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ORIGINAL INVESTIGATION

Identification and characterization of a novel anti-inflammatory lipid isolated from *Mycobacterium vaccae*, a soil-derived bacterium with immunoregulatory and stress resilience properties

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Abstract

Rationale *Mycobacterium vaccae* (NCTC 11659) is an environmental saprophytic bacterium with anti-inflammatory, immunoregulatory, and stress resilience properties. Previous studies have shown that whole, heat-killed preparations of *M. vaccae* prevent allergic airway inflammation in a murine model of allergic asthma. Recent studies also demonstrate that immunization with *M. vaccae* prevents stress-induced exaggeration of proinflammatory cytokine secretion from mesenteric lymph node cells stimulated ex vivo, prevents stress-induced exaggeration of chemically induced colitis in a model of inflammatory bowel disease, and prevents stress-induced anxiety-like defensive behavioral responses. Furthermore, immunization with *M. vaccae* induces anti-inflammatory responses in the brain and prevents stress-induced exaggeration of microglial priming. However, the molecular mechanisms underlying anti-inflammatory effects of *M. vaccae* are not known.

Objectives Our objective was to identify and characterize novel anti-inflammatory molecules from M. vaccae NCTC 11659.

Methods We have purified and identified a unique anti-inflammatory triglyceride, 1,2,3-tri [*Z*-10-hexadecenoyl] glycerol, from *M. vaccae* and evaluated its effects in freshly isolated murine peritoneal macrophages.

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Results The free fatty acid form of 1,2,3-tri [*Z*-10-hexadecenoyl] glycerol, 10(*Z*)-hexadecenoic acid, decreased lipopolysaccharide-stimulated secretion of the proinflammatory cytokine IL-6 ex vivo. Meanwhile, next-generation RNA sequencing revealed that pretreatment with 10(*Z*)-hexadecenoic acid upregulated genes associated with peroxisome proliferator-activated receptor alpha (PPARα) signaling in lipopolysaccharide-stimulated macrophages, in association with a broad transcriptional repression of inflammatory markers. We confirmed using luciferase-based transfection assays that 10(*Z*)-hexadecenoic acid activated PPARα signaling, but not PPARγ, PPARδ, or retinoic acid receptor (RAR) α signaling. The effects of 10(*Z*)-hexadecenoic acid on lipopolysaccharide-stimulated secretion of IL-6 were prevented by PPARα antagonists and absent in PPARα-deficient mice.

Conclusion Future studies should evaluate the effects of 10(*Z*)-hexadecenoic acid on stress-induced exaggeration of peripheral inflammatory signaling, central neuroinflammatory signaling, and anxiety- and fear-related defensive behavioral responses.

Keywords 10(*Z*)-hexadecenoic acid · Bacteria · Inflammation · Interleukin 6 · Lipid · Macrophage · Mycobacteria · PPAR · RNA-seq · *vaccae*

Abbreviations

CD	Cluster of differentiation
CNS	Central nervous system
DC	Dendritic cell
DSM-5	Diagnostic and Statistical Manual of Mental Disorders (5th ed.)
IL	Interleukin
IFN	Interferon
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
MGB	Microbiota–gut–brain
NCTC	National Collection of Type Cultures
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PEA	Palmitoylethanolamide
PPAR	Peroxisome proliferator-activated receptor
PTSD	Posttraumatic stress disorder
RAR	Retinoic acid receptor
TGFβ	Transforming growth factor beta
TLR	Toll-like receptor
Treg	Regulatory T cell

Introduction

The global prevalence of anxiety disorders has been estimated to be 7.3%, ranging from 5.3% in African cultures to 10.4% in Euro/Anglo cultures (Baxter et al. 2013). According to the Diagnostic and Statistical Manual of Mental Disorders (5th ed.) (DSM-5; American Psychiatric Association 2013), anxiety disorders include those that share features of excessive fear and anxiety and related behavioral disturbances, such as generalized anxiety disorder, panic disorder, social anxiety disorder (social phobia), and specific phobia (American Psychiatric Association 2013). Posttraumatic stress disorder (PTSD), although formerly classified as an anxiety disorder, is classified as a trauma- and stressor-related disorder (American Psychiatric Association 2013). Collectively, anxiety and trauma-related disorders are complex and multifactorial, and their differentiation and management are complicated by phenotypic heterogeneity. The etiology and pathophysiology of these disorders are thought to involve interactions among the genome, epigenome, and environment (Nugent et al. 2011). Recently, investigation of the etiology and pathophysiology of psychiatric and neurological diseases has expanded to include a potential role of the microbiota–gut–brain (MGB) axis (Forsythe et al. 2010; Cryan and Dinan 2012, 2015; Leclercq et al. 2016). Of particular interest, evidence from preclinical and clinical studies suggests that exaggerated inflammation, which in some cases may be secondary to dysregulation of the microbiome, may be a risk factor for the development of trauma- and stressor-related disorders (for review, see Langgartner et al. 2018). These studies raise the question of whether or not microbial-based interventions with anti-inflammatory or immunoregulatory properties may have value in the prevention or treatment of trauma- and stressor-related disorders.

Evidence suggests that some common pathogenic and non-pathogenic microorganisms, to which humans have been exposed throughout evolution, drive anti-inflammatory and immunoregulatory mechanisms that inhibit inappropriate immune responses by the host (Rook and Rosa Brunet 2002; Rook 2009, 2010; Okada et al. 2010). Throughout human evolution, the interactions between these ancestral microorganisms, which we have collectively referred to as "old friends," and the innate immune system promoted immunoregulation. These "old friends" included microorganisms that (1) were part of host physiology (human microbiota); (2) were harmless but inevitably contaminating air, food, and water (environmental microbiota); or (3) led to severe host tissue damage when attacked by the host immune system (e.g., helminthic parasites) (Rook 2013; Blaser 2017).

"Old friends" are thought to suppress host inflammation through a variety of mechanisms, including the induction of specific subsets of antigen-presenting cells such as macrophages and dendritic cells (DCs) and modulation of innate

immunity (Le Bert et al. 2011; Garn et al. 2016; Lowry et al. 2016). In their absence, the host may develop inappropriate immune responses to allergens, self-antigens, or gut microbiota. It has been hypothesized that increases in allergies, autoimmune diseases, inflammatory bowel diseases, and psychiatric disorders in modern living conditions may be due, in part, to decreased exposure to "old friends" (Rook 2010; Lyte and Cryan 2014; Bloomfield et al. 2016; Lowry et al. 2016; Stamper et al. 2016). In parallel, individuals with a diagnosis of PTSD have a higher risk of development of any autoimmune disease, relative to those with other psychiatric disorders, or relative to those with no psychiatric disorder (O'Donovan et al. 2015), suggesting that impaired immunoregulation or inappropriate inflammation may confer risk for development of both autoimmune conditions and PTSD. The saprophytic mycobacterium, *Mycobacterium vaccae* (National Collection of Type Cultures (NCTC) 11659), has shown encouraging therapeutic potential in diseases of inflammation and immunodysregulation (Gutzwiller et al. 2007; Rook et al. 2007) and has shown immunoregulatory and stress-protective effects in murine models (Zuany-Amorim et al. 2002; Adams et al. 2004; Lowry et al. 2007; Reber et al. 2016; Fox et al. 2017; Frank et al. 2018). Mycobacteria are abundant in municipal water supplies (Gebert et al. 2018) and are a normal component of the healthy human microbiome of the oral cavity (buccal mucosa and dental plaque) and upper respiratory tract (nostrils and oropharynx) and, therefore, are considered part of the microbiome of the upper airways (Macovei et al. 2015).

The identification of specific microbially derived molecules with anti-inflammatory or immunoregulatory properties may provide novel therapeutic avenues for the treatment of diseases of immunodysregulation or trauma- and stressor-related disorders where exaggerated inflammation is thought to be a risk factor (Lowry et al. 2016; Langgartner et al. 2018). We have previously shown that treatment with a heat-killed preparation of the saprophytic mycobacterium, *M. vaccae*, prevents murine allergic pulmonary inflammation by inducing $CD4^{+}CD45RB^{low}$ Tregs (Zuany-Amorim et al. 2002). These cells are allergen-specific and, upon passive transfer, can protect recipient allergic mice from airway inflammation by significantly reducing eosinophilia in the lungs. In addition, treatment with *M. vaccae* induces a population of pulmonary $CD11c^{+}$ antigen-presenting cells, which are characterized by increased expression of IL-10, transforming growth factor beta (TGFβ) and interferon α (IFNα) (Adams et al. 2004). Furthermore, at least in vitro, priming of human DCs with *M. vaccae* induces strong inhibition of Th2 responses (Le Bert et al. 2011).

Meanwhile, we have shown that immunization of mice with *M. vaccae* promotes a more proactive response to a chronic psychosocial stressor, prevents stress-induced colitis, prevents stress-induced exaggeration of chemically induced colitis in a model of inflammatory bowel disease, and attenuates anxiety-like defensive behavioral responses (Reber et al. 2016). Consistent with these findings, immunization with *M. vaccae* prevents stress-induced exaggeration of interferon gamma and IL-6 secretion from freshly isolated mesenteric lymph node cells stimulated with anti-CD3 antibody ex vivo. Importantly, preimmunization with *M. vaccae*, in stressed mice, resulted in a two orders of magnitude increase in IL-10 secretion from mesenteric lymph node cells stimulated ex vivo. However, until now, specific constituents of *M. vaccae* that suppress inflammation in macrophages in the periphery or central nervous system have not been identified.

