

A Conjugated Linoleic Acid-Enriched Beef Diet Attenuates Lipopolysaccharide-Induced Inflammation in Mice in Part through PPAR γ -Mediated Suppression of Toll-Like Receptor 4¹⁻³

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Abstract

Conjugated linoleic acid (CLA) is a PUFA found in beef and dairy products that has immunoregulatory properties. The level of CLA in beef can be enhanced by feeding cattle fresh grass rather than concentrates. This study determined the effect of feeding a high-CLA beef diet on inflammation in an in vivo model of septic shock. Mice were fed a high-CLA beef (4.3% total fatty acid composition) or low-CLA beef diet (0.84% total fatty acid composition) for 6 wk. Lipopolysaccharide (LPS; 3 μ g) or sterile PBS was injected i.v. and serum was harvested 6 h after injection. Serum interleukin (IL)-1 β , IL-12p70, IL-12p40, and interferon- γ concentrations were significantly reduced in response to the LPS challenge in the high-CLA beef diet group. Bone marrow-derived dendritic cells (BMDC) from the high-CLA beef diet group had significantly less IL-12 and more IL-10 in response to ex vivo LPS stimulation. Furthermore, toll-like receptor 4 (TLR4) and CD14 protein and mRNA expression on BMDC was significantly attenuated in the high-CLA compared with the low-CLA beef diet group. Complimentary in vitro experiments to determine the specificity of the effect showed that synthetic *cis9*, *trans11*-CLA suppressed surface expression of CD14 and TLR4 on BMDC. Treatment with the PPAR γ inhibitor GW9662 partially reversed TLR4 expression in immature BMDC. The results of this study demonstrate that feeding a diet enriched in high-beef CLA exerts profound antiinflammatory effects in vivo within the context of LPS-induced sepsis. In addition, downregulation of BMDC TLR4 is mediated through induction of PPAR γ . J. Nutr. doi: 10.3945/jn.109.113035.

Introduction

Sepsis is an inflammatory condition that is caused by an overwhelming dysregulation of the immune system. Endotoxin or lipopolysaccharide (LPS)⁹ is an essential component of Gram-

negative bacteria and is a causative factor in many cases of sepsis (1). Once released into the circulation LPS has potent effects that can cause overproduction of proinflammatory cytokines, such as tumor necrosis factor- α (TNF α), interleukin (IL)-1 β , IL-6, interferon- γ (IFN γ), and IL-12, which play a major role in the pathogenesis of sepsis. Indeed, circulating levels of these cytokines can be used as biomarkers for predicting the outcome of patients with sepsis (2,3).

Cells of the innate immune system recognize the LPS component of Gram negative bacteria through the cell surface receptor toll-like receptor 4 (TLR4). Once released into the bloodstream, LPS binds to LPS binding protein and mediates its effects by signaling through TLR4 and its coreceptor, CD14 (4). The TLR4 CD14 signaling complex plays a major role in the pathogenesis of sepsis. In addition, TLR4-deficient mice are hyporesponsive to LPS and resistant to endotoxin-induced septic shock (5). In humans, TLR4 polymorphisms have been associated with increased risk of Gram negative infection and sepsis (Asp299Gly; 896G) (6).

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³ Supplemental Figure 1 and Tables 1 and 2 are available with the online posting of this paper at jn.nutrition.org.

⁹ Abbreviations used: BMDC, bone marrow-derived dendritic cell; CLA, conjugated linoleic acid; *c9*, *t11*-CLA, *cis9*, *trans11*-CLA; DC, dendritic cell; DMSO, dimethylsulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony stimulating factor; IFN γ , interferon- γ ; IKK α , I κ B kinase; IL, interleukin; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; TLR4, Toll-like receptor 4; TVA, *trans*-vaccenic acid; TNF α , tumor necrosis factor- α .

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Dendritic cells (DC) provide the first line of defense against pathogens and influence the nature of the subsequent adaptive immune response (7). Their production of IL-12 promotes differentiation of naïve Th cells to Th1 cells, whereas IL-10 production promotes regulatory T cell differentiation (8). DC are critical for initiating the overwhelming cytokine response during the early phase of sepsis (9). There is evidence that the number of DC become depleted during sepsis (10,11), the extent of DC loss correlates with mortality (12), as DC dysfunction and reduced IL-12 impede clearance of infection (13).

