Dietary Fish Oil Increases the Number of Splenic Macrophages Secreting TNF-α and IL-10 But Decreases the Secretion of These Cytokines by Splenic T Cells from Mice[1,2]

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Abstract

Dietary fish oil has immunomodulatory effects that are partly mediated by its effects on cytokine secretion. In this paper, we examine whether dietary fish oil has different effects on cytokine secretion by T cells and macrophages. Female BalbC mice were fed diets supplemented with 18% fish oil + 2% corn oil or 20% corn oil. Concanavalin A (ConA)- and LPS-induced TNF-α and IL-10 secretion by splenocytes was examined using ELISA. Dietary fish oil decreased ConA induced-, but increased LPS-induced, TNF-α and IL-10 secretion by total murine splenocytes. Dietary fish oil increased the number of splenocytes secreting TNF-α and IL-10, following stimulation with LPS, by 123 and 38%, respectively, but did not affect cytokine secretion by each cell, as determined using enzyme-linked immunospot. Spleens from mice fed the fish oil diet had over 2-fold higher proportion of macrophages with high expression of CD11b than spleens from mice fed the corn oil diet. In addition, fish oil increased the proportion of total and $CD11b^+$ splenocytes that expressed the LPS receptor complex molecules, CD14 and toll-like receptor (TLR)4/myeloid differentiation factor-2 (MD-2), by 85 and 28%, respectively. The increased proportion of macrophages expressing the LPS receptor complex molecules, CD14 and TLR4/MD-2, in spleens from mice fed the fish oil diet may explain the increased number of cells that secreted the cytokines after LPS stimulation. J. Nutr. 137: 665–670, 2007.

Introduction

The immunomodulatory effects of dietary fish oil are generally thought to be antiinflammatory (1). This hypothesis is based on results from human studies indicating that dietary fish oil or (n-3) PUFA are beneficial in several inflammatory disorders (2–4). The antiinflammatory effects of dietary fish oil are thought to be mediated in part by decreased proinflammatory cytokine production, shown, for example, by decreased circulating IL-1β levels in patients with rheumatoid arthritis (5) and decreased LPS-induced TNF-α and IL-1β secretion by human peripheral blood mononuclear cells (6–8). Decreased IL-2 secretion by murine T lymphocytes stimulated with concanavalin A (ConA)[3] or anti-CD3/anti-CD28 (9,10) has also been shown following feeding with low- (5 g/100 g) or high- (18 g/100 g) fat diets containing (n-3) PUFA or fish oil (9–11). In addition, a number of studies have shown that dietary fish oil, or (n-3) PUFA, decrease mitogen-induced proliferation by lymphocytes in rodents and men fed low- or high-fat diets (7,10,12–16). The decreased lymphoproliferation may be dependent on dietary fish oil modification of lipid rafts and displacement of signaling proteins and cytokine receptors from lipid rafts (17,18). Displacement of signaling proteins and cytokine receptors from lipid rafts could lead to decreased intracellular signaling and dietary fish oil has indeed been shown to decrease events linked to intracellular signaling, such as formation of diacylglycerol and ceramide (10), tyrosine phosphorylation of protein kinase C-γ (19), and recruitment of protein kinase C-θ to lipid rafts (9).

Although dietary fish oil has antiinflammatory effects on cytokine secretion by circulating monocytes and T cells, as described above, results from several studies show that dietary fish oil has proinflammatory effects on cytokine secretion by macrophages. We and others have shown that feeding mice fish oil increases LPS-induced TNF-α and IL-1β secretion by resident peritoneal macrophages (20–27) but decreases secretion of the antiinflammatory cytokine, IL-10 (25). In addition, dietary fish oil increases TNF-α secretion by murine splenocytes after stimulation with LPS (28,29). These proinflammatory effects were seen in mice fed diets containing 5 (29), 10 (22–24,26), or 20 g/100 g fat (25), with (n-3) PUFA being as low as 1.5 g/100 g in one of these studies (22). The increased TNF-α secretion by resident peritoneal macrophages from mice fed fish oil is partly

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[2] Supplemental Tables 1 and 2 and Supplemental Figure 1 are available with the online posting of this paper at jn.nutrition.org.
[3] Abbreviations used: ConA, concanavalin A; ELISpot, enzyme-linked immunospot; MD-2, myeloid differentiation factor-2; TLR, toll-like receptor.
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explained by decreased prostaglandin production (22,25,27), but other mechanisms at work remain to be elucidated.

As discussed above, dietary fish oil seems to have different effects on cytokine secretion by T cells and circulating monocytes than on tissue macrophages. Thus, the purpose of this study was to determine whether dietary fish oil has different effects on cytokine secretion by splenic T cells and macrophages obtained from the same animals and using similar experimental setup. We chose to monitor the secretion of 1 proinflammatory cytokine, TNF-α, and 1 antiinflammatory cytokine, IL-10, because these cytokines are often monitored following induction of macrophages with LPS (30,31). We also explored a possible mechanism by which dietary fish oil affects cytokine secretion by splenic macrophages.

