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Dietary Docosahexaenoic Acid Suppresses T Cell Protein Kinase Cθ Lipid Raft Recruitment and IL-2 Production[1]

Yang-Yi Fan,* Lan H. Ly,* Rola Barhoumi,†‡ David N. McMurray,*‡§ and Robert S. Chapkin[2]*‡

To date, the proximal molecular targets through which dietary *n*-3 polyunsaturated fatty acids (PUFA) suppress the inflammatory process have not been elucidated. Because cholesterol and sphingolipid-enriched rafts have been proposed as platforms for compartmentalizing dynamically regulated signaling assemblies at the plasma membrane, we determined the in vivo effects of fish oil and highly purified docosahexaenoic acid (DHA; 22:6*n*-3) on T cell microdomain lipid composition and the membrane subdomain distribution of signal-transducing molecules (protein kinase C (PKC)θ, linker for activation of T cells, and Fas/CD95), before and after stimulation. Mice were fed diets containing 5 g/100 g corn oil (control), 4 g/100 g fish oil (contains a mixture of *n*-3 PUFA) plus 1 g/100 g corn oil, or 4 g/100 g corn oil plus 1 g/100 g DHA ethyl ester for 14 days. Dietary *n*-3 PUFA were incorporated into splenic T cell lipid raft and soluble membrane phospholipids, resulting in a 30% reduction in raft sphingomyelin content. In addition, polyclonal activation-induced colocalization of PKCθ with lipid rafts was reduced by *n*-3 PUFA feeding. With respect to PKCθ effector pathway signaling, both AP-1 and NF-κB activation, IL-2 secretion, and lymphoproliferation were inhibited by fish oil feeding. Similar results were obtained when purified DHA was fed. These data demonstrate for the first time that dietary DHA alters T cell membrane microdomain composition and suppresses the PKCθ signaling axis. ***The Journal of Immunology,*** **2004, 173: 6151–6160.**

The anti-inflammatory properties of diets rich in *n*-3 polyunsaturated fatty acids (PUFA),[3] e.g., eicosapentaenoic acid (EPA; $20{:}5^{\Delta5,8,11,14,17}$) and docosahexaenoic acid (DHA; $22{:}6^{\Delta4,7,10,13,16,19}$), on T cell function have been established in both humans and experimental animals (1–9). In contrast, dietary lipids rich in *n*-6 PUFA, found in vegetable oils and animal fats, e.g., linoleic acid ($18{:}2^{\Delta9,12}$) and arachidonic acid ($20{:}4^{\Delta5,8,11,14}$), can be deleterious in some inflammatory diseases (6, 7). This is significant, because the typical Western diet contains 10–20 times more *n*-6 than *n*-3 PUFA (10). Although in some cases, *n*-3 PUFA-enriched diets do not alter human immune function (11), aspects of immune function that have an intimate involvement of the cell membrane, such as oxidative burst and proliferation, appear to be more strongly influenced by fatty acid composition (12). In addition, it has been demonstrated recently that the complex effects of *n*-3 PUFA on cytokine biology can be explained, in part, by polymorphisms/genotypes of the responsive subjects (9, 13). Collectively, these studies emphasize the need to elucidate the precise genetic and epigenetic determinants that influence the effects of foods enriched with *n*-3 PUFA on immune function. Unfortunately, to date, a cogent mechanistic hypothesis that explains the selective effect of *n*-3 PUFA on T cell function is lacking. Therefore, the goal of this study was to identify the molecular targets through which *n*-3 PUFA modulate T cell activation.

In general, increased intake of *n*-3 PUFA is associated with a reduced proinflammatory T cell response, including diminished proliferative capacity in response to mitogenic stimuli and impaired production of a critical T cell growth factor, IL-2 (14–16). We have demonstrated that a short-term feeding paradigm in mice with diets enriched with fish oil (containing EPA and DHA) or EPA and DHA (97% pure) ethyl esters results in suppressed Ag-specific delayed-hypersensitivity reactions and mitogen-induced proliferation of T cells (3, 16). The loss of proliferative activity was accompanied by reduction in IL-2 secretion and IL-2R α-chain mRNA transcription, suggesting that dietary EPA and DHA act, in part, by interrupting the autocrine IL-2 activation pathway (17). In addition, dietary EPA and DHA blunted the production of intracellular second messengers, including diacylglycerol (DAG) and ceramide, following mitogen stimulation ex vivo (18–20). These data indicate that dietary EPA and DHA modulate components of intracellular signaling pathways that regulate T cell activation.

Because dietary lipids are incorporated into T cell membranes (3, 18, 21), we initially investigated the effects of *n*-3 PUFA specifically on cholesterol and sphingolipid-rich plasma membrane microdomains (i.e., rafts) in mouse splenic T cells. A very novel and unexpected outcome of this study was the demonstration that dietary *n*-3 PUFA reduced (by ~45%) lipid raft sphingolipid content and altered raft fatty acid composition (22). These data are noteworthy because rafts compartmentalize the activated TCR and associated signal-transducing molecules upon T cell activation, thus providing an environment conducive to signal transduction (23–25). The earliest mediators of T cell proliferation (i.e., protein

*Molecular and Cell Biology Section, Faculty of Nutrition, †Image Analysis Laboratory, College of Veterinary Medicine, ‡Center for Environmental and Rural Health, Texas A&M University, and §Department of Medical Microbiology and Immunology, Texas A&M University Health Science Center, College Station, TX 77843

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[2] Address correspondence and reprint requests to Dr. Robert S. Chapkin, 442 Kleberg Center, 2471 TAMU, Texas A&M University, College Station, TX 77843-2471. E-mail address: r-chapkin@tamu.edu

[3] Abbreviations used in this paper: PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DAG, diacylglycerol; PKC, protein kinase C; LAT, linker for activation of T cells; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; CTx, cholera toxin; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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kinase C (PKC)θ, phospholipase Cγ, linker for activation of T cells (LAT)) and of T cell apoptosis (i.e., Fas and Fas ligand) translocate to lipid rafts after stimulation (26–30). These cellular responses are consistent with an emerging paradigm that lipid rafts cluster at the T cell:APC interface, ultimately generating platforms specialized for processive and sustained TCR signaling (31). Although the role of the immunological synapse with regard to T cell activation has recently been challenged and the contribution of protein-protein interaction appears critical for signaling (32), there is compelling evidence that lipid raft integrity is a prerequisite for optimized TCR signal transduction and immune response (31, 33–35). Interestingly, conditions that modify raft structure can disrupt these earliest steps of T cell activation (24, 29). In addition, PUFA enrichment selectively modified lipid rafts and suppressed signal transduction in a Jurkat T cell line model (36, 37). Therefore, we have hypothesized that dietary *n*-3 PUFA are unique because of their ability to modulate the composition of plasma membrane microdomains containing the TCR signaling complex. This, we argue, selectively alters TCR macromolecular complex signaling, resulting in the suppression of $CD4^+$ T cells.

The ability of dietary *n*-3 PUFA to influence membrane subdomains, i.e., liquid-ordered rafts (regions where TCR/CD3ζ, CTLA-4, LAT, PKCθ, and Fas reside), in relation to T cell subset activation in vivo has not been determined to date. Therefore, in this study, we examined 1) the in vivo effects of dietary fish oil (enriched in *n*-3 PUFA) on T cell microdomain lipid composition, and 2) the separate effects of fish oil and highly purified DHA ethyl ester on the membrane subdomain distribution of critical signal-transducing molecules (PKCθ, LAT, and Fas/CD95), in T cells before and after stimulation. We found that dietary *n*-3 PUFA suppressed the partitioning of PKCθ into lipid rafts, in association with a reduction in AP-1 and NF-κB activation, the down-regulation of IL-2 secretion, and the loss of normal proliferative responses in T cells upon activation.

