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Isomer-specific effects of conjugated linoleic acid on gene expression in RAW 264.7

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Abstract

Conjugated linoleic acid (CLA) is a mixture of dietary fatty acids that has various beneficial effects including decreasing cancer, atherosclerosis, diabetes and inflammation in animal models. Some controversy exists on the specific isomers of CLA that are responsible for the benefits observed. This study was conducted to examine how different CLA isomers regulate gene expression in RAW 264.7. A mouse macrophage cell line, RAW 264.7, was treated with five different CLA isomers (9*E*,11*E*-, 9*Z*,11*E*-, 9*Z*,11*Z*-, 10*E*,12*Z*- and 11*Z*,13*E*-CLA). Gene expression microarrays were performed, and several significantly regulated genes of interest were verified by a real-time polymerase chain reaction (PCR). Examination of the biological functions of various significantly regulated genes by the five CLA isomers showed distinct properties. Isomers 9*E*,11*E*-, 9*Z*,11*Z*-, 10*E*,12*Z*- and 11*Z*,13*E*-CLA decreased production of proinflammatory cytokines such as interleukin (IL)-1α, IL-1β and IL-6. Many of CLA's effects are believed to be mediated by the fatty acid receptors such as the peroxisome proliferator-activated receptors (PPAR) and retinoid-X-receptors (RXR). Using PPAR and RXR specific antagonists and coactivator recruitment assays, it was evident that multiple mechanisms were responsible for gene regulation by CLA isomers. Coactivator recruitment by CLA isomers showed their distinct properties as selective receptor modulators for PPARγ and RXRα. These studies demonstrate distinct isomer differences in gene expression by CLA and will have important ramifications for determining the potential therapeutic benefit of these dietary fatty acids in prevention of inflammation-related diseases.

Keywords: Conjugated linoleic acid; Gene expression; Peroxisome proliferator-activated receptor; Macrophage; Inflammation

1. Introduction

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid (C18:2) discovered as the component of charcoaled meat that inhibits carcinogenesis in animal models of disease [1]. Main sources of CLA are meats and dairy products of ruminant animals with a negligible amount from fish [2]. The 9-*cis*, 11-*trans* (9*Z*,11*E*)-CLA isomer, which is generated via biohydrogenation of dietary linoleic acid, is the most abundant natural isomer (over 90% of total CLA) in ruminant tissue fats [3]; the 10-*trans*, 12-*cis* (10*E*,12*Z*)-CLA isomer is the next most abundant dietary CLA but is available in much higher concentrations in dietary supplements [4]. CLA has been extensively studied, both as a nutritional component and as a dietary supplement, for its effects in laboratory animals, livestock and humans. The mixture of CLA isomers has been repeatedly demonstrated to possess anticarcinogenic, antiatherogenic, antidiabetic and anti-inflammatory effects in laboratory animals, although some of these effects are not firmly concluded from human clinical studies (reviewed in Ref. [5]).

Cardiovascular disease has a complex etiology with contributions from lipid metabolism and inflammatory pathways affecting the vascular endothelium. In several rodent models including the ApoE$^{-/-}$ mice, the CLA mixture

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or individual CLA isomers (mainly 9*Z*,11*E*- and 10*E*,12*Z*-CLA) reduced the development of atherosclerosis; however, in another study, CLA increased fatty streak development in the aorta in a murine model of atherosclerosis [6]. The difference between these studies may lie in the relative amounts and composition of the CLA used in the studies. The prevention and/or resolution of atherosclerosis by CLA has been proposed to result from a decrease of pro-inflammatory genes [i.e., interleukin (IL) 1β, tumor necrosis factor-α (TNF-α)] and an increase in apoptotic genes (caspase-3) [7]. CLA decreases the production of eicosanoids, particularly the II series of prostaglandins which suppresses the symptoms of fever and pain accompanying inflammation [8–10]. Our laboratory [11,12] and others [13] have shown that CLA decreases leptin and TNF-α levels in vivo and in vitro. Several other observations include CLA-dependent decreases in nuclear factor (NF)-κB activity [14], increased prostaglandin-I_2 levels and enhanced IgA, IgG and IgM formation [5]. In lipopolysaccharide (LPS)-stimulated RAW 264.7 cells, the CLA mixture decreased mRNA and protein levels of iNOS, cyclooxygenase-2 and their subsequent products, nitric oxide and prostaglandin E_2, respectively [15]. In addition, 9*Z*,11*E*-CLA but not 10*E*,12*Z*-CLA reduced LPS-induced TNF-α production in RAW 264.7 cells [16]. Thus, both in vitro and in vivo studies suggest that CLA has effects on cytokine and prostaglandin production, which could affect the inflammatory response. Clinical studies are not as conclusive as animal models and cell line studies, although a recent study in healthy subjects showed that a dietary CLA supplement can reduce IgE, TNF-α and IL-1β and increase IgA and IgM [17]. More clinical studies in various populations are needed to draw a firm conclusion before any recommendations can be made to the public.

Some of the proposed mechanisms for the anti-atherogenic and lipid-lowering effects of CLA include modulating the activity of peroxisome proliferators-activated receptors (PPARs), sterol regulatory element-binding proteins (SREBPs) and stearoyl-CoA desaturase (reviewed in Ref. [18]). Previous studies also showed that PPARα, PPARγ and RXR play an important role in the effects of CLA on gene expression and may be responsible for the effects of this dietary fatty acid on NF-κB activity [11,19,20]; however, it is worthwhile to note that most of CLA studies examine the synthetic mixture (containing mainly the 9*Z*,11*E*- and 10*E*,12*Z*-CLA isomers). There is much less information on the effects of the individual isomers. In this study, we examined gene expression of five different CLA isomers (i.e., 9*E*,11*E*-, 9*Z*,11*E*-, 9*Z*,11*Z*-, 10*E*,12*Z*-, and 11*Z*,13*E*-CLA) in a mouse macrophage cell line, RAW 264.7. Through the use of nuclear receptor (NR) antagonists and coactivator recruitment assays, we observed multiple pathways responsible for gene regulation by CLA isomers. Importantly, the 9*E*,11*E*-, 9*Z*,11*Z*-, 10*E*,12*Z*-, and 11*Z*,13*E*-CLA isomers inhibited production of proinflammatory cytokines. To our knowledge, the distinct regulation of inflammation cytokines by various CLA isomers is a novel finding and provides insight into the use of these isomers as potential therapeutic tools.

2. Methods and materials

2.1. Materials

CLA isomers (9*E*,11*E*)-CLA (98+ % purity), (9*Z*,11*E*)-CLA (98+ % purity), (9*Z*,11*Z*)-CLA (96+ % purity), (10*E*,12*Z*)-CLA (98+ % purity) and (11*Z*,13*E*)-CLA (77% *cis*, *trans*; 2% *cis*, *cis*; 6% *trans*, *trans*) were purchased from Matreya LLC (Pleasant Gap, PA, USA). Methoprene acid was purchased from Biomol (Plymouth Meeting, PA). 15-deoxy-$\Delta^{12,14}$-progstaglandin J_2 (PGJ_2) and GW9662, PPAR antagonist, were purchased from Sigma (St. Louis, MO, USA). UVI-3003, RXR antagonist, was provided by Dr. Angel R. de Lera and has been described [21]. Various NR agonists, 9-*cis* retinoic acid, T0901317, thenoyltrifluoroacetone (TTA), and Rosiglitazone (Rosi) were purchased from Sigma. LPS from *Escherichia coli* was also obtained from Sigma. GW501516 was purchased from Alexis Biochemical (San Diego, CA, USA). All primers for real-time polymerase chain reaction (PCR) were designed by using Primer Express (Applied Biosystems, Foster City, CA, USA), and were purchased from Operon Biotechnologies (Huntsville, AL, USA). Real-time PCR reagent, SybrGreen, and high-capacity cDNA archive kit were purchased from Applied Biosystems. Transfection reagent, LipoFectAMINE (Invitrogen, Carlsbad, CA, USA) was used. DNA plasmid for the transient transfection studies, pRLTK, was purchased from Promega (Madison, WI). CellTiter 96 Aqueous One solution was also purchased from Promega. All media components and fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD, USA). RAW 264.7 and 3T3-L1 cell lines were purchased from ATCC (Manassas, VA, USA).

2.2. Cell culture

RAW 264.7 (RAW) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% heat-inactivated fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM L-glutamine, 100 mM of sodium pyruvate and 100 μM nonessential amino acids [12]. 3T3-L1 cells were cultured in high-glucose (4.5 g/L) DMEM containing 8% FBS, 50 U/ml penicillin, and 50 mg/ml streptomycin. Cells were all maintained in a 5% CO_2 humidified atmosphere at 37°C.

2.3. RNA isolation and real-time PCR

Total RNA was isolated using Tri-Reagent (Sigma) according to the manufacturer's instructions. Reverse transcription was performed using the high capacity cDNA archive kit (Applied Biosystem) according to the provider's instruction. Real-time PCR was performed as described previously [22]. A series of 10-fold dilutions of the pooled cDNA samples were used to make a standard curve. The

housekeeping gene, β-actin, was used to normalize all the tested genes.

