Antigen-Driven Murine CD4$^+$ T Lymphocyte Proliferation and Interleukin-2 Production Are Diminished by Dietary (n-3) Polyunsaturated Fatty Acids[1,2]

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ABSTRACT This study is the first to describe the impact of consuming a diet rich in (n-3) polyunsaturated fatty acids (PUFA) from fish oil on antigen-driven activation of naive CD4$^+$ T lymphocytes. To accomplish this, we used lymphocytes isolated from T cell receptor (TCR) transgenic mice (i.e., DO11.10). A large portion of the T lymphocytes from these mice expresses a TCR specific for a peptide within the ovalbumin (OVA) molecule ($OVA_{323–339}$). When this antigen is presented in the context of major histocompatibility complex I-A^d with costimulation, these naive CD4$^+$ T cells become activated, produce interleukin (IL)-2 and clonally expand. (n-3) PUFA enrichment was accomplished by feeding DO11.10 mice one of two nutritionally complete experimental diets that differed only in the source of fat: lard or menhaden fish oil [high in (n-3) PUFA]. After 2 wk of consuming the experimental diets, lymphocytes were isolated from the spleen of each mouse, then cultured in the presence of antigen (i.e., $OVA_{323–339}$) or concanavalin A (Con A), a nonspecific, polyclonal T cell stimulus. IL-2 production and lymphocyte proliferation were determined after 48 and 72 h, respectively. Naive CD4$^+$ T lymphocytes from fish oil-fed mice stimulated with antigen produced less IL-2 (~33%; $P < 0.001$) and proliferated to a lesser extent (~50%; $P < 0.0001$) than the same cells from lard-fed DO11.10 mice. When stimulated with Con A, (n-3) PUFA did not affect either proliferation or IL-2 production. In summary, we report for the first time that feeding mice a diet enriched with (n-3) PUFA reduces in vitro antigen-stimulated production of IL-2 and subsequent proliferation of naive CD4$^+$ T lymphocytes. J. Nutr. 132: 3293–3300, 2002.

KEY WORDS: • *(n-3) fatty acids* • *mice* • *T lymphocyte* • *interleukin-2* • *proliferation*

Dietary fish and supplemental fish oil have been shown to have a beneficial effect on the clinical course of a number of autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis (1,2). These benefits are associated with an increased intake of the (n-3) polyunsaturated fatty acids (PUFA)[4], eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The most frequently cited mechanisms for the immunomodulation by (n-3) PUFA are reduced proinflammatory eicosanoid and cytokine production and diminished lymphocyte proliferation (3–7). Reduced proliferation of autoreactive T lymphocytes would be expected to improve the clinical course of ongoing autoimmune disease as well as reduce the risk of developing autoimmunity in the first place.

Consumption of fish or fish oil supplements rich in (n-3) PUFA has been shown to reduce lymphocyte proliferation in numerous species, including mice, rats, chickens and humans (8–13). The evidence to date suggests that (n-3) PUFA reduce lymphocyte proliferation by reducing the biosynthesis of interleukin (IL)-2, an essential T cell growth factor. These studies involve the isolation of immune cells from either the peripheral blood or secondary lymphoid tissues such as the spleen and the subsequent culturing of these cells in vitro. Lymphocyte proliferation and cytokine production were induced with a variety of nonantigenic stimuli (e.g., plant lectins, chemicals or cross-linking antibodies). Existing data clearly demonstrate that the impact of (n-3) PUFA observed may vary depending on how cells are stimulated and their culture conditions (reviewed in Ref. 14).

Despite the widespread acceptance that (n-3) PUFA reduce lymphocyte proliferation, our knowledge of how (n-3) PUFA affect T cell responses to antigen stimulation is quite limited. Most of the evidence for (n-3) PUFA reducing antigen-specific lymphocyte responses is based on a few reports in which researchers measured delayed-type hypersensitivity (DTH) re-

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[4] Abbreviations used: 2-ME, 2-mercaptoethanol; APC, antigen-presenting cell; CFSE, carboxyfluorescein diacetate succinimidyl ester; Con A, concanavalin A; DHA, docosahexaenoic acid; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; IL, interleukin; MHC, major histocompatibility complex; OVA, ovalbumin; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid; TBHQ, tertiary butyl-hydroquinone; TCR, T cell receptor.

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sponses to recall antigens. Researchers have reported that (n-3) PUFA from fish oil can either reduce DTH in mice (15,16) and humans (17) or have no effect (18,19). DTH reactions require the coordinated response of both antigen-specific T cells and monocytes/macrophages. This in vivo response involves a complex array of inflammatory mediators, chemokines and cells. Thus, the ability of (n-3) PUFA to reduce DTH responses provides only indirect evidence that antigen-specific T lymphocyte function is altered. Studies of antigen-specific primary lymphocyte responses have been technically difficult due to the relative low frequency of naive lymphocytes to any given antigen (~1 in 10^5–10^6 lymphocytes) (20). To overcome this experimental limitation we used DO11.10 mice that express a transgenic T cell receptor (TCR). This transgenic TCR is specific for a known peptide within ovalbumin (i.e., $OVA_{323–339}$) bound to I-A^d class II major histocompatibility complex (MHC) molecules (21). The use of these mice over the past decade has greatly expanded our understanding of antigen-specific cell signaling and responses of naive $CD4^+$ T lymphocytes. The purpose of this study was to use these transgenic mice to determine the impact of dietary (n-3) PUFA on antigen-specific in vitro responses of naive $CD4^+$ T lymphocytes.

