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Dietary fish oil increases the proportion of a specific neutrophil subpopulation in blood and total neutrophils in peritoneum of mice following endotoxin-induced inflammation☆

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Abstract

Omega-3 polyunsaturated fatty acids may have beneficial effects in inflammation, where neutrophil migration and activation are of importance. The effects of dietary fish oil on neutrophil numbers and subpopulations in healthy mice and mice with endotoxin-induced inflammation were determined. Mice were fed a control diet with or without 2.8% fish oil, and half of them were injected intraperitoneally with endotoxin. Blood, peritoneal lavage, bone marrow and spleen were collected. Expression of cell surface molecules was analyzed by flow cytometry, and chemokine concentrations were determined by enzyme-linked immunosorbent assay. Dietary fish oil did not alter the proportion of total neutrophils in blood but increased the proportion of a specific subpopulation of neutrophils 48 h following endotoxin administration. This subpopulation of neutrophils expressed higher levels of CD11b, Ly6G and major histocompatibility complex-II, suggesting a different role for these neutrophils in the inflammatory response. Dietary fish oil did not affect neutrophil numbers in the peritoneum of healthy mice, but 12 h after endotoxin administration, there were fewer neutrophils in the peritoneum of mice fed the fish oil diet than in mice fed the control diet. However, 48 h after endotoxin administration, mice fed the fish oil diet had more neutrophils in peritoneum than mice fed the control diet. These results indicate that, although dietary fish oil may delay recruitment of neutrophils from blood to the peritoneum early in inflammation, it has the potential to increase the number of peritoneal neutrophils later, which may be of benefit as impaired neutrophil migration and activation have been associated with immunosuppression late in inflammation.

Keywords: Fish oil; LPS; Endotoxin-induced inflammation; Chemokines; Neutrophils; CCL3

1. Introduction

Dietary fish oil, or omega-3 polyunsaturated fatty acids (PUFA), affect inflammatory responses and are thought to have anti-inflammatory effects. Although inflammation is beneficial, uncontrolled or prolonged inflammation can cause harm and needs to be tightly regulated. Inflammation is characterized by a complex sequence of events involving alterations in the inflammatory mediator network as well as rearrangement of innate immune cell populations and changes in their activation status [1]. Neutrophils have an essential role in host defense, but are also thought to be able to promote persistent inflammatory responses and tissue injury, which can be detrimental to the host [2,3]. They are recruited out of the bloodstream in response to chemokines and, after activation, sequentially discharge granules, which contain a large panel of antimicrobial agents [4]. They also recruit other innate cells to the site of inflammation through the release of chemokines and antimicrobial peptides with chemotactic properties [5]. The proinflammatory role of neutrophils has been elucidated in studies using a monoclonal antibody (RB6-8C5) that binds to Ly6G, a specific granulocyte surface marker, but also to Ly6C, an antigen broadly expressed on immune cells, including monocytes, dendritic cells, T cells, natural killer (NK) cells, NKT cells and eosinophils, in addition to neutrophils. Use of the Ly6G-specific antibodies 1A8 and NIMP-R14 has greatly improved identification of mouse neutrophils, and several studies have now shown that neutrophils, in addition to having proinflammatory

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DMEM, Dulbecco's Modified Eagle Medium; EPA, eicosapentaenoic acid; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibodies; MHC, major histocompatibility complex; NK, natural killer; PBS, phosphate-buffered saline; PG, prostaglandin; PUFA, polyunsaturated fatty acids; TNF, tumor necrosis factor.

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effects, also exhibit anti-inflammatory properties, secreting high amounts of anti-inflammatory cytokines, and that neutrophil depletion during a chronic infection promotes inflammation [6–8]. Furthermore, recent studies have shown that subpopulations of neutrophils have the ability to present antigen and provide help for T cells [9] as well as for splenic B cells [10].

In severe inflammation, or sepsis, the initial hyperinflammatory phase is followed by an anti-inflammatory or immunosuppressive phase [11,12]. In the immunosuppressive phase, the neutrophils may become dysregulated, and despite having increased levels of activation markers, such as CD11b, ICAM-1, myeloperoxidase and CD66b, their adherence and transmigration are impaired [13]. Decreased neutrophil recruitment is also, in part, due to down-regulation of the chemokine receptor CXCR2 in response to both lipopolysaccharide (LPS) and high levels of the chemokine CXCL8 (or CXCL1 and CXCL2 in mice) [14]. Failure of neutrophil migration results in impaired bacterial clearance, weakened host defense and increased risk of secondary infections [13,15]. As omega-3 PUFA are considered to have beneficial effects in severe inflammation, it is surprising that, in several *in vitro* studies, omega-3 PUFA have been shown to hinder neutrophil adhesion and migration. In a recent study by Yates et al. [16] docosahexaenoic acid (DHA), although not eicosapentaenoic acid (EPA), inhibited adhesion of neutrophils to the endothelium by reducing surface expression of E-selectin on endothelial cells following cytokine stimulation [16]. In another study from the same laboratory, EPA reduced migration of neutrophils across vascular endothelium by decreasing formation of prostaglandin (PG) D_2, instead generating PGD_3, which antagonizes the action of PGD_2, but PGD_2 provides a necessary signal for neutrophils to traverse the endothelium [17]. In addition, pretreatment of endothelial cells with DHA down-regulated tumor necrosis factor (TNF)-α-induced endothelial cell surface expression of P-selectin and decreased TNF-α-induced neutrophil adhesion [18]. Furthermore, it has been demonstrated that the omega-3-derived resolvin D2 inhibits adherence of neutrophils to activated endothelial cells in a mouse cremaster model [19] and that resolvin E1 reduces infiltration of neutrophils in murine peritonitis [20].

