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

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64 the Illumina sequencing.

65 **CONFLICTS OF INTEREST**

66 Christopher A. Lowry serves on the Scientific Advisory Board of Immodulon Therapeutics Ltd.

67 Dr. Robin Dowell is a founder and scientific advisor of Arpeggio Biosciences.

68

69 **ABSTRACT**

70 *Mycobacterium vaccae* (NCTC 11659) is an environmental saprophytic bacterium with anti-
71 inflammatory, immunoregulatory, and stress resilience properties. Previous studies have shown
72 that whole, heat-killed preparations of *M. vaccae* prevent allergic airway inflammation in a
73 murine model of allergic asthma. Recent studies also demonstrate that immunization with *M.*
74 *vaccae* prevents stress-induced exaggeration of proinflammatory cytokine secretion from
75 mesenteric lymph node cells stimulated *ex vivo*, prevents stress-induced exaggeration of
76 chemically-induced colitis in a model of inflammatory bowel disease, and prevents stress-
77 induced anxiety-like defensive behavioral responses. Furthermore, immunization with *M. vaccae*
78 induces anti-inflammatory responses in brain, and prevents stress-induced exaggeration of
79 microglial priming. However, the molecular mechanisms underlying anti-inflammatory effects of
80 *M. vaccae* are not known. We have purified and identified a unique anti-inflammatory
81 triglyceride, 1,2,3-tri[Z-10-hexadecenoyl]glycerol, from *M. vaccae* and evaluated its effects in
82 freshly isolated murine peritoneal macrophages. The free fatty acid form of 1,2,3-tri[Z-10-
83 hexadecenoyl]glycerol, 10(Z)-hexadecenoic acid, decreased lipopolysaccharide-stimulated
84 secretion of the proinflammatory cytokine IL-6 *ex vivo*. Meanwhile, next generation mRNA
85 sequencing revealed that pretreatment with 10(Z)-hexadecenoic acid upregulated genes
86 associated with peroxisome proliferator-activated receptor alpha (PPAR α) signaling in
87 lipopolysaccharide-stimulated macrophages, in association with a broad transcriptional
88 repression of inflammatory markers. We confirmed using luciferase-based transfection assays
89 that 10(Z)-hexadecenoic acid activated PPAR α signaling, but not PPAR γ , PPAR δ , or retinoic
90 acid receptor (RAR) α signaling. The effects of 10(Z)-hexadecenoic acid on lipopolysaccharide-
91 stimulated secretion of IL-6 were prevented by PPAR α antagonists and absent in PPAR α -

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

95 **KEYWORDS**

96 10(Z)-hexadecenoic acid, bacteria, inflammation, interleukin 6, lipid, macrophage, mycobacteria,
97 PPAR, RNAseq, *vaccae*

98 **ABBREVIATIONS**

99 CD, cluster of differentiation
100 CNS, central nervous system
101 DC, dendritic cell
102 DSM-5, Diagnostic and Statistical Manual of Mental Disorders (5th ed.).
103 IL, interleukin
104 IFN, interferon
105 IRF, interferon regulatory factor
106 LPS, lipopolysaccharide
107 MGB, microbiota–gut–brain
108 NCTC, National Collection of Type Cultures
109 NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells
110 PEA, palmitoylethanolamide
111 PPAR, peroxisome proliferator-activated receptor
112 PTSD, posttraumatic stress disorder
113 RAR, retinoic acid receptor
114 TGF β , transforming growth factor beta
115 TLR, toll-like receptor
116 Treg, regulatory T cell

117 INTRODUCTION

118 The global prevalence of anxiety disorders has been estimated to be 7.3%, ranging from 5.3% in
119 African cultures to 10.4% in Euro/Anglo cultures (Baxter et al. 2013). According to the
120 Diagnostic and Statistical Manual of Mental Disorders (5th ed.) (DSM-5; (American Psychiatric
121 Association 2013)), anxiety disorders include those that share features of excessive fear and
122 anxiety and related behavioral disturbances, such as generalized anxiety disorder, panic disorder,
123 social anxiety disorder (social phobia), and specific phobia (American Psychiatric Association
124 2013). Posttraumatic stress disorder (PTSD), although formerly classified as an anxiety disorder,
125 is classified as a trauma- and stressor-related disorder (APA, 2013). Collectively, anxiety and
126 trauma-related disorders are complex and multifactorial, and their differentiation and
127 management are complicated by phenotypic heterogeneity. The etiology and pathophysiology of
128 these disorders are thought to involve interactions among the genome, epigenome, and
129 environment (Nugent et al. 2011). Recently, investigation of the etiology and pathophysiology of
130 psychiatric and neurological diseases has expanded to include a potential role of the microbiota–
131 gut–brain (MGB) axis (Forsythe et al. 2010; Cryan and Dinan 2012, 2015; Leclercq et al. 2016).
132 Of particular interest, evidence from preclinical and clinical studies suggests that exaggerated
133 inflammation, which in some cases may be secondary to dysregulation of the microbiome, may
134 be a risk factor for the development of trauma- and stressor-related disorders (for review, see
135 (Langgartner et al. 2018)). These studies raise the question of whether or not microbial-based
136 interventions with anti-inflammatory or immunoregulatory properties may have value in the
137 prevention or treatment of trauma- and stressor-related disorders.

138 Evidence suggests that some common pathogenic and non-pathogenic microorganisms, to which
139 humans have been exposed throughout evolution, drive anti-inflammatory and

140 immunoregulatory mechanisms that inhibit inappropriate immune responses by the host (Rook
141 and Rosa Brunet 2002; Rook 2009, 2010; Okada et al. 2010). Throughout human evolution, the
142 interactions between these ancestral microorganisms, which we have collectively referred to as
143 “Old Friends”, and the innate immune system promoted immunoregulation. These “Old Friends”
144 included microorganisms that: 1) were part of host physiology (human microbiota); 2) were
145 harmless but inevitably contaminating air, food and water (environmental microbiota); or 3) led
146 to severe host tissue damage when attacked by the host immune system (e.g., helminthic
147 parasites) (Rook 2013; Blaser 2017).

148 “Old Friends” are thought to suppress host inflammation through a variety of mechanisms,
149 including the induction of specific subsets of antigen-presenting cells such as macrophages and
150 dendritic cells (DCs) and modulation of innate immunity (Le Bert et al. 2011; Garn et al. 2016;
151 Lowry et al. 2016). In their absence, the host may develop inappropriate immune responses to
152 allergens, self-antigens, or gut microbiota. It has been hypothesized that increases in allergies,
153 autoimmune diseases, inflammatory bowel diseases, and psychiatric disorders in modern living
154 conditions may be due, in part, to decreased exposure to “Old Friends” (Rook 2010; Lyte and
155 Cryan 2014; Bloomfield et al. 2016; Lowry et al. 2016; Stamper et al. 2016). In parallel,
156 individuals with a diagnosis of PTSD have a higher risk of development of any autoimmune
157 disease, relative to those with other psychiatric disorders, or relative to those with no psychiatric
158 disorder (O’Donovan et al. 2015), suggesting that impaired immunoregulation or inappropriate
159 inflammation may confer risk for development of both autoimmune conditions and PTSD. The
160 saprophytic mycobacterium, *Mycobacterium vaccae* (National Collection of Type Cultures
161 (NCTC) 11659), has shown encouraging therapeutic potential in diseases of inflammation and
162 immunodysregulation (Gutzwiller et al. 2007; Rook et al. 2007), and has shown

163 immunoregulatory and stress-protective effects in murine models (Zuany-Amorim et al. 2002;
164 Adams et al. 2004; Lowry et al. 2007; Reber et al. 2016; Fox et al. 2017; Frank et al. 2018).
165 Mycobacteria are abundant in municipal water supplies (Gebert et al. 2018) and are a normal
166 component of the healthy human microbiome of the oral cavity (buccal mucosa and dental
167 plaque) and upper respiratory tract (nostrils and oropharynx), and therefore are considered part of
168 the microbiome of the upper airways (Macovei et al. 2015).

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

182 Meanwhile, we've shown that immunization of mice with *M. vaccae* promotes a more proactive
183 response to a chronic psychosocial stressor, prevents stress-induced colitis, prevents stress-
184 induced exaggeration of chemically-induced colitis in a model of inflammatory bowel disease,
185 and attenuates anxiety-like defensive behavioral responses (Reber et al. 2016). Consistent with

186 these findings, immunization with *M. vaccae* prevents stress-induced exaggeration of interferon
187 gamma and IL-6 secretion from freshly isolated mesenteric lymph node cells stimulated with
188 anti-CD3/anti-CD28 *ex vivo*. Importantly, preimmunization with *M. vaccae*, in stressed mice,
189 resulted in a two orders of magnitude increase in IL-10 secretion from mesenteric lymph node
190 cells stimulated *ex vivo*. However, until now, specific constituents of *M. vaccae* that suppress
191 inflammation in macrophages in the periphery or central nervous system have not been
192 identified.

193 Through a screening process of *M. vaccae* NCTC 11659 lipid extracts, a single triglyceride,
194 1,2,3-tri[Z-10-hexadecenoyl]glycerol, was identified with potential immunotherapeutic benefits
195 (Rosa Brunet and Rook 2008). The lipid was demonstrated to prevent allergic airway
196 inflammation, and the lipid recapitulated the therapeutic effects of whole heat-killed *M. vaccae*.
197 The protective phenotype was characterized by increased IL-10, decreased IL-5, and reduced
198 infiltration of eosinophils and macrophages in bronchoalveolar lavage fluid (Rosa Brunet and
199 Rook 2008). It was also shown that the efficacy of the triglyceride was not dependent on the
200 glycerol structure, as the synthetic, constituent free fatty acid, 10(Z)-hexadecenoic acid, was
201 sufficient to suppress pulmonary airway inflammation. The mechanism through which this long-
202 chain, monounsaturated fatty acid was capable of limiting symptoms of inflammation is
203 unknown, but it is explored here in a model of macrophage activation.

204 Notably, it is relatively rare in nature for an organism to naturally produce a fatty acid that is
205 unsaturated at the C10 position, yet several mycobacteria species—including *M. vaccae*, can
206 perform that desaturation (Scheuerbrandt and Bloch 1962; Coyle et al. 1992; Böttger et al. 1993;
207 Springer et al. 1993; Suutari and Laakso 1993; Chou et al. 1998; Tay et al. 1998; Pacífico et al.
208 2018). We successfully synthesized the free fatty acid, 10(Z)-hexadecenoic acid, and using cell-

209 based assays and RNA-seq, revealed that 10(Z)-hexadecenoic acid upregulated genes associated
210 with the peroxisome proliferator-activated receptor (PPAR) signaling pathway and inhibited
211 proinflammatory signaling of activated macrophages *ex vivo*. Furthermore, studies using cultured
212 cells transfected with lipid-regulated transcription factors revealed that both the
213 monoacylglycerol lipid constituent of *M. vaccae* and its free fatty acid form selectively increased
214 PPAR α signaling. The effects of 10(Z)-hexadecenoic acid to inhibit proinflammatory signaling
215 of activated macrophages *ex vivo* were prevented by PPAR α antagonists and absent in PPAR α -
216 deficient mice. This is the first report, to our knowledge, to show that a synthetic *M. vaccae*-
217 derived lipid acts to induce anti-inflammatory responses in host immune cells by acting as an
218 agonist at host PPAR α receptors.

219 MATERIALS AND METHODS

220 Animals

221 Adult male BALB/c mice (BALB/cAnHsd; Cat. No. 047; Harlan, Indianapolis, IN, USA), 6-8
222 weeks old, were used and housed under standard conditions with food and water available *ad*
223 *libitum*. Adult male PPAR α ^{-/-} (B6;129S4-*Ppara*^{tm1Gonz}/J; Cat. No. 008154; Jackson Laboratories,
224 Bar Harbor, ME, USA) and control mice (C57BL/6J; Cat. No. 000664; Jackson Laboratories), 6-
225 8 weeks old, were used and housed under standard conditions with food and water available *ad*
226 *libitum*. Although the C57BL/6J inbred strain is considered an approximate control for the
227 PPAR α ^{-/-} mice (B6;129S4-*Ppara*^{tm1Gonz}/J; Jackson Laboratories) future studies should ideally
228 compare PPAR α ^{-/-} mice to wild type littermates.

229 All experimental protocols were consistent with the National Institutes of Health *Guide for the*
230 *Care and Use of Laboratory Animals*, Eighth Edition (The National Academies Press, 2011) and

231 the Institutional Animal Care and Use Committee at the University of Colorado Boulder
232 approved all procedures. This work was covered under CU Boulder IACUC Protocol Numbers
233 2134-14MAY2018 and 2361-14MAY2018-DT. The research described here was conducted in
234 compliance with The ARRIVE Guidelines: Animal research: reporting of in vivo experiments,
235 originally published in PLOS Biology, June 2010 (Kilkenny and Altman 2010). All possible
236 efforts were made to minimize the number of animals used and their suffering.

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

238 Unless otherwise noted, reagents were obtained commercially and used without further
239 purification. Dichloromethane (CH_2Cl_2) was distilled over calcium hydride (CaH_2) under a
240 nitrogen atmosphere. Tetrahydrofuran (THF; $(\text{CH}_2)_4\text{O}$) was distilled from sodium-benzophenone
241 under a nitrogen atmosphere. Thin-layer chromatography analysis of reaction mixtures was
242 performed on Dynamic Adsorbents, Inc., silica gel F-254 TLC plates. Flash chromatography was
243 carried out on Zeoprep 60 ECO silica gel. ^1H spectra were recorded with a Varian INOVA 500
244 spectrometer. Compounds were detected by monitoring UV absorbance at 254 nm.

245 To a 5 mL sealed tube containing 1-heptene (0.50 mL, 3.55 mmol), methyl 10-undecenoate
246 (0.080 mL, 0.36 mmol) and 0.35 mL THF was added to a Grubbs Z-selective metathesis catalyst
247 (2.2 mg, 3.48 μmol , Cat. No. 771082, Sigma-Aldrich, St. Louis, MO, USA). The reaction was
248 stirred at 45 $^\circ\text{C}$ for 8 h before cooling to room temperature. The slurry was filtrated through a
249 short plug of silica gel and concentrated. The obtained oil was dissolved in 1.0 mL THF. The
250 solution was cooled to 0 $^\circ\text{C}$, then 9-borabicyclo[3.3.1]nonane (9-BBN) solution in THF (1.28
251 mL, 0.50 M, 0.64 mmol) was added. After 2 h stirring at 0 $^\circ\text{C}$, the reaction was quenched with 60
252 μL EtOH, then 1.5 mL pH 7 potassium phosphate buffer and 1.5 mL 30% H_2O_2 . The mixture
253 was stirred at room temperature for 12 h, then extracted with 5 mL EtOAc three times. The

254 combined organic layers were washed with 4 mL saturated Na₂S₂O₃ and 3 mL brine, then dried
255 over Na₂SO₄, filtered and concentrated. To the crude oil in 1.0 mL THF was added LiOH
256 monohydrate (38 mg, 0.90 mmol) in 1.0 mL water. After 2 h, the reaction solution was cooled to
257 0 °C before addition of 0.91 mL 1.0 N HCl. After being concentrated under reduced pressure, the
258 aqueous solution was saturated with NaCl and extracted with 3 mL dichloromethane three times.
259 The combined organic layers were dried over Na₂SO₄, filtered and concentrated. Purification by
260 flash chromatography (2:1:1 hexanes/dichloromethane/diethyl ether) provided (10Z)-hexadec-
261 10-enoic acid (0.022 g, 90%) as a colorless oil. ¹H NMR (500 MHz, CDCl₃): δ 5.48 – 5.22 (m,
262 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,
263 16H), 0.88 (t, J = 6.9 Hz, 3H).