Materials and Methods

Animals and diets. All experimental procedures using laboratory animals complied with the National Research Council's Guide for the Care and Use of Laboratory Animals. Female BalbC mice weighing 18–20 g (Bomholtgaard) were randomly divided into 2 groups of 10 mice each. Mice were housed 5 per cage at 25°C with a 12-h light and dark cycle. We designed experimental diets according to AIN-93 guidelines (32) with modification in fat content. The experimental diets were based on a nutritionally complete diet made for the addition of 200 g/kg of fat (ICN Pharmaceuticals), as previously described (25). The fish oil diet contained 180 g/kg menhaden fish oil and 20 g/kg corn oil (ICN Pharmaceuticals). The corn oil diet contained 200 g/kg corn oil. The antioxidant *tert*-butylhydroquinone (ICN Biomedicals) (1.2 mmol/L) was added to the oils to prevent their deterioration (33). Diets were prepared in bulk and daily portions packed in zip-lock bags, flushed with nitrogen, sealed, and stored at −20°C. Mice consumed water and food ad libitum. The mice were fed the experimental diets for 5 to 6 wk. The length of feeding did not influence the effect of dietary fish oil on the parameters measured. An equal number of animals from each dietary group was killed at each time point.

Fatty acid analysis. Fatty acid composition of the diets was analyzed, as described previously (25, **Supplemental Table 1**). Fatty acid composition of hepatic phospholipids from mice fed the fish oil and corn oil diets was analyzed, as described previously (25, **Supplemental Table 2**). A higher proportion of (n-3) PUFA and a lower proportion of (n-6) PUFA in hepatic phospholipids from mice fed the fish oil diet, compared with that in hepatic phospholipids from mice fed the corn oil diet, demonstrate the effectiveness of the diets in changing tissue fatty acid composition.

Isolation and activation of splenocytes. Mice were anesthetized with isoflurane (Abbot Scandinavia) and blood was collected by axillary bleeding. Serum from 2–3 mice in the same dietary group was pooled, heat inactivated at 56°C for 40 min, and used as homologous serum in cell cultures. The mice were killed by cervical dislocation. Spleens were removed aseptically postmortem, cut, and passed through a wire mesh to obtain a single cell suspension. The cells were treated with lysing buffer (0.15 mol/L NH_4Cl, 1 mmol/L $KHCO_3$, 0.1 mmol/L Na_2EDTA) to lyse red blood cells. For cytokine measurements, 5×10^9 cells/L were cultured in 0.2 mL/well on a 96-well plate with 5% homologous serum at 37°C in an atmosphere of 5% CO_2. The cells were stimulated for 24 h with 2 mg/L LPS, (*E. coli* 055:195, Fluka Chemie) or for 48 h with 8 mg/L ConA (Sigma-Aldrich).

TNF-α and IL-10 quantitation. After incubation of cells, the culture plates were centrifuged, supernatants collected, and stored at −70°C. We measured TNF-α and IL-10 with Duo Set ELISA kits (R&D Systems).

Determination of the numbers of TNF-α and IL-10 secreting cells. Numbers of TNF-α and IL-10 secreting cells were determined by enzyme-linked immunospot (ELISpot; R&D Systems). For TNF-α, 1.25×10^7 cells/L and for IL-10, 2×10^8 cells/L were incubated with or without LPS (2 mg/L) for 24 h on polyvinilidine difluoride microplates (Millipore) coated with anti-mouse TNF-α or IL-10. Spots were developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (R&D Systems) and automatically evaluated with a computer-assisted video image analyzer (KS ELISpot, version 4.8, Zeiss).

Flow cytometric analysis of splenocytes producing TNF-α. Marker expression on TNF-α producing cells from mice receiving standard chow was examined using flow cytometry. Cells (5×10^9 cells/L) were cultured with LPS (2 mg/L) and brefeldin A (1 mL/L) (BD Pharmingen), a protein transport inhibitor, for 2 h. We then incubated the cells with anti-mouse CD3-FITC, CD19-PE, CD11b-PE, CD49b-PE (BD Pharmingen), CD169-FITC, and F4/80-PE (Serotec). The cells were fixed and permeabilized using Leukoperm Permeabilization kit (Serotec) and stained with antibodies against the intracellular markers CD68-PE (FA-11) or MOMA-2-PE (Serotec) and anti-mouse TNF-α-APC (eBioscience). Cells were incubated with anti-mouse CD16/32 (2 μg/10^6 cells) (Mouse Fc Block, BD Pharmingen) prior to incubation with immunoglobulin G antibodies to block unspecific binding. Appropriate isotypic control for each antibody was used to set the quadrants and evaluate background staining. We performed flow cytometry with a Becton Dickinson FACScalibur flow cytometer equipped with an argon ion laser. A total of 100,000 events were collected for each analysis. Data were analyzed using FCS express V3 (de Novo Software).

Flow cytometric analysis of splenocyte subpopulations. Cells were incubated with mouse Fc Block (2 μg/10^6 cells) and stained with 1 or more of the following monoclonal antibodies: CD3-FITC (17A2), CD4-PE (RM4–5), CD8-PerCP (53–6.7), CD19-PE (1D3), CD45-FITC (30-F11), CD49b-PE (DX5), CD11c-PE (HL3) (Pharmingen), F4/80-PE (A3–1), CD169-FITC (3d6.112) (Serotec), CD11b-PE, or PE-Cy5 (M1/70) (eBioscience). Cells were also stained intracellularly with CD68-PE, MOMA-2-PE, CD14-FITC (Sa2–8), and toll-like receptor (TLR)-4/myeloid differentiation factor-2 (MD-2)-PE (MTS510) (eBioscience) using Cytofix/Cytoperm kit (Becton Dickinson) or Leukoperm. Cells were then washed and fixed in 1% paraformaldehyde. Appropriate isotypic control for each antibody was used to set the quadrants and evaluate background staining. We collected a total of 25,000 events.