Through a screening process of *M. vaccae* NCTC 11659 lipid extracts, a single triglyceride, 1,2,3-tri [*Z*-10-hexadecenoyl] glycerol, was identified with potential immunotherapeutic benefits (Rosa Brunet and Rook 2008). The lipid was demonstrated to prevent allergic airway inflammation, and the lipid recapitulated the therapeutic effects of whole heat-killed *M. vaccae*. The protective phenotype was characterized by increased IL-10, decreased IL-5, and reduced infiltration of eosinophils and macrophages in bronchoalveolar lavage fluid (Rosa Brunet and Rook 2008). It was also shown that the efficacy of the triglyceride was not dependent on the glycerol structure, as the synthetic, constituent free fatty acid, 10(*Z*)-hexadecenoic acid, was sufficient to suppress pulmonary airway inflammation. The mechanism through which this long-chain, monounsaturated fatty acid was capable of limiting symptoms of inflammation is unknown, but it is explored here in a model of macrophage activation.

Notably, it is relatively rare in nature for an organism to naturally produce a fatty acid that is unsaturated at the C10 position, yet several mycobacteria species—including *M. vaccae*, can perform that desaturation (Scheuerbrandt and Bloch 1962; Coyle et al. 1992; Böttger et al. 1993; Springer et al. 1993; Suutari and Laakso 1993; Chou et al. 1998; Tay et al. 1998; Pacífico et al. 2018). We successfully synthesized the free fatty acid, 10(*Z*)-hexadecenoic acid, and using cell-based assays and RNA-seq, revealed that 10(*Z*)-hexadecenoic acid upregulated genes associated with the peroxisome proliferator-activated receptor (PPAR) signaling pathway and inhibited proinflammatory signaling of activated macrophages ex vivo. Furthermore, studies using cultured cells transfected with lipid-regulated transcription factors revealed that both the monoacylglycerol lipid constituent of *M. vaccae* and its free fatty acid form selectively increased PPARα signaling. The effects of 10(*Z*)-hexadecenoic acid to inhibit proinflammatory signaling of activated macrophages ex vivo were prevented by PPARα antagonists and absent in PPARα-deficient mice. This is the first report, to our knowledge, to show that a synthetic *M. vaccae*-derived lipid acts to induce anti-inflammatory responses in host immune cells by acting as an agonist at host PPARα receptors.

Materials and methods

Animals

Adult male BALB/c mice (BALB/cAnHsd; Cat. No. 047; Harlan, Indianapolis, IN, USA), 6–8 weeks old, were used and housed under standard conditions with food and water available ad libitum. Adult male PPAR$\alpha^{-/-}$ (B6;129S4-*Ppara*^{tm1Gonz}/J; Cat. No. 008154; Jackson Laboratories, Bar Harbor, ME, USA) and control mice (C57BL/6J; Cat. No. 000664; Jackson Laboratories), 6–8 weeks old, were used and housed under standard conditions with food and water available ad libitum. Although the C57BL/6J inbred strain is considered an approximate control for the PPAR$\alpha^{-/-}$ mice (B6;129S4-*Ppara*^{tm1Gonz}/J; Jackson Laboratories), future studies should ideally compare PPAR$\alpha^{-/-}$ mice to wild-type littermates.

All experimental protocols were consistent with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, Eighth Edition (The National Academies Press 2011), and the Institutional Animal Care and Use Committee at the University of Colorado Boulder approved all procedures. This work was covered under CU Boulder IACUC Protocol Numbers 2134-14MAY2018 and 2361-14MAY2018-DT. The research described here was conducted in compliance with The ARRIVE Guidelines: Animal research: reporting of in vivo experiments, originally published in PLOS Biology, June 2010 (Kilkenny and Altman 2010). All possible efforts were made to minimize the number of animals used and their suffering.

Synthesis of 10(*Z*)-hexadecenoic acid; (10*Z*) -hexadec-10-enoic acid (CAS No. 2511-97-9)

Unless otherwise noted, reagents were obtained commercially and used without further purification. Dichloromethane (CH_2Cl_2) was distilled over calcium hydride (CaH_2) under a nitrogen atmosphere. Tetrahydrofuran (THF; $(CH_2)_4O$) was distilled from sodium-benzophenone under a nitrogen atmosphere. Thin-layer chromatography analysis of reaction mixtures was performed on Dynamic Adsorbents, Inc., silica gel F-254 TLC plates. Flash chromatography was carried out on Zeoprep 60 ECO silica gel. ^{1}H spectra were recorded with a Varian INOVA 500 spectrometer. Compounds were detected by monitoring UV absorbance at 254 nm.

To a 5-mL sealed tube containing 1-heptene (0.50 mL, 3.55 mmol), methyl 10-undecenoate (0.080 mL, 0.36 mmol), and THF (0.35 mL), a Grubbs *Z*-selective metathesis catalyst was added (Grubbs Catalyst(R) C633, 2.2 mg, 3.48 μmol, Cat. No. 771082, Sigma-Aldrich, St. Louis, MO, USA). The reaction was stirred at 45 °C for 8 h before cooling to room temperature. The slurry was filtrated through a short plug of silica gel and concentrated. The obtained oil was dissolved in 1.0 mL THF. The solution was cooled to 0 °C, then 9-borabicyclo[3.3.1] nonane (9-BBN) solution in THF (1.28 mL, 0.50 M, 0.64 mmol) was added. After 2 h stirring at 0 °C, the reaction was quenched with 60 μL EtOH, then 1.5 mL pH 7 potassium phosphate buffer and 1.5 mL 30% H_2O_2. The mixture was stirred at room temperature for 12 h, then extracted with 5 mL EtOAc three times. The combined organic layers were washed with 4 mL saturated $Na_2S_2O_3$ and 3 mL brine, then dried over Na_2SO_4, filtered, and concentrated. To the crude oil in 1.0 mL THF was added LiOH monohydrate (38 mg, 0.90 mmol) in 1.0 mL water. After 2 h, the reaction solution was cooled to 0 °C before the addition of 0.91 mL 1.0 N HCl. After being concentrated under reduced pressure, the aqueous solution was saturated with NaCl and extracted with 3 mL dichloromethane three times. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated. Purification by flash chromatography (2:1:1 hexanes/dichloromethane/diethyl ether) provided (10*Z*)-hexadec-10-enoic acid (0.022 g, 90%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.48–5.22 (m, 2H), 2.35 (t, J = 7.5 Hz, 2H), 2.01 (q, J = 6.6 Hz, 4H), 1.63 (p, J = 7.4 Hz, 2H), 1.35–1.15 (m, 16H), 0.88 (t, J = 6.9 Hz, 3H).

Murine peritoneal macrophage isolation and screening

Murine peritoneal macrophages were isolated and cultured as previously described (Zhang et al. 2008) and used to determine the effects of 10(*Z*)-hexadecenoic acid on lipopolysaccharide (LPS)-induced IL-6 secretion. Briefly, mice received a single injection of 3% thioglycollate medium (1 mL, i.p.; Cat. No. 9000-294, VWR, Radnor, PA, USA). Mice were euthanized 96 h later using cervical dislocation, and macrophages were collected in Dulbecco's phosphate-buffered saline (DPBS; Cat. No. 14190136, Invitrogen, Carlsbad, CA, USA). Cells were centrifuged and resuspended in Dulbecco's modified Eagle medium/Nutrient Mixture F-12 (DMEM/F-12; Cat. No. 10565018, Invitrogen) supplemented with 10% (*v*/*v*) fetal bovine serum (Cat. No. 16000036, Invitrogen) and 1% penicillin/streptomycin (Cat. No. 15140148, Invitrogen). One mouse yielded enough cells for one experimental replicate; 1×10^5 cells/well were allowed to adhere for 1.5 h before being washed with DPBS. 10(*Z*)-hexadecenoic acid was dissolved in DMEM/F-12 with 0.5% (*v*/*v*) dimethyl sulfoxide (Cat. No. D8418, Sigma-Aldrich). The macrophages were incubated with either 10(*Z*)-hexadecenoic acid (0.4, 4, 20, 100, 500, 1000 μM) or DMEM/F-12 for 1 h before being stimulated with either 1 μg/mL LPS (serotype 0127:B8, Sigma-Aldrich, St. Louis, MO, USA) or DMEM/F-12. Culture supernatants were collected at 6, 12, and 24 h post-stimulation.

Cytokine measurements

Cell culture supernatants (10 μL) from freshly isolated peritoneal macrophages were diluted 1:200, and IL-6 was measured using ELISA (Cat. No. 431304, BioLegend, San Diego, CA, USA). The assay has a minimal detectable concentration of 2 pg/mL IL-6. All samples were measured using duplicate wells in the ELISA.

Cytotoxicity assay

Cytotoxicity was determined using the sulforhodamine B (SRB) colorimetric assay, as previously described (Vichai and Kirtikara 2006). Briefly, without removing the culture media, cells were fixed by adding cold trichloroacetic acid and incubated at 4 °C for 1 h. The plates were washed with slow-running tap water and set out to dry overnight. Then, 0.057% SRB (Cat. No. AC333130050, Fisher, Pittsburgh, PA, USA), solubilized in 10 mM Tris (Cat. No. BP153, Fisher), was added to each well. After 30 min, plates were washed with 1% acetic acid and set out to dry overnight. SRB was measured at 490 nm on a Synergy HT microplate reader (Part Number 7091000, Biotek, Winooski, VT, USA). Cell viability was expressed as the ratio of experimental and control growth.

