

Carboxylesterase 1d Inactivation Augments Lung Inflammation in Mice

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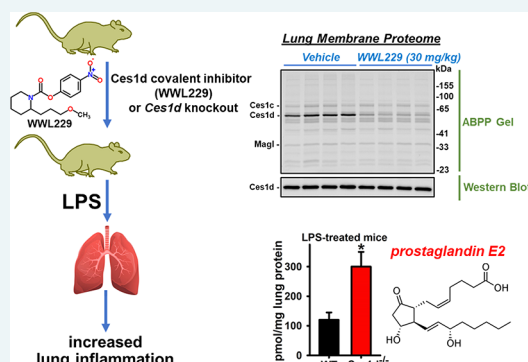


Supporting Information

ABSTRACT: Carboxylesterases are members of the serine hydrolase superfamily and metabolize drugs, pesticides, and lipids. Previous research showed that inhibition of carboxylesterase 1 (CES1) in human macrophages altered the immunomodulatory effects of lipid mediators called prostaglandin glyceryl esters, which are produced by cyclooxygenase-catalyzed oxygenation of the endocannabinoid 2-arachidonoylglycerol (2-AG). Ces1d – the mouse ortholog of human CES1 – is the most abundant Ces isoform in murine lung tissues and alveolar macrophages and a major target of organophosphate poisons. Monoacylglycerol lipase (Magl) is also expressed in murine lung and is the main enzyme responsible for 2-AG catabolism. Several metabolic benefits are observed in *Ces1d*^{-/-} mice fed a high-fat diet; thus, we wondered whether pharmacological and genetic inactivation of *Ces1d* in vivo might also ameliorate the acute inflammatory response to lipopolysaccharide (LPS).

C57BL/6 mice were treated with WWL229 (*Ces1d* inhibitor) or JZL184 (Magl inhibitor), followed 30 min later by either LPS or saline. Wild-type (WT) and *Ces1d*^{-/-} mice were also administered LPS to determine the effect of *Ces1d* knockout. Mice were sacrificed at 6 and 24 h, and cytokines were assessed in serum, lung, liver, and adipose tissues. Lipid mediators were quantified in lung tissues, while activity-based protein profiling and enzyme assays determined the extent of lung serine hydrolase inactivation by the inhibitors. WWL229 was shown to augment LPS-induced lung inflammation in a female-specific manner, as measured by enhanced neutrophil infiltration and *Il1b* mRNA. The marked *Ces* inhibition in female lung by 4 h after drug treatment might explain this sex difference, although the degree of *Ces* inhibition in female and male lungs was similar at 6 h. In addition, induction of lung *Il6* mRNA and prostaglandin E₂ by LPS was more pronounced in *Ces1d*^{-/-} mice than in WT mice. Thus, WWL229 inhibited lung *Ces1d* activity and augmented the female lung innate immune response, an effect observed in part in *Ces1d*^{-/-} mice and *Ces1d*/*CES1*-deficient murine and human macrophages. In contrast, JZL184 attenuated LPS-induced *Il1b* and *Il6* mRNA levels in female lung, suggesting that *Ces1d* and Magl have opposing effects. Mapping the immunomodulatory molecules/pathways that are regulated by *Ces1d* in the context of lung inflammation will require further research.

KEYWORDS: carboxylesterases, cytokines, inflammation, monoacylglycerol lipase



Carboxylesterases are members of the serine hydrolase superfamily and have metabolic functions in vivo.¹ Their gene annotations are denoted *Ces* and *CES* for murine and human species, respectively.² *Ces*/*CES* hydrolyze xenobiotics and lipids that contain carboxylic ester bonds, with pesticides and fatty acylglycerols representing important exogenous and endogenous substrates, respectively.^{2,3} The *Ces*/*CES* genes encode a number of unique isoforms that arose in evolution due to gene duplication events, especially in rodents.² Consequently, mice have a larger number of carboxylesterase-encoding genes than humans (20 total in mice compared to 6 in humans). The murine genome contains eight different *Ces1* genes, and each exhibits a high degree of sequence homology to the human *CES1* gene.⁴ The *Ces1d* protein is the mouse ortholog of human *CES1* protein in terms of both amino acid sequence homology

and enzyme function.² The *Ces*/*CES* isoforms are distributed in several mammalian tissues; for example, *Ces1d*/*CES1* is abundant in liver, lung, and adipose in mice and humans.^{4–6} Further, *CES1* is expressed in human monocytes and macrophages,^{7,8} including human alveolar macrophages,⁹ whereas *Ces1d* mRNA is detected in mouse alveolar macrophages but not in either thioglycolate (TG)-elicited peritoneal macro-

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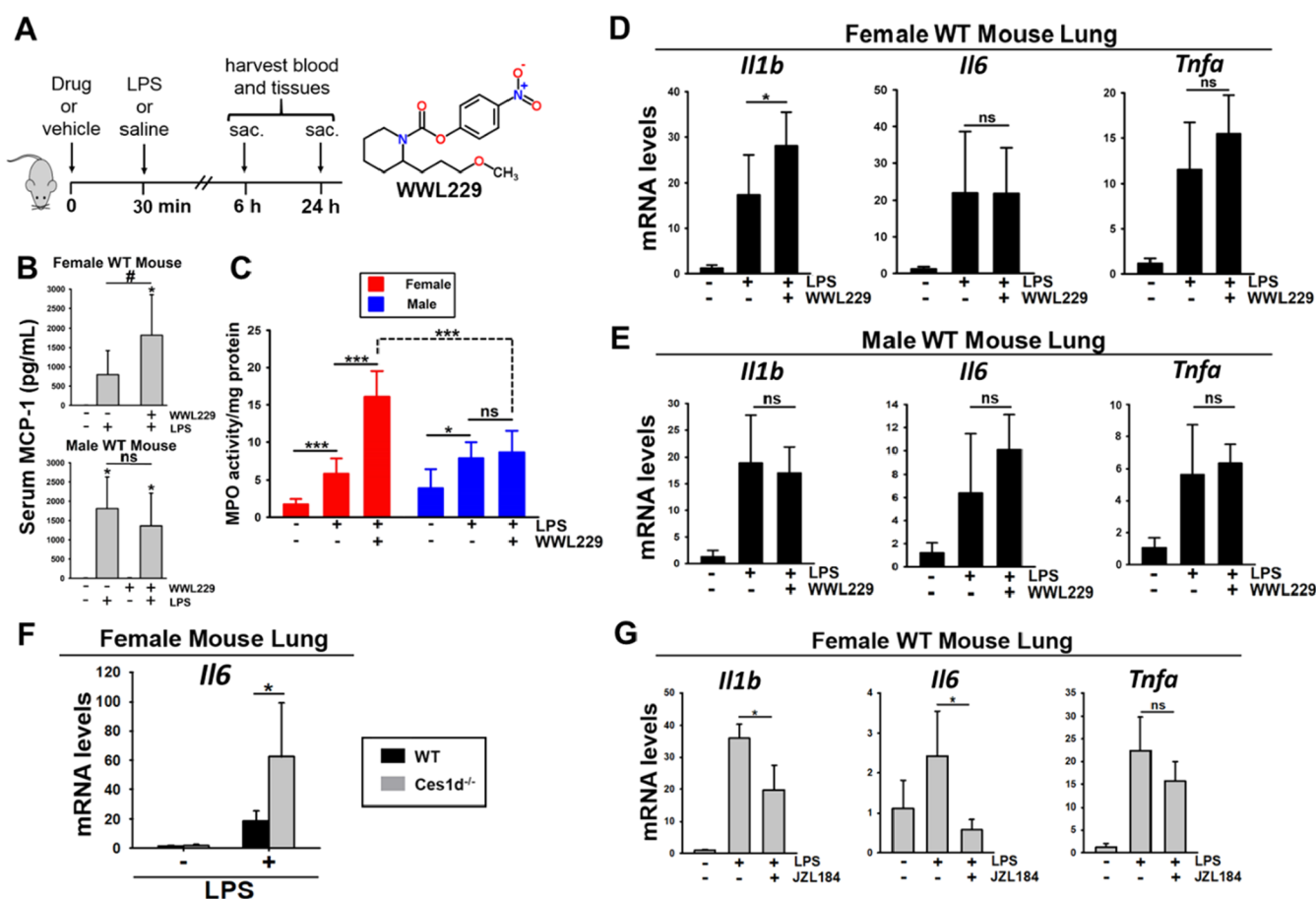


Figure 1. Inflammation was augmented by WWL229 in female WT mouse lung, but not in male WT lung, 6 h after intraperitoneal LPS challenge. (A) Experimental design for the WWL229 study is depicted. (B) Serum MCP-1 levels in female and male WT mice treated with WWL229 and LPS. (C) LPS-induced MPO activity in lung was enhanced by WWL229 in a female-specific manner. LPS-induced *Il1b* cytokine mRNA levels were augmented by WWL229 in female lung (D) but not in male lung (E). (F) LPS-induced *Il6* mRNA levels were enhanced in female *Ces1d*^{-/-} mouse lung relative to WT controls. (G) LPS-induced cytokine mRNA levels were attenuated by JZL184 in female WT mouse lung. The Mag1 study design was the same as the WWL229 study except that mice were sacrificed at 6 h only. Data are expressed as the mean \pm SD ($n = 5$ mice/group). One-way ANOVA (D,E,G) or two-way ANOVA (B,C,F) assessed significant differences between groups. * $p < 0.05$, *** $p < 0.001$, comparisons are indicated; ns, not significant. In panel 1B, * $p < 0.05$ for treatments vs vehicle control; # $p < 0.05$ for indicated comparison.

phages⁵ or bone marrow-derived macrophages (our unpublished data). Together, these findings suggest that *Ces1d*/CES1 enzymes have a role in innate immune responses, particularly in lung tissues.

