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Conjugated linoleic acid suppresses IRF3 activation via modulation of CD14☆

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Abstract

Polyunsaturated fatty acids (PUFA) can modulate the immune response, however the mechanism by which they exert this effect remains unclear. Previous studies have clearly demonstrated that the *cis-9, trans-11* isomer of conjugated linoleic acid (c9,t11-CLA), found predominantly in beef and dairy products, can modulate the response of immune cells to the toll-like receptor (TLR) 4 ligand, lipopolysaccharide (LPS). This study aimed to investigate further the mechanism by which these effects are mediated. Treatment of macrophages with c9,t11-CLA significantly decreased CD14 expression and partially blocked its association with lipid rafts following stimulation with LPS. Furthermore the c9,t11-CLA isomer inhibited both nuclear factor-κB (NF-κB) and IRF3 activation following TLR4 ligation while eicosapentaenoic acid (EPA) only suppressed NF-κB activation. Given that the ability of LPS to activate IRF3 downstream of TLR4 depends on internalisation of the TLR4 complex and involves CD14, we examined TLR4 endocytosis. Indeed the internalisation of TLR4 to early endosomes following activation with LPS was markedly inhibited in c9,t11-CLA treated cells. These effects were not seen with the n-3 fatty acid, EPA, which was used as a comparison. Our data demonstrates that c9,t11-CLA inhibits IRF3 activation via its effects on CD14 expression and localisation. This results in a decrease in the endocytosis of TLR4 which is necessary for IRF3 activation, revealing a novel mechanism by which this PUFA exerts its anti-inflammatory effects.

Keywords: Conjugated linoleic acid; IRF3; NFkappaB; CD14

1. Introduction

It is well established that nutrition plays an important role in health and that dietary fatty acids are essential components of the diet [1]. These include polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), commonly found in high fat fish and marine mammals [2], which have been shown to have beneficial effects in immune-mediated diseases such as rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus and asthma [3–5]. Recent studies indicate strong immunomodulatory potential for another dietary fatty acid, conjugated linoleic acid (CLA) [6]. CLAs are a group of positional and stereoisomers of conjugated dienoic derivatives of linoleic acid and the *cis*-9,*trans*-11 CLA isomer (c9,t11-CLA) is a naturally occurring dietary form of CLA, predominantly found in foods derived from ruminants such as meat and dairy [7]. c9,t11-CLA has been shown to suppress proinflammatory cytokine production in a number of cell types. Indeed, we previously reported that c9,t11-CLA modulates the activation of dendritic cells characterised by a decrease in production of interleukin (IL)-12 and an increase in IL-10 [8]. Furthermore, studies in animal models have demonstrated that feeding CLA has beneficial effects in endotoxin-induced anorexia and on mucosal damage in experimental colitis [9,10].

While the beneficial effects of PUFA are clear, the exact mechanisms through which they exert such effects have still to be elucidated. Numerous studies focus on the cumulative effects of PUFA on eicosanoid production, gene expression and transcription factor activation [11–13]. PUFA are known to modulate activation of nuclear factor-κB (NF-κB) [13] and peroxisome proliferator activated receptors, PPARs [14]. Specifically, EPA and DHA have been shown to suppress NF-κB activation in LPS-stimulated macrophages [15,16]. This suppressive effect on NF-κB in PUFA-treated cells results in decreased secretion of pro-inflammatory cytokines [17–19], which may partly explain their overall anti-inflammatory actions. Babcock et al have demonstrated that EPA suppresses tumor necrosis factor α (TNFα) production in murine macrophages [20], and we have previously reported that c9,t11-CLA suppressed NF-κB in adipose tissue from mice fed on diets of CLA with a concomitant suppression of TNFα [21]. In addition, studies employing human macrophage models report that EPA and DHA inhibit TNFα, IL-6 and IL-1β production [22,23]. We have also demonstrated the suppressive effects of EPA and DHA on NF-κB and subsequent TNFα, IL-6 and IL-1β production in human macrophage [24,25].

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A study showing that DHA fails to inhibit NF-κB activation in the presence of dominant negatives mutants and constitutively active components of upstream signalling, MyD88 and AKT [26] suggests that PUFA may exert their effects at the cell membrane. Indeed, PUFA greatly influence membrane composition, size and distribution [27–29]. Significantly, there is a role for membrane microdomains or "lipid rafts" in immune cell signalling [30,31] and the impact of PUFA on these domains is well reported [32–34]. T-cell activation has been linked with the specific recruitment of signalling complexes to lipid rafts [35]. Changes to the fatty acid composition of these rafts is associated with a decrease in the translocation of PKC to lipid rafts, a key molecule regulating $CD4^+$ T-cell activation [27,36]. Importantly, toll-like receptor (TLR) 2 [37], TLR4 and its associated molecule CD14 [38] are recruited to lipid rafts following stimulation with their respective ligands.

Activation of TLR4 initiates downstream signalling which in turn activates NF-κB and IRF3 via MyD88 dependant and independent pathways, respectively [39,40]. Activation of the MyD88 dependant pathway is mediated at the plasma membrane, while induction of IRF via the MyD88-independent pathway is dependant on the endocytosis of TLR4, a process requiring CD14 [41,42] .

In the present study, we test the hypothesis that c9,t11-CLA may exert its anti-inflammatory effects partly at the plasma membrane by influencing CD14. We demonstrate that c9,t11-CLA modulates CD14 by altering its localisation on the membrane and association to lipid rafts in response to LPS, which was not found to be the case with EPA which was used for comparison. CLA treatment also decreased the internalisation of TLR4 and its localisation to early endosomes in LPS stimulated cells, an event known to be dependent on CD14. Subsequently it suppressed IRF3 activation which could be reversed following overexpression of CD14. These findings provide greater insight into the anti-inflammatory mechanisms used by the c9,t11- CLA isomer while also highlighting a selective inhibition of the inflammatory transcription factor IRF3.

