolism. We next incubated triene tetranor 12(S)-HETE with RAW macrophages, and after 3 h, diene was detected extracellularly (Supplementary Fig. 6b). This indicates that diene
forms via saturation of the triene, confirming the structure as 8(S)-hydroxy-6E,10Z-hexadecadienoic acid.

Next, the impact of CPT1 inhibition on the metabolism of exogenous 12(S)-HETE or 12(R)HETE was tested. Both HETEs were rapidly removed by LPS-stimulated RAW cells, with around 0.15% or 0.4% remaining after 3 h, for the S and R forms, respectively (Fig. 2b, left panel). Cellular and supernatant 12(S)-HETE and 12(R)HETE were both increased by CPT1 inhibition, with 12(R)HETE most strongly impacted. Based on the impact of etomoxir, the primary removal appeared to be non-mitochondrial (Fig. 2b). 12-HETE was converted to tetranor trienes and dienes, which were detected mainly outside the cells (Fig. 2b and Supplementary Fig. 8). These were around 5–8-fold increased by CPT1 blockade, confirming that they are also dynamically
metabolized by mitochondria in LPS-stimulated macrophages (Fig. 2b and Supplementary Fig. 8). In the case of the diene, both S and R enantiomers were similarly elevated by etomoxir treatment, while for the triene, the 12(R)HETE was most strongly impacted.

Since most removals appeared to be non-mitochondrial, we analyzed for esterification of 12(S)-HETE into phospholipid pools via Land’s cycle, as previously described. First, to determine which molecular species were formed, a precursor scanning LC/MS/MS analysis was undertaken, scanning for negative ion precursors of m/z 319.2, the carboxyate anion of HETEs. Cells were supplemented with 12(S)-HETE for 3 h, then harvested and lipid extracts were analyzed. Several precursor ions were found, between m/z 738–810 that were absent in RAW cells not supplemented with 12(S)-HETE (Fig. 3a, b). Their structures were subsequently confirmed using MS/MS, showing they originate from a series of expected HETE-containing PE or PC lipids using MS/MS scans. Next, these lipids were quantified using LC/MS/MS, showing they originate from a series of expected HETE-containing PE or PC lipids using MS/MS scans. Next, these lipids were quantified using LC/MS/MS, showing they originate from a series of expected HETE-containing PE or PC lipids using MS/MS scans.

Inflammation accelerates 12-HETE conversion to triene tetranor 12-HETE via mitochondrial β-oxidation in vivo. In vivo inhibition of CPT1 significantly elevated many oxylipins in the peritoneal cavity following the LPS challenge. To examine β-oxidation of oxylipins via mitochondria in vivo, the formation of tetranor metabolites was next measured. The diene metabolite was absent; however, tetranor 12-HETE triene was significantly reduced by Cpt1a knockdown in LPS-treated but not basal RAW cells (Fig. 5b, c). This indicated that following Cpt1a knockdown, RAW cells showed reduced mitochondrial metabolism of 12-HETE coupled with the lower formation of its tetranor products post LPS stimulation.

Oxylipin metabolism by mitochondria is not sustaining oxidative phosphorylation (OxPhos). Mitochondrial β-oxidation of FA is directly linked with OxPhos. It forms acetyl-CoA, a substrate for the TCA cycle as well as NADH and FADH2, substrates for complexes I and II, and also transfers electrons to flavoprotein-ubiquinone oxidoreductase (ETF-QO). Thus, oxylipins could contribute to OxPhos. However, LPS suppresses mitochondrial respiration, through multiple mechanisms including acacitin inhibition and nitric oxide binding to complex IV, making this unlikely. Here, LPS treatment profoundly decreased cellular respiration consistent with previous data. Importantly, LPS did not impact mitochondrial DNA (mtDNA), or cause organelle damage as measured by DNA lesion frequency. Thus, mitochondria are still present in LPS-treated cells, although β-oxidation of FAs is largely uncoupled from OxPhos.

Blocking CPT1 dampens cytokine/chemokine generation by peritoneal macrophages. CPT1 inhibition doubled the levels of PGs generated by inflammatory activated peritoneal macrophages, in
Fig. 3 Identification of phospholipid-esterified 12-HETE in RAW cells, and CPT1 knockdown reduces PG levels in RAW cells. a–c Cells (10⁶) were incubated for 3 h with 12(S)-HETE ± LPS before lipids were extracted and analyzed as in Methods. Precursor LC/MS/MS was undertaken as described in Methods, scanning for ions that fragment to generate HETE. a Chromatogram showing elution of precursors that generate product ions with m/z 319.2. b MS spectrum from 10–15 min showing PE and PC species that contain 12-HETE. c Quantification of 12-HETE PE and PC species that are formed following incubation of RAW cells with 12(S)-HETE (n = 3, mean ± SEM, separate wells of cells) compared with/without LPS, Student's t-test, two-tailed. Where no bar is shown no significant difference was seen. d Cpt1a knockdown dampens cellular levels of PGs in RAW cells. RAW cells expressing either the non-silencing (RAWnonsil) or Cpt1a knockdown siRNA (RAWCpt1aKD) were treated with LPS (100 ng/ml) for 24 h and cell levels of PGs measured using LC/MS/MS as in Methods (n = 6, mean ± SEM, separate wells of cells). Student's t-test, two-tailed. Where no bar is shown no significant difference was seen.
particular the abundant prostacyclin (PGI$_2$) metabolite 6-keto-PGF$_{1\alpha}$ and PGE$_2$ (Fig. 1c). Thus, we next sought to examine whether this impact PG-mediated autocrine signaling. Several studies have reported that oxylipins such as monohydroxy FAs, PGE$_2$ or iloprost (PGI$_2$ analog) can suppress cytokine and chemokine generation in monocytes or macrophages, including by EP receptor signaling, or PPAR-$\gamma$ activation$^{42–46}$, thus we tested the impact of etomoxir on generation of TNF or RANTES in LPS-stimulated peritoneal macrophages. For both proteins, there was a significant decrease in the generation when etomoxir was present (Fig. 5e, f). This supports the idea that removing oxylipins can dampen their signaling in macrophages.

