

Conjugated Linoleic Acid Suppresses NF- κ B Activation and IL-12 Production in Dendritic Cells through ERK-Mediated IL-10 Induction¹

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Polyunsaturated fatty acids (PUFA) have been shown to modulate immune responses and have therapeutic effects in inflammatory disorders. However, the influence of PUFA on dendritic cells (DC), key cells of the innate immune system in shaping adaptive immune responses, has not yet been defined. In this study, we examine the effects of the *cis*-9, *trans*-11 isomer of conjugated linoleic acid (c9, t11-CLA), a dietary PUFA found in meat and dairy products, on murine DC activation. Treatment of DC with c9, t11-CLA suppressed LPS-induced IL-12, enhanced IL-10R expression, and enhanced IL-10 production at the transcriptional and protein level. The suppression of IL-12 by c9, t11-CLA was found to be IL-10 dependent. We investigated the involvement of the MAPK, ERK, and the transcription factor, NF- κ B, in this IL-10-mediated effect. c9, t11-CLA enhanced ERK activation after LPS stimulation, and inhibition of ERK resulted in abrogation of IL-10 and recovery of IL-12 production. c9, t11-CLA decreased NF- κ B:DNA binding after LPS stimulation, which was concomitant with delayed translocation of NF- κ Bp65 into the nucleus and an increase in I κ B α . These effects were reversed by addition of a neutralizing anti-IL-10 Ab. Our findings demonstrate that c9, t11-CLA suppresses IL-12 production by LPS-stimulated DC by ERK mediated IL-10-induction. Furthermore, these IL-10-mediated effects are dependent on inhibition of NF- κ B activation. This is the first study to demonstrate that c9, t11-CLA can enhance transcription and production of the anti-inflammatory cytokine IL-10, while inhibiting the Th1-promoting cytokine IL-12, and may explain certain of its immunosuppressive properties. *The Journal of Immunology*, 2005, 175: 4990–4998.

It is well established that some classes of dietary polyunsaturated fatty acids (PUFA)⁴ can suppress immune responses and studies focusing on n-3 PUFA, naturally abundant in marine oils, have demonstrated that they can suppress T cell activation and attenuate cytokine production from macrophages (1, 2). Furthermore, feeding fish oils has been shown to ameliorate the symptoms of certain autoimmune diseases in animal models (3). More recently, it has been reported that conjugated linoleic acid (CLA) has immunomodulatory properties (4). CLA is a heterogeneous group of positional and geometric isomers of conjugated dienoic derivatives of linoleic acid, an n-6 PUFA. The *cis*-9, *trans*-11 CLA isomer (c9, t11-CLA) is the naturally occurring dietary form of CLA and is found in the lipid fraction of meat, milk, and dairy products. Lower levels of other isomers, including *trans*-

10, *cis*-12 CLA (t10, c12-CLA), are also present in food sources (4). CLA suppresses proinflammatory cytokine production from a variety of cell types, and studies in animal models have shown that feeding CLA has beneficial effects on inflammatory-induced growth suppression, endotoxin-induced anorexia, and mucosal damage in experimental colitis (5, 6). These data suggest that this fatty acid has considerable potential as an immunomodulatory molecule in the prevention or treatment of inflammatory and immune-mediated diseases. However its role in modulating innate immune responses, in particular dendritic cell (DC) activation, has received little attention.

DC play a critical role in directing adaptive immune responses, including Th cell responses. Activation of DC by inflammatory stimuli, such as LPS, induces maturation and homing to lymph nodes where they present Ag to naive T cells. The differentiation of naive CD4⁺ T cells into Th1, Th2, or regulatory T (Tr) cells is determined in part by the maturation status of the DC, but also by the cytokines produced by DC and other cells of the innate immune system. Production of IL-12, in response to inflammatory stimuli promotes the induction of Th1 cells. In contrast, products of helminth parasites have been shown to activate DC, resulting in the production of IL-4 and induction of Th2 cells (7, 8). Furthermore, other pathogen-derived molecules, such as filamentous hemagglutinin from *Bordetella pertussis*, enhance IL-10 production by DC and promote the induction of type 1 Tr (Tr1) cells (9). The pivotal role of IL-4, IL-10, and IL-12 in directing Th cell responses suggests that modulating their production by DC can affect quantitatively and qualitatively the adaptive immune response. Indeed, molecules that enhance IL-10 and suppress IL-12 production by DC have been shown to promote the induction of Th2 and Tr1 cells (9, 10).

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⁴ Abbreviations used in this paper: PUFA, polyunsaturated fatty acid; CLA, conjugated linoleic acid; c9, t11-CLA, *cis*-9, *trans*-11 CLA; t10, c12-CLA, *trans*-10, *cis*-12 CLA; DC, dendritic cell; EPA, eicosapentaenoic acid; pERK, phosphoERK; Tr, regulatory T; DHA, docosahexaenoic acid.

Expression of IL-12, a heterodimeric cytokine comprising p35 and p40 subunits, is regulated at the level of transcription, and NF- κ B activation is a key factor involved in this process. Indeed inhibition of this transcription factor results in suppression of IL-12 production by APCs (11). Treatment of such cells with a variety of proinflammatory agents, such as LPS, results in phosphorylation, ubiquitination, and degradation of the I κ B. The degradation of I κ B proteins by I κ B kinase results in liberation of NF- κ B from I κ B and subsequent translocation of NF- κ B to the nucleus (12). There is evidence to suggest that certain fatty acids may mediate their immunomodulatory function through effects on NF- κ B. For example, eicosapentaenoic acid (EPA) decreases NF- κ B activation in LPS-stimulated macrophages (13) and docosahexaenoic acid (DHA) inhibits its activation in Jurkat T cells (14). However, the mechanism of inhibition of NF- κ B activation by fatty acids is poorly understood. Studies have indicated that IL-10 can reduce degradation and/or phosphorylation of I κ B proteins and inhibit NF- κ B translocation into the nucleus (12, 15). Furthermore, IL-10 is involved in negative autocrine regulation of IL-12 production in DC. Therefore, IL-10-mediated inhibition of NF- κ B activation may be one mechanism by which immunomodulatory molecules exert their suppressive effect on IL-12 production by activated DC.

The MAPK are important for regulation of survival, maturation, and cytokine secretion by DC. Activation of ERK has been shown to promote IL-10 production and mediate negative feedback regulation of IL-12 production (16). Moreover, the inhibition of ERK activation in DC results in suppression of IL-10 and enhancement of IL-12 production (17). These data suggest that ERK phosphorylation may play an important role in the regulation of two key cytokines involved in the induction of Th cell responses. Although n-3 PUFA have been shown to inhibit phosphorylation of ERK (18), recently it has been demonstrated that CLA isomers can enhance its activation (19).

In the present study we tested the hypothesis that the PUFA, c9, t11-CLA, can enhance anti-inflammatory cytokines by DC and modulate production of key cytokines associated with direction of Th cell responses. We report that CLA suppresses IL-12 production by LPS-stimulated DC by an IL-10-dependent mechanism. We provide evidence for a role in ERK activation in this IL-10-mediated suppression. Furthermore, these IL-10-mediated effects are mediated through inhibition of NF- κ B activation. Given the importance of DC in directing Th cell responses, these results may explain some of the immunomodulatory properties of CLA.

Materials and Methods

Animals and materials

BALB/c mice were purchased from Harlan (U.K.) and were used at 10–14 wk of age. Animals were maintained according to the regulations of the European Union and the Irish Department of Health. c9, t11-CLA was purchased from Alexis Chemicals (Cayman Chemicals). *Escherichia coli* LPS (serotype 127:B8) and the ERK inhibitor, U0126, were purchased from Alexis Biochemicals. The c9, t11-CLA isomer and the ERK inhibitor were dissolved in sterile DMSO (Sigma-Aldrich) and stored at -20°C in the dark.

Isolation and culture of bone marrow-derived DC

Bone marrow-derived immature DC were prepared by culturing bone marrow cells obtained from the femurs and tibia of mice in RPMI 1640 medium with 5% FCS supplemented with 10% supernatant from a GM-CSF-expressing cell line (J558-GM-CSF). The cells were cultured at 37°C for 3 days, and the supernatant was carefully removed without disturbing the cell monolayer and replaced with fresh medium with 10% GM-CSF cell supernatant. On day 7 of culture, cells were collected, counted, and used for assays. For experiments, either DMSO (vehicle control) or c9, t11-CLA was added to the cells on day 1 or 5 of culture.

