(n-3) Polyunsaturated Fatty Acids Promote Activation-Induced Cell Death in Murine T Lymphocytes[1]

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ABSTRACT Previous studies showing dietary (n-3) polyunsaturated fatty acids (PUFA) attenuate T cell immune-mediated inflammatory diseases led us to hypothesize that (n-3) PUFA promote activation-induced cell death (AICD) in T cells. Because T cell subsets display a differential resistance to AICD, we compared the effects of (n-3) PUFA feeding on T cells stimulated in vitro to express different cytokine profiles. Mice were fed either diets lacking (n-3) PUFA (control) or (n-3) PUFA-containing diets for 14 d. Splenic T cells were stimulated with αCD3/αCD28, phorbol myristate acetate (PMA)/Ionomycin or αCD3/PMA for 48 h, followed by reactivation with the same stimuli for 5 h. Apoptosis was measured using Annexin V/propidium iodide. (n-3) PUFA were selectively incorporated into membrane phospholipid pools. Cytokine analyses revealed that (n-3) PUFA enhanced AICD only in T cells expressing a T helper cell (Th)1-like cytokine profile after stimulation with PMA/Ionomycin compared to mice fed the (n-6) PUFA control diet ($P = 0.0008$). In contrast, no increase in apoptosis was seen in T cells stimulated with αCD3/PMA, which exhibited a Th2 cytokine profile. These data demonstrate that the ability of (n-3) PUFA to promote AICD is dependent on the activation stimulus. In conclusion, we have identified a novel mechanism by which (n-3) PUFA modulate T cell−mediated immunity by selective deletion of Th1-like cells while maintaining or enhancing the Th2-mediated humoral immune response. J. Nutr. 133: 496–503, 2003.

KEY WORDS: • *mouse* • *T cells* • *(n-3) fatty acids* • *apoptosis*

Apoptosis is a highly regulated process resulting in cell death without an ensuing inflammatory response, thus playing an important role in maintaining lymphocyte homeostasis and T cell repertoire, and ensuring peripheral tolerance (1). T cells can succumb to apoptosis by two pathways: passive cell death (absence of growth factors) and chronic antigen stimulation [activation-induced cell death (AICD)[3]]. AICD is specifically responsible for the signal-induced elimination of previously activated lymphocytes. The induction of AICD in part is mediated by the coexpression of Fas and its ligand, FasL, on the T cell plasma membrane (1−3). The Fas death receptor is a member of the nerve growth factor receptor/tumor necrosis factor (TNF) receptor family and transmits signals resulting in apoptosis (4). Stimulation upregulates the expression of FasL and other death factors in AICD-sensitive T cells, which in turn induce Fas on the same or neighboring cells. Death receptors are activated by oligomerization by rapidly assembling a number of adaptor proteins, resulting in an irreversible activation of proteases and nucleases that culminates in apoptosis (5). Mutations in Fas or FasL result in lymphoproliferation and manifestations of autoimmunity in both mice and humans (6,7), illustrating the critical role of AICD in the maintenance of self-tolerance.

Not all T cells are equally sensitive to AICD. T cells can be divided into $CD4^+$ and $CD8^+$ subsets based on the mutually exclusive presentation of these molecules on their surfaces and their different effector functions. Mature $CD4^+$ effector cells can be further polarized into Th1 and Th2 subsets according to the cytokines they produce (8). Th1 cells produce interleukin 2 (IL-2), gamma-interferon (IFNγ) and TNFβ and are important in cell-mediated immunity against intracellular pathogens (8,9). Th1 cells are proinflammatory and have been implicated in the pathogenesis of human inflammatory and autoimmune diseases such as rheumatoid arthritis, type 1 diabetes and inflammatory bowel diseases (10,11). Th2 cells produce IL-4, IL-5 and IL-10 and are important in humoral immunity and defense against extracellular pathogens (9). Th1 and Th2 effectors have differential susceptibilities to AICD, with Th1 cells being AICD sensitive and Th2 cells being AICD resistant (12). The selective death of Th1 cells has been attributed to a preferential requirement for phorbol ester-sensitive protein kinase C (PKC) isoforms (13) and the upregulation of FasL expression (12). In addition, the resistance of Th2 cells to

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[3] Abbreviations used: AICD, activation-induced cell death; FACS, fluorescence-activated cell sorting; Fas-L, Fas ligand; FITC, fluorescein isothiocyanate; FO, fish oil; HFFO, high fat (n-3) PUFA diet; IFNγ, gamma-interferon; LFFO, low fat (n-3) PUFA diet; OO, olive oil ethyl ester diet; PPARγ, peroxisome proliferator-activated receptor γ; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI-3-K, phosphatidylinositol-3′-kinase; PKC, protein kinase C; PMA, phorbol myristate acetate; PUFA, polyunsaturated fatty acids; SAF, safflower oil ethyl ester diet; SRA, scavenger receptor type A; TNF, tumor necrosis factor.

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AICD has been linked to expression of FAP-1, an inhibitor of apoptosis (14), and the selective upregulation of phosphatidylinositol-3′-kinase (PI-3-K) activity (15).

Among dietary factors, (n-3) PUFA found in fish oil (FO) have been shown to potently attenuate T cell−mediated inflammatory diseases in humans and experimental model systems (16,17). In contrast, dietary lipids rich in (n-6) PUFA, found in vegetable oils and animal fats, can be deleterious in some inflammatory diseases (16,18−21). This is significant because the Western diet contains 10 to 20 times more (n-6) than (n-3) PUFA (22). Previous studies have demonstrated that (n-3) PUFA decrease T cell proliferation (23,24), cytokine secretion (19,25,26), intracellular enzyme activity (27) and gene transcription (26,28,29). Additionally, we have found that (n-3) PUFA suppress T cell proliferative capacity attributed to a reduction in IL-2 production and/or function, and to enhancement of counterregulatory IL-4−driven Th2 cells (23). Several investigators have examined the effects of (n-3) PUFA on apoptosis in mixed cell populations [e.g., whole splenocytes (30)]. In those studies, (n-3) PUFA increased T cell apoptosis in cultures of whole splenocytes. Since we have recently found that the diet-induced changes in T cell proliferation are ascribed principally to intrinsic alterations in the T cell response to mitogenic stimuli (31), the effects of (n-3) PUFA on apoptosis in purified T cells remained to be examined. Our results indicate that dietary (n-3) PUFA preferentially promote AICD in a T cell subset exhibiting a Th1 cytokine profile. This exciting observation provides yet another unique mechanism by which (n-3) PUFA exert a significant and selective effect on T cell proinflammatory function in the whole animal.

