THE SELECTIVE EFFECT OF DIETARY n-3 POLYUNSATURATED FATTY

ACIDS ON MURINE Th1 AND Th2 CELL DEVELOPMENT

A Dissertation

by

PING ZHANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Nutrition

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Approved by:

Co-Chairs of Committee,	Robert S. Chapkin
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ABSTRACT

The Selective Effect of Dietary n-3 Polyunsaturated Fatty Acids on Murine Th1 and Th2

Cell Development. (August 2005)

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To examine how dietary n-3 polyunsaturated fatty acids affect Th2 cell development, female C57BL/6 mice were fed a washout corn oil (CO) diet for 1 wk followed by 2 wk of either the same CO diet or a fish oil (FO) diet. $CD4^+$ T cells were isolated from spleens and cultured under both neutral (anti-CD3 and phorbol myristate acetate (PMA)) and Th2 polarizing conditions (anti-CD3 and PMA, in presence of rIL-4, rIL-2, and anti-IFN-γ) in the presence of homologous mouse serum (HMS) or fetal bovine serum (FBS) for 2 d. Dietary n-3 PUFA significantly enhanced Th2 cell development and suppressed Th1 development under neutral conditions as assessed by intracellular cytokine staining for IL-4 and IFN-γ as the two prototypic Th2 and Th1 cytokines, respectively. However, under Th2 polarizing conditions, while the suppression of Th1 cells was maintained in FO-fed mice, no dietary effect was observed in Th2 cells. Dietary FO increased the Th2/Th1 ratio under both neutral and Th2 polarizing conditions with HMS in the cultures. To examine the effect of dietary n-3 PUFA on Th1 development, DO11.10 $Rag2^{-/-}$ mice expressing transgenic T cell receptor specific for ovalbumin (OVA) peptide were used. $CD4^+$ T cells were isolated from

spleens and lymph nodes and stimulated with ovalbumin (OVA) peptide and irradiated BALB/c splenocytes in the presence of rIL-12, anti-IL-4, and rIL-2 in HMS for 2d. Cells were expanded for another 3 d in the presence of rIL-2 and rIL-12. Dietary n-3 PUFA did not affect Th1 differentiation as assessed by the proportion of IFN-γ^+, IL-4$^-$ T cells in the cultures, but suppressed rIL-2 induced expansion. The suppressed expansion was due to suppressed proliferation ($p<0.05$). In vivo expansion of antigen-specific T cells was visualized by flow cytometric analysis of CFSE-positive transgenic T cells. Dietary n-3 PUFA did not appear to affect antigen-induced CD4$^+$ T cell cycle progression in vivo. Overall, these results suggest dietary n-3 PUFA have no direct effect on Th2 cell development but do directly suppress Th1 cell development following both mitogenic and antigenic stimulation in vitro.

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CHAPTER I

INTRODUCTION

BACKGROUND

PUFA and chronic inflammatory diseases

Many epidemiological and clinical studies have demonstrated that dietary fish oil

(FO) attenuates immune-mediated inflammatory diseases (1-3). The principal bioactive

constituents of FO are considered to be eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA). Initial evidence for an anti-inflammatory effect of FO

came from an epidemiological study showing a very low incidence of certain chronic

inflammatory diseases in Greenland Eskimos whose diets are rich in EPA and DHA

from fish (4). Subsequently, many clinical trials also showed that FO supplementation

resulted in clinical improvement in a number of Th1-related chronic diseases such as

rheumatoid arthritis (RA), Crohn's disease, ulcerative colitis, psoriasis, lupus, and

multiple sclerosis (5,6). Evidence is strongest in RA, which is accompanied by

production of high levels of inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α

(7,8). These cytokines and arachidonic acid (20:4n-6, AA)-derived eicosanoids such as

PGE2, LTB4, and 5-HETE are found in synovial fluids of joints of RA patients (9,10).

Reduced expression of COX 2, TNF-α, and IL-1 could explain in part the benefits of FO

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This dissertation follows the style and format of The Journal of Nutrition.

in RA (5). Most of the 13 published clinical trials of FO treatment in RA showed a statistically significant benefit (11), thus, justifying the recommendation to include FO in the treatment of RA (12). The benefits of FO also include reduced risky cardiovascular disease (13). The widely used methotrexate and sulfasalazine for RA increase serum levels of homocysteine, which poses a risk for cardiovascular diseases. N-3 PUFA have been proven to reduce mortality in coronary heart disease (14).

Dietary FO has also been shown to be beneficial in various animal models of human Th1-mediated diseases (6). In mice with autoimmune glomerulonephritis (a model of lupus), FO feeding increased survival and decreased proteinuria and auto-antibodies (15,16). In collagen-induced arthritis, FO feeding decreased joint inflammation in rodents (17). In the rat model of colitis, dietary FO reduces inflammation (18). These clinical improvements were accompanied by reduced production of proinflammatory cytokines and increased anti-inflammatory cytokines.

There is currently considerable interest in FO supplementation for asthma, a typical Th2-mediated chronic inflammatory disease (19). There has been a dramatic increase in the prevalence of childhood atopic disease in developed countries over the past 30 years and it is attributed, at least in part, to increased dietary intake of n-6 PUFA (20,21). Supplementation with FO in pregnant women decreased allergy-specific cytokines and the severity of atopic dermatitis in their infants (22). There is also epidemiological evidence to support a protective role of dietary n-3 PUFA in atopic diseases. For example, surveys in the United Stated found that dietary fish oil intake was associated with better lung function (11). School children in Australia who consumed oily fish

regularly had a much lower likelihood to develop asthma than those who did not include oily fish in their diets (20). In asthma, the AA-derived 4-series leukotrienes (LT) produced by mast cells are major mediators of asthmatic bronchoconstriction. Although n-3 PUFA supplementation could reduce 4-series LT, limited clinical effects were observed. The role of n-3 PUFA in asthma still needs to be clarified by well-designed, placebo-controlled intervention studies (11).

T cell activation and differentiation

T cell-dependent immune responses begin with the binding of the T cell receptor (TCR) on a naïve T cell with an antigen presenting cell (APC) which has captured an antigen (23). Soluble antigens must be processed by APC and then re-expressed as peptide fragments on the surface of APCs bound to major histocompatibility complex (MHC) class I and class II molecules. The TCR signal requires additional support by co-stimulatory signals and adhesion receptors to amplify and prolong the activation events. The T cell homodimer, CD28, serves an important role in ensuring maximal clonal expansion and differentiation. In vivo, T cells from mice lacking CD28 (or both B7-1 and B7-2, the ligands for CD28 binding on the APC) showed impaired priming with subsequent weak memory responses (24). In vitro, T cells lacking CD28 costimulation proliferate poorly and showed reduced activation in all transcription factors expressed early in T cell activation. For induction of a primary CD4$^+$ T cell response, two signals are required. One is the antigen-associated MHC class II molecule on the surface of the APCs, and the other is a costimulatory signal delivered by the APCs (25). Upon

activation, "professional APCs" such as dendritic cells (DCs), macrophages and B cells, upregulate surface B7-1 and B7-2, which bind to CD28.

T cells can be divided into $CD4^+$ and $CD8^+$ subsets based on the unique expression of these molecules on the cell surface. Both $CD4^+$ and $CD8^+$ T cells can further differentiate into polarized Th1/Th2 or Tc1/Tc2 effector cells. Mouse Th1 cell cytokines include IL-2 and IFN-γ, whereas Th2 cells express IL-4, IL-5, IL-10, and IL-13 (26). The cytokines of the Th1 and Th2 subsets mutually inhibit the differentiation and functions of the reciprocal phenotype. IFN-γ produced by Th1 cells selectively inhibits proliferation of Th2 cells, and IL-10 produced by Th2 cells inhibits Th1 cytokine synthesis (27). Correlated with their distinctive cytokines, Th1 and Th2 cells have different functions. Th1 cells are pro-inflammatory and are important in cell-mediated immunity against intracellular microorganisms. Th1 cells have been shown to be associated with the pathogenesis of autoimmune diseases such as RA, Crohn's disease, and insulin-dependent diabetes mellitus (IDDM) (26,28). Th2 cells promote antibody secretion, particularly the IgE response, and also enhance eosinophil proliferation and function. Therefore, Th2 cells are often associated with allergic reactions and are important in defense against extracellular pathogens (27).

Many factors have been shown to influence the outcome of Th1/Th2 differentiation. Cytokines are critical inducers of Th subset development (27). IL-12 directs the development of Th1 cells while IL-4 drives Th2 development. IL-12 is produced by macrophages following stimulation by microbial products including LPS and intracellular bacteria such as Listeria monocytogenes and mycobacteria. Specific

dendritic cells in the spleen seem to be able to bias Th1 or Th2 development by eliciting different cytokines (29). CD8α^+ DCs produce IL-12 and induce IFN-γ and IL-2, while CD8α^- DCs induce IL-4 production.

Some other factors which affect Th1 and Th2 differentiation include genetic background of the host, the route and dose of antigen immunization, cell cycle, and nutritional status. Using a murine TCR transgenic system, Hsieh et al showed that genetic background has a profound impact on T helper cell differentiation (30). This TCR transgenic model makes it possible to study Th1/Th2 differentiation under well-controlled conditions. DO11.10 α/β-TCR mice which express a transgenic T cell receptor specific for OVA peptide on BALB/c, B10.D2 or DBA/2J backgrounds were generated and the development of Th1 and Th2 differentiation was examined in vitro by culturing CD4$^+$ T cells from these mice. Purified CD4$^+$ T cells from these mice were stimulated under similar conditions, using BALB/c splenocytes as APC loaded with OVA peptide. Upon restimulation, the IL-4 levels were three to five-fold higher from transgenic T cells on BALB/c background compared with those from B10.D2 background. In contrast, IFN-γ levels were two to three fold higher in transgenic T cells from B10.D2 mice compared with those from BALB/c mice. These cytokine profiles existed even after repeated stimulation of T cells, indicating that the phenotypes were stable. However, the cytokine profiles were not significantly different between different strains after 48 h in culture, suggesting that B10.D2 T cells do not express the Th1 phenotype when isolated fresh, however, they have a predisposition to differentiate into Th1 cells under neutral or default conditions in vitro. Further evidence came from

experiments in which T cells from both backgrounds were primed with APCs from each background (30). BALB/c T cells produced much higher levels of IL-4 and lower levels of INF-γ than T cells from B10.D2 mice, when primed either with APCs from BALB/c mice or B10.D2 mice. Thus, the difference between these two backgrounds for Th phenotype appears to reside within the T cell rather than within the APC (30).

Different adjuvants drive T cell differentiation along the type-1 or type-2 pathway (31). When tested with hen egg white, a prototypic Ag, Complete Freund's Adjuvant (CFA) always induced Th1 cytokines while Incomplete Freund's Adjuvant (IFA) stimulated Th2 cytokine production. These adjuvants can overcome the influence of genetic background and antigen type. For example, in both Th1-prone B10.D2 mice and Th2-biased BALB/c mice, injection of Ag with IFA resulted in IL-5 production upon restimulation, while immunization in CFA induced high levels of IFN-γ production in a recall response. The unipolar cytokine response was consistent with antigens that have intrinsic Th1/Th2 polarizing effects such as Th1-biased Leishmania and Th2-biased Schistosoma egg Ags. Alum, the only adjuvant generally used in human vaccines, is a type-2 adjuvant that stimulates the same humoral but a weaker cellular type-2 immunity. Injection of Alum into both BALB/c and B10.D2 mice resulted in the same concentrations of IgG1 but not IgG2a as IFA did, but the recall response of IL-5 production was much lower than IFA. The intrapertoneal (i.p.) route seems to favor a Type-2 cellular response, as evidenced by more IL-5-producing cells, for both IFA and alum.

Neonates are biased towards type-2 immunity and shift progressively toward type 1 during the first year (32). Peptide dose and affinity influences Th1 or Th2 cytokine production in an opposite way depending on short term or long term stimulation (33). Th1 differentiation increased with cell cycle progression whereas Th2 differentiation only occurred after 3 rounds of cell division (34). Leishmania major infection induces a Th1 response while helminth infection triggers a Th2 response (27).

Some dietary components and nutritional status have been found to affect Th1/Th2 differentiation including intracellular thiols status, vitamins A, D, and E, and dietary ribonucleotides. More intracellular thiols inhibited Th1 cytokine production and increased IL-4 production (35). Vitamin A enhanced Th2 development (36). Vitamin E suppressed IL-4 production in an animal model of allergy and AIDS (37). Dietary ribonucleotides skewed Th2 response to OVA towards Th1 in young BALB/c mice (38). Vitamin D suppressed both Th1 and Th2 cytokines (39). Understanding the role of nutrients in influencing the outcome of Th1/Th2 differentiation could help promote diet as adjunct therapy for Th1/Th2 mediated chronic inflammatory diseases.

The molecular basis for Th1 and Th2 differentiation has been elucidated in the past 20 years (40,41). IL-12 and IL-4 act through STAT-4 and STAT-6 to direct Th1 and Th2 differentiation, respectively. Gata-3 is probably a master regulator of Th2 differentiation (41). Ectopic expression of Gata-3 promotes Th2 differentiation under conditions that otherwise induce Th1 development. Gata-3 can program transcriptionally permissive chromatin structure within the IL-4 locus. Analogous to Gata-3 to direct Th2

differentiation, T-bet is a Th1-specific transcription factor. Ectopic expression of T-bet in developing Th2 cells promotes Th1 differentiation (41).

Beside differences in cytokine profiles, Th1 and Th2 cells have some other distinctive properties. Th1 cells, but not Th2 cells, bind to P and E-selectin and can efficiently enter inflamed skin and arthritic joints (42). Th1 and Th2 cells exhibit different degrees of susceptibility to apoptosis. Th1 cells are more susceptible to activation-induced cell death (AICD) whereas Th2 cells are relatively resistant to AICD (43). The signaling events in polarized Th1 and Th2 cells are quite different (44). Th2 cells showed decreased ZAP-70 phosphorylation and calcium signaling. Th1 and Th2 cells also exhibited differences in the compartmentalization of lipid rafts (45). For example, Th1 cells recruited both the TCR/CD3 complex and CD45 to rafts upon stimulation, whereas the TCR was not stably associated with lipid rafts in Th2 cells.

T cell activation by antigen

A primary CD4$^+$ T cell response can be divided into three distinct phases: activation, proliferation, and differentiation (46). Upon stimulation, CD4$^+$ T cells are activated, and progress through several rounds of cell division in a process called clonal expansion. During the clonal expansion, T cells progressively differentiate into specialized effector cells with the capacity to rapidly produce high levels of cytokines after a reencounter with Ag. However, each of these phases may occur sequentially or simultaneously. Which stimulatory signals are required to trigger each phase and the sequence of the signals during the whole process is still unclear.

The activation phase occurs before division. There is at least a 24-h lag while a resting T cell becomes activated and progresses through the cell cycle. During this period, many genes and their products are expressed to prepare for DNA replication and cell division. Epigenetic remodeling to turn on polarized cytokine genes is among the earliest change which occurs during T cell activation. That is why cytokines which drive polarization are most effective when present at the initiation of T cell activation before the first round of cell division.

Once cell proliferation starts, activated T cells divide rapidly and can continue through multiple rounds of division, resulting in clonal expansion of the initial population. After an initial lag period, stimulated CD4$^+$ T cells divide at a constant rate, with a doubling time of 4-6 h. The APCs present initially in the cell culture were nonviable at 60h. Differentiation also occurs during effector cell generation. The factors influencing the differentiation of naïve CD4$^+$ T cells into polarized effector cells have been discussed above.

A significant level of cell death occurs throughout effector cell generation when CD4$^+$ T cells are stimulated with Ag and APCs in culture, resulting in lower effector recoveries than would be predicted if all of the cells generated survived the 4-day culture (46). Similar cell death also occurred in T cell lines (47).

Much attention has been given to the role of Ag in driving the immune response. Ag concentration is related to both the extent and duration of TCR engagement (46). Extended signaling is necessary for commitment to CD4$^+$ T cell proliferation. Ag

concentration also determines whether differentiation favors effector vs. memory cell development, and affects the Th subset development (46).

While Ag is clearly required to initiate clonal expansion, its role in maintaining the process has been revealed only recently. Lee et al investigated the subsequent effects of Ag on clonal expansion (48). Murine CD4^{+} T cells were labeled with carboxyflurescein diacetate succinimidyl ester (CFSE) and were stimulated with specific peptide Ag. Separation of proliferating cells based on CFSE fluorescence intensity showed that daughter cells from each cell division proliferated after removal of Ag. As short as a 2 h exposure to peptide was sufficient to induce proliferation of OVA-specific DO11.10 CD4^{+} T cells after subsequent removal of OVA. These data suggest that continued antigen stimulation is not required during the clonal expansion of CD4^{+} T cells.

Full development of effector function requires 4 d of in vitro culture and, similarly, 4 or more days are required to generate fully activated T cells in vivo (46). Using vital dyes and FACS analysis, two distinct stages in the transition from naïve CD4^{+} T cells to effector cells have been identified, an early antigen-dependent stage and a late cytokine-driven expansion and differentiation stage. Efficient peptide presentation by professional APC to naïve and effector CD4^{+} T cells is limited to the first 1-2 days of culture and promotes optimal proliferation and effector expansion during culture. A short duration (1-2 d) of TCR stimulation in the presence of growth-promoting and polarizing cytokines is both sufficient and optimal to promote the development of a stable cytokine profile. In fact, prolonging Ag presentation leads to high level of T cell death, decreased effector expansion, and decreased cytokine production by recovered effectors. A TCR

stimulation-independent expansion phase exists during the late phase (d 2-4) of effector

generation and is dependent on IL-2 (46). These studies suggest that optimal CD4$^+$

effector generation occurs when there is an initial 1-2 d of Ag presentation followed by

an Ag-independent and IL-2 driven cell expansion phase lasting an additional 2 d.

Methods to evaluate the effect of fatty acids on lymphocyte function

Various methodologies are available to assess the effect of fatty acids on

lymphocyte function including in vitro, ex vivo, and in vivo studies (6). In the in vitro

studies, fatty acids or other metabolites of lipids such as PGE$_2$ are added to cultured

lymphocytes. Although non-physiological in nature, this kind of study can provide

insight into the mechanisms of fatty acid action on lymphocytes. The ex vivo studies are

so called because experimental diets are fed to animals or humans followed by isolation

and culture of lymphocytes to study the changes induced by feeding. This ex vivo

approach is also somewhat artificial, however, the lymphocytes have undergone diet-

induced changes within the animal or human host, thus better reflecting physiological

changes caused by fatty acids in lymphocytes. In addition, the ex vivo approach also

provides the advantages of examining the function of specific cell types and possible

mechanisms. The challenge for the ex vivo study is to isolate lymphocytes in sufficient

purity and subsequently to culture them. For animal studies, lymphocytes from blood,

spleen, thymus, lymph nodes, lungs, and gut-associated lymphoid tissue. Either mixed

cell population like whole splenocytes or purified lymphocyte subsets (e.g., CD4$^+$ T cells

and CD8$^+$ T cells) have been studied. In human studies, the ex vivo approach is limited

to blood lymphocytes, often as a mixture with monocytes. Preparations of human blood generate a mixture of 85-90% lymphocytes and 10-15% monocytes and are described as mononuclear cell preparations. Finally, certain indicators can be employed to examine diet-induced changes of lymphocyte function in vivo, e.g., the number of different types of lymphocytes, the cell surface expression of certain activation markers such as the adhesion molecules, etc. In animal studies, the weight, size, and cellularity of various lymphoid organs can be determined. Circulating cytokines, cytokine receptors and total Ig and Ig subclasses in the blood can also be quantified. Secretory IgA can be found in extracellular fluids and can be measured in saliva, tears, and intestinal washings. The in vivo cell-mediated immune response can be determined by responses to antigenic challenge (e.g., the delayed-type hypersensitivity (DTH) response). Skin graft rejection has also been used in animal studies as an in vivo indicator of cell-mediated immune function.

Adoptive transfer

The in vivo clonal expansion of T cells of known specificity in response to antigen can be visualized by an adoptive transfer technique that was first developed by Kearney et al. (49). Transgenic T cells from DO11.10 TCR transgenic donor mice were transferred intravenously into normal BALB/c recipient mice. The transferred T cells can be detected by staining lymph nodes cells with anti-CD4 and the KJ1-26 mAb. The transferred T cells represented a very small population but they can increase dramatically after subcutaneous injection of the ovalbumin (OVA) 323-339 peptide in

CFA. The increase was attributed to the expansion of transferred cells and not to recent thymic emigrants. This adoptive transfer protocol has been used to study the behavior of antigen-specific T cells following immunization. It was found that the response following subcutaneous injection could be characterized by marked accumulation of antigen-specific cells only in the draining lymph nodes. Three days after subcutaneous injection of 300 μg of ovalbumin in CFA at three sites on the back, the percentage of CD4$^+$, KJ1-26$^+$ T cells increased markedly in the brachial lymph nodes (49). After 17 days, the CD4$^+$, KJ1-26$^+$ T cells fell to about one third of day 3 level.

In vivo proliferation of these cells can be visualized by adoptive transfer of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled DO11.10 T cells. Without immunization, the KJ1-26$^+$ T cells are identified as a single CFSE-positive peak. In contrast, the KJ1-26$^+$ T cells showed several cell divisions in the immunized host (48).

Using the adoptive transfer protocol, Anderson et al. studied the effect of dietary lipids on in vivo proliferation of antigen-specific naïve CD4$^+$ T cells (50). The expansion of CD4$^+$ KJ1-26$^+$ T cells in draining lymph nodes was detected by flow cytometry. Four weeks of feeding a FO diet (20% total fat) to recipient mice resulted in less in vivo proliferation of CD4$^+$ T cells compared with a soybean oil diet.

Measures for function of isolated lymphocytes

There are a few widely used methods to measure the function of isolated lymphocytes. Proliferation is the most common indicator of lymphocyte function (6).

This is often assessed by incorporation of ^{3}H-labeled thymidine into the DNA of dividing lymphocytes. Since its introduction in 1994, the flow cytometric analysis of lymphocyte proliferation by CFSE labeling has gained popularity and is now widely used in immunological laboratories around the world (51,52). This method allows determination of eight to ten cell divisions, both in vitro and in vivo. The generally used incorporation of tritiated thymidine can only quantify overall division and therefore does not reflect the division history of cells. The fluorescein based dye CFSE has the biochemical properties that make it perfectly suited to this application. CFSE consists of a fluorescein molecule containing two acetate moieties and a succinimidyl ester functional group. It is membrane permeable, colorless and nonfluorescent. After CFSE passively diffuses into cells, its acetate groups are cleaved by endogenous esterase to yield highly fluorescent, carboxyfluorescein succinimidyl ester. The succinimidyl ester groups react with intracellular amines to form fluorescent conjugates, which are well retained in the cells. When cells divide, CFSE is distributed equally between the parent and daughter cells and is not transferred to adjacent cells. Thus, each successive generation in a proliferating population is marked by a 50% decrease in cellular fluorescence intensity that is readily identified by flow cytometry. This technique has been widely used to track in vitro cell proliferation in cell culture and in vivo division of adoptively transferred cells. CFSE has the same spectral characteristics as fluorescein, thus it can be used with monoclonal antibodies conjugated to phycoerythrin (PE) or PE-Cy5 to identify the phenotype of dividing cells.

Agents which stimulate lymphocyte proliferation include mitogens such as

concanavalin A (ConA) and phytohaemagglutinin (PHA), which stimulate T cells,

pokeweed mitogen, which stimulates a mixture of T and B cells, and bacterial

lipopolyssaccharide (LPS) which stimulates B cells (6). T cells can be stimulated with

anti-CD3 or a combination of anti-CD3/anti-CD28, or a combination of phorbol-12-

myristate-13-acetate (PMA) that stimulate protein kinase C and Ionomycin, which

triggers a rise in the intracellular free calcium concentration. Antigen can also be used

to stimulate T cells. However, in the past, this could be done only if the animal or

individual had been sensitized to an antigen. This is due to the incredibly low frequency

of lymphocytes specific for any given antigen in a naïve host (estimated to be < 1 in 10^5

lymphocytes) (53). Immunization of the host causes the expansion of antigen-specific T

cells. The presence and function of antigen-specific T cells can be studied after such in

vivo priming. However, transgenic mice expressing a transgenic T cell receptor for a

specific peptide facilitate the study of antigen-specific T cell activation (50). In general,

TCR transgenic mice contain a much higher number of naïve, antigen-specific T cells,

and the naïve T cells can be stimulated directly with antigen. Among many TCR

transgenic mouse models, the DO11.10 TCR transgenic mouse is widely used to study

antigen-specific T cell activation and differentiation (54). The DO11.10 transgenic

mouse expresses a T cell receptor specific for a peptide from ovalbumin (OVA $_{323-339}$) in

an MHC class II-restricted fashion. Whole splenocytes from DO11.10 mice can be

stimulated with OVA peptide or the purified CD4$^+$ T cells and stimulated with OVA

peptide in the presence of antigen-presenting cells, usually irradiated BALB/c

splenocytes (30). Th1 and Th2 differentiation can be studied by adding Th1 or Th2 polarizing cytokines and antibodies (55). To study antigen-specific T cell activation in vivo, T cells from DO11.10 mice can be adoptively transferred to recipient mice through tail vein injection and the recipient mice are then immunized (49). Upon immunization with the OVA $_{323-339}$ peptide, the transferred T cells undergo a primary T cell response that can be tracked using a monoclonal antibody specific for the DO11.10 T cell receptor (KJ1-26). The draining lymph nodes can be isolated and stained with KJ1-26. Expansion of TCR positive T cells can be visualized by flow cytometric analysis. In addition, cell division can be tracked by the CFSE intensity of KJ1-26^{+} T cells. These features make it possible to accurately study the expansion and differentiation of Ag-stimulated T cells in vivo.

Effect of n-3 PUFA on immune function

PUFA are categorized according to the position of the last double bond relative to their methyl end into two classes: n-3 and n-6. Mammals cannot introduce double bonds between carbon 9 and the methyl end of fatty acids, they can neither synthesize PUFA nor convert n-6 into n-3 PUFA. Thus, they must be obtained through the diet (14). Most common dietary n-6 PUFA include linoleic acid (LA: 18:2n-6), γ-linolenic acid (GLA: 18:3n-6) and arachidonic acid (AA: 20:4n-6). LA is readily converted to AA via GLA. AA is the precursor of eicosanoid messengers, which are potent mediators of inflammation. Among dietary n-3 PUFA, α-linolenic acid (ALNA; 18:3n-3) is found in green leafy vegetables, walnut and flaxseed oils, while EPA (20:5n-3) and DHA (22:6n-

3) are found in cold-water fatty fish such as tuna, salmon, mackerel and herring. Diet can influence the process of chronic inflammatory diseases. The typical Western diet contains 10-20 times more n-6 than n-3 PUFA (56). This imbalance has been associated with progression of cardiovascular disease (19). Thus, this forming the basis for the American Heart Association's recommendation that adult eats fish at least twice a week to prevent cardiovascular diseases.