Effect of c9, t11-CLA on DC cytokine mRNA and protein

On day 7 of culture, after 48 h of treatment with either DMSO or c9, t11-CLA, DC ($1 \times 10^6/\text{ml}$) were cultured in 24-well plates with LPS (100 ng/ml) or medium alone for 0–24 h. In certain experiments, DC were incubated in the presence or absence of a neutralizing anti-IL-10 Ab (10 $\mu\text{g}/\text{ml}$) or the specific ERK inhibitor U0126 (5 μM). At the end of the relevant incubation periods, supernatants were removed and IL-10, IL-12p40, and IL-12p70 concentrations were measured using DuoSet ELISA kits from R&D Systems according to manufacturer's instructions. After removal of the supernatants the cells were harvested and TRIreagent (Molecular Research Center) was added. Total RNA was extracted from the cells according to the manufacturer's instructions. Two micrograms of RNA was DNase treated and reverse transcribed in a 25- μl reaction containing 1 $\times$ reverse transcription buffer (50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl_2 , 10 mmol/L DTT), 500 ng of random primers, 10 mmol/L dNTPs, and 200 U of Moloney murine leukemia virus reverse transcriptase. mRNA expression was quantified by real-time PCR on an ABI 7700 Sequence Detection System (PerkinElmer Applied Biosystems). A negative control was processed from RNA without Moloney murine leukemia virus reverse transcriptase. TaqMan real-time PCR was performed for IL-10, IL-12p40, and IL-12p35 using PreDeveloped Assay Reagent kits. Each 25- μl reaction contained 20 ng of cDNA, 2 $\times$ TaqMan Universal PCR Mastermix, forward and reverse primers, and TaqMan probe. All reactions were performed in duplicate using the following conditions: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For each sample, results were normalized by dividing the amount of target gene by the amount of GAPDH and expressed relative to the vehicle control treatment (DMSO) for the non-LPS-stimulated cells.

Effect of c9, t11-CLA on IL-10R expression

DC were treated with either DMSO or c9, t11-CLA from day 1 of culture and then cultured in 24-well plates (1×10^6 cells) with LPS (100 ng/ml) or medium alone for 24 h. Cells were then washed and used for immunofluorescence analysis. The expression of IL-10R on DC was assessed using an anti-mouse CD11c (hamster IgG1; Caltag Laboratories), IL-10R (rat IgG1; BD Biosciences), and appropriately labeled isotype-matched Abs, which acted as controls. After incubation for 30 min at 4°C , cells were washed and immunofluorescence analysis was performed on a FACScan (BD Biosciences) using CellQuest software.

Effect of c9, t11-CLA on ERK activation

DC were treated with either DMSO or c9, t11-CLA for 48 h and then cultured DC (3×10^6 cells) in 6-well plates with LPS (100 ng/ml) or medium alone for 0–5 h. After this time, supernatants were removed and whole cell extracts were prepared by lysis of the cells in 100 μl of 1 $\times$ sample buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 2% w/v SDS, 0.1% w/v bromophenol blue, 50 mM DTT). Samples were then sonicated using a Vibra Cell sonicator for 10 s at a pulse of 1.5 and amplitude of 40, boiled at 100°C for 5 min, and stored at -80°C for Western blot analysis.

Effect of c9, t11-CLA on NF- κ B activation

After treatment with either DMSO or c9, t11-CLA, DC (3×10^6 cells) were cultured in 6-well plates with LPS (100 ng/ml) or medium alone for 0–5 h. In certain experiments, DC were incubated in the presence or absence of a neutralizing anti-IL-10 Ab (10 $\mu\text{g}/\text{ml}$). Supernatants were removed and cells were washed in ice-cold PBS and lysed in 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl_2 , 10 mmol/L KCl, 0.5 mmol/L PMSF, 0.5 mmol/L DTT, and 0.1% v/v Nonidet P-40. After centrifugation, the supernatant was removed and stored at -80°C as the cytosolic extract. To prepare the nuclear extract, the pellet was resuspended in 20 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl_2 , 420 mmol/L NaCl, 0.2 mmol/L EDTA, 25% glycerol, and 0.5 mmol/L PMSF, incubated on ice for at least 15 min with repeated vortexing, and then centrifuged. To the supernatant, 10 mmol/L HEPES (pH 7.9), 50 mmol/L KCl, 0.2 mmol/L EDTA, 20% glycerol, 0.5 mmol/L DTT, 0.5 mmol/L PMSF was added and samples were mixed well and stored at -80°C as the nuclear extract. The concentration of protein in the nuclear and cytoplasmic samples was quantified by Bradford assay (Bio-Rad). Samples were used for Western blot analysis, NF- κ B activity assay, and EMSAs.

Western blot analysis

Proteins and prestained protein markers (precision plus protein standards; Bio-Rad) were separated by SDS-PAGE and blotted onto 0.45 μm Bio-trace polyvinylidene fluoride transfer membrane (Pall Life Sciences). Membranes were blocked using 10% nonfat dried milk in PBS with 0.05%

Tween (PBS-T) and incubated overnight at 4°C with either anti-phosphoERK (pERK; Santa Cruz Biotechnology; 1:750), anti-ERK (Santa Cruz Biotechnology; 1:1000), anti-NF- κ Bp65 (Santa Cruz Biotechnology; 1:500), or anti-I κ B α (Santa Cruz Biotechnology; 1:500) Abs. Membranes were washed with PBS-T and incubated for 2 h at room temperature with either peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (Sigma-Aldrich; 1:1000). After further washing, protein complexes were visualized with supersignal (Pierce). Membranes were exposed to film for 1–10 min and processed using an Agfa x-ray processor. Protein bands were quantified using the GeneSnap acquisition and GeneTools analysis software (GeneGenius Gel Documentation and Analysis System; Syngene). Where required, membranes were stripped by incubating them in stripping buffer (Tris (pH 6.8), 2-ME, SDS) for 30 min at 50°C before probing with a subsequent Ab. Levels of pERK were normalized to total ERK and expressed in arbitrary units. NF- κ Bp65 and I κ B α were either expressed as arbitrary units or normalized to controls and expressed as fold change relative to control.

EMSA

Two micrograms of nuclear protein was incubated for 30 min at room temperature with 10,000 cpm 32 P-labeled oligonucleotide probe (PerkinElmer Life Sciences), 2 μ g of poly(dI:dC) (Amersham-Pharmacia), 100 mmol/L Tris (pH 7.5), 500 mmol/L NaCl, 40% glycerol (w/v), 5 mmol/L EDTA, 5 mmol/L DTT, and 1 g/L nuclease-free BSA. The probes used in the EMSA contained the consensus binding sequence for NF- κ B 5'-AGT TGA GGG GAC TTT CCC AGG C-3', which had been previously labeled with [32 P]ATP by T4 polynucleotide kinase (Promega). DNA-protein complexes were resolved on a nondenaturing 4% (v/v) polyacrylamide gels and run for ~1.75 h at 140 V in 0.5 $\times$ Tris borate buffer. The gels were dried, exposed to film overnight, and developed using an Agfa x-ray processor.

NF- κ B activity assay

NF- κ B activity was determined by using a TransAM NF- κ B Chemi assay (Active Motif) that measured NF- κ Bp65:DNA binding. The assay was conducted according to the manufacturer's instructions. Briefly, 0.5 μ g of nuclear protein was incubated in a 96-well plate containing the NF- κ B consensus site (5'-GGGACTTTCC-3') for 1 h. After washing, a primary Ab that recognizes an epitope on p65, which is accessible only when NF- κ B is activated and bound to its target DNA, was added for 1 h. After a further washing step, a peroxidase-conjugated secondary Ab was added for 1 h and then a chemiluminescent substrate was applied to give a readout that could be quantified by luminescence. Results are normalized to controls and expressed as fold change relative to control.

Statistics

One-way ANOVA was used to determine significant differences between conditions. When this indicated significance ($p < 0.05$), post hoc Student-Newmann-Keul test analysis was used to determine which conditions were significantly different from each other. Student's unpaired t test was used for analysis when two conditions were compared. There was no significant difference between cells alone and DMSO (vehicle control)-treated cells; therefore, DMSO was used as the reference treatment.