Ligands

For studies using reporter gene assays following transfection of COS1 cells, rosiglitazone, troglitazone, and WY14643 were obtained from Alexis Biochemicals (San Diego, CA, USA); ATRA and AM580 were obtained from Sigma-Aldrich. In addition, GW9662 was a gift from T.M. Willson (GlaxoSmithKline, Brentford, UK). For experiments using freshly isolated peritoneal macrophages, GW 6471 (Cat. No. 4618), GW 9662 (Cat. No. 1508), GSK 0660 (Cat. No. 3433), WY 14643 (Cat. No. 1312), rosiglitazone (Cat. No. 5325), and GW 0742 (Cat. No. 2229) were obtained from Tocris Bioscience (Bristol, UK).

Transfections and reporter gene assays

Cells were transfected with the following receptor and reporter constructs: Gal4-PPARα-LBD, Gal4-PPARγ-LBD, Gal4-PPARδ-LBD, Gal4-RARα-LBD, pMH100-TK-luc, and pCMX-β-galactosidase (Chen and Evans 1995). All transfection experiments were performed with COS1 cells using polyethylenimine (Sigma-Aldrich) reagent (Szatmari et al. 2006). After 6–8 h of the transfection, the medium was replaced with DMEM medium containing the indicated ligands or vehicle (as control) (Chen and Evans 1995; Benko et al. 2003). Cells were lysed and assayed for reporter expression 18 h after transfection. The luciferase assay system (Promega, Madison, WI, USA) was used as described previously (Nagy et al. 1999). Measurements were carried out with a Wallac Victor-2, multilabel counter. Luciferase activity of each sample was normalized to the β-galactosidase activity.

RNA extraction and library preparation

Total RNA content of 1×10^5 macrophages pretreated for 1 h with 200 μM 10(*Z*)-hexadecenoic acid (utilizing separate macrophage preparations from $n = 3$ mice) or vehicle (utilizing separate macrophage preparations from $n = 3$ mice) and stimulated with 1 μg/mL LPS was extracted using TRI Reagent® (Cat. No. T9424, Sigma-Aldrich) according to the manufacturer's instructions. The RNA input was quantified on a Qubit™ 3.0 Fluorometer (Cat. No. Q33216, Thermo Fisher, Waltham, MA, USA) to ensure there was sufficient starting material. The RNA sequencing libraries were generated with the NEBNext rRNA Depletion Kit (Cat. No. E6310, New England BioLabs) in order to enrich the samples in mRNA and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (Cat. No. 7240, New England BioLabs). Briefly, mRNA was purified from 100 ng of total RNA, fragmented, and converted to double-stranded cDNA. Barcodes were ligated to the cDNA fragments, and prior to PCR enrichment of the library, the cDNA product was quantified on a Qubit 3.0 Fluorometer (Thermo Fisher). The integrity of the purified oligo libraries was evaluated on an Agilent Bioanalyzer 2100 (Cat. No. G2939BA, Agilent, Santa Clara, CA, USA).

Sequencing

Libraries were sequenced at the Next Generation Sequencing Facility at the University of Colorado Boulder. The libraries were multiplexed and sequenced on an Illumina HiSeq 2000 Sequencing System (Cat. No. SY-401-1001, Illumina, San Diego, CA, USA). For each sample, paired-end 100-bp reads were sequenced using V3 chemistry.

RNA read processing, mapping, and differential expression

Quality analysis of sequencing data was done using FastQC. The adaptors and low-quality raw reads were cut with Trimmomatic (version 0.32) (Bolger et al. 2014). The reads were aligned to the mouse genome, mm10 (University of California, Santa Cruz, CA, USA), using the TopHat2 sequence aligner (version 2.0.6) (Kim et al. 2013). Reads mapping to exon features were counted using HTseq (version 0.6.1) (Anders et al. 2015). The raw reads and count data have been deposited in the GEO database under accession number GSE125930. Differentially expressed genes were identified

using the R package, DESeq (version 1.28.0) (Anders and Huber 2010).

Statistical analysis

Data are presented as means ± SEM or means + SEM. Data were subjected to a normality test and one-way analysis of variance (ANOVA); Fisher's least significant difference (LSD) tests were performed as appropriate. A two-tailed p value ≤ 0.05 was considered significant. ELISA IL-6 data were analyzed using linear mixed effects models using the software package SPSS (version 21.0, SPSS Inc., Chicago, IL, USA). Network visualizations were created in Cytoscape (version 3.5.1) using an enrichment map plug-in (Merico et al. 2011).

Results

10(*Z*)-hexadecenoic acid decreases LPS-induced secretion of IL-6 in macrophages

To simulate inflammation, freshly isolated mouse peritoneal macrophages were challenged with LPS (1 μg/mL) ex vivo (outlined in Fig. 1). Macrophages that were cultured in the presence of 10(*Z*)-hexadecenoic acid (0, 0.4, 4, 20, 100, 500, 1000 μM) for 1 h prior to 1 μg/mL LPS stimulation secreted less IL-6 relative to macrophages cultured with media alone prior to LPS stimulation (Fig. 2a–c) ($F_{(1,\ 111)} = 15.20$, $p < 0.001$). This difference was observable as early as 6 h after LPS challenge and was sustained for at least 24 h. We selected the 6-, 12-, and 24-h time points for measurement of IL-6 as previous studies have shown increased IL-6 secretion using LPS-stimulated peritoneal macrophage cultures in mice at these time points, with linear increases in IL-6 up to the 24-h time point (Shacter et al. 1993; Wollenberg et al. 1993; Lin and Tang 2007; Lee et al. 2015; Arteaga Figueroa et al. 2017). The effect also appeared to be concentration and time dependent. Using a constrained logistic model on the relative secretion of IL-6, we estimated the EC_{50} to be 823, 115, and 190 μM at the 6-, 12-, and 24-h observations, respectively (Fig. 2). Post hoc pairwise comparisons of raw IL-6 values relative to paired media control values at the same time point ($n = 3$ per group) are presented in Table S1. This time and concentration dependence may indicate that a receptor-mediated transcriptional change is occurring. In contrast to the effects of 10(*Z*)-hexadecenoic acid on LPS-induced IL-6 secretion, it had no detectable effect on IL-6 secretion by itself (IL-6 was undetectable in all conditions; Fig. S1). We cannot exclude the possibility, however, that 10(*Z*)-hexadecenoic acid by itself had effects on IL-6 secretion that were below the limit of detectability of the assay used (i.e., 2 pg/mL). Cell viability was measured to dispel the possibility that senescence or cell death was contributing to reduced IL-6 secretion. Using a high concentration (1 mM) of 10(*Z*)-hexadecenoic acid, less than 40% of macrophages were viable at most time points. However, macrophages cultured with all other concentrations of 10(*Z*)-hexadecenoic acid studied (i.e., 10, 50, 125, 250, and 500 μM) were as viable as media controls (Fig. S2).

Treatment with 10(*Z*)-hexadecenoic acid induces a broad anti-inflammatory transcriptional profile in LPS-stimulated macrophages

To explore the potential effects of 10(*Z*)-hexadecenoic acid on transcriptional responses in LPS-stimulated macrophages, we used RNA-seq. Murine peritoneal macrophages were incubated with 200 μM 10(*Z*)-hexadecenoic acid or a media-only control condition for 1 h prior to stimulation with LPS. Using IL-6 as a measure for the suppressive activity of 10(*Z*)-hexadecenoic acid, we estimated the EC_{50} at 12 h to be 115 μM. The 200-μM concentration was chosen as it was sufficiently larger than the EC_{50}, but less than a concentration that would affect macrophage viability. After a 12 h exposure to LPS, the RNA was extracted and depleted of rRNA. We selected the 12-h time point for measurement of mRNA using RNA-seq as previous studies have shown increased IL-6 secretion using LPS-stimulated peritoneal macrophage cultures in mice at this time point, as well as the ability to suppress IL-6 mRNA expression at this time point by interfering with a TLR4–MyD88–BLT2–Nox1–ROS–NF-κB pathway leading to IL-6 secretion (Lee et al. 2015). The cDNA libraries were sequenced in a 100-bp paired-end experiment generating 51–63 million reads per sample (Table S2; Fig. S3).