Using activity-based protein profiling (ABPP) methods developed by Cravatt and co-workers,¹⁰ *Ces1d* was established to be the most active serine hydrolase in C57BL/6 mouse lung, followed by *Ces1c*.¹¹ Previous research also suggested that the *Ces*/*CES* enzymes in alveolar macrophages and parenchymal lung tissues are involved in pesticide detoxification.^{9,11–13} For example, exposures of mice to chlorpyrifos (CPF), an organophosphate pesticide, inhibited both *Ces1c* and *Ces1d* in neonate lung and *Ces1c* in adult lung due to stoichiometric covalent binding and degradation of the active metabolite chlorpyrifos oxon (CPO).¹¹ Although the lipopolysaccharide (LPS)-evoked pro-inflammatory cytokine levels in adult mouse lung were not altered by CPF exposure, CPF-treated females had augmented levels of LPS-induced eicosanoids (PGE₂, PGD₂, and 12-HETE). Interestingly, inflammation can also influence the extent of *Ces* expression. For example, treatment of isolated mouse alveolar macrophages with LPS caused *Ces1d* mRNA levels to be decreased,¹¹ suggesting that inflammatory pathways can modulate *Ces1d* expression. Similar attenuating effects of

LPS on *Ces2g* mRNA and *Ces2g* activity levels in mouse spleen were observed in vivo.¹⁴ Therefore, because the lung is exposed to noxious chemicals and pathogens that are present in the environment, further studies that examine the role of pulmonary *Ces*/*CES* enzymes during the lung's response to inflammatory stimuli are warranted.

Diseases driven by inflammation often exhibit hallmarks related to deregulated lipid signaling. Two types of lipid mediators involved in cell signaling that are degraded by CES1 in human monocytes/macrophages are 2-arachidonoylglycerol (2-AG),¹⁵ an endocannabinoid produced on demand in activated cells,¹⁶ and prostaglandin glyceryl esters (PG-Gs),¹⁷ which are cyclooxygenase-derived oxygenated metabolites of 2-AG.¹⁸ Although inhibition of CES1 was shown to increase the levels of anti-inflammatory 2-AG in human THP-1 monocytes and macrophages,¹⁵ monoacylglycerol lipase (Magl) has a more important role than *Ces1d* in 2-AG turnover in murine lung.¹¹ In addition, small-molecule inhibitors that target CES1 have only a minor effect on 2-AG hydrolytic activity in human peripheral blood mononuclear cells, whereas Magl inhibitors have a major one.^{8,19,20} (Patho)physiological conditions that augment 2-AG levels in vivo, which is often referred to as increased 'endocannabinoid tone,' can modulate the degree of inflamma-

tion in tissue injury due to autocrine and paracrine signaling evoked by 2-AG.²¹ For example, the drug JZL184, a small-molecule inhibitor of Magl that elevates the local concentration of 2-AG, reduced bronchoalveolar lavage (BAL) cytokine levels and decreased the extent of immune cell infiltration in an LPS-induced acute lung injury model.²² The PG-Gs, on the other hand, exhibit more complex behavior in that they can exert both anti- and pro-inflammatory effects.^{17,23–25} For example, PGE₂-G can augment pro-inflammatory cytokine production by M1 polarized macrophages, whereas PGD₂-G attenuate their levels.²⁴ Similarly, the inhibition of α,β -hydrolase domain 6 (ABHD6), which is the primary 2-AG metabolizing enzyme in TG-elicited mouse peritoneal macrophages, was shown to attenuate the effects of LPS, putatively due to the increased conversion of 2-AG to PGD₂-G. Furthermore, inactivation of CES1 in human THP-1 monocytes/macrophages promoted the anti-inflammatory effects of PGD₂-G, while it diminished the pro-inflammatory effects of PGE₂-G.¹⁷ Although these *in vitro* studies suggested that CES1 inactivation can exert anti-inflammatory effects, the role of Ces1d in murine lung and other tissues in settings of *in vivo* inflammation is largely unexplored. Moreover, studies that directly compare the differential effects of Ces1d and Magl inhibition in settings of inflammation have not been performed.

Inflammation and lipid metabolism are tightly linked together, and enzymes that regulate the levels of bioactive lipids can be targeted by pharmacological agents. In this study, we examined the role of Ces1d in a murine model of LPS-induced inflammation in lung, liver, and adipose tissues. WWL229, a small-molecule mechanism-based covalent inhibitor of Ces1d,²⁶ was used to treat adult mice that were subsequently challenged with LPS. In addition, WT and Ces1d^{-/-} mice were challenged with LPS to determine whether genotype-dependent effects were apparent. The degree of systemic inflammation was assessed by serum cytokine/chemokine levels, and tissue inflammation was determined by the levels of pro-inflammatory cytokines (lung, liver, adipose), bioactive lipids (lung), neutrophil infiltration (lung), and histopathology (liver). We determined whether Ces1d or Magl inactivation (via pharmacological or genetic disruption) altered any of these inflammatory readouts. In addition, activity-based assays established the enzyme target occupancy of the pharmacological inhibitors in the lung, both *in vitro* and *in vivo*. The main findings are that WWL229 augmented LPS-induced lung inflammation in a female-specific manner and Ces1d knockout also promoted inflammation in lung.

MATERIALS AND METHODS

Chemicals. WWL229, (4-nitrophenyl) 2-(3-methoxypropyl)piperidine-1-carboxylate; WWL113, ethyl 4-[4-[methyl-(3-pyridin-4-ylphenyl)methyl]carbamoyl]-oxyphenyl]benzoate; JZL184, (4-nitrophenyl) 4-[bis(2H-1,3-benzodioxol-5-yl) (hydroxy)methyl]piperidine-1-carboxylate; pNPVa, *p*-nitrophenyl valerate; and LPS (*E. coli* O 111:B4) were obtained from Sigma (St. Louis, MO). Authentic standards of lipid mediators were from Cayman (Ann Arbor, MI). LC-MS solvents and the activity probe fluorophosphonate-TAMRA (FP-TAMRA) were from Thermo Fisher.

Animals. Adult C57BL/6 wild-type (WT) mice were from a breeding colony at MSU or from Jackson Laboratories (Bar Harbor, ME). Global Ces1d^{-/-} mice on a C57BL/6 background were established as previously described.²⁷ Mice were housed in temperature- and humidity-controlled AAALAC-approved

facilities (20–25 °C and 40–60% humidity) under a 12 h light cycle and used in accordance with the Mississippi State University Institutional Animal Care and Use Committee.

Drug Treatments and Intraperitoneal LPS Challenge. Adult female and male mice ($n = 5$ /sex/group) were injected with WWL229 (30 mg/kg, *i.p.*) or the drug vehicle (15:1:1 v/v/v saline/ethanol/Brij93) in a volume of 100 μ L/mouse. Thirty minutes after drug injection, the mice were systemically challenged with LPS (1.25 mg/kg, *i.p.*) or saline vehicle in a volume of 100 μ L/mouse. Six or twenty-four hours after LPS injection, mice were sacrificed by exsanguination under isoflurane anesthesia (Figure 1A depicts the experimental protocol). The LPS dose and time points were the same as those in our previous studies.^{11,14} Blood was collected from the inferior vena cava prior to exsanguination, and serum was prepared by centrifuging the clotted blood (4 °C, 10 min, 3,000g). Tissues (lung, liver, and adipose) were immediately flash-frozen in liquid nitrogen and stored at -80 °C until use. In some studies, JZL184 (30 mg/kg, *i.p.*), a Magl inhibitor, was used instead of WWL229, and 0.5% w/v hydroxypropylmethylcellulose in saline was used as the vehicle. In studies involving Ces1d^{-/-} mice (female and male), WT and Ces1d^{-/-} mice ($n = 5$ –6 mice/group) were injected with LPS (1.25 mg/kg, *i.p.*) or saline vehicle in a volume of 100 μ L/mouse and sacrificed 6 h later, as described above. In studies to assess the enzyme target occupancy by inhibitors, mice were injected with drugs only (WWL229, WWL113, and JZL184; 30 mg/kg, *i.p.*) or the vehicle (15:1:1 v/v/v saline/ethanol/Brij93 for WWL229, 0.5% w/v hydroxypropylmethylcellulose in saline for WWL113 and JZL184) in a volume of 100 μ L/mouse and sacrificed after 4 or 6 h. Mice treated for 6 h with WWL229 were subjected to a BAL procedure immediately before harvesting the lungs. Male and female mice were used in most *in vivo* experiments.

Intranasal LPS Challenge. LPS (in saline) or saline vehicle was administered to anesthetized male WT and Ces1d^{-/-} mice by the intranasal (*i.n.*) route (1.25 mg/kg, $n = 5$ /group; <50 μ L per mouse). Mice were sacrificed 6 h later, and BAL was collected in 3 mL of phosphate-buffered saline (PBS) containing 1 mM EDTA, followed by removal of the lungs.