Results

c9, t11-CLA enhances IL-10 but suppresses IL-12 production by DC

Because IL-12 is critical in the induction of Th1 cells and IL-10 can enhance differentiation of Tr1 cells and has also been implicated in inhibiting Th1 responses (9), we examined the possibility that c9, t11-CLA could modulate the transcription and production of these cytokines from DC. Incubation of DC with c9, t11-CLA for 48 h did not induce detectable concentrations of IL-10, IL-12p70, or IL-12p40 (Fig. 1). Activation of DC with LPS resulted in significant production of IL-10, IL-12p70, and IL-12p40, detectable 6 ($p < 0.05$; $p < 0.001$), 12 ($p < 0.001$), and 24 h ($p < 0.001$) after treatment. The production of LPS-induced IL-10 was significantly enhanced ($p < 0.001$) at all three time points examined after pretreatment of the DC with c9, t11-CLA. In contrast, pretreatment of DC with c9, t11-CLA suppressed LPS-induced IL-12p70 ($p < 0.001$) and IL-12p40 ($p < 0.001$) at 12 and 24 h. Similar results were seen with another isomer of CLA, t10, c12-

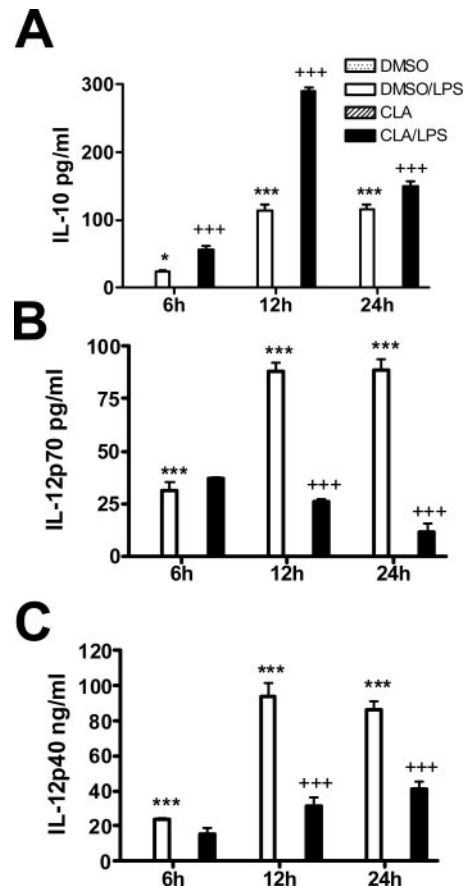


FIGURE 1. c9, t11-CLA modulates cytokine production by DC. DC were treated for 48 h with either c9, t11-CLA (50 μ M) or DMSO vehicle control and then stimulated with LPS (100 ng/ml) for 6–24 h. IL-10 (A), IL-12p70 (B), and IL-12p40 (C) protein concentrations were measured in the supernatants by two-site ELISA. The results are the mean ($\pm$ SEM) for three experiments. ***, $p < 0.001$, ANOVA, comparing unstimulated vs LPS-stimulated groups; + + +, $p < 0.001$, ANOVA, comparing DMSO vs c9, t11-CLA LPS-stimulated groups.

CLA and with the n-3 fatty acids EPA and DHA and not with the saturated fatty acid, stearic acid (data not shown). Treatment of DC with c9, t11-CLA had no effect on TNF- α production (data not shown). To elucidate whether c9, t11-CLA exerted its effects at the level of transcription, we also examined expression of mRNA for these cytokines. Although transcription of IL-12p35 (Fig. 2B) and IL-12p40 (Fig. 2C) were not modulated in resting cells, c9, t11-CLA enhanced IL-10 mRNA expression, although this increase did not reach statistical significance (Fig. 2A). LPS stimulation of DC significantly increased transcription of IL-10 mRNA, IL-12p35 mRNA, and IL-12p40 mRNA at a number of time points over the 48-h period examined ($p < 0.05$; $p < 0.01$; $p < 0.001$). DC pretreated with c9, t11-CLA had significantly enhanced transcription of IL-10 at 2 and 6 h ($p < 0.05$; $p < 0.01$). Conversely, c9, t11-CLA pretreatment significantly suppressed LPS-induced IL-12p35, particularly at 12 h (Fig. 2B; $p < 0.05$). LPS-induced transcription of IL-12p40 was also suppressed by c9, t11-CLA pretreatment at 6, 12, 18, and 24 h (Fig. 2C; $p < 0.05$).

c9, t11-CLA suppression of IL-12 production by DC is IL-10 dependent

Given our data demonstrating that the c9, t11-CLA-induced enhancement of IL-10 transcription and protein production was detectable before the suppression of IL-12, we examined whether the

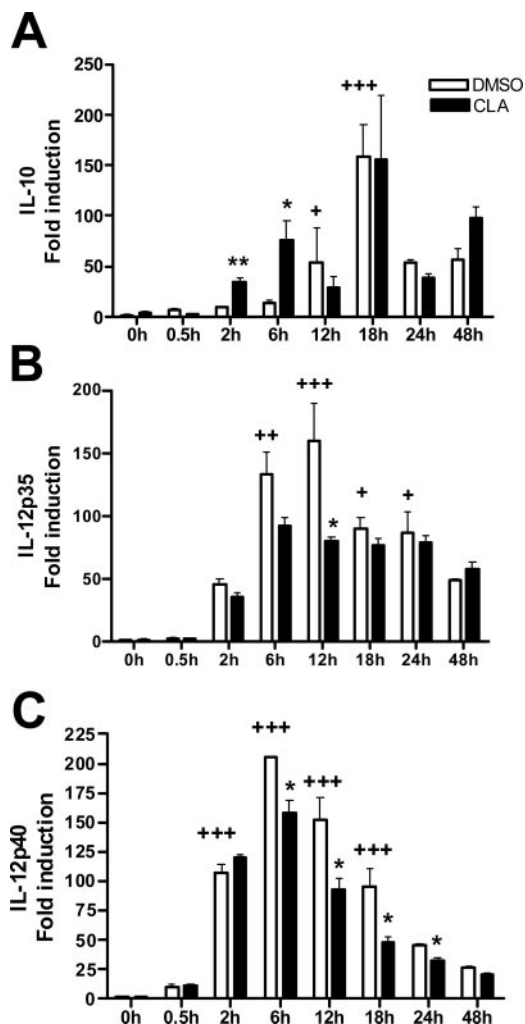


FIGURE 2. Effects of c9, t11-CLA on mRNA expression in DC. DC were treated for 48 h with either c9, t11-CLA (50 μ M) or DMSO vehicle control and then stimulated with LPS (100 ng/ml) for 0–48 h with the 0-h group being the non-LPS-stimulated cells. Expression of IL-10 (A), IL-12p35 (B), and IL-12p40 (C) mRNA were measured using TaqMan real-time PCR. mRNA levels were normalized to GAPDH, and results are expressed as fold induction relative to the DMSO vehicle control for unstimulated cells (0 h). The results are the mean ($\pm$ SEM) for three experiments. +, $p < 0.05$, ++, $p < 0.01$, +++, $p < 0.001$, ANOVA, comparing unstimulated (0 h) with LPS-stimulated DC (0.5–48 h). *, $p < 0.05$, **, $p < 0.01$, Student's t test for unpaired values, comparing DMSO vs c9, t11-CLA at each time point.

suppression of IL-12 by c9, t11-CLA was mediated by IL-10. LPS stimulation of c9, t11-CLA-treated DC was conducted in the presence and absence of a neutralizing anti-IL-10 Ab and IL-12p70 and IL-10 mRNA and protein production were measured. Consistent with the data shown in Fig. 1, c9, t11-CLA significantly suppressed LPS-induced IL-12p70 protein production at 12 h (Fig. 3A; $p < 0.001$). This suppression was significantly reversed by addition of anti-IL-10 Ab ($p < 0.01$). Similarly, LPS-induced IL-12p35 mRNA expression (Fig. 3B) was suppressed by c9, t11-CLA, and significantly reversed in the presence of the anti-IL-10 Ab at 6 ($p < 0.001$) and 12 h ($p < 0.001$). Consistent with the previous data, c9, t11-CLA significantly enhanced LPS-induced IL-10 production at 6 (Fig. 3C; $p < 0.05$) and 12 h ($p < 0.001$) and IL-10 mRNA expression at 6 h (Fig. 3D; $p < 0.05$).