Oxylipin levels detected in vivo regulate leukocyte responses in a concentration-dependent manner. Oxylipins are primarily secreted to act on other cell types in a paracrine manner. Next, to address whether macrophage mitochondrial $\beta$-oxidation impacts wider oxylipin signaling between other cell types, human

**Fig. 4 CPT1 genetic knockdown reduces the secretion of oxylipins.** RAW cells expressing either the non-silencing (RAWnonsil) or Cpt1a knockdown siRNA (RAWCpt1aKD) were treated with LPS (100 ng/ml) for 24 h and secretion of PGs, 17,18-diHETE, or monohydroxy oxylipins were measured using LC/MS/MS as in Methods ($n = 6$, mean ± SEM, separate wells of cells). Student’s t-test, two-tailed. Where no bar is shown no significant difference was seen.
phagocyte and T-cell responses to these lipids were determined. Neutrophils were chosen since they are known to be sensitive to PGE\(_2\), via EP receptor signaling\(^{47,48}\), while T cells can be regulated by PPAR\(\gamma\), a transcription factor that is regulated by many LOX-derived oxylipins\(^{49-55}\). Here, reactive oxygen species (ROS) production elicited by Staphylococcus epidermidis, a common pathogen in human peritonitis was measured by aminophenyl fluorescein (APF) fluorescence of whole blood leukocytes\(^{56}\). Two concentrations were used that represented amounts of 52 individual oxylipins detected in vivo, in the presence or absence of etomoxir (with LPS) (Supplementary Table 1). Here, ROS generation by neutrophils was consistently enhanced by higher or
The enzymes that import oxylipins into mitochondria are not conclusively known, but it is very likely that those importing long-chain FA are involved. These include five acyl-CoA synthetase long-chain family members (ACSL1-3, 6), as well as CPT1a, 1b, 1c, and CPT2. The gene products mediate the formation of −CoA and then carnitines that are required for long-chain FA uptake across mitochondrial membranes (Supplementary Fig. 10 and Supplementary Table 2). This is followed by mitochondrial β-oxidation, which catalyzes sequential removal of 2-carbon fragments to generate acetyl-CoA, and chain shortened metabolites. Genes that encode proteins that metabolize long-chain PUFA include HADHA, HADHB, EC1, DECR1, and ACADVL (Supplementary Fig. 15 and Supplementary Table 2). A set of 33 genes were compiled including also isoforms with a preference for medium or shorter chain FA. Their expression during human infection in vivo was tested using a microarray from human neonatal whole blood cells (35 cases, 26 controls). Here, infants suspected of infection had blood cultures conducted, and a diagnosis of bacterial sepsis was confirmed. Out of the genes tested, 22 were significantly different with 9 up- and 13 downregulated in sepsis. Notably, several upregulated genes encode proteins involved in the generation of long-chain FA-CoAs and their interconversion to acyl-carnitines in mitochondria (Figs. 5 and 6a). For the most relevant, seven were significantly upregulated (ACSL1, 3, 4, CPT1b, CPT2, ACADVL, HADHB) while three were downregulated (ACSL5, EC11, HADHA) (Figs. 5 and 6a). This indicates a high degree of regulation during complex bacterial infection in seriously ill humans, suggesting the specific isoforms that may be involved in oxylipin removal. We also interrogated transcriptomic data from stromal tissues extracted from mice challenged (i.p.) with a cell-free supernatant from a clinical isolate of S. epidermidis (SES)38. Here, there was overall upregulation, showing a similar trend to the human bacterial dataset (Fig. 6b, c). Although most individual genes were not statistically significant, Cpt1a showed around 2-fold induction, which was significant 6 h post infection.

