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Dietary fish oil reduces the acute inflammatory response and enhances resolution of antigen-induced peritonitis☆

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Abstract

Dietary n-3 polyunsaturated fatty acids (PUFA) influence the inductive phase of inflammation but less is known about their effects on the resolution phase. This study examined the effects of dietary fish oil on induction and resolution of antigen-induced inflammation in mice. Mice were fed a control diet with or without 2.8% fish oil, immunized twice with methylated BSA (mBSA) and inflammation induced by intraperitoneal injection of mBSA. Prior to and at different time points after mBSA administration, peritoneal cells were analyzed and expression of surface molecules determined by flow cytometry. Concentration of chemokines, cytokines and soluble cytokine receptors was determined by ELISA. Mice fed the fish oil diet had fewer peritoneal neutrophils, shorter resolution interval and lower levels of pro-inflammatory cytokines and chemokines than mice fed the control diet. In mice fed the fish oil diet there was an early peak in peritoneal levels of the immunosuppressive molecules sIL-6R and TGF-β, that was not seen in mice fed the control diet. In the resolution phase, peritoneal macrophages from mice fed the fish oil diet expressed more of the atypical chemokine receptor D6 and peritoneal TGF-β levels were higher than that in mice fed the control diet. Furthermore, in the late-resolution phase there were more peritoneal eosinophils and macrophages in mice fed the fish oil diet than in mice fed the control diet. These results demonstrate a suppressive effect of n-3 PUFA on the inductive phase of inflammation and indicate an enhancing effect of n-3 PUFA on resolution of inflammation.

Keywords: Fish oil; mBSA; Antigen-induced inflammation; Macrophages; Eosinophils

1. Introduction

Dietary n-3 polyunsaturated fatty acids (PUFA) modulate inflammatory responses and are generally thought to be anti-inflammatory. Inflammation underlies many common conditions and diseases, such as rheumatoid arthritis, atherosclerosis, chronic obstructive pulmonary disease, cancer and Alzheimer's disease. Recent studies indicate that some of these diseases can be ameliorated by increased consumption of n-3 PUFA [1–7].

Inflammation is characterized by a complex sequence of events involving an inductive phase followed by a resolution phase that is important for return to homeostasis. During the inductive phase pro-inflammatory eicosanoids and cytokines are generated and neutrophils are recruited to the inflamed site. Dietary n-3 PUFA have been shown to beneficially affect generation of pro-inflammatory eicosanoids and cytokines [8]. N-3 PUFA have also been shown to hinder neutrophil adhesion and migration in vitro [9–11] and we recently demonstrated fewer neutrophils at the inflamed site during the inductive phase of peritonitis in mice [12].

Resolution of inflammation is an active process and a necessary step in controlling inflammation [13]. Efficient resolution of inflammation depends on inhibition of neutrophil influx, promotion of monocyte recruitment and their development into macrophages, rapid clearance of apoptotic neutrophils and regeneration of disrupted tissue structures [14,15]. Specialized pro-resolving mediators, resolvins (Rvs), protectins (PDs) and maresins aid in the resolution of inflammation [16], and these are generated from the long chain n-3 PUFA, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Therefore, it has been assumed that dietary n-3 PUFA are important for resolution of inflammation, but studies examining their effect as dietary supplement on the resolution phase of inflammation are lacking. Resolution of inflammation is necessary

Abbreviations: DHA, docosahexaenoic acid; C, control; CRP, C-reactive protein; EPA, eicosapentaenoic acid; FO, fish oil; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; i.p, intraperitoneal; mBSA, methylated BSA; mean fluorescence intensity, MFI; NK cells, natural killer cells; PBS, phosphate buffered saline; PDs, protectins; PUFA, polyunsaturated fatty acids; Rvs, resolvins; R_i, resolution interval; SEM, standard error of the mean.

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to minimize inflammation-related collateral damage and to promote tissue healing. Absent or insufficient resolution of inflammation may result in chronic inflammation, which can have serious consequences as chronic inflammation is now being recognized as an important component in the pathogenesis of many of the most prevalent chronic conditions, including atherosclerosis, cancer and Alzheimer's disease, as well as immune-mediated diseases, s.a. rheumatoid arthritis, asthma and inflammatory bowel disease.