Isolation and activation of splenic CD11b positive macrophages. CD11b positive macrophages were isolated using magnetic activated cell sorting (Miltenyi Biotech). After isolation, 1×10^9 cells/L were cultured, 0.2 mL/well, on a 96-well plate with 5% homologous serum, and stimulated with LPS (2 mg/L) for 24 h. Approximately 90% of the isolated cells had high expression of CD11b ($CD11b^{high}$), as determined by flow cytometry in both dietary groups.

Statistical analysis. We analyzed differences between dietary groups by an unpaired Student's *t* test using StatsDirect statistical program, version 2.3.3 (StatsDirect) and differences were significant if $P < 0.05$ (two-tailed).

Results

Mouse growth, spleen weights, and cell counts. Body weights and relative weight gains did not differ between the groups. Mice fed the fish oil diet had heavier spleens (171 ± 6 mg) than those fed the corn oil diet (120 ± 4 mg, $P < 0.0001$). Mice fed the fish oil diet also had a greater spleen cell count (98 ± 4 $\times 10^6$ cells/spleen) than those fed the corn oil diet (71 ± 4 $\times 10^6$ cells/spleen, $P < 0.0001$).

LPS and Con A induced TNF-α and IL-10 secretion by total splenocytes. After stimulation with LPS, total splenocytes from mice fed the fish oil diet secreted significantly more TNF-α and IL-10 than splenocytes from mice fed the corn oil diet (**Fig. 1***A*,*B*). However, when stimulated with ConA, total splenocytes from mice fed the fish oil diet secreted significantly less TNF-α

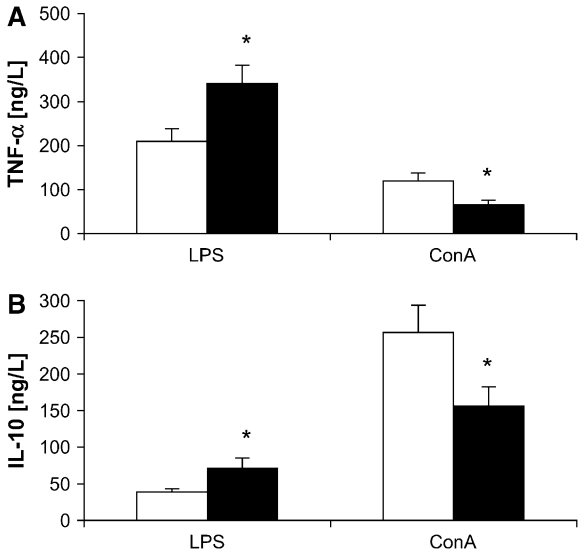

Figure 1 The effects of dietary fish oil on LPS- and ConA-induced TNF-α (*A*) and IL-10 (*B*) secretion by total splenocytes from mice fed the corn oil (open bars) or the fish oil diet (closed bars). Cells were stimulated with LPS (2 mg/L) or ConA (8 mg/L) for 24 h and 48 h, respectively. Values are means ± SEM, $n = 10$. * Different from corn oil, $P < 0.05$.

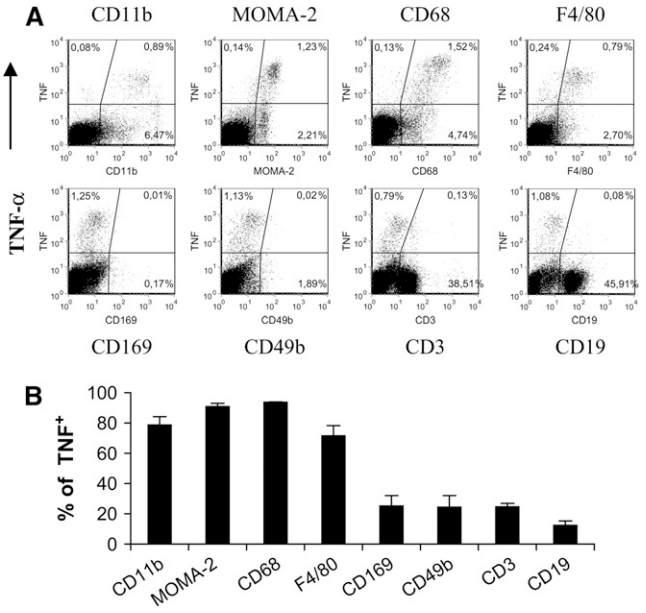

Figure 2 Flow cytometric analysis for identification and characterization of TNF-α-producing splenocytes of BALB/c mice receiving standard chow. Cells were stimulated with LPS (2 mg/L), stained with appropriate markers, and analyzed by flow cytometry. (*A*) Representative dot plots of LPS-induced TNF-α staining of cells and marker expression. Numbers represent the proportion of gated cells in each quadrant. (*B*) The proportion of TNF-α positive splenocytes expressing the indicated markers. Values are mean ± SEM, $n = 3$.

and IL-10 than splenocytes from mice fed the corn oil diet (Fig. 1*A*,*B*).