Consistent upregulation of ACSL isoforms is revealed across multiple human and murine macrophage datasets, and inhibition of ACSLs dampens 12-HETE metabolism by β-oxidation in RAW cells. Next, data from three mouse BMDM or three human PBMCs studies were downloaded from GEO59–64. Several isoforms of ACSL were found to be upregulated, including ACSL1, 3, 4 (human study) or Acsl1, 5 (SES peritonitis and in vitro BMDM experiments). Notably, ACSL1 expression was upregulated in all studies (Figs. 6d, e and 7a, b). For BMDM, Acsl1 was consistently significantly induced on stimulation using either
LPS/IFN-γ or IFN-γ alone (Figs. 6d and 7a). Two of the mouse BMDM datasets also indicated significant increases in Eci1, encoding 3,2-enoyl-CoA isomerase (Supplementary Fig. 15 and Fig. 6d, e). In human studies, ACSL1 was also induced by LPS/IFN-γ or IFN-γ (Fig. 7b). To confirm the functional relevance of ACSL upregulation, the impact of the pharmacological inhibitor triascin C on 12-HETE metabolism was tested. 12-HETE removal was strongly inhibited while the formation of tetranorbs was dampened between 60 and 95% (Fig. 7c). This is consistent with the requirement for CoA-esters for 12-HETE β-oxidation (mitochondrial or peroxisomal), and confirms the involvement of ACSLs. We note that 12-HETE uptake into phospholipid pools...
Fig. 6 Transcriptomics of the mitochondrial β-oxidation pathway reveals ACSL1/Acsl1 as a key checkpoint response to LPS inflammation in human and mouse macrophages. a Plots for the eight significantly upregulated genes in the human neonatal dataset are shown. \( n = 35 \) and \( 26 \) for infection and controls, respectively. Student’s t-test, two-tailed, then adjusted using Benjamini–Hochberg test. b, c Transcriptomics of mouse peritonitis shows significant upregulation of Cpt1a at 6 h post SES. Gene expression data from peritoneal membranes harvested post SES challenge were analyzed for expression of 32 genes (\( n = 3 \) per group) \( p \leq 0.05 \), Student’s t-test, two-tailed, adjusted using Benjamini–Hochberg test. d The plot for Cpt1a expression. e Transcriptomics reveals Asc1 and Eci1 to be highly upregulated in response to LPS/IFN-γ in murine BMDM. Transcriptomics data obtained from GEO database were analyzed as outlined in Methods for expression of 34 genes selected for potential or known involvement in mitochondrial β-oxidation. Samples were BMDM treated with either LPS/IFN-γ or IFNγ alone as indicated. For all genes, the log2fold change was calculated and plotted using Pheatmap in R (d). e Box and whisker plots for normalized expression (using Limma and Oligo Biocomparator packages) of Eci1 in mouse datasets with \( n = 3 \) for all groups, adjusted using Benjamini–Hochberg test (\( n = 3 \) for all groups except for GSE53053 M0 (\( n = 2 \)). Box shows median, and interquartile ranges (IQR). The ends of the whisker are at 1.5 × IQR above the third quartile (Q3) and 1.5 × IQR below the first quartile (Q1). If minimum or maximum values are outside this range, then they are shown as outliers.

via Land’s cycle esterification also requires ACSL activity. Last, we saw a small non-significant suppression of 12-HETE removal by LPS in RAW cells (Fig. 7c). This is consistent with the process being mainly via Land’s cycle esterification, which was in turn slightly suppressed by LPS (Fig. 3a–c).

Arachidonate (20:4) levels increase in response to inflammation and are dampened by CPT1 activity. Pharmacological or genetic inhibition of CPT1/Cpt1a dampened PG generation in RAW cells (Figs. 3d and 4a and Supplementary Fig. 4c). As a potential explanation, we considered whether LPS-driven inflammation leads to elevated PUFAs biosynthesis, which is then dampened by CPT1 inhibition. Specifically, β-oxidation generates acetyl-CoA, which is converted to malonyl-CoA, used to chain elongate and desaturate essential FA to form PUFA via Land

