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Conjugated linoleic acid supplementation reduces peripheral blood mononuclear cell interleukin-2 production in healthy middle-aged males☆

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Abstract

Conjugated linoleic acid (CLA) refers to geometric and positional isomers of linoleic acid. Animal studies have shown that CLA modulates the immune system and suggest that it may have a therapeutic role in inflammatory disorders. This double-blind placebo-controlled intervention trial investigated the effects of CLA supplementation on indices of immunity relating to cardiovascular disease (CVD) in a cohort of healthy middle-aged male volunteers. Subjects were randomly assigned to supplement their diet with 2.2 g 50:50 isomeric blend of *cis 9*, *trans 11* (*c9, t11*)-CLA and *trans 10*, *cis 12* (*t10*, *c12*)-CLA or placebo daily for 8 weeks.

Interleukin (IL) 2, IL-10 and tumour necrosis factor (TNF) α were measured in the supernatant of cultured unstimulated and concanavalin A (Con A)-stimulated peripheral blood mononuclear cells (PBMC) by ELISA. Serum IL-6 and plasma CRP were measured by ELISA and plasma fibrinogen by automated clotting assay. Gene expression was investigated by real-time RT-PCR.

CLA supplementation significantly reduced Con A-stimulated PBMC IL-2 secretion (37.1%; $P=.02$). CLA supplementation had no significant effect on transcription of IL-2. CLA supplementation had no direct significant effects on PBMC TNFα or IL-10 secretion. Other inflammatory markers associated with CVD, including IL-6, CRP and fibrinogen, were not affected by CLA supplementation.

This study showed that CLA supplementation reduced PBMC IL-2 secretion from Con A-stimulated PBMC but lacked effect on other markers of the human inflammatory response.

Keywords: Conjugated linoleic acid; Supplementation; Peripheral blood mononuclear cell; Interleukin-2

1. Introduction

Conjugated linoleic acid (CLA) refers to the geometric and positional conjugated dienoic isomers of linoleic acid. A product of rumen fermentation during microbial biohydrogenation of linoleic and linolenic acid [1], it is naturally found in ruminant meat and dairy products mainly as the *cis*-9, *trans*-11 (*c9, t11*-CLA) isomer [2–4]. CLA is produced synthetically by base-catalysed isomerisation of linoleic acid [5] resulting in a mixture of *c9, t11*-CLA and *trans-10, cis -12* (*t10, c12*-CLA) isomers. Numerous in vitro and animal studies suggest that CLA may have therapeutic effects with respect to cancer [6–8], diabetes [9,10] and atherosclerosis [11,12]. CLA also appears to have intrinsic immune-modulatory properties, particularly with respect to cell-mediated immune function. It has been reported to decrease in vitro porcine lymphocyte [13] and increase ex vivo murine splenocyte IL-2 secretion [14], inhibit macrophage phagocytosis [15,16] and reduce mononuclear cell tumour necrosis factor (TNF) α and IL-6 production [17,18]. Several recent reviews have documented the potential immuno-modulatory effects of CLA in detail [8,19–21].

To date, relatively few studies have investigated the effects of CLA supplementation on inflammatory mediators in man. Kelley et al. [22,23] showed that CLA supplementation had no effect on lymphocyte proliferation, serum antibody titer, delayed type hypersensitivity or ex vivo peripheral blood mononuclear cell (PBMC) cytokine production

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Table 1
Fatty acid composition (w/w%) of the CLA and placebo supplements

	CLA 50:50	Placebo
C18:0	2.28	4.70
C18:1	10.17	34.84
C18:2	1.73	17.89
C20:0	0.35	0.37
C20:1	2.99	0.16
Other saturated fatty acids	8.16	44.97
Total CLA	73.83	0.06
CLA isomers (%)		
*cis*9, *trans*11-CLA	35.62	0.06
*trans*10, *cis*12-CLA	38.21	Undetectable