Dietary lipids can modulate the inflammatory response; SFA are associated with proinflammatory disease states. SFA activate TLR4, thereby exacerbating inflammatory effects in many inflammatory disorders (14). In contrast, monounsaturated fatty acids and PUFA tend to be antiinflammatory (15–17). Indeed, nutritional intervention with both monounsaturated fatty acids and PUFA increases survival in animal models and human cases of septic shock (18,19). Conjugated linoleic acid (CLA) refers to the positional and geometric isomers of linoleic acid (20). It is found in beef and dairy products and may have positive health effects in animal models of inflammation (21,22). *cis*9, *trans*11-CLA (*c*9, *t*11-CLA) is the predominant natural isomer accounting for up to 90% of total dietary CLA (22), which has been shown to have antiinflammatory properties (23–25). Feeding cattle on fresh pasture can enhance the level of CLA and its precursor *trans*-vaccenic acid (TVA) in beef (26,27). Therefore, natural beef-derived CLA may be more potent than synthetic CLA.

*c*9, *t*11-CLA is known to inhibit DC maturation and have antiinflammatory effects on DC following activation with LPS *in vitro* (23,28). CLA is known to be a PPAR γ ligand and it is possible that its antiinflammatory effects can be attributed to this nuclear hormone receptor (29,30). Recent evidence has shown that the PPAR γ agonist pioglitazone inhibits TLR4 expression in human monocytes (31).

In this study, we examined the antiinflammatory effects of a high-CLA beef diet in a murine model of septic shock.

Materials and Methods

Materials. LPS from *Escherichia coli* (serotype 127:B8) and synthetic *c*9, *t*11-CLA were obtained from Alexis Chemicals. The PPAR γ inhibitor GW9662 was purchased from Sigma. *c*9, *t*11-CLA and GW9662 were dissolved in sterile dimethylsulfoxide (DMSO) and stored at -20°C away from light sources.

Mouse experiments and nutritional intervention. All experiments were performed in accordance with current EU and Irish Department of Health guidelines on the use of experimental animals. Female BALB/c mice were purchased from Harlan, UK. Mice were fed an irradiated diet from 6 wk of age. The mice were randomly assigned to either a low-CLA beef diet group or a high-CLA beef diet group for a 6-wk period. Mice consumed food and water *ad libitum*. Food consumption and weight were monitored every second day. Weight and food consumption did not differ between the groups. All diets were stored at 4°C and were provided fresh to the mice every second day.

Generation of beef-derived feeds. Charolais-cross steers were fed a high-concentrate/straw ration to generate the low-CLA beef diet. For the high-CLA beef diet, Charolais-cross heifers were offered grazed perennial ryegrass supplemented with sunflower oil and fish oil. After 150 d, *psoas* muscle, *longissimus* muscle, and subcutaneous adipose tissue were collected and pooled within ration type to yield a 35% fat beef product. This was then freeze-dried and incorporated into the mouse feed.

Fatty acid composition of dietary feeds and adipose tissue. The composition of the diets, in particular the fatty acid composition of the high- and low-CLA beef-enriched diets, is presented in **Supplemental Table 1**. In addition, the adipose tissue fatty acid composition of mice fed the high- or low-CLA beef diets was determined as a biomarker of dietary fat modification. Briefly, lipid was extracted according to Folch *et al.* (32), dried lipid samples were methylated, and acidic *trans*-esterification was carried out as outlined by Kramer and Zhou (33).

LPS shock model. After 6 wk of consuming either a high-CLA or a low-CLA beef diet, LPS shock was induced by injecting mice ($n = 5$ per dietary group) *i.v.* with $3 \mu\text{g}$ LPS or sterile PBS ($n = 5$ per dietary group) (Alexis). After 6 h mice were killed by cervical dislocation. Blood and adipose tissue were collected. The blood was allowed to clot overnight at 4°C and serum removed after centrifugation at $12000 \times g$ for 5 min. Serum samples were stored at -20°C and analyzed for the presence of cytokines using specific immunoassays.

Isolation and culture of bone marrow-derived DC. Bone marrow-derived DC (BMDC) were prepared by culturing bone marrow cells obtained from the femurs and tibia of mice in RPMI 1640 medium in the presence of granulocyte macrophage colony stimulating factor (GM-CSF) (50 nmol/L ; Sigma-Aldrich). Cells from low-CLA and high-CLA beef diet groups were cultured in the presence of GM-CSF (50 nmol/L ; Sigma-Aldrich). Alternatively, DMSO (vehicle control) or *c*9, *t*11-CLA ($50 \mu\text{mol}$) was added to the cells on d 1 of culture. The cells were cultured at 37°C in 5% CO_2 for 3 d, when the supernatant was carefully removed without disturbing the cell monolayer and replaced with fresh medium containing GM-CSF. On d 7 of culture, cells were collected, counted, and used. In other experiments 48 h prior to cell collection, the PPAR γ inhibitor GW9662 (10 nmol/L) was added.