The number of splenocytes that secreted TNF-α and IL-10 following stimulation with LPS. The number of splenocytes that secreted TNF-α following stimulation with LPS was higher in mice fed the fish oil diet (1698 ± 226 spots/10^5 cells) than in mice fed the corn oil diet (760 ± 109 spots/10^5 cells, $P = 0.0025$). The number of splenocytes secreting IL-10 was higher in mice fed the fish oil diet (149 ± 6 spots/10^5 cells) than in mice fed the corn oil diet (108 ± 15 spots/10^5 cells, $P = 0.03$). The mean TNF-α and IL-10 secretion per cell was similar in splenocyte cultures from mice fed the fish oil diet and the corn oil diet (results not shown).

Marker expression on splenocytes that produced TNF-α. A large proportion (~90%) of the cells that produced TNF-α were macrophages expressing CD11b, MOMA-2, and CD68 and ~70% expressed F4/80. Hardly any cells producing TNF-α expressed the macrophage marker CD169, the NK cell marker CD49b, or the T and B cell markers CD3 and CD19 (**Fig. 2**).

Marker expression on splenocytes. Spleens from mice fed the fish oil diet had a higher proportion of macrophages with $CD11b^{high}$ and low expression of CD49b ($CD49b^{low}$) than spleens from mice fed the corn oil diet (**Table 1**). The proportion of MOMA-2 positive macrophages, which are mainly macrophages from the periarteriolar lymphoid sheet and marginal zone (34), was also increased in spleens from mice fed the fish oil diet compared with that in spleens from mice fed the corn oil diet. On the other hand, the proportion of red pulp macrophages, expressing F4/80, was lower in spleens from mice fed the fish oil diet than in spleens from mice fed the corn oil diet. Dietary fish oil did not affect the proportion of macrophages expressing macrosialin (CD68) or the proportion of metallophilic macrophages expressing CD169 (Table 1).

Examination of other surface markers demonstrated that the proportion of NK cells ($CD49b^{high}CD11b^{low}$) was lower in spleens from mice fed the fish oil diet than in spleens from mice fed the corn oil diet, but the proportion of splenic total leukocytes (CD45), B cells (CD19), T cells (CD3, CD4, CD8), and dendritic cells (CD11c) was similar in spleens from mice fed the corn oil diet and spleens from mice fed the fish oil diet (Table 1).

LPS induced TNF-α and IL-10 secretion by $CD11b^+$ splenocytes. Cell yield after isolation of $CD11b^+$ cells was 70% higher in the fish oil group (6.0 ± 0.4 × 10^6 cells) compared with that in the corn oil group (3.6 ± 0.4 × 10^6 cells, $P = 0.0014$). When the same number of $CD11b^+$ cells was cultured and stimulated with LPS, isolated $CD11b^+$ splenocytes from mice fed the fish oil diet secreted more TNF-α and IL-10 than

TABLE 1 Marker expression on splenocytes from mice fed diets containing corn oil or fish oil[1]

Marker	Corn oil	Fish oil
Macrophages	%	
$CD11b^{high}CD49b^{low}$	2.8 ± 0.2	7.3 ± 0.7*
$MOMA\text{-}2^+$	6.3 ± 0.3	10.7 ± 0.9*
$F4/80^+$	10.3 ± 0.8	7.3 ± 0.5*
$CD68^+$	4.2 ± 0.2	4.0 ± 0.3
$CD169^+$	2.7 ± 0.3	3.2 ± 0.4
Other cell types		
$CD11b^{low}CD49b^{high}$	10.7 ± 0.6	7.1 ± 0.4*
$CD45^+$	98.8 ± 1.7	97.8 ± 1.1
$CD45^+CD19^+$	53.7 ± 1.7	57.0 ± 1.1
$CD3^+CD4^+$	24.2 ± 0.7	24.2 ± 0.7
$CD3^+CD8^+$	11.1 ± 0.3	10.9 ± 0.9
$CD11c^+$	3.8 ± 0.6	4.0 ± 1.1

[1] Values are means ± SEM, $n = 5$–8. * Different from corn oil, $P < 0.05$.

$CD11b^+$ splenocytes from mice fed the corn oil diet (**Fig. 3**). TNF-α and IL-10 secretion by total splenocytes from mice fed the corn oil and the fish oil diet in the same experiment is shown for comparison.

Relative expression of the LPS receptor complex. Spleens from mice fed the fish oil diet had a higher proportion (32%) of isolated $CD11b^+$ cells expressing CD14 and TLR4/MD-2 than spleens from mice fed the corn oil diet (**Fig. 4***A*). In comparison, the proportion of total splenocytes expressing CD14 and TLR4/MD-2 was 85% higher in mice fed the fish oil diet than in mice fed the corn oil diet (Fig. 4*B*). Dietary fish oil did not affect the mean fluorescence intensity for CD14 and TLR4/MD-2 on splenocytes from mice fed the different diets (data not shown).

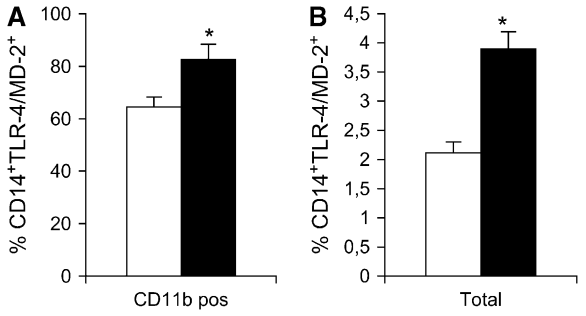

Figure 4 The effects of dietary fish oil on intracellular expression of CD14 and TLR4/MD-2 in isolated $CD11b^+$ splenocytes (*A*) and total splenocytes (*B*). Values are means ± SEM, $n = 4$ for $CD11b^+$ macrophages, $n = 5$ for total splenocytes. * Different from corn oil, $P < 0.05$.