2.4. Transient transfection and reporter assay

The mammalian-2-hybrid system was performed in 3T3-L1. Cells (5×10^5) were plated in 6-well plates and allowed to recover overnight at 37°C. On the next day, the cells were transfected using LipofecAMINE (Invitrogen) according to the manufacturer's protocol. pVP16-mouseRXRα and pVP16-mousePPARγ were made from pM-mouseRXRα and pM-mousePPARγ [23] using standard techniques. pMLXXLL peptide library was kindly provided by Dr. McDonnell [24]. A total of 2 μg of plasmids was used in each well of 6-well plate along with pRLTK plasmid to correct for transfection efficiency. The transfected cells rested overnight before being split to a 96-well plate on the following day. After 4 h, media was replaced with treatment media. At the end of the treatment, media was removed and cells were washed with phosphate-buffered saline. Twenty five μl of passive lysis buffer was added per well. Plates were frozen at −80°C prior to luciferase activity being determined.

2.5. Cell titer assay

Cell viability was measured by cell titer assay using CellTiter 96 AQueous One solution (Promega) according to the manufacturer's protocol. Briefly, RAW cells (500 cells/well) were plated in 96-well plate. On the following day, the cells were treated with treatment compounds (i.e., UVI-3000, GW9662 etc.) for 24 h. After removing the treatment media, 10 μl of CellTiter 96 AQueous One solution and 50 μl of Opti-MEM were added to each well. The plate was incubated for 4 h at 37°C in a humidified and 5% CO_2 atmosphere. The absorbance of the samples was measured using Spectracount (Packard Instrument, Meriden, CT, USA) at 490 nm.

2.6. Microarray analysis

Microarray was performed as previously described with modifications [25]. Briefly, low-intensity signals were removed, and then files (i.e., gpr files) were converted to the format (i.e., tav files) that can be loaded to TM4 (Microarray software suite, http://www.tm4.org/) program. Using TIGER MIDAS (Microarray data analysis system), a normalization was preceded by Locfit (Local regression and likelihood: LOWESS) and S.D. The final filtered files were imported to Tiger MultiExperiment Viewer (TMeV) to perform significance analysis of microarray (SAM). Multi-class SAM was performed to find significantly regulated genes by all CLA isomers compared to PGJ_2 as a positive control, and one-class SAM was also performed to identify regulated genes by each CLA isomer. False discovery rate (%) median was 7.54 for selecting 139 significantly regulated genes. Gene Ontology Tree Machine (GOTM) (http://genereg.ornl.gov.gotm, University of Tennessee and Oak Ridge National Laboratory) was used to cluster the regulated genes by its biological functions.

2.7. Statistical analysis

Data analyzed by one-way analysis of variance (ANOVA) were tested for normality via Ryan-Joiner normality test in terms of outcome variables. The homogeneity of variance was analyzed by Bartletts' test using Minitab (State College, PA, USA) ($P>.05$). Data not meeting the assumptions of the test were normalized by box-cox transformation. Data then were analyzed by one-way ANOVA. Tukey's, family error rate was used for a one-way multiple comparison ($P<.05$). Values with an asterisk are different from the vehicle treated control at the $P<.05$ level.

3. Results

3.1. Identification of genes regulated by CLA isomers in RAW 264.7

RAW cells were treated with five different CLA isomers (9*E*,11*Z*-, 9*E*,11*E*-, 9*Z*,11*E*-, 10*E*,12*Z*-, and 11*Z*,13*E*-CLA) for 24 h, and gene expression microarray studies were performed. Since CLA's effects may be partially due to activation of PPARγ [12], PGJ_2 (15-deoxy-$\Delta^{12,14}PGJ_2$) was used as a reference for activation of this NR. Statistical analysis was performed, and a list of genes (n=139) affected by each of the compounds is shown in Supplemental Table 1. Each CLA isomer resulted in a unique pattern of altered gene expression, although there were several genes that were common among the treatments (Supplemental Figure 1). Compared to PGJ_2, 9*E*,11*E*-CLA was the most divergent from the other CLA isomers. According to the hierarchical tree, the similarity of the CLA isomers to PGJ_2 follows this order: 9Z11E>10E12Z>11Z13E>9Z11Z>9E11E.

Gene ontology tree machine (GOTM; http://genereg.ornl.gov.gotm, University of Tennessee and Oak Ridge National Laboratory) was used to analyze related molecular and biological functions. 9*E*,11*E*-CLA regulated the most genes (n=63), and these genes were related to immune cell activation, DNA replication, lipid transporter activity, protein-tyrosine kinase activity, cell cycle regulation, chemokine activity and inflammatory response. The genes (n=24) regulated by 9*Z*,11*E*-CLA are related to embryonic development, signal transduction, immune response, receptor binding and extracellular space. The genes (n=31) regulated by 9*Z*,11*Z*-CLA are related to cell proliferation, antioxidant activity (oxidoreductase activity), positive regulation of cellular physiological process and transcription factor complex. The genes (n=25) regulated by 10*E*,12*Z*-CLA are related to morphogenesis, organ development, and DNA binding. The genes (n=19) regulated by 11*Z*,13*E*-CLA are related to transmembrane receptor protein tyrosine kinase signaling pathway, ubiquitin cycle, morphogenesis, embryonic development and protein binding. Thus, CLA isomers

have distinct isomer-specific gene expression on mouse macrophage cells.

3.2. Verification of regulated genes by CLA isomers in RAW 264.7

Real-time PCR was used to verify twelve genes that were significantly regulated from the microarray results with an emphasis on those genes, with relatively high statistical significance ($P<.05$) and affects on immune function and cell cycle regulation. Fig. 1 depicts relative mRNA levels of the selected genes and in general there was good concordance with the microarray data. Treatment with 9*E*,11*E*-CLA resulted in a threefold increase in the relative mRNA levels of a disintegrin-like and metallopeptidase (ADAMTS) 1, but the rest of the CLA isomers had a twofold decrease in its mRNA levels compared to vehicle control. Catalase1 mRNA levels were increased by 9*E*,11*E*-, 9*Z*,11*Z*-CLA and PGJ_2, five-, two- and 20-fold, respectively. CD36 (scavenger receptor) mRNA levels were increased approximately 2.5-fold and 8.3-fold by 9*E*,11*E*-CLA and PGJ_2 respectively, but decreased approximately fourfold by 9*Z*,11*E*-, 9*Z*,11*Z*- and 11*Z*,13*E*-CLA compare to vehicle control. CD44 mRNA levels were decreased approximately fourfold by 9*Z*,11*E*-, 9*Z*,11*Z*-, 11*Z*,13*E*-CLA and PGJ_2 compared to vehicle control. 9*E*,11*E*-CLA decreased mRNA levels of cyclin E (CycE) and EskK (also known as TtK) 10-fold compared to control. Fatty acid binding protein 5 (FABP5) mRNA levels were increased only by 9*E*,11*E*-CLA approximately 3-fold compared to control, but 9*Z*,11*E*-, 10*E*,12*Z*- and 11*Z*,13*E*-CLA decreased mRNA levels. IL-1Ra mRNA levels were strongly increased by 9*E*,11*E*-CLA but decreased by 10*E*,12*Z*-, 11*Z*,13*E*- and PGJ_2. Lipin1 and RGS1 (regulator of G-protein signaling 1) mRNA levels were increased approximately 2.5-fold by 9*E*,11*E*-CLA but decreased by the rest of the CLA isomers including the positive control. Only 9*E*,11*E*-CLA increased RhoC (Aplysia ras-related homolog 9) mRNA levels fivefold, but 9*Z*,11*E*-, 10*E*,12*Z*- and 11*Z*,13*E*-CLA decreased its mRNA levels. Usp18 (ubiquitin specific protease 18) mRNA was decreased by all CLA isomers except 9*E*,11*E*-CLA. These results confirm that all of the CLA isomers act differently when regulating gene expression and also showed small structural/geometric changes in fatty acid structure can dramatically affect gene expression in macrophages.

3.3. Anti-inflammatory effects of CLA isomers

CLA has anti-inflammatory effects in animal and human cell models. Our microarray data suggested that some of CLA isomers have immunological functions via regulation of genes such as CD36, CD44 and IL-1Ra. Since CLA isomers alone did not alter pro-inflammatory cytokines in RAW cells, LPS was utilized to induce the proinflammatory cytokines to test whether CLA isomers can alter the cytokine levels. RAW cells were treated with CLA isomers and then challenged with LPS. The mRNA for the pro-inflammatory genes (IL-1α, IL-1β and IL-6) were decreased by all of the CLA isomers except 9*Z*,11*E*-CLA (Fig. 2). There was no significant alteration on TNF-α mRNA levels by CLA isomers in RAW cells. Among the five different CLA isomers, 9*E*,11*E*-CLA showed the most definitive decrease in mRNA levels of the proinflammatory cytokines in RAW cells.

3.4. Effects of PPAR and RXR antagonist in the regulated genes by CLA isomers in RAW 264.7

Previous studies have shown that CLA isomers activate the PPARs as well as RXRα. To determine if these NR(s) are involved in the gene regulation by the CLA isomers, PPAR and RXR antagonists were employed. Cell titer assay was performed as described in Materials and Methods to determine toxicity of the two antagonists in RAW cells (data not shown). Cells were pretreated with GW9662 (pan-PPAR antagonist) or UVI-3003 (RXR antagonist) and were then cotreated with CLA isomers (Fig. 3). These studies showed that some of the genes are in fact regulated by the PPAR/RXR heterodimer, whereas other genes exhibit other mechanisms for their effects on mRNA accumulation. For instance, the increase of mRNA of CD36, FABP5, lipin1, IL-1Ra, and RhoC by 9*E*,11*E*-CLA was decreased by PPAR or RXR antagonists. The results of this experiment on individual genes are summarized in Supplemental Table 2.