Quantification of Serum Cytokines by Multiplex Assay. A custom premixed Luminex xMAP multiplex assay kit (Millipore Sigma) was used to measure cytokines and inflammatory markers in mouse serum following the manufacturer's instructions on a Luminex 200 system.

Preparation of Lung Total Membrane Fraction. Lungs were rinsed in cold PBS and Dounce-homogenized in sucrose buffer (50 mM Tris-HCl, 0.32 M sucrose, pH 7.4) and then centrifuged at a low speed (4 °C, 5 min, 1000g) to remove tissue debris, followed by centrifugation of the supernatant at high speed (4 °C, 60 min, 100,000g). The resulting pellet was washed by sonication in the sucrose buffer, followed again by centrifugation (4 °C, 30 min, 100,000g). The washed pellet (total membrane fraction) was resuspended by sonication in 200–400 μ L of sucrose buffer and stored at -80 °C. Protein concentrations were determined using the BCA reagent (Thermo Pierce) with bovine serum albumin standards following the manufacturer's instructions.

Lung Myeloperoxidase Activity. Aliquots of lung total membranes from male and female mice treated with WWL229 and LPS were assayed for myeloperoxidase (MPO) activity. In a 96-well microtiter plate, the following assay components (final concentrations indicated) were combined in 200 μ L of 5 mM

Table 1. Quantitect Primers and Primer Sequences Used for RT-qPCR Analysis

quantitect primers	mouse	
Gapdh	Mm_Gapdh 3_SG QuantiTect Primer Assay—QT01658692	
Il1b	Mm_Il1b 2_SG QuantiTect Primer Assay—QT01048355	
Il6	Mm_Il6 1_SG QuantiTect Primer Assay—QT00098875	
Tnfa	Mm_Tnf 1_SG QuantiTect Primer Assay—QT00104006	
	mouse	
Gene	5'-Forward Sequence-3'	5'-Reverse Sequence-3'
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Il1b	GCAACTGTTCTCTGAACTCAACT	ATCTTTTGGGGTCCGCTCAACT
Il6	TAGTCCTTCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Tnfa	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
Ces1c	CTCAGATATGTTTCAGCACCAGAA	GCTTTTTGTCAAATCGGCAGG
Ces1d	ATATGGCTTTCTCTTGTCGG	CCCAGGACTTTGCCTTTAACAGT
Ces2g	TCTCTGAGGTGTTTTACCAAACG	CCTCTCAGACAGCGCACCAG
	human	
Gene	5'-Forward Sequence-3'	5'-Reverse Sequence-3'
IL6	AGCCACTCACCTCTTCAGAAC	GCCTCTTTGCTGCTTTCACAC
TNFa	CCCATGTTGTAGCAAACCT	TGAGGTACAGGCCCTCTGAT
CCL3	TTCCGTACCTGCTCAGAAT	CAGCAGCAAGTGATGCAGAGA
GAPDH	GAPDH 1_SG QuantiTect Primer Assay—QT00079247	

sodium acetate (pH 5.0) containing 75 mM NaCl and 0.005% v/v Triton X-100: H₂O₂ (1 mM), lung membranes (~10–25 μg protein), and tetramethyl benzidine (500 μM; added last to begin reactions). The absorbance (λ = 650 nm) in each well was measured for 5 min in a Molecular Devices plate reader (at 37 °C) to assess the rates of MPO-catalyzed oxidation of tetramethyl benzidine.

Reverse Transcriptase Quantitative Polymerase Chain Reaction Analysis. Total RNA from adipose, liver, and lung tissues was extracted with an RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. A NanoDrop ND-1000 spectrophotometer was utilized to quantify RNA. cDNA was prepared with a RevertAid First Strand cDNA synthesis kit (Thermo Scientific). Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed on a Stratagene Mx3005P thermal cycler with a QuantiFast SYBR Green PCR master mix (Qiagen) using QuantiTect primers (Qiagen) or custom oligonucleotides (Invitrogen or Eurofins) (see Table 1). The comparative cycle threshold ($\Delta\Delta C_T$) was used to determine gene expression changes using GAPDH as the reference gene, with the results compared to the control condition $[(2^{-\Delta\Delta C_T})]$ as previously described.²⁸

Quantitation of Ces Activity in Lung Membranes. Carboxylesterase activity in lung membranes was evaluated by two methods. The first used a continuous kinetic assay with pNPVa substrate (λ = 405 nm) in a Molecular Devices plate reader.²⁹ Enzyme activities were normalized on protein to give specific activities. The second employed gel-based activity-based protein profiling (ABPP) of the membranes with FP-TAMRA to evaluate the activities of multiple serine hydrolases in parallel, including Ces, as previously described.¹⁷ In some cases, following fluorescent visualization of in-gel proteins that had been labeled by FP-TAMRA, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with a rabbit monoclonal anti-human CES1 antibody (Abcam Cat# 2312-1, RRID/AB 1266968; the antibody cross-reacts with mouse Ces1d isoform).

Quantitation of Lipid Mediators in Lung Tissues and Bronchoalveolar Lavage Fluid. Eicosanoids were extracted from lungs as previously described.³⁰ One-hundred microliters

of methanol was added to the dried extracts and transferred to LC vials with volume-reducing inserts for analysis by targeted LC-MS/MS as previously described.³¹ Bronchoalveolar lavage fluid (BALF) (1.5 mL volume) was spiked with deuterated internal standards and extracted twice with an equal volume hexane/ethyl acetate (1:1, v/v) containing 0.2% acetic acid. The pooled organics were dried down under nitrogen and reconstituted in methanol (100 μL) for LC-MS/MS. Aliquots of BALF (25 μL) were taken to determine their total protein concentration for normalization purposes. There was no significant difference in the BALF protein concentrations due to genotype or LPS treatment (2-way ANOVA).

In Vitro Treatments of Murine and Human Macrophages with Inflammatory Stimuli. Alveolar macrophages were harvested from male and female WT and Ces1d^{-/-} mice lungs in cold PBS and 1 mM EDTA and pooled according to the genotype. The cells were washed and counted with a hemocytometer and then plated overnight in Dulbecco's modified Eagle's medium containing antibiotics and 10% FBS in an atmosphere of 5% CO₂ in air (37 °C). The next day, the attached cells were gently washed and overlaid with the serum-free medium containing LPS (100 ng/mL) and incubated for 24 h. Total RNA was extracted from cells for RT-qPCR, and PGE₂ levels in the culture media were quantified by LC-MS/MS. In addition, human THP-1 macrophage lines with normal and attenuated CES1 protein expression³² – termed control macrophages and CES1KD macrophages, respectively – were also treated with LPS (1 μg/mL, 6 h) or TNFα/IFNγ (100 ng/mL and 20 ng/mL, respectively, 12 h) and the RNA was extracted for RT-qPCR.

Histochemistry of Liver Sections. A portion of the liver tissue was fixed in 10% neutral-buffered formalin (10% NBF). Fixed liver was processed and embedded in paraffin using standard histologic techniques. Paraffin-embedded liver was cut into 5 μm sections and stained with hematoxylin and eosin on an automatic stainer (Leica). The number of immune cells within 4 layers of hepatocytes surrounding 20 portal zones in each mouse were quantified and averaged per portal zone.

Statistical Analysis. GraphPad Prism (Version 7, San Diego, CA) or SigmaPlot (Version 11.0, San Jose, CA) was used