Discussion

The results from this study demonstrate that dietary fish oil has different effects on TNF-α and IL-10 secretion by splenocytes stimulated with ConA or LPS (Fig. 1). The TNF-α and IL-10 measured, following stimulation with ConA, was most likely secreted by T cells, as splenocytes depleted of $CD90.1^+$ T cells secreted no detectable TNF-α and IL-10 following stimulation with ConA (results not shown). ConA is a plant lectin and a well-characterized mitogen that stimulates T cells by binding to the CD3 molecule and perhaps also via a pathway dependent on the T cell surface protein, CD2 (35). On the other hand, TNF-α and IL-10 measured following stimulation with LPS was most likely secreted by macrophages, as LPS binds to CD14 (36) expressed on monocytes and macrophages (37,38) and signals into the cell through the TLR4/MD-2 complex (39). LPS also stimulates B cells through a complex containing TLR4 and the TLR protein, RP105 (40), leading to enhanced antigen-presenting capacity, enhanced proliferation, and secretion of LPS-neutralizing antibodies (41,42), but TNF-α and IL-10 secretion by B cells has been shown at later time points and using higher concentration of LPS than in this study (43,44). In addition, characterization of the cells secreting TNF-α after LPS stimulation in our study revealed that few cells expressing the B cell marker, CD19, produced TNF-α (Fig. 2). Thus, our results indicate that dietary fish oil differently affects cytokine secretion by T cells and macrophages, decreasing TNF-α and IL-10 secretion by T cells and increasing the secretion of these cytokines by splenic macrophages.

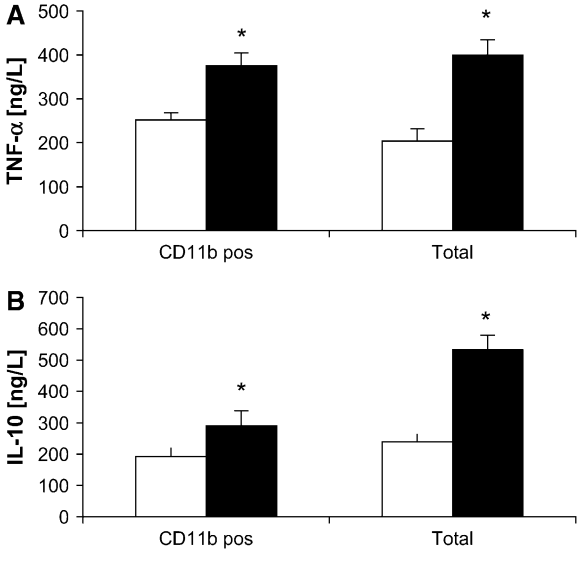

Figure 3 The effects of dietary fish oil on LPS-induced TNF-α (*A*) and IL-10 (*B*) secretion by isolated $CD11b^+$ splenic macrophages and total splenocytes from mice fed the corn oil diet (open bars) or the fish oil diet (closed bars). Macrophages expressing CD11b (1×10^9 cells/L) and total splenocytes (5×10^9 cells/L) were stimulated with LPS (2 mg/L) for 24 h. Values are means ± SEM, $n = 10$. * Different from corn oil, $P < 0.05$.

Decreased ConA-induced TNF-α secretion by splenocytes from mice fed (n-3) PUFA has been shown previously (45) and we have also seen decreased TNF-α secretion by total splenocytes and isolated CD90 positive T cells, following stimulation with anti-CD3/anti-CD28 (our unpublished results). Our results are also in agreement with results by Ly et al. (46) who showed decreased anti-CD3/anti-CD28 induced IL-10 secretion by $CD4^+$ T cells from mice fed DHA. Yaqoob et al. (47) showed no effect of dietary fish oil on IL-10 secretion by splenic lymphocytes stimulated with ConA, which can possibly be explained by the shorter stimulation time (24 h) used in that study, compared with the stimulation time (48 h) in this study and the one by Ly et al. (46). A number of studies also showed decreased ConA and anti-CD3/anti-CD28 induced IL-2 secretion and proliferation of splenic lymphocytes from mice fed (n-3) PUFA (9–11,48). Together, these results indicate that dietary (n-3) PUFA have antiinflammatory effects on cytokine secretion and proliferation of T cells.