3.5. Similarity of 9E,11E-CLA vs. various NR agonists

To verify the effects observed with chemical antagonists and to further explore the unique properties of the 9*E*,11*E*-CLA isomer, the effects on gene expression were compared to that of relatively selective NR agonists. The agonists were Wy-14643 (PPARα agonist), GW501516 (PPARβ agonist), tetradecylthioacetic acid [PPAR pan-agonist (TTA)], rosiglitazone [PPARγ agonist (Rosi)], 9-cis retinoic acid [RXR agonist (RA)], methoprene acid [RXR agonist (MA)], T0901317 (LXR agonist) and LPS [toll-like receptor 4 (TLR4) agonist] (Fig. 4). TTA decreased ATP-binding cassette transporter A1 (ABCA1) mRNA levels, but Rosi, MA, RA, T0901317 and LPS increased its mRNA levels. The increase in this gene by Rosi was expected [26]. All of the NR agonists including 9*E*,11*E*-CLA, with the notable exception of Rosi, increased mRNA levels of adipose differentiation related protein (ADRP), which is considered as a nonselective PPAR target. 9*E*,11*E*-CLA and TTA increased ADRP mRNA levels 12-fold compared to vehicle control. TTA and LPS decreased the LXR target apolipoprotein E (ApoE) mRNA expression, 25% and 90%, respectively, while T0901317 increased its levels. CD36 is a scavenger receptor that is increased by PPARγ activators [27]. Wy-14643, TTA, PGJ_2 and LPS increased CD36 mRNA levels. FABP5 mRNA levels were increased by 9*E*,11*E*-CLA, TTA and LPS. Interferon (IFN)-β is a TLR4 target gene, and its mRNA levels were decreased by TTA and PGJ_2 but increased by LPS. 9*E*,11*E*-CLA, TTA and LPS

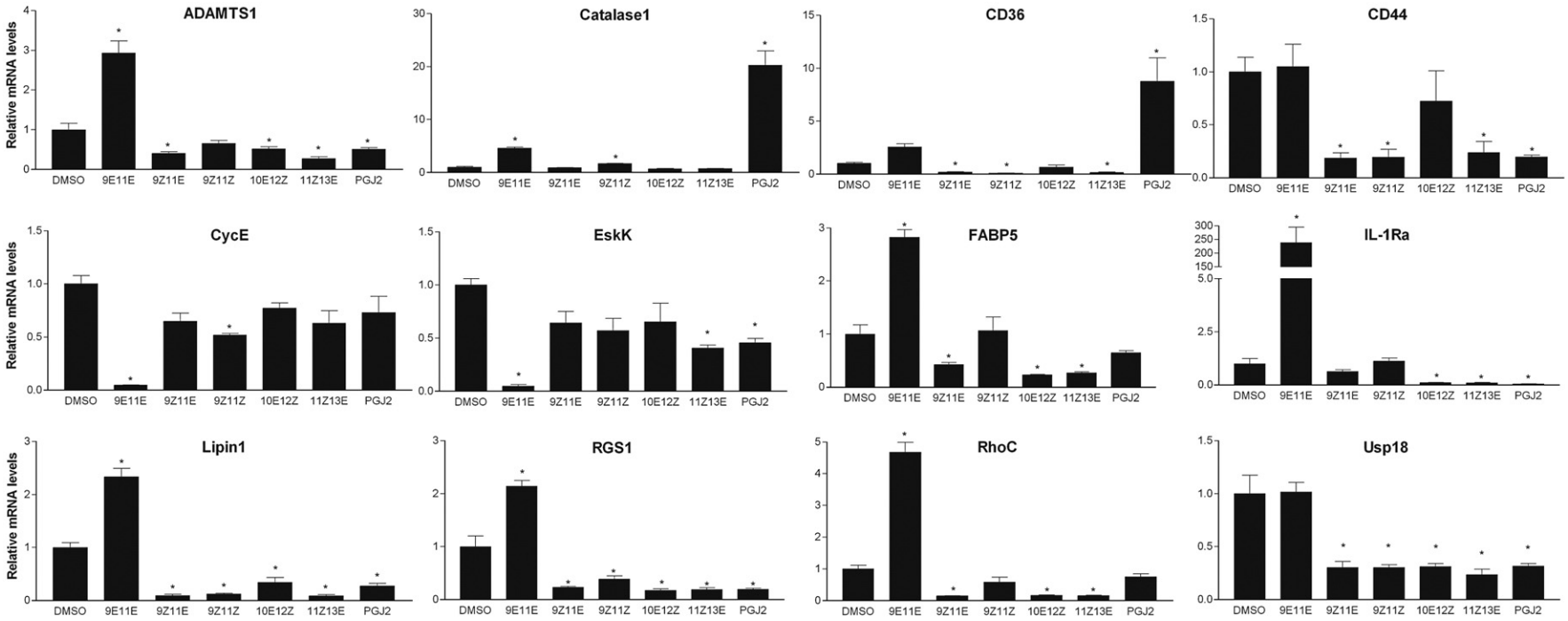


Fig. 1. Regulation of gene expression by CLA isomers in RAW 264.7: representative genes from microarray. RAW cells were incubated in serum starved media for 24 h and then treated with 200 μM of CLA isomers for 24 h. Total RNA was isolated, and real-time PCR was performed as described in Materials and methods. The values represent three independent experiments with triplicate samples. Asterisks indicate a significant difference compared to vehicle control group ($P<.05$).

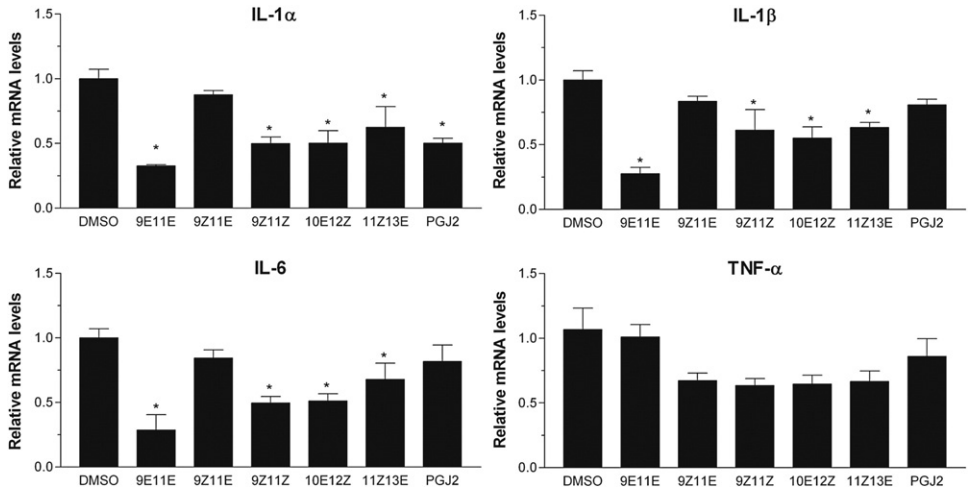


Fig. 2. Regulation of gene expression by CLA isomers in RAW 264.7: inflammatory cytokines. RAW cells were starved for 24 h and treated with vehicle control or 200 μM 9*E*,11*E*-CLA for 24 hrs with LPS (1 μg/ml) challenge for last 6 h. RNA was isolated and real-time PCR was performed as described in Methods. Asterisks indicate a significant difference compared to vehicle control group ($P<.05$). Data represent three independent studies with triplicate samples.

increased IL-1Ra mRNA levels, but MA and T0901317 decreased its mRNA levels. As shown in Fig. 4, 9*E*,11*E*-CLA and TTA tended to produce similar effects on gene expression, in particular for IL-1Ra, ADRP and FABP5 mRNA. The similarity of 9*E*,11*E*-CLA to various NRs tested in this study was: TTA>GW501516>PGJ_2>Wy-14643>LPS >MA>RA>T0901317>Rosi.

3.6. CLA is a selective receptor modulator: Coactivator recruitment study

The previous studies demonstrate that differential gene expression for various fatty acids and xenobiotics is observed despite the fact that they share similar mechanisms of action. These studies suggest that the CLAs are selective receptors modulators (SRMs) where they activate the NRs but enhance the regulation of genes in an isomer-specific manner. A library of 16 different coactivator peptides was used to determine whether the CLA isomers act as SRMs for RXRα and/or PPARγ. These experiments were done using the mammalian-2 hybrid system in which LXXLL peptide library [24] is linked to GAL4 DNA binding domain and RXRα and PPARγ are linked to the VP16 activation domain. Class I peptides (i.e., D2, D11, and D30) contain a conserved serine at the -2 position and a positively charged residue at the -1 position. Class II peptides (i.e., D14, D47 and C33) contain a conserved proline at the −2 position and hydrophobic leucine residue directly preceding the LXXLL motif. Class III peptides (i.e., F6, D22, D48, D43, D17, D41, D26, D40, D15 and F4) contain a conserved serine or threonine at the −2 position followed by a hydrophobic leucine or isoleucine at the −1 position. As shown in Fig. 5, the clustering analysis reveals different recruitment affinity of LXXLL peptides upon ligand binding to RXRα and PPARγ. This is a hallmark of SRMs and shows that CLA isomers result in a conformational shape of RXRα and/or PPARγ that is distinct from prototypical agonists. In addition, each CLA isomer results in a different affinity profile for LXXLL peptides, with the 9*E*,11*E*-CLA being the most divergent. This may in part explain the isomer-specific as well as tissue- and cell-type specific effects on gene expression.

