Reduced ex Vivo Interleukin-6 Production by Dietary Fish Oil Is Not Modified by Linoleic Acid Intake in Healthy Men¹⁻³

Camilla T. Damsgaard,¹ Lotte Lauritzen,²⁺ Philip C. Calder,³ Tanja R. Kjær,⁴ and Hanne Frøkiær⁵

¹Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, Frederiksberg DK-1958, Denmark; ²Institute of Human Nutrition, School of Medicine, University of Southampton, Southampton SO16 6YD, UK; ³Center of Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Lyngby DK-2800, Denmark

Abstract

Fish oil (FO) is considered antiinflammatory, but evidence regarding its effect on human cytokine production is conflicting. High linoleic acid (LA) intake may impair any effects of FO. The aim of this study was to investigate how FO combined with high or low LA intake affected cytokine production from cultures of whole blood, peripheral blood mononuclear cells (PBMC), and monocytes in healthy men. The study was a double-blinded, controlled, 2 × 2 factorial 8-wk intervention. Sixty-four healthy men were randomized to 5 mL/d FO or olive oil (OO) provided in capsules and to spreads and foods with high or low LA content, resulting in LA intakes of 7 ± 2% and 4 ± 1% energy, respectively. We measured eicosapentaenoic acid (EPA) in PBMC and stimulated cytokine production in whole blood and PBMC 24-h cultures before and immediately after intervention and after an 8-wk wash-out period, and in monocyte cultures immediately after intervention. PBMC-EPA was markedly increased by FO (P < 0.001). LA intake did not modify the incorporation of FO and tended to have only a slight effect on PBMC-EPA by itself (P = 0.06). Lipopolysaccharide (LPS)-stimulated whole-blood interleukin (IL)-6 production immediately after intervention was lower with FO than OO (P = 0.02) but did not correlate with PBMC-EPA in the FO groups (r = −0.12; P = 0.53; n = 31). The LA intake did not modify IL-6 production or the effect of FO. Neither FO nor LA intake affected the production of tumor necrosis factor-α, IL-10, or interferon-γ in any of the cultures. In conclusion, FO intake reduced IL-6 production from LPS-stimulated whole blood in healthy men compared with OO, but the effect was not modified by the LA intake. J. Nutr. 139: 1410–1414, 2009.

Introduction

It is widely considered that (n-3) long-chain PUFA (LCPUFA)⁷ from fish oil (FO) are antiinflammatory and decrease the production of immunomodulating cytokines. This perception is partly based on trials showing that FO reduces pain and use of antiinflammatory drugs in patients with rheumatoid arthritis (1). Furthermore, FO-feeding studies in animals have shown reductions in ex vivo production of tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1 from monocyte/macrophages (2,3) and of T helper type 1 cytokines such as interferon (IFN)-γ (4). However, the evidence of an effect of FO on cytokine production in healthy humans is not as convincing. Some intervention studies showed decreased ex vivo cytokine production with FO supplementation, but many placebo-controlled, randomized trials found no effect (5). Different immune cell types may respond differently to FO and there is a lack of studies that concurrently evaluate the effects on cytokine production from stimulated whole blood, peripheral blood mononuclear cells (PBMC; a mixture of monocytes, T-, B-, and natural killer-cells), and isolated monocytes.

Eicosapentaenoic acid (EPA) can be converted to eicosanoids that regulate inflammation, but the immunomodulating effect of FO may also relate to changes in cell membrane characteristics as a result of increased incorporation of docosahexaenoic acid (DHA) (6). PUFA of the (n-6) family compete with (n-3) PUFA for incorporation into and release from cell membranes and for conversion to eicosanoids and other bioactive derivates (6). It has been argued that a high consumption of (n-6) PUFA, typically linoleic acid (LA) in the Western diet, reduces the
effects of (n-3) LCPUFA (7), but to our knowledge no study has investigated how FO in combination with different LA intakes affects ex vivo cytokine production in humans.

In this double-blinded, randomized, controlled 2 × 2 factorial intervention, we investigated how 8-wk FO supplementation in combination with a high or low LA intake affected ex vivo cytokine production from whole blood, PBMC, and isolated monocytes in healthy men.

Materials and Methods

Participants. The study protocol was approved by the Scientific Ethical Committee of Frederiksberg and Copenhagen and registered in the U.S. NIH clinical trial database (ClinicalTrials.gov, no. NCT00266292). Participants were recruited as previously described (8). In short, eligible persons were apparently healthy 18- to 40-y-old males who smoked <5 cigarettes/wk, exercised strenuously <7 h/wk, ate homemade meals ≥5 d/wk, and consumed spreads or cooking oils daily. All participants gave written informed consent to participate. Sixty-four participants (median age, 25 [range 19–40] y; BMI, 22.8 ± 1.9 kg/m²) completed the intervention period, but 1 withdrew during the wash-out period. The intervention groups did not differ at baseline in age or body size or in consumption of fish, energy, or macronutrients (8).