The aim of this study was to determine the effects of dietary fish oil on the induction and resolution of inflammation in a methylated BSA (mBSA)-induced peritonitis, a model for subacute/chronic inflammation [17].

2. Methods and materials

2.1. Animals and diets

Female C57BL/6 mice, weighing 18–20 g, were purchased from Taconic Europe (Ejby, Denmark). All animal procedures were approved by the Experimental Animal Committee, Ministry for the Environment in Iceland and complied with National Research Council's Guide for the Care and Use of Laboratory Animals [18]. Mice were housed 8 per cage with a 12 h light/dark cycle at 23–25°C and 45–55% humidity. Mice were acclimated for 1 week prior to initiation of the experiments, whereafter they were divided into two groups that were fed either a control (C) diet or a fish oil (FO) diet, starting 1 week before the immunization protocol and continuing throughout the experiment (4–5 weeks). The composition of the control diet was based on a typical American diet, i.e., the "US17" diet formulated by Monsanto (St. Louis, MO, USA) and Research Diets Inc (D07121302; Research Diets, New Brunswick, NJ, USA) with minor modification by the authors, as previously described [19]. Energy distribution of the diet was as follows: carbohydrate, 44%; fat, 35%; protein, 21%. The FO 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, Newark, NJ, USA). Arachidonic acid ethyl ester (Nu-Check-Prep, Elysian, MN, USA) (0.5 g/kg) was added to the control diet to adjust for the arachidonic acid content in the FO diet. In brief, the FO diet contained 10.6 g/kg n-3 PUFA (4.0 g/kg EPA and 2.5 g/kg DHA) and the C diet 3.4 g/kg n-3 PUFA (undetectable levels of EPA and DHA). All mice were provided fresh food daily and had free access to food and water. We have previously shown that healthy mice receiving the FO diet had a higher proportion of n-3 fatty acids and a higher ratio of n-3:n-6 PUFA in hepatic phospholipids than healthy mice receiving the C diet [19].

2.2. Antigen-induced peritonitis

Mice were immunized subcutaneously at the base of the tail with an emulsion containing 100 μg of mBSA (Sigma Aldrich, St. Louis, MO, USA) in an equal volume of complete Freund's adjuvant (Sigma Aldrich). Booster injection of 100 μg of mBSA in incomplete Freund's adjuvant (Sigma Aldrich) was given 2 weeks later. Three weeks after the initial immunization, peritonitis was induced by intraperitoneal (i.p.) injection of 100 μg of mBSA in saline. Prior to and at several time points after mBSA administration (3 h, 6 h, 12 h, 24 h, 48 h, 5 days and 10 days), mice were anesthetized with a (1:1) mixture of hypnorm (VetaPharma Ltd, Leeds, UK) and dormicum (Roche, Basel, Switzerland) and killed by cervical dislocation.

2.3. Collection of peritoneal lavage and spleen

Peritoneal fluid and cells were collected in 1.5 ml of cold phosphate buffered saline (PBS) without calcium or magnesium. Cells and fluid were separated by centrifugation and the peritoneal fluid stored at −70°C until analysis. The cells were washed twice with PBS and resuspended in FACS buffer (PBS containing 1% BSA, 0.01% NaN_3). Peritoneal cells were counted by Countess automated cell counter (Invitrogen, Paisley, UK).

Spleens were removed postmortem and passed through a cell strainer (BD Bioscience, San Jose, CA, USA) to obtain 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 were washed and resuspended in 5 ml Dulbecco's modified Eagle medium (Invitrogen). Spleen cells were counted by Countess automated cell counter.

