Dietary (n-3) Polyunsaturated Fatty Acids Modulate Murine Th1/Th2 Balance toward the Th2 Pole by Suppression of Th1 Development[1,2]

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ABSTRACT We showed that dietary long-chain (n-3) PUFAs present in fish oil (FO) affect $CD4^+$ T cell proliferation and cytokine production in C57BL/6 mice. To test the hypothesis that the anti-inflammatory effect of dietary (n-3) PUFAs could be due to the indirect suppression of T helper (Th)1 cells by cross-regulation of enhanced Th2 activation, mice were fed a wash-out control diet [5% corn oil (CO), (n-6) PUFA] for 1 wk, followed by the control diet or a fish oil diet [1% CO + 4% FO, (n-3) PUFA] for 2 wk. Splenic $CD4^+$ T cells were cultured under both neutral and Th2 polarizing conditions for 2 d. Cells were reactivated and analyzed for interleukin-4 and interferon-γ by intracellular cytokine staining. Dietary fish oil significantly increased the percentage of Th2 polarized cells and suppressed Th1 cell frequency under neutral conditions. However, under Th2 polarizing conditions, although the suppression of Th1 cells was maintained in FO-fed mice, no effect was observed in Th2 cells. Dietary fish oil increased the Th2/Th1 ratio in the presence of homologous mouse serum under both neutral ($P = 0.0009$) and Th2 polarizing conditions ($P = 0.0185$). The FO diet did not significantly affect proliferation under Th2 polarizing conditions. Thus, the anti-inflammatory effects of FO may be explained in part by a shift in the Th1/Th2 balance, due to the direct suppression of Th1 development, and not by enhancement of the propensity of $CD4^+$ T cells to be polarized toward a Th2 phenotype, at least in vitro. J. Nutr. 135: 1745–1751, 2005.

KEY WORDS: • *(n-3) fatty acids* • *fish oil* • *Th1/Th2 balance*

Two polarized $CD4^+$ T cell subsets have been identified by their signature cytokines and their mutually exclusive helper functions. T-helper 1 (Th1)[4] effector cells produce interleukin (IL)-2, interferon-γ (IFN-γ), and lymphotoxin; Th2 cells produce IL-4, IL-5, IL-10, and IL-13 (1). The pathogenic role of Th1 and the protective role of Th2 cells have been described for certain autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), and insulin-dependent diabetes mellitus (2). In some cases, the Th1/Th2 balance was an important indicator of the disease state (3). A shift from Th1 to Th2 cytokine profiles was observed in many clinical interventions that resulted in improvement in these diseases (4,5). Modulation of the Th1/Th2 balance has provided a new paradigm for immunomodulatory therapy in some autoimmune diseases (2).

The beneficial effects of dietary fish oil (FO) on Th1-mediated diseases have been well documented (6,7). The principal bioactive constituents of FO are considered to be eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Epidemiologic studies showed decreased incidence of autoimmune/inflammatory diseases in Greenland Eskimos whose diets are rich in EPA and DHA from fish (8). Clinical dietary intervention trials also showed a protective effect of FO in a number of Th1-mediated autoimmune diseases such as RA, Crohn's disease, and ulcerative colitis (7). A shift away from a Th1 response was considered to be the mechanism that resulted in the benefits of FO (9).

The anti-inflammatory effect of (n-3) PUFAs in FO may be attributed in part to direct suppression of T-cell function (10). FO was shown to inhibit T cell proliferation, IL-2 secretion, IL-2 receptor expression, and CD28 function (11–13). To date, the effects of dietary (n-3) PUFAs on individual Th cell subsets has not been determined. Very limited data exist on how FO affects Th1 and Th2 cytokines (3,14–16). Wallace et al. (9) reported decreased IFN-γ in splenocytes from FO-fed mice. Suppressed IFN-γ gene expression was found in the Peyer's patches in FO-fed BB rats (3). In human MS patients, FO supplementation resulted in reduced IFN-γ secretion by

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[4] Abbreviations used: CO, corn oil; Con A, concanavalin A; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FO, fish oil; HMS, homologous mouse serum; IFN-γ, interferon-γ; IL, interleukin; JNK, c-Jun NH_2-terminal kinase; LAT, linker for activation of T cells; MBP, myelin basic protein; MS, multiple sclerosis; NFAT, nuclear factor of activated T cells; PE, phycoerythrin; PMA, phorbol myristate acetate; RA, rheumatoid arthritis; Th1/2, T-helper 1/2.