264 **Murine peritoneal macrophage isolation and screening**

265 Murine peritoneal macrophages were isolated and cultured as previously described (Zhang et al.
266 2008) and used to determine the effects of 10(Z)-hexadecenoic acid on lipopolysaccharide-
267 induced IL-6 secretion. Briefly, mice received a single injection of 3% thioglycollate medium (1
268 mL, i.p.; Cat. No. 9000-294, VWR, Radnor, PA, USA). Mice were euthanized 96 h later using
269 cervical dislocation, and macrophages were collected in Dulbecco's phosphate-buffered saline
270 (DPBS; Cat. No. 14190136, Invitrogen, Carlsbad, CA, USA). Cells were centrifuged and
271 resuspended in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Cat.
272 No. 10565018, Invitrogen) supplemented to be 10% (v/v) fetal bovine serum (Cat. No.
273 16000036, Invitrogen) and 1% penicillin/streptomycin (Cat. No. 15140148, Invitrogen). One
274 mouse yielded enough cells for one experimental replicate. 1 x 10⁵ cells/well were allowed to
275 adhere for 1.5 h before being washed with DPBS. 10(Z)-hexadecenoic acid was dissolved in
276 DMEM/F-12 with 0.5% (v/v) dimethyl sulfoxide (Cat. No. D8418, Sigma-Aldrich). The

277 macrophages were incubated with either 10(*Z*)-hexadecenoic acid (0.4 μ M, 4 μ M, 20 μ M, 100
278 μ M, 500 μ M, 1000 μ M) or DMEM/F-12 for 1 h before being stimulated with either 1 μ g/ml
279 lipopolysaccharide (serotype 0127:B8, Sigma-Aldrich, St. Louis, MO, USA) or DMEM/F-12.
280 Culture supernatants were collected at 6, 12, and 24 h post-stimulation.

281 **Cytokine measurements**

282 Cell culture supernatants (10 μ l) from freshly isolated peritoneal macrophages were diluted
283 1:200, and IL-6 was measured using ELISA (Cat. No. 431304, BioLegend, San Diego, CA,
284 USA). The assay has a minimal detectable concentration of 2 pg/ml IL-6. All samples were
285 measured using duplicate wells in the ELISA.

286 **Cytotoxicity assay**

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

296 **Ligands**

297 For studies using reporter gene assays following transfection of COS1 cells, rosiglitazone,
298 troglitazone, and WY14643 were obtained from Alexis Biochemicals (San Diego, CA, USA);

299 ATRA and AM580 were obtained from Sigma-Aldrich. In addition, GW9662 was a gift from
300 T.M. Willson (GlaxoSmithKline, Brentford, United Kingdom). For experiments using freshly
301 isolated peritoneal macrophages, GW 6471 (Cat. No. 4618), GW 9662 (Cat. No. 1508), GSK
302 0660 (Cat. No. 3433), WY 14643 (Cat. No. 1312), rosiglitazone (Cat. No. 5325), GW 0742 (Cat.
303 No. 2229) were obtained from Tocris Bioscience (Bristol, United Kingdom).

304 **Transfections and reporter gene assays**

305 Cells were transfected with the following receptor and reporter constructs: Gal4-PPAR α -LBD,
306 Gal4-PPAR γ -LBD, Gal4-PPAR δ -LBD, Gal4-RAR α -LBD, pMH100-TK-luc, and pCMX- β -
307 galactosidase (Chen and Evans 1995). All transfection experiments were performed with COS 1
308 cells using polyethylenimine (Sigma-Aldrich) reagent (Szatmari et al. 2006). After 6–8 h of the
309 transfection, the medium was replaced with DMEM medium containing the indicated ligands or
310 vehicle (as control) (Chen and Evans 1995; Benko et al. 2003). Cells were lysed and assayed for
311 reporter expression 18 h after transfection. The luciferase assay system (Promega, Madison, WI,
312 USA) was used as described previously (Nagy et al. 1999). Measurements were carried out with
313 a Wallac Victor-2, multilabel counter. Luciferase activity of each sample was normalized to the
314 β -galactosidase activity.

315 **RNA extraction and library preparation**

316 Total RNA content of 1×10^5 macrophages pretreated for 1 h with 200 μ M 10(Z)-hexadecenoic
317 acid (utilizing separate macrophage preparations from $n = 3$ mice) or vehicle (utilizing separate
318 macrophage preparations from $n = 3$ mice) and stimulated with 1 μ g/ml LPS was extracted using
319 TRI Reagent® (Cat. No. T9424, Sigma-Aldrich) according to manufacturer's instructions. The
320 RNA input was quantified on a Qubit™ 3.0 Fluorometer (Cat. No. Q33216, Thermo Fisher,
321 Waltham, MA, USA) to ensure there was sufficient starting material. The RNA sequencing

322 libraries were generated with the NEBNext rRNA Depletion Kit (Cat. No. E6310, New England
323 BioLabs) in order to enrich the samples in mRNA, and NEBNext Ultra Directional RNA Library
324 Prep Kit for Illumina (Cat. No. 7240, New England BioLabs). Briefly, mRNA was purified from
325 100 ng of total RNA, fragmented, and converted to double stranded cDNA. Barcodes were
326 ligated to the cDNA fragments, and prior to PCR enrichment of the library, the cDNA product
327 was quantified on a Qubit 3.0 Fluorometer (Thermo Fisher). The integrity of the purified oligo
328 libraries was evaluated on an Agilent Bioanalyzer 2100 (Cat. No. G2939BA, Agilent, Santa
329 Clara, CA, USA).

330 **Sequencing**

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

335 **RNA read processing, mapping, and differential expression**

336 Quality analysis of sequencing data was done using FastQC. The adaptors and low quality raw
337 reads were cut with Trimmomatic (version 0.32) (Bolger et al. 2014). The reads were aligned to
338 the mouse genome, mm10 (University of California, Santa Cruz, CA, USA), using the TopHat2
339 sequence aligner (version 2.0.6) (Kim et al. 2013). Reads mapping to exon features were counted
340 using HTseq (version 0.6.1) (Anders et al. 2015). The raw reads and count data have been
341 deposited in the GEO database under accession number GSE125930. Differentially expressed
342 genes were identified using the R package, DESeq (version 1.28.0) (Anders and Huber 2010).

343 **Statistical analysis**

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

350 RESULTS

351 10(Z)-hexadecenoic acid decreases LPS-induced secretion of IL-6 in macrophages

352 To simulate inflammation, freshly isolated mouse peritoneal macrophages were challenged with
353 LPS (1 $\mu\text{g}/\text{mL}$) *ex vivo* (outlined in Fig. 1). Macrophages that were cultured in the presence of
354 10(Z)-hexadecenoic acid (0.4 μM , 4 μM , 20 μM , 100 μM , 500 μM , 1000 μM) for 1 h prior to 1
355 $\mu\text{g}/\text{mL}$ LPS stimulation secreted less IL-6 relative to macrophages cultured with media alone
356 prior to LPS stimulation (Fig. 2A-C) ($F_{(1,111)} = 15.20$, $p < 0.001$). This difference was observable
357 as early as 6 h after LPS challenge, and was sustained for at least 24 h. We selected the 6 h, 12 h,
358 and 24 h time points for measurement of IL-6 as previous studies have shown increased IL-6
359 secretion using LPS-stimulated peritoneal macrophage cultures in mice at these time points, with
360 linear increases in IL-6 up to the 24 h time point (Shacter et al. 1993; Wollenberg et al. 1993; Lin
361 and Tang 2007; Lee et al. 2015; Arteaga Figueroa et al. 2017). The effect also appeared to be
362 concentration and time dependent. The lowest concentration of 10(Z)-hexadecenoic acid (0.4
363 μM) was ineffective at 6 h, but reduced IL-6 secretion to 40% of control levels at 24 h. Using a
364 constrained logistic model on the relative secretion of IL-6, we estimated the EC50 to be 823
365 μM , 115 μM , and 190 μM at the 6h, 12h, and, 24h observations, respectively (Fig. 2). Post hoc
366 pairwise comparisons of raw IL-6 values relative to paired media control values at the same time

367 point ($n = 3$ per group) are presented in Table S1. This time and concentration dependence may
368 indicate that a receptor-mediated transcriptional change is occurring. In contrast to the effects of
369 10(Z)-hexadecenoic acid on LPS-induced IL-6 secretion, it had no detectable effect on IL-6
370 secretion by itself (IL-6 was undetectable in all conditions; Fig. S1). We cannot exclude the
371 possibility, however, that 10(Z)-hexadecenoic acid by itself had effects on IL-6 secretion that
372 were below the limit of detectability of the assay used (i.e., 2 pg/mL). Cell viability was
373 measured to dispel the possibility that senescence or cell death was contributing to reduced IL-6
374 secretion. Using a high concentration (1 mM) of 10(Z)-hexadecenoic acid, less than 40% of
375 macrophages were viable at most time points. However, macrophages cultured with all other
376 concentrations of 10(Z)-hexadecenoic acid studied (0.4 μ M, 4 μ M, 20 μ M, 100 μ M, 500 μ M)
377 were as viable as media controls (Fig. S2).

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

380 To explore the potential effects of 10(Z)-hexadecenoic acid on transcriptional responses in LPS-
381 stimulated macrophages, we used RNA-seq. Murine peritoneal macrophages were incubated
382 with 200 μ M 10(Z)-hexadecenoic acid for 1 h prior to stimulation with LPS. Using IL-6 as a
383 measure for the suppressive activity of 10(Z)-hexadecenoic acid, we estimated the EC_{50} at 12h to
384 be 115 μ M. The 200 μ M concentration was chosen as it was sufficiently larger than the EC_{50} , but
385 less than a concentration that would affect macrophage viability. After 12 h, the RNA was
386 extracted and depleted of rRNA. We selected the 12 h time point for measurement of mRNA
387 using RNAseq as previous studies have shown increased IL-6 secretion using LPS-stimulated
388 peritoneal macrophage cultures in mice at this time point, as well as the ability to suppress IL-6
389 mRNA expression at this time point by interfering with a TLR4-MyD88-BLT2-Nox1-ROS-NF-

390 κ B pathway leading to IL-6 secretion (Lee et al. 2015). The cDNA libraries were sequenced in a
391 100bp paired-end experiment generating 51-63 million reads per sample (Table S2; Fig. S3).
392 For differential expression, we examined LPS-stimulated macrophages pretreated with either
393 10(Z)-hexadecenoic acid or vehicle (GSE125930). Differentially expressed transcripts were
394 identified using the R package, DESeq (Anders and Huber 2010). A total of 203 genes were
395 found to be differentially expressed with an FDR-adjusted $p < 0.1$ (Table S3). Of the 203
396 differentially expressed genes, 109 were downregulated in the 10(Z)-hexadecenoic acid
397 condition, and 20% of those genes were associated with proinflammatory processes (Table S4).
398 The top 20 differentially expressed genes are reported in Fig. 3A. Consistent with the *ex vivo*
399 macrophage experiments measuring IL-6 protein with ELISA, the second most significantly
400 differentially expressed transcript was IL-6 (Table S3).

401 ***PPAR α regulated genes are associated with 10(Z)-hexadecenoic acid treatment in LPS-***
402 ***stimulated macrophages***

403 To better understand the pathways affected by 10(Z)-hexadecenoic acid treatment, the list of 203
404 differentially expressed genes were queried against the Database for Annotation, Visualization
405 and Integrated Discovery (DAVID) (Huang et al. 2009). Within the top 40 most significantly
406 enriched KEGG pathways, 34 (i.e., 85%) were related to disease or inflammation (Table S5). In
407 addition, 32 of the top 40 most significantly enriched KEGG pathways (i.e., 82%) were
408 exclusively enriched for genes that were significantly downregulated by treatment with 10(Z)-
409 hexadecenoic acid. Among these most significantly affected pathways, there was a wide scope of
410 immunological context, which included infections, diseases, cytokine signaling, and various
411 inflammatory pathways. The top 5 pathways with genes that were exclusively downregulated by
412 treatment with 10(Z)-hexadecenoic acid are reported in Fig. 3C.

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

425 In a secondary analysis, rather than rely on a subset of genes for biological interpretation, we
426 used all expression data in Gene Set Enrichment Analysis (GSEA). We queried all detected
427 transcripts against the KEGG pathways database (c2.cp.kegg.v6.2), and the top 5 enriched gene
428 sets for the 10(Z)-hexadecenoic acid phenotype were: “peroxisome” (KEGG: hsa04146), a main
429 site of fatty acid oxidation via the β -oxidation cycle, “ppar_signaling_pathway” (KEGG:
430 hsa03320), “citrate_cycle_tca_cycle” (KEGG: hsa00020), “fatty_acid_metabolism” (KEGG:
431 hsa00071), and “propanoate_metabolism” (KEGG: hsa00640) (Table S6). Of potential interest,
432 four of these KEGG pathways, “peroxisome” (KEGG: hsa04146), “ppar_signaling_pathway”
433 (KEGG: hsa03320), “fatty_acid_metabolism” (KEGG: hsa00071), and
434 “propanoate_metabolism” (KEGG: hsa00640) were also found to be enriched in livers from 24 h
435 fasted PPAR α ^{+/+} relative to PPAR^{-/-} mice, while “peroxisome” (KEGG: hsa04146),

436 “ppar_signaling_pathway” (KEGG: hsa03320), and “fatty_acid_metabolism” (KEGG:
437 hsa00071), were found to be enriched in livers from wild type mice treated with the PPAR α
438 agonist Wy14643, relative to vehicle (Kersten 2014). Together, these studies support a
439 convergence of 10(Z)-hexadecenoic acid effects on PPAR α signaling pathways induced by
440 physiological or pharmacological stimuli. Propionate is one of the short-chain fatty acids, which
441 are emerging as key mediators and regulators of host-microbe cross-talk, with a significant
442 impact on host metabolism, including as an energy source (Hoyles et al. 2018). All 5 gene sets
443 were significant with an unadjusted p value < 0.05 , but failed to reach significance using FDR-
444 adjusted p values; nevertheless, the overall pattern is consistent with a modulation of lipid
445 metabolism. In a network visualization of gene set overlap between all detected pathways using
446 DAVID, PPARs and peroxisomal lipid metabolism were prominent vertices (Fig. 3D). We also
447 searched against the collection of transcription factor binding motifs (c3.tft.v6.0), which revealed
448 enrichment for CREB, Gfi1, and PPAR α *cis*-regulatory motifs upstream of the genes upregulated
449 with 10(Z)-hexadecenoic acid treatment in LPS-stimulated macrophages (Table S7). Again, these
450 were nominally significant (i.e. $p < 0.05$; $q > 0.05$), but these findings bolster PPARs, and
451 specifically PPAR α , as a potential receptor mediating anti-inflammatory effects of 10(Z)-
452 hexadecenoic acid.