4. Discussion

Since its discovery as an anti-cancer agent in 1990 [1], CLA has been intensively studied in animals and humans due to its various potential beneficial effects (reviewed in [5]). In this study, distinctly different effects on gene expression of five different CLA isomers (9*E*,11*E*-, 9*Z*,11*E*-, 9*Z*,11*Z*-, 10*E*,12*Z*-, and 11*Z*,13*E*-CLA) was demonstrated in mouse macrophage cell line, RAW 264.7. Comprehensive gene expression analysis by microarray clearly showed how different the five CLA isomers are in their ability to regulate gene expression in macrophages. The cluster analysis and GOTM of the genes regulated by the five CLA isomers supported their distinctively different regulation. Among the studied CLA isomers, 9*E*,11*E*-CLA showed the most significantly regulated genes, which were related to immune cell activation, chemokine activity and inflammatory response.

Twelve genes were selected for further verification by real-time PCR and were of interest due to their role in inflammation and atherogenesis. ADAMTS-1 is a member of the ADAM (A disintegrin and metalloprotease) gene family containing thrombospondin anchoring to the extracellular matrix and plays a crucial role in regulating bone turnover in a type I motifs and response of parathyroid hormone partially [28]. ADAMTS-1 is necessary for normal growth, fertility and organ morphology and function [29]. Interestingly, recent studies have shown that CLA has beneficial effects on bone health (reviewed in Ref. [18]); whether the ADAMTS-1 regulation by CLA isomers is playing a role in this beneficial effect is not known. It is

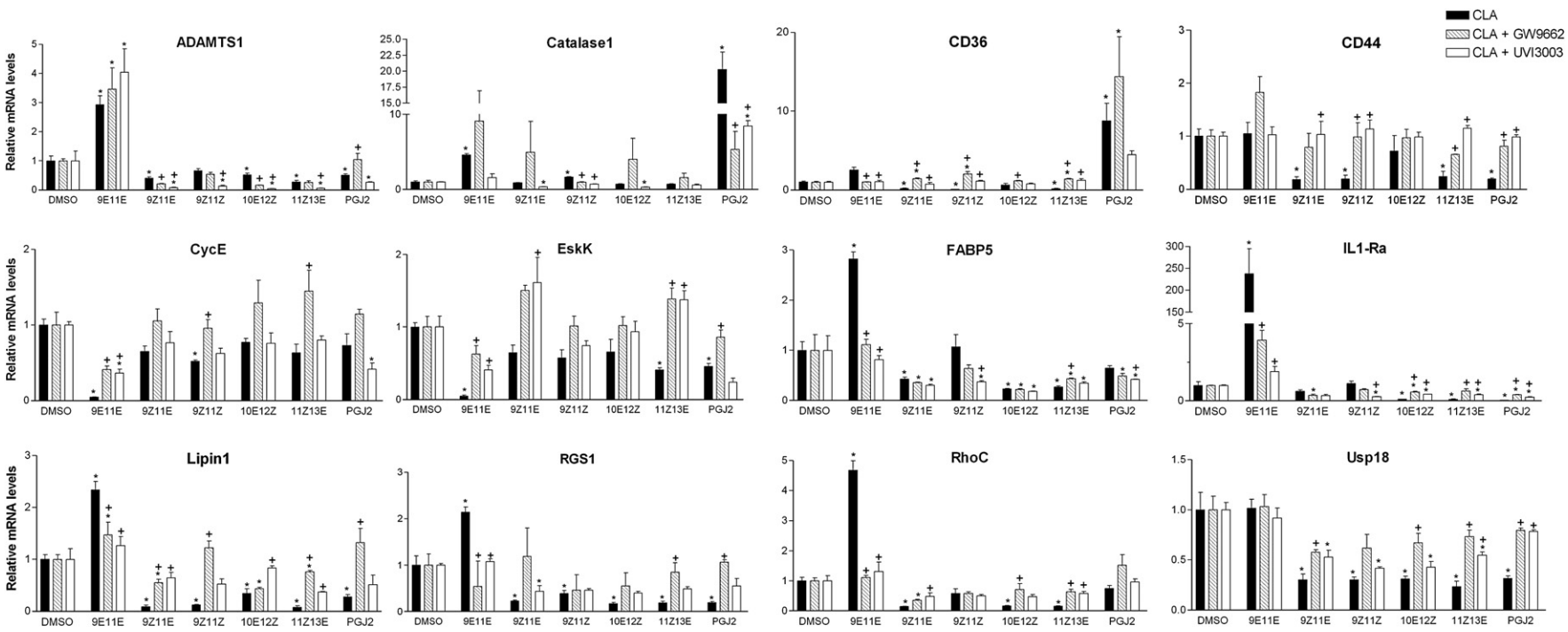
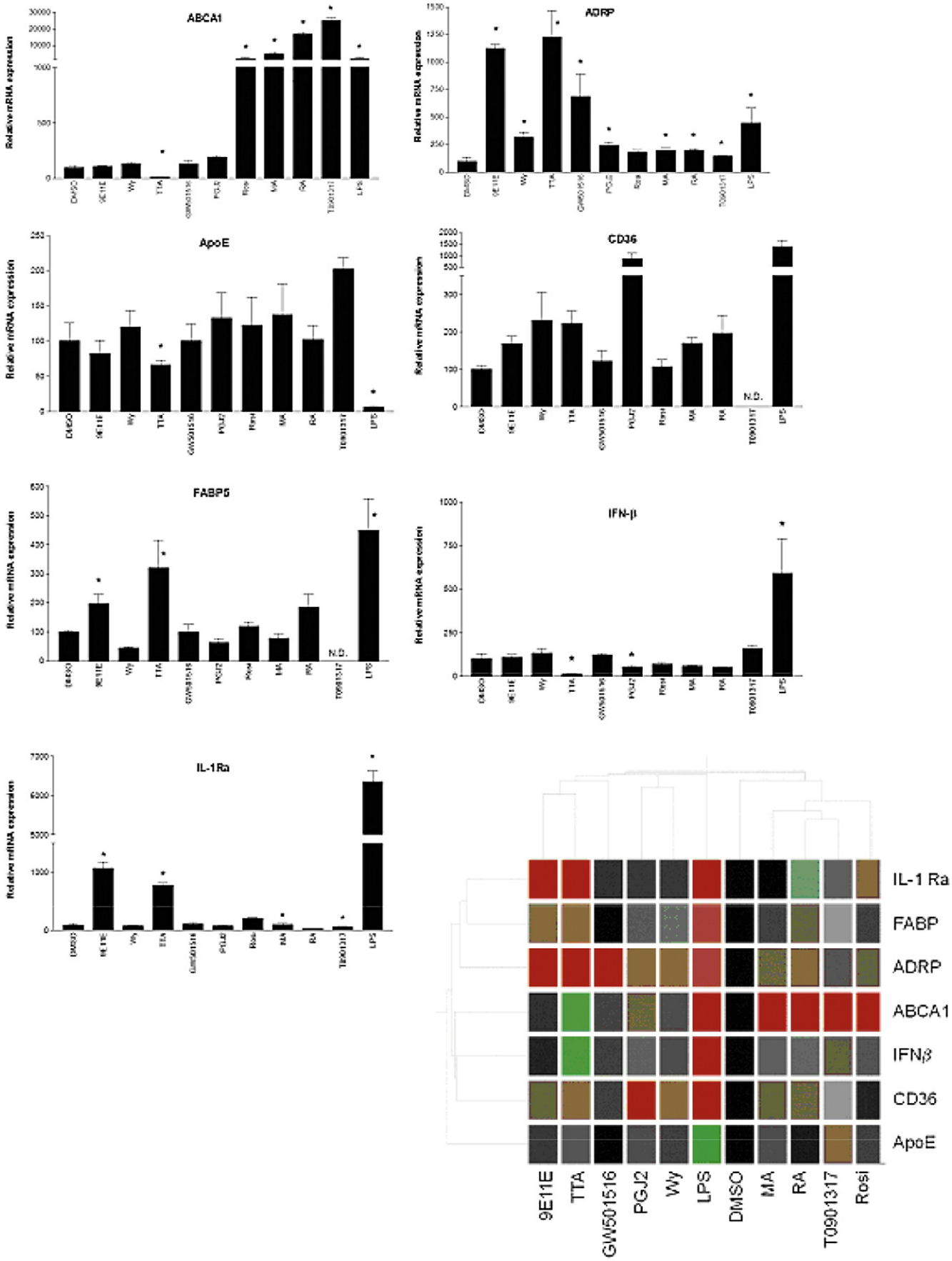


Fig. 3. Effects of cotreatment of PPAR or RXR antagonists with CLA isomers on gene expression. RAW cells were cultured as described in Materials and methods. Antagonists were pretreated for 30 min and cotreated with 200 μM of CLA isomers for 24 h. Black bar represents CLA only, slashed bar represents CLA with 50 μM of GW9662 and clear bar represents CLA with 1 μM of UVI-3003. Asterisks indicate a significant difference compared to each DMSO group, and plus signs indicate a significant difference compared to CLA only treated group ($P<.05$).


