Dietary lipids modify the cytokine response to bacterial lipopolysaccharide in mice

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SUMMARY

To investigate the effect of dietary lipids with different fatty acid compositions upon the *in vivo* cytokine response to bacterial lipopolysaccharide (LPS), mice were fed for 5 weeks on a low-fat diet or on one of four high-fat diets that contained 20%, by weight, of coconut oil (CO), olive oil (OO), safflower oil (SO) or fish oil (FO). The mice were injected intraperitoneally with a non-lethal dose of *Escherichia coli* LPS (100 µg/20 g body weight) and killed 90 or 180 min later. Plasma tumour necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-6 and IL-10 concentrations were measured by enzyme-linked immunosorbent assay (ELISA). Plasma TNF-α and IL-10 concentrations were higher 90 min postinjection than after 180 min, whereas plasma IL-1β and IL-6 concentrations were higher 180 min postinjection than after 90 min. Peak plasma TNF-α, IL-1β and IL-6 concentrations were lower in the CO- and FO-fed mice than in those fed the SO diet. Peak plasma IL-10 concentrations were higher in CO-fed mice than in those fed some of the other diets. These observations suggest that, relative to the *n*-6 polyunsaturated fatty acid-rich SO diet, CO and FO diminish production of proinflammatory cytokines *in vivo*. This indicates that these fatty acids might be useful therapies in acute and chronic inflammatory diseases. The enhanced production of IL-10 following CO feeding appears to be an additional antiinflammatory effect of this oil, which could give added benefit in various clinical conditions.

INTRODUCTION

Severe bacterial infections or sepsis can result in profound physiological changes, including hypotension, fever, tissue breakdown, widespread organ dysfunction and, ultimately, death.[1,2] In the case of Gram-negative bacteria this toxicity is caused by endotoxin, a lipopolysaccharide (LPS) component of the bacterial cell wall.[3,4]

Circulating concentrations of the proinflammatory mediators tumour necrosis factor (TNF) and interleukin (IL)-1 are elevated in patients with sepsis and after LPS administration in humans and laboratory animals.[5] These cytokines mimic the effects of LPS,[6–8] and animals can be protected from bacterial- and LPS-induced shock by neutralization of these cytokines.[9–11] Furthermore, mice deficient in the 55×10^3 MW TNF-α receptor are resistant to endotoxic shock.[12] Thus, the proinflammatory cytokines play a key role in the progression of sepsis, suggesting that inhibition of their production might prevent the pathology of endotoxic shock.

LPS injection in experimental animals also leads to an

increase in plasma IL-10 concentrations.[13,14] IL-10 suppresses LPS-induced production of TNF-α, IL-1α, IL-1β and IL-6 by human monocytes[15,16] and murine peritoneal macrophages[17,18] *in vitro*. Neutralization of IL-10 by specific antibodies in LPS-injected mice leads to markedly elevated levels of circulating TNF-α and IL-6 and enhances LPS toxicity.[13,14] Administration of IL-10 reduces circulating TNF-α concentrations post-LPS administration[19–21] and prevents the onset of septic shock.[19,20] These observations suggest that IL-10 protects against the lethal effects of LPS by suppressing the production of the damaging proinflammatory cytokines.

Fish oil (FO) feeding significantly enhances the survival of guinea-pigs following LPS challenge[22,23] and decreases the accompanying lactic acidosis,[24] fever[25] and anorexia.[26,27] Likewise, FO feeding to humans results in a slightly reduced febrile response to LPS.[28] These effects of FO are often attributed to the changes in eicosanoid metabolism induced by the eicosapentaenoic acid (20:5n-3) found in FO: this fatty acid decreases the production of the proinflammatory eicosanoids, such as prostaglandin E_2 (PGE$_2$), generated from arachidonic acid (20:4n-6) and gives rise to an alternative family of eicosanoids that are less biologically active than those produced from arachidonic acid.[29]