The effects of dietary fish oil, or omega-3 PUFA, on neutrophil populations or neutrophil migration *in vivo* have not been examined. In the present study, we show that dietary fish oil may delay neutrophil migration to the peritoneum of mice *in vivo*, but increase their numbers in the peritoneum late in the inflammation, as well as increase the proportion of a specific subpopulation of neutrophils in the circulation that may have a different role in the inflammatory process than other neutrophils.

2. Methods

2.1. Mice and diets

Female C57BL/6 mice weighing between 18 and 20 g (Taconic Europe, Ejby, Denmark) were housed five or eight per cage in a humidity-controlled (45%–55%) and temperature-controlled (23°C–25°C) environment with a 12-h light and dark cycle. All procedures with animals complied with NRC's Guide for the Care and Use of Laboratory Animals and were approved by the Experimental Animal Committee, Ministry for the Environment, in Iceland. Mice were acclimated for 1 week prior to initiation of the experiment. They were randomly assigned to either a group fed control diet (D07121302; Research Diets Inc., New Brunswick, NJ, USA) or a group fed fish oil diet (D07121303; Research Diets Inc.) for 6 weeks. The composition of the diets was based on a typical American diet, i.e., the "US17" diet formulated by Monsanto (St. Louis, MO, USA) and Research Diets Inc. with minor modification by the authors (Table 1). Energy distribution of the diets was as follows: carbohydrate, 44%; fat, 35%; and protein, 21%. The fish oil diet contained 28 g/kg menhaden fish oil (Omega Protein, Reedville, VA, USA), which was added at the expense of safflower oil (Welch, Holme & Clark Co. Inc., Newark, NJ, USA). To adjust for the arachidonic acid (AA) content in the fish oil diet, AA ethyl ester (Nu-Check-Prep, Elysian, MN, USA) (0.5 g/kg) was added to the control diet. The fatty acid composition of the diets is shown in Table 2. In brief, the fish oil diet contained 10.6 g/kg omega-3 PUFA (4.0 g/kg EPA and 2.5 g/kg DHA), and the control diet contained 3.4 g/kg omega-3 PUFA (undetectable levels of EPA and DHA). To prevent oxidation, the diets were aliquoted into daily portions and were stored under an atmosphere of nitrogen at −20°C. All mice were provided fresh food daily and had free access to food and water.

Table 1
Composition of the control and fish oil diets

Ingredients	Diet (g/kg)	
	Control	Fish oil
Casein	229	229
L-Cystine	3	3
Cornstarch	274	274
Maltodextrin 10	86	86
Sucrose	114	114
Cellulose	57	57
Cocoa butter	43	43
Linseed oil, RBD	5	5
Palm oil, bleached, deodorized	60	60
Safflower oil, USP	32.5	4.5
High-oleic sunflower, Trisun Extra	31	31
Fish oil	0	28
20:4 n-6 ethyl ester	0.5	0
Mineral mix S10026[a]	11	11
Dicalcium phosphate	15	15
Calcium carbonate	6	6
Potassium citrate	19	19
Vitamin mix V13401[b]	11	11
Choline bitartrate	2	2
α-Vitamin E acetate[c]	0.15	0.15
t-BHQ	0.03	0.03

RBD, refined, bleached, deodorized; USP, United States Pharmacopeia.

[a] Containing the following (g/kg mineral mix): sodium chloride, 259; magnesium oxide, 41.9; magnesium sulfate $7H_2O$, 257.6, chromium KSO_4 $12H_2O$, 1.925; cupric carbonate, 1.05; sodium fluoride, 0.2; potassium iodate, 0.035; ferric citrate, 21; manganous carbonate, 12.25; ammonium molybdate $4H_2O$, 0.3; sodium selenite, 0.035; zinc carbonate, 5.6; sucrose 399.105.

[b] Containing the following (g/kg vitamin mix): retinyl palmitate, 0.8, cholecalciferol, 1.0; menadione sodium bisulfate, 0.08; biotin (1.0%), 2.0; cyanocobalamin (0.1%), 1.0; folic acid, 0.2; nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine-HCl, 0.7; riboflavin, 0.6; thiamin-HCl, 0.6; sucrose 988.42.

[c] 500 IU/g.

Table 2
Fatty acid composition of control and fish oil diets

Fatty acid	Diet (g/kg)	
	Control	Fish oil
10:0	ND	ND
12:0	0.2	0.2
14:0	0.5	2.4
15:0	ND	0.1
16:0	36.8	39.2
16:1	0.1	2.9
18:0	19.5	19.5
18:1	70.1	69.5
18:2 (n-6)	36.2	14.9
18:3 (n-3)	3.4	3.8
20:0	0.7	0.8
20:1	ND	0.4
20:2	ND	0.1
20:3	ND	0.1
20:4 (n-6)	0.6	0.6
20:5 (n-3)	ND	4.0
22:1	ND	0.1
22:4	ND	0.1
22.6 (n-3)	ND	2.5
24:0	ND	0.1
Total SFA	57.7	62.5
Total MUFA	70.2	72.9
Total PUFA	40.2	26.5
PUFA/SFA	0.7	0.4
Total (n-3)	3.4	10.6
Total (n-6)	36.8	15.6

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; ND, not detected.