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

454 NF- κ B is one of three major transcription factors downstream of LPS-induced activation of toll-
455 like receptor 4 (TLR4), the other two being IRF3 and AP-1 (Kawasaki and Kawai 2014). Using
456 all expression data in GSEA, we found that pretreatment with 10(Z)-hexadecenoic acid, relative
457 to treatment with vehicle, prior to LPS stimulation, downregulated signaling pathways
458 downstream from TLR4, such as NF- κ B and IRF3, but not AP-1. In a network visualization of

459 the most significant pathways (FDR < 0.1), there is a bifurcation at the
460 “ZHOU_INFLAMMATORY_RESPONSE_LPS” node representing the NF- κ B and IRF3
461 responses (Fig. 4A). Among all nodes in this network, we counted and ranked the occurrence of
462 enriched transcripts. The counts for the highest ranking transcripts were categorized into either
463 NF- κ B regulated responses or IRF regulated responses (Fig. 4B). We also examined enrichment
464 for transcription factor motifs and detected enrichment for NF- κ B, IRF1, IRF2, and IRF_Q6
465 among others, in transcripts associated with the vehicle treated, LPS stimulated group (Table
466 S8). Alternatively, this can be understood to mean that mRNA transcripts that are located near
467 those transcription factor binding sites are downregulated in 10(Z)-hexadecenoic acid treated,
468 LPS-stimulated macrophages. To better understand the classification of the IRF_Q6 gene set (N
469 = 242 genes) and differentially expressed genes ($q < 0.1$, $N = 203$ genes), they were both queried
470 against the Interferome database (Rusinova et al. 2013). There are three types of interferons
471 (IFNs), namely type I (composed of α , β , κ , ϵ , and ω subtypes), type II (IFN γ) and type III
472 (IFN λ ; also called IL28 and IL29), which are distinguished by having distinct genetic loci, amino
473 acid sequence homology and specific cognate receptors (Pestka et al. 2004). This analysis
474 revealed that a vast majority of the differentially expressed genes are regulated by both type I
475 and II interferon responses (Fig. 4C), consistent with the hypothesis that 10(Z)-hexadecenoic
476 acid alters TLR4, IRF3, and interferon signaling. Of note, cells infected with *Mycobacterium*
477 *tuberculosis* induce type I interferons, including IFN α and IFN β , which are thought to promote
478 infection with *M. tuberculosis* (Travar et al. 2016). Using enrichment tools, like GSEA and
479 DAVID, these RNA-seq data suggest that both NF- κ B and IRF3 pathways are downregulated in
480 LPS-stimulated macrophages when treated with 10(Z)-hexadecenoic acid.

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

482 ***10(Z)-hexadecenoic acid specifically activates PPAR α***

483 Fatty acids can modulate inflammation via the activation of nuclear hormone receptors (Chinetti
484 et al. 2000; Kidani and Bensinger 2012). Therefore, we assessed the nuclear receptor activation
485 capacity of: 1) the triacylglycerol (TAG), 1,2,3-tri[Z-10-hexadecenoyl]glycerol; 2) the
486 monoacylglycerol (MAG), 1-[Z-10-hexadecenoyl]glycerol; and 3) the free fatty acid (FFA),
487 10(Z)-hexadecenoic acid. We conducted reporter gene assays via the transfection of COS1 cells
488 using GAL4-fusion ligand binding domains (LBDs) of various lipid-activated nuclear receptors
489 (PPAR α -LBD, PPAR γ -LBD, PPAR δ -LBD and RAR α -LBD) along with a plasmid carrying
490 MH100-TK-luciferase reporter (Chen and Evans 1995). Transfected cells were incubated with
491 TAG, MAG, or FFA for 18 h and relative luciferase activity, normalized to β -galactosidase
492 activity, was measured. Each reporter transfection was validated with the respective receptor
493 agonist (PPAR α , WY-14643; PPAR γ , rosiglitazone (RSG); PPAR δ , GW1516; RAR α , AM580).
494 Both the MAG and FFA, at concentrations of 80 μ M, reliably increased PPAR α -, but not
495 PPAR γ -, PPAR δ , or RAR α -regulated reporter expression (Fig. 5A-D). The triglyceride had no
496 effect (Fig. 5A-D). Together, these results demonstrate that 10(Z)-hexadecenoic acid and its
497 monoacylglycerol form selectively activate the PPAR α receptor.

498 ***PPAR α is required for anti-inflammatory effects of 10(Z)-hexadecenoic acid***

499 Next, we investigated if this interaction was necessary for inhibiting LPS-stimulated release of
500 IL-6. Agonists and antagonists of each PPAR were used to test if PPAR α has a singular role in
501 this process. The agonists and antagonists and their receptor specificities are listed in Table S9
502 Macrophages were incubated with a single PPAR antagonist for 1 h prior to treatment with either
503 200 μ M 10(Z)-hexadecenoic acid or a PPAR agonist complementary to its respective PPAR
504 antagonist. After another 1 h incubation period, the cells were stimulated with LPS (1 μ g/ml),

505 and IL-6 was measured 12 h later. Only with the PPAR α antagonist, GW 6471, could the anti-
506 inflammatory effects of 10(Z)-hexadecenoic acid be significantly reversed (Fig. 6A). The effects
507 of the PPAR γ and $-\delta$ antagonists were comparable to media (Fig. 6A). These results suggest a
508 selective interaction between 10(Z)-hexadecenoic acid and PPAR α , as the PPAR α antagonist,
509 GW 6471, had no effect on macrophage viability (Fig. S4), while it was effective in reversing the
510 anti-inflammatory effects of the PPAR α agonist, WY-14643, as measured by IL-6 secretion in
511 LPS-stimulated macrophages (Fig. S5). To further explore the role of PPAR α in the anti-
512 inflammatory effects of 10(Z)-hexadecenoic acid, we repeated the assay with freshly isolated
513 peritoneal macrophages from adult male C57BL/6J wild type and PPAR α ^{-/-} mice. As expected,
514 10(Z)-hexadecenoic acid suppressed LPS-stimulated IL-6 in macrophages from wild type
515 C57BL/6J mice, but this effect was absent in macrophages from PPAR α KO mice (Fig. 6B).
516 This indicated a full reversal of the anti-inflammatory effect of 10(Z)-hexadecenoic acid and the
517 necessity of PPAR α in mediating the effect.

518 **DISCUSSION**

519 Here we characterized the monounsaturated C16 free fatty acid, 10(Z)-hexadecenoic acid,
520 derived from *M. vaccae* NCTC 11659, a saprophytic bacterium with anti-inflammatory and
521 immunoregulatory properties that previously has been shown to prevent stress-induced
522 exaggeration of peripheral inflammation and neuroinflammation, and to prevent stress-induced
523 exaggeration of anxiety- and fear-related defensive behavioral responses. In addition, we showed
524 that 10(Z)-hexadecenoic acid induced a broad transcriptional repression of inflammatory gene
525 markers (see, for example, Table S10-11) and suppressed IL-6 secretion from freshly isolated,
526 LPS-stimulated, murine peritoneal macrophages. Furthermore, we showed that both the
527 monoacylated glycerol, 1-[Z-10-hexadecenoyl]glycerol and 10(Z)-hexadecenoic acid activated

528 PPAR α signaling, as measured by transfection assays. Finally, we showed that PPAR α
529 antagonists prevented the anti-inflammatory effects of 10(Z)-hexadecenoic acid in macrophages,
530 while the *ex vivo* effects of the lipid were absent in macrophages isolated from PPAR α -deficient
531 mice.

532 Here we focused on effects of 10(Z)-hexadecenoic acid on peritoneal macrophages. Based on a
533 number of lines of evidence, effects of 10(Z)-hexadecenoic acid actions on peritoneal
534 macrophages may have important implications for CNS immunity and subsequent behavioral
535 outcomes. Intraperitoneal administration of lipopolysaccharide is known to induce priming of
536 hippocampal microglia and worsen CNS outcomes (Cunningham 2005, 2013; Cunningham et al.
537 2009). Although the mechanisms through which peripheral inflammation signals to the CNS to
538 induce microglial priming and neuroinflammatory responses are not entirely clear, a number of
539 potential signaling mechanisms have been proposed. These include: 1) entry of cytokines into the
540 brain at circumventricular organs that have a reduced blood-brain barrier; 2) binding of cytokines
541 to cerebral vascular endothelium, inducing the secretion of central neuroinflammatory mediators;
542 3) carrier-mediated transport of immune signals into the brain, across the blood-brain barrier; 4)
543 migration of proinflammatory monocytes from the periphery to the CNS; and 5) activation of
544 peripheral afferent nerves, including vagal and non-vagal pathways (Watkins et al. 1995; Maier
545 et al. 1998; Maier 2003; Miller et al. 2010; Miller and Raison 2016).

546 Together, these data support the hypothesis that bacterially-derived 10(Z)-hexadecenoic acid
547 may induce a form of macrophage “inflammation anergy” (i.e., a condition characterized by an
548 absence of the normal immune response to a particular antigen, see, for example, (Smythies et al.
549 2005, 2010) through actions on PPAR α . Peroxisome proliferator-activated receptors, PPAR α ,
550 PPAR γ , and PPAR δ are ligand-activated nuclear receptors, each of which acts as a heterodimer

551 with retinoid X receptor (RXR), with potent anti-inflammatory properties, through interference
552 with proinflammatory transcription factor pathways (Chinetti et al. 2003). PPAR α ^{-/-} mice have
553 increased vulnerability to chemically-induced colitis, experimental autoimmune encephalitis
554 (EAE, a model of multiple sclerosis) and experimentally-induced allergic asthma, consistent with
555 the hypothesis that endogenous PPAR α suppresses inflammatory signaling in these models (for
556 review, (Bensinger and Tontonoz 2008)). Activation of PPAR α in macrophages inhibits the
557 production of proinflammatory response markers, including IL-6, IL-1 β , TNF, and inducible
558 nitric oxide synthase (Xu et al. 2005; Paukkeri et al. 2007). Interaction between PPAR α and
559 TLR4 signaling has been observed in other endogenous systems, like vascular smooth muscle
560 cells, where responses to activation of TLR4 with LPS are mitigated by a PPAR α agonist (Ji et
561 al. 2010). The anti-inflammatory effects were mediated, in part, by a reduction of tissue inhibitor
562 of metalloprotease-1 (TIMP-1), which was also reduced in our study (Table S3). PPAR α -
563 mediated inhibition of TLR4 signaling has also been shown in enteric glial cells (Esposito et al.
564 2014), and a potential downstream target of PPAR α -mediated suppression, TRIF, is required for
565 LPS-induced activation of microglia (Burfeind et al. 2018). TRIF KO mice have attenuated
566 expression of *Il6*, *Ccl2*, and *Cxcl2*, which were all also suppressed in our study (Table S3), in the
567 hypothalamus after peripheral LPS stimulation (Burfeind et al. 2018). Furthermore, bacterially-
568 derived agonists of PPARs have potential for modulation of host-acquired immunity; PPARs
569 have been found to regulate T cell survival, activation, and CD4⁺ T helper cell differentiation
570 into the Th1, Th2, Th17, and Treg lineages (Choi and Bothwell 2012).

571 Synthesis of 10(Z)-hexadecenoic acid by mycobacteria may be an example of molecular mimicry
572 of eukaryotic signaling. Endogenous host-derived agonists of PPAR α include 16:1 isoforms of
573 palmitoleic acid (Kliwer et al. 1997; Kota et al. 2005), a lipokine released from adipose cells.

574 Palmitoleic acid localizes predominantly to nuclear fractions, consistent with a nuclear
575 mechanism of action in host cells (Foryst-Ludwig et al. 2015), and is potently anti-inflammatory
576 (Chan et al. 2015). In addition, the endocannabinoid, palmitoylethanolamide (PEA), acts as an
577 agonist at PPAR α (Verme et al. 2005; Guida et al. 2017). Of interest to trauma- and stressor-
578 related psychiatric disorders, PEA induces potent antidepressant-like behavioral responses (Yu et
579 al. 2011) and, through induction of cannabinoid 2 receptors, alters the phenotype of macrophages
580 and microglia (Guida et al. 2017). Recent studies have demonstrated PEA increases biosynthesis
581 of allopregnanolone, an endocannabinoid, in the spinal cord, brainstem, hippocampus and
582 amygdala, effects that are associated with faster fear extinction learning and improvement of
583 aggression in socially-isolated mice (Sasso et al. 2012; Locci and Pinna 2017; Pinna 2018).
584 Future studies should determine if 10(*Z*)-hexadecenoic acid is sufficient to induce the enhanced
585 fear extinction learning previously demonstrated using whole, heat-killed *M. vaccae* (Fox et al.
586 2017), and to what extent these effects are mediated by PPAR α .

587 Mycobacteria are unique in that they accumulate triacylglycerols as intracellular lipophilic
588 inclusions. For example, *M. smegmatis* accumulates triacylglycerols and the acyl chain
589 composition varies depending on the growth medium (Garton et al. 2002). Monounsaturated
590 fatty acids, C_{16:1} hexadecenoic acid and C_{18:1} octadecenoic acid were found to be high when
591 bacteria were grown in nutrient rich Middlebrook 7H9 broth, relative to low-nitrogen Youmans'
592 broth, but highest when bacteria were grown in Youmans' broth with monounsaturated oleic acid
593 ((*9Z*)-octadec-9-enoic acid) supplementation. Thus, it is possible that mycobacteria synthesize
594 and store triacylglycerols using environmental fatty acids as substrates, potentially for export to
595 the cell envelope and release. If so, it may be possible to modify the immunoregulatory and anti-
596 inflammatory potential of mycobacteria through modification of growth conditions.

597 Of potential importance, conjugated linoleic acids are bacterial metabolites. For example,
598 specific members of the genus *Lactobacillus*, including *Lactobacillus reuteri*, and *L. plantarum*,
599 as well as bifidobacteria, mediate the conversion of dietary linoleic acid into immunomodulatory
600 conjugated linoleic acids (Coakley et al. 2003; Lee et al. 2003; Ogawa et al. 2005; Kishino et al.
601 2013). Most of the conjugated linoleic acid produced is located in the extracellular space (~98%)
602 (Lee et al. 2003; Roman-Nunez et al. 2007), suggesting that bacterially-derived conjugated
603 linoleic acids may be metabolic signaling molecules that modulate the host immune response.
604 These bacterially-derived fatty acid metabolites include 10-hydroxy-*cis*-12-octadecenoic acid
605 (HYA), *cis*-9,*trans*-11-linoleic acid, *trans*-9,*cis*-11-linoleic acid, and *cis*-10,*trans*-12-linoleic
606 acid (Lee et al. 2003; Miyamoto et al. 2015), among many others (Ogawa et al. 2005). Several of
607 these bacterially-derived fatty acid metabolites are potent PPAR α agonists (IC₅₀ values from 140
608 nM to 400 nM) (Moya-Camarena et al. 1999). Perhaps the closest analogue of 10(*Z*)-
609 hexadecenoic acid identified here is *trans*-10-octadecenoic acid, produced by *L. plantarum* from
610 linoleic acid (Kishino et al. 2013) or γ -linolenic acid (Ogawa et al. 2005). Although, to the best
611 of our knowledge, the efficacy of *trans*-10-octadecenoic acid at PPAR α receptors is not known,
612 production of 10(*Z*)-hexadecenoic acid and diverse conjugated linoleic acids, which then act at
613 host PPAR α receptors, may be a general strategy of commensal organisms to suppress host
614 immune responses, and promote symbiotic relationships with the host. Consistent with this
615 hypothesis, macrophages lining the gut mucosa are anergic, characterized by an inability to
616 mount proinflammatory responses, despite avid phagocytic activity (Smythies et al. 2005), while
617 lung airway macrophages are immunoregulatory (Strickland et al. 1996; Soroosh et al. 2013;
618 Duan and Croft 2014). Recent studies have also identified α -linolenic acid-derived bacterial
619 metabolites, 13-hydroxy-9(*Z*),15(*Z*)-octadecadienoic acid (13-OH) and 13-oxo-9(*Z*),15(*Z*)-

620 octadecadienoic acid (13-oxo), that induce differentiation of anti-inflammatory M2 macrophages
621 through activation of G protein-coupled receptor 40 (GPR40) (Ohue-Kitano et al. 2018).
622 Together, these data support the hypothesis that bacterially-derived “postbiotic” compounds,
623 including fatty acid metabolites, have important beneficial effects on the host via diverse host
624 receptor signaling mechanisms.