TABLE 1

Fatty acid composition of experimental diets[1]

Fatty acids[2]	Dietary treatment groups	
	Low PUFA (lard)	(n-3) PUFA (fish oil)
	g/100 g total fatty acids	
14:0	—	4.6
16:0	15.9	17.7
16:1(n-7)	1.1	9.9
18:0	19.0	3.6
18:1(n-7) & (n-9)	51.9	16.8
18:2(n-6)	10.8	12.7
18:3(n-3)	0.4	1.6
20:5(n-3) (EPA)	—	15.9
22:5(n-3)	—	2.9
22:6(n-3) (DHA)	—	12.0

[1] The two semipurified diets used in this study contained 18 g/100 g lard or 16 g/100 g menhaden fish oil and 2 g/100 g corn oil (i.e., fish oil).

[2] Fatty acids are denoted by the number of carbons: the number of double bonds, followed by the position of the first double bond relative to the terminal methyl group (n-).

MATERIALS AND METHODS

Mice. DO11.10 mice (a generous gift from Dr. Marc Jenkins, University of Minnesota, Minneapolis, MN) were bred and maintained at the Office of Animal Resources, University of Missouri (Columbia, MO). While in this barrier facility, DO11.10 mice were housed in autoclaved individually ventilated polycarbonate cages containing autoclaved recycled paper bedding (Paperchip; Canbrands International, Ontario, Canada). The room was maintained on a 12:12 h light:dark cycle at 23°C and 40–50% relative humidity. Mice had free access to commercial irradiated rodent diet (PicoLab Rodent Diet 20; Purina Mills, Richmond, IN) and acidified water in drilled water bottles. The mice were serologically negative for the following pathogens: mouse hepatitis virus, minute mouse virus, mouse parvovirus, Sendai virus, *Mycoplasma pulmonis*, Theiler's murine encephalomyelitis virus, mouse rotavirus, pneumonia virus of mice, reovirus 3, lymphocytic choriomeningitis virus, ectromelia virus, mouse adenovirus 1 and 2 and polyomavirus. Mice were negative by culture for the following bacteria: *Pasteurella pneumotropica, M. pulmonis, Salmonella* spp. and *Pseudomonas aeruginosa*. Mice were free of external and internal parasites. At onset of study, mice (8–10 wk of age) were transferred to the Animal Sciences Research Center. In this animal facility mice were housed in autoclaved microisolator cages and were provided with free access to food (Autoclavable Laboratory Rodent Diet 5010; Purina Mills, St. Louis, MO) and sterile water. This research was approved by the University of Missouri Animal Care and Use Committee (protocol no. 3390).

Experimental diets. At the onset of a given experiment, mice were switched from the commercial diet to one of two semipurified, nutritionally complete AIN-93 diets (22). These diets were modified to accommodate a higher level of fat (18 g/100 g) and differed only in fat source: lard or menhaden fish oil. Corn oil was added to the fish oil (125 g/kg) to match the EFA (i.e., linoleic acid) content of lard. The menhaden fish oil was supplied by Omega Protein (Reedville, VA) and stabilized with a synthetic antioxidant [0.2 g/kg tertiary butyl-hydroquinone (TBHQ)] and 1000 mg/kg mixed tocopherols to protect it from auto-oxidation. We added 0.2 g/kg TBHQ, but not tocopherols, to the lard. **Table 1** displays a summary of the fatty acid profiles of these experimental diets. Other ingredients in the diets were purchased from ICN and include the following (unit/kg diet): 390 g of corn starch, 253 g of casein, 110 g of sucrose, 63.2 g of fiber, 44.2 g of AIN-93 mineral mix, 12.7 g of AIN-93 vitamin mix, 3.7 g of L-cysteine and 3.2 g of choline bitartrate.

Immune cell isolation. After being anesthetized with an intramuscular injection of ketamine (200 mg/kg) and xylazine (16 mg/kg), mice were humanely killed by exsanguination. After they were bled, spleens were aseptically removed and placed in 5 mL of sterile phosphate-buffered saline (PBS) at room temperature. Spleens were forced through a sterile tissue sieve into a single cell suspension. Erythrocytes in the spleen-derived lymphocyte preparation (hereafter referred to as splenocytes) were removed by gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) as described previously (23). Immune cells were enumerated electronically with a Coulter Counter (model ZM; Beckman Coulter, Fullerton, CA), then resuspended in HEPES-buffered RPMI medium (GIBCO-BRL, Grand Island, NY) containing 50,000 U/L penicillin, 50 mg/L streptomycin, 2 mmol/L L-glutamine and 50 mL/L fetal bovine serum (herein referred to as complete medium) at 1×10^9 cells/L.

Homologous serums collection. Blood that had been collected by cardiac puncture using a 1-ml insulin syringe (BD Biosciences, Franklin Lakes, NJ) was immediately placed in 4 mL clot-activator serum-separator tubes (BD Biosciences). Blood was allowed to clot in the tube at room temperature for ~1 h, then was centrifuged (ThermoIEC, Needham Heights, MA) at $1000 \times g$ for 10 min. In preparation for its use in our in vitro lymphocyte cultures, serum was pooled according to diet groups, heat-inactivated (56°C for 30 min) and then sterile filtered through a 0.2-μm filter (Corning, Corning, NY).