In general, PUFA exert immunomodulatory and anti-inflammatory effects, and the effects of n-3 PUFA are considered more pronounced compared to those of n-6 series. N-3 PUFA exert anti-inflammatory effects through a variety of mechanisms (14). First, alterations of lipid messengers by n-3 PUFA have been regarded as a primary mechanism of action of n-3 PUFA on immune faction. Fatty acids in the diet are incorporated into the plasma membranes of immune cells. The changes in fatty acid composition of lymphocyte membranes directly affect some lipid messengers that are derived from membrane fatty acids. Usually, AA is liberated from the membrane in response to stimuli. AA is metabolized by cyclooxygenases (COX) to generate prostaglandins (PGs) and thromboxanes of the 2-series. N-3 PUFA can interfere with eicosanoid production from AA and generate less effective messengers, the 3-series of PGs. Dietary n-3 PUFA are incorporated into the plasma membranes at the expense of AA. PUFA compete with AA for the same enzymes, but they are poor substrates. In addition to inhibition at the enzymatic level, n-3 PUFA also inhibit gene expression of COX-2. Although lymphocytes are a poor source of eicosanoids, the PGs have a

profound impact on their function. For example, PGE2 derived from macrophages inhibits lymphocyte proliferation and Th1 cytokines (11,57,58).

PUFA also regulate gene expression directly through binding nuclear receptors or indirectly by altering signaling pathways. Peroxisome proliferation-activated receptor (PPAR) was originally found to be a key regulator of adipocyte differentiation. Its role in the regulation of lymphocyte function was only recently reported (59). PPARγ is the most abundant isoform expressed in myeloid cells, whereas PPARα is predominantly expressed in lymphocytes (59). It is noteworthy that PPARγ is up regulated during T cell activation at the expense of PPARα. Both PPARγ and PPARα agonists inhibit IL-2 and IFN-γ in murine and human lymphocytes, although the PPARα agonists is less potent than PPARγ agonists (59). PPAR α can be activated by many fatty acids whereas PPAR γ has selectivity for some unsaturated fatty acids and their derivatives. However, nuclear receptor involvement does not explain the stronger anti-inflammatory effects of n-3 compared to n-6 PUFA (14).

Recently, modulation of lipid rafts by n-3 PUFA was revealed as a novel mechanism for their immunosuppressive effect (14). Membrane lipid rafts are specific detergent-resistant domains within the plasma membrane that play an essential role in T cell signal transduction. Lipid rafts are characterized by a high content of cholesterol and sphingolipids, such as sphingomyelin and glycolipids (60). Numerous proteins involved in T cell signaling such as the linker for activation in T cells (LAT) are highly enriched in rafts (61). Early mediators of T cell proliferation, such as PKCθ, and of T cell apoptosis such as Fas and Fas-ligand, translocate to lipid rafts after stimulation. There is

overwhelming evidence that lipid raft integrity is a prerequisite for optimized TcR signal transduction and the subsequent immune response. Conditions that modify raft structure can disrupt the earliest steps of T cell activation. Polyunsaturated fatty acids such as EPA have been shown by in vitro studies using a Jurkat T cell line to displace acylated signaling proteins from membrane lipid rafts predominantly due to altered raft lipid composition. Treatment of cultured T cells with n-3 PUFA causes incorporation of n-3 PUFA into lipids of the cytoplasmic leaflet of lipid rafts, resulting in displace acylated proteins (62). Treatment of Jurkat and human peripheral T cells with n-3 PUFA results in inhibition of LAT phosphorylation and downstream signaling events such as calcium response, JNK and NF-AT activation (63). However upstream events, such as CD3δ phosphorylation and ZAP-70 recruitment to the CD3 complex, are not affected. LAT displacement from lipid rafts has been shown to be a major molecular mechanism by which n-3 PUFA inhibits T cells (63). Recent studies indicate that the macromolecular complex organization in lipid rafts is distinct in Th1 and Th2 subsets (45). It is thought that the TcR may be pre-committed to respond differently depending on the differentiative state of the T cell and the regulation of T cell activation may be distinct in different T helper subsets. We have demonstrated that dietary n-3 PUFA remodel both raft and non-raft membrane composition in murine splenic T cells (64). However, the ability of n-3 PUFA to influence partitioning of signaling proteins in relation to T-cell subset activation in vivo has not been determined to date.

The role of lipid rafts in regulating IL-2R signaling

IL-2 is produced by T cells upon activation and is a potent T cell growth factor. IL-2 binds to the high affinity IL-2 receptor, which consists of an α (CD25), a β (CD122), and a γ chain (CD132) (65). The α chain is only expressed after stimulation through TCR or with IL-2 and is distinct from other cytokine receptors. Although it is not directly involved in IL-2 signaling, the IL-2 Rα chain enhances the affinity of the receptor. In contrast, although the β and γ chains mediate signal transduction, they are members of the hematopoietin-cytokine receptor superfamily and participate in receptors for other cytokines.

Recently several lines of evidence indicated the involvement of lipid rafts in the regulation of IL-2 R signaling. First, the importance of the rafts in IL-2 signaling was demonstrated by attenuation of the IL-2 signal in spleen cells of complex ganglioside-lacking mice (66). Mutant mouse lines were established that completely lacked GM2/GD2 synthase activity and, thus, expressed no complex gangliosides. The sizes and cellularities of spleen and thymus of the mutant mice were significantly reduced. Spleen cells from these mice exhibited a dramatic decrease in proliferation when stimulated with IL-2, but not with ConA or anti-CD3. Expression of IL-2 Rα, β and γ chains was not different between the mutant mice and wild type mice. Up-regulation of IL-2 Rα was also normal in mutant mice. Activation of JAK1, JAK3 and STAT5 after IL-2 stimulation was reduced. Also, c-fos expression was delayed and reduced in mutant mice, but c-myc expression appeared to be normal compared with wild type mice. The

attenuated IL-2 signaling in mutant mice could be attributed to modulation of IL-2 receptors by the lack of complex gangliosides.

Second, it has been demonstrated that antibody or ligand-mediated immobilization of components of lipid rafts, glycosyl-phosphatidyl-inositol (GPI)-anchored proteins and the GM1 ganglioside inhibit IL-2 induced T cell proliferation (67). IL-2 Rα is constitutively enriched in rafts and further enriched upon raft immobilization with anti-Thy-1. In contrast, IL-2 Rβ and IL-2 Rγ were localized to the soluble fractions. JAK1 and JAK3, which are constitutively associated with the IL-2 Rβ and IL-2 Rγ, also occurred in soluble membranes. In addition, IL-2-induced tyrosine phosphorylation of JAK1 and JAK3 are also localized to soluble fractions. IL-2 induced heterotrimerization of IL-2 R chains occurs within soluble membrane fractions. Disruption of lipid raft integrity has little effect on IL-2 induced signaling (67). Using ^{125}I-labeled IL-2, all three IL-2 R chains were identified in soluble membranes. In addition, disruption of the integrity of lipid rafts by methyl-β-cyclodextrin (MCD) did not inhibit IL-2 induced tyrosine phosphorylation of JAK1 and JAK3 in CTLL-2 cells. TCR-induced tyrosine phosphorylation of PLCγ-1 was ablated by MCD (68). These results suggest that IL-2R signaling occurs in soluble fractions of the plasma membrane (67). Lipid rafts may regulate IL-2 R signaling by segregating elements of the receptor complex in the plasma membrane. IL-2 binding may result in the dissociation of IL-2 Rα from lipid rafts and its interaction with IL-2 Rβ and IL-2 Rγ chains in soluble membranes to initiate signaling. Immobilization of raft components impairs the mobility of IL-2 Rα and prevents the dissociation from rafts. It is therefore likely, that IL-2R α is in a dynamic equilibrium

between rafts and soluble membranes, and immobilization of raft components may shift the equilibrium by trapping the IL-2 Rα in lipid rafts (67). In contrast with above findings, IL-2 Rβ was found selectively enriched in rafts in untreated human T cells, but exposure to IL-2 resulted in partial translocation into soluble fractions (67). Disruption of rafts by MCD decreased surface expression of IL-2 Rβ. Several IL-2 Rβ associated kinase and adaptor molecules colocalize with IL-2 Rβ chain before and after IL-2 stimulation.

Third, the distribution of IL-2 R on the T cell plasma membrane is not random. Using immunogold staining and electron microscopy, IL-2 Rα has been found to exhibit nonrandom surface distribution on human lymphoma cells and this clustering is independent of IL-2 (69). Clustering was also confirmed by confocal microscopy. IL-2 Rα was found to be cocluster with MHC and CD48, which are known to localize in lipid rafts (69). Thus, IL-2 Rα was localized to cell surface clusters that appear to be lipid rafts. Disruption of rafts with filipin or MCD resulted in blurring of cluster boundaries and appeared to disperse the clusters for all four proteins. The three IL-2 R chains appear to form pre-existing complexes on the surface of T cells, brought closer together upon IL-2 binding, resulting in the aggregation of β and γ and the initiation of signaling (69).

n-3 PUFA regulation of T cell function and rational for proposed studies

The significant effects of dietary n-3 PUFA on T cell function have been well documented in both human and experimental animals (3,6,70-72). In general, increased intake of n-3 PUFA is associated with reduced T cell proliferation in response to

mitogenic stimuli, and impaired production of a critical growth factor of T cells, interleukin-2 (IL-2). For example, dietary supplementation with 2.4-18 g/d EPA and DHA significantly reduced mitogen-stimulated proliferation and IL-2 production in human peripheral blood mononuclear cells (PBMC) (71). Mice fed 10g/kg EPA or DHA for as short as 10 d exhibited a significant decrease in ConA-stimulated splenocyte proliferation accompanied by a decrease in IL-2, and IL-2 Rα expression, and a reduction in DAG and ceramide (73). More recently, Arrington et al. reported decreased IL-2 secretion from purified CD4^{+} T cells from mice fed diets containing 1% DHA when cultures were stimulated with anti-CD3/anti-CD28. In contrast, FO feeding increased CD4^{+} T cell proliferation when cells were stimulated with anti-CD3 plus PMA (74).

Interestingly, murine CD4^{+} T cells stimulated with anti-CD3/anti-CD28 displayed a Th1-like cytokine profile while cells stimulated with anti-CD3/PMA displayed a Th2-like cytokine profile (74). These data suggest that the anti-inflammatory effect of dietary n-3 PUFA could be due to a combination of direct suppression of Th1 responses and an indirect regulation through enhanced Th2 activation. To date, the effects of dietary n-3 PUFA on individual Th subsets has not been determined. There is only very limited data on how FO affects Th1 and Th2 cytokine production. Wallace et al reported a decrease in IFN-γ production in splenocytes from FO-fed mice (75). Suppressed IFN-γ gene expression was detected in the Peyer's patches of FO-fed BB rats (76). In addition, in human MS patients, FO supplementation resulted in reduced IFN-γ secretion by blood lymphocytes (77). In contrast to these studies, no effect on IFN-γ production by murine spleen lymphocytes was seen in mice fed a diet enriched in n-3 PUFA (78). FO

exhibited little effect on IL-4 secretion or gene expression in spleens from FO-fed mice

(75). While FO feeding increased IL-10 gene expression in Peyer's patches in BB rats

(76), FO supplements for pregnant women resulted in decreased neonatal IL-10

secretion (79). These conflicting results could be explained by important differences

between the study parameters. First, different models were used. Cytokine production

was examined in either murine splenocytes, gut-associated immune cells or human

peripheral lymphocytes. Second, the cytokines were examined after a short period of

stimulation. It has been shown that IL-4 expression depends on cell cycle progression

and only increases dramatically and acquires a stable phenotype after 48 h in culture

(80). Third, mixed cell populations were used, which means that the cytokine

concentrations may not truly reflect the balance between Th1 and Th2 cells. In order to

conclusively determine how dietary n-3 PUFA influence the Th1/Th2 balance,

experiments using purified $CD4^+$ T cells that have been classically polarized to Th1 or

Th2 effector cells are required.

This laboratory has used C57BL/6 mice as a model to study the effects of dietary n-

3 PUFA on T cell function. In the past 10 yr, we have shown that dietary n-3 PUFA

suppress mitogen stimulated splenocyte proliferation and anti-CD3/anti-CD28

stimulated proliferation of purified $CD4^+$ T cells (3,74,81). We have also demonstrated

that dietary n-3 PUFA suppress surface CD3/CD28 function, modulate lipid composition

of $CD4^+$ T cells, in the lipid rafts and inhibit the recruitment of PKC θ to lipid rafts

resulting in the down-regulation of AP1 and NF-κB activation (82). In addition, we have

also shown that dietary n-3 PUFA enhanced AICD in purified $CD4^+$ T cells and

polarized Th1 cells (83,84). C57BL/6 mice are Th1-prone, thus, making this strain a suitable choice to study the possible role of dietary n-3 PUFA on the regulation of Th1/Th2 differentiation. However, antigen specific T cell activation has not been extensively studied. Pompos et al. reported reduced proliferation and IL-2 secretion in OVA stimulated splenocytes from transgenic DO11.10 mice fed a FO diet (53). No study has examined the effect of dietary n-3 PUFA on antigen stimulated purified CD4^{+} T cells. In addition, the precise effects of dietary n-3 PUFA on antigen stimulated Th1/Th2 differentiation has not been studied.

Previous studies from our lab have shown that low dose, short-term n-3 PUFA feeding can modulate T cell function in C57BL/6 mice. Feeding as short as 10 d with 5% FO results in a significant effect on T cells (74). Because regular mouse chow contains low amounts of n-3 PUFA, in order to control exposure to dietary fatty acids, mice were placed on a control baseline CO diet (5% by weight) for 1 wk to wash out n-3 PUFA from T-cell membranes. Mice were subsequently fed the experimental diets for 2 wk containing either CO or FO.

The overall objective of the project was to determine the effect of dietary n-3 PUFA on murine Th1 and Th2 development using both C57 BL/6 mice and the DO11.10 transgenic mice. The results of these experiments will contribute to the understanding of the mechanisms by which dietary n-3 PUFA modulate T cell functions and provide data which will support the rational application of dietary n-3 PUFA as adjunct therapy in T-cell mediated inflammatory diseases.

CHAPTER II

DIETARY n-3 POLYUNSATURATED FATTY ACIDS MODULATE MURINE Th1/Th2 BALANCE TOWARDS THE Th2 POLE BY SUPPRESSION OF Th1 DEVELOPMENT*

We have shown that dietary long chain n-3 polyunsaturated fatty acids (PUFA) present in fish oil (FO) affect $CD4^+$ T cell proliferation and cytokine production in C57BL/6 mice. To test the hypothesis that the anti-inflammatory effect of dietary n-3 PUFA could be due to the indirect suppression of Th1 cells by cross-regulation of enhanced Th2 activation, mice were fed a wash out control diet (5% corn oil (CO), n-6 PUFA) for 1 wk, followed by maintenance on the control diet or a fish oil diet (1% CO + 4% FO, n-3 PUFA) for 2 wk. Splenic $CD4^+$ T cells were cultured under both neutral and Th2 polarizing conditions for 2 d. Cells were reactivated and analyzed for IL-4 and interferon-γ production by intracellular cytokine staining. Dietary FO increased the percentage of Th2 polarized cells and suppressed Th1 cell frequency under neutral conditions. However, under Th2 polarizing conditions, while the suppression of Th1 cells was maintained in FO-fed mice, no effect was observed in Th2 cells. Dietary FO

increased the Th2/Th1 ratio in the presence of homologous mouse serum under both neutral (p=0.0009) and Th2 polarizing conditions (p=0.0185). The FO diet did not significantly affect proliferation under Th2 polarizing conditions. Thus, the anti-inflammatory effects of FO may be explained, in part, by a shift of the Th1/Th2 balance, due to the direct suppression of Th1 development, and not by enhancement of the propensity of CD4^{+} T cells to be polarized toward a Th2 phenotype, at least *in vitro*.

INTRODUCTION

Two polarized CD4^{+} T cell subsets have been identified by their signature cytokines and their mutually exclusive helper functions. T-helper 1 effector (Th1) cells produce IL-2, IFN-γ, and lymphotoxin; T-helper 2 (Th2) cells produce IL-4, IL-5, IL-10 and IL-13 (26). The pathogenic role of Th1 and the protective role of Th2 cells have been described for certain autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), and insulin-dependent diabetes mellitus (IDDM) (28). In some cases, the Th1/Th2 balance was an important indicator of the disease state (76). A shift from Th1 to Th2 cytokine profiles was observed in many clinical interventions that resulted in improvement in these diseases (85,86). Modulation of the Th1/Th2 balance has provided a new paradigm for immunomodulatory therapy in some autoimmune diseases (87).

The beneficial effects of dietary fish oil (FO) on Th1 mediated diseases have been well documented (5,6). The principal bioactive constituents of FO are considered to be EPA and DHA. Epidemiological studies showed decreased incidence of autoimmune/inflammatory diseases in Greenland Eskimos whose diets are rich in EPA

and DHA from fish (4). Clinical dietary intervention trials also showed a protective effect of FO in a number of Th1-mediated autoimmune diseases such as RA, Crohn's disease, and ulcerative colitis (6). A shift away from a Th1 response was considered to be the mechanism that resulted in the benefits of FO (75).

The anti-inflammatory effect of n-3 PUFA in FO may be attributed, in part, to direct suppression of T cell function (3). FO has been shown to inhibit T cell proliferation, IL-2 secretion, IL-2 receptor expression, and CD28 function (71,73,88). To date, the effects of dietary (n-3) PUFA on individual T helper cell subsets has not been determined. There are only very limited data on how FO affects Th1 and Th2 cytokines (78). Wallace et al reported decreased IFN-γ in splenocytes from FO-fed mice (75). Suppressed IFN-γ gene expression was found in the Peyer's patches in FO-fed BB rats (76). In human MS patients, FO supplementation resulted in reduced IFN-γ secretion by blood lymphocytes (77). In contrast to these studies, no effect on IFN-γ production by murine spleen lymphocytes was seen in mice fed a diet enriched in (n-3) PUFA (78). FO exhibited little effect on IL-4 secretion or gene expression in spleens from FO-fed mice (75). While FO feeding increased IL-10 gene expression in Peyer's patches in BB rats (76), FO supplements for pregnant women resulted in decreased neonatal IL-10 secretion (79). In order to conclusively determine how dietary (n-3) PUFA influence the Th1/Th2 balance, experiments using purified CD4$^+$ T cells which have been polarized into Th1/Th2 cells are necessary.

Previous data from our laboratory revealed that purified mouse CD4+ T cells from C57BL/6 mice stimulated with different agonists displayed different cytokine profiles,

corresponding to Th1-like and Th2-like cells (74). The increased proliferation of Th2-like cells and the suppressed IL-2 secretion by Th1-like cells from FO-fed mice suggested that the anti-inflammatory effects of dietary n-3 PUFA may be the combined result of direct suppression of IL-2 induced Th1 activation and indirect suppression of Th1 cells through cross-regulation of enhanced Th2 activation. There is ample evidence that diminished Th1 responses following therapy of some Th1-mediated autoimmune diseases are accompanied by enhanced Th2 responses (28,76,85). Therefore, in the current study, we tested the hypothesis that the anti-inflammatory effects of dietary fish oil are due, in part, to enhanced Th2 activation. Our results indicated that dietary (n-3) PUFA alter the Th1/Th2 balance towards the Th2 pole by selectively suppressing Th1 development rather than by enhancing Th2 development.

MATERIALS AND METHODS

Animals and diets

All experimental procedures using laboratory animals were approved by the University Laboratory Animal Care Committee of Texas A&M University. Pathogen-free, female, young C57BL/6 mice (12-14 g) were purchased from Frederick National Cancer Research Facility, Frederick, MD. Mice were housed 5 per microisolator cage, and had free access to water and diet. Mice were fed a control semi-purified diet containing 5% corn oil (CO) during the 7-d acclimation period followed by 2 wk of feeding with either the same control diet or a fish oil (FO) diet (1% CO + 4% FO). The diets met NRC nutrition requirements and varied only in lipid content (21). The basic

diet composition, expressed as g/100g was: casein, 20; sucrose, 42; cornstarch, 22;

cellulose, 6; AIN-76 mineral mix, 3.5; AIN-76 vitamin mix, 1; DL-methionine, 0.3;

choline chloride, 0.2; Tenox 20A, 0.1; and oil, 5. The fatty acid composition of the diets,

as determined by gas chromatography, is shown in **Table 1**.

TABLE 1

Dietary fatty acid composition[1,2,3]

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

[1]Mean values of two analyses.

[2]Abbreviations used: CO, 5% corn oil; FO, 4% fish oil + 1% corn oil (w/w);

[3]Only the major fatty acids (> 1 g/100 g) are listed.

[4]Tr, trace amount (< 0.1 g/100 g).

Chemical compounds, cytokines and antibodies

Phorbol myristate acetate (PMA) was purchased from Sigma (St. Louis, MO).

Ionomycin was purchased from Calbiochem-Novabiochem (San Diego, CA).

Recombinant murine IL-2 was purchased from R&D Systems (Minneapolis, MN).

Hamster anti-mouse CD3e monoclonal antibody (clone145-2C11), neutralizing rat mAb

for murine IFN-γ (clone R4-6A2), R-Phycoerythrin (PE)-labeled anti-IL-4 (clone

11B11) and Fluorescein isothiocyanate (FITC)-labeled anti-IFN-γ (clone XMG1.2) were

purchased from BD PharMingen (San Diego, CA).

Cell culture

CD4$^+$ T cells were purified from the splenocytes of C57BL/6 mice by a negative

selection column method which we have published (74). Cells were adjusted to 5×10^6

cells/ml and cultured in 24-well plates containing 2 mL complete RPMI medium [RPMI

1640 medium with 25 mM HEPES (Irvine Scientific, Santa Ana, CA) supplemented

with 5% FBS, 1×10^5 U/L penicillin and 100 mg/L streptomycin (Irvine Scientific), 2

mM L-glutamine, and 10 μM 2-mercaptoethanol]. To drive Th2 development, cells were

cultured for 48 h with plate bound anti-CD3 (10 μg/mL) and PMA (0.5 ng/mL) in the

presence of IL-4 (10 ng/mL), anti-IFN-γ (10 μg/mL) and IL-2 (20 ng/mL). For

stimulation under neutral conditions, cells were cultured only with anti-CD3 (10 μg/mL)

and PMA (0.5 ng/mL). Select cultures were incubated with homologous mouse serum

(HMS) (complete RPMI 1640 with 2.5% FBS and 2.5% HMS). The HMS was collected

according to the method described by Pompos et al. (53), and was included to preserve

the diet-induced changes in the lipid composition of cell membranes during the in vitro

culture period, as we have published previously (83).

Proliferation assay

Purified CD4$^+$ T cells were cultured at 2×10^5 cells per well in 96-well round-

bottomed microtitre plates (Falcon, Becton-Dickenson, Lincoln Park, NJ). Cells were

cultured under Th2 polarizing conditions (described above) for 48 h at 37 ^{o}C with 5%

CO_2. For the final 6 h, 1.0 μCi ^{3}[H]-thymidine was added to the cultures. Cells were

harvested and counted as we have previously described (74).

Intracellular cytokine staining

Intracellular cytokine staining was quantified as we have described previously with

modifications (83). After a 48 h incubation, live cells were separated by Lympholyte-M

and cultured (10^6 cells/mL) with PMA (10 ng/mL) and Ionomycin (1 μg/mL) for 5 h at

37 ^{o}C. GolgiStop (BD PharMingen, San Diego, CA) was added during the last 2 h of

incubation at the concentration recommended by the manufacturer. Cells were

subsequently collected, stained with Fc block, fixed and permeabilized for 20 min at 4

^{o}C in 250 μL Perm/Fix (BD PharMingen, San Diego, CA). Cells were washed in

Perm/Wash (BD PharMingen, San Diego, CA) followed by staining with PE-labeled

mAb to murine IL-4 and FITC-labeled mAb to murine IFN-γ. Following a final wash in

Perm/Wash, cells were resuspended in 500 μL staining buffer (PBS with 0.05% FCS and

0.01% sodium azide) for analysis on a FACS-Calibur flow cytometer (Becton Dickinson

Immunocytometry systems, San Jose, CA). In preliminary studies, isotype controls for

the anti-IL-4-PE and anti-IFN-γ were performed. These samples stained minimally (IL-4

<0.1%, IFN-γ <0.2%) and were similar to nonstained samples (see Appendix A-2). Thus,

in the dietary study, isotype controls were omitted and nonstained samples were used to

set the quadrant.

Statistical analysis

Significance of main treatment effects was assessed using PROC GLM in SAS. The

differences between means were analyzed for significance by Tukey's test. A 95% level

of probability was accepted as being statistically significant. Results are from 1-3

separate experiments.