MATERIALS AND METHODS

Diet and mice. All experimental procedures using laboratory mice were approved by the University Laboratory Animal Care Committee of Texas A&M University. Female, pathogen-free (12–14 g) C57BL/6 mice were purchased from Frederick National Cancer Research Facility (Frederick, MD). Mice were housed in autoclaved polycarbonate microisolator cages and were maintained at room temperature (~25°C) on a 12-h light:dark cycle. Mice were initially fed standard mouse diet (Teklad 9F Sterilizable Rodent diet; Madison, WI) for a 1-wk acclimation period and had free access to autoclaved water and diet. The study was completed in two phases. For the first experiment, mice were assigned to one of three semipurified diets containing: 2% safflower oil ethyl ester [SAF, (n-6) PUFA control], 2% olive oil ethyl esters [OO, monounsaturated fatty acid devoid of both (n-3) and (n-6) PUFA] or 2% Menhaden fish oil [FO, (n-3) PUFA] for 14 d. For the second experiment, mice were assigned to one of the following diets: SAF, 4% Menhaden fish oil [low fat fish oil (LFFO)] or 9% Menhaden fish oil [high fat fish oil (HFFO)] for 14 d. The purified diets met National Research Council nutrition requirements and varied only in lipid composition as previously described (32).

The diet composition, expressed in g/kg of complete diet, was as follows for expt. 1: 200 g casein, 420 g sucrose, 219.8 g starch, 60 g cellulose, 35 g AIN-76 mineral mix, 10 g vitamin mix AIN-76, 3 g DL-methionine, 2 g choline chloride and 0.2 g tertiary butyl hydroquinone. The lipid content of the three diet groups was as follows: SAF, 30 g/kg corn oil (CO) combined with 20 g/kg safflower oil ethyl esters; OO, 30 g/kg CO combined with 20 g/kg olive oil ethyl esters; FO, 30 g/kg CO combined with 20 g/kg menhaden fish oil. For expt. 2, the basic diet composition differed only in the reduced amount of sucrose for HFFO (370 g/kg) to compensate for the increase in fat content. The lipid content of the three diet groups was as follows: SAF, 30 g/kg CO combined with 20 g/kg safflower oil ethyl esters; LFFO, 10 g/kg CO combined with 40 g/kg menhaden fish oil; HFFO, 10 g/kg CO combined with 90 g/kg menhaden fish oil. The fatty acid composition, expressed in g/kg of complete diet, is detailed in **Table 1**. The linoleic acid [18:2(n-6)] content from CO was 2−7% of total energy and thus met the minimum 1–2% essential fatty acid (EFA) requirement for mice (32). Levels of vitamin A, D and E were approximately equal and exceeded the minimum requirement. Safflower oil [70.5% as 18:2(n-6)] was obtained in ethyl ester form from the National Institutes of Health Test Materials Program (Charleston, SC). Control dietary lipids lacking (n-3) PUFA, olive oil and corn oil [57.3% as 18:2(n-6)] were obtained from Degussa Bioactives (Champaign, IL); Menhaden fish oil [13.1% as 20:5(n-3), 9.7% as 22:6(n-3)] was provided by the National Institutes of Health Test Materials Program. Diets were analyzed by gas chromatography (Table 1), aliquoted and stored at −80°C. Diets were provided ad libitum and were changed daily to prevent peroxidation. The analysis confirmed the enrichment of 18:2(n-6) in the SAF diet (53.3% of total lipid), 20:5(n-3) and 22:6(n-3) in the FO diet (6.1 and 4.3% of total lipid, respectively), 20:5(n-3) and 22:6(n-3) in the LFFO diet (11.7 and 8.1% of total lipid, respectively) and 20:5(n-3) and 22:6(n-3) in the HFFO diet (13.4 and 9.4% of total lipid, respectively). Food intake did not differ between dietary groups, and weight gain was similar in all groups (data not shown).

Isolation and preparation of splenic lymphocytes. Mice were killed by CO_2 asphyxiation. Spleens were placed in 3 mL of RPMI-complete medium [RPMI 1640 with 25 mmol/L HEPES (Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS (Irvine Scientific), 1×10^5 U/L penicillin and 100 mg/L streptomycin (Irvine Scientific), 2 mmol/L L-glutamine and 10 μmol/L 2-mercaptoethanol] (24). Spleens were dispersed with glass homogenizers and passed through a 149-μm wire mesh filter to create single-cell suspensions. Cells were subsequently washed with RPMI-complete medium before T cell enrichment.

T cell purification. Total lymphocytes were initially enriched by a density gradient centrifugation method using Lympholyte-M (Cedarlane, Toronto, Canada) (32) in accordance with the manufacturer's protocol. Subsequently, $60–90 \times 10^6$ mononuclear cells were loaded onto a negative-selection mouse T cell purification column (R&D Systems, Minneapolis, MN) and incubated for 10 min at room temperature. Nonadherent cells were eluted for assay. The purity of the T cell population as analyzed by flow cytometry was determined to be $90.3 \pm 1.4\%$, $n = 3$.