Cytokine analysis. Supernatants from the DC activation experiments were analyzed for IL-12p70 and IL-10 concentrations using commercial DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. Serum from the LPS shock model experiments was analyzed for IL-12p70, IL-12p40, IL-1 β , and IFN γ using commercial DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions.

mRNA analysis of BMDC. BMDC were isolated from mice fed either a high-CLA or low-CLA beef diet and cultured in the presence of GM-CSF (50 nmol/L ; Sigma-Aldrich). On d 7 of culture, DC (2×10^6 cells) were cultured in 6-well plates with LPS (100 nmol/L) or medium alone for 0–12 h. After removal of the supernatants, the cells were harvested and TRI Reagent (Molecular Research Centre) was added. Total RNA was extracted from the cells according to the manufacturer's instructions. RNA ($2 \mu\text{g}$) was treated with RNase-free DNase to remove contaminating genomic DNA (DNase I Amplification Grade; Invitrogen). Single-stranded cDNA was prepared using the High Capacity cDNA archive kit (Applied Biosystems). mRNA expression was quantified by real-time PCR on an ABI 7700 Sequence Detection system (Perkin-Elmer Applied Biosystems). TaqMan real-time PCR was performed for TLR4, IL-10, and IL-12p35 using Pre-Developed Assay Reagent kits. For each sample, results were normalized by dividing the amount of target gene by the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed relative to the low-CLA beef control treatment for the non-LPS-stimulated cells.

Analysis of protein expression in BMDC by Western blotting. BMDC were plated at 2×10^6 cells/well in 6-well tissue culture plates. These cells were then stimulated for 0, 2, or 5 h with 100 nmol/L LPS. Cytosolic and nuclear extracts were prepared using Nuclear extract kit (Active Motif). The concentration of protein in the nuclear and cytoplasmic samples was quantified by Bradford assay (Bio-Rad Laboratories). A total of $15 \mu\text{g}$ of protein was separated by SDS-PAGE for Western immunoblotting. Antibodies were used to detect nuclear factor-kappa B (NF- κB) p65, I κB kinase- α (IKK α), PPAR γ (Santa Cruz Biotechnology; 1/750–1000 dilution), and TLR4 (Abcam; 1/1000 dilution) in nuclear and/or cytosolic extracts. Membranes were washed with PBS-T and incubated for 1 h at room temperature with anti-rabbit IgG (Santa Cruz Biotechnology; 1:1000–

2000). 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). Where required, membranes were stripped by incubating them in stripping buffer (Tris, pH 6.8, β -mercaptoethanol, SDS) for 30 min at 50°C before probing with a subsequent antibody.

Transcription factor assay. NF- κ B activity was determined using a TransAM NF- κ B assay (Active Motif), which measured NF- κ Bp65:DNA binding. The assay was used in accordance with the manufacturer's instructions. For each kit, 2 μ g of nuclear protein was incubated in a 96-well plate containing the consensus sequence for NF- κ B (5'-GGGACTTCC-3') for 1 h. After washing, a primary antibody that recognizes an epitope on NF- κ Bp65 upon DNA binding was applied for 1 h. After subsequent washing, a secondary antibody conjugated to HRP was added for 1 h, followed by the addition of a chemiluminescent substrate, the color product of which was quantified by luminescence.

Effect of c9, t11-CLA on surface marker expression by flow cytometry. The expression of CD14 and TLR4 on DC was assessed using an anti-mouse CD14 (anti-mouse, eBioscience), TLR4-MD2 (anti-mouse, eBioscience), and appropriately labeled isotype-matched antibodies, which acted as controls. After incubation for 30 min at 4°C, cells were washed and immunofluorescence analysis was performed on a FACScalibur (BD Biosciences) using Cell Quest software.

Statistics. Data are presented as means \pm SEM. Data that were not normally distributed were log-transformed before analysis. ANOVA was used to determine significant differences between conditions. Two-way ANOVA was used to determine significant differences between dietary conditions and LPS-dependant effects and their interaction. When this indicated significance ($P < 0.05$), we used post hoc Bonferroni test analysis to determine which conditions were significantly different from each other. Cells alone and DMSO (vehicle control) treated cells did not differ; therefore, DMSO was used as the reference treatment.