RESULTS

Dietary fish oil alters Th1/Th2 cytokine profiles

In order to determine conclusively how dietary n-3 PUFA affect Th2 polarization,

purified splenic CD4$^+$ T cells from FO- and CO-fed mice were cultured with anti-CD3

and PMA in the presence of IL-4, IL-2 and anti-IFN-γ with FBS or homologous mouse

serum (HMS). Our previous data showed that CD4$^+$ T cells cultured with anti-CD3 and

PMA produce both IL-4 and IFN-γ (74), therefore, we chose these stimuli as neutral

conditions for the induction of cytokines. The immunofluorescent detection of IFN-γ and

IL-4 by intracellular cytokine staining has proven to be the appropriate method of

characterizing polarized Th1 and Th2 cell populations and has been validated in
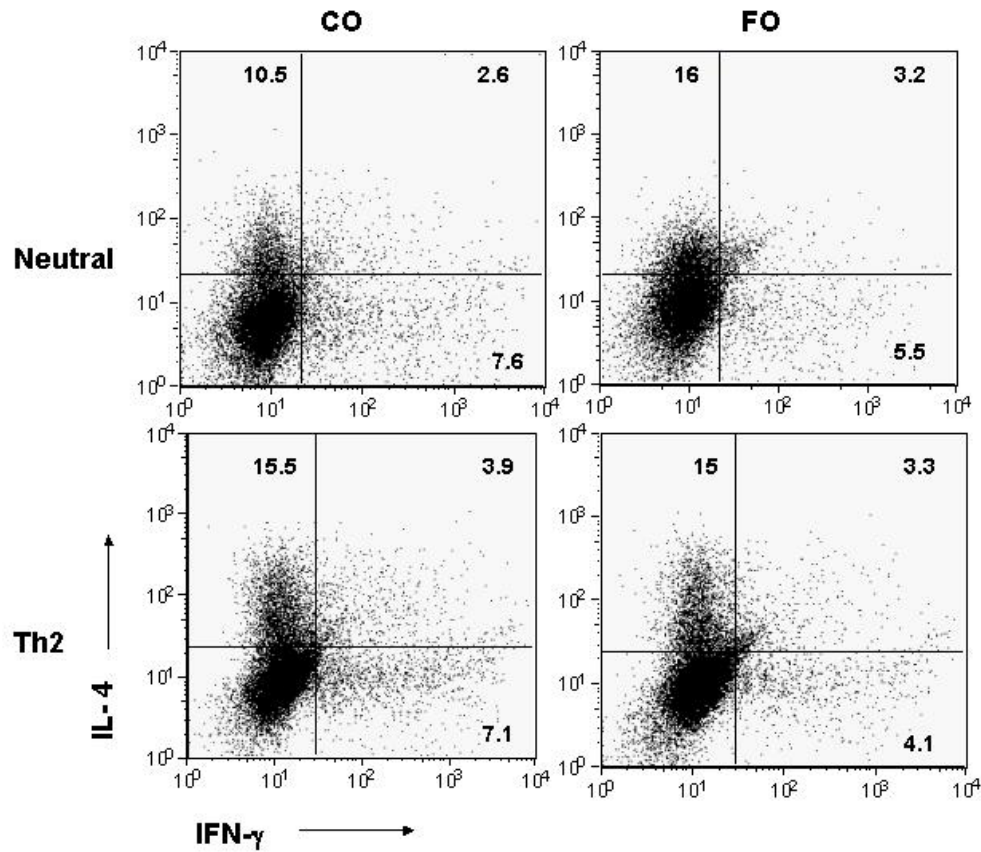
Figure 1. Representative fluorescence-activated cell sorting (FACS) images of cytokine profiles obtained by intracellular cytokine staining of polarized CD4^{+} T cells from C57BL/6 mice cultured under either neutral or Th2 polarizing conditions. Splenic CD4^{+} T cells were purified from mice fed test diets and cultured with anti-CD3 and PMA (neutral conditions) or with anti-CD3, PMA, IL-4, anti-IFN-γ and IL-2 (Th2 polarizing conditions) for 48 h in the presence of homologous mouse serum (HMS). Living cells were isolated and cytokines were determined by intracellular cytokine staining. The number in the upper left quadrant of each dot plot represents the percentage of Th2 cells (IL-4^{+}, IFN-γ[-]) and values in lower right quadrant of each dot plot represents Th1 cells (IFN-γ[+], IL-4^{-}). The isotype controls were tested in preliminary studies and showed no difference from unstained samples (data not shown), thus, unstained samples were used to set the quadrant. CO, corn oil; FO, fish oil.

numerous publications (80,89,90). We followed the standard published protocol for intracellular cytokine staining (83). Briefly, cells were reactivated for 5 h in the presence of a protein inhibitor, GolgiStop^{TM}, followed by incubation with FITC- conjugated monoclonal antibody to IFN-γ and PE-conjugated monoclonal antibody to IL-4. Stained cells were analyzed by flow cytometry. Representative FACS images of cytokine profiles obtained by intracellular cytokine staining of polarized cells from FO- or CO- fed mice cultured with HMS are shown in **Figure 1**. Th2 cells were defined as cells producing IL-4, but not IFN-γ, and are represented by the IL-4^{+}, IFN-γ^{-} population in the upper left quadrant of each dot plot. Th1 cells were defined as cells producing IFN-γ, but not IL-4, and are represented by the IFN-γ^{+}, IL-4^{-} cells in the lower right quadrant of each dot plot.

Using the flow cytometry data for all animals, similar to the data illustrated in **Figure 1,** the mean percentage of Th1 and Th2 cells generated under neutral and Th2-polarizing conditions were calculated and presented in **Figure 2**. Panel 2A shows the percentage of IL-4^{+}, IFN-γ^{-} Th2 cells generated after 48 h culture in the presence of HMS. Under neutral conditions, dietary FO significantly increased the proportion of Th2 cells by 46% (14.9% for FO-fed mice vs. 10.2% for CO-fed mice, p=0.0049). Following culture under Th2 polarizing conditions, there was no difference (p > 0.05) in the proportion of Th2 cells between the FO (15.3%) and CO (16.2%) groups.
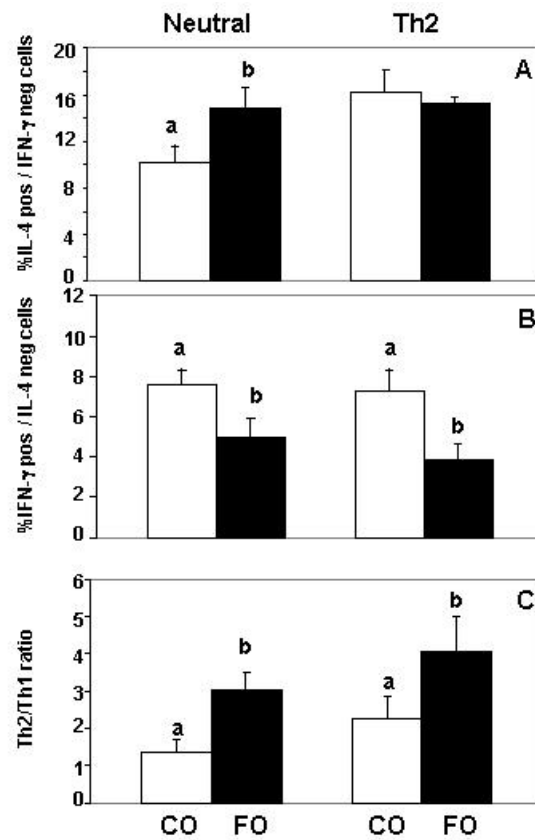
Figure 2. Dietary FO shifts the Th1/Th2 balance by suppressing Th1 development in C57BL/6 mice. Purified CD4^{+} T cells from CO- or FO-fed mice were cultured under either neutral or Th2 polarizing conditions in the presence of homologous mouse serum (HMS) for 48 h as described in the Materials and Methods. Cytokine production was assessed by intracellular cytokine staining. Panels represent IL-4^{+} cell frequency (A) and IFN-γ[+] cell frequency (B) and the Th2/Th1 ratio (C). Values identified by different letters are significantly different (p<0.05). Data represent means ± SEM; n = 4 replicates per diet group, and 5 mice were pooled per analysis. CO, corn oil; FO, fish oil.

Panel 2B shows the percentage of IFN-γ^+, IL-4$^-$ Th1 cells generated after 48 h culture in the presence of HMS. Dietary FO significantly decreased the proportion of Th1 cells following culture under both neutral (p=0.0053) and Th2 polarizing conditions (p=0.0017). Under neutral conditions, 7.6% of the CD4$^+$ T cells were Th1 polarized in CO-fed mice, while 5.0% of the CD4$^+$ T cells exhibited a Th1 phenotype in FO-fed mice. Similarly, under Th2 conditions, 7.3% of the CD4$^+$ T cells were Th1 polarized in CO-fed mice, compared to 3.9% in FO-fed mice. Thus, dietary FO suppressed Th1 cell development following culture under both neutral and Th2 polarizing conditions in the presence of HMS.

Panel 2C shows the ratio of Th2/Th1 cells after 48 h culture in the presence of HMS. Dietary FO significantly increased the ratio following culture under both neutral (p=0.0009) and Th2 polarizing conditions (p=0.0185). This indicates a clear shift in the Th1/Th2 balance towards a Th2 response in mice fed FO.

Figure 3 shows the percentage of IL-4$^+$, IFN-γ^- Th2 cells and IFN-γ^+, IL-4$^-$ Th1 cells after 48 h culture under Th2 polarizing conditions in the presence of FBS. Without HMS, there was no difference in the Th1 and Th2 cell frequency between FO- and CO-fed mice. Similar percentages of Th2 cells (9.4% for FO, 9.0% for CO, p>0.05, **Fig. 3A**) and Th1 cells (6.4% for FO, 6.5% for CO, p>0.05, **Fig. 3B**) were generated. Likewise, there was no difference (p>0.05) in the Th2/Th1 ratio of CD4$^+$ T cells from mice fed FO (1.5) and CO (1.3) when HMS was not present in the cultures.
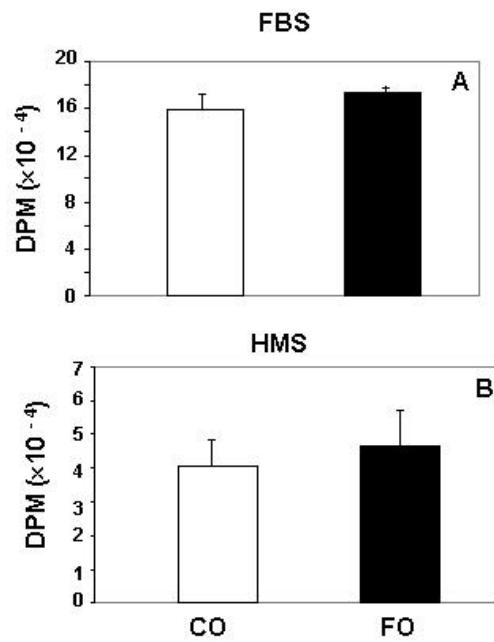
Figure 3. Dietary FO does not affect Th2 or Th1 cell frequency under Th2 polarizing conditions in the presence of FBS in C57BL/6 mice. Splenic CD4^{+} T cells were isolated from CO- or FO-fed mice and polarized to Th2 cells in presence of FBS as described in the Materials and Methods. Cytokines were determined by intracellular cytokine staining. Data represent the mean ± SEM; n = 3 replicates per diet group, and 5 mice were pooled per analysis. The experiments were repeated three times. A representative experiment is shown. Panel A represent IL-4^{+} Th2 cells; Panel B represent IFN-γ^{+} Th1 cells. CO, corn oil; FO, fish oil.
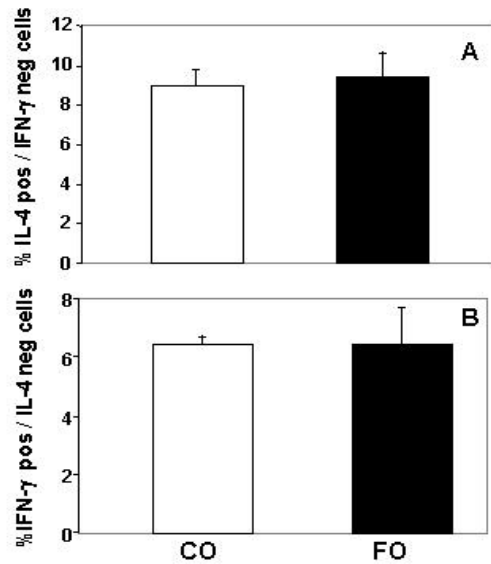
Figure 4. Lack of effect of dietary FO on proliferation of CD4^{+} T cells under Th2 polarizing

conditions. Splenic CD4^{+} T cells were isolated from CO- or FO-fed mice and cultured under Th2

polarizing conditions for 48 h in presence with either FBS (Panel A) or homologous mouse

serum (HMS) (Panel B). Cell proliferation was measured by uptake of ^{3}H-Thymidine during the

last 6 h of culture. Values represent the means ± SEM of net thymidine uptake (DPM) (n = 4 for

HMS, n = 3 for FBS). Results are from 1-3 separate experiments.

Dietary fish oil does not affect proliferation under Th2 polarizing conditions. Previously, we have shown that dietary n-3 PUFA enhance proliferation of Th2- like cells (74). To extend this observation, we measured the proliferation of purified splenic CD4$^+$ T cells following culture under Th2 polarizing conditions in the presence of either FBS or HMS. The proliferative response to anti-CD3/PMA of cells from FO- and CO-fed mice is shown in **Figure 4**. There was no significant difference in the degree of [^{3}H]-thymidine uptake between the FO and CO cells cultured either in the presence of FBS (**Fig. 4A**) or HMS (**Fig. 4B**). The overall proliferation was somewhat lower in the presence of HMS compared with FBS in both diet groups.

DISCUSSION

The work from our lab in the past ten years has demonstrated clearly that the anti-inflammatory effects of dietary fish oil was due, at least in part, to suppression of various T cell functions in C57BL/6 mice (64,73,74,82-84,88). Recently, we showed that CD4$^+$ T cells stimulated in vitro with anti-CD3/anti-CD28 displayed a Th1-like cytokine profile whereas those stimulated with anti-CD3/PMA showed a Th2-like cytokine profile (74). The observation that dietary FO suppressed IL-2 secretion of Th1-like cells and enhanced proliferation of Th2-like cells suggested that the anti-inflammatory effect of dietary n-3 PUFA could be the combined result of direct suppression of Th1 activation and indirect suppression of Th1 response by enhanced Th2 cross-regulation (74). Therefore, the main purpose of this study was to test the hypothesis that the anti-inflammatory effect of dietary n-3 PUFA was due to indirect Th1 suppression by

enhanced Th2 activation. We polarized murine splenic CD4^{+} T cells to Th2 cells in vitro

using the same polarizing conditions as those reported by many investigators to study

Th1/Th2 responses (55,91). The cytokines chosen for our study are among the strongest

determinants of T cell differentiation. IL-4 induces Th2 development and reduces the

Th1 response (27). IL-2 is also important for Th2 development (92). The common

polarizing protocol employs anti-CD3/anti-CD28, or PMA/Ionomycin as the stimuli,

plus rIL-2, rIL-4 and anti-IFN-γ for 2 or 3 d. The cells are then expanded for another 3 d

(89,90,93). An incubation period of 48 h has also been reported to be sufficient for naïve

CD4^{+} T cells to acquire the Th2 phenotype in vitro (87). Since we have shown that anti-

CD3/PMA promoted Th2 cytokine production (74) we chose these stimuli plus rIL-2,

rIL-4 and anti-IFN-γ for Th2 polarization. The dietary effect of n-3 PUFA depends, at

least in part, on diet-induced changes in membrane lipid composition, and long-term

culture in fetal bovine serum resulted in loss of effect of dietary PUFA on T cell function

(83). Therefore, we incorporated HMS and contracted the culture period. Among several

polarizing protocols tested, we found that incubation with anti-CD3/PMA plus rIL-2,

rIL-4, and anti-IFN-γ for 48 h gave the highest proportion of IL-4 positive cells.

Expanding the culture for another 3 d did not increase the percentage of IL-4 positive

CD4^{+} T cells (data not shown). We have elected not to include data on the mean

fluorescence intensity (MFI) of the IL-4 and IFN-γ positive cells for two reasons. First,

the question we addressed in this paper relates to the effect of dietary fish oil on Th2

development in vitro. Therefore, we determined the percentage of IL-4 and IFN-γ

positive cells which represent the Th2 and Th1 cell phenotype, respectively. Second,

most papers examining Th1/Th2 development only report the percentage of positive cells (89,90). Although the intracellular cytokine staining method provides information on cytokine synthesis at the single cell level, these data are relatively qualitative in nature. Therefore, we elected not to compare the cytokine level on per cell basis.

In vitro Th2 polarization is always more difficult than Th1 polarization. The majority of CD4$^+$ T cells do not polarize under Th2 polarizing conditions regardless of background of mice or the culture conditions (80). Genetic background is one determinant of Th1/Th2 development (30) and C57BL/6 mice are known to be Th1-prone (55). This may explain why less than 20% of the total cell population in our study was classified as IL-4$^+$, IFN-γ^- under Th2 polarizing conditions, and a small proportion of Th1 cells was also generated. Although repeated in vitro stimulation also seems to help enhance the yield of Th2 cells, IL-4 producing cells were always detected at lower frequency in established Th2 clones or short term Th2 polarized CD4$^+$ T cells perhaps due to transient IL-4 synthesis (80). The proportion of Th2 cells generated in our culture was within the typical range found in the literature and comparable to reported IL-4 positive cells under Th2 polarizing conditions from mice of similar background (94). For example, naïve CD4$^+$ T cells from C57BL/6 mice cultured with anti-CD3 and anti-CD28, in the presence of similar concentrations of IL-4, anti-IFN-γ and IL-2 for 1 wk, resulted in 34% Th2 cells (93). Only 9% Th2 cells were generated following stimulation with the Ac1-11 peptide from myelin basic protein (MBP) together with twice the IL-4 and similar anti-IFN-γ concentrations in splenocytes from Th1-prone B10.PL MBP TCR transgenic mice (94). In transgenic DO11.10 mice on the Th2-prone BALB/c

background, only 14% Th2 cells were induced when naïve CD4^{+} T cells were stimulated with OVA peptide and antigen-presenting cells in the presence of IL-4 for 1 wk (80). Even in established Th2 clones, synthesis of intracellular IL-4 could only be detected in approximately 40% of cells (80).

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

We have demonstrated that dietary n-3 PUFA do not affect either in vitro Th2 development or proliferation under Th2 polarizing conditions. However, dietary n-3 PUFA did increase the Th2/Th1 ratio by suppression of Th1 development in the presence of HMS. Splenic CD4^{+} T cells from FO-fed mice exhibited decreased Th1 differentiation following culture under both neutral and Th2 polarizing conditions (**Fig. 2B**). Under neutral conditions, the simultaneous suppression of Th1 cells and enhancement of Th2 cells in FO-fed mice could be explained by the cross-regulation of Th1 and Th2 cells (95). It is possible that FO acts on Th1 cells, or Th2 cells, or both. Under Th2 conditions, although FO maintained the suppression of Th1 development,

there was no difference in Th2 differentiation between FO and CO. Because cross-regulation of Th2 cells by Th1 cells was diminished in the presence of Th2 polarizing conditions, i.e., IL-4 and anti-IFN-γ, the cells were pushed toward the Th2 pole to their maximum capacity. The similarity in the number of Th2 cells between FO and CO under polarizing conditions suggests the lack of a direct effect on Th2 development (**Fig. 2A**). Therefore, dietary n-3 PUFA does not appear to enhance Th2 polarization but does suppress Th1 cells, thus creating a shift in the Th1/Th2 balance (**Fig. 2C**).

Our findings confirm the observation of decreased IFN-γ production by T cells from animals fed diets enriched with FO or human patients taking FO supplements. Fish oil feeding inhibited Con A stimulated IFN-γ production by whole splenocytes in C57BL/6 mice but had little effect on Con A stimulated IL-4 production (75). Similar findings on IL-4 were observed in MF1 mice (78). In addition, fish oil supplements decreased IFN-γ production and IL-2 production by peripheral blood mononuclear cells harvested from MS patients (77). Previously we showed that there was no difference in IL-4 secretion in CD4^{+} T cells cultured with anti-CD3/PMA (74). Here, we show that dietary FO shifts the Th1/Th2 balance towards the Th2 pole by direct suppression of Th1 development, as evidenced by reduced IFN-γ production (**Fig. 2B and 2C**).

Suppression by dietary n-3 PUFA of Th1 development in CD4^{+} T cells cultured under Th2 polarizing conditions was only observed when T cells were cultured in the presence of HMS (**Fig. 2B**). When the cells were polarized in cultures with FBS, there was no difference in either Th2 or Th1 development from CD4^{+} T cells from FO- and CO-fed mice (**Fig. 3**). The suppression of Th1 cells by FO feeding could be due to

changes in lipid rafts. Our lab has demonstrated that FO feeding modulates the lipid composition of raft microdomains in murine $CD4^+$ T lymphocytes (64). HMS in culture has been shown in our experimental system to be essential to preserve the diet-induced in vivo changes in T cell function and microdomain lipid composition (83). Considering that activated Th1 and Th2 cells have distinct patterns of membrane receptor compartmentalization into lipid rafts (45), it is possible that dietary n-3 PUFA differentially modulate membrane microdomains in Th1 and Th2 cells and selectively inhibit recruitment of certain signaling molecules important for Th1 differentiation and activation. In support of this hypothesis, we have shown that dietary DHA inhibits recruitment of PKC-θ into lipid rafts in $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28, resulting in reduced NF-κB and AP-1 activation and IL-2 secretion (82). Differentiation of $CD4^+$ T cells into Th1 cells requires the activation of a MAP kinase, JNK2, while differentiation into Th2 cells does not require JNK2 activation (96). In addition, NFAT promotes Th1 differentiation (97). Treatment of human Jurkat cells with EPA significantly inhibited JNK activity and NFAT (98). This may also occur in FO-fed mice, and explain why dietary FO only affects Th1 differentiation. The selective inhibition of JNK and NFAT could be explained by modulation of rafts by PUFA. The translocation of the palmitoylated and acylated adapter protein, LAT, has been shown to be an important upstream event for NFAT activation (61). Treatment of human Jurkat cells with EPA resulted in the displacement of LAT from rafts (63). It is therefore possible that alteration in lipid raft composition could regulate JNK activation. Taken together, the data suggest that the suppression of Th1 development by FO feeding could

be due to changes in the molecular interactions of lipids in rafts. These hypothetical mechanisms will be tested in future experiments.

The lower proliferation of Th2-polarized $CD4^+$ T cells observed in the presence of HMS but not FBS (**Fig. 4**), is consistent with previous findings (53). We have previously observed enhanced proliferation in Th2-like $CD4^+$ T cells from FO-fed mice (74), which was not recapitulated in this study. This may be attributable to differences in stimuli and culture conditions. Since a heterogeneous $CD4^+$ T cell population (Th1, Th2, and unpolarized) existed in the present studies, the proliferation measured represented the sum of all cells present. Only experiments using an isolated Th2 population will provide a definitive conclusion about the precise effect of diet on Th2 proliferation. These results differ from the well-documented suppression of lymphocyte proliferation by FO observed by us and others (71,73). However, considering that FO only affected Th1 development, it is possible that the reported suppression of lymphocyte proliferation was observed in cell cultures where Th1 cells dominated.

In summary, we found that dietary n-3 PUFA shift the Th1/Th2 balance towards the Th2 pole by suppression of Th1 development rather than enhancement of Th2 development. The results here are directly relevant to the therapeutic use of dietary FO in many Th1-mediated autoimmune and hypersensitivity diseases. Fish oil has the capacity to suppress Th1 development and reduce IFN-γ production without a concomitant effect on Th2 development. This may be beneficial because n-3 PUFA will not affect Th2-related antibody production, which may be important for resistance against some infections (28). Although Th2 cells were initially thought to be protective

in some autoimmune diseases, subsequent research showed that a deviation in the Th1 to Th2 effector population might result in more complications in many Th1-mediated diseases (99-101). Therefore, the lack of a direct effect on Th2 cells has advantages for FO as an adjunct therapy for Th1-mediated autoimmune diseases.

CHAPTER III

DIETARY N-3 POLYUNSATURATED FATTY ACIDS INHIBIT ANTIGEN-SPECIFIC Th1 CELL DEVELOPMENT BY SUPPRESSION OF CLONAL EXPANSION

Dietary fish oil (FO) supplementation has been shown to be beneficial in certain Th1-mediated autoimmune diseases. The purpose of this study was to determine how dietary FO affects antigen-stimulated Th1 cell development in vitro. DO11.10 Rag2$^{-/-}$ TCR-transgenic mice were fed a control diet (5% corn oil (CO), n-6 PUFA) for 1 week, followed by the same control diet or a FO diet (1% CO + 4% FO, n-3 PUFA) for 2 wk. CD4$^+$ T cells were isolated from spleens and lymph nodes, stimulated with OVA peptide and irradiated BALB/c splenocytes in the presence of rIL-12, αIL-4, and rIL-2 for 2 d, followed by expansion with rIL-2 and rIL-12 for 3 d. After reactivation with PMA and Ionomycin, cells were surface stained with the clonotypic mAb KJ1-26, followed by intracellular staining for IFN-γ and IL-4. Flow cytometric analysis showed no difference (p>0.05) in the percentage of KJ1-26$^+$, IFN-γ^+, IL-4$^-$ Th1 cells between CO-fed (98.8$\pm$0.7%) and FO-fed mice (97.4$\pm$0.8%). However, the absolute number of Th1 cells in polarized cultures from FO-fed mice was 61% lower than that observed in CO-fed mice (p=0.03). Cell proliferation, as assayed by ^{3}H-thymidine uptake, showed a 15% decrease in Th1 cells from the FO group (p=0.01). Therefore, an attenuated Th1 response in clinical trials of FO supplementation may be explained, at least in part, by suppression of clonal expansion during Th1 differentiation.

INTRODUCTION

Antigen stimulation elicits clonal expansion of $CD4^+$ T cells, during which, the cells may differentiate into two distinct types of effector cells, Th1 and Th2, based on cytokine secretion profiles. Th1 cells produce IL-2, IFN-γ, and lymphotoxin; Th2 cells produce IL-4, IL-5, IL-10 and IL-13 (26,102). Although there is controversy regarding the role of Th2 cells in some autoimune diseases such as insulin-dependent diabetes mellitus (IDDM), multiple sclerosis (MS), the pathogenic role of Th1 cells in such diseases is well established (28,99). Elucidation of the molecular basis for Th1 and Th2 development could contribute to the modulation of Th1/Th2 ratios as therapy for treating Th1 cell- mediated chronic inflammation (40,41,90). Cytokines play crucial roles in mediating the Th cell differentiation outcome. For example, IL-12 promotes Th1 development, while IL-4 favors Th2 development (55). Many other factors have been found to influence Th1/Th2 differentiation: genetic background, antigen dose, and the strength of TCR and costimulatory signaling (27,30,33). Interestingly, some nutrients such as Vitamins A, D, E, and dietary component ribonucleotides have also been shown to strongly influence Th1/Th2 differentiation (36-39,94). Therefore, defining the mechanisms by which nutrients modulate Th1/Th2 development could facilitate the use of diet as adjunct therapy for treating T cell-mediated autoimmune diseases.

Among dietary factors, n-3 polyunsaturated fatty acids (PUFA) found in fish oil (FO) have been shown to attenuate many T cell-mediated inflammatory diseases (6,103). The primary effector molecules are considered to be eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Clinical dietary intervention trials demonstrated a

protective effect of FO in rheumatoid arthritis (RA), Chrohn's disease, and ulcerative colitis (5). Dietary FO has also been shown to have beneficial effects in various animal models of human chronic diseases, including decreased joint inflammation in rodents with collagen-induced arthritis, reduced inflammation in rat models of colitis, and decreased proteinuria in mice with autoimmune glomerulonephritis (5).

We and others have recently demonstrated that dietary n-3 PUFA affect a variety of immune responses by modulating T cell function (6). Specifically, FO suppressed proliferation of splenocytes and purified $CD4^+$ T cells in response to mitogen, reduced IL-2 secretion and IL-2 receptor mRNA, increased AICD of $CD4^+$ T cells and polarized Th1 cells, and suppressed T cell signaling, in part via changes in lipid rafts. (64,73,74,82,84). However, studies investigating the effects of dietary n-3 PUFA on Th1 and Th2 effector cells and their cytokines are limited. Wallace et al reported decreased IFN-γ protein in splenocytes from FO-fed mice (75). Suppressed IFN-γ gene expression was also found in the Peyer's patches of FO-fed BB rats (76). Similarly, in human MS patients, FO supplementation reduced IL-2 and IFN-γ secretion by peripheral blood mononuclear cells (77). In addition, FO supplementation of healthy human volunteers decreased DTH responses to seven recall antigens (6). It is known that the DTH responses are mediated by Th1 subsets (104). Consistent with these human studies, FO-fed mice showed reduced DTH responses when compared to those fed the control n-6 PUFA or olive oil-rich diets (105). Taken together, these studies strongly suggest that FO may suppress the immune response by inhibiting proinflammatory cytokine production by Th1 cells.

