Dietary Conjugated Linoleic Acid Decreased Cachexia, Macrophage Tumor Necrosis Factor-α Production, and Modifies Splenocyte Cytokines Production[1]

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The effect of conjugated linoleic acid (CLA) on macrophage functions were studied *in vitro*, *in vivo*, and *ex vivo*. In RAW macrophage cell line, CLA (mixed isomers) was shown to inhibit lipopolysaccharide (LPS)-stimulated tumor necrosis factor-α (TNF-α) production. Two CLA isomers, c9,t11 and t10,c12, were tested on RAW cells and it was found that the c9,t11 was the isomer responsible for the inhibition of LPS-induced TNF-α production. BALB/c mice were used to determine the effect of dietary CLA on body weight wasting and feed intake after LPS injection. CLA was protective against LPS-induced body weight wasting and anorexia. Plasma TNF-α levels after LPS injection were lower in the CLA group compared with the corn oil-fed control group 2 hr post-LPS injection. In a separate experiment, 30 mice were fed a CLA-supplemented diet or a corn oil-supplemented diet for 6 weeks and peritoneal resident macrophages were obtained for measuring TNF-α and nitric oxide production after *in vitro* exposure to interferon-γ (IFN-γ) and/or LPS. TNF-α production was not found to be different in peritoneal macrophages from mice fed the dietary treatments, but less nitric oxide was produced in macrophages from CLA-fed mice upon stimulation when compared with macrophages from control-fed mice. Splenocytes were also collected from the mice fed the dietary treatments and stimulated to produce cytokines in culture. Supernatant was used to run cytokine enzyme-linked immunoabsorbant assays. Interleukin-4 (IL-4) was decreased in CLA-fed mice when splenocytes were stimulated with concanavalin A (Con A) for 44 hr; however, IL-2 and the IL-2-to-IL-4 ratio were elevated. Exp Biol Med 228:51–58, 2003

Key words: CLA; cachexia; macrophage; cytokines

A biological important role of conjugated linoleic acid (CLA) as an anticarcinogen was first reported by Pariza and Hargraves (1). It was later found that CLA had physiological properties that differed from LA. Synthetic CLA is a mixture of isomers, and it is difficult and costly to purify pure isomers, hence, until recently, most reports on the physiological effects of CLA have used mixed isomers (predominantly c9,t11 and t10,c12). Among these studies, CLA has been shown to decrease carcinogenesis (2, 3), decrease atherosclerosis (4, 5), and change body composition by increasing body protein and water content and decreasing body fat (6–8). In addition to the above-mentioned properties, CLA also modulates the immune system by increasing lymphocyte blastogenesis, lymphocyte cytotoxic activity, and macrophage killing ability, as well as protecting against end-stage body wasting in autoimmune disease (9–12). Other fatty acids also affect immunity as well (13–15).

It has been shown that several dietary oils modulate macrophage function. For example, dietary fish oil decreased interleukin-1 (IL-1) release by peritoneal macrophages when compared with corn oil-fed controls (16). Macrophages are believed to be the principal sources of tumor necrosis factor (TNF)-α produced *in vivo*, and lipopolysaccharide (LPS) is the most potent stimulus of macrophages for TNF-α production. The activity of TNF-α was originally shown to kill tumor cells, but it also has a profound effect in causing body wasting, or cachexia. Direct infusion of TNF-α into rats has been shown to promote muscle degradation (17). Our laboratory has shown that feeding CLA to chicks reduces LPS-induced body weight wasting and feed intake (12, 18). Because macrophages are immune cells known to produce TNF-α upon LPS stimulation, the effect of CLA at inhibiting LPS-induced wasting could be due to decreased TNF-α production by macrophages.

T lymphocytes play an important role in the immune system. Upon activation, naive T cells (Th0) differentiate into either T helper 1 (Th1) or Th2 effector cells as they secret different cytokines and mediate very different im-

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mune responses (19). Th1 type responses are primarily cell-mediated immunity and inflammation, and Th2 cells and cytokines mediate humoral immunity. Upon activation, development of Th0 cells into either Th1 or Th2 cells depends on the local cytokine milieu. For example, in the presence of IL-12, Th1 clone development is preferred (20, 21). On the other hand, IL-4 is indispensable for Th0 cells to polarize into Th2 clones (22, 23). Moreover, Th1 and Th2 responses are cross-regulated. For example, IFN-γ, a cytokine produced by Th1 cells, inhibits IL-4 production and suppresses Th2 development (24, 25). Conversely, IL-4 and IL-10 produced by Th2 cells block differentiation of Th0 to Th1 (26, 27). Sugano *et al.* (11) showed dietary CLA increased immunoglobulin (Ig) A, IgG, and IgM in both rat serum and cultured lymph node cells, whereas IgE was reduced. This work implied that CLA may promote Th1 cytokine and inhibit Th2 cytokine production, as Ig class switch from IgG to IgE would not occur without IL-4 or IL-13, both which are potent Th2 cytokines (28, 29). In a guinea pig asthma model, allergen-induced trachea histamine production was reduced in CLA-fed guinea pigs (30). IgE-induced histamine release was enhanced by IL-5, a Th2 cytokine (31). It was our interest to test the effect of CLA on the helper T cell cytokines profile modification as suggested by previous studies.

