THE EFFECT OF DIETARY n-3 POLYUNSATURATED FATTY ACIDS ON T CELL SUBSET ACTIVATION-INDUCED CELL DEATH

A Dissertation

by

KIRSTEN COLLETTE SWITZER

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

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Nutrition

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Approved as to style and content by:

Robert S. Chapkin (Co-Chair of Committee)	David N. McMurray (Co-Chair of Committee)
Joanne R. Lupton (Member)	Roger Smith III (Member)
Robert S. Chapkin (Chair of Nutrition Faculty)	John W. McNeill (Head of Department)

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ABSTRACT

The Effect of Dietary n-3 Polyunsaturated Fatty Acids on T Cell Subset Activation-Induced Cell Death. (August 2004)

Kirsten Collette Switzer, B.S., University of Nebraska, Lincoln

Co-Chairs of Advisory Committee: Dr. Robert S. Chapkin
Dr. David N. McMurray

Dietary n-3 polyunsaturated fatty acids (PUFA) have been shown to potently attenuate T cell-mediated inflammation, in part, by suppressing T cell activation and proliferation. Apoptosis is an important mechanism for preventing chronic inflammation by maintaining T cell homeostasis through the contraction of populations of activated T cells. We hypothesized that dietary n-3 PUFA would promote T cell apoptosis, thus, providing an additional mechanism to explain the anti-inflammatory effects. We specifically examined activation-induced cell death (AICD) since it is the form of apoptosis associated with peripheral T cell deletion involved in immunological tolerance and T cell homeostasis. Female C57BL/6 mice were fed diets containing either n-6 PUFA (control) or n-3 PUFA for 14 d. Splenic T cells were stimulated with αCD3/αCD28, αCD3/PMA, or PMA/Ionomycin for 48 h followed by reactivation with the same stimuli for 5 h. Apoptosis was measured using Annexin V/propidium iodide and flow cytometry. Cytokine analyses revealed that n-3 PUFA enhanced AICD only in T cells expressing a Th1-like cytokine profile (high IFNγ, low IL-4) compared to mice fed the n-6 PUFA control diet. Dietary n-3 PUFA significantly altered the fatty acid composition of phosphatidylcholine and phosphatidylethanolamine in T cell membranes.

To examine the apparently selective effect of dietary n-3 PUFA on AICD in Th1 cells, $CD4^+$ T cells were polarized *in vitro* to a Th1 phenotype by culture with αIL-4, IL-2, and IL-12 for 2 d, followed by culture with IL-2 and IL-12 for 3 d in the presence of diet-matched homologous mouse serum (MS) to prevent loss of cell membrane fatty acids. Following polarization and reactivation, we observed that n-3 PUFA enhanced Th1 polarization and AICD only in cells cultured in the presence of MS, but not in fetal bovine serum. The n-3 PUFA enhancement of Th1 polarization and AICD was associated with the maintenance of diet-induced changes in EPA (20:5n-3) and DHA (22:6n-3) in plasma T cell membrane lipid rafts. Overall, these results suggest that dietary n-3 PUFA enhance both the polarization and deletion of pro-inflammatory Th1 cells, possibly as a result of alterations in lipid raft fatty acid composition.

To the memory of my dad.

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CHAPTER I

INTRODUCTION

Autoimmune diseases (e.g., rheumatoid arthritis, inflammatory bowel disease, Type I diabetes) are associated with severe morbidity and mortality, affecting more than 3% of the U.S. population (1). The chronic nature of many of these diseases results in a significant impact in terms of medical intervention and costs, and quality of life (1). Autoimmune diseases can be classified as either organ-specific or systemic (2). The defining feature is the damage to tissues and organs that arises due to an adaptive immune response to self antigens. The maintenance of self tolerance prevents the body from mounting an immune response against itself. Immunological tolerance is a state of unresponsiveness to a particular antigen that is induced by prior exposure to that antigen (3). There are a number of ways in which self-reactive lymphocytes are prevented from responding to self antigens: (a) ignorance-which occurs when the antigen is present in sub-stimulatory concentrations or is sequestered anatomically from the self-reactive lymphocytes; (b) anergy-which occurs when self-reactive cells see antigen in the absence of costimulation resulting in an inability to produce interleukin-2 (IL-2) and proliferate; (c) programmed cell death (apoptosis)-which occurs as a result of repeated antigenic stimulation; or (d) immune deviation-the activation of an antagonistic or

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suppressive arm of the immune system (3,4). With this in mind, most of the etiological mechanisms leading to autoimmunity result from a failure of one of the above processes. Genetic factors may contribute to self-antigen reactivity, e.g., defects of apoptotic genes and genes affecting cytokine expression may lead to T cell population imbalances (5).

The induction of autoimmune disease is usually triggered by an exogenous factor. Infectious agents may initiate autoreactivity through molecular mimicry, polyclonal activation, or release of previously sequestered antigens (2). Drugs can induce autoreactivity by acting as haptens and rendering auto-antigens immunogenic and inducing anti-nuclear antibodies (2). Finally, cytokine dysregulation may also lead to activation of autoreactive cells.

Most autoimmune diseases are associated with chronic inflammation. Inflammation is a response that is caused by tissue damage or injury, characterized by redness, heat, pain, and swelling ("rubor, calor, dolor, and tumor") (6). The inflammatory response involves three major stages: (i) capillary dilation to increase blood flow; (ii) increase in vascular permeability and release of chemokines and other chemotactic molecules; and (iii) leukocyte migration across the endothelium and accumulation at the site of injury (6). The inflammatory process is usually tightly regulated, involving both mediators that initiate and maintain inflammation and mediators that shut the process down (7). In states of chronic inflammation, an imbalance between the two types of mediators leaves inflammation unchecked, resulting in damage (7). $CD4^+$ T cells are involved in the induction of inflammation, orchestrating the cell-mediated response by stimulating monocytes and macrophages to secrete

inflammatory cytokines such as IL-1, IL-6, and TNFα (7). Activated $CD4^+$ T cells also stimulate B cells to produce immunoglobulins. As will be discussed in more detail later, differentiated $CD4^+$ T cells are functionally heterogeneous as defined by their different cytokine profiles. T helper 1 (Th1) cells, important in cell-mediated immunity, secrete interferon γ (IFNγ) and IL-2, while Th2 cells, associated with humoral responses, secrete IL-4, IL-5, and IL-10 (8). While a Th1 response is appropriate for the eradication of some microbial pathogens, if this response is elicited inappropriately against self antigens, inflammation, tissue destruction, and pathology may result (4,9). Th2 cells can downregulate the development and potentiation of Th1 driven cell-mediated immune responses (8). That cell-mediated and humoral immune responses are often mutually exclusive (10), suggests that a switch from a Th1- to a Th2-type response could explain the immune deviation from cell-mediated immunity to tolerance (4,9). However, the proposal that Th1 cells are pathogenic and Th2 cells are regulatory in the context of autoimmune disease may be too simplistic (4). Recent evidence suggests that a regulatory population distinct from Th2 cells exists in normal mice, but is absent in immunodeficient mice (11). A number of T cell subpopulations with regulatory or suppressor activities have been described. Among them are $CD4^+CD25^+$ T cells, Tr1/Th3, NK T cells, γδ T cells, and $CD8^+CD28^-$ T cells (12). These cells are thought to mediate their immune responses through direct cell-cell contact and/or secretion of TGFβ and IL-10 (13).

CURRENT THERAPEUTIC STRATEGIES

Current pharmacological means of reducing inflammation include non-steroidal anti-inflammatory drugs (NSAIDs), inhibitors of pro-inflammatory cyclooxygenases; cytotoxic drugs; corticosteroids; antibodies against cytokines (e.g., the anti-TNFα agents etanercept and infliximab) and cytokine receptor antagonists (2). There are drawbacks to the use of some of these substances. For example, infliximab must be used in combination with methotrexate to be effective (14). For other molecules, there is frequently a short half-life in the plasma which necessitates frequent daily treatment and high doses (15). Most importantly, the long-term safety of these new agents, particularly with respect to the risk of infections, cancer, and other autoimmune diseases limits their use in all patients.

In the late 1970s, epidemiological studies revealed that Greenland Inuits had substantially reduced incidence of inflammatory diseases compared to Western populations (16). Similar observations were made in the Japanese, which led to the correlation between a lower incidence of inflammatory disease and a high consumption of cold-water fatty fish (17). The overwhelming evidence from these and other epidemiological studies led to a number of clinical trials using fish oil (FO), which is enriched with the two n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The majority of clinical studies have focused on the use of FO supplementation in rheumatoid arthritis. Inflammatory bowel disease, systemic lupus erythematosus (SLE), and psoriasis have also been examined. Placebo-controlled, double blind studies of n-3 PUFA to treat inflammatory diseases have used

between 1 and 7 g/d of EPA plus DHA over a 12-52 week duration (18). As a point of reference, the traditional Inuit diet provides 6-12 g/d of EPA and DHA while the average American diet provides approximately 0.1 g/d (19,20). All studies in rheumatoid arthritis reported improvements in various clinical measurements including reduced tender or swollen joints, increased grip strength, and reduced joint pain (21-23). Furthermore, Skoldstam et al. (24) reported that NSAID usage was significantly decreased following 6 months of supplementation with 3.0 g/d of EPA and DHA. Aslan et al. (25) demonstrated beneficial effects of FO in patients with ulcerative colitis and Alimallah et al. (26) demonstrated clinical improvements of patients with procto-colitis. Additionally, a one-year double-blind placebo controlled study of remission maintenance in patients with Crohn's disease reported significantly greater remission rates in the group receiving 2.7 g/d of FO versus the placebo group (27). Furthermore, studies have shown decreased total serum PUFA, as well as deficiencies of both n-3 and n-6 PUFA in patients with chronic intestinal disorders (28). Symptoms of spontaneous lupus erythematosus (SLE) are also alleviated by FO supplementation (29). n-3 PUFA treatment hasn't always been successful. A study by Soyland et al. (30) provided 6 g/d of EPA and DHA to patients with psoriasis and saw no clinical improvements.

Studies have also employed animal models of inflammation in order to elucidate the mechanism(s) of the anti-inflammatory properties of n-3 PUFA. In (NZB X NZW)F_1 mice, which spontaneously develop a disease similar to human SLE, a diet composed of 25% FO (wt/wt) increased survival from renal complications (31). Using the same animal model, Fernandes et al. (32) found similar results, i.e., increased

survival and decreased proteinuria, using a 10% FO by weight diet. Vilaseca et al. (33) pretreated rats with n-6 and n-3 PUFA prior to trinitrobenzenesulfonic acid-induced colitis. The n-3 PUFA supplemented group had significantly less colonic damage than did the n-6 PUFA supplemented group. The effects of n-3 PUFA supplementation are more dramatic in animal models than in human clinical trials. This may be due to the fact that n-3 PUFA treatment in animals begins prior to disease onset, whereas human trials begin supplementation after disease onset.

EFFECT OF n-3 PUFA ON CELL FUNCTION

PUFA are divided into two major classes: n-3 and n-6. Fatty acid nomenclature indicates the number of carbon atoms in the hydrocarbon chain, the number of double bonds, and the position of the first double bond from the methyl end in the chain. For example, 22:6n-3 (DHA) has 22 carbons, 6 double bonds, and the first double bond is on the third carbon from the methyl end. Of the n-3 PUFA class, EPA (20:5n-3) and DHA are found in cold-water fatty fish while αlinolenic acid (ALA, 18:3n-3) is found in green leafy vegetables, and walnut and flaxseed oils. n-6 PUFA are primarily found in animal fats and plant-based oils, with linoleic acid (LA, 18:2n-6) being the most abundant. As described above, there is strong evidence for a protective effect of n-3 PUFA on autoimmune and inflammatory diseases. In contrast, n-6 PUFA can be deleterious with respect to the incidence and severity of such diseases (34-36). This is significant because the typical Western diet contains 10-20 times more n-6 than n-3 PUFA (37).

n-3 PUFA may exert their anti-inflammatory effects through a variety of mechanisms. Most importantly, n-3 PUFA affect the fatty acid composition of membrane phospholipids. This, in turn, could affect eicosanoid synthesis, membrane structure and function, and signal transduction. In addition, n-3 PUFA may directly regulate gene expression. Eicosanoids are signaling molecules that include prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), and hydroxy and hydroperoxy derivatives (HETE, HPETE). Non-esterified arachidonic acid (AA, 20:4n-6) released from membrane phospholipids by phospholipases acts as a substrate for cyclooxygenases (COX-1, a constitutive enzyme or COX-2, an inducible enzyme) and lipoxygenases (LOX). Cyclooxygenase products of AA give rise to PGs and TXs, which are mostly pro-inflammatory due to vascular permeability and vasodilation enhancement. Lipoxygenase products give rise to LT, HETE and HPETE, which are involved in vascular permeability and vasoconstriction (38). Compared to AA, EPA and DHA are poor substrates for COX and LOX, thus the cell's ability to produce eicosanoids and resulting inflammatory responses is strongly influenced by the fatty acid composition of the membrane phospholipids. Increasing dietary n-3 PUFA will decrease AA substrate availability for eicosanoid synthesis as shown by Endres et al. (39) who saw decreased PGE_2 production following n-3 PUFA feeding.

The plasma membrane is an asymmetrical lipid bilayer composed of phospholipids, cholesterol, sphingolipids, and integral proteins. In 1972, Singer and Nicolson proposed the fluid-mosaic model which described the fluid nature of the membrane bilayer (40). The fluidity of the membrane affects the organization and

dynamics of both lipids and proteins within the membrane and, thus, its biological function. Fluidity of the membrane depends, in part, on its composition. The length and saturation of the fatty acyl chain of phospholipids influences the ability of the phospholipid molecules to pack against one another. A shorter chain length reduces the tendency of the acyl chains to interact with one another, and *cis*-double bonds create kinks in the chains that prevent them from packing together. The presence of cholesterol and sphingolipids promotes rigidity. Saturation is more prevalent in sphingolipid acyl chains and the sterol structure of cholesterol causes the fatty acyl chains to become closely packed. It is also known that cholesterol preferentially interacts with sphingolipids (41) and, together with glycosylphosphatidylinositol (GPI)-anchored proteins, they form liquid-ordered microdomains that float in the liquid-disordered bulk membranes (42). These "rafts" are resistant to solubilization by non-ionic detergents, facilitating isolation for study (43).

Lipid rafts have been implicated in an ever-increasing number of biologically important phenomena such as signaling. Lipid raft domains play an important role in cell signaling, particularly through the organization of surface receptors and adaptor molecules into complexes at specific sites in the plasma membrane (44). Binding of proteins by an extracellular ligand can induce rapid lateral translocation to rafts and clustering, both events which are important for signal transduction. In T cells, lipid rafts are important in the formation and stabilization of macromolecular complexes containing the T cell receptor (TCR), CD4 and CD45, acting as platforms that facilitate intramolecular associations and propagation of signal transduction cascades (44).

Changes in raft lipid and/or protein composition can alter cell function and phenotype. Fan et al. (45) have recently shown that dietary n-3 PUFA remodel the phospholipid composition of murine T cell rafts. In addition, it has been reported that Jurkat T cells cultured in vitro with PUFA undergo modification of lipid raft composition and suppression of signal transduction (46,47).

Many cellular proteins are modified by fatty acid acylation with myristate (14:0) and palmitate (16:0) (48). The Src kinase family of protein tyrosine kinases plays an important role in T cell activation and their myristoylation and palmitoylation are required for localization to the plasma membrane and rafts (49). Proteins acylated with saturated fatty acids have a higher affinity for rafts and the two Src kinases Lck and Fyn are concentrated on the cytoplasmic side of rafts. The profile of Fyn acylation parallels the composition of fatty acids available to the cell. PUFA treatment of T cells results in PUFA acylation of Fyn, most-likely due to the promiscuous nature of the palmitoyl acyltransferase enzyme that will covalently attach a broad spectrum of fatty acids to proteins. Inhibition of Fyn palmitoylation by AA and EPA blocked Fyn localization to rafts (49). Therefore, changes in membrane phospholipid composition as well as protein acylation can affect signaling from the plasma membrane and, thus, the effect of PUFA on these phenomena is likely to have a broad impact on signaling pathways.

In addition to its effects on membrane composition, PUFA can directly affect gene expression. A number of inflammatory genes have been identified as targets for n-3 PUFA including COX-2, 5-LOX, adhesion molecules ICAM-1, VCAM-1 and E-selectin, and proinflammatory cytokines IL-1α, IL-1β, TNFα, and IL-6 (50-53). In

contrast, constitutively expressed COX-1 and anti-inflammatory genes were unaffected (50). Nuclear factor κB (NFκB) is a transcription factor that is involved in the induction of a number of inflammatory genes. NFκB exists in a latent form comprising a transcriptionally active dimer bound to an inhibitor protein, IκB. Following stimulation, IκB is phosphorylated, which targets it for ubiquitination and subsequent degradation. The released NFκB can then translocate to the nucleus and activate genes by binding to κB response elements in their promoters (54). Decreased nuclear NFκB has been demonstrated following n-3 PUFA feeding (55) and this may be due to decreased phosphorylation of IκB (56). Another group of transcription factors regulated by n-3 PUFA are the nuclear receptors (57). Nuclear receptors function as ligand-activated transcription factors that regulate the expression of target genes. PUFA have been shown to serve as ligands for peroxisome proliferators-activated receptors (57) and retinoid X receptors (58,59).

In summary, n-3 PUFA may act through a variety of mechanisms to suppress inflammation, including indirect effects via alterations in membrane composition, or direct effects via regulation of gene expression.

T CELL ACTIVATION AND DIFFERENTIATION

T cell activation is essential for an efficient immune response. A primary stimulus is generated upon interactions between a foreign antigen presented in the context of self major histocompatibility complex (MHC) class I or II and the TCR/CD3 complex. This signal is on its own insufficient to induce a T cell response. A second, or

costimulatory, signal delivered by an antigen presenting cell (APC) is necessary. The principal costimulatory molecules are B7.1 and B7.2 expressed on the APC that bind to CD28 and CTLA-4 on the T cell. This second signal appears to be fundamental since, in its absence, TCR stimulation may lead to anergy (60). CTLA-4 binds B7 molecules with about 20 times more avidity than CD28 and delivers an inhibitory signal which effectively shuts down the proliferative phase of the response (61). The net effect of stimulatory signals delivered to the TCR/CD3 complex and the CD28 co-receptor is the synthesis and secretion of IL-2. NF-AT, NFκB, and AP-1 are all necessary transcription factors for the expression of IL-2 and IL-2 receptor α chain (IL-2Rα) (60). IL-2 is the most powerful autocrine and paracrine T cell growth factor and activator and its production determines whether a T cell will proliferate and become an armed effector cell.

T cells can be divided into $CD4^+$ and $CD8^+$ subsets based on the mutually exclusive presentation of these molecules on their surfaces and their different effector functions. $CD4^+$ and $CD8^+$ T cells can further differentiate in a highly polarized manner into Th1/Tc1 or Th2/Tc2 effector cells (8,62). Th1 cells are characterized by the production of IL-2, IFNγ, and TNFβ and are required to mount a cell-mediated immunological response against intracellular pathogens. Th2 cells are characterized by the production of IL-4, IL-5, and IL-10 and are important in humoral immunity and defense against extracellular pathogens (8). In addition to their protective roles in host defense, both Th subsets have been implicated in pathological responses. Th1 cells are proinflammatory and can mediate autoimmune and inflammatory diseases, while Th2

cells have been implicated in the pathogenesis of asthma and allergy (63). The two helper subsets cross-regulate each other to maintain a balanced heterogeneous response using cytokines; Th1 cells inhibit Th2 responses with IFNγ, and Th2 cells inhibit Th1 responses with IL-10 (64).

Many factors influence the cell's decision to become a Th1 or Th2 effector. It has been proposed that antigen dose, strength of signal through the TCR, costimulators, and genetic modifiers all influence the dominance of a Th cell response, but cytokines released from accessory cells have emerged as critical inducers of Th subset development (64). The major accessory cells appear to be dendritic cells (DC) which display unique properties aimed at inducing an immune response against antigens from invading organisms (65). Specific DC subsets located in distinct splenic micro-environments appear to bias Th subset development by secreting critical cytokines. $CD8\alpha^+$ DCs produce IL-12 in response to microbes, whereas $CD8\alpha^-$ DCs induce IL-4 production. Signaling through the IL-12 receptor (IL-12R) on the T cell surface activates Stat-4 (signal transducer and activator of transcription 4) which induces T-bet (T-box expressed in T cells) and promotes Th1 lineage commitment. In contrast, signaling through the IL-4 receptor activates Stat-6 which induces GATA-3 leading to Th2 differentiation (64). T-bet is a Th1-specific factor that is involved in chromatin remodeling of the IFNγ gene promoter and induces IL-12Rβ2-subunit expression. Transcriptionally inactive genes have regions of condensed chromatin, unmodified histones, and are densely methylated. Histone acylation and DNA de-methylation favor chromatin decondensation, providing an open conformation for transcription factor

access (66). GATA-3 seems to be crucial for inducing some, but perhaps not all, key attributes of Th2 cells. GATA-3 has been shown to directly regulate IL-5 expression, and indirectly affect IL-4 transcription. GATA-3 remodels the chromatin of the IL-4 promoter in a manner similar to that by which T-bet remodels the IFNγ promoter. GATA-3 also upregulates c-Maf, an acute transcription factor for IL-4 (63).

In addition to the functional differences of Th1 and Th2 cells, there are also differences in their patterns of membrane compartmentalization into lipid rafts (44). Leitenberg et al. (67) have recently shown that Th1 cells recruit both the TCR/CD3 complex and CD45 to rafts following stimulation, while Th2 cell stimulation does not result in stable association of TCR complex components with raft domains. Possibly as a result, Th2 cells have been shown to exhibit defects in proximal signaling events such as ZAP-70 phosphorylation, Fyn phosphorylation, and calcium mobilization (68). Thus, it is evident that Th1 and Th2 cells have unique ways of organizing molecules at the plasma membrane, which is likely to impact the cell's ability to signal and ultimately function.

n-3 PUFA REGULATION OF T CELL FUNCTION

There have been numerous investigations measuring a variety of outcomes on n-3 PUFA and T cell function in humans, mice, rats, and cell culture systems. Several studies have examined lymphoproliferation. Three different clinical trials of dietary supplements ranging from 2.4-18 g/d of EPA and DHA reported significant reductions in mitogen-induced proliferation and IL-2 production in human peripheral blood

mononuclear cells (PBMC) (69-71). Another human study found similar reductions in proliferation and IL-2 secretion when 180 g/d of fish was used rather than oil (72). Additionally, Grimble et al. (73) found that 6 g/d of FO was able to decrease TNFα production.

Similar studies have been conducted in rodents. Jolly et al. (74,75) fed mice 10g/kg EPA or DHA for 10 days and reported a significant decrease in ConA-stimluated splenocyte proliferation in both diet groups. This was accompanied by a decrease in IL-2, IL-2Rα expression, and reductions in DAG and ceramide, lipid second messengers. Feeding rats a diet containing 20% (wt/wt) FO for 10 weeks, a much higher level of fat and longer feeding duration, also suppressed splenocyte proliferation (76). Additional cytokine analyses have reported decreased IFNγ (77,78) and IL-12 (77) in mitogen-stimulated or *Listeria monocytogenes*-infected splenocytes from n-3 PUFA-fed mice. Interestingly, Fritsche et al. (79) found that 18% dietary FO by weight enhanced *in vivo* IFNγ in serum and the spleen from *Listeria*-infected mice. They suggested that the FO-induced increase in circulating IFNγ could be a consequence of alterations in the expression and/or function of the IFNγ receptor (IFNγR). In a follow-up study, Feng et al. (80) showed a decrease in IFNγR-1 expression in FO-fed mouse splenocytes and peritoneal macrophages infected with *Listeria*. More recently, Arrington et al. (81) noted decreased splenocyte IL-2 secretion from mice fed diets containing 1% DHA by weight only when cultures were stimulated with antibodies against CD3 and CD28 (αCD3/αCD28). Thus, in that experimental system, dietary n-3 PUFA appear to alter TCR-dependant or costimulatory (CD28) signal transduction. Using the same set of

stimuli, Sasaki et al. (82) showed a decrease in CD4 and CD8 and an increase in CD28 surface expression in DHA-fed mouse splenocytes. In a further attempt to elucidate a mechanism of action, Arrington et al. (83) isolated $CD4^+$ and $CD8^+$ T cell populations from mice following a 14 day diet of 2% (wt/wt) FO. They found that an effect on proliferation was dependant on the stimulus used in culture. FO suppressed $CD8^+$ T cell proliferation in cells stimulated with αCD3/αCD28. In contrast, FO increased $CD4^+$ T cell proliferation when cells were stimulated with αCD3 plus phorbal-12-myristate-13-acetate (PMA), an agonist that stimulates PKC (protein kinase C). Interestingly, this stimulation combination directs cells towards a Th2-like phenotype (83,84). Another study saw a decrease in reduction in PLCγ tyrosine phosphorylation in mitogen-stimulated rat splenocytes fed 20% (wt/wt) FO (85). Fan et al. (submitted) have recently shown that $CD3^+$ T cells from 4% FO (wt/wt)-fed mice had decreased PKCθ colocalization with lipid rafts after αCD3/αCD28 stimulation. PKCθ is an important molecule in the regulation of T cell proliferation and apoptosis and requires translocation to lipid rafts to be active (86). These data indicate that changes in membrane composition induced by dietary n-3 PUFA have functional consequences with respect to the membrane distribution of T cell signaling proteins.

In cell culture experiments, mouse splenocytes and human PBMC cultured in DHA or EPA had reduced levels of proliferation, IL-2, and IL-2Rα expression following mitogen stimulation (78,87,88). In more mechanistic studies, Denys et al. (89) noted a decrease in PMA-stimulated PKC activation and ERK1/ERK2 signaling in Jurkat T cells that were cultured in the presence of up to 60 μM EPA and DHA. Diaz et al. (90)

showed that mitogen-stimulated human PBMC had increased phospholipase D (PLD) activity, a regulator of T cell proliferation and apoptosis, following culture in 5-15 μM DHA. They also found greater DHA incorporation in the phospholipids of rafts than non-rafts. Therefore, they concluded that DHA stimulates PLD by an indirect mechanism involving lipid modification of rafts.

In an attempt to explain the suppressive effect of n-3 PUFA on T cell proliferation, studies have examined the effect of dietary n-3 PUFA on T cell apoptosis. Avula et al. (91) showed that feeding young (4 months) and old (9 months) mice 5% (wt/wt) FO resulted in increased splenocyte apoptosis in both age groups. Fernandes et al. (92) reported an increase in mouse splenocyte apoptosis following a 10% (wt/wt) FO diet, and this was associated with an increase in the surface expression of Fas, a death receptor and mediator of apoptosis. In a similar study, FasL, the molecule that binds to and activates Fas, was upregulated and Bcl-2, an anti-apoptotic molecule, was decreased in mouse splenocytes after a 4% (wt/wt) FO diet (93). Additionally, Jurkat T cells cultured in 0-90 μM DHA for up to 48 hours produced more caspase 3, a cysteine protease involved in the apoptotic process (94).

APOPTOSIS

Apoptosis refers to a morphologically defined mode of programmed cell death, which shapes the lymphocyte repertoire though selection of maturing T cells in the thymus and maintains homeostasis in the mature lymphocyte pool after antigenic expansion. The dysregulation of apoptosis plays a major role in the pathogenesis of

disease. The fundamental morphological features of apoptosis are chromatin condensation and cell shrinkage, accompanied by membrane blebbing, culminating in the break up into membrane-bound apoptotic bodies (95). During this process, phosphatidylserine flips to the outer leaflet of the cell membrane and is recognized and phagocytosed by macrophages or adjacent cells. Thus, apoptosis does not trigger an inflammatory response (95,96). Due to the distinctive characteristics, apoptosis can easily be detected using a variety of assays. For example, DNA fragmentation can be detected by TUNEL (terminal-deoxynucleotidyl-transferase-mediated dUTP-digoxygenin nick end labeling) or subdiploid assays, and phosphatidylserine externalization can be detected by Annexin V binding assays (95).

There are at least two major pathways of T cell apoptosis. One pathway, known as passive cell death, results from the absence of growth factors and other survival stimuli (97). Passive cell death is important in the elimination of cells that fail to undergo positive selection in the thymus (98). The second pathway, known as activation-induced cell death (AICD), is induced by chronic antigen stimulation (97) and plays an essential role in peripheral deletion involved in tolerance and homeostasis (96,99). Additionally, AICD is important in negative selection in the thymus. Negative selection occurs when the TCR of a thymocyte binds a peptide-MHC ligand with high affinity, leading to apoptotic death of the cell (98). The biochemical mechanisms of passive cell death and AICD are distinct. However, both are mediated by the action of caspases, cysteine proteases with specificity for aspartate residues. Caspases exist as inactive zymogens (proenzymes) in the cytoplasm until activated by proteolytic

cleavage. They are organized in cascades that amplify the initial death signal (100). Passive cell death results from activity of the intrinsic or the mitochondrial pathway. AICD occurs following events which follow the extrinsic or death receptor pathway.

When cells are deprived of survival stimuli, there is an increase in mitochondrial permeability and breakdown of mitochondrial membrane potential. As a consequence, cytochrome c is released into the cytoplasm and binds to Apaf-1. Pro-caspase 9 associates with this complex, leading to its proteolytic activation. Active caspase 9 subsequently activates downstream caspases including caspase 3, leading to cell death (97,101). Mitochondrial membrane permeability is highly regulated by members of the Bcl-2 family. Anti-apoptotic members of the Bcl-2 family, including Bcl-2 and Bcl-x_L, prevent membrane permeability and release of cytochrome c. Pro-apoptotic members, including Bax, Bak, Bid, and Bad, enhance membrane permeability and resulting cytochrome c release (95).

In the immune system, AICD acts as feedback mechanism for terminating an ongoing immune response and serves to maintain peripheral tolerance. T cells responding to an antigenic stimulus first expand in number and then decrease as a result of AICD. Repeated antigen stimulation leads to the expression of death receptors that link extracellular signals to apoptosis. Death receptors belong to the tumor necrosis factor receptor (TNFR) superfamily and include Fas, TNFR1, and TRAIL-R (102). They are characterized by multiple cysteine-rich repeats in their extracellular domain and a protein-protein interaction death domain (DD) in their cytoplasmic tail (103). Fas (CD95, APO-1) is the best-studied receptor and is the principal mediator of $CD4^+$ T cell

AICD (97). Fas-mediated apoptosis is initiated by its interaction with FasL (CD95L), which is a TNF-related type II transmembrane molecule (103). Unlike Fas, which is expressed in wide variety of tissues including thymus, spleen, lymph node, kidney, liver, and heart (104), FasL is expressed primarily in activated T cells and NK cells (103,105). Interestingly, while IL-2 plays a critical role in cell growth, it also sensitizes the same cells to apoptosis. When high levels of IL-2 are present, this cytokine promotes FasL expression (106). Fas trimerization, necessary for apoptosis transduction, is triggered by FasL binding and immediately recruits a complex of associated proteins known as the death-inducing signaling complex (DISC). The adaptor molecule, Fas-associated death domain protein (FADD), binds to Fas via its DD. In addition, FADD also has a death-effector domain (DED) with which it recruits the DED-containing pro-caspase 8 into the DISC (103). Pro-caspase 8 is then cleaved and active caspase 8 dissociates from the DISC to cleave pro-caspase 3, initiating the caspase cascade that results in cell death. The importance of Fas/FasL in AICD is illustrated by the development of lymphoproliferative disorders in *lpr/lpr* and *gld/gld* mice due to mutations in the genes encoding Fas and FasL, respectively (97).

Recent studies have shown that Fas and components of the DISC translocate into lipid rafts following TCR restimulation and/or Fas ligation (107-109). Furthermore, they reported that rafts are required for efficient propagation of apoptotic signals. Grassme et al. (110) have implicated ceramide generation in Fas clustering in membrane microdomains. They showed that ligation of Fas results in the activation of acidic

sphingomyelinase (ASM) which hydrolyzes sphingomyelin into ceramide. Ceramide is able to coalesce and form membrane microdomains.

Two pathways of Fas-mediated apoptosis have been described, dependent on the quantity of caspase 8 activated (101). In type I cells (e.g., lymphocytes), a high amount of caspase 8 is released from the DISC leading to direct caspase 3 activation. However, in type II cells (e.g., hepatocytes), despite similar expression levels of Fas and signaling molecules, the DISC is poorly formed and the amount of caspase 8 activated is insufficient to process caspase 3, but sufficient to cleave the Bcl-2 family member Bid (101,111). Bid cleavage results in a pro-apoptotic fragment called tBid that mediates mitochondrial cytochrome c release and resulting apoptosis.

T cells are not equally sensitive to AICD (112). In addition to their differential cytokine production and functions, Th1 and Th2 cells have different susceptibilities to AICD, with Th1 cells reported to be AICD sensitive and Th2 cells reported to be AICD resistant (112,113). The selective death of Th1 cells has been attributed to a preferential requirement for phorbol ester-sensitive PKC isoforms (114), and the upregulation of Fas (114) and FasL (114,115). In contrast, the resistance of Th2 cells to AICD has been linked to expression of c-FLIP, an enzymatically inert homologue of caspase 8 (116), and PI3-K activity (117).

RATIONALE AND EXPERIMENTAL MODEL

Major observations in our lab have demonstrated that dietary n-3 PUFA suppress splenic and T cell proliferation (74,75,81,83,118), and diminish delayed-type

hypersensitivity (118). Additionally, our lab has found that dietary n-3 PUFA suppress Th1 proliferative capacity in mice due to a reduction in IL-2 production and/or function, and enhance counter-regulatory IL-4-driven Th2 cells (83). These observations may implicate a role for apoptosis in these processes. An increase in AICD may explain a loss of proliferative capacity of T cells from n-3 PUFA-fed mice and explain the immunosuppressive effects described above. There are relatively little data on the effects of n-3 PUFA on lymphocyte apoptosis. The few studies that have addressed this issue have examined mixed cell populations, i.e., whole splenocytes (92,93). Avula et al. (93) did examine $CD4^+$ and $CD8^+$ T cell subset apoptosis, however this study did not culture purified $CD4^+$ or $CD8^+$ T cells, but rather used flow cytometry to quantify the subset populations after stimulation of the mixture. We believe that AICD is a critical apoptotic pathway to examine since it is the form of apoptosis associated with the deletion of cells involved in chronic inflammation and autoimmune disease. Consistent with this thinking, we will focus on the $CD4^+$ Th1 subset, as it is the cell mediator of chronic inflammation.

Previous studies from our lab have demonstrated that low dose, short-term n-3 PUFA dietary supplementation can modulate specific functions and responses of murine splenic lymphocytes (118). That study found that a 10 day feeding period using 1% (wt/wt) of highly purified EPA or DHA was effective at modulating lymphocyte membrane composition and proliferation. Thus, we have continued to use a similar feeding paradigm.

Our lab has used the C57BL/6 mouse strain as the model to study n-3 PUFA and immune function. The C57BL/6 mouse is the most widely used mouse strain and is extensively used in immunology. In addition, it is Th1 biased, making it a suitable choice to study inflammatory processes and other Th1-mediated pathologies (119).

We have chosen to use three main sets of agonists to activate T cells. Previous experiments have determined the optimal concentrations for effectiveness (81). These agonists include a pair of monoclonal antibodies that engage the TCR/CD3 complex and the important costimulatory molecule CD28 (αCD3/αCD28); a pair of stimuli that bypasses the membrane to stimulate intracellular PKC and calcineurin (PMA/Ionomycin); and two agonists which act on membrane and intracellular components (αCD3/PMA). Interestingly, the nature of the stimulus can result in T cell bias towards specific cytokine secretion profiles (83,84). Noble et al. (84) showed that when unprimed T cells received a strong calcium signal and a weak PKC signal they preferentially shifted towards Th1 effector cells. In contrast, strong PKC combined with a weaker calcium signal resulted in a Th2 shift. Comparably, Arrington et al. (83) showed that our PMA/Ionomycin pair directed T cells towards a Th1 phenotype and the αCD3/PMA pair directed T cells towards a Th2 phenotype.

The overall objective of these experiments is to determine the extent to which dietary n-3 PUFA modulate AICD in the Th1 subset of $CD4^+$ murine T cells. The results of these experiments, combined with our previous studies, will contribute to the elucidation of the mechanisms by which dietary n-3 PUFA suppress chronic inflammation mediated by T cells.

CHAPTER II

n-3 POLYUNSATURATED FATTY ACIDS PROMOTE ACTIVATION-INDUCED CELL DEATH IN MURINE T LYMPHOCYTES*

Previous studies showing dietary n-3 polyunsaturated fatty acids (PUFA) attenuate T cell immune-mediated inflammatory diseases led us to hypothesize that n-3 PUFA promote activation-induced cell death (AICD) in T cells. Since T cell subsets display a differential resistance to AICD, we compared the effects of n-3 PUFA feeding on T cells stimulated *in vitro* to express different cytokine profiles. Mice were fed either diets lacking n-3 PUFA (control) or n-3 PUFA-containing diets for 14 days. Splenic T cells were stimulated with αCD3/αCD28, PMA/Ionomycin, or αCD3/PMA for 48 h, followed by reactivation with the same stimuli for 5 h. Apoptosis was measured using AnnexinV/propidium iodide. n-3 PUFA were selectively incorporated into membrane phospholipid pools. Cytokine analyses revealed that n-3 PUFA enhanced AICD only in T cells expressing a Th1-like cytokine profile following stimulation with PMA/Ionomycin compared to mice fed the n-6 PUFA control diet ($p=0.0008$). In contrast, no increase in apoptosis was seen in T cells stimulated with αCD3/PMA, which exhibited a Th2 cytokine profile. These data demonstrate that the ability of n-3 PUFA to promote AICD is dependent upon the activation stimulus. In conclusion, we have

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identified a novel mechanism by which n-3 PUFA modulate T cell mediated immunity by selective deletion of Th1-like cells while maintaining or enhancing the Th2 mediated humoral immune response.