in healthy women aged 20–41 years. Volunteers were supplemented with 3.9 g CLA daily for 63 days, receiving 0.88 g *t10, c12*-CLA, 0.69 g *c9, t11*-CLA and 2.3 g of other CLA isomers. Albers et al. [24] showed that CLA beneficially affected the initiation of an immune response to hepatitis B vaccination, but had no effect on ex vivo PBMC cytokine and eicosanoid secretion in a group of healthy men aged 31–69 years. CLA supplemented volunteers received either a 50:50 blend (containing 1.7 g CLA fatty acids) or an 80:20 blend (containing 1.6 g CLA glycerides) of the *c9, t11*-CLA and *t10, c12*-CLA isomers daily for 12 weeks. The control group of these studies received sunflower oil fatty acids [22–24]. Tricon et al. [25] demonstrated, in an 8-week crossover study, that *c9, t11*-CLA (0.59, 1.19 and 2.39 g daily) and *t10, c12*-CLA (0.63, 1.26 and 2.52 g daily) single-isomer supplementation resulted in a dose-dependent reduction in the mitogen-induced activation of T lymphocytes but had no consistent significant effect on ex vivo cytokine production. Our group recently reported that CLA supplementation had little effect on immune function in young healthy subjects, males and females (31.5±9.9 years), receiving 1.9 g 50:50 or 1.7 g 80:20 *c9, t11*-CLA and *t10, c12*-CLA supplements daily for 8 weeks. The control group received linoleic acid supplements [26]. Differences in the amount and composition of CLA and placebo consumed, the age and gender of volunteers and length of supplementation must be considered when interpreting the results of these studies.

Since age is a critical factor in the immune response [27,28], the primary objective of the present study was to investigate the effects of CLA supplementation on cytokine secretion in concanavalin A (Con A)-stimulated PBMC and on systemic markers of inflammation associated with cardiovascular disease (CVD) in middle-aged men. Secondary aims were to investigate the effects of CLA supplementation on systemic inflammatory mediators and lipids.

2. Methods and materials

2.1. Subjects and intervention details

This double-blind placebo-controlled study was approved by the ethics committee of the Federated Dublin Voluntary Hospitals, Ireland. Thirty healthy male volunteers aged 40–60 years, recruited to typify a cohort at risk of CVD, gave written, informed consent and completed the trial. Volunteers had no history of inflammatory disorders or prescribed medications. All, but one, of the volunteers were nonsmokers. No volunteer was vegetarian, adhering to a special diet or consumed dietary supplements prior to or throughout the study period. All volunteers habitually consumed meat and dairy products. A short food frequency questionnaire was conducted at recruitment. The amount of total fat contributed to the diet by CLA-rich foods was calculated using standard food composition tables [29]. Content of CLA (g/100 g fat) of these foods [19] was used to estimate volunteer intake of CLA. Intakes of CLA were estimated to be 197±94 mg/day, comparable to intakes reported in other studies [30–32]. Subjects were randomly assigned to receive 2.2 g of CLA or placebo daily for eight weeks, presented as six 0.5-g capsules per day. The composition of the CLA and placebo supplements is presented in Table 1. Supplements were provided by Loders Croklann (Wormeveer, The Netherlands). The placebo contained a blend of fatty acids typical of the fatty acid composition of the Irish and UK diet [33].

2.2. Blood sampling and PBMC preparation

Fasting blood samples were drawn from volunteers (Vacutainer, Becton Dickinson, Oxford, UK) at the beginning and end of the supplementary period. Citrated plasma and serum were harvested from blood by centrifugation, snap frozen and stored (−80°C) until subsequent analysis of fibrinogen, CRP and IL-6. Peripheral blood mononuclear cells from heparinised blood were isolated by density gradient centrifugation. Briefly, blood diluted (1:1) in Hepes-buffered HBSS (Gibco, Grand Island, NY) was gently layered onto Lymphoprep (density 1.077 g/ml, Nycomed Pharma, Oslo, Norway), centrifuged slowly and PBMC were removed from the buffy interface. Cells were washed twice with HBSS and resuspended in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 2 mmol L-glutamine/L, 100 mg streptomycin/ml and 100 μg penicillin/ml (Sigma, St. Louis, MO, USA). Cell viability and concentration were assessed by fluorescent microscopy using ethidium bromide/acridine orange stain. Cells were plated and incubated at a concentration of 1×10^6 cells/ml in RPMI medium supplemented with 2.5% v/v autologous serum [29,34,35], with or without the lectin Con A at a concentration of 10 μg/ml culture. Con A (12.5–25 μg/ml) has been used to stimulate 1×10^6 PBMC in other similar studies [25,36,37].

