Docosahexaenoic Acid Suppresses Function of the CD28 Costimulatory Membrane Receptor in Primary Murine and Jurkat T Cells¹

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ABSTRACT  (n-3) Polyunsaturated fatty acids (PUFA) have been widely documented to reduce inflammation in diseases such as rheumatoid arthritis. This study sought to elucidate the mechanism whereby (n-3) PUFA downregulate T-cell proliferation. We hypothesized that membrane incorporation of dietary PUFA would alter membrane structure and consequently membrane receptor function. Female C57BL/6 mice were fed for 14 d one of three diets containing arachidonic acid (AA), fish oil or docosahexaenoic acid (DHA) that varied in lipid composition only. Spleens were harvested and T cells (~ 90% purity) were activated with agonists that stimulated proliferation at the receptor level [anti-CD3 (αCD3)/anti-CD28 (αCD28)], intracellularly [phorbol-12-myristate-13-acetate (PMA)/ionomycin] or with a combined receptor/intracellular agonist (αCD3/PMA). Although there was no significant difference (P > 0.05) in proliferative response across dietary groups within each agonist set, interleukin (IL)-2 secretion was significantly reduced (P = 0.05) in cells from DHA-fed mice stimulated with αCD3/αCD28. In parallel in vitro experiments, Jurkat T cells were incubated with 50 μmol/L linoleic acid, AA, or DHA. Similar agonists sets were employed, and cells incubated with DHA and AA had a significantly reduced (P < 0.05) IL-2 secretion in three of the agonist sets. However, only when the CD28 receptor was stimulated was there a significant difference (P < 0.05) between DHA and AA. The results of this study suggest the involvement of the CD28 receptor in reducing IL-2 secretion in DHA-fed mice and DHA-incubated Jurkat cells and that purified T cells from DHA-fed mice require accessory cells to modulate proliferative suppression.

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KEY WORDS: • T cell • Jurkat cell • docosahexaenoic acid • fish oil • arachidonic acid • mice

Rheumatoid arthritis affects ~2.1 million Americans and is the number one cause of disability in the United States (Centers for Disease Control and Prevention 2000). In addition, by the year 2020, the number of cases of arthritis will increase to 60 million Americans. The inflammation experienced in this disease is due in part to hyperproliferation of T cells and the subsequent production of proinflammatory cytokines. Current disease is due in part to hyperproliferation of T cells and the subsequent production of prostaglandins. Current pharmacologic means to reduce this inflammation include nonsteroidal anti-inflammatory drugs (NSAIDS).3 These drugs function by blocking the action of cyclooxygenases, thereby decreasing prostaglandin formation and the subsequent inflammatory response. However, due to side effects, NSAIDS generally have to be discontinued after ~2 y (Kremer 1991, Pincus and Callahan 1993). In contrast, human clinical and epidemiologic studies, as well as murine disease models, have clearly shown dietary fish oil to possess anti-inflammatory properties, thus mediating the same net result as NSAIDS without the associated side effects (Cathcart et al. 1987, Geusens et al. 1994, Kremer 1991). In fact, double-blind, placebo-controlled clinical trials showed that supplementation over a 12-wk period with fish oil containing 2.7–3.6 g/d of eicosapentaenoic acid (EPA) [20:5(n-3)] and docosahexaenoic acid (DHA) [22:6(n-3)], the two major (n-3) polyunsaturated fatty acids (PUFA) found in fish oil, led to significant improvement in the number of tender and swollen joints, grip strength and global disease activity (Kremer 1991, Nielsen et al. 1992). Another trial showed that supplementation for 6 mo with 3.0 g/d of EPA and DHA resulted in a significant decrease in NSAID usage (Skoldstam et al. 1992). Fish oil (FO) trials have also proven beneficial in additional inflammatory diseases such as autoimmune nephritis (Prickett et al. 1983) and systemic lupus erythematoses (Watson et al. 1988).