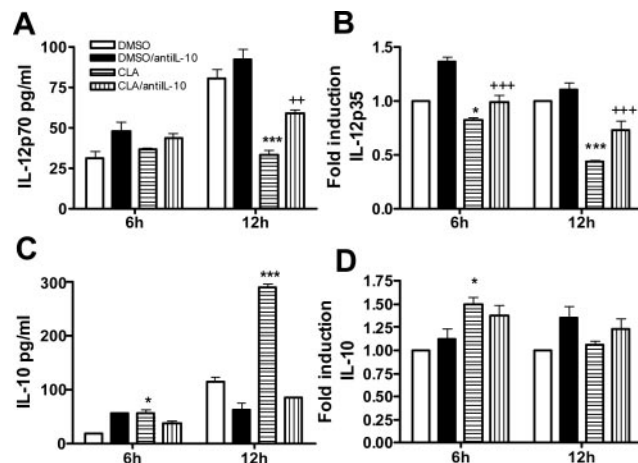


FIGURE 3. The suppression of LPS-induced IL-12 by c9, t11-CLA is mediated by IL-10. DC were treated for 48 h with either c9, t11-CLA (50 μ M) or DMSO vehicle control and then stimulated with LPS (100 ng/ml) in the presence or absence of a neutralizing anti-IL-10 Ab (10 μ g/ml) for 6–12 h. Supernatants were assayed for IL-12p70 (A) or IL-10 (C) protein production by two-site ELISA. The results are the mean ($\pm$ SEM) for three experiments. *, $p < 0.05$, **, $p < 0.01$, ANOVA, comparing DMSO vs c9, t11-CLA; ++, $p < 0.01$, ANOVA, comparing c9, t11-CLA vs c9, t11-CLA with anti-IL-10 Ab. Expression of IL-12p35 (B) and IL-10 (D) mRNA were measured by TaqMan real-time PCR. mRNA levels were normalized to GAPDH and are expressed relative to DMSO vehicle control. The results are the mean ($\pm$ SEM) for three experiments. *, $p < 0.05$, **, $p < 0.01$, ANOVA, comparing DMSO vs c9, t11-CLA; +++, $p < 0.001$, ANOVA, comparing c9, t11-CLA vs c9, t11-CLA with anti-IL-10 Ab.

c9, t11-CLA enhances IL-10R expression on DC

Having demonstrated enhanced production of IL-10 by DC, we examined the possibility that c9, t11-CLA may also modulate IL-10R expression on these cells. Incubation of bone marrow-derived DC with c9, t11-CLA increased the percentage of DC expressing IL-10R (48.8% in DMSO-treated DC vs 68.7% in CLA-treated DC) and enhanced the level of expression on CD11c⁺ DC (Fig. 4, top panel). Stimulation of DC with LPS decreased IL-10R expression, and treatment with CLA partially reversed this effect (Fig. 4, bottom panel).

c9, t11-CLA activation of ERK is necessary for IL-10-mediated suppression of IL-12 in DC

Having demonstrated that c9, t11-CLA enhances IL-10 mRNA expression in immature DC and significantly increases LPS-induced IL-10 production, we examined whether ERK activation was involved in this enhancement and the subsequent suppression of IL-12 production. To address this question, we first determined whether c9, t11-CLA could modulate ERK phosphorylation in both resting DC and after stimulation with LPS at both early and later time points. Activation of ERK was significantly increased in resting DC after treatment with c9, t11-CLA treatment (Fig. 5, A and B; $p < 0.01$; $p < 0.001$). After LPS stimulation, c9, t11-CLA significantly enhanced phosphorylation of ERK at 5, 10, and 20 min (Fig. 5A; $p < 0.001$; $p < 0.05$). This increase in ERK activation in c9, t11-CLA-treated DC was sustained at 1, 2, and 5 h (Fig. 5B; $p < 0.05$; $p < 0.01$). We then tested whether the effect of c9, t11-CLA on IL-10 production and IL-12 suppression was dependent on ERK activation by using the specific ERK inhibitor, U0126. Consistent with the data in Fig. 1, c9, t11-CLA enhanced LPS-induced IL-10 production (Fig. 5C; $p < 0.05$) and suppressed IL-12p70 production (Fig. 5D; $p < 0.001$). Inhibition of ERK activation by U0126 resulted in an almost total abrogation of IL-10

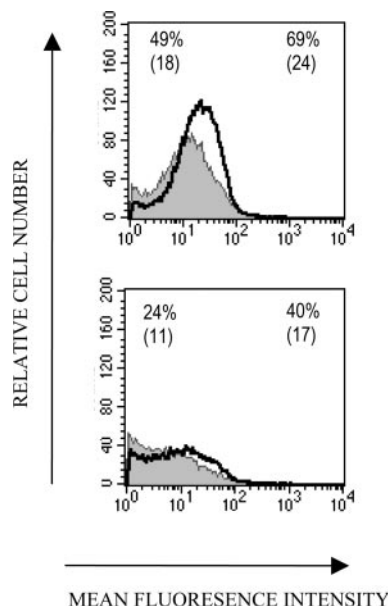


FIGURE 4. c9, t11-CLA up-regulates IL-10R expression on DC. DC were treated with c9, t11-CLA or DMSO vehicle control for 7 days and then stimulated with LPS (100 ng/ml). Cells were assessed for IL-10R expression by FACS analysis. Immunofluorescence is shown for c9, t11-CLA-treated DC (black line) compared with DMSO control (gray histogram). The numbers on the left of each histogram refer to the mean fluorescence intensity of the DMSO control cells and the number on the right of each histogram refers to the mean fluorescence intensity of the c9, t11-CLA-treated DC. The numbers in parentheses on the left and right of the histograms refer to the percentage of cells expressing IL-10R in DMSO and c9, t11-CLA-treated DC, respectively.

production in both DMSO- and c9, t11-CLA-treated DC (Fig. 5C; $p < 0.001$), demonstrating that ERK activation is necessary for IL-10 production in DC. Furthermore, the suppression of LPS-induced IL-12p70 production by c9, t11-CLA was significantly reversed in the presence of U0126 (Fig. 5D; $p < 0.001$), indicating that ERK activation and subsequent production of IL-10 is necessary for c9, t11-CLA-induced IL-12p70 suppression.

c9, t11-CLA decreases NF- κ B activity

Having demonstrated that c9, t11-CLA suppresses LPS-induced IL-12 mRNA expression, and that this effect was mediated in part through IL-10 production, we examined the role of NF- κ B. Given that translocation of NF- κ Bp65 into the nucleus and degradation of I κ B α are two events involved in NF- κ B activation, we examined the effects of c9, t11-CLA on NF- κ B:DNA binding and on NF- κ Bp65 and I κ B α levels in the cytosol and the nucleus. c9, t11-CLA significantly decreased NF- κ B activity in resting DC (Fig. 6A; $p < 0.001$) and treatment of DC with c9, t11-CLA significantly decreased LPS-induced NF- κ B activity at 2 ($p < 0.001$) and 5 h ($p < 0.001$). c9, t11-CLA had no effect on cytosolic or nuclear NF- κ Bp65 levels in resting cells (Fig. 6, B and C). A kinetic study showed that c9, t11-CLA significantly increased cytosolic NF- κ Bp65 (Fig. 6B; $p < 0.05$) and decreased nuclear NF- κ Bp65 levels (Fig. 6C; $p < 0.01$) at 5 h. These data suggest that c9, t11-CLA delays LPS-stimulated nuclear NF- κ Bp65 translocation for NF- κ B activation. c9, t11-CLA also marginally increased nuclear I κ B α expression in resting DC (Fig. 6E). After LPS stimulation, c9, t11-CLA increased I κ B α in the cytosol from 0.5 h and significantly at 1 (Fig. 6D; $p < 0.01$) and 5 h ($p < 0.01$). I κ B α was also significantly increased after LPS stimulation by c9, t11-CLA in the nucleus at 0.5 (Fig. 6E; $p < 0.001$), 1, 2, and 5 h ($p < 0.05$).

These results indicate that c9, t11-CLA prevents the degradation of I κ B α in both resting cells and after LPS stimulation. These data, together with the delay in nuclear NF- κ Bp65 translocation, may explain how c9, t11-CLA decreases NF- κ B activation.

The effect of c9, t11-CLA on NF- κ B activity is mediated by IL-10

Because the suppression of IL-12 by c9, t11-CLA is IL-10 dependent and IL-10 can inhibit NF- κ B activity, we examined whether the effect of CLA on NF- κ B activity was mediated by IL-10. c9, t11-CLA-treated DC were stimulated with LPS in the presence and absence of a neutralizing anti-IL-10 Ab for 5 h, a time point at which c9, t11-CLA modulated expression of both NF- κ Bp65 and I κ B α (shown in Fig. 6). c9, t11-CLA significantly decreased LPS-induced NF- κ B activity (Fig. 7A; $p < 0.05$) that was significantly reversed in the presence of an anti-IL-10 Ab ($p < 0.01$). These results were confirmed by EMSA (Fig. 7B). NF- κ Bp65 and I κ B α levels were assessed in the cytosolic and the nuclear fractions by Western blotting using specific Abs. Consistent with the data in Fig. 6, Western blot analysis revealed that c9, t11-CLA significantly increased cytosolic NF- κ Bp65 (Fig. 7C; $p < 0.01$) and decreased nuclear NF- κ Bp65 (Fig. 7D; $p < 0.05$) when assessed 5 h after LPS stimulation. These effects were significantly reversed in the presence of the anti-IL-10 Ab ($p < 0.01$; $p < 0.05$). Furthermore, c9, t11-CLA significantly increased cytosolic and nuclear I κ B α levels (Fig. 7, E and F; $p < 0.05$) after LPS stimulation, and these effects were significantly reversed in the presence of an anti-IL-10 Ab ($p < 0.05$). These data suggest that the IL-10-dependent suppression of LPS-induced IL-12 by c9, t11-CLA is mediated by inhibition of NF- κ B activation.