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blood lymphocytes (15). In contrast to these studies, no effect on IFN-γ production by murine spleen lymphocytes was seen in mice fed a diet enriched in (n-3) PUFAs (14). FO had little effect on IL-4 secretion or gene expression in spleens from FO-fed mice (9). Although FO feeding increased IL-10 gene expression in Peyer's patches in BB rats (3), FO supplements for pregnant women resulted in decreased neonatal IL-10 secretion (16). These conflicting results could be explained by important differences among the study parameters. First, different models were used. Cytokine production was examined in murine spleen lymphocytes, gut-associated immune cells, or human peripheral lymphocytes. Second, the cytokines were examined after a short period of stimulation. It was shown that IL-4 expression depends on cell cycle and increases dramatically and acquires a stable phenotype only after 48 h in culture (17,18). Third, mixed cell populations were used, which means that the cytokine concentrations may not truly reflect the balance between Th1 and Th2 cells. To determine conclusively how dietary (n-3) PUFAs influence the Th1/Th2 balance, experiments using purified $CD4^+$ T cells that have been polarized into Th1/Th2 cells are necessary.

Previous data from our laboratory revealed that purified mouse $CD4^+$ T cells from C57BL/6 mice stimulated with different agonists displayed different cytokine profiles, corresponding to Th1-like and Th2-like cells (19). The increased proliferation of Th2-like cells and the suppressed IL-2 secretion of Th1-like cells from FO-fed mice suggested that the anti-inflammatory effects of dietary (n-3) PUFAs may be the combined result of direct suppression of IL-2–induced Th1 activation and indirect suppression of Th1 cells through cross-regulation of enhanced Th2 activation. There is ample evidence that diminished Th1 responses after therapy for some Th1-mediated autoimmune diseases are accompanied by enhanced Th2 responses (2–4). Therefore, in the current study, we tested the hypothesis that the anti-inflammatory effects of dietary fish oil are due in part to enhanced Th2 activation. Our results indicated that dietary (n-3) PUFAs alter the Th1/Th2 balance toward the Th2 pole by selectively suppressing Th1 development rather than by enhancing Th2 development.

MATERIALS AND METHODS

Mice and diets. All experimental procedures using laboratory animals were approved by the University Laboratory Animal Care Committee of Texas A&M University. Pathogen-free female young C57BL/6 mice (12–14 g) were purchased from the Frederick National Cancer Research Facility. Mice were housed 5/microisolator cage, and had free access to water and food. Mice were fed a control semipurified diet containing 5% corn oil (CO, DeGussa Bioactives) during the 7-d acclimation period followed by 2 wk of consumption of either the same control diet or a FO (Omega Protein) diet (1% CO + 4% FO). The diets met NRC nutrition requirements and varied only in lipid content. The concentrations of vitamin A and vitamin D of the 2 diets were approximately equal (20). The basic diet composition, expressed as g/100 g, was: casein, 20; sucrose, 42; cornstarch, 22; cellulose, 6; AIN-76 mineral mix, 3.5; AIN-76 vitamin mix, 1; DL-methionine, 0.3; choline chloride, 0.2; Tenox 20A (containing 32% glycerol, 30% corn oil, 20% *tert*-butyl-hydroquinone, 15% propylene glycol, 3% citric acid), 0.1; and oil, 5. The fatty acid composition of the diets, as determined by GC, is shown in **Table 1**.

Chemical compounds, cytokines and antibodies. Phorbol myristate acetate (PMA) was purchased from Sigma. Ionomycin was purchased from Calbiochem-Novabiochem. Recombinant murine IL-2 was purchased from R&D systems. Hamster anti-mouse CD3e monoclonal antibody (clone 145–2C11), neutralizing rat mAb for murine IFN-γ (clone R4–6A2), mouse Fc block™ (clone 2.4G2), R-phycoerythrin (PE)-labeled anti-IL-4 (clone 11B11), and fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ (clone XMG1.2) were purchased from BD PharMingen.

TABLE 1

Dietary fatty acid composition[1–3]

Fatty acid	CO	FO
	g/100 g fatty acids	
14:0	tr[4]	7.4
16:0	11.9	18.6
16:1(n-7)	tr	10.2
18:0	2.1	2.2
18:1(n-9)	30.1	13.3
18:2(n-6)	54.1	14.9
18:3(n-3)	1.1	2.0
20:5(n-3)	tr	8.1
22:5(n-3)	tr	1.5
22:6(n-3)	tr	8.5

[1] Mean of 2 analyses.
[2] CO, 5% corn oil; FO, 4% fish oil + 1% corn oil (wt:wt).
[3] Only the major fatty acids (>1 g/100 g) are listed.
[4] tr, trace amount (<0.1 g/100 g).