Although the primary effector molecules in fish oil are thought to be the (n-3) PUFA, EPA and DHA, the mechanisms involved in defining the ability of EPA or DHA to modulate T-cell function have not been elucidated. In addition, a comparison of the overall effects of FO vs. specific (n-3) and (n-6) PUFA are seldom addressed within the same dietary study.
We demonstrated previously that short-term dietary exposure to highly purified EPA and DHA suppresses mitogen-induced mouse T-cell proliferation by inhibiting interleukin-2 (IL-2) secretion and IL-2 receptor α mRNA expression, and these events are accompanied by reductions in the production of essential lipid second messengers, diacylglycerol (DAG) and ceramide (Jolly et al. 1997 and 1998). Because DAG and ceramide are part of the intracellular T-cell signaling cascade and lipids can alter membrane domain organization (Anel et al. 1993, Conroy et al. 1986, Malis et al. 1990), we hypothesized that dietary FO and DHA suppress T-cell activation by altering T-cell receptor (TCR)-mediated function, subsequently downregulating intracellular signaling.

Because our previous studies utilizing (n-3) PUFA esters involved either whole mixed populations of splenocytes (Hosack-Fowler et al. 1993b, Jolly et al. 1996, 1997 and 1998) or splenic lymphocytes enriched by nylon wool columns (Hosack-Fowler et al. 1993a), it is essential that the effect of dietary FO and DHA on purified T lymphocytes (where the TcR has been quantified) activated in the virtual absence of accessory cells, be determined. Thus, in this study, we probed TCR-mediated events by measuring DNA synthesis and IL-2 production to determine the ability of select PUFA to modulate T-cell receptor vs. intracellular costimulation in purified splenic T lymphocytes and Jurkat human T cells. Because T lymphocytes require a dual signal stimulation via both the TcR and CD28 to become fully activated (Schlom and Hodge 1999), we selected pairs of agonists that would either provide this dual receptor stimulation or bypass it. Therefore, if dietary PUFA affect TCR function, bypassing the receptor would abrogate the dietary effect. This information will allow us to draw mechanistic conclusions concerning the specific mode of action of (n-3) PUFA on the T lymphocyte, which is critical for the eventual development of recommendations to the public regarding FO supplementation.

MATERIALS AND METHODS

Diet and animals. All experimental procedures using laboratory animals were approved by the University Laboratory Animal Care Committee of Texas A&M University. Female pathogen-free young (12–14 g) C57BL/6 mice were purchased from Frederick National Cancer Research Facility, Frederick, MD. Mice were assigned randomly to one of three diets and housed in polycarbonate microisolator cages behind an RO1 pathogen barrier. Mice had free access to manger diet composition, expressed in g/kg, was as follows: 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 β-tocopherol, 2 g choline chloride, 0.2 g tertiary butyl hydroquinone, 40 g corn oil (CO) and 10 g PUFA (AIN 1977). The three diet groups varied by PUFA lipid source onl

| Fatty acid composition of the experimental diets |
|-----------------------------------------------|
| Fatty acid | AA | DHA | FO |
|-----------|----|-----|----|
| 14:0      | 0.00 | 0.00 | 3.54 |
| 16:0      | 12.46 | 11.86 | 15.60 |
| 16:1(n-7) | 0.70 | 0.00 | 0.88 |
| 18:0      | 3.55 | 2.33 | 2.41 |
| 18:1(n-7) | 28.21 | 29.16 | 25.92 |
| 18:2(n-6) | 44.18 | 46.67 | 44.01 |
| 18:3(n-6) | 0.73 | 0.00 | 0.00 |
| 18:3(n-3) | 1.23 | 0.00 | 1.05 |
| 20:0      | 0.96 | 0.00 | 0.00 |
| 20:1(n-9) | 0.93 | 0.00 | 0.00 |
| 20:3(n-6) | 0.00 | 0.00 | 0.00 |
| 20:4(n-6) | 7.04 | 0.00 | 0.00 |
| 20:5(n-3) | 0.00 | 0.00 | 2.10 |
| 22:5(n-3) | 0.00 | 0.00 | 1.18 |
| 22:6(n-3) | 0.00 | 9.98 | 1.05 |
| Total SFA | 16.97 | 14.19 | 21.55 |
| Total MUFAs | 29.84 | 29.16 | 26.80 |
| Total (n-6) PUFA | 51.95 | 46.67 | 44.01 |
| Total (n-3) PUFA | 1.23 | 9.98 | 3.15 |

1 Only the major fatty acids are listed.

TABLE 1

Isolation and preparation of splenic lymphocytes. Mice were killed via CO2 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% fetal bovine serum (Irvine Scientific), 1 × 10^5 U/L penicillin and 100 μg/mL streptomycin (Irvine Scientific), 2 mmol/L L-glutamine, and 10 μmol/L 2-mercaptoethanol] (Jolly et al. 1997). Spleens were dispersed with glass homogenizers and passed through a 149-μm wire mesh filter to create single-cell suspensions. Splenocytes were 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) in accordance with the manufacturer’s protocol. Subsequently, 60–90 × 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 purity analysis, proliferation or cytokine assays. The purity of the T-cell population was analyzed by flow cytometry (FACScan; Becton-Dickenson, Bedford, MA) as previously described by Darzynkiewicz and Crissman (1990). Using anti-CD3 (aCD3) antibody conjugated to fluorescein isothiocyanate, the T-cell population purity was determined to be ~90%.