INTRODUCTION

Apoptosis is a highly regulated process resulting in cell death without an ensuing inflammatory response, thus playing an important role in maintaining lymphocyte homeostasis and T cell repertoire, and ensuring peripheral tolerance (96). T cells can succumb to apoptosis by two pathways; passive cell death (absence of growth factors) and chronic antigen stimulation (activation-induced cell death, AICD). AICD is specifically responsible for the signal-induced elimination of previously activated lymphocytes. The induction of AICD, in part, is mediated by the co-expression of Fas and its ligand, FasL, on the T cell plasma membrane (96,120,121). The Fas death receptor is a member of the nerve growth factor receptor/TNF receptor family and transmits signals resulting in apoptosis (122). Stimulation upregulates the expression of FasL and other death factors in AICD-sensitive T cells, which, in turn, induce Fas on the same or neighboring cells. Death receptors are activated by oligomerization by rapidly assembling a number of adaptor proteins resulting in an irreversible activation of proteases and nucleases which culminate in apoptosis (123). Mutations in Fas or FasL result in lymphoproliferation and manifestations of autoimmunity in both mice and humans (124,125), illustrating the critical role of AICD in the maintenance of self-tolerance.

T cells can be divided into $CD4^+$ and $CD8^+$ subsets based on the mutually exclusive presentation of these molecules on their surfaces and their different effector functions. Mature $CD4^+$ effector cells can be further polarized into Th1 and Th2 subsets according to the cytokines they produce (8). Th1 cells produce IL-2, IFNγ, and TNFβ and are important in cell-mediated immunity against intracellular pathogens (8,66). Th1 cells are proinflammatory and have been implicated in the pathogenesis of human inflammatory and autoimmune diseases such as rheumatoid arthritis, type-1 diabetes, and inflammatory bowel diseases (9,126). Th2 cells produce IL-4, IL-5, and IL-10 and are important in humoral immunity and defense against extracellular pathogens (66). Th1 and Th2 effectors have differential susceptibilities to AICD, with Th1 cells being AICD sensitive and Th2 cells being AICD resistant (115). The selective death of Th1 cells has been attributed to a preferential requirement for phorbol ester-sensitive protein kinase C (PKC) isoforms (114) and the upregulation of FasL expression (115). In addition, the resistance of Th2 cells to AICD has been linked to expression of FAP-1, an inhibitor of apoptosis (112), and the selective upregulation of phosphatidylinositol-3'-kinase (PI-3-K) activity (117).

Among dietary factors, n-3 polyunsaturated fatty acids (PUFA) found in fish oil (FO) have been shown to potently attenuate T cell-mediated inflammatory diseases in humans and experimental model systems (34,127). In contrast, dietary lipids rich in n-6 PUFA, found in vegetable oils and animal fats, can be deleterious in some inflammatory diseases (34-36,70,128). This is significant because the Western diet contains 10 to 20 times more n-6 than n-3 PUFA (37). Previous studies have demonstrated that n-3 PUFA

decrease T cell proliferation (74,83), cytokine secretion (70,77,129), intracellular enzyme activity (130), and gene transcription (75,77,131). Additionally, we have found that n-3 PUFA suppress T cell proliferative capacity due to a reduction in IL-2 production and/or function, and to enhancement of counter-regulatory IL-4-driven Th2 cells (83). Several investigators have examined the effects of n-3 PUFA on apoptosis in mixed cell populations, e.g. whole splenocytes (132). In those studies, n-3 PUFA increased T cell apoptosis in cultures of whole splenocytes. Since we have recently found that the diet-induced changes in T cell proliferation are due principally to intrinsic alterations in the T cell response to mitogenic stimuli (133), the effects of n-3 PUFA on apoptosis in purified T cells remained to be examined. Our results indicate that dietary n-3 PUFA preferentially promote AICD in a T cell subset exhibiting a Th1 cytokine profile. This exciting observation provides yet another unique mechanism by which n-3 PUFA exert a significant and selective effect on T cell proinflammatory function in the whole animal.

MATERIALS AND METHODS

Diet and animals

All experimental procedures using laboratory animals were approved by the University Laboratory Animal Care Committee of Texas A&M University. Female, pathogen-free (12–14g) C57BL/6 mice were purchased from Frederick National Cancer Research Facility, Frederick, MD. Animals were housed in autoclaved polycarbonate microisolator cages and were maintained at room temperature (~25°C) on a 12 h

light:dark cycle. Mice were initially fed standard mouse chow (Teklad 9F Sterilizable Rodent diet, Madison, WI) during a 1 week acclimation period and had free access to autoclaved water and diet. The study was completed in two phases. For the first experiment, mice were assigned to one of three semi-purified diets containing: 2% safflower oil ethyl ester (SAF, n-6 PUFA control), 2% olive oil ethyl esters (OO, monounsaturated fatty acid devoid of both n-3 and n-6 PUFA) or 2% Menhaden fish oil (FO2, n-3 PUFA) for 14 d. For the second experiment, mice were assigned to one of the following diets: SAF, 4% Menhaden fish oil (FO4) or 9% Menhaden fish oil (FO9) for 14 d. In a third study, mice were assigned to a 5% corn oil (CO) or 1% DHA diet. The purified diets met National Research Council nutrition requirements and varied only in lipid composition (81,134). The diet composition, expressed in g/kg of complete diet, was as follows for experiment I: 200 g casein, 420 g sucrose, 219.8 g starch, 60 g cellulose, 35 g AIN-76 mineral mix, 10 g vitamin mix AIN-76, 3 g dl-methionine, 2 g choline chloride, and 0.2 g tertiary butyl hydroquinone. The lipid content of the three diet groups was: SAF, 30g/kg CO combined with 20 g/kg safflower oil ethyl esters; OO, 30g/kg CO combined with 20 g/kg olive oil ethyl esters; FO2, 30g/kg CO combined with 20 g/kg menhaden fish oil. For experiment II, the basic diet composition differed only in the reduced amount of sucrose for FO9 (370 g/kg) to compensate for the increase in fat content. The lipid content of the three diet groups was: SAF, 30g/kg CO combined with 20 g/kg safflower oil ethyl esters; FO4, 10 g/kg CO combined with 40 g/kg menhaden fish oil; FO9, 10 g/kg CO combined with 90 g/kg menhaden fish oil. For the third study, the lipid content of the two diets was: CO, 50g/kg CO; DHA, 10g/kg DHA ethyl esters

combined with 40g/kg CO. The fatty acid composition, expressed in g/kg of complete diet, is detailed in **Table 1**. The linoleic acid (18:2 n-6) content from CO was 2-7% of total energy and, thus, met the minimum 1–2% essential fatty acid requirement for rodents (134). The vitamin A, D, and E levels were approximately equal and exceeded the minimum requirement. Safflower oil (70.5% as 18:2 n-6) was obtained in ethyl ester form from the National Institute of Health Test Materials Program (Charleston, SC). Control dietary lipids lacking n-3 PUFA, olive oil and corn oil (57.3% as 18:2 n-6), were obtained from Degussa Bioactives (Champaign, IL), Menhaden fish oil (13.1% as 20:5 n-3, 9.7% as 22:6 n-3) was provided by the National Institutes of Health Test Materials Program. Diets were analyzed by gas chromatography (Table 1), aliquoted, and stored at -80°C. Diets were provided ad libitum and were changed daily to prevent peroxidation. The analysis confirmed the enrichment of 18:2 (n-6) in the SAF diet (53.3% of total lipid), 20:5 (n-3) and 22:6 (n-3) in the FO2 diet (6.1% and 4.3% of total lipid, respectively), 20:5 (n-3) and 22:6 (n-3) in the FO4 diet (11.7% and 8.1% of total lipid, respectively), and 20:5 (n-3) and 22:6 (n-3) in the FO9 diet (13.4% and 9.4% of total lipid, respectively). There was no significant difference in food intake between dietary groups, and weight gain was similar in all groups (data not shown).

Isolation and preparation of splenic lymphocytes

Mice were euthanized by CO_2 asphyxiation. Spleens were placed in 3 ml of RPMI-complete medium [RPMI 1640 with 25 mM HEPES (Irvine Scientific, Santa Ana, CA) supplemented with 10% FBS (Irvine Scientific), 1 x 10^5 U/L penicillin and 100

mg/L streptomycin (Irvine Scientific), 2 mM L-glutamine, and 10 μM 2-mercaptoethanol] (74). Spleens were dispersed with glass homogenizers and passed through a 149 micron wire mesh filter (Pall Gelman, Fisher Scientific) to create single-cell suspensions. Cells were subsequently washed with RPMI-complete medium prior to T cell enrichment.

Table 1. Fatty Acid Composition of Experimental Diets

Fatty Acid	CO	SAF	OO	FO2	FO4	FO9	DHA
14:0	tr	0.3	0.4	3.7	7.4	8.2	tr
16:0	12.2	12.4	14.8	13.2	16.1	16.9	10.0
16:1 (n-7)	0.1	tr	0.9	4.8	9.6	11.0	0.2
18:0	2.0	2.8	5.1	2.6	3.0	3.1	2.1
18:1 (n-7 + n-9)	30.0	28.4	37.4	20.8	13.8	11.8	25.9
18:2 (n-6)	54.3	53.3	34.1	35.5	13.5	8.0	46.6
18:3 (n-3)	1.1	1.0	2.0	1.2	1.4	1.2	1.0
20:5 (n-3)	tr	tr	tr	6.1	11.7	13.4	tr
22:5 (n-3)	tr	tr	tr	1.1	1.7	1.9	tr
22:6 (n-3)	tr	tr	tr	4.3	8.1	9.4	14.0
Total SFA	14.2	16.0	21.2	20.1	26.9	28.7	12.1
Total MUFA	30.1	28.8	39.0	26.4	24.0	23.5	52.0
Total (n-6) PUFA	54.3	53.3	34.1	35.5	14.3	9.4	46.6
Total (n-3) PUFA	1.1	1.0	3.0	12.8	22.9	25.9	15.0

Only the major fatty acids are listed. CO, corn oil-containing diet; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SAF, linoleic acid-containing diet; OO, oleic acid-containing diet; FO2, fish oil (2%, w/w)-containing diet; FO4, fish oil (4%, w/w)-containing diet; FO9, fish oil (9%, w/w)-containing diet; DHA, docosahexanoic acid-containing diet; tr, trace amount (<0.1 g/100 g). Values are expressed as g/100g of total fatty acids in each diet.

T cell purification

Total lymphocytes were initially enriched by a density gradient centrifugation method using Lympholyte-M (Cedarlane, Toronto, Canada) (81) in accordance with the manufacturer's protocol. Subsequently, 60–90 x 10^6 mononuclear cells were loaded onto

a negative-selection mouse T cell purification column (R&D Systems, Minneapolis, MN) and incubated for 10 min at room temperature. Nonadherent cells were eluted for assay. The purity of the T cell population as analyzed by flow cytometry was determined to be 90.3 ± 1.4%, n=3 (81).

T cell apoptosis assay

T cells, 5 x 10^5 cells/well from each mouse, were cultured in the presence of each of five sets of stimuli: (a) 1 μg/ml plate-bound purified hamster anti-mouse CD3ε (αCD3) monoclonal antibody (PharMingen, San Diego, CA) with 5 μg/ml soluble purified hamster anti-mouse CD28 (αCD28) monoclonal antibody (PharMingen); (b) 0.5 ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma, St Louis, MO) with 1 μg/ml αCD3; (c) 1 ng/ml PMA with 500 nM Ionomycin (Calbiochem-Novabiochem, San Diego, CA); (d) 50 ng/ml recombinant human soluble FasL fused to a FLAG-tag (FasL) (Alexis Biochemicals, San Diego, CA) with 1 μg/ml Enhancer (αFLAG) (Alexis Biochemicals); or (e) 330 ng/ml dexamethasone (Sigma). Choice of concentrations were determined in previous experiments (81). Cells were incubated at 37°C, 5% CO_2 for 24 h. Apoptosis was assessed via flow cytometry (see below).

Induction of AICD

AICD was induced as previously described by Dao et al. (122) with slight modifications. T cells, 5 x 10^6 cells/well, were cultured in duplicate in the presence of either stimuli (a), (b), or (c) as described above for 48 h at 37°C, 5% CO_2 (**Figure 1**).

Cells were harvested, washed and recultured at 3 x 10^6 cells/well in RPMI-complete medium overnight at 37°C, 5% CO_2. Cultures were reharvested and dead cells were removed using Lympholyte-M in accordance with the manufacturer's protocol (81). Subsequently, 1 x 10^6 cells/well were recultured either in the presence of the initial stimuli ("reactivated") or in RPMI-complete medium ("unreactivated") for 5 h at 37°C, 5% CO_2. Following reactivation, apoptosis was assessed via flow cytometry (see below).

Staining and flow cytometric analysis for apoptotic cells

Harvested T cells (1 x 10^6 cells) were washed with cold 1X PBS and stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V (PharMingen) and propidium iodide (PharMingen) for 15 min according to PharMingen's Annexin V Kit I. Stained cells were subsequently analyzed for apoptosis via flow cytometry (FACSCalibur; Becton-Dickinson, Bedford, MA). Controls consisted of single staining for Annexin V-FITC only and PI only. T cell population purity was assessed by staining 5 x 10^5 cells with 5 μg/ml of a FITC-conjugated anti-mouse CD3ε monoclonal antibody (PharMingen) following 5 μg/ml anti-mouse CD16/CD32 monoclonal antibody (Fc Block; PharMingen) treatment.

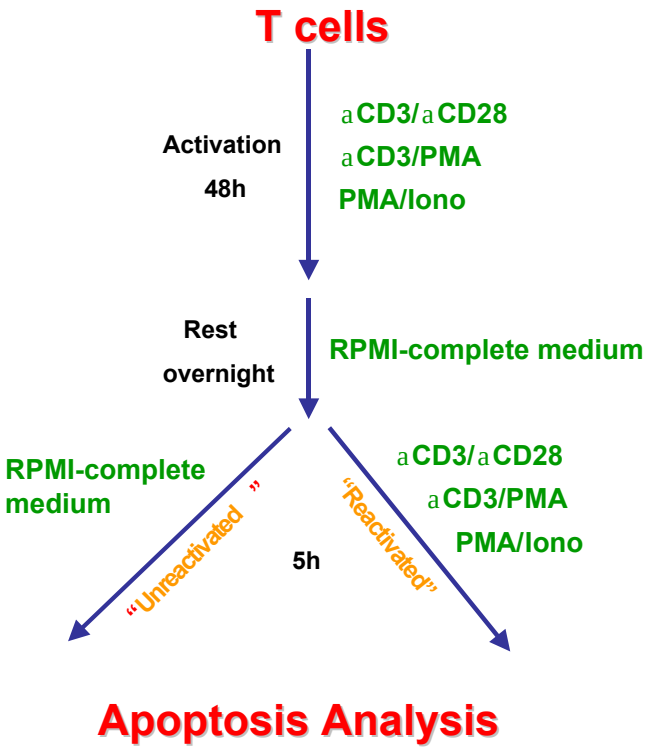

Figure 1. Experimental design for AICD induction. Purified splenic T cells from mice fed the test diets were activated as follows: αCD3/αCD28, PMA/αCD3, or PMA/Ionomycin for 48 h. Following an overnight rest period in RPMI-complete medium, T cells were either reactivated with the initial stimuli or cultured in RPMI-complete medium for 5 h. Apoptosis was analyzed with Annexin V/PI as described in the *Materials and Methods*.

Cytokine ELISAs

For cytokine analyses, cells were cultured for 48 h as described in Figure 1. Cell culture supernatants from duplicate wells of T cells for each stimulus were harvested, pooled, and stored at -80°C. After thawing, supernatants were assayed in duplicate for IL-2, IL-4, IL-10, and IFNγ using Mouse Immunoassay (ELISA) Kits (R&D Systems). Results are expressed as pg/3 x 10^6 cells as previously described (74).

T cell membrane phospholipid analysis

T cell lipids were extracted by the method of Folch et al. (135). The individual phospholipid classes were separated by thin layer chromatography (TLC) on silica gel 60 plates using chloroform/ methanol/ acetic acid/ water (50: 37.5: 3.5: 2, v/v) (136). Bands were detected under ultraviolet light after spraying with 0.1% 8-anilino-naphthalene-sulfonic acid. Fractions were scraped from the plates, transesterified, and fatty acid methyl esters were extracted using hexane and 0.1 M potassium chloride. The fatty acid composition of sphingomyelin, phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and cardiolipin were analyzed by gas chromatography as previously described (136).

Assessment of systemic oxidative stress

Livers were removed from mice at the time of necropsy, flash frozen in liquid nitrogen and RNA was isolated using Ambion's Totally RNA Kit (Austin, TX) according to the manufacturer's protocol. The isolated RNA was subsequently treated with DNase Inactivation Reagent (Ambion) to remove contaminating DNA. Reverse transcription was performed with 2 μg RNA in a 50 μl reaction using Superscript II (Gibco BRL, Rockville, MD). Reactions in the absence of reverse transcriptase enzyme served as negative controls. Real time PCR was performed using an ABI 7700 unit (Applied Biosystems, Foster City, CA). Primer pairs for mouse CD36, peroxisome proliferator-activated receptor γ (PPARγ), and scavenger receptor type A (SRA) were designed with Primer Express software, version 1.5. The sequences of the primers were

as follows: CD36, forward, 5'-CAA GCT CCT TGG CAT GGT AGA-3', and reverse, 5'-TGG ATT TGC AAG CAC AAT ATG AA-3'; PPARγ, forward, 5'-GAT GAA TAA AGA TGG AGT CCT CAT CTC-3', and reverse, 5'-CCG CAG GCT TTT GAG GAA-3'; and SRA, forward, 5'-CAG GAA TAA GAG GTA TTC CAG GTG TTA-3', and reverse, 5'-TCC TGG TGC TCC TGG GTT T-3'. Primer sequences were checked for sequence homology against known genes using BLAST (http://www.ncbi.nlm.nih.gov/blast/). Each PCR reaction consisted of 25 µl 2X SYBR Green master mix (Applied Biosystems), 300 nM final concentration forward and reverse primers, and 2 µl reverse transcription reaction in a 50 µl final reaction volume. To assess assay reproducibility, select RT reactions were performed in triplicate followed by PCR. Values for cycle threshold (C_T), the point at which exponential amplification of the PCR products is detected, were obtained from the Applied Biosystems software.

Fluorescence microscopy

Morphological assessment of apoptosis was performed by 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) staining on a TE 300 Nikon Eclipse microscope equipped with a Princeton Instruments MicroMax 5 MHz cooled digital CCD camera. Images were processed using Metamorph software, version 4.6r3 (Universal Imaging Corp., Downing, PA).

Statistical analysis

A mixed model was used to assess whether the dietary effects differed among the various stimuli. This mixed model is essentially a 2-way ANOVA with factors diet and stimulus, but accounts for the correlation among multiple measurements coming from the T cells taken from a single mouse. The procedure MIXED in SAS software was used for the analysis (SAS, Cary NC). When diet effects were significantly different among stimuli, comparisons between the diet groups were conducted using Fisher's protected Least Significant Difference (LSD) test. In that case, two diets were deemed statistically different if their p-value was less than α=0.05. Values presented in the text are means ± SEM.

RESULTS

n-3 PUFA differentially affect T cell apoptosis depending on stimulus

Since previous studies have indicated a suppressive effect of n-3 PUFA on T cell proliferation (74), we determined the effect of n-3 PUFA on T cell apoptosis. In order to validate our system, we stimulated T cells from chow-fed mice with an apoptotic positive control. **Figure 2** shows the 4 h (**A**) and 24 h (**B**) early and late apoptotic T cell response following stimulation with rhsFasL fused to a FLAG-tag plus an αFLAG antibody that crosslinks the FasL-FLAG (FasL + αFLAG) or FasL + αFLAG plus Z-VAD-fmk (a pan-caspase inhibitor). As expected, FasL + αFLAG increased late apoptosis by 34% and 40% compared to RPMI levels for 4 h and 24 h, respectively (**Fig. 2**).

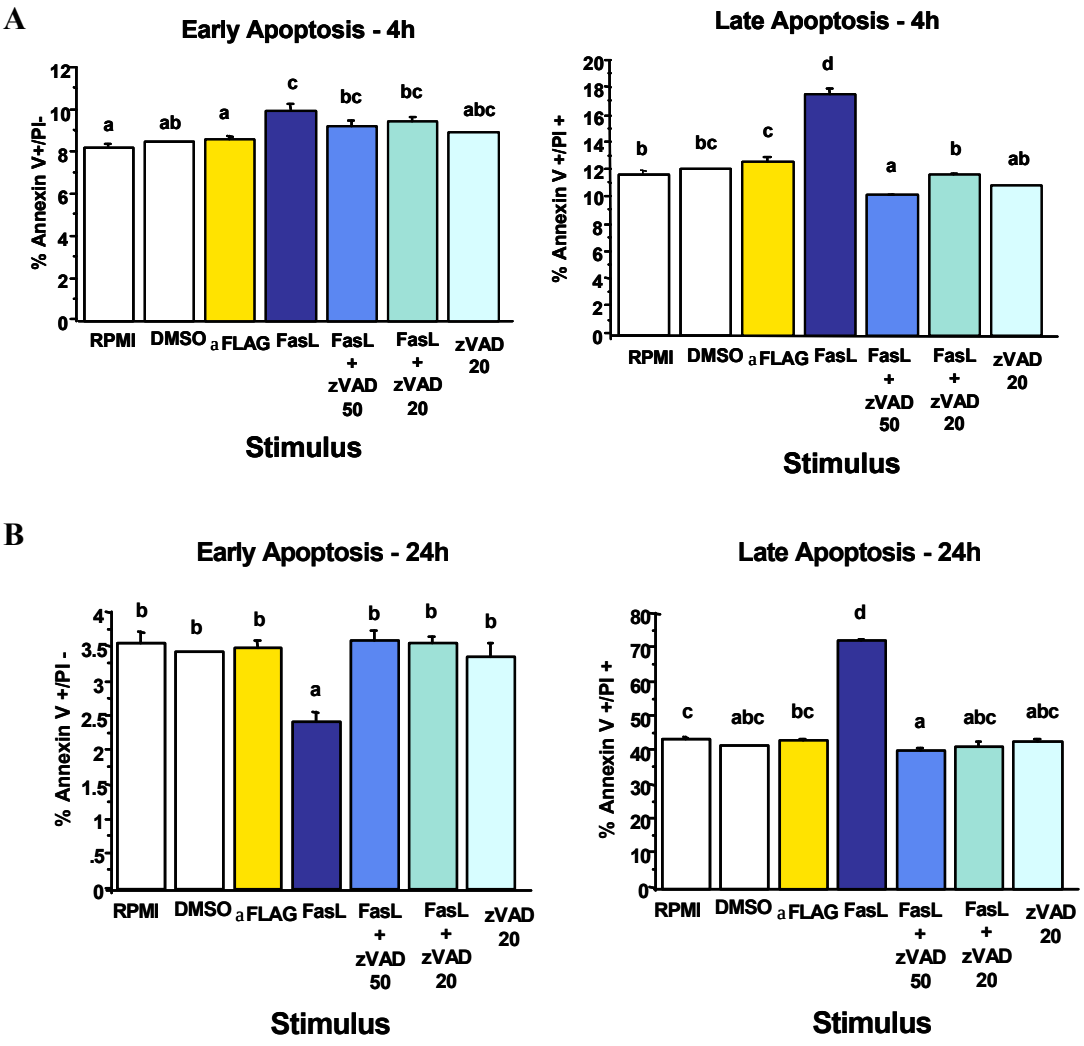

Figure 2. Validation of apoptosis model. Purified splenic T cells from chow-fed mice were cultured for 4 h (**A**) and 24 h (**B**) in the presence of RPMI-complete medium, 1% DMSO, an αFLAG antibody, FasL + αFLAG, FasL + αFLAG + 50 μM Z-VAD-fmk, FasL + αFLAG + 20 μM Z-VAD-fmk, or 20 μM Z-VAD-fmk followed by Annexin V/PI staining and FACS analysis. Data shows cells classified as either early apoptotic or late apoptotic and represent the mean ± SEM, n=3 per group. Different letters denote significant differences between stimuli ($p<0.05$).

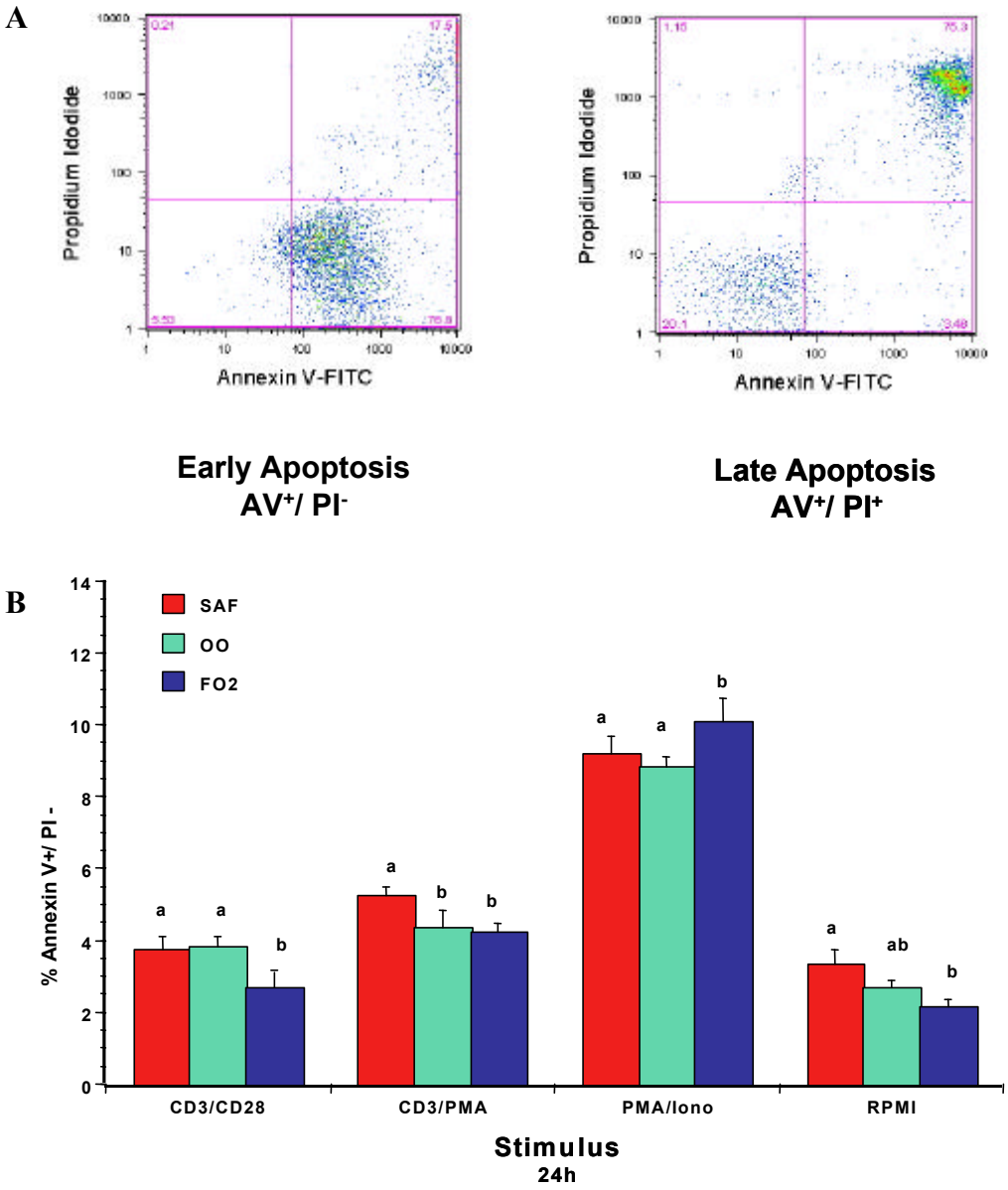

Figure 3. n-3 PUFA differentially affect T cell apoptosis depending on stimulus. **A**, Representative 2 parameter flow cytometric dot plots of early and late stage apoptosis. Cells were distinguished as being early apoptotic or late apoptotic by Annexin V positive, PI negative (left panel) or Annexin V positive/PI positive (right panel) staining, respectively. **B**, T cells (5 x 10^5/well) from animals fed the test diets were cultured in the presence of αCD3/αCD28, PMA/αCD3, PMA/Ionomycin, or RPMI-complete medium for 24 h, followed by Annexin V/PI staining and FACS analysis.

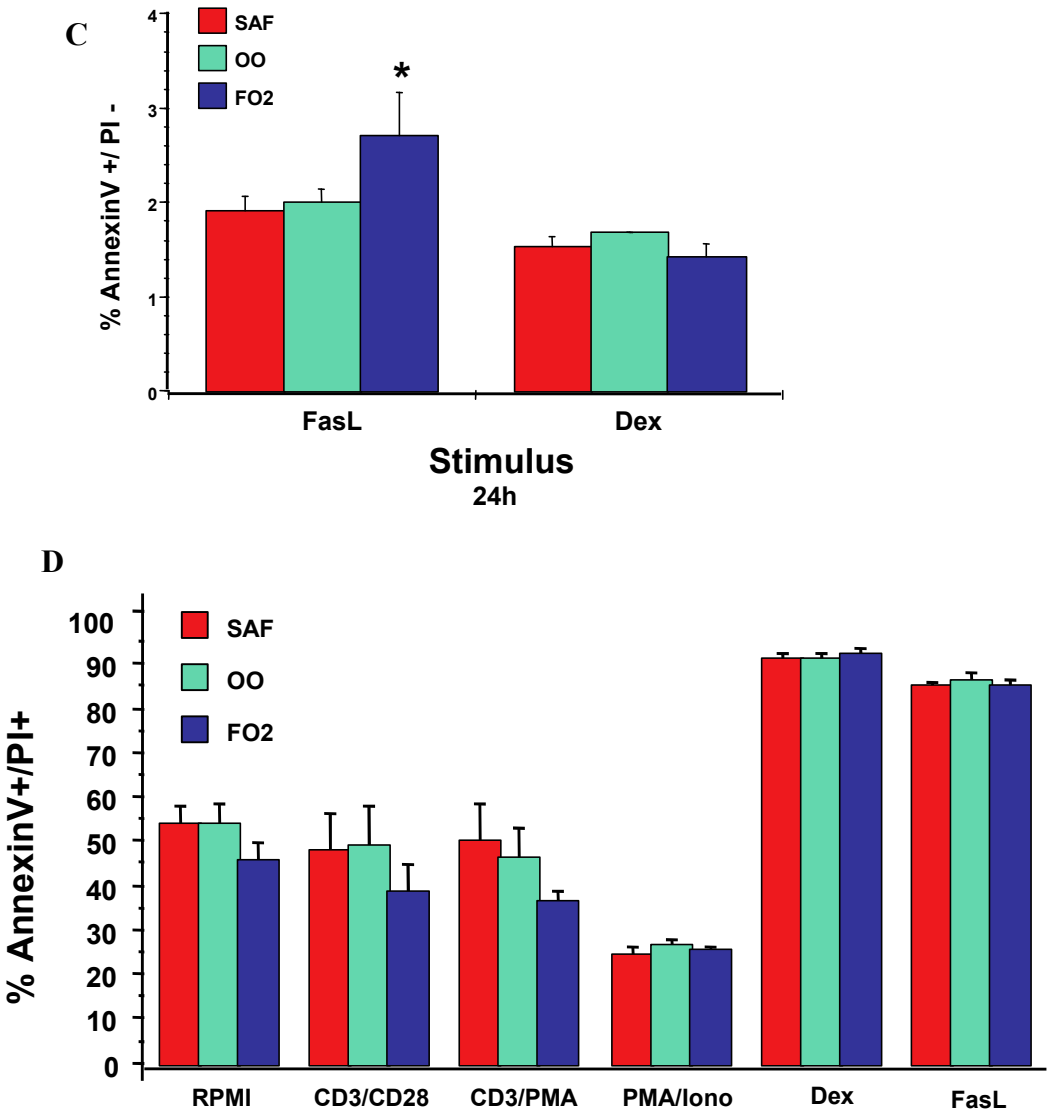

Figure 3. Continued.
C, T cells (5 x 10^5/well) from animals fed the test diets were cultured in the presence of FasL + αFLAG or dexamethasone for 24 h, followed by Annexin V/PI staining and FACS analysis. Data shows cells classified as early apoptotic (**B & C**) or late apoptotic (**D**) and represent the mean ± SEM, n=6 per group. Different letters denote significant differences within each stimulus group ($p<0.05$). SAF, safflower oil; OO, olive oil; FO2, fish oil (2%, w/w).

Because signaling downstream from Fas is mediated by caspases, Z-VAD-fmk is expected to inhibit apoptosis. **Figure 2A** shows that when Z-VAD-fmk was added to FasL + αFLAG apoptosis was decreased by as much as 45%, reducing apoptosis to RPMI levels. Culture in 1% DMSO (the vehicle for Z-VAD-fmk and at the level of the highest concentration of Z-VAD-fmk) did not affect T cell apoptosis.

Purified splenic T cells from n-3 PUFA-fed mice were stimulated with various agonists which act at the plasma membrane receptor level (αCD3/αCD28), intracellular level (PMA/Ionomycin), or at both the receptor and intracellular levels (αCD3/PMA). **Figure 3A** shows a representative 2 parameter flow cytometric histogram of early and late stage apoptosis following a 24 h stimulation period. Cells were classified as early stage apoptotic (Annexin V positive, PI negative) or late stage apoptotic (Annexin V positive, PI positive). **Figure 3B** shows the early apoptotic response of 24 h single activated T cells taken from mice fed SAF, OO, or FO2 (containing n-3 PUFA). Since the diet effects were different depending on stimulus group ($p = 0.001$), differences among the diets were investigated separately for the individual stimuli. FO2 increased the percentage of apoptosis relative to SAF ($p = 0.04$) and OO ($p = 0.005$) only in PMA/Ionomycin stimulated cells. In contrast, FO2 decreased apoptosis in the αCD3/αCD28 [FO2 vs SAF and OO ($p = 0.01$)], αCD3/PMA [FO2 vs SAF ($p = 0.02$)],

and RPMI-complete medium (negative control) [FO2 vs SAF ($p = 0.007$)] stimulated groups. These data indicate that the effect of n-3 PUFA is dependent on the nature of the stimulus. **Figure 3C** shows the early apoptotic response of T cells from SAF, OO, or FO2-fed mice activated for 24 h with known apoptotic inducers. T cells were incubated with dexamethasone or FasL + αFLAG for 24 h in order to induce apoptosis either pharmacologically or physiologically, respectively. Of interest, FO2 produced a modest increase in apoptosis ($p = 0.068$) in FasL + αFLAG stimulated cells, suggesting that n-3 PUFA may modulate Fas signaling pathways. The late apoptotic T cell response is shown in **Figure 3D**. Both sets of stimuli promoted substantial levels of apoptosis when late apoptosis was examined (dexamethasone, 93.1 ± 0.6% and FasL + αFLAG, 87.9 ± 0.6%) indicating that the reagents were effective (**Fig. 3D**).

Figure 4 illustrates the use of DAPI staining to assess morphological evidence of apoptosis. In some experiments, T cells were stimulated for 24 h followed by DAPI staining and examined for the presence of apoptotic bodies. In general, the number of apoptotic bodies present in the cultures was proportional to the levels of apoptosis as determined by Annexin V/PI staining.

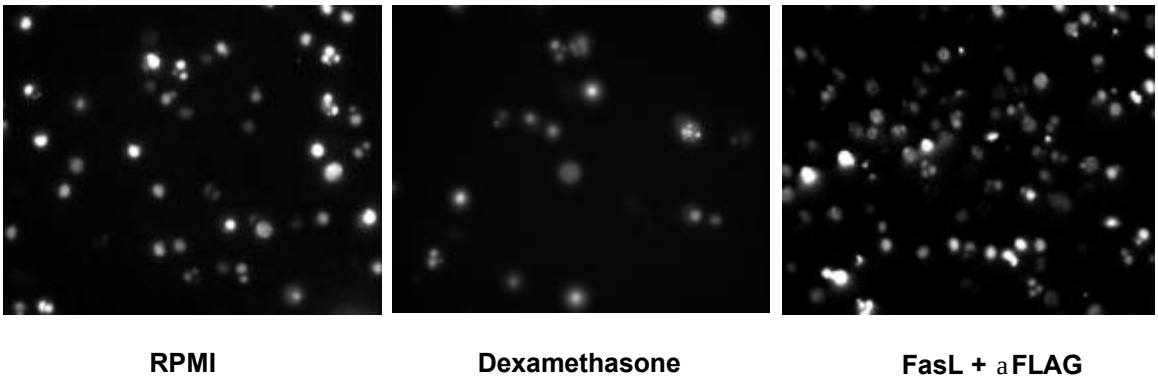

Figure 4. Morphological assessment of apoptosis. Purified T cells (2 x 10^6/ml) from chow-fed mice were cultured in the presence of RPMI-complete medium, FasL/αFLAG, or dexamethasone for 24 h followed by DAPI staining and subsequent examination of apoptotic bodies. Magnification, 600x.