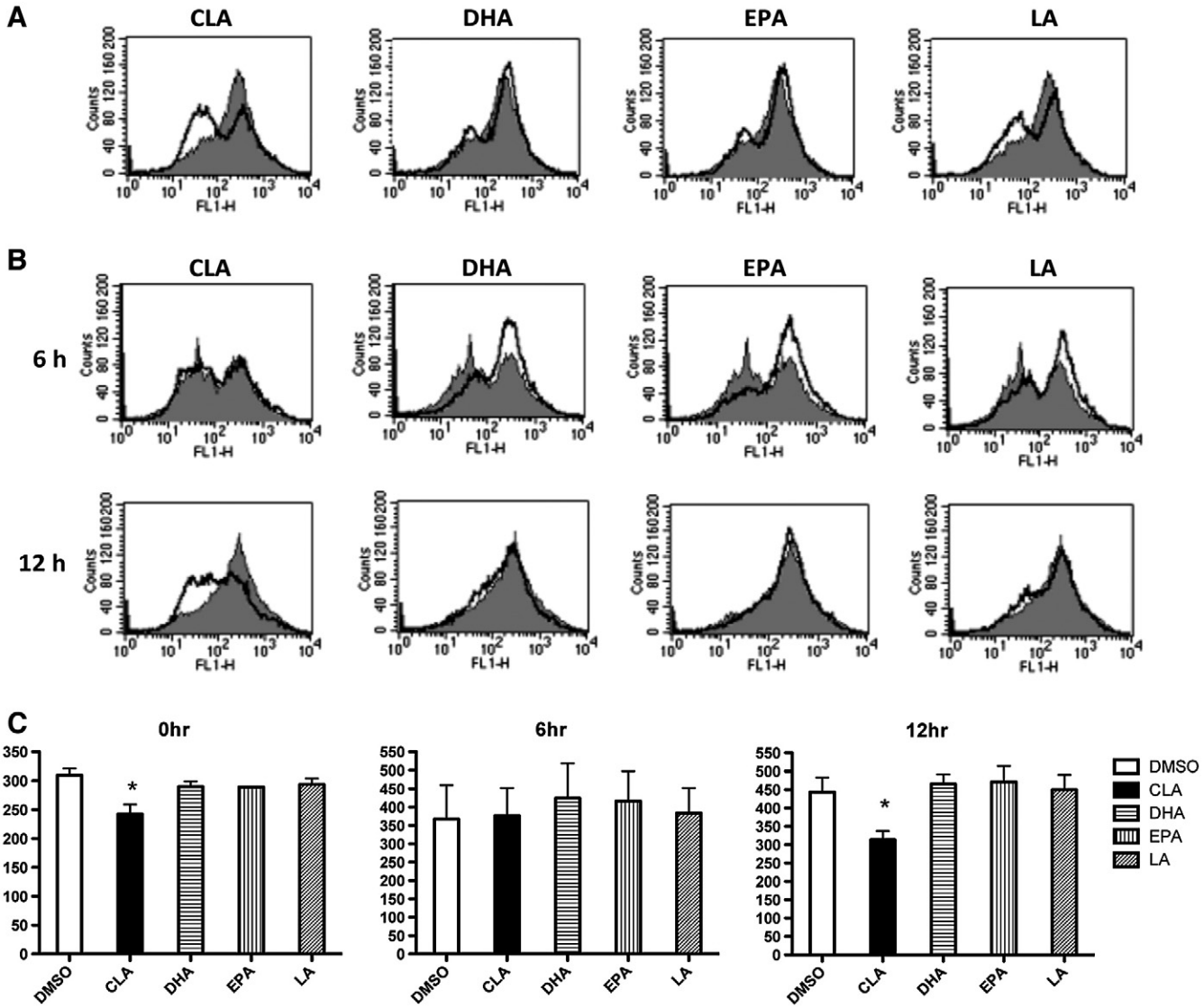

Fig. 1. PUFA modulate the surface expression of CD14 on LPS-stimulated J774 macrophage. Cells were cultured for 7 days in DMSO (vehicle control), c9,t11-CLA (50 μM), DHA (25 μM), EPA (25 μM) or LA (50 μM)and then assessed for surface levels of CD14 using flow cytometry. Expression of CD14 in DMSO (grey filled histogram) or c9,t11-CLA,DHA, EPA or LA treated cells (black line) in resting cells (A) and cells stimulated with LPS (100ng/ml) for 6 h and 12h (B) is shown. Histograms shown are representative of three experiments. Statistical analysis was carried out using mean fluorescence intensity values. Values are MFI values (±S.E.M.). *P<.05, ANOVA, comparing all groups.

2. Materials and methods

2.1. Plasmids and reagents

c9,t11-CLA was purchased from Alexis Chemicals (Cayman Chemicals). Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and lauric acid (LA) were all purchased from Sigma-Aldrich. Escherichia coli LPS (serotype R515) was purchased from Alexis Biochemicals. The c9,t11-CLA isomer, EPA, DHA and LA were dissolved in sterile DMSO (Sigma-Aldrich) and stored at −20°C in the dark. The interferon stimulated response element (ISRE) and NF-κB luciferase plasmids were purchased from Clontech and the pcDNA3.1 empty vector was from Invitrogen.

2.2. Cell culture and transient transfection

J774A.1 were purchased from the ECACC and maintained in RPMI supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution (v/v). HEK293Tand HEK-293TLR4-CD14-MD-2 were grown in DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution (v/v) with the addition of 50 μg/ml Hygrogold (Invitrogen) and 1 μg/ml Blasticidin (Invitrogen) to maintain expression of TLR4, CD14 and MD-2. U373 astrocytoma cells and those stably transfected with CD14 (U373-CD14) were a kind gift from Katherine Fitzgerald (University of Massachusetts Medical School, Worcester, MA, USA) and were grown in complete DMEM with the addition of 250 μg/ml neomycin analog G418 to maintain CD14 expression. For luciferase assays, cell lines were seeded in 24-well plates and incubated overnight prior to transfection using geneJuice transfection reagent (Novagen, Madison, WI, USA).

2.3. Western Blotting

Pre-stained protein molecular weight marker (Bio-Rad laboratories) and protein samples were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto nitrocellulose membranes. Membranes were blocked in 5% (w/v) nonfat dried milk in TBS-T and incubated overnight at 4°C with either anti-CD14 (AbCam), anti-FL-1 (BD), or anti-β-actin (Sigma) antibodies. Membranes were washed and incubated for 2 h at room temperature with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Sigma-Aldrich) before being developed by enhanced chemiluminescence (Cell Signaling Technology).

2.4. Flow cytometry

J774 macrophages were treated with PUFA (as indicated in the figure legends) and left resting or stimulated with LPS (100 ng/ml) for 6 h and 12 h. Cells were slightly agitated into suspension and the surface expression of CD14 was assessed using an anti-mouse CD14-FITC (BD) and appropriately labelled isotype-matched antibody control (data not shown for isotype). Immunofluorescence analysis was performed on a BD FACSCalibur using CellQuest software.