T cell apoptosis assay. T cells, 5×10^5 cells/well from each mouse, were cultured in the presence of each of five sets of stimuli: (1) 1 mg/L plate-bound purified hamster anti-mouse CD3ε (αCD3) monoclonal antibody (PharMingen, San Diego, CA) with 5 mg/L

TABLE 1

Fatty acid composition of experimental diets[1]

Fatty acid	SAF	OO	FO	LFFO	HFFO
	g/100 g fatty acids				
14:0	0.3	0.4	3.7	7.4	8.2
16:0	12.4	14.8	13.2	16.1	16.9
16:1(n-7)	0.0	0.9	4.8	9.6	11.0
18:0	2.8	5.1	2.6	3.0	3.1
18:1(n-7) + 18:1(n-9)	28.4	37.4	20.8	13.8	11.8
18:2(n-6)	53.3	34.1	35.5	13.5	8.0
18:3(n-3)	1.0	2.0	1.2	1.4	1.2
20:5(n-3)	0.0	0.0	6.1	11.7	13.4
22:5(n-3)	0.0	0.0	1.1	1.7	1.9
22:6(n-3)	0.0	0.0	4.3	8.1	9.4
Total SFA	16.0	21.2	20.1	26.9	28.7
Total MUFA	28.8	39.0	26.4	24.0	23.5
Total (n-6) PUFA	53.3	34.1	35.5	14.3	9.4
Total (n-3) PUFA	1.0	3.0	12.8	22.9	25.9

[1] Only the major fatty acids are listed. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SAF, linoleic acid–containing diet; OO, oleic acid-containing diet; FO, fish oil (2 g/100 g)–containing diet; LFFO, fish oil (4 g/100 g)–containing diet; HFFO, fish oil (9 g/100 g)–containing diet. Values are expressed as g/100 g of total fatty acids in each diet.

soluble purified hamster anti-mouse CD28 (αCD28) monoclonal antibody (PharMingen); (2) 0.5 μg/L phorbol-12-myristate-13-acetate (PMA) (Sigma, St. Louis, MO) with 1 mg/L αCD3; (3) 1 μg/L PMA with 500 nmol/L Ionomycin (Calbiochem-Novabiochem, San Diego, CA); (4) 50 μg/L recombinant human soluble FasL fused to a FLAG-tag (FasL) (Alexis Biochemicals, San Diego, CA) with 1 mg/L Enhancer (αFLAG) (Alexis Biochemicals); or (5) 330 μg/L dexamethasone (Sigma). Choice of concentrations were determined in previous experiments (32). Cells were incubated at 37°C, 5% CO_2 for 24 h. Apoptosis was assessed via flow cytometry (see below).

Induction of AICD. T cells, 5×10^6 cells/well, were cultured in duplicate in the presence of either stimuli (1), (2) or (3) as described above for 48 h at 37°C, 5% CO_2 (**Fig. 1**). Cells were harvested, washed and recultured at 3×10^6 cells/well in RPMI-complete medium overnight at 37°C, 5% CO_2. Cultures were reharvested and dead cells were removed using Lympholyte-M. Subsequently, 1×10^6 cells/well were recultured either in the presence of the initial stimuli ("reactivated") or in RPMI-complete medium ("unreactivated") for 5 h at 37°C, 5% CO_2 as previously described (4). After reactivation, apoptosis was assessed via flow cytometry (see below).

Staining and fluorescence-activated cell sorting (FACS) analysis for apoptotic cells. Harvested T cells (1×10^6 cells) were washed with cold 1× PBS and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V (PharMingen) and propidium iodide (PharMingen) for 15 min according to PharMingen's Annexin V Kit I. Stained cells were subsequently analyzed for apoptosis via flow cytometry (FACScan; Becton-Dickinson, Bedford, MA). Controls consisted of single staining for Annexin V-FITC only and PI only. T cell population purity was assessed by staining 5×10^5 cells with 5 mg/L of a FITC-conjugated anti-mouse CD3ε monoclonal antibody (PharMingen) after 5 mg/L anti-mouse CD16/CD32 monclonal antibody (FC Block; PharMingen) treatment.

Cytokine ELISAs. For AICD analyses, cells were cultured for 48 h as described in Figure 1. Cell culture supernatants from duplicate wells of T cells for each stimulus were harvested, pooled and stored at −80°C. After thawing, supernatants were assayed in duplicate for IL-2, IL-4, IL-10 and IFNγ using Mouse Immunoassay (ELISA) Kits (R&D Systems). Results are expressed as pg/3×10^6 cells as previously described (24).

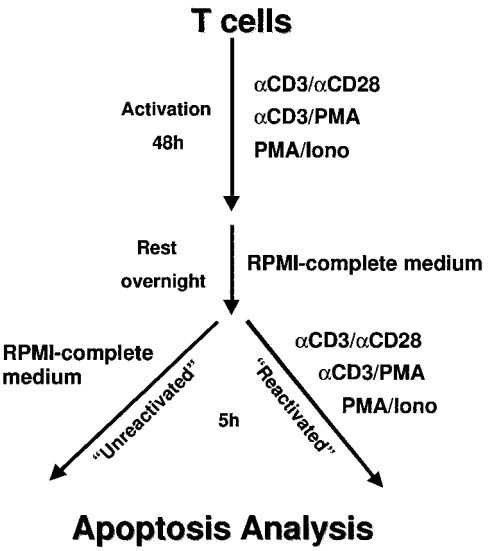

FIGURE 1 Experimental design for activation-induced cell death (AICD) induction. After a 2-wk feeding period, purified T cells from safflower oil ethyl ester (SAF), low fat (n-3) PUFA (LFFO) and high fat (n-3) PUFA (HFFO) fed mice were activated as follows: αCD3/αCD28, phorbol myristate acetate (PMA)/αCD3 or PMA/Ionomycin for 48 h. After an overnight rest period in RPMI-complete medium, T cells were either reactivated with the initial stimuli or cultured in RPMI-complete medium for 5 h. Apoptosis was analyzed with Annexin V/propidium iodide (PI) as described under Materials and Methods.