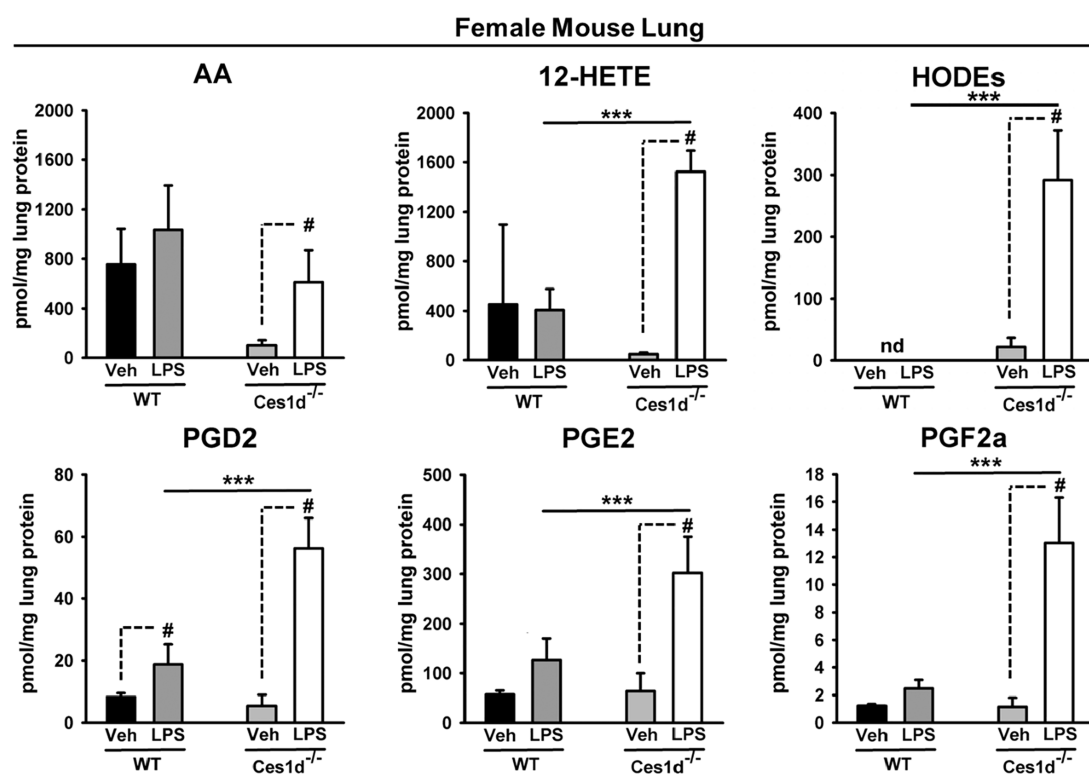


Figure 2. Eicosanoids were quantified in lungs of female WT and *Ces1d*^{-/-} mice 6 h after intraperitoneal treatment with saline (Veh) or LPS. *Ces1d*^{-/-} mice were more responsive to the effects of LPS than WT mice. HODEs represent the combined levels of 9-HODE and 13-HODE because they were not chromatographically resolved. Data are expressed as mean \pm SD ($n = 5$ mice/group). Two-way ANOVA assessed significant differences between groups. # $p < 0.05$ and *** $p < 0.001$, comparisons are indicated; nd, not detected.

for statistical analyses. The results are presented as the mean \pm standard deviation (SD). Data from RT-qPCR were transformed to linearized $\Delta\Delta C_T$ values as previously described.²⁸ Differences between groups were assessed with either an unpaired Student *t*-test, one-way analysis of variance (ANOVA), or two-way ANOVA as appropriate. Post-hoc testing was performed using the Student–Newman–Keuls method. Outliers were identified by Grubb's outlier test. p -values < 0.05 were considered significant.

RESULTS

WWL229 had Minimal Effect on Serum Cytokines/Chemokines Following LPS Challenge Except for MCP-1.

Cytokines and chemokines in WT mouse serum were evaluated by a Luminex assay at 6 h (male and female) and 24 h (female only). LPS challenge induced a robust increase in these molecules regardless of sex (Figure S1). WWL229 enhanced the amount of serum monocyte chemoattractant protein-1 (MCP-1) induced by LPS in females, but not in males, at 6 h (Figure 1B), although this effect was not apparent at 24 h ($p > 0.05$) (Figure S1). In general, the levels of the other serum cytokines/chemokines induced by LPS were not significantly altered by WWL229 (Figure S1). In addition, WWL229 treatment in the absence of LPS challenge had no impact on serum cytokines/chemokines; thus, it did not cause overt inflammation.

WWL229 Augmented Inflammation in Female Lung Following LPS Challenge. Elevated MPO activity is a surrogate measure of tissue neutrophil infiltration during inflammation.³³ MPO activity in WT mouse lungs was determined at 6 h to assess the degree of inflammation induced

by either LPS alone or LPS combined with WWL229 (Figure 1C). LPS caused a significant increase in MPO activity in both females and males, indicative of inflammation-dependent neutrophil infiltration into the lung. Interestingly, WWL229 augmented the LPS-induced MPO activity in females but not in males, suggesting that WWL229 enhanced female lung inflammation (Figure 1C).

We next evaluated cytokine expression in lungs by RT-qPCR. Six hours after LPS treatment, a robust increase in the expression of *Il1b*, *Il6*, and *Tnfa* mRNA was noted in both females and males (Figure 1D,E). Interestingly, the *Ces1d* inhibitor WWL229 augmented the levels of *Il1b* in female lungs (Figure 1D) but not in male lungs (Figure 1E). Based on gene expression and ABPP data, it is apparent that *Ces1d* is robustly expressed and enzymatically active in WT murine lung (Figure S2A–C). To extend these findings, we challenged female *Ces1d*^{-/-} mice with LPS to assess the effect of *Ces1d* knockout. After 6 h, LPS-induced lung *Il6* mRNA levels in this model were significantly higher than those in WT lung (Figure 1F), whereas no differences in LPS-induced *Il1b* and *Tnfa* levels were noted between WT and *Ces1d*^{-/-} lungs (Figure S3). Thus, pharmacologic and genetic inactivation of *Ces1d* appears to modulate the degree of lung inflammation that is induced by LPS in female mice.

Next, we examined the effects of the MagI inhibitor JZL184 on lung inflammation. The LPS-induced expression of *Il1b* and *Il6* mRNA after 6 h was significantly attenuated by JZL184 (Figure 1G). JZL184 also decreased *Tnfa* levels, although this did not reach statistical significance ($p > 0.05$). Thus, the MagI inhibitor exerted effects on lung inflammation that were opposite to those of the *Ces1d* inhibitor.

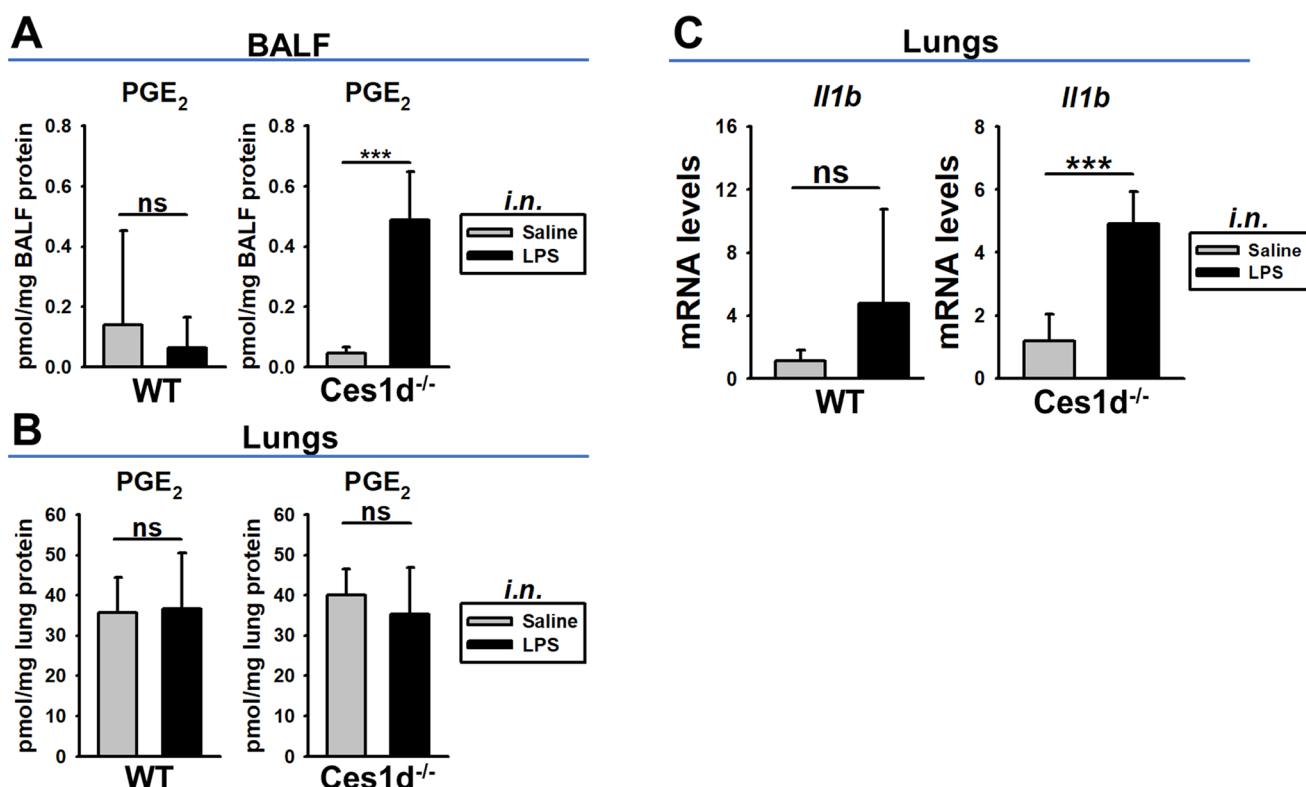


Figure 3. PGE₂ and Il1b mRNA levels in lungs of male WT and Ces1d^{-/-} mice 6 h after i.n. treatment with saline or LPS. (A) PGE₂ levels in BALF. (B) PGE₂ levels in lung tissues. (C) Il1b mRNA levels in lung tissues. Data are expressed as mean ± SD (n = 5 mice/group). Two-way ANOVA assessed significant differences between groups. ****p* < 0.001; ns, not significant; comparisons are indicated.