Differential effect of cell stimulus on T cell cytokine profiles

Because the nature of the stimulus can result in T cell bias toward specific cytokine secretion profiles (83,84), we analyzed cell culture supernatant cytokines. **Figure 5** shows the ability of selected stimuli to induce naive T cells to produce different cytokine profiles. The data show that T cells in all diet groups stimulated with PMA/Ionomycin for 48 h secreted significantly ($p = 0.0001$) more IL-2 than αCD3/αCD28 or αCD3/PMA stimulated T cells. Stimulation with PMA/Ionomycin resulted in cytokine ratios as follows: IFNγ:IL-4=2560; IFNγ:IL-2=4.5; and IL-4:IL-2=0.002. Since Th1 cells secrete high levels of IL-2 and IFNγ, PMA/Ionomycin appears to have generated a Th1-like phenotype (66). In comparison, αCD3/PMA stimulated T cells secreted significantly ($p = 0.0001$) more IL-10 and IL-4 compared to αCD3/αCD28 or PMA/Ionomycin (**Fig. 5**), and produced cytokine ratios of: IFNγ:IL-4=167.1; IFNγ:IL-2=383.6; and IL-4:IL-2=2.3. IL-4 and IL-10 are secreted by Th2 polarized cells and are also cross-regulatory cytokines which inhibit Th1 polarization (66). Therefore, these data indicate that αCD3/PMA induced T cells to express a Th2-like cytokine profile. Stimulation with αCD3/αCD28 produced the following cytokine ratios: IFNγ:IL-4=278.6; IFNγ:IL-2=72.1; and IL-4:IL-2=0.26.

Figure 6 shows the effect of diet on cytokine secretion. The data show that for the two Th2 cytokines, IL-4 and IL-10, dietary FO enhanced secretion only in the αCD3/PMA stimulated T cells (Th2-like). In contrast, whereas there was no diet effect on IFNγ secretion, dietary FO enhanced IL-2 secretion only in PMA/Ionomycin

stimulated T cells (Th1-like). These data suggest that FO promotes T cells to differentiate into either Th1 or Th2 cells when induced to do so.

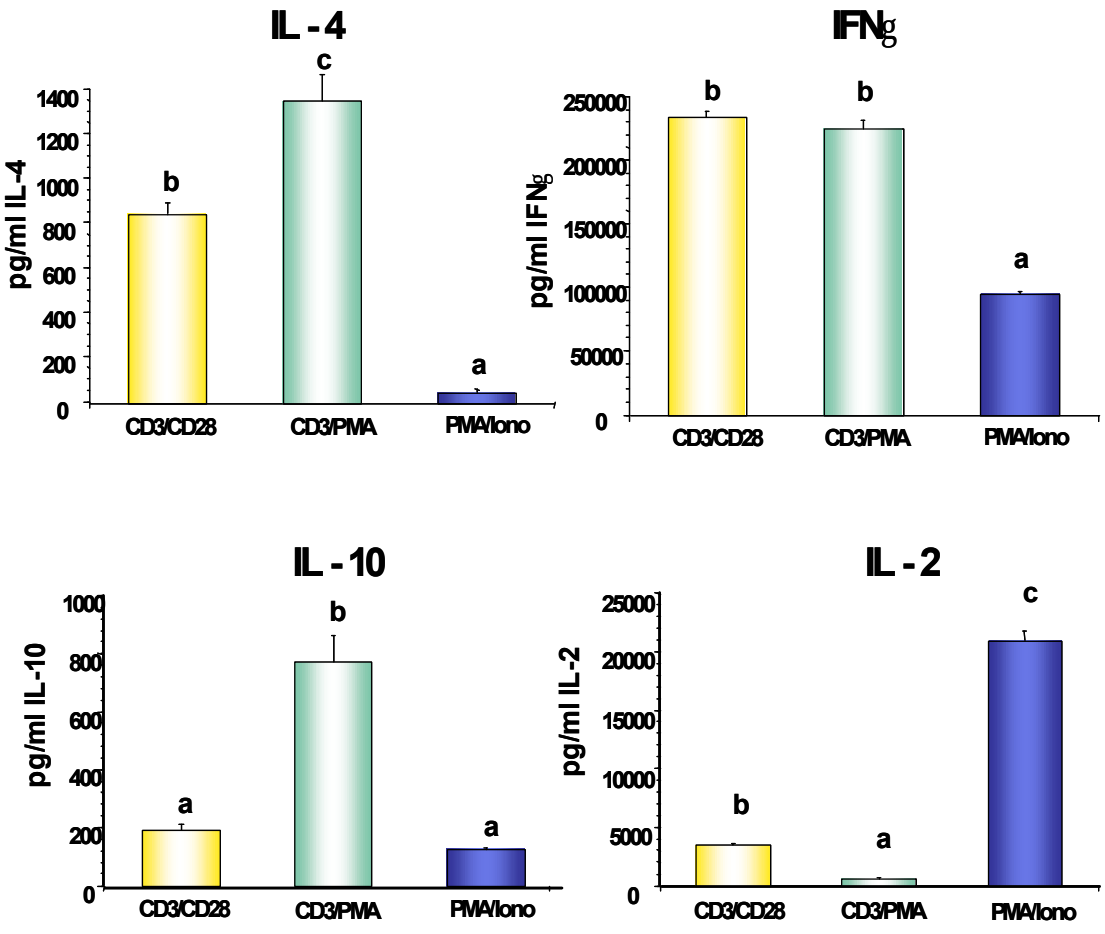

Figure 5. Cytokine profiles of T cells from diet-fed mice incubated with selected stimuli. Following 48 h of stimulation with αCD3/αCD28, PMA/αCD3, or PMA/Ionomycin, supernatants were collected and IL-2, IL-4, IL-10 and IFNγ were quantified as described in *Materials and Methods*. Data represent the mean ± SEM, n=18 per group. Different letters denote significant differences between stimulus groups ($p < 0.05$).

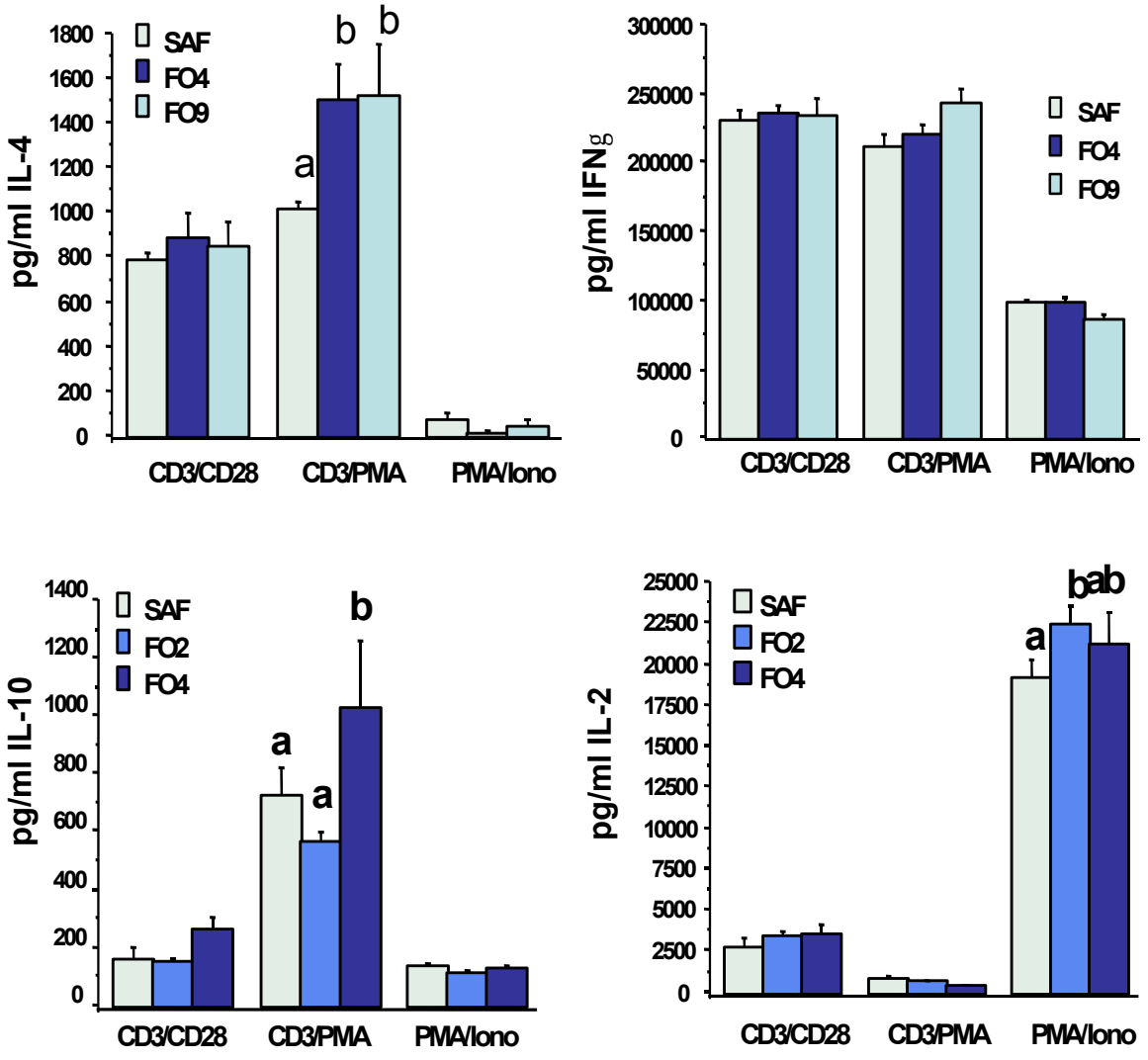

Figure 6. n-3 PUFA enhancement of cytokine secretion is dependent upon the stimulus. Purified splenic T cells from mice fed diets containing SAF, FO2 (2%, w/v), FO4 (4%, w/v), or FO9 (9%, w/v) were stimulated for 48 h with αCD3/αCD28, αCD3/PMA, or PMA/Ionomycin. Supernatants were collected and IL-2, IL-4, IL-10, and IFNγ were quantified as described in *Materials and Methods*. Data represent the mean ± SEM, n=6 per diet (for IL-2 and IL-10) or n=3 (for IL-4 and IFNγ). Different letters denote significant differences between diet groups ($p<0.05$).

Dietary lipids alter T cell membrane phospholipid composition

Dietary lipids can be incorporated into cellular membranes and thereby regulate cell function (137). For this reason, we determined the effect of dietary lipid on the fatty acid composition of phospholipid classes in T cell membranes. **Tables 2-5** show the PC, PE, PS, and PI fatty acid compositions of purified T cell membranes from SAF, OO, and FO2-fed mice. Gas chromatographic analysis revealed fatty acid differences in the PC and PE pools only. In the PC class (**Table 2**), FO-fed mice had significantly less 18:1n-7 and 20:4n-6 and significantly more 18:2n-6. 18:1n-7 was significantly enriched in the PC class from OO-fed mice. Docosahexanoic acid (22:6n-3), one of the major fatty acids found in fish oil, and eicosapentanoic acid (22:5n-3) were significantly enriched ($p < 0.05$) in the PE class from FO2-fed mice (**Table 3**). No significant dietary influence was observed on the fatty acid composition of PS (**Table 4**) and PI (**Table 5**) pools. These data indicate a remodeling of T cell membrane composition by dietary n-3 PUFA.

n-3 PUFA significantly enhance AICD

Since dietary FO may enhance Fas-mediated apoptosis (see above), we determined the effect of n-3 PUFA on T cell AICD, a process largely regulated by the expression of Fas and FasL (96,120,121). T cells were initially activated and subsequently restimulated or incubated in RPMI-complete medium as described in **Figure 1**. The experimental design was effective at inducing AICD. In αCD3/αCD28-stimulated cells, there was a highly statistically significant 2.5-fold increase in total apoptosis (early and late stage apoptosis values of all diet groups) following reactivation

Table 2. Fatty Acid Composition of T Cell Phosphatidylcholine (%mol)

Fatty Acid	SAF	OO	FO2
14:0	3.68 ± 0.16	3.12 ± 0.77	3.08 ± 0.83
14:1	4.08 ± 0.60	2.33 ± 0.67	1.88 ± 1.88
16:0	34.82 ± 1.38	34.95 ± 1.27	40.65 ± 3.44
16:1n-7	2.39 ± 0.21	2.50 ± 0.40	2.38 ± 0.90
18:0	11.33 ± 0.52	10.24 ± 0.64	13.26 ± 3.92
18:1n-9	11.11 ± 0.79	12.03 ± 0.13	9.42 ± 0.53
18:1n-7	1.45 ± 1.45 [b]	5.55 ± 0.29 [a]	tr[b]
18:2n-6	5.37 ± 0.21 [b]	4.13 ± 0.44 [b]	7.66 ± 1.29 [a]
18:3n-6	0.82 ± 0.69	0.96 ± 0.96	1.11 ± 0.15
18:3n-3	0.16 ± 0.16	0.23 ± 0.23	4.71 ± 4.71
20:1n-9	tr	tr	tr
20:2n-6	tr	tr	0.42 ± 0.42
20:3n-6	2.01 ± 0.23	1.83 ± 0.26	1.74 ± 1.21
20:4n-6	20.88 ± 1.24 [a]	19.58 ± 0.24 [a]	9.35 ± 5.72 [b]
20:5n-3	tr	0.07 ± 0.07	0.44 ± 0.44
22:0	tr	tr	tr
22:1n-9	tr	tr	tr
22:4n-6	1.53 ± 0 .80	1.09 ± 0.55	tr
22:5n-3	tr	tr	1.70 ± 1.09
22:6n-3	0.37 ± 0.37	1.36 ± 0.16	2.21 ± 1.37

Only the major fatty acids are listed. Mol % values are expressed as means ± SEM, n=6 per group. Different letters denote significant differences between diet groups ($p<0.05$).
tr, trace (<0.01%); SAF, safflower oil; OO, olive oil; FO, fish oil.

Table 3. Fatty Acid Composition of T Cell Phosphatidylethanolamine (%mol)

Fatty Acid	SAF	OO	FO2
14:0	tr	tr	tr
14:1	tr	tr	tr
16:0	4.38 ± 3.41	2.87 ± 2.87	2.71 ± 2.71
16:1n-7	1.54 ± 0.78	0.76 ± 0.76	0.56 ± 0.56
18:0	24.53 ± 1.54	21.95 ± 1.01	25.42 ± 1.80
18:1n-9	7.45 ± 0.72	6.90 ± 0.45	5.94 ± 0.40
18:1n-7	1.27 ± 0.65	2.56 ± 0.30	1.56 ± 0.18
18:2n-6	3.45 ± 0.95	2.12 ± 0.25	2.87 ± 0.11
18:3n-6	0.49 ± 0.49	tr	tr
18:3n-3	0.30 ± 0.30	tr	tr
20:1n-9	tr	tr	tr
20:2n-6	0.57 ± 0.57	tr	tr
20:3n-6	0.25 ± 0.25	0.96 ± 0.60	1.78 ± 0.22
20:4n-6	23.25 ± 1.53	24.02 ± 2.50	20.57 ± 1.82
20:5n-3	tr	tr	tr
22:0	tr	tr	tr
22:1n-9	tr	1.95 ± 1.95	4.36 ± 1.25
22:4n-6	1.34 ± 1.34	3.38 ± 0.33	tr
22:5n-3	tr	tr	4.70 ± 0.27 [a]
22:6n-3	2.05 ± 1.13 [c]	5.40 ± 0.75 [b]	11.65 ± 0.19 [a]

Refer to *Table 2* for legend details.

Table 4. Fatty Acid Composition of T cell Phosphatidylserine (%mol)

Fatty Acid	SAF	OO	FO2
14:0	3.23 ± 0.13 [ab]	0.01 ± 0.01 [a]	8.86 ± 3.81 [b]
14:1	23.35 ± 5.05 [b]	tr[a]	4.06 ± 3.10 [a]
16:0	tr	tr	17.46 ± 10.65
16:1n-7	2.65 ± 2.65	tr	4.43 ± 3.14
18:0	39.37 ± 1.04	49.67 ± 6.20	24.40 ± 7.60
18:1n-9	9.91 ± 3.40	14.34 ± 2.97	13.50 ± 0.84
18:1n-7	1.63 ± 1.63	0.59 ± 0.59	1.65 ± 1.65
18:2n-6	8.19 ± 4.64	6.31 ± 2.37	9.37 ± 1.60
18:3n-6	1.58 ± 1.58	tr	0.03 ± 0.03
18:3n-3	tr	tr	0.15 ± 0.15
20:1n-9	tr	tr	0.37 ± 0.37
20:2n-6	tr	tr	tr
20:3n-6	tr	2.77 ± 1.82	0.77 ± 0.77
20:4n-6	10.61 ± 5.56	11.88 ± 0.19	9.89 ± 1.46
20:5n-3	tr	tr	3.26 ± 3.02
22:0	tr	tr	tr
22:1n-9	tr	tr	tr
22:4n-6	tr	4.28 ± 2.78	0.11 ± 0.11
22:5n-3	tr	tr	0.57 ± 0.57
22:6n-3	tr	4.28 ± 2.29	0.86 ± 0.86

Refer to *Table 2* for legend details.

Table 5. Fatty Acid Composition of T cell Phosphatidylinositol (%mol)

Fatty Acid	SAF	OO	FO2
14:0	tr	tr	tr
14:1	tr	tr	tr
16:0	tr	tr	tr
16:1n-7	tr	tr	tr
18:0	19.50 ± 2.73	26.63 ± 4.69	16.20 ± 0.23
18:1n-9	15.35 ± 4.50	13.00 ± 2.94	14.59 ± 1.18
18:1n-7	3.57 ± 3.57	3.90 ± 3.90	tr
18:2n-6	6.04 ± 4.72	5.83 ± 1.66	3.76 ± 3.76
18:3n-6	6.35 ± 6.35	tr	4.42 ± 4.42
18:3n-3	1.38 ± 1.38	tr	tr
20:1n-9	10.00 ± 10.00	7.56 ± 7.56	7.48 ± 1.44
20:2n-6	tr	tr	tr
20:3n-6	tr	tr	tr
20:4n-6	36.91 ± 10.00	38.03 ± 16.42	33.57 ± 3.72
20:5n-3	0.90 ± 0.90	tr	tr
22:0	tr	tr	tr
22:1n-9	tr	tr	tr
22:4n-6	tr	tr	tr
22:5n-3	tr	tr	tr
22:6n-3	tr	tr	tr

Refer to *Table 2* for legend details.

[reactivated, 37.4 ± 0.9% vs. unreactivated, 20.9 ± 0.4%, p=0.0001, n=48]. In PMA/Ionomycin-stimulated cells, there was a nearly 2-fold increase in total apoptosis, which was highly statistically significant [reactivated, 12.5 ± 0.8% vs. unreactivated, 7.5 ± 0.2%, p=0.0001, n=48].

Figure 7 shows further validation of our AICD model. T cells were stimulated with PMA/Ionomycin for the initial 48 h and restimulated with PMA/Ionomycin plus soluble Fas-Fc, PMA/Ionomycin plus the pan-caspase inhibitor Z-VAD-fmk, or

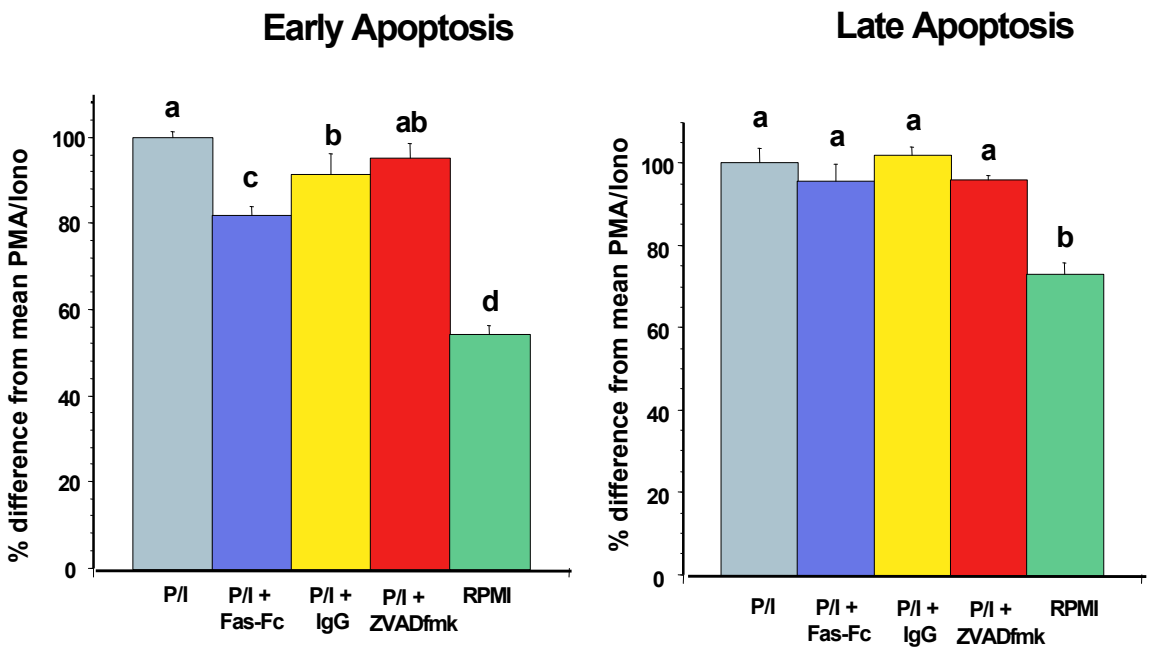

Figure 7. Validation of AICD model. Purified splenic T cells from chow-fed mice were initially activated with PMA/Ionomycin and then restimulated, as described in *Figure 1*, with PMA/Ionomycin, PMA/Ionomycin + soluble Fas-Fc, PMA/Ionomycin + IgG (isotype control for Fas-Fc), PMA/Ionomycin + Z-VAD-fmk, or RPMI-complete medium. Data are expressed as the percent difference from the mean PMA/Ionomycin value for either early or late apoptosis and represent the mean ± SEM, n=4-7 per group. Different letters denote significant differences between diet groups ($p<0.05$).

incubated with RPMI-complete medium. Cells left unreactivated underwent nearly 50% less early apoptosis than PMA/Ionomycin restimulated T cells. Fas-Fc decreased early apoptosis by 18%. Unexpectedly, Z-VAD-fmk only decreased apoptosis 4.7%. This could be explained by the fact that this reagent was most-likely not added early enough to cultures to inhibit substantial caspase activation and thus suppress apoptosis.

Figure 8A shows the effect of stimulus among all diet groups on AICD. Each stimulus was significantly different from the other ($p < 0.05$) in early stage apoptosis following reactivation, with αCD3/PMA stimulating the greatest degree of apoptosis ($42.9 \pm 0.5\%$), and PMA/Ionomycin stimulating the least ($8.2 \pm 0.3\%$). **Figures 8B-E** illustrate the effect of each diet on early (**B-D**) and late (**E**) apoptosis within particular stimulus groups, αCD3/αCD28, αCD3/PMA, and PMA/Ionomycin-stimulated, respectively. In PMA/Ionomycin-stimulated (Th1-like) T cells, dietary FO, independent of dose, was accompanied by a significantly ($p=0.0008$) greater percentage of early stage apoptosis than was observed in T cells from control mice fed SAF (**Fig. 8D**). In comparison, no dietary effect on early apoptosis was seen when T cells were restimulated with either αCD3/αCD28 or αCD3/PMA (**Fig. 8B & 8C**).

Previously, we have shown that DHA is the active component in FO for reducing T cell proliferation (81). Therefore, we determined the effect of DHA on T cell AICD. **Figure 9** shows the effect of DHA and CO on T cell AICD. There were no dietary differences among all stimulus pairs in early (**Fig. 9**) and total apoptosis (data not shown). These results suggest that DHA is not the active molecule in FO with respect to AICD. It remains to be determined what effect EPA has on T cell AICD.

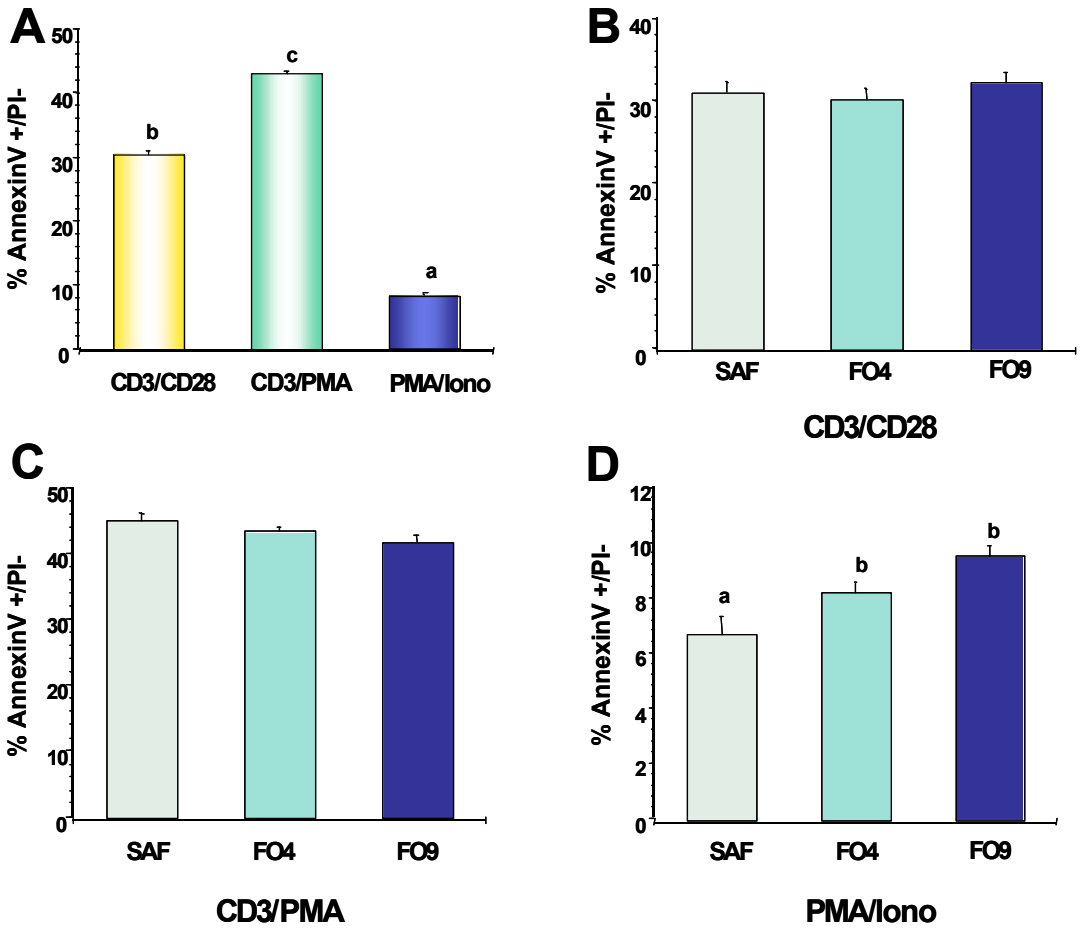

Figure 8. n-3 PUFA significantly enhance AICD in PMA/Ionomycin-stimulated T cells. A-D, Splenic T cells from SAF, FO4 (4%, w/w), and FO9 (9%, w/w)-fed mice were initially activated and restimulated as described in *Figure 1*. **A**, The effect of stimulus among all diet groups on AICD. Data represent the mean ± SEM, n=48 per stimulus. Different letters denote significant differences between stimulus groups ($p<0.05$). **B-D** show the effect of diet on αCD3/αCD28-stimulated, αCD3/PMA-stimulated, and PMA/Ionomycin-stimulated T cell early apoptosis, respectively.

E

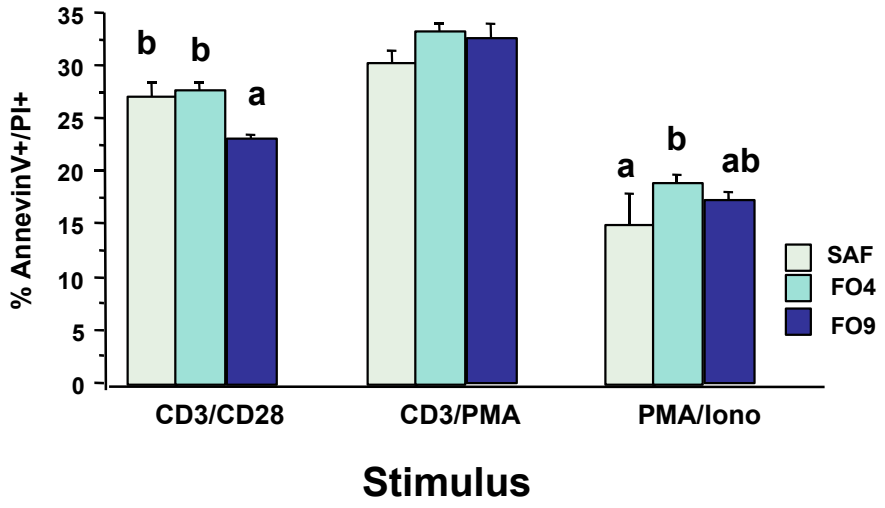

Figure 8. Continued.
E, The late apoptotic response of αCD3/αCD28-stimulated, αCD3/PMA-stimulated, and PMA/Ionomycin-stimulated T cells. Data represent the mean ± SEM, n=12 per diet group. Different letters denote significant differences between diet groups (p=0.0008).

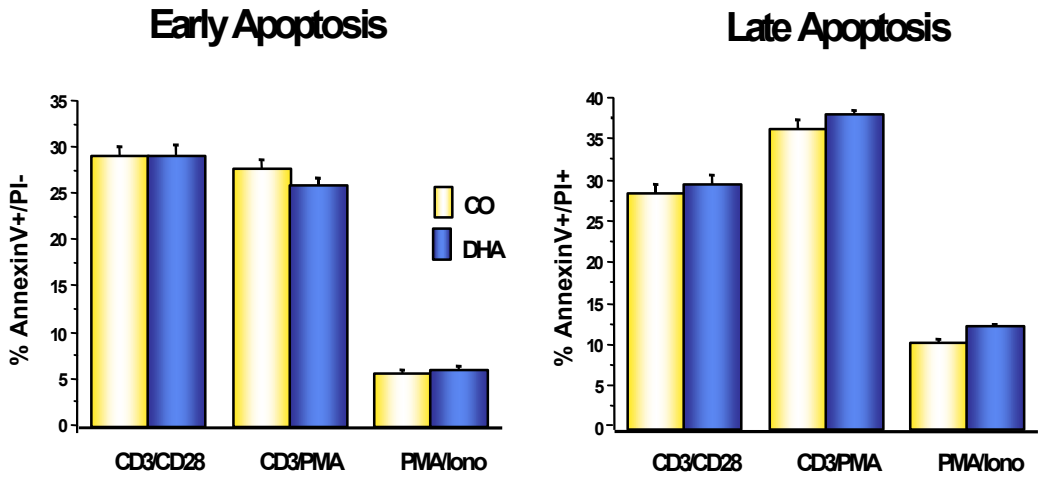

Figure 9. DHA does not affect T cell AICD. Splenic T cells from CO (5%, w/v) and DHA ethyl ester (1%, w/v)-fed mice were initially activated and restimulated to induce AICD as described in *Figure 1*. Data represent the mean ± SEM, n=6 per diet group.

n-3 PUFA do not induce systemic oxidative stress

In order to determine whether the ingestion of different lipid sources altered oxidative stress, three known markers of systemic oxidative stress were evaluated: PPARγ, SRA, and CD36 (138). **Table 6** shows the mRNA expression levels from the liver for PPARγ, SRA, and CD36. There were no statistically significant differences between mice fed either the SAF, OO, or FO2 diets with respect to the levels of liver mRNA for PPARγ, SRA, or CD36 (**Table 6**). These results indicate that short-term feeding of n-3 PUFA does not elevate biomarkers of systemic lipid peroxidation in our model.

Table 6: mRNA Expression of PPARg, CD36, and SRA

	SAF	OO	FO2
PPARg	26.40 ± 0.35	26.63 ± 0.33	27.14 ± 0.32
CD36	24.18 ± 0.50	24.09 ± 0.34	24.78 ± 0.35
SRA	25.75 ± 0.47	25.51 ± 0.28	26.34 ± 0.34

Liver RNA was isolated and expression of PPARγ, CD36, and SRA mRNA was quantified by real time RT-PCR. Values represent cycle threshold (C_T) as described in *Materials and Methods* and are expressed as means ± SEM, n = 6 per group. SRA, scavenger receptor type A; Diet groups: SAF, safflower oil; OO, olive oil; FO2, fish oil (2%).

DISCUSSION

Among dietary factors, there is considerable evidence for a protective effect of n-3 PUFA in autoimmune/inflammatory diseases (34,35,70,127). Additionally, the significant effects of diets rich in n-3 PUFA on a variety of T cell functions have been firmly established in both humans and animal models (34,35,70,127). The primary dietary effector molecules are thought to be eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) (74,81). However, the precise effects of dietary n-3 PUFA on $CD4^+$ cells, and the mechanisms by which dietary PUFA influence the maintenance of appropriate T cell subset balance to promote a healthy immune system, have not been elucidated. Previous experiments have demonstrated a blunting effect of n-3 PUFA on T cell proliferation (74,83). Since the suppressive effects of dietary n-3 PUFA on the accumulation of inflammatory T cells could result from either reduced proliferation or enhanced apoptosis of activated T cells, or both, we investigated the effect of dietary n-3 PUFA on T cell apoptosis. Our data clearly show that dietary n-3 PUFA enhanced T cell apoptosis following *in vitro* incubation with selective stimuli (**Fig. 3B**). With regard to the biological relevance of this effect, it is now clear that small changes in apoptosis can profoundly alter downstream events such as cancer and autoimmune disease risk (139). For example, when multipotential-stage (undifferentiated) cells fail to undergo AICD, exponential growth in cell numbers may occur, perturbing the balance between responsiveness and nonresponsiveness (tolerance) (126,140). n-3 PUFA effects on these mechanisms are consistent with a wealth of literature supporting the contention that diet in general, and dietary fat in particular, is an

important determinant of the quantity and quality of the host's immune responses (34,35,127,132).

Apoptotic death of T cells is an important mechanism for regulating immune responses. There are two distinct pathways of apoptosis (a) *passive cell death*, mediated in part by the loss of Bcl-2 proteins, and (b) *AICD*, primarily regulated by *extrinsic* signals from death receptors, such as Fas (96,120-123). Passive cell death results from the absence of growth factors and is important for loss of immature T cells. AICD occurs as a result of repeated stimulation through the TCR, upregulating plasma membrane expression of Fas and its ligand (FasL), thereby acting as a feedback mechanism for terminating an ongoing immune response. It has been shown that T cell subsets differ in their propensity to undergo AICD (112,114,117). Interestingly, in our experimental system, the selected stimuli influenced the profile of cytokines produced by the cells in culture (**Fig. 5**). T cells stimulated with αCD3/PMA secreted significantly more IL-4 and IL-10, Th2 cytokines, while PMA/Ionomycin-stimulated T cells secreted much more IFNγ and much less IL-4, indicative of a Th1-like phenotype. Because these cells had not been subjected to the selective effects of long-term culture, they likely represent physiologically polarized T cell effector precursors which arise in primary immune responses (112). These data suggest a means by which T cell functional subclasses could be individually analyzed following dietary treatment.

When naive T cells were stimulated with PMA/Ionomycin there was an increase in AICD in cells from mice fed both of the n-3 PUFA-containing diets (FO4 and FO9) (**Fig. 8D**). Therefore it is conceivable that the ability of n-3 PUFA to enhance AICD

following PMA/Ionomycin stimulation could be due to the induction of a biased pattern of cytokine production in the T cells. These data are consistent with our previous observations showing an increase in IL-4 dependent Th2 proliferation following n-3 PUFA feeding (83), and indicate that dietary n-3 PUFA act differently on distinct T cell subsets. These important findings provide evidence for both direct and indirect mechanisms whereby selected T cell subset functions would be suppressed by dietary n-3 PUFA.

The elucidation of the mechanisms which regulate apoptosis is important because the failure of T cells to undergo appropriate AICD is associated with a variety of immunopathological diseases, including inflammatory bowel diseases (IBD) (140). With regard to diet, a critical question remaining to be addressed is the identity of the relevant cellular targets in Th1-like cells which mediate the apoptogenic effects of n-3 PUFA. AICD depends, in part, on a FasL-dependent pathway (96,120,121) and FasL is expressed at higher levels on the surface of activated Th1 cells than Th2 cells (114,115). The fact that n-3 PUFA enhanced Th1-like AICD in our experiments, combined with a report indicating a 30% increase in FasL expression in splenocytes from n-3 PUFA-fed mice (93), support the notion that certain dietary lipids upregulate FasL expression in Th1 cells, rendering them more susceptible to AICD. Additional studies are underway in order to test this hypothesis.