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Arachidonate (20:4) levels increase in response to inflammation and are dampened by CPT1 activity. Pharmacological or genetic inhibition of CPT1/Cpt1a dampened PG generation in RAW cells (Figs. 3d and 4a and Supplementary Fig. 4c). As a potential explanation, we considered whether LPS-driven inflammation leads to elevated PUFAs biosynthesis, which is then dampened by CPT1 inhibition. Specifically, β-oxidation generates acetyl-CoA, which is converted to malonyl-CoA, used to chain elongate and desaturate essential FA to form PUFA via Land
for oxylipin uptake and mitochondrial metabolism during infection (Supplementary Fig. 15). A consistent pattern across all studies was upregulation of ACSL1/Acsl1, while we also saw upregulation of ACSL3,4 (human) and Acsl5 (mice) (Figs. 5j, 6a–e, and 7a, b). Consistent with this, the pan-ACSL inhibitor, Triascin C was highly effective at preventing 12-HETE metabolism to the diene and triene tetranor metabolites in RAW cells (Fig. 7c). ACSL1 is already known to shuttle FAs to mitochondria for \( \beta \)-oxidation and lipid synthetic pathways although others may also be involved\(^{70–76} \). As yet, the specific ACSLs that acylate oxylipins are not known, although Klett found that all six isoforms are active in vitro\(^ {77} \). Given our findings, characterizing which specific ACSLs support the import of oxylipins into mitochondria warrants study. ACSL1 is known to be critical for shuttling FA into mitochondria for \( \beta \)-oxidation in the heart, skeletal muscle, and adipose tissues\(^ {70–74} \). Furthermore, in the liver, ACSL1 and CPT1 were shown to be physically associated\(^ {75, 76} \). Thus, ACSL/CPT1 may be a critical checkpoint enabling higher rates of oxylipin metabolism during infection. Indeed, a critical role for ACSL1 in sepsis outcome has been proposed\(^ {78} \). Regulating import of long-chain FAs into the mitochondrial matrix, we found increased CPT1a or b, dependent on...
Fig. 7 Elevated Acsl1/ACSL1 in mouse and human GEO datasets, triascin C significantly inhibits 12-HETE removal by cells, and prevents generation of tetranor diene or triene HETE metabolites, while etomoxir alters AA levels in RAW cells. a Plots for normalized expression (using Limma and Oligo Bioconductor packages) of Acsl1 in the mouse datasets, with n = 3 for all groups except for GSE53053 M0 (n = 2) adjusted using Benjamini–Hochberg test. b ACSL1 is strongly induced in human M1 macrophages. Human transcriptomics data for ACSL1 expression were downloaded from GEO and normalized expression level plotted. GSE46903 (n = 3, 10 for M0, M1 respectively), GSE35499 (n = 7), GSE5099 (n = 3). Data were normalized using Limma and Oligo Bioconductor packages as outlined in Methods, then adjusted using the Benjamini–Hochberg test. Box shows median, and interquartile ranges (IQR). The ends of the whisker are at 1.5 × IQR above the third quartile (Q3) and 1.5 × IQR below the first quartile (Q1). If minimum or maximum values are outside this range, then they are shown as outliers. c Triascin C alters metabolism of 12-HETE. RAW cells were cultured for 3 h in serum/phenol red-free medium with 12(S)-HETE (1.4 μg/10⁵ cells), with/without 100 ng/ml LPS with/without 7 μM Triascin C. Cells and supernatant were harvested and 12-HETE and its tetranor metabolites measured using LC/MS/MS (n = 3, mean ± SEM, separate wells of cells). Significance was tested using ANOVA with Tukey post hoc test. The impact of LPS (either with or without Triascin C) was not significant for any conditions or lipids, except for diene. d Etomoxir modulates levels of AA in RAW cells. RAW cells were cultured for 3 h in serum/phenol red-free medium with 12(S)-HETE (1.4 μg/10⁵ cells), with/without 100 ng/ml LPS with/without 25 μM etomoxir. Cells and supernatant were harvested and 16:0, 18:0, and 20:4 measured using LC/MS/MS (n = 3, mean ± SEM, separate wells of cells). Significance was tested using ANOVA with Tukey post hoc test. For 16:0 and 18:0, there were no significant differences found.

The model (Fig. 6a–c), along with CPT2 induction in human sepsis (Fig. 6a). Murine BMDM stimulated with IFN-γ also upregulated Ecil (Fig. 6d, e). This encodes DCI, which converts 3-cis or trans-enoyl-CoA to 2-trans-enoyl-CoA during mitochondrial β-oxidation and was previously shown to be induced during Hepatitis C virus infection and required for virus replication.79,80 Last, upregulation of the β subunit of the mitochondrial trifunctional protein (encoded by HADHB) and very long-chain acyl-CoA dehydrogenase (encoded by ACADVL) are also fully consistent with their proposed role in oxylipin β-oxidation (Fig. 6a and Supplementary Fig. 15). Macrophages rely primarily on glycolysis for energy when stimulated with LPS/IFN-γ, and conversely use FAO to supply OxPhos with substrates when exposed to immune-modulatory stimuli such as IL-4.81 However, recent studies have uncovered a significant role for lipid metabolism in “M1” cells even in the absence of OxPhos, with FA synthetic pathways (FAS) being upregulated by LPS.82 Our observation that etomoxir partially suppressed 18:0 and 16:0 levels in macrophages, which is elevated during in vivo and in vitro stimulation, suggests an alternative role in lipid metabolism. In contrast, monohydroxy oxylipins were themselves synthesized via chain elongation and desaturation, and reduced OxPhos. We show for the first time that CPT1 mediates exogenous and endogenously generated oxylipin β-oxidation by macrophages, which is elevated during inflammation. The impact of blocking CPT1 varied by sub-family of oxylipins, based on either:

(i) Cell type: etomoxir consistently elevated LOX-derived oxylipins and various other monohydroxy-oxylipins, while suppressing CYP7E1 metabolites in all macrophages. However, COX-derived PGs increased in vivo and in primary macrophages, but were suppressed in RAW cells.