Materials and Methods

Materials

RPMI 1640 medium and heat-inactivated FBS were obtained from Irvine Scientific (Santa Ana, CA). Lymphocyte-M was purchased from Cedarlane (Toronto, Ontario, Canada). T cell purification columns, goat anti-mouse Fas Ab, and Quantikine mouse IL-2 Immunoassay kits were obtained from R&D Systems (Minneapolis, MN). FITC-conjugated cholera toxin B subunit and poly-L-lysine solution were purchased from Sigma-Aldrich (St. Louis, MO). Alexa Fluor 568-conjugated rabbit anti-goat IgG, goat anti-rabbit IgG Abs, ProLong Antifade kit, and Amplex Red Cholesterol Assay kit were purchased from Molecular Probes (Eugene, OR). Rabbit anti-mouse PKCθ Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-LAT Ab was obtained from Upstate Biotechnology (Lake Placid, NY). Brij-58 was obtained from Fisher Scientific (Fair Lawn, NJ). Silica gel 60 G plates and all organic solvents were purchased from EM Science (Gibbstown, NJ). Fatty acid methyl ester standards were purchased from NuChek Prep (Elysian, MN). Mouse anti-CD3 and anti-CD28 Abs were purchased from BD Pharmingen (Los Angeles, CA). Nuclear Extract kit, Trans AM NF-κB p65 Chemi Transcription Factor Assay kit, and Trans AM AP-1 c-Jun Transcription Factor Assay kit were purchased from Active Motif (Carlsbad, CA). [^{3}H]Thymidine was purchased from New England Nuclear (North Bellerica, MA). Corn oil was obtained from Degussa BioActives (Champaign, IL). DHA ethyl ester (95% purity) was obtained from Martek Biosciences (Columbia, MD). Menhaden fish oil was provided by the National Institutes of Health (Fish Oil Test Material Program, Washington, DC).

Animals and diets

All experimental procedures using laboratory animals were approved by the University Laboratory Animal Care Committee of Texas A&M University. Three separate dietary studies were conducted. In studies I and II, pathogen-free female C57BL/6 mice (Frederick Research Facility, Frederick, MD), weighing 16–18 g, were randomly divided into two groups. For 2 wk, mice were given free access to one of the two semipurified diets that were adequate in all nutrients (38). Diets varied only in the oil composition: either corn oil (CO) or an (*n*-3) PUFA-enriched fish-corn oil (FO) mixture (4:1, w/w) at 5% of the diet by weight. In study III, mice were fed diets containing either 5% corn oil (CO) or a mixture of 95% pure DHA ethyl ester and corn oil (1:4, w/w) (DHA) for 2 wk. The basic diet composition for all studies, expressed as g/100 g, was as follows: 20 casein, 41.9 sucrose, 22 corn starch, 6 cellulose, 3.5 AIN-76 mineral mix, 1 AIN-76 vitamin mix, 0.3 DL-methionine, 0.2 choline chloride, 0.1 Tenox 20A (containing 32% glycerol, 30% corn oil, 20% *tert*-butylhydroquinone, 15% propylene glycol, 3% citric acid), and 5 dietary oil.

T cell purification

Following a 2-wk feeding period, mice were killed by CO_2 asphyxiation. T cells were isolated from spleens as described previously (14, 21). Briefly, spleens were homogenized in complete RPMI medium (RPMI 1640 with 25 mmol/L HEPES supplemented with 2.5% heat-inactivated FBS, 2.5% homologous mouse serum, 1×10^5 U/L penicillin, 100 mg/L streptomycin, 2 mmol/L L-glutamine, and 10 μmol/L 2-ME), followed by passage through a 149-μm wire mesh filter to create single-cell suspensions. Erythrocytes were removed by density gradient centrifugation over Lymphocyte-M. Total lymphocytes were loaded onto a negative-selection affinity column to purify T cells. $CD3^+$ T cells from study I were used for raft lipid analysis and colocalization measurements. $CD4^+$ T cells from studies II and III were used for transcription factor, cytokine, and proliferation assays.

Density gradient centrifugation and isolation of lipid rafts

Raft microdomains were isolated from mouse T cells as previous described (22, 39). Naive $CD3^+$ T cells from corn oil- and fish oil-fed mice were lysed in buffer (100 mmol/L NaCl, 2 mmol/L EDTA, 4.1 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.2 mmol/L Na_3VO_4, 50 μmol/L NaF, 25 mmol/L HEPES, 3.2 μmol/L aprotinin, 88 μmol/L leupeptin, 160 μmol/L bestain, 60 μmol/L pepstain A, and 56 μmol/L E-64 (pH 6.9)) supplemented with 1% Brij-58. Cell lysates were passed through a 27G needle once, followed by a 30-min incubation on ice. A solution containing 85% sucrose (w/v in lysis buffer) was added to the lysate and mixed by pipetting to generate a 45% sucrose lysate. Cell lysates were transferred to the bottom of a 2-ml polyallomer ultracentrifuge tube, which was subsequently overlaid with 35% sucrose and 5% sucrose, respectively. After centrifugation at 200,000 $\times$ *g* (Beckman Coulter (Fullerton, CA) Optima Max-E Ultracentrifuge, TLS 55 rotor) for 16 h at 4°C, aliquots from the top (liquid-ordered glycolipid-enriched raft fraction), and from the bottom (liquid-disordered soluble fraction) of the tube were collected for lipid analysis.

Sphingomyelin and cholesterol analysis

Total lipids in liquid-ordered membrane raft and liquid-disordered soluble fractions from corn oil- and fish oil-fed mice were extracted by the method of Folch et al. (40). Individual phospholipid classes were separated by one-dimensional TLC on silica gel 60 G plates using chloroform-methanol-acetic acid-water (50:37.5:3.5:2, v/v) as the developing solvent (22). Isolated sphingomyelin was spiked with 50 ng of heptadecanoic acid (C17:0) as an internal standard and transesterified in the presence of 6% methanolic HCl. Sphingolipid mass was determined by quantifying the mass of the *N*-acyl linked fatty acyl groups derived exclusively from the *sn*-2 position of sphingomyelin. Molar amounts of the fatty acid methyl esters were used to determine the molar levels of sphingomyelin as previously described (41, 42). Cholesterol levels in the total lipid extracts were subsequently measured. Briefly, aliquots of lipid extract were dried under nitrogen, redissolved in reaction buffer from the Amplex Red Cholesterol Assay kit (Molecular Probes), and processed according to the manufacturer's instructions (43).

Isolation of lipid raft glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE) subclasses

GPC and GPE, the two major phospholipids classes in cell membranes, were isolated from total lipids by TLC as described above. Each phospholipid class was initially hydrolyzed by phospholipase C treatment and converted to benzoate derivatives as previously described (44–46). The resultant diradylglycerobenzoates were extracted and separated on silica gel 60 G plates using benzene/hexane/diethyl ether (50:45:4, v/v/v) as the developing solvent (45). The alkenylacyl, alkylacyl, and diacylglycerobenzoates were extracted using ethanol/water/hexane (1:1:1, v/v/v) (44). Isolated GPC and GPE subclasses were transesterified in the presence of 6% methanolic HCl. Fatty acid methyl esters were subsequently analyzed by capillary gas chromatography as previously described (41).