Results

Adipose tissue c9, t11-CLA. Enrichment of diet for 6 wk with CLA lead to a 4.1-fold increase in adipose tissue c9, t11-CLA and TVA concentrations ($P < 0.01$). This increase was at the expense of oleic acid 18:1 (n-9) and palmitic acid (16:0) (Supplemental Table 2).

LPS-induced serum and BMDC cytokine responses. We examined the effect of high-CLA and low-CLA beef diets in a murine model of septic shock. An i.v. injection of LPS significantly increased circulating IL-12p70, IL-12p40, IL-1 β , and IFN γ concentrations in both the low-CLA beef diet group ($P <$

TABLE 1 A high-CLA beef diet attenuates LPS-induced septic shock in BALB/c mice¹

	Low-CLA beef diet	High-CLA beef diet	Low-CLA beef diet + LPS	High-CLA beef diet + LPS
	<i>pmol/L</i>			
IL-12p70	5.5 \pm 2	1.22 \pm 2	33.6 \pm 14 ^b	21.2 \pm 7*
IL-12p40	29.1 \pm 6	25.4 \pm 8	1201.3 \pm 170 ^c	183 \pm 49** ^a
IL-1 β	ND	ND	38.4 \pm 12 ^c	12.3 \pm 10*** ^c
IFN γ	19.4 \pm 0.04	19.1 \pm 0.7	114.8 \pm 74 ^c	22.4 \pm 2** ^b

¹ Values are means \pm SEM, $n = 4-5$. Asterisks indicate different from the corresponding low-CLA: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.002$. Letters indicate different from corresponding control (no LPS): ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$. ND, not determined.

0.01) and the high-CLA beef diet group ($P < 0.05$) (Table 1). Following LPS injection, circulating concentrations of IL-12p70 ($P < 0.05$), IL-12p40 ($P < 0.001$), IL-1 β ($P < 0.001$), and IFN γ ($P < 0.01$) were lower in the high-CLA compared with the low-CLA beef diet group (Table 1).

This study focused on how the high-CLA diet modulated the inflammatory response of DC. In unstimulated DC, IL-10 and IL-12p70 mRNA concentrations did not differ between the low- and high-CLA beef diet-fed mice. Cytokine mRNA expression increased significantly 6 and 12 h post-LPS stimulation in both groups. DC from the high-CLA beef diet group had greater IL-10 mRNA expression 6 h post-LPS ($P < 0.05$), which normalized to equivalent levels as the low-CLA diet 12 h post-LPS stimulation (Fig. 1A). Conversely, IL-12p70 mRNA expression decreased 12 h following LPS stimulation in DC from the high-CLA beef diet group ($P < 0.05$) (Fig. 1B).

TLR4 expression in BMDC. TLR4 and CD14 are involved in the recognition of LPS (34), therefore their expression was determined on DC from the high- and low-CLA beef diets following stimulation with LPS for 0–12 h. LPS-stimulated DC from the high-CLA beef diet group had significantly lower TLR4 protein (Fig. 2A; $P < 0.05$) and mRNA (Fig. 2C; $P < 0.05$) expression compared with the low-CLA beef diet group. In addition, TLR4 protein concentrations were also decreased in unstimulated DC (Fig. 2A; $P < 0.01$).

Prior to LPS stimulation, CD14 protein expression was less on DC from the high-CLA compared with the low-CLA beef diet group (Fig. 2B; $P < 0.05$). Furthermore, when examined 5 h after stimulation with LPS, there was a significant reduction in

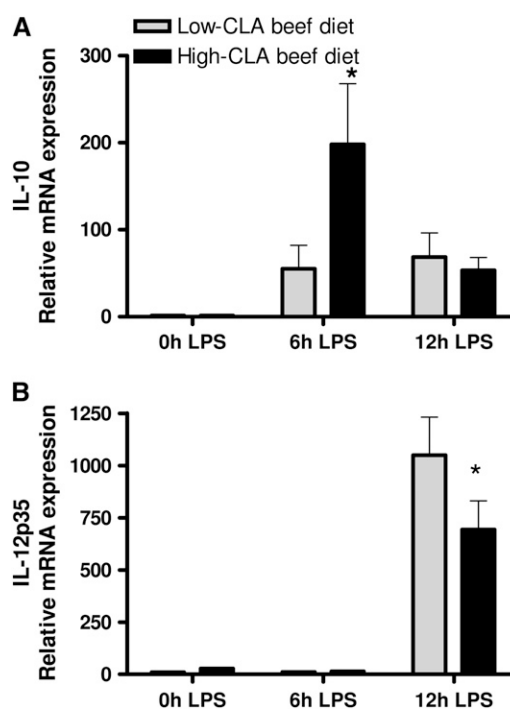


FIGURE 1 A high-CLA beef diet modulates IL-10 (A) and IL-12p35 (B) expression in BMDC from BALB/c mice. Expression of IL-10 (A) and IL-12p35 (B) were measured using Taqman RT-PCR. mRNA levels were normalized to GAPDH and the results expressed as fold induction relative to unstimulated DC from the low-CLA beef diet group. The results represent the mean \pm SEM, $n = 3$. *Different from low CLA, $P < 0.05$.