2.5. Membrane fractionation

Macrophages were treated with PUFA (as indicated in the figure legends), seeded at 2×10^5 cell/ml in a six-well plate and stimulated with LPS (100 ng/ml) for 0 and 2 h. Following stimulation cells were resuspended in 300 μl membrane fractionation buffer (MFB; 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 250 μM sucrose, 200 μM PMSF) containing protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 100 μM sodium orthovanidate and 0.1 mM PMSF). Cells were lysed with 50 strokes of a dounce homogenizer (Sigma) and spun in thick wall polycarbonate Beckman tubes at 425,000 *g* for 1 h at 4°C. The resulting supernatant (cytosolic fraction) was removed to a fresh tube, and the pellet (membrane fraction) was resuspended in 60 μl of sample buffer [125 mM Tris, 1M Tris HCl pH 6.8, 10% glycerol, 2% SDS, 0.05 % (w/v) bromophenol blue and 0.25 M dithiothreitol]. Protein within the cytosolic fraction was concentrated by MeOH/chloroform precipitation and resuspended in 60 μl sample buffer. Equal volumes of samples were run on 10 % (v/v) SDS-PAGE gels.

2.6. Localisation of CD14 by confocal microscopy

J774 cells were grown in c9,t11-CLA (50 μM) or DMSO (vehicle control) for 7 days. Cells were then plated on cover slips (1×10^5 cells/ml) and left to rest overnight. The following day cells were left unstimulated or treated with LPS (100 ng/ml) as indicated. Cells were incubated with a CellMask plasma membrane stain (Deep Red C10046, Molecular Probes, Invitrogen) at 2.5 μg/ml for 3 min at 37°C. After membrane staining, cells were washed with cold phosphate-buffered saline (PBS) solution three times and fixed with warm 3.75% formaldehyde in PBS at 37°C for 10 min. Samples were washed 3×5 min in PBS-baths, cover slips were incubated with

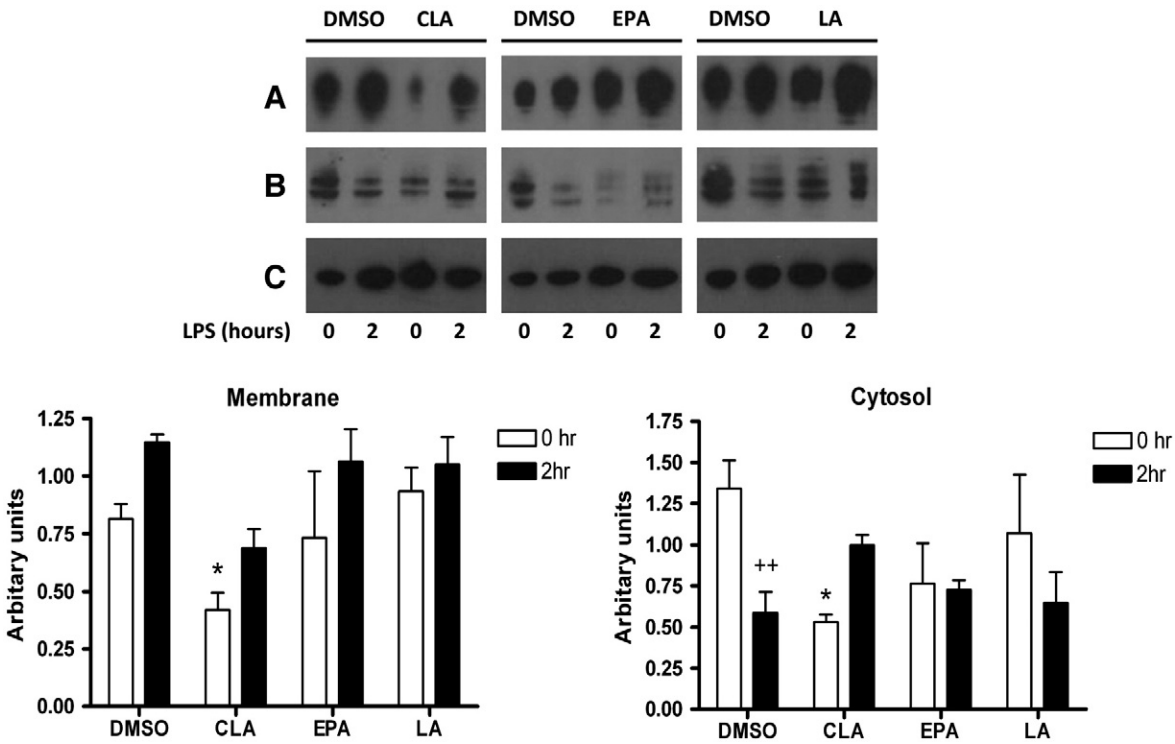

Fig. 2. c9,t11-CLA modulates membrane and cytosolic CD14 in J774 macrophages. Cells were cultured for 7 days with either DMSO (vehicle control), c9,t11-CLA (50 μM), EPA (25 μM) or LA (50 μM). Cells were plated at 2×10^5 cell/ml, left to rest overnight and left unstimulated or stimulated with LPS (100 ng/ml) for 2 h. Cell lysates were harvested and membrane fractionation performed. Western blotting was used to determine the expression of CD14 in the cytosolic and membrane fractions of cells treated with DMSO, c9,t11-CLA, EPA and LA. Total cellular levels of β-actin were used as a loading control. Immunoblots shown are representative of 3 experiments. Densitometric analysis was carried out on immunoblots and CD14 is expressed as arbitrary units (±S.E.M.) after normalising to β-actin. $^{*}P<.05$, ANOVA, comparing DMSO vs. PUFA-treated groups at 0 h, $^{++}P<.01$, ANOVA, comparing DMSO vs. PUFA-treated groups at 2 h.

100 mM glycine PBS-1.2% fish gelatin blocking buffer and incubated with a rabbit polyclonal anti-CD14 (M305, Santa Cruz) over night at 4°C. The next day, cell preparations were washed and incubated with an AlexaFluor 488 donkey anti-rabbit secondary antibody (Molecular Probes, Invitrogen) for 1 h at 37°C. Finally, cover slips were washed and mounted on slides with antifade medium (Dako). Slide preparations were observed using a Zeiss Axio Observer. Z1 equipped with a Zeiss 710 and ConfoCor 3 laser scanning confocal head (Carl Zeiss, Germany). Images were analysed using ZEN 2008 software.