Cell culture. $CD4^+$ T cells were purified from the splenocytes of C57BL/6 mice by a previously published negative selection column method (19). Cells were adjusted to 5×10^9 cells/L and cultured in 24-well plates containing 2 mL complete RPMI medium [RPMI 1640 medium with 25 mmol/L HEPES (Irvine Scientific) supplemented with 5% fetal bovine serum (FBS), 1×10^5 U/L penicillin and 100 mg/L streptomycin (Irvine Scientific), 2 mmol/L L-glutamine, and 10 μmol/L 2-mercaptoethanol]. To drive Th2 development, cells were cultured for 48 h with plate bound anti-CD3 (10 mg/L) and PMA (0.5 μg/L) in the presence of IL-4 (10 μg/L), anti-IFN-γ (10 mg/L), and IL-2 (20 mg/L). For stimulation under neutral conditions, cells were cultured only with anti-CD3 (10 mg/L) and PMA (0.5 μg/L). Select cultures were incubated with homologous mouse serum (HMS; complete RPMI 1640 with 2.5% FBS and 2.5% HMS). The HMS was collected according to the method described by Pompos et al. (21) and was included to preserve the diet-induced changes in the lipid composition of cell membranes during the in vitro culture period, as published previously (22).

Proliferation assay. Purified $CD4^+$ T cells were cultured at 2×10^5 cells/well in 96-well round-bottomed microtiter plates (Falcon, Becton-Dickenson). Cells were cultured under Th2 polarizing conditions (described above) for 48 h at 37°C with 5% CO_2. For the final 6 h, 1.0 μCi [^{3}H]-thymidine was added to the cultures. Cells were harvested and counted as described previously (19).

Intracellular cytokine staining. Intracellular cytokine staining was quantified as described previously (22) with modifications. After 48 h of incubation, live cells were separated by Lympholyte-M and reactivated (10^9 cells/L) with PMA (10 μg/L) and Ionomycin (1 mg/L) for 5 h at 37°C. GolgiStop (BD PharMingen), a protein transport inhibitor, was added during the last 2 h of incubation at the concentration recommended by the manufacturer. Cells were subsequently collected, stained with mouse Fc block™ (BD PharMingen), fixed, and permeabilized for 20 min at 4°C in 250 μL Perm/Fix (BD PharMingen). Cells were washed in Perm/Wash (BD PharMingen) followed by staining with PE-labeled mAb to murine IL-4 and FITC-labeled mAb to murine IFN-γ. After a final wash in Perm/Wash, cells were resuspended in 500 μL staining buffer (PBS with 0.05% fetal calf serum and 0.01% sodium azide) for analysis on a fluorescence-activated cell sorting (FACS)-Calibur flow cytometer (Becton Dickinson Immunocytometry systems). In preliminary studies, isotype controls for the anti-IL-4-PE and anti-IFN-γ were performed. These samples stained minimally (IL-4 < 0.1%, IFN-γ < 0.2%) and were similar to unstained samples. Thus, in the dietary study, isotype controls were omitted and unstained samples were used to set the quadrant.

Statistical analysis. Significance of main treatment effects was assessed using PROC General Linear Model in SAS. Data were analyzed using one-way ANOVA and Tukey's test. A 95% level of probability was accepted as being significant. Results are from 1 to 3 separate experiments.

RESULTS

Dietary fish oil alters Th1/Th2 cytokine profiles. To determine conclusively how dietary (n-3) PUFAs affect Th2 polarization, purified splenic $CD4^+$ T cells from FO- and CO-fed mice were cultured with anti-CD3 and PMA in the presence of IL-4, IL-2, and anti-IFN-γ with FBS or HMS. Our previous data showed that $CD4^+$ T cells cultured with anti-CD3 and PMA produce both IL-4 and IFN-γ (19); therefore, we chose these stimuli as neutral conditions for the induction of cytokines. The immunofluorescent detection of IFN-γ and IL-4 by intracellular cytokine staining has proven to be the appropriate method for characterizing polarized Th1 and Th2 cell populations and has been validated in numerous publications (23–25). In the representative FACS images of cytokine profiles obtained by intracellular cytokine staining of polarized cells from FO- or CO-fed mice cultured with HMS (**Fig. 1**), Th2 cells were defined as cells producing IL-4, but not IFN-γ, and are represented by the $IL\text{-}4^+$, $IFN\text{-}\gamma^-$ population in the upper left quadrant of each dot plot. Th1 cells were defined as cells producing IFN-γ, but not IL-4, and are represented by the $IFN\text{-}\gamma^+$, $IL\text{-}4^-$ cells in the lower right quadrant of each dot plot.