Discussion

The significant findings of this study are that the PUFA, c9, t11-CLA, suppresses LPS-induced IL-12 production in DC and that this suppression is dependent on activation of ERK, enhanced IL-10 production, and inhibition of NF- κ B activation. This is the first study to demonstrate that c9, t11-CLA can modulate transcription and production of key cytokines by DC involved in controlling inflammatory responses and directing Th cell responses, and may explain certain of its immunomodulatory properties.

The effects of PUFA on cytokine production by immune cells, such as T cells and macrophages are well documented. For example, EPA reduces production of IL-2 and IL-13 from T cells (2) and CLA suppresses TNF- α , IL-1 β , and IL-6 production in IFN- γ -stimulated RAW macrophages (20). In this study, we demonstrate that the c9, t11 isomer of CLA suppresses LPS-induced IL-12p40 and IL-12p70 production by murine DC. We also found similar suppressive effects with EPA, DHA, and with another CLA isomer, t10, c12-CLA (data not shown). IL-12, produced by innate immune cells, is a key cytokine in the development of Th1 responses and has been implicated in the pathogenesis of infectious, inflammatory, and autoimmune diseases, such as inflammatory bowel disease (21, 22). Our findings are consistent with studies demonstrating that PUFA can inhibit Th1 responses. Specifically, EPA and DHA reduce circulating and splenic IL-12p70 and IL-12p40 production in mice after infection with *Listeria monocytogenes* (23) and decrease adaptive Th1-driven responses in a murine sensitization model (24). Furthermore, a recent report demonstrated that EPA suppresses IL-12p40 and TNF- α concentrations in human DC (25). Interestingly, we found that c9, t11-CLA had no effect on TNF- α production (data not shown). This suggests that individual PUFA exert their effects on specific cytokines and, therefore, may have distinct mechanisms of action. The selective suppression of IL-12 we have observed in DC suggests the potential use of c9, t11-CLA in Th1-mediated diseases. Furthermore, the

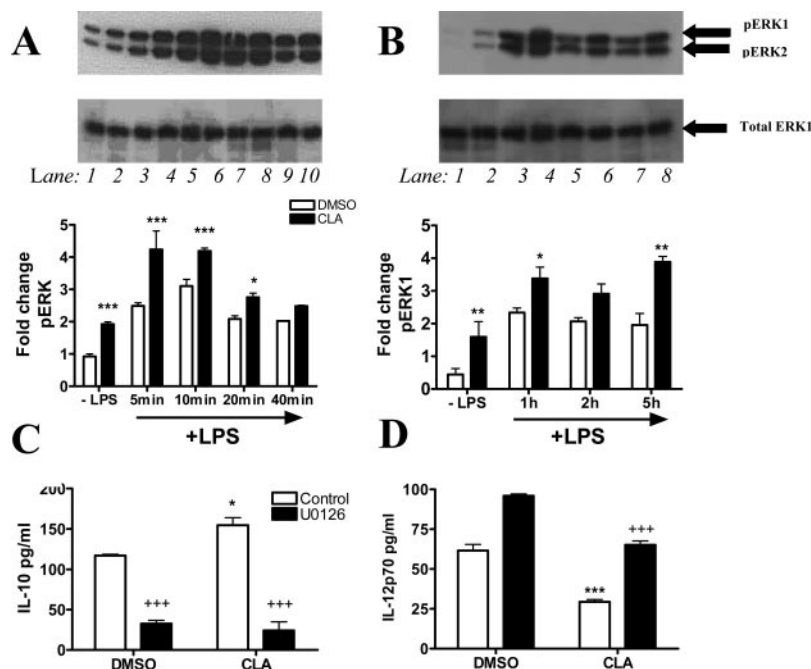


FIGURE 5. The IL-10-mediated suppression of LPS-induced IL-12 by c9, t11-CLA is dependent on ERK activation. *A* and *B*, DC were treated for 48 h with either c9, t11-CLA (50 μ M) or DMSO vehicle control and then stimulated with LPS (100 ng/ml) or medium alone in the presence or absence of the specific ERK inhibitor, U0126 (5 μ M), which was added 1 h before LPS stimulation. Cells were harvested 0–5 h after LPS stimulation, and total and phosphorylated ERK was determined in whole cell lysates by Western blotting for both early (*A*) and later (*B*) time points. Densitometric analysis was conducted on immunoblots and pERK is expressed as arbitrary units ($\pm$ SEM) after normalizing to total ERK. A representative immunoblot from four experiments is shown. *A*, Lane 1, 0 h DMSO; lane 2, 0 h CLA; lane 3, 5 min DMSO; lane 4, 5 min CLA; lane 5, 10 min DMSO; lane 6, 10 min CLA; lane 7, 20 min DMSO; lane 8, 20 min CLA; lane 9, 40 min DMSO; lane 10, 40 min CLA. *B*, Lane 1, 0 h DMSO; lane 2, 0 h CLA; lane 3, 1 h DMSO; lane 4, 1 h CLA; lane 5, 2 h DMSO; lane 6, 2 h CLA; lane 7, 5 h DMSO; lane 8, 5 h CLA. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ANOVA, comparing DMSO vs c9, t11-CLA. In a parallel experiment, DC were treated with c9, t11-CLA or DMSO as described above and then stimulated with LPS (100 ng/ml) for 24 h in the presence or absence of U0126 (5 μ M). Supernatants were then assayed for IL-10 (*C*) or IL-12p70 (*D*) protein production by immunoassay. The results are the mean ($\pm$ SEM) for three experiments. *, $p < 0.05$, ***, $p < 0.001$, ANOVA, comparing DMSO vs c9, t11-CLA; + + +, $p < 0.001$, ANOVA, comparing control vs U0126-treated groups.

effects of c9, t11-CLA on IL-12p40 suggest that it may also modulate production of IL-23, which shares the p40 subunit with IL-12 and is involved in the maintenance of Th1 responses (26).

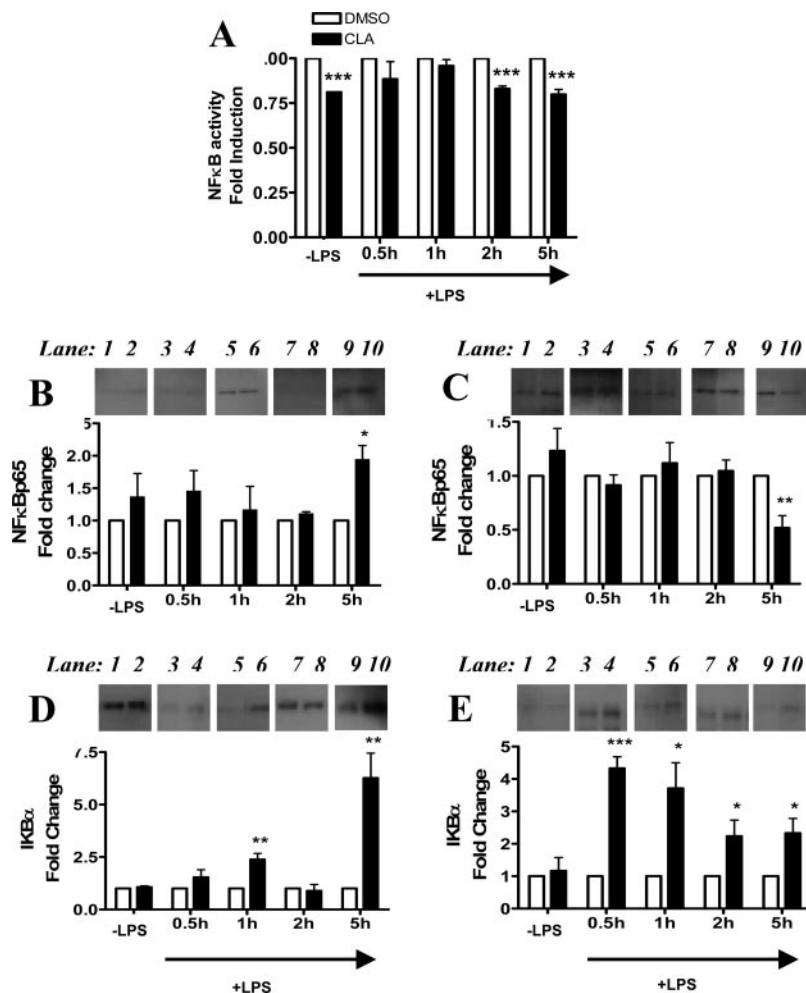
IL-10 is a regulatory cytokine with anti-inflammatory properties, potently inhibiting the capacity of innate immune cells to produce inflammatory mediators such as IL-12, TNF- α , and IL-1 (27). We report that c9, t11-CLA enhanced LPS-induced IL-10 transcription and production by DC. Furthermore, c9, t11-CLA enhanced IL-10R expression on both resting and LPS-stimulated DC. This enhanced IL-10R expression would allow the DC to have an increased response to the enhanced levels of IL-10 being secreted by these cells. These data indicate a possible role for IL-10 in mediating the effects of CLA. PUFA have previously been shown to enhance IL-10; treatment of mice with EPA results in increased production of IL-10 by whole blood cells and T cells (28, 29) and also enhances IL-10 levels in the brain in response to gamma irradiation (30). A recent study reported that EPA had no effect on IL-10 production from human DC (25). However, we found EPA, DHA, and the t10, c12 isomer of CLA significantly enhanced IL-10 production by murine DC (data not shown). This provides further evidence of different mechanisms of action of individual PUFA or differential effects on human and murine cells.