Results from this study showing increased TNF-α secretion by splenic macrophages from mice fed fish oil are in agreement with results by others (28,29), but, to our knowledge, the effects of dietary fish oil on LPS-induced IL-10 secretion by splenocytes has not been investigated previously. The increase in LPS-induced TNF-α and IL-10 secretion by splenocytes in this study proved to be due to an increase in the number of cells secreting these cytokines, but not to increased TNF-α and IL-10 secretion by each cell (demonstrated by ELISpot). Thus, the mechanism by which dietary fish oil affects cytokine secretion by splenic macrophages may be very different from the mechanism by which they affect cytokine secretion by T cells, which has been attributed, e.g., to the ability of (n-3) PUFA to affect the production of lipid messengers, to modify action of nuclear receptors, and to affect lipid rafts in cell membranes [reviewed in (49)]. Whether increased LPS-induced TNF-α secretion by splenocytes (28,29) or resident peritoneal macrophages (20–25) from mice fed fish oil seen in previous studies is due to an increase in the number of cells secreting the cytokine is not known. Results from our laboratory show that dietary fish oil does not affect the number of resident peritoneal macrophages secreting TNF-α, but, rather,

increases TNF-α secretion per cell (I. Skuladottir, D. Petursdottir, I. Hardardottir, unpublished results). Thus, the mechanism by which dietary fish oil increases TNF-α secretion by resident peritoneal macrophages may be different from the mechanism by which dietary fish oil increases cytokine secretion by splenic macrophages. In fact, the increased TNF-α secretion by resident peritoneal macrophages from mice fed the fish oil diet has been partly explained by decreased prostaglandin (PG)-E_2 production (22,25,27), but decreased PGE_2 production does not explain the increase in TNF-α secretion by splenic macrophages in this study, because blocking prostaglandin production with indomethacine had no effect on LPS-induced TNF-α and IL-10 secretion (results not shown). Further underlining that dietary fish oil has different effects on cytokine secretion by macrophages from different body compartments, our previous results showed that dietary fish oil decreased IL-10 secretion by resident peritoneal macrophages (25), which is in contrast to the increase in IL-10 secretion by splenic macrophages from mice fed the fish oil diet in this study.

Most of the cells producing TNF-α, following stimulation with LPS, were macrophages expressing $CD11b^{high}$, MOMA-2, and CD68 (Fig. 2). Spleens from mice fed the fish oil diet had a higher proportion of cells expressing $CD11b^{high}$ and MOMA-2 than spleens from mice fed the corn oil diet (Table 1), with these markers being predominantly expressed on the same cells (results not shown). Thus, the increase in the number of cells secreting TNF-α and IL-10, demonstrated by ELISpot, could be explained by the increase in the proportion of these cells in spleens from mice fed the fish oil diet. However, when $CD11b^{+}$ cells were isolated (~90% were $CD11b^{high}$) and the same number of $CD11b^{+}$ cells from mice fed the fish oil diet and the corn oil diet plated and stimulated with LPS, isolated $CD11b^{+}$ splenocytes from mice fed the fish oil diet secreted significantly more TNF-α and IL-10 than isolated $CD11b^{+}$ splenocytes from mice fed the corn oil diet (Fig. 3), indicating that that the increase in the proportion of $CD11b^{high}$ cells in spleens from mice fed the fish oil diet is not sufficient to explain the increase in TNF-α and IL-10 secretion. Because there was not an increase in cytokine secretion per cell, there must be a higher proportion of cells producing TNF-α and IL-10 within the $CD11b^{high}$ population in spleens from mice fed the fish oil diet than in spleens from mice fed the corn oil diet. That this could be the case was demonstrated by showing that a higher proportion of isolated $CD11b^{+}$ cells from mice fed the fish oil diet expressed the LPS receptor complex molecules, CD14 and TLR4/MD-2, compared with that in $CD11b^{+}$ cells from mice fed the corn oil diet (Fig. 4). Furthermore, the increase in the proportion of cells expressing CD14 and TLR4/MD-2 among isolated $CD11b^{+}$ cells and total splenocytes from mice fed the fish oil diet correlated with the increase in cytokine secretion by isolated $CD11b^{+}$ and total splenocytes, respectively (Figs. 4 and 3). Thus, we conclude that the increase in the proportion of cells expressing CD14 and TLR4/MD-2 from mice fed the fish oil diet was probably responsible for the increase in TNF-α and IL-10 secretion by splenocytes from mice fed the fish oil diet.

The increased proportion of $CD11b^{high}$ macrophages in spleens from mice fed fish oil in this study may be caused by infiltration of monocytes from blood, because there was an increase in the proportion of circulating monocytes expressing $CD11b^{high}$ in mice fed the fish oil diet compared with that in mice fed the corn oil diet (results not shown). An increase in the proportion of macrophages in the spleen and an increase in the proportion of circulating monocytes in mice fed fish oil has been shown previously (50). In that study, there was an increase in anti-F4/80 staining in the red pulp area in spleens from mice fed fish oil, whereas the results from this study show an increase in cells expressing other macrophage markers, with a modest decrease in the proportion of red pulp macrophages. The reason for the discrepancy in the results from the 2 studies may be due to the different methods used.

The decreased proportion of F4/80 red pulp macrophages and NK-cells expressing $CD49b^{high}$ in spleens from mice fed the fish oil diet probably reflects no change in absolute number of these cells, as there is an increase in total cell count in spleens from mice fed the fish oil diet. Whether this proportional decrease in F4/80 macrophages and NK-cells is likely to affect immune function is difficult to predict.

In summary, results from this study demonstrated that dietary fish oil had different effects on cytokine secretion by splenocytes stimulated with ConA and LPS, probably reflecting different effects of dietary fish oil on cytokine secretion by T cells and macrophages. Furthermore, the results demonstrated that dietary fish oil increased the number of cells secreting TNF-α and IL-10 after LPS stimulation and this increase is probably due to an increase in the number of splenocytes from mice fed the fish oil diet expressing the LPS receptor complex proteins, CD14 and TLR4/MD-2. Increased proportion of macrophages expressing the LPS receptor molecules may be important for primary defenses and immunosurveillance.