T cell membrane phospholipid analysis. T cell lipids were extracted by the method of Folch et al. (33). The individual phospholipid classes were separated by thin layer chromatography (TLC) on silica gel 60 plates using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, v/v/v/v) (34). Bands were detected under ultraviolet light after spraying with 0.1% 8-anilino-naphthalene-sulfonic acid. Fractions were scraped from the plates and transesterified, and fatty acid methyl esters (FAME) were extracted using hexane and 0.1 mol/L potassium chloride. The fatty acid composition of sphingomyelin, phosphatidylcholine (PC), phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine (PE) and cardiolipin were analyzed by gas chromatography as previously described (34).

Assessment of systemic oxidative stress. Livers were removed from mice at the time of necropsy and flash frozen in liquid nitrogen, and RNA was isolated using Ambion's Totally RNA Kit (Austin, TX) according to the manufacturer's protocol. The isolated RNA was subsequently treated with DNase Inactivation Reagent (Ambion) to remove contaminating DNA. Reverse transcription was performed with 2 μg RNA in a 50-μL reaction using Superscript II (Gibco BRL, Rockville, MD). Reactions in the absence of reverse transcriptase enzyme served as negative controls. Real-time PCR was performed using an ABI 7700 unit (Applied Biosystems, Foster City, CA). Primer pairs for mouse CD36, peroxisome proliferator-activated receptor γ (PPARγ) and scavenger receptor type A (SRA) were designed with Primer Express software, version 1.5. The sequences of the primers were as follows: CD36, forward, 5′-CAA GCT CCT TGG CAT GGT AGA-3′, and reverse, 5′-TGG ATT TGC AAG CAC AAT ATG AA-3′; PPARγ, forward, 5′-GAT GAA TAA AGA TGG AGT CCT CAT CTC-3′, and reverse, 5′-CCG CAG GCT TTT GAG GAA-3′; and SRA, forward, 5′-CAG GAA TAA GAG GTA TTC CAG GTG TTA-3′, and reverse, 5′-TCC TGG TGC TCC TGG GTT T-3′. Primer sequences were checked for sequence homology against known genes using BLAST (http://www.ncbi.nlm.nih.gov/blast/). Each PCR reaction consisted of 25 μL 2× Sybr Green master mix (Applied Biosystems), 300 nmol/L final concentration forward and reverse primers, and 2 μL reverse transcription reaction in a 50-μL final reaction volume. To assess assay reproducibility, select RT reactions were performed in triplicate followed by PCR. Values for cycle threshold (C_T), the point at which exponential amplification of the PCR products is detected, were obtained from the Applied Biosystems software.

Statistical analysis. A mixed model was used to assess whether the dietary effects differed among the various stimuli. This mixed model is essentially a two-way ANOVA with factors diet and stimulus, but accounts for the correlation among multiple measurements coming from the T cells taken from a single mouse. The procedure MIXED in SAS software was used for the analysis (SAS Institute, Cary NC). When diet effects were different among stimuli, comparisons between the diet groups were conducted using Fisher's protected least significant difference (LSD) test. In that case, two diets were deemed statistically different if their *P*-value was less than $\alpha = 0.05$. Values presented in the text are means ± SEM.

RESULTS

(n-3) PUFA differentially affect T cell apoptosis depending on stimulus. Given that previous studies indicated a suppressive effect of (n-3) PUFA on T cell proliferation (24), we determined the effect of (n-3) PUFA on T cell apoptosis. Purified splenic T cells from (n-3) PUFA-fed mice were stimulated with various agonists that act at the plasma membrane receptor level (αCD3/αCD28), intracellular level (PMA/Ionomycin) or at both the receptor and intracellular levels (αCD3/PMA). Representative FACS images of early- and late-stage apoptosis after a 24-h stimulation period are shown in **Figure 2**A. Cells were classified as early-stage apoptotic (Annexin V positive, PI negative) or late-stage apoptotic (Annexin V positive, PI positive). To rule out the possibility of primary necrosis, Z-VAD-fmk (50 μmol/L), a pan-caspase inhibitor, or soluble Fas-Fc were added to select cultures. Consistent with an apoptotic phenotype, the addition of Z-