(ii) How CPT1 was targeted: in RAW cells, etomoxir or gene silencing suppressed COX-derived PGs and CYP7E1 oxylipins. In contrast, monohydroxy oxylipins were increased by etomoxir but suppressed by CPT1 knockdown in RAW cells. Etomoxir was used at 25 μM, well below concentrations found to induce off-target effects on adenine nucleotide translocase.36,37 Indeed, previous studies using high levels of etomoxir led to FAO being incorrectly proposed as required for alternative activation of M2 macrophages (100–200 μM).83 Nowadays, lower concentrations are recommended that block ~90% of β-oxidation without side-effects (e.g., 10–25 μM).83 As a second approach, shRNA knockdown stably reduced expression of CPT1a. However, this causes adaptive changes to the metabolic status of macrophage mitochondria, beyond acute inhibition of CPT1. Recent publications implicate CPT1 in maintaining mitochondrial health due to its requirement for importing FAs for maintaining mitochondrial cardiolipin and phospholipid pools.83 Thus, cells lacking CPT1 do not proliferate normally and may have adapted to a deficiency in OxPhos through alterations in other lipid and energy metabolism pathways. Thus, constitutive knockdown may directly impact the balance between mitochondrial and peroxisomal β-oxidation and the ability to support elongase activities in the cells. Nevertheless, both inhibitory approaches caused significant changes to oxylipin secretion, both for PGs, 12-HETE and its two tetranor metabolites, supporting the hypothesis that mitochondria can be a significant site of oxylipin regulation in macrophages.

Our data reveal complex modulation of oxylipin removal and formation by CPT1 as follows:

(i) Removal: oxylipins and their metabolites can be degraded by two separate β-oxidation pathways, with only mitochondrial enzymes relying on CPT1. However, peroxisomes and mitochondria display metabolic interplay where FA degradation intermediates are transferred for metabolism between the organelles.82 Thus, stable knockdown of CPT1 may require compensatory increases in peroxisomal β-oxidation. It has been proposed that truncated unsaturated FA metabolites may need to transfer to mitochondria for complete metabolism.82 Indeed, 12-HETE-triene was suggested as the terminal peroxisomal metabolite for 12-HETE, with further metabolism proposed to require COX-derived PGs. However, this causes adaptive changes to oxylipin secretion, both for PGs, 12-HETE and its two tetranor metabolites, supporting the hypothesis that mitochondria can be a significant site of oxylipin regulation in macrophages.

(ii) Generation: oxylipins are generated from PUFA, which are themselves synthesized via chain elongation and desaturation of linoleic and α-linolenic acids. This process requires...
malonyl-CoA, which is formed from acetyl-CoA, a product of which themselves depend at least in part on CPT1 for formation. Thus, while both synthesis and degradation of oxylipins simultaneously occur in macrophages, steady-state levels will depend on the cells’ metabolic status, and whether CPT1 is mainly supporting degradation (by β-oxidation) or synthesis (by supplying TCA intermediates) of individual oxylipins. Thus, CPT1 could either increase or decrease levels of oxylipins and their metabolites concomitantly, as seen in our study. Detailed flux analysis of this phenomenon is required to further our understanding since a role for regulation of elongation/desaturation was not clearly seen in our study.

Here, we show that mitochondrial oxypinic removal takes place during inflammation on a background of reduced OxPhos, a well-known response to LPS stimulation by macrophages28. However, β-oxidation requires a basal level of OxPhos both to regenerate NAD+ and oxidized flavin cofactors in the electron transferring flavoprotein complex, which reduces ubiquinone. While OxPhos was suppressed, there was a low residual activity remaining in both RAW and peritoneal cells that appeared sufficient to sustain β-oxidation, observed by the formation/metabolism of oxylipins and HETE tetranors. Our experiments using exogenous 12-HETE suggest that non-mitochondrial pathways such as peroxisomal oxidation and also esterification is probably responsible for removing most. This agrees with previous studies where exogenous 12-HETE added to mammalian cells was removed by peroxisomes forming a series of metabolites, down to C12:1

Further studies are required to test this idea in vivo, including C12:1

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were killed by incubation at 65 °C for 20 min, then washed by
RNA was recovered using the RNeasy Mini Kit (QIAGEN, 74104) according to the
volume of 20 µL for 1 h at 50 °C, and the reaction was terminated at 70 °C for
reverse-transcribed using superscript III reverse transcriptase (Invitrogen) in a total
μ

supplemented were (per 10

serum-free RPMI, with 100 ng/ml LPS, with/without 25 µM etomoxir. Amounts
Metabolism of exogenous 12-HETE and 12-HETE-d8 by RAW cells