ABCA1
ADRP
ApoE
CD36
FABP5
IFN-β
IL-1Ra
Relative mRNA expression
N.D.
IL-1 Ra
FABP
ADRP
ABCA1
IFNβ
CD36
ApoE
9E11E
TTA
GW501516
PGJ2
Wy
LPS
DMSO
MA
RA
T0901317
Rosi

worthwhile to note that 9*Z*,11*E*-, 10*E*,12*Z*-, 11*Z*,13*E*-CLA and PGJ_2 decreased ADAMTS-1 mRNA levels, but 9*E*,11*E*-CLA caused its accumulation. Catalase1 is an antioxidant enzyme that forms the first line of defence against free radical damage. The 9*E*,11*E*- and 9*Z*,11*Z*-CLA isomers as well as PGJ_2 induced mRNA levels of this gene. In support, a diet containing 3% and 5% CLA mixture increased the activity of catalase in adult male Sprague–Dawley rats [30]. In MCF-7 breast cancer cells, CLA-treated cells have a higher activity of superoxide dismutase, catalase and glutathione peroxidase [31]. Unlike these two studies using the CLA mixture, we showed only two minor CLA isomers (9*E*,11*E*- and 9*Z*,11*Z*-CLA) increased the catalase1 mRNA expression. CD36 is a scavenger receptor that recognizes oxidized low-density lipoprotein (reviewed in Ref. [32]). This scavenger receptor is present on the surface of monocytes, platelets and endothelial cells. When the macrophages are treated with PPARγ ligands, CD36 was increased, but TNF-α, gelatinase B and other inflammatory mediators were inhibited [33]. In addition, recent reports indicated that CD36 may play a role in the development of atherosclerosis [34]. 9*Z*,11*E*-, 9*Z*,11*Z*- and 11*Z*,13*E*-CLA isomers decreased CD36 mRNA levels in this present study, but 9*E*,11*E*-CLA increased its mRNA level, although the increase was not significantly different from the control. This result represents that CLA may not be an efficacious activator for PPARγ, which is supported by our own unpublished data as well as direct binding experiments [35]. 9*Z*,11*E*-, 9*Z*,11*Z*-, 11*Z*,13*E*-CLA isomers and PGJ_2 decreased CD44 (homing cellular adhesion molecule, PGP-1, Hermes antigen, and HUTCH-1) mRNA levels to approximately one third of control values. Depending on tumor differentiation, stage and size, CD44 expression is altered in colorectal cancer patients, and CD44 is associated with the tumor suppressor gene, p53 [36]. Patients who had lower levels of CD44 had a higher survival rate than patients with higher levels of CD44. Whether the decrease of CD44 mRNA levels by several of CLA isomers can play a role in preventing tumorogenesis requires more studies.

Cyclin E (cycE) is involved in cell cycle regulation and is highly related to tumorigenesis (reviewed in Ref. [37]). Strictly regulated cycE levels in normal cells are disrupted in cancer cells making it an attractive cancer therapeutic target. CLA increased tumor suppressor products such as p57, p27 and p21 protein, but decreased cyclin D1, E, Rb protein in MCF-7 cells and HCT116 cells, breast cancer and colon cancer cell line, respectively [38]. Unlike previous studies, the 10*E*,12*Z*-CLA isomer did not alter cycE levels, as was previously observed in Caco-2 cells [39]. In the present study, 9*E*,11*E*- and 9*Z*,11*Z*-CLA isomers decreased cycE mRNA levels. EskK is a protein kinase that appears in all proliferative human cells and tissues and is also involved in a regulation of cell cycle [40]. 9*E*,11*E*- and 11*Z*,13*E*-CLA as well as PGJ_2 decreased EskK (Ttk) mRNA expression. FABP5 is an intracellular lipid transport protein that is a marker for prostate cancer and psoriatic skin [41,42]; however, its regulation by fatty acids and CLA has not been established. In this study using mouse macrophages, 9*E*,11*E*-CLA increased FABP5 mRNA expression, but 9*Z*,11*E*-, 10*E*,12*Z*- and 11*Z*,13*E*-CLA isomers decreased its mRNA abundance. The mRNA levels of Lipin1, RGS1 and RhoC showed a similar pattern of regulation by CLA isomers with only the 9*E*,11*E*-CLA increasing their mRNA expression levels. Lipin1 is an important target gene for insulin resistance [43] and has an antiproliferative function in pro-B cells [44]. RGS1 (regulator of G-protein signaling) is involved in chemokine signal transduction and is a guanosine triphosphatase (GTPase)-activating protein [45]. RhoC is a Ras-related GTPases and belongs to Rho-GTPase family (i.e., RhoA, RhoB, RhoC, Rac1 and Cdc42) (reviewed in Ref. [46]). Since RhoC level is detected at the early tumor stages with potential metastasis, it has been used as a biological marker of cancer.

Anti-inflammatory effects of CLA are not conclusive. In vivo and in vitro studies from our laboratory [12] and others [18] have demonstrated anti-inflammatory effects of CLA. On the other hand, it was reported 9*Z*,11*E*- and/or 10*E*,12*Z*-CLA similarly or dissimilarly increased pro-inflammatory factors such as IL-6 and TNF-α in mice [47,48]. Thus, more studies are required to conclude the anti-inflammatory effects of CLA, especially CLA isomers existing minimal amounts in the CLA mixture and their roles in this effect, are not well understood. In the present study, we tested the five different CLA isomers in a mouse macrophage cell line; however, it has to be noted that our observation of CLA isomers in RAW cells could be over reaching to be compared with their effects in in vivo models. Among the five isomers, 9*E*,11*E*-, 9*Z*,11*Z*-, 10*E*,12*Z*- and 11*Z*,13*E*-CLA inhibited mRNA levels of LPS-induced proinflammatory cytokines IL-1α, IL-1β and IL-6. In addition, 9*E*,11*E*-CLA enhanced the anti-inflammatory cytokine IL-1Ra. In contrast to our previous study [12], TNF-α in the present study was not significantly decreased by CLA isomers in RAW cells. An explanation for this discrepancy is a difference of macrophage activator, IFN-γ vs. LPS. The anti-inflammatory cytokine IL-1Ra is an IL-1 family member which includes IL-1α, IL-1β and IL-18 [49–51]. IL-1Ra binds to IL-1 receptor with similar affinity as IL-1, but it does not induce any intracellular response (reviewed in Ref. [52]) and, hence, is a competitive antagonist of this pathway. IL-$1Ra^{-/-}$ mice

Fig. 4. Comparison of 9*E*,11*E*-CLA to PPAR, LXR and RXR agonists. RAW cells were treated with 9*E*,11*E*-CLA (200 μM) or various NR agonists [TTA (10 μM), GW051516 (100 nM), PGJ_2 (10 μM), Wy-14643 (10 μM), LPS (1 μg/ml), MA (25 μM), RA (10 μM), T0901317 (1 μM) and Rosi (10 μM)] for 24 h. Messenger RNA was isolated, and real-time PCR was performed as described in Materials and methods. Asterisks denote a significant difference ($P<.05$) compared to vehicle control. In the heat map, the red color indicates increased, black and no change, and green decreased expression of the target gene.

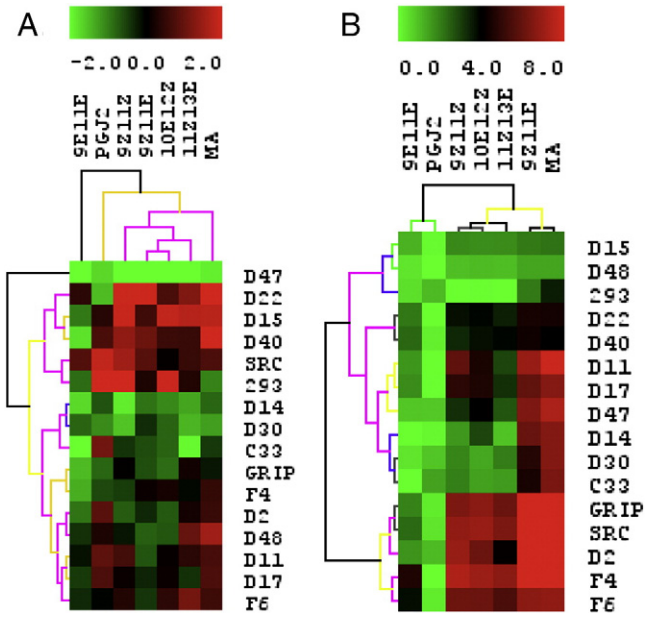


Fig. 5. CLA isomers are a SRM of PPARγ and RXRα. (A) 3T3-L1 cells were transiently transfected with PPARγ and LXXLL peptides and treated with 200 μM of CLA isomers, 10 μM of PGJ_2 and 25 μM of MA for 24 h as described in Materials and methods. The normalized luciferase activity values were transformed to Log_2 scale to generate this support tree by using TMeV. (B) pVP16 RXRα and pMLXXLL peptide library were transiently transfected in 3T3-L1 cells. The treatments were same as described above.

have increased IL-1 levels and susceptibility of various kinds of inflammatory diseases [52,53]. The regulation of anti-inflammatory effect by CLA is at least in part involved in NF-κB pathway [15]. Although IL-1Ra$^{-/-}$ mice study showed that IL-1Ra deficiency increased and prolonged nuclear translocation of NF-κB p65, further study is required to determine whether the induction of IL-1Ra by 9*E*,11*E*-CLA is regulated via the NF-κB pathway [54]. Isomer-specific effects of CLA have been shown in various studies (reviewed in Ref. [55]), although the majority of the focus is with the two main isomers, 9*Z*,11*E*- and 10*E*,12*Z*-CLA. The distinctly different regulation of 9*E*,11*E*-CLA from the rest of the CLA isomers has been observed previously. For example, a recent paper showed that 9*E*,11*E*-CLA has the strongest anti-carcinogenic effects on human colon cancer cells (Caco-2, HT-29 and DLD-1) among four different CLA isomers tested (i.e., 9*E*,11*E*-, 9*Z*,11*E*-, 9*Z*,11*Z*- and 10*E*,12*Z*-CLA) [56]. Another study showed that CLA isomers have distinctly different gene expression in human macrophages and only 9*E*,11*E*-CLA activates ABCG-1 (ATP-binding cassette transporter) via SREBP-1c [57].