Because macrophages are very sensitive to LPS stimulation, it seemed that macrophages can be a potential target of CLA to decrease LPS-induced responses. In this study, we investigated the effect of CLA on macrophage TNF-α production, as it was hypothesized that CLA inhibits TNF-α production by macrophages. In addition, splenocyte cytokines IL-2 and IL-4 were also measured.

Material and Methods

CLA. CLA used in the *in vivo* and *ex vivo* feeding trials was obtained from Natural Lipids Inc. (Hovdebygda, Norway) and contained approximately 90% CLA (CLA-90) with the following C18:2 conjugated isomer distribution: 43.5% t10,c12; 41.9% c9,t11 and t9,c11; 1.5% t9,t11 and t10,t12; 0.9% c9,c11; and 0.9% c10,c12. Other fatty acids in CLA-90 were 5.6% oleate, 1.4% palmitate, 0.5% linoleate, 0.4% stearate, and 3.4% unidentified compounds. For *in vitro* studies, LA was purchased from Nu-Check Prep (>99% pure; Elysian, MN). The c9,t11 CLA isomer was purchased from Matreya Inc. (Pleasant Gap, PA). The c9,t11 CLA isomer was 96.3% pure and had 2.6% of t9,t11/t10,t12 and 1.1% of other CLA isomers. The t10,c12 isomer was from Natural Lipids, and it had 92.8% of t10,c12; 1.6% of c9,t11; 1.2% of t9,t11/t10,t12; and 2.8% of other CLA isomers.

Macrophages. RAW 264.7 cells were a gift from M.W. Pariza (Food Research Institute, University of Wisconsin, Madison, WI). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) media (Life Technologies, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS) and 1% antibiotics solution (A9909; Sigma, St. Louis, MO). Macrophages were plated in the density of 100,000 cells/well in a 24-well plate for 24 hr, followed by culture in fresh media containing fatty acid-albumin complex. The final fatty acid concentration was 100 μM. Cells were cultured with fatty acid-albumin complex for 24 hr. Fresh media (containing corresponding fatty acid-albumin complex) containing 500 ng/ml LPS (from *E. coli*, serotype 055:B5; L-4005; Sigma) in phosphorus-buffered saline (PBS) was then added for 16 hr to stimulate TNF-α secretion. Control cells were treated with PBS. Media was then collected, frozen, and subsequently analyzed for TNF-α as described below. Cell viability was checked under microscope and by total nonwashable protein measurement (cell protein concentration was measured after removing supernatants for TNF-α analysis and washing cells with PBS three times) to confirm that viability of macrophages cultured under the 100 μM fatty acid-albumin complex did not differ from macrophages cultured with albumin alone.

Preparation of Fatty Acid-Albumin Complex. To make fatty acid-albumin complexes for treating macrophages *in vitro*, 2.8 mg of free fatty acid, already dissolved in 0.5 ml of KOH (0.1 *M*), was transferred to a scintillation vial, and 4 ml of 2.5 m*M* bovine albumin PBS solution (LPS free, A-8806; Sigma) was added and gassed with nitrogen. The vial was then wrapped with foil and refrigerated overnight. The pH was then adjusted to 7.2 using 0.1 *M* NaOH solution, and the volume was brought to 5 ml with PBS. The solution was filtered using a 0.22-μm syringe filter for use in cell culture. The fatty acid concentration of the preparation was 2 m*M*.

Diet. A semipurified powdered diet was purchased from Harlen-Teklad (TD94060, 99% basal mix; Madison, WI). The diet had 5% corn oil and it was supplemented with either 0.5% CLA or corn oil and 0.5% sugar. Hence, the control diet had 5.5% corn oil, and the CLA diet had 5% corn oil and 0.5% CLA (10). Fresh diets were prepared every other week and were kept refrigerated. Fresh diet was provided to mice three times a week. Both diets and water were provided *ad libitum*.

Animals. Weanling BALB/c mice were purchased from Harlan-Sprague Dawley (Indianapolis, IN). In experiment one, 12 mice were immediately divided into two groups of six and were fed either CLA or the control diet. Three mice were housed together in a shoebox cage during dietary treatment and were then individually caged right before LPS injection (see below). In the *ex vivo* study, 30 mice were used in the study with 15 mice who were fed the control diet and 15 mice who were fed the CLA diet. Three mice were housed per cage in a temperature- and humidity-controlled room with a 12:12-hr light:dark cycle. The protocol for animal care and use was approved by the institutional animal care and use committee at the College of Agriculture and Life Sciences, University of Wisconsin-Madison.