Several intracellular second messengers (e.g., ceramide) have been implicated in the generation of the Fas/FasL death signal and may mediate the n-3 PUFA enhanced AICD in Th1 cells. Ceramide is a lipid second messenger cleaved from membrane

sphingomyelin by sphingomyelinases. Interaction of FasL with membrane-bound Fas results in a transient and weak activation of a limited number of Fas trimers insufficient to trigger apoptosis but sufficient for acidic sphingomyelinase (ASM) translocation (110). Translocated ASM subsequently hydrolyzes sphingomyelin into ceramide which spontaneously self-aggregates into membrane microdomains, thereby inducing Fas receptor clustering and apoptosis (110). Interestingly, we have previously demonstrated (74) that dietary n-3 PUFA are capable of modulating ceramide levels in murine splenic T cells. In addition, our data indicate that n-3 PUFA modify the fatty acid composition of membrane PC and PE (**Tables 2 & 3**) in purified T cells. Since membrane fatty acid content can influence sphingomyelin hydrolysis (141), these data suggest that ceramide formation, and hence AICD, could be modulated via the incorporation of n-3 PUFA into specific membrane phospholipid pools.

Within the T cell plasma membrane, there are specific detergent-resistant domains in which key signal transduction proteins are localized (142). These "lipid rafts" are composed mostly of cholesterol and sphingolipids and do not integrate well into the fluid phospholipid bilayers, thereby forming microdomains. Upon T cell activation, rafts compartmentalize the activated TCR and associated signal-transducing molecules, thus providing an environment conducive to signal transduction (67). With respect to apoptosis, Fas and FasL translocate to lipid rafts after stimulation (110). Our data demonstrating that dietary PUFA remodel the membrane composition of T cells (**Tables 2 & 3**) provide evidence for a direct diet effect on membrane properties. This is supported by recent *in vitro* studies using a Jurkat T cell line, where n-3 PUFA

enrichment selectively modified lipid rafts and suppressed signal transduction (47). Interestingly, conditions that modify raft structure can disrupt T cell signaling events (142). Recent studies indicate that the macromolecular complex organization in lipid rafts is distinct in T cell subsets (44,67), suggesting that these subsets could respond differently to dietary PUFA-induced perturbation. Experiments are currently underway to determine the effects of dietary n-3 PUFA on the lipid composition of T cell rafts in our model.

Long chain n-3 PUFA are highly susceptible to lipid peroxidation (143). This is significant because the formation of reactive oxygen species and/or glutathione depletion can regulate signals involved in AICD which contribute to T cell deletion (144). Interestingly, we saw no change in systemic oxidative stress following ingestion of different lipid sources (**Table 6**). This indicates that short-term feeding of n-3 PUFA did not promote the generation of proapoptotic reactive oxygen species in our model.

Taken together, these data support our hypothesis that dietary n-3 PUFA preferentially suppress functions of a T cell subset induced to secrete a biased cytokine pattern resembling Th1 cells in mice, in part, by increasing AICD in these cells. Furthermore, a comparison of FO4 *versus* FO9 demonstrated that the effect seen on AICD can occur efficiently at low n-3 PUFA intakes. These novel findings contribute significantly to the elucidation of the mechanisms by which dietary n-3 PUFA selectively modulate T cell subset function. Studies such as these could result in the establishment of dietary guidelines designed to promote a balanced immune system, so that protective host responses (e.g., to infectious agents) can be maintained, while

potentially detrimental host responses (e.g. chronic inflammation and hypersensitivity) can be controlled appropriately. Further studies are needed to determine the precise cellular and molecular mechanisms by which dietary n-3 PUFA differentially modulate AICD in Th subsets.

CHAPTER III

DIETARY n-3 POLYUNSATURATED FATTY ACIDS PROMOTE ACTIVATION-INDUCED CELL DEATH IN Th1-POLARIZED MURINE $CD4^+$ T CELLS*

Dietary n-3 polyunsaturated fatty acids (PUFA) have been shown to attenuate T cell-mediated inflammation. To investigate whether dietary n-3 PUFA promote activation-induced cell death (AICD) in $CD4^+$ T cells induced *in vitro* to a polarized Th1 phenotype, C57BL/6 mice were fed diets containing either 5% corn oil (n-6 PUFA control) or 4% fish oil + 1% corn oil (n-3 PUFA) for 2 wk. Splenic $CD4^+$ T cells were cultured with αIL-4, IL-12, and IL-2 for 2 d and then rIL-12 and rIL-2 for 3 d in the presence of diet-matched homologous mouse serum (MS) to prevent loss of cell membrane fatty acids, or fetal bovine serum. Following polarization, Th1 cells were reactivated and analyzed for IFNγ and IL-4 by intracellular cytokine staining, and for apoptosis by Annexin V/PI. Dietary fish oil (FO) enhanced Th1 polarization by 49% ($p=0.0001$) and AICD by 24% ($p=0.0001$) only in cells cultured in the presence of MS. FO enhancement of Th1 polarization and AICD following culture was associated with

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the maintenance of EPA (20:5n-3) and DHA (22:6n-3) in plasma membrane lipid rafts. In conclusion, n-3 PUFA enhance the polarization and deletion of proinflammatory Th1 cells, possibly as a result of alterations in membrane microdomain fatty acid composition.

INTRODUCTION

The differentiation of naive $CD4^+$ T cells into helper (Th) 1 or Th2 effector cells is a critical process during immune responses (8). Th1 cells are characterized by the production of interleukin-2 (IL-2), interferon γ (IFNγ) and tumor necrosis factor β (TNFβ) and are required to mount a cell-mediated immunological response against intracellular pathogens (8). Th1 cells are proinflammatory and have been implicated in the pathogenesis of human inflammatory and autoimmune diseases such as rheumatoid arthritis, Type I diabetes, and inflammatory bowel disease (9). In contrast, Th2 cells are characterized by the production of IL-4, IL-5, and IL-10 and are important in humoral immunity and defense against extracellular pathogens, but can promote allergy (8). Additionally, Th1 and Th2 effectors apparently have different susceptibilities to activation-induced cell death (AICD), with Th1 cells reported to be AICD sensitive and Th2 cells reported to be AICD resistant (112). The selective death of Th1 cells has been attributed to a preferential requirement for phorbol ester-sensitive protein kinase C (PKC) isoforms (114) and the upregulation of FasL expression (115). In contrast, the resistance of Th2 cells to AICD has been linked to expression of c-FLIP and FAP-1,

inhibitors of apoptosis (112), and the selective upregulation of phosphatidylinositol-3'-kinase (PI-3-K) activity (117).

AICD is a form of apoptosis resulting from chronic antigen stimulation and is responsible for the peripheral deletion of previously activated lymphocytes. Apoptosis is a highly regulated process resulting in cell death without an ensuing inflammatory response, thus playing an important role in maintaining lymphocyte homeostasis and a balanced T cell repertoire (96). The inability of T cells to undergo AICD is associated with a variety of immunopathological diseases. The induction of AICD in $CD4^+$ T cells is mediated by interactions between Fas and FasL. The Fas death receptor is a member of the TNF/nerve growth factor-receptor superfamily (120). The binding of Fas by its ligand (FasL) results in receptor trimerization, recruitment of the Fas-associated death domain (FADD) to the death domain of Fas, and binding of caspase 8 to the death-effector domain of FADD (108). This process results in the formation of the death-inducing signaling complex (DISC). Activation of caspase 8 leads to the induction of a cascade of caspases culminating in apoptosis.

Recent studies on non-transformed human T cells have shown that Fas and components of the DISC are recruited to lipid rafts following Fas ligation and that raft structures are required for efficient propagation of apoptotic signals (108). Rafts are highly organized plasma membrane microdomains rich in cholesterol and sphingolipids and their polar lipids are predominantly composed of saturated fatty acyl chains (145). These lipids form liquid-ordered membrane regions that are insoluble in non-ionic detergents facilitating raft isolation as detergent-resistant membrane domains (145).

Lipid raft domains play an important role in the localization and distribution of key receptors and associated signal-transducing molecules (44), and are crucial in regulating the susceptibility of T cells to AICD (107). Interestingly, Th1 and Th2 cells have distinct resident protein/lipid membrane microdomain compositions (67). This is likely to impact T cell signaling and thereby modulate effector function, which may help explain the differential susceptibility to AICD in the two T helper subsets.

We have recently shown that dietary n-3 polyunsaturated fatty acids (PUFA) remodel the phospholipid composition of membrane rafts in murine T cells (45). In addition, Stulnig et al. (47) have shown that Jurkat T cells cultured *in vitro* with PUFA are capable of modifying lipid rafts and suppressing signal transduction. The anti-inflammatory properties of diets rich in n-3 PUFA on T cell function have been firmly established in human and animal models (34,35,127). The primary effector molecules are thought to be eicosapentaenoic acid [20:5n-3, EPA] and docosahexaenoic acid [22:6n-3, DHA]. In general, consumption of diets rich in n-3 PUFA is associated with a reduced pro-inflammatory T cell response due, in part, to a decreased proliferative capacity attributed to a reduction in IL-2 production and/or function and an increase in T cell apoptosis (83,132,146). Previously, we have shown that n-3 PUFA promote AICD in T cells induced to secrete a biased cytokine pattern resembling Th1 cells (146). However, the effect of n-3 PUFA on polarized Th1 cell function has not been determined. In addition, previous research has shown that cell culture conditions, with respect to the lipid content in the serum added to tissue culture medium, have a significant influence on cell fatty acid composition (147,148). Using homologous MS in

the cultures, we examined the effect of dietary n-3 PUFA on Th1 AICD and membrane microdomain fatty acid composition. Our results indicate that dietary n-3 PUFA enhance both the polarization and deletion of pro-inflammatory Th1 cells, possibly as a result of alterations in membrane microdomain fatty acid composition.

MATERIALS AND METHODS

Diet and animals

All experimental procedures using laboratory animals were approved by the University Laboratory Animal Care Committee of Texas A&M University. Female, pathogen-free weanling (12–14g) C57BL/6 mice (Frederick National Cancer Research Facility, Frederick, MD) were housed in autoclaved polycarbonate microisolator cages and were maintained at room temperature (~25°C) on a 12 h light:dark cycle. Mice were fed standard nonpurified diet (Teklad 9F Sterilizable Rodent diet) during a 1 wk acclimation period and had free access to autoclaved water and diet. Thereafter, animals were randomly assigned to one of two semi-purified diets (24-30 mice/diet group): corn oil (CO, n-6 PUFA) or an n-3 PUFA-enriched menhaden fish:corn oil (FO) mixture (4:1, w/w) at 50 g/kg diet for 14 d (146). The purified diets met NRC requirements and varied only in lipid composition (81,134). The diet composition, expressed in g/kg of complete diet, was: 200 g casein, 420 g sucrose, 219.8 g cornstarch, 60 g cellulose, 35 g AIN-76 mineral mix, 10 g AIN-76 vitamin mix, 3 g DL-methionine, 2 g choline chloride, 0.2 g tertiary butyl hydroquinone, and 5 g oil (146). Diets were aliquoted and stored at -80°C. Diets were provided ad libitum and were changed daily to prevent

peroxidation. The fatty acid composition as assessed by gas chromatography, expressed in g/kg of complete diet, is detailed in **Table 7**. The linoleic acid (18:2 n-6) content was 5.5% and 1.4% of total energy in the CO and FO diets, respectively, and thus, met the minimum 1–2% essential fatty acid requirement for rodents (134). Vitamin A, D, and E levels were approximately equal and exceeded the minimum requirement (146). Corn oil was obtained from Degussa Bioactives (Champaign, IL), and menhaden fish oil was provided by the National Institutes of Health Test Materials Program. There was no significant difference in food intake between dietary groups, and weight gain was similar in all groups (data not shown).

Table 7. Fatty Acid Composition of Experimental Diets

Fatty Acid	CO	FO4
	g/100g fatty acids	
14:0	tr	7.4
16:0	12.4	16.4
16:1 (n-7)	tr	9.2
18:0	2.2	3.0
18:1 (n-7 + n-9)	32.8	11.0
18:2 (n-6)	50.2	12.5
18:3 (n-3)	1.1	1.2
20:5 (n-3)	tr	12.2
22:5 (n-3)	tr	2.1
22:6 (n-3)	tr	9.2

Only the major fatty acids are listed. CO, 5 g/100 g corn oil; FO, 4 g/100 g fish oil + 1 g/100 g corn oil; tr, trace amount (<0.1 g/ 100 g). Values are expressed as g/100g of total fatty acids in each diet.

Splenocyte isolation and CD4$^+$ T cell enrichment

Mice were euthanized by CO_2 asphyxiation. Spleens were placed in 3 ml RPMI complete medium [(RPMI 1640 with 25 mmol/l HEPES (Irvine Scientific, Santa Ana, CA) supplemented with 5% heat-inactivated fetal bovine serum (Irvine Scientific), 1 x 10^5 U/L penicillin and 100mg/L streptomycin (Irvine Scientific), 2 mmol/l L-glutamine, and 10 μmol/l 2-mercaptoethanol]. Spleens were dispersed with glass homogenizers and passed through a 149 μm wire mesh filter to create single-cell suspensions. Total lymphocytes were initially enriched by density gradient centrifugation using Lympholyte-M (Cedarlane, Toronto, Canada) as previously described (146). Subsequently, approximately 120 x 10^6 mononuclear cells were loaded onto negative-selection CD4$^+$ T cell purification columns (R&D Systems, Minneapolis, MN). Nonadherent cells were eluted for assay. The purity of the T cell population as analyzed by flow cytometry was determined to be 90.3 ± 1.4%, n=3 (83).

Th1 polarization

Th1 cells were induced *in vitro* using standardized polarization methodology (149). In general, CD4$^+$ T cells, 5 x 10^6 cells/well, were cultured in the presence of 1 ng/ml phorbol-12-myristate-13-acetate (PMA) (Sigma, St Louis, MO) with 500 nM Ionomycin (Calbiochem-Novabiochem, San Diego, CA) combined with 10 μg/ml αIL-4 monoclonal antibody (National Cancer Institute), 20 ng/ml recombinant IL-2 (R&D Systems), and 5 ng/ml recombinant IL-12 (BD PharMingen, San Diego, CA) (Th1 Cocktail) in either 5% FBS-complete medium (FBS) or 2.5% homologous mouse serum

+ 2.5% FBS-complete medium (MS) for 2 d at 37°C, 5% CO_2. Cells were harvested and recultured in 20 ng/ml rIL-2 and 5 ng/ml rIL-12 for an additional 3 d at 37°C, 5% CO_2 in the presence of FBS or MS. The serum was changed each time cytokines and antibodies were provided, i.e., on days 2 and 3. Polarized Th1 cells were subsequently analyzed for intracellular cytokine expression and AICD.

Intracellular cytokine staining

Following Th1 polarization, cultures were harvested, dead cells were removed by density gradient centrifugation using Lympholyte-M, and 1 x 10^6 cells/well were reactivated with PMA/Ionomycin with 3 μM monensin for 5 h at 37°C, 5% CO_2. Cells were then washed, fixed and permeabilized using a Cytofix/Cytoperm Kit (BD PharMingen) before staining with αIL-4-PE (BD PharMingen) and αIFNγ–FITC (BD PharMingen). Cytokine staining was assessed via flow cytometry (FACSCalibur; Becton Dickinson, Bedford, MA) as previously described (146).

Induction of AICD

Following Th1 polarization, cultures were harvested, dead cells were removed by density gradient centrifugation using Lympholyte-M, and 1 x 10^6 cells/well were reactivated with PMA/Ionomycin for 5 h at 37°C, 5% CO_2 (122,146). Cells were then washed with cold 1 X PBS and stained with 5 μl FITC-conjugated Annexin V and 2 μl Propidium Iodide for 15 min followed by flow cytometry analysis. Controls consisted of single staining for Annexin V-FITC only and PI only.

Density gradient centrifugation and isolation of lipid rafts

Raft microdomains were isolated from $CD4^+$ T cells as previously described by Tamir et al. (150) and Fan et al. (45) with slight modification immediately following isolation (Day 0), after 5 days in culture with FBS, or after 5 days in culture with homologous MS. Cells were resuspended in lysis buffer [100 mmol/L NaCl, 2 mmol/L EDTA, 0.14 mmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 0.2 mmol/L Na_3VO_4, 50 μmol/L aprotinin, 88 μmol/L leupeptin, 160 μmol/L bestain, 60 μmol/L pepstain A, and 56 μmol/L E-64, pH 6.9] supplemented with 1% Brij-58. Cell lysates were passed through a 27G needle twice, followed by a 30 min incubation on ice. An 850 g/L sucrose solution in lysis buffer was added to the homogenate and mixed by pipetting to generate a 450 g/L sucrose lysate. Cell lysates were transferred to the bottom of a 2 ml polyallomer ultracentrifuge tube, which was subsequently overlaid with 350 and 50 g/L sucrose, respectively. After centrifugation at 200,000 x g (Beckman Coulter Optima Max-E Ultracentrifuge, TLS 55 rotor) for 16 h at 4°C, aliquots from the top (low density detergent insoluble glycerolipid enriched raft fraction, liquid ordered membrane rafts) and from the bottom (cytosol-high density membrane detergent soluble fraction, liquid disordered soluble fractions) of each tube were collected for lipid analysis. We have previously shown that the protein distribution patterns are consistent with the features of lipid rafts, i.e., enrichment of ganglioside GM-1 and exclusion of CD3, etc. (45).

Analysis of mouse sera and membrane fraction phospholipid fatty acid composition

Total lipids from mouse sera and in liquid ordered membrane rafts and liquid disordered soluble fractions were extracted by the method of Folch et al. (135). Total phospholipids from the membrane fractions were separated by thin layer chromatography (TLC) on silica gel 60 G plates using chloroform/ methanol/ acetic acid/ water (90, 8, 1, 0.8, v/v) as the developing solvent. Bands were detected under ultraviolet light after spraying with 0.1% 8-anilino-naphthalene-sulfonic acid. Total phospholipids were scraped from the plates, spiked with heptadecanoic acid (17:0) and transesterified in the presence of 6% methanolic HCl (45). Fatty acid methyl esters from serum lipids and phospholipids of membrane fractions were extracted using hexane and 0.1 M potassium chloride and analyzed by capillary gas chromatography as previously described (45).

Cholesterol analysis

Following lipid extraction, cholesterol was analyzed using the Amplex Red Cholesterol Assay Kit (Molecular Probes, Eugene, OR). Total lipids in liquid ordered membrane rafts and liquid disordered soluble fractions were dried down under nitrogen and redissolved in Reaction Buffer. Samples were assayed in duplicate using a 300 μM solution of Amplex Red reagent mix containing 2 U/ml horseradish peroxidase, 2 U/ml cholesterol oxidase, and 2 U/ml cholesterol as per kit instructions.

Statistical analysis

Membrane lipid data were analyzed using a robust ANOVA method (151). A cell mean model was fitted using S-PLUS software (Insightful, Seattle, WA). Huber's weights were implemented to downweight potentially outlying observations. This approach enables all data points to be considered without the drawback of having one or few outlying data points dominate and bias the outcomes. For the group containing no outlying observations, the cell means are identical to those obtained by the regular ANOVA. The corresponding T/F tests were then used to test the existence of differences between treatment groups. Remaining data was analyzed using one-way ANOVA. Differences of $p<0.05$ were considered significant.

RESULTS

Dietary lipids differentially affect Th1 polarization

Th1 cells were induced *in vitro* using standardized polarization methodology (149). To verify that $CD4^+$ T cells were polarized towards a Th1 phenotype, cells were analyzed by flow cytometry for coexpression of IFNγ and IL-4 using intracellular cytokine staining. Representative 2 parameter flow cytometric histograms of IFNγ-FITC and IL-4-PE labeled cells are shown in **Figure 10A**. The numbers in each quadrant represent the percentage of cells positive for IFNγ and/or IL-4. **Figure 10B** shows the effect of diet and culture conditions on cells positively expressing IFNγ but not IL-4. A majority of the cells were $IFN\gamma^+IL\text{-}4^-$ and less than 1% of the cells were $IL\text{-}4^+IFN\gamma^-$ producing T cells, indicating successful Th1 polarization. With respect to the effect of

dietary treatment on Th1 polarization status, $CD4^+$ T cells from mice fed CO had significantly fewer $IFN\gamma^+$ cells than FO-fed mice (36.9% in MS-CO *vs.* 55.1% in MS-FO, p = 0.0001), but the change was only observed in cells cultured in the presence of homologous MS **(Fig. 10)**.

The fatty acid composition of MS and FBS, as shown in **Table 8**, revealed that FBS was relatively devoid of n-3 PUFA (20:5n-3, 22:5n-3, 22:6n-3). With respect to the effect of dietary source on MS, serum from FO *vs* CO-fed mice had an elevated n-3/n-6 PUFA molar ratio, markedly lower levels of saturated and monounsaturated fatty acids, and n-6 PUFA (18:2n-6, 20:4n-6), and was highly enriched in 20:5n-3.

n-3 PUFA promote Th1 AICD

In order to assess the effect of diet on AICD following Th1 polarization, cells were reactivated and analyzed for apoptosis using AnnexinV-FITC/PI labeling. Annexin V binding is an excellent marker for apoptotic cells because the early distribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus (107,152,153). To rule out the possibility of primary necrosis, Fas-Fc, an inhibitor of the Fas death receptor, and Z-VAD-fmk (50 μmol/L), a pan-caspase inhibitor, were added to select cultures (146). Unreactivated $CD4^+$ T cells from both diet groups had equivalent levels of apoptosis (13.53 ± 0.77 in FO *vs* 13.32 ± 0.72 % in CO treatments, n=36 per diet) after 3 days in culture. Upon restimulation (reactivation), apoptosis was increased two-fold (data not shown), consistent with our previously

published results (146). These data indicate that T cells were not compromised in any way prior to restimulation.

A

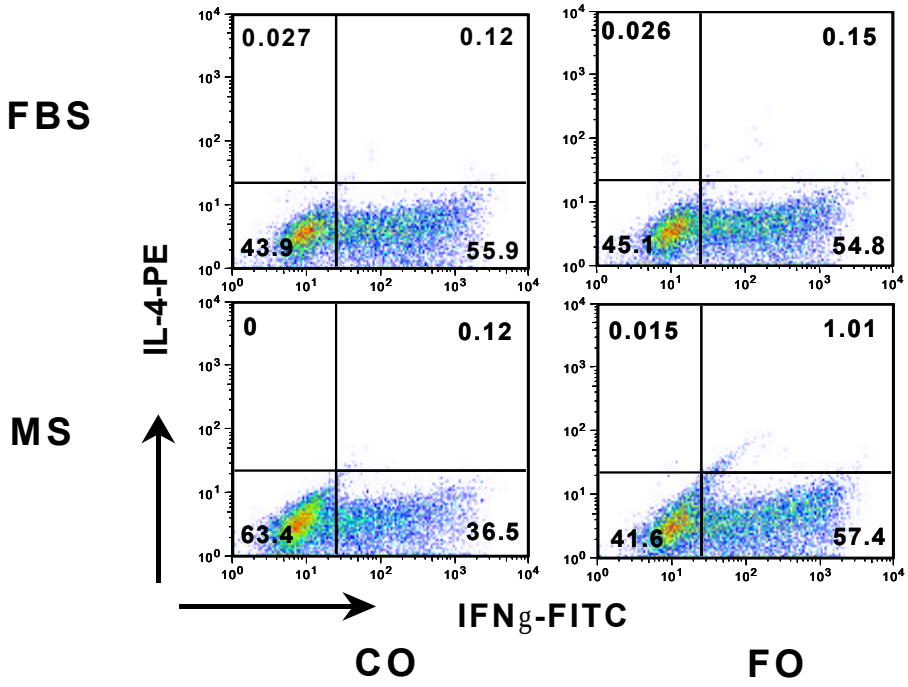

Figure 10. Diet-induced alteration of Th1 cell polarization is observed only in the presence of homologous mouse serum. Splenic $CD4^+$ T cells were isolated from mice fed CO or FO-containing diets for 2 weeks followed by polarization towards Th1 in the presence of MS or FBS. After 5 days in culture, cells were reactivated with PMA/Ionomycin in the presence of monensin and assessed for the coexpression of IFNγ and IL-4 by intracellular cytokine staining as described in the *Materials and Methods*. (**A**) Representative 2 parameter flow cytometric histograms of MS *vs* FBS-cultured cells. Numbers in each quadrant represent the percentage of cells positive for IFNγ and/or IL-4.

B

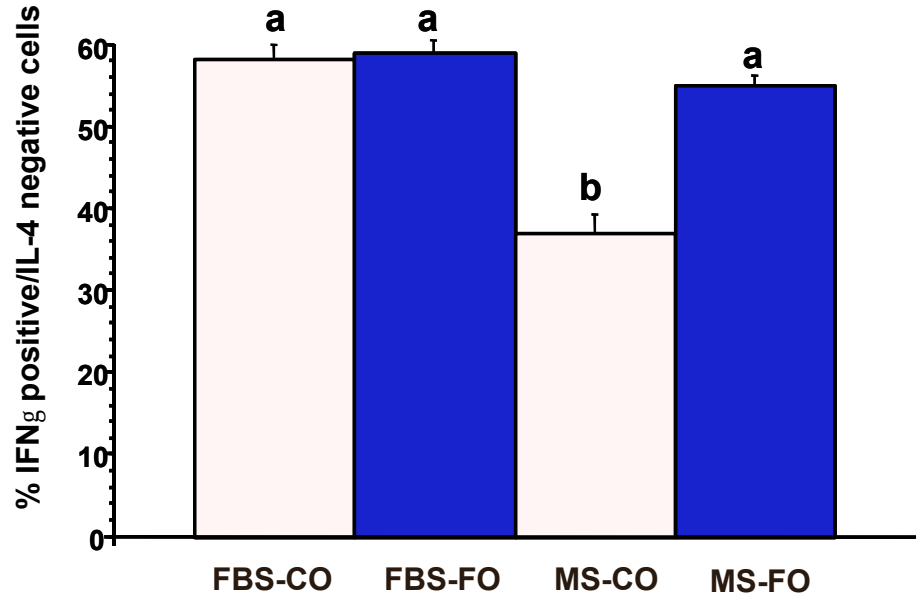

Figure 10. Continued.
(**B**) Bars with different letters denote significant differences ($p<0.05$). Data represent the mean ± SEM, n=6 replicates per diet group, 4 mice pooled per analysis.

Table 8. Fatty acid composition of sera used for cell culture

Fatty Acid	FBS	MS-CO	MS-FO
16:0	1.71 ± 0.02	14.20 ± 0.55	5.03 ± 0.22
18:0	0.80 ± 0.02	7.93 ± 0.20	1.84 ± 0.07
18:1n-9/n-7	1.63 ± 0.05	15.54 ± 0.22	3.82 ± 0.15
18:2n-6	0.40 ± 0.01	17.72 ± 0.37	2.10 ± 0.09
20:4n-6	0.53 ± 0.01	12.32 ± 0.63	1.12 ± 0.07
20:5n-3	tr	tr	2.99 ± 0.07
22:5n-3	0.15 ± 0.0	tr	0.22 ± 0.02
22:6n-3	0.17 ± 0.01	2.24 ± 0.24	2.22 ± 0.13
n-3/n-6	0.34 ± 0.00	0.07 ± 0.01	0.76 ± 0.04

Mouse serum from CO and FO-fed mice were extracted and analyzed to assess fatty acid composition. FBS is shown for comparison. Values are expressed as μg fatty acid/ml serum and represent means ± SEM, n=3. FBS, fetal bovine serum; MS-CO, serum from corn oil fed mice; MS-FO, serum from fish oil fed mice; tr, trace amount (<0.1 μg/ml).

Representative 2 parameter flow cytometric histograms of Annexin V-FITC and PI labeled cells are shown in **Figure 11A**. The numbers in each quadrant represent the percentage of cells positive for Annexin V-FITC and/or PI. The relative number of apoptotic cells (indicated by Annexin V positive, PI negative and Annexin V positive, PI positive) from $CD4^+$ T cells from CO and FO-fed mice polarized in FBS or homologous MS is detailed in **Figure 11B**. $CD4^+$ T cells from FO-fed mice exhibited a statistically significant enhancement in AICD (55.9% in MS-CO *vs*. 69.3% in MS-FO, p = 0.0001). Similar to the polarization results illustrated above (**Fig. 10**), this effect only occurred in

MS-cultured cells, as this phenotype was lost when cells were cultured in FBS. In other words, dietary changes in $CD4^+$ T cell function were maintained in cells cultured in homologous MS, but were lost when cells were cultured in FBS during the 5 day polarization period.

To determine the relationship between cell polarization status and the amount of AICD induced, a correlation analysis of $IFN\gamma^+$ cells versus apoptotic cells was performed. **Figure 12** shows the correlation of MS-CO and MS-FO $CD4^+$ T cells. Both the Spearman and the Pearson correlation tests indicated that there was a significant positive correlation ($p=0.0119$ and $p < 0.0005$, respectively) between $IFN\gamma^+$ cells and the number of apoptotic cells when the data from the MS-CO and MS-FO cultures were analyzed together. There was also a statistically significant positive correlation, $p=0.0139$ (Spearman test) and $p=0.0073$ (Pearson test), in FBS-cultured cells (data not shown).

A

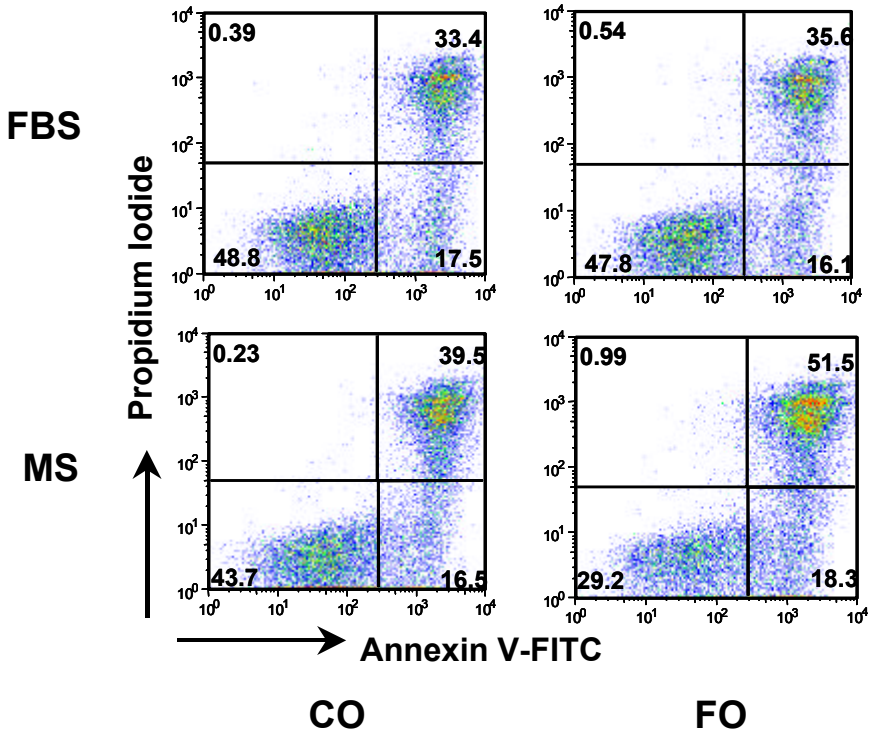

Figure 11. Dietary n-3 PUFA promote CD4$^+$ T cell AICD only in the presence of homologous mouse serum. Splenic CD4$^+$ T cells were isolated from mice fed CO or FO-containing diets for 2 weeks followed by polarization towards Th1 in the presence of MS or FBS. After 5 days in culture, cells were reactivated with PMA/Ionomycin and assessed for apoptosis by Annexin V-FITC and propidium iodide staining as described in the *Materials and Methods*. (**A**) Representative 2 parameter flow cytometric histograms of MS *vs* FBS-cultured cells. Numbers in each quadrant represent the percentage of cells positive for Annexin V-FITC and/or PI.

B

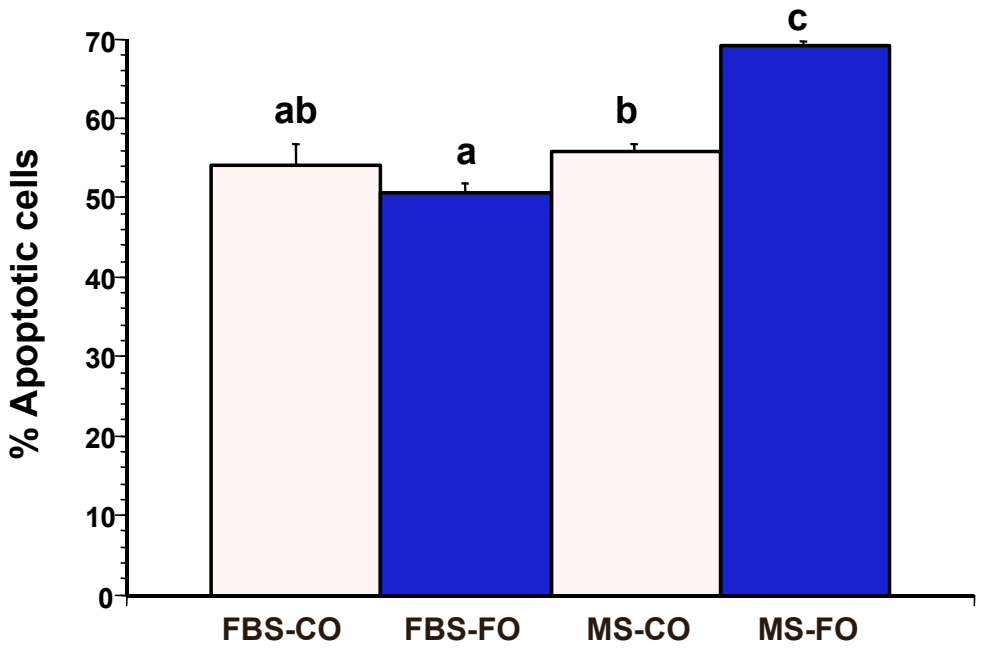

Figure 11. Continued.
(B**)** Bars with different letters denote significant differences ($p<0.05$). Data represent the mean ± SEM, n=6 replicates per diet group, 4 mice pooled per analysis**.**

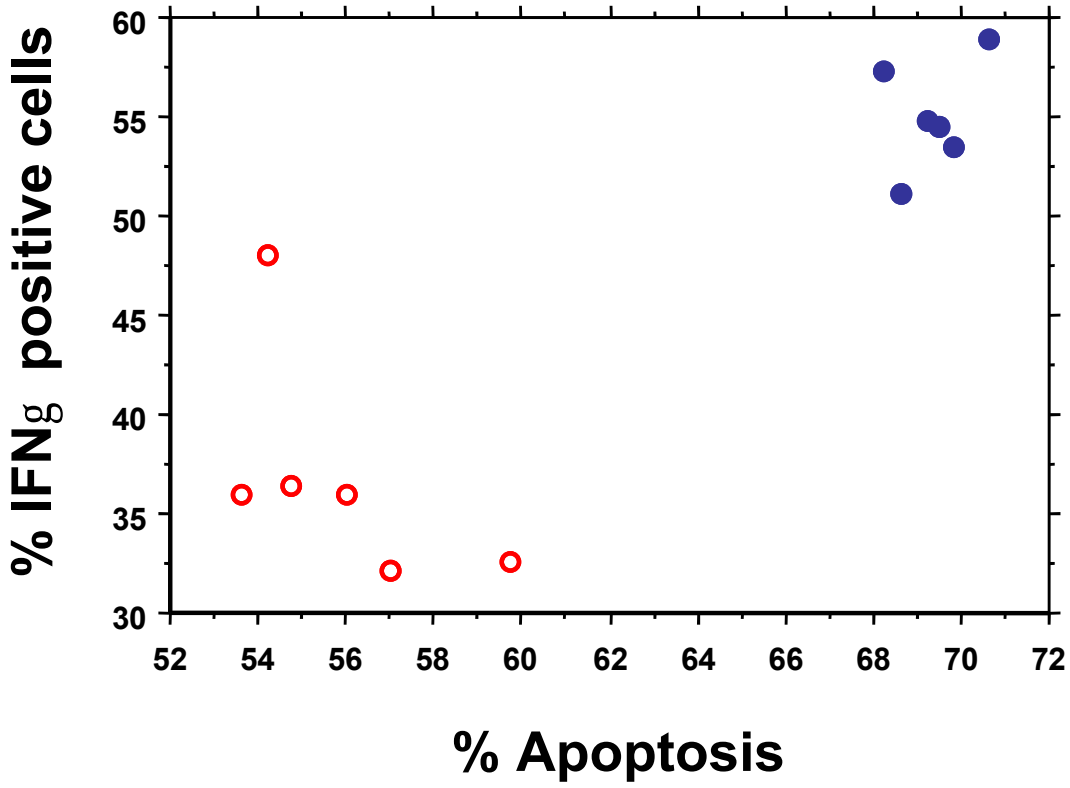

Figure 12. AICD and Th1 differentiation are highly correlated in mouse serum-cultured CD4$^+$ T cells. The relationship between AICD and intracellular IFNγ levels was examined by both Spearman and Pearson tests of IFNγ $^+$ cells versus apoptotic cells. Filled circles represent MS-cultured cells isolated from FO-fed animals and open circles represent MS-cultured cells isolated from CO-fed animals. Data represent the mean ± SEM, n=6 replicates per diet group, 4 mice pooled per analysis ($p<0.05$).