Mouse Lung Membrane Fractions

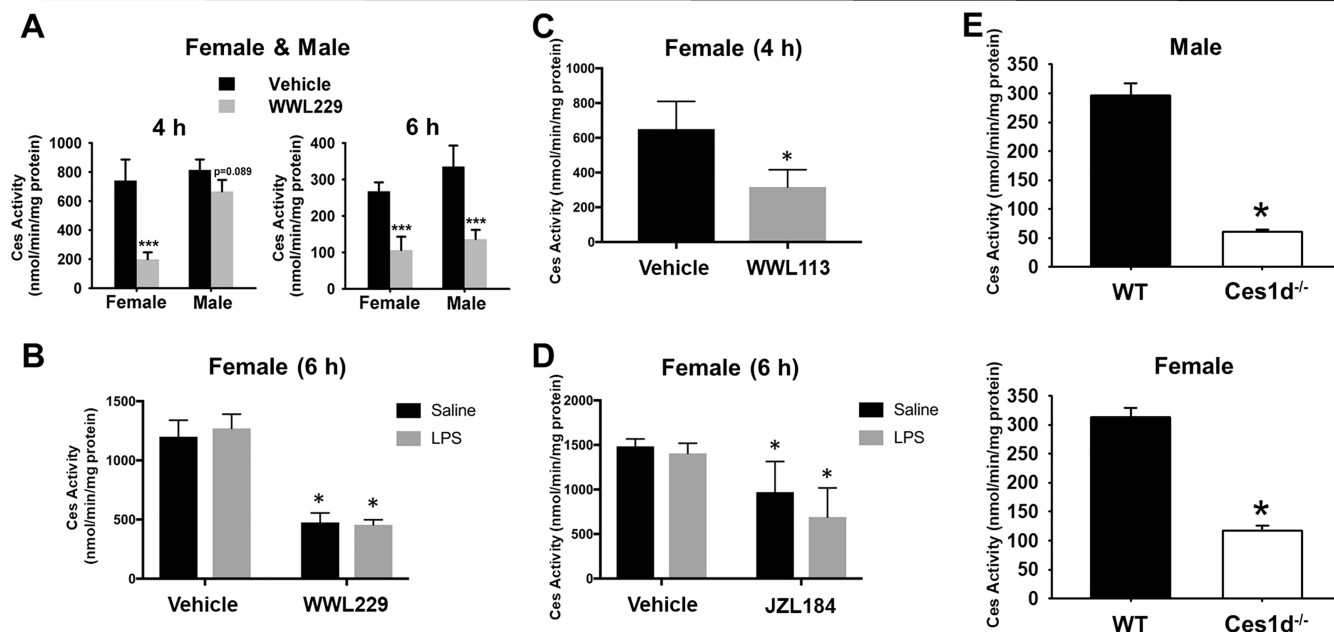


Figure 4. Ces activity in lung membranes from vehicle- and inhibitor-treated WT mice and Ces1d^{-/-} mice. (A) Lung Ces activity was reduced in WT mice by WWL229 in a sex-dependent and -independent manner at 4 h and 6 h, respectively. (B) Intraperitoneal LPS challenge had no effect on lung Ces activity in female mice, and it did not alter WWL229-mediated Ces inhibition. Lung Ces activity was inhibited in female mice by WWL113 (C) and JZL184 (D). Lung Ces activity in Ces1d^{-/-} mice were markedly lower than those in WT mice (E). Data are expressed as the mean ± SD [n = 5 mice, n = 3 male mice at 4 h, (A); n = 3 mice, (B,C); n = 5 mice, (D,E)]. One-way ANOVA (C,E) or two-way ANOVA (A,B,D) assessed significant differences between groups. **p* < 0.05, ****p* < 0.001 for treatments vs vehicle or Ces1d^{-/-} vs WT.

Levels of Lung Tissue Lipid Mediators in Ces1d^{-/-} Mice Following LPS Challenge Were Dependent on Their

Sex. Ces enzymes have roles in the regulation of certain lipid mediators,^{15,17} so we next evaluated eicosanoid levels in lung

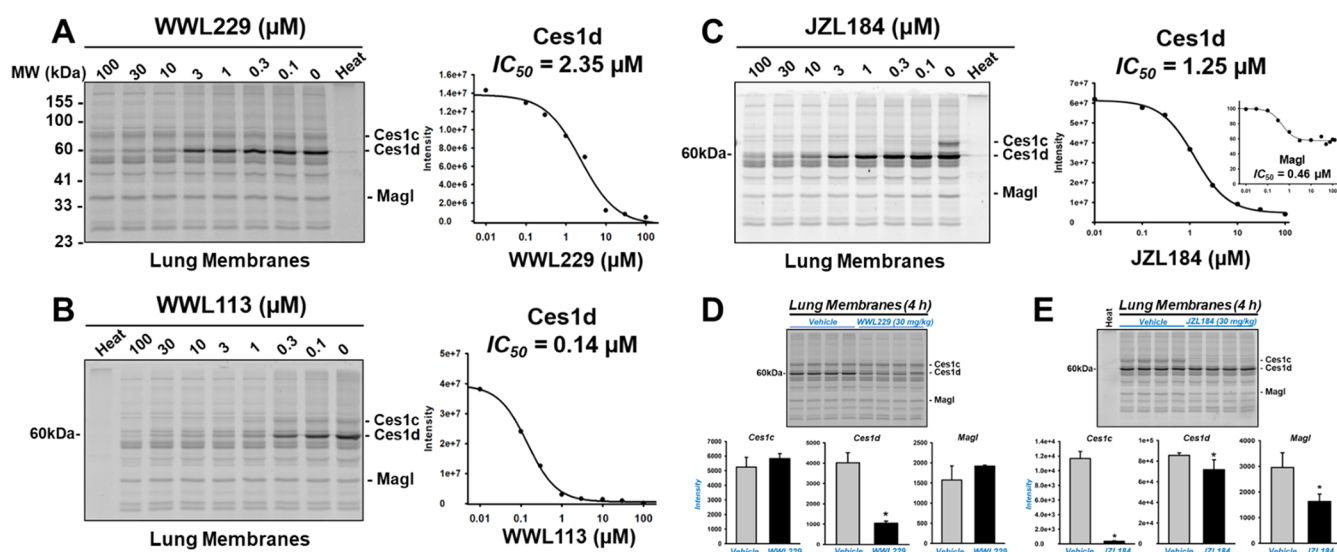


Figure 5. Gel-based activity-based protein profiling of lung serine hydrolases. Lung membranes from naïve WT mice were treated *in vitro* with the indicated concentrations of WWL229 (A), WWL113 (B), and JZL184 (C) for 30 min at 37 °C before incubation with the activity probe FP-TAMRA (0.2 μM). The IC_{50} s for the inhibition of Ces1d are indicated. Lung membranes were also prepared from WT mice 4 h after *in vivo* treatments with WWL229 (D) and JZL184 (E). WWL229 selectively inhibited lung Ces1d, whereas JZL184 was less selective in that it also inhibited Ces1c, Ces1d, and Magl (its canonical target). Data are expressed as the mean \pm SD ($n = 4$ mice, D,E). Student's *t*-test assessed significant differences between groups, * $p < 0.05$ for treatments vs vehicle. The lanes designated as "Heat" indicate that lung membranes were heat-denatured (95 °C, 3 min) prior to adding the activity probe FP-TAMRA.

tissues of male and female WT and *Ces1d*^{-/-} mice in the context of LPS-induced inflammation. In male mice, there was no genotype-dependent difference in PGD₂, PGE₂, and PGF_{2 α} levels during baseline and LPS-induced conditions (Figure S4, top), whereas AA, 12-HETE, and HODEs were generally higher in *Ces1d*^{-/-} lungs than in WT lungs under either condition (Figure S4, bottom). In addition, the 12-HETE levels were lower in LPS-challenged *Ces1d*^{-/-} mice than in unchallenged *Ces1d*^{-/-} mice, an effect not observed in WT mice. In contrast, there was a genotype-dependent difference in the magnitude of lung lipid mediators in female mice that were induced by LPS challenge (Figure 2). For example, 12-HETE, HODEs, and PGs were markedly higher in *Ces1d*^{-/-} mice than in WT mice following LPS treatment. Thus, with respect to eicosanoid production, the female *Ces1d*^{-/-} lung appears to be more active than female WT lung in settings of inflammation. These results also correlate with the higher Il6 mRNA levels that were observed in female *Ces1d*^{-/-} lung. Increased eicosanoid levels were also observed in female WT mice treated with both WWL229 and LPS for 24 h (Figure S5), again suggesting the augmentation of inflammatory molecules when Ces1d is inactivated.

Ces1d Deficiency Augmented Localized Inflammation in Lungs. Male WT and *Ces1d*^{-/-} mice were treated with LPS intranasally to induce a localized inflammation in the alveolar space. Mice were sacrificed 6 h later and PGE₂ was quantified in BALF and lung tissues, while Il1b mRNA expression was determined in lung tissues. The total protein concentration in BALF, a measure of capillary leakage, was unaffected by treatment or genotype (2-way ANOVA; treatment, $p = 0.638$; genotype, $p = 0.303$); thus, overt lung injury at 6 h was not yet apparent. However, the PGE₂ levels in BALF from *Ces1d*^{-/-} mice were ~10-fold more than those in BALF from WT mice following the LPS challenge (Figure 3A). PGE₂ levels were not induced by i.n. LPS administration in either WT or *Ces1d*^{-/-} lung tissues (Figure 3B), which is similar to the situation

following i.p. LPS administration in male WT and *Ces1d*^{-/-} mice (Figure S4). In addition, Il1b mRNA was also significantly induced (~5-fold) in *Ces1d*^{-/-} lung by LPS, whereas no significant increase was noted in the LPS-treated WT mice (Figure 3C). These findings again suggest that *Ces1d* deficiency causes a more pronounced LPS-induced inflammatory response in the lung.