Our laboratory recently demonstrated that purified mouse CD4$^+$ T cells stimulated in vitro with various agonist combinations display distinct cytokine profiles that correspond with Th1 and Th2 cells (74). Splenic CD4$^+$ T cells cultured with phorbol ester (PMA) and Ionomycin expressed a Th1-like cytokine profile while those activated with anti-CD3/PMA exhibited a Th2-like profile. The suppressed IL-2 secretion of Th1-like cells from FO-mice compared to CO-fed mice suggested that FO may alter Th1 development. Indeed, we recently confirmed that the anti-inflammatory properties of dietary FO were due, in part, to a direct effect on Th1 development as opposed to the indirect regulation of Th2 activation (106). Unfortunately, to date, previous studies of n-3 PUFA on T cell function have used non-physiologic, polyclonal stimuli such as ConA or anti-CD3/CD28. In addition, little is known regarding how dietary n-3 PUFA affect antigen-induced T cell activation and antigen-stimulated Th1 development. Therefore, in the current study, we investigated the effects of dietary n-3 PUFA on antigen-specific CD4$^+$ T cell activation and differentiation into Th1 cells in vitro using the DO11.10 Rag2$^{-/-}$ transgenic mouse system, a widely used model to study antigen-specific T cell activation and differentiation. The DO11.10 mouse expresses a transgenic T cell receptor (TCR), which recognizes a peptide, OVA $_{323-339,}$ within ovalbumin and can be identified by using the clonotypic mAb KJ1-26 (54). Besides antigen-specificity, this model has another advantage due to the rag 2 gene deletion. Virtually all peripheral CD4$^+$ T cells from these mice are TCR transgenic, whereas only about 25% of the CD4$^+$ T cells express the transgenic TCR in the periphery of the conventional DO11.10 mouse. Using

this model, we report that dietary n-3 PUFA inhibits antigen-specific CD4$^+$ T cell proliferation and clonal expansion of polarized Th1 cells.

MATERIALS AND METHODS

Animals and diets

All experimental procedures using laboratory animals were approved by the University Laboratory Animal Care Committee of Texas A&M University. Pathogen-free, female, young BALB/c (4-6 wk of age) were purchased from Frederick National Cancer Research Facility, Frederick, MD. DO11.10 Rag2$^{-/-}$ mice were purchased from Taconic, bred and maintained at Texas A & M University. At the onset of the study, DO11.10 Rag2$^{-/-}$ mice (8-10 wk) were housed 5 per microisolator cage, and had free access to water and diet. DO11.10 Rag2$^{-/-}$ mice were fed a control semi-purified diet containing 5% corn oil (CO) by weight during the 7-d acclimation period followed by a 2-wk feeding period with the same control diet or a fish oil (FO) diet (1% CO + 4% FO). All diets met the NRC nutrition requirements and varied only in lipid content (84). The basic diet composition, expressed as g/100g was: casein, 20; sucrose, 42; cornstarch, 22; cellulose, 6; AIN-76 mineral mix, 3.5; AIN-76 vitamin mix, 1; DL-methionine, 0.3; choline chloride, 0.2; Tenox 20A, 0.1; and oil, 5. The fatty acid composition of the diets, as determined by gas chromatography, is shown in Table 1. BALB/c mice were fed standard mouse chow.

Chemical compounds, cytokines and antibodies

Phorbol myristate acetate (PMA) was purchased from Sigma (St. Louis, MO).

Ionomycin was purchased from Calbiochem-Novabiochem (San Diego, CA).

Recombinant murine IL-2 was purchased from R&D Systems (Minneapolis, MN).

Neutralizing rat mAb for murine IL-4 was obtained from the National Cancer Institute.

Hamster anti-mouse CD3e monoclonal antibody (clone145-2C11), recombinant murine

IL-12, PE-labeled anti-IL-4 (clone 11B11) and FITC-labeled anti-IFN-γ (clone

XMG1.2) was purchased from BD PharMingen (San Diego, CA). $OVA_{323-339}$ peptide

was synthesized by Biosource, International, Camrillo, CA using the following

sequence: ISQAVHAAHAEINEAGR. Mouse monclonal antibody to DO11.10 TCR

KJ1-26 was purchased from Caltag Laboratories, Burlingame, CA.

T cells

Spleens and lymph nodes (brachial and mesenteric) were taken from DO11.10

$Rag2^{-/-}$ mice and placed in 3 mL RPMI complete media (RPMI 1640 medium with 25

mM HEPES (Irvine Scientific, Santa Ana, CA) supplemented with 5% FBS or

homologous mouse serum (HMS), 1×10^5 U/L penicillin and 100 mg/L streptomycin

(Irvine Scientific), 2 mM L-glutamine, and 10 μM 2-mercaptoethanol). The spleens and

lymph nodes were homogenized and filtered as described earlier (74). Cells were then

combined and total lymphocytes enriched by density gradient centrifugation using

Lympholyte-M (Cedarlane, Toronto, Canada). $CD4^+$ T cells were purified by a negative

selection column method as previously described (74). For Th1 polarization, cells were

adjusted to 2.5×10^5 cells/mL and cultured in 24-well plates in 2 mL complete media, with 5×10^6 irradiated BALB/c splenoctyes. Homologous serum was collected according to the method of Pompos et al. (53).

Th1 polarization

To drive Th1 development, $CD4^+$ T cells were cultured with the OVA peptide (0.3 μM) in the presence of irradiated BALB/c splenocytes (2600 rads) combined with rIL-12 (5 ng/mL), anti-IL-4 (10 μg/mL) and rIL-2 (20 ng/mL). After culture at 37 °C with 5% CO_2 for two days, the cells were expanded with 20 ng/mL rIL-2 and 5 ng/mL rIL-12 for an additional three days. Cytokine expression was analyzed via Flow cytometry (FACScan; Becton Dickinson, Bedford, MA).

Intracellular cytokine staining

Following Th1 polarization, live cells were separated by Lympholyte-M and cultured (10^6 cells/mL) with PMA (10 ng/mL) and Ionomycin (1μg/mL) for 5 h at 37 °C. A protein transport inhibitor, GolgiStop^{TM} (BD Farmington, San Diego, CA), was added according to the manufacturer's recommendations during the last 2 h of incubation. Cells were subsequently isolated by centrifugation, stained with Fc block, washed and stained with KJ1-26-TC (Caltag laboratories, Burlingame, CA). After fixation and permeabilization steps for 20 min at 4 °C in 250 μl Perm/Fix (BD Farmington, San Diego, CA), cells were washed in Perm/Wash (BD PharMingen, San Diego, CA) twice followed by staining with PE-labeled mAb to murine IL-4 and FITC-labeled mAb to

murine IFN-γ in 100 μL Perm/Wash. The cells were washed twice in Perm/Wash and resuspended in 500 μL staining buffer (PBS with 0.05% FBS and 0.01% sodium azide) for analysis on a FACS-Calibur (Becton Dickinson Immunocytometry systems, San Jose, CA).

T cell proliferation

For proliferation of CD4$^+$ T cells under neutral conditions, CD4$^+$ T cells were cultured with OVA peptide (0.3 μM) in the presence of irradiated BALB/c splenocytes (2600 rads) in 96-well round-bottomed microtitre plates (Falcon, Becton-Dickinson, Lincoln Park, NJ). Cells were labeled with 1.0 μCi [^{3}H]thymidine and cultured for 5 h at 37 ^{o}C with 5% CO_2. Cells were harvested and counted as described previously (74). For proliferation of polarized Th1 cells, following Th1 polarization, cells were resuspended and a 200 μL aliquot was transferred to 96-well round-bottomed microtitre plates (Falcon, Becton-Dickinson, Lincoln Park, NJ). Cell proliferation was measured as described above.

IL-2 receptor assay

CD4$^+$ T cells from diet-fed DO11.0 Rag2$^{-/-}$ mice were cultured with OVA peptide (0.3 μM) in the presence irradiated BALB/c splenocytes (2600 rads) for 24 h. For quantitative surface IL-2 receptor staining, 1×10^6 cells were stained with vital dye 7-amino-actinomycin D (7-AAD) and anti-CD25-PE (BD PharMingen). Quantification was performed using PE-labeled beads (Becton-Dickinson) (details see Appendix B-12).

Apoptosis

CD4$^+$ T cells from diet-fed DO11.0 Rag2$^{-/-}$ mice were Th1 polarized as described above. Cells were harvested and stained with Annexin-V and PI (BD PharMingen) and analyzed by flow cytometry as previously described (84) (details see Appendix B-13).

Statistical analysis

Significance of main treatment effects was assessed using PROC GLM in SAS. The differences between means were determined by Tukey's test. A 95% level of probability was accepted as being statistically significant.

RESULTS

Lymphocyte yield from DO11.10 Rag 2$^{-/-}$ mice

Spleens from DO11.10 Rag2$^{-/-}$ mice were much smaller than spleens from B10.D2 wild type mice. Total splenic lymphocyte yield (1.7 x 10^6) was much lower than that in the wild type mice (20-30 x 10^6). Approximately 40-70% of the cells was TCR positive in whole blood from the transgenic mice, while greater than 90% cells from lymph nodes were CD4$^+$ and TCR$^+$ (Appendix A-3).

Dietary n-3 PUFA suppress antigen specific CD4$^+$ T cell proliferation

To determine how dietary n-3 PUFA affect antigen specific CD4$^+$ T cell proliferation, CD4$^+$ T cells from CO and FO-fed DO11.10 Rag2$^{-/-}$ mice were isolated and stimulated with OVA peptide in the presence of irradiated splenocytes from chow-

Figure 5. Dietary FO suppresses antigen-specific CD4^{+} T cell proliferation. Purified CD4^{+} T cells from DO11.10 Rag2^{-/-} mice were cultured with OVA peptide and irradiated BALB/c splenocytes in 96-well plates in the presence of either FBS or homologous mouse serum (HMS). The cells were labeled with [³H]Thymidine for the last 5 h of the 72 h incubation. Values represent the mean ± SEM of the net thymidine uptake (DPM); n=4 replicates per diet group, and 5 mice were pooled per analysis. Different letters indicate significant differences between the two diet groups.

fed BALB/c mice. Preliminary studies demonstrated that CD4^{+} T cells from DO11.10 Rag2^{-/-} mice proliferate in a manner dependent both on the dose of OVA peptide and the number of antigen-presenting cells (APCs) (Appendix A-5). The optimum OVA dose

was in the range of 0.1-1 μM and two concentrations of APC were selected to examine

optimal and suboptimal stimulation of CD4$^+$ T cells. When cultured in the presence of

homologous mouse serum, FO significantly suppressed [^{3}H]thymidine incorporation at

two APC concentrations (**Fig. 5**). However, when cultured with FBS, there was no

significant difference in T-cell proliferation under either optimal or suboptimal

conditions from CO and FO-fed mice ($p > 0.05$).

Dietary n-3 PUFA do not affect in vitro Th1 differentiation

To determine whether dietary n-3 PUFA affect in vitro antigen-stimulated Th1

differentiation, CD4$^+$ T cells harvested from CO and FO-fed DO11.10 Rag2$^{-/-}$ mice were

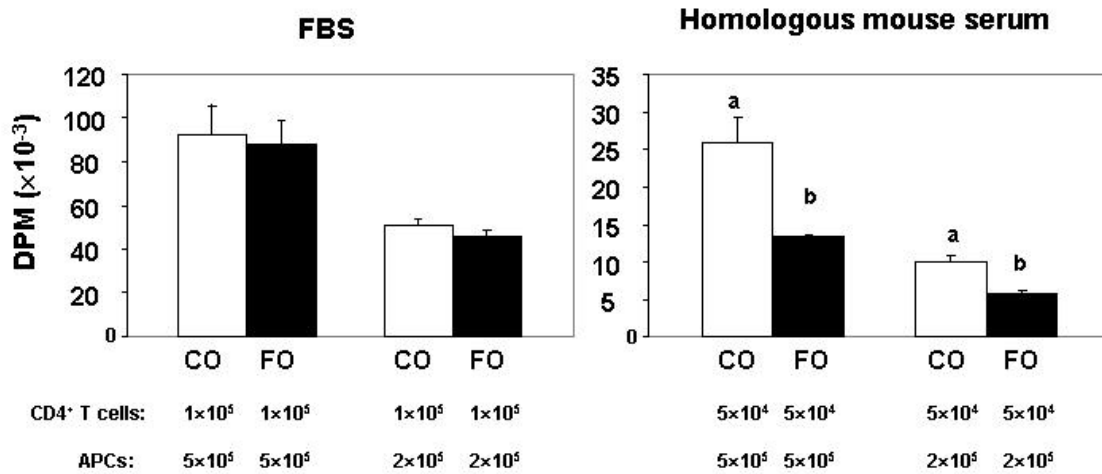stimulated with OVA peptide in the presence of irradiated splenocytes from chow-fed

BALB/c mice and rIL-12, rIL-2, and anti-IL-4. The cytokine patterns of differentiated

Th1 cells were very similar for CO and FO-fed mice. Virtually all cells were IFN-γ

positive, IL-4 negative Th1 cells following the 5-d in vitro polarization regimen, FO

(97%) and CO (99%) (**Fig. 6**). Only Th1 cells were generated and no Th2
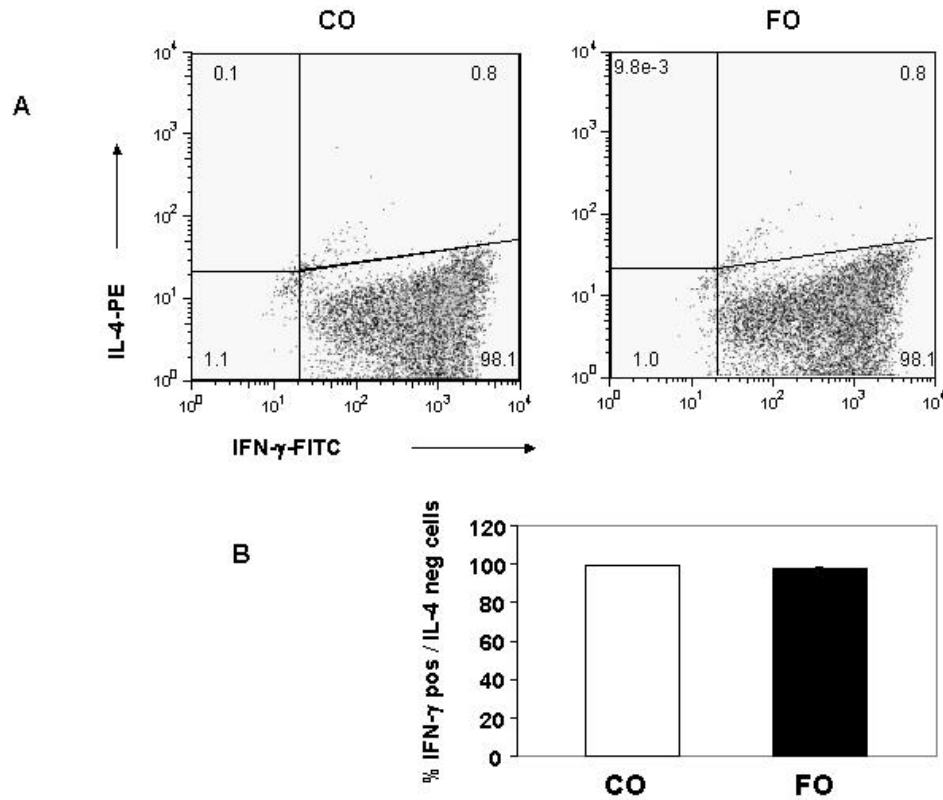
Figure 6. Dietary FO does not affect antigen-specific Th1 differentiation. Purified CD4^{+} T cells from DO11.10 Rag2^{-/-} mice (different than **Fig. 5**) were cultured with OVA peptide in the presence of irradiated BALB/c splenocytes with IL-12, IL-2, and anti-IL-4. After 2 d, the cell culture was expanded for another 3 d. At d 5, cells were harvested, restimulated for 5 h with PMA/Ionomycin for intracellular IFN-γ and IL-4 staining. A: Representative dot plots from cultures from FO and CO-fed mice. The numbers in the lower right quadrant of the dot plot are the percentage of IFN-γ-producing cells (Th1) and the numbers in the upper left quadrant are the percentage of IL-4-producing cells (Th2). B: Bars represented the percentage of Th1 cells generated under above conditions. Data represent means ± SEM; n=4 replicates per diet group, and 5 mice were pooled per analysis.
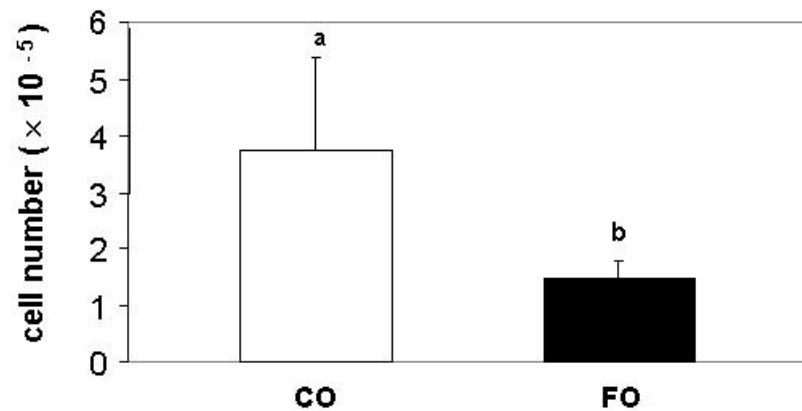
Figure 7. Dietary FO suppresses IL-2 induced clonal expansion of Th1 cells. Purified

$CD4^+$ T cells from DO11.10 $Rag2^{-/-}$ mice (same as **Fig.6**) were cultured with OVA

peptide in the presence of irradiated BALB/c splenocytes with IL-12, IL-2, anti-IL-4.

After 2 d, the cell culture was expanded for another 3 d. At d 5, cells were harvested,

viable cells separated by Lympholyte-M, and the number of living cells was determined

by trypan blue exclusion using a hemocytometer. Letters indicate significant differences

between the two diet groups. Data represent means ± SEM; n=4 replicates per diet

group, and 5 mice were pooled per analysis.

cells were detected. These data indicated that FO feeding did not influence in vitro Th1 differentiation.

Dietary n-3 PUFA suppress IL-2 induced clonal expansion of antigen-specific Th1 cells

Although dietary FO did not affect Th1 differentiation, a difference in media color was noticed between cultures of cells from FO and CO-fed mice. The color of cultures from CO-fed mice was yellowish by d 5 but the color of FO was still pink.

This suggests that the dynamics of cell growth were different between the two groups during the last 3-d expansion period. Analysis of cell counts showed a significant difference between FO and CO cultures (p=0.0335, **Fig. 7**). The absolute total number of viable cells harvested from FO-fed mice cultures (1.5×10^6 cells per well) was 61% lower than that from CO-fed mice (3.7×10^6 cells per well). These data indicate that FO feeding suppressed IL-2 induced clonal expansion of differentiated Th1 cells. In addition, with regard to [^{3}H]thymidine incorporation, the proliferative response was significantly decreased at d 4 (p=0.042) and d 5 (p=0.0115, **Fig. 8**).
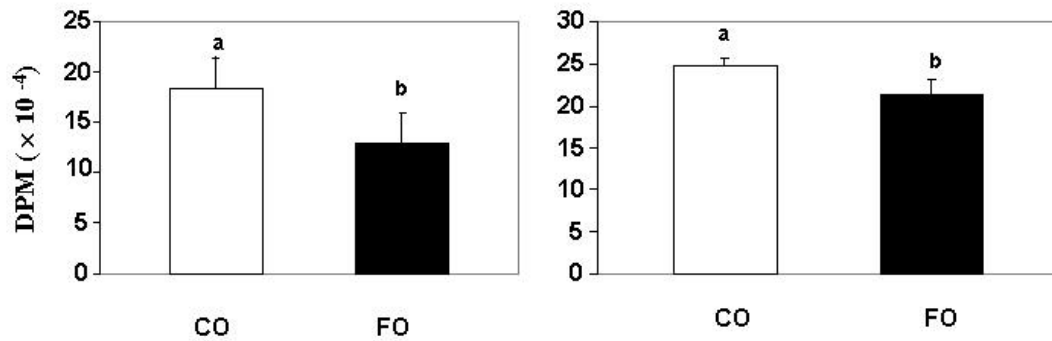
Figure 8. Dietary FO suppresses IL-2 induced cell proliferation of polarized Th1 cells.

Purified CD4$^+$ T cells from DO11.10 Rag2$^{-/-}$ mice (different from **Fig. 7**) were cultured

with OVA peptide in presence of irradiated BALB/c splenocytes with IL-12, IL-2, anti-

IL-4. After 2 d, the cell culture was expanded for another 3 d. At d 4 and 5, cells were

transferred to 96-well plates and labeled with [^{3}H]thymidine for 5 h. Values represent

the mean ± SEM of the net thymidine uptake (DPM); n=4 replicates per diet group, and

5 mice were pooled per analysis. Different letters indicate significant differences

between the two diet groups.
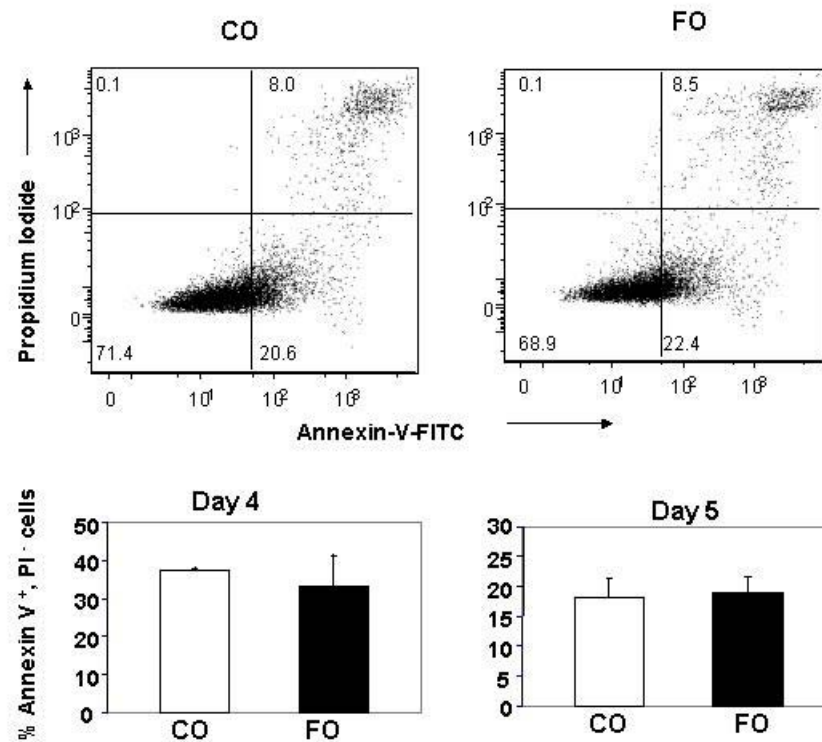
Figure 9. Dietary FO does not affect early apoptosis in IL-2 expanded Th1 effector cells. Purified CD4$^+$ T cells from DO11.10 Rag2$^{-/-}$ mice (different from **Fig. 8**) were cultured with OVA peptide in presence of irradiated BALB/c splenocytes with IL-12, IL-2, anti-IL-4. After two days, the cell culture was expanded for another three days. At day 4 and 5, cells were harvested and live cells separated by Lympholyte-M. Cells were stained with Annexin-V-FITC and propidium iodide and analyzed by flow cytometry. Data represent means ± SEM; n=4 replicates per diet group, and 5 mice were pooled per analysis.

Dietary n-3 PUFA does not affect apoptosis of polarized Th1 cells

To determine whether apoptosis contributed to decreased cell number in Th1 polarized cultures from FO-fed mice, Th1 cells were stained using Annexin-V-FITC and

PI. Flow cytometric analysis revealed no difference in early apoptosis (Annexin-V positive and PI negative cells) between FO and CO-fed mice at day 4 and day 5 in culture (**Fig. 9**).