***In Vivo* Mouse Trial Treatment.** After 6 weeks on the dietary treatments, three mice from each dietary group

were injected intraperitoneally with LPS (0.1 mg/ml in sterile PBS, 1 ml LPS solution/100 g of body weight) and the remaining three were injected with sterile PBS (injection control). Body weight and feed intake were recorded at 0, 24, 48, and 72 hr postendotoxin injection on the individually housed mice. Blood samples were obtained retro-orbitally at 0, 1, and 2 hr after LPS injection (32). Plasma samples were taken from the blood samples after centrifugation and were subsequently analyzed for TNF-α as described below.

Isolation and Culture of Mouse Resident Peritoneal Macrophages. In the *ex vivo* study, 30 mice were either fed the CLA (15 mice) or control diet (15 mice) for 6 weeks. Two or four mice were then sacrificed daily to obtain resident peritoneal macrophages and splenocytes. Peritoneal resident macrophages were collected posteuthanasia by injecting 10 ml of ice-cold DMEM media containing 10% FBS and 1% of antibiotics solution (Sigma) into the peritoneal cavities, and then recovering the fluid (33). The fluid was collected and gently laid on top of 3 ml of Histopaque 1081 (Sigma) and was centrifuged at 400*g* for 15 min at ambient temperature. Mononuclear cells were transferred to a clean centrifuge tube and were washed twice in Mg^{2+} Ca^{2+}-free PBS. Viable cell numbers, as determined by trypan blue exclusion, were greater than 95%. The cells were suspended and adjusted to 1×10^6 viable cells/ml in RPMI 1640 medium with 10% FBS and 1% antibiotics solution (Sigma). One hundred microliters of cell suspension was added to individual wells in a 96-well plate (100,000 cells/well) for 2 hr. Nonadherent cells were removed by washing the monolayer twice with fresh medium. The adherent peritoneal exudate cells are hereafter referred to as macrophages. Macrophages were immediately primed with 5 unit/ml IFN-γ for 4 hr before being stimulated for TNF-α or nitrite production (33). Macrophages were cultured with LPS (500 ng/ml) for 16 hr and the supernatants were collected for TNF-α assay. For nitric oxide assay, phenol-red free DMEM was used, and 500 ng/ml LPS and 5 unit/ml IFN-γ were added to the macrophages and incubated for 44 hr before the culture supernatants were removed for nitrite assay (33).

Splenocyte Isolation. Spleens were removed from the CLA- or control-fed mice and were placed in a petri dish with 10 ml of RPMI 1640 media. A 10-ml sterile syringe plunger was used to disperse the spleen into a single-celled suspension. The media containing the suspended splenocytes were then collected and layered atop 3 ml of Histopaque 1081 (Sigma) in a 15-ml centrifuge tube and were centrifuged at 400*g* for 15 min at ambient temperature. Mononuclear cells at the interface were collected using a transfer pipette, placed in another centrifugation tube, and 10 ml of media was added. Cells were centrifuged and the pellet was then washed twice with fresh media by centrifugation. Splenocytes were then resuspended to a cell density of 2×10^6/ml, and 500 μl was applied into each well in a 24-well plate. Concanavalin A (Con A) at 4 μg/ml was added into splenocyte culture, and supernatant was collected after 48 hr and frozen for subsequent cytokine analysis.

Cytokine Assay. TNF-α-, IL-2-, and IL-4- OptEIA enzyme-linked immunoabsorbant assay (ELISA) kits (PharMingen, San Diego, CA) were used to determine the serum TNF-α level as well as culture supernatant IL-2, IL-4, and TNF-α concentration. A standard procedure of cytokine ELISA was performed according to the manufacture's instructions. Briefly, a plastic plate was coated overnight with a capture antibody for a specific cytokine, followed by washing and blocking the plate. Diluted samples and standards (provided in the kit) were then added and incubated. An extensive wash was applied before secondary antibody and enzyme conjugates were added. Plates then went through another round of incubation and extensive washing. Developing reagent was then added to the plate for 15 min. Color development was stopped during linear increases in substrate utilization by adding 0.5 *M* sulfuric acid to disrupt enzymatic activity. OD readings of samples were converted to concentration based on the reference curve. At lease duplicate samples were analyzed for each cytokine ELISA. The TNF-α kit used in the present study only detects free-form TNF-α but not receptor-bound form. The detection limit for TNF-α, IL-2, and IL-4 is 20, 10, and 10 pg/ml, respectively.