Immunofluorescence and confocal microscopy

Naive CD3$^+$ T cells isolated from corn oil- or fish oil-fed mice were seeded onto poly-L-lysine-precoated coverglasses, followed by stimulation with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml), or left unstimulated in a 37°C incubator. At the end of the 30-min incubation period, cells were washed with PBS and immediately fixed in freshly prepared 4% paraformaldehyde for 20 min at room temperature (47, 48). Following fixation, adherent cells on Lab-Tek II chamber coverglass slides (Nalge Nunc International, Naperville, IL) were rinsed with PBS, and incubated with 10 mM glycine in PBS for 10 min at room temperature to quench the aldehyde groups (49). Samples were permeabilized using PBS/0.2% Triton X-100 for 5 min at room temperature, followed by PBS washing (39). Samples were then covered with blocking solution (1% BSA/0.1% NaN_3 in PBS) and incubated at 4°C overnight. In some cases, cells were incubated with IgG as a negative control. Slides were subsequently rinsed with PBS and incubated with FITC-cholera toxin (CTx) (10 μg/ml) and primary Abs (either rabbit anti-PKCθ, anti-LAT, or goat anti-Fas diluted in blocking solution (10 μg/ml)) at room temperature for 1 h in a dark, humidity-controlled chamber (39, 50, 51). Following incubation, samples were rinsed with PBS and blocking solution. Samples were then incubated with secondary Abs (either Alexa 568 goat anti-rabbit IgG or Alexa 568 rabbit anti-goat IgG diluted in blocking solution (10 μg/ml)) at room temperature for 1 h in a dark, humidity-controlled chamber. Subsequently, samples were washed with PBS, followed by a series of ethanol dehydration steps, and mounted onto glass slides with ProLong Antifade reagent (Molecular Probes). Slides were stored at −20°C in the dark until analysis by confocal microscopy. Fluorescence images were acquired at ×126 magnification on a Bio-Rad (Hercules, CA) Radiance 2000 MP confocal microscope. For FITC detection, an excitation wavelength was set to 488 nm and emission was collected with a 550LP dichroic mirror and 515/28-nm BP filter. For Alexa 568 detection, an excitation of 568 nm was used, and emission was collected using a 600-nm LP filter. Ten images, with a minimum of four cells per image, were collected per treatment group. Four animals per group were studied. For data analysis, regions of interest were selected by drawing polygons around each cell boundary. Colocalization of PKCθ, LAT, or Fas with the raft marker in each cell was quantified as percentage of colocalized pixel area (both green and red) over total raft pixel area (green) according to the algorithm provided with the Meridian Ultima work station (Meridian Instruments, Okemos, MI) (52). Colocalization values with different superscript were considered significantly different at $p < 0.05$.

Nuclear extraction and quantification of NF-κB and AP-1 activation

Splenic CD4$^+$ T cells isolated from mice fed corn oil, fish oil, or DHA-containing diets, were stimulated with anti-CD3/anti-CD28 for 24 and 48 h as previously described (16). Subsequently, nuclei were pelleted and extracted using a Nuclear Extract kit (Active Motif). The levels of active NF-κB and AP-1 were measured using an ELISA-based Trans AM NF-κB p65 and AP-1 c-Jun Transcription Factor Assay kit (Active Motif). For these assays, biotinylated double-stranded oligonucleotides containing either the NF-κB consensus site or 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element were precoated onto 96-well plates. Induction of NF-κB or AP-1 in the nuclear extracts was quantified by nucleotide (5′-GGGACTTTC-3′ for NF-κB, or 5′-TGA(C/G)TCA-3′ for AP-1) binding using specific Abs to NF-κBp65 or c-Jun followed by secondary HRP-conjugated Abs and chromogenic substrate (53, 54). A sensitive colorimetric readout was quantified by spectrophotometry at 450 nm with a reference wavelength of 655 nm. Competition experiments were performed by incubating the extracts with the labeled probe in the presence of 100-fold excess (20 pmol) of unlabeled wild-type NF-κB or AP-1 oligonucleotide. TPA and calcium ionophore-stimulated Jurkat cell extracts were used as a positive control, resulting in a 43-fold activation of NF-κB in comparison to the negative control (Jurkat cell extract plus NF-κB wild-type oligonucleotide competitor). Similarly, a 17-fold activation of AP-1 was observed in the positive control (TPA-stimulated K-562 nuclear extract) relative to the negative control (K-562 nuclear extract plus AP-1 wild-type oligonucleotide competitor).

Cytokine analysis

IL-2 secretion was measured in cell culture supernatants. Splenic CD4$^+$ T cells isolated from mice fed corn oil, fish oil, or DHA-containing diets, were stimulated using anti-CD3/anti-CD28 for 24 and 48 h. Culture medium was harvested and stored at −80°C until analysis by Quantikine mouse IL-2 ELISA kit (R&D Systems) as previously described (20).

Cell proliferation

Splenic CD4$^+$ T cells isolated from mice fed corn oil, fish oil, and or DHA-containing diets were cultured at the density of 2×10^5 cells/well in round-bottom 96-well plates. Cells were either unstimulated (basal) or stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) (stimulated) for 62 h, and then pulsed with [^{3}H]thymidine for an additional 6 h. Cells were then harvested onto glass fiber filter paper discs (Whatman, Maidstone, U.K.) using a multiple automated sample harvest unit (MASH II; MA Bioproducts, Walkersville, MD). Cellular uptake of [^{3}H]thymidine was measured using a liquid scintillation counter (LS 8000; Beckman Instruments, Irvine, CA).

Statistical analysis

Data were analyzed using one-way ANOVA and Duncan's New Multiple Range procedure for comparing treatment means. Differences of $p < 0.05$ were considered statistically significant.

Table I. *GPC subclass fatty acid composition in splenic T cell raft and soluble membrane fractions from corn oil- and fish oil-fed mice*[a]

	Raft		Soluble	
Fatty Acid[b]	CO	FO	CO	FO
Diacyl[c]				
16:0	12.98 ± 3.96	52.33 ± 8.00*	39.88 ± 4.22	37.00 ± 1.52
18:0	4.72 ± 1.55	14.12 ± 3.18*	10.82 ± 0.96	10.56 ± 0.91
18:1(*n*-7,9)	3.22 ± 1.64	5.32 ± 1.33	14.69 ± 1.19	17.44 ± 2.61
18:2(*n*-6)	1.04 ± 0.83	7.12 ± 3.98	8.70 ± 1.12	10.25 ± 2.70
20:4(*n*-6)	76.95 ± 7.80	17.45 ± 15.22*	19.93 ± 5.06	15.19 ± 3.40
20:5(*n*-3)	ND	ND	ND	0.84 ± 0.32*
22:5(*n*-3)	ND	ND	ND	1.52 ± 0.24*
22:6(*n*-3)	ND	ND	ND	1.91 ± 0.30*
Alkylacyl				
16:0	7.86 ± 1.26	60.70 ± 0.98*	41.24 ± 7.96	37.69 ± 0.54
18:0	1.90 ± 0.42	11.57 ± 1.11*	9.54 ± 1.58	9.98 ± 0.29
18:1(*n*-7,9)	1.04 ± 0.33	9.60 ± 3.24*	16.62 ± 1.01	14.14 ± 1.08
18:2(*n*-6)	1.21 ± 0.38	9.85 ± 1.88*	10.75 ± 2.51	7.16 ± 0.77
20:4(*n*-6)	87.50 ± 2.51	2.62 ± 1.39*	26.46 ± 5.00	16.67 ± 2.38
20:5(*n*-3)	ND	ND	ND	1.64 ± 0.22*
22:5(*n*-3)	ND	ND	ND	2.14 ± 0.58*
22:6(*n*-3)	ND	ND	1.31 ± 0.15	2.99 ± 0.37*

[a] Values represent mean ± SE (n = 3–4). Fifteen mice were pooled per analysis. *, Significantly different from CO in the same fraction/subclass, $p < 0.05$. Abbreviations used: ND, Not detectable; CO, corn oil diet; FO, fish oil diet.