More recently it has become appreciated that FO can alter production of proinflammatory cytokines (see reference 30 for a review). For example, production of TNF-α and IL-1 by Kupffer cells was decreased after feeding rats a FO-rich diet compared with feeding safflower oil (SO)- or corn oil-rich diets, which are rich in the *n*-6 polyunsaturated fatty acid

linoleic acid (18:2n-6).[31] Likewise, FO feeding decreased *ex vivo* production of TNF-α, IL-1 and IL-6 by murine thiogly-collate-elicited peritoneal macrophages[32,33] and of TNF-α and IL-6 by rat peripheral blood mononuclear cells.[34] In humans, FO supplementation has been shown to decrease *ex vivo* production of TNF,[35–38] IL-1[35–39] and IL-6[36,37,39] by peripheral blood mononuclear cells. Such effects might account for the prolongation of survival and the decreased pathology in response to LPS which accompanies FO feeding. However, to our knowledge, only one study has examined the effect of FO feeding on cytokine production *in vivo*[40] and that study examined TNF only.

Therefore, the current study set out to compare the effect of feeding mice fish and other oils upon plasma cytokine concentrations in response to LPS. In doing so, this is the first study to examine the effect of dietary lipids on the *in vivo* production of IL-1β, IL-6 and IL-10.

MATERIALS AND METHODS

Animals and diets

Male C57B16 mice (weighing ≈15 g) were purchased from Charles River (Margate, Kent, UK). They were housed individually in plastic cages in controlled environmental conditions (21·5±0·5°; 45±2% humidity; 12 hr light/12 hr dark cycle). After acclimatization for 1 week, the mice were randomly allocated to receive one of five diets. They were maintained on this diet for 5 weeks until killed. The diets used were a low-fat (LF) diet and four high-fat diets (all prepared by ICN Biomedicals, High Wycombe, Bucks, UK); the LF diet most closely resembled normal mouse chow and contained 2·5% by weight of corn oil. The high-fat diets contained 20% by weight of the lipid under study (coconut oil (CO), olive oil (OO), SO or FO), plus 1% by weight corn oil to prevent essential fatty acid deficiency. All diets contained 20% protein, 20% starch, 29·6% sucrose and 0·12% vitamin E (250 U/g). The fatty acid composition of the diets is shown in Table 1. Apart from the FO diet, which was stored frozen, all diets were stored at 20°. Diets were provided fresh to the animals every 2 days. Mice fed on standard laboratory chow were used for preliminary experiments. All procedures involving experimental animals were approved under the Animals (Scientific Procedures) Act 1986, by the Home Office.

LPS administration and blood collection

Mice were injected intraperitoneally (i.p.) with 1 ml of sterile phosphate-buffered saline (PBS) containing *Escherichia coli* serotype 0111:B4 LPS, purchased from Sigma Chemical Co. (Poole, Dorset, UK; see the Results for the amounts used); control mice received sterile PBS alone. The mice were killed with an overdose of CO_2 (see the Results for timing of killing). Blood was collected by cardiac puncture into heparin (≈20 μl heparin/ml blood) and plasma was prepared by centrifugation (4000 *g* for 5 min). Plasma was aliquoted under sterile conditions and was stored frozen until analysis.

Cytokine measurements

Plasma TNF-α, IL-1α, IL-6 and IL-10 concentrations were measured using Cytoscreen enzyme-linked immunosorbent assay (ELISA) kits purchased from BioSource International (Camarillo, CA). All measurements were made according to the instructions given by the manufacturers.

Statistics

Data are presented as mean±SEM for the indicated number of separate animals. Statistically significant effects of diet were determined by one-way analysis of variance followed by Duncan's multiple range test. Statistical analyses were performed using SPSS for Windows (SPSS Inc., Chicago, IL).

RESULTS

### Timing and dose dependence of the cytokine response to LPS

Prior to investigating the effects of different types of dietary lipids on the cytokine response to i.p. LPS, it was important to define the time course of this response and its dependence upon LPS concentration.

To investigate the time course of the response, chow-fed mice (n=3 per time point) were injected with 100 μg LPS/20 g body weight and killed at 30, 60, 90, 120 and 240 min postinjection; blood was collected from non-injected mice to provide data for time 0. The concentration of TNF-α in the plasma had increased by 60 min and peaked 90 min postinjection (Fig. 1a). Thereafter the TNF-α concentration decreased, returning to 0-time values by 180 min (Fig. 1a). IL-1β and IL-6 concentrations in plasma were also elevated by 60 min postinjection and these peaked at 180 min postinjection before decreasing sharply (Fig. 1b, 1c). Plasma IL-10 concentrations were elevated by 30 min postinjection, peaked at 90 min postinjection and decreased slightly thereafter, but still remained elevated relative to baseline levels (Fig. 1d).