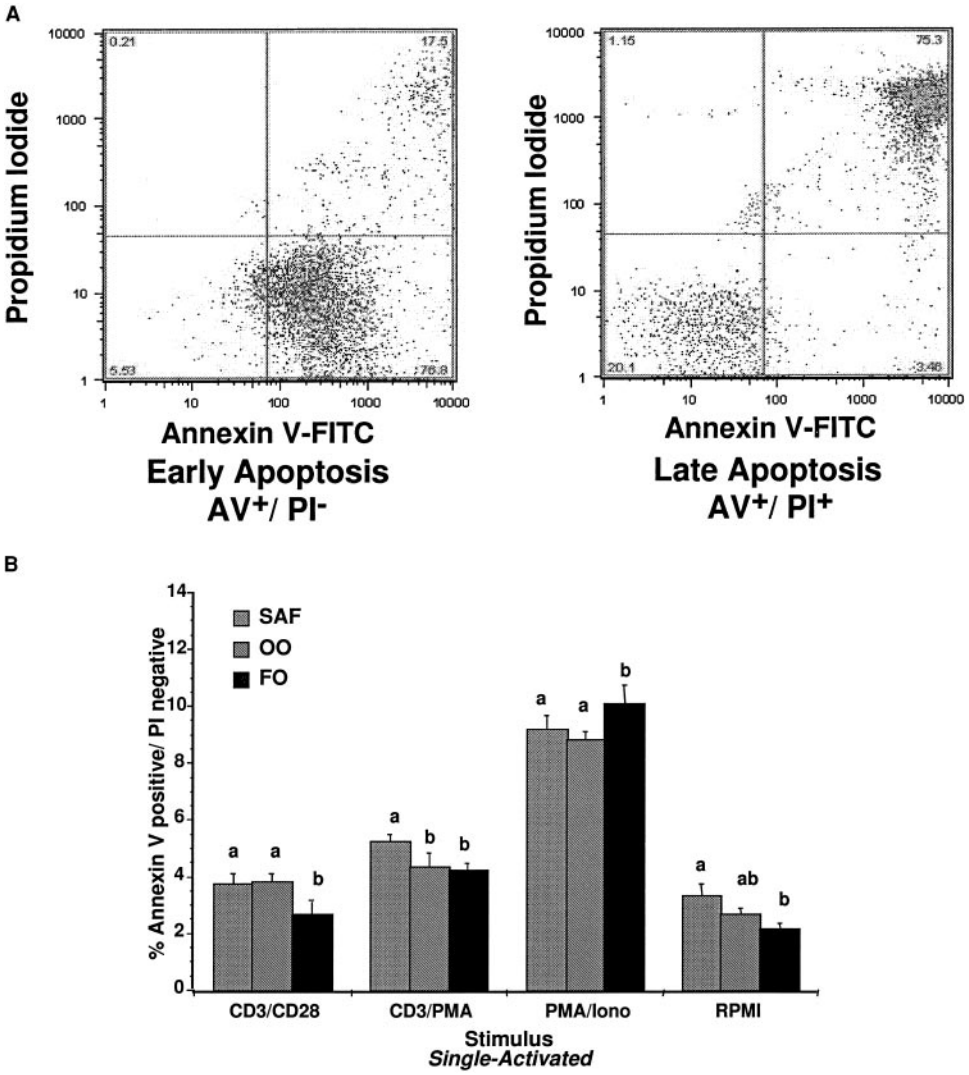

FIGURE 2 Dietary (n-3) PUFA differentially affect T cell apoptosis depending on stimulus. (*A*) Representative fluorescence-activated cell sorting (FACS) images of early- and late-stage apoptosis. Cells were distinguished as being early apoptotic or late apoptotic by Annexin V positive, PI negative (*left panel*) or Annexin V positive/PI positive (*right panel*) staining, respectively. (*B*) T cells (5×10^5/well) from mice fed the test diets were cultured in the presence of αCD3/αCD28, phorbol myristate acetate (PMA)/αCD3, PMA/Ionomycin or RPMI-complete medium for 24 h, followed by Annexin V/PI staining and FACS analysis. Data show cells classified as early apoptotic and represent the means ± SEM, $n = 6$ per group. Different letters denote significant differences within each stimulus group ($P < 0.05$). SAF, safflower oil; OO, olive oil; FO, fish oil [containing (n-3) PUFA].

VAD-fmk and Fas-Fc reduced the number of apoptotic cells by 58.7 and 18.2%, respectively ($P < 0.05$, $n = 3$) (data not shown).

The early apoptotic responses of 24-h single activated T cells taken from mice fed SAF, OO or FO [containing (n-3) PUFA] are shown in Figure 2*B*. Given that the diet effects were different depending on stimulus group ($P = 0.001$), differences among the diets were investigated separately for the individual stimuli. FO increased the percentage of apoptosis relative to SAF ($P = 0.04$) and OO ($P = 0.005$) only in PMA/Ionomycin-stimulated cells. In contrast, FO decreased apoptosis in the αCD3/αCD28 [FO vs. SAF and OO ($P = 0.01$)], αCD3/PMA [FO vs. SAF ($P = 0.02$)], and RPMI-complete medium (negative control) [FO vs. SAF ($P = 0.007$)] stimulated groups. These data indicate that the effect of (n-3) PUFA is dependent on the nature of the stimulus. Additionally, T cells from SAF-, OO- or FO-fed mice were incubated with dexamethasone or FasL ± αFLAG for 24 h to induce apoptosis either pharmacologically or physiologically, respectively. Of interest, FO produced a modest increase in apoptosis ($P = 0.068$) in FasL ± αFLAG-stimulated cells, suggesting that (n-3) PUFA may modulate Fas signaling pathways (data not shown).

Differential effect of cell stimulus on T cell cytokine profiles. Because the nature of the stimulus can result in T cell bias toward specific cytokine secretion profiles (23,35), we analyzed cell culture supernatant cytokines. The ability of selected stimuli to induce naive T cells to produce different cytokine profiles is represented in **Figure 3**. The data show that T cells in all diet groups stimulated with PMA/Ionomycin for 48 h secreted significantly ($P = 0.0001$) more IL-2 than αCD3/αCD28 or αCD3/PMA stimulated T cells. Stimulation with PMA/Ionomycin resulted in cytokine ratios as follows: IFNγ:IL-4 = 2560; IFNγ:IL-2 = 4.5; and IL-4:IL-2 = 0.002. Because Th1 cells secrete high levels of IL-2 and IFNγ, PMA/Ionomycin appears to have generated a Th1-like phenotype (9). In comparison, αCD3/PMA-stimulated T cells secreted significantly ($P = 0.0001$) more IL-10 and IL-4 compared to αCD3/αCD28 or PMA/Ionomycin (Fig. 3), and produced cytokine ratios of: IFNγ:IL-4 = 167.1; IFNγ:IL-2 = 383.6; and IL-4:IL-2 = 2.3. IL-4 and IL-10 are secreted by Th2 polarized cells and are also cross-regulatory cytokines that inhibit Th1 polarization (9). Therefore, these data indicate that αCD3/PMA induced T cells to express a Th2-like cytokine profile. Stimulation with αCD3/αCD28 produced the following cytokine ratios: IFNγ:IL-4 = 278.6; IFNγ:IL-2 = 72.1; and IL-4:IL-2 = 0.26. There were no effects of diet on cytokine production (data not shown).