To determine which NR(s) played a role in regulation of the significantly regulated genes by CLA isomers, PPAR and RXR antagonists were employed. The genes regulated by 9*E*,11*E*-CLA fall into a group regulated by either PPAR or RXR and a group regulated by neither PPAR nor RXR. The genes regulated by the rest of the CLA isomers were well distributed in four different categories, such as PPAR only, RXR only, either PPAR or RXR or neither PPAR nor RXR. Upon examination of similar of the response of 9*E*,11*E*-CLA to prototypical NR agonists, the fatty acid analogue TTA showed the most commonality. TTA is a thio-fatty acid which cannot undergo beta-oxidation. It has a proapoptosis and antiproliferation function [58,59] as well as prevention of insulin resistance [60]. TTA activates rodent PPARs with rank order PPARβ>PPARα>PPARγ [61]. One possibility for the distinct effects of 9*E*,11*E*-CLA, despite affecting the same NRs as the other CLAs, is the concept of selective receptor modulation. Each ligand-receptor combination results in a different conformation change and hence altered affinity of recruiting coactivator and/or corepressor [24]. As shown in the mammalian 2-hybrid system, CLA isomers indeed act as a SRM for RXRα or PPARγ.

In conclusion, we demonstrated that CLA isomers regulate gene expression in RAW 264.7, a mouse macrophage cell line in an isomer-specific manner. We further showed that isomer-specific anti-inflammatory effects and characterized them by the alteration of various inflammatory cytokines. Among the CLA isomers tested, 9*E*,11*E*-, 9*Z*,11*Z*-, 10*E*,12*Z*-, 11*Z*,13*E*-CLA isomers showed anti-inflammatory effects in RAW cells. Understanding the effects of the individual CLA isomers helps us to make a combination of the CLA isomers in dietary supplements to maximize its healthful effects and minimize its harmful effects. Taken together, this is the first report to show isomer-specific anti-inflammatory effects on a mouse macrophage cell line and CLA isomers have a potential to be used as a therapeutic purpose.

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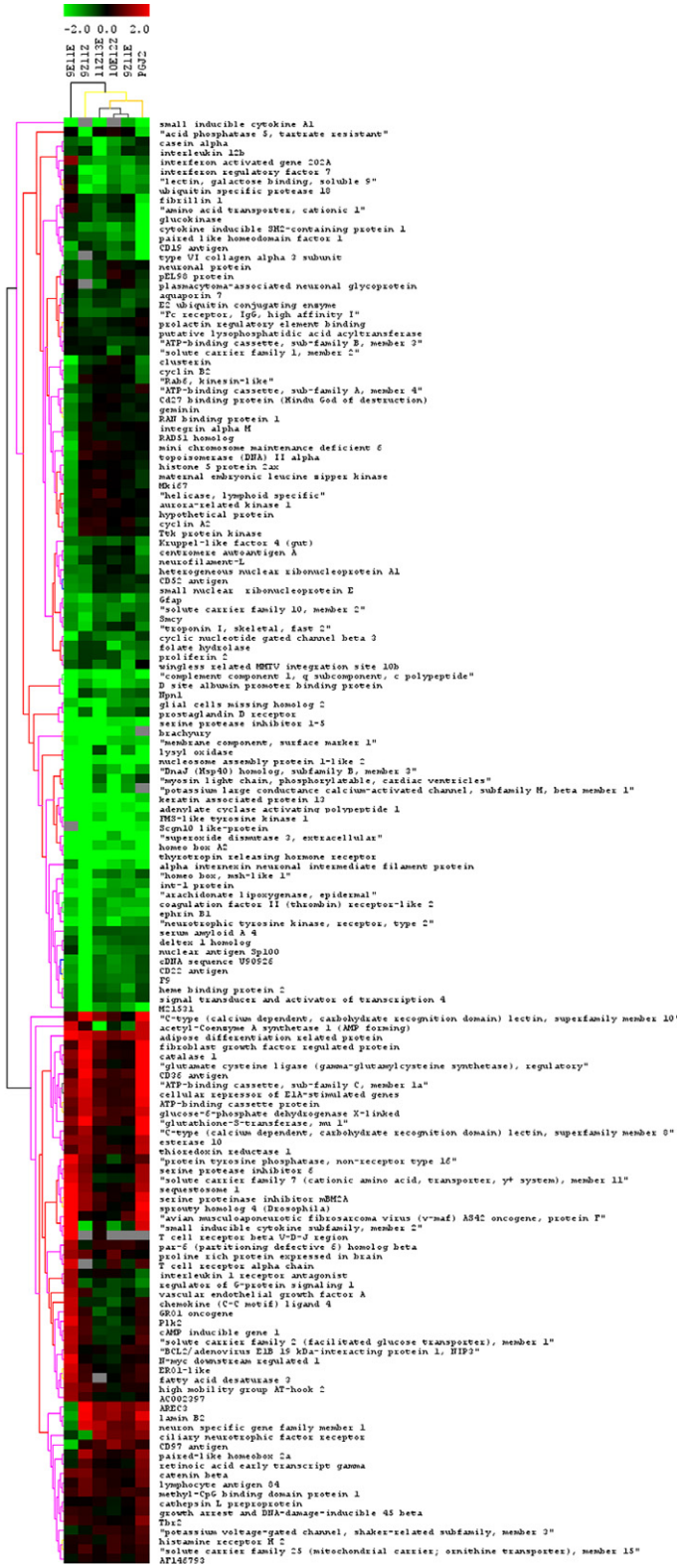
Appendix A. Supplementary data



Supplemental Figure 1. Differentially-regulated gene expression by CLA isomers in RAW 264.7, a mouse macrophage line. Significantly-regulated genes from microarray are shown as gene tree by TMeV. Color scale is from −2.0 (green) to 2.0 (red) that represent decrease and increase respectively, relative to the vehicle control.