The present study is the first to demonstrate that c9, t11-CLA can enhance LPS-induced IL-10 in any cell type. In addition, our data demonstrate for the first time that suppression of IL-12 by a PUFA is at least in part mediated through IL-10 induction. Our findings are consistent with studies on a number of pathogen-derived molecules, including filamentous hemagglutinin from *B. per-*

tussis, which suppresses IL-12 production by DC through an IL-10-dependent mechanism (9). Interestingly suppression of IL-12 by the t10, c12-CLA isomer was not IL-10 dependent (data not shown). This strengthens the argument that individual PUFA may use different mechanisms through which they exert their immunosuppressive effects. IL-12 production by DC can also be down-regulated by a number of other factors including PGE₂ (31). Although c9, t11-CLA enhances production of PGE₂ in DC, it is not involved in IL-12 suppression, as inhibition of PGE₂ using specific inhibitors did not restore IL-12 production in LPS-stimulated DC (C. E. Loscher, K. H. Mills, and H. M. Roche, unpublished observation). This is consistent with other studies, which have demonstrated that the inhibitory effects of PUFA are not dependent on production of PGE₂ (25, 32); however, the involvement of other prostaglandins has not yet been investigated.

We also demonstrated c9, t11-CLA induction of IL-10 and suppression of IL-12 at the transcriptional level. Furthermore, IL-10 appears to exert its suppressive effect on IL-12 through inhibition of IL-12 transcription. This suggested the involvement of transcription factors, particularly NF- κ B, which is a key factor involved in production of IL-12. Previous studies have demonstrated that specific inhibitors of NF- κ B suppress IL-12 production (33) and anti-inflammatory molecules such as the glucocorticoid dexamethasone inhibit IL-12 production in LPS-stimulated monocytic cells by inhibiting NF- κ B activity (34). Moreover, IL-10 has recently been shown to exert its anti-inflammatory effects through inhibition of NF- κ B. Specifically, inhibition of NF- κ B activation in DC is associated with suppressed I κ B kinase activity (15), and

FIGURE 6. c9, t11-CLA modulates NF- κ B activation. DC were treated for 48 h with either c9, t11-CLA (50 μ M) or DMSO vehicle control and then stimulated with LPS (100 ng/ml) or medium alone for 0–5 h. Cells were harvested at a number of time points, and the cytosolic and nuclear fractions were extracted. NF- κ B activation (A) was measured in nuclear fractions by an NF- κ B binding assay. The mean luminescence value for DMSO vehicle control cells was normalized to a value of 1.0, and the fold difference for the CLA-treated cells were then calculated. NF- κ Bp65 and I κ B α proteins in the cytosolic (B and D) and nuclear (C and E) fractions were measured by Western blotting. Densitometric analysis was conducted on immunoblots, and results are expressed as fold change ($\pm$ SEM) relative to control (DMSO) levels for five replicates. A representative immunoblot is shown for four experiments. Lane 1, 0 h DMSO; lane 2, 0 h CLA; lane 3, 0.5 h DMSO; lane 4, 0.5 h CLA; lane 5, 1 h DMSO; lane 6, 1 h CLA; lane 7, 2 h DMSO; lane 8, 2 h CLA; lane 9, 5 h DMSO; lane 10, 5 h CLA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's t test for unpaired values, comparing c9, t11-CLA to DMSO control at each time point.



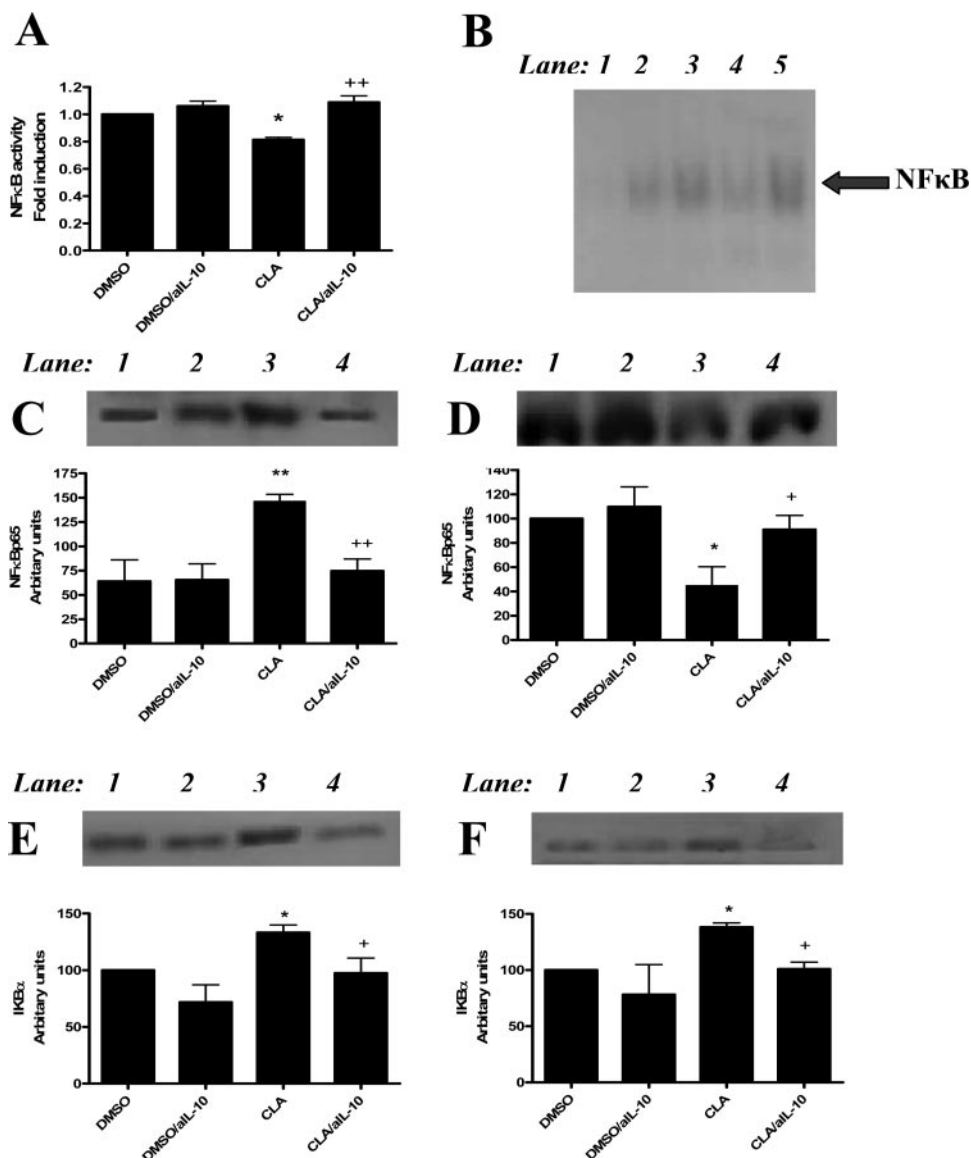
IL-10 can delay translocation of NF- κ Bp65 into the nucleus in monocytic cells (12). The present study clearly demonstrates that c9, t11-CLA suppresses activation of NF- κ B in both resting and LPS-stimulated DC. Analysis of cytosolic and nuclear NF- κ Bp65 and I κ B α revealed that this suppression was associated with inhibition of I κ B α degradation and delay of NF- κ Bp65 translocation into the nucleus. This is consistent with a recent study, which demonstrated that capsaicin, an anti-inflammatory nutrient, can inhibit I κ B α degradation after LPS stimulation in peritoneal macrophages (35). Although few studies have reported the effects of CLA on NF- κ B, our findings are consistent with a limited number of studies that have suggested that other PUFA can down-regulate NF- κ B activity. Treatment of murine RAW macrophages with a PUFA lipid emulsion resulted in reduced LPS-stimulated NF- κ B activity and I κ B phosphorylation (36). Recently, EPA was shown to have no effect on NF- κ B in human DC and T cells (2, 25). In contrast, it significantly inhibits NF- κ B activation in monocytic THP-1 cells, endothelial cells and Jurkat T cells (13, 14, 37). These conflicting data again indicate that individual PUFA may have distinct effects on different transcription factors or work in a cell-specific manner. Although these studies suggest that PUFA exert their effects through inhibition of transcription factors, clear evidence of the mechanism involved is lacking. We report that the inhibitory effect of c9, t11-CLA on NF- κ B was reversed in the presence of anti-IL-10 Abs indicating that this is an IL-10-dependent mechanism. Furthermore, we clearly demonstrate that IL-10 is directly involved in inhibition of I κ B α degradation and NF- κ Bp65 translocation into the nucleus. Although the ability of IL-10