2.7. Lipid Raft Isolation

For the isolation of lipid rafts $1\times10^7$of DMSO or PUFA-treated cells were stimulated with 100 ng/ml LPS for 30 min prior to membrane preparation. Cells were then washed, pelleted and resuspended in 2 ml membrane extraction buffer (MEB; 20 mM MES, 150 Mm NaCl, pH 6.5) containing 5 mM iodoacetamide, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin and left on ice for 30 min. For mechanical cell disruption, lysates were freeze-thawed in liquid nitrogen three times followed by 40 strokes with a dounce homogeniser and passage through a 25G 1-in. syringe (BD Microlance). Lysates were spun at 100,000 g for 1 h at 4°C. The membrane pellet was resuspended in 500 μl MEB containing 0.5 % (v/v) Triton X-`100. For the isolation of lipid rafts the membrane preparation was mixed with an equal volume (500 μl) of 90 % (w/v) sucrose/MEB (with protease inhibitors as described) and applied to a discontinuous sucrose gradient (30% (w/v) sucrose/MEB, 5% (w/v) sucrose/MEB). Samples were spun for 18 h at 175,000 g in a TH-641 swinging bucket rotor in a Sorvall WX ultracentrifuge. After centrifugation 1 ml fractions were carefully collected from the top of the gradient and designated fractions 1–11. A light refracting band (typically in fractions 5–6) represented fractions enriched for lipid rafts. A flotillin-1 antibody (BD) was used to confirm this.

2.8. Luciferase assays

For ISRE and NF-κB luciferase assays, 75 ng of ISRE/NF-κB luciferase plasmid, 30 ng of *Renilla* luciferase, and empty pcDNA3.1 vector made up to a total of 220 ng of DNA were transfected into each well. Empty pcDNA3.1 vector alone was also transfected into cells and as expected gave no detectable response. Cells were left to rest overnight and then stimulated with LPS (100 ng/ml) as indicated in the figure legends. After stimulation cells were lysed in 100 μl of passive lysis buffer (Promega, Southampton, UK) for 15 min. Firefly luciferase activity was assayed by the addition of 40 μl of luciferase assay mix (20 mm Tricine, 1.07 mm $(MgCO_3)_4Mg(OH)_2\cdot5H_2O$, 2.67 $MgSO_4$, 0.1 m EDTA, 33.3 mm dithiothreitol, 270 mm coenzyme A, 470 mm luciferin, 530 mm ATP) to 20 μl of the lysed sample. *Renilla* luciferase was read by the addition of 40 μl of a 1:1000 dilution of Coelentrazine (Argus Fine Chemicals) in phosphate-buffered saline. Luminescence was read using the Reporter microplate luminometer (Turner Designs). The *Renilla* luciferase plasmid was used to normalize for transfection efficiency in all experiments.

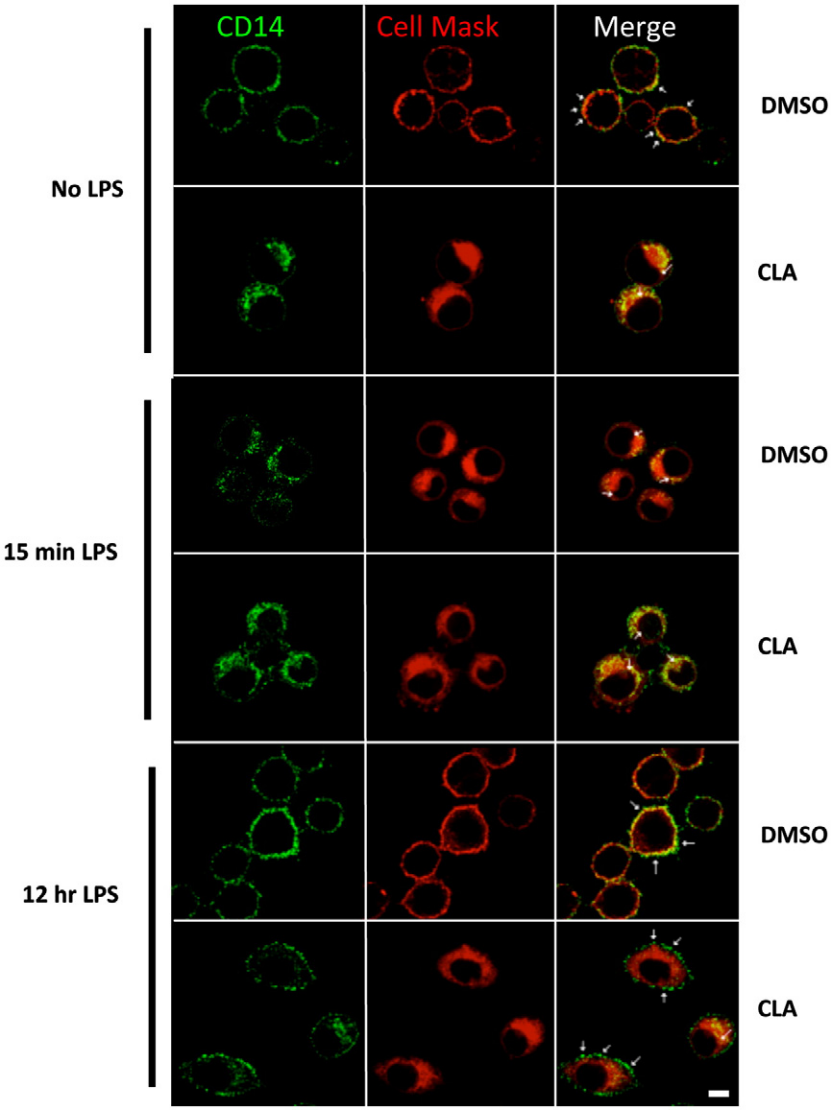

Fig. 3. Localisation of CD14 in J774 macrophages pre-treated with c9,t11-CLAboth prior to and following stimulation with LPS was examined by confocal microscopy. Cells were grown in DMSO (vehicle control) or c9,t11-CLA (50 μM) for 7 days and then stimulated with LPS (100 ng/ml) for the time indicated. The plasma membrane was stained using CellMask (Invitrogen), red, and CD14 with anti-CD14 antibody (Santa Cruz), green. Arrows indicate CD14 localising on the membrane or into the cytoplasm. Bar represents 10 μm. Results shown are representative of three experiments.