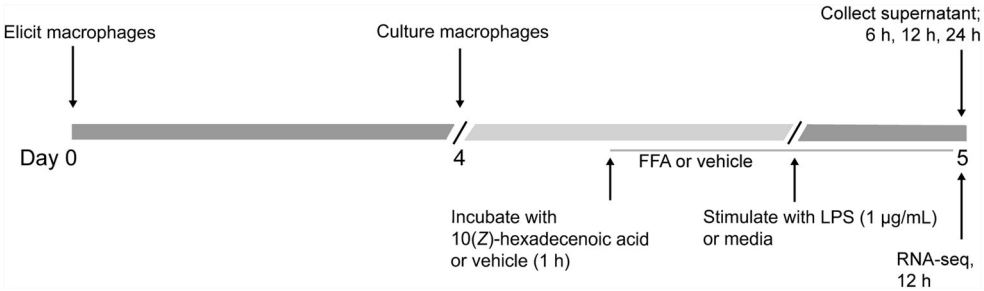

Fig. 1 Experimental timeline for ex vivo macrophage stimulation. Abbreviations: FFA, free fatty acid; LPS, lipopolysaccharide

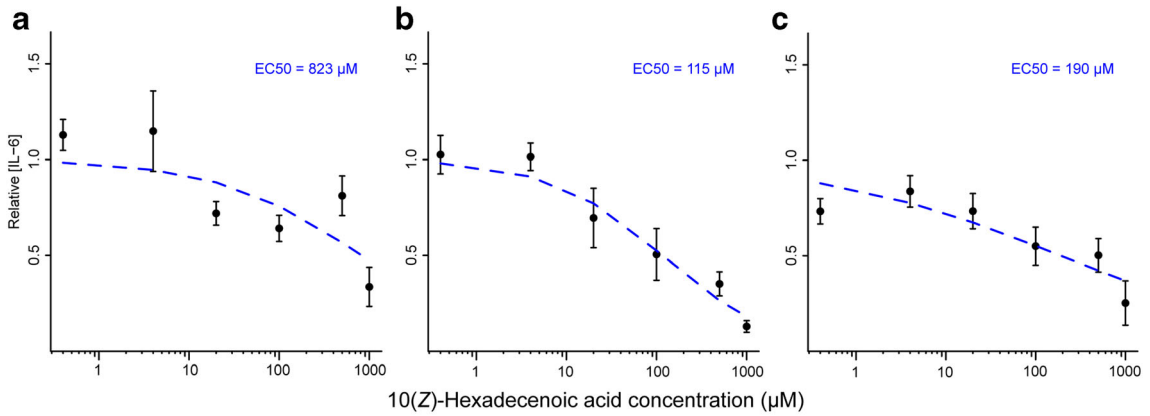

Fig. 2 Anti-inflammatory effects of 10(*Z*)-hexadecenoic acid in freshly isolated murine peritoneal macrophages. Freshly isolated murine peritoneal macrophages were incubated for 1 h with synthetic 10(*Z*)-hexadecenoic acid (0, 0.4, 4, 20, 100, 500, 1000 μM), then challenged with lipopolysaccharide (LPS; 1 μg/mL). Cell supernatants were collected at **a** 6 h, **b** 12 h, and **c** 24 h after LPS challenge. Interleukin (IL) 6 concentrations in the supernatant were determined using enzyme-linked immunosorbent assay (ELISA) and reported relative to media-only controls ($n = 6$ replicates, with each replicate using different freshly isolated peritoneal macrophages; each sample was run using duplicate wells in the ELISA). Data were fit with a logistic function, which was used to estimate the EC_{50}. Data are expressed as mean ± SEM

For differential expression, we examined LPS-stimulated macrophages pretreated with either 10(*Z*)-hexadecenoic acid or vehicle (GSE125930). Differentially expressed transcripts were identified using the R package, DESeq (Anders and Huber 2010). A total of 203 genes were found to be differentially expressed with an FDR-adjusted $p < 0.1$ (Table S3). Of the 203 differentially expressed genes, 109 were downregulated in the 10(*Z*)-hexadecenoic acid condition, and 20% of those genes were associated with proinflammatory processes (Table S4). The top 20 differentially expressed genes are reported in Fig. 3a. Consistent with the ex vivo macrophage experiments measuring IL-6 protein with ELISA, the second most significantly differentially expressed transcript was IL-6 (Table S3).

PPARα-regulated genes are associated with 10(*Z*)-hexadecenoic acid treatment in LPS-stimulated macrophages

To better understand the pathways affected by 10(*Z*)-hexadecenoic acid treatment, the list of 203 differentially expressed genes was queried against the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al. 2009). Within the top 40 most significantly enriched KEGG pathways, 34 (i.e., 85%) were related to disease or inflammation (Table S5). In addition, 32 of the top 40 most significantly enriched KEGG pathways (i.e., 80%) were exclusively enriched for genes that were significantly downregulated by treatment with 10(*Z*)-hexadecenoic acid. Among these most significantly affected pathways, there was a wide scope of immunological context, which included infections, diseases, cytokine signaling, and various inflammatory pathways. The top 5 pathways with genes that were exclusively downregulated by treatment with 10(*Z*)-hexadecenoic acid are reported in Fig. 3c.

While the majority of pathways with genes affected by 10(*Z*)-hexadecenoic acid involved genes that were exclusively downregulated by 10(*Z*)-hexadecenoic acid, some pathways involved genes that were exclusively upregulated by 10(*Z*)-hexadecenoic acid. Of the top 40 pathways, 7 (i.e., 17.5%) pathways were exclusively enriched for genes that were significantly upregulated by treatment with 10(*Z*)-hexadecenoic acid. Overall, of 203 genes that were differentially expressed following treatment with 10(*Z*)-hexadecenoic acid, 93 genes (46%) were upregulated. The pathways with detectable enrichment involved regulation of lipolysis in adipocytes, glycerolipid metabolism, circadian entrainment, PPAR signaling pathway, and extracellular matrix–receptor interaction (Fig. 3b). The top 5 pathways with genes that were exclusively upregulated by treatment with 10(*Z*)-hexadecenoic acid are reported in Fig. 3b. The PPAR signaling pathway was among the top 5 most-enriched KEGG pathways with genes that were exclusively upregulated by treatment with 10(*Z*)-hexadecenoic acid (Fig. 3b).

In a secondary analysis, rather than rely on a subset of genes for biological interpretation, we used all expression data in Gene Set Enrichment Analysis (GSEA). We queried all detected transcripts against the KEGG pathways database (c2.cp.kegg.v6.2), and the top 5 enriched gene sets for the 10(*Z*)-hexadecenoic acid phenotype were as follows: "peroxisome" (KEGG: hsa04146), a main site of fatty acid oxidation via the β-oxidation cycle; "ppar_signaling_pathway" (KEGG: hsa03320); "citrate_cycle_tca_cycle" (KEGG: hsa00020); "fatty_acid_metabolism" (KEGG: hsa00071); and "propanoate_metabolism" (KEGG: hsa00640) (Table S6). Of potential interest, four of these KEGG