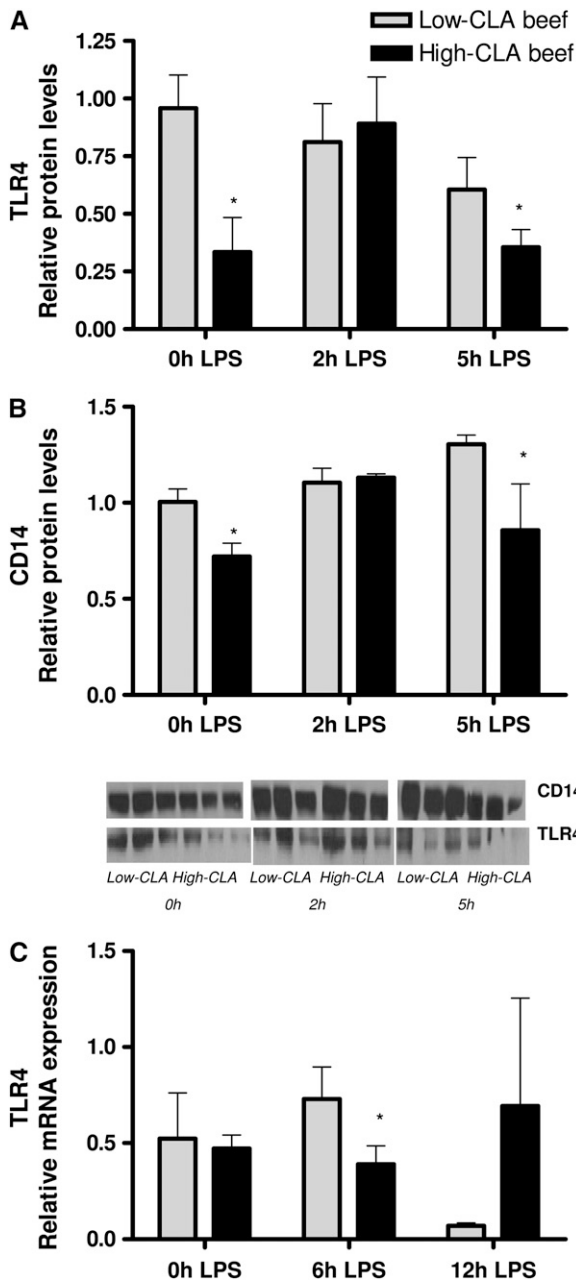


FIGURE 2 Feeding a high-CLA beef diet suppresses TLR4 (A,C) and CD14 (B) expression in BMDC from BALB/c mice. Expression of TLR4 (A) and CD14 (B) protein was measured 0–5 h post-LPS stimulation by Western blot. Densitometric analysis was conducted on immunoblots. Results are expressed \pm SEM, $n = 3$, relative to unstimulated DC from the low-CLA beef diet group. A representative immunoblot is shown. (C) TLR4 mRNA expression was measured using Taqman PCR. mRNA levels were normalized to GAPDH and the results expressed relative to unstimulated DC from mice fed the low-CLA beef diet group. The results represent the mean \pm SEM, $n = 3$. *Different from low CLA, $P < 0.05$.

CD14 protein expression in DC from the high- compared with the low-CLA beef diet group (Fig. 2C; $P < 0.05$).

NF- κ B activation in BMDC. We examined expression of nuclear and cytosolic NF- κ B and cytosolic IKK α . Cytosolic NF- κ Bp65 expression did not differ between the low- and high-CLA beef diet groups in either unstimulated DC or in DC stimulated for 2 h with LPS (Fig. 3A). However, following 5 h

LPS activation, there was greater cytosolic NF- κ Bp65 expression in DC from mice in the high-CLA beef diet group (Fig. 3A; $P < 0.05$). Nuclear NF- κ Bp65 was not detected in resting DC. Stimulation with LPS induced nuclear NF- κ Bp65 expression in DC from the mice in the high- and low-CLA beef diet groups; however, the groups did not differ after LPS stimulation (Fig. 3B). A TransAM assay determined that NF- κ Bp65 DNA binding did not differ between diet groups in unstimulated DC. Following stimulation with LPS for 5 h, there was less NF- κ Bp65:DNA binding activity in the high-CLA beef diet mice ($P < 0.05$; Fig. 3C). This reduction in NF- κ Bp65:DNA binding activity was accompanied by a significant reduction in IKK α protein levels in both resting and LPS stimulated DC from mice fed the high-CLA beef diet (Fig. 3D; $P < 0.05$).