2.9. Endocytosis of TLR4 by confocal microscopy

PUFA treated HEK293-TLR4-MD-2-CD14 cells were plated on poly-L-lysine-treated glass cover slips and left to rest overnight. For the investigation of TLR4 and EEA1 localisation, TLR4-YFP and EEA1-CFP constructs were kind gifts from Douglas Gollenbock (University of Massachusetts Medical School, Worcester, MA, USA) and Terje Espeviks (Norwegian University of Science and Technology, Trondheim, Norway), respectively. Cells were transfected with TLR4-YFP (0.75 μg) and EEA1-CFP (0.25 μg) using geneJuice according to manufacturer's instructions. Cells were left to rest for 24 h before stimulation with 250 ng/ml LPS for 7.5 and 15 min. Cell preparations were washed, fixed and mounted to glass coverslips and analysed using an Olympus FluoView FV1000 and FV1000 Viewer Software version 1.7.

2.10. Statistics

One-way analysis of variance (ANOVA) was used to determine significant differences between conditions. When this indicated significance ($P<.05$), post hoc Student-Newmann-Keul test analysis was used to determine which conditions were significantly different from each other.

3. Results

3.1. c9,t11-CLA modulates CD14 expression in macrophages

The ligation of LPS with TLR4 activates downstream signalling pathways that initiate inflammatory responses and CD14 is important in this process. Therefore, we examined whether the c9,t11-CLA isomer could modulate the expression of CD14 using flow cytometry. We examined the surface expression of CD14 following LPS stimulation of J774 macrophages pre-treated with either c9,t11-CLA, EPA or DHA. c9,t11-CLA suppressed the surface expression of CD14 in both resting macrophages (Fig. 1A) and in LPS-stimulated macrophages (Fig. 1B). This suppressive effect was

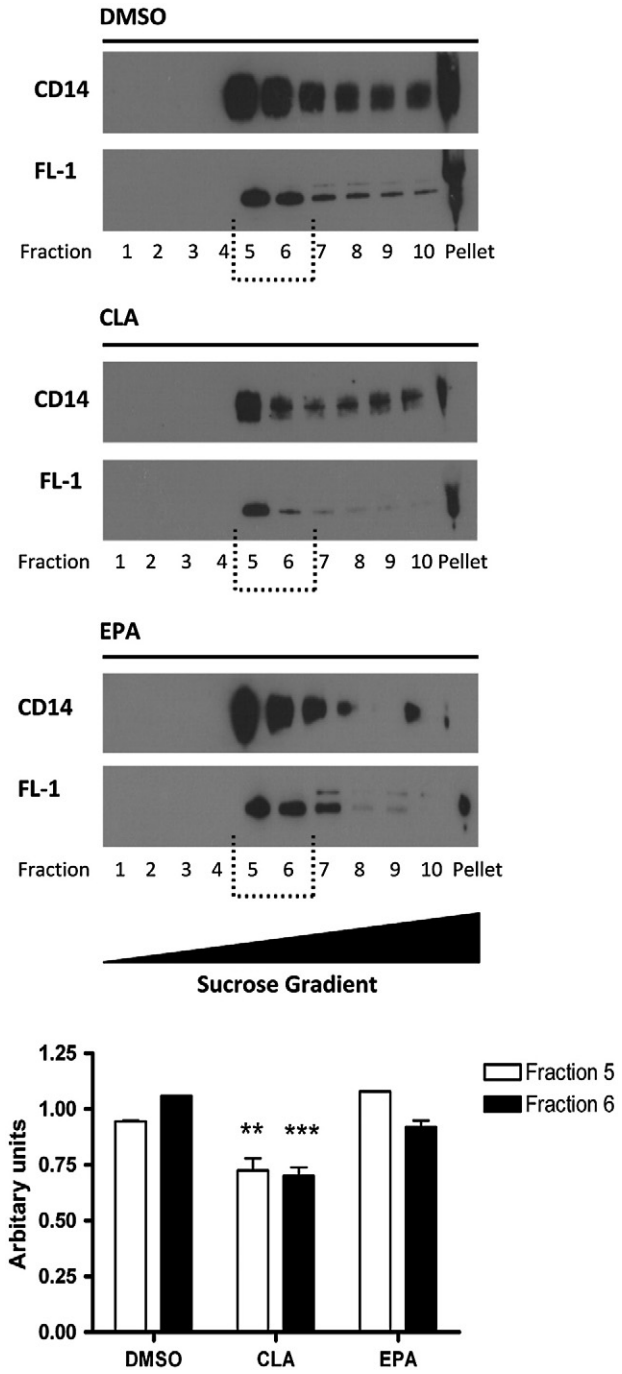

Fig. 4. c9,t11-CLA decreases expression of CD14 in lipid rafts in J774 macrophage following stimulation with LPS. Cells were cultured for 7 days with either DMSO (vehicle control), c9,t11-CLA (50 μM) or EPA (25 μM). 1×10^7 cells were stimulated with LPS (100ng/ml) for 30 min. Cells were then lysed with buffer containing 0.5% Triton X-100 and layered on the bottom of a sucrose gradient. Following centrifugation 1 ml fractions were collected and analysed by SDS-PAGE. Lipid raft fractions were identified by localisation of constitutive raft protein flotilin-1 (FL-1) in fractions 5 and 6. Similarly, fractions were analysed for CD14 content by SDS-PAGE using a CD14 specific antibody (AbCam). Immunoblots shown are representative of 3 experiments. Densitometric analysis was carried out on immunoblots and CD14 is expressed as arbitrary units (±S.E.M.) after normalising to FL-1. ** $P<.01$, *** $P<.001$, determined by one-way ANOVA test comparing all groups.