[b] Only selected major fatty acids (>1 mol%) are reported.

[c] Results are expressed as moles per 100 mol of total fatty acids.

Results

Dietary n-*3 PUFA alter raft lipid fatty acyl composition*

An emerging theme in the concept of lipid rafts in T cell signaling is that exogenous lipids capable of altering membrane microdomain composition may influence signaling complexes and modulate cell activation (22, 36, 37). Therefore, we examined the fatty acyl composition in $CD3^+$ T cell raft and soluble membrane GPC and GPE phospholipids classes. In addition, because lipid rafts are enriched in plasmenylethanolamine, i.e., 1-*O*-alk-1′enyl-2 acyl GPE, particularly molecular species containing PUFA (55), we fractionated GPC and GPE into subclasses dependent upon the covalent linkage of the aliphatic side chain at the *sn*-1 position of the glycerol backbone. As shown in Tables I and II, dietary *n*-3 PUFA were effectively incorporated into liquid-disordered T cell-soluble membranes. The incorporation of EPA and DHA was quite extensive in all lipid fractions with one exception. EPA was undetectable in 1-*O*-alk-1′enyl-2 acyl GPE following fish oil feeding (Table II). With respect to lipid rafts, EPA and DHA were not incorporated into either diacyl or 1-*O*-alkyl-2 acyl GPC. In contrast, both diacyl and 1-*O*-alk-1′enyl-2 acyl GPE were enriched with *n*-3 PUFA (Table II). Upon examination of the choline-containing subclasses (Table I), it was noted that fish oil supplementation selectively reduced diacyl and 1-*O*-alkyl-2 acyl GPC arachidonic acid (20:4*n*-6) levels by 77 and 97%, respectively, in lipid rafts.

Dietary *n*-3 PUFA also altered the composition of a critical structural phospholipid that preferentially localizes to the plasma membrane. Specifically, raft sphingomyelin content was decreased significantly ($p < 0.05$) by 30% in T cells from mice fed fish oil (Fig. 1*A*). In contrast, no change in the raft or liquid-disordered soluble (bulk) membrane cholesterol levels were detected (Fig. 1*B*). Because cholesterol and sphingomyelin promote the formation of hydrophobic liquid-ordered molecular packing (56, 57), these data indicate that *n*-3 PUFA are incorporated into the plasma membrane bilayer and differentially alter the microenvironment of lipid rafts and bulk membranes.

Dietary n-*3 PUFA partially displace PKC*θ *from membrane rafts*

To determine whether the diet-induced perturbation of lipid raft composition (Fig. 1*A*; Tables I and II) is associated with a disruption in T cell function, we examined the ability of dietary fish oil to modify the plasma membrane partitioning of signaling proteins. For this purpose, splenic $CD3^+$ T cells isolated from mice fed diets enriched with either fish oil or corn oil were stimulated via the TCR/CD3 complex to investigate changes in microdomain localization. PKCθ, LAT, and Fas/CD95 were selected, because T cell activation and apoptosis require their translocation to rafts (27, 35, 58). PKCθ, LAT, and Fas/CD95 were homogeneously distributed at the plasma membrane in unstimulated cells; however, they became concentrated in distinct patches in a large proportion of stimulated cells. As shown in Fig. 2*A*, overlay images of green (GM1) and red (Alexa 568-PKCθ, LAT, or Fas) fluorescent signals show stimulation-induced clustering of membrane rafts and colocalization of PKCθ, LAT, and Fas with the raft marker. In contrast, when cells were incubated with IgG as a negative control, no patch formation was observed (data not shown). This is consistent with previous reports indicating that polyclonal stimulation is capable of inducing cell polarization and reorganization of the cytoskeleton in T cells (59). In support of our central hypothesis, the data indicate that the changes in membrane composition induced by *n*-3 PUFA feeding have functional consequences with respect to subdomain distribution of T cell signaling proteins. Specifically, dietary fish oil significantly ($p < 0.05$) suppressed the recruitment of PKCθ to lipid rafts in nonpolarized T cells following mitogenic stimulation (Fig. 2*B*). Interestingly, under basal conditions, Fas colocalization with GM-1, a lipid raft marker, was elevated significantly ($p < 0.05$) in T cells from fish oil-fed mice. In contrast, LAT colocalization was significantly ($p < 0.05$) reduced in naive cells from fish oil-fed mice compared with stimulated cells from fish oil-fed mice.

*Dietary DHA suppresses PKC*θ *effector pathways*

Because PKCθ and lipid rafts both integrate and amplify TCR/CD28 costimulatory signals, we determined whether dietary fish

Table II. *GPE subclass fatty acid composition in splenic T cell raft and soluble membrane fractions from corn oil- and fish oil-fed mice*[a]

	Raft		Soluble	
Fatty Acid[b]	CO	FO	CO	FO
Diacyl[c]				
16:0	45.31 ± 4.15	31.43 ± 8.09	31.10 ± 2.67	31.72 ± 2.63
18:0	15.04 ± 2.20	20.08 ± 2.74	23.28 ± 2.15	27.51 ± 1.94
18:1(*n*-9,7)	8.89 ± 2.12	7.64 ± 2.79	21.49 ± 6.45	11.45 ± 1.25
18:2(*n*-6)	7.67 ± 0.86	7.97 ± 3.83	5.49 ± 0.78	8.36 ± 1.51
20:4(*n*-6)	15.68 ± 3.81	25.19 ± 14.20	10.28 ± 4.23	10.87 ± 2.30
20:5(*n*-3)	ND	0.76 ± 0.08*	ND	1.43 ± 0.18*
22:5(*n*-3)	ND	0.93 ± 0.51*	ND	2.33 ± 0.36*
22:6(*n*-3)	ND	2.47 ± 0.64*	1.70 ± 1.01	4.12 ± 0.72*
Alkenylacyl				
16:0	38.33 ± 4.05	25.33 ± 7.85	24.78 ± 1.89	20.66 ± 4.01
18:0	10.84 ± 0.91	10.02 ± 2.56	15.67 ± 4.89	6.86 ± 0.58
18:1(*n*-9,7)	8.65 ± 3.08	8.66 ± 3.18	14.40 ± 3.23	4.96 ± 1.10*
18:2(*n*-6)	6.12 ± 0.72	6.47 ± 2.73	13.80 ± 5.65	13.82 ± 6.76
20:4(*n*-6)	22.25 ± 3.23	24.62 ± 10.84	11.35 ± 5.13	33.39 ± 17.32
20:5(*n*-3)	ND	0.89 ± 0.40*	ND	ND
22:5(*n*-3)	ND	2.13 ± 0.56*	ND	1.79 ± 0.19*
22:6(*n*-3)	ND	3.68 ± 1.32*	2.98 ± 0.78	2.78 ± 0.93

[a] Values represent mean ± SE (n = 3–4). Fifteen mice were pooled per analysis. *, Significantly different from CO in the same fraction/subclass, $p < 0.05$. Abbreviations used: ND, Not detectable; CO, corn oil diet; FO, fish oil diet.
[b] Only selected major fatty acids (>1 mol%) are reported.
[c] Results are expressed as moles per 100 mol of total fatty acids.