Effect of culture conditions on Th1 cell membrane microdomains

In order to clarify how dietary n-3 PUFA membrane enrichment is influenced by the 5 day culture period necessary for Th1 polarization, we examined lipid raft (liquid ordered) and soluble (liquid disordered) membrane fractions from $CD4^+$ T cells immediately following isolation (Day 0), and after 5 days in culture with either FBS or homologous MS. **Table 9** shows the effect of diet and culture conditions on the fatty acid composition of raft and soluble membrane domains. Consistent with previous reports (47,154), the raft membrane fraction had a 36% greater cholesterol to phospholipid molar ratio (chol/PL) compared to the soluble fractions ($p = 0.07$) (shown in **Figure 13**). In addition, the total phospholipid unsaturation index was lower in rafts than in soluble membrane fractions (**Table 9**). Collectively, these data indicate that proper fractionization was accomplished.

Gas chromatographic analysis of the fatty acid composition of cell phospholipids showed that the amount of DHA (22:6n-3) in both raft and soluble fractions was decreased in FBS cultures of $CD4^+$ T cells from FO-fed mice 5 days after culture (**Table 9**). In contrast, the inclusion of MS prevented the culture-induced rearrangement of T cell membrane lipids. This phenomenon was observed with respect to other PUFA and fatty acid classes in both diets and both membrane fractions (**Table 9**). Examination of membrane microdomain distributions of EPA (20:5n-3) and DHA (22:6n-3) from CO and FO-fed mouse $CD4^+$ T cells (shown in **Figure 14**) revealed that the significant enhancement of these two n-3 PUFA in the $CD4^+$ T cells of FO-fed, compared to CO-fed, mice was lost in the rafts from $CD4^+$ T cells in the FBS cultures.

Table 9. Fatty acid composition in raft and soluble membrane fractions of CO and FO-fed mouse CD4$^+$ T cells

CO	RAFT			SOLUBLE		
Fatty acid	**Day 0**	**Day 5 (FBS)**	**Day 5 (MS)**	**Day 0**	**Day 5 (FBS)**	**Day 5 (MS)**
SFA	70.57 ± 0.76	69.33 ± 2.04	68.97 ± 1.30	55.13 ± 0.81	53.69 ± 1.46	54.88 ± 6.25
MUFA	10.72 ± 0.43^{a*}	20.30 ± 1.79^{b}	12.57 ± 0.38^{a}	10.27 ± 1.60^{a}	31.09 ± 2.19^{b}	14.82 ± 1.43^{a}
PUFA	18.70 ± 1.01^{b}	10.38 ± 1.26^{a}	18.46 1.58^{b}	34.59 ± 1.87^{b}	15.22 ± 1.87^{a}	30.30 ± 5.15^{b}
n-6 PUFA	15.65 ± 2.43^{b}	8.58 ± 1.45^{a}	15.29 ± 1.71^{b}	28.07 ± 1.27^{b}	12.78 ± 1.66^{a}	25.67 ± 5.45^{b}
n-3 PUFA	3.05 ± 2.50	1.79 ± 0.30	3.17 ± 0.71	6.52 ± 1.38^{b}	2.44 ± 0.35^{a}	4.64 ± 0.36^{ab}
n-3/n-6	0.26 ± 0.23	0.23 ± 0.06	0.21 ± 0.06	0.23 ± 0.05	0.19 ± 0.03	0.21 ± 0.06
UI**	54.79 ± 3.44^{ab}	36.81 ± 3.62^{a}	65.35 ± 4.36^{b}	124.24 ± 8.25^{b}	53.47 ± 5.80^{a}	99.58 ± 17.26^{b}
18:2n-6	3.76 ± 1.96	2.61 ± 0.42	4.82 ± 0.69	6.44 ± 0.33^{b}	3.52 ± 0.49^{a}	12.74 ± 0.77^{c}
20:3n-6	3.32 ± 1.50^{b}	0.59 ± 0.35^{a}	2.05 ± 0.61^{ab}	8.36 ± 0.94^{b}	2.48 ± 1.2^{a}	1.11 ± 0.56^{a}
20:4n-6	4.69 ± 0.68	3.72 ± 0.45	6.49 ± 0.58	11.43 ± 1.88^{b}	5.14 ± 0.26^{a}	8.33 ± 3.39^{ab}
20:5n-3	tr	tr	0.29 ± 0.16	0.45 ± 0.45	tr	tr
22:6n-3	0.61 ± 0.31	1.01 ± 0.17	1.95 ± 0.12	2.42 ± 0.50^{ab}	1.29 ± 0.14^{a}	3.80 ± 0.24^{b}

FO	RAFT			SOLUBLE		
Fatty acid	**Day 0**	**Day 5 (FBS)**	**Day 5 (MS)**	**Day 0**	**Day 5 (FBS)**	**Day 5 (MS)**
SFA	71.22 ± 3.07	76.52 ± 5.01	71.02 ± 1.58	65.16 ± 1.57	53.15 ± 0.83	54.39 ± 2.79
MUFA	12.64 ± 2.51	13.50 ± 4.07	13.62 ± 1.66	10.87 ± 0.79^{a}	30.23 ± 0.29^{c}	19.16 ± 3.61^{b}
PUFA	16.14 ± 1.25	9.98 ± 1.22	15.36 ± 0.35	23.97 ± 1.37^{ab}	16.63 ± 0.67^{a}	26.45 ± 6.37^{b}
n-6 PUFA	13.93 ± 1.39^{b}	7.80 ± 1.20^{a}	9.03 ± 0.15^{ab}	16.28 ± 0.64	12.52 ± 0.56	14.26 ± 2.93
n-3 PUFA	2.21 ± 0.29^{a}	2.18 ± 0.04^{a}	6.34 ± 0.49^{b}	7.69 ± 1.92^{a}	4.10 ± 0.68^{a}	12.19 ± 3.44^{b}
n-3/n-6	0.17 ± 0.03^{a}	0.30 ± 0.05^{a}	0.70 ± 0.07^{b}	0.48 ± 0.13^{a}	0.33 ± 0.06^{a}	0.82 ± 0.09^{b}
UI	54.24 ± 3.67	36.96 ± 3.99	60.75 ± 2.21	97.36 ± 10.03^{b}	63.42 ± 3.92^{a}	109.55 ± 27.50^{b}
18:2n-6	3.39 ± 0.28	2.00 ± 0.46	4.15 ± 0.55	4.78 ± 1.48^{ab}	2.78 ± 0.42^{a}	6.01 ± 1.02^{b}
20:3n-6	2.43 + 0.85	1.42 ± 0.22	0.81 ± 0.81	0.82 ± 0.42	1.66 ± 0.09	1.04 ± 0.52
20:4n-6	5.20 ± 2.02	3.48 ± 0.68	2.54 ± 0.20	10.30 ± 0.86^{b}	6.43 ± 0.74^{ab}	5.56 ± 1.27^{a}
20:5n-3	0.83 ± 0.66	tr	0.26 ± 0.14	1.14 ± 0.62	0.26 ± 0.13	0.96 ± 0.50
22:6n-3	2.47 ± 1.27^{ab}	1.17 ± 0.06^{a}	3.61 ± 0.38^{b}	4.54 ± 0.34^{b}	1.92 ± 0.04^{a}	6.26 ± 1.31^{c}

Splenic CD4+ T cells were isolated from mice fed CO or FO-containing diets for 2 weeks followed by membrane preparation (Day 0) or cultured in the presence of either 5% FBS-complete medium (Day 5 (FBS)) or 5% homologous mouse serum-complete medium (Day 5 (MS)) for 5 days. Rafts and soluble membrane fractions were isolated and fatty acid composition was analyzed.

Values are expressed as mol/100 mol fatty acids (mol%) and represent means ± SEM, n=3, 10 mice pooled per analysis.

* Letters indicate significant differences between culture conditions of the same diet and membrane fraction ($p<0.05$).

** Unsaturation Index (UI) represents the summed mol/100 mol multiplied by the number of double bonds.

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; tr, trace amounts (<0.1 mol%).

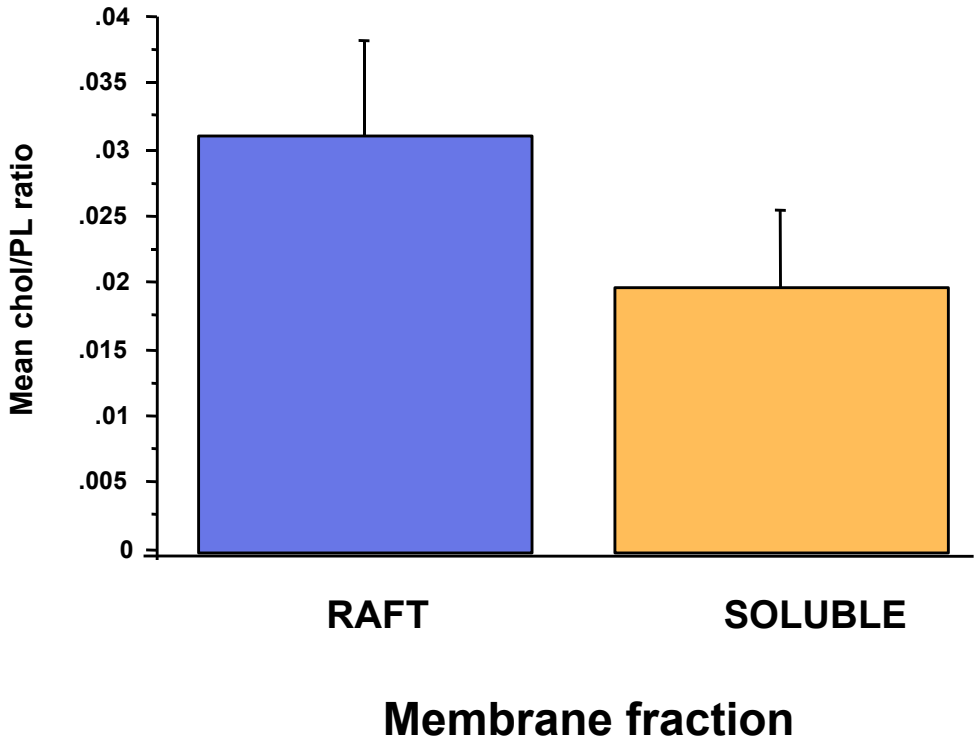

Figure 13. Characterization of membrane microdomain fractions. Splenic $CD4^+$ T cells were isolated from mice fed CO or FO-containing diets for 2 weeks followed by membrane isolation (Day 0) or after culture in the presence of either 5% FBS-complete medium (FBS) or homologous mouse serum (MS) for 5 days. Raft and soluble membrane fractions were isolated and total phospholipid and cholesterol levels were expressed as a mole percentage ratio. Data represent the mean ± SEM, n = 18 replicates per membrane fraction.

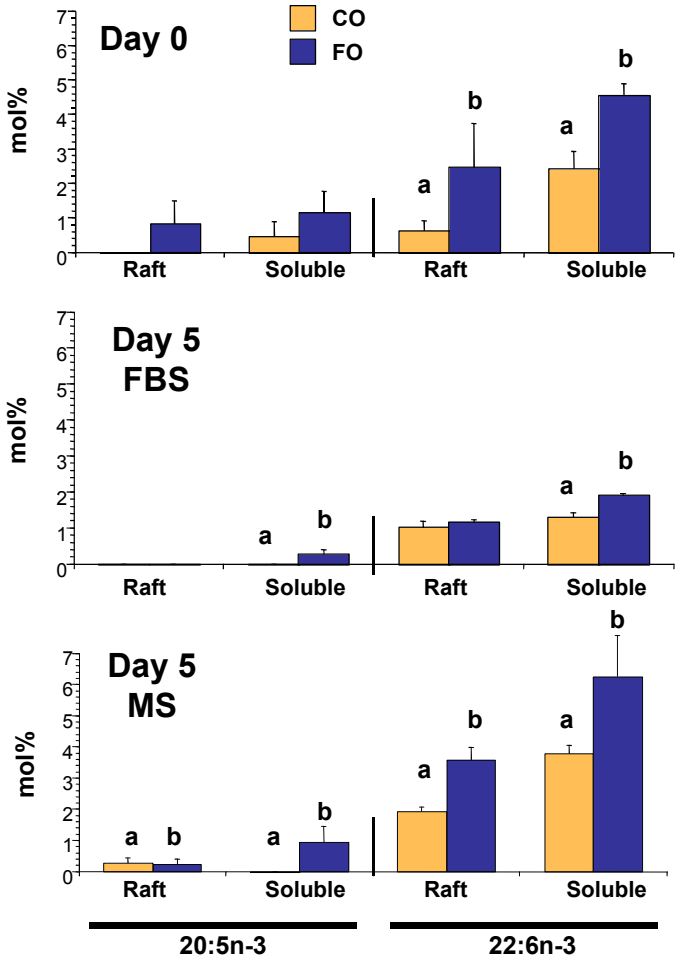

Figure 14. Membrane microdomain distribution of EPA and DHA in CO and FO-fed mouse CD4$^+$ T cells. Splenic CD4$^+$ T cell membrane fractions were isolated from mice fed CO or FO-containing diets for 2 weeks either immediately (Day 0) or after culture in the presence of either 5% FBS-complete medium (FBS) or homologous mouse serum (MS) for 5 days. Raft and soluble membrane fractions were isolated and 20:5n-3 (EPA) and 22:6n-3 (DHA) were analyzed. Data represent the mean ± SEM, n = 3 replicates per diet group, 10 mice pooled per analysis. Letters indicate significant differences between diets for the same membrane fraction and fatty acid ($p<0.05$).

Since cholesterol is critical for raft integrity (145), we examined the cholesterol level by assessing the molar chol/PL ratio in the various cell membrane fractions. **Figure 15** shows the effect of diet and culture conditions on the molar ratio of cholesterol to total phospholipid. In both diets groups, chol/PL significantly dropped from day 0 to day 5-FBS ($p < 0.02$) in $CD4^+$ T cell membrane rafts. However, in cultures of $CD4^+$ T cells from FO-fed, but not CO-fed mice, the addition of MS prevented culture-induced loss of cholesterol. In comparison, in soluble membrane fractions, a significant reduction ($p < 0.05$) in the chol/PL ratio occurred overtime regardless of culture conditions (**Fig. 15**).

DISCUSSION

Among dietary factors, there is overwhelming evidence for a protective effect of n-3 PUFA on autoimmune/inflammatory diseases (34,35,127). In contrast, dietary lipids rich in n-6 PUFA can be deleterious with respect to the incidence and severity of inflammatory diseases. This is significant because the typical Western diet contains 10-20 times more n-6 than n-3 PUFA (37). Additionally, the immunosuppressive effects of diets rich in n-3 PUFA on a variety of T cell functions has been firmly established in both human and animal models (17,34,35,70,127,155). With respect to the physiological relevance of the diets used in our study, the FO diet contained approximately 1.4 and 1.0 energy % as EPA and DHA, respectively. As a point of reference, the Japanese typically consume n-3 PUFA at 1-2% of energy in their diet (156), while most European countries

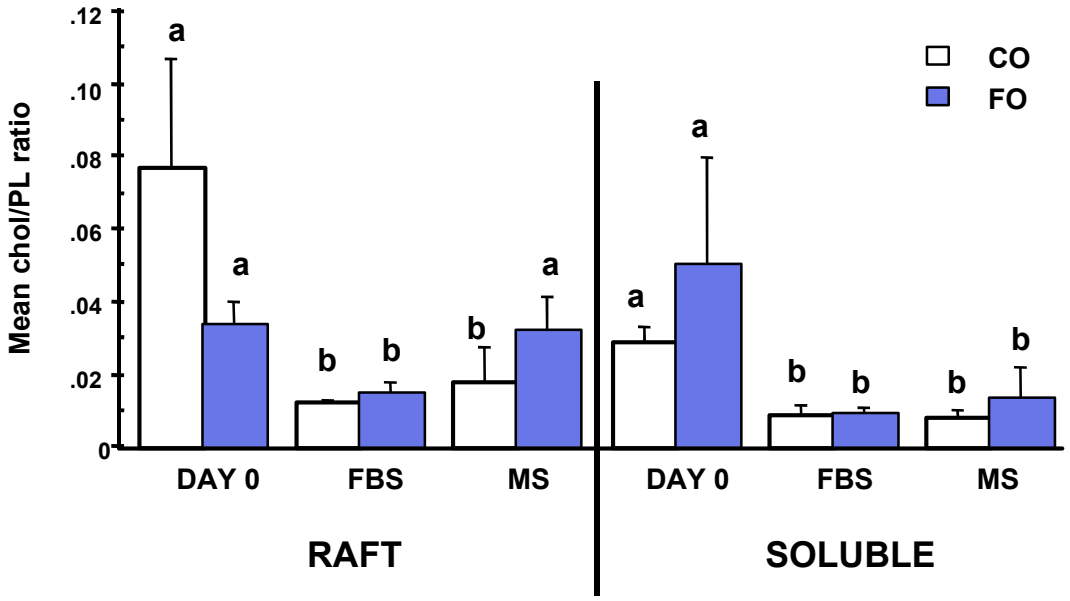

Figure 15. CD4$^+$ T cells from n-3 PUFA-fed mice cultured in the presence of homologous MS maintain the cholesterol content of lipid rafts. Splenic CD4$^+$ T cells were isolated from mice fed CO or FO-containing diets for 2 weeks followed by membrane isolation (Day 0) or after culture in the presence of either 5% FBS-complete medium (FBS) or homologous mouse serum (MS) for 5 days. Raft and soluble membrane fractions were isolated and total phospholipid and cholesterol levels were expressed as a mole percentage ratio. Data represent the mean ± SEM, n = 3 replicates per diet group, 10 mice pooled per analysis. Letters indicate significant differences between culture conditions within the same membrane fraction and diet ($p<0.05$).

and the U.S. consume 0.1-0.2% of energy as n-3 PUFA (157). Therefore, the n-3 PUFA content of the experimental diets used in this study was well within the range consumed by humans.

We have observed previously that the dietary effect of n-3 PUFA on T cell proliferation and apoptosis depended upon the propensity of the *in vitro* stimulus to bias T cells towards a predominantly IL-2/IFNγ producing population (a putative Th1-like subset) or an IL-4/IL-10 producing population (a putative Th2-like subset) (83,146). Specifically, n-3 PUFA increased proliferation only in $CD4^+$ T cells stimulated with αCD3/PMA, a Th2-biasing stimulus (83). With respect to apoptosis, we have previously shown that n-3 PUFA enhanced AICD in T cells induced to secrete a biased cytokine pattern, resembling Th1 cells, following stimulation with PMA/Ionomycin (146). Therefore, we hypothesized that n-3 PUFA would promote AICD in polarized Th1 cells. $CD4^+$ T cells were driven to differentiate *in vitro* towards a Th1 cytokine pole using standard polarization methodology (149). Due to the extended time in culture to achieve polarization (5 days), a set of cells from each diet group were cultured in diet-matched homologous MS, rather than fetal bovine serum, to prevent culture-induced loss of n-3 PUFA from membrane phospholipids. Fatty acid quantitation of medium containing MS or FBS revealed that there were significant differences in fatty acid composition between MS-CO, MS-FO, and FBS complete medium (**Table 8**). Previous research has shown that cell culture conditions have a significant influence on lymphocyte bulk membrane fatty acid composition (147,148). Yaqoob et al. (147) reported that culturing lymphocytes in medium containing autologous serum, which had

a fatty acid composition closely resembling that of the diet, allowed maintenance of diet-induced changes in fatty acid composition. In addition, culturing in autologous or homologous serum was shown to have a significant effect on lymphocyte function. Yaqoob et al. (76) and Pompos et al. (148) reported that n-3 PUFA decreased T cell proliferation when cells were cultured in autologous/homologous serum, but had little effect when the same cells were cultured in standard medium. These results provided the rationale to include homologous MS in our cultures.

To verify that $CD4^+$ T cells were polarized towards a Th1 phenotype, cells were analyzed for the coexpression of IFNγ and IL-4. Consistent with previous reports (149), a majority of the cells were $IFN\gamma^+$ $IL\text{-}4^-$, indicating a predominant Th1 phenotype (**Fig. 10**). A noteworthy outcome from these analyses was that dietary CO suppressed Th1 polarization relative to FO, however, this effect only occurred in cells cultured in the presence of homologous MS (**Fig. 10**). These results are not consistent with the findings from other researchers who have shown that n-3 PUFA decrease IFNγ (77,78). Specifically, Fritsche et al. (77) reported decreased serum IFNγ and splenic IFNγ mRNA levels in mice fed FO compared to n-6 PUFA control diets. In contrast, other studies indicate that n-3 PUFA increase IFNγ production (79,158,159). There are several reasons for these apparent discrepancies. Fritsche et al. (77) and Wallace et al. (78), who also reported decreased IFNγ following FO-feeding, analyzed whole splenocyte populations and cultured cells in the presence of fetal bovine serum prior to IFNγ analysis. In contrast, Oarada et al. (158) and Fritsche et al. (79) detected enhanced IFNγ when samples were assayed directly from n-3 PUFA fed animals, i.e., cells were not

maintained in culture. The fact that a number of *in vivo* experiments show an increase in IFNγ production following n-3 PUFA feeding support the conclusion that diet-induced changes in T cell cytokine production can be masked by culture conditions.

Since dietary lipids are incorporated into T cell membrane phospholipids (146), we investigated the effect of culture conditions on $CD4^+$ T cell membranes. Recent data generated in our lab have shown that n-3 PUFA remodel T cell lipid rafts (45), therefore we examined plasma membrane microdomains, i.e., rafts. Interestingly, only in rafts isolated from $CD4^+$ T cells from FO-fed mice did the addition of homologous MS prevent the culture-induced loss of cholesterol (**Fig. 15**). Since cholesterol is critical for raft integrity (145), these results indicate that culture conditions have a profound effect on lipid raft composition and structure. These data are noteworthy because perturbations in lipid raft integrity/composition directly mediate T cell AICD (107). Rafts play an important role in cell signaling, particularly through the organization and distribution of surface receptors, including Fas, at specific sites in the plasma membrane (107,109). Recent studies have shown that the formation of macromolecular complexes containing the T cell receptor, CD4, and CD45 is believed to contribute to sustained TCR interaction with its ligand (44). Lipid rafts are important for the formation and stabilization of these TCR signaling complexes, acting as platforms that facilitate intramolecular associations and propagation of signal transduction cascades (44). Interestingly, conditions that modify raft structure can disrupt early steps in T cell activation (160). Rafts appear to differ depending on the developmental state of the T cell and these differences probably contribute to markedly different outcomes of

signaling (67). Effector and memory T cells have more surface rafts compared to naïve T cells and activated Th1 cells differ from activated Th2 cells in raft organization. Stimulation of Th1 cells results in a stable association of TCR components with raft domains, while Th2 stimulation fails to form these signaling complexes (67). Therefore, our data are noteworthy, because this is one of the first studies to examine the effect of diet on raft modification and its relationship to T cell activation.

With respect to AICD, raft structures are required for efficient propagation of apoptotic signals. The death receptor and primary initiator of AICD, Fas, has been shown to require clustering and capping at the membrane to effectively signal to downstream apoptotic molecules (110). This clustering and capping occurs in rafts; a location that best facilitates the trapping of Fas, recruitment of additional intracellular molecules of the DISC, and exclusion of inhibitory pathways. Th1 and Th2 cells have different susceptibilities to AICD possibly explained, in part, by their distinct lipid raft compositions (67). Our analysis showed that $CD4^+$ T cells from FO-fed mice cultured in the presence of homologous MS exhibited significantly enhanced AICD (**Fig. 11**), i.e., the diet and culture conditions that promoted the greatest number of Th1 cells also enhanced AICD to the greatest extent. These data extend our earlier findings where we demonstrated that alterations in dietary lipid composition can directly influence AICD (146). In these experiments, following a 2 week feeding period, T cells were isolated from mice on diet and subsequently cultured for 3 days in the presence of FBS. Only T cells from n-3 PUFA-fed mice had increased levels of AICD. These observations clearly indicate that the tendency to undergo AICD is already established *in vivo*. Therefore, the

use of homologous mouse serum does not create a new phenotype. Interestingly, there was a significant positive correlation between the polarization status of the cell and the amount of AICD induced in homologous MS cells (**Fig. 12**). The enhancement of AICD was not observed in FBS cultures where IFNγ was suppressed. Since Th1 cells are susceptible to AICD (112), our data suggest that n-3 PUFA may *indirectly* enhance AICD via promotion of a Th1 phenotype.

It has been reported that IFNγ is required for T cell AICD (161). IFNγ signaling results in activation of Stat-1, which translocates to the nucleus and induces expression of caspase 8, the initiator caspase associated with the DISC. Thus, IFNγ and Stat-1 are involved in apoptosis mediated by death receptors. Refaeli et al. (161) showed that T cells from IFNγ -/- or Stat-1 -/- mice are unable to undergo apoptosis. Our data indicating that dietary FO enhances the number of IFNγ-producing $CD4^+$ T cells relative to dietary CO (**Fig. 10**) suggests that IFNγ may mediate the n-3 PUFA enhancement of AICD. Figure 16 illustrates the putative mechanisms by which IFNγ may mediate the n-3 PUFA enhancement of AICD.

The IFNγ receptor (IFNγR) has recently been found to be recruited to raft-like domains in T cells following IFNγ stimulation (162). IFNγ binding and receptor relocalization to rafts is followed by IFNγR endocytosis and translocation of the IFNγ/IFNγR/Stat-1 complex to the nucleus (162). Since our data clearly show that dietary n-3 PUFA remodel $CD4^+$ T cell lipid rafts (**Fig. 14 & 15, Table 9**), it is possible that n-3 PUFA modulate IFNγ signaling by enhancing IFNγR raft localization due to

alterations in raft composition (**Fig. 16**). Experiments are in progress in order to test this hypothesis.

Alterations in the methylation status of the IFNγ promoter plays a critical role in regulating IFNγ production (163). For example, DNA methylation may interfere with transcription by preventing the recruitment of acetylase to the local chromatin, thereby inhibiting acquisition of the open conformation suitable for recruitment of transcription factors (163). Recently, it has been shown that the IFNγ promoter undergoes differential methylation during *in vitro* differentiation, with the promoter being in a hypomethylated state in Th1 cells, whereas it is hypermethylated in Th2 cells (163). Interestingly, prostaglandin E_2 (PGE_2) inhibits IFNγ promoter hypomethylation (164). This is noteworthy, because n-3 PUFA decrease PGE_2 production (39), whereas n-6 PUFA promote the synthesis of PGE_2 (165). Overall, these data suggest that the reduced IFNγ expression in n-6 PUFA-fed mice in our studies (Fig. 10) might be due to reduced IFNγ hypomethylation mediated by PGE_2 (**Fig. 16**). Additional studies to address the involvement of this mechanism are in progress.

Another mechanism by which n-3 PUFA could alter $CD4^+$ T cell polarization might involve peroxisome proliferator-activated receptors (PPARs), specifically PPARγ. PPARγ has been shown to inhibit IL-4 production in $CD4^+$ T cells (166). However, PPARγ also reduces IFNγ and IL-2 in human T cells (167), indicating that PPARγ is not selective in T cell subset repression. Additionally, PPARγ binds both n-3 and n-6 PUFA with equal affinity and lacks fatty acid class specificity (59,168). Finally, we have already shown that our short-term feeding of n-3 PUFA does not induce increased

PPARγ mRNA (**Table 6**). Therefore, the effects of n-3 PUFA observed in this study are likely not mediated by PPARs.

In conclusion, our data support the hypothesis that dietary n-3 PUFA promote AICD in $CD4^+$ T cells which are polarized toward the Th1 phenotype *in vitro*. Susceptibility to AICD in T cells from FO-fed mice appears to be an indirect result of the propensity of those cells to be induced to acquire a Th1 phenotype, i.e., produce IFNγ. We demonstrated the requirement for homologous MS in long-term cultures in order to preclude the loss of diet-derived n-3 PUFA from lipid rafts. Furthermore, we have shown for the first time that the maintenance of diet-induced membrane alterations *in vitro* is required to observe the biological impact of diet on $CD4^+$ T cell function.

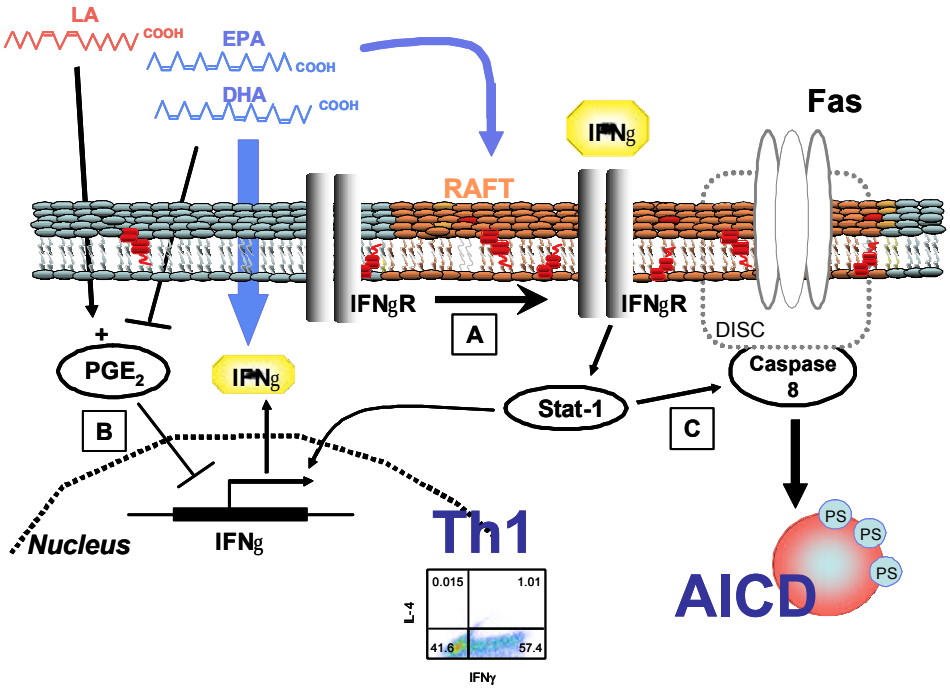

Figure 16. Scheme depicting the putative mechanisms by which IFNg may mediate the n-3 PUFA enhancement of AICD. [**A**] n-3 polyunsaturated fatty acid (PUFA) induced alteration of lipid raft composition may promote IFNγ receptor (IFNγR) localization to rafts, thereby permitting endocytosis and nuclear translocation of the IFNγ/IFNγR/Stat-1 complex (162), resulting in enhanced IFNγ expression (Th1 polarization). [**B**] Alternatively, n-3 PUFA may promote IFNγ expression through the reduction of prostaglandin E_2 (PGE_2), an inhibitor of IFNγ transcription (164). [**C**] The enhancement of IFNγ expression by n-3 PUFA may promote the activation of caspase 8, resulting in increased activation-induced cell death (AICD) (161). Plasma membrane represents the phospholipid-rich phase of the membrane. Lipid raft represents the sphingolipid and cholesterol enriched portion of the membrane. LA, linoleic acid (n-6 PUFA); EPA, eicosapentaenoic acid (n-3 PUFA); DHA, docosahexaenoic acid (n-3 PUFA); DISC, death-inducing signaling complex.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Among dietary factors, n-3 PUFA found in fish oil (FO) have been shown to attenuate T cell-mediated inflammatory diseases in humans and experimental model systems (34,35,127). The primary effector molecules are thought to be eicosapentaenoic acid [20:5n-3, EPA] and docosahexaenoic acid [22:6n-3, DHA]. In general, consumption of diets rich in n-3 PUFA are associated with a reduced pro-inflammatory T cell response due, in part, to a decreased proliferative capacity attributed to a reduction in IL-2 production and/or function (74,75,83). Additionally, we have previously observed that the dietary effect of n-3 PUFA on T cell proliferation depends upon the propensity of the *in vitro* stimulus to bias T cells towards a predominant IL-2/IFNγ producing population (a putative Th1-like subset) or an IL-4/IL-10 producing population (a putative Th2-like subset) (83). Since the suppressive effects of n-3 PUFA on the accumulation of inflammatory T cells could result from either reduced proliferation, enhanced apoptosis of activated T cells, or both, we investigated the effect of dietary n-3 PUFA on T cell apoptosis. We specifically examined AICD since it is the form of apoptosis associated with the deletion of cells involved in chronic inflammation and autoimmune disease. Consistent with this thinking, we focused on the $CD4^+$ Th1 cell subset, as it is the cell mediator of chronic inflammation.

Our initial investigation of the effect of dietary n-3 PUFA on T cell apoptosis showed that n-3 PUFA enhanced T cell apoptosis following *in vitro* incubation with selective stimuli. Purified splenic T cells from SAF, OO, and FO2-fed mice were cultured in αCD3/αCD28, αCD3/PMA, or PMA/Ionomycin for 24 h. FO2 significantly increased the percentage of apoptotic cells relative to both SAF and OO only in PMA/Ionomycin stimulated T cells (**Fig. 3B**). Cytokine analyses of cell culture supernatants revealed that αCD3/PMA stimulated T cells secreted significantly more IL-4 and IL-10, Th2 cytokines (**Fig. 5**). In comparison, PMA/Ionomycin stimulated T cells secreted significantly more IL-2, and much less IL-4, indicative of a Th1 phenotype. Thus, in agreement with previous studies (83), αCD3/PMA drove T cells towards a Th2-like cytokine profile, while PMA/Ionomycin drove T cells towards a Th1-like cytokine profile. Additionally, splenic T cells from FO2-fed mice had a modest increase in apoptosis following incubation in FasL + αFLAG (**Fig. 3C**), suggesting that n-3 PUFA are capable of modulating Fas signaling pathways.

Since AICD is primarily regulated by Fas and FasL (96,120,121), we next determined the effect of n-3 PUFA on T cell AICD. Similar to our initial apoptosis experiment, n-3 PUFA significantly increased AICD only following PMA/Ionomycin stimulation, or in Th1-like cells (**Fig. 8D**). Furthermore, a comparison of dose (4% FO *vs* 9% FO) demonstrated that there was no difference in AICD, thus T cell AICD can occur just as efficiently at low n-3 PUFA intakes. Th1 effectors are more susceptible to AICD (112,113), therefore we hypothesized that the ability of n-3 PUFA to enhance AICD following PMA/Ionomycin stimulation was due to the induction of a biased

pattern of cytokine production in T cells. In an attempt to determine whether the pro-apoptotic effects of dietary FO were mediated by DHA, we fed mice diets composed of 1% DHA ethyl esters or CO. To our surprise, DHA did not enhance apoptosis (**Fig. 9**), suggesting that DHA, by itself, is not the active molecule in FO with respect to AICD.

Next, we wanted to determine whether the ingestion of different lipid sources altered systemic oxidative stress, since long chain n-3 PUFA are highly susceptible to peroxidation. This is significant because the formation of reactive oxygen species and/or glutathione depletion can regulate signals involved in AICD (144). Our results indicate that there was no change in oxidative status (**Table 6**), suggesting that short-term feeding of n-3 PUFA did not promote the generation of pro-apoptotic reactive oxygen species in our model.

Based on our results that n-3 PUFA enhanced AICD in T cells induced to secrete a Th1-biased cytokine profile, we hypothesized that n-3 PUFA would also promote AICD in $CD4^+$ T cells driven to differentiate *in vitro* towards a Th1 cytokine pole using standard polarization methodology. Due to the extended time in culture to achieve polarization, a set of cells from each diet group were cultured in diet-matched homologous MS, rather than fetal bovine serum (FBS). This approach prevented culture-induced loss of n-3 PUFA from membrane phospholipids. Following verification that the cells were appropriately polarized towards a Th1 phenotype, it was demonstrated that dietary CO suppressed Th1 polarization relative to FO and that this effect only occurred in cells cultured in the presence of homologous MS (**Fig. 10**). Analysis of AICD in $CD4^+$ T cells from FO-fed mice cultured in the presence of

homologous MS had significantly enhanced AICD (**Fig. 11**). Therefore the diet and culture conditions that promoted the greatest number of Th1 cells also enhanced AICD to the greatest extent. Since Th1 cells are susceptible to AICD (112,113), our data suggest that n-3 PUFA may *indirectly* enhance AICD via promotion of a Th1 phenotype.

That the phenotypes were lost when cells were cultured in FBS led us to hypothesize that homologous MS maintained diet-induced changes in membrane fatty acid composition. In addition, recent data have shown that n-3 PUFA remodel T cell lipid rafts (45), therefore we examined the effect of culture conditions on $CD4^+$ T cell plasma membrane microdomains, i.e., rafts. Analysis of the fatty acid composition of cell phospholipids showed that the inclusion of MS prevented the culture-induced rearrangement of T cell lipids (**Table 9**). Detailed examination of membrane microdomains revealed that the significant enhancement of EPA and DHA in the $CD4^+$ T cells from FO-fed mice, compared to CO-fed mice, was lost in cells cultured in FBS (**Fig. 14**). Cholesterol analysis revealed that only in rafts isolated from $CD4^+$ T cells from FO-fed mice did the addition of homologous MS prevent the culture-induced loss of cholesterol (**Fig. 15**). Since cholesterol is critical for raft integrity (145), these results indicate that culture conditions have a profound effect on lipid raft composition and structure.