primer mix (0.5

in some experiments. After 24 h supernatant was recovered and frozen at

article for lipid extraction and analysis.
In this assay, identi-

minimum of two points across each peak. Where lipids fell below LOQ, a zero value was recorded, but replaced with
50%LOQ for statistical analysis. Example chromatograms for all lipids analyzed are provided in Supplementary File.
Targeted lipidomics analysis of 12(S)-HETE, 12(R)-HETE, 12(S)-HETE-d8, or
tetranor 12(S)-HETE metabolites was performed on a Nexera LC coupled to a 4000
QTRAP (AB Sciex). Briefly, liquid chromatography was performed at 40 °C using a
Spherisorb ODS2 C18 column (4.6 × 150 mm, 5 µm, Waters) at a flow rate of 1 ml/
min over 30 min. Mobile phase A was 75% HPLC water/25% acetonitrile; v/v and
0.1% acetic acid) and mobile phase B was 60% methanol/40% acetonitrile; v/v and
1.0% acetic acid. The following gradient for mobile phase B was applied: 50–90% B
over 20 min, then held at 90% B for 5 min, followed by re-equilibration to 50% B
from 25 to 30 min. The injection volume was 10 µL. Ionization was performed using
electrospray ionization in the positive mode with a nebulizer pressure of 40 psi, curtain gas
40 °C, GS1 60 psi, GS2 30 psi, curtain gas 35 psi, ESI voltage –4.5 kV. Cycle time was 1.46 s.
Peak areas for lipids were integrated and quantification was performed using an external calibration curve with 15-HETE-d8 as
standard for all investigated compounds. As no primary standard is available for the tetranor HETE and HETE triene, 1.2 µg. Following incubation, the supernatant was harvested, centrifuged to remove any dead/dying cells and then snap-frozen. Cells were gently rinsed with PBS to remove any dead/dying cells. Following this, the cells were scraped in PBS and snap-frozen. Samples were stored at –80 °C.
Lipids were extracted as described below, using solid-phase C18 columns, resus-
pended in small volumes of methanol and stored at –80 °C. Lipids were analyzed by liquid

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formate. The lipids were vacuum dried, resuspended in methanol and stored at

Heatmap method. For the generation of heatmaps, amounts (ng/106 cells) of each
lipid were averaged, then log10 was applied to the mean value. Heatmaps were

cells/well and peritoneal macrophages were seeded at 5 × 10^5 cells/well (and incubated with LPS (100 ng/ ml)).
Mitochondrial function was assessed using inhibitors (all from Sigma) injected in the following order: oligomycin (1 µM), FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazono), 2 or 4 µM), and 2 µM antimycin A (10 µM for peritoneal macrophages; 1 µM for RAW/Raw/alex15 cells). OCR is described as

Determination of reactive oxygen species generation by peripheral blood cells

Human blood from healthy workplace volunteers was obtained with ethical approval from School of Medicine (SMREC 16/02) with informed consent. Volunteers were male or female, healthy, between the ages of 25 and 55, with no current use of oral contraceptives. There were no significant differences in age and BMI between the groups. Peripheral blood mononuclear cells (PBMC) (5 ml) was drawn using a butterfly syringe into 50 ml heparin sodium; 12 µl of this was then placed in a microcentrifuge tube and 1 ml cold PBS added. Following centrifugation (350 × g, 5 min, 4 °C) the cell pellet was recovered and resuspended, then stained with anti-CD11B BV510 (1:50 in 0.5 ml PBS, W6/32, Becton Dickinson, 563141) for 30 min on ice. Cells were centrifuged (350 × g, 5 min, 4 °C) and resuspended in DMEM media (2 mM glutamine) containing APF (1:1000, cell concentration = cells from 5 ml blood per ml of APF media). S. epidermidis bacteria (clinical isolate)36 were killed by incubation at 65 °C for 20 min, then washed by centrifugation and resuspended in PBS three times (12,000 x g, 5 min). Bacteria were counted by flow cytometry using an Attune NXT; bacteria were excluded from other particles by size (Forward-scatter vs Side-scatter). Bacteria were first added to blood cells at varying doses, to determine optimal reactivity per donor isolate
Transcriptional analysis of GEO datasets. A set of genes relevant to mitochondrial β-oxidation were collated, and then examined for differential gene expression (Supplementary Table 2). Published transcriptome data (NCBI GEO DataSets accession numbers GSE50553, GSE69607, GSE48517, GSE46903; GSE50999) were compared against our dataset (using different microarrays for sample processing). mtDNA copy numbers, relative to gDNA, was done as previously described. Differential gene expression analysis was performed using DEseq2 (Bioconductor, 1.24.0). For the specific genes of interest, Student’s t-test was used. Significance: adj.pval < 0.05, Benjamin–Hochberg correction for multiple testing.