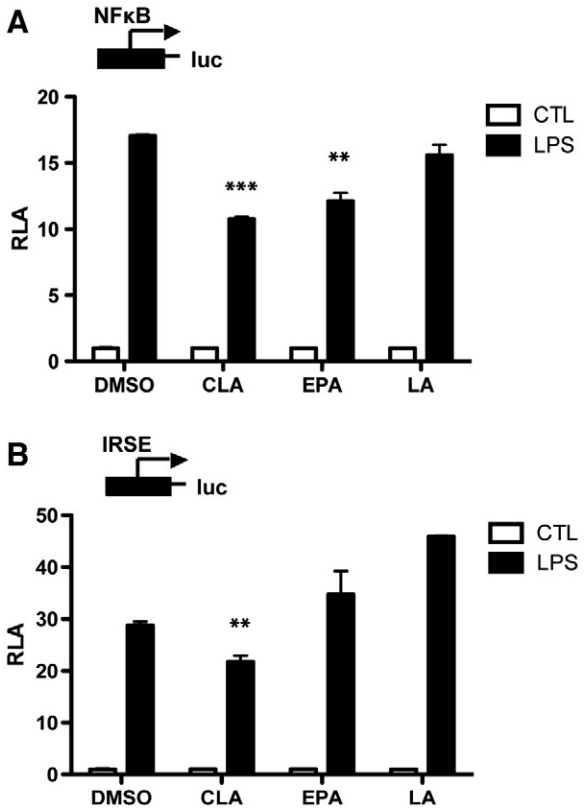

Fig. 5. c9,t11-CLA suppresses NF-κB and IRF3. HEK-TLR4-CD14-MD-2 cells were cultured for 7 days with either DMSO (vehicle control), c9,t11-CLA (50 μM),EPA (25 μM) or LA (50 μM). Subsequently, cells were transiently transfected with either an NF-κB (A) or ISRE (B) luciferase reporter plasmid. Induction of both transcription factors was assessed following 6 hr stimulation with LPS (100 ng/ml). Results are expressed as ± SEM for triplicate determinants and are representative of 3 experiments. **$P<.01$; ***$P<.001$; determined by one-way ANOVA test comparing all groups.

not evident in either DHA or EPA-treated cells, indeed they slightly increased CD14 expression following activation of macrophages with LPS.

3.2. c9,t11-CLA modulates CD14 localisation following stimulation with LPS

In order to confirm the effect of c9,t11-CLA on membrane expression of CD14 we isolated both the membrane and cytosolic fractions of J774 macrophages treated with either c9,t11-CLA, EPA or LA and used western blotting to determine the levels of CD14 on the membrane and in the cytosol at early points after LPS stimulation. We also used confocal microscopy to further establish the location of CD14. Exposure of DMSO control cells to LPS increased CD14 levels in membrane fractions which was concomitant with a decrease in cytosolic CD14 (Fig. 2A). Pre-treatment of cells with c9,t11-CLA resulted in a decreased level of membrane CD14 in both resting cells and 2 h post stimulation with LPS (Fig. 2A). In contrast to DMSO, the c9,t11-CLA-treated cells showed an increase in cytosolic CD14 after LPS stimulation. Neither EPA nor LA suppressed membrane levels of CD14 (Fig. 2B and C) when normalised to beta-actin levels. Indeed the levels of membrane CD14 are increased by EPA, which concurs with the flow cytometry data. Furthermore, investigation by confocal microscopy using double-staining of DMSO or c9,t11-CLA treated cells with anti-CD14 and the plasma membrane marker, CellMask, demonstrated that the cellular trafficking of CD14 in the presence of c9, t11-CLA was altered. Fig. 3 shows that in resting DMSO-treated cells CD14 clearly localises at the plasma membrane. In contrast, c9,t11-CLA-treated cells had significantly reduced cell surface expression of CD14 and it was primarily confined to the cytoplasm. Following 15 min of stimulation with LPS, CD14 leaves the membrane and moves into the cytoplasm in DMSO-treated cells. This movement is not obvious in the c9,t11-CLA-treated cells due to the low levels of CD14 on the membrane in these cells. After a longer exposure to LPS (12 h) CD14 re-circulated to the cell surface in DMSO-treated cells which was reduced in c9,t11-CLA-treated cells.

3.3. c9-t11- CLA decreases the expression of CD14 in lipid rafts

The recruitment of CD14 to lipid rafts following activation of cells with LPS is an important event [38,43]. Our data clearly demonstrates that c9,t11-CLA suppresses expression of CD14 at the membrane; therefore, we examined whether c9,t11-CLA altered the expression of CD14 in lipid rafts following LPS stimulation. We used EPA as a comparison given that it did not suppress CD14 expression. Anti-FL-1 was used as a marker for lipid rafts. Fractions 4–6 were designated to be lipid raft fractions with the expression of FL-1 predominant in these samples. Fig. 4 demonstrates the presence of CD14 in raft fractions following LPS stimulation of DMSO-treated cells. The levels of CD14 in these lipid raft fractions was decreased in c9,t11-CLA-treated cells which was not seen with EPA-treated cell.

3.4. c9,t11-CLA selectively inhibits IRF3 activation downstream of TLR4

Given the role of CD14 in endocytosis of TLR4 and subsequent signalling to IRF3, we next examined whether c9,t11-CLA could modulate activation of IRF3. It is well established that dietary fatty acids can suppress NF-κB; therefore, we also examined the effect of c9,t11-CLA on this transcription factor. EPA was again used as a comparison as it does not suppress CD14 expression. Treatment of HEK-TLR4-MD2-CD14 cells with EPA and c9,t11-CLA resulted in suppression of NF-κB following LPS stimulation ($P<.01$; $P<.001$; Fig. 5A). Furthermore, treatment with c9,t11-CLA suppressed LPS-induced IRF3 activation ($P<.01$; Fig. 5B) which was indicated by its effect on the ISRE plasmid. In contrast, treatment of cells with EPA had no effect on IRF3 activation.

3.5. Overexpression of CD14 reverses the inhibitory effect of c9,t11-CLA on IRF3 activation in response to LPS

Our data demonstrated that c9,t11-CLA selectively suppressed the surface expression of CD14 and subsequently inhibited activation of IRF3. CD14 is necessary for endocytosis of the TLR4 complex and subsequent activation of IRF3 [41,42]. Therefore, in order to determine whether the effect of c9,t11-CLA on CD14 expression could be responsible for its effect on IRF3 activation, we employed U373 cell lines, one of which stably overexpressed CD14. Cells were exposed to c9,t11-CLA and then LPS-induced IRF3 activation was measured. c9, t11-CLA suppressed activation of IRF3 in U373 cells (Fig. 6A; $P<.001$); however, this inhibitory effect was abrogated in U373 stably over expressing CD14 (Fig. 6B).