2.3. Protein assays

IL-2, IL-10 and TNFα concentrations from PBMC supernatant were measured using commercial ELISA kits (R&D Systems, Oxon, UK) after 24 h of incubation at 37°C and 5% CO_2. This time point was deemed appropriate for optimal investigation of cytokine secretion from PBMC [36,38]. Serum IL-6 and plasma CRP assays were

Table 2
Baseline characteristics of placebo and CLA supplemented volunteers

	CLA	Placebo
Age (years)	51.0 *(2.01)*	47.8 *(1.59)*
Weight (kg)	83.4 *(2.37)*	84.5 *(3.19)*
Height (m)	1.78 *(0.02)*	1.8 *(0.01)*
BMI (kg/m^2)	26.2 *(0.58)*	26.0 *(0.85)*
Total cholesterol (mmol/L)	5.31 *(0.17)*	5.20 *(0.22)*
HDL cholesterol (mmol/L)	1.17 (0.07)	1.06 (0.06)
LDL cholesterol (mmol/L)	4.01 (0.27)	4.32 (0.26)
Triglycerides (mmol/L)	1.31 *(0.14)*	1.27 *(0.14)*
Glucose (mmol/L)	5.12 (0.13)	5.29 (0.14)
GGT (IU)	35.0* *(4.76)*	20.7 *(2.17)*
Haemoglobin (g/d L)	14.5 *(0.09)*	14.4 *(0.26)*

Values are means with standard error in parenthesis. All biochemical analyses were completed in the fasted state. Values in italics are the SEM for each characteristic between groups.

* Significant difference from placebo group $P \leq .05$.

performed using high sensitivity ELISA kits (Biosource, California, USA, and BioCheck, Inc., California, USA, respectively) following manufacturers instructions. Fibrinogen clotting activity was measured using an automated assay, as previously described [39].

2.4. Gene expression analysis

Total RNA was extracted from PBMC after 4 h of incubation using TRIZOL reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. RNA yields were quantified spectrophotometrically, and integrity was confirmed by the visualisation of the 18s and 28s bands following formaldehyde denaturing agarose gel electrophoresis (GeneSnap, Syngene, Cambridge, UK). RNA was treated with deoxyribonuclease I, amplification grade as per manufacturer's protocol to remove contaminating genomic DNA. Reactions containing 2 μg of RNA were reversed transcribed and 1:2 serial dilutions performed to generate a standard curve for the subsequent PCR analysis (ABI Prism 7700 Sequence Detector, Applied Biosystems, Foster City, CA, USA) with glyceraldehyde dehydrogenase as an endogenous control.

2.5. Fatty acid analysis

Total plasma lipid fatty acid composition was determined using a Shimadzu GC-14A gas liquid chromatograph (Mason Technologies, Dublin, Ireland) and a Shimadzu CR6A integrator, fitted with a CP Sil 88 fused Silica Column (50×0.22 m file thickness; Chrompack, Ltd., Middleburg, The Netherlands). The method used was specifically designed and validated wherein it detected both the *c9, t11*-CLA and *t10, c12*-CLA isomers [40]. Briefly, total plasma lipids were isolated using a method derived from that of Folch et al. [41]. Methyl esters of total plasma lipid were prepared by adding 0.5 ml 0.01 M NaOH in dry methanol followed by boron trifluoride (0.5 ml). Nitrogen was used as the carrier gas. The column initial temperature (180°C) was increased (5°C/min) to 195°C, held for 40 min and then increased (2°C/min) to 220°C, and held for 20 min. Peaks were identified using a fatty acid methyl ester standard spiked with known concentrations of the *c9, t11*-CLA and *t10, c12*-CLA isomers (Cayman Chemical, Michigan, USA). Fatty acids were identified by comparison with the retention times of the standard. Plasma fatty acid compositions were calculated as a percentage of the total fatty acids.