Study design. The details of this randomized, double-blinded, 2 × 2 factorial study design have been described previously (8). In short, participants were provided with olive oil (OO) and butter for a 2-wk prebaseline, run-in period. They were then randomized to capsules with either 5 mL/d FO (n-3) LCPUFA as FFA or OO (unrefined extra virgin as triacylglycerols) for 8 wk. Within each group, the men were also randomly allocated to replace their household spreads and oils with LA-rich types (sunflower oil and Becel 60 margarine (S/B)) or types with a low LA content [rapeseed oil and the rapeseed oil-enriched butterspread (version 15.0). Normal distribution was checked with the Shapiro-Wilk procedure (11) and transmethylated with boron trifluoride in methanolic butylated hydroxytoluene. Total lipids were extracted by the Folch procedure (10) using an ELISA kit from R&D Systems with a detection limit of 0.10 µg/L, but no IL-12 was detected. CpG-DNA did not induce detectable cytokine concentrations. IL-10 was detected in only 8% of the L. acidophilus-stimulated whole-blood samples (and thus not reported) and IFNγ was detected in 80% of these samples. For all other cytokine determinations, the total sample size was 59–64 participants.

PBMC fatty acid analysis. PBMC were frozen at −80°C with 0.01% butylated hydroxytoluene. Total lipids were extracted by the Folch procedure (11) and transmethylated with boron trifluoride in methanolic NaOH and the fatty acids were determined by GC as described in (8). The relative content of specific fatty acids is expressed as area percentage relative to all fatty acids in a chromatogram.

Statistics. All statistical analyses were performed with SPSS software (version 15.0). Normal distribution was checked with the Shapiro-Wilk test and visual inspection of histograms. The production of TNFα in LPS-stimulated whole blood and L. acidophilus-stimulated PBMC was normally distributed as were docosapentaenoic acid (DPA) and DHA in PBMC. All other cytokine variables and PBMC-EPA were not normally distributed and were log10-transformed before analysis. At baseline, the 4 intervention groups were compared in 1-way ANOVA. Cytokine data obtained immediately after the intervention and after the wash-out period were analyzed in ANCOVA with fat and capsule type as fixed factors and baseline values as covariate, after checking for significant fat × capsule interactions. Furthermore, dose-response relations with PBMC-EPA were tested by Pearson correlation and regression analysis to check if significant findings were supported by differences in compliance. The analyses were conducted with and without participants with deviating culture procedures (4 with 66 h of PBMC stimulation and 4 with <1.7 × 10⁹ cells/L in the monocyte cultures). Analyses of cytokines in whole blood were conducted with and without adjustment for total leukocyte counts. Due to the many statistical tests for outcome variables reflecting different aspects of the same outcome, P < 0.025 was considered significant for the cytokine variables, P < 0.05 was used for all other variables. Values in the text are given as means ± SEM or median (range) unless otherwise stated.

Results

The fat and capsule intervention did not interact significantly with the incorporation of (n-3) LCPUFA in PBMC (Table 1),
indicating that the incorporation of (n-3) LCPUFA from FO was not affected by the LA intake (8). FO supplementation strongly increased the PBMC content of (n-3) LCPUFA compared with OO (Table 1). The low LA fats (R/K) resulted in a slightly higher DPA content in PBMC and tended to have a higher EPA content (8). FO supplementation strongly indicated that the incorporation of (n-3) LCPUFA from FO was lower in the FO group after adjusting for the total number of intervention (P = 0.06), whereas DHA and total (n-3) LCPUFA (P = 0.11) were unaffected by the fat intervention.