625 Although we did not assess the effects of 10(Z)-hexadecenoic acid on DCs or immunoregulation,
626 defined as the balance between regulatory and effector T cells, conjugated linoleic acid
627 suppresses NF- κ B signaling and IL-12 production in DCs through IL-10 production (Loscher et
628 al. 2005). Exposure of murine DCs to conjugated linoleic acid suppresses their ability to promote
629 differentiation of naïve T cells into Th1 and/or Th17 cells *in vitro* following their adoptive
630 transfer *in vivo* (Draper et al. 2014). Future studies should investigate the effects of 10(Z)-
631 hexadecenoic acid on inflammatory signaling in macrophages, DCs, as well as on T cell
632 differentiation and function, the potential role of PPAR α in these effects, and consequences for
633 stress-induced exaggeration of anxiety- and fear-related behavioral responses.

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

641 **AUTHOR CONTRIBUTIONS**

642 G.S.B and P.A.I. isolated and synthesized 1,2,3-tri[Z-10-hexadecenoyl]glycerol. W.X. and X.W.
643 developed a synthesis for 10(Z)-hexadecenoic acid and synthesized the compound. Experimental
644 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
645 designed the PPAR experiments. *In vivo* screening and experimentation was performed by R.M.,
646 and L.R.B. *In vitro* experiments using freshly isolated murine peritoneal macrophages were
647 performed by D.G.S. Transfections and reporter gene assays were performed by I.S. and P.B.
648 RNA-seq data processing and analysis was done by D.G.S., R.D.D., M.A.A. Experimental
649 design and preparation of the manuscript were done by D.G.S., R.M., G.S.B., L.N., G.A.W.R.,
650 L.R.B., and C.A.L.

651

652 **FIGURE LEGENDS**

653 **Fig. 1 Experimental timeline for *ex vivo* macrophage stimulation.**

654 Abbreviations: FFA; free fatty acid, LPS, lipopolysaccharide.

655 **Fig. 2 Anti-inflammatory effects of 10(Z)-hexadecenoic acid in freshly-isolated murine**
656 **peritoneal macrophages.**

657 Freshly isolated murine peritoneal macrophages were incubated for 1 h with synthetic 10(Z)-
658 hexadecenoic acid (0.4 μ M, 4 μ M, 20 μ M, 100 μ M, 500 μ M, 1000 μ M), then challenged with
659 lipopolysaccharide (LPS; 1 μ g/mL). Cell supernatants were collected at (A) 6 h, (B) 12 h, and
660 (C) 24 h after lipopolysaccharide (LPS) challenge. Interleukin (IL) 6 concentrations in the
661 supernatant were determined using enzyme-linked immunosorbent assay (ELISA) and reported
662 relative to media-only controls ($n = 6$ replicates, with each replicate using different freshly
663 isolated peritoneal macrophages; each sample was run using duplicate wells in the ELISA). Data
664 were fit with a logistic function, which was used to estimate the EC50. Data are expressed as
665 mean \pm SEM.

666 **Fig. 3 Gene networks upregulated following pretreatment with 10(Z)-hexadecenoic acid in**
667 **LPS-stimulated macrophages suggest anti-inflammatory effects are mediated by PPAR α .**

668 Murine peritoneal macrophages were treated with either 10(Z)-hexadecenoic acid (200 μ M) or
669 vehicle. Following a 12 h period after stimulation with lipopolysaccharide (LPS), total RNA
670 content was measured using RNA-seq. (A) Heat map of the top 20 differentially expressed
671 transcripts. (B) and (C) Genes significantly (B) upregulated or (C) downregulated with treatment
672 of 10(Z)-hexadecenoic acid were separately queried on the Database for Annotation,
673 Visualization and Integrated Discovery (DAVID). (B) The top five Kyoto Encyclopedia of

674 Genes and Genomes (KEGG) pathways enriched for genes upregulated following pretreatment
675 of LPS-stimulated macrophages with 10(Z)-hexadecenoic acid, relative to media pretreated,
676 LPS-stimulated macrophages. (C) The top five KEGG pathways enriched for genes
677 downregulated following pretreatment of LPS-stimulated macrophages with 10(Z)-hexadecenoic
678 acid, relative to media pretreated, LPS-stimulated macrophages. (D) Pathway analysis using the
679 entire transcriptional data set was performed with Gene Set Enrichment Analysis (GSEA).
680 Pathways enriched for genes upregulated following pretreatment of LPS-stimulated macrophages
681 with 10(Z)-hexadecenoic acid, relative to media-pretreated, LPS-stimulated macrophages, were
682 visualized in a network built by their gene set overlap. The size of the network node represents
683 the number of genes shared between the particular gene set and the transcription data. The
684 weight of network edges represents the degree of gene set overlap. In the largest cluster of
685 pathways enriched in genes upregulated with 10(Z)-hexadecenoic acid, lipid metabolism and
686 peroxisome proliferator-activated receptors (PPARs) were implicated as some of the more salient
687 pathways. Abbreviations: Adamtsl4, thrombospondin repeat-containing protein 1; AMPK, 5'
688 AMP-activated protein kinase; Ch25h, cholesterol 25-hydroxylase; Cish, cytokine inducible SH2
689 containing protein; Ctla2b, cytotoxic T-lymphocyte-associated protein 2-beta; Cyp26b1,
690 cytochrome P450 family 26 subfamily B member 1; Dusp1, dual specificity phosphatase 1;
691 ECM, extracellular matrix; F3, coagulation factor III; Flrt3, fibronectin leucine rich
692 transmembrane protein 3; Hdc, histidine decarboxylase; Hp, haptoglobin; Il1b, interleukin 1
693 beta; Il6, interleukin 6; LKB1, liver kinase B1; Mir5105, microRNA 5105; MTOR, mechanistic
694 target of rapamycin kinase; Plbd1, phospholipase B domain containing 1; Plin2, perilipin 2;
695 PPAR, peroxisome proliferator activated receptor; PPARA, peroxisome proliferator activated
696 receptor alpha; Ptgs2, prostaglandin-endoperoxide synthase 2; RORA, RAR related orphan

697 receptor A; TNF, tumor necrosis factor; Tns1, tensin 1; Tsc22d3, Tsc22 domain family member
698 3; Vnn3, vanin 3.

699

700 **Fig. 4. 10(Z)-hexadecenoic acid suppresses expression of transcription factors downstream**
701 **of TLR4.**

702 Murine peritoneal macrophages were treated with either 10(Z)-hexadecenoic acid (200 μ M) or
703 vehicle for 1 h, then challenged with lipopolysaccharide (LPS; 1 μ g/ml). Following a 12 h period
704 after stimulation with LPS, mRNA was measured using RNA-seq. (A) From Gene Set
705 Enrichment Analysis (GSEA; c2.all.v6.2), pathways enriched with genes downregulated
706 following pretreatment of LPS-stimulated macrophages with 10(Z)-hexadecenoic acid, relative to
707 media-pretreated, LPS-stimulated macrophages, were visualized in a network built by their gene
708 set overlap. The size of the network node indicates the number of genes shared between the
709 particular gene set and the transcription data from our study. The weight of network edges
710 indicates the degree of gene set overlap between nodes. The color of the node indicates whether
711 the genes in the gene set are upregulated in NF- κ B pathways (blue), upregulated in IRF pathways
712 (purple), ambiguously upregulated (gray), or downregulated (red). (B) Among the leading edges
713 of enriched pathway gene sets, the occurrence of high ranking genes in either the NF- κ B-
714 regulated network (blue) or IRF-regulated network (purple) (corresponding to data illustrated in
715 panel A) are reported. (C) Genes included in the IRF_Q6 gene set (left; i.e., genes having at least
716 one occurrence of the transcription factor binding site V\$IRF_Q6 (v7.4 TRANSFAC) in the
717 regions spanning up to 4 kb around their transcription starting sites) and the significant 10(Z)-
718 hexadecenoic acid-dependent differentially expressed genes with $q < 0.1$ (right) were queried
719 against the Interferome database (v2.0) to identify their association with known interferon
720 responses. The majority of genes in both gene sets are attributed to both Type I and Type II

721 interferon responses. Abbreviations: CCL2, C-C motif chemokine ligand 2; CXCL1, C-X-C
722 motif chemokine ligand 1; CXCL2, C-X-C motif chemokine ligand 2; IER3, immediate early
723 response 3; IFN, interferon; IFNA, interferon alpha; IFNB1, interferon beta 1; IKK, inhibitor of
724 nuclear factor kappa B kinase; INHBA, inhibin subunit beta A; IL1A, interleukin 1 alpha; IL1B,
725 interleukin 1 beta; IL1R, interleukin 1 receptor; IL6, interleukin 6; JUNB, junB proto-oncogene,
726 AP-1 transcription factor subunit; LPS, lipopolysaccharide; NFKB, nuclear factor kappa B;
727 NFKB1, nuclear factor kappa B subunit 1; PLAUR, plasminogen activator, urokinase receptor;
728 PSMB8, proteasome subunit beta 8; PSMB9, proteasome subunit beta 9; PSMB10, proteasome
729 subunit beta 10; PSME1, proteasome activator subunit 1; PTGS2, prostaglandin-endoperoxide
730 synthase 2; STAT3, signal transducer and activator of transcription 3; TNF, tumor necrosis
731 factor.

732

733 **Fig. 5 Analysis of the effects of *M. vaccae*-derived lipids on peroxisome proliferator-**
734 **activated receptor (PPAR) α , PPAR γ , PPAR δ , and retinoic acid receptor (RAR) α signaling**
735 **in transfection assays using COS-1 cells.**

736 (A) Relative activity of PPAR α following incubation with the 1,2,3-tri[Z-10-
737 hexadecenoyl]glycerol (PI-70; TAG), monoacylglycerol, 1-[Z-10-hexadecenoyl]glycerol (PI-69;
738 MAG), or 10(Z)-hexadecenoic acid (PI-71; FFA) for 18 h, expressed as relative luciferase
739 activity, normalized to β -galactosidase activity. (B) Relative activity of PPAR γ . (C) Relative
740 activity of PPAR δ . (D) Relative activity of RAR α . Abbreviations and concentrations: AM580
741 (RAR α -specific agonist, 100 nM), GW1516 (PPAR δ agonist, 1 μ M), RSG, rosiglitazone
742 (PPAR γ agonist, 2.5 μ M), troglitazone (PPAR γ agonist, 10 μ M), WY-14643 (PPAR α agonist, 2
743 μ M). Data are representative of 2-3 replicates per experiment.

744 **Fig. 6 PPAR α is required for suppression of LPS-induced inflammation in macrophages.**

745 A peroxisome proliferator-activated receptor (PPAR) α , $-\gamma$, or $-\delta$ antagonist (GW 6471, GW
746 9662, GSK 0660 respectively) or vehicle was applied to murine peritoneal macrophages
747 followed by treatment with either 10(Z)-hexadecenoic acid (200 μ M), vehicle, or dexamethasone
748 (Dex; 10 μ M), then stimulated with lipopolysaccharide (LPS; 1 μ g/ml). (A) After 12 h,
749 interleukin (IL) 6 was measured in the cell supernatant and reported relative to vehicle controls.
750 (B) The necessity of PPAR α was shown in a PPAR α knock out (KO) model. Murine peritoneal
751 macrophages from PPAR α ^{-/-} or WT mice were incubated with either 10(Z)-hexadecenoic acid
752 (50 μ M or 200 μ M) or vehicle, then stimulated with LPS (1 μ g/ml). # p < 0.05, Fisher's least
753 significant difference (LSD), relative to cells only treated with 10(Z)-hexadecenoic acid. * p <
754 0.05 relative to KO.

755 **SUPPORTING INFORMATION TITLES AND CAPTIONS**

756 **Table S1. Dose- and time-dependent effects of 10(Z)-hexadecenoic acid on secretion of IL-6**
757 **from freshly isolated murine peritoneal macrophages stimulated with lipopolysaccharide.**

758 **Table S2. Descriptive statistics of cDNA libraries and RNA-sequencing.**

759 **Table S3. List of 203 differentially expressed genes between treatment with 10(Z)-**
760 **hexadecenoic acid and vehicle in LPS-stimulated macrophages (FDR-adjusted p < 0.1)**

761 **Table S4. KEGG pathways and GO biological processes with associated genes that are**
762 **significantly downregulated in LPS-stimulated murine macrophages preincubated with**
763 **10(Z)-hexadecenoic acid, relative to LPS-stimulated murine macrophages preincubated**
764 **with media.**

765 **Table S5. Top scoring KEGG pathways enriched for differentially expressed genes ($q <$**
766 **0.1).**

767 **Table S6. Top scoring KEGG pathway enrichment scores of 10(Z)-hexadecenoic acid**
768 **treatment**

769 **Table S7. Transcription factor binding site enrichment scores of 10(Z)-hexadecenoic acid**
770 **treatment**

771 **Table S8. Transcription factor binding site enrichment scores of DMEM (i.e., LPS**
772 **exposure, in the absence of 10(Z)-hexadecenoic acid treatment)**

773 **Table S9. Selective peroxisome proliferator-activated receptor (PPAR) antagonists and**
774 **agonists.**

775 **Table S10. Lipopolysaccharide-induced proinflammatory cytokine and chemokine ligand**
776 **mRNAs downregulated by preincubation of freshly isolated murine peritoneal**
777 **macrophages with 10(Z)-hexadecenoic acid (selected from 203 differentially expressed**
778 **mRNAs)**

779 **Fig. S1. 10(Z)-hexadecenoic acid alone has no detectable effect on IL-6 release.**

780 After isolation of peritoneal macrophages and incubation with 10(Z)-hexadecenoic acid for 1 h,
781 macrophages were challenged with either lipopolysaccharide (LPS) or Dulbecco's Modified
782 Eagle Medium (DMEM; as control). There was no detectable effect of 10(Z)-hexadecenoic acid
783 on interleukin (IL) 6 secretion in the cultures that did not receive LPS. Abbreviations: IL-6,
784 interleukin 6; LPS, lipopolysaccharide. Data are representative of 3 replicates per condition.