Immune cell stimulation. Splenocytes were cultured in triplicate at 5×10^4 cells per well in complete medium in 96-well cell culture plates (Costar, Corning, NY). Cells were stimulated with OVA peptide ($OVA_{323–339}$) at 0.02, 0.05 or 0.2 μmol/L, or with concanavalin A (Con A; Sigma-Aldrich) at 0.5, 1.0 or 5.0 mg/L. OVA peptide was synthesized with a PE Applied Biosystems 432 Peptide Synthesizer (PE Applied Biosystems, Foster City, CA) using the following primary amino sequence: ISQAVHAAHAEINEAGR.

We investigated the impact of homologous serums and 2-mercaptoethanol (2-ME) on in vitro lymphocyte proliferation and IL-2 production and our ability to discern diet-induced changes in these parameters. Homologous serums (50 mL/L) were added to diet-matched lymphocyte cultures with a duplicate set of cells receiving no additional serum. Also, 2-ME (50 μmol/L) was added to a replicate set of samples. One set of cells was cultured in the presence of both homologous serums and 2-ME. After addition of all additives and stimuli, splenocytes were incubated at 37°C in a 5% CO_2 and 95% humidified air incubator.

IL-2 production. After culturing splenocytes for 48 h, plates were centrifuged and 20-μL aliquots of supernatants per well (<10% of total volume) were collected. Immediately after supernatant collection, plates were returned to the incubator to complete the 72-h experiment. Supernatants were stored at −70°C until IL-2 concentration was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Pierce-Endogen, Rockford, IL). The limit of detection for the kit was 15 ng/L. Samples were assayed in duplicate. The intra-assay variation was <10%.

Lymphocyte proliferation assays. Proliferative response to stimulation was determined by [^{3}H]thymidine incorporation. Cells in 96-well plates were cultured in triplicate for 72 h; ~1 μCi/well [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) was added for the last 6 h of the culture. Cells were harvested with a PHD cell harvester (Cambridge Technologies, Cambridge, MA), and radioactivity incorporated into cellular DNA was counted in a scintillation counter.

Generational analysis by flow cytometry. To investigate the impact of dietary fat source on the proliferative history of naive CD4$^+$ T lymphocytes after antigen-stimulated clonal expansion in vitro we used a novel cell dye, carboxyfluorescein diacetate succinimidyl ester (CFSE) (24). Splenocytes were labeled with the dye according to the manufacturer's instructions (Vybrant CFDA SE Cell Tracer Kit; Molecular Probes, Eugene, OR). Briefly, CFSE was suspended in dimethylsulfoxide and diluted to 5 μmol/L in PBS. Splenocytes were pelleted and resuspended in 1 mL of the freshly made prewarmed CFSE solution. Cells were labeled at 37°C for 15 min, then washed and reincubated at 37°C for 30 min. After a second wash, the labeled cells were resuspended in complete medium with 50 μmol/L 2-ME at 2×10^9 cells/L. CFSE-labeled cells were transferred into a 24-well plate (10^6 cells per well). Diet-matched homologous serum was added to wells (5% final volume). Antigen-specific stimulation was provided by the addition of optimal and submaximal concentrations of OVA (i.e., 0.02, 0.05 and 0.2 μmol/L) to duplicate wells of cell preparations from each diet treatment group. Cells were incubated at 37°C in a 5% CO_2 and 95% humidified air incubator. After 72 h, cells from duplicate wells were pooled, washed with PBS containing 10 g/L BSA (i.e., staining buffer) then fixed with 3.7% formaldehyde for 15 min. After another wash, the fixed cells were stained with anti-CD4 (a rat anti-mouse CD4*APC conjugate; Caltag Laboratories, Burlingame, CA) and KJ1-26 (a gift from Dr. Marc Jenkins) monoclonal antibodies. KJ1-26 antibodies are specific for the transgenic TCR expressed on T cells from DO11.10 mice (25). CFSE fluorescence data were collected on CD4$^+$ and KJ1-26$^+$ dual positive cells. Cell division results in sequential halving of the CFSE; thus, fluorescence histograms may show up to eight peaks that represent successive generations of cells. Two independent experiments were conducted in which data collected were from pooled cell preparations from three to four mice within each diet group.

Fatty acid analysis. The effect of dietary fat source on lymphocyte fatty acid composition was determined as described in detail elsewhere (26). Briefly, total cellular lipids were extracted from lymphocytes with chloroform and methanol (2:1). Fatty acid methyl esters (FAME) were prepared by base-catalyzed methylation, isolated by thin layer chromatography, then analyzed by gas chromatography. The gas chromatograph (model 5890; Hewlett-Packard, Norwalk, CT) was equipped with a 30-m × 0.25-mm inner diameter fused silica capillary column (Supelco, Bellefonte, PA). FAME were identified by comparing relative retention times with commercial standards.