Quantification of Nitrite in Culture Supernatant. Measurement of nitrite has been commonly used as an indirect measurement of nitric oxide formation because nitrite is a stable end product of the highly reactive nitric oxide (34). In the *ex vivo* experiment, 50 μl of cultured resident macrophages supernates was taken from each well and mixed with 50 μl of Griess reagent [1 part of 1% sulfanilamide and 1 part of 0.1% N-(1-naphthyl) ethylenediaminedihydrochloride, mixed immediately before use] in a different 96-well plate. The plate was gently shaken at ambient temperature for 5 min, and OD was measured at 562 nm. A standard curve was established using known $NaNO_2$ preparations. Sample nitrite concentration was extrapolated from the OD based on the standard curve. Duplicate samples were used in every nitrite microplate assay. The typical standard curve had a correlation coefficient of more than 99% between 0 and 20 μM. Samples were diluted to this range and were analyzed again if they were found to be higher than the upper limit of standards at first analysis.

Colorimetric Tetrazolium Assay. The linear relationship between number of macrophages or splenocytes and cleavage of tetrazolium was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) assay (35). MTT was dissolved in PBS (5 mg/ml) and filter sterilized through a 0.22-μm syringe filter. Twenty-five microliters of MTT solution was added to each culture well and these plates were incubated at 37°C for 3 hr. One hundred microliters stop reagent was added to each well and mixed. Stop reagent was prepared by dissolving sodium dodecyl sulfate 20% (w/v) at ambient temperature in 50% N,N-dimethylformamide in demineralized wa-

ter, and pH was adjusted to 4.7 using 80% acetic acid and 2.5% 1 *N* HCl. The plates were incubated overnight at 37°C to ensure the complete solubilization of cells and the blue crystals of formazan. OD was read on a microplate reader at wavelength of 562 nm. MTT values were determined after Con A stimulation and were used to correct IL-2 and IL-4 data based on cell numbers.

Statistical Analysis. Both nonrepeated and repeated data were analyzed to determine CLA treatment effect by PROC MIX in SAS computer program, version 8 (SAS Institute, Cary, NC) (36). For Figures 1 and 2, data were analyzed by one-way analysis of variance (ANOVA) with two treatments, with repeated measures on experiment unit (mouse). A type "ARH(1)" error structure was used to account for auto-correlated errors. For Figures 3 and 4, data were analyzed by one-way ANOVA with diet as treatment and mice(diet) as error. In addition, the model included a blocking factor to account for variations due to different days of animal sacrifice. For Figure 5, data were analyzed by randomized complete block design with experiment unit

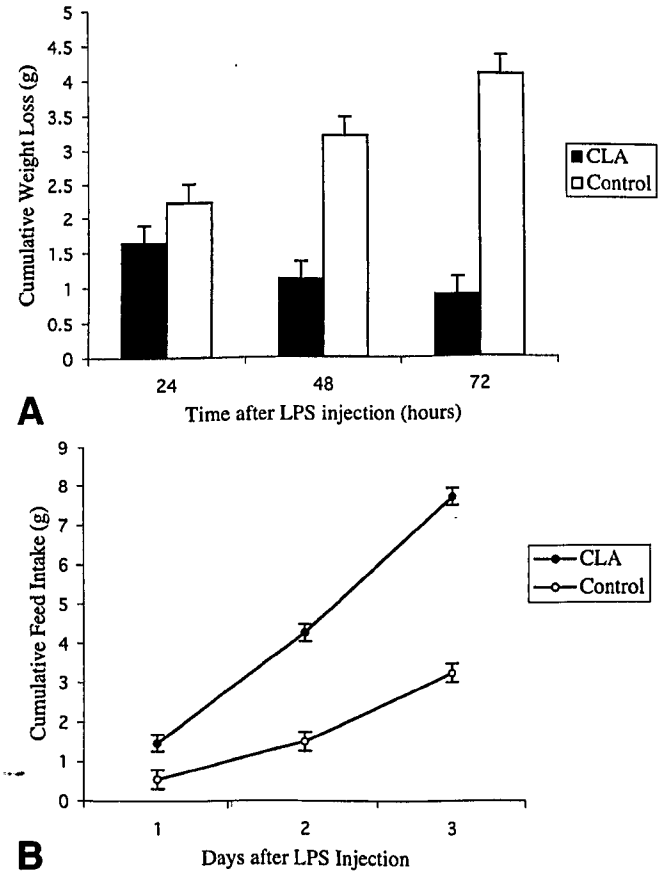

Figure 1. The influence of dietary CLA on LPS-induced body weight loss and feed intake. After feeding CLA or control diet for 6 weeks, mice were injected with either LPS (1 mg/kg) or PBS (data not shown). Cumulative body weight change and feed intake were monitored for 3 days. Each point represents least square mean with pooled error. There were three mice in each group. (a) Repeated data analysis showed dietary CLA was protecting mice against weight loss compared with mice fed the control (corn oil) diet ($P < 0.01$). (b) CLA-fed mice ate more diet after LPS injection compared with control-fed mice ($P < 0.01$).