2.2. Body mass and food intake

Weight of the mice was monitored weekly throughout the experiments. Food was provided daily, and any remaining food from the previous day was discarded. Food intake was determined as the difference between the food supplied and the amount of food left.

2.3. Induction of inflammation

Mice were injected intraperitoneally with LPS (0.5 mg/kg; *Escherichia coli*, serotype 055:B5, Sigma Aldrich) in phosphate-buffered saline (PBS) (Invitrogen, Paisley, UK). At indicated time points, they were anesthetized with a (1:1) mixture of Hypnorm (VetaPharma Ltd., Leeds, UK) and Dormicum (Roche, Basel, Switzerland). Blood was collected via axillary bleeding, and the mice were killed by cervical dislocation.

2.4. Collection of blood and peritoneal lavage

Blood was collected into EDTA-K coated tubes (Sarstedt, Nümbrect, Germany) for flow cytometry analysis or Eppendorf tubes for whole blood collection. Serum was collected and stored at −70°C until analyzed. For collection of peritoneal lavage, cold PBS without calcium or magnesium was injected into the peritoneum, it was massaged gently, and peritoneal lavage was collected. Cells were spun down, and the supernatant was collected and stored at −70°C. Peritoneal cells were washed with PBS and resuspended in Dulbecco's modified Eagle medium (DMEM) with Gluta-MAX-I (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Invitrogen).

2.5. Collection of bone marrow and spleen cells

Right femurs and tibiae were cut at the diaphyses, and bone marrow cells were flushed out by repeated injections of DMEM supplemented with 10% fetal bovine serum (Invitrogen). Spleens were passed through a cell strainer (BD Bioscience, San Jose, CA, USA) to acquire a single cell suspension. Red blood cells were lysed with ACK lysing buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA), and the cells washed and resuspended in 5 ml DMEM.

2.6. Cell count and phenotypic characterization of leukocytes by flow cytometry

For total blood cell numbers and number of neutrophils, 50 μl of blood was measured precisely into Trucount tubes (BD Biosciences) and stained with monoclonal antibodies (mAb) against Ly6G. Peritoneal, spleen and bone marrow cells were counted using trypan blue and Countess automated cell counter (Invitrogen).

For phenotypic analysis of leukocytes, 100 μl of blood or 0.3×10^6 bone marrow, spleen or peritoneal cells were preincubated with a (1:1) mixture of normal rat:normal mouse serum (2%) (AbD Serotec, Kidlington, UK). The cells were stained with fluorochrome-labeled mAb against CD11b (Mac-1), CD14, CD62L (L-selectin), major histocompatibility complex (MHC)-II (eBioscience, San Diego, CA, USA), Ly6G (clone 1A8 that only binds to Ly6G but not Ly6C, BD Bioscience) and CXCR2 (R&D Systems, Abington, UK). Bone marrow, spleen and peritoneal cells were additionally stained with mAb against B220, CD90.2 and NK1.1 (eBioscience) for exclusion of lymphocytes. Red blood cells in blood samples were lysed with FACS Lysing Solution (BD Bioscience). All samples were washed and resuspended in FACS staining buffer (PBS with 0.5% bovine serum albumin, 2 mM EDTA, 0.1% sodium azide). Appropriate isotypic controls were used to set the quadrants and evaluate background staining. Samples were collected on FACScalibur (BD Biosciences), and data were analyzed using Cell Quest (BD Biosciences) and FlowJo (Tree Star Inc., Ashland, OR, USA). Blood, bone marrow, spleen and peritoneal neutrophils were identified as cells expressing the granulocyte marker Ly6G (clone 1A8) and the chemokine receptor CXCR2 but lacking expression of the lymphocyte markers B220, CD90.2 and NK1.1. The neutrophils also expressed CD11b, CD14, CD62L and MHC-II.

2.7. Activation of resident peritoneal cells ex vivo and intracellular cytokine staining by flow cytometry

Peritoneal cells in DMEM (2.5×10^5 cells/250 μl/well) were cultured in a 48-well flat-bottom plate at 37°C with 5% CO_2. Nonadherent cells were discarded, and the adherent cells washed twice with PBS and cultured further in 250 μl DMEM supplemented with 5% autologous serum for 24 h in the absence (negative control) or presence of 1 μg/ml LPS (*E. coli* 055:195; Difco Laboratories, Detroit, MI, USA). For cytokine measurements, the cells were spun down after 24-h culture, and the supernatant was collected and stored at −70°C until analyzed with enzyme-linked immunosorbent assay (ELISA). For intracellular staining, brefeldin A (3 μg/ml; eBioscience) was added to the cell culture for the last 6 h of the 24-h LPS stimulation. The supernatant was removed, and the cells were scraped from the plate after being treated with cold PBS containing 20 mM EDTA for 15 min. The cells were stained with mAb against CD11b and against B220, NK1.1 and CD90.2 (for exclusion of contaminating lymphocytes) and then, after fixation in 4% formaldehyde, permeabilization with 0.1% saponin in FACS staining buffer and blocking with a 1:1 normal mouse:normal rat serum (20%), with mAb against CCL3 (eBioscience). Cells were analyzed on FACScalibur as described above.

2.8. Chemokine and cytokine analysis

Concentrations of chemokines and cytokines in serum and peritoneal fluid were measured with Duo Set ELISA kits (R&D Systems).

2.9. Data analysis

Data are expressed as mean and standard error of the mean (SEM) and were analyzed using two-way analysis of variance and Tukey's post hoc test except in Fig. 3 in which unpaired Student's *t* test was used to determine whether differences between the two dietary groups were statistically significant at a single time point. Statistical analysis was performed using SPSS software, version 17 (SPSS Inc., Chicago, IL, USA). A P value of $<.05$ was considered statistically significant. All experiments were performed at least three times.