In summary, our results add to the wealth of clinical and experimental data describing the anti-inflammatory properties of n-3 PUFA. Our novel findings contribute significantly to the elucidation of mechanisms by which dietary n-3 PUFA selectively modulate T cell subset function. Furthermore, we have shown that cell culture

conditions (e.g., serum source) are critical to observe the biological impact of diet on cell function *in vitro*. Studies such as these will aid in the establishment of dietary guidelines designed to promote a balanced immune system, so that protective host responses (e.g., to infectious agents) can be maintained, while potentially detrimental host responses (e.g., chronic inflammation and hypersensitivity) can be controlled.

Future studies are needed to determine the precise cellular and molecular mechanisms by which dietary n-3 PUFA promote Th1 polarization and subsequent AICD.

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APPENDIX A

EXPERIMENTAL DATA

APPENDIX A-1

CHAPTER II - MEANS ± SEM

Means ± SEM for Fig. 2

	Early apoptosis 4h	Late apoptosis 4h
RPMI	8.18 ± 0.17	11.54 ± 0.32
DMSO	8.46 ± 0.00	12.02 ± 0.00
aFLAG	8.56 ± 0.17	12.56 ± 0.32
FasL	9.89 ± 0.35	17.52 ± 0.38
FasL + ZVAD 50	9.24 ± 0.23	10.13 ± 0.04
FasL + ZVAD 20	9.45 ± 0.20	11.58 ± 0.06
ZVAD 20	8.97 ± 0.00	10.83 ± 0.00

	Early apoptosis 24h	Late apoptosis 24h
RPMI	3.55 ± 0.18	43.07 ± 0.54
DMSO	3.41 ± 0.00	41.70 ± 0.00
aFLAG	3.49 ± 0.10	42.74 ± 0.56
FasL	2.42 ± 0.13	71.75 ± 0.51
FasL + ZVAD 50	3.61 ± 0.12	39.73 ± 0.88
FasL + ZVAD 20	3.56 ± 0.08	41.04 ± 1.15
ZVAD 20	3.35 ± 0.20	42.32 ± 0.79

values expressed as % AV+/PI- or %AV+/PI+

Means ± SEM for Fig. 3

Early apoptosis	SAF	OO	FO2
RPMI	3.35 ± 0.39	2.68 ± 0.22	2.13 ± 0.24
CD3/CD28	3.74 ± 0.35	3.80 ± 0.32	2.67 ± 0.50
CD3/PMA	5.24 ± 0.24	4.35 ± 0.50	4.21 ± 0.27
PMA/Iono	9.19 ± 0.50	8.84 ± 0.28	10.09 ± 0.65
Dexamethasone	1.53 ± 0.11	1.68 ± 0.10	1.42 ± 0.15
FasL + aFLAG	1.91 ± 0.15	2.01 ± 0.14	2.71 ± 0.46

values expressed as % AV+/PI-

Late apoptosis	SAF	OO	FO2
RPMI	54.06 ± 3.82	53.83 ± 4.56	46.07 ± 3.46
CD3/CD28	48.23 ± 8.04	48.94 ± 9.17	38.65 ± 6.02
CD3/PMA	50.29 ± 8.01	46.11 ± 6.73	36.43 ± 2.23
PMA/Iono	24.55 ± 1.55	26.42 ± 1.47	25.82 ± 0.30
Dexamethasone	91.41 ± 0.66	91.04 ± 1.49	92.28 ± 1.23
FasL + aFLAG	85.29 ± 0.37	86.26 ± 1.77	85.42 ± 0.64

values expressed as % AV+/PI+

CHAPTER II - MEANS ± SEM (cont.)

Means ± SEM for Fig. 5

	CD3/CD28		CD3/PMA		PMA/Iono	
IFNg	233302.50	± 5021.16	224857.7	± 6193.46	94209.2	± 2420.00
IL-10	200.63	± 19.01	752.35	± 94.26	125.47	± 4.30
IL-2	3424.87	± 207.50	586.16	± 83.88	20920.6	± 827.41
IL-4	837.39	± 48.17	1345.62	± 116.16	36.79	± 15.45

values expressed as pg/ml

Means ± SEM for Fig. 6

	CD3/CD28		CD3/PMA		PMA/Iono	
IL-4						
SAF	780.33	± 31.04	1010.73	± 37.37	64.90	± 35.13
FO4	882.93	± 114.94	1503.53	± 155.28	11.03	± 6.16
FO9	848.90	± 104.58	1522.60	± 228.49	34.43	± 29.37
IFNg						
SAF	230270.0	± 7707.49	210515.3	± 9866.03	98070.2	± 2420.34
FO4	235705.8	± 5258.06	220641.6	± 6428.63	97628.3	± 3903.71
FO9	233931.7	± 12924.3	242713.4	± 10725.2	85473.2	± 4576.23
IL-10						
SAF	155.82	± 39.62	719.79	± 97.32	133.79	± 8.49
FO2	148.84	± 8.90	564.13	± 32.64	113.31	± 4.94
FO4	263.81	± 35.12	1026.91	± 226.10	129.32	± 6.77
IL-2						
SAF	3228.25	± 358.63	848.54	± 131.79	19106.9	± 1070.33
FO2	3432.54	± 288.70	580.12	± 129.26	22421.5	± 1118.47
FO4	3581.04	± 458.19	329.81	± 103.77	21233.3	± 1859.70

values expressed as pg/ml

Means ± SEM for Fig. 7

	Early apoptosis		Late apoptosis	
PMA/Iono	99.92	± 1.31	100.01	± 3.34
P/I + Fas-Fc	81.77	± 1.99	95.48	± 4.27
P/I + IgG	91.32	± 4.88	101.91	± 2.05
P/I + ZVAD	95.25	± 3.21	96.10	± 0.94
RPMI	54.27	± 2.00	72.80	± 2.86

values expressed as % AV+/PI- or %AV+/PI+

CHAPTER II - MEANS ± SEM (cont.)

Means ± SEM for Fig. 8

	SAF	FO4	FO9
CD3/CD28	30.32 ± 2.57	26.62 ± 1.43	29.65 ± 1.42
CD3/PMA	44.33 ± 1.76	43.45 ± 1.05	42.33 ± 1.15
PMA/Iono	6.66 ± 0.66	8.14 ± 0.42	9.47 ± 0.38

	TOTAL
CD3/CD28	30.24 ± 0.62
CD3/PMA	42.93 ± 0.52
PMA/Iono	8.23 ± 0.27

values expressed as % AV+/PI-

Means ± SEM for Fig. 9

	CO	DHA
CD3/CD28	29.02 ± 1.10	29.02 ± 1.28
CD3/PMA	27.62 ± 0.98	25.92 ± 0.64
PMA/Iono	5.55 ± 0.34	6.02 ± 0.27

values expressed as % AV+/PI-

APPENDIX A-2

CHAPTER III - MEANS ± SEM

Means ± SEM for Fig. 10B

	Intracellular cytokines		
FBS-CO	58.18	±	2.14
FBS-FO	58.85	±	1.64
MS-CO	36.93	±	2.36
MS-FO	55.05	±	1.12

values are expresssed as %IFNγ +/IL-4 -

Means ± SEM for Fig. 11B

	Total apoptosis		
FBS-CO	54.27	±	2.45
FBS-FO	50.83	±	0.99
MS-CO	55.87	±	0.92
MS-FO	69.32	±	0.35

values are expresssed as % apoptotic cells (AV+/PI- + AV+/PI+)

Means ± SEM for Fig. 13

	chol/PL		
Raft	0.031	±	0.007
Soluble	0.020	±	0.006

values are expressed as a cholesterol to phospholipid molar ratio (chol/PL)

CHAPTER III - MEANS ± SEM (cont.)

Means ± SEM for Fig. 14

20:5n-3	Day 0	Day 5 FBS	Day 5 MS
CO			
raft	0.00 ± 0.00	0.00 ± 0.00	0.29 ± 0.16
soluble	0.45 ± 0.45	0.00 ± 0.00	0.00 ± 0.00
FO			
raft	0.83 ± 0.66	0.00 ± 0.00	0.26 ± 0.14
soluble	1.14 ± 0.62	0.26 ± 0.13	0.96 ± 0.50

22:6n-3	Day 0	Day 5 FBS	Day 5 MS
CO			
raft	0.61 ± 0.31	1.01 ± 0.17	1.95 ± 0.12
soluble	2.42 ± 0.50	1.29 ± 0.14	3.80 ± 0.24
FO			
raft	2.47 ± 1.27	1.17 ± 0.06	3.61 ± 0.38
soluble	4.54 ± 0.34	1.92 ± 0.04	6.26 ± 1.31

values are expressed as mol%

Means ± SEM for Fig. 15

	Day 0	Day 5 FBS	Day 5 MS
CO			
raft	0.077 ± 0.030	0.012 ± 0.001	0.018 ± 0.009
soluble	0.029 ± 0.004	0.008 ± 0.003	0.008 ± 0.002
FO			
raft	0.033 ± 0.006	0.015 ± 0.002	0.032 ± 0.010
soluble	0.050 ± 0.029	0.009 ± 0.002	0.014 ± 0.008

values are expressed as mean chol/PL ratio

APPENDIX A-3

EXPT. 1 – APOPTOSIS DATA

Diet	Mouse	Stimulus	%Early	%Late	%Total
SAF	1	CD3/CD28	2.72	26.65	29.37
SAF	2	CD3/CD28	4.11	46.36	50.47
SAF	3	CD3/CD28	3.4	56.05	59.45
SAF	4	CD3/CD28	3.92	66.74	70.66
SAF	5	CD3/CD28	5.14	69.83	74.97
SAF	6	CD3/CD28	3.13	23.77	26.9
OO	7	CD3/CD28	5.25	63.91	69.16
OO	8	CD3/CD28	3.49	39.75	43.24
OO	9	CD3/CD28	2.89	18.95	21.84
OO	10	CD3/CD28	3.76	67.88	71.64
OO	11	CD3/CD28	3.87	73.27	77.14
OO	12	CD3/CD28	3.54	29.9	33.44
FO	13	CD3/CD28	5.05	63.07	68.12
FO	14	CD3/CD28	1.94	29.92	31.86
FO	15	CD3/CD28	2.24	35.06	37.3
FO	16	CD3/CD28	2.68	29.29	31.97
FO	17	CD3/CD28	2.41	49.72	52.13
FO	18	CD3/CD28	1.71	24.84	26.55

Diet	Mouse	Stimulus	%Early	%Late	%Total
SAF	1	CD3/PMA	5.1	57.96	63.06
SAF	2	CD3/PMA	5.91	69.76	75.67
SAF	3	CD3/PMA	5.41	62.32	67.73
SAF	4	CD3/PMA	5.86	23.05	28.91
SAF	5	CD3/PMA	4.46	28.07	32.53
SAF	6	CD3/PMA	4.71	60.6	65.31
OO	7	CD3/PMA	3.42	35.75	39.17
OO	8	CD3/PMA	6.17	64.38	70.55
OO	9	CD3/PMA	4.14	27.67	31.81
OO	10	CD3/PMA	3.04	36.59	39.63
OO	11	CD3/PMA	3.88	68.06	71.94
OO	12	CD3/PMA	5.44	44.23	49.67
FO	13	CD3/PMA	4.76	28.34	33.1
FO	14	CD3/PMA	3.57	40.16	43.73
FO	15	CD3/PMA	4.13	37.1	41.23
FO	16	CD3/PMA	5.11	31.98	37.09
FO	17	CD3/PMA	4.24	37.66	41.9
FO	18	CD3/PMA	3.45	43.36	46.81

EXPT. 1 – APOPTOSIS DATA (cont.)

Diet	Mouse	Stimulus	%Early	%Late	%Total
SAF	1	PMA/Iono	8.85	23.3	32.15
SAF	2	PMA/Iono	9.89	27.08	36.97
SAF	3	PMA/Iono	9.46	19.96	29.42
SAF	4	PMA/Iono	11.06	21.85	32.91
SAF	5	PMA/Iono	8.3	30.56	38.86
SAF	6	PMA/Iono	7.56	24.53	32.09
OO	7	PMA/Iono	9.67	31.76	41.43
OO	8	PMA/Iono	8.65	30.06	38.71
OO	9	PMA/Iono	8.13	25.02	33.15
OO	10	PMA/Iono	9.21	22.73	31.94
OO	11	PMA/Iono	7.97	24.76	32.73
OO	12	PMA/Iono	9.38	24.21	33.59
FO	13	PMA/Iono	11.11	26.44	37.55
FO	14	PMA/Iono	10.14	25.32	35.46
FO	15	PMA/Iono	10.27	24.85	35.12
FO	16	PMA/Iono	10.78	26.48	37.26
FO	17	PMA/Iono	11.27	26.48	37.55
FO	18	PMA/Iono	6.98	25.35	32.33

Diet	Mouse	Stimulus	%Early	%Late	%Total
SAF	1	RPMI	3.43	57.6	61.03
SAF	2	RPMI	3.68	56.16	59.84
SAF	3	RPMI	4.99	69.03	74.02
SAF	4	RPMI	2.45	42.97	45.42
SAF	5	RPMI	2.43	53.11	55.54
SAF	6	RPMI	3.09	45.5	48.59
OO	7	RPMI	1.82	38.41	40.23
OO	8	RPMI	3.44	71.14	74.58
OO	9	RPMI	2.95	59.39	62.34
OO	10	RPMI	2.45	49.82	52.27
OO	11	RPMI	2.57	56.15	58.72
OO	12	RPMI	2.87	48.05	50.92
FO	13	RPMI	3.13	47.12	50.25
FO	14	RPMI	1.75	40.57	42.32
FO	15	RPMI	2.52	59.25	61.77
FO	16	RPMI	1.97	52.09	54.06
FO	17	RPMI	1.77	40.9	42.67
FO	18	RPMI	1.65	36.49	38.14

EXPT. 1 – APOPTOSIS DATA (cont.)

Diet	Mouse	Stimulus	%Early	%Late	%Total
SAF	1	Dexameth	1.5	92.18	93.68
SAF	2	Dexameth	2.04	90.33	92.37
SAF	3	Dexameth	1.42	88.79	90.21
SAF	4	Dexameth	1.53	93.45	94.98
SAF	5	Dexameth	1.44	91.88	93.32
SAF	6	Dexameth	1.24	91.81	93.05
OO	7	Dexameth	1.63	94.17	95.8
OO	8	Dexameth	1.81	91.96	93.77
OO	9	Dexameth	1.69	89.04	90.73
OO	10	Dexameth	1.28	92.47	93.75
OO	11	Dexameth	1.67	93.97	95.64
OO	12	Dexameth	2.01	84.6	86.61
FO	13	Dexameth	2.13	89.01	91.14
FO	14	Dexameth	1.34	94.65	95.99
FO	15	Dexameth	1.44	91.1	92.54
FO	16	Dexameth	1.23	89.7	90.93
FO	17	Dexameth	1.05	96.86	97.91
FO	18	Dexameth	1.31	92.35	93.66

Diet	Mouse	Stimulus	%Early	%Late	%Total
SAF	1	FasL/FLAG	2.25	84.17	86.42
SAF	2	FasL/FLAG	2.22	86.14	88.36
SAF	3	FasL/FLAG	1.81	84.07	85.88
SAF	4	FasL/FLAG	2.16	85.82	87.98
SAF	5	FasL/FLAG	1.71	85.85	87.56
SAF	6	FasL/FLAG	1.29	85.67	86.96
OO	7	FasL/FLAG	1.78	86.63	88.41
OO	8	FasL/FLAG	1.96	86.9	88.86
OO	9	FasL/FLAG	1.88	81.5	83.38
OO	10	FasL/FLAG	1.61	92.81	94.42
OO	11	FasL/FLAG	2.32	88.33	90.65
OO	12	FasL/FLAG	2.53	81.41	83.94
FO	13	FasL/FLAG	2.43	83.67	86.1
FO	14	FasL/FLAG	4.07	85.32	89.39
FO	15	FasL/FLAG	1.86	87.03	88.89
FO	16	FasL/FLAG	2.54	83.51	86.05
FO	17	FasL/FLAG	4.05	86.99	91.04
FO	18	FasL/FLAG	1.33	86.01	87.34

APPENDIX A-4

EXPT. 2 – APOPTOSIS DATA

Diet	Mouse	Stimulus	% Early	% Late	% Total
SAF	1	RPMI	1.7	68.7	70.4
SAF	2	RPMI	1.44	64.9	66.3
SAF	3	RPMI	1.97	72.9	74.9
SAF	4	RPMI	1.72	70.8	72.5
SAF	5	RPMI	0.82	61	61.8
SAF	6	RPMI	2.32	74.5	76.8
OO	7	RPMI	0.88	70.5	71.4
OO	8	RPMI	1.02	70.5	71.5
OO	9	RPMI	0.97	71.5	72.5
OO	10	RPMI	0.75	66.5	67.3
OO	11	RPMI	0.97	58.2	59.2
OO	12	RPMI	0.97	69.1	70.1
FO	13	RPMI	0.98	69.2	70.2
FO	14	RPMI	0.92	71.9	72.8
FO	15	RPMI	0.97	69.1	70.1
FO	16	RPMI	0.94	68.2	69.1
FO	17	RPMI	0.67	69.4	70.1
FO	18	RPMI	2.07	66.8	68.9

Diet	Mouse	Stimulus	% Early	% Late	% Total
SAF	1	CD3/CD28	3.89	36.8	40.7
SAF	2	CD3/CD28	1.62	15.4	17.0
SAF	3	CD3/CD28	2.37	28.8	31.2
SAF	4	CD3/CD28	3.95	28.8	32.8
SAF	5	CD3/CD28	2.6	32	34.6
SAF	6	CD3/CD28	3.19	31.1	34.3
OO	7	CD3/CD28	2.93	27.5	30.4
OO	8	CD3/CD28	1.84	28.1	29.9
OO	9	CD3/CD28	1.68	26.8	28.5
OO	10	CD3/CD28	4.3	37.3	41.6
OO	11	CD3/CD28	2.01	23.9	25.9
OO	12	CD3/CD28	1.63	32.6	34.2
FO	13	CD3/CD28	3.5	32.8	36.3
FO	14	CD3/CD28	1.66	23.3	25.0
FO	15	CD3/CD28	2.11	27.3	29.4
FO	16	CD3/CD28	2.12	25.5	27.6
FO	17	CD3/CD28	1.91	27.1	29.0
FO	18	CD3/CD28	2.21	32	34.2

EXPT. 2 – APOPTOSIS DATA (cont.)

Diet	Mouse	Stimulus	% Early	% Late	% Total
SAF	1	PMA/Iono	2.19	17.2	19.4
SAF	2	PMA/Iono	1.61	15.5	17.1
SAF	3	PMA/Iono	1.28	14.5	15.8
SAF	4	PMA/Iono	2.51	17.8	20.3
SAF	5	PMA/Iono	2.31	22.3	24.6
SAF	6	PMA/Iono	1.84	19.7	21.5
OO	7	PMA/Iono	1.38	11.7	13.1
OO	8	PMA/Iono	5.84	26.2	32.0
OO	9	PMA/Iono	1.47	15.2	16.7
OO	10	PMA/Iono	1.21	13	14.2
OO	11	PMA/Iono	1.18	13.4	14.6
OO	12	PMA/Iono	4.14	20.5	24.6
FO	13	PMA/Iono	0.93	12.8	13.7
FO	14	PMA/Iono	1.37	16.2	17.6
FO	15	PMA/Iono	1.19	15	16.2
FO	16	PMA/Iono	1.25	15.2	16.5
FO	17	PMA/Iono	1.74	17.1	18.8
FO	18	PMA/Iono	1.13	15.9	17.0

Diet	Mouse	Stimulus	% Early	% Late	% Total
SAF	1	CD3/PMA	3	21	24.0
SAF	2	CD3/PMA	6.12	30.3	36.4
SAF	3	CD3/PMA	4.49	22.4	26.9
SAF	4	CD3/PMA	4.47	24.2	28.7
SAF	5	CD3/PMA	4.63	24.4	29.0
SAF	6	CD3/PMA	3.15	24.2	27.4
OO	7	CD3/PMA	3.62	19.9	23.5
OO	8	CD3/PMA	4.73	28	32.7
OO	9	CD3/PMA	3.43	22.3	25.7
OO	10	CD3/PMA	6.91	32.9	39.8
OO	11	CD3/PMA	4.75	21.7	26.5
OO	12	CD3/PMA	2.9	24.7	27.6
FO	13	CD3/PMA	0.087	0.52	0.6
FO	14	CD3/PMA	0.68	4.88	5.6
FO	15	CD3/PMA	13.2	41.4	54.6
FO	16	CD3/PMA	5.39	26	31.4
FO	17	CD3/PMA	5.03	27.1	32.1
FO	18	CD3/PMA	2.99	24.1	27.1

EXPT. 2 – APOPTOSIS DATA (cont.)

Diet	Mouse	Stimulus	% Early	% Late	% Total
SAF	1	FasL/FLAG	0.63	78.7	79.3
SAF	2	FasL/FLAG	0.66	71.4	72.1
SAF	3	FasL/FLAG	0.61	80.3	80.9
SAF	4	FasL/FLAG	0.54	78.7	79.2
SAF	5	FasL/FLAG	0.47	85.9	86.4
SAF	6	FasL/FLAG	1.02	76.2	77.2
OO	7	FasL/FLAG	0.58	74	74.6
OO	8	FasL/FLAG	0.7	76.3	77.0
OO	9	FasL/FLAG	0.46	77.6	78.1
OO	10	FasL/FLAG	0.56	82	82.6
OO	11	FasL/FLAG	0.44	74.2	74.6
OO	12	FasL/FLAG	0.53	85.4	85.9
FO	13	FasL/FLAG	0.32	82.2	82.5
FO	14	FasL/FLAG	0.37	77.2	77.6
FO	15	FasL/FLAG	0.54	83.5	84.0
FO	16	FasL/FLAG	0.49	77.6	78.1
FO	17	FasL/FLAG	0.47	82.7	83.2
FO	18	FasL/FLAG	0.53	79.2	79.7

Diet	Mouse	Stimulus	% Early	% Late	% Total
SAF	1	P/I/F/F	3.13	18.1	21.2
SAF	2	P/I/F/F	2.86	31.3	34.2
SAF	3	P/I/F/F	3.3	18	21.3
SAF	4	P/I/F/F	3.16	15.1	18.3
SAF	5	P/I/F/F	1.05	10.6	11.7
SAF	6	P/I/F/F	2.95	18.3	21.3
OO	7	P/I/F/F	2.65	14.6	17.3
OO	8	P/I/F/F	4.26	21.8	26.1
OO	9	P/I/F/F	4.13	19	23.1
OO	10	P/I/F/F	1.49	15.6	17.1
OO	11	P/I/F/F	2.74	18.5	21.2
OO	12	P/I/F/F	1.75	13.3	15.1
FO	13	P/I/F/F	2.38	17.2	19.6
FO	14	P/I/F/F	2.7	20.1	22.8
FO	15	P/I/F/F	3.42	22.7	26.1
FO	16	P/I/F/F	3.58	20.5	24.1
FO	17	P/I/F/F	3.21	21	24.2
FO	18	P/I/F/F	4.68	24.9	29.6

EXPT. 2 – APOPTOSIS DATA (cont.)

Diet	Mouse	Stimulus	% Early	% Late	% Total
SAF	1	3/P/F/F	10.1	42.4	52.5
SAF	2	3/P/F/F	7.85	37.2	45.1
SAF	3	3/P/F/F	9.46	38.8	48.3
SAF	4	3/P/F/F	10.9	41.5	52.4
SAF	5	3/P/F/F	10	42.6	52.6
SAF	6	3/P/F/F	8.02	42.8	50.8
OO	7	3/P/F/F	8.75	38.8	47.6
OO	8	3/P/F/F	7.91	43.3	51.2
OO	9	3/P/F/F	9.6	39.7	49.3
OO	10	3/P/F/F	9.32	44.7	54.0
OO	11	3/P/F/F	10.6	36.9	47.5
OO	12	3/P/F/F	7.58	46.1	53.7
FO	13	3/P/F/F	7.25	39.8	47.1
FO	14	3/P/F/F	8.52	38.9	47.4
FO	15	3/P/F/F	8.72	41.7	50.4
FO	16	3/P/F/F	9.74	44.9	54.6
FO	17	3/P/F/F	8.67	45.8	54.5
FO	18	3/P/F/F	8.36	46	54.4

APPENDIX A-5

EXPT. 3 – APOPTOSIS DATA

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Stimulus	Mouse	%CD3+	CD3+ AV-/7AAD- LIVE	CD3+ AV+/7AAD- EARLY	CD3+ AV+/7AAD+ LATE	CD3+ AV-/7AAD+ DEAD	Median CD3 Fluor.
RPMI	1	40.1	42.8	2.2	49.4	5.6	703.0
RPMI	2	33.7	42.1	1.1	51.1	5.7	455.0
RPMI	3	40.1	54.9	1.2	40.5	3.4	765.0
RPMI	4	36.9	45.2	1.1	46.7	7.0	527.0
RPMI	5	35.4	46.5	1.5	48.7	3.3	677.0
RPMI	6	32.8	39.4	1.3	55.7	3.6	735.0
RPMI	7	36.5	44.3	1.5	50.0	4.3	780.0
RPMI	8	36.6	36.3	1.1	53.1	9.5	716.0
RPMI	9	35.1	48.2	0.9	45.7	5.2	744.0
RPMI	10	39.5	47.7	1.2	47.2	4.0	593.0
RPMI	11	34.5	46.3	1.0	48.2	4.5	667.0
RPMI	12	33.9	41.6	1.5	48.2	8.7	610.0
RPMI	13	36.8	53.0	1.5	40.7	4.8	615.0
RPMI	14	37.7	41.6	1.0	43.2	14.3	607.0
RPMI	15	31.3	49.2	1.0	45.2	4.7	506.0
RPMI	16	32.5	48.9	1.2	44.5	5.5	524.0
RPMI	17	32.1	41.8	0.8	49.9	7.5	621.0
RPMI	18	39.3	42.7	0.6	49.9	6.7	503.0

Stimulus	Mouse	%CD3+	LIVE	EARLY	LATE	DEAD	Median CD3 Fluor.
CD3/CD28	1	32.5	64.4	17.4	12.5	5.6	175.0
CD3/CD28	2	26.9	63.4	21.8	9.9	5.0	128.0
CD3/CD28	3	28.7	74.3	12.8	10.0	2.9	127.0
CD3/CD28	4	27.2	66.8	14.8	11.7	6.7	140.0
CD3/CD28	5	26.5	70.1	16.0	9.5	4.4	150.0
CD3/CD28	6	27.7	68.1	15.4	11.0	5.5	148.0
CD3/CD28	7	27.3	70.1	12.3	12.5	5.2	158.0
CD3/CD28	8	28.1	63.4	14.4	11.3	10.8	126.0
CD3/CD28	9	28.9	73.8	11.9	10.3	3.9	145.0
CD3/CD28	10	30.4	72.0	13.2	10.4	4.5	115.0
CD3/CD28	11	30.2	70.2	16.1	9.3	4.4	147.0
CD3/CD28	12	29.5	63.5	16.3	12.8	7.4	146.0
CD3/CD28	13	29.2	65.9	15.4	12.3	6.5	166.0
CD3/CD28	14	31.7	61.6	15.9	9.6	13.0	115.0
CD3/CD28	15	28.7	67.4	16.2	10.0	6.5	129.0
CD3/CD28	16	27.9	71.4	12.4	9.3	6.8	129.0
CD3/CD28	17	30.1	69.8	12.7	11.0	6.4	160.0
CD3/CD28	18	27.8	68.2	10.2	12.5	9.1	145.0

EXPT. 3 – APOPTOSIS DATA (cont.)

Stimulus	Mouse	%CD3+	LIVE	EARLY	LATE	DEAD	Median CD3 Fluor.
CD3/PMA	1	30.5	65.1	14.1	12.9	7.9	151.0
CD3/PMA	2	25.2	66.2	19.3	9.4	5.1	109.0
CD3/PMA	3	25.8	73.3	13.5	8.9	4.3	132.0
CD3/PMA	4	28.2	68.7	13.2	10.3	7.8	141.0
CD3/PMA	5	25.8	72.8	11.3	9.7	6.3	174.0
CD3/PMA	6	24.2	73.9	11.0	8.7	6.5	116.0
CD3/PMA	7	26.3	64.4	16.0	12.3	7.4	167.0
CD3/PMA	8	29.1	64.0	15.2	13.5	7.3	150.0
CD3/PMA	9	26.9	68.7	14.8	10.3	6.2	141.0
CD3/PMA	10	31.2	71.2	15.8	7.3	5.7	124.0
CD3/PMA	11	27.0	70.2	17.0	7.8	5.0	139.0
CD3/PMA	12	26.3	58.9	18.9	14.5	7.7	181.0
CD3/PMA	13	27.8	60.8	19.5	14.1	5.7	166.0
CD3/PMA	14	27.8	55.4	18.0	12.6	13.9	129.0
CD3/PMA	15	25.6	66.6	15.4	13.2	4.8	172.0
CD3/PMA	16	28.9	71.1	12.0	11.6	5.3	233.0
CD3/PMA	17	25.6	64.5	16.9	11.8	6.8	158.0
CD3/PMA	18	29.1	63.3	11.9	14.1	10.6	195.0

Stimulus	Mouse	%CD3+	LIVE	EARLY	LATE	DEAD	Median CD3 Fluor.
ConA	1	38.2	42.7	16.9	36.5	4.0	282.0
ConA	2	35.7	42.9	19.4	33.5	4.1	279.0
ConA	3	36.0	47.4	18.4	31.3	2.9	303.0
ConA	4	34.0	41.0	13.7	40.2	5.1	265.0
ConA	5	31.8	49.3	13.2	32.9	4.7	280.0
ConA	6	31.4	45.1	14.1	37.3	3.5	289.0
ConA	7	33.4	43.3	12.5	40.2	3.9	300.0
ConA	8	36.3	43.1	12.6	37.7	6.7	290.0
ConA	9	33.8	44.3	17.7	34.2	3.7	305.0
ConA	10	40.2	43.0	15.1	37.7	4.3	299.0
ConA	11	36.2	43.6	15.6	35.2	5.6	293.0
ConA	12	33.4	40.7	19.0	33.9	6.3	283.0
ConA	13	33.8	39.3	21.4	33.4	5.9	263.0
ConA	14	39.8	40.8	17.1	34.1	8.1	307.0
ConA	15	35.0	42.8	19.5	32.8	4.9	332.0
ConA	16	36.9	47.4	18.3	30.8	3.5	275.0
ConA	17	34.7	44.7	16.0	33.8	5.5	302.0
ConA	18	33.9	42.6	10.6	40.8	6.1	305.0

EXPT. 3 – APOPTOSIS DATA (cont.)

Accessory Cells

Diet	Stimulus	Mouse	%CD3-	CD3- AV-/7AAD- LIVE	CD3- AV+/7AAD- EARLY	CD3- AV+/7AAD+ LATE	CD3- AV-/7AAD+ DEAD	Median CD3 Fluor.
SAF	RPMI	1	59.3	25.4	5.0	68.2	1.4	2.2
SAF	RPMI	2	66.0	25.7	3.2	69.7	1.5	1.9
SAF	RPMI	3	59.1	25.5	2.4	71.3	0.8	1.7
SAF	RPMI	4	62.6	22.8	2.9	73.7	0.5	1.6
SAF	RPMI	5	64.2	25.5	5.0	68.7	0.7	1.7
SAF	RPMI	6	66.6	14.7	2.0	82.4	0.9	1.7
FO2	RPMI	7	62.9	17.5	2.1	79.8	0.6	1.6
FO2	RPMI	8	63.0	15.1	2.1	81.6	1.2	1.7
FO2	RPMI	9	64.3	26.2	2.2	70.8	0.8	2.2
FO2	RPMI	10	60.0	20.2	3.3	75.8	0.8	2.1
FO2	RPMI	11	65.1	20.3	2.3	76.1	1.3	1.9
FO2	RPMI	12	65.5	27.2	3.4	68.0	1.5	2.4
FO4	RPMI	13	62.7	24.3	3.9	70.5	1.3	1.7
FO4	RPMI	14	61.9	23.1	2.2	72.8	1.9	2.2
FO4	RPMI	15	68.4	26.3	3.0	69.4	1.3	2.2
FO4	RPMI	16	67.1	25.5	3.4	70.1	1.0	2.0
FO4	RPMI	17	67.3	23.2	2.8	72.6	1.4	2.4
FO4	RPMI	18	60.4	12.6	1.6	84.8	1.0	1.6

Diet	Stimulus	Mouse	%CD3-	LIVE	EARLY	LATE	DEAD	Median CD3 Fluor.
SAF	CD3/CD28	1	67.0	55.6	5.0	37.7	1.8	3.1
SAF	CD3/CD28	2	72.8	54.6	5.6	38.9	0.9	3.8
SAF	CD3/CD28	3	70.8	53.5	4.8	41.1	0.7	2.9
SAF	CD3/CD28	4	72.2	47.9	5.0	46.2	0.9	3.0
SAF	CD3/CD28	5	73.1	52.3	5.7	40.5	1.6	2.6
SAF	CD3/CD28	6	72.0	39.6	4.2	55.2	1.1	2.2
FO2	CD3/CD28	7	72.1	46.3	3.9	48.6	1.1	2.9
FO2	CD3/CD28	8	71.2	41.0	4.7	52.6	1.7	2.6
FO2	CD3/CD28	9	70.5	57.4	3.9	37.4	1.4	3.8
FO2	CD3/CD28	10	69.1	47.3	4.1	47.8	0.7	3.4
FO2	CD3/CD28	11	69.2	49.3	5.1	44.3	1.2	3.0
FO2	CD3/CD28	12	70.1	49.4	6.3	42.9	1.4	3.7
FO4	CD3/CD28	13	70.2	52.6	4.4	42.3	0.7	3.2
FO4	CD3/CD28	14	67.8	51.6	6.4	40.4	1.6	2.9
FO4	CD3/CD28	15	70.8	50.5	6.1	41.9	1.5	2.8
FO4	CD3/CD28	16	71.7	53.5	4.9	40.2	1.3	3.2
FO4	CD3/CD28	17	69.3	50.1	5.6	42.6	1.8	2.7
FO4	CD3/CD28	18	71.6	36.6	3.8	58.6	0.9	2.6

EXPT. 3 – APOPTOSIS DATA (cont.)