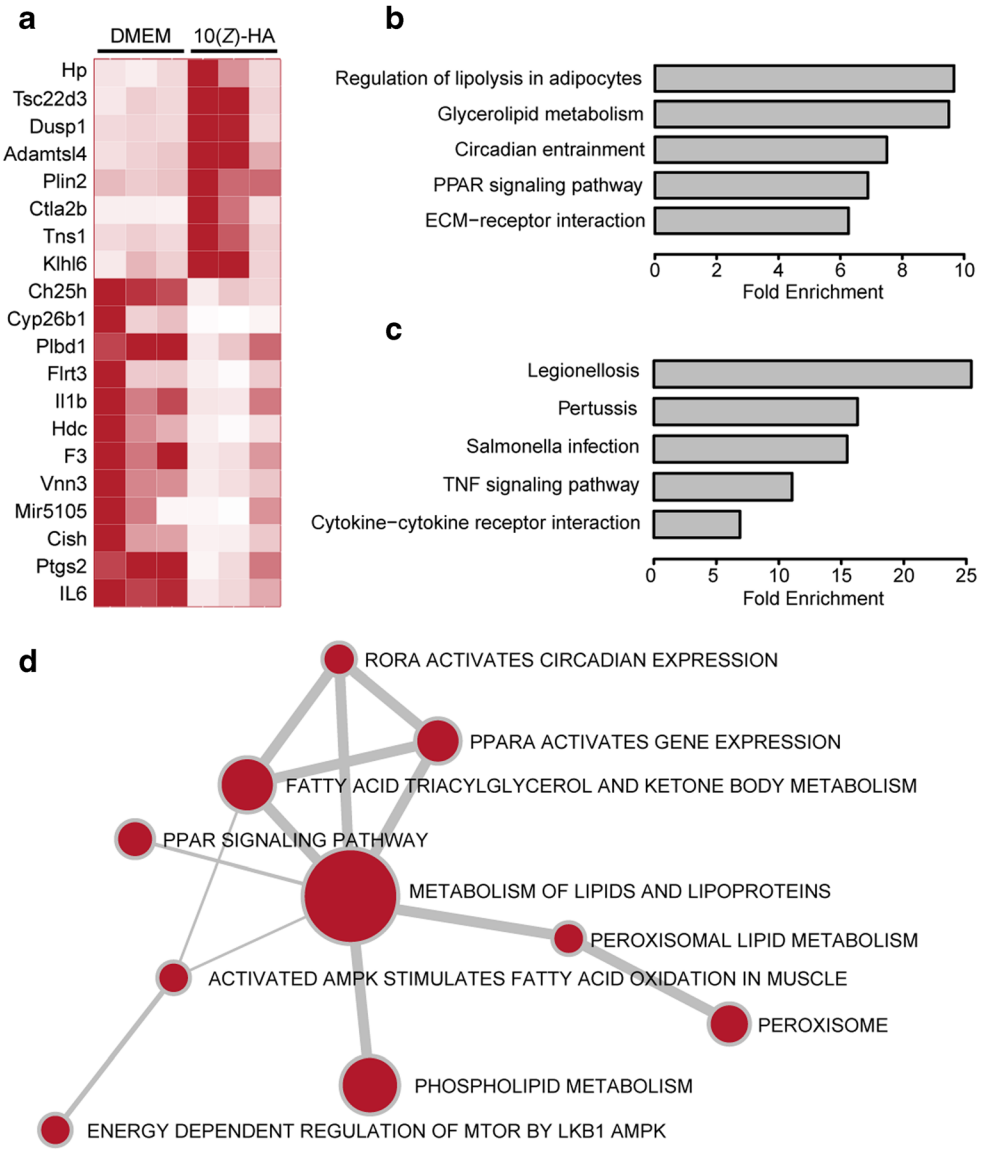

Fig. 3 Gene networks upregulated following pretreatment with 10(*Z*)-hexadecenoic acid in LPS-stimulated macrophages suggest anti-inflammatory effects are mediated by PPARα. Murine peritoneal macrophages were treated with either 10(*Z*)-hexadecenoic acid (200 μM) or vehicle. Following a 12-h period after stimulation with lipopolysaccharide (LPS), total RNA content was measured using RNA-seq. **a** Heat map of the top 20 differentially expressed transcripts. **b**, **c** Genes significantly **b** upregulated or **c** downregulated following treatment with 10(*Z*)-hexadecenoic acid were separately queried on the Database for Annotation, Visualization and Integrated Discovery (DAVID). **b** The top 5 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched for genes upregulated following pretreatment of LPS-stimulated macrophages with 10(*Z*)-hexadecenoic acid, relative to media pretreated, LPS-stimulated macrophages. **c** The top 5 KEGG pathways enriched for genes downregulated following pretreatment of LPS-stimulated macrophages with 10(*Z*)-hexadecenoic acid, relative to media pretreated, LPS-stimulated macrophages. **d** Pathway analysis using the entire transcriptional data set was performed with Gene Set Enrichment Analysis (GSEA). Pathways enriched for genes upregulated following pretreatment of LPS-stimulated macrophages with 10(*Z*)-hexadecenoic acid, relative to media-pretreated, LPS-stimulated macrophages, were visualized in a network built by their gene set overlap. The size of the network node represents the number of genes shared between the particular gene set and the transcription data. The weight of network edges represents the degree of gene set overlap. In the largest cluster of pathways enriched in genes upregulated with 10(*Z*)-hexadecenoic acid, lipid metabolism and peroxisome proliferator-activated receptors (PPARs) were implicated as some of the more salient pathways. Abbreviations: Adamtsl4, thrombospondin repeat-containing protein 1; AMPK, 5′ AMP-activated protein kinase; Ch25h, cholesterol 25-hydroxylase; Cish, cytokine-inducible SH2 containing protein; Ctla2b, cytotoxic T-lymphocyte-associated protein 2-beta; Cyp26b1, cytochrome P450 family 26 subfamily B member 1; Dusp1, dual specificity phosphatase 1; ECM, extracellular matrix; F3, coagulation factor III; Flrt3, fibronectin leucine-rich transmembrane protein 3; Hdc, histidine decarboxylase; Hp, haptoglobin; Il1b, interleukin 1 beta; Il6, interleukin 6; LKB1, liver kinase B1; Mir5105, microRNA 5105; MTOR, mechanistic target of rapamycin kinase; Plbd1, phospholipase B domain containing 1; Plin2, perilipin 2; PPAR, peroxisome proliferator-activated receptor; PPARA, peroxisome proliferator-activated receptor alpha; Ptgs2, prostaglandin-endoperoxide synthase 2; RORA, RAR-related orphan receptor A; TNF, tumor necrosis factor; Tns1, tensin 1; Tsc22d3, Tsc22 domain family member 3; Vnn3, vanin 3

pathways, "peroxisome" (KEGG: hsa04146), "ppar_signaling_pathway" (KEGG: hsa03320), "fatty_acid_metabolism" (KEGG: hsa00071), and "propanoate_metabolism" (KEGG: hsa00640), were also found to be enriched in livers from 24 h fasted PPARα$^{+/+}$ relative to PPAR$^{-/-}$ mice, while "peroxisome" (KEGG: hsa04146), "ppar_signaling_pathway" (KEGG: hsa03320), and "fatty_acid_metabolism" (KEGG: hsa00071) were found to be enriched in livers from wild-type mice treated with the PPARα agonist Wy14643, relative to vehicle (Kersten 2014). Together, these studies support a convergence of 10(*Z*)-hexadecenoic acid effects on PPARα signaling pathways induced by physiological or pharmacological stimuli. Propionate is one of the short-chain fatty acids, which are emerging as key mediators and regulators of host–microbe cross-talk, with a significant impact on host metabolism, including as an energy source (Hoyles et al. 2018). All five gene sets were significant with an unadjusted p value < 0.05 but failed to reach significance using FDR-adjusted p values; nevertheless, the overall pattern is consistent with a modulation of lipid metabolism. In a network visualization of gene set overlap between all detected pathways using GSEA, PPARs and peroxisomal lipid metabolism were prominent vertices (Fig. 3d). We also searched against the collection of transcription factor binding motifs (c3.tft.v6.0), which revealed enrichment for CREB, Gfi1, and PPARα *cis*-regulatory motifs upstream of the genes upregulated with 10(*Z*)-hexadecenoic acid treatment in LPS-stimulated macrophages (Table S7). Again, these were nominally significant (i.e., $p < 0.05$; $q > 0.05$), but these findings bolster PPARs, and specifically PPARα, as a potential receptor mediating anti-inflammatory effects of 10(*Z*)-hexadecenoic acid.

Downstream signaling of TLR4 is inhibited with 10(*Z*)-hexadecenoic acid treatment

NF-κB is one of three major transcription factors downstream of LPS-induced activation of toll-like receptor 4 (TLR4), the other two being IRF3 and AP-1 (Kawasaki and Kawai 2014). Using all expression data in GSEA, we found that pretreatment with 10(*Z*)-hexadecenoic acid, relative to treatment with vehicle, prior to LPS stimulation, downregulated signaling pathways downstream from TLR4, such as NF-κB and IRF3, but not AP-1. In a network visualization of the most significant pathways (FDR < 0.1), there is a bifurcation at the "ZHOU_INFLAMMATORY_RESPONSE_LPS" node representing the NF-κB and IRF3 responses (Fig. 4a). Among all nodes in this network, we counted and ranked the occurrence of enriched transcripts. The counts for the highest ranking transcripts were categorized into either NF-κB-regulated responses or IRF-regulated responses (Fig. 4b). We also examined enrichment for transcription factor motifs and detected enrichment for NF-κB, IRF1, IRF2, and IRF_Q6 among others, in transcripts associated with the vehicle-treated, LPS stimulated group (Table S8). Alternatively, this can be understood to mean that mRNA transcripts that are located near those transcription factor binding sites are downregulated in 10(*Z*)-hexadecenoic acid-treated, LPS-stimulated macrophages. To better understand the classification of the IRF_Q6 gene set ($N = 242$ genes) and differentially expressed genes ($q < 0.1$, $N = 203$ genes), they were both queried against the Interferome database (Rusinova et al. 2013). There are three types of interferons (IFNs), namely type I (composed of α, β, κ, ε, and ω subtypes), type II (IFNγ), and type III (IFNλ; also called IL-28 and IL-29), which are distinguished by having distinct genetic loci, amino acid sequence homology, and specific cognate receptors (Pestka et al. 2004). This analysis revealed that a vast majority of the differentially expressed genes are regulated by both type I and II interferon responses (Fig. 4c), consistent with the hypothesis that 10(*Z*)-hexadecenoic acid alters TLR4, IRF3, and interferon signaling. Of note, cells infected with *Mycobacterium tuberculosis* induce type I interferons, including IFNα and IFNβ, which are thought to promote infection with *M. tuberculosis* (Travar et al. 2016). Using enrichment tools, like DAVID and GSEA, these RNA-seq data suggest that both NF-κB and IRF3 pathways are downregulated in LPS-stimulated macrophages when treated with 10(*Z*)-hexadecenoic acid.

The anti-inflammatory effects of 10(*Z*)-hexadecenoic acid are mediated through PPARα

10(*Z*)-hexadecenoic acid specifically activates PPARα

Fatty acids can modulate inflammation via the activation of nuclear hormone receptors (Chinetti et al. 2000; Kidani and Bensinger 2012). Therefore, we assessed the nuclear receptor activation capacity of (1) the triacylglycerol (TAG), 1,2,3-tri [*Z*-10-hexadecenoyl]glycerol; (2) the monoacylglycerol (MAG), 1-[*Z*-10-hexadecenoyl]glycerol; and (3) the free fatty acid (FFA), 10(*Z*)-hexadecenoic acid. We conducted reporter gene assays via the transfection of COS1 cells using GAL4-fusion ligand binding domains (LBDs) of various lipid-activated nuclear receptors (PPARα-LBD, PPARγ-LBD, PPARδ-LBD, and RARα-LBD) along with a plasmid carrying MH100-TK-luciferase reporter (Chen and Evans 1995). Transfected cells were incubated with TAG, MAG, or FFA for 18 h, and relative luciferase activity, normalized to β-galactosidase activity, was measured. Each reporter transfection was validated with the respective receptor agonist (PPARα, WY-14643; PPARγ, rosiglitazone (RSG); PPARδ, GW1516; RARα, AM580). Both the MAG and FFA, at concentrations of 80 μM, reliably increased PPARα-, but not PPARγ-, PPARδ-, or RARα-regulated reporter expression (Fig. 5a–d). The triglyceride had no effect