3. Results

3.1. Mouse growth and dietary intake

There was no difference in the mean daily food intake, weight gain of mice not receiving LPS or weight loss of mice receiving LPS between mice in the two dietary groups (Supplemental Table 1).

3.2. Effects of dietary fish oil on blood neutrophils in healthy mice and mice with endotoxin-induced inflammation

Dietary fish oil did not affect the number of total neutrophils in healthy mice or mice with endotoxin-induced inflammation, although at 12 and 24 h, there was a trend towards more neutrophils in blood from mice fed the fish oil diet compared with that in blood from mice fed the control diet (Fig. 1A). Dietary fish oil did not alter the proportion of total neutrophils (of total blood cells) prior to or following LPS administration (data not shown). Neutrophils from healthy mice were homogenous in size and granularity, but 8, 12 and 48 h after administration of LPS, two distinct populations of neutrophils were present in blood: one resembling the population present in blood prior to LPS administration (N1) and another population consisting of less granular and slightly larger neutrophils (N2) (Fig. 1B and C) that had higher expression of CD11b, Ly6G and MHC-II than the N1 neutrophils, but similar levels of CXCR2 and CD62L (data not shown). At 8 and 12 h after administration of LPS, there was no difference in the proportion of the two neutrophil populations in mice fed the different diets (Fig. 1D). However, at 48 h following LPS administration, the N2 subpopulation was larger in proportion in mice fed the fish oil diet (Fig. 1C and D) than in mice fed the control diet (Fig. 1B and D). N2 neutrophils from mice fed the fish oil diet had higher expression levels of Ly6G 12 h after administration of LPS (2342 ± 205) than N2 neutrophils from mice fed the control diet (1640 ± 108, $P<.05$). There was no difference in expression levels of Ly6G on N2 neutrophils from mice fed the different diets at 8 or 48 h after induction of inflammation. Dietary fish oil did not affect the Ly6G expression levels on N1 neutrophils in healthy mice or mice with endotoxin-induced inflammation, or the expression of other surface markers on N1 or N2 neutrophils at any of the time points examined (data not shown).

3.3. Effects of dietary fish oil on bone marrow and spleen neutrophils in healthy mice and mice with endotoxin-induced inflammation

Dietary fish oil did not affect the number or the proportion of neutrophils, or the expression levels of surface markers on neutrophils in either bone marrow or spleen in healthy mice or in mice with endotoxin-induced inflammation (data not shown).