2.6. Statistical analysis

Statistical tests were performed with DataDesk 6.0 (Data Description, Inc., Ithaca, NY, USA). A pooled *T*-test investigated differences in the baseline screening characteristics of the placebo and CLA supplementation groups. The effects of placebo and CLA supplementation on study inflammatory mediators were investigated using a two-way ANOVA (subject and time were the independent variables) and results were confirmed for all mediators by repeated measure ANOVA. Data were normalised and transformed where necessary. Results are expressed as mean±S.E.M. A P value $\leq .05$ was considered statistically significant.

3. Results

3.1. Subject details and study compliance

Thirty volunteers participated in and completed the study, with 15 subjects in each supplementation group. Both groups were similar in respect of all screening markers except the hepatic enzyme gamma-glutamyltransferase measured in serum, which was significantly higher in the CLA group ($P=.02$). The baseline characteristics of the CLA and placebo groups are presented in Table 2.

Study compliance was assessed by capsule count and total plasma fatty acid composition. The control and CLA groups consumed 96.5% and 95.9% of supplements, respectively, according to the return of extra capsules following the supplementation period. Total plasma fatty acid *c9, t11*-CLA levels were significantly increased after CLA supplementation (2.3±0.6 and 7.1±0.6 mg/g total plasma fatty acids pre- and post-supplementation, respectively; $P<.001$). In contrast, the *t10, c12*-CLA levels were not detectable following CLA supplementation. The placebo group showed no significant levels of *c9, t11*-CLA or *t10, c12*-CLA pre- and post-intervention.

3.2. PBMC markers of inflammation

Secretion of IL-2 from unstimulated PBMC was not significantly affected by CLA or placebo supplementation (data not shown). Con A-stimulated IL-2 secretion was significantly lowered (37.1%) following CLA supplementation ($P=.02$), whereas the placebo had no effect (Fig. 1A). This result was confirmed by repeated measures ANOVA ($P=.03$). Con A-stimulated PBMC IL-2 transcription, investigated by real-time RT-PCR, was not significantly affected by CLA or placebo supplementation (Fig. 1B).

Secretion of the pro-inflammatory cytokine TNFα by unstimulated and Con A-stimulated PBMC was not affected

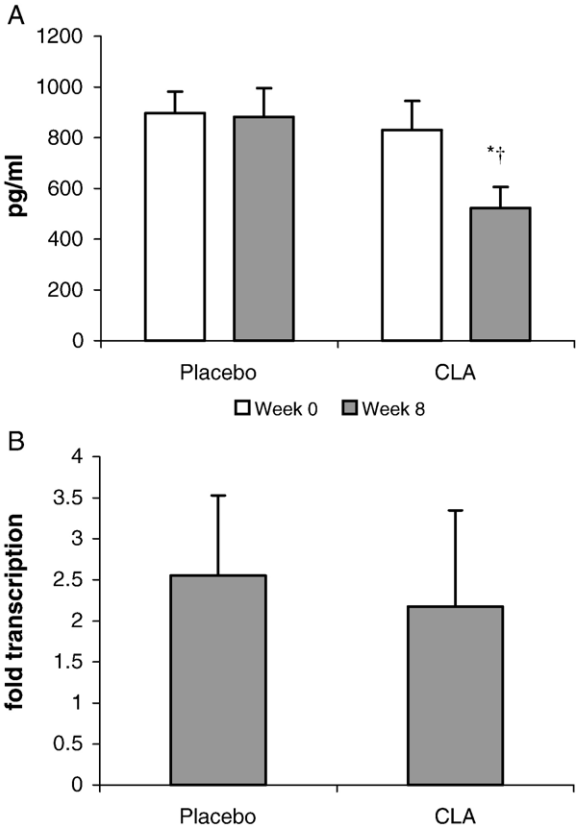

Fig. 1. The effect of CLA supplementation on (A) Con A-stimulated PBMC IL-2 cytokine secretion (pg/ml); and (B) Con A-stimulated PBMC IL-2 mRNA expression. Values represent group mean±S.E.M. IL-2 mRNA data are normalised to GAPDH and expressed relative to the control group. * Significant difference within group ($P=.05$). †Significant difference between groups ($P=.02$).

by CLA or placebo supplementation. The anti-inflammatory cytokine IL-10 was undetectable by ELISA in the supernatants of unstimulated PBMC. IL-10 secretion from Con A-stimulated PBMC was not altered by either CLA or placebo supplements. The concentrations of these cytokines before and after supplementation are presented in Table 3. Observations of significant changes in TNFα secretion following volunteer ranking by baseline plasma CRP concentrations are presented in the discussion.