PPAR γ expression in BMDC. PPAR γ is a nuclear hormone receptor that is known to have antiinflammatory effects and has been shown to reduce NF- κ B activation in DC (35). In the cytosolic fraction, DC from the 2 diet groups did not differ prior to LPS stimulation; however, PPAR γ expression was greater 2 and 5 h after LPS stimulation in DC from the mice fed the high-CLA beef diet (Fig. 4B; $P < 0.05$). Nuclear PPAR γ expression in unstimulated DC was greater in the high-CLA beef diet mice (Fig. 4A; $P < 0.05$). Furthermore, the nuclear PPAR γ protein level was greater in the LPS-activated DC from the high-compared with the low-CLA beef diet mice (Fig. 4B; $P < 0.05$).

Suppression of TLR4 but not CD14 by *c9*, *t11*-CLA is PPAR γ dependent. It has been demonstrated that activation of PPAR γ with the specific agonist pioglitazone can suppress expression of TLR4 in macrophages from db/db mice and in peripheral blood monocytes (PBMC) from healthy volunteers (31). Therefore, we determined whether PPAR γ activation played a role in the suppression of TLR4 expression in DC treated with *c9*, *t11*-CLA, which suppressed TLR4 (MFI 61 vs. MFI 42) in unstimulated DC; this was reversed by the PPAR γ inhibitor, GW9662 (MFI 42 vs. MFI 72). CLA also decreased the expression of CD14 in resting DC (MFI 125 vs. MFI 64); however, this effect was not reversed in the presence of the PPAR γ inhibitor, GW9662 (Supplemental Fig. 1).

Discussion

Sepsis is a biological syndrome that can occur following infection or injury and is characterized by an initial surge of proinflammatory cytokines, including TNF α , IL-1 β , IL-12, and IFN γ , in the bloodstream (36). This leads to vasodilation, vascular and endothelial permeability, and hypotension, which can result in multiple organ failure and death (37). A range of pharmaceutical treatments, including recombinant human activated protein C (38), and anti-TNF α monoclonal antibodies (39) dampen the inflammatory responses and improve survival. Nevertheless, patient mortality is still very high.

Several studies have demonstrated better clinical outcomes and reduced mortality following addition of the dietary fatty acids eicosapentanoic acid and γ -linolenic acid to the feeding regimen of sepsis patients (19,40). However, little is known in relation to the potential role of another fatty acid, CLA, which is naturally enriched in beef from animals fed on pasture rather than concentrates or straw. In addition, there is also significant enrichment with TVA, which can be endogenously converted to the *c9*, *t11*-CLA isomer in mammals (41). The potential antiinflammatory benefits of feeding synthetic CLA have been