Supplemental Table 1
List of regulated genes by CLA isomers

CLA isomers	Common Gene Name	Gene bank	9E11E	9Z11E	9Z11Z	10E12Z	11Z13E	PGJ2
Increased by 9E11E	Vascular endothelial growth factor A	M95200	2.68	0.93	1.37	0.68	0.68	0.65
	Esterase 10	AB025408	2.14	1.00	2.22	0.95	1.36	2.75
	Sequestosome 1	U40930	8.23	1.23	2.16	1.09	0.94	2.65
	T cell receptor beta V-D-J region	AF093870	4.73				1.16	
	Mouse chromosome 6 BAC-284H12	AC002397	2.34	0.86	1.49	0.94	0.92	1.07
	High mobility group AT-hook 2	X99915	2.28	1.17	1.75	0.88	1.29	1.17
	cAMP inducible gene 1	AF121080	2.70	0.77	1.50	0.74	0.72	1.42
	Proline rich protein expressed in brain	AF085348	2.33	1.07	1.72	1.71	0.99	0.98
	Avian musculoaponeurotic fibrosarcoma virus (v-maf) AS42 oncogene, protein F	AB009694	4.69	1.60	1.14	0.94	1.00	3.61
	Solute carrier family 2 (facilitated glucose transporter), member 1	M23384	2.77	0.99	1.41	0.75	0.78	1.58
	Fatty acid desaturase 3	AB041560	2.82	1.24	1.42	0.98	1.40	fatty acid desaturase 3
	par-6 (partitioning defective 6) homolog beta	AF252291	2.59	1.38	0.85	1.32	1.31	1.16
	Acetyl-Coenzyme A synthetase 1 (AMP forming)	AF216873	3.24	0.51	1.28	0.84	0.19	2.96
	Serine protease inhibitor 6	U96700	3.13	1.01	1.86	0.99	0.95	2.44
	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3	AF041054	2.59	1.35	0.97	0.85	1.08	1.31
	Interleukin 1 receptor antagonist	M64404	2.37	0.92	0.94	1.10	1.11	0.56
	Plk2	M96163	3.52	0.90	1.47	0.66	0.81	1.28
	N-myc downstream regulated 1	U60593	3.72	1.10	1.07	0.81	0.90	1.15
	GRO1 oncogene	J04596	2.87	1.00	1.30	0.79	0.57	1.36
	Solute carrier family 7 (cationic amino acid, transporter, y+ system), member 11	AB022345	3.44	0.85	2.03	0.84	0.77	2.47
	Serine proteinase inhibitor mBM2A	U96701	4.78	0.97	2.31	1.10	1.44	3.60
	Small inducible cytokine subfamily, member 2	X53798	4.34	0.77	0.31	0.42	1.16	3.26
	T cell receptor alpha chain	X72904	3.29	0.75		1.47	0.82	1.51
	Chemokine (C-C motif) ligand 4	NM_013652	2.94	0.84	0.81	0.58	0.81	1.32
	Adipose differentiation related protein	M93275	5.16	2.42	3.32	1.82	2.41	2.98
	protein tyrosine phosphatase, non-receptor type 16	X61940	3.81	1.20	2.02	0.66	1.16	1.56
	Sprouty homolog 4 (Drosophila)	NM_011898	4.53	1.13	2.28	0.97	1.37	5.54
	ERO1-like	AF144695	3.10	1.17	1.38	0.93	1.03	1.43
Decreased by 9E11E	Cyclin B2	X66032	0.38	0.89	0.83	1.05	1.03	0.52
	Rab6, kinesin-like	Y09632	0.36	1.02	1.21	1.04	1.07	0.55
	Proliferin 2	K03235	0.50	0.38	0.76	0.67	0.75	0.54
	CD97 antigen	Y18365	0.33	1.56	1.22	2.18	1.43	1.25
	Myosin light chain, phosphorylatable, cardiac ventricles	M91602	0.11	0.45	0.03	0.14	0.61	0.74
	Centromere autoantigen A	AF012709	0.40	0.99	0.55	0.88	0.91	0.42
	RAN binding protein 1	X56045	0.47	0.84	0.82	0.90	1.10	0.80
	Mki67	X82786	0.43	0.92	1.10	1.02	1.11	0.74
	Maternal embryonic leucine zipper kinase	X95351	0.40	1.05	1.10	1.02	1.29	0.74
	Neurotrophic tyrosine kinase, receptor, type 2	M33385	0.27	0.41	0.15	0.26	0.40	0.44
	CD19 antigen	NM_009844	0.37	0.71	0.82	0.51	0.66	0.18
	Hypothetical protein	AJ237585	0.41	1.00	1.08	0.99	1.16	0.95
	Neurofilament-L	M20480	0.45	0.88	0.72	0.77	0.90	0.54
	Integrin alpha M	X07640	0.49	0.98	1.12	1.08	0.87	1.05
	Histone 5 protein 2ax	X58069	0.38	0.93	1.00	1.02	0.96	0.82
	Small nuclear ribonucleoprotein E	X65704	0.44	0.77	0.48	0.81	0.88	0.68
	Topoisomerase (DNA) II alpha	D12513	0.35	0.98	1.34	1.07	1.10	0.89
	Heterogeneous nuclear ribonucleoprotein A1	D86729	0.52	0.89	0.50	0.96	0.80	0.55
	Clusterin	L05670	0.25	1.03	0.69	1.17	0.76	0.48
	ATP-binding cassette, sub-family A, member 4	AF000149	0.59	0.84	0.76	0.96	0.93	1.27
	Mini chromosome maintenance deficient 6	D86726	0.31	0.91	1.17	1.15	1.29	0.97
	Geminin	AF068780	0.49	0.81	0.83	1.02	1.05	0.92
	Aurora-related kinase 1	U69106	0.46	1.01	1.18	1.18	1.12	0.94
	Coagulation factor II (thrombin) receptor-like 2	U92972	0.37	0.38	0.14	0.35	0.48	0.38
	Helicase, lymphoid specific	U25691	0.42	0.89	1.14	1.00	1.44	1.01
	Cd27 binding protein (Hindu God of destruction)	NM_013929	0.42	0.92	0.69	1.10	1.01	0.87
	Gfap	M25937	0.37	0.35	0.30	0.57	0.53	0.50
	Ttk protein kinase	M86377	0.44	1.09	1.31	0.96	1.24	0.90
	Cyclin A2	Z26580	0.48	1.23	1.26	1.00	1.31	0.76
	Ephrin B1	Z48781	0.34	0.32	0.13	0.38	0.44	0.31

(continued on next page)

Supplemental Table 1 (*continued*)

CLA isomers	Common Gene Name	Gene bank	9E11E	9Z11E	9Z11Z	10E12Z	11Z13E	PGJ2
	Homeo box, msh-like 1	X14759	0.27	0.41	0.07	0.54	0.47	0.63
	Signal transducer and activator of transcription 4	U09351	0.50	0.52	0.18	0.62	0.66	0.71
	RAD51 homolog	D13473	0.37	0.92	1.18	0.88	0.90	0.98
	Serum amyloid A 4	U02554	0.45	0.60	0.09	0.64	0.64	0.61
Decreased by	Ephrin B1	Z48781	0.34	0.32	0.13	0.38	0.44	0.31
9Z11E	Adenylate cyclase activating polypeptide 1	AB010149	0.16	0.29	0.06	0.16	0.28	0.24
	D site albumin promoter binding protein	U29762	0.03	0.35	0.43	0.10	0.29	0.41
	Wingless related MMTV integration site 10b	U30464	0.61	0.34	0.76	0.66	0.52	1.00
	Folate hydrolase	AF026380	0.50	0.50	0.75	0.29	0.73	0.57
	Small inducible cytokine A1	M23501	0.01	0.39			0.04	0.01
	Coagulation factor II (thrombin) receptor-like 2	U92972	0.37	0.38	0.14	0.35	0.48	0.38
	Complement component 1, q subcomponent, c polypeptide	X66295	0.02	0.37	0.30	0.10	0.19	0.17
	Ubiquitin specific protease 18	NM_011909	1.40	0.40	0.25	0.55	0.33	0.50
	Int-1 protein	M11943	0.30	0.39	0.07	0.42	0.30	0.45
	Lysyl oxidase	NM_010728	0.10	0.49	0.05	0.04	0.70	0.06
	Arachidonate lipoxygenase, epidermal	X99252	0.22	0.48	0.11	0.43	0.40	0.31
	Serine protease inhibitor 1-5	M75717	0.17	0.30	0.08	0.30	0.01	0.15
	alpha internexin neuronal intermediate filament protein	L27220	0.49	0.37	0.12	0.30	0.12	0.26
	CD22 antigen	L02844	0.44	0.50	0.15	0.46	0.54	0.65
	Keratin associated protein 13	AF031485	0.18	0.18	0.06	0.17	0.13	0.34
	Nucleosome assembly protein 1-like 2	X92352	0.16	0.23	0.08	0.04	0.29	0.22
	Interferon regulatory factor 7	U73037	0.80	0.35	0.18	0.46	0.28	0.48
	Npn1	Z31360	0.04	0.56	0.71	0.40	0.50	0.63
	ATP-binding cassette, sub-family B, member 3	U60087	0.96	0.68	0.87	0.67	0.88	0.76
	Lectin, galactose binding, soluble 9	U55060	1.14	0.39	0.35	0.48	0.26	0.43
	Homeo box, msh-like 1	X14759	0.27	0.41	0.07	0.54	0.47	0.63
	Aquaporin 7	AB010100	0.98	0.61	0.63	0.79	0.82	0.67
Increased by	Methyl-CpG binding domain protein 1	AF072240	1.37	1.39	1.75	1.21	1.34	1.31
9Z11Z	Catenin beta	M90364	1.83	1.13	1.94	1.18	1.23	1.86
	Serine proteinase inhibitor mBM2A	U96701	4.78	0.97	2.31	1.10	1.44	3.60
	Cellular repressor of E1A-stimulated genes	AF084524	2.22	1.36	3.33	1.24	1.48	4.57
	AREC3	D50418	0.47	1.85	6.52	2.43	2.09	2.40
	Thioredoxin reductase 1	AB027565	1.54	1.13	2.30	1.30	1.35	2.83
	Glutathione-S-transferase, mu 1	J04632	2.16	1.12	6.34	1.40	1.47	4.08
	Catalase 1	L25069	2.14	1.14	4.52	1.19	1.66	6.64
	Glucose-6-phosphate dehydrogenase X-linked	Z11911	1.64	1.44	2.93	1.37	1.84	3.21
	ATP-binding cassette, sub-family C, member 1a	AF022908	2.04	1.28	3.46	1.30	1.34	4.28
	Fibroblast growth factor regulated protein	U04204	1.45	1.10	3.55	1.13	1.74	10.23
	Paired-like homeobox 2a	X75014	0.79	1.21	2.53	1.12	1.40	2.20
	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 10	AF240357	1.43	0.96	3.85	1.41	2.10	34.24
	CD36 antigen	L23108	1.40	1.49	4.12	1.33	1.88	5.38
	Lamin B2	X54098	0.40	1.72	3.91	2.33	2.10	3.55
	ATP-binding cassette protein	AF213389	1.78	1.29	2.93	1.27	1.55	3.78
	Ciliary neurotrophic factor receptor	AF068615	0.74	1.69	0.42	1.79	2.38	2.06
	Neuron specific gene family member 1	AF035683	0.50	1.60	3.38	1.70	2.64	2.50
Decreased by	Neuronal protein	AB031291	0.76	0.87	0.50	1.12	0.74	0.93
9Z11Z	Casein alpha	M36780	0.60	0.59	0.48	0.42	0.09	0.84
	Acid phosphatase 5, tartrate resistant	M99054	1.00	1.02	0.08	1.19	1.07	0.30
	CD52 antigen	M55561	0.46	0.74	0.51	0.84	0.87	0.83
	Superoxide dismutase 3, extracellular	U38261	0.15	0.21	0.14	0.38	0.10	0.14
	F9	M23109	0.41	0.51	0.15	0.51	0.55	0.63
	pEL98 protein	D00208	0.70	1.08	0.50	1.31	0.73	0.78
	Troponin I, skeletal, fast 2	J04992	0.45	0.88	0.31	0.82	0.66	0.47
	E2 ubiquitin conjugating enzyme	AF159230	0.86	0.55	0.56	0.76	0.75	0.73
	Nuclear antigen Sp100	U83630	0.79	0.54	0.12	0.50	0.42	0.56
	Prostaglandin D receptor	NM_008962	0.15	0.53	0.57	0.54	0.04	0.74
	Regulator of G-protein signaling 1	AF215667	2.37	0.73	0.57	0.67	0.87	0.48
	Centromere autoantigen A	AF012709	0.40	0.99	0.55	0.88	0.91	0.42
Decreased by	alpha internexin neuronal intermediate filament protein	L27220	0.49	0.37	0.12	0.30	0.12	0.26
10E12Z	Paired like homeodomain factor 1	U77946	0.74	0.49	0.61	0.40	0.61	0.21
	Brachyury	X51683	0.19	0.34	0.01	0.09	0.15	
	Superoxide dismutase 3, extracellular	U38261	0.15	0.21	0.14	0.38	0.10	0.14
	Nuclear antigen Sp100	U83630	0.79	0.54	0.12	0.50	0.42	0.56