Analysis of mouse in vivo bacterial peritonitis gene expression. The SES model was carried out and lavaged harvested at 3 or 6 h as described, data are available as: GSE25504. Eight to twelve weeks old mixed-genotype wild-type C57Bl/6 mice were from Charles River. Parietal peritoneal tissue was extracted at 3 and 6 h post SES administration, and collected using aseptic techniques. Two sections of lining were immediately snap-frozen in liquid N2 and stored at –80°C prior to total RNA extraction. For this, peritoneal membrane sections (80 mg) were dissociated in 1 ml binding buffer (Qiagen) supplemented with 0.02% (v/v) Tween 80. Genes were identified using a handheld electric homogenizer (Benchmark Scientific). Lysate was diluted 1:3 in distilled water and digested in 0.2 mg/ml protease K (Invitrogen; 25350049) for 10 min at 55°C. Lysate was cleared and RNA precipitated in 70% ethanol. Total RNA was extracted using the RNeasy mini kit (QIAGEN; 24136) in accordance with the manufacturer’s instructions. RNA was eluted in 50 μl RNase-free water and quantified using a nanodrop 2000. The integrity and quality of RNA preparations was assessed using an Agilent 2100 bioanalyzer. Samples with an RNA integrity number exceeding 8 were used for library preparation (2–4 μg input). Cytoplasmic, mitochondrial, and ribosomal RNA was depleted using the RiboMinus transcription subtraction kit (Ambion; K11501). Libraries were prepared using the RNA-seq kit v2 (Life technologies; 475936) and sequencing on an Ion torrent (T throat; Fisher Scientific, cat. No. 10053293) according to the manufacturer’s instructions and quantified using a Nanodrop (Thermo Scientific). Assessment of mtDNA copy numbers, relative to gDNA, was done as previously described. Briefly, quantitative PCR (qPCR) analysis was performed using PerfeCTaq™ SYBR Green FastMix™, low ROX™ (Quanta Biosciences) and primers specific for mouse mtDNA (mouse mtDNA forward: 5′-CCCGACGACACTACCATGATAGTAAC-T3′, mouse mtDNA reverse: 5′-GCGGAAAGTTCAAGACGATGGC-3′) or the genomic 18S ribosome gene (mouse 18S forward: 5′-AACAGGCTACCACTATCTCAG-3′, mouse 18S reverse: 5′-GCCGTCGAACGTCTC-3′). Genes were identified using a handheld electric homogenizer (Benchmark Scientific). Lysate was diluted 1:3 in distilled water and digested in 0.2 mg/ml protease K (Invitrogen; 25350049) for 10 min at 55°C. Lysate was cleared and RNA precipitated in 70% ethanol. Total RNA was extracted using the RNeasy mini kit (QIAGEN; 24136) in accordance with the manufacturer’s instructions. RNA was eluted in 50 μl RNase-free water and quantified using a nanodrop 2000. The integrity and quality of RNA preparations was assessed using an Agilent 2100 bioanalyzer. Samples with an RNA integrity number exceeding 8 were used for library preparation (2–4 μg input). Cytoplasmic, mitochondrial, and ribosomal RNA was depleted using the RiboMinus transcription subtraction kit (Ambion; K11501). Libraries were prepared using the RNA-seq kit v2 (Life technologies; 475936) and sequencing on an ion torrent (T throat; Fisher Scientific). Raw data were mapped using Torrent Suite™ (STAR and Bowtie2 (2.3.4.3) aligners) using mm10 as the reference genome. Library quality was assessed using FastQC (0.11.8). Differential gene expression analysis was completed with DEseq2 (Bioconductor, 1.24.0). For the specific genes of interest (Supplementary Table 2), Student’s t-test was used, significance: adj.pval < 0.05, Benjamin–Hochberg correction for multiple testing.

Assessment of mitochondrial DNA in LPS-treated cells. RAW cells were seeded in 6-well plates at 1 x 10^6 cells/well and grown in DMEM with 10% FCS, 2 mM glutamine and 100 U/ml of penicillin/streptomycin for 24 h. Next, the medium was aspirated and 1 ml (0.5 ml ml for 12-well plates) of 1 mg/ml LPS (Invivogen, 0111-22) and 100 U/ml of penicillin/streptomycin were added and cells were incubated for 24 h. Total DNA was extracted from cells with PureLink Genomic DNA kit (Fisher Scientific, cat. No. 10053293) according to the manufacturer’s instructions and quantified using a Nanodrop (Thermo Scientific). Assessment of mtDNA copy numbers, relative to gDNA, was done as previously described. Briefly, quantitative PCR (qPCR) analysis was performed using PerfeCTaq™ SYBR Green FastMix™, low ROX™ (Quanta Biosciences) and primers specific for mouse mtDNA (mouse mtDNA forward: 5′-CCCGACGACACTACCATGATAGTAAC-T3′, mouse mtDNA reverse: 5′-GCGGAAAGTTCAAGACGATGGC-3′) or the genomic 18S ribosome gene (mouse 18S forward: 5′-AACAGGCTACCACTATCTCAG-3′, mouse 18S reverse: 5′-GCCGTCGAACGTCTC-3′). Genes were identified using a handheld electric homogenizer (Benchmark Scientific). Lysate was diluted 1:3 in distilled water and digested in 0.2 mg/ml protease K (Invitrogen; 25350049) for 10 min at 55°C. Lysate was cleared and RNA precipitated in 70% ethanol. Total RNA was extracted using the RNeasy mini kit (QIAGEN; 24136) in accordance with the manufacturer’s instructions. RNA was eluted in 50 μl RNase-free water and quantified using a nanodrop 2000. The integrity and quality of RNA preparations was assessed using an Agilent 2100 bioanalyzer. Samples with an RNA integrity number exceeding 8 were used for library preparation (2–4 μg input). Cytoplasmic, mitochondrial, and ribosomal RNA was depleted using the RiboMinus transcription subtraction kit (Ambion; K11501). Libraries were prepared using the RNA-seq kit v2 (Life technologies; 475936) and sequencing on an ion torrent (T throat; Fisher Scientific). Raw data were mapped using Torrent Suite™ (STAR and Bowtie2 (2.3.4.3) aligners) using mm10 as the reference genome. Library quality was assessed using FastQC (0.11.8). Differential gene expression analysis was completed with DEseq2 (Bioconductor, 1.24.0). For the specific genes of interest (Supplementary Table 2), Student’s t-test was used, significance: adj.pval < 0.05, Benjamin–Hochberg correction for multiple testing.