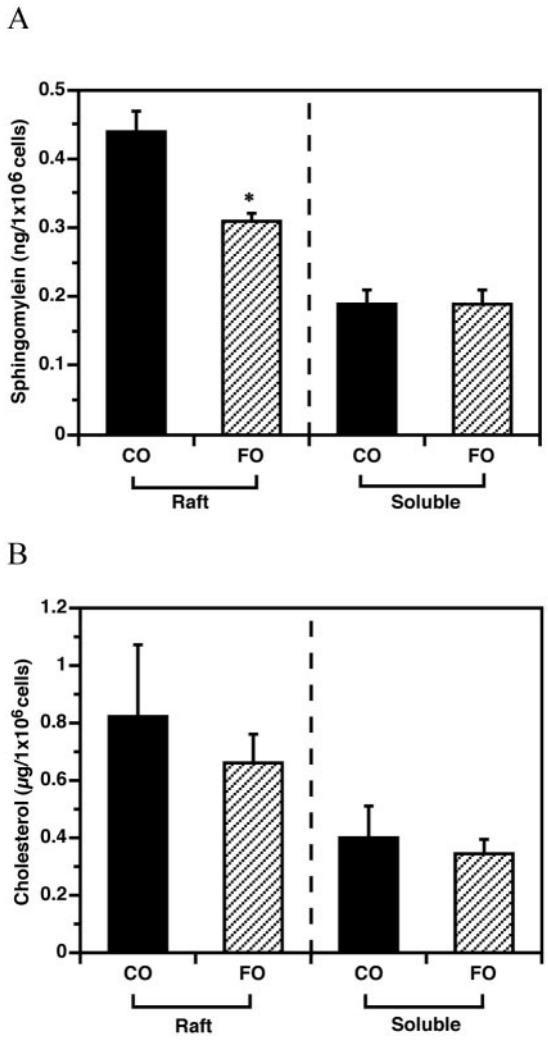

FIGURE 1. Dietary fish oil reduces sphingomyelin, but not cholesterol, levels in T cell lipid rafts. Splenic $CD3^+$ T cells were isolated from C57BL/6 mice fed either a 5% corn oil (CO) control diet or a 4% fish oil plus 1% corn oil (FO) *n*-3 PUFA-enriched diet for 2 wk. Cell membrane raft and soluble fractions were isolated by sucrose gradient centrifugation as described in *Materials and Methods*. Total lipids were extracted using chloroform/methanol (2:1, v/v), and sphingomyelin was isolated from total phospholipids by TLC. *A*, Levels of sphingomyelin were quantified by gas chromatography. *B*, Cholesterol levels in total lipid extracts were analyzed using the Amplex Red Cholesterol Assay kit (Molecular Probes). ∗, Indicates a significant difference ($p < 0.05$). Values represent mean ± SE ($n = 4$).

oil or DHA specifically impair receptor-induced activation of the transcription factors AP-1 and NF-κB. DNA binding activities were assessed by ELISA using nuclear extracts from $CD4^+$ splenic T cells from mice fed either corn oil or fish oil for 2 wk. There was no significant dietary effect on body weight at the end of the study (data not shown). As shown in Figs. 3*A* and 4*A*, NF-κB and AP-1, respectively, were activated upon incubation with anti-CD3 and anti-CD28. The specificity of the DNA-binding activity of NF-κB and AP-1 was verified using an excess of unlabeled probes for competition (data not shown). The kinetics of Ab-induced stimulation was analyzed from 0 to 48 h (Figs. 3*A* and 4*A*). At both the 24- and 48-h time points, NF-κB and AP-1 DNA binding activity was inhibited by dietary fish oil. Similar results were obtained when purified DHA, a fatty acid constituent of fish oil, was fed. Specifically, DHA feeding reduced NF-κB (Fig. 3*B*) and AP-1 (Fig. 4*B*) activation by 52 and 56% at 48 h, respectively.

Effect of dietary lipid on mitogen-induced T cell function

PKCθ is essential for coupling TCR signaling to the activation of AP-1 and NF-κB in T cells (60, 61). In addition, several lines of evidence point to AP-1 and NF-κB as critical transcription factors for IL-2 regulation (62, 63). Therefore, we separately investigated the effect of dietary fish oil and DHA on the $CD4^+$ T cell proliferative response relative to corn oil (control). Because IL-2 is a potent autocrine and paracrine T cell growth factor, we initially determined whether dietary fish oil or purified DHA influenced mitogen-induced T cell IL-2 production. As shown in Fig. 5, the $CD4^+$ T cells from mice fed diets enriched in either fish oil (Fig. 5*A*) or DHA ethyl ester (*B*) had significantly ($p < 0.05$) less IL-2 in the culture supernatants at both 24 and 48 h poststimulation with anti-CD3/anti-CD28. Consistent with their effects on IL-2 synthesis and secretion, both dietary fish oil and dietary DHA suppressed significantly ($p < 0.05$) the anti-CD3/anti-CD28-induced proliferation of $CD4^+$ T cells (Fig. 6).

Discussion

In the present study, we have shown for the first time that dietary DHA, an effector molecule capable of attenuating immune-mediated inflammatory diseases (2, 4, 7), down-regulates the PKCθ signaling axis in murine T cells (Fig. 7). DHA is a unique fatty acid, because it significantly alters basic properties of cell membranes, including acyl chain order and fluidity, phase behavior, elastic compressibility, ion permeability, fusion, rapid flip-flop, and resident protein function (64). Because of its polyunsaturation, DHA is sterically incompatible with sphingolipid and cholesterol and, therefore, is believed to alter lipid raft behavior and protein function (65). Therefore, we hypothesized that dietary DHA would alter T cell plasma membrane microdomain lipid composition and influence signaling complexes that regulate T cell activation in vivo.

The concept that lipid domains in membranes could be modified by exogenous fatty acids was first described over 20 years ago (66). Recently, a number of in vitro studies using model membrane and cell culture systems have demonstrated that unsaturated fatty acids greatly reduce raft formation (67) and can displace signaling proteins (36, 37). Because DHA is preferentially incorporated into GPE and GPC with lesser amounts in other phospholipid classes (41, 68), we initially examined the effect of short-term dietary exposure to fish oil (a natural source of DHA) on the fatty acyl composition of these phospholipid classes. Our data indicate for the first time that T cell lipid rafts are highly enriched in *sn*-1 ether-linked phospholipids, i.e., 1-*O*-alk-1′enyl-2 acyl GPE and 1-*O*-alkyl-2 acyl GPC, particularly those containing arachidonic acid (20:4*n*-6). This is consistent with previous reports indicating that phospholipids with long ether-linked alkyl chains congregate to the liquid-ordered phase of lipid rafts in the plasma membrane (69). In addition, our results demonstrate that lipid rafts exhibit a lipid composition that is distinct from that seen in isolated bulk membrane fractions (Tables I and II). This is likely to contribute to the specialized functions of these domains.