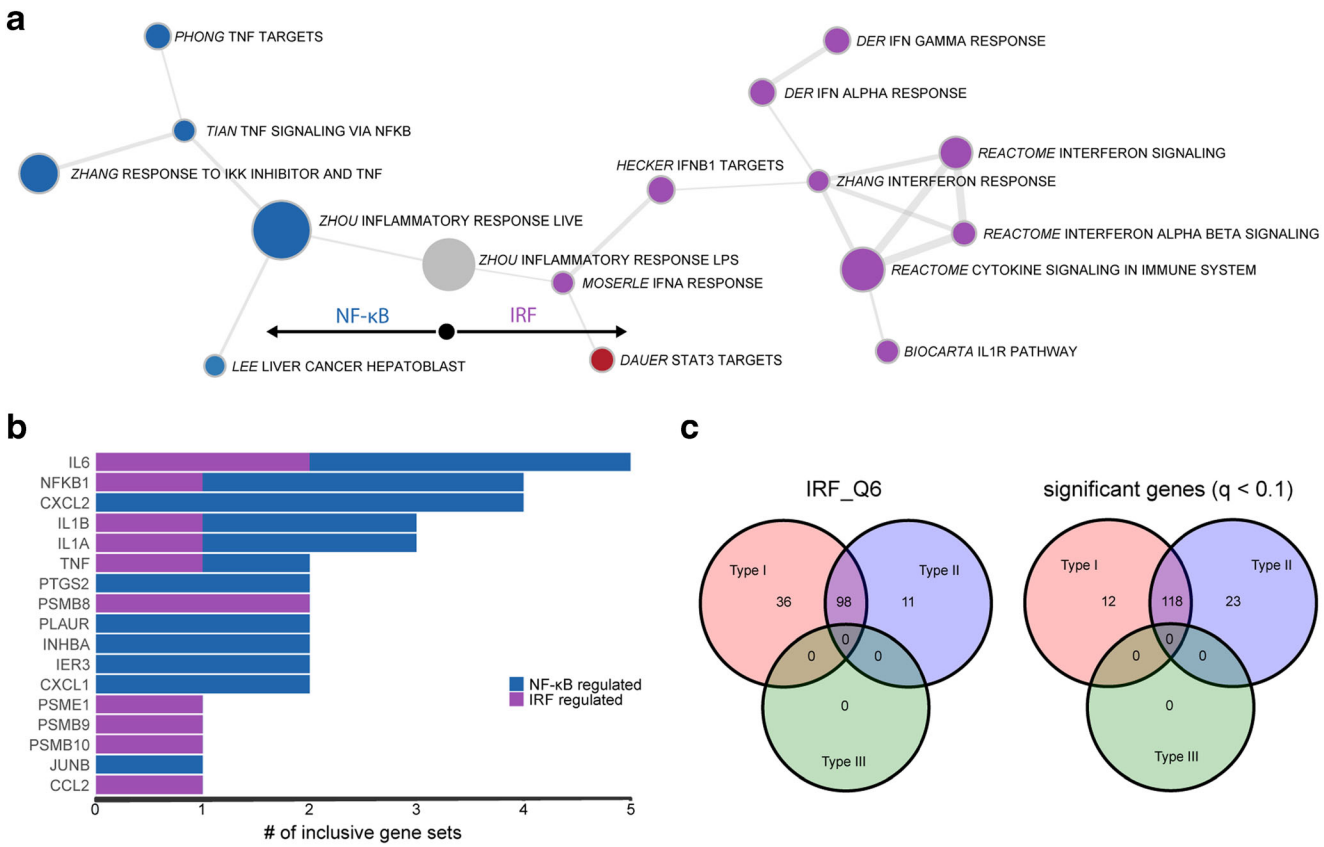

Fig. 4 10(*Z*)-hexadecenoic acid suppresses expression of transcription factors downstream of TLR4. Murine peritoneal macrophages were treated with either 10(*Z*)-hexadecenoic acid (200 μM) or vehicle for 1 h, then challenged with lipopolysaccharide (LPS; 1 μg/mL). Following a 12-h period after stimulation with LPS, mRNA was measured using RNA-seq. **a** From the Gene Set Enrichment Analysis (GSEA; c2.all.v6.2), pathways enriched with genes downregulated following pretreatment of LPS-stimulated macrophages with 10(*Z*)-hexadecenoic acid, relative to media-pretreated, LPS-stimulated macrophages, were visualized in a network built by their gene set overlap. The size of the network node indicates the number of genes shared between the particular gene set and the transcription data from our study. The weight of network edges indicates the degree of gene set overlap between nodes. The color of the node indicates whether the genes in the gene set were downregulated in NF-κB pathways (blue), downregulated in IRF pathways (purple), ambiguously downregulated (gray), or upregulated (red) in the 10(*Z*)-hexadecenoic acid condition. **b** Among the leading edges of enriched pathway gene sets, the occurrence of high ranking genes in either the NF-κB-regulated network (blue) or IRF-regulated network (purple) (corresponding to data illustrated in panel **a**) are reported. **c** Genes included in the IRF_Q6 gene set (left; i.e., genes having at least one occurrence of the transcription factor binding site V$IRF_Q6 (v7.4 TRANSFAC) in the regions spanning up to 4 kb around their transcription starting sites) and the significant 10(*Z*)-hexadecenoic acid-dependent differentially expressed genes with $q < 0.1$ (right) were queried against the Interferome database (v2.0) to identify their association with known interferon responses. The majority of genes in both gene sets are attributed to both type I and type II interferon responses. Abbreviations: CCL2, C-C motif chemokine ligand 2; CXCL1, C-X-C motif chemokine ligand 1; CXCL2, C-X-C motif chemokine ligand 2; IER3, immediate early response 3; IFN, interferon; IFNA, interferon alpha; IFNB1, interferon beta 1; IKK, inhibitor of nuclear factor kappa B kinase; INHBA, inhibin subunit beta A; IL1A, interleukin 1 alpha; IL1B, interleukin 1 beta; IL1R, interleukin 1 receptor; IL6, interleukin 6; JUNB, junB proto-oncogene, AP-1 transcription factor subunit; LPS, lipopolysaccharide; NFKB, nuclear factor kappa B; NFKB1, nuclear factor kappa B subunit 1; PLAUR, plasminogen activator, urokinase receptor; PSMB8, proteasome subunit beta 8; PSMB9, proteasome subunit beta 9; PSMB10, proteasome subunit beta 10; PSME1, proteasome activator subunit 1; PTGS2, prostaglandin-endoperoxide synthase 2; STAT3, signal transducer and activator of transcription 3; TNF, tumor necrosis factor

(Fig. 5a–d). Together, these results demonstrate that 10(*Z*)-hexadecenoic acid and its monoacylglycerol form selectively activate the PPARα receptor.

PPARα is required for anti-inflammatory effects of 10(*Z*)-hexadecenoic acid

Next, we investigated if this interaction was necessary for inhibiting LPS-stimulated release of IL-6. Agonists and antagonists of each PPAR were used to test if PPARα has a singular role in this process. The agonists and antagonists and their receptor specificities are listed in Table S9. Macrophages were incubated with a single PPAR antagonist for 1 h prior to treatment with either 200 μM 10(*Z*)-hexadecenoic acid or a PPAR agonist complementary to its respective PPAR antagonist. After another 1 h incubation period, the cells were stimulated with LPS (1 μg/mL), and IL-6 was measured 12 h later. Only