T-lymphocyte proliferation assay. Purified splenic T cells from mice fed the test lipids were cultured at 2 × 10^3 cells per well in 96-well round-bottomed microtiter plates (Falcon, Becton-Dickenson, Lincoln Park, NJ) with triplicate wells for each diet treatment. Cells were cultured in the presence of the following agonists: 2.5 mg/L T-cell mitogenic lectin concanavalin A (Con A) (Sigma, St. Louis, MO); 1 mg/L plate-bound purified hamster anti-mouse CD3ε (aCD3) monoclonal antibody (MCA) (Pharmingen, San Diego, CA) with 5 mg/L soluble purified hamster anti-mouse CD28 (aCD28) MCA (Pharmingen); 1 μg/L phorbol-12-myristate-13-acetate (PMA) (Sigma) with 10 mg/L bound purified hamster anti-mouse CD3ε MCA; and 10 μg/mL PMA with 50 mmol/L ionomycin (Calbiochem-Novabiochem, San Diego, CA). Concentrations were form from the NIH Test Materials Program (Charleston, SC); AA (44.3% fatty acid purity) was obtained in triglyceride form from Martek Biosciences (Columbia, MD); vacuum-deodorized menhaden oil [13.1% as 20:5(n-3), 9.7% as 22:6(n-3)] was provided by the NIH Test Materials Program; and CO [57.3% as 18:2(n-6)] was obtained from Traco Labs (Champaign, IL).
DHA SUPPRESSES CD28 RECEPTOR FUNCTION

RESULTS

Lipid extraction and fatty acid analysis. To monitor the in vivo incorporation of dietary fatty acids into cellular lipids during feeding, livers were removed from each mouse during necropsy, flash frozen in liquid nitrogen and stored at −80°C. For analysis, livers were thawed, homogenized in 0.1 M KCl, mixed with chloroform/methanol (2:1, v/v), centrifuged at 500 × g and the lower phase transferred to a leak-proof 12-ml tube. Samples were dried under nitrogen and methylated at 76°C for 15 h in 6% methanolic HCl. Resultant fatty acid methyl esters were dried under nitrogen, dissolved in methylene chloride and analyzed by GC (Chapkin and Cole 1991).

Statistical analysis. One-way ANOVA was used to evaluate between-subjects effect of diet (AA, DHA, FO) for each agonist pair and their potential interaction on proliferation and IL-2 production in purified splenic T lymphocytes. Analyses were computed using superANOVA statistical software (Berkeley, CA), and significance was accepted at α = 0.05 for all tests. Analysis of the effect of DHA concentration on proliferation was analyzed using the regression option in Minitab Release 13 for Windows (www.minitab.com; State College, PA). Values in the text are means ± SEM.

Livers were removed from each mouse during necropsy, flash frozen in liquid nitrogen and stored at −80°C. For analysis of fatty acid incorporation, lipid was extracted as described in Materials and Methods. Values (g/100 g) represent means ± SEM, n = 6 (AA) or 4 (DHA and FO). Means denoted by different letters are significantly different (P = 0.0001) within each fatty acid.

In vivo incorporation of (n-3) fatty acids. Food intakes and weight gains did not differ among groups [final body weights: AA, 19.2 ± 0.6 g (n = 6); DHA, 19.6 ± 0.9 g (n = 5); FO, 19.8 ± 1.0 g (n = 6)]. In vivo incorporation of dietary lipids was verified by liver total lipid analysis (Fig. 1). As expected, the AA content was higher (P < 0.05) in mice fed the AA-enriched diet relative to mice fed the DHA or FO diets. Similarly, DHA content was significantly higher (P < 0.05) in mice fed the DHA-enriched diet, and the DHA and EPA contents were significantly higher (P < 0.05) in mice fed FO relative to those fed the AA diet.