The incorporation of dietary *n*-3 PUFA into both raft and soluble membrane phospholipids is noteworthy, in view of the fact that DHA-containing phospholipids partition into cholesterol-poor domains and may therefore perturb microdomain/raft formation (65). Along these lines, it was interesting to note that raft sphingomyelin content was decreased in T cells isolated from *n*-3 PUFA-fed mice (Fig. 1). Complex sphingolipids (sphingomyelin and glycosphingolipids) are required to facilitate raft formation and T cell activation (27, 56, 70). Although caution is needed when drawing conclusions based solely on the capacity of detergent to

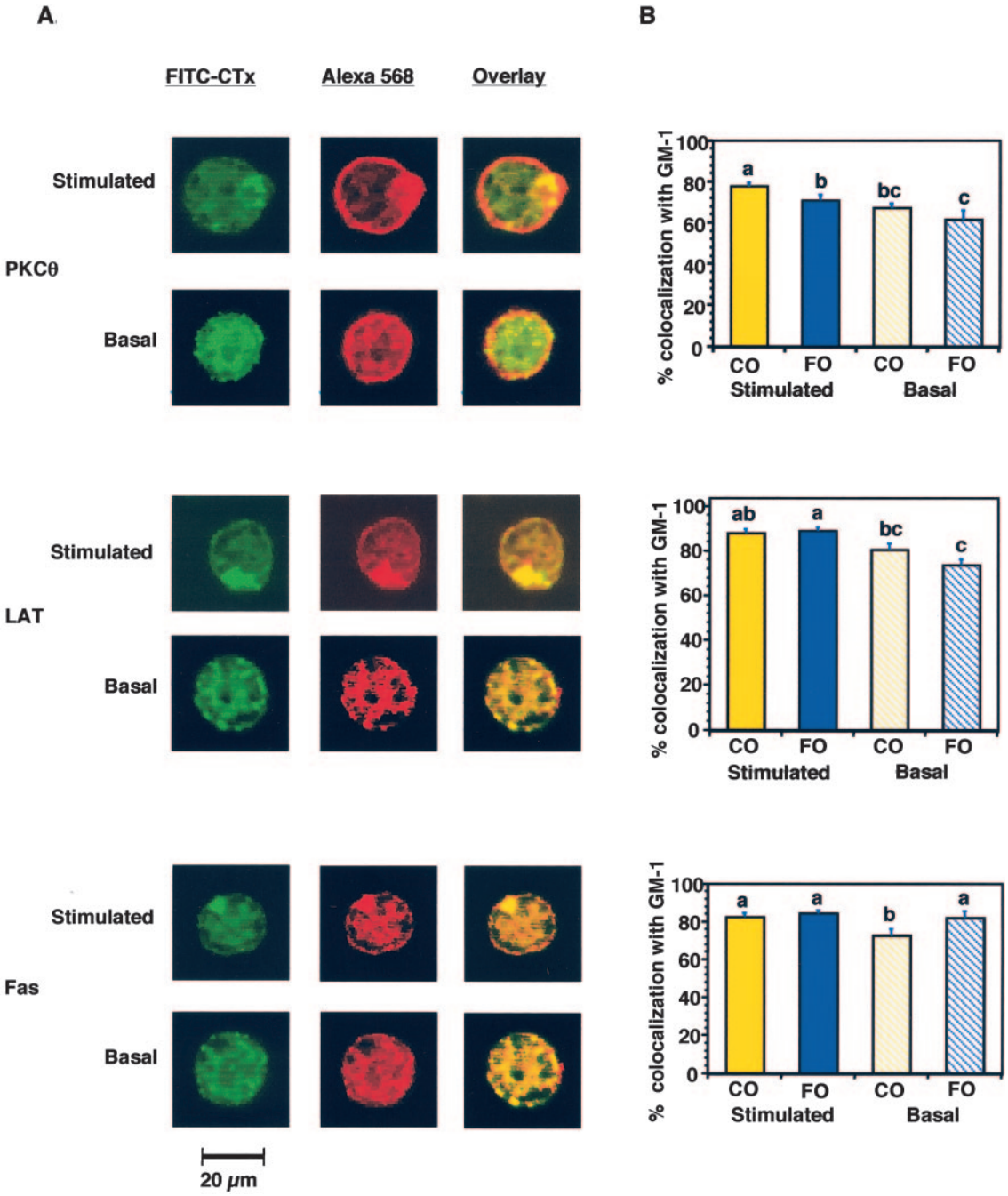

FIGURE 2. Colocalization of raft markers (GM1) with select signaling proteins is affected by diet. Splenic $CD3^+$ T cells were isolated from C57BL/6 mice fed either a 5% corn oil (CO) control diet or a 4% fish oil plus 1% corn oil (FO) *n*-3 PUFA-enriched diet for 2 wk. Cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml), or unstimulated for 30 min at 37°C. Following immunostaining with FITC-CTx and PKCθ, LAT, or Fas, respectively, signal and two-color confocal analyses were performed at $\times$126 magnification. *A*, Overlay images of green (FITC-CTx binds the raft marker, GM1) and red (Alexa 568-PKCθ, LAT, or Fas) fluorescent signals shows stimulation-induced clustering of membrane rafts and colocalization of PKCθ, LAT, or Fas with the raft marker in yellow. *B*, Colocalization of PKCθ, LAT, or Fas with the raft marker was quantified as percentage of colocalized area (both green and red) over total raft area (green). Images were randomly captured from a minimum of 10 fields per slide, and a minimum of 40 cells from four separate animals ($n = 4$) were evaluated per treatment group. Values not sharing the same superscript are significantly different ($p < 0.05$).

solubilize lipids, collectively, these data support our hypothesis that *n*-3 PUFA alter specific lipid microdomains that are required for T cell activation and proliferation.

With respect to the physiological relevance of the diets used in our studies, the fish oil diet contained ~1.4 energy percentage as DHA. Diets using purified DHA contained ~2.2 energy percentage ethyl ester. In contrast, the corn oil diet contained only trace amounts of EPA and DHA. As a point of reference, the Japanese typically consume DHA at 1–2% of energy in the diet (71), whereas those in most European countries and the United States consume ~0.1–0.2% of energy as *n*-3 PUFA (72). Therefore, our experimental diets were within the range consumed by humans.

There is strong evidence that lipid raft integrity is a prerequisite for optimized TCR signal transduction and immune response (31,

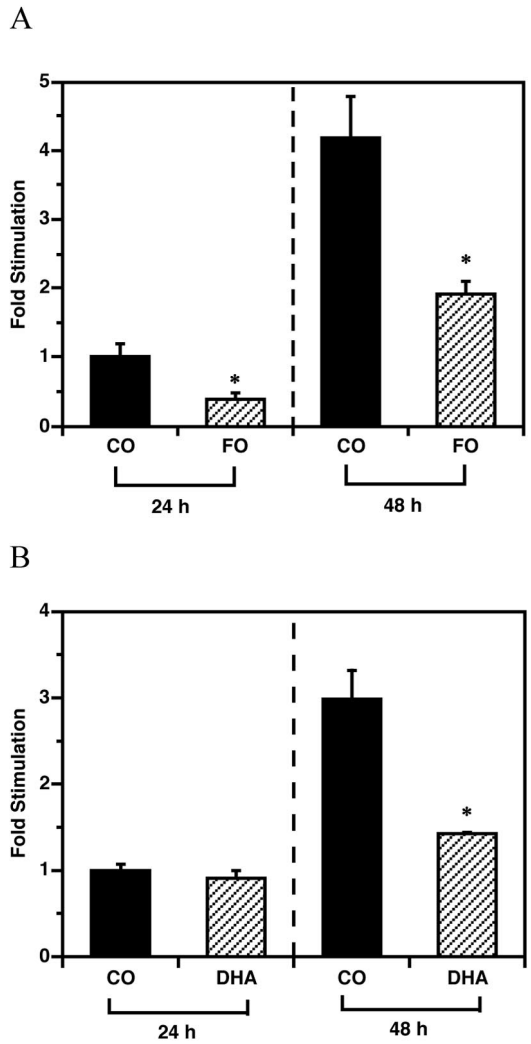

FIGURE 3. Dietary *n*-3 PUFA reduces nuclear NF-κB activation in T cells. Two separate dietary studies were conducted. *A*, Splenic $CD4^+$ T cells were isolated from C57BL/6 mice fed either a 5% corn oil (CO) control diet or a 4% fish oil plus 1% corn oil (FO) *n*-3 PUFA-enriched diet for 2 wk. *B*, Splenic $CD4^+$ T cells were isolated from C57BL/6 mice fed either a 5% corn oil (CO) control diet or a 4% corn oil plus 1% DHA ethyl ester (DHA) diet for 2 wk. Cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) for 24 and 48 h. Nuclear fractions were isolated, and the levels of activated NF-κB were quantified as described in *Materials and Methods*. Data are expressed as fold stimulation relative to that observed at 24 h in $CD4^+$ T cells from corn oil-fed mice. ∗, Indicates a significant dietary effect ($p < 0.05$) within the same time interval. Values represent mean ± SE ($n = 5$).