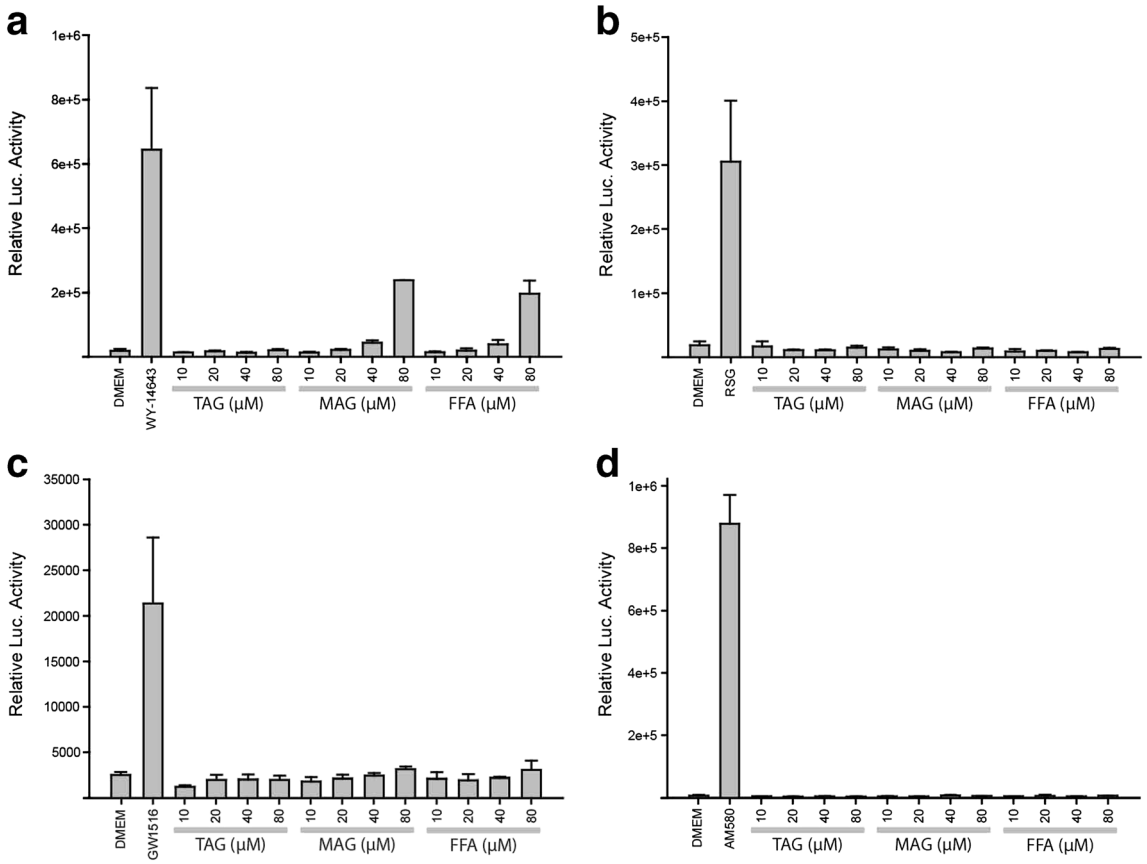

Fig. 5 Analysis of the effects of *M. vaccae*-derived lipids on peroxisome proliferator-activated receptor (PPAR) α, PPARγ, PPARδ, and retinoic acid receptor (RAR) α signaling in transfection assays using COS-1 cells. **a** Relative activity of PPARα following incubation with the 1,2,3-tri [*Z*-10-hexadecenoyl] glycerol (PI-70; TAG), monoacylglycerol, 1-[*Z*-10-hexadecenoyl] glycerol (PI-69; MAG), or 10(*Z*)-hexadecenoic acid (PI-71; FFA) for 18 h, expressed as relative luciferase activity, normalized to β-galactosidase activity. **b** Relative activity of PPARγ. **c** Relative activity of PPARδ. **d** Relative activity of RARα. Abbreviations and concentrations: AM580 (RARα-specific agonist, 100 nM); GW1516 (PPARδ agonist, 1 μM); RSG, rosiglitazone (PPARγ agonist, 2.5 μM); troglitazone (PPARγ agonist, 10 μM); WY-14643 (PPARα agonist, 2 μM). Data are representative of two to three replicates per experiment

with the PPARα antagonist, GW 6471, could the anti-inflammatory effects of 10(*Z*)-hexadecenoic acid be significantly reversed (Fig. 6a). The effects of the PPARγ and PPARδ antagonists were comparable to media (Fig. 6a). These results suggest a selective interaction between 10(*Z*)-hexadecenoic acid and PPARα, as the PPARα antagonist, GW 6471, had no effect on macrophage viability (Fig. S4), while it was effective in reversing the anti-inflammatory effects of the PPARα agonist, WY-14643, as measured by IL-6 secretion in LPS-stimulated macrophages (Fig. S5). To further explore the role of PPARα in the anti-inflammatory effects of 10(*Z*)-hexadecenoic acid, we repeated the assay with freshly isolated peritoneal macrophages from adult male C57BL/6J wild-type and PPARα$^{-/-}$ mice. As expected, 10(*Z*)-hexadecenoic acid suppressed LPS-stimulated IL-6 in macrophages from wild-type C57BL/6J mice, but this effect was absent in macrophages from PPARα KO mice (Fig. 6b). This indicated a full reversal of the anti-inflammatory effect of 10(*Z*)-hexadecenoic acid and the necessity of PPARα in mediating the effect.

Discussion

Here, we characterized the monounsaturated C16 free fatty acid, 10(*Z*)-hexadecenoic acid, derived from *M. vaccae* NCTC 11659, a saprophytic bacterium with anti-inflammatory and immunoregulatory properties that previously has been shown to prevent stress-induced exaggeration of peripheral inflammation and neuroinflammation and to prevent stress-induced exaggeration of anxiety- and fear-related defensive behavioral responses. In addition, we showed that 10(*Z*)-hexadecenoic acid induced a broad transcriptional repression of inflammatory gene markers (see, for example, Tables S10–11) and suppressed IL-6 secretion from freshly isolated, LPS-stimulated, murine peritoneal macrophages. Furthermore, we showed that

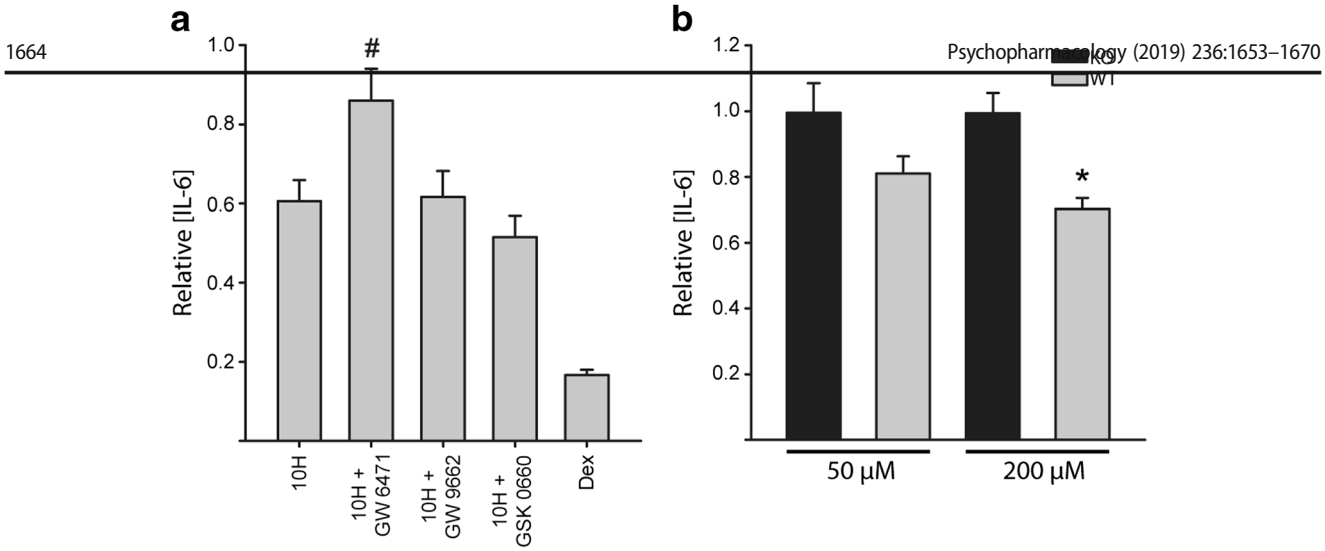

Fig. 6 PPARα is required for suppression of LPS-induced inflammation in macrophages. A peroxisome proliferator-activated receptor (PPAR) α, γ, or δ antagonist (GW 6471, GW 9662, GSK 0660, respectively) or vehicle was applied to murine peritoneal macrophages followed by treatment with either 10(*Z*)-hexadecenoic acid (200 μM), vehicle, or dexamethasone (Dex; 10 μM), then stimulated with lipopolysaccharide (LPS; 1 μg/mL). **a** After 12 h, interleukin (IL) 6 was measured in the cell supernatant and reported relative to vehicle controls. **b** The necessity of PPARα was shown in a PPARα knockout (KO) model. Murine peritoneal macrophages from PPARα$^{-/-}$ or WT mice were incubated with either 10(*Z*)-hexadecenoic acid (50 or 200 μM) or vehicle, then stimulated with LPS (1 μg/mL). #$p < 0.05$, Fisher's least significant difference (LSD), relative to cells only treated with 10(*Z*)-hexadecenoic acid. *$p < 0.05$ relative to KO. Abbreviations: 10H, 10(Z)-hexadecenoic acid; Dex, dexamethasone; IL-6, interleukin 6; KO, PPARα knockout; WT, wild type

both the monoacylated glycerol, 1-[*Z*-10-hexadecenoyl] glycerol and 10(*Z*)-hexadecenoic acid, activated PPARα signaling, as measured by transfection assays. Finally, we showed that PPARα antagonists prevented the anti-inflammatory effects of 10(*Z*)-hexadecenoic acid in macrophages, while the ex vivo effects of the lipid were absent in macrophages isolated from PPARα-deficient mice.

Here, we focused on the effects of 10(*Z*)-hexadecenoic acid on peritoneal macrophages. Based on a number of lines of evidence, the effects of 10(*Z*)-hexadecenoic acid actions on peritoneal macrophages may have important implications for CNS immunity and subsequent behavioral outcomes. Intraperitoneal administration of LPS is known to induce priming of hippocampal microglia and worsen CNS outcomes (Cunningham 2005, 2013; Cunningham et al. 2009). Although the mechanisms through which peripheral inflammation signals to the CNS to induce microglial priming and neuroinflammatory responses are not entirely clear, a number of potential signaling mechanisms have been proposed. These include (1) entry of cytokines into the brain at circumventricular organs that have a reduced blood–brain barrier; (2) binding of cytokines to cerebral vascular endothelium, inducing the secretion of central neuroinflammatory mediators; (3) carrier-mediated transport of immune signals into the brain, across the blood–brain barrier; (4) migration of proinflammatory monocytes from the periphery to the CNS; and (5) activation of peripheral afferent nerves, including vagal and nonvagal pathways (Watkins et al. 1995; Maier et al. 1998; Maier 2003; Miller et al. 2010; Miller and Raison 2016).