Literature Cited

1. Calder PC. Immunoregulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. Braz J Med Biol Res. 1998;31:467–90.
2. Belluzzi A, Brignola C, Campieri M, Pera A, Boschi S, Miglioli M. Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. N Engl J Med. 1996;334:1557–60.
3. Donadio JV Jr, Larson TS, Bergstralh EJ, Grande JP. A randomized trial of high-dose compared with low-dose omega-3 fatty acids in severe IgA nephropathy. J Am Soc Nephrol. 2001;12:791–9.
4. Kremer JM, Bigauoette J, Michalek AV, Timchalk MA, Lininger L, Rynes RI, Huyck C, Zieminski J, Bartholomew LE. Effects of manipulation of dietary fatty acids on clinical manifestations of rheumatoid arthritis. Lancet. 1985;1:184–7.
5. Kremer JM, Lawrence DA, Petrillo GF, Litts LL, Mullaly PM, Rynes RI, Stocker RP, Parhami N, Greenstein NS, et al. Effects of high-dose fish oil on rheumatoid arthritis after stopping nonsteroidal antiinflammatory drugs. Clinical and immune correlates. Arthritis Rheum. 1995;38:1107–14.
6. Endres S, Ghorbani R, Kelley VE, Georgilis K, Lonnemann G, van der Meer JW, Cannon JG, Rogers TS, Klempner MS, et al. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med. 1989;320:265–71.
7. Meydani SN, Endres S, Woods MM, Goldin BR, Soo C, Morrill-Labrode A, Dinarello CA, Gorbach SL. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J Nutr. 1991;121:547–55.
8. Trebble T, Arden NK, Stroud MA, Wootton SA, Burdge GC, Miles EA, Ballinger AB, Thompson RL, Calder PC. Inhibition of tumour necrosis factor-alpha and interleukin 6 production by mononuclear cells following dietary fish-oil supplementation in healthy men and response to antioxidant co-supplementation. Br J Nutr. 2003;90:405–12.
9. Fan YY, Ly LH, Barhoumi R, McMurray DN, Chapkin RS. Dietary docosahexaenoic acid suppresses T cell protein kinase C theta lipid raft recruitment and IL-2 production. J Immunol. 2004;173:6151–60.
10. Jolly CA, Jiang YH, Chapkin RS, McMurray DN. Dietary (n-3) polyunsaturated fatty acids suppress murine lymphoproliferation, interleukin-2 secretion, and the formation of diacylglycerol and ceramide. J Nutr. 1997;127:37–43.
11. Pompos LJ, Fritsche KL. Antigen-driven murine CD4+ T lymphocyte proliferation and interleukin-2 production are diminished by dietary (n-3) polyunsaturated fatty acids. J Nutr. 2002;132:3293–300.