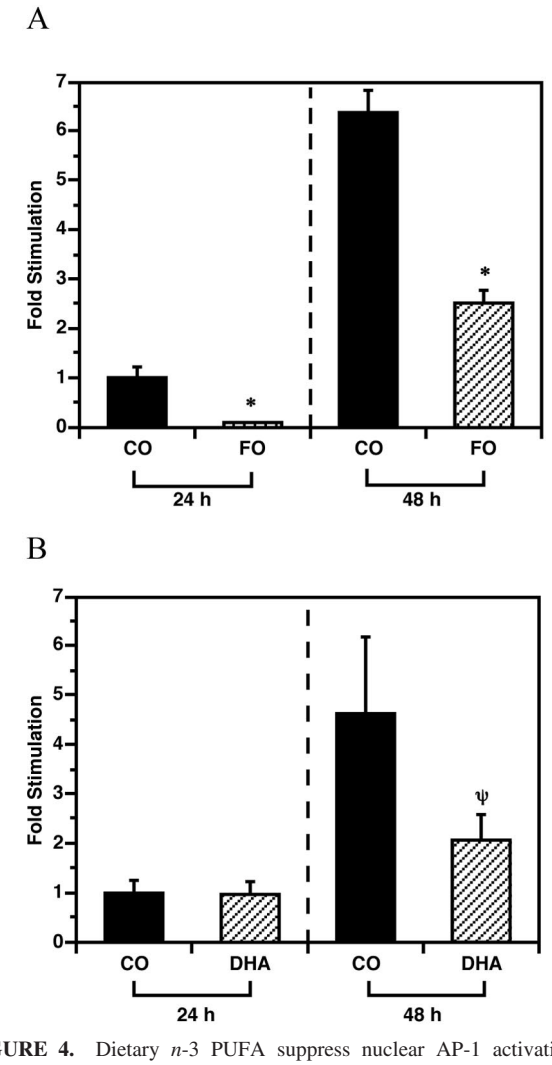

FIGURE 4. Dietary *n*-3 PUFA suppress nuclear AP-1 activation in mouse T cells. Splenic $CD4^+$ T cells isolated from mice on experimental diets (refer to Fig. 3 for details) were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) for 24 and 48 h. The levels of activated AP-1 in isolated nuclei were quantified as described in *Materials and Methods*. Data are expressed as fold stimulation relative to that observed at 24 h in $CD4^+$ T cells from corn oil-fed mice. ∗, Indicates a significant dietary effect ($p < 0.05$) within the same time interval. ψ, $p = 0.06$. Values represent mean ± SE ($n = 5$).

33–35). Upon T cell activation, rafts compartmentalize the activated TCR and associated signal-transducing molecules, thus providing an environment conducive to signal transduction (23). For example, translocation of PKCθ to lipid rafts is required for peripheral T cells to secrete IL-2 and mount typical proliferative responses upon activation (30, 62). Therefore, it was important to note that dietary fish oil reduced the localization of PKCθ to lipid rafts following polyclonal stimulation (Fig. 2). Interestingly, although both groups exhibited an increase in PKCθ colocalization with lipid rafts following stimulation, the absolute poststimulation level was 10% lower in the fish oil group. Because small changes in the oligomerization and conformation state of membrane proteins may stabilize transient rafts and induce the formation of larger domains (73), it is conceivable that a modest change in PKC localization could alter the threshold setting required to initiate maximal signal transduction and contribute to the observed failure to induce AP-1 and NF-κB activation in DHA-fed mice (Figs. 3 and 4). Although the physiological substrates of PKCθ have not been defined, T cells lacking PKCθ fail to activate NF-κB and AP-1, and to express IL-2 (30, 61). Interference with IL-2 production would be expected to have a major suppressive effect on T cell proliferation (74), and this is exactly what we observed in $CD4^+$ T cells from mice fed diets enriched in fish oil or DHA (Figs. 5 and 6). These data suggest that the PKCθ-dependent signaling axis is an important molecular target of DHA. We have proposed a molecular model that links diet-induced plasma membrane alterations to downstream signaling events in T cells (Fig. 7). At present, it is difficult to predict what the physiological consequences of reduced PKCθ localization in naive cells is. PKCθ is the only isoform of PKC recruited to the immunological synapse after TCR stimulation and localized to the supramolecular activation cluster (62). Its function in naive cells has not been addressed to date. In addition,

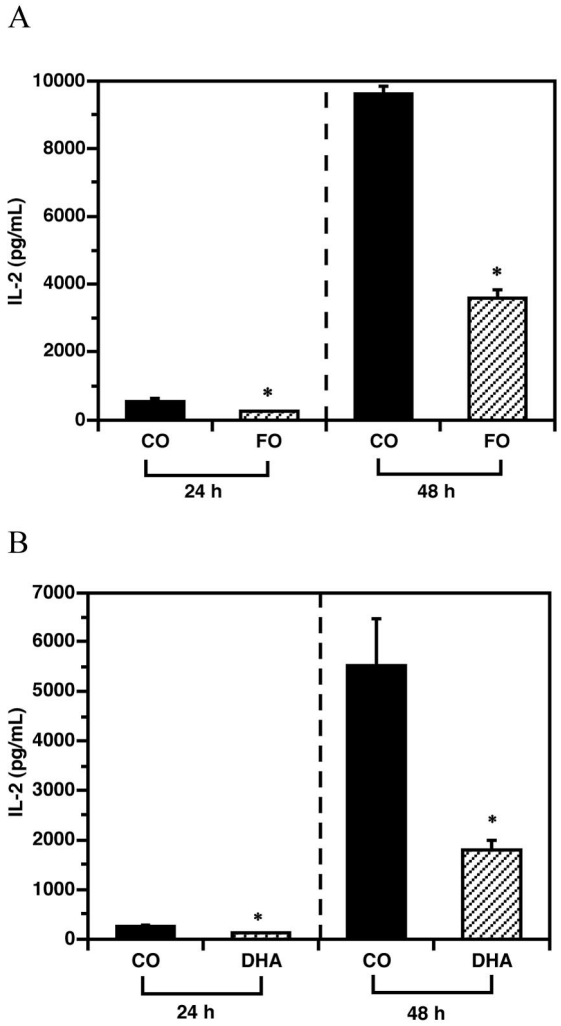

FIGURE 5. Suppression of IL-2 secretion by dietary fish oil and DHA in murine CD4$^+$ T cells. Splenic CD4$^+$ T cells isolated from mice on experimental diets (refer to Fig. 3 for details) were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) for 24 and 48 h. Cell culture supernatant IL-2 levels were quantified by Quantikine mouse ELISA as described in *Materials and Methods*. Data represent the concentration of IL-2 secretion (picograms per milliliter; mean ± SE; n = 5) per culture (originally seeded at a density of 200,000 cells/well). *, Indicates a significant dietary effect ($p < 0.05$) within the same time interval.