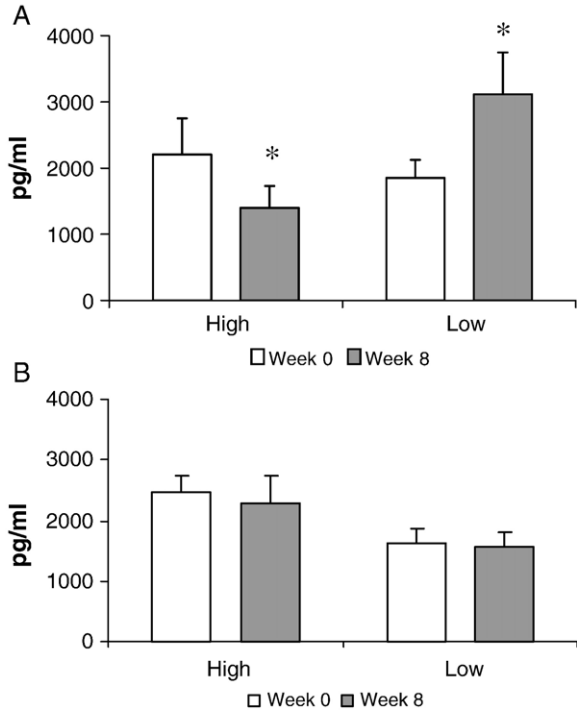

Fig. 2. The effect of supplementation on Con A-stimulated PBMC TNFα secretion (pg/ml) in the CLA test (A) and placebo (B) groups on the basis of baseline "high" (CRP ≥0.8 mg/L) and "low" (CRP <0.8 mg/L) plasma CRP concentrations. Values represent group mean±S.E.M. *Significant difference within groups (high, $P=.01$; low, $P=.03$).

3.3. Systemic markers of inflammation

Serum IL-6, plasma CRP and fibrinogen were not significantly altered by either the placebo or CLA supplements. Serum IL-6 and plasma CRP concentrations were nonsignificantly higher in the placebo group than in the CLA group throughout the study. The concentrations of these inflammatory mediators before and after supplementation are presented in Table 3.

4. Discussion

In the present study, ex vivo PBMC cytokine secretion was measured to investigate the effects of CLA supplemen-

Table 3
Mean concentrations of inflammatory mediators in placebo and CLA supplemented groups

Mediator	Units	CLA		Placebo	
		Week 0	Week 8	Week 0	Week 8
[a]IL-10	pg/ml	587.43 (177.88)	384.83 (123.46)	517.90 (115.51)	487.72 (116.32)
TNFα	pg/ml	226.17 (77.37)	218.06 (61.63)	182.19 (59.52)	213.01 (91.01)
[a]TNFα	pg/ml	2041.49 (309.52)	2200.70 (401.33)	2015.76 (212.93)	1920.42 (243.43)
IL-6	pg/ml	2.75 (2.12)	2.10 (1.47)	3.48 (2.28)	3.94 (2.49)
CRP	mg/l	1.10 (0.27)	1.17 (0.30)	1.75 (0.54)	0.97 (0.25)
Fibrinogen	mg/dl	279.85 (7.85)	278.43 (5.34)	281.99 (8.66)	284.15 (6.85)

IL-10 and TNFα measured in PBMC supernatant.
IL-6 measured in serum.
CRP and fibrinogen measured in plasma.
Values are means with standard errors in parenthesis.
[a] Con A-stimulated.