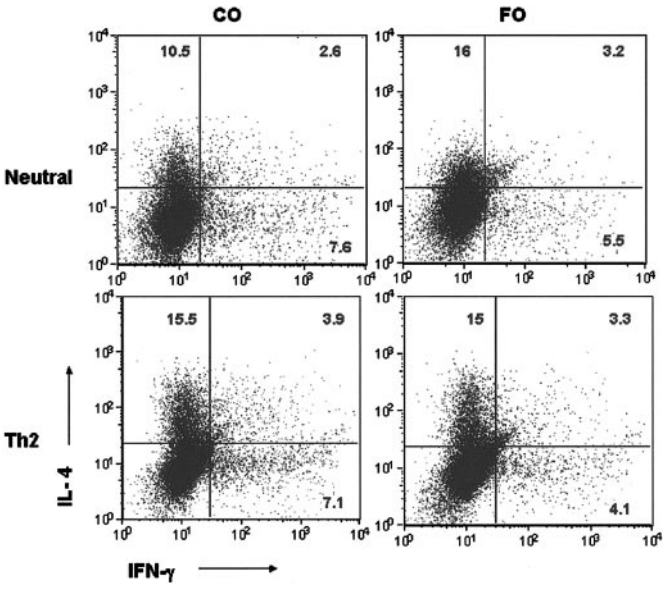

FIGURE 1 Representative FACS images of cytokine profiles obtained by intracellular cytokine staining of polarized $CD4^+$ T cells from C57BL/6 mice fed CO or FO diets cultured under either neutral or Th2 polarizing conditions. Splenic $CD4^+$ T cells were purified from mice fed test diets and cultured with anti-CD3 and PMA (neutral conditions) or with anti-CD3, PMA, IL-4, anti-IFN-γ, and IL-2 (Th2 polarizing conditions) for 48 h in the presence of HMS. Living cells were isolated and cytokines were determined by intracellular cytokine staining. The number in the upper left quadrant of each dot plot represents the percentage of Th2 cells ($IL\text{-}4^+$, $IFN\text{-}\gamma^-$) and values in the lower right quadrant of each dot plot represent Th1 cells ($IFN\text{-}\gamma^+$, $IL\text{-}4^-$). The isotype controls were tested in preliminary studies and did no differ from unstained samples (data not shown); thus, unstained samples were used to set the quadrant.

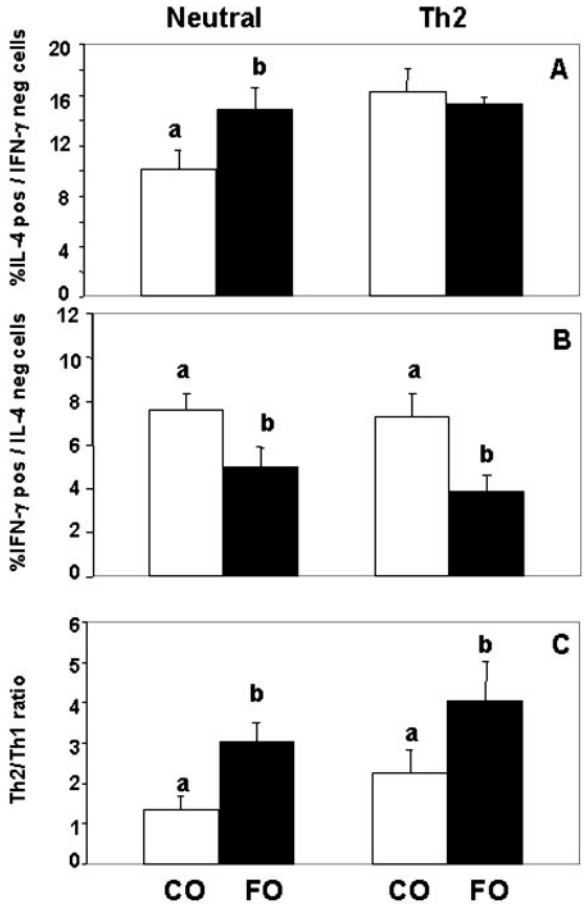

FIGURE 2 Dietary FO shifts the Th1/Th2 balance by suppressing Th1 development in C57BL/6 mice. Purified $CD4^+$ T cells from CO- or FO-fed mice were cultured under either neutral or Th2 polarizing conditions in the presence of HMS for 48 h as described in the Materials and Methods. Cytokine production was assessed by intracellular cytokine staining. Panels represent $IL\text{-}4^+$ cell frequency (*A*) and $IFN\text{-}\gamma^+$ cell frequency (*B*) and the Th2:Th1 ratio (*C*). Values are means ± SEM, $n = 4$ replicates per diet group, and 5 mice were pooled per analysis. Values identified by different letters differ, $P < 0.05$.