2.4. Phenotypic characterization of leukocytes by flow cytometry

Peritoneal cells (3×10^5) and spleen cells (2.5×10^5) were incubated with a 1:1 mixture of 2% normal rat: normal mouse serum (AbD Serotec, Kidlington, UK) for 20 min. Peritoneal cells were stained with fluorochrome-labeled monoclonal antibodies against CD11b, F4/80, B220, CD90.2, NK1.1, CD117 (eBioscience, San Diego, CA, USA), Ly6G (clone 1A8), CXCR2, CD11b, F4/80, CCR7 (BD Biosciences), and CCR3, D6, CXCR2 (R&D Systems, Abington, UK). Antibodies against B220 (B cells), CD90.2 (T cells), NK1.1 (NK cells) were used for lymphocyte exclusion. NK cells were identified as $NK1.1^+$ cells, neutrophils as $Ly6G^+CXCR2^+$ cells, eosinophils as $CCR3^+$ cells, macrophages as $F4/80^{high}CD11b^+$ or $F4/80^{low}CD11b^+$ cells, and mast cells as $CD117^+$ cells. Eosinophils in spleens were stained with CCR3 and lymphocytes excluded as before. All samples were washed and resuspended in FACS buffer. Appropriate isotypic controls were used to set the quadrants and evaluate background staining. Samples were collected on FACScalibur (BD Biosciences) and data analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA). The results were expressed as percentage positive cells, mean fluorescence intensity (MFI) or total number of positive cells. The resolution interval (R_i) was determined as the time interval within which peak number of inflammatory cells in the peritoneum were reduced by 50% [20].

2.5. Chemokine and cytokine analysis

Cytokines/growth factors (IL-1β, IL-6, G-CSF and TGF-β), soluble cytokine receptors/agonists (IL-1ra, sIL-6R and sTNFR) and chemokines [CCL2, CCL3, CCL11 (eotaxin-1), CXCL1 (KC), and CXCL2] were measured in peritoneal fluid using Duoset ELISA kits (R&D Systems).

2.6. Data analysis

Data are expressed as mean values±standard error of the mean (S.E.M.). As the data were not distributed normally, Kruskal-Wallis nonparametric ANOVA was used to calculate overall P values. The nonparametric Mann–Whitney Rank Sum 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 SigmaStat software, version 3.2 (Systat software Inc., Chicago, IL, USA). $P<.05$ was considered significant. Values are means±S.E.M., $n=4$ for the 6 h and the 10 day time points and 7 for all other time points, making the total number of mice 100. Two other experiments with selected time points gave similar results.

3. Results

3.1. Mouse growth, dietary intake and peritoneal cell count

There was no difference in body weights of the mice or in daily food intake between the two dietary groups. There were more peritoneal cells in mice fed the FO diet ($15.6\pm1.3\times10^6$) than in mice fed the C diet ($11.0\pm0.9\times10^6$, $P<.05$) 5 days after administration of mBSA but no difference in total peritoneal cell number at other time points (data not shown).

3.2. Effects of dietary fish oil on peritoneal mast cells

Mast cells were present in the peritoneum of mice prior to induction of inflammation. There was a tendency towards more mast cells in the peritoneum of mice fed the FO diet than in peritoneum of mice fed the C diet ($5.7\pm0.6\times10^4$ vs. $4.1\pm0.5\times10^4$, $P=.06$).

3.3. Effects of dietary fish oil on peritoneal neutrophils

Administration of mBSA resulted in an influx of neutrophils into the peritoneum. Mice fed the FO diet had fewer neutrophils in their peritoneum than mice fed the C diet, at the time when the number of neutrophils in the peritoneum peaked (3 h for mice fed the FO diet, 6 h for mice fed the C diet) (Fig. 1A). The R_i was also shorter for mice receiving the FO diet (3 h) than for mice receiving the C diet (5 h) (Fig. 1A). Neutrophils from mice fed the FO diet had higher mean expression levels of the integrin CD11b 6 h after administration of mBSA than neutrophils from mice fed the C diet, although there were similar CD11b expression levels on neutrophils from mice in both dietary groups at 3 h, the time point at which CD11b expression peaked (Fig. 1B). At 24 h neutrophils from mice fed the fish oil diet did not express CD11b, whereas neutrophils from mice fed the control diet had similar expression of CD11b at 24 h as they had at 12 h after administration of mBSA.

The peritoneal neutrophils formed two distinct populations that differed in size and granularity. The majority (~90%) of the neutrophils belonged to a population (N1) that was smaller (FSC: 376±3) and less granular (SSC: 389±5) than the other population (N2) (FSC: 621±2, $P<.001$; SSC: 621±7, $P<.001$). The N2 neutrophils expressed more of the surface molecules CXCR2 (MFI: 102±5 vs. 66±