3.6. c9,t11-CLA decreases endocytosis of TLR4 following stimulation with LPS

Given the requirement of CD14 for endocytosis of the TLR4 complex and subsequent activation of IRF3 [41,42] we assessed whether CLA could alter endocytosis of the TLR4 complex following LPS stimulation. Again we compared this to EPA, one of the PUFA which did not have a suppressive effect on either CD14 or IRF3 activation. PUFA treated HEK-TLR4-MD2-CD14 cells were transiently transfected with TLR4-YFP and EEA1-CFP (early endosomal antigen) constructs. Localisation of TLR4 with EEA1 was examined following stimulation with LPS for 7.5 and 15 min. As expected, following 7.5 min

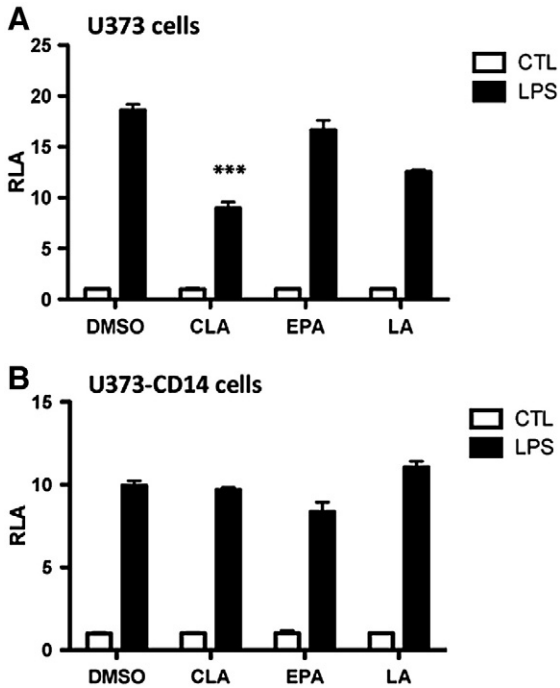

Fig. 6. The suppressive effect of c9,t11-CLA on IRF3 activation is reversed by overexpression of CD14. U373 cells (A) and U373-CD14 cells (B) were cultured for 7 days with either DMSO (vehicle control), c9,t11-CLA (50 μM), EPA (25 μM) or LA (50 μM). Cells were then transiently transfected with an ISRE luciferase reporter plasmid and the induction of IRF3 was assessed 6 h after stimulation with LPS (100 ng/ml). Results are expressed as ±SEM for triplicate determinants and are representative of 3 experiments. ***$P<.001$; determined by one-way ANOVA test comparing all groups.

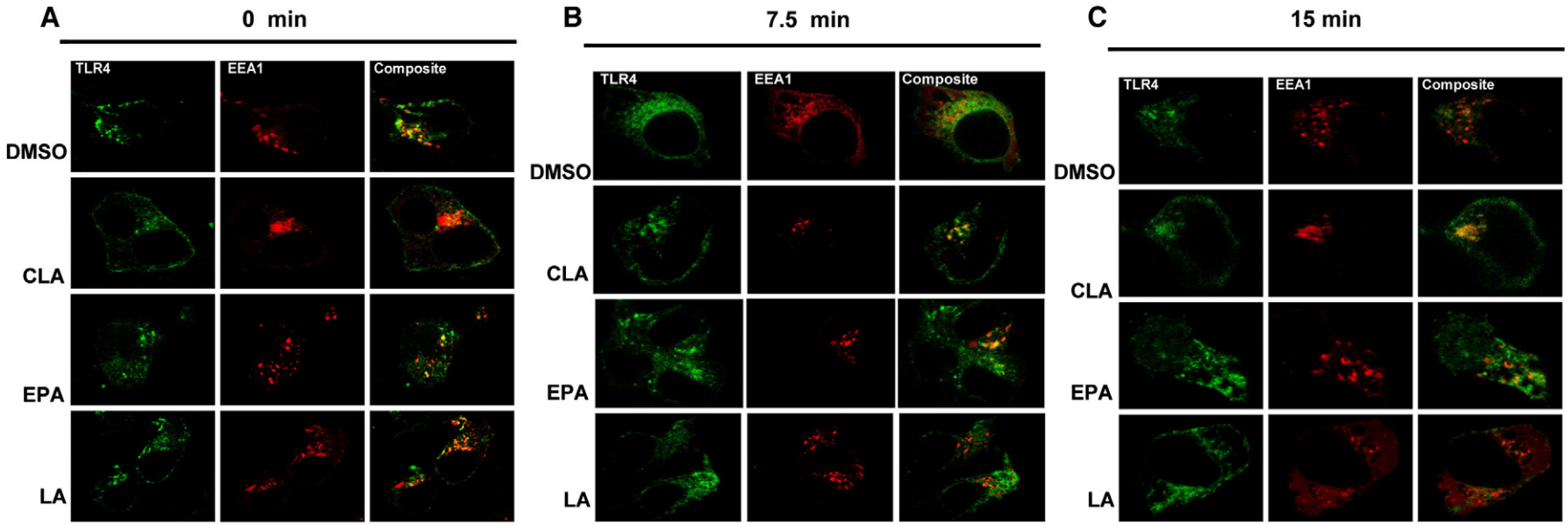

Fig. 7. c9,t11-CLA decreases endocytosis of TLR4 following LPS stimulation. HEK-TLR4-MD-2-CD14 cells were cultured for 7 days in either DMSO (vehicle control), c9,t11-CLA (50 μM) (EPA (25 μM) or LA (50 μM). Subsequently, cells were transiently transfected with TLR4-YFP (green) and EEA1-CFP (red) plasmids. Endocytosis of TLR4 and its localisation relevant to early endosomal marker EEA1 was assessed using confocal microscopy following stimulation with LPS for 7.5 min (B) and 15 min (C) compared to resting cells (A). Colocalisation of TLR4 and EEA1 is shown in the composite image (yellow). Results shown are representative of three experiments.

stimulation with LPS in DMSO-treated cells, TLR4 was rapidly endocytosed and is almost completely inside the cell with early endosome formation and co-localisation of EEA1 with TLR4 being quite prominent (Fig. 7A and B, top panel). After 15 min stimulation there appears to be less co-localisation of the two molecules and less endosomes present (Fig. 7C, top panel). In direct comparison to DMSO, c9,t11-CLA-treated cells displayed distinct patterns of TLR4 localisation and endocytosis. Upon stimulation more TLR4 appears to be retained at the plasma membrane in these cells (Fig. 7B and C, second panels down). Conversely, almost all TLR4 was observed to be inside cells and co-localised with EEA1 in EPA treated cells stimulated with LPS (Fig. 7B, third panel down). In addition, at 15 min stimulation there is a distinct movement of TLR4 away from endosomes in these cells (Fig. 7C, third panel down). It was noted that more co-localisation was observed at this time point in EPA treated cells compared to DMSO (Fig. 7B, third panel down). Cells treated with the saturated fat, LA, displayed patterns of TLR4 endocytosis and early endosome formation similar to those of DMSO-treated cells (Fig. 7B and C, bottom panels).