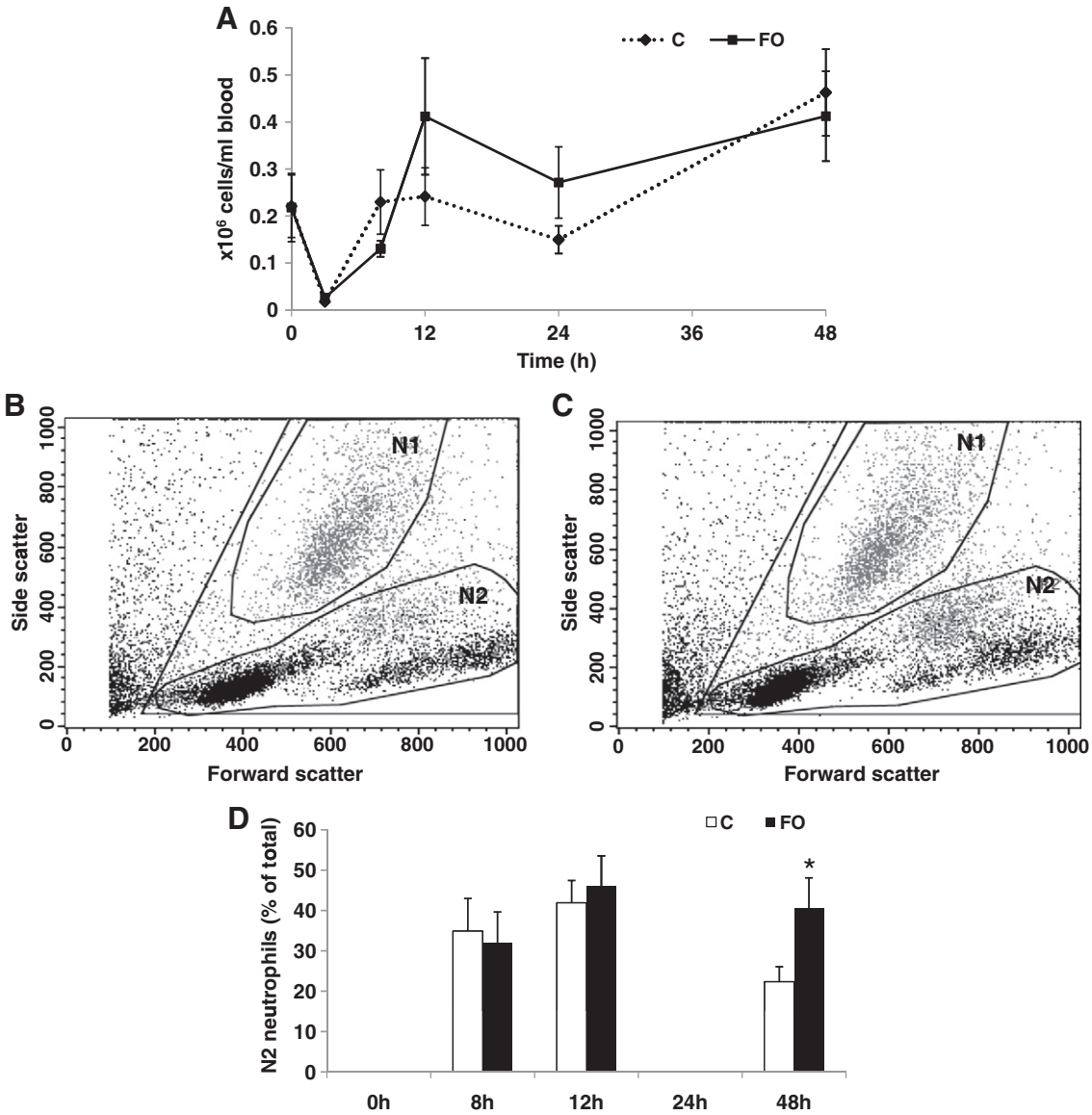

Fig. 1. Effects of dietary fish oil on the number of total neutrophils (A), size and granularity of the neutrophils (B–C) and proportion of N2 neutrophils of total neutrophils (D) prior to and/or following LPS administration. Mice were fed a control diet (dashed line, open bar) or a diet supplemented with 2.8% fish oil (solid line, black bar) for 6 weeks. They were injected with LPS (0.5 mg/kg) or not and sacrificed at indicated time points (A, D) or at 48 h (B, C). Blood was collected, and cells were stained with monoclonal antibodies, counted using TruCount (A) and analyzed by flow cytometry (A–D). Values are means±SEM, $n=3$–5 (A), $n=8$ (D). Representative forward and side scatter dot plots of blood cells from mice fed the control (B) and the fish oil (C) diets 48 h after administration of LPS: grey dots, neutrophils (N1 and N2); black dots, other cells. *Different from control, $P<.05$.

3.4. Effects of dietary fish oil on serum concentrations of neutrophil chemoattractants in mice with endotoxin-induced inflammation

Murine CXCL1 and CXCL2 are potent neutrophil chemoattractants that bind to the chemokine receptor CXCR2 and recruit neutrophils to the site of infection or inflammation, and CCL3 has also been implicated to play a role in the recruitment of neutrophils [21,22]. Dietary fish oil did not affect serum levels of CXCL1 in healthy mice or mice killed 3 h or 48 h after endotoxin-induced inflammation (Table 3). CXCL2 and CCL3 were not detected in serum from healthy mice or in serum from mice that were killed 48 h after endotoxin-induced inflammation (Table 3). However, fish-oil-fed mice that were killed 3 h after endotoxin-induced inflammation had lower serum concentrations of CXCL2 but higher serum concentrations of CCL3 than mice fed the control diet (Table 3). The effects of dietary fish oil on serum concentrations of the cytokines granulocyte colony-stimulating factor (G-CSF) and interleukin (IL)-33 were examined as G-CSF stimulates the release of neutrophils from the bone marrow and IL-33 prevents down-regulation of CXCR2 on neutrophils during acute inflammation and thus enhances neutrophil migration to the site of infection [23]. G-CSF was not detected in serum from healthy mice, and there was no difference in G-CSF levels in serum from mice in the two dietary groups killed 3 and 48 h after induction of inflammation (Table 3). IL-33 was not detected in serum from healthy mice or mice with endotoxin-induced inflammation (data not shown).

3.5. Effects of dietary fish oil on peritoneal neutrophils in healthy mice and mice with endotoxin-induced inflammation

The number of total neutrophils was similar in the peritoneum of healthy mice fed the fish oil diet and healthy mice fed the control diet (Fig. 2A). Following administration of LPS, there was a delay in the increase in the number of neutrophils in the peritoneum of mice fed

Table 3
Chemokine and cytokine concentrations in serum and peritoneal fluid from mice prior to and following administration of LPS[a]

Chemokine/ cytokine	No LPS (0 h)		LPS (3 h)		LPS (48 h)	
	Control	Fish oil	Control	Fish oil	Control	Fish oil
Serum						
CXCL1 (μg/L)	0.17±0.02	0.15±0.01	405±12	401±16	0.23±0.03	0.21±0.02
CXCL2 (μg/L)	ND	ND	24.9±3.6	13.6±2.1*	ND	ND
CCL3 (μg/L)	ND	ND	1.69±0.14	2.77±0.28*	ND	ND
G-CSF (μg/L)	ND	ND	393±30	450±39	28±18	39±15
Peritoneum						
CXCL1 (μg/L)	ND	ND	7.53±0.68	7.27±0.53	0.03±0.00	0.04±0.00
CXCL2 (μg/L)	28.2±2.9	20.8±2.0	1084±120	1052±166	18.7±1.3	20.1±1.7
CCL3 (ng/L)	ND	ND	25.1±1.4	30.9±2.0*	ND	ND

[a] Values are means±SEM, $n=8$–18 for chemokines, $n=3$–6 for cytokines.
* Different from control, $P<.05$.

the fish oil diet compared with that of mice fed the control diet where, at 12 h after LPS administration, fewer neutrophils were observed in the peritoneum of mice fed the fish oil diet than in mice fed the control diet (Fig. 2A). However, at 48 h, there were more neutrophils present in the peritoneum of mice fed the fish oil diet compared with that of mice fed the control diet (Fig. 2B), and the proportion of neutrophils of total peritoneal cells was significantly greater in mice fed the fish oil diet (Figs. 2D and E) than in mice fed the control diet (Figs. 2C and E).