Dietary effect on agonist-induced murine T-cell proliferation. To determine the effect of dietary (n-3) PUFA on T-cell activation, purified murine splenic T cells were stimulated with the following: αCD3/αCD28 for receptor-mediated stimulation; αCD3/αCD28, in which PMA would activate intracellular protein kinase C, thus bypassing the need for the CD28 receptor; or PMA/ionomycin in which ionomycin would increase intracellular calcium levels and combine with PMA to abrogate the need to engage either the CD3 or CD28 membrane receptors. All agonist pairs were added with and without exogenous recombinant murine IL-2. Proliferation was assessed by [3H]-thymidine incorporation within each agonist, across all dietary groups (Fig. 2). When exogenous IL-2 was added to PMA/ionomycin, T cells from FO-fed mice exhibited a significantly lower level of proliferation relative to cells from AA- (P = 0.02) and DHA-fed mice (P = 0.02). All other proliferative responses within each agonist group between diets were not significantly different from each other (P > 0.05). When comparisons were made between agonist groups

determined by conducting proliferation assays at various concentrations of agonist pairs. Concentrations listed are those that produced maximal proliferation without compromising viability (>90% viable cells) (data not shown). All agonists, with the exception of Con A and the RPMI negative control, were incubated with and without 1 × 105 U/L recombinant murine IL-2 (R&D Systems).

Cells were incubated at 37°C in an atmosphere of 5% CO2 in air for 72 h. For the final 6 h, cells were incubated in the presence of 1.0 μCi [3H]-thymidine/well (New England Nuclear, North Bellerca, MA). Cells were harvested onto glass fiber filter paper discs (Whatman, Maidstone, England) using a multiple automated sample harvester unit (MASH II; MA Bioproducts, Walkersville, MD). Cellular uptake of [3H]-thymidine was measured using a liquid scintillation counter (LS 8000, Beckman Instruments, Irvine, CA). Results are expressed as the mean disintegrations per minute (dpm) (stimulated − control) of triplicate cultures (Hosack-Fowler et al. 1993a).

Mouse and human T-cell interleukin-2 quantitation. Cells were cultured for 48 h as previously described (Jolly et al. 1997). Supernatants from splenic T cells or Jurkat cells were harvested and the RPMI negative control, were incubated with and without 1 pg/200,000 cells. All agonists, with the exception of Con A, were supplemented with one of three fatty acids (FA), i.e., LA, AA or DHA complexed to bovine serum albumin (BSA) at a mole ratio of 1:3 (FA/BSA) to a final concentration of 50 μmol/L (Lynch 1990). This concentration is physiologically relevant because it lies well within the range of blood levels in human subjects supplemented with DHA (Conquer and Holub 1998). Fresh medium containing FA-BSA was provided daily. After a 72-h incubation, cells were resuspended in FA-free medium (2 × 106 cells/well) of a 96-well plate) containing one of the following five stimuli for 16 h: S1, medium only; S2, PMA (1 μg/L) + ionomycin (0.5 μmol/L); S3, PMA (1 μg/L) + ionomycin (0.5 μmol/L); S4, PMA (1 μg/L) + ionomycin (0.5 μmol/L) + αCD28 (5 mg/L); S5, αCD3 (25 mg/L) + αCD28 (5 mg/L) + αCD28 (5 mg/L). Concentrations were based on the findings of Sadra et al. (1999) and pilot studies to determine maximal IL-2 production (data not shown). At the end of the 16-h stimulation period, supernatants were harvested by centrifugation at 200 × g, and IL-2 levels in the supernatants were measured using the Hu IL-2 Cytokine Direct ELISA Kit from R&D Systems. Results are shown as net (stimulated − control) IL-2 levels in pg/200,000 cells.

Lipid extraction and fatty acid analysis. To monitor the in vivo incorporation of dietary fatty acids into cellular lipids during feeding, livers were removed from each mouse during necropsy, flash frozen in liquid nitrogen and stored at −80°C. For analysis, livers were thawed, homogenized in 0.1 M KCl, mixed with chloroform/methanol (2:1, v/v), centrifuged at 500 × g and the lower phase transferred to a leak-proof 12-ml tube. Samples were dried under nitrogen and methylated at 76°C for 15 h in 6% methanolic HCl. Resultant fatty acid methyl esters were dried under nitrogen, dissolved in methylene chloride and analyzed by GC (Chapkin and Cole 1991).