Dietary lipids alter T cell membrane phospholipid composition. Dietary lipids can be incorporated into cellular mem-

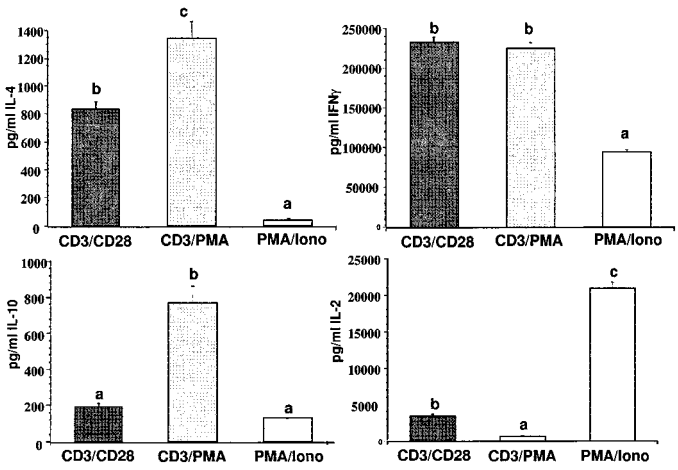

FIGURE 3 Cytokine profiles of T cells from diet-fed mice incubated with selected stimuli. Following 48 h of stimulation with αCD3/αCD28, phorbol myristate acetate (PMA)/αCD3 or PMA/Ionomycin, supernatants were collected and IL-2, IL-4, IL-10 and IFNγ were quantified as described under Materials and Methods. Data represent the means ± SEM from mice pooled across all diets, $n = 18$ per group. Different letters denote significant differences between stimulus groups ($P < 0.05$).

branes and thereby regulate cell function (36). For this reason, we determined the effect of dietary lipid on the fatty acid composition of phospholipid classes in T cell membranes. The PC and PE fatty acid compositions of purified T cell membranes from SAF-, OO- and FO-fed mice are shown in **Table 2** and **Table 3**, respectively. Gas chromatographic analysis revealed fatty acid differences in the PC and PE pools only. In the PC class (Table 2), FO-fed mice had significantly less 18:1(n-7) and 20:4(n-6) and significantly more 18:2(n-6). 18:1(n-7) was significantly enriched in the PC class from OO-fed mice. Docosahexanoic acid [22:6(n-3)], one of the major fatty acids found in fish oil, and 22:5(n-3) were significantly enriched ($P < 0.05$) in the PE class from FO-fed mice (Table 3). These data indicate a remodeling of T cell membrane composition by dietary (n-3) PUFA.

(n-3) PUFA significantly enhance AICD. Because dietary FO may enhance Fas-mediated apoptosis (see above), we determined the effect of (n-3) PUFA on T cell AICD, a process largely regulated by the expression of Fas and FasL (1–3). T cells were initially activated and subsequently restimulated or incubated in RPMI-complete medium as described in Figure 1. The experimental design was effective at inducing AICD. In αCD3/αCD28-stimulated cells, there was a significant 1.5-fold increase in total apoptosis (early- and late-stage apoptosis values of all diet groups) after reactivation [reactivated, 27.2 ± 0.5% vs. unreactivated, 11.3 ± 0.3%, $P = 0.0001$, $n = 46$]. In αCD3/PMA-stimulated cells, there was a significant doubling of total apoptosis [reactivated, 37.4 ± 0.9% vs. unreactivated, 20.9 ± 0.4%, $P = 0.0001$, $n = 48$]. In PMA/Ionomycin-stimulated cells, there was a nearly a doubling in total apoptosis, which was significant [reactivated, 12.5 ± 0.8% vs. unreactivated, 7.5 ± 0.2%, $P = 0.0001$, $n = 48$].

The effect of stimulus among all diet groups on AICD is shown in **Figure 4***A*. Each stimulus was significantly different from the other ($P < 0.05$) in early-stage apoptosis after reactivation, with αCD3/PMA stimulating the greatest degree of apoptosis (42.9 ± 0.5%), and PMA/Ionomycin stimulating the least (8.2 ± 0.3%). The effects of each diet on early apoptosis within particular stimulus groups, αCD3/αCD28, αCD3/PMA and PMA/Ionomycin-stimulated, are illustrated in Figure 4*B–D*, respectively. In PMA/Ionomycin-stimulated (Th1-like) T cells (Fig. 4*D*), dietary FO, independent of dose, was accompanied by a significantly ($P = 0.0008$) greater percentage of early-stage apoptosis than was observed in T cells from control mice fed SAF (Fig. 4*D*). In comparison, no dietary effect was seen when T cells were restimulated with either αCD3/αCD28 or αCD3/PMA (Fig. 4*B*, *C*).

(n-3) PUFA do not induce systemic oxidative stress. To determine whether the ingestion of different lipid sources altered oxidative stress, the liver expression levels of mRNA for three known markers of systemic oxidative stress were evaluated: PPARγ, SRA and CD36 (37). There were no differences among mice fed either the SAF, OO or FO diets with respect to the levels of liver mRNA for PPARγ, SRA or CD36 (data not shown). These results indicate that short-term feeding of (n-3) PUFA does not elevate biomarkers of systemic lipid peroxidation in our model.

DISCUSSION

Among dietary factors, there is overwhelming evidence for a protective effect of (n-3) PUFA in autoimmune/inflammatory diseases (16–19). Additionally, the effects of diets rich in (n-3) PUFA on a variety of T cell functions have been firmly established both in humans and in animal models (16–19). The primary dietary effector molecules are thought to be eicosapentaenoic acid (EPA) [20:5(n-3), EPA] and docosahexaenoic acid (DHA) [22:6(n-3), DHA] (24,32). However, the precise effects of dietary (n-3) PUFA on $CD4^+$ cells, and the mechanisms by which dietary PUFA influence the maintenance of appropriate T cell–subset balance to promote a healthy immune system, have not been elucidated. Previous experiments demonstrated a blunting effect of (n-3) PUFA on T cell proliferation (23,24). Given that the suppressive effects of dietary (n-3) PUFA on the accumulation of inflammatory T cells could result from either reduced proliferation or enhanced apoptosis of activated T cells, or both, we investigated the effect of dietary (n-3) PUFA on T cell apoptosis. Our data clearly show that dietary (n-3) PUFA enhanced T cell apoptosis after in vitro incubation with selective stimuli (Fig. 2*B*).