Diet	Stimulus	Mouse	%CD3-	LIVE	EARLY	LATE	DEAD	Median CD3 Fluor.
SAF	CD3/PMA	1	69.1	59.6	5.0	33.4	1.9	3.8
SAF	CD3/PMA	2	74.5	59.6	6.3	33.0	1.2	3.6
SAF	CD3/PMA	3	73.7	56.0	4.2	39.3	0.6	3.2
SAF	CD3/PMA	4	71.2	59.1	4.1	36.1	0.7	3.8
SAF	CD3/PMA	5	73.6	62.2	4.6	32.1	1.1	4.0
SAF	CD3/PMA	6	75.4	61.7	3.1	33.9	1.2	3.9
FO2	CD3/PMA	7	73.3	57.6	4.9	36.9	0.6	3.4
FO2	CD3/PMA	8	70.3	62.2	5.9	30.3	1.6	4.3
FO2	CD3/PMA	9	72.7	64.8	4.0	30.1	1.0	4.5
FO2	CD3/PMA	10	68.0	55.7	5.3	38.4	0.7	3.3
FO2	CD3/PMA	11	72.5	60.7	4.9	33.2	1.2	4.0
FO2	CD3/PMA	12	73.0	58.1	6.3	34.4	1.1	4.0
FO4	CD3/PMA	13	71.8	62.3	5.4	31.2	1.1	4.5
FO4	CD3/PMA	14	71.7	59.0	5.5	32.8	2.8	4.7
FO4	CD3/PMA	15	73.8	59.9	4.5	33.4	2.2	4.5
FO4	CD3/PMA	16	70.3	62.1	5.0	31.3	1.6	5.0
FO4	CD3/PMA	17	73.9	63.2	5.2	30.0	1.6	4.5
FO4	CD3/PMA	18	70.4	56.0	4.2	38.9	0.9	4.0

Diet	Stimulus	Mouse	%CD3-	LIVE	EARLY	LATE	DEAD	Median CD3 Fluor.
SAF	ConA	1	61.1	50.4	3.8	43.7	2.1	3.9
SAF	ConA	2	63.8	51.0	3.8	43.9	1.4	3.8
SAF	ConA	3	63.6	46.3	4.4	48.4	0.9	2.9
SAF	ConA	4	65.4	42.8	3.5	52.9	0.8	2.8
SAF	ConA	5	67.7	50.9	3.7	43.7	1.6	3.5
SAF	ConA	6	68.0	39.4	2.7	56.7	1.3	3.1
FO2	ConA	7	66.0	40.7	3.5	54.8	1.0	3.1
FO2	ConA	8	63.2	36.6	2.6	58.8	2.0	3.3
FO2	ConA	9	65.6	49.4	3.3	46.0	1.3	4.0
FO2	ConA	10	59.2	40.0	4.6	54.5	0.9	2.8
FO2	ConA	11	63.5	50.6	3.4	44.3	1.7	4.1
FO2	ConA	12	66.1	50.2	4.5	43.6	1.7	3.9
FO4	ConA	13	65.7	45.7	4.4	49.0	0.8	3.4
FO4	ConA	14	59.6	45.6	3.9	48.5	2.0	4.0
FO4	ConA	15	64.4	44.7	4.1	49.4	1.8	3.4
FO4	ConA	16	62.8	49.3	3.8	45.5	1.4	3.4
FO4	ConA	17	64.8	51.4	4.4	42.9	1.3	3.7
FO4	ConA	18	65.4	33.0	3.2	62.4	1.4	3.2

APPENDIX A-6

EXPT. 4 – APOPTOSIS DATA

			AV-/ PI-	AV+/ PI-	AV+/ PI+	AV-/ PI+
Diet	**Stimulus**	**Mouse**	**%Live**	**%Early**	**%Late**	**%Dead**
SAF	αCD3/ αCD28	**1**	69.0	3.9	22.4	4.7
SAF	αCD3/ αCD28	**2**	66.4	12.5	19.2	1.8
SAF	αCD3/ αCD28	**3**	65.5	15.9	16.2	2.5
SAF	αCD3/ αCD28	**4**	62.7	21.5	14.8	1.1
SAF	αCD3/ αCD28	**5**	62.2	18.1	18.2	1.5
SAF	αCD3/ αCD28	**6**	62.1	17.7	18.0	2.2
FO2	αCD3/ αCD28	**7**	67.6	11.8	18.7	1.9
FO2	αCD3/ αCD28	**8**	67.0	15.6	16.0	1.4
FO2	αCD3/ αCD28	**9**	60.3	16.9	20.2	2.5
FO2	αCD3/ αCD28	**10**	58.4	21.7	18.5	1.4
FO2	αCD3/ αCD28	**11**	68.1	14.3	15.5	2.0
FO2	αCD3/ αCD28	**12**	57.8	24.5	16.4	1.3
FO4	αCD3/ αCD28	**13**	64.7	17.1	16.8	1.4
FO4	αCD3/ αCD28	**14**	65.1	16.3	17.2	1.5
FO4	αCD3/ αCD28	**15**	65.6	13.9	17.7	2.8
FO4	αCD3/ αCD28	**16**	58.6	19.4	20.2	1.8
FO4	αCD3/ αCD28	**17**	63.8	16.7	17.8	1.7
FO4	αCD3/ αCD28	**18**	45.7	33.3	20.0	1.1

Diet	**Stimulus**	**Mouse**	**%Live**	**%Early**	**%Late**	**%Dead**
SAF	αCD3/ PMA	**1**	65.3	14.5	18.9	1.3
SAF	αCD3/ PMA	**2**	61.9	15.6	20.7	1.7
SAF	αCD3/ PMA	**3**	63.6	12.4	21.8	2.2
SAF	αCD3/ PMA	**4**	61.8	13.9	20.2	4.1
SAF	αCD3/ PMA	**5**	60.0	16.7	21.6	1.8
SAF	αCD3/ PMA	**6**	53.3	20.4	23.5	2.9
FO2	αCD3/ PMA	**7**	65.4	12.7	20.0	1.9
FO2	αCD3/ PMA	**8**	61.1	16.2	20.2	2.6
FO2	αCD3/ PMA	**9**	54.6	21.5	22.5	1.5
FO2	αCD3/ PMA	**10**	54.2	20.5	23.5	1.8
FO2	αCD3/ PMA	**11**	55.7	22.1	21.0	1.2
FO2	αCD3/ PMA	**12**	59.6	14.9	23.5	2.0
FO4	αCD3/ PMA	**13**	62.6	13.7	22.4	1.4
FO4	αCD3/ PMA	**14**	59.0	18.6	20.8	1.5
FO4	αCD3/ PMA	**15**	61.3	18.4	18.1	2.2
FO4	αCD3/ PMA	**16**	52.0	20.9	24.5	2.6
FO4	αCD3/ PMA	**17**	47.3	26.5	24.2	2.1
FO4	αCD3/ PMA	**18**	47.6	24.3	26.2	1.9

EXPT. 4 – APOPTOSIS DATA (cont.)

Diet	Stimulus	Mouse	%Live	%Early	%Late	%Dead
SAF	PMA/Iono	**1**	78.0	5.6	15.5	0.9
SAF	PMA/Iono	**2**	80.7	3.5	14.8	1.0
SAF	PMA/Iono	**3**	74.7	4.9	19.3	1.1
SAF	PMA/Iono	**4**	77.5	4.3	16.8	1.4
SAF	PMA/Iono	**5**	75.3	4.4	18.9	1.3
SAF	PMA/Iono	**6**	75.7	6.8	16.2	1.3
FO2	PMA/Iono	**7**	79.3	4.0	15.9	0.8
FO2	PMA/Iono	**8**	77.5	4.1	17.0	1.4
FO2	PMA/Iono	**9**	78.7	4.6	15.7	1.0
FO2	PMA/Iono	**10**	76.2	4.4	18.3	1.1
FO2	PMA/Iono	**11**	79.5	4.1	15.3	1.1
FO2	PMA/Iono	**12**	75.3	3.9	18.7	2.2
FO4	PMA/Iono	**13**	74.7	4.5	19.4	1.5
FO4	PMA/Iono	**14**	75.8	4.3	17.9	2.0
FO4	PMA/Iono	**15**	72.5	6.5	19.8	1.3
FO4	PMA/Iono	**16**	72.9	4.3	21.2	1.6
FO4	PMA/Iono	**17**	74.9	4.6	18.8	1.7
FO4	PMA/Iono	**18**	70.1	4.6	23.6	1.7

APPENDIX A-7

EXPT. 5 – APOPTOSIS DATA

PART I

SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD
S1-1	41	32.9	24.9	1.17
S1-2	45.8	24	29.2	1.04
S1-3	41.1	31.8	26	1.11
S1-4	41.6	25.1	31.1	2.18
S1-5	37.3	37.8	24.4	0.49
S1-7	49.8	23.3	25.4	1.47
S1-8	42.8	27.2	28.5	1.58
S1-9	39.3	28.5	31.2	0.97
S1-10	46.6	23.6	28.1	1.72
S1-11	46.7	24.7	27.1	1.45
S1-12	41.3	32.4	25	1.32
S1-13	45.8	27.3	25.3	1.66
S1-14	41	32.8	24.7	1.45
S1-15	42.2	28.4	26.8	2.53
S1-16	44.7	27.5	25.3	2.53
S1-17	42.9	27.4	27.4	2.36
S1-18	48.1	26.8	23.5	1.57
S1-19	43.1	32	24	0.94
S1-20	46.8	29.4	22.7	1.1
S1-21	50.5	25.2	23.7	0.67
S1-22	42.1	32.2	23.7	2.04
S1-23	42.2	33.3	23.1	1.39
S1-24	51.8	25.8	21.1	1.28
S2-1	28	42.3	29.3	0.34
S2-2	30.6	39.1	29.7	0.51
S2-3	28.9	42	28.7	0.38
S2-4	17.1	50.7	31.9	0.34
S2-5	29.4	43.7	26.6	0.29
S2-6	16.2	48.2	34.8	0.77
S2-7	21.2	43.3	34.1	1.37
S2-8	23.1	41.7	33.9	1.29
S2-9	17.7	47.2	34.7	0.42
S2-10	20.2	44.3	34.9	0.53
S2-11	30	39.7	28.9	1.49
S2-12	22.8	44.5	32.3	0.42
S2-13	26.7	38.1	33.9	1.25
S2-14	23.8	43.4	32.2	0.59
S2-15	22.5	43.5	33.4	0.57
S2-16	21.9	44	33.3	0.78
S2-17	28	38	32.8	1.25
S2-18	30.2	40.3	28.8	0.73
S2-19	23	45.3	31.1	0.62
S2-20	21.9	44.1	33	0.99
S2-21	16.8	43.2	38.6	1.37
S2-22	22.7	43.5	33.1	0.65
S2-23	31.1	40.1	28.2	0.6
S2-24	30.8	37.8	30.4	0.97

SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD
U1-1	75.7	9.74	14.2	0.43
U1-2	77.3	10.3	12.1	0.36
U1-3	77.9	8.35	13.1	0.63
U1-4	78.1	9.83	11.4	0.65
U1-5	75.5	11.5	12.5	0.53
U1-7	76.7	9.44	13.6	0.28
U1-8	77.7	9.38	12.1	0.83
U1-9	77.6	9.76	12.2	0.43
U1-10	76.6	10.1	13	0.44
U1-11	77.1	8.58	13.7	0.69
U1-12	76.9	10.1	12.6	0.34
U1-13	77	9.93	12.4	0.65
U1-14	77.2	9.38	12.8	0.63
U1-15	76.8	8.5	13.6	1.06
U1-16	77.1	8.9	12.9	1.05
U1-17	75.8	9.54	13.7	0.96
U1-18	73.7	10.7	14.6	0.94
U1-19	76.3	10.4	12.9	0.38
U1-20	78.9	7.67	12.9	0.55
U1-21	80.7	7.61	11.3	0.32
U1-22	75.3	9.9	14.2	0.61
U1-23	74.1	11.5	13.8	0.57
U1-24	75.2	9.89	14.3	0.63
U2-1	54.7	20.8	24.2	0.33
U2-2	55.9	21.2	22.7	0.29
U2-3	56.6	20.9	22.3	0.18
U2-4	60.2	18.5	21.1	0.16
U2-5	63.8	16.7	19.2	0.31
U2-6	57	19	23.7	0.33
U2-7	59.5	16.8	23.1	0.57
U2-8	63.8	16.7	19.1	0.39
U2-9	59.5	20.7	19.5	0.35
U2-10	52.2	22.4	25.2	0.2
U2-11	58.1	19.5	21.8	0.6
U2-12	53.1	21	25.7	0.12
U2-13	57.3	19.1	23.1	0.45
U2-14	54.8	20.2	24.8	0.23
U2-15	52.8	21.1	25.8	0.37
U2-16	58.6	21.8	19.3	0.39
U2-17	61.7	17.5	20.6	0.31
U2-18	57.7	20	21.9	0.35
U2-19	59	17.7	23	0.35
U2-20	59.6	18.3	21.7	0.41
U2-21	56.1	17.2	26.1	0.59
U2-22	54.7	20.5	24.5	0.29
U2-23	60	18.5	21	0.41
U2-24	60.9	17.5	21.1	0.51

EXPT. 5 – APOPTOSIS DATA (cont.)

SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD
S3-1	75.1	5.94	15.7	3.28
S3-2	72.9	5.81	19.3	2
S3-3	78.2	0.059	0.3	21.5
S3-4	70.8	8.34	19.5	1.34
S3-5	75.4	7.66	15.1	1.77
S3-6	71.6	8.25	19.4	0.69
S3-7	75.4	7.76	15.6	1.3
S3-8	73.8	6.94	18	1.23
S3-9	67.5	9.63	21.7	1.15
S3-10	72	8.71	17.6	1.7
S3-11	68	9.48	21.2	1.26
S3-12	71.4	8.9	18.6	1.09
S3-13	75.7	7.46	15.6	1.23
S3-14	73.8	8.49	16.8	0.97
S3-15	73.1	7.49	18.2	1.17
S3-16	73.1	8.82	17.4	0.75
S3-17	72.2	7.8	18.9	1.09
S3-18	71.7	7.88	19.6	0.89
S3-19	76.1	7.92	14.9	1.11
S3-20	74.3	9.08	15.8	0.73
S3-21	72.1	9.43	18.1	0.38
S3-22	72.7	8.02	18.5	0.79
S3-23	75.3	8.71	15.2	0.75
S3-24	70.1	9.08	20	0.85

SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD
U3-1	83.9	6.95	8.9	0.22
U3-2	82.5	8.69	8.39	0.48
U3-3	83.5	6.45	9.35	0.75
U3-4	84.7	6.12	9.02	0.18
U3-5	83.8	6.38	8.78	1.01
U3-6	85.2	6.2	8.42	0.2
U3-7	86.6	5.66	6.84	0.85
U3-8	86.6	5.7	7.35	0.38
U3-9	83.8	6.39	9.43	0.4
U3-10	82.2	6.62	10.5	0.65
U3-11	83.3	6.7	9.46	0.58
U3-12	83	6.26	10.2	0.57
U3-13	85.2	5.66	8.81	0.36
U3-14	83.7	5.97	9.92	0.44
U3-15	83.7	6.12	9.67	0.48
U3-16	85.4	5.91	8.23	0.48
U3-17	85.3	6.01	8.41	0.24
U3-18	85.1	6.17	8.28	0.4
U3-19	85	5.3	9.33	0.42
U3-20	85.4	5.86	8.28	0.44
U3-21	87.2	4.74	7.94	0.1
U3-22	85.9	5.64	8.12	0.3
U3-23	84.7	5.68	9.23	0.36
U3-24	84.9	5.24	9.46	0.38

PART II

SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD
S1-1	45.3	31.3	22.4	1.03
S1-2	43.5	27.1	27.4	1.96
S1-3	41.4	33.1	24.6	0.92
S1-4	42.9	32	24.4	0.71
S1-5	35	34.1	29.8	1.03
S1-7	33	36.2	30.2	0.55
S1-8	41.1	31.7	26.5	0.63
S1-9	35.4	36.2	27.7	0.63
S1-10	40.8	30.3	28.3	0.59
S1-11	40.5	31.7	26.8	1
S1-12	40.2	35.1	24.1	0.55
S1-13	44.9	25.3	28.3	1.44
S1-14	42.6	26.5	29.7	1.21
S1-15	43	28.5	27.2	1.28
S1-16	39.1	33.1	27	0.86
S1-17	45.3	24.7	28.8	1.22
S1-18	43.5	27.2	28.2	1.06
S1-19	39.4	31.6	28	1.02
S1-20	39.2	29.6	30.3	0.9
S1-21	38.8	32.3	28.1	0.77
S1-22	33.6	36.5	29	0.86
S1-23	31.7	39.7	28	0.63
S1-24	37.2	37.7	24.5	0.66

SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD
U1-1	71.3	11.1	17	0.59
U1-2	71.1	11.6	16.9	0.53
U1-3	75	9.81	14.5	0.61
U1-4	78.1	7.82	13.4	0.67
U1-5	76.7	9.63	13.2	0.46
U1-7	74	11.1	14.6	0.28
U1-8	76.4	8.95	14.2	0.49
U1-9	74.7	9.72	15.3	0.28
U1-10	73.2	11.3	15.2	0.24
U1-11	74.5	11.7	13.5	0.28
U1-12	70.9	14	14.9	0.18
U1-13	70.1	11.3	18	0.59
U1-14	73.5	9.61	16.4	0.45
U1-15	72.1	11.8	15.3	0.77
U1-16	73.5	10.6	15.4	0.48
U1-17	75.1	10.9	13.4	0.49
U1-18	74.4	10	14.9	0.65
U1-19	69	12.9	17.7	0.41
U1-20	71.3	12	16.4	0.28
U1-21	70.4	12.8	16.3	0.57
U1-22	68.7	13.4	17.5	0.41
U1-23	66.6	13.9	19.1	0.43
U1-24	70.7	12.6	16.2	0.49

EXPT. 5 – APOPTOSIS DATA (cont.)

SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD
S2-1	14.1	46.9	38.8	0.27
S2-2	17	45.5	36.9	0.65
S2-3	23.8	40.1	35	1.02
S2-4	15.6	45.1	38.6	0.74
S2-5	21.2	43.6	34.5	0.75
S2-6	19.6	51.8	27.9	0.65
S2-7	19	44.6	35.6	0.84
S2-8	24.1	39.9	35.2	0.82
S2-9	20.8	41.3	37.6	0.3
S2-10	18.6	44.8	36.2	0.36
S2-11	20.5	42.8	36.2	0.47
S2-12	17.3	45.9	36.4	0.36
S2-13	25.1	37.4	36.3	1.12
S2-14	16	45.8	37.8	0.4
S2-15	17.5	44.6	37.7	0.23
S2-16	22	42.2	35.3	0.5
S2-17	26.5	39.6	33	0.96
S2-18	19.3	44.7	35.5	0.47
S2-19	23.4	41	35.1	0.5
S2-20	27.6	38.7	32.8	0.92
S2-21	12.3	49.1	38.3	0.4
S2-22	20.8	45.9	32.7	0.59
S2-23	27.8	34.5	35	2.66
S2-24	32	36.6	30.4	0.94
S3-1	73.5	8.27	17.5	0.67
S3-2	80.7	5.88	12.7	0.71
S3-3	75.8	8.16	14.9	1.13
S3-4	79.3	6.51	13.1	1.13
S3-5	77.6	7.57	14.6	0.28
S3-6	74.4	7.49	17.5	0.67
S3-7	76.7	7.44	14.7	1.18
S3-8	83.6	6.13	9.97	0.28
S3-9	71.8	8.1	19.2	0.91
S3-10	74.7	7.61	16.8	0.83
S3-11	71.1	6.02	22	0.89
S3-12	70.2	10.9	18.3	0.51
S3-13	73.1	9.93	16.4	0.49
S3-14	75.7	9.04	14.8	0.43
S3-15	72.4	9.46	17.7	0.37
S3-16	79	8.43	12	0.52
S3-17	74.9	10.2	14.3	0.63
S3-18	74	8.85	16.6	0.53
S3-19	74.4	9.63	15.4	0.53
S3-20	73.8	9.64	16.2	0.36
S3-21	74	8.9	16.4	0.73
S3-22	74.6	10.3	14.7	0.43
S3-23	74.2	9.93	15.3	0.53
S3-24	70.2	13	16.4	0.36

SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD
U2-1	55.2	16.4	27.5	0.87
U2-2	57.7	12.5	29.3	0.43
U2-3	62.1	14	23.5	0.43
U2-4	62.3	14	23.3	0.35
U2-5	61.5	13.5	24.5	0.47
U2-6	61.6	14.5	23.5	0.31
U2-7	63.8	13.1	22.8	0.31
U2-8	59.3	13.8	26	0.86
U2-9	61.3	15.7	22.9	0.18
U2-10	58.2	17.1	24.3	0.41
U2-11	61	14.2	24	0.76
U2-12	54.9	16.1	28.5	0.52
U2-13	59.1	14.5	26.1	0.37
U2-14	57.1	18.1	24.4	0.39
U2-15	56.9	17.2	25.6	0.31
U2-16	61.3	14.5	23.8	0.37
U2-17	63.5	14.1	21.9	0.59
U2-18	60.3	17.2	22.1	0.35
U2-19	56.7	15.9	27.1	0.23
U2-20	61.9	14.5	23.1	0.49
U2-21	61.4	15.1	22.9	0.63
U2-22	58.7	15.5	25.4	0.45
U2-23	57	15.6	26.9	0.41
U2-24	60	15.5	24	0.51
U3-1	84	6.63	9.02	0.3
U3-2	85	5.32	9.52	0.18
U3-3	83.5	6.47	9.69	0.3
U3-4	90	0.02	0.2	9.81
U3-5	85.4	6.57	7.93	0.12
U3-6	86.7	5.93	7.24	0.18
U3-7	85.8	5.97	8.03	0.18
U3-8	85.4	6.69	7.7	0.16
U3-9	84.1	5.99	9.68	0.22
U3-10	86.6	5.03	8.19	0.16
U3-11	84.5	6.1	9.31	0.099
U3-12	83.4	6.32	10.2	0.099
U3-13	84.1	7.06	8.71	0.18
U3-14	86.3	5.63	7.94	0.12
U3-15	84.9	5.98	8.86	0.24
U3-16	86	5.3	8.48	0.22
U3-17	85.2	5.86	8.65	0.34
U3-18	86	5.13	8.54	0.34
U3-19	85.5	5.16	9.15	0.24
U3-20	84.8	6.22	8.82	0.14
U3-21	86.2	5.27	8.19	0.36
U3-22	84.9	5.43	9.35	0.28
U3-23	86	4.4	9.41	0.22
U3-24	83.5	7.5	8.8	0.18

APPENDIX A-8

EXPT. 6 – APOPTOSIS DATA

SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD	TOTAL Apop.
1-1	39.7	29.1	30.9	0.3	60.0
1-2	41.7	26.8	30.8	0.7	57.6
1-3	39.2	31.3	29.1	0.4	60.4
1-4	42.5	32.6	24.6	0.3	57.2
1-5	43.9	28.9	26.9	0.3	55.8
1-6	45.3	25.4	28.5	0.8	53.9
1-7	46.2	23.9	29.1	0.7	53.0
1-8	43.7	29.8	26.2	0.3	56.0
1-9	38.4	27.0	34.1	0.5	61.1
1-10	40.0	30.0	29.7	0.2	59.7
1-11	39.0	32.9	27.9	0.2	60.8
1-12	39.7	30.5	29.6	0.3	60.1
2-1	38.0	29.3	32.3	0.4	61.6
2-2	31.3	28.8	39.0	0.9	67.8
2-3	35.4	29.8	34.6	0.2	64.4
2-4	36.9	27.7	34.8	0.6	62.5
2-5	34.9	26.9	37.7	0.6	64.6
2-6	36.8	23.2	38.6	1.3	61.8
2-7	37.1	25.1	37.3	0.5	62.4
2-8	38.3	24.2	36.9	0.6	61.1
2-9	34.2	25.5	39.8	0.6	65.3
2-10	34.3	27.7	37.7	0.3	65.4
2-11	33.9	28.0	37.6	0.4	65.6
2-12	36.2	25.0	38.5	0.3	63.5
3-1	82.8	6.3	10.7	0.2	17.0
3-2	83.5	4.9	11.4	0.3	16.3
3-3	84.3	6.0	9.6	0.1	15.6
3-4	83.0	5.8	11.1	0.1	16.9
3-5	84.1	6.1	9.6	0.2	15.7
3-6	86.2	4.2	9.4	0.2	13.6
3-7	83.7	5.3	10.8	0.2	16.1
3-8	83.1	5.5	11.2	0.2	16.7
3-9	81.4	6.1	12.3	0.3	18.4
3-10	81.9	6.1	11.9	0.1	18.0
3-11	80.3	5.9	13.6	0.3	19.5
3-12	79.7	7.2	13.0	0.2	20.2

APPENDIX A-9

EXPT. 7 – DATA

APOPTOSIS

	SAMPLE	AV-/PI- LIVE	AV+/PI- EARLY	AV+/PI+ LATE	AV-/PI+ DEAD	TOTAL Apop.
FBS-CO	1	43.8	18.9	36.7	0.5	55.6
	2	41.3	20.2	37.9	0.6	58.1
	3	35.8	19.8	43.9	0.5	63.7
	4	48.8	17.5	33.4	0.4	50.9
	5	51.4	16.8	31.3	0.4	48.1
	6	50.7	15.4	33.8	0.2	49.2
FBS-FO	7	51.8	14.0	32.8	1.3	46.8
	8	47.8	16.1	35.6	0.5	51.7
	9	47.5	15.2	36.4	0.9	51.6
	10	48.1	15.2	35.7	1.0	50.9
	11	49.4	15.2	34.7	0.6	49.9
	12	44.9	15.7	38.4	1.0	54.1
MS-CO	1	43.7	16.5	39.5	0.2	56.0
	2	45.1	15.4	39.3	0.2	54.7
	3	45.6	18.3	35.9	0.2	54.2
	4	42.7	14.9	42.1	0.3	57.0
	5	45.9	14.9	38.7	0.5	53.6
	6	39.6	15.3	44.4	0.6	59.7
MS-FO	7	30.8	19.5	49.1	0.5	68.6
	8	30.2	19.4	49.8	0.5	69.2
	9	30.5	17.5	50.7	1.3	68.2
	10	29.2	18.3	51.5	1.0	69.8
	11	27.6	19.1	51.5	1.8	70.6
	12	29.0	19.1	50.4	1.4	69.5

EXPT. 7 – DATA (cont.)

INTRACELLULAR CYTOKINES

	SAMPLE	IFNg-/IL4-	IFNg+/IL4-	IFNg-/IL4+	IFNg+/IL4+
FBS-CO	1	42.1	57.8	0.0	0.1
	2	36.6	63.3	0.0	0.1
	3	35.0	64.8	0.0	0.1
	4	42.9	56.9	0.1	0.1
	5	43.9	55.9	0.0	0.1
	6	49.5	50.4	0.0	0.1
FBS-FO	7	41.8	58.1	0.0	0.1
	8	37.4	62.6	0.0	0.1
	9	45.1	54.8	0.0	0.2
	10	41.6	58.2	0.0	0.1
	11	45.2	54.8	0.0	0.1
	12	35.3	64.6	0.0	0.1
MS-CO	1	63.8	36.0	0.0	0.2
	2	63.4	36.5	0.0	0.1
	3	51.8	48.1	0.0	0.2
	4	67.7	32.2	0.0	0.1
	5	63.8	36.1	0.0	0.1
	6	67.2	32.7	0.0	0.1
MS-FO	7	48.2	51.2	0.0	0.6
	8	44.4	54.8	0.0	0.8
	9	41.6	57.4	0.0	1.0
	10	45.6	53.5	0.0	0.9
	11	40.0	58.9	0.0	1.0
	12	44.5	54.5	0.0	1.0

APPENDIX A-10

EXPT. 8 – DATA

Moles FA

Cell #	19 x 10^6	19 x 10^6	30 x 10^6	30 x 10^6	38 x 10^6	38 x 10^6
			DAY 0			
	1 raft	1 sol.	2 raft	2 sol.	3 raft	3 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
14:10	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
15:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
16:00	2.41E-06	3.36E-06	1.77E-06	3.55E-06	2.86E-06	3.75E-06
16:1N-7	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:00	6.60E-07	1.18E-06	5.73E-07	1.49E-06	1.62E-06	2.29E-06
18:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:1N-7	4.89E-07	9.57E-07	3.65E-07	9.12E-07	5.08E-07	8.15E-07
18:2N-6	0.00E+00	6.43E-07	2.14E-07	5.40E-07	3.00E-07	7.36E-07
18:3N-6	1.59E-07	1.77E-07	1.34E-07	2.18E-07	2.49E-07	1.42E-07
18:3N-3	2.38E-07	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:4N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:2N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:3N-6	1.89E-07	8.56E-07	1.22E-08	5.93E-07	3.39E-07	1.06E-06
20:4N-6	1.54E-07	7.00E-07	1.54E-07	1.19E-06	3.74E-07	1.56E-06
20:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:5N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.57E-07
22:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:4N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-3	7.52E-08	5.95E-07	0.00E+00	1.38E-07	1.50E-08	3.36E-07
22:6N-3	4.20E-08	1.50E-07	2.85E-08	2.08E-07	0.00E+00	3.87E-07
24:0	0.00E+00	3.78E-07	0.00E+00	1.44E-07	0.00E+00	3.02E-07
24:1N-9	0.00E+00	1.34E-07	0.00E+00	1.65E-07	1.22E-07	0.00E+00
Total	4.42E-06	9.13E-06	9.15E-06	9.66E-06	6.38E-06	1.15E-05

EXPT. 8 – DATA (cont.)

Moles FA

Cell #	54 x 10^6	54 x 10^6	47 x 10^6	47 x 10^6	72 x 10^6	72 x 10^6
			DAY 0			
	4 raft	4 sol.	5 raft	5 sol.	6 raft	6 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
14:10	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
15:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
16:00	2.90E-06	5.16E-06	5.71E-06	8.14E-06	2.30E-06	1.75E-06
16:1N-7	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:00	1.13E-06	2.64E-06	2.95E-06	3.79E-06	8.71E-07	8.06E-07
18:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:1N-7	3.22E-07	1.07E-06	2.09E-06	2.21E-06	5.55E-07	4.06E-07
18:2N-6	1.50E-07	3.53E-07	4.77E-07	7.24E-07	3.81E-07	2.90E-07
18:3N-6	1.17E-07	0.00E+00	5.06E-07	2.24E-07	0.00E+00	0.00E+00
18:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:4N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:2N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:3N-6	2.12E-07	1.70E-07	2.99E-07	2.04E-07	0.00E+00	0.00E+00
20:4N-6	1.71E-07	1.45E-06	4.16E-07	1.80E-06	3.81E-07	3.63E-07
20:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:5N-3	0.00E+00	1.55E-07	4.66E-08	4.13E-07	0.00E+00	0.00E+00
22:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:4N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-3	4.91E-08	3.78E-07	1.49E-07	5.67E-07	0.00E+00	0.00E+00
22:6N-3	5.93E-08	5.71E-07	1.72E-07	9.75E-07	8.30E-08	1.46E-07
24:0	7.93E-08	7.07E-08	1.72E-07	2.33E-07	0.00E+00	0.00E+00
24:1N-9	1.33E-07	8.53E-08	1.73E-07	1.71E-07	0.00E+00	0.00E+00
Total	5.32E-06	1.21E-05	1.32E-05	1.94E-05	4.57E-06	3.76E-06

EXPT. 8 – DATA (cont.)

Moles FA

Cell #	14×10^6	14×10^6	17×10^6	17×10^6	15×10^6	15×10^6
	DAY 5 (FBS)					
	1 raft	1 sol.	2 raft	2 sol.	3 raft	3 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
14:10	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
15:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
16:00	4.51E-06	7.80E-06	5.47E-06	4.61E-06	4.29E-06	6.85E-06
16:1N-7	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:00	1.84E-06	5.40E-06	2.63E-06	2.94E-06	2.03E-06	4.46E-06
18:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:1N-7	1.34E-06	8.89E-06	2.59E-06	3.89E-06	1.28E-06	5.83E-06
18:2N-6	2.09E-07	6.68E-07	2.68E-07	5.22E-07	3.23E-07	9.13E-07
18:3N-6	5.61E-08	7.63E-07	9.65E-08	0.00E+00	3.40E-07	4.38E-07
18:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:4N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:2N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:3N-6	5.10E-08	2.85E-07	1.58E-07	1.99E-07	0.00E+00	1.07E-06
20:4N-6	2.57E-07	1.23E-06	5.30E-07	7.61E-07	3.96E-07	1.12E-06
20:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:5N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:4N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-3	7.09E-08	1.89E-07	1.30E-07	1.66E-07	5.22E-08	3.32E-07
22:6N-3	9.51E-08	2.66E-07	1.64E-07	2.04E-07	6.56E-08	2.97E-07
24:0	2.44E-07	2.71E-07	3.79E-07	9.93E-08	2.11E-07	3.64E-07
24:1N-9	3.84E-07	3.41E-07	4.84E-07	1.50E-07	4.12E-07	3.66E-07
Total	9.06E-06	2.61E-05	1.29E-05	1.35E-05	9.40E-06	2.20E-05

EXPT. 8 – DATA (cont.)

Moles FA

Cell #	12 x 10^6	12 x 10^6	14 x 10^6	14 x 10^6	21 x 10^6	21 x 10^6
	DAY 5 (FBS)					
	4 raft	4 sol.	5 raft	5 sol.	6 raft	6 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
14:10	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
15:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
16:00	3.65E-06	2.83E-06	5.49E-06	6.10E-06	4.93E-06	4.84E-06
16:1N-7	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:00	1.94E-06	1.55E-06	2.72E-06	4.55E-06	2.33E-06	3.19E-06
18:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:1N-7	5.06E-07	2.33E-06	4.74E-07	6.19E-06	2.01E-06	4.39E-06
18:2N-6	1.41E-07	2.41E-07	1.29E-07	4.26E-07	3.19E-07	5.23E-07
18:3N-6	9.97E-08	2.99E-07	1.85E-08	1.56E-07	1.32E-07	9.58E-08
18:3N-3	0.00E+00	0.00E+00	0.00E+00	7.81E-08	0.00E+00	0.00E+00
18:4N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:2N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:3N-6	7.77E-08	1.29E-07	1.85E-07	3.90E-07	1.55E-07	2.39E-07
20:4N-6	3.26E-07	4.56E-07	2.20E-07	1.68E-06	4.32E-07	8.98E-07
20:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:5N-3	0.00E+00	0.00E+00	0.00E+00	7.19E-08	0.00E+00	6.61E-08
22:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:4N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-3	7.20E-08	9.61E-08	1.09E-07	5.92E-07	1.11E-07	2.22E-07
22:6N-3	8.46E-08	1.57E-07	1.10E-07	3.97E-07	1.44E-07	3.04E-07
24:0	1.94E-07	9.69E-08	3.36E-07	2.95E-07	2.86E-07	1.81E-07
24:1N-9	2.60E-07	1.41E-07	3.94E-07	3.26E-07	4.16E-07	2.00E-07
Total	7.35E-06	8.33E-06	1.02E-05	2.13E-05	1.13E-05	1.51E-05

EXPT. 8 – DATA (cont.)

Moles FA

Cell #	19 x 10^6	19 x 10^6	16 x 10^6	16 x 10^6	17 x 10^6	17 x 10^6
	DAY 5 (MS)					
	1 raft	1 sol.	2 raft	2 sol.	3 raft	3 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
14:10	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
15:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
16:00	1.06E-04	1.21E-05	4.42E-06	3.04E-06	3.67E-06	4.73E-06
16:1N-7	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:00	6.24E-05	7.56E-06	1.77E-06	2.41E-06	1.96E-06	4.41E-06
18:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:1N-7	2.12E-05	7.19E-06	8.26E-07	1.04E-06	7.24E-07	2.05E-06
18:2N-6	1.52E-05	6.32E-06	4.58E-07	1.01E-06	2.80E-07	2.00E-06
18:3N-6	9.87E-06	3.92E-06	8.55E-08	6.50E-08	7.90E-08	1.44E-07
18:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:4N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:2N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:3N-6	2.85E-06	6.92E-07	2.95E-07	0.00E+00	1.44E-07	3.05E-07
20:4N-6	1.93E-05	3.98E-06	5.16E-07	1.78E-07	4.96E-07	2.36E-06
20:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:5N-3	0.00E+00	0.00E+00	3.15E-08	0.00E+00	4.16E-08	0.00E+00
22:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:4N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-3	1.13E-06	2.75E-07	2.16E-07	8.77E-08	0.00E+00	1.41E-07
22:6N-3	4.57E-06	1.48E-06	1.74E-07	3.31E-07	1.72E-07	6.90E-07
24:0	3.05E-06	2.06E-07	9.05E-08	0.00E+00	0.00E+00	0.00E+00
24:1N-9	8.87E-06	6.61E-07	3.64E-07	5.93E-08	3.00E-07	2.36E-07
Total	2.55E-04	4.44E-05	9.25E-06	8.21E-06	7.86E-06	1.71E-05

EXPT. 8 – DATA (cont.)

Moles FA

Cell #	10×10^6	10×10^6	10×10^6	10×10^6	21×10^6	21×10^6
	DAY 5 (MS)					
	4 raft	4 sol.	5 raft	5 sol.	6 raft	6 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
14:10	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
15:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
16:00	2.01E-06	2.69E-06	3.39E-06	7.14E-06	3.91E-06	3.81E-06
16:1N-7	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:00	8.79E-07	1.65E-06	1.63E-06	5.47E-06	1.76E-06	2.94E-06
18:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:1N-7	7.20E-07	1.91E-06	6.61E-07	3.66E-06	6.42E-07	1.71E-06
18:2N-6	2.21E-07	2.91E-07	2.39E-07	1.66E-06	3.17E-07	9.66E-07
18:3N-6	3.71E-08	8.50E-08	8.85E-08	4.90E-07	1.99E-07	2.38E-07
18:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
18:4N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:2N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:3N-6	0.00E+00	0.00E+00	1.72E-07	3.71E-07	0.00E+00	2.16E-07
20:4N-6	1.25E-07	2.33E-07	1.62E-07	1.90E-06	1.95E-07	7.72E-07
20:3N-3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
20:5N-3	0.00E+00	0.00E+00	2.33E-08	3.01E-07	3.67E-08	2.19E-07
22:00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:1N-9	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:4N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-6	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00
22:5N-3	8.87E-08	1.19E-07	1.70E-07	1.68E-06	2.35E-07	8.57E-07
22:6N-3	1.73E-07	2.64E-07	2.04E-07	1.94E-06	3.16E-07	9.72E-07
24:0	0.00E+00	0.00E+00	1.72E-07	1.85E-07	1.61E-07	9.81E-08
24:1N-9	0.00E+00	0.00E+00	1.92E-07	3.41E-07	3.23E-07	2.79E-07
Total	4.25E-06	7.24E-06	7.11E-06	2.51E-05	8.10E-06	1.31E-05

EXPT. 8 – DATA (cont.)