Using the flow cytometry data for all mice, the mean percentages of Th1 and Th2 cells generated under neutral and Th2-polarizing conditions in the presence of HMS were calculated (**Fig. 2**). Under neutral conditions, dietary FO significantly increased the proportion of $IL\text{-}4^+$, $IFN\text{-}\gamma^-$ Th2 cells by 46% (14.9 ± 1.6% for FO-fed mice vs. 10.2 ± 1.4% for CO-fed mice, $P = 0.0049$). After culture under Th2 polarizing conditions, the proportion of Th2 cells did not differ between the FO (15.3 ± 0.5%) and CO (16.2 ± 1.8%) groups (Fig. 2*A*).

Dietary FO significantly decreased the proportion of $IFN\text{-}\gamma^+$, $IL\text{-}4^-$ Th1 cells after culture with HMS under both neutral ($P = 0.0053$) and Th2 polarizing conditions ($P = 0.0017$) (Fig. 2*B*). Under neutral conditions, 7.6 ± 0.8% of the $CD4^+$ T cells of CO-fed mice were Th1 polarized, whereas 5.0 ± 0.9% of the $CD4^+$ T cells of FO-fed mice exhibited a Th1 phenotype. Similarly, under Th2 polarizing conditions, 7.3 ± 1.0% of the $CD4^+$ T cells of CO-fed mice were Th1 polarized compared with 3.9 ± 0.7% of the cells from FO-fed mice. Thus, dietary FO suppressed Th1 cell development after culture under both neutral and Th2 polarizing conditions in the presence of HMS.

Dietary FO significantly increased the ratio of Th2:Th1 cells after 48 h of culture in the presence of HMS under both neutral (3.0 ± 0.4 for FO vs. 1.4 ± 0.3 for CO, $P = 0.0009$) and Th2 polarizing conditions (4.1 ± 0.9 for FO vs. 2.3 ± 0.6 for CO, $P = 0.0185$) (Fig. 2C), indicating a clear shift in the Th1/Th2 balance toward a Th2 response in mice fed FO.

In the presence of FBS, the IFN-γ^+, IL-4$^-$ Th1 and IL-4$^+$, IFN-γ^- Th2 cell frequency did not differ between the groups after 48 h of culture under Th2 polarizing conditions. Similar percentages of Th2 cells (9.4 ± 1.2% for FO, 9.0 ± 0.8% for CO, $P > 0.05$, **Fig. 3***A*) and Th1 cells (6.5 ± 0.25% for FO, 6.5 ± 1.2% for CO, $P > 0.05$, Fig. 3*B*) were generated. Similarly, the Th2:Th1 ratio of CD4$^+$ T cells from mice fed FO (1.5 ± 0.4) and CO (1.3 ± 0.1) did not differ ($P > 0.05$) when HMS was not present in the cultures.

Dietary fish oil does not affect proliferation under Th2 polarizing conditions. The proliferative response of CD4$^+$ T cells under Th2 polarizing conditions, as indicated by the degree of [^{3}H]-thymidine uptake when cultured either in the presence of FBS (**Fig. 4***A*) or HMS (Fig. 4*B*), did not differ between the groups ($P > 0.05$). The overall proliferation was lower ($P < 0.0001$) in the presence of HMS compared with FBS in both diet groups.

DISCUSSION

The work from our laboratory in the past 10 years has demonstrated clearly that the anti-inflammatory effects of dietary fish oil are due, at least in part, to suppression of various T cell functions in C57BL/6 mice (10,12,13,19). Recently, we showed that CD4$^+$ T cells stimulated in vitro with anti-CD3/anti-CD28 displayed a Th1-like cytokine profile, whereas those stimulated with anti-CD3/PMA had a Th2-like cytokine profile (19). The observation that dietary FO suppressed IL-2 secretion of Th1-like cells and enhanced proliferation of Th2-like cells suggested that the anti-inflammatory effect of dietary (n-3) PUFAs could be the combined result of direct suppression of Th1 activation and indirect suppression of Th1 response by enhanced Th2 cross-regulation (19). Therefore, the main purpose of this study was to test the hypothesis that the anti-inflammatory effect of dietary (n-3) PUFAs was due to indirect Th1 suppression by enhanced Th2 activation. We polarized murine splenic CD4$^+$ T cells to Th2 cells in vitro using the same polarizing conditions as those reported by many investigators to study Th1/Th2 responses (26,27). The cytokines chosen for our study are among the strongest determinants of T cell differentiation. IL-4 induces Th2 development and reduces the Th1 response (28). IL-2 is also important for Th2 development (29). The common polarizing protocol employs anti-CD3/anti-CD28, or PMA/Ionomycin as the stimuli, plus recombinant (r)IL-2, rIL-4 and anti-IFN-γ for 2 or 3 d. The cells are then expanded for another 3 d (24,25,30). An incubation period of 48 h was also reported to be sufficient for naïve CD4$^+$ T cells to acquire the Th2 phenotype in vitro (18). Because we showed that anti-CD3/PMA promoted Th2 cytokine production (19), we chose these stimuli plus rIL-2, rIL-4, and anti-IFN-γ for Th2 polarization. The dietary effect