Statistical analysis. One-way ANOVA was used to evaluate between-subjects effect of diet (AA, DHA, FO) for each agonist pair and their potential interaction on proliferation and IL-2 production in purified splenic T lymphocytes. Analyses were computed using superANOVA statistical software (Berkeley, CA), and significance was accepted at α = 0.05 for all tests. Analysis of the effect of DHA concentration on proliferation was analyzed using the regression option in Minitab Release 13 for Windows (www.minitab.com; State College, PA). Values in the text are means ± SEM.
across all dietary groups, T cells stimulated with PMA-containing agonists proliferated most vigorously regardless of diet (P < 0.05). The net dpm for the three agonists were as follows: PMA/ionomycin = 405,134 ± 11,161 (n = 15); PMA/αCD3 = 369,309 ± 11,410 (n = 15); αCD3/αCD28 = 244,866 ± 9,552 (n = 15). Con A stimulation was lower relative to other agonist pairs as expected, due to the reduction of accessory cells during purification. All agonist treatments with the exception of IL-2 add-backs (i.e., antibodies vs. antibodies + rIL-2) exhibited significantly different (P < 0.05) levels of stimulation from each other. IL-2 was added back to these cultures to attempt to reverse the expected diet-induced decrease in T-cell proliferation.

**Effect of dietary lipid on purified murine T-cell interleukin-2 (IL-2) production.** IL-2 is a potent autocrine and paracrine polyclonal T-cell growth factor. Therefore, concentrations of immunoreactive IL-2 protein were measured. T-cells from DHA-fed mice secreted less IL-2 relative to AA- and FO-fed mice when T cells were activated via the TcR and CD28 receptors (Fig. 3; P = 0.05). Conversely, αCD3/PMA- and PMA/ionomycin-stimulated T cells secreted levels of IL-2 that were not different. The use of PMA-containing agonists, irrespective of diet, caused T cells to secrete approximately twofold greater levels of IL-2 (P < 0.001) than did stimulation with αCD3/αCD28 [αCD3/PMA, 5240 ± 228 pg/200,000 cells (n = 10); PMA/ionomycin, 4954 ± 227 pg/200,000 cells (n = 14); αCD3/αCD28, 2339 ± 250 pg/200,000 cells (n = 12)]. As expected, all stimuli increased IL-2 secretion relative to the unstimulated (RPMI) control cells.

**Correlation between T-cell proliferation and IL-2 production.** The precise nature of the relationship between IL-2 production and T-cell proliferation was determined in T-cell cultures by performing a regression analysis (Fig. 4). The relationship was positively correlated at IL-2 levels below ~4000 pg/200,000 cells. However, as IL-2 concentrations increased above 5400 pg/200,000 cells, proliferation reached a plateau. The $R^2$ value for this quadratic is 0.93 ($P < 0.0001$). Linear regression analyses were conducted within each diet group and produced the following $R^2$ values: AA, 0.91; DHA, 0.81; and FO, 0.87 ($P < 0.001$ for each). Thus, proliferation and IL-2 production were highly correlated within each diet group.

**Effect of in vitro fatty acid incubation on IL-2 secretion in Jurkat cells.** To determine whether the effect of dietary (n-3) PUFA on IL-2 production by purified murine T-cell splenocytes could be reproduced under in vitro culture conditions, we conducted a similar study using Jurkat cells, a human leukemia T-cell line, which serves as a model for T-cell activation (Weiss et al. 1984). Jurkat cells were incubated in media containing the same purified fatty acids as described in Materials and Methods. The enrichment of culture medium with AA or DHA significantly decreased IL-2 protein secretion ($P < 0.05$), relative to control treatments (media-only, or LA), when cells were stimulated with PMA-containing agonists (Fig. 5). Furthermore, DHA-enriched cultures stimulated with PMA/ionomycin/αCD28 secreted significantly less IL-2 protein ($P < 0.05$) relative to AA-enriched cultures.

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**FIGURE 2** Effect of dietary fatty acids on T-cell proliferation in splenic T lymphocytes of mice fed diets enriched in arachidonic acid (AA; dotted bars), docosahexaenoic acid (DHA; hatched bars) or fish oil (FO; grey bars) for 14 d. Cells were isolated, purified and stimulated with various agonists as described in Materials and Methods. Values (AA, n = 6; DHA, n = 5; FO, n = 4) represent the mean ± SEM of net thymidine uptake (dpm). Different letters denote significant differences within each agonist group (P < 0.05). Con A, concanavalin A; Abs, αCD3/αCD28 antibodies; 3/P, αCD3/phorbol-12-myristate-13-acetate (PMA); P/I, PMA/ionomycin.