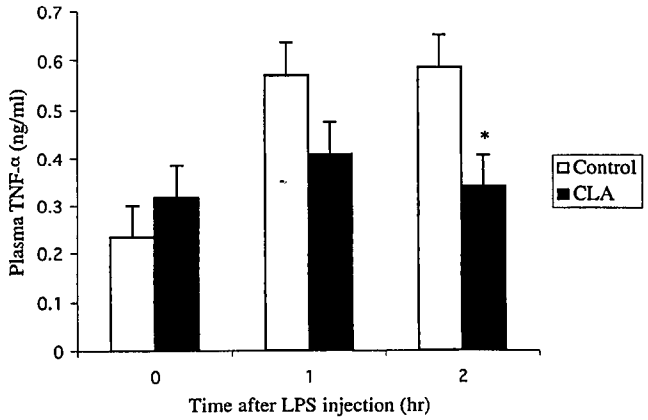

Figure 2. Influence of dietary CLA on LPS-induced release of plasma TNF-α. Mice were fed a CLA or control diet for 6 weeks before LPS (1 ng/g body weight) or PBS injection. Blood samples were obtained from mice 0, 1, and 2 hr after LPS injection. ELISA was used to determine plasma TNF-α level. Each bar represents least square mean + pooled error (SEM). Plasma TNF-α was unchanged over time in the PBS-injected mice (data not shown). An asterisk indicates significantly different from control at $P < 0.01$.

was mean of quadraplicate wells. Data from five independent experiments were used in the analysis where experiment was treated as block.

Results

Mice fed CLA had significantly less endotoxin-induced body weight loss (0.9 g) over 3 days compared with control-fed endotoxin-injected mice (4.1 g; Fig. 1A). Feed intake after LPS injection was also significantly affected by CLA. After LPS injection, CLA-fed mice ate more feed (2.6 g/day) compared with control-fed mice (1.1 g/day) over a 3-day period (Fig. 1B). In PBS injection treatment, feed intake was 2.8 g/d for the CLA group and 2.6 g/d for the control group. Weight loss in PBS injection treatment was 1.7 g in the control group and 0.1 g in the CLA group (data not shown). In the *in vivo* trial, CLA had a potent inhibitory effect on LPS-induced anorexia and body weight loss.

The evidence that CLA protects against LPS-induced cachexia and the role of dietary CLA on LPS-induced plasma TNF-α was investigated. After mice were fed CLA for 6 weeks, the plasma TNF-α levels were significantly lower in the CLA-fed mice compared with the control-fed mice 2 hr after LPS injection (Fig. 2). The TNF-α production from PBS injection control was unchanged (data not shown).

In the *ex vivo* trial, we studied peritoneal resident macrophage activity after mice were fed the dietary treatments for 6 weeks. We found that even though CLA decreased plasma TNF-α level *in vivo*, CLA did not statistically decreased LPS-induced release of TNF-α in peritoneal resident macrophage in this *ex vivo* system (Fig. 3A). Nitric oxide production, however, was reduced by CLA. Peritoneal resident macrophages from CLA-fed mice had less nitric oxide production when stimulated by IFN-γ and LPS when compared with cells from control-fed mice (Fig. 3B).