Surface CD25 expression on antigen-specific CD4$^+$ T cells is not altered by diet

To further elucidate the influence of dietary n-3 PUFA on IL-2 induced proliferation of polarized Th1 cells, surface CD25 expression was determined on CD4$^+$ T cells from diet fed mice stimulated with OVA. As expected, stimulation with OVA increased surface CD25 expression compared to non-stimulated cells (**Fig. 10 A**). Since the same amount of IL-2 was present in the Th1 polarizing cultures during the last three days expansion period, the decreased proliferation could be explained by a suppression of IL-2 receptor signaling. It is known that the polarizing conditions did not alter cell proliferation in similar cultures of antigen-specific CD4$^+$ T cells (48). Indeed, we also observed decreased proliferation of CD4$^+$ T cells under non-polarizing conditions. Therefore, we decided to measure IL-2 R α chain (CD25) expression under non-polarizing conditions, i.e., after 24 h stimulation with OVA peptide and APCs. Since the
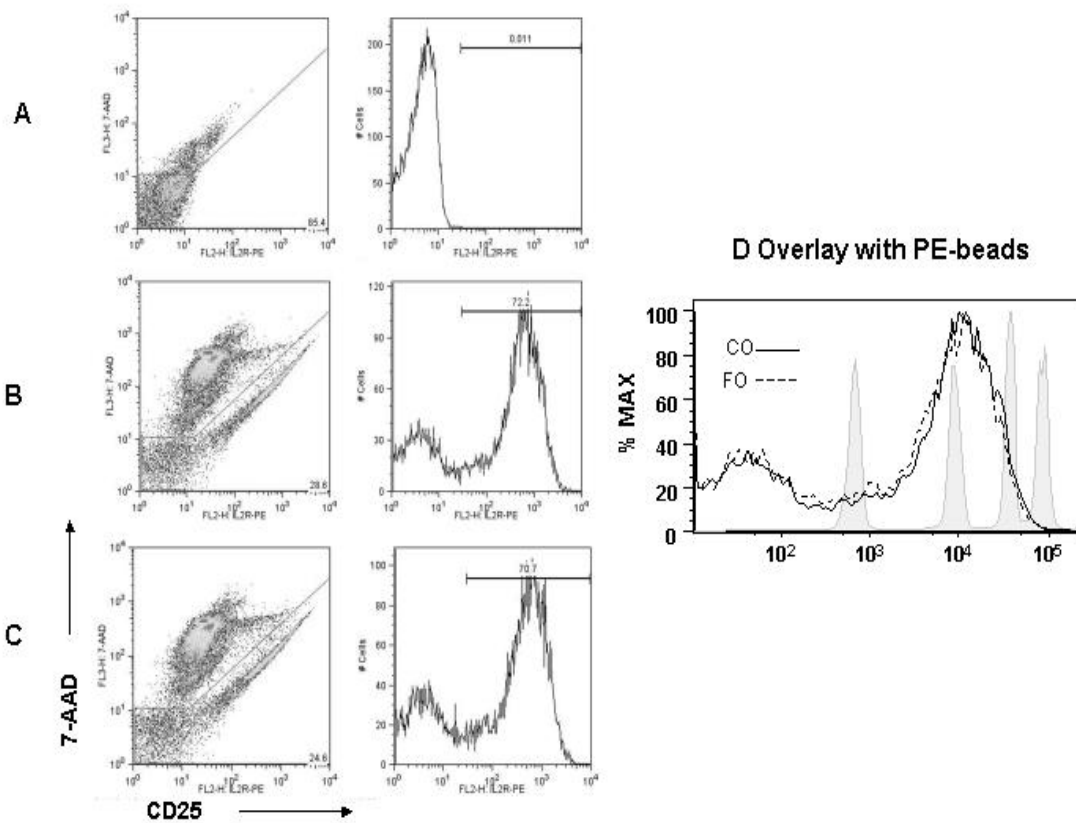
**Figure 10.** Dietary FO does not affect surface CD25 expression on antigen-specific CD4^{+} T cells. Dietary n-3 PUFA suppressed IL-2 induced cell proliferation of Th1 cells. Purified CD4^{+} T cells from DO11.10 Rag2^{-/-} mice (different from **Fig. 9**) were cultured with OVA peptide in the presence of irradiated BALB/c splenocytes. After 24 h, cells were harvested, stained with 7-AAD and anti-CD25-PE, and analyzed by flow cytometry. PE beads were used to quantify CD25. (A) Unstimulated sample; (B) CD25 levels on CD4^{+} T cells from CO-fed mice; (C) CD25 levels on CD4^{+} T cells from FO-fed mice; (D) Overlay of the histogram of CD25 expression with PE beads. n=4 per diet group, and 5 mice were pooled per analysis.

culture period was relatively short, we omitted homologous mouse serum from the

culture. CD25 was only measured on living cells as defined by non-staining of 7-AAD.

Surprisingly, there was no difference (p >0.05) in the surface expression of CD25

between FO and CO-fed mice (**Fig.10**). Cultures from CO and FO-fed mice exhibited a

similar percentage of viable cells, CD25 positive cells with approximately same median

fluorescence of intensity (**Table 2**).

TABLE 2

Dietary FO does not affect CD25 expression on antigen-stimulated CD4^{+} T cells

	CO	FO	p
% viable cells	31.9 (3.8)	29.1 (4.0)	0.35
% CD25 positive cells	66.7 (5.5)	65.8 (4.8)	0.81
Median α-CD25-PE intensity	8811 (1382)	8488 (1410)	0.72

Purified CD4^{+} T cells from CO or FO-fed DO11.10 Rag 2[-/-] mice were cultured with OVA

peptide in the presence of irradiated splenocytes from chow-fed BALB/C mice. After 24 hour,

the cells were harvested, stained with 7-AAD and anti-CD25-PE, and analyzed by flow

cytometry. PE beads were used to quantify CD25 expression.

DISCUSSION

A reduction in IFN-γ production by FO feeding has been reported by several

investigators (75-77). Therefore, we hypothesized that FO suppresses Th1 development.

Since genetic background determines default Th1/Th2 development of naïve Th cells

under neutral culture conditions, and the DO11.10 mice we used are on a Th1- prone B10.D2 background (30), it is not surprising that almost all cells were polarized to a Th1 phenotype, i.e., >90% (**Fig. 6**). This is comparable to what has been achieved using similar Th1 polarizing conditions (90). Surprisingly, FO did not affect the frequency of Th1 cells generated under Th1 polarizing conditions, which strongly suggests that dietary FO does not affect the Th1 differentiation process. However, we did notice that the total number in culture by d 5 was significantly lower in FO-fed compared to CO-fed mice. In this type of culture, the BALB/c splenocytes actually die after d 2 (46,48), and the $CD4^+$ T cells acquire the Th1 phenotype after 48 h (87). Since there was only rIL-2 and rIL-12 in the culture media after d 2, and rIL-12 does not contribute to cell expansion (107), the modulation in cell number is likely due to the difference in the expansion of polarized Th1 cells. This is noteworthy, because an effective immune response depends not only on the acquisition of Th1 and Th2 phenotypes but also requires the survival and clonal expansion of differentiated effector cells. In addition, autoreactive Th1 cells are major players in some chronic inflammatory conditions. Therefore, inhibition of clonal expansion of Th1 cells could be a major mechanism to explain the beneficial anti-inflammatory effects of dietary FO.

In Th1 polarized cultures, two active processes exist: cell proliferation and apoptosis. With regard to apoptosis, we have previously shown that dietary FO promotes activation-induced cell death (AICD) of polarized Th1 cells (83). In antigen stimulated $CD4^+$ T cells, a high level of apoptosis were observed in primary cultures and similarly in mouse T cell lines (47). Apoptotic cells were generated within each cell cycle. We

found that in IL-2 expanded Th1 cultures, and cells from FO and CO-fed mice displayed a similar percentage of apoptotic cells, indicating that IL-2 induced apoptosis did not play a role in diet-mediated differences in cell number (**Fig. 8**).

The finding of suppression of proliferation of polarized Th1 cells by dietary n-3 PUFA is novel. Although suppressed T cell proliferation has been a widely reported effect of n-3 PUFA, this is the first study that clearly demonstrates the suppression of fully differentiated Th1 cells. Along these lines, previous reports about diminished IFN-γ production by n-3 PUFA could be due to a reduction in proliferation of Th1 cells rather than an impairment of Th1 differentiation (75,77). We demonstrated a reduction in the proliferation of antigen-specific CD4^{+} T cells and fully polarized Th1 cells, suggesting a main effect of dietary n-3 PUFA on the molecular machinery for proliferation as opposed to differentiation. In fact, there are similarities between the cultures of CD4^{+} T cells and polarized Th1 cells. Specifically, there are two distinct stages for the transition of CD4^{+} T cells to effectors (46). In cultures of antigen stimulated CD4^{+} T cells, efficient peptide presentation was limited to the first 1-2 d, with the late stage being IL-2 driven antigen-independent expansion. Therefore, the proliferation of CD4^{+} T cells which we measured at day 3 was truly the proliferative response to IL-2.

Many studies have investigated the suppression of T cell proliferation by n-3 PUFA. Reduced IL-2 secretion by n-3 PUFA has been widely reported. The addition of EPA decreased IL-2 secretion by cultures of human peripheral blood mononuclear cells (PBMC) (108) and human Jurkat T cells (98). We have shown that dietary FO decreased IL-2 secretion in CD4^{+} T cells stimulated with anti-CD3/anti-CD28 (74). In a similar

antigen-specific model (74), Pompos et al. reported that dietary n-3 PUFA reduced OVA peptide stimulated IL-2 secretion from splenocytes from TCR transgenic mice (53). This could be true in our culture of CD4$^+$ T cells. However, no study to date has determined whether exogenous rIL-2 is capable of rescuing suppression of proliferation by n-3 PUFA. This is precisely what occurred in our Th1 polarized cultures, in the presence of the same amount of IL-2, dietary fish oil still suppressed proliferation of polarized Th1 cells (**Fig.9**). This clearly suggests a new mechanism by which n-3 PUFA interfere with T cell clonal expansion, namely, by suppression of IL-2 R signaling.

IL-2 R signaling is regulated at both the transcriptional level and the post-transcriptional level. AP-1 and NFAT regulate IL-2R transcription. EPA has been shown to suppress CD25 expression 24 h after stimulation of human PBMC stimulated with phytohemagglutinin (PHA) (108). EPA also suppressed CD25 expression in human Jurkat cells compared with saturated fatty acids (98). In contrast to these findings, we did not find any difference in surface CD25 expression in CD4$^+$ T cells isolated from CO-and FO-fed mice. This is the first time that the effect of n-3 PUFA on CD25 expression in antigen-stimulated CD4$^+$ T cell activation has been examined; all previous studies have used mitogenic stimuli (98,108). The reported CD25 decrease in previous studies could be due to reduced IL-2 secretion. The similarity of expression of CD25 on the plasma membrane of CD4$^+$ T cells from FO or CO-fed mice indicated the absence of regulation at the transcription level. It is possible, therefore, that n-3 PUFA exert an effect at the post-transcriptional level, possibly by modulating lipid rafts. Recently, the functional role of lipid rafts in the regulation of IL-2 R signaling has been revealed (67).

Similar to many receptors, IL-2R is not randomly distributed in the lipid bilayer of the plasma membrane. IL-2 receptor α, β, γ chains form preexisting complexes on the surface of T cells. IL-2 R α has been shown to cluster in lipid rafts on T cells, while the β and γ chains are in soluble fractions. IL-2 binding triggers dissociation of the α chain from lipid rafts and interaction with the β and γ chains, thus, initiating signal transduction (67). Disruption of lipid rafts in GM2/GD2 knockout mice resulted in inhibited T cell proliferation without changing the surface expression of IL-2 receptor (66). The suppression was found to be the result of a decrease in JAK1, JAK3 and STAT5 activation and delayed and reduced c-fos expression. We have previously shown that dietary n-3 PUFA suppress anti-CD3/anti-CD28 stimulated CD4$^+$ T cell proliferation by modulating lipid rafts, inhibiting recruitment of PKC-θ to lipid rafts, and suppressing NF-κB and AP1 activation (82). This could also be true in antigen stimulated CD4$^+$ T cells. It is possible that the n-3 PUFA incorporated into lipid rafts affect the interaction of IL-2 Rα chain with β and γ chains, thus, inhibiting IL-2 R signaling. It is also known that NF-κB is essential for post-differentiation IFN-γ production and clonal expansion in a Th1 response (109). Further investigation into the role of NF-κB activation in the suppression of antigen-stimulated CD4$^+$ T cells by n-3 PUFA is warranted. The possible involvement of changes in cell cycle by dietary n-3 PUFA could also be investigated in culture systems using the vital dye CFSE (34).

In conclusion, we investigated how dietary n-3 PUFA affect OVA stimulated CD4$^+$ T cell activation and differentiation into Th1 effector cells. We showed that dietary FO inhibits Th1 development by suppression of clonal expansion of polarized Th1 cells. We

also demonstrated that dietary n-3 PUFA inhibits proliferation of both antigen-stimulated CD4$^+$ T cells and IL-2 induced proliferation of antigen-specific Th1 cells. We propose that these changes could be due to modifications in lipid rafts by dietary n-3 PUFA that could ultimately affect IL-2 receptor down-stream signaling. The exact mechanisms of involvement of lipid rafts in inducing these changes will be the subject of further investigation and will provide insight into why dietary n-3 PUFA have beneficial effects in Th1-mediated autoimmune diseases.

CHAPTER IV

EFFECT OF DIETARY n-3 PUFA ON IN VIVO ANTIGEN SPECIFIC T CELL

ACTIVATION AND Th1 DEVELOPMENT

INTRODUCTION

Since we observed that dietary n-3 PUFA inhibited expansion of antigen-specific

Th1 cells (chapter II), we designed an experiment to evaluate the outcome in vivo. Since

CFA is an adjuvant which is known to promote Th1 differentiation (31), CFA plus OVA

peptide was used to activate $CD4^+$ T cells from DO11.10 $Rag2^{-/-}$ mice in vivo. We

hypothesized that dietary FO would inhibit the expansion of $CD4^+$ T cells from mice in

response to immunization with OVA peptide in the presence of CFA.

Previously, studies with the original TCR transgenic mice on a BALB/c background

showed that the intact TCR transgenic mice cannot be immunized directly to examine in

vivo antigen-specific T cell activation because these mice mount an abnormal immune

responses to antigen challenge in vivo due to the artificially high frequency of anti-

specific T cells (49). T cells from unimmunized TCR transgenic mice proliferate

vigorously in response to OVA peptide in vitro, although die easily after direct

immunization with OVA (Dr. Stephensen, U.C. Davis, personal communications). To

avoid this complication, Kearney et al. developed an adoptive transfer system in which

TCR transgenic T cells were injected into a non-transgenic recipient, and the transgenic

T cells could be reliably detected using KJ1-26 mAb (49). This adoptive transfer

protocol has been used to study the behavior of antigen-specific T cells following

immunization for a wide range of experimental conditions (48,50,110). It has been

documented that the response following subcutaneous injection is characterized by marked accumulation of antigen-specific transgenic T cells only in the draining lymph nodes. We used this adoptive transfer approach to study the effects of dietary n-3 PUFA on antigen-driven T cell activation and differentiation into Th1 cells in vivo.

In addition to physically tracking the distribution and number of antigen-specific T cells, the in vivo proliferation of these cells was visualized by adoptive transfer of CFSE-labeled DO11.10 cells (52). Without immunization, the KJ1-26[+] transgenic T cells remain as a single peak, whereas, KJ1-26 positive T cells undergo several cell divisions in the OVA-immunized host.

Using the adoptive transfer protocol, Anderson et al. (50) examined the effect of dietary lipids on in vivo proliferation of antigen-specific naïve CD4^{+} T cells. Using the original TCR transgenic mice on a BALB/c background, following 4 wk the feeding a FO diet (20% total fat) to recipient mice reduced the in vivo proliferation of CD4^{+} T cells compared with a soybean oil diet. The mechanisms of action are unknown. Here for the first time we demonstrate that dietary n-3 PUFA suppress in vivo antigen-specific Th1 development, but do not affect cell cycle progression.

MATERIALS AND METHODS

Animals and diets

All experimental procedures using laboratory animals were approved by the University Laboratory Animal Care Committee of Texas A& M University. Pathogen-free, female, young 4-6 wk of age BALB/c mice were purchased from the Frederick National Cancer Research Facility, Frederick, MD. DO11.10 Rag2$^{-/-}$ breeder mice were purchased from Taconic, bred and maintained at the LARR, Texas A & M University. Male B10.D2 mice were purchased from Taconic and were used as recipient mice in the adoptive transfer experiments. DO11.10 Rag2$^{-/-}$ mice (8-10 wk) were housed 5 per microisolator cage, and had free access to water and diet. They were fed a control semi-purified diet containing 5% corn oil (CO) during the 7-d acclimation period, followed by 2 wk feeding of either the same control diet or a fish oil (FO) diet (1% CO + 4% FO). Two groups of B10.D2 recipient mice were fed in parallel to donor DO11 mice. The diets met the NRC nutrition requirements and varied only in lipid content. The basic diet composition, expressed as g/100g was: casein, 20; sucrose, 42; cornstarch, 22; cellulose, 6; AIN-76 mineral mix, 3.5; AIN-76 vitamin mix, 1; DL-methionine, 0.3; choline chloride, 0.2; Tenox 20A, 0.1; and oil, 5. The fatty acid composition of the diets, as determined by gas chromatography, is shown in Table 1.

Chemical compounds and antibodies

Phorbol myristate acetate (PMA) was purchased from Sigma (St. Louis, MO). Ionomycin was purchased from Calbiochem-Novabiochem (San Diego, CA). KJ1-26-

TC was purchased from Caltag Laboratories (Burlingame, CA). Hamster anti-mouse CD3e monoclonal antibody (clone145-2C11) was purchased from PharMingen (San Diego, CA). $OVA_{323-339}$ peptide was synthesized by Biosource, International, Camrillo, CA using the following sequence: ISQAVHAAHAEINEAGR. CFA was purchased from Sigma (St. Louis, Missouri). Lympholyte-M was purchased from Cedarlane (Ontario, Canada).

CD4^{+} T cell isolation, CFSE labeling and adoptive transfer

Spleens and lymph nodes (brachial and mesenteric lymph nodes) were taken from diet-fed DO11.10 $Rag2^{-/-}$ mice and placed in 3 mL RPMI complete media (RPMI 1640 medium with 25 mM HEPES (Irvine Scientific, Santa Ana, CA) supplemented with 5% FBS, 1×10^5 U/L penicilin and 100 mg/L streptomycin (Irvine Scientific), 2 mM L-glutamine, and 10 μM 2-mercaptoethanol). The spleens and lymph nodes were homogenized and filtered as described earlier (74). Total lymphocytes were enriched by density gradient centrifugation using Lympholyte-M. CD4^{+} T cells were purified from splenocytes by a negative selection column method described by Arrington et al. (74). Since preliminary data showed that greater than 90% of the lymph node cells from DO11.10 $Rag2^{-/-}$ mice were CD4^{+} T cells, the cells from lymph nodes were used directly after Lympholyte-M separation. CD4^{+} T cells from spleens were combined with lymphocytes from lymph nodes and labeled with CFSE as described (48). Briefly, cells were incubated with CFSE (10 μM) in PBS for 10 min at 37 ^{o}C. After washing three times with PBS, the labeled cells were adjusted to 3.0×10^7 cells/mL in sterile PBS and

100 µL were injected into each B10.D2 recipient mouse via the tail vein. Aliquots of labeled cells were cultured with PMA/Ionomycin to serve as the CFSE positive control for later flow cytometric analysis.

Immunization

One day after adoptive transfer, selected recipient mice were immunized with OVA $_{323-339}$ (300 µg) s.c. in CFA while others were not immunized (For details, see Appendix B-10).

Flow cytometry

Three days after immunization, the brachial and inguinal lymph nodes were removed from individual mice, and processed into single cell suspensions. Living cells were counted, then stained with KJ1-26-PECy5 and analyzed by flow cytometry.
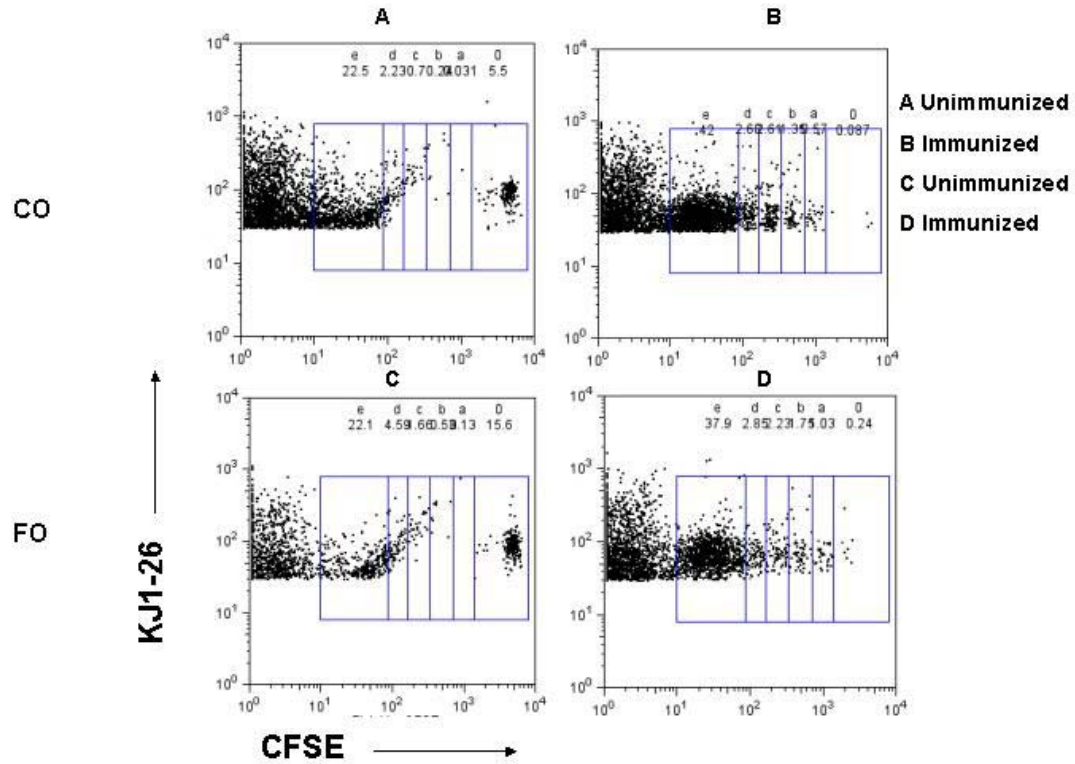
Figure 11. Dietary FO does not affect in vivo cell cycle progression of antigen-specific T cells. DO11.10 Rag2$^{-/-}$ mice and B10.D2 mice were fed one week a CO diet followed by 2 wk of either the same CO diet or a FO diet. CD4$^+$ T cells were isolated, labeled with CFSE and adoptively transferred to B10.D2 mice. B10.D2 mice were immunized 1 d after adoptive transfer with OVA peptide and CFA and maintained on the respective diets. Three days after immunization, draining lymph nodes were harvested, stained with KJ1-26 and analyzed by flow cytometry. A: unimmuned B10.D2 recipient mice fed the CO diet; B: immunized B10.D2 recipient mice fed the CO diet; C: unimmunized B10.D2 recipient mice fed the FO diet; D: immunized B10.D2 recipient mice fed the FO diet.

RESULTS

In unimmunized B10.D2 mice, adoptively transferred CD4$^+$ T cells (KJ1-26$^+$) were

identified as one cluster of cells, indicating a non-dividing resting state. **Figure 11**

showed the CFSE pattern of transgenic T cells from lymph nodes in these mice. The mice fed either the CO or FO diet had similar number of cells (**Fig.11** A&C). In one of three immunized B10.D2 mice in each diet group, the KJ1-26^{+} T cells showed several cell divisions indicated by CFSE labeling. This would indicate a similar number of cell divisions in CO vs. FO-fed mice (**Fig.11** B &D). Interestingly, the size of draining lymph nodes were smaller in immunized FO-fed B10.D2 mice compared with those fed the CO diet. Accordingly, total viable cells harvested from the lymph nodes were significantly lower in immunized FO-fed mice relative to CO-fed mice (**Fig.12**).

DISCUSSION

To date, most studies examining the effects of n-3 PUFA on T cell function has utilized model systems employing transformed T cell lines or ex vivo methodology. We present evidence that dietary n-3 PUFA suppress T cell proliferation and the Th1 response in vivo. Anderson et al. used a similar TCR transgenic model to study the effect of dietary n-3 PUFA on T cell activation in vivo, and reported no dietary effect on the frequency of CD4^{+} T cells (50). Similar to these data, we also observed similar numbers of transgenic T cells in unimmunized mice. Interestingly, Anderson et al. reported that the adoptively transferred CD4^{+} T cells in recipient mice fed an n-6 PUFA-enriched diet for 4 wk exhibited more proliferation cells in the recipient mice fed the FO diet.
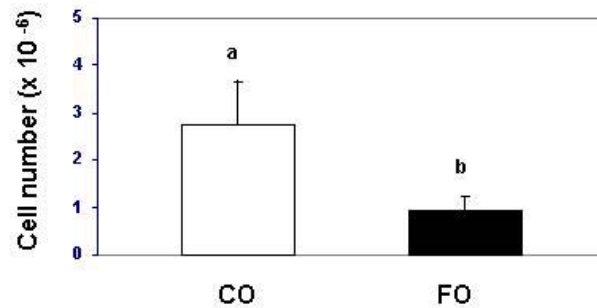
Figure 12. Dietary FO suppresses in vivo antigen-specific T cell clonal expansion. DO11.10 Rag2^{-/-} mice and B10.D2 mice were fed 1 wk a CO diet followed by 2 wk of either the same CO diet or a FO diet. CD4^{+} T cells were isolated, labeled with CFSE and adoptively transferred to B10.D2 mice. B10.D2 mice were immunized 1 d after adoptive transfer with OVA peptide and CFA and maintained on the respective diets. Three days after immunization, living cells counted. Values of different letters indicate significant difference (p=0.032).

We also observed that CO and FO-fed mice displayed similar numbers of cell divisions as assessed by CFSE staining, but that there were more cells in each cycle in CO-fed compared with FO-fed mice. Overall, the total number of antigen-specific CD4$^+$ T cells was lower in FO-fed mice. In addition, we showed that the dietary effect on the in vivo expansion of antigen-specific CD4$^+$ T cells was not due to inhibition of cell cycle progression. This is in agreement with another study by Pompos et al. (53), in which he showed there was no difference in cell cycle progression in cultures of splenocytes from DO11.10 mice stimulated with OVA peptide. Thus, reduced number of antigen-specific CD4$^+$ T cells in FO-fed mice must be explained by other mechanisms.

One alternative explanation could be enhanced apoptosis of Th1 cells by dietary FO. Switzer et al. showed that dietary FO enhanced AICD of purified CD4$^+$ T cells and polarized Th1 cells in C57 BL/6 mice (83,84). However, we did not observe a diet effect on apoptosis in CD4$^+$ T cells stimulated by antigen in vitro (see **Fig. 8**). Nonetheless, in vivo apoptosis in n-3 PUFA-fed mice is worthy of further investigation in this model. Another reason could be reduced IL-2 secretion. Pompos et al. (53) showed in cell culture that, CD4$^+$ T cells from fish-oil fed mice proliferated less and secreted less IL-2 compared with CO-fed mice in response to OVA.

With respect to future experiments, a couple of things could be examined. It is known that the DTH response is a typical Th1 response (104). So the DTH response could be examined in recipient mice following injecting OVA into the footpads (104). In addition, the recipient mice could be maintained on diet for more than 2 wk and the

serum levels of OVA-specific IgG2a and IgG1 could be measured to confirm a dietary effect on the Th1 response to OVA.

In summary, we established the adoptive transfer approach to study the effect of dietary n-3 PUFA on in vivo T cell activation and Th1 development. We have some interesting preliminary observations, however further work is needed to verify our initial findings and to investigate the mechanisms by which dietary n-3 PUFA appear to limit the accumulation of antigen-specific CD4$^+$ Th1 cells in DO11.10 cell transfer recipients. Reciprocal adoptive transfers between DO11.10 and B10.D2 recipients fed each of the diets might clarify the effects of n-3 PUFA on the transferred T cells themselves, as opposed to a diet effect on the accessory factors and co-stimulatory environment provided by the recipient.