Table 1. Fatty acid composition of the diets used

| Diet | Fatty acid (g/100 g total fatty acids) | | | | | | | | | | |
	10:0	12:0	14:0	16:0	16:1 *n*-7	18:0	18:1 *n*-9	18:2 *n*-6	18:3 *n*-3	20:5 *n*-3	22:6
LF	ND	3·4	3·5	13·7	ND	3·3	21·9	54·3	ND	ND	ND
CO	6·8	56·5	17·6	7·8	ND	5·1	2·0	2·3	ND	ND	ND
OO	ND	ND	ND	14·9	2·2	3·7	60·0	19·1	ND	ND	ND
SO	1·6	4·3	1·1	8·7	ND	3·9	19·4	61·0	ND	ND	ND
FO	ND	ND	10·3	22·0	14·5	4·2	11·8	9·0	3·5	10·6	10·1

CO, coconut oil; FO, fish oil; LF, low fat; OO, olive oil; SO, safflower oil.
ND, not detected.

S. Sadeghi *et al.*
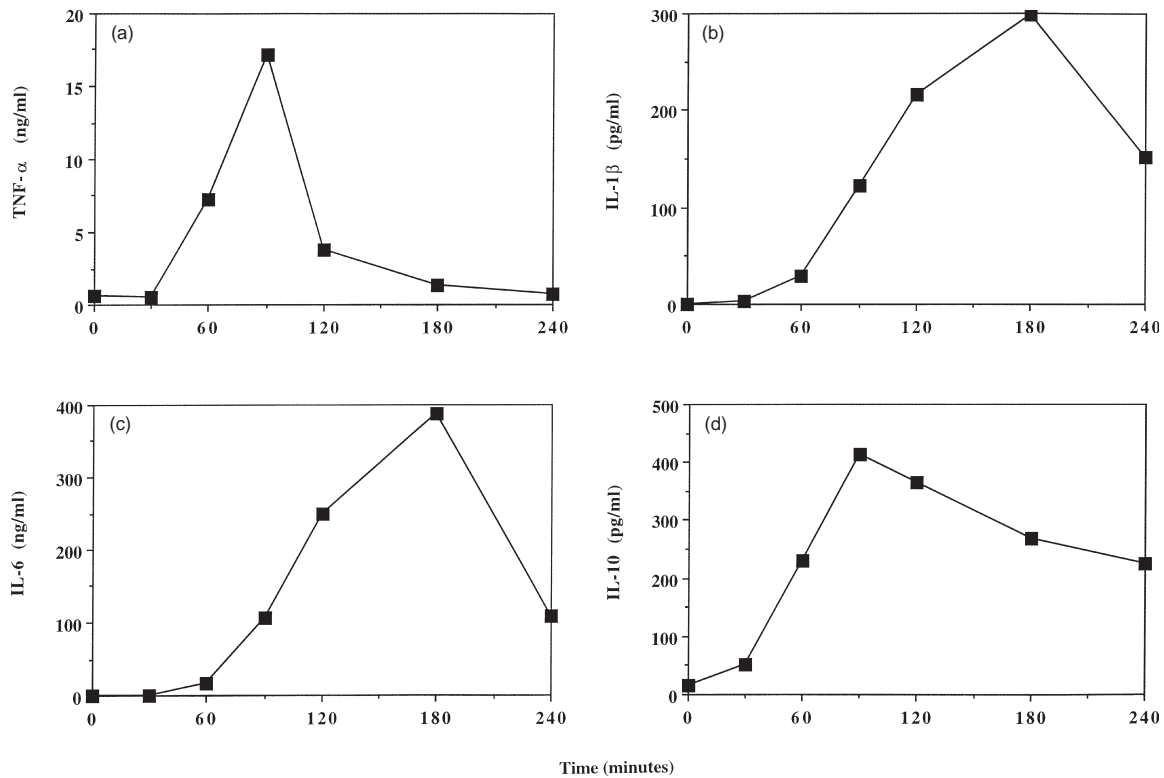
Figure 1. Time course of plasma concentrations of (a) tumour necrosis factor-α (TNF-α), (b) interleukin (IL)-1β, (c) IL-6, or (d) IL-10 following intraperitoneal (i.p.) lipopolysaccharide (LPS) injection. Chow-fed mice were injected with 100 μg LPS/20 g body weight. Data represent the mean value from three animals.