785 **Fig. S2. Effect of 10(Z)-hexadecenoic acid on macrophage cell viability.**

786 Sulforhodamine B (SRB) was used to assess cytotoxic effects of various concentrations of
787 synthetic 10(Z)-hexadecenoic acid (10 μ M, 50 μ M, 125 μ M, 250 μ M, 500 μ M, 1000 μ M) after
788 0, 6, 12, 24, 48, and 72 h of incubation with freshly isolated murine peritoneal macrophages.
789 Percent control growth is expressed as % viability and is a ratio of the amount of growth that
790 occurred with treatment over the amount of growth that occurred in media. One hundred percent
791 indicates no differences in cell growth between treatment and media, whereas values below
792 100% indicate that growth was impaired with treatment. Data are expressed as mean \pm SEM of
793 3-7 mice per condition.

794

795 **Fig. S3. BioAnalyzer electropherograms of cDNA libraries used for RNA-sequencing.** Total
796 RNA content of 1×10^5 macrophages was prepared for each sample utilizing separate macrophage
797 preparations from $n = 3$ mice treated with vehicle (Dulbecco's Modified Eagle Medium
798 (DMEM; upper row) then challenged with 1 μ g/mL lipopolysaccharide (LPS) or $n = 3$ mice
799 treated with 200 μ M 10(Z)-hexadecenoic acid for 1 h, then challenged with 1 μ g/mL LPS.
800 Macrophages were harvested 12 h following LPS challenge. Peaks at 35 bp and 10,380 bp are
801 gel migration markers.

802

803 **Fig. S4. Effect of PPAR agonists and antagonists on macrophage cell viability.**

804 Sulforhodamine B (SRB) was used to assess cytotoxic effects of PPAR agonists (PPAR α , WY-
805 14643; PPAR γ , rosiglitazone (RSG); PPAR δ , GW0742) or PPAR agonists and antagonists
806 (PPAR α , GW6471; PPAR γ , GW9662; PPAR δ , GSK0660). The agonists and antagonists were
807 incubated with freshly isolated murine peritoneal macrophages at 2x their respective EC50 or
808 IC50 (see Table S9). Percent control growth is expressed as % viability and is a ratio of the

809 amount of growth that occurred with treatment over the amount of growth that occurred in
810 media. One hundred percent indicates no differences in cell growth between treatment and
811 media, whereas values below 100% indicate that growth was impaired with treatment. Data are
812 expressed as mean \pm SEM of 3-7 mice per condition.

813 **Figure S5. Suppression of IL-6 in LPS-stimulated macrophages is achieved through**
814 **activation of PPAR α and reversed by addition of a PPAR α antagonist.**

815 Murine peritoneal macrophages were incubated with peroxisome proliferator-activated receptor
816 (PPAR) α antagonist (GW 6471), PPAR γ antagonist (GW 9662), PPAR δ antagonist (GSK
817 0660), or Dulbecco's Modified Eagle Medium (DMEM)/F-12. After a 1-h incubation, the cells
818 were treated with the complementary agonist (PPAR α : WY-14643, PPAR γ : rosiglitazone; Rosi.,
819 PPAR δ : GW 0742). For each agonist, four concentrations were assayed, 1x, 2x, 5x, and 10x the
820 half-maximal effective concentration (EC₅₀). The immune response was measured as the
821 concentration of interleukin (IL) 6 in the cell supernatant relative to vehicle controls. # $p < 0.05$
822 main effect of agonist + antagonist condition relative to agonist alone condition in a multifactor
823 ANOVA. * $p < 0.05$, Fisher's least significant difference (LSD), pairwise comparison relative to
824 antagonist-treated cells.

825

826

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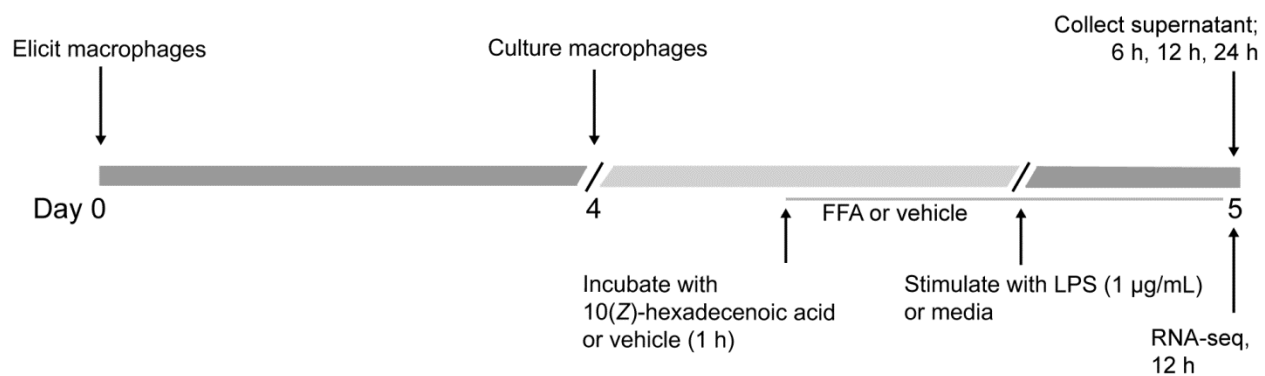
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1121 **FIGURE 1**

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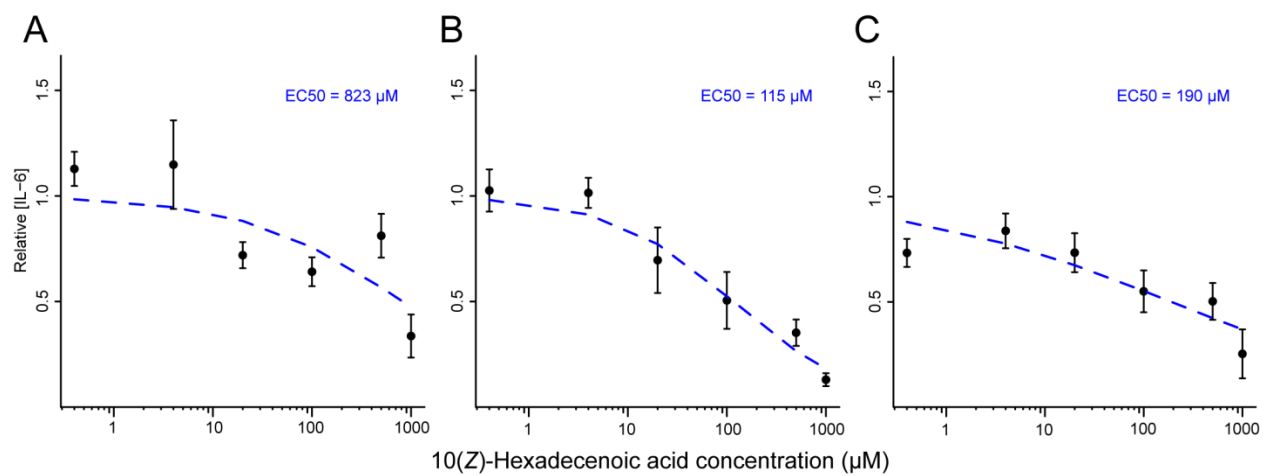
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1136 **FIGURE 2**

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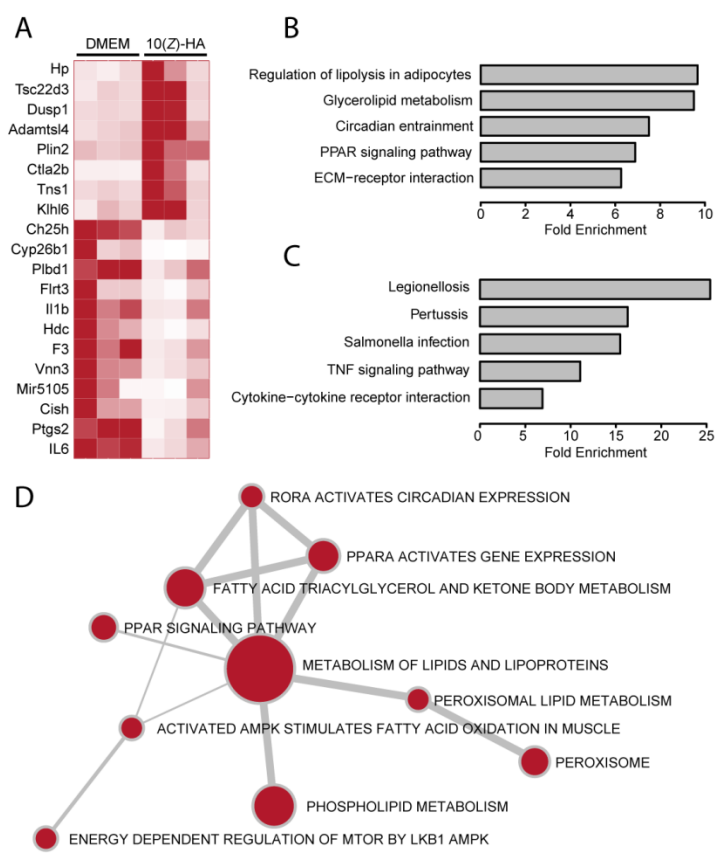
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1150 **FIGURE 3**



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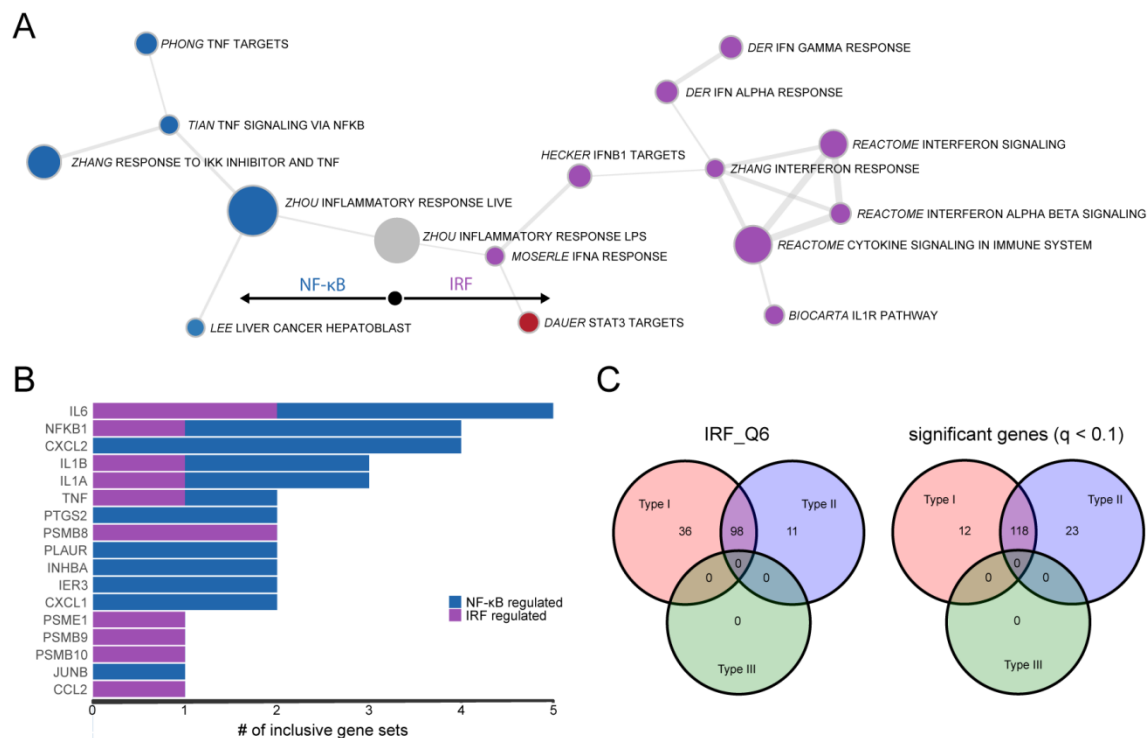
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1160 **FIGURE 4**



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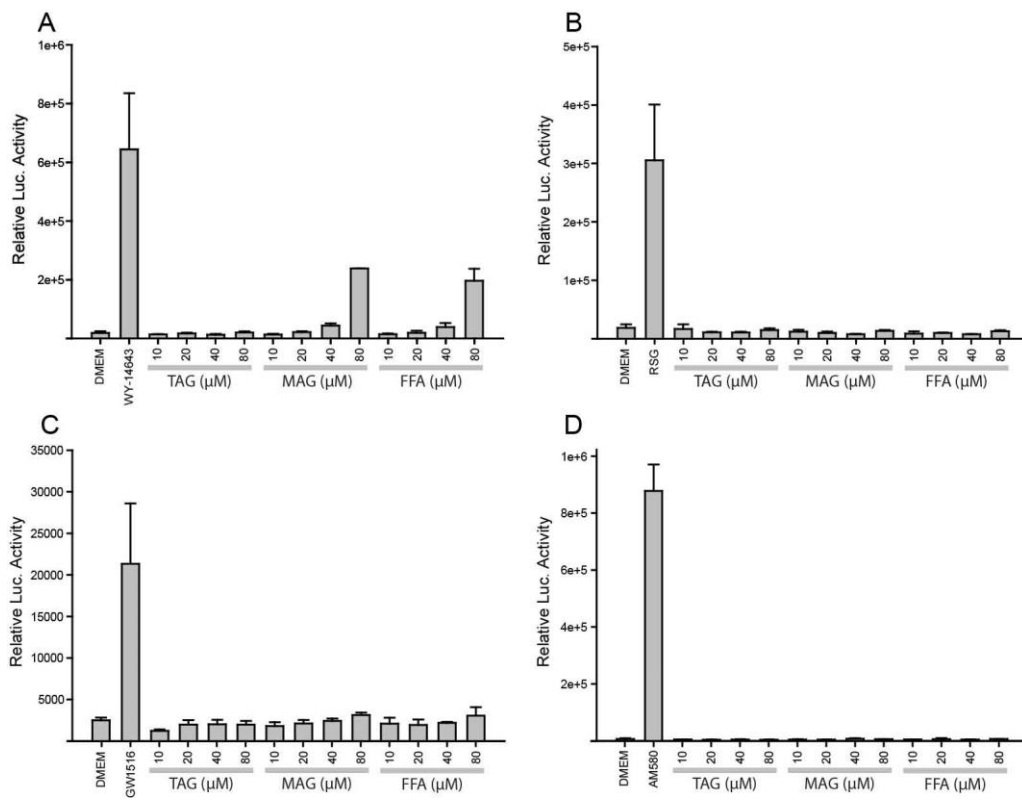
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1171 **FIGURE 5**

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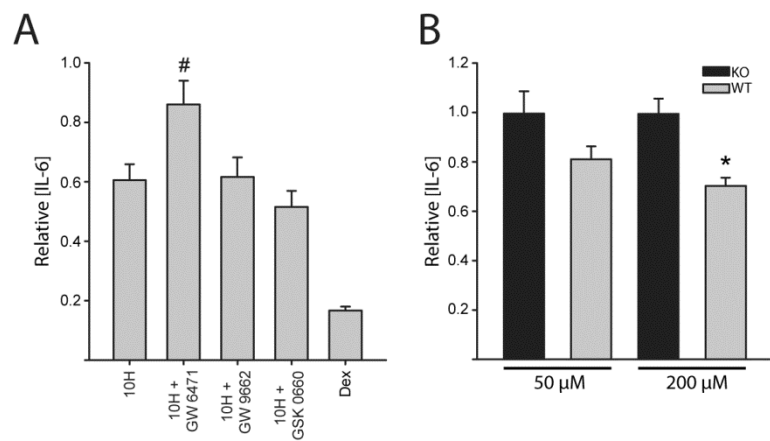
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1181 **FIGURE 6**