CHAPTER V

SUMMARY AND CONCLUSIONS

A large body of epidemiological and experimental studies shows that high dietary intake of n-3 PUFA ameliorates or decreases the risk for a variety of chronic inflammatory diseases (2,11,111). The two principal n-3 PUFA found in FO, EPA and DHA, are considered to be responsible for these beneficial effects. Extensive studies have been carried out to understand the mechanisms by which dietary n-3 PUFA exert their anti-inflammatory effects. In the past 10 y, this laboratory and others have shown that the T cell is a primary target for n-3 PUFA-mediated immunomodulation (3,64,73,74,82-84,88). Using murine models, we have demonstrated that feeding mice diets enriched in n-3 PUFA resulted in reduced T cell proliferation, reduced IL-2 secretion, and diacylglycerol (73,74,81). There are at least two subsets of $CD4^+$ T cells: Th1 and Th2 cells which are defined by their distinctive cytokine profiles. Th1 cells produce IFN-γ, IL-2 and lymphotoxin, Th2 cells produce IL-4, IL-5, and IL-10 (26). Evidence indicates that the Th1/Th2 balance is an important indicator for some autoimmune and inflammatory disease states (28,76,85). The effect of dietary n-3 PUFA on Th1 and Th2 development has not been studied. Previous findings in our lab indicate that $CD4^+$ T cells from C57BL/6 mice stimulated with different agonists display distinct cytokine profiles, corresponding to Th1-like and Th2-like cells (74). The increased proliferation of Th2-like cells and the suppressed IL-2 secretion by Th1-like cells following FO feeding suggest that the anti-inflammatory effects of dietary n-3 PUFA could be due to the combination of suppressed Th1 function and enhanced Th2

activation (74). Therefore, we hypothesized that dietary n-3 PUFA alter Th1/Th2 balance by enhancing Th2 development.

Our study on in vitro Th2 polarization of CD4$^+$ T cells from C57BL/6 mice showed that dietary fish oil did not affect the percentage of Th2 cells generated under Th2 polarizing conditions either with FBS or HMS (see **Fig.3**), indicating dietary n-3 PUFA has no direct effect on Th2 development. Interestingly, dietary fish oil significantly decreased the number of Th1 cells when cultured with HMS (see **Fig.2**). In addition, dietary n-3 PUFA significantly decreased Th1 cells and increased Th2 cells under neutral culture conditions (**Fig.2**). Thus, dietary n-3 PUFA shifted the Th1/Th2 balance towards the Th2 pole through suppression of Th1 cells (**Fig.2**).

To investigate how dietary n-3 PUFA affects Th1 development, we chose a model that allowed us to study the antigen-specific T cell response, DO11.10 Rag2$^{-/-}$ mice. The DO11.10 transgenic mouse expresses a T cell receptor specific for the 323-339 aa peptide from ovalbumin (OVA$_{323-339}$) in an MHC class II-restricted fashion (54). This model is widely used as a model to study Th1/Th2 differentiation in vitro. Because it is Rag2 knockout, virtually all CD4$^+$ T cells are TCR positive in this mouse strain (112). Preliminary studies were carried out to identify the optimal dose of OVA peptide and the proportion of antigen-presenting cells to stimulate CD4$^+$ T cell proliferation in vitro.

We first tested the effect of dietary FO on proliferation of CD4$^+$ T cells from DO11.10 Rag2$^{-/-}$ mice under neutral conditions. In agreement with our previous findings, we noted that dietary FO significantly suppressed antigen-specific proliferation of CD4$^+$ T cells (**Fig.5**). However, the suppression was only seen when culture contained HMS

(**Fig.5**). This confirmed our earlier findings that the inclusion of HMS could maintain ex vivo lipid content of the membranes of cultured T cells and preserve the diet-induced changes during prolonged culture in vitro (83).

Following culturing CD4$^+$ T cells from the DO11.10 mice under Th1 polarizing conditions, we found that almost all cells exhibited Th1 phenotype. FO and CO-fed mice displayed similar percentages of Th1 cells (**Fig.6**), indicating that n-3 PUFA do not affect Th1 differentiation. However, we noticed that the total number of T cells from FO-fed mice was significantly lower (**Fig.7**). We showed that the difference in the number was not due to an enhancement in apoptosis (**Fig.8**), but due to reduced proliferation of polarized Th1 cells (**Fig.9**). The reduced proliferation did not appear to be due to altered surface expression of IL-2 Rα (CD25) (**Fig.10** and **Table 2**).

Our novel observation that dietary FO suppressed clonal expansion of Th1 cells suggested that this could be an important mechanism for beneficial effects of FO in autoimmune and inflammatory diseases. We wished to confirm in vitro findings in whole animals. Therefore, we established an adoptive transfer system to examine antigen-specific in vivo T cell activation and Th1 development. In agreement with the in vitro findings, we observed significantly fewer antigen-specific CD4$^+$ Th1 cells in FO-fed recipient mice (**Fig.12**). This indicates that dietary FO inhibits Th1 development in vivo. However, the cell cycle analysis by CFSE staining showed that dietary FO did not inhibit cell cycle progression (**Fig.11**). This would suggest that the suppressed Th1 clonal expansion by dietary FO must be due to other mechanisms such as effects on apoptosis in vivo or lymphocyte trafficking.

In summary, we have demonstrated that dietary n-3 PUFA have different effects on Th1 and Th2 development, with selective inhibition of Th1 development, leading to a shift in the Th1/Th2 balance towards the Th2 pole. The suppression of antigen-specific Th1 development was not due to an inhibition of Th1 differentiation but due to suppression of clonal expansion. This is a novel finding because it indicates that dietary FO mainly affects the molecular machinery involved in cell proliferation, even though clonal expansion includes both cell proliferation and differentiation. Furthermore, we established the adoptive transfer system to study the effects of dietary n-3 PUFA on in vivo antigen-specific T cell activation and Th1 differentiation. This system could be utilized to examine the immunological properties of other dietary components.

Further studies are needed to elucidate the mechanisms by which dietary n-3 PUFA suppress proliferation of antigen-specific Th1 cells. These studies could focus on (1) how dietary n-3 PUFA affect lipid rafts and IL-2 receptor signaling; (2) how dietary n-3 PUFA affect antigen-specific TCR signaling; (3) how dietary n-3 PUFA affect dendritic cell maturation; and (4) how dietary PUFA affect regulatory T cell function. These studies will contribute to our understanding of how Th1 responses can be modulated by dietary n-3 PUFA and elucidate possible targets of chronic inflammation in Th1-mediated diseases. In addition, they will provide useful data for the establishment of dietary guidelines for dietary n-3 PUFA in terms of promoting optimal immune function.

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APPENDIX A

OTHER DATA

A-1. Summary of Th2 polarization-method development

1. PMA+anti-CD3

Samples	%IL-4$^+$, IFN-γ^-	%IL-4$^-$, IFN-γ^+	%IL-4$^+$, IFN-γ^+
1	14.5	0.25	0.46
2	12.4	0.061	0.22
3	0.07	9.03	0.42
4	0.02	9.25	0.64
Mick-2	33.4	0.095	0.89
Mick-2	39	0	1.52

2. anti-CD3, PMA, IL-2, IL-4, anti-IFN-γ (48 h)

Samples	%IL-4$^+$, IFN-γ^-	%IL-4$^-$, IFN-γ^+	%IL-4$^+$, IFN-γ^+
1	21.5	2.54	1.75
2	17.6	2.78	1.4
3	20.1	4.61	4.91

3. PMA, Ionomycin, rIl-2, IL-4 (48 h)

Samples	%IL-4$^+$, IFN-γ^-	%IL-4$^-$, IFN-γ^+	%IL-4$^+$, IFN-γ^+
1	12.9	4.03	1.84
2	12.3	4.47	1.82

4. anti-CD3, PMA, IL-2, IL-4, anti-IFN-γ (5 d)

Samples	%IL-4$^+$, IFN-γ^-	%IL-4$^-$, IFN-γ^+	%IL-4$^+$, IFN-γ^+
1	7.22	17.2	2.33
2	8.08	16.1	2.6

5. anti-CD3, anti-CD28, rIl-2, IL-4, anti-IFN-γ (5 d)

Samples	%IL-4$^+$, IFN-γ^-	%IL-4$^-$, IFN-γ^+	%IL-4$^+$, IFN-γ^+
1	6.65		0.046
2	6.19		0.064
3		6.11	0.081
4		6.34	0.71

6. PMA, Ionomycin, rIl-2, rIL-4 (5 d)

Samples	%IL-4$^+$, IFN-γ^-	%IL-4$^-$, IFN-γ^+	%IL-4$^+$, IFN-γ^+
1	4.45	14.5	1.39
2	3.95	14.4	1.31

Objective: To determine the optimal culture conditions for Th2 polarization.

Methodology: CD4$^+$ T cells were isolated from chow-fed C57BL/6 mice and cultured as follows:
(1) PMA (0.5 ng/ml) + anti-CD3 (10 μg/ml) for 48 h;
(2) PMA (0.5 ng/ml) + anti-CD3 (10 μg/ml), with rIL-2 (20 ng/ml), IL-4 (10 ng/ml), anti-IFN-γ (10 μg/ml) for 48 h;
(3) PMA (30 ng/ml) + Ionomycin (0.3 μM), with rIL-2 (20 ng/ml), rIL-4 (10 ng/ml) for 48 h;
(4) PMA (0.5 ng/ml) + anti-CD3 (10 μg/ml), rIL-2 20 ng/ml), rIL-4 (10 ng/ml), anti-IFN-γ (10 μg/ml) for 2 d, expanded with rIL-2 (20 ng/ml), rIL-4 (10 ng/ml) for anther 3 d;
(5) anti-CD3 (1 μg/ml) + antiCD28 (5 μg/ml), rIL-2 (20 ng/ml), IL-4 (10 ng/ml), anti-IFN-γ (10 μg/ml) for 2 d, expanded with rIL-2 (20 ng/ml), rIL-4 (10 ng/ml) for another 3 d;
(6) PMA (30 ng/ml)+Ionomycin (0.3 μM), rIL-2 (20 ng/ml), rIL-4 (10 ng/ml) for 2 d, expanded with rIL-2 (20 ng/ml), rIL-4 (10 ng/ml) for another 3 d.

Cells were harvested and restimulated with PMA and Ionomycin for analysis of intracellular cytokine staining for IL-4 and IFN-γ by flow cytometry as described in Appendix B-3.

Conclusion: Among all tested conditions, No.2 seem to be optimal for Th2 polarization, so this condition was chosen for dietary studies on Th2 polarization.

A-2. Isotype controls for intracellular cytokine staining

Samples	% IL-4 pos cells	%IFN-γ pos cells	%IL-4 neg, IFN-γ neg cells
Nonstained	0.09	0.18	99.3
IgG-PE	0.06	0.16	99.3
IgG-FITC	8.82e-3	8.82e-3	99.3

A-3. Genotyping the DO11.10 Rag 2$^{-/-}$ mice using whole blood

KJ1-26-TC   **KJ1-26-TC**   **KJ1-26-TC**
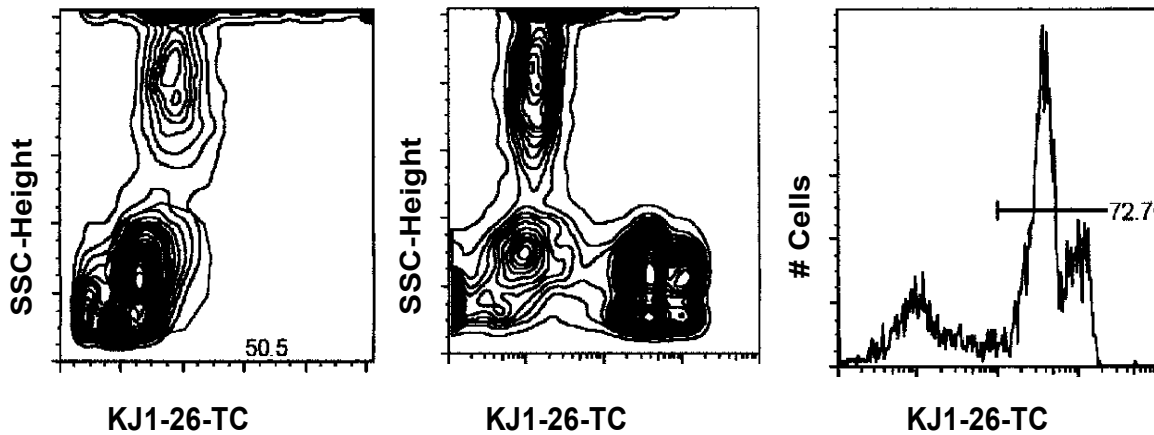
Objective: To confirm the presence of transgenic T cells in breeder DO11.10 Rag2$^{-/-}$ mice.

Methodology: Whole blood was taken from the tail vein of four breeder mice and stained with KJ1-26-PECY5 and analyzed by flow cytometry. Results from four separate analysis showed that the transgenic T cells in the whole blood were 39.3%, 40.4%, 60.3%, 72.7%.

Conclusion: The breeders have 39-73% transgenic T cells among lymphocytes in the blood.

A-4. Characterization of lymph node cells from DO11.10 Rag2$^{-/-}$ mice
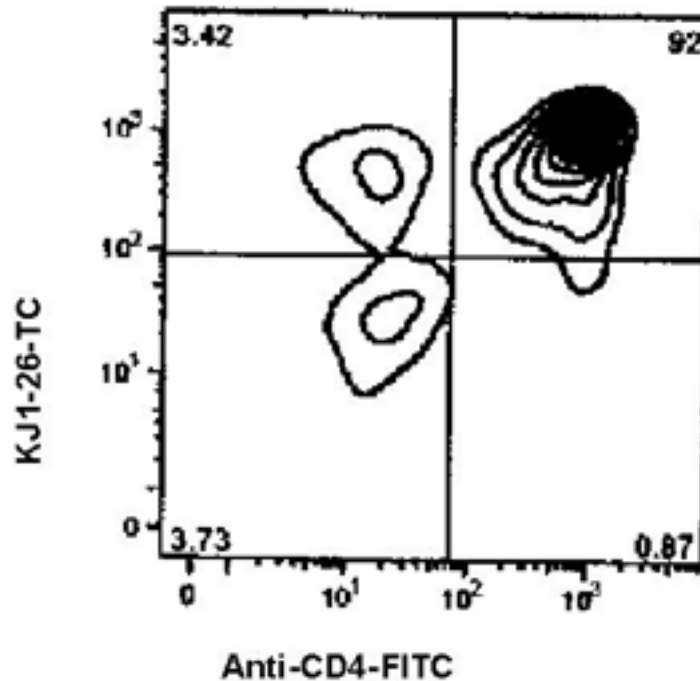
Objective: To determine the percentage of CD4$^+$, TCR$^+$ T cells in the lymph nodes of DO11.10 Rag2$^{-/-}$ mice.

Methodology: Brachial and mesenteric lymph nodes were isolated from chow-fed DO11.10 Rag2$^{-/-}$ mice. Lymphocytes were enriched by Lympholyte-M then stained with anti-CD4-FITC and KJ1-26-CyPE5.

Conclusion: Greater than 90% of lymphocytes in the lymph nodes of DO11.10 Rag2$^{-/-}$ mice were CD4$^+$, TCR$^+$, confirming the phenotype of the mice. These data support our decision to obtain purified CD4$^+$ T cells, lymph nodes cells were used directly without using an additional column purification step.

A-5. Proliferation of whole splenocytes from DO 11.10 Rag2$^{-/-}$ mice
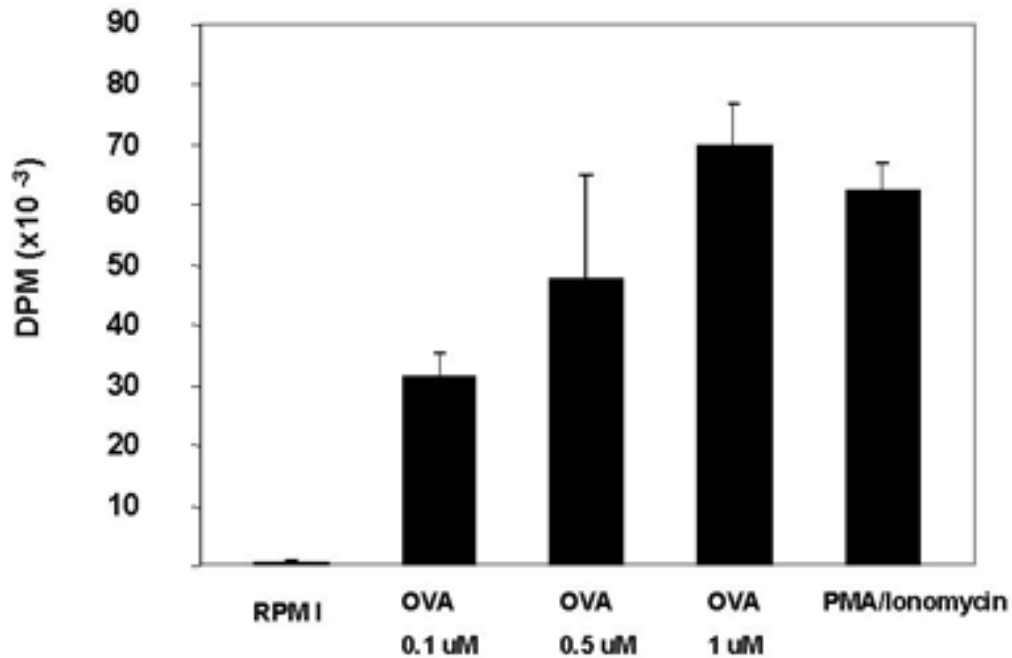
Objective: To determine the optimal dose of OVA peptide to stimulate whole splenocytes from DO11.10 Rag2$^{-/-}$ mice.

Methodology: Spleens were isolated from DO11.10 Rag2$^{-/-}$ mice and CD4$^+$ T cells were isolated. Cells were cultured with different doses of OVA peptide for 72 h. Proliferation was measured by ^{3}H-Thymidine uptake as described.

Conclusion: Whole splenocytes from DO11.10 Rag2$^{-/-}$ mice proliferate in response to OVA in a dose dependent manner. The optimal dose of OVA peptide was 1 μM.

A-6. Proliferation of CD4$^+$ T cells from DO11.10 Rag2$^{-/-}$ mice

Stimuli :	1 RPMI	2 OVA	3 P/I	4 OVA	5 OVA	6 OVA	7 OVA	8 OVA	9 OVA
CD4$^+$ T cells:	2×10^5	2×10^5	2×10^5	1×10^5	1×10^5	2×10^5	2×10^5	2×10^5	2×10^5
APC :	-	-	-	5×10^5	2×10^5	5×10^5	2×10^5	1×10^5	5×10^4
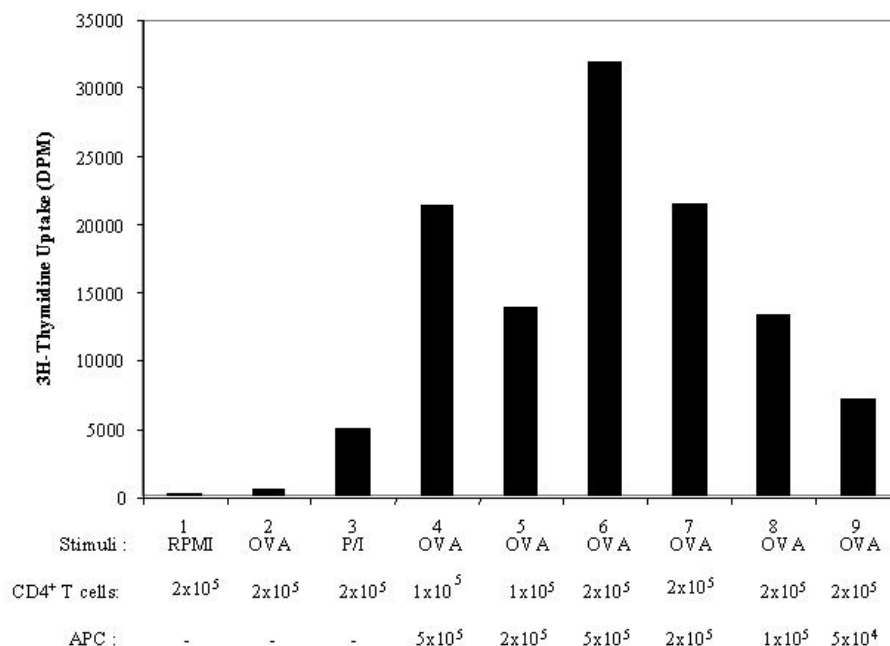
Objective: To determine the optimal condition for proliferation of purified CD4$^+$ T cells from DO11.10 Rag2$^{-/-}$ mice.

Methodology: CD4$^+$ T cells were isolated from both spleens and lymph nodes as described as Appendix B-4 then cultured with irradiated BALB/c splenocytes for 3 d. Proliferation was measured by ^{3}H-Thymidine uptake. To be continued on next page.

Conclusion: Proliferation of CD4$^+$ T cells from DO11.10 Rag2$^{-/-}$ mice was dependent on both the number of CD4$^+$ T cells and the number of APCs. Optimal conditions (4 and 5) were chosen for further experiments.

A-7. Visualization of in vitro cell division of antigen-stimulated CD4^{+} T cells from DO11.10 Rag2^{-/-} mice
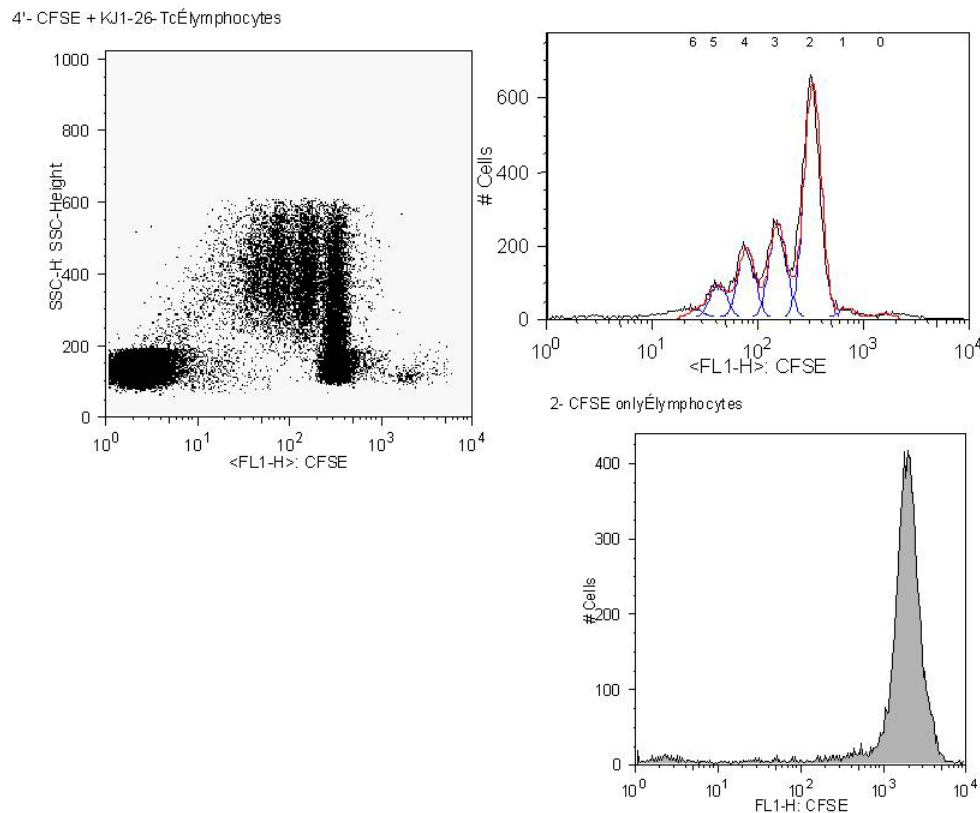
Objective: To detect cell proliferation of in vitro cultured cells by using CFSE.

Methodology: CD4^{+} T cells were isolated from both lymph nodes and spleens of DO11.10 Rag2^{-/-} mice, labeled with CFSE, and cultured with OVA peptide and irradiated BALB/c splenocytes for 3 d. Cells were harvested, stained with KJ1-26PECy5 and analyzed by flow cytometry. A. CFSE staining of transgenic T cells; B. Histogram showing cell devisions; C Fresh isolated cells labeled with CFSE showed a single peak.

Conclusion: In vitro cell division can clearly be detected with CFSE labeling.

APPENDIX B

PROTOCOLS

B-1. CD4$^+$ T CELL ISOLATION

1. Spleen Isolation

Materials*:*
Sterile tools (scissors, forceps, etc)
70% Ethanol (EtOH)
RPMI 1640 with 25 mM HEPES buffer, Irvine Scientific, #9159
Labeled 15 ml conical tube filled with 3 ml complete RPMI (RPMI 1640 with 5% FBS, 2 mmol/L L-glutamine, 1 x 10^5 U/L penicillin, 100 mg/L streptomycin, and 10 μM 2-mercaptoethanol (2-ME))

Methods:
1. Sacrifice mice by CO_2.
2. Place mice on their right side so the left side is facing up.
3. Apply 70% alcohol to the abdomen area.
4. Grab the skin of the abdomen with forceps and make a small incision.
5. Peel the skin to expose the membrane underneath.
6. Grab the membrane with forceps and cut the membrane to expose the organs.
7. Remove the spleen with forceps and trim the fat tissue with scissors.
8. Carefully remove as much fat as possible.
9. Place the spleens in a conical tube containing complete RPMI.

2. Splenocyte preparation

Materials:
Sterile 7 ml glass-in-glass homogenizers, Kontes glass co.
Sterile wire filters
5 cc syringes
20 gauge needles
RPMI media
15 ml conical tubes

Methods:
1. Transfer spleen and RPMI solution into a glass-in-glass homogenizer.
2. Homogenize spleens until completely broken up (5-7 strokes).
3. Assemble a 5 cc syringe, wire filter and 20 gauge needle unit and place on top of a newly labeled 15 ml conical tube.
4. Remove the syringe plunger and transfer the appropriate spleen suspension into the assembled unit.
5. Slowly reinsert the plunger to filter the suspension into the corresponding tube (be gentle with this step!).
6. Fill each tube with RPMI for washing and centrifuge at 200g (rcf=200, rpm=1096, acc/bcc=6) for 5 min at RT.
7. After the cells are done spinning, carefully aspirate the RPMI. Now the splenocytes are ready for next step of lymphocyte isolation.