In Vivo WWL229 Treatment Inhibited Lung Ces Activity. Ces enzymatic activity in lung membranes was determined using the pan-Ces substrate pNPVa.²⁹ When WT mice were treated with WWL229 for 4 h, lung Ces activity in females, but not males, was inhibited (Figure 4A). By 6 h, however, lung Ces activity was inhibited in both females and males. Treatment with LPS had no effect on the degree of inhibition caused by WWL229 in female mice (Figure 4B). Ces activity in lung was also inhibited when female mice received WWL113, another Ces1d inhibitor (Figure 4C). The extent of inhibition caused by WWL229 was slightly greater than that caused by WWL113. Although not as potent as the other inhibitors, JZL184 also decreased lung Ces activity *in vivo* (Figure 4D). LPS challenge also had no impact on JZL184-dependent Ces inhibition (Figure 4D). Furthermore, lung Ces activity in male and female *Ces1d*^{-/-} mice was also markedly lower than that in WT mice, with Ces1d responsible for up to ~80% of the total lung Ces activity (Figure 4E). These data again indicate that functional Ces1d enzyme is present in mouse lung and can be inactivated *in vivo* by covalent inhibitors.

As indicated by the ABPP gel in Figure S2B, the major 60 kDa protein detected in WT lung membranes had almost disappeared in *Ces1d*^{-/-} lung membranes, enabling it to be annotated as Ces1d. Ces1c protein was also detected at ~70 kDa, which is due to this soluble protein being more extensively glycosylated than other Ces isoforms. It should be noted that Ces1c mRNA is detected along with Ces1d and Ces2g mRNA in lung tissues; thus, it is expressed in lung too (Figure S2A). However, Ces1c protein is abundant in mouse plasma; thus,

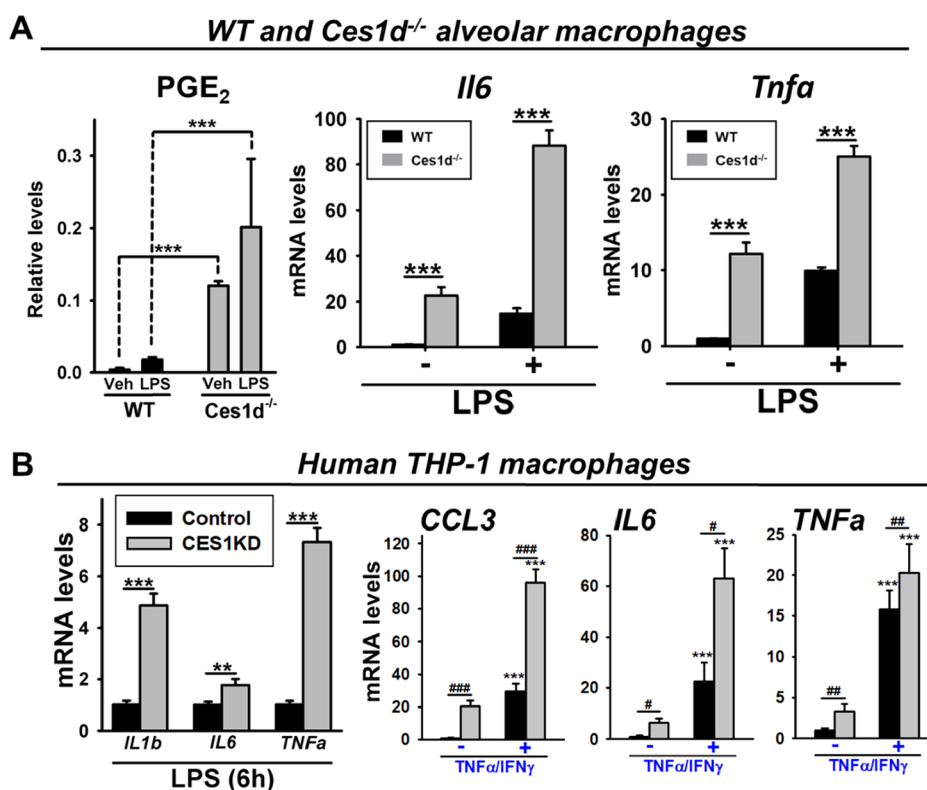


Figure 6. Mouse alveolar macrophages and human THP-1 macrophages are more responsive to inflammatory stimuli when *Ces1d* and *CES1* expression are ablated. (A) Alveolar macrophages from WT and *Ces1d*^{-/-} mice (male and female) were isolated and pooled to obtain enough cells, which were then treated with LPS. The levels of PGE₂, the most abundant prostaglandin in lung tissues, were determined in culture supernatants and normalized on cell protein content, left. The extent of murine *Il6* and *Tnfa* mRNA expression was also determined, right. (B) Human THP-1 macrophages with ablated *CES1* expression were treated with either LPS (left) or TNF α /IFN γ (right), and their inflammatory responses were compared to control THP-1 macrophages (no *CES1* knockdown). The expression levels of human *IL1b*, *IL6*, *TNFA*, and *CCL3* (*MIP-1 α*) mRNA are shown. Data are expressed as mean \pm SD ($n = 3$ –4 replicate wells), and the results are representative of two independent experiments (alveolar macrophages) or three independent experiments (THP-1 macrophages). Two-way ANOVA assessed significant differences between groups. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, * $p < 0.01$, and *** $p < 0.001$; comparisons are indicated. In panel B, *** $p < 0.001$ for TNF α /IFN γ treatment vs corresponding untreated controls.

residual plasma contamination might have also contributed to its presence in lung membranes, despite the extensive washing protocol used. Therefore, the *Ces1c* band detected in the ABPP gels might come from both lung tissues and plasma.

To examine the potency and selectivity of the inhibitors WWL229, WWL113, and JZL184 toward the serine hydrolases found in mouse lung, we employed the gel-based ABPP approach in a competitive format (Figure 5). Each inhibitor bears an electrophilic carbamate functional group that covalently modifies the nucleophilic serine residue in the active site of its target serine hydrolase,²⁶ thereby preventing the covalent labeling of the enzyme by the FP-TAMRA probe. Lung membranes obtained from naïve WT mice were pretreated in vitro with increasing concentrations of small-molecule inhibitors for 30 min, followed by addition of FP-TAMRA. Of the serine hydrolases detected following gel electrophoresis, the intensity of the major 60 kDa protein band represented by *Ces1d* was reduced by WWL229 in a concentration-dependent manner ($IC_{50} = 2.4 \mu M$), whereas WWL113 and JZL184 inhibited *Ces1d* with greater potency but with less selectivity than WWL229 (Figure 5A–C). This is evident from the protein bands besides *Ces1d* that were also reduced in intensity by WWL113 and JZL184 in a concentration-dependent manner.

Next, we examined lung membranes from female WT mice 4 h after receiving the vehicle, WWL229 (30 mg/kg, i.p.), or JZL184

(30 mg/kg, i.p.) by gel-based ABPP. Densitometry of band intensities indicated WWL229 inhibited *Ces1d* in vivo by $\sim 75\%$, whereas it did not inhibit either *Ces1c* or *Mag1* (Figure 5D). A similar degree of *Ces1d* inhibition by WWL229 in both female and male lungs was seen at 6 h (Figure S6A,B), consistent with the 6 h data in Figure 4A. Western blot analysis of the 6 h female lung membranes also indicated that *Ces1d* protein abundance was not altered by the drug treatment (Figure S6A).

In contrast, JZL184 only slightly inhibited *Ces1d* activity in vivo, whereas it strongly inactivated *Ces1c* by $>90\%$ and inactivated the ~ 35 kDa band denoted *Mag1* by $>50\%$ (Figure 5E). JZL184 is a potent inhibitor of *Mag1*,³⁴ but the band intensity attributed to *Mag1* in Figure 5E was not completely ablated as expected. In vitro treatment of naïve lung membranes with JZL184 indicated a similar situation (Figure 5C). Quantitation of this “*Mag1*” band suggested the presence of two serine hydrolase activities that migrate in the gels with similar R_f ; one is sensitive to JZL184 ($IC_{50} = 0.46 \mu M$), while the other is insensitive even at $100 \mu M$ JZL184 (Figure 5C, inset).

Based on these in vitro and in vivo results, we can conclude that WWL229 is the more selective covalent inhibitor of *Ces1d* in mouse lung and a good tool to probe *Ces1d* functions in vivo. Furthermore, these biochemical data indicate that *Ces1d* is the major carboxylesterase isoform expressed in murine lung and WWL229 can selectively inactivate lung *Ces1d* activity in vivo.