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1195 **SUPPLEMENTAL MATERIAL**1196 **Supplemental Tables**

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1198 **Table S1. Dose- and time-dependent effects of 10(Z)-hexadecenoic acid on secretion of IL-6**
1199 **from freshly isolated murine peritoneal macrophages stimulated with lipopolysaccharide.**

Concentration	P-values ¹					
	6h		12h		24h	
	LMER	<i>t</i> -test	LMER	<i>t</i> -test	LMER	<i>t</i> -test
0.4	0.45488	0.1957	0.97506	0.6117	0.172346	0.08114
4	0.12283	0.2779	0.754265	0.4239	0.810008	0.07019
20	0.16382	0.04546*	0.011331*	0.07472	0.096179	0.103
100	0.0616	0.03446*	0.000424***	0.02735*	0.00377**	0.03797*
500	0.62085	0.1786	0.000159***	0.0195*	0.003926**	0.032*
1000	0.01311*	0.002494**	2.12E-05***	0.01586*	0.000405***	0.028502*

1200 Statistical tests consisted of pairwise comparisons of raw IL-6 values relative to paired media
1201 control values at the same time point ($n = 6$ per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,
1202 two-tailed LMER or Student's *t*-test. ¹Abbreviations: LMER, linear mixed effects in R.
1203

1204 **Table S2. Descriptive statistics of cDNA libraries and RNA-sequencing.**

Sample	Library	Avg. frag.	counts
	Conc. (nM)	length (bp)	(M)
DMEM(1)	144.9	378	56.6
DMEM(2)	121	411	60.8
DMEM(3)	204.6	394	47.4
10H(1)	246	388	51.4
10H(2)	280.9	402	62.8
10H(3)	249.6	404	63.6

1205 Abbreviations: 10H, 10(Z)-hexadecenoic acid; DMEM, Dulbecco's Modified Eagle Medium

1206 (DMEM)/F-12

1207

1208 **Table S3. List of 203 differentially expressed genes between treatment with 10(Z)-**

1209 **hexadecenoic acid and vehicle in LPS-stimulated macrophages (FDR-adjusted $p < 0.1$).**

Log2Fold Change	padj	gene	Ensembl ID	Log2Fold Change	padj	gene	
2.155269	2.26E-21	Hp	ENSMUSG00000031722	4.175519	0.006465	Gm807	ENSMUSG00000097848
-2.09473	4.37E-19	Il6	ENSMUSG00000025746	0.866752	0.006465	Cpt1a	ENSMUSG00000024900
2.132848	3.67E-17	Tsc22d3	ENSMUSG00000031431	1.201891	0.006658	Havcr2	ENSMUSG00000020399
-1.81185	8.08E-16	Ptgs2	ENSMUSG00000032487	-0.84047	0.006831	Zyx	ENSMUSG00000029860
-2.30575	4.38E-15	Cish	ENSMUSG00000032578	1.679239	0.006937	Wee1	ENSMUSG00000031016
1.986963	1.67E-14	Dusp1	ENSMUSG00000024190	1.318917	0.007903	Adrb2	ENSMUSG00000045730
2.012712	1.86E-12	Adamts14	ENSMUSG00000015850	-1.02689	0.008108	Timp1	ENSMUSG00000001131
-1.6455	1.98E-12	Mir5105	ENSMUSG00000093077	1.166393	0.008496	Foxred2	ENSMUSG00000016552
-2.04621	2.63E-12	Vnn3	ENSMUSG00000020010	1.245321	0.009034	Jdp2	ENSMUSG00000034271
1.578041	2.63E-12	Plin2	ENSMUSG00000028494	-0.8805	0.009042	Pim1	ENSMUSG00000024014
-1.93629	5.09E-12	F3	ENSMUSG00000028128	0.881662	0.009058	Sgms1	ENSMUSG00000040451
-2.8558	5.31E-12	Hdc	ENSMUSG00000027360	1.272806	0.00933	Rims3	ENSMUSG00000032890
-1.5767	6.48E-12	Il1b	ENSMUSG00000027398	0.915219	0.009572	Prkar2b	ENSMUSG00000002997
-2.15506	7.97E-12	Flrt3	ENSMUSG00000051379	-1.60497	0.009607	Rnf180	ENSMUSG00000021720
3.024984	2.40E-11	Ctla2b	ENSMUSG00000074874	-2.14325	0.009652	Syt7	ENSMUSG00000024743
1.811323	4.79E-11	Tns1	ENSMUSG00000055322	-1.6082	0.009895	Hspa1a	ENSMUSG00000091971
1.82332	1.45E-10	Klhl6	ENSMUSG00000043008	-1.02922	0.010112	Tfec	ENSMUSG00000029553
-1.53809	2.78E-10	Plbd1	ENSMUSG00000030214	-1.32561	0.010112	Zfp558	ENSMUSG00000074500
-4.06225	4.95E-10	Cyp26b1	ENSMUSG00000063415	-0.78966	0.010112	Il1rn	ENSMUSG00000026981

-2.35715	5.70E-10	Ch25h	ENSMUSG00000050370	0.801393	0.010902	Dock10	ENSMUSG00000038608
-1.4212	3.35E-09	Ccl7	ENSMUSG00000035373	0.858932	0.010902	Tgfb1	ENSMUSG00000035493
-1.34039	4.91E-09	Ccl2	ENSMUSG00000035385	0.788151	0.010902	Pla2g7	ENSMUSG00000023913
-3.35822	6.81E-09	Car4	ENSMUSG00000000805	1.794042	0.010902	Srgap3	ENSMUSG00000030257
1.750436	1.16E-08	Susd2	ENSMUSG00000006342	0.983421	0.010993	Abcc3	ENSMUSG00000020865
-3.51502	2.92E-08	Adm	ENSMUSG00000030790	-1.03832	0.012028	Dennd3	ENSMUSG00000036661
-1.41666	4.57E-08	Il12a	ENSMUSG00000027776	0.78379	0.013193	Man2a1	ENSMUSG00000024085
1.394626	5.98E-08	Ppp1r12b	ENSMUSG00000073557	1.561095	0.013193	Frm4b	ENSMUSG00000030064
1.317669	7.23E-08	Dennd4c	ENSMUSG00000038024	1.181418	0.013404	Per2	ENSMUSG00000055866
-1.56573	7.86E-08	Gm10499	ENSMUSG00000073403	1.920189	0.013773	Kenk13	ENSMUSG00000045404
-1.76168	1.01E-07	Hbegf	ENSMUSG00000024486	-0.80185	0.014517	Plk2	ENSMUSG00000021701
1.619779	2.04E-07	Ms4a8a	ENSMUSG00000024730	1.39729	0.014584	Nrg4	ENSMUSG00000032311
-1.29736	2.22E-07	Itgax	ENSMUSG00000030789	1.946616	0.016015	Fabp4	ENSMUSG00000062515
-1.48148	3.26E-07	Ccl22	ENSMUSG00000031779	0.814942	0.018239	Adam8	ENSMUSG00000025473
1.312963	5.18E-07	Glul	ENSMUSG00000026473	-0.99463	0.018957	Slamf1	ENSMUSG00000015316
-1.29094	6.75E-07	Iigp1	ENSMUSG00000054072	0.896952	0.01948	Plpp1	ENSMUSG00000021759
1.426376	7.14E-07	Lpxn	ENSMUSG00000024696	0.778795	0.01948	Cd47	ENSMUSG00000055447
-1.30554	8.49E-07	Slc1a2	ENSMUSG00000005089	3.257959	0.019865	Saxo1	ENSMUSG00000028492
1.555412	8.59E-07	Fos	ENSMUSG00000021250	-2.11589	0.023245	Csf2	ENSMUSG00000018916
-1.73463	1.06E-06	Scimp	ENSMUSG00000057135	-0.83357	0.024354	Gm14023	ENSMUSG00000085498
1.307021	1.18E-06	Slc43a2	ENSMUSG00000038178	1.256371	0.026556	Cdo1	ENSMUSG00000033022
2.671207	1.26E-06	Ly6c2	ENSMUSG00000022584	-0.81499	0.027117	Ier3	ENSMUSG00000003541
1.168314	2.02E-06	Lcn2	ENSMUSG00000026822	0.806704	0.029122	Dock5	ENSMUSG00000044447
1.578367	2.97E-06	Fkbp5	ENSMUSG00000024222	-0.71877	0.029122	Ccl3	ENSMUSG00000000982
1.370214	4.86E-06	Sepp1	ENSMUSG00000064373	0.851011	0.03319	Rassf2	ENSMUSG00000027339
1.299991	5.32E-06	Sort1	ENSMUSG00000068747	-1.53448	0.03319	Schip1	ENSMUSG00000027777
-1.24524	1.79E-05	Upp1	ENSMUSG00000020407	0.943748	0.034299	Alox5ap	ENSMUSG00000060063

-1.1367	2.36E-05	Ccl6	ENSMUSG00000018927	0.738488	0.039546	Sdc4	ENSMUSG00000017009
-1.35404	2.68E-05	Cnn3	ENSMUSG00000053931	-1.05575	0.043357	Olfm1	ENSMUSG00000026833
-2.22194	2.86E-05	Gm5483	ENSMUSG00000079597	0.734737	0.044388	Mt2	ENSMUSG00000031762
1.131171	3.65E-05	Ecm1	ENSMUSG00000028108	-0.71991	0.044388	Inhba	ENSMUSG00000041324
1.297072	3.65E-05	Cacna1d	ENSMUSG00000015968	0.833112	0.044388	Ift57	ENSMUSG00000032965
-1.28314	3.90E-05	Olr1	ENSMUSG00000030162	1.775472	0.044388	Fabp7	ENSMUSG00000019874
-1.54507	4.24E-05	Ahr	ENSMUSG00000019256	-0.78572	0.044388	Trim30c	ENSMUSG00000078616
-1.96156	4.34E-05	Car2	ENSMUSG00000027562	-0.68344	0.044485	Rpph1	ENSMUSG00000092837
-2.66361	5.55E-05	Hspa1b	ENSMUSG00000090877	0.75981	0.047915	Ezr	ENSMUSG00000052397
-1.0926	6.25E-05	Plaur	ENSMUSG00000046223	-0.87194	0.048952	Cd83	ENSMUSG00000015396
-1.22776	7.12E-05	Procr	ENSMUSG00000027611	0.802102	0.048952	Gpcpd1	ENSMUSG00000027346
1.463418	8.54E-05	Nav2	ENSMUSG00000052512	-0.72003	0.050936	Nfkb2	ENSMUSG00000025225
-1.03592	0.000104	Tnf	ENSMUSG00000024401	-1.8997	0.050936	Lrrc32	ENSMUSG00000090958
-1.12432	0.000105	Serpib2	ENSMUSG00000062345	0.746734	0.051435	Itga4	ENSMUSG00000027009
-1.08005	0.000134	Clec7a	ENSMUSG00000079293	-0.92303	0.051789	Cd86	ENSMUSG00000022901
-3.0819	0.000134	Ccl17	ENSMUSG00000031780	-0.73811	0.051789	Cmklr1	ENSMUSG00000042190
2.207158	0.000208	Orml	ENSMUSG00000039196	-1.11436	0.051885	Il12b	ENSMUSG00000004296
1.323308	0.000232	Cd300a	ENSMUSG00000034652	-1.98017	0.053401	Gm13872	ENSMUSG00000087185
0.972281	0.00028	Saa3	ENSMUSG00000040026	0.784546	0.056288	Gm12840	ENSMUSG00000086320
-1.20636	0.000281	Egr2	ENSMUSG00000037868	-0.70065	0.05698	Oasl1	ENSMUSG00000041827
-0.95987	0.000319	Il1a	ENSMUSG00000027399	-0.70774	0.05698	Irf8	ENSMUSG00000041515
-1.62852	0.00032	Tnc	ENSMUSG00000028364	-1.19175	0.058415	Frmd6	ENSMUSG00000048285
1.004165	0.000329	Xdh	ENSMUSG00000024066	0.849896	0.058415	Per1	ENSMUSG00000020893
-0.97547	0.000451	Cmpk2	ENSMUSG00000020638	0.735235	0.059193	Hal	ENSMUSG00000020017
1.213513	0.000585	Htra1	ENSMUSG00000006205	-0.72158	0.059548	Wars	ENSMUSG00000021266
-1.53878	0.000656	Irf4	ENSMUSG00000021356	1.006913	0.062477	Mmp19	ENSMUSG00000025355
-1.42269	0.000656	AA467197	ENSMUSG00000033213	-0.57969	0.062658	Gm10800	ENSMUSG00000075014

-2.07631	0.000696	Kazn	ENSMUSG00000040606	-0.67963	0.065954	Nos2	ENSMUSG00000020826
0.956982	0.000867	Cd24a	ENSMUSG00000047139	-0.83535	0.066479	Csrnp1	ENSMUSG00000032515
-0.89122	0.000958	Rmrp	ENSMUSG00000088088	0.712094	0.068155	Gm26809	ENSMUSG00000097815
-0.9802	0.001016	Ccl4	ENSMUSG00000018930	-0.78044	0.068155	Insig1	ENSMUSG00000045294
3.903311	0.001194	Dnah12	ENSMUSG00000021879	-0.61444	0.070733		ENSMUSG00000045999
1.169473	0.001524	Rin3	ENSMUSG00000044456	1.704424	0.071129	Bpife	ENSMUSG00000050108
1.169244	0.001741	F13a1	ENSMUSG00000039109	0.701972	0.071129	Pdxk	ENSMUSG00000032788
-0.949	0.002022	Chst11	ENSMUSG00000034612	0.994597	0.071577	Trim29	ENSMUSG00000032013
-0.9961	0.002166	Casp7	ENSMUSG00000025076	-0.8871	0.076468	Mmp13	ENSMUSG00000050578
3.931076	0.002435	Bach2os	ENSMUSG00000086150	0.991755	0.078103	Wipi1	ENSMUSG00000041895
0.885144	0.002435	Lox	ENSMUSG00000024529	1.00311	0.079388	Serinc5	ENSMUSG00000021703
-1.66375	0.002486	Rhoh	ENSMUSG00000029204	-1.25174	0.079543	Il1f9	ENSMUSG00000044103
-1.24964	0.002515		ENSMUSG00000092773	-0.67556	0.080434	Nfkb1	ENSMUSG00000028163
-1.08726	0.002887	Socs1	ENSMUSG00000038037	0.942972	0.080838	Syt11	ENSMUSG00000068923
1.302178	0.003251	Paqr7	ENSMUSG00000037348	1.324205	0.080838	Klra2	ENSMUSG00000030187
-0.90079	0.003254	Mmp12	ENSMUSG00000049723	-0.71767	0.082101	Tmem2	ENSMUSG00000024754
-1.06355	0.003509	Csf3	ENSMUSG00000038067	0.734669	0.082101	Ergic1	ENSMUSG00000001576
-2.35111	0.003653	Il11	ENSMUSG00000004371	-0.80694	0.08253	Wfs1	ENSMUSG00000039474
-0.84287	0.003941	Rsad2	ENSMUSG00000020641	-0.68387	0.082531	Isg15	ENSMUSG00000035692
-1.12922	0.004208	Timp3	ENSMUSG00000020044	-0.67778	0.083148	Cxcl3	ENSMUSG00000029379
-2.03218	0.004208	Destamp	ENSMUSG00000022303	-0.90013	0.086117	Fst	ENSMUSG00000021765
1.386263	0.00424	Mgll	ENSMUSG00000033174	1.157447	0.087469	Apoc2	ENSMUSG00000002992
-0.89309	0.004485	Dab2	ENSMUSG00000022150	0.80021	0.090822	Cyth3	ENSMUSG00000018001
1.14163	0.005073	Mafb	ENSMUSG00000028874	1.07939	0.090822	Col18a1	ENSMUSG00000001435
0.940402	0.005073	Fgr	ENSMUSG00000074622	-1.25793	0.091248	Osmr	ENSMUSG00000022146
-0.81843	0.006355	Cxcl2	ENSMUSG00000058427	-1.42935	0.091568	Alpk2	ENSMUSG00000032845
-1.24059	0.006465	Ptgs2os2	ENSMUSG00000097754	-0.72425	0.09327	Axl	ENSMUSG00000002602