To investigate the response to different concentrations of LPS, chow-fed mice ($n = 3$ per LPS dose) were injected with 25, 50, 100 or 250 μg LPS/20 g body weight and were killed at 90 or 180 min postinjection; mice injected with PBS alone were used as controls. The plasma concentrations of all four cytokines under study were highest following injection of LPS at a dose of 100 μg/20 g body weight (Fig. 2).

Effects of dietary lipids on plasma cytokine concentrations

None of the cytokines investigated were present in the plasma of PBS-injected mice ($n = 6$ per diet), irrespective of the diet fed (data not shown). Diet had a significant effect on post-LPS concentrations of TNF-α ($P = 0.019$), IL-1β ($P = 0.045$), IL-6 ($P < 0.0001$) and IL-10 ($P = 0.041$).

Ninety minutes after i.p. LPS (100 μg/20 g body weight), TNF-α concentrations were significantly lower in the plasma of CO- or FO-fed mice than in the plasma of those fed the LF or SO diets (Fig. 3a). There were no differences in plasma IL-1β concentrations 90 min after LPS injection among mice fed the different diets (data not shown). In contrast, after 180 min, plasma IL-1β concentrations were significantly higher in mice fed the SO diet than in those fed the LF, CO or FO diets (Fig. 3b). FO-fed mice had significantly lower plasma IL-6 concentrations 180 min after LPS injection than any other group (Fig. 3c). Plasma IL-6 concentrations were also significantly lower in CO-fed mice than in SO-fed mice (Fig. 3c). Plasma IL-10 concentrations were significantly higher in CO-fed mice 90 min post-LPS injection than in those fed the LF or OO diets (Fig. 3d).

As a result of the effects of diet on the plasma concentrations of TNF-α and IL-10, the ratio between these cytokines 90 min post-LPS was significantly affected ($P = 0.042$). The TNF-α:IL-10 ratio was significantly lower in mice fed the CO diet (9.3 ± 1.1) compared with those fed the LF diet (24.4 ± 4.1). In mice fed the other diets, this ratio was 18.5 ± 3.1 (OO), 18.0 ± 3.0 (SO) and 18.5 ± 5.5 (FO).

DISCUSSION

This study investigated the effect of different dietary lipids upon the cytokine response to bacterial LPS *in vivo*. The lipids studied were selected because the fatty acids they contain represent the principal classes of fatty acids found in the human diet and/or because it has been proposed that the fatty acids they contain possess pro- or anti-inflammatory activities.[41,42] Furthermore, the types of oils used in this study are the basis of the lipid emulsions found in licensed or experimental parenteral formulae designed for use in patients in trauma situations. As such patients are at risk of sepsis, it seems important to determine the effects of the lipids that are administered upon components of the acute inflammatory response.

The time course of the plasma cytokine responses seen in this study agree with those reported previously in mice,[13,14] as are the concentrations observed.[14,21] Thus, the cytokine response to LPS observed in the current study appears to be very similar to that reported by others.