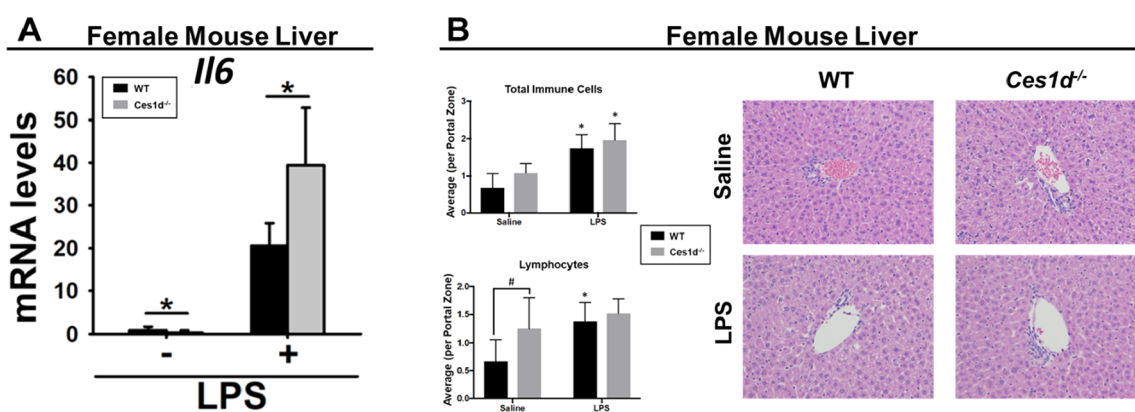


Figure 7. LPS-induced Il6 mRNA levels were enhanced in female *Ces1d*^{-/-} mouse liver compared to WT mouse liver at 6 h following intraperitoneal LPS challenge (A). Hematoxylin and eosin (H&E) histology and immune cell quantification of female WT and *Ces1d*^{-/-} mouse livers 6 h following LPS challenge (B). Representative H&E-stained images from each treatment group are shown. Total immune cells were quantified within 4 layers of hepatocytes surrounding 20 portal zones from each mouse. The average number of cells per portal zone is represented in the bar graphs. Immune cells were further distinguished by cell type, and the lymphocyte numbers are shown. Data are expressed as mean \pm SD ($n = 5$ –6 mice/group). Two-way ANOVA assessed significant differences between groups. In panel A, * $p < 0.05$, comparisons are indicated. In panel B, # $p < 0.05$, comparison is indicated; * $p < 0.05$ for LPS vs saline.

Macrophages Deficient in *Ces1d*/CES1 are More Responsive to LPS-Induced Inflammation. *Ces1d* is expressed in mouse alveolar macrophages.¹¹ In vitro experiments with these cells showed that baseline and LPS-induced levels of PGE₂ and cytokine mRNA (Il6 and TNF α) were higher in alveolar macrophages from *Ces1d*^{-/-} mice compared to those from WT mice (Figure 6A). Consistent with these findings, when CES1 expression in human THP-1 macrophages – a cell line that strongly expresses CES1 mRNA and protein³⁵ – was knocked down by an shRNA-containing lentivirus³² (Figure S2D), the levels of inflammatory cytokines were greater than those expressed by control THP-1 macrophages in the setting of LPS exposure (Figure 6B, left). Similar effects were also observed when the inflammatory stimulus was TNF α /IFN γ instead of LPS (Figure 6B, right). TLR4 mRNA transcript levels were also found to be slightly higher in CES1KD macrophages than in control macrophages under baseline conditions (1.6-fold, $p < 0.05$). Similarly, the baseline IL1b, IL6, and TNF α mRNA levels in CES1KD macrophages were 3–5-fold higher than those in control macrophages (Figure 6B, right; data not shown). Thus, these results suggested, regardless of whether an immune stimulus is present or not, that macrophages deficient in *Ces1d*/CES1 are more poised toward the M1 phenotype than those that express *Ces1d*/CES1. From another perspective, due to the higher basal cytokine levels in the enzyme-deficient cells, the fold change in cytokine levels is more pronounced in the WT/control cells than in the *Ces1d*/CES1-deficient cells following an immune stimulus (Figure 6).

***Ces1d* Knockout Augmented IL-6 Expression in Female Liver Following LPS Challenge.** Because *Ces1d* is also expressed in other metabolically active tissues such as liver and adipose, inflammation was evaluated in these tissues in female WT mice treated with WWL229 and in female *Ces1d*^{-/-} mice (Figures S7 and 7). Systemically administered LPS significantly induced Il1b, Il6, and Tnfa mRNA in WT liver (Figure S7A), as expected, but their expression was not altered by WWL229. However, although female WT and *Ces1d*^{-/-} mouse liver expressed similar levels of Il1b and Tnfa following LPS challenge (Figure S7B), Il6 was significantly higher in *Ces1d*^{-/-} liver than in WT liver (Figure 7A). Histology of liver portal zones from female WT and *Ces1d*^{-/-} mice showed that

LPS increased the number of total immune cells per portal zone regardless of the genotype (Figure 7B); however, no genotype \times LPS treatment interaction was noted ($p > 0.05$). When further stratified by immune cell type, baseline lymphocyte numbers were significantly increased in *Ces1d*^{-/-} liver compared to those in WT liver (Figure 7B), suggesting that the *Ces1d*^{-/-} liver is poised toward higher numbers of T- and/or B-cells under homeostatic conditions than the WT liver.

LPS challenge also significantly induced Il1b, Il6, and Tnfa mRNA in WT adipose tissues, but WWL229 did not modulate their levels (Figure S7C). Although the baseline expression of Il1b in *Ces1d*^{-/-} adipose tissues was lower than that in WT adipose tissues, no significant differences were found in the inflammatory cytokines in adipose tissues from WT and *Ces1d*^{-/-} mice following LPS challenge (Figure S7D). Finally, the effects of JZL184 were also examined in female WT liver and adipose tissues. However, JZL184 also did not alter the levels of LPS-induced cytokines in either tissue (Figure S8); thus, its immune modulating effects appeared to be confined to the lung in our animal model.

DISCUSSION

Carboxylesterases play an important role in the metabolism of xenobiotics and lipids. In addition to their abundant expression in mammalian liver and lung, CES1 is found in human monocytes and macrophages, including alveolar macrophages, and *Ces1d* in murine alveolar macrophages.^{5,7,8,15} Our previous work identified *Ces1d* as the most abundant serine hydrolase in mouse lung, although Magl was more important in the catabolism of 2-AG.¹¹ Here, we investigated the roles of lung *Ces1d* and Magl in an LPS model of inflammation. The main findings are as follows: 1) inhibition of *Ces1d* by WWL229 augmented LPS-induced inflammation in female lung, whereas it did not alter the degree of inflammation in male lung, suggesting a sex-specific effect for this compound in mice; 2) the levels of LPS-induced Il6 mRNA in female *Ces1d*^{-/-} lung and liver were higher than those in WT lung and liver; 3) in contrast to the potentiating effects of *Ces1d* inactivation, inhibition of Magl by JZL184 attenuated LPS-induced inflammatory cytokines in female lungs, suggesting that *Ces1d* and Magl exert opposing effects in the setting of lung inflammation.

The inflammatory effects made more potent by the Ces1d inhibitor WWL229 in female lungs were observed in part in Ces1d^{-/-} mice, which was evident from the higher expression of Il6 mRNA and eicosanoids in Ces1d^{-/-} lung compared to WT lung following LPS challenge. In support of this, the LPS-induced PGE₂ and Il6 mRNA response was stronger in Ces1d^{-/-} alveolar macrophages than in WT alveolar macrophages. In addition, CES1 knockdown in a human macrophage cell line also resulted in a stronger response to immune stimuli. Our previous studies using cultured macrophages have also identified immunomodulatory effects caused by the inhibition of CES1.^{15,17,32} For example, CES1 inactivation by either reactive pesticide metabolites or lentivirus-mediated knockdown resulted in an increase in cellular levels of endogenous 2-AG and PG-Gs due to the more limited degradation of these lipid mediators.^{15,32} These bioactive lipids can modulate inflammation.^{36–38} In addition, we showed that CES1 knockdown decreased the production of the oxysterol 27-hydroxycholesterol (27-OHC) in cholesterol-loaded macrophages, which was due to marked downregulation of the 27-OHC biosynthetic enzyme CYP27A1.³² 27-OHC is another lipid mediator that exerts immune effects and is an endogenous ligand for LXR α .³⁹ Furthermore, combined treatment of M1 polarized macrophages with WWL113 and PGD₂-G attenuated IL-6 levels more strongly than that of PGD₂-G alone,¹⁷ which suggested that blocking CES1 hydrolysis of PGD₂-G potentiated the anti-inflammatory effect of this lipid mediator. Because of the instability and transient nature of PG-Gs, they are difficult to detect in vivo. We attempted to measure these endogenous compounds in lung extracts by LC–MS/MS but to no avail. Indeed, it was previously shown that PG-Gs could only be detected in tissues of genetically engineered animal models under specific conditions.⁴⁰ Specifically, they were identified in brains of transgenic COX2-overexpressing mice treated with LPS, but only when MAGL was concomitantly inactivated. Therefore, it remains technically challenging to detect the PG-Gs in (patho)physiological settings.