**FIGURE 3** Effect of dietary fatty acids on interleukin (IL)-2 production by purified murine splenic T cells from mice fed diets enriched in arachidonic acid (AA; dotted bars), docosahexaenoic acid (DHA; hatched bars) or fish oil (FO; grey bars) for 14 d. Cells were cultured with various agonists for 72 h. Interleukin (IL)-2 in the culture supernatant was quantitated by ELISA as described in Materials and Methods. Values (AA, n = 6; DHA, n = 4; FO, n = 3) represent the mean ± SEM in pg/200,000 cells. Different letters denote significant differences within each agonist group (P < 0.05). R, RPMI; Abs, αCD3/αCD28 antibodies; 3/P, αCD3/phorbol-12-myristate-13-acetate (PMA); P/I, PMA/ionomycin.

**FIGURE 4** Relationship between interleukin (IL)-2 secretion and cell proliferation in purified murine splenic T-cell cultures from mice fed diets enriched in arachidonic acid (AA), docosahexaenoic acid (DHA) or fish oil (FO) for 14 d. Data from all agonists and all diet groups were combined and analyzed. The regression analysis was conducted using Minitab for Windows ($y = -0.013x^2 + 140.73x + 5110.4; R^2 = 0.93$ and $P < 0.0001$).
A plethora of studies demonstrate an anti-inflammatory effect of dietary (n-3) PUFA in humans and rodents. Researchers have conducted experiments using whole oils (Avula et al. 1999, Fernandes 1994, Fernandes and Jolly 1998), whole oils and purified ethyl esters (Soyland et al. 1993a, b) or (n-9), (n-6) and (n-3) PUFA (Emken et al. 1999, Stulnig et al. 1998). Previously, we compared the effects of purified (n-3) and (n-6) ethyl esters (Hosack-Fowler et al. 1993a, Jolly et al. 1997 and 1998). Clinical trials have employed either whole oils (Cleland et al. 1988, Kremer et al. 1990, Lau et al. 1993, van der Tempel et al. 1990) or (n-3) vs. (n-6) PUFA (Nielsen et al. 1992). Other investigators have altered the ratio of EPA/DHA (Sasaki et al. 1993, Volker et al. 2000) and have shown that EPA is more effective than DHA in suppressing the inflammatory response. Because it is difficult to reconcile the variable results of studies employing such widely varying sources of (n-3) PUFA, we have attempted to incorporate many of these dietary aspects [purified ethyl esters, (n-6) vs. (n-3), whole oil vs. ethyl ester] into one comprehensive study. On the basis of previous work, which documented that (n-3) fatty acids mediate suppression of T-cell proliferation (Jolly et al. 1998) compared with (n-6) PUFA, we selected AA [20:4(n-6)] as our control lipid.