There was no significant interaction between the fat and capsule intervention on stimulated cytokine production. LPS-stimulated IL-6 production was also lower in the FO groups compared with the OO groups after the capsule intervention on stimulated cytokine production. LPS-induced IL-6, TNF, or IL-10 (Supplemental Table 1). The fat intervention did not affect ex vivo production of any of the cytokines, although LPS-induced TNF production from PBMC tended to be higher in the high LA groups (S/B) than in the low LA groups (R/K) (P = 0.05) (Supplemental Table 1). The results did not change when the whole-blood analyses were adjusted for leukocyte count or when data for PBMC incubated for 66 h or for monocytes seeded at densities < 1.7 × 10⁹ cells/L were excluded (data not shown). None of the cytokines from any type of TNF, IFN, or IL-10 (

### Table 1
(n-3) LCPUFA in PBMC total lipids in healthy men at baseline and after 8-wk of FO or OO supplementation at 2 levels of LA intake

<table>
<thead>
<tr>
<th></th>
<th>EPA</th>
<th>DPA</th>
<th>DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Intervention</td>
<td>Baseline</td>
</tr>
<tr>
<td></td>
<td>FA%</td>
<td>FA%</td>
<td>FA%</td>
</tr>
<tr>
<td><strong>FO</strong></td>
<td><strong>Baseline</strong></td>
<td><strong>Intervention</strong></td>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td><strong>High LA (S/B)</strong></td>
<td>0.4 (0.4–0.5)</td>
<td>0.5 (0.4–0.6)</td>
<td>0.5 (0.4–0.6)</td>
</tr>
<tr>
<td><strong>Low LA (R/K)</strong></td>
<td>0.3 (0.3–0.5)</td>
<td>0.4 (0.4–0.5)</td>
<td>2.6 (2.1–2.2)</td>
</tr>
<tr>
<td><strong>DPA</strong></td>
<td>2.6 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td><strong>Intervention</strong></td>
<td>2.6 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td><strong>DHA</strong></td>
<td>2.9 ± 0.2</td>
<td>2.7 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td><strong>Intervention</strong></td>
<td>2.7 ± 0.1</td>
<td>2.6 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM or median (25th–75th percentile).

### Table 2
Ex vivo IL-6 production in response to L. acidophilus or LPS in healthy men at baseline and after 8-wk FO or OO supplementation at 2 levels of LA intake

<table>
<thead>
<tr>
<th></th>
<th>IL-6, µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FO</strong></td>
<td><strong>Baseline</strong></td>
</tr>
<tr>
<td><strong>Whole blood, 3 mg/L L. acidophilus</strong></td>
<td>4.2 (1.9–7.0)</td>
</tr>
<tr>
<td><strong>Intervention</strong></td>
<td>6.0 (3.3–10.0)</td>
</tr>
<tr>
<td><strong>Whole blood, 1 mg/L LPS</strong></td>
<td>25.6 (16.4–33.0)</td>
</tr>
<tr>
<td><strong>Intervention</strong></td>
<td>28.4 (18.6–42.9)</td>
</tr>
<tr>
<td><strong>PBMC, 1 mg/L L. acidophilus</strong></td>
<td>20.5 (16.6–48.2)</td>
</tr>
<tr>
<td><strong>Intervention</strong></td>
<td>24.3 (11.5–32.0)</td>
</tr>
<tr>
<td><strong>PBMC, 0.1 mg/L LPS</strong></td>
<td>35.5 (26.0–54.4)</td>
</tr>
<tr>
<td><strong>Intervention</strong></td>
<td>28.5 (20.1–38.1)</td>
</tr>
<tr>
<td><strong>Monocytes, 3 mg/L L. acidophilus</strong></td>
<td>63.3 (29.4–54.8)</td>
</tr>
<tr>
<td><strong>Intervention</strong></td>
<td>154.7 (106.6–243.7)</td>
</tr>
</tbody>
</table>

1 Values are medians (25th–75th percentile).

2 There were no capsule × fat interactions, P = 0.07. Immediately after the intervention, and no effects of either capsule × fat, capsule, or fat after an 8-wk wash-out period, P = 0.05 (data not shown). Due to multiple comparisons, statistical significance was established at P < 0.025.
of culture differed significantly between the capsule or fat groups after the 8-wk wash-out period (data not shown).

Within the FO groups, the change from baseline to 8 wk in PBMC-EPA was not associated with the change in LPS-induced whole-blood IL-6 during the intervention \( (r = -0.12; P = 0.53; n = 31) \) or the change in that induced by \( L. \) acidophilus \( (r = -0.23; P = 0.22; n = 31) \).

**Discussion**

This is the first study, to our knowledge, to investigate whether FO affects ex vivo cytokine production from whole blood, PBMC, and monocytes at 2 levels of LA intake in healthy men. The production of IL-6 in whole blood was lower immediately after FO supplementation compared with control. However, this effect showed no dose-response-relationship with EPA incorporation in PBMC and none of the other cytokines or ex vivo cultures were affected by FO. A number of trials have investigated the effects of FO on cytokine production in healthy humans, but the evidence is conflicting (5). Apart from the many effects in nonrandomized studies and studies without control groups, most randomized controlled trials found no effects of FO supplementation on cytokine production (12–17). Consistent with our findings, a few randomized, controlled trials reported significant reductions in IL-6 production only (18–20).