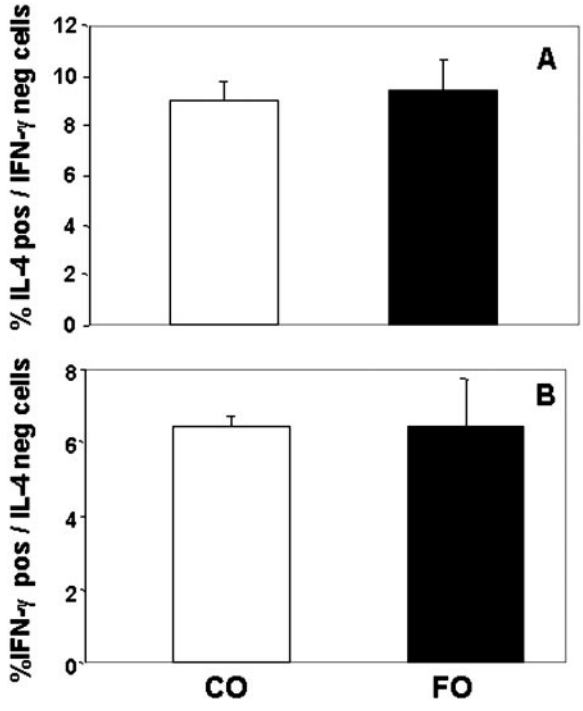

FIGURE 3 Dietary FO does not affect IL-4$^+$ (*Panel A*) or IFN-γ^+ (*Panel B*) CD4$^+$ T cell frequency under Th2 polarizing conditions in the presence of FBS in C57BL/6 mice. Splenic CD4$^+$ T cells were isolated from CO- or FO-fed mice and polarized to Th2 cells in the presence of FBS as described in the Materials and Methods. Cytokines were determined by intracellular cytokine staining. Values are means ± SEM, $n = 3$ replicates per diet group, and 5 mice were pooled per analysis. The experiments with FBS were repeated 3 times. A representative experiment is shown.

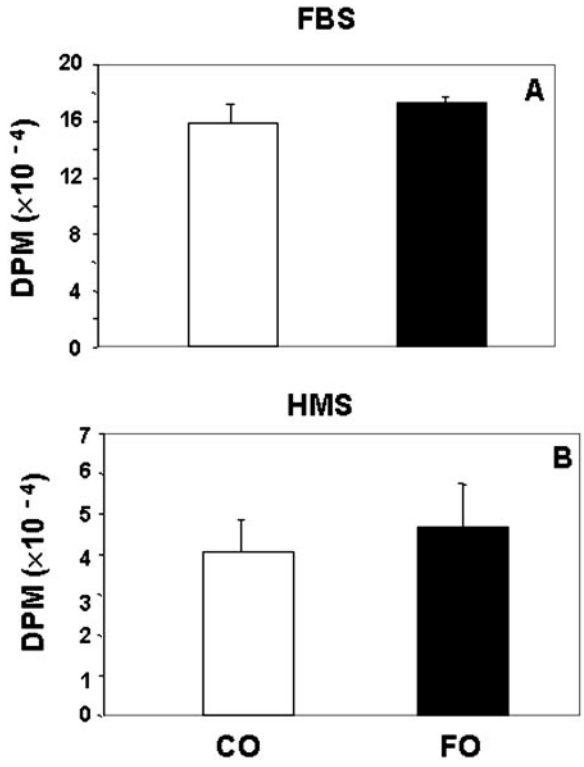

FIGURE 4 Lack of an effect of dietary FO on proliferation of CD4$^+$ T cells from C57BL/6 mice under Th2 polarizing conditions. Splenic CD4$^+$ T cells were isolated from CO- or FO-fed mice and cultured under Th2 polarizing conditions for 48 h in presence of either FBS (*Panel A*) or HMS (*Panel B*). Cell proliferation was measured by uptake of ^{3}H-thymidine during the last 6 h of culture. Values are means ± SEM of net thymidine uptake (DPM; $n = 4$ for HMS, $n = 3$ for FBS). Results are from 1 to 3 separate experiments.