Cell gating strategies are shown in Supplementary Fig. 11d-f. This included forward- vs side- scatter profile (FSC vs SSC) that differentiates neutrophils and monocytes from lymphocytes. Neutrophils were further separated from eosinophils and monocytes via their high expression of CD15. The bacteria were resuspended in 25 μl lysis buffer (350 x g, 5 min, 4°C) then analyzed by flow cytometry. Cell gating was performed above and of assessment of mtDNA damage was performed using qPCR as previously described. A common reverse primer (mouse mtDNA reverse: 5′-GGCCAAA-AATCCAGAAGACGATGC-3′) in combination with primers called either forward short PCR primer (5′-GGCCAAA-AATCCAGAAGACGATGC-3′) or long PCR primer (5′-CCCCAGCTACATCCTGATCAGTAG-3′). The reverse primer with the forward shorter primer gives rise to a 16.2 kb product, or with the long forward primer gives rise to a 80 bp product. The lesion frequency of the mtDNA is calculated using the equation below. Chloroquine (CQ) at 40 μM was used as a positive control (Supplementary Table 5).

Lesion frequency per 16kb = – log 2 [lesion copy number/total mtDNA copy number]
Analysis of 12-HETE incorporation into PLs by RAW264 cells. For MS precursor scanning, 1 × 10^6 RAW264 cells were seeded in a 6-well plate in DMEM supplemented with 10% FBS. The next day, cells were washed twice with PBS and 2 ml serum-free RPMI (no phenol red) was added to each well. Cells were treated for 3 h with 100 ng/ml LPS, with and without 2.8 μg 12(S)-HETE/10^5 cells. Following 3-h incubation, cells were scraped into their culture media and immediately transferred to 2.5 ml extraction solvent and extracted using the hexane:isopropanol method, as described in the Methods section. Lipids were analyzed on a Sciex 6500 QTRAP, with chromatography as follows: Luna 3 μm C18 150 × 2 mm column (Phenomenex, Torrance, CA) with a gradient of 50–100% B over 10 min followed by 20 min at 100% B (A, methanol:acetonitrile:water, 1 mM ammonium acetate, 60:20:20; B, methanol, 1 mM ammonium acetate) with a flow rate of 200 μl/min. Source and MS conditions were as follows: CUR 35, IS-4500, TEM 500, GS1 40, GS2 30, DP-50, CE-38, CXP-11. First, a precursor scan (PREC 319.2) was performed in negative mode to determine a list of precursor PE ions present in the cell extract, scanning a mass range of 600–950 Da at a scan rate of 1000 Da/s. Following this, ten ions of interest were identified and EPI scans were performed for each precursor ion to obtain MS/MS spectra. Source and chromatography conditions were identical to the above. Product ions were scanned at 1000 Da/s with a mass range of 100–820 Da, using a dynamic fill time in the trap. Once candidate lipids were identified, product ions were extracted in MRM mode, monitored against a calibration curve obtained against a calibration curve obtained using 18:0/12-HETE-PE and 18:0/12-HETE-PC as primary standards. MRM traces were processed using MRMquant (SCIEX) software. MS/MS data were quantified against a calibration curve (fully quantified ions 12:6 179.1, 724.6–179.1, 766.6–179.1, 786.8–179.1, 782.6–179.1, 808.7–179.1, 810.7–179.1). Chromatography and mass conditions were the same as above. Peak inclusion criteria were those that had at least 7 points across the peak and exceeded a signal-to-noise ratio of 5:1.

LC-MS/MS of free fatty acids (FFAs). FFAs were extracted from cell culture supernatants (1 ml) using the hexanes-isopropanol method described above. An internal standard mix was added (10 μl) to samples prior to extraction (Supplementary Table 7). FFAs were derivatized in sample extracts according to Han et al. with modifications10. Dried extracts were reconstituted in methanol (100 μl), then 50 μl 3NPH (200 mM 3-nitrophenyl-hydroxazidine, 50/50 Methanol/H2O) and 50 μl EDC/PyR (120 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide and 6% pyridine, 50/50 Methanol/H2O) were added. Samples were vortexed then incubated for 30 min at 40°C. Excess derivatization reagents were quenched by the addition of 0.5% formic acid (100 μl; 75/25 Methanol/H2O) and incubation at 40°C for 30 min. Samples were aliquoted into HPLC vials for LC/MS/MS in MRM mode on a Nexera liquid chromatography system (Shimadzu) with chromatography as follows: Luna 3 μm C18 150 × 2 mm column (Phenomenex, Torrance, CA) with a gradient of 0–100% B over 10 min followed by 20 min at 100% B (A, methanol:acetonitrile:water, 1 mM ammonium acetate, 60:20:20; B, methanol, 1 mM ammonium acetate) with a flow rate of 200 μl/min.

Data availability

The raw numbers for charts and graphs are available in the Source Data file whenever possible. All data are included in the article and its Supplementary Information, or are available from the authors upon reasonable requests. Source Data are provided with this paper.

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Competing interests

P.K. is an employee of Cayman Chemical. All other authors declare no competing interests.

Additional information

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