tation on markers of the T helper lymphocyte inflammatory responses. CLA reduced Con A-stimulated PBMC IL-2 secretion by 37.1%. IL-2 mRNA expression was not significantly affected by CLA supplementation, but a large degree of subject variability at the transcriptional level was observed. Elevated IL-2 levels are associated with chronic pro-inflammatory states and have been observed in patients with unstable angina [42]. Interestingly, recent research has demonstrated that the common hypolipidemic drugs, statins and fibrates, inhibit the release of the T helper (Th) 1 cytokines IL-2 and interferon (IFN) γ from lymphocytes of patients with primary type II dyslipidemia [43]. It has been demonstrated that injection of recombinant IL-2 induces a greater pro-atherogenic burden, and injection of anti-IL-2 has a profound anti-atherogenic effect in apo-E knockout mice [44]. A pro-atherogenic role for IL-2 in sensitising vascular smooth muscle cells to provocative agents was suggested by Nabata et al. [45] when IL-2 increased intracellular Ca^{2+} and stimulated glycosaminoglycan and prostacyclin synthesis in response to angiotensin II. There is increasing evidence that IL-2, an important classical Th1 inflammatory cytokine, may potentate atherosclerosis. Therefore, nutritional interventions, which attenuate IL-2 secretion, as observed in the present CLA supplementation trial, may be considered beneficial.

IL-10 is a Th2 cytokine that inhibits Th1 proliferation, chemotaxis and expression of IL-2 and IFN-γ [46]. It is considered anti-inflammatory and protective against atherosclerosis [47]. In a model of porcine colitis, CLA supplementation suppressed lymph node IL-10 expression, maintaining it at noninfected state levels [48]. Our study demonstrated that CLA had no effect on Con A-induced PBMC IL-10 secretion. This finding agrees with a recent publication which showed that neither *c9, t11*-CLA nor *t10, c12*-CLA had a significant effect on PBMC IL-10 production [25].

TNFα promotes pro-inflammatory cytokine expression, activates monocytes and macrophages, and increases host defences [49]. In accordance with data from other human trials [23–25], our study showed that CLA did not directly affect PBMC TNFα secretion. However, upon further analysis, when volunteers were ranked according to baseline plasma CRP concentrations, CLA significantly modulated TNFα secretion. CRP has been proposed, albeit not substantiated, as a predictive risk factor for CVD [50,51]. CLA supplementation had no effect on plasma CRP concentrations in this healthy male cohort not suffering from any pro-inflammatory diseases. Nevertheless, when volunteers were ranked into low and high CRP groups according to baseline CRP concentrations (CRP <0.8 mg/L and CRP ≥ 0.8 mg/L), TNFα secretion was significantly reduced (36.5%; $P=.01$) after CLA supplementation in the high CRP group but significantly increased (40.5%; $P=.03$) by CLA supplementation in the low CRP group (Fig. 2). This effect of CLA on TNFα according to baseline CRP levels was not evident in the placebo-supplemented groups. Interpretation of these trends may be limited by the number of volunteers in the high and low CRP groups ($n=16$ and $n=12$, respectively). Nevertheless, this analysis indicates that subtleties in the anti-inflammatory effects of CLA, or any nutrient-based immune regulator, may be affected by a background inflammatory profile that reflects an individual's genotype and/or other environmental exposures.

To date, relatively few studies have investigated the effects of CLA supplementation on markers of human immunity. Kelley et al. [22,23] showed no significant effect of CLA supplementation on PBMC TNFα, IL-1β, prostaglandin E_2 (PGE_2) and IL-2 production. This study, in comparison to others cited, supplemented subjects with less *c9, t11*-CLA and *t10, c12*-CLA (0.7 and 0.9 g/day, respectively). Albers et al. [24] demonstrated that CLA may beneficially affect the initiation of an immune response to hepatitis B vaccination but had no effects on ex vivo PBMC secretion of TNFα, IL-1β, IL-6, IFNγ, IL-2, IL-4 and PGE_2. Nugent et al. [26] used supplements (1.7 g 50:50 and 1.6 g 80:20 active isomers per day) similar to those used by Albers et al. (1.9 g 50:50 and 1.7 g 80:20 active isomers per day) and also demonstrated no effects on PBMC secretion of cytokines. Each of these studies used n-6 PUFA-based placebo. The present study used a placebo containing a blend of fatty acids representative of those found in a typical Western diet. The %w/w of linoleic acid in the placebo was 17.89%; the %w/w of polyunsaturated fatty acids of a "prudent" Irish diet has been reported to be 17.89%. Saturated and monounsaturated fat content of placebo were also comparable to that of the Irish diet [33]. It is possible that effects of CLA on cytokine expression may be obscured by the use of linoleic acid as a placebo [26]. The fatty acid composition of a placebo in a supplementation trial should reflect that of the indigenous diet. In a recent cross-over trial where no placebo was used, Tricon et al. [25] showed that *c9, t11*-CLA and *t10, c12*-CLA single-isomer supplementation resulted in a dose-dependent reduction in the mitogen-induced activation of T lymphocytes but had inconsistent effects on a range of cytokines. The minimal effects of CLA on immune function were observed in human supplementation trials despite significant increase in the CLA content of PBMC [23,24,52] following 8–12 weeks CLA supplementation. The present study demonstrated a significant increase in the *c9, t11*-CLA composition of plasma fatty acids following 8 weeks of supplementation, indicating that an 8-week supplementary period is sufficient to achieve CLA incorporation into plasma and immune cells, with subsequent possible effects on markers of inflammation.