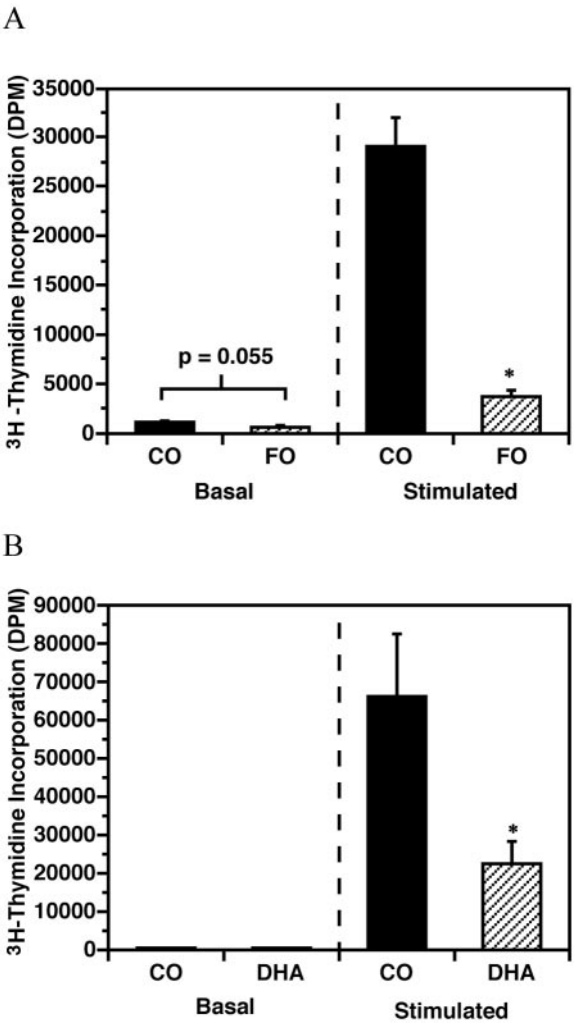

FIGURE 6. Dietary fish oil and DHA suppress polyclonal lymphoproliferation in murine CD4$^+$ T cells. Following diet supplementation (refer to Fig. 3 for details), splenic CD4$^+$ T cells isolated from corn oil- or fish oil-fed mice (*A*), and corn oil- or DHA-fed mice (*B*) were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (5 μg/ml) or left unstimulated (basal) for 62 h, and then pulsed with [^{3}H]thymidine for an additional 6 h as described in *Materials and Methods*. Results are expressed as disintegrations per minute (DPM) per culture. Data represent mean ± SE (n = 4). *, Indicates a significant dietary effect ($p < 0.05$).

with respect to lipid microdomains in resting T cells, small lipid microdomains may be forming and dissolving continually, and raft-preferring signaling molecules, e.g., PKCθ, may reside only transiently in rafts (75).

Although fish oil feeding did not alter either Fas or LAT lipid raft colocalization in stimulated T cells, a significant fraction of LAT was displaced from rafts in naive cells (Fig. 2). These results are consistent with previous in vitro studies that demonstrated that select n-3 PUFA are capable of displacing LAT from lipid rafts in Jurkat cells (36, 37). Although LAT function per se was not examined in the present study, fish oil- vs corn oil-fed mouse T cells had comparable levels of LAT colocalization upon stimulation. Therefore, we do not believe that LAT-dependent signaling would be significantly altered. In contrast, Fas colocalization with lipid rafts was elevated in naive T cells isolated from fish oil-fed mice (Fig. 2). Because dietary n-3 PUFA are capable of enhancing Fas-mediated activation-induced cell death in T cells (76), Fas microdomain relocation may also be a molecular mechanism by which EPA and DHA inhibit T cell signaling. Additional studies are required to assess the biological impact of Fas and LAT mislocalization in vivo.

A wealth of published literature supports the contention that diet in general, and n-3 PUFA in particular, is an important determinant of the quantity and quality of the host's immune responses (2–5, 7, 13). Despite this clinical evidence, the precise mechanism(s) of n-3 PUFA regulation of T cell function has not been elucidated. The data in this study clearly indicate that DHA plays an important role in mediating the immunosuppressive properties of dietary fish oil. We demonstrate that dietary fish oil, containing a mixture of n-3 PUFA, modulates T cell lipid raft microdomain composition and the localization of PKCθ, a key mediator of cell proliferation. Interestingly, DHA feeding has been shown to suppress DAG generation in mouse splenic T cells (20). Although DAG serves as a PKC-activating second messenger in vivo, its role in the translocation and activation of PKCθ is still unclear. Reconciling how

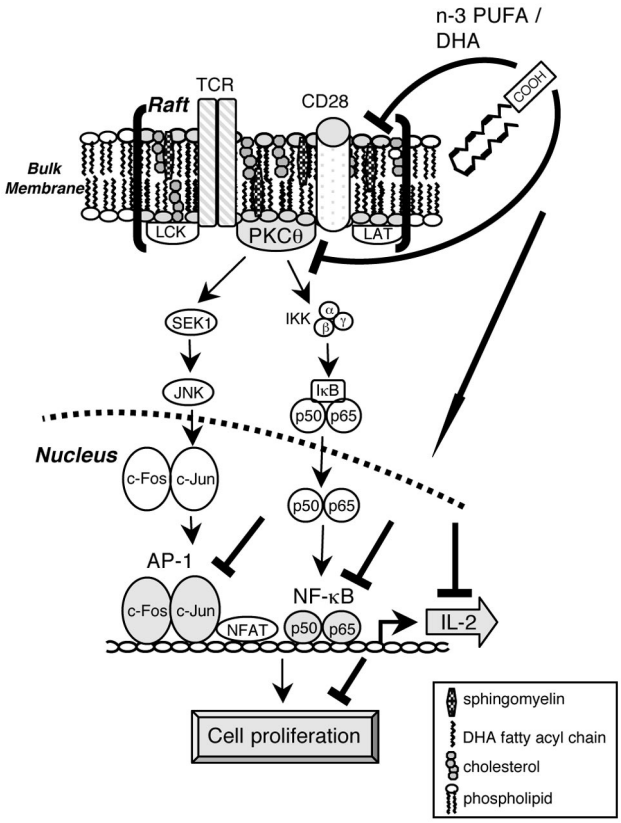

FIGURE 7. Proposed molecular model of dietary *n*-3 PUFA on lipid rafts and PKCθ-dependent signal transduction in murine T cells. The incorporation of *n*-3 PUFA into T cell membrane phospholipids alters lipid rafts by reducing sphingomyelin levels and altering GPC and GPE fatty acyl composition. This directly or indirectly suppresses the recruitment of PKCθ into lipid rafts (black bar indicates inhibition), thereby down-regulating PKCθ-mediated signaling cascades. Together, these effects culminate in the reduction of NF-κB and AP-1 activation, decreased IL-2 secretion, and impaired lymphoproliferation.

lipid rafts link membrane-proximal activation cascades to transcriptional responses is crucial to understanding how DHA suppresses typical T cell-proliferative responses upon activation.

In conclusion, we have demonstrated for the first time that dietary *n*-3 PUFA suppress the partitioning of PKCθ into murine T cell lipid rafts, and that this was associated with a reduction in AP-1 and NF-κB activation, the down-regulation of IL-2 secretion, and the failure to mount a normal proliferative response upon polyclonal activation.

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