4. Discussion

In this study we explored whether the CLA isomer, c9,t11-CLA, exerts its anti-inflammatory actions via the TLR4 receptor complex. We observed suppressed expression of CD14 both on the cell membrane and in lipid rafts following stimulation with LPS in c9, t11-CLA-treated cells. This effect on CD14 resulted in a decrease in endocytosis of TLR4 to early endosomes following activation with LPS and subsequent suppression of IRF3 activation. These effects were not seen with EPA, which was assessed as a comparison, and suggests a novel mechanism by which c9,t11-CLA exerts its anti-inflammatory effects.

Activation of TLR4 at the plasma membrane initiates downstream signalling which in turn activates NF-κB and IRF3 via MyD88-dependent and -independent pathways, respectively [39,40]. Knowing that the incorporation of PUFA to the plasma membrane modifies its fluidity and indeed its function via receptors, proteins and enzymes bound to it, we firstly examined the effect of the c9,t11-CLA isomer on the components of the TLR4 complex. CD14 is a 56 kDa, GPI-anchored protein lacking a transmembrane and intracellular domain and acts as an accessory molecule for both TLR4 and TLR2 signalling [41,44]. c9,t11-CLA was the only fatty acid to suppress surface expression of CD14 in both resting and LPS-activated macrophages. Furthermore, analysis by western blot revealed suppressed levels of CD14 at the membrane in c9,t11-CLA treated cells which was concomitant with enhanced levels of CD14 in the cytosol suggesting that CD14 may be retained in the cytosol in these cells. This finding was supported by confocal microscopy which showed that in c9,t11-CLA-treated cells, CD14 was mainly confined to the cytoplasm in both resting cells and after 15 min of stimulation with LPS, as well as after 30 min, 1 h and 6 h LPS (data not shown). It was only after prolonged stimulation, some but not all of CD14 was seen to return to the plasma membrane. In contrast EPA did not alter CD14 in this way. These findings suggested a distinct mechanism by which c9,t11-CLA may exert its anti-inflammatory effects.

CD14 is recruited to lipid rafts in order to complex with TLR4 and this is a crucial element in subsequent signalling by the complex [30,38,45,46]. Therefore we examined whether c9,t11-CLA altered the localisation of CD14 in these lipid rafts and whether this had consequences for endocytosis of TLR4. We found CD14 to be markedly suppressed in lipid raft fractions isolated from LPS-stimulated macrophages treated with c9,t11-CLA. On the other hand, macrophages treated with the n-3 PUFA, EPA for comparison, displayed enhanced levels of CD14 in lipid raft fractions compared to control. There is currently no other published data available on the effects of c9,t11-CLA on lipid rafts. The most studied fatty acid to date is DHA which has been shown to modulate the recruitment of TLR4 into lipid rafts [47] and also modify the clustering and size of lipid rafts [48].

GPI-anchored proteins are known to contain two saturated fatty acid chains in their phosphatidylinositol moiety allowing them to be incorporated to lipid rafts [49]. The remodelling of the GPI anchor takes place between the ER and Golgi with the unsaturated sn-2 chain being removed and a saturated one added. This process is mediated by post GPI-attachment proteins (PGAP2 and PGAP3). An increase in unsaturated fatty acids following exposure of the cells to c9,t11-CLA may affect the availability of saturated fatty acids for this process. Furthermore, S-acylation of proteins with heterogeneous unsaturated fatty acids has been proposed as a mechanism by which cells regulate signal transduction by altering the association of proteins with rafts [50,51]. Our finding that c9,t11-CLA reduced the localization of CD14 in lipid rafts, may suggest that this fatty acid could interfere with these processes.

In agreement with a number of published studies, here we have also observed a suppressive effects of c9,t11-CLA and EPA on NF-κB [17–19]. We previously demonstrated that c9,t11-CLA can suppress NF-κB in vitro in dendritic cells [8] and in vivo in adipose tissue from mice fed on diets of CLA [21]. TLR4 signalling is unique in its ability to activate both NF-κB and IRF3 [39,40]. Interestingly, our data demonstrates a novel and selective inhibition of IRF3 in HEK-TLR4-CD14-MD2 cells exposed to c9,t11-CLA but not to EPA. This is the first study to demonstrate a suppressive effect of a dietary fatty acid on IRF3 in any cell type. Given that activation of IRF3 downstream of TLR4 requires endocytosis of the TLR4 complex [42] and is a process dependent on CD14 [52], our findings suggested that the distinct modulation of CD14 by c9,t11-CLA could be responsible for the subsequent suppression of IRF3 we observed. Further evidence is provided by the experiments using U373 cells which demonstrated that the inhibitory effect of c9,t11-CLA on IRF3 was completely reversed by over-expressing CD14. Our data on the effect of c9,t11-CLA on CD14 is consistent with our recent study demonstrating that bone-marrow derived dendritic cells isolated from mice fed on a high-CLA diet show decreased levels of CD14 [53].

In order to assess whether the suppression of CD14 was associated with reduced endocytosis of TLR4, which would explain the decrease in IRF3 activation, confocal microscopy was employed examining TLR4 expression at the membrane and in early endosomes in HEK-TLR4-MD2-CD14 cells treated with c9,t11-CLA, EPA or LA. In this study we demonstrate retention of TLR4 on the plasma membrane following stimulation with LPS in c9,t11-CLA treated cells indicating a decrease in endocytosis of TLR4 compared to the DMSO control. This was not the case in EPA-treated cells which were used as a comparison due to the fact that EPA did not decrease CD14 expression or IRF3 activation. This data reveals a novel mechanism which explains how c9,t11-CLA may exert its anti-inflammatory effects.

Previously, research has focussed on downstream signalling components [8,53] and suggested these as key targets through which PUFA exert their effects. This study demonstrates a novel mechanism through which c9,t11-CLA exerts its effects at the membrane. With emerging roles for CD14 in the exacerbation of infection and inflammatory disease our finding with regard to c9,t11-CLA may have implications for the possible use of this fatty acid as a complementary treatment.

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