TABLE 2

Fatty acid composition of T cell phosphatidylcholine in linoleic acid–containing diet (SAF), oleic acid–containing diet (OO) and fish oil–containing diet (FO)[1,2]

Fatty acid	SAF	OO	FO
	mol/100 mol fatty acids		
16:0	34.82 ± 1.38	34.95 ± 1.27	40.65 ± 3.44
18:0	11.33 ± 0.52	10.24 ± 0.64	13.26 ± 3.92
18:1(n-9)	11.11 ± 0.79	12.03 ± 0.13	9.42 ± 0.53
18:1(n-7)	1.45 ± 1.45[b]	5.55 ± 0.29[a]	tr[b]
18:2(n-6)	5.37 ± 0.21[b]	4.13 ± 0.44[b]	7.66 ± 1.29[a]
20:4(n-6)	20.88 ± 1.24[a]	19.58 ± 0.24[a]	9.35 ± 5.72[b]
20:5(n-3)	tr	0.07 ± 0.07	0.44 ± 0.44
22:5(n-3)	tr	tr	1.70 ± 1.09
22:6(n-3)	0.37 ± 0.37	1.36 ± 0.16	2.21 ± 1.37

[1] Only the major fatty acids are listed. Mol percentage values are expressed as means ± SEM, $n = 6$ per group. Different letters denote significant differences between diet groups ($P < 0.05$).

[2] tr, trace (<0.01%); SAF, safflower oil; OO, olive oil; FO, fish oil.

TABLE 3

Fatty acid composition of T cell phosphatidylethanolamine in linoleic acid–containing diet (SAF), oleic acid–containing diet (OO) and fish oil–containing diet (FO)[1]

Fatty acid	SAF	OO	FO
	mol/100 mol fatty acids		
16:0	4.38 ± 3.41	2.87 ± 2.87	2.71 ± 2.71
18:0	24.53 ± 1.54	21.95 ± 1.01	25.42 ± 1.80
18:1(n-9)	7.45 ± 0.72	6.90 ± 0.45	5.94 ± 0.40
18:1(n-7)	1.27 ± 0.65	2.56 ± 0.30	1.56 ± 0.18
18:2(n-6)	3.45 ± 0.95	2.12 ± 0.25	2.87 ± 0.11
20:4(n-6)	23.25 ± 1.53	24.02 ± 2.50	20.57 ± 1.82
20:5(n-3)	tr	tr	tr
22:5(n-3)	tr	tr	4.70 ± 0.27[a]
22:6(n-3)	2.05 ± 1.13[c]	5.40 ± 0.75[b]	11.65 ± 0.19[a]

[1] Refer to Table 2 for legend details.

With regard to the biological relevance of this effect, it is now clear that small changes in apoptosis can profoundly alter downstream events such as cancer and autoimmune disease risk (38). For example, when multipotential-stage (undifferentiated) cells fail to undergo AICD, exponential growth in cell numbers may occur, perturbing the balance between responsiveness and nonresponsiveness (tolerance) (11,39). (n-3) PUFA effects on these mechanisms are consistent with a wealth of literature supporting the contention that diet in general, and dietary fat in particular, is an important determinant of the quantity and quality of the host's immune responses (16–18,30).

Apoptotic death of T cells is an important mechanism for regulating immune responses. There are two distinct pathways of apoptosis: (1) *passive cell death*, mediated in part by the loss of Bcl-2 proteins and (2) *AICD*, primarily regulated by *extrinsic* signals from death receptors, such as Fas (1–5). Passive cell death results from the absence of growth factors and is important for loss of immature T cells. AICD occurs as a result of repeated stimulation through the T cell receptor (TCR), upregulating plasma membrane expression of Fas and its ligand (FasL), thereby acting as a feedback mechanism for terminating an ongoing immune response. It has been shown that T cell subsets differ in their propensity to undergo AICD (13–15). Interestingly, the selected stimuli influenced the spectrum of cytokines produced by the cells in culture (Fig. 3). T cells stimulated with αCD3/PMA secreted more IL-4 and IL-10, Th2 cytokines, whereas PMA/Ionomycin-stimulated T cells secreted IFNγ ≫ IL-4, indicative of a Th1-like phenotype. Because these cells had not been subjected to the selective effects of long-term culture, they likely represent physiologically polarized T cell effector precursors that arise in primary immune responses (14). These data suggest a means by which T cell functional subclasses could be individually analyzed after dietary treatment. The lack of an effect of (n-3) PUFA on cytokine production, although distinct from previous reports (23,24), likely reflects the differences in stimuli and the T cell subset populations examined.

When naive T cells were stimulated with PMA/Ionomycin there was an increase in AICD in cells from mice fed both of the (n-3) PUFA-containing diets (LFFO and HFFO) (Fig. 4*D*). Therefore it is conceivable that the ability of (n-3) PUFA to enhance AICD after PMA/Ionomycin stimulation could be attributable to the induction of a biased pattern of cytokine production in the T cells. These data are consistent with our previous observations showing an increase in IL-4–dependent Th2 proliferation after (n-3) PUFA feeding (23), and indicate that dietary (n-3) PUFA act differently on distinct T cell subsets. These important findings provide evidence for both direct and indirect mechanisms whereby T cell subset functions would be suppressed by dietary (n-3) PUFA.

The elucidation of the mechanisms that regulate apoptosis is important because the failure of T cells to undergo appropriate AICD is associated with a variety of immunopathological diseases, including inflammatory bowel diseases (39). With regard to diet, a critical question remaining to be addressed is the identity of the relevant cellular targets in Th1-like cells that mediate the apoptogenic effects of (n-3) PUFA. AICD depends in part on a FasL-dependent pathway (1–3) and FasL is expressed at higher levels on the surface of activated Th1 cells than Th2 cells (12,13). The fact that (n-3) PUFA enhanced Th1-like AICD in our experiments, combined with a report indicating a 30% increase in FasL expression in splenocytes from (n-3) PUFA-fed mice (40), support the notion that certain dietary lipids upregulate FasL expression in Th1 cells, rendering them more susceptible to AICD. Additional studies are under way to test this hypothesis.