of (n-3) PUFAs depends, at least in part, on diet-induced changes in membrane lipid composition, and long-term culture in fetal bovine serum resulted in loss of effect of dietary PUFAs on T-cell function (22). Therefore, we incorporated HMS and contracted the culture period. Among several polarizing protocols tested, we found that incubation with anti-CD3/PMA plus rIL-2, rIL-4, and anti-IFN-γ for 48 h gave the highest proportion of IL-4 positive cells. Expanding the culture for another 3 d did not increase the percentage of IL-4 positive $CD4^+$ T cells (data not shown). We elected not to include data on the mean fluorescence intensity of the IL-4– and IFN-γ–positive cells for 2 reasons. First, the question we addressed in this paper related to the effect of dietary fish oil on Th2 development in vitro. Therefore, we determined the percentage of IL-4 and IFN-γ positive cells that represents the Th2 and Th1 cell phenotype, respectively. Second, most papers examining Th1/Th2 development report only the percentage of positive cells (24,25). Although the intracellular cytokine staining method provides information on cytokine synthesis at the single-cell level, these data are relatively qualitative in nature. Therefore, we elected not to compare the cytokine level on a per cell basis.

In vitro Th2 polarization is always more difficult than Th1 polarization. The majority of $CD4^+$ T cells do not polarize under Th2 polarizing conditions regardless of the background of the mice or the culture conditions (23). Genetic background is one determinant of Th1/Th2 development (2,31) and C57BL/6 mice are Th1-prone (27). This may explain why <20% of the total cell population in our study was classified as IL-4^+, IFN-γ^- under Th2 polarizing conditions, and a small proportion of Th1 cells was also generated. Although repeated in vitro stimulation also seemed to help enhance the yield of Th2 cells, IL-4–producing cells were always detected at lower frequency in established Th2 clones or short-term Th2 polarized $CD4^+$ T cells perhaps due to transient IL-4 synthesis (23). The proportion of Th2 cells generated in our culture was within the typical range found in the literature and comparable to reported IL-4 positive cells under Th2 polarizing conditions from mice of a similar background (32). For example, naïve $CD4^+$ T cells from C57BL/6 mice cultured with anti-CD3 and anti-CD28, in the presence of similar concentrations of IL-4, anti-IFN-γ, and IL-2 for 1 wk, resulted in 34% Th2 cells (30). Only 9% Th2 cells were generated after stimulation with the Ac1–11 peptide from myelin basic protein (MBP) together with twice the IL-4 and similar anti-IFN-γ concentrations in splenocytes from Th1-prone B10.PL MBP T-cell receptor transgenic mice (31,32). In transgenic DO11.10 mice on the Th2-prone BALB/c background, only 14% Th2 cells were induced when naïve $CD4^+$ T cells were stimulated with ovalbumin peptide and antigen-presenting cells in the presence of IL-4 for 1 wk (23). Even in established Th2 clones, synthesis of intracellular IL-4 could be detected in only ~40% of cells (23).

The success of Th2 polarization was manifested by an increased Th2:Th1 ratio compared with culture under neutral conditions for $CD4^+$ T cells from mice fed both diets. In the FO group, there was a modest increase in the absolute frequency of IL-4–producing Th2 cells and a small decrease in IFN-γ–producing Th1 cells under Th2 polarizing conditions compared with neutral conditions. The changes resulted in a 33% increase in the Th2:Th1 ratio. For the CO group, the increase of Th2 cells was more pronounced under Th2 polarizing conditions, with little change in the number of Th1 cells. This resulted in a significant 66% increase ($P = 0.035$) in the Th2:Th1 ratio in FO-fed mice (Fig. 2C).

We demonstrated that dietary (n-3) PUFAs do not affect either in vitro Th2 development or proliferation under Th2 polarizing conditions. However, dietary (n-3) PUFAs did increase the Th2:Th1 ratio by suppression of Th1 development in the presence of HMS. Splenic $CD4^+$ T cells from FO-fed mice exhibited decreased Th1 differentiation after culture under both neutral and Th2 polarizing conditions (Fig. 2*B*). Under neutral conditions, the simultaneous suppression of Th1 cells and enhancement of Th2 cells in FO-fed mice could be explained by the cross-regulation of Th1 and Th2 cells (33). It is possible that FO acts on Th1 cells, or Th2 cells, or both. Under Th2 conditions, although FO maintained the suppression of Th1 development, there was no difference in Th2 differentiation between FO and CO. Because cross-regulation of Th2 cells by Th1 cells was diminished in the presence of Th2 polarizing conditions, i.e., IL-4 and anti-IFN-γ, the cells were pushed toward the Th2 pole to their maximum capacity. The similarity in the number of Th2 cells between FO and CO under polarizing conditions suggests the lack of a direct effect on Th2 development (Fig. 2*A*). Therefore, dietary (n-3) PUFAs do not appear to enhance Th2 polarization but do suppress Th1 cells, thus creating a shift in the Th1/Th2 balance (Fig. 2*C*).