12. Endres S, Meydani SN, Ghorbani R, Schindler R, Dinarello CA. Dietary supplementation with n-3 fatty acids suppresses interleukin-2 production and mononuclear cell proliferation. J Leukoc Biol. 1993;54:599–603.
13. Fowler KH, Chapkin RS, McMurray DN. Effects of purified dietary n-3 ethyl esters on murine T lymphocyte function. J Immunol. 1993;151: 5186–97.
14. Oarada M, Furukawa H, Majima T, Miyazawa T. Fish oil diet affects on oxidative senescence of red blood cells linked to degeneration of spleen cells in mice. Biochim Biophys Acta. 2000;1487:1–14.
15. Ly LH, Smith R, Switzer KC, Chapkin RS, McMurray DN. Dietary eicosapentaenoic acid modulates CTLA-4 expression in murine CD4+ T-cells. Prostaglandins Leukot Essent Fatty Acids. 2006;74:29–37.
16. Triboulot C, Hichami A, Denys A, Khan NA. Dietary (n-3) polyunsaturated fatty acids exert antihypertensive effects by modulating calcium signaling in T cells of rats. J Nutr. 2001;131:2364–9.
17. Li Q, Wang M, Tan L, Wang C, Ma J, Li N, Li Y, Xu G, Li J. Docosahexaenoic acid changes lipid composition and interleukin-2 receptor signaling in membrane rafts. J Lipid Res. 2005;46:1904–13.
18. Stulnig TM, Berger M, Sigmund T, Raederstorff D, Stockinger H, Waldhausl W. Polyunsaturated fatty acids inhibit T cell signal transduction by modification of detergent-insoluble membrane domains. J Cell Biol. 1998;143:637–44.
19. Sanderson P, Calder PC. Dietary fish oil appears to prevent the activation of phospholipase C-gamma in lymphocytes. Biochim Biophys Acta. 1998;1392:300–8.
20. Blok WL, Vogels MT, Curfs JH, Eling WM, Buurman WA, van der Meer JW. Dietary fish-oil supplementation in experimental gram-negative infection and in cerebral malaria in mice. J Infect Dis. 1992; 165:898–903.
21. Chang HR, Arsenijevic D, Pechere JC, Piguet PF, Mensi N, Girardier L, Dulloo AG. Dietary supplementation with fish oil enhances in vivo synthesis of tumor necrosis factor. Immunol Lett. 1992;34:13–7.
22. Hardardottir I, Kinsella JE. Tumor necrosis factor production by murine resident peritoneal macrophages is enhanced by dietary n-3 polyunsaturated fatty acids. Biochim Biophys Acta. 1991;1095:187–95.
23. Hardardottir I, Kinsella JE. Increasing the dietary (n-3) to (n-6) polyunsaturated fatty acid ratio increases tumor necrosis factor production by murine resident peritoneal macrophages without an effect on elicited peritoneal macrophages. J Nutr. 1992;122:1942–51.
24. Lokesh BR, Sayers TJ, Kinsella JE. IL-1 and TNF synthesis by mouse peritoneal macrophages is enhanced by dietary n-3 polyunsaturated fatty acids. Immunol Lett. 1990;23:281–6.
25. Petursdottir DH, Olafsdottir I, Hardardottir I. Dietary fish oil increases tumor necrosis factor secretion but decreases interleukin-10 secretion by murine peritoneal macrophages. J Nutr. 2002;132:3740–3.
26. Watanabe S, Hayashi H, Onozaki K, Okuyama H. Effect of dietary alpha-linolenate/linoleate balance on lipopolysaccharide-induced tumor necrosis factor production in mouse macrophages. Life Sci. 1991;48: 2013–20.
27. Ertel W, Morrison MH, Ayala A, Chaudry IH. Modulation of macrophage membrane phospholipids by n-3 polyunsaturated fatty acids increases interleukin 1 release and prevents suppression of cellular immunity following hemorrhagic shock. Arch Surg. 1993;128:15–20.
28. Albers R, Bol M, Bleumink R, Willems A, Blonk C, Pieters R. Effects of dietary lipids on immune function in a murine sensitisation model. Br J Nutr. 2002;88:291–9.
29. Barber MD, Fearon KC, Ross JA. Eicosapentaenoic acid modulates the immune response but has no effect on a mimic of antigen-specific responses. Nutrition. 2005;21:588–93.
30. Gratchev A, Kzhyshkowska J, Kothe K, Muller-Molinet I, Kannookadan S, Utikal J, Goerdt S. Mphi1 and Mphi2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. Immunobiology. 2006;211:473–86.
31. Meng F, Lowell CA. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. J Exp Med. 1997;185:1661–70.
32. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr. 1993;123:1939–51.
33. Fritsche KL, Johnston PV. Rapid autoxidation of fish oil in diets without added antioxidants. J Nutr. 1988;118:425–6.
34. Lang T, Ave P, Huerre M, Milon G, Antoine JC. Macrophage subsets harbouring Leishmania donovani in spleens of infected BALB/c mice: localization and characterization. Cell Microbiol. 2000;2:415–30.
35. Wimer BM, Mann PL. Mitogen information summaries. Cancer Biother Radiopharm. 2002;17:569–97.
36. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 1990;249:1431–3.
37. Griffin JD, Ritz J, Nadler LM, Schlossman SF. Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest. 1981;68:932–41.
38. Todd RF III, Nadler LM, Schlossman SF. Antigens on human monocytes identified by monoclonal antibodies. J Immunol. 1981;126:1435–42.
39. Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci USA. 1998;95:588–93.
40. Ogata H, Su I, Miyake K, Nagai Y, Akashi S, Mecklenbrauker I, Rajewsky K, Kimoto M, Tarakhovsky A. The toll-like receptor protein RP105 regulates lipopolysaccharide signaling in B cells. J Exp Med. 2000;192:23–9.
41. Kearney JF, Lawton AR. B lymphocyte differentiation induced by lipopolysaccharide. I. Generation of cells synthesizing four major immunoglobulin classes. J Immunol. 1975;115:671–6.
42. Oliver AM, Martin F, Kearney JF. IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. J Immunol. 1999;162:7198–207.
43. Gieni RS, Umetsu DT, DeKruyff RH. Ly1- (CD5-) B cells produce interleukin (IL)-10. Cell Immunol. 1997;175:164–70.
44. O'Garra A, Stapleton G, Dhar V, Pearce M, Schumacher J, Rugo H, Barbis D, Stall A, Cupp J, et al. Production of cytokines by mouse B cells: B lymphomas and normal B cells produce interleukin 10. Int Immunol. 1990;2:821–32.
45. Sun D, Krishnan A, Zaman K, Lawrence R, Bhattacharya A, Fernandes G. Dietary n-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. J Bone Miner Res. 2003;18:1206–16.
46. Ly LH, Smith R III, Chapkin RS, McMurray DN. Dietary n-3 polyunsaturated fatty acids suppress splenic CD4(+) T cell function in interleukin (IL)-10(−/−) mice. Clin Exp Immunol. 2005;139:202–9.
47. Yaqoob P, Calder PC. The effects of dietary lipid manipulation on the production of murine T cell-derived cytokines. Cytokine. 1995;7: 548–53.
48. Arrington JL, Chapkin RS, Switzer KC, Morris JS, McMurray DN. Dietary n-3 polyunsaturated fatty acids modulate purified murine T-cell subset activation. Clin Exp Immunol. 2001;125:499–507.
49. Stulnig TM. Immunomodulation by polyunsaturated fatty acids: mechanisms and effects. Int Arch Allergy Immunol. 2003;132:310–21.
50. Blok WL, de Bruijn MF, Leenen PJ, Eling WM, van Rooijen N, Stanley ER, Buurman WA, van der Meer JW. Dietary n-3 fatty acids increase spleen size and postendotoxin circulating TNF in mice; role of macrophages, macrophage precursors, and colony-stimulating factor-1. J Immunol. 1996;157:5569–73.