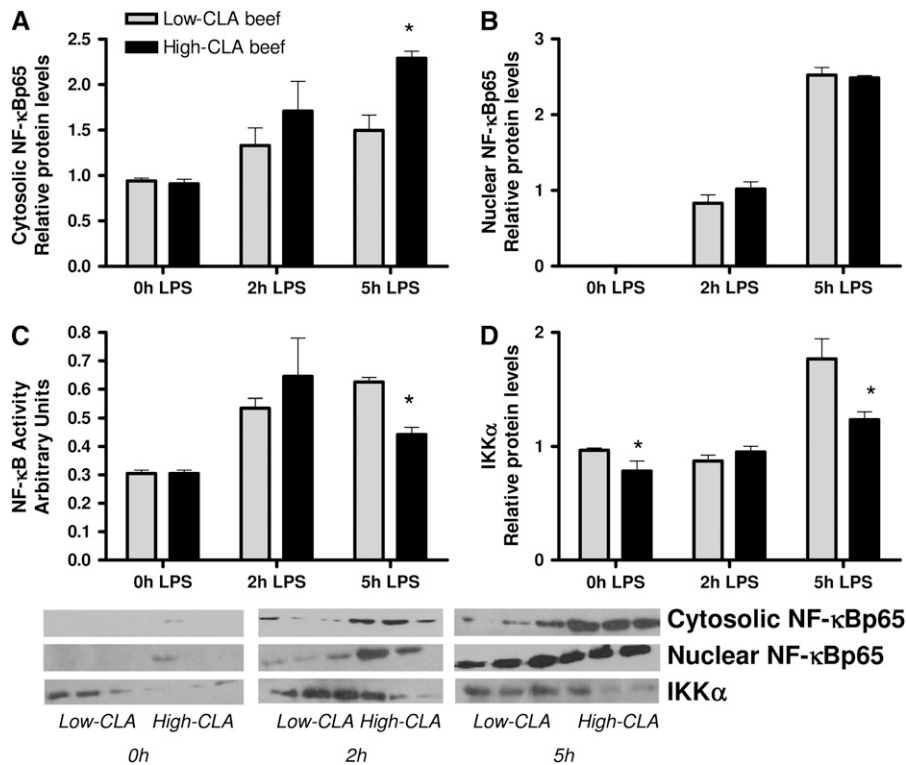


FIGURE 3 A high-CLA beef diet decreases NF- κ B activation (A,B,D) and IKK α (C) expression in BMDC from BALB/c mice. NF- κ Bp65 in the cytosolic (A) and nuclear (B) fractions and IKK α cytosolic protein (D) were measured by Western blotting. Densitometric analysis was conducted on immunoblots. Results are expressed \pm SEM, $n = 3$. A representative immunoblot is shown. (C) NF- κ B activation was measured in nuclear fractions by an NF- κ B binding assay. Values were expressed relative to the mean luminescence value for unstimulated DC from the low-CLA beef diet group. *Different from low CLA, $P < 0.05$.

demonstrated in vitro and in vivo and within the context of intestinal inflammation (25,42). The present study demonstrated that feeding mice with a natural source of CLA derived from beef could modulate LPS-induced immune responses and suggested that food products with a high CLA content can act as a viable, functional nutrient for the prevention and treatment of septic shock syndromes.

IFN γ , IL-12, and IL-1 β are elevated in the serum during septic syndromes and play a key role in the pathogenesis of sepsis (43). This study showed that prefeeding mice with a high-CLA beef diet reduced these proinflammatory cytokines in response to LPS-induced septic shock. This concurs with the effects of dietary supplementation with synthetic *c9*, *t11*-CLA, which reduced serum IL-1 β in pigs (44) and other cytokines in a range of cell types, including macrophages (45), epithelial cells (46), and smooth muscle cells (47).

DC play a critical role in inducing and directing the adaptive immune response. Furthermore, regulatory cytokines produced by DC and other cells of the innate immune system in response to pathogens or their products influence T helper cell responses (43). Given the key role of the DC in innate and adaptive immune response, we examined whether a low-CLA and high-CLA beef diet affected the response of DC to LPS, in particular its ability to produce IL-10 and IL-12. The active form of IL-12, IL-12p70, is produced by DC following stimulation with TLR agonists and promotes naive T cells to differentiate into Th1 cells (44). We found that IL-12p35 mRNA (the functional subunit of IL-12p70) was significantly reduced in BMDC isolated from mice fed the high-CLA diet, which concurs with our previous in vitro studies in BMDC (14). The antiinflammatory cytokine IL-10 regulates IL-12 production (45) and has an important role in controlling septic shock. The balance between these cytokines has a major influence on the nature of the immune response mounted following infection. A polymorphism in the promoter region of IL-10 (-1082A) reduces IL-10

production and increases susceptibility to sepsis in humans (46). Furthermore, overexpression of IL-10 improves survival in a mouse model of sepsis (47). The present study showed that IL-10 mRNA expression was higher in LPS-stimulated DC from mice fed the high-CLA beef diet 6 h post-LPS stimulation, indicating early induction of the antiinflammatory effect. This concurs with evidence that a synthetic 50:50 mixture of *c9*, *t11*-CLA and *t10*, *c12*-CLA increased IL-10 levels in the serum, spleen, and thymus of pigs challenged with LPS (48) and our previous in vitro finding that synthetic *c9*, *t11*-CLA enhanced secretion of IL-10 from DC (14).

TLR4 is a pattern recognition receptor that, along with the coactivator CD14, is responsible for detecting LPS, a component of Gram negative bacteria (4). The present study demonstrated that CLA downregulates TLR4 mRNA and protein expression in BMDC of mice fed a high-CLA beef diet. Furthermore, TLR4 expression was reduced in immature DC, demonstrating that CLA may act by dampening cellular responsiveness to LPS.