Our findings confirm the observation of decreased IFN-γ production by T cells from mice fed diets enriched with FO or human patients taking FO supplements. Fish oil feeding inhibited concanavalin A (Con A)-stimulated IFN-γ production by whole splenocytes in C57BL/6 mice but had little effect on Con A-stimulated IL-4 production (9). Similar findings for IL-4 were observed in MF1 mice (14). In addition, fish oil supplements decreased IFN-γ production and IL-2 production by peripheral blood mononuclear cells harvested from MS patients (15). We showed previously that there was no difference in IL-4 secretion in $CD4^+$ T cells cultured with anti-CD3/PMA (19). In this study, dietary FO shifted the Th1/Th2 balance toward the Th2 pole by direct suppression of Th1 development, as evidenced by reduced IFN-γ production (Fig. 2*B* and 2*C*).

Suppression by dietary (n-3) PUFA of Th1 development in $CD4^+$ T cells cultured under Th2 polarizing conditions was observed only when T cells were cultured in the presence of HMS (Fig. 2*B*). When the cells were polarized in cultures with FBS, there was no difference in either Th2 or Th1 development from $CD4^+$ T cells from FO- and CO-fed mice (Fig. 3). The suppression of Th1 cells by FO feeding could be due to changes in lipid rafts. Our laboratory demonstrated that FO feeding modulates the lipid composition of raft microdomains in murine $CD4^+$ T lymphocytes (34). HMS in culture was shown in our experimental system to be essential to preserve the diet-induced in vivo changes in T-cell function and microdomain lipid composition (22). Considering that activated Th1 and Th2 cells have distinct patterns of membrane compartmentalization into lipid rafts (35), it is possible that dietary (n-3) PUFAs differentially modulate membrane microdomains in Th1 and Th2 cells and selectively inhibit recruitment of certain signaling molecules important for Th1 differentiation and activation. In support of this hypothesis, we showed that dietary DHA inhibits recruitment of protein kinase C-θ into lipid rafts in $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28, resulting in reduced nuclear factor-κB and activator protein-1 activation and IL-2 secretion (36). Differentiation of $CD4^+$ T cells into Th1 cells requires the activation of a mitogen-activated protein kinase, c-Jun NH_2-terminal kinase (JNK), whereas differentiation into Th2 cells does not require JNK2 activation (37). In addition,

nuclear factor of activated T cells (NFAT) promotes Th1 differentiation (38). Treatment of human Jurkat cells with EPA significantly inhibited JNK activity and NFAT (39). This may also occur in FO-fed mice and explain why dietary FO affects only Th1 differentiation. The selective inhibition of JNK and NFAT could be explained by modulation of rafts by PUFAs. The translocation of palmitoylated and acylated adapter protein linker for activation of T cells (LAT) was shown to be an important upstream event for NFAT activation (40). Treatment of human Jurkat cells with EPA resulted in the displacement of LAT from rafts (41). It is therefore possible that alteration in lipid raft composition could regulate JNK activation. Taken together, the data suggest that the suppression of Th1 development by FO feeding could be due to changes in the molecular interactions of lipids in rafts. These hypothetical mechanisms will be tested in future experiments.

The lower proliferation of Th2-polarized $CD4^+$ T cells observed in the presence of HMS but not FBS (Fig. 4) is consistent with previous findings (20). We previously observed enhanced proliferation in Th2-like $CD4^+$ T cells from FO-fed mice (19), which was not recapitulated in this study. This may be attributable to differences in stimuli and culture conditions. Because a heterogeneous $CD4^+$ T cell population (Th1, Th2, and unpolarized) existed in the present studies, the proliferation measured represented the sum of all cells present. Only experiments using an isolated Th2 population will provide a definitive conclusion about the precise effect of diet on Th2 proliferation. These results differ from the well-documented suppression of lymphocyte proliferation by FO observed by us and others (10,11). However, considering that FO affected only Th1 development, it is possible that the reported suppression of lymphocyte proliferation was observed in cell cultures in which Th1 cells dominated.

In summary, we found that dietary (n-3) PUFAs shift the Th1/Th2 balance toward the Th2 pole by suppression of Th1 development rather than enhancement of Th2 development. The results here are directly relevant to the therapeutic use of dietary FO in many Th1-mediated autoimmune and hypersensitivity diseases. Fish oil has the capacity to suppress Th1 development and reduce IFN-γ production without a concomitant effect on Th2 development. This may be beneficial because (n-3) PUFAs will not affect Th2-related antibody production, which may be important for resistance against some infections (2). Although Th2 cells were initially thought to be protective in some autoimmune diseases, subsequent research showed that a deviation in the Th1 to Th2 effectors population might result in more complications in many Th1-mediated diseases (42–44). Therefore, the lack of a direct effect on Th2 cells has advantages for FO as an adjunct therapy for Th1-mediated autoimmune diseases.

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