Together, these data support the hypothesis that bacterially derived 10(*Z*)-hexadecenoic acid may induce a form of macrophage "inflammation anergy" (i.e., a condition characterized by the absence of the normal immune response to a particular antigen, see, for example, Smythies et al. 2005, 2010) through actions on PPARα. Peroxisome proliferator-activated receptors, PPARα, PPARγ, and PPARδ, are ligand-activated nuclear receptors, each of which acts as a heterodimer with retinoid X receptor (RXR), with potent anti-inflammatory properties, through interference with proinflammatory transcription factor pathways (Chinetti et al. 2003). PPARα$^{-/-}$ mice have increased vulnerability to chemically induced colitis, experimental autoimmune encephalitis (EAE, a model of multiple sclerosis), and experimentally induced allergic asthma, consistent with the hypothesis that endogenous PPARα suppresses inflammatory signaling in these models (for review, Bensinger and Tontonoz 2008). Activation of PPARα in macrophages inhibits the production of proinflammatory response markers, including IL-6, IL-1β, TNF, and inducible nitric oxide synthase (Xu et al. 2005; Paukkeri et al. 2007). Interaction between PPARα and TLR4 signaling has been observed in other endogenous systems, like vascular smooth muscle cells, where responses to activation of TLR4 with LPS are mitigated by a PPARα agonist (Ji et al. 2010). The anti-inflammatory effects were mediated, in part, by a reduction of tissue inhibitor of metalloprotease-1 (TIMP-1), which was also reduced in our study (Table S3). PPARα-mediated inhibition of TLR4 signaling has also been shown in enteric glial cells (Esposito et al. 2014), and a potential downstream target of PPARα-mediated suppression, TRIF,

is required for LPS-induced activation of microglia (Burfeind et al. 2018). TRIF KO mice have attenuated expression of *Il6*, *Ccl2*, and *Cxcl2*, which were all also suppressed in our study (Table S3), in the hypothalamus after peripheral LPS stimulation (Burfeind et al. 2018). Furthermore, bacterially derived agonists of PPARs have potential for modulation of host-acquired immunity; PPARs have been found to regulate T-cell survival, activation, and $CD4^+$ T-helper cell differentiation into the Th1, Th2, Th17, and Treg lineages (Choi and Bothwell 2012).

Synthesis of 10(*Z*)-hexadecenoic acid by mycobacteria may be an example of molecular mimicry of eukaryotic signaling. Endogenous host-derived agonists of PPARα include 16:1 isoforms of palmitoleic acid (Kliewer et al. 1997; Kota et al. 2005), a lipokine released from adipose cells. Palmitoleic acid localizes predominantly to nuclear fractions, consistent with a nuclear mechanism of action in host cells (Foryst-Ludwig et al. 2015), and is potently anti-inflammatory (Chan et al. 2015). In addition, the endocannabinoid, palmitoylethanolamide (PEA), acts as an agonist at PPARα (Verme et al. 2005; Guida et al. 2017). Of interest to trauma- and stressor-related psychiatric disorders, PEA induces potent antidepressant-like behavioral responses (Yu et al. 2011) and, through induction of cannabinoid 2 receptors, alters the phenotype of macrophages and microglia (Guida et al. 2017). Recent studies have demonstrated PEA increases the biosynthesis of allopregnanolone, an endocannabinoid, in the spinal cord, brainstem, hippocampus, and amygdala, effects that are associated with faster fear extinction learning and improvement of aggression in socially isolated mice (Sasso et al. 2012; Locci and Pinna 2017; Pinna 2018). Future studies should determine if 10(*Z*)-hexadecenoic acid is sufficient to induce the enhanced fear extinction learning previously demonstrated using whole, heat-killed *M. vaccae* (Fox et al. 2017), and to what extent these effects are mediated by PPARα.

Mycobacteria are unique in that they accumulate triacylglycerols as intracellular lipophilic inclusions. For example, *Mycobacterium smegmatis* accumulates triacylglycerols and the acyl chain composition varies depending on the growth medium (Garton et al. 2002). Monounsaturated fatty acids, $C_{16:1}$ hexadecenoic acid and $C_{18:1}$ octadecenoic acid, were found to be high when bacteria were grown in nutrient-rich Middlebrook 7H9 broth, relative to low-nitrogen Youmans' broth, but highest when bacteria were grown in Youmans' broth with monounsaturated oleic acid ((9*Z*)-octadec-9-enoic acid) supplementation. Thus, it is possible that mycobacteria synthesize and store triacylglycerols using environmental fatty acids as substrates, potentially for export to the cell envelope and release. If so, it may be possible to modify the immunoregulatory and anti-inflammatory potential of mycobacteria through modification of growth conditions.

Of potential importance, conjugated linoleic acids are bacterial metabolites. For example, specific members of the genus *Lactobacillus*, including *Lactobacillus reuteri* and *L. plantarum*, as well as bifidobacteria, mediate the conversion of dietary linoleic acid into immunomodulatory conjugated linoleic acids (Coakley et al. 2003; Lee et al. 2003; Ogawa et al. 2005; Kishino et al. 2013). Most of the conjugated linoleic acid produced is located in the extracellular space (~98%) (Lee et al. 2003; Roman-Nunez et al. 2007), suggesting that bacterially derived conjugated linoleic acids may be metabolic signaling molecules that modulate the host immune response. These bacterially derived fatty acid metabolites include 10-hydroxy-*cis*-12-octadecenoic acid (HYA), *cis*-9,*trans*-11-linoleic acid, *trans*-9,*cis*-11-linoleic acid, and *cis*-10,*trans*-12-linoleic acid (Lee et al. 2003; Miyamoto et al. 2015), among many others (Ogawa et al. 2005). Several of these bacterially derived fatty acid metabolites are potent PPARα agonists (IC_{50} values from 140 to 400 nM) (Moya-Camarena et al. 1999). Perhaps the closest analogue of 10(*Z*)-hexadecenoic acid identified here is *trans*-10-octadecenoic acid, produced by *L. plantarum* from linoleic acid (Kishino et al. 2013) or γ-linolenic acid (Ogawa et al. 2005). Although, to the best of our knowledge, the efficacy of *trans*-10-octadecenoic acid at PPARα receptors is not known, production of 10(*Z*)-hexadecenoic acid and diverse conjugated linoleic acids, which then act at host PPARα receptors, may be a general strategy of commensal organisms to suppress host immune responses, and promote symbiotic relationships with the host. Consistent with this hypothesis, macrophages lining the gut mucosa are anergic, characterized by an inability to mount proinflammatory responses, despite avid phagocytic activity (Smythies et al. 2005), while lung airway macrophages are immunoregulatory (Strickland et al. 1996; Soroosh et al. 2013; Duan and Croft 2014). Recent studies have also identified α-linolenic acid-derived bacterial metabolites, 13-hydroxy-9(*Z*),15(*Z*)-octadecadienoic acid (13-OH) and 13-oxo-9(*Z*),15(*Z*)-octadecadienoic acid (13-oxo), that induce differentiation of anti-inflammatory M2 macrophages through activation of G protein-coupled receptor 40 (GPR40) (Ohue-Kitano et al. 2018). Together, these data support the hypothesis that bacterially derived "postbiotic" compounds, including fatty acid metabolites, have important beneficial effects on the host via diverse host receptor signaling mechanisms.

Although we did not assess the effects of 10(*Z*)-hexadecenoic acid on DCs or immunoregulation, defined as the balance between regulatory and effector T cells, conjugated linoleic acid suppresses NF-κB signaling and IL-12 production in DCs through IL-10 production (Loscher et al. 2005). Exposure of murine DCs to conjugated linoleic acid suppresses their ability to promote differentiation of naïve T cells into Th1 and/or Th17 cells

in vitro following their adoptive transfer in vivo (Draper et al. 2014). Future studies should investigate the effects of 10(*Z*)-hexadecenoic acid on inflammatory signaling in macrophages, DCs, as well as on T-cell differentiation and function, the potential role of PPARα in these effects, and consequences for stress-induced exaggeration of anxiety- and fear-related behavioral responses.

Overall, our data suggest that chemical mimicry of eukaryotic signaling molecules may be common among environmental bacteria, including mycobacteria (Gebert et al. 2018), that are abundant in host mucosal surfaces (Macovei et al. 2015), and bacterially derived anti-inflammatory lipids have potential as a novel approach to therapeutic intervention in inflammatory disease and stress-related psychiatric disorders, where immunodysregulation and inappropriate inflammation have been identified as risk factors (Rohleder 2014; Langgartner et al. 2018).

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Author contributions G.S.B. and P.A.I. isolated and synthesized 1,2,3-tri[*Z*-10-hexadecenoyl]glycerol. W.X. and X.W. developed a synthesis for 10(*Z*)-hexadecenoic acid and synthesized the compound. Experimental design was done by D.G.S., R.M., G.S.B., G.A.W.R., L.R.B., and C.A.L. L.N. and P.A.I designed the PPAR luciferase-based transfection assay experiments. In vivo screening and experimentation was performed by R.M. and L.R.B. In vitro experiments using freshly isolated murine peritoneal macrophages were performed by D.G.S. Transfections and reporter gene assays were performed by I.S. and P.B. RNA-seq data processing and analysis was done by D.G.S., R.D.D., and M.A.A. Experimental design and preparation of the manuscript were done by D.G.S., R.M., G.S.B., L.N., G.A.W.R., L.R.B., and C.A.L.

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Compliance with ethical standards

All experimental protocols were consistent with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, Eighth Edition (The National Academies Press 2011), and the Institutional Animal Care and Use Committee at the University of Colorado Boulder approved all procedures. This work was covered under CU Boulder IACUC Protocol Numbers 2134-14MAY2018 and 2361-14MAY2018-DT. The research described here was conducted in compliance with The ARRIVE Guidelines: Animal research: reporting of in vivo experiments, originally published in PLOS Biology, June 2010 (Kilkenny and Altman 2010).

Conflict of interest Christopher A. Lowry serves on the Scientific Advisory Board of Immodulon Therapeutics Ltd. Dr. Robin Dowell is a founder and scientific advisor of Arpeggio Biosciences.

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