Supplemental Table 1 (*continued*)

CLA isomers	Common Gene Name	Gene bank	9E11E	9Z11E	9Z11Z	10E12Z	11Z13E	PGJ2
	Membrane component, surface marker 1	L15429	0.28	0.36	0.01	0.33	0.09	0.33
	Glial cells missing homolog 2	D88611	0.35	0.49	0.27	0.40	0.05	0.47
	Interferon regulatory factor 7	U73037	0.80	0.35	0.18	0.46	0.28	0.48
	F9	M23109	0.41	0.51	0.15	0.51	0.55	0.63
	Smcy	AF127244	0.47	0.75	0.30	0.45	0.59	0.56
	Solute carrier family 1, member 2	AB007810	0.90	0.87	0.92	0.47	0.98	0.78
	Thyrotropin releasing hormone receptor	M94384	0.18	0.18	0.08	0.20	0.23	0.09
	Type VI collagen alpha 3 subunit	AF064749	0.46	0.53		0.47	0.84	0.22
	DnaJ (Hsp40) homolog, subfamily B, member 3	U95607	0.07	0.26	0.08	0.36	0.48	0.43
	Interleukin 12b	M86671	0.68	0.83	0.84	0.47	0.19	0.77
	Cyclic nucleotide gated channel beta 3	AJ243572	0.24	0.44	0.67	0.47	0.53	0.75
	Int-1 protein	M11943	0.30	0.39	0.07	0.42	0.30	0.45
	Interferon activated gene 202A	M31418	2.12	0.40	0.49	0.43	0.39	0.60
	Solute carrier family 10, member 2	AB002693	0.43	0.48	0.37	0.46	0.55	0.56
	Prostaglandin D receptor	NM_008962	0.15	0.53	0.57	0.54	0.04	0.74
	Heme binding protein 2	AF117614	0.50	0.39	0.18	0.55	0.61	0.69
	Neurotrophic tyrosine kinase, receptor, type 2	M33385	0.27	0.41	0.15	0.26	0.40	0.44
	Kruppel-like factor 4 (gut)	AF117109	0.56	0.76	0.58	0.56	0.64	0.47
	cDNA sequence U90926	U90926	0.39	0.51	0.17	0.45	0.56	0.66
	Scgn10 like-protein	AB007912		0.17	0.10	0.11	0.25	0.28
Decreased by 11Z13E	Potassium large conductance calcium-activated channel, subfamily M, beta member 1	AF020711	0.21	0.44	0.06	0.09	0.48	
	Plasmacytoma-associated Neuronal glycoprotein	L01991	1.08	1.11		0.95	0.49	0.72
	Cytokine inducible SH2-containing protein 1	NM_009896	0.77	0.54	0.44	0.35	0.48	0.12
	Homeo box A2	M95599	0.09	0.36	0.12	0.30	0.34	0.14
	Coagulation factor II (thrombin) receptor-like 2	U92972	0.37	0.38	0.14	0.35	0.48	0.38
	Arachidonate lipoxygenase, epidermal	X99252	0.22	0.48	0.11	0.43	0.40	0.31
	Interferon regulatory factor 7	U73037	0.80	0.35	0.18	0.46	0.28	0.48
	FMS-like tyrosine kinase 1	D88689	0.14	0.20	0.10	0.19	0.34	0.35
	Ubiquitin specific protease 18	NM_011909	1.40	0.40	0.25	0.55	0.33	0.50
	Fc receptor, IgG, high affinity I	M31314	0.78	0.67	0.55	0.61	0.56	0.98
	Adenylate cyclase activating polypeptide 1	AB010149	0.16	0.29	0.06	0.16	0.28	0.24
	Interferon activated gene 202A	M31418	2.12	0.40	0.49	0.43	0.39	0.60
	Amino acid transporter, cationic 1	M26687	1.41	0.90	0.80	0.85	0.53	0.16
	Glucokinase	L41631	0.81	0.71	0.82	0.66	0.54	0.12
	int-1 protein	M11943	0.30	0.39	0.07	0.42	0.30	0.45
	Deltex 1 homolog	U38252	0.64	0.64	0.17	0.57	0.48	0.63
	Mus musculus calbindin	M21531	0.55	0.38	0.14	0.54	0.42	0.17
	Homeo box, msh-like 1	X14759	0.27	0.41	0.07	0.54	0.47	0.63
	Fibrillin 1	L29454	0.99	1.03	0.76	0.79	0.65	0.29
Increased by PGJ2	Tbr2	AB031037	1.44	1.07	1.28	1.03	1.15	2.07
	Fibroblast growth factor regulated protein	U04204	1.45	1.10	3.55	1.13	1.74	10.23
	Growth arrest and DNA-damage-inducible 45 beta	X54149	1.32	1.00	1.25	0.88	0.93	1.58
	Retinoic acid early transcript gamma	D64162	0.83	0.92	1.61	1.03	1.11	1.98
	Prolactin regulatory element binding	AF193017	1.02	0.96	0.74	0.92	0.79	1.15
	Cathepsin L preproprotein	X06086	1.04	1.06	1.05	0.89	1.13	1.67
	ATP-binding Cassette protein	AF213389	1.78	1.29	2.93	1.27	1.55	3.78
	solute carrier family 25 (mitochondrial carrier; ornithine transporter), member 15	NM_011017	1.13	1.14	1.48	1.41	1.08	1.34
	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 8	AF061272	1.61	0.90	2.47	0.90	1.17	5.43
	Potassium voltage-gated channel, shaker-related subfamily, member 3	M30441	0.77	1.13	0.93	1.30	1.30	1.58
	Glutamate cysteine ligase (gamma-glutamylcysteine synthetase), regulatory	U95053	1.88	1.09	3.67	0.92	1.31	6.56
	Lymphocyte antigen 84	D13695	1.20	1.04	1.76	1.17	1.34	1.76
	Putative lysophosphatidic acid acyltransferase	AF015811	0.87	0.87	0.80	0.82	0.75	1.01
	Histamine receptor H 2	D50096	1.15	1.20	1.20	1.39	0.91	1.51
	Catalase 1	L25069	2.14	1.14	4.52	1.19	1.66	6.64
	Mus musculus Neuromedin U precursor (Nmu) gene	AF146793	0.91	0.92	1.21	1.20	1.08	1.29

RAW cells were starved for 24 hrs, and then treated with 200 μM of CLA isomers for 24 h. Total RNA was isolated and microarray analysis was performed as described in Materials and methods. Data represent three independent studies, and values are represented as Log_{10} scale. *P* value less than .05 was considered as a significant difference.

Supplemental Table 2
Summary of effects of chemical antagonists of PPAR and RXR on gene expression by CLA isomers.

	9*E*,11*E*-CLA	9*Z*,11*E*-CLA	9*Z*,11*Z*-CLA	10*E*,12*Z*-CLA	11*Z*,13*E*-CLA	PGJ_2
PPAR only		IL-1Ra	CycE	CD36	CycE	ADAMTS1
		RGS1	Lipin1	Usp18	IL-1Ra	CD36
		Usp18	Usp18		Usp18	EskK
RXR only		Catalase1	ADAMTS1	Catalase1	ADAMTS1	CycE
		EskK	FABP5	Lipin1	Lipin1	
		RhoC	IL-1Ra			
Either PPAR or RXR	Catalase1	ADAMTS1	Catalase1	ADAMTS1	CD36	Catalase1
	CD36	CD36	CD36	RGS1	CD44	CD44
	CycE	CD44	CD44	RhoC	EskK	FABP5
	EskK (Ttk)	Lipin1	RGS1		RGS1	Lipin1
	FABP5				RhoC	RGS1
	IL-1Ra					Usp18
	Lipin1					
	RGS1					
	RhoC					
Neither PPAR nor RXR	ADAMTS1	CycE	EskK	CD44	Catalase1	IL-1Ra
	CD44	FABP5	RhoC	CycE	FABP5	RhoC
	Usp18			EskK		
				FABP5		
				IL-1Ra		

Summary of the involved nuclear receptor(s) for each CLA isomers in the verified regulated genes in RAW 264.7. Data in Fig. 3 were categorized in four groups: PPAR only, RXR only, either PPAR or RXR, or neither PPAR nor RXR to show the complexity of the regulation of CLA isomers in mouse macrophages.