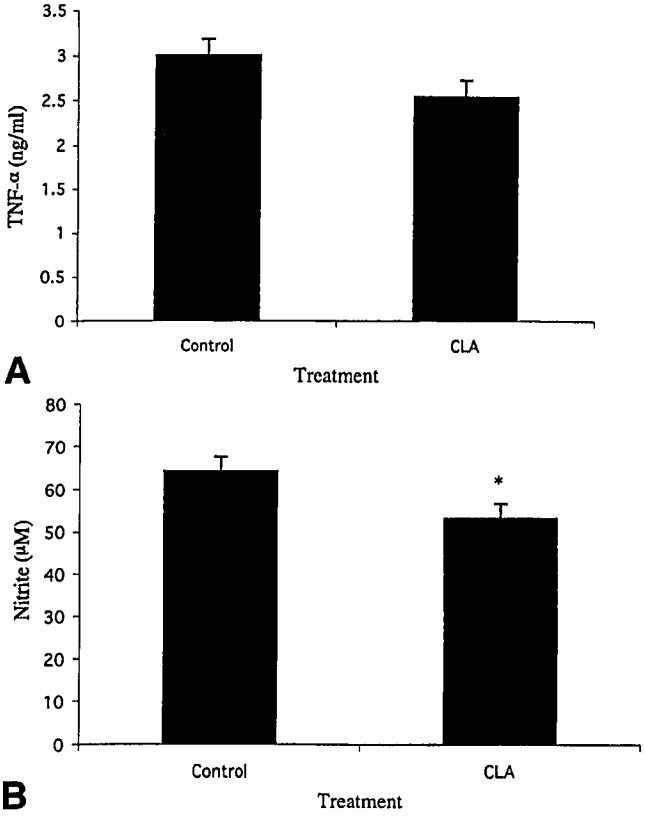

Figure 3. Nitric oxide and TNF-α production by resident peritoneal macrophages were affected by CLA feeding. Mice were fed CLA or control diet for 6 weeks before sacrifice. Resident peritoneal macrophages were obtained and primed with IFN-γ. (a) Macrophages were later stimulated with LPS for TNF-α production. (b) Macrophages were later stimulated with LPS and IFN-γ for nitric oxide production. Macrophages from mice fed a CLA diet produced a significantly lower amount of nitrite (53.3 μM) compared with macrophages from mice fed a control diet (64.2 μM). TNF-α production, however, was not different between CLA and control groups. Each bar represents least square mean with pooled error. There were 15 mice in each diet treatment. An asterisk indicates significantly different from control at $P < 0.05$.

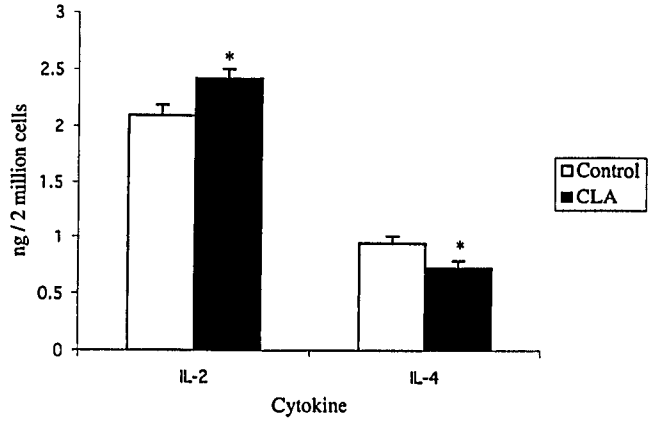

Figure 4. Cytokine production by splenocytes from mice fed CLA or control diet. After mice were fed CLA or control diet for 6 weeks, splenocytes were harvested and stimulated with concanavalin A for cytokine production. Supernatants were then harvested from the cultured splenocytes and analyzed by ELISA. Cytokine production from each well was corrected by numbers of cells presented at the end of the culture that was determined by colorimetric tetrazolium assay as described in "Materials and Methods." Each bar represents least square mean with pooled error. There are 15 mice in each diet treatment. The IL-2-to-IL-4 ratio is 3.3 for the CLA group and 2.2 for the control group. An asterisk indicates significantly different from control at $P < 0.05$.

Spleens were also obtained for lymphocyte cytokine analysis. Splenocytes were treated with Con A for 48 hr before collecting supernatant for cytokine analysis. Splenocytes from CLA-fed mice did show a higher IL-2 production after LPS stimulation and lower IL-4 production than splenocytes from control-fed mice (Fig. 4). The ratio of IL-2 to IL-4 was significantly ($P < 0.05$) higher (ratio = 3.3) for splenocytes from the CLA-fed mice compared with the control-fed mice (ratio = 2.2).

In the *in vitro* experiment, after RAW macrophages were cultured with fatty acid-albumin complexes, they were stimulated with LPS to produce TNF-α. Our data showed that CLA mixture had an inhibitory effect on TNF-α production compared with the LA control. Among isomers, the c9,t11 isomer was responsible for this inhibitory effect (Fig. 5A), but not the t10,c12 isomer (Fig. 5B). In average, 100 μ*M* c9,t11 CLA isomer decreased macrophage TNF-α production by 60% compared with LA control. This c9,t11 isomer effect was dose responsive as 50 μ*M* c9,t11 CLA alone was 40% inhibitory to macrophage TNF-α production. On the other hand, t10,c12 isomer did not show any effect on macrophage TNF-α production.

Discussion

Dietary fatty acids have been shown to be potent immune regulators. In general, high dietary levels of PUFA or n-3 fatty acids have an inhibitory effect on T cell proliferation and natural killer (NK) cell activity (37). Fish oil, rich in n-3 fatty acids, decreased murine and human LPS-stimulated TNF-α production (38, 39). It has been shown that dietary fish oil prevents endotoxin-induced death in guinea pigs (40), possibly by inhibition of cytokines produced by macrophages (41).