to inhibit NF- κ B activation has been previously reported, this is the first study to demonstrate a role for IL-10 in suppression of NF- κ B activity by any PUFA; however, whether this mechanism is unique to c9, t11-CLA is yet to be defined.

The role of ERK in DC function remains unclear, but recent studies have highlighted its importance in the initiation of responses and in cell survival (38). This study demonstrates that culture with c9, t11-CLA results in significant activation of ERK in resting DC and enhances ERK activation in LPS-stimulated DC. Although the effects of c9, t11-CLA on LPS-induced ERK phosphorylation may be additive, it is still clear that treatment of DC with c9, t11-CLA results in early and sustained enhancement of ERK activation, which is important given the role of ERK in production of IL-10. The few attempts to address the role of PUFA on MAPK have demonstrated that EPA and DHA decrease phosphorylation of ERK in T cells and can selectively modulate JNK (2, 18). However, EPA has no effect on ERK or JNK activation in human DC (25). In contrast, our findings are consistent with a recent study, which demonstrated that CLA enhanced ERK activation in stromal vascular cells, which was associated with enhanced production of IL-6 and IL-8 (19). These observations provide further evidence for cell-specific and fatty acid-specific effects of individual PUFA.

The specific role of ERK in IL-10 production remains to be elucidated; however, it is clear from some studies that ERK activation is important for IL-10 production in DC (16). Furthermore, studies using specific ERK inhibitors have shown that ERK activation suppresses LPS-mediated IL-12 production in both macrophages (39)

FIGURE 7. The modulation of NF- κ B activation by c9, t11-CLA is mediated by IL-10. DC were treated for 48 h with either c9, t11-CLA (50 μ M) or DMSO vehicle control and then stimulated with LPS (100 ng/ml) in the presence or absence of a neutralizing anti-IL-10 Ab (10 μ g/ml) for 5 h. Cells were harvested and the cytosolic and nuclear fractions were extracted. NF- κ B activation (A) was measured in nuclear fractions by an NF- κ B binding assay. This was confirmed by EMSA and a representative autorad is shown (B). Lane 1, Unstimulated control; lane 2, DMSO; lane 3, DMSO/anti-IL-10; lane 4, CLA; lane 5, CLA/anti-IL-10. NF- κ Bp65 and I κ B α proteins in the cytosolic (C and E) and nuclear (D and F) fractions were measured by Western blotting. Densitometric analysis was conducted on immunoblots and results are expressed as arbitrary units ($\pm$ SEM). A representative immunoblot is shown for four experiments. Lane 1, DMSO; lane 2, DMSO plus anti-IL-10; lane 3, CLA; lane 4, CLA plus anti-IL-10. *, $p < 0.05$, **, $p < 0.01$, ANOVA, comparing c9, t11-CLA to DMSO; +, $p < 0.05$, ++, $p < 0.01$, ANOVA, comparing c9, t11-CLA vs c9, t11-CLA with anti-IL-10 Ab.



and DC (17). We report that ERK activation is essential for IL-10 production in DC, as inhibition of ERK using a specific inhibitor completely abrogated IL-10 production. Furthermore, inhibition of ERK activation also reverses the c9, t11-CLA-induced suppression of IL-12 in LPS-stimulated DC. These data clearly demonstrate that ERK-mediated induction of IL-10 is involved in the suppression of IL-12 production by murine DC after treatment with c9, t11-CLA. This conclusion is consistent with a report that demonstrated that *Leishmania* lipophosphoglycan exerts its inhibitory effects on IL-12 production by macrophages through activation of ERK (39) and inhibition of ERK using specific inhibitors, results in enhancement of IL-12 production by DC (17). The mechanism of ERK induction in DC has not yet been fully defined nor has the mechanism by which PUFA modulate its activation. It has been suggested that PGJ₂ can enhance ERK activation (40). This may be important in determining the mechanism of action of PUFA given that they readily induce production of a variety of prostaglandins from cells, including PGJ₂. Although we have ruled out the involvement of PGE₂ in suppression of IL-12 by c9, t11-CLA, it may be important to examine the involvement of other prostaglandins in mediating this effect.

Our findings demonstrate that c9, t11-CLA can suppress IL-12 and enhance IL-10 production by DC, a cytokine profile that has

been associated with suppression of Th1 and enhancement of Tr cell responses. It has previously been reported that PUFA promotes activation-induced cell death in T cells that are polarized toward the Th1 phenotype in vitro (41). Furthermore, CLA has been shown to protect against inflammatory bowel disease, a disease mediated by Th1 cells (5). Our study demonstrates that c9, t11-CLA can act at the level of the DC, inhibiting cytokines that promote Th2 or Tr cells, and thereby provides a possible mechanism whereby it may be capable of protecting against Th1-mediated diseases. This is the first study to provide clear evidence of a mechanism whereby PUFA may exert their anti-inflammatory effects.

Disclosures

The authors have no financial conflict of interest.

References

- Lo, C. J., K. C. Chiu, M. Fu, R. Lo, and S. Helton. 1999. Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NF κ B activity. *J. Surg. Res.* 82: 216–221.
- Zeyda, M., A. B. Szekeres, M. D. Saemann, R. Geyeregger, H. Stockinger, G. J. Zlabinger, W. Waldhausl, and T. M. Stulnig. 2003. Suppression of T cell