Statistical analysis. Data are expressed as mean ± SEM. Lymphocyte proliferation is expressed as the mean disintegrations per min of the triplicate wells ± SEM. The impact of dietary fat source, stimulus dose and culture conditions (i.e., the presence or absence of homologous serums and 2-ME) were tested by multifactor ANOVA for each stimulus tested (i.e., OVA and Con A). Significance of differences was accepted when $P < 0.05$. When a significant interaction between diet and stimulus concentration or culture condition and stimuli concentration was identified ($P < 0.05$), treatment effects were analyzed using the Bonferroni post hoc test. All analyses were conducted using GraphPad Prism and InStat (GraphPad Software, San Diego, CA).

RESULTS

Body weights, spleen weights and immune cell yield. Feeding DO11.10 mice a high-fat semipurified AIN-93G diet for 2 wk had no apparent adverse effects on growth or overall health of these transgenic mice. The source of fat in these diets did not influence final body weights of the mice (data not shown). Mice fed the (n-3) PUFA diet had heavier spleens than lard-fed mice (133 ± 6 versus 111 ± 6 mg, respectively; $P < 0.01$). Also, the total number of mononuclear cells (e.g., T and B lymphocytes, macrophages, dendritic cells) obtained from the spleens of mice fed the high (n-3) PUFA diet was >30% greater than from the spleens of lard-fed mice (4.6 ± 0.2 versus $3.2 \pm 0.5 \times 10^7$ cells, respectively; $P < 0.01$). However, flow cytometric analysis showed that dietary fat source did not affect the proportion of CD4$^+$ TCR transgenic cells (i.e., KJ1-26$^+$) in the spleen of DO11.10 mice.

(n-3) PUFA incorporation into DO11.10 immune cells. The fatty acid profiles of splenocytes illustrated that immune cells from DO11.10 mice can incorporate dietary (n-3) PUFA into their cellular membranes. For example, the EPA content of splenocytes from fish oil-fed mice was >4 g/100 g total fatty acids, whereas this fatty acid was not detectable in splenocytes isolated from mice fed lard. As expected, the immune cell DHA content more than doubled and arachidonic acid levels decreased ~50% in response to high dietary (n-3) PUFA (data not shown).

IL-2 production. The stimulation of DO11.10 splenocytes with OVA peptide or Con A resulted in a stimuli dose-dependent accumulation of IL-2 in the cell supernatants 48 h poststimulation. Dietary fat source significantly affected ex vivo IL-2 production by antigen-stimulated DO11.10 splenocytes (**Fig. 1**A). The IL-2 production by OVA-stimulated splenocytes isolated from DO11.10 mice fed the high (n-3) PUFA diet was 33–50% lower compared with mice fed the lard diet ($P < 0.05$). Diet-induced differences in IL-2 production between diet treatment groups were of a similar magnitude in the presence or absence of 5% homologous serums (Fig. 1A), even though the inclusion of 5% homologous mouse serums in the culture medium greatly diminished the production of IL-2 to levels that were one-fifth to one-tenth those in the absence of mouse serums ($P < 0.001$). In contrast to antigen-specific activation of CD4$^+$ T lymphocytes, dietary fat source did not affect Con A-stimulated IL-2 production (Fig. 1*B*).

Lymphocyte proliferation. Many researchers have reported that culture conditions can have a substantial impact on the functional response of lymphocytes in vitro. We found that, when 2-ME was omitted from the culture medium, lymphocyte proliferation was substantially diminished such that [^{3}H]thymidine incorporation was typically 1/50–1/200 of the control value when cells were stimulated with OVA or Con A (data not shown). Therefore, all lymphocyte proliferation data shown here were from cells cultured in the presence of 2-ME. Furthermore, in contrast to IL-2 production, we noted that the inclusion of 5% homologous mouse serums to the culture medium had a very modest impact on in vitro lymphocyte proliferation but did affect our ability to observe diet-induced differences in proliferation.

Feeding DO11.10 mice a diet enriched with (n-3) PUFA from fish oil significantly reduced ex vivo antigen-driven lymphocyte proliferation of naive CD4$^+$ T lymphocytes (**Fig. 2**A). This inhibitory action of (n-3) PUFA was evident at all three concentrations of antigen tested. This included two suboptimal concentrations as well as one that gave a maximal response (i.e., 0.2 μmol/L). The (n-3) PUFA-induced impair-