Several intracellular second messengers (e.g., ceramide) have been implicated in the generation of the Fas/FasL death signal and may mediate the (n-3) PUFA-enhanced AICD in Th1 cells. Ceramide is a lipid second messenger cleaved from membrane sphingomyelin by sphingomyelinases. Interaction of FasL with membrane-bound Fas results in a transient and weak activation of a limited number of Fas trimers insufficient to trigger apoptosis but sufficient for acidic sphingomyelinase (ASM) translocation (41). Translocated ASM subsequently hydrolyzes sphingomyelin into ceramide, which spontaneously self-aggregates into membrane microdomains, thereby induc-

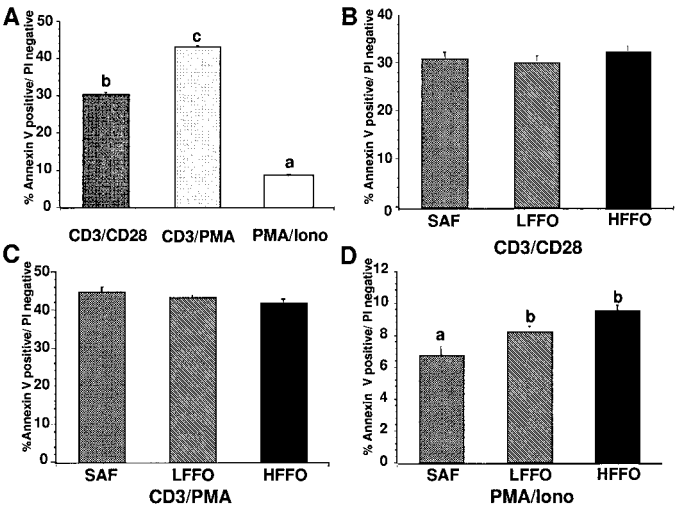

FIGURE 4 (n-3) PUFA significantly enhance activation-induced cell death (AICD) in phorbol myristate acetate (PMA)/Ionomycin–stimulated T cells. (*A–D*) T cells from safflower oil ethyl ester (SAF) 45 , low fat (n-3) PUFA (LFFO) 45 and high fat (n-3) PUFA (HFFO) 45 fed mice were initially activated and restimulated as described in Figure 1. (*A*) The effect of stimulus among all diet groups on AICD. Data represent the means ± SEM, $n = 48$ per stimulus. Different letters denote significant differences between stimulus groups ($P < 0.05$). (*B–D*) The effect of diet on αCD3/αCD28-stimulated, αCD3/PMA-stimulated and PMA/Ionomycin-stimulated T cells, respectively. Data represent the means ± SEM, $n = 12$ per diet group. Different letters denote significant differences between diet groups ($P = 0.0008$).

ing Fas receptor clustering and apoptosis (41). Interestingly, we have previously demonstrated (24) that dietary (n-3) PUFA are capable of modulating ceramide levels in murine splenic T cells. In addition, our data indicate that (n-3) PUFA modify the fatty acid composition of membrane PC and PE (Tables 2 and 3) in purified T cells. Because membrane fatty acid content can influence sphingomyelin hydrolysis (42), these data suggest that ceramide formation, and hence AICD, could be modulated via the incorporation of (n-3) PUFA into specific membrane phospholipid pools.

Within the T cell plasma membrane, there are specific detergent-resistant domains in which key signal transduction proteins are localized (43). These so-called lipid rafts are composed mostly of cholesterol and sphingolipids and do not integrate well into the fluid phospholipid bilayers, thereby forming microdomains. Upon T cell activation, rafts compartmentalize the activated TCR and associated signal-transducing molecules, thus providing an environment conducive to signal transduction (44). With respect to apoptosis, Fas and FasL translocate to lipid rafts after stimulation (41). Our data demonstrating that dietary PUFA remodel the membrane composition of T cells (Tables 2 and 3) provide evidence for a direct diet effect on membrane properties. This is supported by recent in vitro studies using a Jurkat T cell line, where (n-3) PUFA enrichment selectively modified lipid rafts and suppressed signal transduction (45). Interestingly, conditions that modify raft structure can disrupt T cell signaling events (43). Recent studies indicate that the macromolecular complex organization in lipid rafts is distinct in T cell subsets (44,46), suggesting that these subsets could respond differently to dietary PUFA-induced perturbation. Experiments are currently under way to determine the effects of dietary (n-3) PUFA on the lipid composition of T cell rafts in our model.

Long-chain (n-3) PUFA are highly susceptible to lipid peroxidation (47). This is significant because the formation of reactive oxygen species and/or glutathione depletion can regulate signals involved in AICD that contribute to T cell deletion (48). Interestingly, we saw no change in systemic oxidative stress after ingestion of different lipid sources. This indicates that short-term feeding of (n-3) PUFA did not promote the generation of proapoptotic reactive oxygen species in our model.

Taken together, these data support our hypothesis that dietary (n-3) PUFA preferentially suppress functions of a T cell subset induced to secrete a biased cytokine pattern resembling Th1 cells in mice in part by increasing AICD in these cells. Furthermore, a comparison of LFFO vs. HFFO demonstrated that the effect seen on AICD can occur efficiently at low (n-3) PUFA intakes. These novel findings contribute importantly to the elucidation of the mechanisms by which dietary (n-3) PUFA selectively modulate T cell subset function. Our results may contribute to the establishment of dietary guidelines designed to promote a balanced immune system, so that protective host responses (e.g., to infectious agents) can be maintained, while potentially detrimental host responses (e.g., chronic inflammation and hypersensitivity) can be controlled appropriately. Further studies are needed to determine the precise cellular and molecular mechanisms by which dietary (n-3) PUFA differentially modulate AICD in Th subsets.

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