- signaling by polyunsaturated fatty acids: selectivity in inhibition of mitogen-activated protein kinase and nuclear factor activation. *J. Immunol.* 170: 6033–6039.
3. Calder, P. C., and R. B. Zurier. 2001. Polyunsaturated fatty acids and rheumatoid arthritis. *Curr. Opin. Clin. Nutr. Metab. Care* 4: 115–121.
 4. Roche, H. M., E. Noone, A. Nugent, and M. J. Gibney. 2001. Conjugated linoleic acid: a novel therapeutic nutrient? *Nutr. Res. Rev.* 14: 173–187.
 5. Bassaganya-Riera, J., K. Reynolds, S. Martino-Catt, Y. Cui, L. Hennighausen, F. Gonzalez, J. Rohrer, A. U. Benninghoff, and R. Hontecillas. 2004. Activation of PPAR γ and δ by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. *Gastroenterology* 127: 777–791.
 6. MacDonald, H. B. 2000. Conjugated linoleic acid and disease prevention: a review of current knowledge. *J. Am. Coll. Nutr.* 19: 111S–118S.
 7. Moser, M., and K. M. Murphy. 2000. Dendritic cell regulation of TH1-TH2 development. *Nat. Immunol.* 1: 199–205.
 8. Whelan, M., M. M. Harnett, K. M. Houston, V. Patel, W. Harnett, and K. P. Rigley. 2000. A filarial nematode-secreted product signals dendritic cells to acquire a phenotype that drives development of Th2 cells. *J. Immunol.* 164: 6453–6460.
 9. McGuirk, P., C. McCann, and K. H. Mills. 2002. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. *J. Exp. Med.* 195: 221–231.
 10. Lavelle, E. C., E. McNeela, M. E. Armstrong, O. Leavy, S. C. Higgins, and K. H. Mills. 2003. Cholera toxin promotes the induction of regulatory T cells specific for bystander antigens by modulating dendritic cell activation. *J. Immunol.* 171: 2384–2392.
 11. Vandenbroeck, K., I. Alloza, M. Gadina, and P. Matthys. 2004. Inhibiting cytokines of the interleukin-12 family: recent advances and novel challenges. *J. Pharm. Pharmacol.* 56: 145–160.
 12. Driessler, F., K. Venstrom, R. Sabat, K. Asadullah, and A. J. Schottelius. 2004. Molecular mechanisms of interleukin-10-mediated inhibition of NF- κ B activity: a role for p50. *Clin. Exp. Immunol.* 135: 64–73.
 13. Zhao, Y., S. Joshi-Barve, S. Barve, and L. H. Chen. 2004. Eicosapentaenoic acid prevents LPS-induced TNF- α expression by preventing NF- κ B activation. *J. Am. Coll. Nutr.* 23: 71–78.
 14. Denys, A., A. Hichami, and N. A. Khan. 2005. N-3 PUFAs modulate T-cell activation via protein kinase C- α and - ϵ and NF- κ B signaling pathway. *J. Lipid Res.* 46: 752–758.
 15. Bhattacharyya, S., P. Sen, M. Wallet, B. Long, A. S. Baldwin, Jr., and R. Tisch. 2004. Immunoregulation of dendritic cells by IL-10 is mediated through suppression of the PI3K/Akt pathway and of I κ B kinase activity. *Blood* 104: 1100–1109.
 16. Yi, A. K., J. G. Yoon, S. J. Yeo, S. C. Hong, B. K. English, and A. M. Krieg. 2002. Role of mitogen-activated protein kinases in CpG DNA-mediated IL-10 and IL-12 production: central role of extracellular signal-regulated kinase in the negative feedback loop of the CpG DNA-mediated Th1 response. *J. Immunol.* 168: 4711–4720.
 17. Puig-Kroger, A., M. Relloso, O. Fernandez-Capetillo, A. Zubiaga, A. Silva, C. Bernabeu, and A. L. Corbi. 2001. Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. *Blood* 98: 2175–2182.
 18. Denys, A., A. Hichami, and N. A. Khan. 2001. Eicosapentaenoic acid and docosahexaenoic acid modulate MAP kinase (ERK1/ERK2) signaling in human T cells. *J. Lipid Res.* 42: 2015–2020.
 19. Brown, J. M., M. S. Boysen, S. Chung, O. Fabiyi, R. F. Morrison, S. Mandrup, and M. K. McIntosh. 2004. Conjugated linoleic acid induces human adipocyte delipidation: autocrine/paracrine regulation of MEK/ERK signaling by adipocytokines. *J. Biol. Chem.* 279: 26735–26747.
 20. Yu, Y., P. H. Correll, and J. P. Vanden Heuvel. 2002. Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPAR γ -dependent mechanism. *Biochim. Biophys. Acta* 1581: 89–99.
 21. Liu, Z., K. Geboes, H. Heremans, L. Overbergh, C. Mathieu, P. Rutgeerts, and J. L. Ceuppens. 2001. Role of interleukin-12 in the induction of mucosal inflammation and abrogation of regulatory T cell function in chronic experimental colitis. *Eur. J. Immunol.* 31: 1550–1560.
 22. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3: 133–146.
 23. Fritsche, K. L., M. Byrge, and C. Feng. 1999. Dietary omega-3 polyunsaturated fatty acids from fish oil reduce interleukin-12 and interferon- γ production in mice. *Immunol. Lett.* 65: 167–173.
 24. Albers, R., M. Bol, R. Bleumink, A. Willems, C. Blonk, and R. Pieters. 2002. Effects of dietary lipids on immune function in a murine sensitisation model. *Br. J. Nutr.* 88: 291–299.
 25. Zeyda, M., M. D. Saemann, K. M. Stuhlmeier, D. G. Mascher, P. N. Nowotny, G. J. Zlabinger, W. Waldhausl, and T. M. Stulnig. 2005. Polyunsaturated fatty acids block dendritic cell activation and function independently of NF- κ B activation. *J. Biol. Chem.* 280: 14293–14301.
 26. Trinchieri, G., S. Pflanz, and R. A. Kastelein. 2003. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 19: 641–644.
 27. Conti, P., D. Kempuraj, K. Kandere, M. Di Gioacchino, R. C. Barbacane, M. L. Castellani, M. Felaco, W. Boucher, R. Letourneau, and T. C. Theoharides. 2003. IL-10, an inflammatory/inhibitory cytokine, but not always. *Immunol. Lett.* 86: 123–129.
 28. Ly, L. H., R. S. Smith III, R. S. Chapkin, and D. N. McMurray. 2005. Dietary n-3 polyunsaturated fatty acids suppress splenic CD4⁺ T cell function in interleukin (IL)-10^{-/-} mice. *Clin. Exp. Immunol.* 139: 202–209.
 29. Song, C., X. Li, B. E. Leonard, and D. F. Horrobin. 2003. Effects of dietary n-3 or n-6 fatty acids on interleukin-1 β -induced anxiety, stress, and inflammatory responses in rats. *J. Lipid Res.* 44: 1984–1991.
 30. Lynch, A. M., M. Moore, S. Craig, P. E. Lonergan, D. S. Martin, and M. A. Lynch. 2003. Analysis of interleukin-1 β -induced cell signaling activation in rat hippocampus following exposure to γ irradiation: protective effect of eicosapentaenoic acid. *J. Biol. Chem.* 278: 51075–51084.
 31. Harizi, H., M. Juzan, V. Pitard, J. F. Moreau, and N. Gualde. 2002. Cyclooxygenase-2-induced prostaglandin E₂ enhances the production of endogenous IL-10, which down-regulates dendritic cell functions. *J. Immunol.* 168: 2255–2263.
 32. Costabile, M., C. S. Hii, M. Melino, C. Easton, and A. Ferrante. 2005. The immunomodulatory effects of novel β -oxa, β -thia, and γ -thia polyunsaturated fatty acids on human T lymphocyte proliferation, cytokine production, and activation of protein kinase C and MAPKs. *J. Immunol.* 174: 233–243.
 33. Kabashima, K., T. Honda, Y. Nunokawa, and Y. Miyachi. 2004. A new NF- κ B inhibitor attenuates a Th1 type immune response in a murine model. *FEBS Lett.* 578: 36–40.
 34. Ma, W., K. Gee, W. Lim, K. Chambers, J. B. Angel, M. Kozlowski, and A. Kumar. 2004. Dexamethasone inhibits IL-12p40 production in lipopolysaccharide-stimulated human monocytes by down-regulating the activity of c-Jun N-terminal kinase, the activation protein-1, and NF- κ B transcription factors. *J. Immunol.* 172: 318–330.
 35. Kim, C. S., T. Kawada, B. S. Kim, I. S. Han, S. Y. Choe, T. Kurata, and R. Yu. 2003. Capsaicin exhibits anti-inflammatory property by inhibiting I κ B- α degradation in LPS-stimulated peritoneal macrophages. *Cell Signal.* 15: 299–306.
 36. Novak, T. E., T. A. Babcock, D. H. Jho, W. S. Helton, and N. J. Espat. 2003. NF- κ B inhibition by omega-3 fatty acids modulates LPS-stimulated macrophage TNF- α transcription. *Am. J. Physiol.* 284: L84–L89.
 37. Chaudhary, A., A. Mishra, and S. Sethi. 2004. Oxidized omega-3 fatty acids inhibit pro-inflammatory responses in glomerular endothelial cells. *Nephron Exp. Nephrol.* 97: e136–e145.
 38. Ballif, B. A., and J. Blenis. 2001. Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ.* 12: 397–408.
 39. Feng, G. J., H. S. Goodridge, M. M. Harnett, X. Q. Wei, A. V. Nikolaev, A. P. Higson, and F. Y. Liew. 1999. Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide-mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: *Leishmania phosphoglycans* subvert macrophage IL-12 production by targeting ERK MAP kinase. *J. Immunol.* 163: 6403–6412.
 40. Ruiz, P. A., S. C. Kim, R. B. Sartor, and D. Haller. 2004. 15-Deoxy- δ 12,14-prostaglandin J₂-mediated ERK signaling inhibits gram-negative bacteria-induced RelA phosphorylation and interleukin-6 gene expression in intestinal epithelial cells through modulation of protein phosphatase 2A activity. *J. Biol. Chem.* 279: 36103–36111.
 41. Switzer, K. C., Y. Y. Fan, N. Wang, D. N. McMurray, and R. S. Chapkin. 2004. Dietary n-3 polyunsaturated fatty acids promote activation-induced cell death in Th1-polarized murine CD4⁺ T-cells. *J. Lipid Res.* 45: 1482–1492.