3.6. Effects of dietary fish oil on peritoneal concentrations and peritoneal cell production of neutrophil chemoattractants

Dietary fish oil did not affect peritoneal concentrations of CXCL1 or CXCL2 prior to or 3 or 48 h after induction of inflammation (Table 3). CCL3 was not detected in peritoneal fluid of healthy mice or mice killed 48 h after endotoxin-induced inflammation (Table 3). However, dietary fish oil increased the concentration of CCL3 in peritoneal fluid from mice killed 3 h after endotoxin-induced inflammation (Table 3; Fig. 3A). When peritoneal cells from mice fed control and fish oil diet were collected and stimulated with LPS *ex vivo*, peritoneal cells from mice fed the fish oil diet secreted more CCL3 than peritoneal cells from mice fed the control diet (Fig. 3B), and the amount of CCL3 produced by each cell was higher among peritoneal cells from mice fed the fish oil diet than mice fed the control diet (Fig. 3C). In contrast, dietary fish oil decreased the number of $CD11b^+$ resident peritoneal macrophages secreting CCL3 in the peritoneum (Fig. 3D).

4. Discussion

Although omega-3 PUFA may be of benefit in inflammation, their effects on migration and dysregulation of neutrophils, which are thought to be of importance in defense failure during the immunosuppressive phase observed late in the inflammatory process, have not been reported. The results from the current study show that dietary fish oil affects the proportion of a subpopulation of neutrophils in blood and the number and proportion of total neutrophils in peritoneum of mice with endotoxin-induced inflammation. The most striking finding is that, late in the inflammation or 48 h after endotoxin administration, there were around 40% more neutrophils in the peritoneum of mice fed the fish oil diet than in mice fed the control diet, and the proportion of neutrophils of total cells in the peritoneum was around 80% higher in mice fed the fish oil diet than in mice fed the control diet. At this time point, there was no difference in expression levels of any of the surface markers examined, indicating that the activation state of the neutrophils was not affected by the fish oil diet. These results indicate that dietary fish oil has the potential to enhance migration of neutrophils to the site of inflammation late in the inflammatory phase without affecting their activation status, which may be of benefit during the immunosuppressive phase of severe inflammation.

The higher number of neutrophils in the peritoneum of mice fed the fish oil diet 48 h following induction of inflammation was preceded by fewer neutrophils in the peritoneum at 12 h (and perhaps also 24 h), indicating that infiltration of neutrophils into the peritoneum was delayed in mice fed the fish oil diet compared with that in mice fed the control diet. The tendency towards more neutrophils being present in blood at 12 and 24 h after induction of inflammation in mice fed the fish oil diet compared with that in mice fed the control diet supports the notion that there may have been a delay in the recruitment of neutrophils from the blood to the peritoneum in mice fed the fish oil diet and that they accumulated in the circulation at that time. The delayed recruitment of neutrophils from blood to the peritoneum may be explained by the omega-3 PUFA inhibiting adhesion or transmigration of the neutrophils as results from several *in vitro* and *in vivo* studies indicate that omega-3 PUFA, or lipid mediators derived from them, can affect endothelial adhesion and/or migration of neutrophils [16-20]. These studies all demonstrate that omega-3 PUFA, or mediators derived from them, affect neutrophil endothelial adhesion or transendothelial migration, although the mechanism by which they do so differ. Therefore, in the present study, the effects of fish oil to decrease neutrophil migration to the peritoneum at early time points in the inflammatory phase may be explained by any of these mechanisms. That the reduction in neutrophil migration in mice fed the fish oil diet at 12 and 24 h after induction of inflammation was overcome at 48 h indicates that other mechanisms may mediate neutrophil migration later in the inflammation.

The only other study that has examined the effects of dietary fish oil on neutrophil recruitment into the peritoneum *in vivo* did so following infection with *Listeria monocytogenes* and showed no effect of fish oil on neutrophil numbers in peritoneum 24 h following infection [24]. It is not surprising that the results from that study differ from the results from the present study as the mechanism of neutrophil recruitment to the peritoneum in response to *L. monocytogenes* differs from the mechanism of neutrophil recruitment in response to *E. coli* (or LPS), the former being CD11b/CD18-independent but the latter being CD11b/CD18-dependent [25].

The effects of the fish oil diet on peritoneal concentrations of the chemokines involved in neutrophil recruitment did not shed light on the mechanism by which fish oil affected neutrophil numbers in the peritoneum. Peritoneal concentrations of the neutrophil chemoattractants CXCL1, CXCL2 and CCL3 peaked within 4 h and were back to almost undetectable levels by 8 h (data not shown). The increase in peritoneal concentration of CCL3 in mice fed the fish oil diet was evident at 3 h, when there were few neutrophils in the peritoneum and no difference in their numbers between mice fed the different diets. When the number of neutrophils started to increase, there were