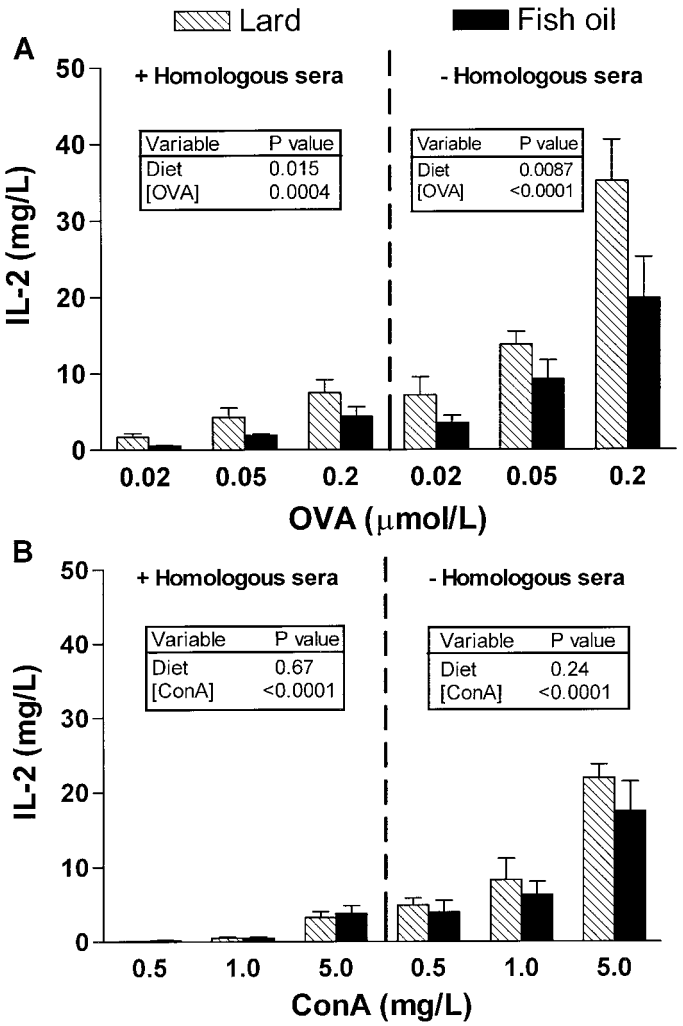

FIGURE 1 Effect of dietary (n-3) polyunsaturated fatty acid (PUFA) on in vitro interleukin (IL)-2 production in lymphocytes from DO11.10 mice. Cells from mice fed lard or fish oil were harvested from the spleen and cultured (10^{10}/L in 96-well plates at a final volume of 0.2 mL/well) in the presence of various concentrations of ovalbumin (OVA) peptide (*A*) or concanavalin A (Con A) (*B*) in triplicate wells. The presence or absence of 5% homologous serums is indicated above the corresponding data. After 48 h, supernatants were harvested and analyzed by enzyme-linked immunosorbent assay (ELISA) in duplicate. Values represent the mean ± SEM (n = 5 mice per diet). The statistical analysis of the data by two-factor ANOVA is summarized in the insets.

ment in lymphocyte proliferation was more pronounced in the presence of 5% homologous serums. Under these conditions, ex vivo lymphocyte proliferation was reduced at all three antigen concentrations tested and inhibition ranged from 50 to 75% ($P < 0.0001$). In contrast, when homologous mouse serum was not included in the culture medium the (n-3) PUFA-mediated reductions were not significant.

Dietary (n-3) PUFA did not affect the proliferation of DO11.10 lymphocytes stimulated with the polyclonal activator Con A (Fig. 2*B*). Interestingly, when lymphocyte preparations were cultured without 2-ME and stimulated with Con A, we observed a significant difference between the diet treatment groups such that proliferation was only half as much for cells from (n-3) PUFA-fed mice compared with cells from lard-fed mice (data not shown). However, the impact of (n-3) PUFA was not consistent across all concentrations of Con A tested. Furthermore, the use of homologous mouse serums did not accentuate the effect of dietary treatments when Con A was the cell stimulus.

Generational analysis by flow cytometry. **Figure 3** is a representative flow cytometric histogram showing the TCR transgenic lymphocytes (i.e., $CD4^+$ and $KJ1\text{-}26^+$ dual positive cells; Fig. 3, upper right quadrant) used for CFSE analysis. When DO11.10 splenocytes were unstimulated for 72 h, $CD4^+$ and $KJ1\text{-}26^+$ showed a fairly uniform bell-shaped distribution of fluorescence staining (data not shown). **Figure 4** illustrates representative histograms of CFSE fluorescence 72 h after stimulation with 0.05 μmol/L OVA peptide. A majority of the OVA-specific $CD4^+$ T cells from the DO11.10 mice