It has been proposed that PPAR γ activation may mediate the antiinflammatory effects of CLA (21,24,30). PPAR γ ligands, including pioglitazone and 15-deoxy- Δ (12,14)-prostaglandin J₂, can attenuate LPS-induced TLR4 expression in murine macrophages, human PBMC, and the colonic cell line HT-29 (31,49). PPAR γ ligands also have positive effects in the treatment of sepsis (50,51). In the present study, PPAR γ expression on DC was significantly higher in mice fed the high-CLA beef diet. Furthermore, *c9*, *t11*-CLA significantly reduced CD14 and TLR4 surface expression on DC in vitro. The addition of the specific PPAR γ inhibitor, GW9662, reversed the effect of *c9*, *t11*-CLA in lowering TLR4 but not CD14 expression in immature DC. This suggests that the antiinflammatory effects of CLA are partly mediated by PPAR γ -induced TLR4 downregulation.

NF- κ B regulates the inflammatory cytokine response following LPS-induced TLR4 activation (52). Previous studies have shown that CLA mediates its antiinflammatory effects through

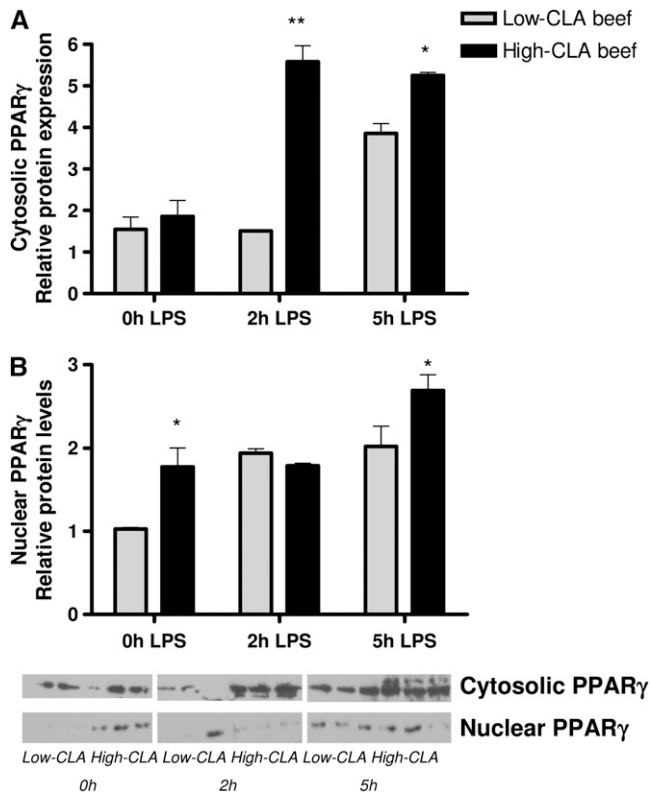


FIGURE 4 A high-CLA beef diet enhances PPAR γ expression (A,B) in BMDC from BALB/c mice. PPAR γ protein in the cytosolic (A) and nuclear (B) fractions were measured by Western blotting. Densitometric analysis was conducted on immunoblots. Results are expressed \pm SEM, $n = 3$. A representative immunoblot is shown. *Different from low CLA, $P < 0.02$, ** $P < 0.01$.

the NF- κ B pathway (53–55). The present study suggests that CLA may inhibit nuclear translocation of NF- κ Bp65. There was a small but significant increase in IKK α in DC following stimulation with LPS for 2 h, with significantly lower expression in DC from mice fed the high-CLA beef diet. In addition, the results of a TransAM assay demonstrated that whereas there was no reduction in nuclear NF- κ Bp65, there was a significant reduction in NF- κ Bp65 DNA binding. This concurs with a previous in vitro analysis of BMDC treated with synthetic *c9*, *t11*-CLA (14).

The results of this study demonstrate that naturally derived beef CLA has potent antiinflammatory effects at both a systemic and cellular level in a mouse model of sepsis. We demonstrated that feeding mice a high-CLA beef diet dramatically reduced LPS-induced proinflammatory cytokine concentrations, which are important in the pathogenesis of septic shock syndromes. DC are the primary antigen presenting cells in the initial immune response to infection. Our results suggest that reduced ex vivo expression of TLR4 on DC after the high-beef CLA diet limits their ability to sense LPS, which in turn attenuates the NF- κ B pathway. It is also clear that PPAR γ activation plays a role by reducing TLR4 expression. Overall, these findings provide clear evidence that naturally derived CLA has a powerful antiinflammatory effect. Further studies should help to underscore the potential importance of CLA as a viable, functional food in the treatment and prevention of sepsis.

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