				0.815505	0.095702	Aldh9a1	ENSMUSG00000026687
				0.925759	0.095702	Cav1	ENSMUSG00000007655
				3.350338	0.099786	Glyctk	ENSMUSG00000020258

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1223 **Table S4. KEGG pathways and GO biological processes with associated genes that are significantly**
 1224 **downregulated in LPS-stimulated murine macrophages preincubated with 10(Z)-hexadecenoic**
 1225 **acid, relative to LPS-stimulated murine macrophages preincubated with media.**

NF-κB (KEGG PATHWAY: mmu04064)	Jak-STAT (KEGG PATHWAY: mmu04630)	Inflammatory response (GO:0006954)	
chemokine (C-C motif) ligand 4 (Ccl4)	colony stimulating factor 2 (granulocyte- macrophage)(Csf2)	AXL receptor tyrosine kinase (Axl)	chemokine (C-X-C motif) ligand 3 (Cxcl3)
interleukin 1 beta (Il1b)	colony stimulating factor 3 (granulocyte) (Csf3)	C-type lectin domain family 7, member a (Clec7a)	cytochrome P450, family 26, subfamily b, polypeptide 1 (Cyp26b1)
nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105 (Nfkb1)	cytokine inducible SH2- containing protein (Cish)	chemokine (C-C motif) ligand 17 (Ccl17)	interleukin 1 alpha (Il1a)
nuclear factor of kappa light polypeptide gene enhancer in B cells 2, p49/p100 (Nfkb2)	interleukin 11 (Il11)	chemokine (C-C motif) ligand 2 (Ccl2)	interleukin 1 beta (Il1b)
prostaglandin- endoperoxide synthase 2 (Ptgs2)	interleukin 12a (Il12a)	chemokine (C-C motif) ligand 22 (Ccl22)	interleukin 1 family, member 9 (Il1f9)
tumor necrosis factor (Tnf)	interleukin 12b (Il12b)	chemokine (C-C motif) ligand 3 (Ccl3)	interleukin 6 (Il6)
	interleukin 6 (Il6)	chemokine (C-C motif) ligand 4 (Ccl4)	nitric oxide synthase 2, inducible (Nos2)
	oncostatin M receptor (Osmr)	chemokine (C-C motif) ligand 6 (Ccl6)	nuclear factor of kappa light polypeptide gene

			enhancer in B cells 2, p49/p100 (Nfkb2)
	proviral integration site 1 (Pim1)	chemokine (C-C motif) ligand 7 (Ccl7)	oxidized low density lipoprotein (lectin-like) receptor 1 (Olr1)
	suppressor of cytokine signaling 1(Socs1)	chemokine (C-X-C motif) ligand 2(Cxcl2)	prostaglandin- endoperoxide synthase 2 (Ptgs2)
			tumor necrosis factor (Tnf)

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1233 **Table S5. Top scoring KEGG pathways enriched for differentially expressed genes ($q <$** 1234 **0.1).**

Term	Count	% genes in pathwa y	Fold Enrichme nt	Benjami ni	FDR

mmu05134:Legionellosis	12	6.18556 7	15.33267	3.36E-08	2.33E- 07
mmu05133:Pertussis	11	5.67010 3	10.82611	4.33E-06	6.01E- 05
mmu05132:Salmonella infection	11	5.67010 3	10.27092	4.83E-06	1.01E- 04
mmu05140:Leishmaniasis	10	5.15463 9	11.37972	7.38E-06	2.05E- 04
mmu05323:Rheumatoid arthritis	10	5.15463 9	8.88173	5.13E-05	0.00177 9
mmu04668:TNF signaling pathway	11	5.67010 3	7.349836	5.77E-05	0.00240 2
mmu04620:Toll-like receptor signaling pathway	10	5.15463 9	7.21091	2.11E-04	0.01023 4
mmu05142:Chagas disease (American trypanosomiasis)	10	5.15463 9	7.070892	2.17E-04	0.01202 5
mmu04640:Hematopoietic cell lineage	9	4.63917 5	7.803235	3.27E-04	0.02039 6
mmu04060:Cytokine-cytokine receptor interaction	14	7.21649 5	4.161725	4.43E-04	0.03073 7
mmu05164:Influenza A	11	5.67010	4.684983	0.001651	0.12604

		3			3
mmu05321:Inflammatory bowel disease (IBD)	7	3.60824	8.64087	0.00203	0.16908
		7			5
mmu04630:Jak-STAT signaling pathway	10	5.15463	5.022772	0.002005	0.18089
		9			3
mmu05146:Amoebiasis	9	4.63917	5.602322	0.002267	0.22024
		5			9
mmu04062:Chemokine signaling pathway	11	5.67010	4.087409	0.003697	0.38483
		3			4
mmu05144:Malaria	6	3.09278	9.103774	0.005023	0.55760
		4			3
mmu05162:Measles	9	4.63917	4.819645	0.005203	0.61348
		5			1
mmu05152:Tuberculosis	10	5.15463	4.138079	0.006105	0.76199
		9			7
mmu05145:Toxoplasmosis	8	4.12371	5.15612	0.007753	1.02102
		1			4
mmu05143:African trypanosomiasis	5	2.57732	10.40431	0.010775	1.49226
					5
mmu04940:Type I diabetes mellitus	6	3.09278	7.048083	0.012405	1.80263
		4			2

mmu05168:Herpes simplex infection	10	5.15463	3.501451	0.016134	2.45259
		9			2
mmu05332:Graft-versus-host disease	5	2.57732	7.002903	0.040085	6.32105
					7
mmu05205:Proteoglycans in cancer	9	4.63917	3.228925	0.045359	7.43982
		5			6
mmu04621:NOD-like receptor signaling pathway	5	2.57732	6.502695	0.047851	8.15499
					9
mmu04380:Osteoclast differentiation	7	3.60824	4.046122	0.048295	8.54390
		7			8
mmu04010:MAPK signaling pathway	10	5.15463	2.878664	0.047148	8.65159
		9			7
mmu04064:NF-kappa B signaling pathway	6	3.09278	4.50496	0.062425	11.772
		4			
mmu04622:RIG-I-like receptor signaling pathway	5	2.57732	5.355161	0.079744	15.4004
					9
mmu00910:Nitrogen metabolism	3	1.54639	12.85239	0.122031	23.7337
		2			
mmu05330:Allograft rejection	4	2.06185	5.202156	0.209372	39.6728
		6			1
mmu00340:Histidine metabolism	3	1.54639	9.103774	0.209628	40.6909

		2			
mmu04923:Regulation of lipolysis in adipocytes	4	2.06185	5.11089	0.206209	41.0717
		6			
mmu04915:Estrogen signaling pathway	5	2.57732	3.715826	0.209529	42.5792
mmu04623:Cytosolic DNA-sensing pathway	4	2.06185	4.551887	0.253222	50.7955
		6			1
mmu04932:Non-alcoholic fatty liver disease (NAFLD)	6	3.09278	2.783319	0.271611	54.6942
		4			3
mmu05020:Prion diseases	3	1.54639	6.82783	0.29259	58.8836
		2			5
mmu04917:Prolactin signaling pathway	4	2.06185	3.990695	0.310953	62.5492
		6			3
mmu05410:Hypertrophic cardiomyopathy (HCM)	4	2.06185	3.687604	0.355616	69.5583
		6			7
mmu03320:PPAR signaling pathway	4	2.06185	3.641509	0.357001	70.6484
		6			7

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1236 **Table S6. Top scoring KEGG pathway enrichment scores of 10(Z)-hexadecenoic acid**
 1237 **treatment.**

NAME	SIZE	ES	NES	NOM p- val	FDR q- val	FWER p- val
KEGG_PEROXISOME	70	0.455491	1.776987	0	0.206677	0.202
KEGG_PPAR_SIGNALING_PATHWAY	50	0.405869	1.523902	0.022688	0.965135	0.877

KEGG_CITRATE_CYCLE_TCA_CYCLE	26	0.458822	1.441984	0.062157	1	0.976
KEGG_FATTY_ACID_METABOLISM	35	0.410082	1.418581	0.066784	1	0.985
KEGG_PROPANOATE_METABOLISM	29	0.381988	1.262367	0.171735	1	1

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1239 **Table S7. Transcription factor binding site enrichment scores of 10(Z)-hexadecenoic acid**
1240 **treatment.**

NAME	SIZE	ES	NES	NOM p- val	FDR q- val	FWER p- val
MYB_Q3	193	0.354277	1.630786	0	0.4669	0.462
CREBP1_01	138	0.353955	1.554846	0.001592	0.570198	0.79
MYB_Q5_01	199	0.327401	1.527497	0.003185	0.502861	0.861
TGATTRY_GFI1_01	196	0.329035	1.51785	0	0.417293	0.885
PPARA_01	31	0.448403	1.497608	0.024433	0.41565	0.938

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1242 **Table S8. Transcription factor binding site enrichment scores of DMEM (i.e., LPS**
1243 **exposure, in the absence of 10(Z)-hexadecenoic acid treatment).**

NAME	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p- val
YAATNANRNNNCAG_UNKNOWN	111	-0.38331	-1.73728	0	0.188651	0.134
GGAMTNNNNNTCCY_UNKNOWN	45	-0.45405	-1.72149	0	0.105558	0.147
NFKB_Q6_01	196	-0.34847	-1.68059	0	0.110536	0.223
NFKB_Q6	214	-0.33542	-1.63828	0	0.137538	0.345
GGCNKCCATNK_UNKNOWN	110	-0.35338	-1.56007	0.002342	0.235213	0.583
IRF_Q6	200	-0.31662	-1.53823	0.007916	0.237864	0.654
NFKB_C	216	-0.30917	-1.53386	0	0.214951	0.676
NFKAPPAB65_01	194	-0.30235	-1.46879	0.002611	0.363113	0.888
NFKAPPAB_01	210	-0.29933	-1.4586	0.002786	0.356037	0.915
IRF2_01	105	-0.32533	-1.4371	0.012019	0.39177	0.951

2	ENSMUSG0000 0025746	1302	305	0.23	-2.09	4.19E- 23	4.37E-19	Il6
13	ENSMUSG0000 0027398	15,957	5349	0.34	-2.16	4.03E- 15	6.48E-12	Il1b
21	ENSMUSG0000 0035373	1182	441	0.37	-1.42	3.37E- 12	3.35E-9	Ccl7
22	ENSMUSG0000 0035385	5656	2234	0.39	-1.34	5.18E- 12	4.91E-9	Ccl2
26	ENSMUSG0000 0027776	714	268	0.37	-1.42	5.70E- 11	4.57E-8	Il12a
33	ENSMUSG0000 0031779	327	117	0.36	-1.48	5.15E- 10	3.26E-7	Ccl2 2
47	ENSMUSG0000 0018927	810	368	0.45	-1.14	5.31E- 8	2.36E-5	Ccl6
59	ENSMUSG0000 0024401	2098	1023	0.49	-1.04	2.95E- 7	1.04E-4	Tnf
61	ENSMUSG0000 0031780	24	3	0.12	-3.08	3.97E- 7	1.34E-4	Ccl1 7
67	ENSMUSG0000 0027399	16533	8500	0.51	-0.96	1.02E- 6	3.19E-4	Il1a
77	ENSMUSG0000 0018930	603	305	0.51	-0.98	3.75E- 6	1.02E-3	Ccl4
91	ENSMUSG0000 0004371	26	5	0.20	-2.35	1.59E- 5	3.7E-3	Il11
99	ENSMUSG0000 0058427	4726	2680	0.57	-0.82	3.01E- 5	6.4E-3	Cxcl 2 ¹
142	ENSMUSG0000	6693	4067	0.61	-0.72	1.99E-	2.9E-2	Ccl3

	0000982					4		
158	ENSMUSG00000025225	916	556	0.61	-0.72	3.85E-4	5.09E-2	Nfkb 2
161	ENSMUSG00000042190	789	473	0.60	-0.74	4.02E-4	5.18E-2	Cmkl r1
163	ENSMUSG00000004296	77	35	0.46	-1.11	4.05E-4	5.19E-2	Il12b
167	ENSMUSG00000041515	775	475	0.61	-0.71	4.56E-4	5.70E-2	Irf8
185	ENSMUSG00000044103	50	21	0.42	-1.25	7.05E-4	7.95E-2	Il1f9
186	ENSMUSG00000028163	1109	694	0.63	-0.68	7.16E-4	8.04E-2	Nfkb 1
193	ENSMUSG00000029379	8341	5214	0.62	-0.68	7.68E-4	8.31E-2	Cxcl 3

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¹Cxcl2 is a functional homologue of human IL-8

Table S11. Significant enrichment of “immune system process” (GO: 0006955) in lipopolysaccharide-induced mRNAs downregulated by preincubation of freshly isolated murine peritoneal macrophages with 10(Z)-hexadecenoic acid.