This study found that dietary lipids affect the cytokine response to bacterial LPS *in vivo*. The key effects were: lower
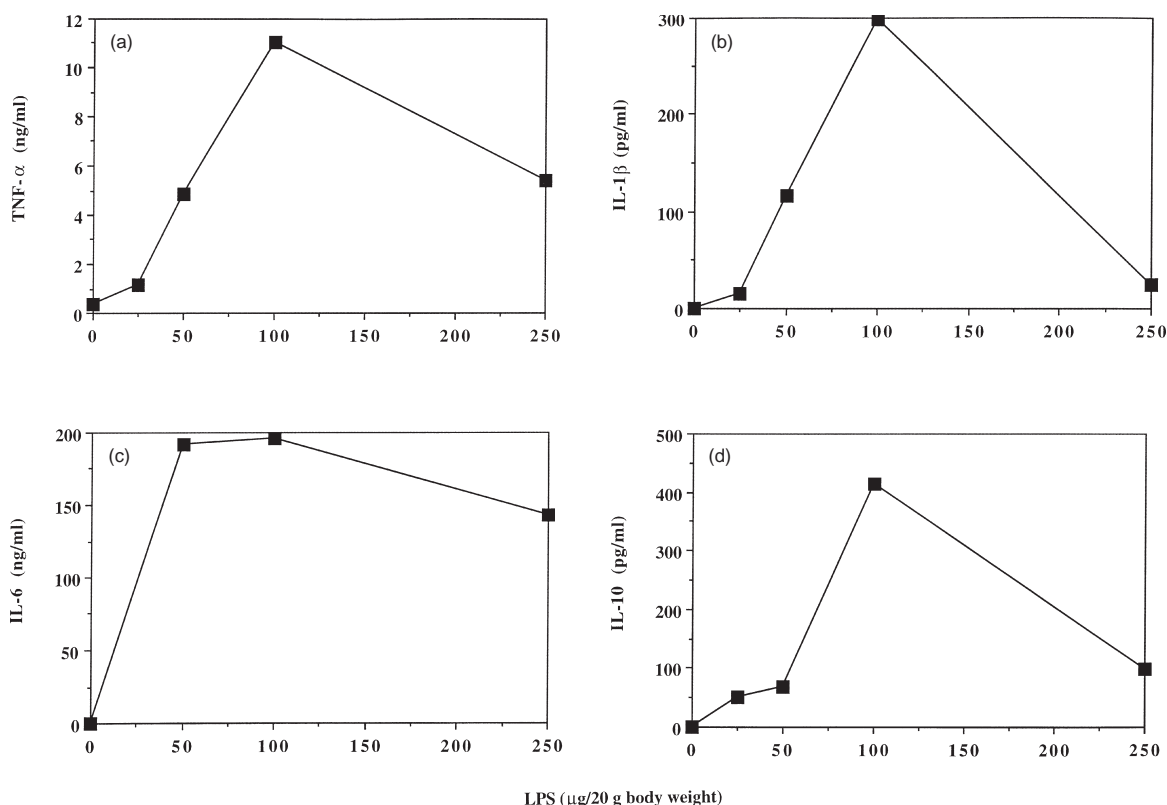
Figure 2. Dependence of plasma concentrations of (a) tumour necrosis factor-α (TNF-α), (b) interleukin (IL)-1β, (c) IL-6, or (d) IL-10 upon dose of lipopolysaccharide (LPS) injected. Chow-fed mice were injected with 0, 25, 50, 100 or 250 μg LPS/20 g body weight. Data represent the mean value from three animals killed at 90 min (a, d) or 180 min (b, c) postinjection.

TNF-α, IL-1β and IL-6 concentrations following CO or FO feeding than after feeding the SO diet; and higher IL-10 concentrations following CO feeding than after feeding some other oils. These observations suggest that, relative to the *n*-6 polyunsaturated fatty acid-rich SO diet, CO and FO diminish production of proinflammatory cytokines *in vivo*. The lower plasma concentrations of TNF-α, IL-1β and IL-6 in LPS-injected mice fed FO might account for the significantly enhanced survival of guinea-pigs given high doses of LPS[22,23] and for the decreased pathophysiological responses to LPS.[24–28] Taken together, these observations indicate that inclusion of FO, or CO, or the fatty acids they contain, in regimens for intravenous use in patients at risk of developing acute inflammatory responses, might be beneficial. Furthermore, inclusion of these fatty acids in the diet of patients with chronic inflammatory diseases might also be beneficial. The enhanced production of IL-10 following CO feeding appears to be an additional anti-inflammatory effect of this oil, which could convey added benefit in various clinical conditions.