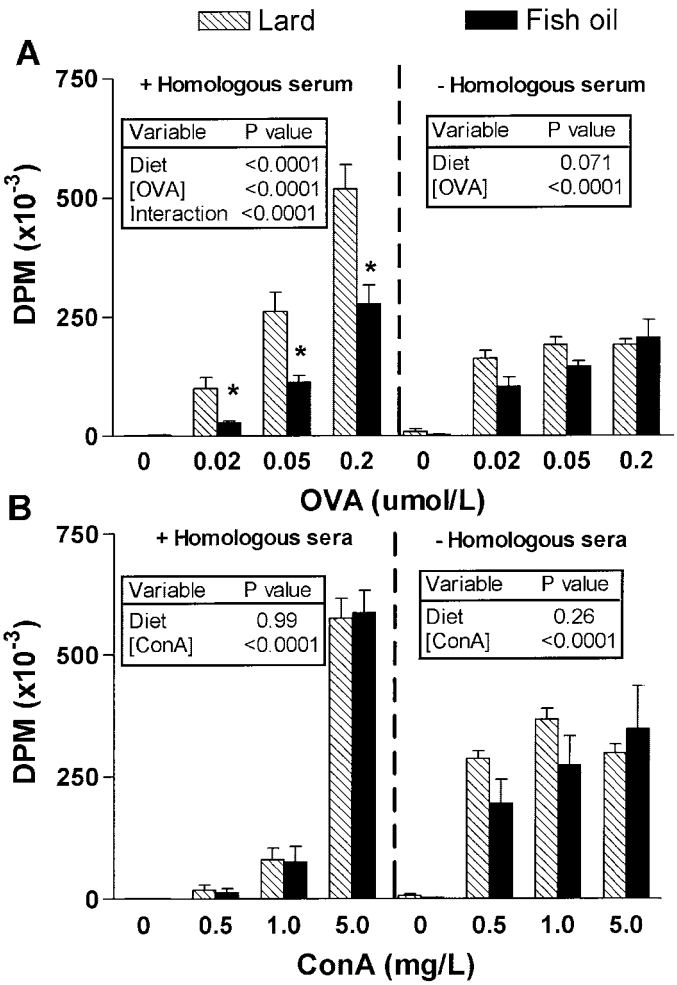

FIGURE 2 Effect of dietary (n-3) polyunsaturated fatty acid (PUFA) on in vitro lymphocyte proliferation in lymphocytes from DO11.10 mice. Cells from mice fed lard or fish oil were isolated from the spleen and cultured in the presence of 2-mercaptoethanol (2-ME) and ovalbumin (OVA) peptide (*A*) or concanavalin A (Con A) (*B*) in triplicate. After 66 h in a 37°C incubator [^{3}H]thymidine was added (1 μCi/well), then 6 h later cells were harvested and [^{3}H]thymidine incorporation was measured. Values represent the mean ± SEM of triplicates (n = 5 mice per diet). The presence or absence of 5% homologous serums is indicated above corresponding data. The statistical analysis by two-factor ANOVA (where * indicates significant differences between treatments using Bonferroni post hoc test when interaction was $P < 0.05$) is summarized in the insets.

proceeded through two to three cell divisions within the 72-h incubation period. Dietary fat treatment did not alter the pattern of asynchronous cell division after antigen-driven stimulation of $CD4^+$ T lymphocytes. Similar data were obtained with the other two concentrations of OVA peptide investigated (data not shown).

DISCUSSION

The impact of (n-3) PUFA and lymphocyte proliferation has been studied for nearly two decades and has been the subject of several reviews (27–29). Most previous studies of (n-3) PUFA and lymphocyte proliferation have relied on nonphysiologic, polyclonal activators such as plant lectins, chemicals or cross-linking antibodies to initiate T cell activation and subsequent IL-2 production and proliferation. Although there is no doubt that such agents are potent activators of T lymphocytes, it is unclear whether such stimuli accurately mimic antigen-induced T cell activation. To our knowledge this study is the first demonstration that consumption of a diet rich in (n-3) PUFA from fish oil significantly reduces antigen-driven IL-2 production and proliferation of naive $CD4^+$ T lymphocytes. Importantly, we observed that feeding DO11.10 mice an (n-3) PUFA-enriched diet did not alter the frequency of naive $CD4^+$ T lymphocytes that were present in splenocyte preparations before antigen stimulation. This is consistent with previous reports from our laboratory as well as others (8,30). Thus, it seems unlikely that (n-3) PUFA-mediated reductions in antigen-specific responses of $CD4^+$ T cells were a consequence of reduced precursor frequency in our DO11.10 cell preparations.

Before the development of TCR transgenic mice, the only practical way to study antigen-specific immune responses was via recall responses. This was a consequence of the incredibly low precursor frequency of lymphocytes specific for any given antigen in a naive host (i.e., estimated to be <1 in 10^5 lymphocytes). Immunization of the naive host causes the rapid expansion of antigen-specific T cell clones, which enhances their frequency substantially (20). The presence and function of antigen-specific T cells could be studied after such in vivo or in vitro priming. However, primed T cells no longer have a naive phenotype, but differentiate into effector and memory cells. In contrast, TCR transgenic mice provide researchers access to unlimited numbers of naive, antigen-specific T cells. However, the absence of clonal diversity in the T cell population is one shortcoming of using TCR transgenic mice. The diversity of binding affinities may be important in determining the subsequent response of various T cells that would respond to any given antigen in normal mice (31). Despite this possible shortcoming we contend that our data with T cells from DO11.10 mice are representative of T cells from nontransgenic mice. Specifically, Harbige and Fisher (32) showed that feeding mice an (n-3) PUFA-rich diet significantly diminished OVA-specific lymphocyte proliferation in response to restimulation ex vivo with antigen. In their study BALB/c mice were fed a low fat, commercial mouse diet to which 10 g/kg fish oil or borage oil had been added. After 21 d, mice were immunized with OVA in Freund's complete adjuvant. After 7 d, splenocytes were isolated and restimulated with OVA ex vivo. Lymphocyte proliferation (reported as stimulation index) was 50% lower in fish oil-fed mice compared with mice fed the low fat, low (n-3) PUFA control diet.