The difference in the reported effects of CLA supplementation on IL-2 production between the present and other human studies may relate to a number of experimental differences, including the nature of the pro-inflammatory mitogen and the age profile of the cohort. Con A was employed in the present study; other studies used PHA or LPS [22,23,25]. Both Con A and PHA bind to the $T_{idotypic}$

portion of the T-cell receptor (TCR). However, the effect of mitogens on CD3, a complex of five invariant polypeptide chains that form the non-antigenic-related portion of the TCR, is controversial [53]. While Con A binds to CD3, there is some debate as to whether PHA interacts with CD3. Also, Con A binds more effectively with CD2, another T lymphocyte surface glycoprotein, than PHA [54]. This suggests different affinities for the TCR and other T-cell surface molecules and thus mitogenic potencies. Indeed, our group has shown that PHA-induced cytokine secretion was not significantly altered by CLA supplementation [26]. But in the present study, Con A-induced IL-2 secretion was affected by CLA supplementation. It is also worthy to note that the mean age (31.5±1.3 years) of the subjects in our previous study [26] was considerably younger than that of the present group (49.4±1.3 years). As age affects immune function and reactivity, this could also account for differences between studies [27,28].

CLA had no significant effects on the systemic inflammatory mediators associated with CVD investigated in this study. IL-6 is produced by a variety of cell types including fibroblasts, endothelial cells, monocytes, T- and B- lymphocytes. It increases basal glucose uptake, alters insulin sensitivity, increases the release of adhesion molecules from the endothelium, increases hepatic release of fibrinogen and regulates hepatic synthesis of CRP [55]. While it has been shown that CLA suppressed IFN-γ induced IL-6 production in RAW macrophages [56], our study showed no effect. This finding agrees with those of recent studies which have shown active isomeric blend, and *t10, c12*-CLA supplementation had no effect on plasma IL-6 concentration in adults with the metabolic syndrome or diabetes [57,58].

Although a causal role of CRP in CVD remains unsubstantiated, the association between concentrations of CRP and CVD risk factors, and its predictive value for CVD, appears strong [51,59]. Riserus et al. [58] demonstrated that *t10, c12*-CLA markedly increased CRP concentrations in men with the metabolic syndrome. Smedman et al. [60] have shown that supplementation with a 50:50 CLA isomer mix (4.2 g/day for 3 months) significantly increased circulating CRP concentrations in apparently healthy volunteers. Tricon et al. [25] found neither the *c9, t11*-CLA nor *t10, c12*-CLA isomer affected serum CRP in healthy volunteers. In subjects at high risk of coronary heart disease, supplementation with the individual CLA isomers (3 g/day for 13 weeks) had no effect on CRP concentrations [61]. Moloney et al. [57] also demonstrated no effect of supplementation with 3 g/day CLA active isomer mix for 8 weeks on type II diabetic subjects. No effect of the CLA isomeric mix on CRP concentrations was observed in the present study. Also, the lack of effect of CLA on CRP and fibrinogen may have been in consequence of the regulatory role of IL-6 on their hepatic synthesis and release. Interestingly, IL-6 and CRP concentrations were notably, although nonsignificantly, higher in the placebo group at baseline and the end of the supplementary period. This may represent a greater presence of inflammatory genotype within the placebo group. The −174G/C allele of the IL-6 promoter, for example, is associated with an increased transcription of IL-6, elevated CRP concentrations and genetic predisposition to systemic inflammation [62,63].