To determine the relative effect of diet on membrane receptors and intracellular components of the T-cell activation pathway, we measured the effects of PUFA and FO on T-cell membrane-receptor function by stimulating T cells at the receptor level vs. stimulation with agonists that upregulate intracellular components of the T-cell signaling cascade by selecting agonist pairs that distinguish between activating pathways. The antibodies (αCD3/αCD28) stimulated the T cell via the TcR/CD3 complex and the CD28 membrane costimulatory ligand. Alternatively, we used a combined receptor/intracellular agonist, αCD3/αCD28, to bypass the CD28 membrane receptor. PMA is an agonist that mimics DAG (Manger et al. 1987) and thus can activate T lymphocytes. Finally, we used an agonist pair that bypassed the need for a membrane receptor entirely, PMA/ionomycin. Ionomycin is a calcium ionophore, which can activate calcineurin directly (Stoffel et al. 1998). In the proliferation experiment, there was no significant dietary effect on T-cell proliferation within each agonist pair. This result was surprising because we reported a dramatic suppression of Con A–induced lymphocyte proliferation in cultures containing whole splenocytes from mice fed DHA and EPA (Jolly et al. 1998). In addition, Hosack-Fowler (1993a) conducted an experiment in which lymphocytes from each diet group and macrophages from each diet group were combined for a total of nine different treatments. The resulting data indicated that dietary (n-3) fatty acids alter Con A–induced T-cell proliferation by influencing lymphocytes only, and it was concluded that the role of accessory cells in PUFA-mediated suppression of T-cell proliferative responses to Con A was minimal. However, other research has shown that accessory cells are indeed critical to the (n-3) PUFA response. Soyland et al. (1993) showed that preincubation of human T cells with accessory cells was necessary to promote an in vitro inhibitory effect by DHA on superantigen-induced T-cell proliferation. Nishiyama-Naruke and Curi (2000) recently demonstrated that the presence of macrophages was necessary to mediate a PUFA effect on proliferation in Con A–stimulated cultures, and that phosphatidylcholine, a lymphocyte inhibitor, was transferred from macrophages to T cells. It is important to note, however, that both Con A and super antigens require direct accessory cell-to-T-cell contact to mediate a response. Thus, the systems employed by Jolly et al. (1998), Hosack-Fowler et al. (1993a), Soyland et al. (1993) and Nishiyama-Naruke and Curi (2000) are accessory-cell dependent. In addition, accessory cells potentially have two roles, i.e., as contact-dependent antigen-presenting cells, and as contact-independent bystander cells secreting soluble mediators to modulate T-cell proliferation. In our current experiment, we used agonists that do not require accessory cells for T-cell proliferation; thus, our results reflect an accessory cell–independent system. However, because the removal of accessory cells essentially eliminated the effect of PUFA-induced T-cell suppression observed in previous studies, we hypothesize that accessory cells are necessary to provide a “bystander effect.” The bystander role of accessory cells (including both macrophages and B cells) in (n-3) PUFA-mediated T-cell suppression will be examined in future experiments.

The ability of DHA to suppress T-cell IL-2 production (Figs. 3 and 5) is consistent with results seen using unenriched splenic mononuclear cells (Jolly et al. 1997) and in human studies (Endres et al. 1993). This effect is seen when αCD28 is employed, i.e., Figure 3 shows that IL-2 secretion is suppressed in antibody-stimulated (αCD3/αCD28) cells from DHA-fed mice, but not in cells stimulated by αCD3/αCD28 and F M 2 (1993a) conducted an experiment in which lymphocytes from each diet group and macrophages from each diet group were combined for a total of nine different treatments. The resulting data indicated that dietary (n-3) fatty acids alter Con A–induced T-cell proliferation by influencing lymphocytes only, and it was concluded that the role of accessory cells in PUFA-mediated suppression of T-cell proliferative responses to Con A was minimal. However, other research has shown that accessory cells are indeed critical to the (n-3) PUFA response. Soyland et al. (1993) showed that preincubation of human T cells with accessory cells was necessary to promote an in vitro inhibitory effect by DHA on superantigen-induced T-cell proliferation. Nishiyama-Naruke and Curi (2000) recently demonstrated that the presence of macrophages was necessary to mediate a PUFA effect on proliferation in Con A–stimulated cultures, and that phosphatidylcholine, a lymphocyte inhibitor, was transferred from macrophages to T cells. It is important to note, however, that both Con A and super antigens require direct accessory cell-to-T-cell contact to mediate a response. Thus, the systems employed by Jolly et al. (1998), Hosack-Fowler et al. (1993a), Soyland et al. (1993) and Nishiyama-Naruke and Curi (2000) are accessory-cell dependent. In addition, accessory cells potentially have two roles, i.e., as contact-dependent antigen-presenting cells, and as contact-independent bystander cells secreting soluble mediators to modulate T-cell proliferation. In our current experiment, we used agonists that do not require accessory cells for T-cell proliferation; thus, our results reflect an accessory cell–independent system. However, because the removal of accessory cells essentially eliminated the effect of PUFA-induced T-cell suppression observed in previous studies, we hypothesize that accessory cells are necessary to provide a “bystander effect.” The bystander role of accessory cells (including both macrophages and B cells) in (n-3) PUFA-mediated T-cell suppression will be examined in future experiments.