Feeding 0.5% CLA to animals resulted in up to 10 mg of CLA per gram of body fat in several different tissues within 30 days (42), and it took between 2 and 4 weeks to reduce CLA to baseline levels after its withdrawal from the diet (43). Several physiological changes, including body composition change (6), anticarcinogenesis (44), and antiatherosclerosis (4), were also shown when using up to 0.5% CLA in animal studies.

CLA has been shown to be a potent immune regulator. CLA decreased the LPS-induced cachectic response in chicken, rats, and mice, but at the same time, increased lymphocyte blastogenesis (12, 18). Other groups also reported CLA-increased lymphocyte blastogenesis as well as increased IL-2 production (9, 45, 46). To the best of our knowledge, this is the first report of an elevated Th1/Th2 cytokine ratio in CLA-fed mice. The increased IL-2 production (shown here, and by others) and the decreased IL-4 production associated with dietary CLA may explain the

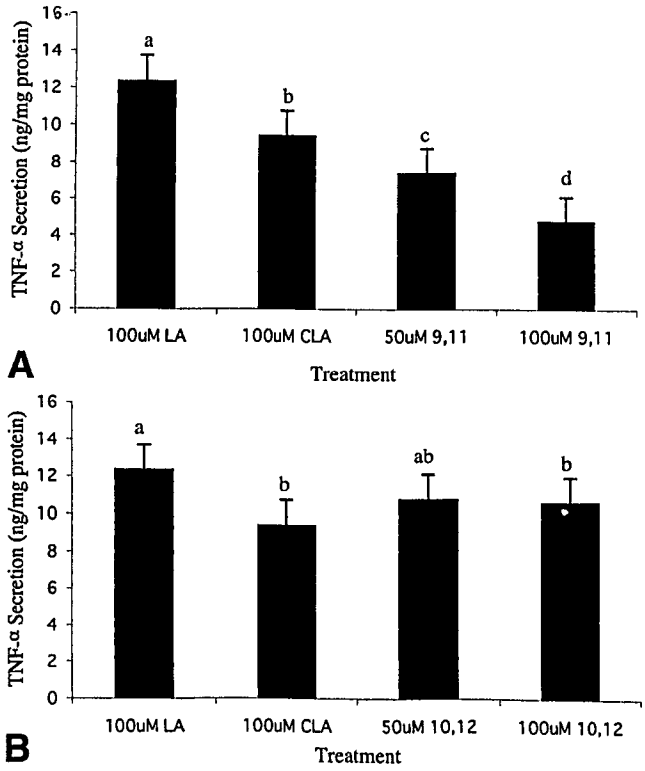

Figure 5. CLA inhibited TNF-α production from cultured RAW macrophage cell line (a and b). RAW cells were plated (100,000 cells/well) in 24-well plate for 24 hr. Then they were cultured with fatty acid-albumin complex for 24 hr before LPS was added into culture to stimulate TNF-α secretion for 16 hr. Each bar represents least square mean with pooled error (SEM). There were 4 wells for each treatment in a 24-well plate. Bar with a different letter indicates significantly difference at $P < 0.05$. (a) Compared among LA, CLA, and c9, t11 CLA isomer. (b) Compared among LA, CLA, and t10, c12 CLA isomer. Mixed isomers, approximately 42% c9,t11 and 44% t10,c12. Figure shown here represented means of five independent experiments.

reduced IgE production in CLA fed rats (11) as compared with rats fed diets largely based on LA.

Because CLA seems to enhance immunological responses, which may pose a potential risk on immune hypersensitivity, our laboratory has been studying how dietary CLA may affect type I and type III immune hypersensitivity. In a type I hypersensitivity model, CLA reduced antigen-induced histamine and PGE_2 release from tracheae of CLA-fed guinea pigs (30). In an autoimmune (type III hypersensitivity) model, CLA feeding not only protected against end-stage body wasting, but also increased survival days 1.5-fold after the onset of proteinuria in mice (10). Shifted cytokine profiles toward Th1 may interfere with Ig class switch that is needed in producing IgE in immune type I hypersensitivity responses (47). The reduced IgE production by CLA-fed rats (11) would certainly result in a reduced type I hypersensitivity reaction upon challenge, as demonstrated in a guinea pig hypersensitivity model (30). The development of experimentally inducible lupus in mice seems to involve two stages: increased Th1 cytokines followed by elevated Th2 cytokines later in life (48). Increased Th2 cytokine production correlates well to disease progression. Hence, a CLA-induced shift toward Th1 cytokines could explain, at least in part, increased days of survival post-proteinuria in lupus-prone mice. Inhibited production of TNF-α may also help to explain the reduction in body weight loss in CLA-fed lupus mice at the end stage of systemic lupus disease (10).