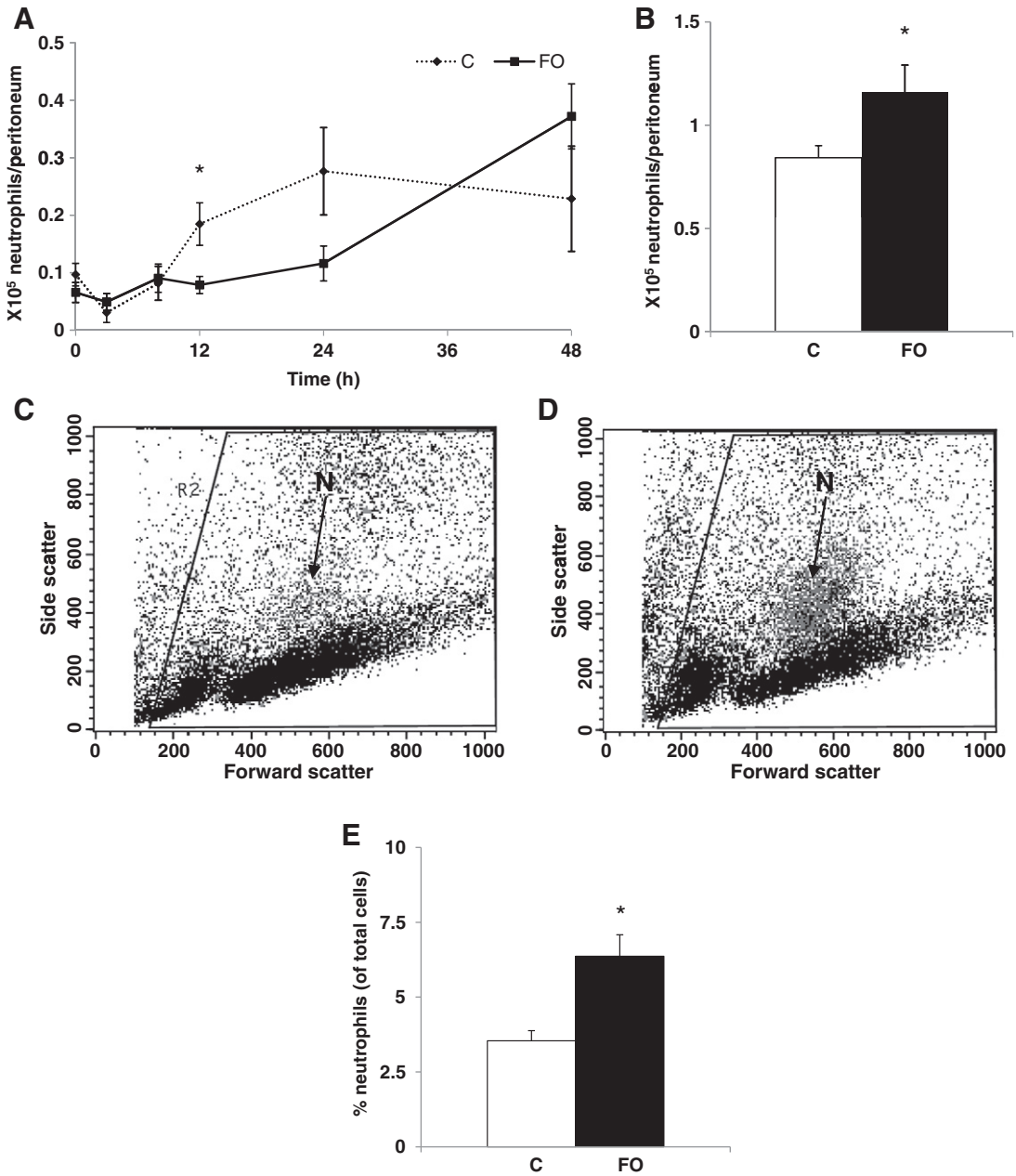

Fig. 2. Effects of dietary fish oil on the number of total peritoneal neutrophils (A, B), size and granularity of the neutrophils (C–D) and proportion of neutrophils of total peritoneal cells (E) prior to and/or following administration of LPS. Mice were fed a control diet (dashed line, open bar) or a diet supplemented with 2.8% fish oil (solid line, black bar) for 6 weeks. They were injected with LPS (0.5 mg/kg) or not and sacrificed at indicated time points (A) or at 48 h (B–E). Peritoneal cells were collected, stained with monoclonal antibodies and analyzed with flow cytometry. Values are means±SEM, $n=3$–5 (A), $n=11$ (B, E). Representative forward and side scatter dot plots of peritoneal cells from mice fed control (C) or fish oil (D) diets 48 h after administration of LPS: grey dots, neutrophils (N); black dots, other cells. *Different from control, $P<.05$.

fewer neutrophils in the peritoneum of mice fed the fish oil diet, which is in contrast to the higher concentration of CCL3 in the peritoneum a few hours earlier. Neither did expression levels of CXCR2 on the neutrophils explain the delay in neutrophil recruitment to the peritoneum, as there was no difference in the expression levels of CXCR2 on neutrophils from mice fed the different diets.

Although there was no difference in neutrophil numbers in bone marrow from mice with endotoxin-induced inflammation fed the different diets, there was a trend towards higher serum levels of G-CSF in mice fed the fish oil diet compared with that in mice fed the control diet 12 h after induction of inflammation. G-CSF stimulates the release of neutrophils from the bone marrow into the circulation [26], and the trend towards an increase in G-CSF levels in blood is consistent with the trend towards increased numbers of neutrophils in blood from mice fed the fish oil diet at 12 and 24 h after induction of inflammation. Serum levels of CCL3 were also higher in mice fed the fish oil diet compared with those in mice fed the control diet, but serum levels of CCL3 peaked around 2 h after administration of LPS, long before the trend towards higher neutrophil numbers appeared in blood from mice fed the fish oil diet.