The ability of DHA to suppress T-cell IL-2 production (Figs. 3 and 5) is consistent with results seen using unenriched splenic mononuclear cells (Jolly et al. 1997) and in human studies (Endres et al. 1993). This effect is seen when αCD28 is employed, i.e., Figure 3 shows that IL-2 secretion is suppressed in antibody-stimulated (αCD3/αCD28) cells from DHA-fed mice, but not in cells stimulated by αCD3/αCD28 and PMA/ionomycin; Figure 5 shows that only when PMA/ionomycin is used in conjunction with an antibody to CD28 can robust suppression of IL-2 secretion by the AA compared with DHA treatment be observed. Thus, one mechanism of action of PUFA on T cells may be to alter the function of the CD28 costimulatory receptor. The inability of FO to suppress IL-2 secretion may be related to its relatively low content of DHA, 1 vs. 10 g/100 g fat (acid) in the DHA diet.

Interestingly, there was a selective dietary effect in antibody-stimulated cells on IL-2 secretion (>50% suppression) (Fig. 3) and proliferation (no suppression) (Fig. 2). Because
IL-2 is the primary autocrine and paracrine growth factor for T cells, we expected parallel results when comparing IL-2 suppression to proliferation. Additionally, a strong positive correlation ($R^2 = 0.93$) existed when IL-2 secretion was plotted vs. proliferation. This complex phenotype may be explained by the fact that the cultures contained a mixture of CD4 Th1 and Th2 cells plus CD8 cells, all of which could respond to the agonists employed. Because CD4 Th1 and CD8 memory cells secrete primarily IL-2 and CD4 Th2 cells secrete IL-4 as their principal growth factor, the IL-2 data do not represent all of the cell types that are contributing to the proliferation data (Esser et al. 1997).

Jurkat cells are malignant transformed (Weiss et al. 1984) and they proliferate without stimulation. Thus, the relationship between IL-2 production and proliferation could not be evaluated in this cell line. However, a reduction in IL-2 secretion in FA-supplemented Jurkat cells was evident. Interestingly, a difference between (n-6) and (n-3) PUFA was seen only when the agonist involved the CD28 receptor. This correlates with our ex vivo data in which decreased IL-2 secretion was noted in the αCD3/αCD28 agonist pair from DHA-fed mice. Taken together, these data suggest that PUFA modulate CD28 receptor function of human and murine T cells.

In our earlier studies, PUFA suppressed Con A–induced T-cell proliferation in unenriched splenic mononuclear cells (Jolly et al. 1997). Jolly and colleagues (1998) also demonstrated that these cells produced less IL-2 protein and less mRNA for the IL-2 receptor α-chain. Therefore, we attempted to rescue (n-3) PUFA-induced T-cell suppression by adding back rIL-2. Surprisingly, a reduction in proliferation was seen in FO-fed mice in the PMA/ionomycin/IL-2 group when we added back exogenous rIL-2 (Fig. 2). Because IL-2 can induce apoptosis (Ayroldi et al. 1998, Van Parijs et al. 1999), the response of T cells from FO-fed mice to apoptotic stimuli should be addressed in subsequent studies.

Our results indicate consistently that feeding an (n-3) PUFA-enriched diet results in downregulation of IL-2 production in purified T cells stimulated with αCD3/αCD28, and in Jurkat cells stimulated with PMA/ionomycin/αCD28. The apparent critical role of the membrane costimulatory ligand (CD28) in the diet-induced suppression of IL-2 production supports the role of lipid incorporation and modification of membrane-receptor function as a mechanism. This finding has ramifications beyond the scope of inflammatory diseases. Recently, IL-2 has been shown to regulate transcription of the protooncogene c-myc (Grigorieva et al. 2000), via an IL-2 responsive element within the 5′-flanking region of the c-myc gene. Additionally, Lord et al. (2000) showed that the IL-2 receptor is capable of inducing the c-myc, bcl-2 and bcl-x genes. Thus, the ability of DHA to decrease IL-2 secretion could potentially reduce induction of the protooncogenes c-myc, bcl-2 and bcl-x. Because bcl-2 inhibits apoptosis (Scaffidi et al. 1998), downregulation of this gene would lead to increased apoptosis and thus have important implication in various cancers.

In conclusion, DHA decreases IL-2 secretion in purified murine T lymphocytes and in Jurkat cells via modulation of TcR-CD28 signaling. Furthermore, on the basis of our proliferation experiment, it appears that accessory cells are necessary to modulate T-cell proliferation via a “bystander effect.” Thus, our data suggest that select dietary lipids may modify discrete plasma membrane domains enriched in immunoreceptors, thereby altering immune cell signaling.

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