The observations made in the current study may be compared with those of previous studies. FO feeding has been reported to decrease *ex vivo* production of proinflammatory cytokines by rat Kupffer cells,[31] by mouse thioglycollate-elicited macrophages,[32,33] by rat blood mononuclear cells[34] and by human blood mononuclear cells.[35–39] In contrast, some other studies show no effect of FO feeding or even enhanced production (see reference 30 for appropriate references). The outcome from *ex vivo* measurements might be, in part, affected by the experimental protocols used (e.g. the type of macrophage used and its state of activation at harvesting, the agent used to elicit macrophages where used, the type of serum present in *ex vivo* cultures and its concentration, the duration of *ex vivo* culture, etc.). The current study used plasma cytokine concentrations as a measure of the response of the intact physiological system in order to avoid the problems associated with *ex vivo* culture of purified cells. The lower plasma cytokine concentrations observed after FO feeding agree with some studies of *ex vivo* production of TNF,[31–38] IL-1[31–33,35–39] and IL-6[32–34,36,37,39] by LPS-stimulated rodent and human mononuclear cells. Studies that have used CO have not reported any significant differences in *ex vivo* cytokine production by macrophages compared with those from animals fed other diets such as OO, SO or corn oil.[33,40] This indicates a different series of events occurring *in vivo* compared with those occurring in cell culture. One component that will be absent in cell culture is the interaction between different cell types. No published studies have investigated the effect of dietary lipids on IL-10 production by macrophages or monocytes.

The only previous study reporting the effect of dietary lipids upon the cytokine response to LPS *in vivo* found enhanced peak plasma TNF concentrations in mice fed 20% FO compared with those fed LF, or 20% corn oil or CO diets.[40] The reason for the discrepancy between this observation and those of the current study is unclear.

How the lipids used in the current study act to influence cytokine production is not clear and was not investigated.
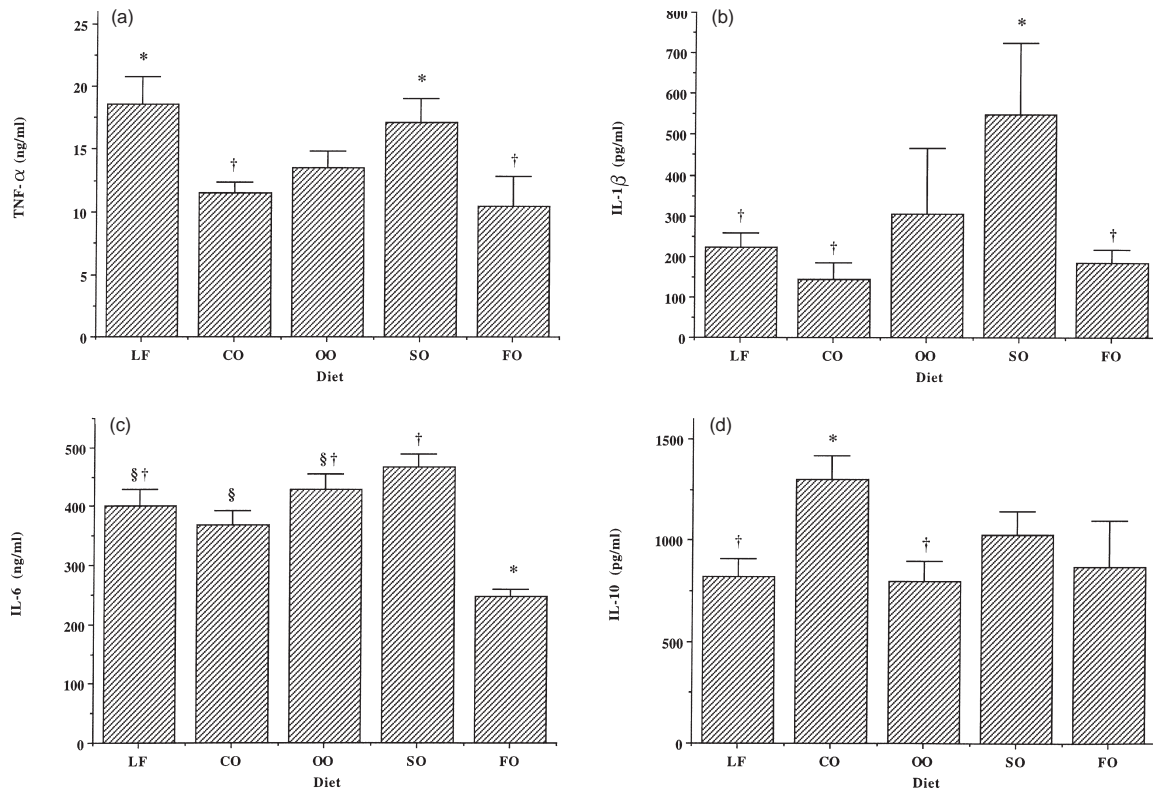
Figure 3. Effect of dietary lipids on post-lipopolysaccharide (LPS) plasma cytokine concentrations. Plasma was collected 90 (tumour necrosis factor-α (TNF-α), interleukin (IL)-10) or 180 (IL-1β, IL-6) min after intraperitoneal (i.p.) injection of LPS (100 μg/20 g body weight). Data are expressed as mean ± SEM for six mice fed on each diet. Bars not sharing a common symbol are significantly different from one another.