Doses ranged from \( < 1 \) to \( > 5 \) g/d (n-3) LCPUFA, but the inconsistent findings seem unrelated to dose or participant age (5,21). No other studies, to our knowledge, have investigated whether FO-induced changes in ex vivo IL-6 production persist after supplementation ends, but long-term effects on other cytokines have been examined. Gallai et al. (22) found that production of TNF\( \alpha \), IL-1\( \beta \), IFN\( \gamma \), and IL-2 was suppressed after 26 wk of FO supplementation but returned to presupplementation levels after a 13-wk wash-out period, whereas Endres et al. (23) observed that TNF\( \alpha \) and IL-1 were further suppressed after a 10 wk wash-out period. However, neither of these studies included a control group. Many of the previous studies used LPS to induce ex vivo cytokine production from monocytes. LPS acts via nuclear factor \( \kappa B \), which has been shown to be specifically modulated by (n-3) LCPUFA (24). To our knowledge, we are the first to use UV-killed Lactobacilli as the cytokine inducer, but with this polyclonal stimulant, we did not find a definitive effect of the intervention.

The fat intervention, which increased the LA intake in the S/B groups, did not affect cytokine production and did not modify the effect of FO. However, the fat intervention had no pronounced effect on (n-3) LCPUFA in PBMC, either alone or in combination with FO. This may explain why the LA intake had no immunomodulating effects in the present study. The LA intake of the 2 groups differed by a factor of 2, but we cannot exclude that larger differences or a different range of intakes would have been more effective. Cell studies have shown that LA alone is able to induce nuclear factor \( \kappa B \) in endothelial cells, leading to increased expression of inflammatory molecules (25), and diets with a very high LA content may impair cell-mediated and antibody responses in rodents (26). However, the few trials investigating the effects of dietary LA on cytokine production and immune function in healthy humans did not demonstrate proinflammatory or immunomodulating effects (27,17).

Monocyte isolation is very laborious and expensive and requires a lot of blood, so we only did this immediately after the intervention. As a result, we could not adjust for baseline values for this cell type, which is essential because of the large interindividual variation in cytokine responses (28). This may explain why FO did not affect IL-6 production from monocytes but only from whole blood. Also, in contrast to the culture medium used for isolated cells, whole blood contains nutrients, growth factors, and other cells in physiological, person-specific proportions that may be important for the immunomodulating effects of FO. Although we accounted for the large number of statistical tests by allowing only a 2.5% chance of type I errors, we cannot exclude the risk of a chance finding in the case of IL-6.

The study was not specifically powered to investigate effects on cytokine production. However, it is one of the largest studies within the field and post hoc power calculation showed sufficient power at least for whole-blood IL-6. We recently showed that the whole-blood assays had greater reproducibility than the PBMC assays, indicating a better power and ability to detect differences between intervention groups (9). Variation in the other cell cultures and cytokines gives rise to a greater chance of type II errors on these outcomes, which may explain why the FO intervention affected cytokine production in the whole-blood assays only.

The difference in IL-6 production between the capsule groups was due to an increase in the OO groups rather than a decrease in the FO groups. However, in this experimental design, the control group is considered a reference group that represents habitual changes in the variables. The choice of unrefined OO as the control oil can be criticized, because it has been shown to contain antiinflammatory compounds (29). However, this does not explain the apparent increase in IL-6 in the OO groups in the present study. Thus, this study indicates that FO counteracts an elevation in IL-6 production over time.

IL-6 has numerous proinflammatory effects such as promotion of fever and induction of hepatic acute-phase proteins, but potential antiinflammatory or inflammation-resolving roles are now being discovered (30). A small lowering of the potential to produce IL-6 may become important if sustained for longer time periods and may be more pronounced in inflammatory disease states. Whether the observed effects of FO would be of any importance for the ability of healthy individuals to resist infections is beyond the scope of this study. The potential functional implications need larger, more long-term investigations with focus on other, functional aspects of the immune system than ex vivo cytokine production.

We conclude that apart from a lower IL-6 production from LPS-stimulated whole blood, 8-wk FO supplementation did not affect ex vivo cytokine production in a large group of healthy men. Increased LA intake did not affect cytokine production, nor did it modify the effect of FO.

**Acknowledgments**

We thank technicians Anni Mehlisen and Elin Skytte for excellent laboratory work.

**Literature Cited**