3. Isolation of lymphocytes by Lympholyte-M and cell preparation for the column elution steps

Materials:
Lympholyte-M (1x500 ml, Cedarlane, Ontario, Canada), # CL5031 warm to RT
R&D antibody cocktail (from R&D Mouse CD4 Subset Mini Column Kit, R&D Systems, Inc, Cat # MCD4C-1000)
RPMI 1640 media with 25 mM HEPES buffer, Irvine Scientific, #9159

1X column wash buffer (diluted from 10X in the R&D Mouse CD4 Subset Mini Column Kit with sterile water (1500 ml, Baxter Healthcare Corporation, #2F7115))

Methods:
1. Add RPMI to the washed cells (add 2.0 ml for 30-40 × 10^6 cells or 3 ml for 60-80 × 10^6 cells according to Lan) and resuspend cells.
2. Add an equal amount of Lympholyte-M by layering it beneath the suspended cells (insert the pipet tip into the bottom of the conical tube then dispense the Lympholyte-M slowly and carefully in order to see 2 distinct layers).
3. Centrifuge the cells at 500g for 15 min (deactivate centrifuge break, for example, set acc/dcc=0 for the Beckman CS-15centrifuge) at RT.
   Meanwhile, begin T-cell column preparation.
   When samples are done, 2 distinct layers (white on top and pink on bottom) and an interface band should be seen.
4. Carefully remove the interface band with a 2 ml pipette and dispense it into a new 15 ml conical tube.
5. Fill the conical tube with RPMI to wash and centrifuge at 300g for 5 min at RT (set acc/dcc back to 6).
6. Aspirate off the RPMI and resuspend the cells in 1 ml 1X column wash buffer.
7. Add 1 vial of monoclonal antibody cocktail (1ml) to suspend the cells and mix gently.
8. Incubate cells at RT for 15 min.
9. Wash cells in 10 ml 1X column wash buffer and spin at 300g for 5 min.
10. Aspirate and repeat wash as step 9.
11. Resuspend final cell pellet in 2 ml 1X column wash buffer.

4. CD4$^+$ T cell purification

Materials:
R&D Mouse T cell CD4 Subset column kit (Cat# MCD4C-1000)
R&D column rack
70% EtOH
waste receptacle
timers
sterile 15 conical tubes
1X column wash buffer (diluted from 10X in the R&D Mouse CD4 Subset Mini Column Kit with sterile water (1500 ml, Baxter Healthcare Corporation, #2F7115))
RPMI 1640 media with 25 mM HEPES buffer, Irvine Scientific, #9159

Methods:
1. Place the T-cell columns in the R&D column rack and place a waste receptacle beneath the columns.
2. To prepare the columns, remove the top cap first and then remove the bottom cap.
3. Allow the fluid within the column to drain until dripping stops; meanwhile rinse the outside of the column tip with 70% EtOH.
4. Wash the column with 10 ml of 1X column buffer.
5. Replace the waste receptacle with a newly labeled sterile15 ml conical tube.
6. Apply 2 ml cell suspension to the top of the column.
7. Allow the cells to sit in the column for 10 min at RT (start the timer when the dripping stops from the columns).
8. Apply 10 ml of 1X column wash buffer to the column to elute the CD4$^+$ T cells.
9. Once the dripping from columns stopped again, centrifuge at 300 g for 5 min at RT.

10. Aspirate the supernatant and resuspend in 1 ml complete RPMI. Now cells are ready to be counted.

5. Cell counting by hemacytometer

Materials:
Trypan blue (0.4%), Sigma, #T8154
0.6 ml snap cap microcentrifuge tube, Nepture, # 3435.AS
Hemacytometer
Cell counter

Methods:
1. Add 198 µl of Trypan blue to a 0.6 ml epi-tube.
2. Suspend the T cells and remove 2 µl and add to a sample tube with Trypan blue.
3. Mix by pipetting up and down several times.
4. Remove about 20 ul from this suspension and put in a hemacytometer.
5. Count the viable white cells (count 200 cells at least).

$$\text{Cell number} = \frac{\text{Total cells}}{\text{squares}} \times 10^4 \times 10^2 \text{ cells/ml}$$

Suspend the cells in complete RPMI at the concentration of 5×10^6 cells/ml.

LITERATURE CITED:

(74) Arrington, J. L., Chapkin, R. S., Switzer, K. C., Morris, J. S. & McMurray, D. N. (2001) Dietary n-3 polyunsaturated fatty acids modulate purified murine T-cell subset activation. Clin Exp Immunol 125: 499-507.

B-2. Th2 Polarization

Materials:

Sterile 24-well plates, Corning Incorporated, #3524

Th2 polarizing agents final concentrations diluted in complete RPMI except anti-CD3:

- 10 μg/ml αIFNγ (R46A2) Pharmingen Cat#554430
- 10 ng/ml rIL-4   Pharmingen Cat#550067
- 20 ng/ml rIL-2   R&D Systems, Inc, Cat#402-ML
- 1 μg/ml anti-CD3   Pharmingen Cat#55305, diluted in sterile PBS
- 0.5 ng/ml PMA   Sigma, St Louis, MO   Cat#P-8139
- 500 nM Ionomycin   Calbiochem-Novabiochem, San Diego, CA
  Cat# 407952
- 5% complete RPMI 1640 media (with 5% FBS, or 5% homologous mouse serum (HMS) (2.5% FBS+2.5% HMS))

Methods:

1. The day before the experiment, prepare anti-CD3 solution in sterile PBS. Coat the 24-well plate with 300 μl of 1 μg/ml anti-CD3 per well, incubate at 4 °C overnight.
2. Prepare the Th2 cocktail at double concentration of the final one in complete RPMI with 5% FBS or HMS, i.e., PMA, 1 ng/ml; rIL-4, 20 ng/ml; rIL-2, 40 ng/ml;α-IFNγ, 20 ug/ml.
3. Add 1 ml Th2 cocktail in each well of the 24-well plate.
4. Add 1 ml cell suspension (5 x 10^6 cells/ml) in each well.
5. Incubate at 37°C for 2 days (day0 and day1). The cytokines are analyzed as described in Appendix B-3.

LTIERATURE CITED:

(83) Switzer, K. C., Fan, Y. Y., Wang, N., McMurray, D. N. & Chapkin, R. S. (2004) Dietary n-3 polyunsaturated fatty acids promote activation-induced cell death in Th1-polarized murine CD4+ T-cells. J Lipid Res 45: 1482-1492.

(90) Kato, Y., Negishi, T., Furusako, S., Mizuguchi, K. & Mochizuki, H. (2003) An orally active Th1/Th2 balance modulator, M50367, suppresses Th2 differentiation of naive Th cell in vitro. Cell Immunol 224: 29-37.

(93) Wurster, A. L., Rodgers, V. L., Satoskar, A. R., Whitters, M. J., Young, D. A., Collins, M. & Grusby, M. J. (2002) Interleukin 21 is a T helper (Th) cell 2 cytokine that specifically inhibits the differentiation of naive Th cells into interferon gamma-producing Th1 cells. J Exp Med 196: 969-977.

B-3. Intracellular Cytokine Staining

Materials:
50 ml conical tube
12×75 Polystyrene tubes for flow cytometry, Falcon, #352054
Aluminum foil
Lympholyte-M (1x500 ml, Cedarlane, ontario, Canada), # CL5031 warm to RT
0.2 uM HT Tuffryn^R membrane filter, PALL life scientific, #4192
Complete RPMI 1640 with 5% FBS
PMA final concentration 10 ng/ml   Sigma, St Louis, MO   Cat#P-8139
Ionomycin final concentration 500 nM   Calbiochem-Novabiochem, San Diego, CA
                                                                              Cat# 407952
BD Cytofix/Cytoperm solution   from BD Cytofix/Cytoperm Kit   Cat#554715
BD Perm/Wash solution from BD Cytofix/Cytoperm Kit, Cat#554715, 10X (dilute 1:10 in distilled water prior to use)
Golgistop from BD Cytofix/Cytoperm Kit, Cat#554715 (8 μg in 6 ml complete media) to inhibit intracellular protein transport processes
(Golgistop should be in cell culture no more than 12 h due to toxicity)
Staining antibodies and controls
- αIL-4-PE   Pharmingen Cat#554435
- αIFNγ-FITC   Pharmingen Cat#554411
- IgG-PE   Pharmingen Cat#554685
- IgG-FITC   Pharmingen Cat#554684

Fc Block, Pharmingen Cat#553142, (stock 0.5 mg/ml, final concentration 5 μg/ml in staining buffer)

Staining buffer
- Dulbecco's PBS without Mg2$^+$ or Ca2$^+$, Invitrogen Corporation 14190-144
- 1% heat-inactivated fetal bovine serum (FBS)
- 0.09% (w/v) sodium azide, Sigma, #S-2002
- Adjust pH to 7.4-7.6, filter through 0.2 μm pore membrane and store at 4 ^{o}C

Methods:
1. Cell stimulation at day2 following Th2 polarization as described in Appendix B-2.
    1.1 Media preparation: prepare complete RPMI with PMA (2ng/ml), Ionomycin (1 μM), and Golgistop (8 μl in 6 ml complete media).
    1.2 Harvest cells by pipeting up and down for a few times, then deliver cells into a conical tube.
    1.3 Fill the tube with RPMI for washing and centrifuge at 200 g for 5 min at RT.
    1.4 Resuspend in 2 ml RPMI.
    1.5 Add equal amount of Lympholyte-M by layering it beneath the suspended cells (insert the pipet tip into the bottom of the conical tube then dispense the Lympholyte-M slowly and carefully in order to see 2 distinc layers).
    1.6 Centrifuge at 500g for 15 min with break off.
    1.7 Remove the interface into a new 15 ml conical tube, fill with RPMI, centrifuge at 300g for 5 min with break on.
    1.8 Aspirate and resuspend cells in complete media.
    1.9 Cell counting.
    1.10 Resuspend cells at 2×10^6 cells/ml in complete media.
    1.11 Add 0.5 ml complete RPMI with PMA, Ionomycin and Golgistop to each well; add 0.5 ml cells to each well.
    1.12 For nonstimulated cells, add 0.5 ml complete RPMI into each well and add 0.5 ml cells.
    1.13 Incubate the cells for 5h at 37 ^{o}C incubator.

1.14 Meanwhile prepare anti-cytokines dilutions in staining buffer. These antibody conjugates are light sensitive, always dim light when prepare these solutions.

αIL-4-PE: prepare 2.7 μg/ml, 0.9μg/ml, 0.3μg/ml

αIFNγ-FITC: 4.8μg/ml, 2.4μg/ml, 0.6μg/ml

IgG-PE: same as αIL-4-PE

IgG-FITC: same as αIFNγ-FITC

Keep the prepared solutions on ice and cover with aluminum foil.

2. Harvest cells

Harvest cells from each well into corresponding 1.5 ml microcentrifuge tubes and spin down the medium containing Golgistop by centrifuging at 200 g for 5 min at 4 ^{o}C.

3. Block Fc receptors

Add 100 μl Fc Block to each tube, resuspend the cells, incubate for 15 min at 4 ^{o}C, then wash cells with staining buffer by adding 1 ml staining buffer and centrifuge at 200g for 5 min at 4 ^{o}C.

4. Fix and permeabilize cells

Resuspend the cells in 250 μl BD Cytofix/Cytoperm solution, incubate for 20 min at 4 ^{o}C, then wash cells 2 times in 1 ml 1X BD Perm/Wash solution by centrifuging at 200 g for 5 min at 4 ^{o}C.

5. Staining for intracellular cytokines

Thoroughly resuspend fixed/permeabilized cells in 100 μl anti-cytokine antibody or appropriate negative control

Negative controls: Unstained, IgG-PE, IgG-FITC.

Incubate cells at 4 ^{o}C for 30 min in the dark. Wash cells 2 times in 1 ml 1X BD Perm/Wash solution by centrifuging at 200 g for 5 min at 4 ^{o}C and resuspend in 500 μl Staining buffer

6. Flow cytometry

Right before flow cytometric analysis, transfer the cells into 12×75 Polystyrene tubes for flow cytometry at room temperature, wrap in aluminum foil and bring to Dr.Smith's lab (contact information: rosmith@cvm.tamu.edu).

Samples to be analyzed: unstained, stained with αIL-4-PE only, stained with αIFN-γ-FITC only, stained with αIL-4-PE and αIFN-γ-FITC. End point: intracellular cytokine profiles.

LITERATURE CITED:

(83) Switzer, K. C., Fan, Y. Y., Wang, N., McMurray, D. N. & Chapkin, R. S. (2004) Dietary n-3 polyunsaturated fatty acids promote activation-induced cell death in Th1-polarized murine CD4+ T-cells. J Lipid Res 45: 1482-1492.

B-4. Proliferation Assay of CD4$^+$ T cells from DO11.10 mice

1. Antigen presenting cells (APC) preparation

1.1. Spleen Isolation

Materials*:*
Sterile tools (scissors, forceps, etc)
EtOH
RPMI 1640 with 25 mM HEPES buffer, Irvine Scientific, #9159
Labeled 15 ml conical tube filled with 3 ml complete RPMI (RPMI 1640 with 5% FBS, 2 mmol/L L-glutamine, 1 x 10^5 U/L penicillin, 100 mg/L streptomycin, and 10 μM 2-mercaptoethanol (2-ME))

Methods:
1. Sacrifice one BALB/C mouse by CO_2.
2. Place mouse on the right side so the left side is facing up.
3. Apply 70% alcohol to the abdomen area.
4. Grab the skin of the abdomen with forceps and make a small incision.
5. Peel the skin to expose the membrane underneath.
6. Grab the membrane with forceps and cut the membrane to expose the organs.
7. Remove the spleen with forceps and trim the fat tissue with scissors.
8. Carefully remove as much fat as possible.
9. Place the spleen in a conical tube containing complete RPMI.

1.2. BALB/c spleen cells preparation

Materials:
Sterile glass-in-glass homogenizers
Sterile wire filters
5 cc syringes
20 gauge needles
complete RPMI (RPMI 1640 with 5% FBS, 2 mmol/L L-glutamine, 1 x 10^5 U/L penicillin, 100 mg/L streptomycin, and 10 μM 2-mercaptoethanol (2-ME))
15 ml conical tubes
Ovalbumin (OVA) peptide, synthesized by Biosource, Inc.

Methods:
1. Transfer spleen and RPMI solution into a glass-in-glass homogenizer
2. Homogenize spleen until completely broken up (5-7 strokes)
3. Assemble a 5 cc syringe, wire filter and 20 gauge needle unit and place on top of a newly labeled 15 ml conical tube
4. Remove the syringe plunger and transfer the appropriate spleen suspension into the assembled unit
5. Slowly reinsert the plunger to filter the suspension into the corresponding tube (be gentle with this step!)
6. Fill the tube with RPMI for washing and centrifuge at 200g (rcf=200, rpm=1096, acc/bcc=6) for 5 min at RT
7. After the cells are done spinning, carefully aspirate the RPMI. Fill the tube with complete RPMI to top and put on ice. (Air will reduce irradiation in next step so there should be no air in the tube)
8. Take the tube to Dr.Walker (979-412-3128) or Vickie Weir (979-412-3130) at large animal clinic for irradiation.
9. Irradiate the tube at 2600 rads.

10. Centrifuge the tube at 300g for 5min. Aspirate off the RPMI. Resuspend in 1 ml complete RPMI and count the cells. Centrifuge again and resuspend the cells with complete media with OVA (0.1 uM-1uM) at desired concentration (for example, $5x10^6$ cells/ml).

2.DO11.10 CD4$^+$ T cells preparation

2.1. Spleen and lymph nodes Isolation

Materials*:*
Sterile tools (scissors, forceps, etc)
EtOH
Labeled 15 ml conical tube filled with 3 ml complete RPMI (RPMI 1640 with 5% FBS, 2 mmol/L L-glutamine, 1 x 10^5 U/L penicillin, 100 mg/L streptomycin, and 10 μM 2-mercaptoethanol (2-ME))

Methods:
1. Sacrifice mouse by CO_2
2. Place a mouse with abdomen facing up
3. Apply 70% alcohol to the abdomen area
4. Grab the skin of the abdomen with forceps and make a small incision
5. Peel the skin to expose the membrane underneath
6. Find the lymph nodes under the arms and hind legs. Pinch the lymph nodes off and tease off the connecting fat, put them in a tube containing complete RPMI.
7. Make an incision in the membrane at abdomen and find the mesenteric node. Get it and put in the above tube.
6. Turn the mouse around and find the spleen.
7. Remove the spleen with forceps while cutting the fat tissue with scissors
8. Carefully remove as much fat as possible
9. Place the spleen in a conical tube containing complete RPMI

2.2. Spleen and lymph node cell preparation

Materials:
Sterile glass-in-glass homogenizers
Sterile wire filters
5 cc syringes
20 gauge needles
RPMI media
15 ml conical tubes

Methods:
1. Transfer spleen and lymph nodes into corresponding glass-in-glass homogenizers
2. Homogenize spleens until completely broken up (5-7 strokes) and homogenize the lymph nodes the same way (only 3-4 strokes will be enough).
3. Assemble a 5 cc syringe, wire filter and 20 gauge needle unit and place on top of a newly labeled 15 ml conical tube
4. Remove the syringe plungers and transfer the appropriate spleen and lymph node suspension into the assembled units
5. Slowly reinsert the plunger to filter the suspension into the corresponding tube (be gentle with this step!)
6. Fill each tube with RPMI for washing and centrifuge at 200g (rcf=200, rpm=1096, acc/bcc=6) for 5 min at RT

7. After the cells are done spinning, carefully aspirate the RPMI.

2.3. Isolation of lymphocytes by Lympholyte-M

Materials:
Lympholyte-M (1x500 ml, Cedarlane, Ontario, Canada), # CL5031 warm to RT
RPMI media

Methods:
1. Add RPMI to the washed cells (add 3ml for cells combined from 5 spleens or lymph nodes from 5 DO11.10 mice, cells from spleens and lymph nodes from 5 mice are just above the required minimum number of cells for optimum output for the column) and resuspend cells.
2. Add an equal amount of Lympholyte-M by layering it beneath the suspended cells (insert the pipet tip into the bottom of the conical tube then dispense the Lympholyte-M slowly and carefully in order to see 2 distinct layers)
3. Centrifuge the cells at 500g for 15 min (deactivate centrifuge break by setting acc/dcc=0) at RT When samples are done, 2 distinct layers (white on top and pink on bottom) and an Interface band should be seen.
4. Carefully remove the interface band with a pipette and dispense it into a new conical tube
5. Fill the conical tube with RPMI, suspend the cells and centrifuge at 300g for 5 min at RT
6. Aspirate off the RPMI and wash again
7. Resuspend cells in 1 ml complete RPMI. Combine the cells prepared from spleens and lymph nodes. .

2.4 CD4$^+$ T cell purification

Materials:
R&D CD4$^+$ columns (Cat# MCD43) at RT
R&D column rack
70% EtOH
waste receptacle
timers
sterile 15 ml conical tubes
1X column wash buffer (diluted from 10X with sterile water)
complete RPMI 1640 media

Methods:
1. Place the T-cell columns in the R&D column rack and place a waste receptacle beneath the columns.
2. To prepare the columns, remove the top cap first and then remove the bottom cap.
3. Allow the fluid within the column to drain until dripping stops; meanwhile rinse the outside of the column tip with 70% EtOH.
4. Wash the column with 6 ml of 1X column buffer.
5. Replace the waste receptacle with a newly labeled sterile15 ml conical tube.
6. Apply 2 ml cell suspension to the top of the column.
7. After the dripping stopped, allow the cells to sit in the column for 10 min at RT.
8. When the dripping stopped again, apply 8 ml of 1X column wash buffer to the column to elute the CD4$^+$ T-cells.
9. Once all the T cells have been collected, centrifuge at 300 g for 5 min at RT.
10. Aspirate the supernatant and resuspend in 1 ml complete RPMI. Now cells are ready to be counted.

2.5. Cell counting by hemacytometer

Materials:
Trypan blue
Small sample tubes
Hemacytometer
Cell counter

Methods:
6. Add 198 µl of Trypan blue to the empty sample tubes
7. Suspend the T cells and remove 2µl and add to a sample tube with Trypan blue
8. Mix by pipetting up and down several times
9. Remove about 20 µl from this suspension and put in a hemacytometer
10. Count the viable white cells (count at 200 cells at least)

$$\text{Cell number} = \frac{\text{Total cells}}{\text{squares}} \times 10^4 \times 10^2 \quad \text{cells/ml}$$

Suspend the cells in complete RPMI at the concentration of 10^6 cells/ml.

3. ^{3}H-Thymidine uptake

Materials:
Sterile 96 well round bottom plates
Complete RPMI
Lyophilized OVA peptide, synthesized by Biosource
OVA peptide solution final concentration: 0.1µM, 1µM
Acetic acid, HPLC grade, Fisher Scientific, #A35-500
1.5 ml ultra low retention microcentrifuge microtubes, PHENIX research products, #MH-815 SA
50 ml sterile filtration system with 0.22 um Millipore Express Plus membrane, Millipore, #SCGP00525
Falcon polypropylene tubes
Multi-petter
Sterile solution basins

Preparation of OVA peptide stock solution (1 mg/ml in 0.05% acetic acid)
1. Dissolve 1 mg lyophilized peptide in 0.5 ml sterile water
2. Preare 0.5% acetic acid solution: 500 µl high purity acetic acid is added to 100 ml
   sterile water, then filtered through a 50 ml sterile filter
3. To a low retention microcentrifuge tube, add 360 µl sterile water and 90 µl 0.5% acetic acid
4. Take 450 µl of the above peptide in step 1 to this low retention epp (this is the stock), the rest for peptide concentration analysis by Dr. Larry dangott's lab on campus (the report says the peptide concentration is 32.30 µg/15 µl = 608.6 µM for stock)
5. aliquote to 20 µl and store <-70°C

Methods:
Day 0
1. Prepare OVA peptide solution in complete RPMI containing the irradiated BALB/c spleen cells.
2. Label the wells of a sterile 96 well plate for RPMI and respective concentrations of OVA peptide (3 for each)
3. Add 100 µl of OVA peptide and BALB/c splenocyte suspension in complete RPMI to the designated wells

4. Add 100 μl of CD4+ T cells.
5. Thoroughly mix the cells by pipetting up and down.
6. Tape the lid and place in 37 $^{\circ}$C incubator for 72 h

Day3
1. Remove the plate from the incubator and check under microscope to ensure cells have proliferated (notice the expansion of cell mass)
2. Pipette 1600μl complete RPMI into a 15 ml conical tube
3. Make a plate map by numbering each well of the plate
4. Bring the plate and RPMI over to Dr.McMurray's lab
5. In the room containing the cell harvester, find the conical tube of 100 μCi ^{3}H-Thymidine solution in the refrigerator.
6. Dilute the ^{3}H-Thymidine 1:5 to 20 μCi
   Add 400 μl of 100 μCi ^{3}H-Thymidine solution +1600 μl complete RPMI
7. Add 50 μl of 20 μCi ^{3}H-Thymidine to each well
8. Incubate the cells in 37 $^{\circ}$C incubator for 5-6 h
9. After incubation, prepare a filter paper
10. Turn on vaccum pump
11. Run H$_2$O through the harvester by pushing COLD button and ensure that all probes are aspirating and dispensing well and wash the system using HOT vaccum.
12. Place a filter paper on the top compartment of the cell harvester and place sample plate underneath the probes
13. Suck up contents using HOT vaccum and wash with water at least 7 times
14. Remove top compartment with vaccum on to help dry the filter
15. Once filter looks dry, remove the template with filter on it
16. Label scintillation vials with designated numbers of each filter disc
17. Using forceps, gently push out the perforated discs of filter paper and place them in appropriate scintillation vial
18. Then fill each scintillation vial with 10 ml scintillation fluid
19. Bring scintillation vials over to beta/gammer counter at Kleberg Biochemistry Lab
20. Load scintillation vials in rack from left to right and place a red STOP rack behind the last rack
21. Use Counter User1 and AutoCount (about1 min for each vial)

LITERATURE CITED:
(30) Hsieh, C. S., Macatonia, S. E., O'Garra, A. & Murphy, K. M. (1995) T cell genetic background determines default T helper phenotype development in vitro. J Exp Med 181: 713-721.
(74) Arrington, J. L., Chapkin, R. S., Switzer, K. C., Morris, J. S. & McMurray, D. N. (2001) Dietary n-3 polyunsaturated fatty acids modulate purified murine T-cell subset activation. Clin Exp Immunol 125: 499-507.

B-5. Protocol for Genotyping of DO11.10 Rag2$^{-/-}$ Mice

Materials:
Fc Block (stock 0.5 mg/ml) Pharmingen #553142
KJ1-26-TC specific for the transgenic T cell receptor (stock 0.1 mg/0.5 ml) Caltag Laboratories, Burlingame, CA, MM7504
Mouse Erythrocyte Lysing Kit R&D systems Cat#WL-2000
10 X concentration lysis buffer from Mouse Erythocyte Lysing Kit, R&D systems, #WL-2000
10 X concentration fixative from Mouse Erythocyte Lysing Kit, R&D system, #WL-2000
10 X washing buffer from Mouse Erythrocyte Lysing Kit R&D systems, #WL-2000
12x75 mm 5 ml polystyrene round-bottom tube, Falcon, #352054
2.0 ml microtubes, Fisher Scientific, #02-681-266
PBS, Invitrogen Corporation, #14190-250
BSA, IgG free, Boehringer Mannheim, #100018
Microhematocrit capillary tubes, non heparinized blue coded tip, Fisher Scientific, #02-668-68
Heparin, 5000 units/ml, Elkins-simn, Inc, #NDC0641-2460-41
Miniseal^R microplate, DADE, #B4424-1

Protocol:

1. Nick the tail of a DO11.10 mouse to collect a few drops of blood into a 2.0 ml microtubes, Fisher Scientific, #02-681-266 as described in Appendix B-6.
2. Add about 20 µl heparin in microhematocrit capillary tubes, then fill with blood, seal with the Miniseal^R microplate without inversion mixing, put on ice, bring back to lab.
3. Break the tube and release the blood to a new 2.0 ml microtubes, Fisher Scientific, #02-681-266, take 50 ul into a 12x75 mm 5 ml polystyrene round-bottom tube.
4. Add Pharmingen Fc block (1 µl stock/50 µl blood), vortex, incubate at room temperature for 10 mins.
5. In the dark, add 10 µl KJ1-26 monoclonal antibody MM7504 (200 µg/ml stock diluted to 5 µg/ml with 1 X washing buffer) and incubate in the dark for 30 mins on ice.
6. Lyse the cells by incubation with 100 µl 1X M-lysis (diluted from 10X M-lyse buffer from Mouse Erythrocyte Lysing Kit R&D systems Cat#WL-2000) buffer for 10 mins at room temperature.
7. Wash one time with 1 ml PBS, 200g, 5 min, 4 ^{o}C.
8. Repeat lysis two times until the pellet is no more red.
9. Resuspend in 300 µl PBS containing 1% BSA at room temperature, bring to Dr. Smith's lab.
10. If the flow cytometry detection is delayed for 1 hour, fix the cells with the 10X fixative from the Mouse Erythrocyte Lysing Kit R&D systems Cat#WL-2000 (add 100 µl 10X fixative to 1 ml cells).

    End point: to detect percentage of TCR positive cells in the whole blood. Unstained samples and stained samples with KJ1-26-TC.

Follow instructions from the Mouse Erythrocyte Lysing Kit R&D systems Cat#WL-2000.