Ensemble ID	Mean #Reads LPS (n =3)	Mean #Reads s148.2ffa +LPS (n = 3)	Log2 Fold-change	p value	Adjusted p value	Gene
ENSMUSG00000019256	124.9318	42.81153	-1.54507	1.08E-07	4.24E-05	Ahr
ENSMUSG00000029379	8341.778	5214.664	-0.67778	0.000768	0.083148	Cxcl3
ENSMUSG00000029204	49.42187	15.5984	-1.66375	1.01E-05	0.002486	Rhoh
ENSMUSG00000041827	1097.95	675.5632	-0.70065	0.000454	0.05698	Oasl1
ENSMUSG00000041515	775.8443	475.0327	-0.70774	0.000456	0.05698	Irf8

ENSMUSG0000004296	77.84137	35.95454	-1.11436	0.000405	0.051885	Il12b
ENSMUSG00000042190	789.4998	473.3228	-0.73811	0.000402	0.051789	Cmklr1
ENSMUSG00000027398	15956.74	5349.469	-1.5767	4.03E-15	6.48E-12	Il1b
ENSMUSG00000027399	16533.28	8499.829	-0.95987	1.02E-06	0.000319	Il1a
ENSMUSG00000058427	4726.055	2679.948	-0.81843	3.01E-05	0.006355	Cxcl2
ENSMUSG00000035692	773.1049	481.2528	-0.68387	0.000759	0.082531	Isg15
ENSMUSG00000022901	143.9578	75.92354	-0.92303	0.0004	0.051789	Cd86
ENSMUSG00000018916	26.48043	6.109115	-2.11589	0.000154	0.023245	Csf2
ENSMUSG00000038067	215.195	102.9609	-1.06355	1.51E-05	0.003509	Csf3
ENSMUSG00000044103	50.60669	21.25181	-1.25174	0.000705	0.079543	Il1f9
ENSMUSG00000000982	6693.382	4066.999	-0.71877	0.000199	0.029122	Ccl3
ENSMUSG00000015316	164.228	82.4202	-0.99463	0.000122	0.018957	Slamf1
ENSMUSG00000002602	383.6876	232.2509	-0.72425	0.000893	0.09327	Axl
ENSMUSG00000054072	609.7951	249.2138	-1.29094	1.13E-09	6.75E-07	Iigp1
ENSMUSG00000022303	33.02525	8.074181	-2.03218	1.89E-05	0.004208	Dstamp
ENSMUSG00000027776	714.3374	267.5763	-1.41666	5.7E-11	4.57E-08	Il12a
ENSMUSG00000018930	602.5693	305.4475	-0.9802	3.75E-06	0.001016	Ccl4
ENSMUSG00000021356	87.38355	30.07534	-1.53878	2.27E-06	0.000656	Irf4
ENSMUSG00000035373	1181.631	441.2235	-1.4212	3.37E-12	3.35E-09	Ccl7
ENSMUSG00000020641	2750.461	1533.472	-0.84287	1.74E-05	0.003941	Rsad2
ENSMUSG00000018927	809.8237	368.3064	-1.1367	5.31E-08	2.36E-05	Ccl6
ENSMUSG00000035385	5656.07	2233.662	-1.34039	5.18E-12	4.91E-09	Ccl2
ENSMUSG00000030789	1173.119	477.3067	-1.29736	3.41E-10	2.22E-07	Itgax
ENSMUSG00000031780	24.3371	2.874252	-3.0819	3.97E-07	0.000134	Ccl17
ENSMUSG00000030162	279.057	114.6645	-1.28314	9.71E-08	0.000039	Olr1
ENSMUSG00000031779	326.9606	117.0914	-1.48148	5.15E-10	3.26E-07	Ccl22
ENSMUSG00000025225	916.256	556.2449	-0.72003	0.000385	0.050936	Nfkb2
ENSMUSG00000050370	100.3056	19.57727	-2.35715	5.46E-13	5.7E-10	Ch25h
ENSMUSG00000026981	3855.465	2230.31	-0.78966	5.76E-05	0.010112	Il1rn
ENSMUSG00000024401	2098.107	1023.257	-1.03592	2.95E-07	0.000104	Tnf

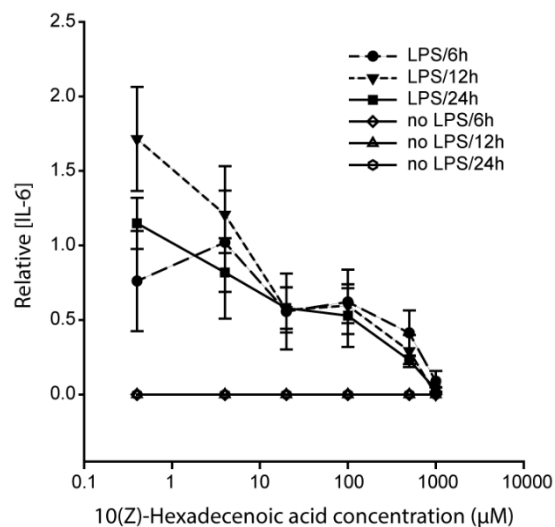
ENSMUSG00000025746	1302.137	304.8466	-2.09473	4.19E-23	4.37E-19	Il6
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1266 **Supplemental Figures**

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1269 **Fig. S1. 10(Z)-hexadecenoic acid alone has no detectable effect IL-6 release.**

1270 After isolation of peritoneal macrophages and incubation with 10(Z)-hexadecenoic acid for 1 h,
1271 macrophages were challenged with either lipopolysaccharide (LPS) or Dulbecco's Modified
1272 Eagle Medium (DMEM; as control). There was no detectable effect of 10(Z)-hexadecenoic acid
1273 on interleukin (IL) 6 secretion in the cultures that did not receive LPS. Abbreviations: IL-6,
1274 interleukin 6; LPS, lipopolysaccharide. Data are representative of 3 replicates per condition.

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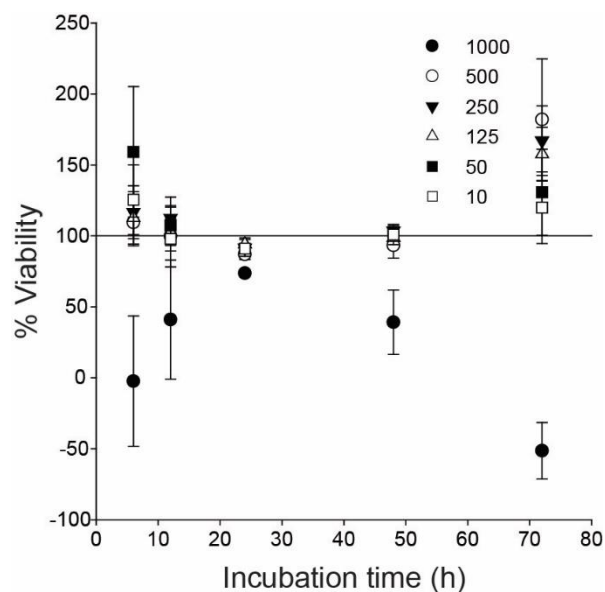
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1283 **Fig. S2. Effect of 10(Z)-hexadecenoic acid on macrophage cell viability.**

1284 Sulforhodamine B (SRB) was used to assess cytotoxic effects of various concentrations of
1285 synthetic 10(Z)-hexadecenoic acid (10 μ M, 50 μ M, 125 μ M, 250 μ M, 500 μ M, 1000 μ M) after
1286 0, 6, 12, 24, 48, and 72 h of incubation with freshly isolated murine peritoneal macrophages.
1287 Percent control growth is expressed as % viability and is a ratio of the amount of growth that
1288 occurred with treatment over the amount of growth that occurred in media. One hundred percent
1289 indicates no differences in cell growth between treatment and media, whereas values below
1290 100% indicate that growth was impaired with treatment. Data are expressed as mean \pm SEM of
1291 3-7 mice per condition.

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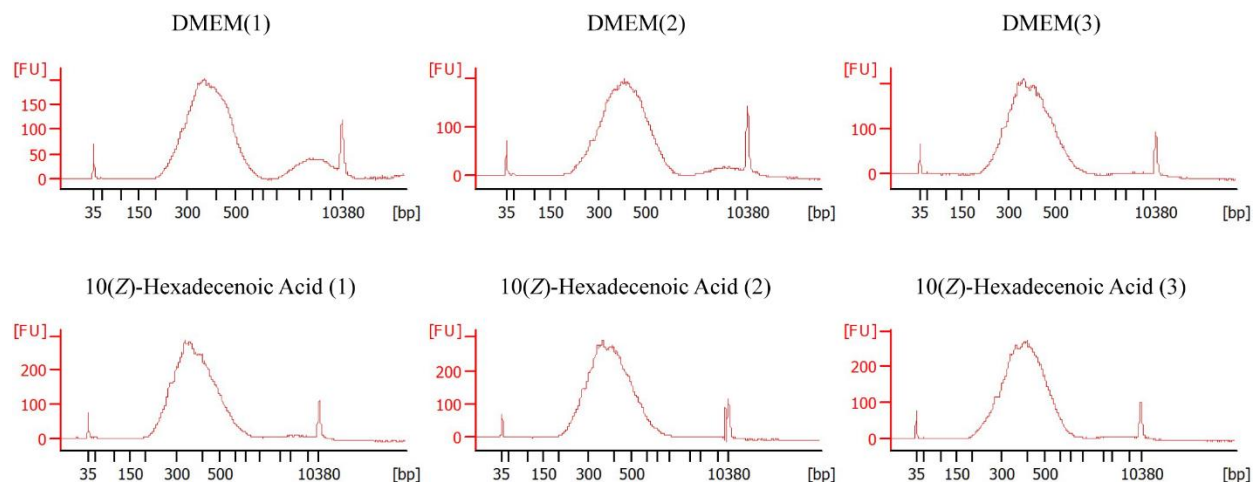
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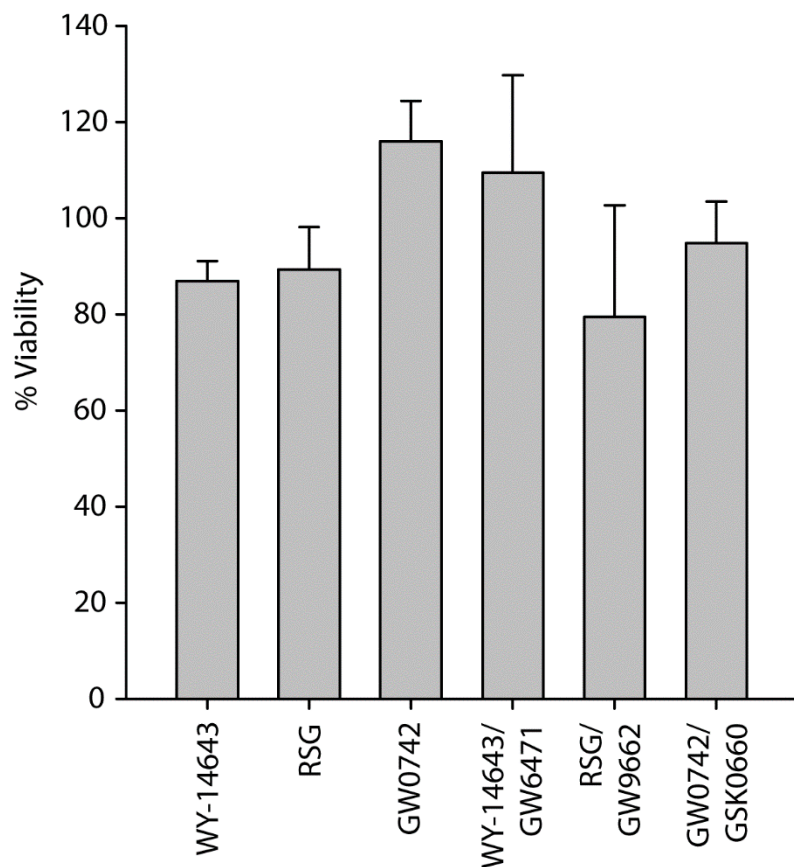
1305 **Fig. S3. BioAnalyzer electropherograms of cDNA libraries used for RNA-sequencing.**

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1307 Total RNA content of 1×10^5 macrophages was prepared for each sample utilizing separate
 1308 macrophage preparations from $n = 3$ mice treated with vehicle (Dulbecco's Modified Eagle
 1309 Medium (DMEM; upper row) then challenged with $1 \mu\text{g}/\text{mL}$ lipopolysaccharide (LPS) or $n = 3$
 1310 mice treated with $200 \mu\text{M}$ 10(Z)-hexadecenoic acid for 1 h, then challenged with $1 \mu\text{g}/\text{mL}$ LPS.
 1311 Macrophages were harvested 12 h following LPS challenge. Peaks at 35 bp and 10,380 bp are
 1312 gel migration markers. For concentrations of cDNA and average fragment length for each
 1313 sample, see Table S2.

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1318 **Fig. S4. Effect of PPAR agonists and antagonists on macrophage cell viability.**

1319 Sulforhodamine B (SRB) was used to assess cytotoxic effects of PPAR agonists (PPAR α , WY-

1320 14643; PPAR γ , rosiglitazone (RSG); PPAR δ , GW0742) or PPAR agonists and antagonists

1321 (PPAR α , GW6471; PPAR γ , GW9662; PPAR δ , GSK0660). The agonists and antagonists were

1322 incubated with freshly isolated murine peritoneal macrophages at 2x their respective EC50 or

1323 IC50 (see Table S9). Percent control growth is expressed as % viability and is a ratio of the

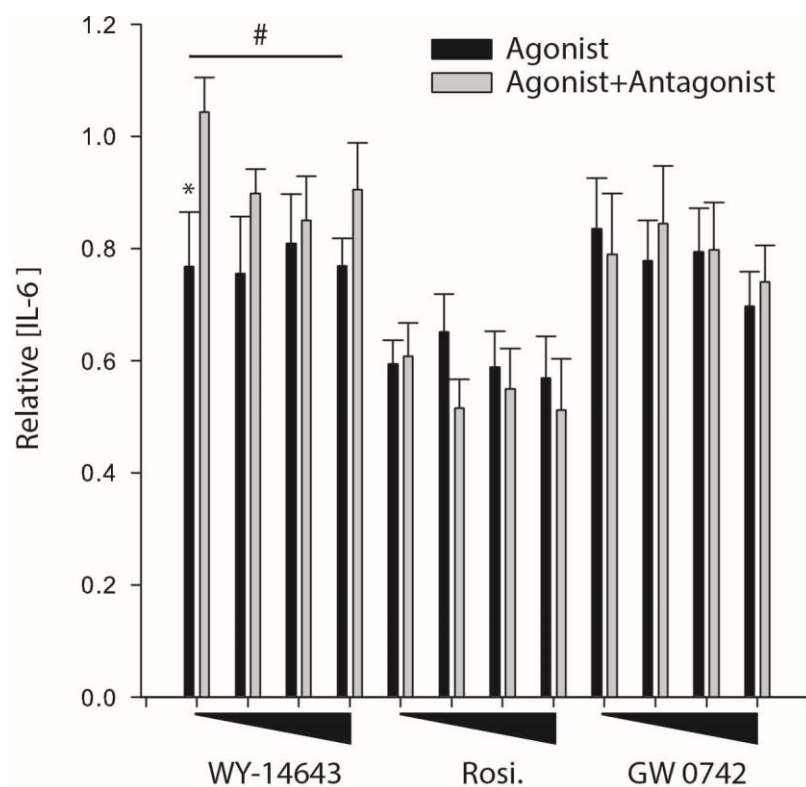
1324 amount of growth that occurred with treatment over the amount of growth that occurred in

1325 media. One hundred percent indicates no differences in cell growth between treatment and

1326 media, whereas values below 100% indicate that growth was impaired with treatment. Data are
 1327 expressed as mean \pm SEM of 3-7 mice per condition

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1331 **Fig. S5. Suppression of IL-6 in LPS-stimulated macrophages is achieved through activation**
 1332 **of PPAR α and reversed by addition of a PPAR α antagonist.**

1333 Murine peritoneal macrophages were incubated with peroxisome proliferator-activated receptor

1334 (PPAR) α antagonist (GW 6471), PPAR γ antagonist (GW 9662), PPAR δ antagonist (GSK

1335 0660), or Dulbecco's Modified Eagle Medium (DMEM)/F-12. After a 1-h incubation, the cells

1336 were treated with the complementary agonist (PPAR α : WY-14643, PPAR γ : rosiglitazone; Rosi.,

1337 PPAR δ : GW 0742). For each agonist, four concentrations were assayed, 1x, 2x, 5x, and 10x the

1338 half-maximal effective concentration (EC₅₀). The immune response was measured as the

1339 concentration of interleukin (IL) 6 in the cell supernatant relative to vehicle controls. #p < 0.05
1340 main effect of agonist + antagonist condition relative to agonist alone condition in a multifactor
1341 ANOVA. *p < 0.05, Fisher's least significant difference (LSD), pairwise comparison relative to
1342 antagonist-treated cells.

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