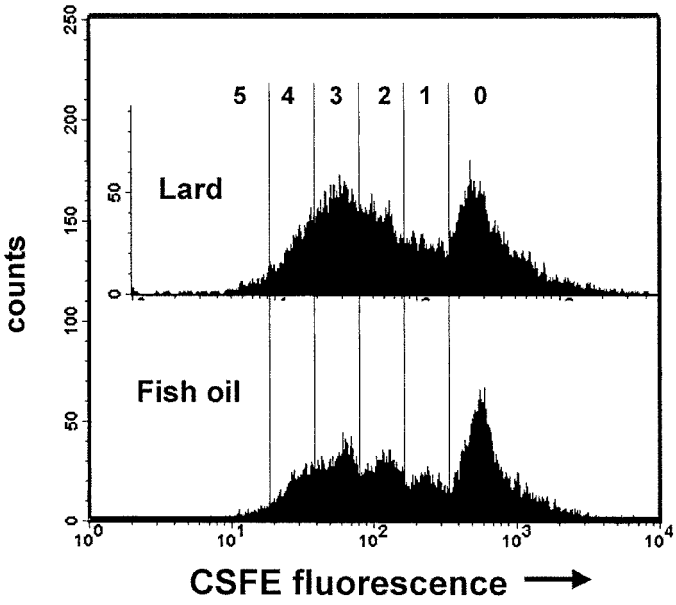

FIGURE 4 Effect of dietary (n-3) polyunsaturated fatty acid (PUFA) on antigen-driven clonal expansion of $CD4^+$ T cells in mice fed lard or fish oil. Splenocytes from DO11.10 mice were isolated, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultured in the presence of 2-mercaptoethanol (2-ME) and 5% homologous serums, then stimulated with ovalbumin $(OVA)_{323–339}$ peptide at one of three concentrations (0.02, 0.05 and 0.2 μmol/L). After 72 h, asynchronous cell division using CFSE fluorescence was measured by flow cytometry. Data are gated to show CFSE staining on viable CD4 and KJ1-26 dual-positive cells. The numbers at the top of the histogram represent the relative number of divisions cells have undergone. The histograms represent cells pooled from three DO11.10 mice fed lard (upper histogram) or fish oil (lower histogram) stimulated with 0.2 μmol/L OVA peptide. Similar results were observed at other concentrations of OVA.

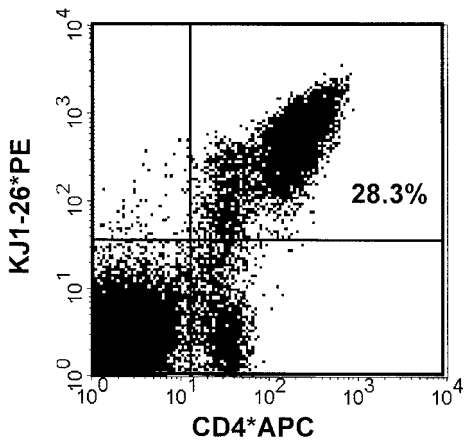

FIGURE 3 A representative two-parameter flow cytometric scattergram of splenocytes from DO11.10 mice fed lard or fish oil. Cells were isolated, labeled with mAb against murine CD4 and the DO11.10 transgenic T cell receptor (TCR) (i.e., KJ1-26), then analyzed by flow cytometry (FACSVantage; BD Biosciences, San Diego, CA). The gates were established based on background staining using isotype-matched nonspecific mAb. The number in the upper right quadrant represents the percentage of $CD4^+$ T cells that express the DO11.10 clonotypic TCR specific for the ovalbumin $(OVA)_{323–339}$ peptide relative to the total number of viable nucleated cells.

The most common approach for measuring antigen-specific T cell responses is the DTH test to recall antigens. In general, (n-3) PUFA intake is associated with diminished DTH recall responses (15–17). DTH reactions require the coordinated response of both antigen-specific $CD4^+$ T cells and monocyte/macrophages (33). The induration associated with a DTH test is a consequence of the in situ responses of several different cell types and numerous humoral factors, including proinflammatory cytokines and chemokines (34). Although the DTH test is a valuable in vivo measure of immune responsiveness, it provides only an indirect measure of dietary (n-3) PUFA modulation of lymphocyte function. Thus, we believe our study is the first to directly examine (n-3) PUFA on antigen-specific T cell activation and subsequent responses of naive lymphocytes.

In contrast to these recall studies just described, Byleveld et al. (35) recently reported that feeding mice a high (n-3) PUFA diet enhanced in vitro lymphocyte proliferation. In this study mice were immunized against influenza virus, then proliferation was measured in vitro after exposure to the live virus. The (n-3) PUFA effect was seen with lymphocytes isolated from the bronchial lymph nodes 5 d, but not 12 d, after an intranasal challenge with influenza virus. Similar results were seen with splenic lymphocytes from mice challenged intraperitoneally with influenza virus 7 d before cell isolation. In this case, the (n-3) PUFA enhancement of in vitro lymphocyte proliferation was noted for viral (i.e., antigen)-driven as well as for Con A-driven T cell activation. (n-3) PUFA did not affect ex vivo viral-specific proliferation from nonimmunized mice. Because host responses to viruses are dependent upon $CD8^+$ T cell responses, it is tempting to speculate that these data, in conjunction with our data, suggest that (n-3) PUFA may alter $CD4^+$ and $CD8^+$ T cell responses differently. In support of such a hypothesis, Arrington et al. (36) reported that proliferation of spleen-derived mouse $CD8^+$, but not $CD4^+$, T cells was reduced by dietary (n-3) PUFA. In this study the authors used purified T cell populations and various polyclonal stimuli. That they observed (n-3) PUFA effects with only certain stimuli and not others raises several unanswered questions. We cannot explain why they found no effect of (n-3) PUFA on $CD4^+$ T cell proliferation, while we found a significant impairment. In short, these findings along with our data suggest that further studies are warranted to better define the impact of (n-3) PUFA on antigen-specific responses of $CD4^+$ and $CD8^+$ T cells.