Similar effects to those observed in the current study were seen with the lipolytic enzyme acyloxyacyl hydrolase (AOAH) in alveolar macrophages.⁴¹ AOAH can disarm LPS by removing the secondary acyl chains from it, and AOAH inactivation was shown to augment the expression of LPS-induced Il1b and Tnfa. Thus, an explanation for the in vivo effects in our study is that Ces1d functions (directly or indirectly) to reduce the levels of inflammatory molecules; therefore, its enzymatic inhibition may stabilize those compounds, whether they are host- or pathogen-derived, that increase the production of inflammatory cytokines. Along these lines, lipid mediators, such as PGE₂, help shape the course of inflammation by stimulating immune cells to synthesize cytokines. For example, we previously showed that the potentiating effect of PGE₂ on IL-6 production by M1 macrophages was strongly augmented by a CES1/Ces1d inhibitor.¹⁷ In the current study, within the setting of LPS-induced inflammation, PGE₂ levels were elevated in female Ces1d^{-/-} lungs compared to WT lungs, while Ces1d^{-/-} alveolar macrophages produced more PGE₂ than their WT counterparts. Thus, the elevated tissue levels of PGE₂ might be a mechanistic driver of the augmented inflammation that results following Ces1d inactivation. Increased levels of PGE₂ were also seen in male Ces1d^{-/-} mice after i.n. LPS administration, where alveolar macrophages in the lung lumen are directly activated by the endotoxin. The higher levels of LPS-induced PGE₂ in the BALF

of Ces1d^{-/-} mice compared to that of WT mice also correlated with the higher levels of Il1b mRNA in Ces1d^{-/-} lungs.

Other negative impacts of Ces1d inactivation have been reported in the context of metabolic syndrome.⁴² Ces1d ablation in adipose tissues led to a local unhealthy microenvironment in metabolically active tissues. Specifically, an adipose tissue-specific Ces1d knockout mouse, placed on a high-fat diet, exhibited a more pronounced lipotoxicity and proinflammatory phenotype in adipose and liver tissues as compared to control mice, resulting in greater metabolic dysregulation and systemic insulin resistance. These effects were linked to the absence of Ces1d-dependent triacylglycerol lipase activity in adipose tissues, causing ectopic accumulation of triacylglycerols in peripheral tissues. Whether Ces1d has a role in triacylglycerol metabolism within lung tissues, however, will require further study.

Another important issue is that the WWL229-dependent augmentation of lung inflammation seen in female mice was not observed in male mice. This is congruent with the female-specific inactivation of lung Ces activity by WWL229 observed at 4 h in vivo. However, lung Ces activity was decreased by WWL229 equally in females and males by 6 h (Figures 4A and S6), suggesting a differential rate of drug uptake into the lung. Because the levels of Ces activity in male and female lungs are comparable, and Ces1d accounts for most of this enzymatic activity, this implies that lung Ces1d concentrations are similar in both sexes. Indeed, this appears to be borne out by gel-based ABPP of male and female mouse lung membranes (Figure S2C). It is possible that the time-dependent effects on Ces inhibition in females and males might be related to differences in the pharmacokinetic behavior of WWL229, that is, it may be inferred from the enzyme activity data that the biologically effective concentration of WWL229 required to inhibit Ces1d was achieved earlier in female lung than in male lung. It is known that sex-related differences in drug metabolism exist, especially in the liver;^{43,44} males often have higher levels of xenobiotic metabolism enzymes (e.g., CYPs and Ces) than females.^{11,45} Therefore, it is possible that WWL229 is inactivated in vivo more efficiently in males by either hepatic oxidative metabolism or covalent adduction of Ces enzymes, altering the rate of compound uptake into the lung. This also could be the reason that WWL113, a more potent in vitro inhibitor of Ces1d, did not inhibit Ces activity to the same extent as WWL229 in female lung in vivo.

The observed in vivo effects caused by Ces inhibition might also be specific to mice. For instance, recent work showed that the inhibition of Ces in porcine alveolar macrophages by bis(4-nitrophenyl) phosphate (BNPP) reduced the expression of Il1b, Il6, and Tnfa mRNA,⁴⁶ which is opposite to our findings. Furthermore, combined treatment of pigs with BNPP and LPS in vivo led to decreased levels of AA and other pro-inflammatory lipid mediators, which was attributed to inhibited Ces-dependent endocannabinoid hydrolysis. We had previously suggested that the LPS-dependent reductions of Ces1d and Ces2g activity in murine alveolar macrophages, lung, and spleen might be part of a negative feedback mechanism to control inflammation.^{11,14} However, our current data seem to suggest that Ces inactivation in lung induces a pro-inflammatory response rather than an anti-inflammatory one. The identification of those endogenous substrates that are possibly metabolized by Ces1d in the murine lung will help to unravel the species-related differences observed in these different models. In addition, as shown by the significantly altered levels

of CYP27A1 and its metabolite 27-OHC in human CES1KD macrophages,³² the inactivation of CES1 (and its mouse counterpart Ces1d) may indirectly regulate the levels of immune-modulating lipid mediators, such as PGE₂ and other eicosanoids. Further research on the signaling pathways that are involved is needed.

Magl is the primary hydrolytic enzyme of the endocannabinoid 2-AG in murine lung despite the fact that the catalytic efficiencies of recombinant human CES1 and MAGL enzymes toward 2-AG are equally comparable.^{11,14,15,47} Furthermore, the *in vitro* hydrolysis rates of 2-AG by WT and Ces1d^{-/-} lung membranes were not significantly different (data not shown), indicating that other enzymes, notably Magl, are responsible. Therefore, we treated mice with JZL184, a Magl inhibitor with some known off-targets, for example, Ces/CES isoforms⁴⁸ (Figure 5C,E), and found that it could attenuate lung cytokines induced by LPS challenge. These findings are opposite to those of WWL229 but are in line with others that showed JZL184 could exert anti-inflammatory effects.^{22,49–52} Two of these studies examined the murine lung. The first showed that JZL184 reduced IL-6 and TNF- α levels in BAL fluid after *i.n.* LPS challenge.²² JZL184 also reduced MCP-1, an effect opposite to that in our study when female mice were treated with WWL229. The authors attributed the JZL184-mediated effects to increased 2-AG levels. The second study showed that JZL184 reduced TNF- α and IL-1 β in BAL.⁴⁹ Lung 2-AG was also found to be significantly increased by JZL184. Thus, our JZL184 findings are consistent with other reports that indicate that Magl inactivation in settings of lung inflammation has benefits. One caveat to this is that Ces1c can also be potently inhibited by JZL184 *in vivo*, but whether this contributes to the immune-dampening effects of JZL184 in the lung is not currently known. Another study has demonstrated that the ABHD6 inhibitor WWL70 decreased LPS-induced murine lung inflammation to an even greater extent than JZL184 did, which was attributed to increases in 2-AG levels and some lysoglycerophospholipids following ABHD6 inactivation.⁵³ ABHD6 adds another node to the intricate lipid biochemical pathways that can be targeted to alleviate inflammation during lung injury.

Finally, the effects observed in our study also have implications regarding mechanisms of immunotoxicity. Our previous work showed that lung Ces1d had a protective role against CPF-mediated lung immune effects.¹¹ For example, in the setting of inflammation, a CPF-dependent increase in TNF α mRNA was revealed in adult Ces1d^{-/-} mouse lung, an effect not seen in WT mice. In addition, Ces1c expression in male Ces1d^{-/-} LPS-treated lungs was lower than that in LPS-treated WT lungs, which could have compounded the CPF-dependent immune effects in Ces1d^{-/-} mice because Ces1c can also detoxify the bioactive metabolite of CPF (*i.e.*, CPO). These findings, which were obtained with a chemical toxicant instead of a drug, also suggested that the inactivation or downregulation of Ces functions in lungs can exaggerate immune effects;¹¹ thus, they are consistent with the changes seen in the present study.

CONCLUSIONS

Here, we showed that opposing effects on inflammation can occur when Ces1d and Magl are inhibited in mouse lungs. Although the effects of the Magl inhibitor are most likely due to increased levels of inflammation-dampening 2-AG, the identification of the effector molecules responsible for the Ces1d-dependent effects will require further research. For example, it will be of interest to examine the connections between the

transcription factors (*e.g.*, NF- κ B and AP-1) activated by LPS and the signaling pathways that are unmasked when Ces1d is inhibited or knocked out. Overall, our study further supports the benefits that Magl inhibitors might offer in inflammatory settings. Moreover, it suggests that functional Ces1d has a role, albeit undefined, in regulating the degree of acute inflammation in the lung. The mechanisms that explain this regulation will be of interest to explore further.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acspsci.2c00098>.

Mouse serum cytokine/chemokine levels; Ces1d expression/activity in WT mouse lung; *Il1b* and *Tnfa* mRNA levels in female WT and Ces1d^{-/-} mouse lungs; eicosanoids in lungs from male WT and Ces1d^{-/-} mice treated with saline and LPS; eicosanoids in lungs from female WT mice treated with WWL229 and LPS; cytokine mRNA levels in female mouse liver and adipose resulting from Ces1d inhibition or Ces1d genetic knockout; and cytokine mRNA levels in female mouse liver and adipose after Magl inhibition (PDF)

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Author Contributions

B.N.S. and A.B. contributed equally. B.N.S., A.B., B.L.F.K., and M.K.R. designed the study. B.N.S., A.B., H.S., A.M.M., J.E.C., A.O., C.B.W., and M.K.R. carried out experiments, data analyses, and interpretation. R.L. provided *Ces1d*^{-/-} mice and interpretation. The manuscript was drafted by B.N.S. and M.K.R., and revised by B.L.F.K. and M.K.R. All authors approved the final manuscript.

Notes

The authors declare no competing financial interest.

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This paper was published ASAP on September 12, 2022, with an error in the Abstract and also the Macrophages Deficient in Ces1d/CES1 are More Responsive to LPS-Induced Inflammation section. The corrected version was reposted on September 14, 2022.