B-6. Collect Blood from Mice – Tail Nick

Materials:
red light , 250 w, Underwriters Laboratories portable lamp, BD-19, 935
restrainer, hand made from an empty pipit tip box with a hole on the edge
70% alcohol
scalpel
2-ml microcentrifuge tube, Fisher Scientific, #02-681-266
gauze

Protocol:
1. Put a mouse into a plastic container, heat it with red light until it jumps.
2. Put the mouse into a restrainer
3. Clean the tail with 70% alcohol
4. Using a scalpel blade make a small nick on the underside of the tail
5. Hold the tail so that the tip points to the floor
6. Allow blood to drip into a collection tube
7. When finished apply slightly pressure with a piece of gauze about 15 sec to insure homeostasis

Follow instructions from LARR.

B-7. Protocol for DO11.10 Th1 Polarization

1. CD4$^+$ T cells isolation. Same as described in Appendix B-4. BALB/c splenocytes were prepared as described in Appendix B-4.

2. Cell counting by hemacytometer

Materials:
Trypan blue (0.4%), Sigma, #T8154
0.6 ml snap cap microcentrifuge tubes, Neptune, #3435.AS
Hemacytometer
Cell counter

Methods:
1. Add 195 μl of Trypan blue to the empty sample tubes.
2. Suspend the T cells and remove 5 μl and add to a sample tube with Trypan blue.
3. Mix by pipetting up and down several times.
4. Remove about 20 ul from this suspension and put in a hemacytometer.
5. Count the viable white cells (count at 200 cells at least)

$$\text{Cell number} = \frac{\text{Total cells}}{\text{squares}} \times 4 \times 10^5 \text{ cells/ml}$$

Suspend the cells in complete RPMI at the concentration of 5×10^5 cells/ml.

Th1 polarization

Materials:
   Sterile 24-well plates
   Th1 polarizing agents final concentration diluted in complete media:
   - 10 μg/ml αIL-4 (11B.11)   gift from NCI
   - 5 ng/ml rIL-12   PharMingen #554592
   - 20 ng/ml rIL-2   R&D Systems #402-ML
   - T cell media (complete RPMI1640 with 5% FBS or 5% HMS)

Methods:
1. Prepare the Th1 cocktail at double concentration of the final one in complete RPMI (with 5% FBS).
2. Resuspend the irradiated BALB/c cells in Th1 cocktail at the concentration of 2.5×10^5 cells/ml.
3. Add 1 ml BABL/c (2.5×10^5 cells) in Th1 cocktail in each well of the 24-well plate.
4. Add 1 ml DO11.10 CD4$^+$ T cells (5.0×10^6 cell/ml) suspension in each well.
5. Incubate at 37°C for 2 days.
6. At day2 and day3, harvest cells and wash once with RPMI.
7. Resuspend cells in fresh 10% FBS containing only rIL-2 and rIL-12.
8. Incubate at 37 ^{o}C until day 5.

Intracellular cytokine staining

Materials:
   50 ml conical tube

12×75 Polystyrene tubes for flow cytometry
Aluminum foil
Lympholyte-M
Complete RPMI with 5% FBS
PMA 10 ng/ml   Sigma, St Louis, MO   Cat#P-8139
Ionomycin 1 μg (1.33 μM)   Calbiochem-Novabiochem, San Diego, CA
                        Cat# 407952
BD Cytofix/Cytoperm solution   from BD Cytofix/Cytoperm Kit   Cat#554715
BD Perm/Wash solution, 10X (dilute 1:10 in distilled water prior to use) Cat#554715
Golgistop (final concentration: 4 μg in 6 ml media) to inhibit intracellular transport
 processes   #554715
(Golgistop should be in cell culture no more than 12 h due to toxicity)
Staining antibodies and controls
   - αIL-4-PE   Pharmingen Cat#554435
   - αIFNγ-FITC   Pharmingen Cat#554411
   - IgG-PE   Pharmingen Cat#554685
   - IgG-FITC   Pharmingen Cat#554684
   - Mick-2 cells (Mouse intracellular cytokine positive control cells) Pharmingen Cat #554653
Fc Block (dilute stock to final concentration 5 μg/ml in staining buffer)   Pharmingen Cat#553142
KJ1-26-PE-TC monoclonal antibody to transgenic TcR, Caltag Laboratories #MM7506 (diluted to 2
   ug/ml in staining buffer before use)
Staining buffer
   - Dulbecco's PBS without $Mg2^+$ or $Ca2^+$
   - 1% heat-inactivated FBS
   - 0.09% (w/v) sodium azide
   - Adjust pH to 7.4-7.6, filter through 0.2 μm pore membrane and store at 4 ^{o}C

Methods:

1. Cell stimulation
   1.1 Media preparation: prepare complete RPMI with PMA (20ng/ml), Ionomycin (2.66 μM),
        and Golgistop (100 times of the concentration of 4 μl in 6 ml media).
   1.2 Harvest cells into a conical tube.
   1.3 Fill the tube with RPMI for washing and centrifuge at 200 g for 5 min at RT.
   1.4 Resuspend in 2 ml RPMI.
   1.5 Add equal amount of Lympholyte-M, as described in Appendix B-1 step 3.
   1.6 Centrifuge at 500g for 15 min with break off.
   1.7 Remove the interface into a new tube, fill with RPMI, centrifuge at 300g for 5 min with
        break on.
   1.8 Cell counting.
   1.9 Resuspend cells at 2×106 cells/ml.
   1.10 Add 0.5 ml complete RPMI with PMA, Ionomycin to each well; add 0.5 ml cells to each
        well.
   1.11 For nonstimulated cells, add 0.5 ml complete RPMI into each well and add 0.5 ml cells
   1.12 Incubate the cells for 3h.
   1.13 After 3 h, add 10 μl of prepared GolgiStop (66.7 ul/ml) to each well of stimulated cells.
        Incubate for another 2 h.
   1.14 Meanwhile prepare anti-cytokine dilutions in staining buffer. Dim the light in the process.
        αIL-4-PE: 3 μg/ml
        αIFNγ-FITC: 2.4μg/ml

2   Harvest cells

Harvest cells from each well into corresponding small tubes and spin down the medium containing Golgistop.

3 Block Fc receptors

Add 100 µl Fc Block to each tube for 15 min at 4 $^{\circ}$C, then wash cells with 1 ml staining buffer.

4 Add 100 ul KJ1-26-PE-TC in staining buffer and incubate for 30 min at 4 $^{\circ}$C in the dark.

5 Wash cells two times with 1 ml staining buffer and pellet by centrifuging at 200g, 5 min, 4 $^{\circ}$C.

6 Fix and permeabilize cells

Resuspend the cells in 250 µl BD Cytofix/Cytoperm solution for 20 min at 4 $^{\circ}$C, then wash cells 2 times in 1 ml 1X BD Perm/Wash solution, 200g, 5 min, 4 $^{\circ}$C.

7 Staining for intracellular cytokines

Thoroughly resuspend fixed/permeabilized cells in 100 µl anti-cytokine antibody or appropriate negative control

Negative controls: unstimulated and unstained

Positive controls: Mick-2 cells, (Mouse intracellular cytokine positive control cells)

Fc Block (dilute stock to final concentration 5 µg/ml in staining buffer)   Pharmingen Cat#553142

KJ1-26-PE-TC monoclonal antibody to transgenic TcR, Caltag Laboratories #MM7506 (diluted to 2 ug/ml in staining buffer before use)

Incubate cells at 4 $^{\circ}$C for 30 min in the dark. Wash cells 2 times with 1 ml BD Perm/Wash and resuspend in 500 µl Staining buffer.

8 Flow cytometry

Right before flow cytometric analysis, transfer the cells into 12×75 Polystyrene tubes for flow cytometry at room temperature.

Samples: nonstained, stained with αIL-4-PE only, stained with αIFN-γ-FITC only, stained with αIL-4-PE and αIFN-γ-FITC. End point: cytokine profiles.

LITERATURE CITED:

(30) Hsieh, C. S., Macatonia, S. E., O'Garra, A. & Murphy, K. M. (1995) T cell genetic background determines default T helper phenotype development in vitro. J Exp Med 181: 713-721.

(36) Stephensen, C. B., Rasooly, R., Jiang, X., Ceddia, M. A., Weaver, C. T., Chandraratna, R. A. & Bucy, R. P. (2002) Vitamin A enhances in vitro Th2 development via retinoid X receptor pathway. J Immunol 168: 4495-4503.

(83) Switzer, K. C., Fan, Y. Y., Wang, N., McMurray, D. N. & Chapkin, R. S. (2004) Dietary n-3 polyunsaturated fatty acids promote activation-induced cell death in Th1-polarized murine CD4+ T-cells. J Lipid Res 45: 1482-1492.

B-8. Determination of Percentage of Transgenic CD4$^+$ T cells from Lymph Nodes in DO11.10 Mice by Flow Cytometry

Materials:
Staining buffer:
- DPBS, Invitrogen Corporation, 14190-144
- 1% heat-inactivated FBS
- 0.09% sodium azide
- adjust to pH7.4-7.6, filter, store at 4 ^{o}C
Fc Block (0.5 mg/ml)   Pharmingen #553142
- dilute to 5 μg/ml in staining buffer
anti-CD4-FITC (stock 0.5 mg/ml)   Pharmingen #553729
anti-TcR-TC   (stock 0.2 mg/ml)   Caltag Laboratories #MM7506
2.0 ml microtubes, Fisher Scientific, #02-681-266
12 × 75mm Polystyrene FACS tubes, Falcon, #352054
Aluminum foil

Protocol:
1.   prepare cell suspensions from lymph nodes as Appendix B-4.
2.   Resuspend cells at 2×10^6 cells/ml in PBS.
3.   Add 500 μl cells to 1.5 ml centrifuge tubes.
4.   Centrifuge at 200g for 5 min at 4 ^{o}C.
5.   Aspirate.
6.   Resuspend cells in 100 μl Fc Block (5 μg/ml) and and incubate at 4 ^{o}C for 15 min.
7.   Add 1 ml staining buffer and centrifuge at 200g for 5 min at 4 ^{o}C.
8.   Aspirate and resuspend cell pellets in 100 ul antibody diluted in staining buffer (anti-CD4 dilute to 5 μg/ml, anti-TcR-Tc dilute to 2 μg/ml) and incubate in dark at 4 ^{o}C for 30 min.
9.   Wash with 1 ml staining buffer twice.
10.   Resuspend in 500 μl staining buffer and transfer to FACS tubes at room temperature.
11.   Promptly take samples for flow cytometer analysis. End point: percentage of CD4$^+$, TCR$^+$ cells.

Follow the technical data sheet.

B-9. CFSE Labeling to analyze cell proliferation

Materials:
PBS/0.1% BSA
Carboxyfluorescein Diacetate, Succinimidyl Ester (CFSE) (Molecular probes V-12883)
RPMI 1640/10% FBS
2.0 ml microtubes
12 × 75 mm Polystyrene FACS tubes for flow cytometer
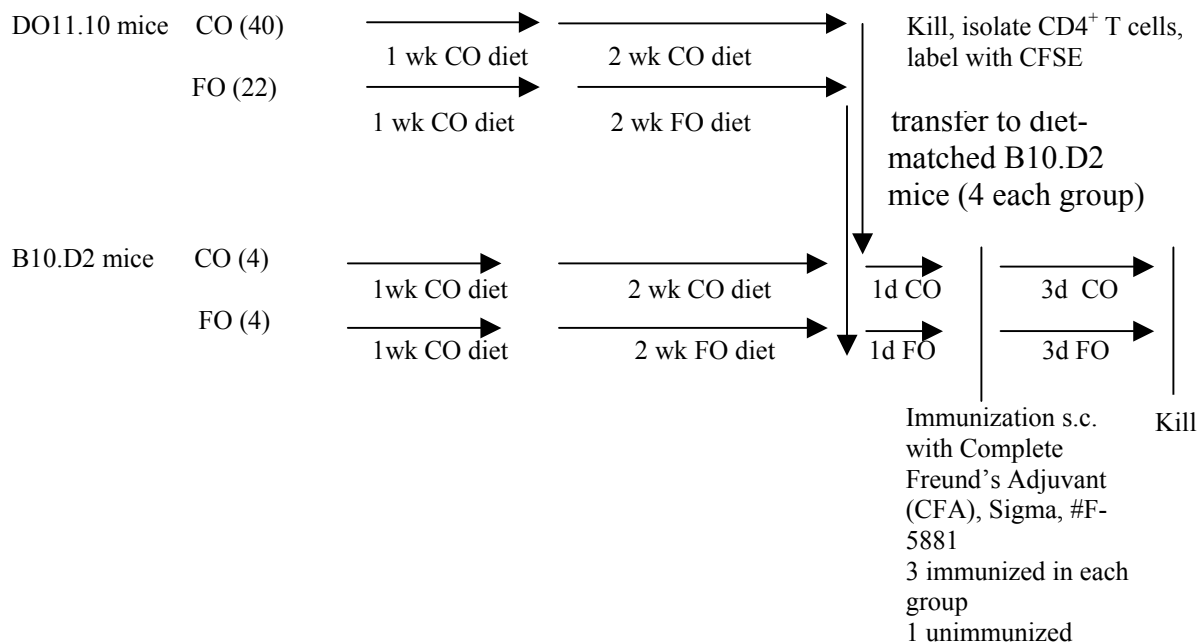Aluminum foil

Protocol:
1. Resuspend purified CD4$^+$ T cells in PBS/0.1% BSA at a final concentration of 2×10^6 cells/ml in a 15 ml conical tube.
2. Add 1 µl of 10 mM CFSE to the tube. Vortex immediately for a few seconds. Incubate at 37 ^{o}C for 10 min (to prepare 10 mM CFSE, add 90 µl of DMSO into one vial of CFSE).
3. Add 5 vol ice-cold RPMI 1640/10% FBS according to the vol used as step 1 and incubate for 5 min on ice.
4. Wash cells three times with PBS, centrifuge at 200g for 5 min at room temperature.
5. Resuspend cells at 2×10^6 cells/ml in PBS/0.1% BSA and add 500 µl to the FACS tubes, wrap with aluminum foil, keep at room temperature, bring to Dr. Smith's lab. End point: to detect CFSE positive cells.

Follow the technical data sheet from the company.

B-10. Diagram for Adoptive Transfer Dietary Study

Experimental Design:

DO11.10 mice   CO (40)

→ 1 wk CO diet → 2 wk CO diet → Kill, isolate CD4$^+$ T cells, label with CFSE

FO (22)

→ 1 wk CO diet → 2 wk FO diet → transfer to diet-matched B10.D2 mice (4 each group)

B10.D2 mice   CO (4)

→ 1wk CO diet → 2 wk CO diet → 1d CO → 3d CO →

FO (4)

→ 1wk CO diet → 2 wk FO diet → 1d FO → 3d FO →

Immunization s.c. with Complete Freund's Adjuvant (CFA), Sigma, #F-5881
3 immunized in each group
1 unimmunized

Kill

LITERATURE CITED:

(49) Kearney, E. R., Pape, K. A., Loh, D. Y. & Jenkins, M. K. (1994) Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. Immunity 1: 327-339.

1. CD4$^+$ T cells isolation. Same as described in Appendix B-4 and resuspended at $3x10^7$ cells/ml in sterile PBS.

2. CFSE lableing of CD4$^+$ T cells

Materials:
BSA: IgG free, Boehringer Mannheim Laboratory Reagents, #100018
PBS/0.1% BSA
CFSE (Molecular probes V-12883)
½ cc U100 insulin syringe, BD.
RPMI 1640/10% FBS
2.0 ml microtubes
12 × 75 mm Polystyrene FACS tubes for flow cytometer
Aluminum foil

Methods:
1. Resuspend cells ($3x10^7$ cells/ml) in PBS containing 0.1% BSA.
2. Add 1 μl of 10 mM CFSE in the tube. Incubate at 37 ^{o}C for 10 min (to prepare 10 mM CFSE, add 90 μl of DMSO into one vial of CFSE).
3. Add 5 vol ice-cold RPMI 1640/10% FBS and incubate for 5 min on ice.
4. Wash cells three times with culture medium or PBS.
5. Resuspend cells at 3×10^7 cells/ml in PBS/0.1% BSA and load 100 μl into 30 gauge insulin syringes.
6. Put syringes on ice and bring over to LARR for tail injection.

3. Immunization

Materials:
Sterile PBS
OVA peptide
Complete Freund's adjuvant (CFA),   Sigma #F-5881
Micro-emulsifying needles 73 mm, Popper & Sons, Inc, New Hyde Park, NY #7973
3ml BD Luer-Lok syringe, #309585
1ml BD Luer Lok syringe, #309628
25G 5/8 inch needle BD #305122

Methods:
1. One day after tail injection, prepare 0.5 ml of OVA peptide with sterile PBS (final concentration 6mg/ml)
2. Emulsify with 0.5 ml of CFA (Add 0.5 ml of OVA peptide in a syringe at one end of the mixing tube, then add 0.5 ml of CFA to another syringe attached to the other end of the mixing tube, push through the micro-emulsifying needle several times until it is really hard to push-which means it is ready).
3. Inject subcutaneously in a total volume of 0.1 ml distributed between two dorsal sites on the back with 25G 5/8 inch needle.

4. Flow detection of antigen-specific cells

Materials:
0.2 um Nylon membrane filter, Gelman Laboratory, #4433

Staining buffer:
- DPBS
- 1% heat-inactivated FBS
- 0.09% sodium azide
- adjust to pH7.4-7.6, filter, store at 4 ^{o}C
Fc Block (0.5 mg/ml)   Pharmingen #553142
- dilute to 5 μg/ml in staining buffer
anti-TcR-TC   (0.2 mg/ml)   Caltag Laboratories #MM7506
1.5 ml microtubes
12 × 75mm Polystyrene FACS tubes
Aluminum foil

Methods:
1. Harvest brachical and inguinal lymph nodes from recipient B10.D2 mice
2. Homogenize and wash once as Appendix 4.
3. Resuspend cells at 2×10^6 cells/ml in PBS.
4. Add 500 μl cells to 1.5 ml centrifuge tubes.
5. Centrifuge at 200g for 5 min at 4 ^{o}C.
6. Aspirate.
7. Resuspend cells in 100 μl Fc Block (5 μg/ml) and incubate at 4 ^{o}C for 15 min.
8. Add 1 ml staining buffer and centrifuge.
9. Resuspend in 100 μl antibody diluted in staining buffer (anti-CD4 dilute to 5 μg/ml, anti-TcR-Tc dilute to 2 μg/ml) and incubate in dark at 4 ^{o}C for 30 min.
10.Wash with 1 ml staining buffer twice.
11.Resuspend in 500 μl staining buffer and transfer to FACS tubes at room temperature, wrap in aluminum foil.
12.   Promptly take samples for flow cytometer analysis.

LITERATURE CITED:
(49) Kearney, E. R., Pape, K. A., Loh, D. Y. & Jenkins, M. K. (1994) Visualization of peptide-specific T
         cell immunity and peripheral tolerance induction in vivo. Immunity 1: 327-339.

(52) Lyons, A. B. (2000) Analysing cell division in vivo and in vitro using flow cytometric measurement
         of CFSE dye dilution. J Immunol Methods 243: 147-154.

B-11. Protocol for Autologous Mouse Serum Collection
(from Dr. Fritsche-----12/02)

Materials:
B-D vacutainer SST tubes, 2.5 mL w/clot activator (Fisher cat # 02-688-29)
Costar 8110 mStar 0.22 micro sterile filter (Fisher # 07-200-370)
Sterile 1.5 mL microfuge tubes w/ snap-on lids
Sterile syringes w/o needles, 3 mL
1 cc tuberculin syringes w/ fixed 25 G needle

Protocol:
1. Sacrifice mice with CO_2. Collect blood from mice via cardiac puncture using paster pipets.
2. Immediately transfer blood into one of the B-D SST tubes.
3. Add blood from other mice within the same dietary treatment group to the same tube as space will allow.
4. Turn on waterbath and set the temperature to 56 ^{o}C.
5. Allow blood to clot in tubes for 1-2 hr at room temperature.
6. Spin tubes (1000 g for 15 min).
7. In the laminar flow hood, attach a 0.22 mm filter to a 3 mL syringe after the plunger has been removed.
8. Carefully decant the sera from each tube into a syringe, then use the plunger to slowly force the sera through the filter and into a sterile microfuge tube.
9. The sterile filtered sera should be heat-inactivated by placing in the 56 ^{o}C water bath for 30 min.
10. After inactivation, the sera should be cooled on ice, then used or stored at –80 ^{o}C (Sera should be good for up to 6 months, but the fresher the sera the better, particularly when mice are fed high PUFA diets.

B-12. Staining of Surface CD25 (IL-2 receptor α chain)

Materials:
Staining buffer:
- DPBS
- 1% heat-inactivated FBS
- 0.09% sodium azide
-adjust to pH7.4-7.6, filter, store at 4 °C
0.2 μM HT Tuffryn low protein binding membrane filter, PALL life scientific, #4192
Fc Block (stock 0.5 mg/ml)   PharMingen #553142
- dilute to 5 μg/ml in staining buffer
anti-CD25-PE (stock 0.2 mg/ml, dilute to 2 ug/ml in staining buffer before use)   PharMingen   #553866
7-amino-actinomycin D (7-AAD, use stock directly, do not need dilution)   PharMingen # 559925
1.5 ml microtubes
12 × 75mm Polystyrene tubes
Aluminum foil

Methods:
   Controls:
    Unstained,
    Stained with 7-AAD only
    Stained with anti-CD25-PE only

1.   Resuspend cells at 2×10^6 cells/ml in PBS .
2.   Add 500 μl cells to 1.5 ml centrifuge tube.
3.   Centrifuge at 200g for 5 min.
4.   Aspirate.
5.   Resuspend in 100 μl Fc Block, incubate at 4 °C for 15 min.
6.   Add 1 ml staining buffer, centrifuge at 200g for 5 min, aspirate.
7.   Add 100 μl anti-CD25-PE (diluted to 2 μg/ml final concentration in staining buffer, incubate at 4 °C for 30 min in dark.
8.   Wash twice with 1 ml staining buffer.
9.   Resuspend cells in 80 μl staining buffer, then add 20 μl stock 7-AAD, gently vortex, incubate at RT in dark for 10 min.
10. Add 400 μl staining buffer.
11. Transfer to FACS tubes, at room temperature, wrap in aluminum foil, bring to Dr.Smith's lab. End point: living CD25$^+$ cells .

Follow the technical data sheet from BD Phaemingen

B-13. Apoptosis assayed by Annexin V-FITC/PI staining for flow Cytometry

Materials:
AnnexinV Kit, BD PharMingen, #6693KK
1X PBS, stored at 4 ^{o}C
1.5 ml tubes
12x75mm Polystyrene tubes (for flow cytometry)
Aluminum foil

Methods:
1. Harvest cells following Th1 polarization, wash once and resuspend cells at $2x10^6$ cells/ml in PBS, add 1ml to 1.5 ml tube.
2. Centrifuge at 200g, 5 min.
3. Aspirate supernatant.
4. Resuspend cells in 100 ul of 1x Binding buffer from the kit and transfer to 12x75 mm polystyrene tubes.
5. In the dark, add to each tube: 5 µl of Annexin V-FITC, 2 µl of Propidium Iodide.
6. Resuspend after adding each solution.
7. Wrap tubes in aluminum foil and incubate in the dark for 15 min at room temperature
8. After 15 min incubation, add 400 µl of 1 X Binding Buffer to each tube.
9. Cover tubes in aluminum foil.
10. Promptly take samples to Dr. Smith for flow analysis. (Samples should be analyzed within 1 hour of staining)

LITERATURE CITED:
(84) Switzer, K. C., McMurray, D. N., Morris, J. S. & Chapkin, R. S. (2003) (n-3) Polyunsaturated fatty acids promote activation-induced cell death in murine T lymphocytes. J Nutr 133: 496-503.

B-14. Dietary Lipid Extraction
(from Kirsten C. Switzer**)**

1. Obtain prepared 12 ml leak-proof tubes.
   If not available: Put acetone into 12 ml glass screw-top tubes. Mark the acetone level. Close lids tightly and place in 80 °C oven for 1 h. Remove and note the acetone level. Do not use the tubes that evaporated acetone.
2. Pour off acetone from tubes and drain upside down.
3. Put each diet (0.5-1 g, amount not critical) into 25 ml glass screw-cap tubes.
4. Add 5 ml Folch reagent ($CHCl_3$/Methanol – 2:1 v:v) to each tube.
5. Add 1 ml 0.1 M cold KCl to each tube.
6. Vortex for 1 min.
7. Centrifuge at 4000 rpm for 5 min at 4 °C. Make sure to put glass tubes in a rubber holder before placing in the centrifuge.
8. Transfer the lower phase to a 12 ml leak-proof tube-use a Pasteur pipet bubble through the top layer so as not to contaminate the extraction.
9. Dry down with Nitrogen.
   -wipe each needle
   -do not put the needle into the solution
   -do not turn the Nitrogen on too high otherwise the solution will bubble out
   -dry until no liquid remains
10. Add 3 ml fresh 6% HCl/MeOH to each tube (for 20 ml, use 1.2 ml concentrated HCl in 18.8 ml MeOH. This solution is light sensetive, so put foil around the container).
11. Flush with Nitrogen and cap tightly.
12. Vortex for 1 min.
13. Put in an 80 °C oven for 14-16 h (overnight).
14. Take tubes out of oven, put on ice.
15. Add 1 ml of 0.1 M KCl and 2 ml Hexane to each tube.
16. Vortex for 1 min.
17. Label 4 ml glass screw-top vials: "FAME" for Fatty Acid Methyl Esters. Make sure to replace normal black lids with green lids with Teflon for organic solvents.
18. Transfer upper phase to 4 ml glass vials.
19. Dry down with Nitrogen-see step 9.
20. Redissolve the FAME extraction into 500 μl of CH_2Cl_2. Use extra if the FAME looks concentrated (yellowish). Remove 100 μl of the concentrated oil and add it to 1 ml of CH_2Cl_2.
21. Flush with Nitrogen.
22. Store at –20 °C or analyse immediately on GC.

VITA

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EDUCATION

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AWARDS

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2000-2001	Reagent fellowship from Texas A&M University
2004	Travel award from Faculty of Nutrition to attend 2004 Experimental Biology meeting in Washington DC.
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GRADUATE ACTIVITES

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2003-2005 Research assistant in Department of Animal Science

PUBLICATIONS

Zhang, P., Smith, R., Chapkin, R.S. & McMurray, D.N. 2005. Dietary n-3 polyunsaturated fatty acids modulate Th1/Th2 balance in vitro towards the Th2 pole by suppression of Th1 development. Journal of Nutrition. 135, 1745-1751.

Zhang, P., Smith, R., Ly, L.H., Kim, W., Chapkin, R.S. & McMurray, D.N. 2005. Dietary fish oil suppresses antigen-stimulated Th1 development. Submitted to Journal of Leukocyte Biology.