Our results with the polyclonal T cell activator Con A were not consistent with our findings with OVA-specific responses or with the findings of some other researchers. For example, Jolly et al. (8) reported that feeding C57BL/6 mice a diet containing 1% of either EPA or DHA as ethyl esters resulted in a substantial reduction in splenocyte Con A-stimulated proliferation (75–80% reduction) and IL-2 production (50% reduction). However, several other researchers have failed to find a significant reduction in Con A-stimulated proliferation in association with the consumption of experimental diets or supplements high in (n-3) PUFA (19,36–38). One possible explanation for the discrepancy between (n-3) PUFA effects on antigen-specific and mitogen-induced IL-2 production and lymphocyte proliferation is that Con A stimulates $CD4^+$ and $CD8^+$ T lymphocytes, whereas in our system the OVA peptide selectively stimulated $CD4^+$ T cells. Furthermore, many other possible explanations have been offered to account for the apparent contradictory findings, including differences in test subjects, the amount and length of (n-3) PUFA feeding, the nature and concentration of immune stimuli, as well as variations in the cell culture media. To a limited extent we addressed the latter two variables and we contend that our data make a contribution to this research area.

In this study we showed how culture conditions affect dietary (n-3) PUFA modulation of lymphocyte response in vitro. Others have reported that lipoproteins in murine serum, such as HDL and LDL, have a suppressive effect on in vitro lymphocyte proliferation (39,40). However, lipoproteins also are a major source of diet-derived fatty acids for cells. Immune cells from (n-3) PUFA-fed subjects lose a substantial portion of these fatty acids during prolonged in vitro culturing (41,42). The inclusion of autologous or homologous serums helps maintain the fatty acid profiles of cells. In this study our data illustrate the importance of using homologous mouse serums in the cell culture medium. In this regard our findings are consistent with reports by others (41,43).

Another example that culture conditions can affect in vitro lymphocyte responses was clearly demonstrated with 2-ME. Others have reported that the inclusion of reducing agents (e.g., 2-ME, glutathione, cysteine) in culture media enhances a number of in vitro immune cell responses, including T cell proliferation (44–46). Our data showing that exclusion of 2-ME from our culture medium dramatically impaired in vitro lymphocyte proliferation are consistent with these previous reports. We believe our data showing that diet (n-3) PUFA-mediated reduction of antigen-driven $CD4^+$ T cell proliferation in the presence or absence of 2-ME suggest that enhanced lipid peroxidation was not important in determining how (n-3) PUFA were altering T cell responses to antigen in our system. However, unlike antigen-driven proliferation, 2-ME did affect whether we observed an effect of (n-3) PUFA on lymphocyte proliferation induced by Con A. In the presence of 2-ME there was no (n-3) PUFA effect, but without 2-ME we noted a significantly lower response in (n-3) PUFA-enriched DO11.10 cells stimulated at some, but not at all, Con A concentrations tested. Our results in the absence of 2-ME are similar to some of the reports in the literature that showed (n-3) PUFA reducing in vitro lymphocyte proliferation in response to Con A stimulation. Thus, we contend that much of the variability in the (n-3) PUFA lymphocyte proliferation literature may be explained by variations in the cell culture conditions.

The extent of antigen-driven lymphocyte proliferation is generally thought to be proportional to the number of molecules of MHC class II expressed on antigen-presenting cells (APC) (47). One possible mechanism by which (n-3) PUFA may reduce antigen-stimulated lymphocyte proliferation in our study is by reducing the expression of MHC class II on the surface of splenic APC, and thus diminishing OVA peptide presentation to naive $CD4^+$ T cells. We and others have demonstrated that (n-3) PUFA can reduce MHC class II expression on APC, such as macrophages and dendritic cells (30,48–50). However, we have also previously shown that dietary (n-3) PUFA do not affect MHC class II expression on murine splenic APC. Therefore, we do not believe that the diminished lymphocyte proliferation observed in this study is a consequence of reduced antigen presentation. Further support for this conclusion was provided by Fowler et al. (9). Using macrophage-lymphocyte cocultures, they showed that (n-3) PUFA affect T cell proliferation independent of APC source.

Finally, we used a cell-tracking dye, CFSE, to conduct generational analysis of antigen-stimulated lymphocytes. This

approach can been used to follow up to eight successive generations of cell division (24). We found that most of the TCR transgenic $CD4^+$ T cells had undergone two to three cell divisions by 72 h after in vitro OVA stimulation. Furthermore, $CD4^+$ TCR transgenic T cells from the mice fed the (n-3) PUFA diet had a pattern of cell divisions similar to cells from lard-fed mice. However, not all TCR transgenic $CD4^+$ T cells responded to antigen stimulation. In fact, ~25% of the cells failed to divide even at the highest concentration of antigen tested. The existence of this nonproliferating subpopulation has been observed by others (51). These findings suggest that (n-3) PUFA do not change the normal cell program that controls progression through numerous cell divisions after antigen stimulation.

In conclusion, we have demonstrated for the first time that feeding mice a diet enriched with (n-3) PUFA from fish oil is associated with a significant reduction in antigen-driven IL-2 production and lymphocyte proliferation by naive $CD4^+$ T lymphocytes in vitro.

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