Macrophages are sensitive to dietary fatty acid supplementation, as its fatty acid profile reflects such dietary intervention (49, 50). These works also showed that dietary fatty acids also dictate macrophage physiology. Dietary fish oil has been shown to decrease macrophage antigen presentation (51, 52) and to decrease cytokine production as well as mRNA expression (38, 53). In the present study, macrophages were tested as one of the target cells on which CLA modulates immune function. Our data showed that CLA, more specifically c9,t11 CLA, decreased TNF-α production in RAW macrophage cell line. LPS-induced anorexia and cachexia responses were reduced in CLA-fed animals. The plasma TNF-α level after LPS injection was also suppressed in CLA-fed mice. It was unanticipated that mice fed CLA did not suppress *ex vivo* TNF-α production in resident peritoneal macrophages. However, both *in vitro* and plasma samples showed that CLA has an inhibitory effect on TNF-α production. Fatty acid turnover rate in cultured peritoneal resident macrophages may have played a role in the lack of TNF-α response observed in this model. In the cell culture study, CLA was kept at a constant concentration during the *in vitro* experiments, and when blood was drawn for TNF-α analysis, mice were still fed the treatment diet. However, when culture supernatants were collected in the *ex vivo* study, peritoneal macrophages had already been cultured *in vitro* for 24 hr without exogenous exposure to CLA. Moreover, the fetal bovine serum used in the system might not have been as effective as autologous serum (15) in demonstrating CLA's effects on TNF-α production by resident peritoneal macrophages.

The two main isomers of the CLA mixture (c9,t11 and t10,c12) used in this study were considered to have different properties on cytokine regulation. In fact, c9,t11 CLA increased feed efficiency (54, 55), whereas the t10,c12 was the isomer shown to affect body composition (56). In the present study, c9,t11 CLA isomer inhibited TNF-α production from *in vitro* macrophage cultures (Fig. 5a). The slight decrease of TNF-α in 100 μ*M* t10,c12 CLA isomer treatment probably was thought to be due to c9,t11 CLA isomer contamination of the t10,t12 CLA isomer used (Fig. 5b). Moreover, a recent study reported decreased hepatic TNF-α mRNA expression by c9,t11 CLA in mice (57). It remains to be shown which isomers downregulate IL-4 production and increase IL-2 production. Even though the *in vitro* data suggested that c9,t11 CLA is the active isomer in reducing LPS-induced TNF-α production, further *in vivo* studies with pure CLA isomers are required to validate the *in vitro* data on TNF-α production and determine the effects of different CLA isomers on immune function.

TNF-α was shown to be involved in endotoxin-induced

weight loss and cancer cachexia (58). Muscle degradation was enhanced by TNF-α. Blocking TNF-α function by either injection of anti-TNF-α antibody or TNF-α-binding proteins reduced the cachexia reaction (59, 60). The decreased LPS-induced TNF-α production by CLA may provide at least one mechanism by which CLA enhanced the rate of growth and improved feed efficiency in animals (12, 54, 55).

Thromboxane A_2 (TXA_2) is a mediator of renal damage (61). In murine lupus, TXA_2 inhibitor prolonged survival of NZB/W F1 mice, and renal TXA_2 production was elevated in F1 mice (62). In a study comparing TXB_2 (stable end product of TXA_2) production and COX gene expression of peripheral blood mononuclear cells (PBMC) among patients with active or inactive lupus nephritis and healthy individuals as a control, COX-2 expression and TXB_2 production in PBMC was only elevated in patients with active lupus nephritis (63). Immunostaining of kidney biopsies showed no difference in COX-1, but COX-2 staining in patients with active lupus nephritis was increased compared with patients with inactive lupus nephritis or healthy controls. Double staining of kidney biopsies with anti-COX-2 and anti-CD68 antibodies (macrophage marker) demonstrated that upregulated COX-2 enzyme expression was within the macrophages of glomeruli in patients with active lupus nephritis (63).

Infiltrating macrophages in glomeruli, with upregulated COX-2 enzyme expression, may contribute to the elevated TXA_2 level in active lupus nephritis. Regulation of macrophage activities and eicosanoid profiles by CLA may play a role in lupus nephritis. Because COX-2 is the inducible form and the COX-1 is constitutively expressed, decreased antigen-induced TXB_2 production but not the basal level of TXB_2 production in CLA-fed guinea pigs provides an explanation that CLA may preferentially inhibit COX-2 activity (64). Decreased macrophage activities including TNF-α and nitric oxide productions seen in this study may help to explain the effect of CLA in prolonging survival of NZB/W F1 mice.

In conclusion, these data suggest that cytokine regulation by CLA could be responsible for previous reports demonstrating CLA's anticachectic effects, increased lymphocyte blastogenesis, decreased immune type-1 hypersensitivity, decreased IgE production, and prolonged life post proteinuria in the NZB/W F1 autoimmune mouse.

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