However, dietary FO has been shown to significantly lower TNF-α mRNA levels in LPS-stimulated murine peritoneal macrophages,[32] IL-1β mRNA levels in LPS-stimulated murine spleen lymphocytes,[43] and TNF-α, IL-1β and IL-6 mRNA levels in the kidneys of lupus-prone mice.[44] These observations suggest that one or more components of FO can alter proinflammatory cytokine gene expression, perhaps by altering the intercellular signalling mechanisms that lead to activation of proinflammatory cytokine genes. This might occur through inhibition of activation of transcription factors, such as nuclear factor κB (NFκB), which regulate activation of the TNF-α, IL-1β and IL-6 genes. NFκB is activated by phosphorylation, often by protein kinase C, and subsequent dissociation of its inhibitory subunit. The n-3 polyunsaturated fatty acids found in FO have been shown to directly inhibit protein kinase C from brain,[45] spleen lymphocytes[46] and macrophages[47] and so these fatty acids might prevent the activation of NFκB by this mechanism. This effect could account for the reduced plasma TNF-α, IL-1β and IL-6 concentrations, seen in the current study, in FO-fed mice following LPS administration.

Eicosanoids derived from the n-6 polyunsaturated fatty acid, arachidonic acid, might activate NFκB via protein kinase C.[48] This might account for the fact that plasma TNF-α, IL-1β and IL-6 concentrations were highest in mice fed diets rich in the arachidonic acid precursor linoleic acid (i.e. the LF and SO diets). Furthermore, the concentrations of these cyokines were lowest in the plasma of mice fed diets that were poor in linoleic acid (i.e. the CO and FO diets). FO is known to

diminish production of arachidonic acid-derived eicosanoids[29,33] and this effect could account for the reduced plasma TNF-α, IL-1β and IL-6 concentrations, seen in the current study, in FO-fed mice following LPS administration.

IL-10 suppresses LPS-induced production of TNF-α, IL-1β and IL-6 in vitro,[15–18] neutralization of IL-10 in LPS-injected mice leads to markedly elevated levels of circulating TNF-α and IL-6,[13,14] and administration of IL-10 reduces circulating TNF-α concentrations post-LPS administration.[19–21] IL-10 concentrations were not elevated in the plasma of FO-fed mice post-LPS, suggesting that the decreased plasma TNF-α, IL-1β and IL-6 concentrations in these animals were not a result of enhanced IL-10 production. In contrast, feeding CO elevated plasma IL-10 concentrations such that the ratio of TNF-α:IL-10 was lowered in mice fed this diet. Thus, CO might reduce proinflammatory cytokine production post-LPS by enhancing production of IL-10. How this might occur is not clear but, given the important protective role of IL-10 in LPS-induced shock, this is an exciting observation.

The observations made in the current study are of relevance to acute and chronic inflammatory conditions. With respect to the former, critically ill patients are often provided with artificial nutritional support, although the nature of the precise components of this remain controversial. The most widely used formulae for parenteral nutrition include lipid emulsions based upon n-6 polyunsaturated fatty acid-rich oils, such as soybean oil or SO. Concern has been expressed that the high levels of n-6 polyunsaturated fatty acids in these emulsions

may be responsible for some of the observed deleterious effects on reticuloendothelial system and immune function during total parenteral nutrition.[49–51] The findings of the current study support this concern because they suggest that such oils might favour enhanced proinflammatory cytokine production in patients already at risk of acute overproduction of such cytokines and the resulting septic shock and multiorgan failure. This study supports the idea of using alternative lipid sources in such formulae, particularly CO or FO. Lipid emulsions that include FO or the medium-chain saturated fatty acids typical of CO, or mixtures of the two, are available[52] and warrant study in experimental and pathological acute inflammation.

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