It is important to note that CLA has been associated with potentially adverse effects, particularly with respect to fasting glucose, insulin resistance, lipid peroxidation and inflammatory markers in obese and type 2 diabetic subjects [57,58]. Tricon et al. [64] recently reported a small increase in the ratio of LDL to HDL in healthy volunteers consuming dairy products fortified with both isomers, although there was no significant effect on fasting glucose, triacylglycerol, LDL, HDL or total cholesterol. Elsewhere, Tricon et al. [25] reported that supplementation of healthy volunteers with highly purified isomers had opposing effects; *c9, t11*-CLA decreased the ratios of LDL and total cholesterol to HDL, whereas *t10, c12*-CLA increased the ratios. Moloney et al. [57] reported a decrease in the ratio of LDL to HDL following supplementation with a 50:50 blend of active CLA isomers. Interestingly, it has been shown that the significant reduction in plasma TAG concentrations induced by supplementation with 3 g/day of 50:50 *c9, t11*-CLA and *t10, c12*-CLA isomers for 8 weeks was lost when an 80:20 *c9, t11*-CLA and *t10, c12*-CLA supplement was used [40]. The results of these papers, demonstrating effects of CLA on blood lipids, are incongruent. Other studies have shown no effect of CLA supplementation or fortification on fasting glucose and blood lipids in healthy subjects [60,65,66] and those at greater risk of CVD [67]. In the present study, we measured the effect of CLA supplementation on the classical lipid-related risk factors (plasma cholesterol, triacyglycerol, high-density lipoprotein, low-density lipoprotein cholesterol and glucose concentrations; data not shown). None of these markers was adversely affected by CLA, an effect which probably reflects this cohort's normoglycaemic state and low risk of impaired glucose tolerance and insulin resistance. On the basis of the parameters investigated in the present study, there were no indications of potential adverse metabolic effects of supplementation with 2.2 g CLA mix in a cohort of middle-aged healthy men.

Human supplementation trials and animal feeding studies have given opacity to the effects of CLA on inflammatory mediators and circulating lipids. The use of single and mixed isomeric blends, placebos atypical of dietary fatty acid composition, a variety of agents that stimulate ex vivo PBMC, and cohorts varying in age and health status leaves many published studies incomparable. Furthermore, genetic variation between individuals is increasingly recognised as a distorting factor for interpretation of the effects of fatty acids on cytokine gene expression. Recently, however, a number of cellular studies have regenerated confidence in the anti-inflammatory potential of CLA. It has long been established that CLA isomers are ligands for the anti-inflammatory PPAR family of transcription factors [68]. The *c9, t11*-CLA

isomer has recently been shown to reduce the transcriptional activity of the pro-inflammatory NF-κB family of proteins in prostate cancer cells [69], murine carcinogenic skin cells [70] and human colon cancer cells [71]. CLA feeding has been shown to reduce NF-κB activation in ex vivo and in vivo LPS-activated porcine PBMC [18]. In addition to reducing NF-κB activity and nuclear translocation, *c9, t11*-CLA has suppressed IL-12, enhanced IL-10R and enhanced IL-10 production at transcriptional and protein level in LPS-activated dendritic cells. The suppression of IL-12 was IL-10-dependent and mechanistically occurred with enhanced activation of ERK [72].

In conclusion, this study showed that CLA decreased PBMC IL-2 secretion from Con A-stimulated PBMC. This suggests that CLA may modulate components of the Th1 inflammatory response. The potential benefits of an immuno-modulatory agent represent a fine balance between attenuating a chronic pro-inflammatory response, whilst not adversely affecting immune defences. A potential limitation of the present study is the number of subjects. Ideally, to measure such an effect of CLA supplementation on the immune response a minimum of 20 subjects are required to demonstrate statistical differences at 80% power. Furthermore, analysis of CLA incorporation into PBMC phospholipid membrane following supplementation may strengthen the evidence for mechanistic effects of this fatty acid in T-lymphocyte populations. More work is required to determine whether CLA could have beneficial effects in models of Th1-mediated inflammatory conditions. Also, the isomer specific, and possibly age-dependent, effects of CLA on inflammatory mediators associated with human disease need to be investigated in vivo.

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