In the present study, mice fed the fish oil diet had a higher proportion of N2 neutrophils in the circulation than mice fed the control diet. The N2 neutrophils expressed higher levels of CD11b, Ly6G and MHC-II than the N1 population. The higher expression levels of CD11b on the N2 neutrophils suggest that they may be in a more activated state than the N1 neutrophils [27,28] or have

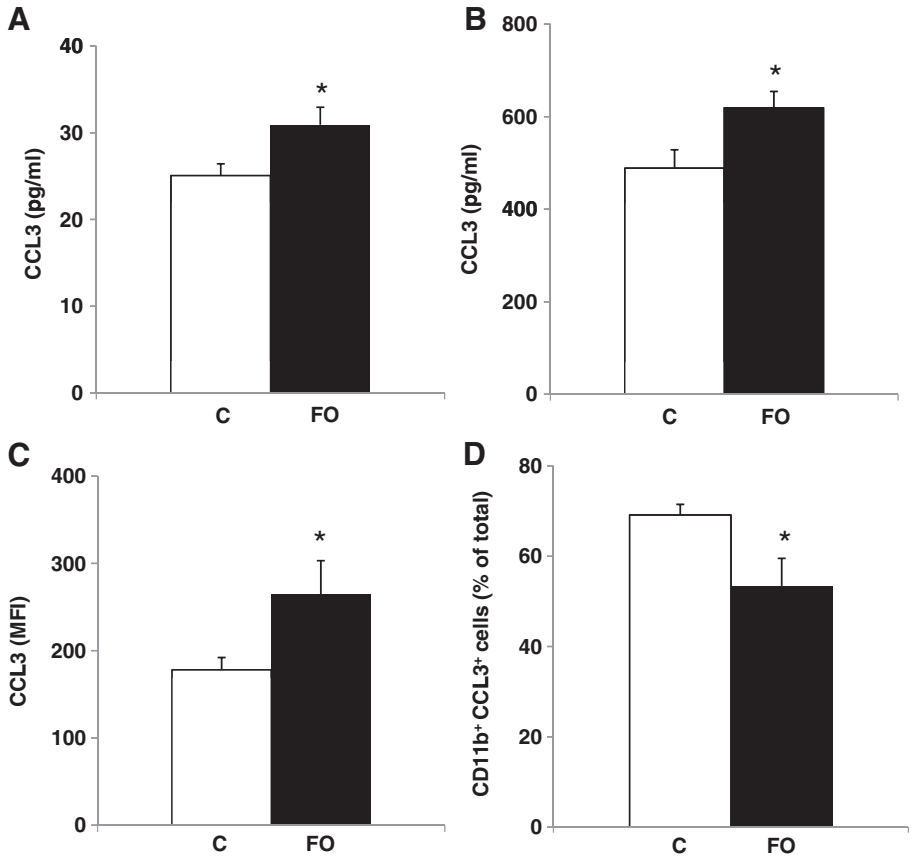

Fig. 3. Effects of dietary fish oil on peritoneal concentrations of CCL3 (A), LPS-induced CCL3 secretion by resident peritoneal cells *ex vivo* (B), expression levels of CCL3 on peritoneal cells (C) and the proportion of CCL3-secreting cells of total CD11b$^+$ peritoneal cells (D). Mice were fed a control diet (open bar) or a diet supplemented with 2.8% fish oil (black bar) for 6 weeks. Mice were injected with LPS (0.5 mg/kg), and peritoneal fluid was collected 3 h later (A). Peritoneal cells (1×10^6 cells/ml) were collected from healthy mice and stimulated with LPS (1 μg/ml) for 24 h, supernatant was collected (B), and chemokine concentrations were measured with ELISA (A, B). Brefeldin A was added to peritoneal cells in culture for the last 6 h of the stimulation, and expression levels of CCL3 on CD11b$^+$ peritoneal cells (C) and the percentage of CD11b$^+$ peritoneal cells secreting CCL3 (D) were measured with flow cytometry. Values are means±S.E.M., $n=14$–17 (A), $n=17$ (B), $n=13$–16 (C–D). *Different from control, $P<.05$.

immunosuppressive properties similar to human neutrophils with high expression levels of CD11b [29]. Their expression of MHC-II also indicates that they can be involved in antigen presentation to T cells as has been shown for mouse neutrophils expressing MHC-II [9]. The presence of two neutrophil populations in the circulation upon induction of inflammation was intriguing as several recent studies have emerged showing that neutrophils are far from being a homogeneous population with only the traditional phagocytic and bactericidal function. These studies have shown neutrophils to be able to secrete high levels of IL-10, suppress T cell responses, possess antigen-presenting function and provide help for T cells, and a population of neutrophils located in the marginal zone of spleen has been shown to induce immunoglobulin class switching, somatic hypermutation and antibody production by activating marginal zone B cells [6–10].

The results from the present study show that dietary fish oil increases the proportion of a specific subpopulation of circulating neutrophils that may have a different role in the inflammatory process than other neutrophils. They also show that, although dietary fish oil may delay the recruitment of neutrophils from blood to the peritoneum at early time points after onset of inflammation, it increases the number of peritoneal neutrophils at a later time point. The increased number of neutrophils at the site of inflammation at a late time point in the inflammatory process may shed light on the mechanism by which omega-3 PUFA can have beneficial effects in severe inflammation, in which impaired neutrophil migration and activation have been associated with immunosuppression and defense failure. In addition, although neutrophils are the main effector cells during inflammation, they can also control excessive inflammatory responses by secreting anti-inflammatory cytokines and may have a previously unsuspected regulatory role during inflammation [6,7]. Whether these late-arriving neutrophils observed in the mice fed dietary fish oil are of this anti-inflammatory type remains to be investigated.

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