Mole %

Cell #	19 x 10^6	19 x 10^6	30 x 10^6	30 x 10^6	38 x 10^6	38 x 10^6
			DAY 0			
	1 raft	1 sol.	2 raft	2 sol.	3 raft	3 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00	0.00	0.00	0.00	0.00	0.00
14:10	0.00	0.00	0.00	0.00	0.00	0.00
15:00	0.00	0.00	0.00	0.00	0.00	0.00
16:00	54.60	36.77	54.43	38.76	44.77	32.48
16:1N-7	0.00	0.00	0.00	0.00	0.00	0.00
18:00	14.94	12.94	17.63	16.30	25.35	19.83
18:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
18:1N-7	11.06	10.49	11.23	9.97	7.96	7.06
18:2N-6	0.00	7.04	6.58	5.90	4.70	6.38
18:3N-6	3.59	1.93	4.14	2.38	3.90	1.24
18:3N-3	5.39	0.00	0.00	0.00	0.00	0.00
18:4N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:00	0.00	0.00	0.00	0.00	0.00	0.00
20:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
20:2N-6	0.00	0.00	0.00	0.00	0.00	0.00
20:3N-6	4.28	9.38	0.38	6.48	5.31	9.21
20:4N-6	3.49	7.67	4.73	13.05	5.86	13.56
20:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:5N-3	0.00	0.00	0.00	0.00	0.00	1.36
22:00	0.00	0.00	0.00	0.00	0.00	0.00
22:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
22:4N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-3	1.70	6.52	0.00	1.50	0.23	2.92
22:6N-3	0.95	1.64	0.88	2.27	0.00	3.35
24:0	0.00	4.14	0.00	1.57	0.00	2.61
24:1N-9	0.00	1.47	0.00	1.81	1.90	0.00

EXPT. 8 – DATA (cont.)

Mole %

Cell #	54 x 10^6	54 x 10^6	47 x 10^6	47 x 10^6	72 x 10^6	72 x 10^6
			DAY 0			
	4 raft	4 sol.	5 raft	5 sol.	6 raft	6 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00	0.00	0.00	0.00	0.00	0.00
14:10	0.00	0.00	0.00	0.00	0.00	0.00
15:00	0.00	0.00	0.00	0.00	0.00	0.00
16:00	54.49	42.64	43.40	41.84	50.28	46.51
16:1N-7	0.00	0.00	0.00	0.00	0.00	0.00
18:00	21.23	21.78	22.38	19.49	19.07	21.44
18:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
18:1N-7	6.05	8.84	15.91	11.38	12.16	10.80
18:2N-6	2.83	2.92	3.62	3.72	8.34	7.71
18:3N-6	2.19	0.00	3.84	1.15	0.00	0.00
18:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
18:4N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:00	0.00	0.00	0.00	0.00	0.00	0.00
20:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
20:2N-6	0.00	0.00	0.00	0.00	0.00	0.00
20:3N-6	3.98	1.40	2.27	1.05	0.00	0.00
20:4N-6	3.21	12.01	3.16	9.24	8.34	9.65
20:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:5N-3	0.00	1.28	0.35	2.13	0.00	0.00
22:00	0.00	0.00	0.00	0.00	0.00	0.00
22:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
22:4N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-3	0.92	3.13	1.13	2.92	0.00	0.00
22:6N-3	1.11	4.72	1.30	5.01	1.82	3.88
24:0	1.49	0.58	1.31	1.20	0.00	0.00
24:1N-9	2.50	0.70	1.31	0.88	0.00	0.00

EXPT. 8 – DATA (cont.)

Mole %

Cell #	14 x 10^6	14 x 10^6	17 x 10^6	17 x 10^6	15 x 10^6	15 x 10^6
	DAY 5 (FBS)					
	1 raft	1 sol.	2 raft	2 sol.	3 raft	3 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00	0.00	0.00	0.00	0.00	0.00
14:10	0.00	0.00	0.00	0.00	0.00	0.00
15:00	0.00	0.00	0.00	0.00	0.00	0.00
16:00	49.79	29.87	42.43	34.08	45.61	31.07
16:1N-7	0.00	0.00	0.00	0.00	0.00	0.00
18:00	20.31	20.69	20.37	21.69	21.60	20.23
18:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
18:1N-7	14.81	34.06	20.07	28.70	13.64	26.44
18:2N-6	2.31	2.56	2.08	3.85	3.43	4.14
18:3N-6	0.62	2.92	0.75	0.00	3.62	1.99
18:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
18:4N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:00	0.00	0.00	0.00	0.00	0.00	0.00
20:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
20:2N-6	0.00	0.00	0.00	0.00	0.00	0.00
20:3N-6	0.56	1.09	1.22	1.47	0.00	4.87
20:4N-6	2.83	4.72	4.11	5.62	4.22	5.09
20:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:5N-3	0.00	0.00	0.00	0.00	0.00	0.00
22:00	0.00	0.00	0.00	0.00	0.00	0.00
22:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
22:4N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-3	0.78	0.72	1.01	1.23	0.56	1.51
22:6N-3	1.05	1.02	1.27	1.51	0.70	1.35
24:0	2.69	1.04	2.94	0.73	2.24	1.65
24:1N-9	4.24	1.31	3.75	1.11	4.38	1.66

EXPT. 8 – DATA (cont.)

Mole %

Cell #	12 x 10^6	12 x 10^6	14 x 10^6	14 x 10^6	21 x 10^6	21 x 10^6
	DAY 5 (FBS)					
	4 raft	4 sol.	5 raft	5 sol.	6 raft	6 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00	0.00	0.00	0.00	0.00	0.00
14:10	0.00	0.00	0.00	0.00	0.00	0.00
15:00	0.00	0.00	0.00	0.00	0.00	0.00
16:00	49.68	34.01	53.91	28.72	43.73	31.94
16:1N-7	0.00	0.00	0.00	0.00	0.00	0.00
18:00	26.37	18.59	26.69	21.39	20.69	21.05
18:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
18:1N-7	6.88	28.00	4.66	29.14	17.86	28.99
18:2N-6	1.92	2.89	1.26	2.01	2.83	3.45
18:3N-6	1.36	3.59	0.18	0.73	1.17	0.63
18:3N-3	0.00	0.00	0.00	0.37	0.00	0.00
18:4N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:00	0.00	0.00	0.00	0.00	0.00	0.00
20:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
20:2N-6	0.00	0.00	0.00	0.00	0.00	0.00
20:3N-6	1.06	1.55	1.82	1.84	1.37	1.58
20:4N-6	4.43	5.48	2.16	7.89	3.84	5.93
20:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:5N-3	0.00	0.00	0.00	0.34	0.00	0.44
22:00	0.00	0.00	0.00	0.00	0.00	0.00
22:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
22:4N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-3	0.98	1.15	1.07	2.79	0.99	1.47
22:6N-3	1.15	1.89	1.08	1.87	1.28	2.01
24:00:00	2.64	1.16	3.30	1.39	2.54	1.19
24:1N-9	3.53	1.69	3.87	1.53	3.70	1.32

EXPT. 8 – DATA (cont.)

Mole %

Cell #	19 x 10^6	19 x 10^6	16 x 10^6	16 x 10^6	17 x 10^6	17 x 10^6
	DAY 5 (MS)					
	1 raft	1 sol.	2 raft	2 sol.	3 raft	3 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00	0.00	0.00	0.00	0.00	0.00
14:10	0.00	0.00	0.00	0.00	0.00	0.00
15:00	0.00	0.00	0.00	0.00	0.00	0.00
16:00	41.73	27.29	47.78	36.99	46.66	27.73
16:1N-7	0.00	0.00	0.00	0.00	0.00	0.00
18:00	24.50	17.04	19.18	29.31	24.89	25.81
18:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
18:1N-7	8.34	16.19	8.93	12.65	9.21	12.03
18:2N-6	5.95	14.24	4.95	12.27	3.57	11.70
18:3N-6	3.88	8.82	0.92	0.79	1.01	0.84
18:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
18:4N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:00	0.00	0.00	0.00	0.00	0.00	0.00
20:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
20:2N-6	0.00	0.00	0.00	0.00	0.00	0.00
20:3N-6	1.12	1.56	3.19	0.00	1.83	1.78
20:4N-6	7.58	8.97	5.58	2.17	6.31	13.85
20:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:5N-3	0.00	0.00	0.34	0.00	0.53	0.00
22:00	0.00	0.00	0.00	0.00	0.00	0.00
22:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
22:4N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-3	0.45	0.62	2.34	1.07	0.00	0.83
22:6N-3	1.79	3.32	1.88	4.03	2.19	4.04
24:0	1.20	0.46	0.98	0.00	0.00	0.00
24:1N-9	3.48	1.49	3.93	0.72	3.82	1.39

EXPT. 8 – DATA (cont.)

Mole %

Cell #	10×10^6	10×10^6	10×10^6	10×10^6	21×10^6	21×10^6
	DAY 5 (MS)					
	4 raft	4 sol.	5 raft	5 sol.	6 raft	6 sol.
Fatty Acid						
17:0 ISD						
14:00	0.00	0.00	0.00	0.00	0.00	0.00
14:10	0.00	0.00	0.00	0.00	0.00	0.00
15:00	0.00	0.00	0.00	0.00	0.00	0.00
16:00	47.26	37.15	47.71	28.38	48.34	29.12
16:1N-7	0.00	0.00	0.00	0.00	0.00	0.00
18:00	20.65	22.75	22.98	21.77	21.70	22.51
18:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
18:1N-7	16.93	26.38	9.29	14.56	7.93	13.05
18:2N-6	5.20	4.02	3.35	6.61	3.91	7.39
18:3N-6	0.87	1.17	1.24	1.95	2.46	1.82
18:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
18:4N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:00	0.00	0.00	0.00	0.00	0.00	0.00
20:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
20:2N-6	0.00	0.00	0.00	0.00	0.00	0.00
20:3N-6	0.00	0.00	2.42	1.47	0.00	1.65
20:4N-6	2.93	3.22	2.28	7.57	2.40	5.90
20:3N-3	0.00	0.00	0.00	0.00	0.00	0.00
20:5N-3	0.00	0.00	0.33	1.20	0.45	1.68
22:00	0.00	0.00	0.00	0.00	0.00	0.00
22:1N-9	0.00	0.00	0.00	0.00	0.00	0.00
22:4N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-6	0.00	0.00	0.00	0.00	0.00	0.00
22:5N-3	2.08	1.65	2.40	6.70	2.91	6.55
22:6N-3	4.07	3.65	2.86	7.71	3.90	7.43
24:0	0.00	0.00	2.42	0.74	1.99	0.75
24:1N-9	0.00	0.00	2.70	1.36	3.99	2.14

EXPT. 8 – DATA (cont.)

Cholesterol Data

	ug chol.	ug chol/1x106	mol chol	molchol/1x106	FA (total mol)	PL (mol)	molPL/1x106	chol/ PL (mol)
Day 0								
1 R	44.9856	2.37	1.16E-07	6.12E-09	4.42E-09	2.21E-09	1.16E-10	52.65
1 S	43.0272	2.26	1.11E-07	5.86E-09	9.13E-09	4.56E-09	2.40E-10	24.39
2 R	85.9008	2.86	2.22E-07	7.41E-09	3.25E-09	1.62E-09	5.41E-11	136.85
2 S	64.7184	2.16	1.67E-07	5.58E-09	9.15E-09	4.57E-09	1.52E-10	36.60
3 R	51.1968	1.35	1.32E-07	3.48E-09	6.38E-09	3.19E-09	8.40E-11	41.50
3 S	57.9408	1.52	1.50E-07	3.94E-09	1.15E-08	5.77E-09	1.52E-10	25.99
4 R	21.9168	0.41	5.67E-08	1.05E-09	5.32E-09	2.66E-09	4.93E-11	21.31
4 S	69.4848	1.29	1.80E-07	3.33E-09	1.21E-08	6.05E-09	1.12E-10	29.70
5 R	92.8848	1.98	2.40E-07	5.11E-09	1.32E-08	6.58E-09	1.40E-10	36.51
5 S	51.096	1.09	1.32E-07	2.81E-09	1.94E-08	9.72E-09	2.07E-10	13.59
6 R	37.2192	0.52	9.63E-08	1.34E-09	4.57E-09	2.28E-09	3.17E-11	42.17
6 S	78.1536	1.09	2.02E-07	2.81E-09	3.76E-09	1.88E-09	2.61E-11	107.64

	ug chol.	ug chol/1x106	mol chol	molchol/1x106	FA (total mol)	PL (mol)	molPL/1x106	chol/ PL (mol)
Day 5 FBS								
1 R	18.2352	1.30	4.72E-08	3.37E-09	9.06E-09	4.53E-09	3.23E-10	10.42
1 S	31.9296	2.28	8.26E-08	5.90E-09	2.61E-08	1.31E-08	9.33E-10	6.33
2 R	33.9456	2.00	8.78E-08	5.17E-09	1.32E-08	6.58E-09	3.87E-10	13.34
2 S	14.4336	0.85	3.73E-08	2.20E-09	1.37E-08	6.87E-09	4.04E-10	5.44
3 R	21.384	1.43	5.53E-08	3.69E-09	9.40E-09	4.70E-09	3.13E-10	11.77
3 S	58.0464	3.87	1.50E-07	1.00E-08	2.20E-08	1.10E-08	7.35E-10	13.62
4 R	19.92	1.66	5.15E-08	4.29E-09	7.35E-09	3.68E-09	3.06E-10	14.02
4 S	17.2752	1.44	4.47E-08	3.72E-09	8.33E-09	4.16E-09	3.47E-10	10.73
5 R	38.2176	2.73	9.89E-08	7.06E-09	1.02E-08	5.09E-09	3.64E-10	19.42
5 S	44.8704	3.21	1.16E-07	8.29E-09	2.13E-08	1.06E-08	7.59E-10	10.92
6 R	24.8496	1.18	6.43E-08	3.06E-09	1.13E-08	5.63E-09	2.68E-10	11.41
6 S	15.3456	0.73	3.97E-08	1.89E-09	1.51E-08	7.57E-09	3.61E-10	5.24

	ug chol.	ug chol/1x106	mol chol	molchol/1x106	FA (total mol)	PL (mol)	molPL/1x106	chol/ PL (mol)
Day 5 MS								
1 MS R	17.0976	0.90	4.42E-08	2.33E-09	2.55E-07	1.27E-07	6.70E-09	0.35
1 MS S	28.6224	1.51	7.40E-08	3.90E-09	4.44E-08	2.22E-08	1.17E-09	3.34
2 MS R	37.32	2.33	9.65E-08	6.03E-09	9.25E-09	4.63E-09	2.89E-10	20.87
2 MS S	14.6928	0.92	3.80E-08	2.38E-09	8.21E-09	4.11E-09	2.57E-10	9.25
3 MS R	48.9024	2.88	1.26E-07	7.44E-09	7.86E-09	3.93E-09	2.31E-10	32.19
3 MS S	36.0384	2.12	9.32E-08	5.48E-09	1.71E-08	8.53E-09	5.02E-10	10.92
4 MS R	41.7504	4.18	1.08E-07	1.08E-08	4.25E-09	2.13E-09	2.13E-10	50.77
4 MS S	41.7696	4.18	1.08E-07	1.08E-08	7.24E-09	3.62E-09	3.62E-10	29.85
5 MS R	26.3568	2.64	6.82E-08	6.82E-09	7.11E-09	3.55E-09	3.55E-10	19.18
5 MS S	45.2832	4.53	1.17E-07	1.17E-08	2.51E-08	1.26E-08	1.26E-09	9.32
6 MS R	40.5744	1.93	1.05E-07	5.00E-09	8.10E-09	4.05E-09	1.93E-10	25.92
6 MS S	4.248	0.20	1.10E-08	5.23E-10	1.31E-08	6.54E-09	3.11E-10	1.68

APPENDIX A-11

CELL COUNTS

Cell numbers (1 x 10^6 cells/ml) from all diet studies

	Splenocytes	T Cells	$CD4^+$ T Cells
SAF	64.75 ± 7.23	12.39 ± 0.55	ND
HFSAF	ND	12.40 ± 0.66	ND
OO	ND	14.39 ± 1.47	ND
CO	ND	6.26 ± 0.38	7.74 ± 0.67
FO 2	85.88 ± 7.33	15.20 ± 1.60	ND
FO 4	92.75 ± 6.15	13.90 ± 1.70	8.92 ± 0.44
HFFO	ND	13.80 ± 0.95	ND
DHA	ND	8.65 ± 0.38	ND

Cell counts were obtained at Day 0. Numbers represent 1 x 10^6 cells/ml per mouse. SAF, 2% Safflower oil + 3% corn oil; HFSAF, 4% safflower oil + 6% corn oil; OO, 2% olive oil + 3% corn oil; CO, 5% corn oil; FO2, 2% menhaden fish oil (fish oil) + 3% corn oil; FO4, 4% fish oil + 1% corn oil; HFFO, 9% fish oil + 1% corn oil; DHA, 1% DHA ethyl esters + 4% corn oil.

APPENDIX B

EXPERIMENTAL PROTOCOLS

APPENDIX B-1

EXPT. 1: T cell Apoptosis - Experiment Description

Diets:

1. Safflower Oil Ethyl Esters (2% safflower oil + 3% corn oil)
2. Olive Oil Ethyl Esters (2% olive oil + 3% corn oil)
3. Fish Oil (2% menhaden fish oil + 3% corn oil)

n = 6 mice per diet = 18 mice total

Stimuli:

- Untreated
- αCD3/αCD28 (1μg/ml / 5μg/ml)
- PMA/Ionomycin (1ng/ml / 500nM)
- PMA/ αCD3 (0.5ng/ml / 1μg/ml)
- Dexamethasone (330ng/ml)
- FasL/αFLAG (50ng/ml / 1μg/ml)

6 treatments per mouse
culture 500,000 cells per well (treatment)
so, need 3 x 10^6 cells per animal

Purified T cells from column yields ~ 10 X 10^6 cells per animal
Remaining T cells (~ 7 x 10^6) will be used for lipid extraction
Pool 2 mice for n = 3 per diet group to have enough cells for lipid analysis

Culture 1 treatment per 24-well plate = 6 plates
Will use 18 wells per plate — 1 for each animal
so, will have 108 total wells (samples)

Analysis:

1. **Annexin V/ PI** - at 24h for 108 samples
2. **Lipid extraction** - at 0h (collect and pool remaining T cells from T cell columns) for 9 samples

APPENDIX B-2

EXPT. 1 - Diet Composition and Preparation

Diet Composition:

14g/mouse/day for 2 weeks
6 mice per group x 14 days x 14g/m/day_____
1176g per diet ⇒ make 1400g each diet

3 Groups:
1. 2% Olive Oil + 3% CO
2. 2% Saf + 3% CO
3. 2% FO + 3% CO

Lot # L90325WF *Lot* # L93271YF *Lot* # L95108BB

	g/100g	OO 1.4kg	Saf 1.4kg	FO 1.4kg	Total
Casein	20	280	280	280	840
Sucrose	42	588	588	588	1764
Starch	21.98	307.7	307.7	307.7	923.1
Cellulose	6	84	84	84	252
Mineral	3.5	49	49	49	147
Vitamin	1	14	14	14	42
Met.	0.3	4.2	4.2	4.2	12.6
Chol. Cl	0.2	2.8	2.8	2.8	8.4
Tenox-20A	0.1	1.4	1.4	1.4	4.2
OO	2	28			28
Saf	2		28		28
FO	2			28	28
CO	3	42	42	42	126

Tenox-20A = Source of **TBHQ**
32% Glycerol
30% Corn Oil
20% **TBHQ**
15% Propylene glycol
3% Citric acid
Amount of TBHQ = 0.02g/100g diet

Sources of other dietary ingredients:
- Sucrose: product # 3900, Bioserv, lot # 1979.08, 10kg
- Casein: product # 1100, Bioserv, lot # 1589.01, 10kg
- Choline Chloride: product # 30200, Harlan-Teklad, 500g
- Tenox-20A: B&D Nutritional Ingredients, Inc., lot # 90114, 4oz
- Salt Mix, AIN-76: product # F8505, Harlan-Teklad, lot # 30230, 4kg
- Vitamin Mix, AIN-76A: product # 40077, Harlan-Teklad, Rx 993083, 1kg
- Methionine: product # 1340, Bioserv, lot # 1806.02, 0.5kg
- Cellulose: product # 3425, Bioserv, lot # 2667.01, 10kg
- Corn Starch: product # 160170, Harlan-Teklad, lot # 96420, 25kg
- Corn Oil: product # BLKC40, Traco Labs, Inc., lot # 99-017-22

Vitamin E content of diets

A mouse requires 22mg Vit. E/ kg diet – NRC, *Nutrient Requirements of Laboratory Animals*, 1995

Vitamin Mix:

10g vit E/kg Vitamin mix
= 1% vit E
= 0.01g vit E/100g diet
= 100mg vit E/kg diet

Olive Oil Ethyl Ester:

2.05mg vit E/g OO
= 2050mg vit E/kg OO
since 2% of diet
= 41mg vit E/kg diet

Safflower Oil Ethyl Ester:

1.9mg vit E/g Saf
= 1900mg vit E/kg Saf
since 2% of diet
= 38mg vit E/kg diet

Fish Oil:

1.9mg vit E/g FO
= 1900mg vit E/kg FO
since 2% of diet
= 38mg vit E/kg diet

Corn Oil:

896ppm = 896 mg vit E/kg CO
since 3% of diet
= 26.88mg vit E/kg diet

Total Vitamin E in each diet:

OO diet:
= 2% OO + 3% CO + Vitamin Mix
= 41mg + 26.88mg + 100mg
= 167.88mg vit E/kg diet

Saf diet:
= 2% Saf + 3% CO + Vitamin Mix
= 38mg + 26.88mg + 100mg
= 164.88mg vit E/kg diet

FO diet:
=2% FO + 3% CO + Vitamin Mix
= 38mg + 26.88mg + 100mg
= 164.88mg vit E/kg diet

Diet Preparation

Remove Menhaden oil and Olive oil from freezer and place in warm H_2O bath to thaw

- ❑ Bring downstairs: scale, bowls, mixing bowl w/ beaters, plastic containers, tape, markers, spoons, scoops, gloves, scissors, vitamin, methionine, choline chloride, detergent, EtOH
- ❑ Weigh 5kg mixing bowl before adding any ingredients. WEIGHT_____

Label large containers as follows:
Casein 840g
Sucrose 1764g
Starch 923.1g
Cellulose 252g

Label small containers as follows:
Mineral 147g
Vitamin 42g
Met 12.6g
Chol. Cl 8.4g

Label containers as follows:
___OO (***green***), Saf (***red***), FO (***blue***)
___Add 42g **CO** to each of these containers
___Cover and put on ice

****Note: Flush all containers with Nitrogen after use**

OO Prep:
Add the following to the **CO**:
___**Olive Oil** – 28g
___Tenox-20A – 1.4g
___ Cover and put on ice

Saf Prep:
Add the following to the **CO**:
___**Safflower Oil** – 28g
___ Tenox-20A – 1.4g
___ Cover and put on ice

FO Prep:
Add the following to the **CO**:
___**Menhaden Oil** – 28g
___ Tenox-20A – 1.4g
___ Cover and put on ice

Weigh out the following dry ingredients and mix together in one large mixing bowl:
___ Casein 840g
___Sucrose 1764g
___Starch 923.1g
___Cellulose 252g

*Mix with the 5kg Hobart mixer for **5 min***

Weigh out the following dry ingredients. Sift the ingredients and mix together in one small bowl:

___Mineral 147g
___Vitamin 42g
___Met 12.6g
___Chol. Cl 8.4g

Once these ingredients are mixed, add them to the large mixing bowl containing the other dry ingredients.
*Mix for **10 min** with the Hobart mixer*

- ❑ Weigh the total diet (should be 3989g + weight of bowl).
- ❑ Divide the diet three ways into labeled plastic containers marked "OO", "Saf", or "FO"
- ❑ Add all of the **OO** mixture to 1/3 the dry diet. *Mix for 5 min with Hobart mixer*
- ❑ Remove **OO** diet from bowl and put into labeled plastic container. Cover.
- ❑ Wash mixing bowl well
- ❑ Add all of the **Saf oil** mixture to 1/3 the dry diet. *Mix for 5 min with Hobart mixer*
- ❑ Remove **Saf** diet from bowl and put into labeled plastic container. Cover.
- ❑ Wash mixing bowl well
- ❑ Add all of the **FO** mixture to 1/3 the dry diet. *Mix for 5 min with Hobart mixer*
- ❑ Remove **FO** diet from bowl and put into labeled plastic container. Cover.
- ❑ Wash mixing bowl well
- ❑ Aliquot diets into plastic cups at 84g per cup (enough for 6 mice per day)

APPENDIX B-3

DIETARY LIPID EXTRACTION

To extract lipid from mixed diet which will be analyzed via Gas Chromatography

- ❑ Obtain prepared 12ml leak-proof vials
- ❑ *If not available: Put acetone into 12ml glass screw-top vials.*
  - *Mark the acetone level. Put lids on and place in 80°C oven for 1h.*
  - *Remove and note the acetone level. Discard vials that evaporated acetone.*
- ❑ Pour off acetone from vials and drain upside down
- ❑ Put each diet (0.5-1g, amount not critical) into 25ml glass screw-top vials.
- ❑ Add 5ml Folch ($CHCl_3$/ Methanol – 2:1 v:v) to each tube
- ❑ Add 1ml 0.1 M KCl to each tube
- ❑ Vortex for 1 min
- ❑ Centrifuge at 4000rpm for 5 min at 4°C *Make sure to put glass tubes in a rubber holder before placing in the centrifuge*
- ❑ Transfer the **lower** phase to a 12ml leak-proof vial
- ❑ -Using a Pasteur pipet bubble through the fat layer so as not to contaminate the extraction
- ❑ Dry down with Nitrogen
  - -Wipe each needle with EtOH
  - -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
- ❑ Add 3ml fresh 6% HCl/MeOH to each tube
  For 20ml, use 1.2ml concentrated HCl in 18.8ml MeOH. This solution is light sensitive, so put foil around the container
- ❑ Flush with Nitrogen and cap tightly
- ❑ Vortex for 1 min
- ❑ Put in an 80°C oven for 14-16h (overnight)

- ❑ Take tubes out of oven, put on ice
- ❑ Add 1ml of 0.1 M KCl and 2ml Hexane to each tube
- ❑ Vortex for 1 min
- ❑ Label 4ml glass screw-top vials: "FAME" for Fatty Acid Methyl Esters
  Make sure to replace normal black lids with green lids for organic solvents
- ❑ Transfer **upper** phase to 4ml glass vials
- ❑ Vortex for 1 min
- ❑ Dry down with Nitrogen - see above description
- ❑ Redissolve the FAME extract into 500ul of CH_2Cl_2.
  Use extra if the oil looks concentrated (yellowish). Remove 100ul of the concentrated oil and add it to 1ml of CH_2Cl_2
- ❑ Flush with Nitrogen
- ❑ Store at -20°C or analyze immediately on GC

APPENDIX B-4

EXPT. 1 Cell Preparation

*Take out **T cell columns and buffer, Lympholyte M**, and **10% FBS-RPMI** and warm to room temperature.*

Necropsy

⇒ Fill 15ml tubes (one for each animal) with 3ml of 10% FBS-RPMI
⇒ Assemble necessary equipment to take over to LARR:
- __labcoat
- __sterile scissors and forceps
- __gloves
- __EtOH
- __15ml tubes
- __keys, ID card

⇒ Go to LARR, room 202 to get mice
⇒ Get scale and take to necropsy room
⇒ Asphyxiate mice (1 cage at a time) with CO_2, then weigh each individually
⇒ Remove spleens aseptically and place in 15ml tubes

Homogenization

⇒ Have the following equipment ready and in the hood:
- __sterile glass homogenizers (1 per spleen)
- __10cc syringes
- __20 gauge needles
- __wire filters

⇒ Take out the bottom portion of the homogenizer from the sterile pouch –be careful to not touch any part of the homogenizer that will come in contact with the spleen
⇒ Pour the spleen and RPMI into the homogenizer
⇒ Homogenize with the top portion of the homogenizer until no spleen is visible (~5-7 strokes)
⇒ Pass homogenate through wire filter and 20 gauge needle to produce a single cell suspension
- Screw a 20 gauge needle into the smaller opening of the filter
- Screw a 10cc syringe into the larger opening of the filter
- Remove the syringe plunger
- Place assembly over 15cc tube
- Pour homogenate into syringe and reinsert plunger to push all cells *gently* through the filter

⇒ Fill tube with RPMI to wash
⇒ Centrifuge at 200g for 5 min
⇒ Aspirate supernatant

RBC Removal

⇒ Resuspend cells in 3ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500g for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge

⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15ml tube, fill with RPMI and spin at 300g for 5 min
⇒ Aspirate
⇒ Resuspend cells in 2ml of 1x R&D wash buffer (see below)

Column Loading for T Cell Purification

Begin preparing columns during 15 min spin from above

⇒ Dilute 10x R&D wash buffer to 1x using sterile H_2O (Will need 18ml/column)
⇒ Place columns in the R&D column rack
⇒ Remove the TOP cap first – to avoid drawing air into the bottom of the column
⇒ Remove the bottom cap
⇒ Rinse tip with EtOH while column drains into a waste receptacle (a 50ml tube)
⇒ Wash the column with 8ml of 1x wash buffer by applying the buffer to the top of the column
⇒ Replace the waste receptacle with a sterile 15cc tube
⇒ Apply the 2ml cell suspension to the top of the column. The cells will enter the column and displace the wash buffer in the column. This is collected in the 15cc tube.
⇒ Incubate the cells within the column at room temperature for 10 min
⇒ Elute the T cells from the column with 8ml of 1x wash buffer
⇒ Centrifuge the collected T cells at 200g for 5min
⇒ Aspirate and resuspend in 1ml of RPMI for counting

Cell Counting with Trypan Blue

⇒ Add 198ul of Trypan Blue to a 12x75mm tube with cap
⇒ Mix the cells by pipetting
⇒ Add 2ul of cells to 198ul Trypan Blue and resuspend
⇒ Resuspend the cells without forming bubbles and add to one side of a hemocytometer
⇒ Add the next sample to the other side of the hemocytometer
⇒ Using a 10x or 20x magnification look at the entire grid to make sure the cells are evenly distributed
⇒ Count the viable cells in one of the four 16-square grids
⇒ Write down number and repeat
⇒ 1 16-square grid = ___x10^6 cells/ml. Take the average of the two counts
⇒ Culture cells at 2 x 10^6/ml
Calculation:
(___x10^6 cells/ml)(1ml) = (2x10^6 cells/ml)(X) – 1ml

Example:
Count 7x10^6 cells/ml
(7x10^6 cells/ml)(1ml) = (2x10^6 cells/ml)(X)
X = 3.5 – 1ml (volume cells are presently in), so add 2.5ml 10%FBS-RPMI to cell suspension

Cell Culture

⇒ Label 24-well plates (one plate per stimulus)
⇒ Rinse plates containing incubating αCD3 from day before (day before add 300ul 1ug/ml αCD3 to each well of plates using the αCD3 stimulus)
- Forcefully flick out αCD3 solution
- Add 300ul RPMI to each well with a repeator pipet
- Shake briefly and flick out
- Repeat 2 more times

⇒ Add stimuli solutions in the appropriate volumes to the appropriate wells
⇒ Add the cells at the 2x10^6 cells/ml concentration to the wells with the solutions

⇒ Place in 37°C incubator for 24h incubation
⇒ Perform day 0 lipid extraction on remaining cells set aside for that purpose

STIMULI CALCULATIONS

αCD3 1ug/ml
-Need 12ml, make 1ug/ml sol'n
(1000ug/ml)(x) = (1ug/ml)(12000ul)
12ul stock into 12,000ul PBS Þ 300ul/well

αCD28 5ug/ml
-Need 6ml, make 10ug/ml sol'n
(1000ug/ml)(x) = (10ug/ml)(6000ul)
60ul stock into 5940ul RPMI Þ 250ul/well

Ionomycin 500nM
-Need 5ml, make 2000nM sol'n
(1000uM)(x) = (2uM)(5000ul)
10ul stock into 5000ul RPMI Þ add to PMA (1ng/ml) Þ add 250ul/well

PMA 1ng/ml
-Need 5ml, make 4ng/ml sol'n
[1] (1000ug/ml)(x) = (2ug/ml)(2000ul)
4ul stock into 2000ul RPMI
[2] (2000ng/ml)(x) = (4ng/ml)(5000ul)
10ul [1] into 5000ul RPMI Þ add to Ionomycin Þ add 250ul/well

PMA 0.5ng/ml
-Need 6ml, make 1ng/ml sol'n
[1] See Above
[2] (2000ng/ml)(x) = (1ng/ml)(6000ul)
3ul [1] into 6000ul RPMI Þ add 250ul/well

Dexamethasone 330ng/ml
-Need 6ml, make 660ng/ml sol'n
[1] (1000ug/ml)(x) = (10ug/ml)(1000ul)
10ul stock into 990ul RPMI
[2] (10000ng/ml)(x) = (660ng/ml)(6000ul)
396ul [1] into 5600ul RPMI Þ add 250ul/well

FasL 50ng/ml
-Need 3ml, make 200ng/ml sol'n
(100,000ng/ml)(x) = (200ng/ml)(3000ul)
6ul stock into 3000ul RPMI Þ add to aFLAG Þ add 250ul/well

αFLAG 1ug/ml
-Need 3ml, make 4ug/ml sol'n
(1000ug/ml)(x) = (4ug/ml)(3000ul)
12ul stock into 2988ul RPMI Þ add to FasL Þ add 250ul/well

APPENDIX B-5

Annexin V-FITC/ PI Protocol for Flow Cytometry

Solutions and Equipment:

- *Following 3 reagents from Pharmingen's Annexin V Kit 1 (# 6693KK*)
  - FITC-conjugated Annexin V
  - Annexin V Binding Buffer, 10x concentrate (0.1M HEPES, pH 7.4; 1.4M NaCl; 25nM $CaCl_2$). *Dilute to 1x with sterile H_2O prior to use.*
  - Propidium Iodide Staining Solution (50ug/ml stock in 1x PBS)
- 1xPBS, stored at 4°C
- 1.5ml tubes
- 12x75mm Polystyrene tubes (for the flow cytometer)
- Aluminum foil

**** Make a flow cytometry appointment with Roger Smith:** rosmith@tamu.edu

Protocol:

1. Remove 24-well plates containing 24h-stimulated lymphocytes at a concentration of $2x10^6$ cells/ml in 500ul from incubator.
2. For each well:
   a. Resuspend cells in well several times *forcefully* and aspirate using a p1000 pipettor set at **500ul**.
   b. Transfer aspirated cells to a 1.5ml tube.
   c. Rinse wells by resuspending *forcefully* several times with **500ul** cold 1xPBS and add to the1.5ml tube.
3. Centrifuge cells at 200xg, 5 min.
4. Aspirate supernatant leaving cell pellets behind.
5. Resuspend pellets in **100ul** of 1x Binding Buffer and transfer to 12x75mm polystyrene tubes. (This should be ~$1x10^5$ cells)
6. *In the dark* add to each tube:
   5ul of Annexin V-FITC
   2ul of Propidium Iodide
7. Resuspend after adding each solution
8. Wrap tubes in aluminum foil and incubate in the dark for 15 min at room temperature.
9. After the 15 min. incubation, add **400ul** of 1x Binding Buffer to each tube. *No need to mix—samples will be vortexed before loading into flow cytometer*
10. Cover tubes in aluminum foil again
11. Promptly take samples to the Vet. School flow cytometer in Dr. Smith's lab.

Samples need to be analyzed within 1 hour of staining

APPENDIX B-6

Thin Layer Chromatography (TLC)
Quantitating fatty acid mass of major phospholipids of T cells

After lipid extraction of the samples:

⇒ Prepare 12ml glass screw-top leak-proof vials (will need 7/sample)
- Put 1ml Acetone into each vial
- Mark Acetone level and tightly screw a black vinyl lid on
- Place at 80°C for 1h
- Use only vials that didn't evaporate

⇒ Pre-activate TLC plate by heating at 110°C for 1h

⇒ Remove extracts from -20°C and place on ice

⇒ Dry the samples under N_2

⇒ Redissolve the sample in 25ul FOLCH (MeOH/$CHCl_3$, 1:2, v:v) and place on ice
- Use a 25ul Wiretrol to add the FOLCH, then vortex thoroughly (make sure to vortex along sides of vial)
- Flush with N_2

⇒ Make-up TLC system solvents
($CHCl_3$/MeOH/Acetic Acid/H_2O, 50 : 37.5 : 3.5 : 2, v/v)
- Make ~100ml per TLC tank
- Use HPLC-grade H_2O and Tracepur Acetic Acid, MeOH and $CHCl_3$
- *Use glass pipets to add all solvents*

⇒ Pour solvent mixture into a TLC tank

⇒ Gently place 1/3 sheet of filter paper into tank width-side up to equilibrate the solvents

⇒ Place glass lid onto the top of tank and a heavy object (e.g. the top from the N_2 tank) on top of the lid to prevent evaporation

⇒ Allow solvents to equilibrate for at least 30 min

⇒ **Spot** the TLC plate after it has cooled from the pre-activation
- Lie plate silica-side up
- Using the extra wiretrol needle scrape a line across the plate 1/2" from the top
- Place the TLC "plate grid" on top of the plate and mark evenly spaced lines for the number of lanes needed. Scrape lines from top to bottom of the plate. (make sure to leave ~1/2" on both sides of the plate)

- Put "plate grid" back onto plate and use the shadow of the edge of the grid to spot the sample (make sure the shadow falls ~1" from the bottom of the plate)
- Vortex sample well and aspirate sample into the 25ul Wiretrol
- Spot the sample in one lane by releasing droplets, one-after-another, onto shadow-line
  -Spot in the center of the lane, staying away from the edges
  -Be sure not to touch the plate with tip of the Wiretrol
  -If needed, respot over previous spot once dry
- After spotting add 25ul additional FOLCH to sample, vortex and repot in same lane
- Wipe Wiretrol needle with FOLCH between each sample
- Include a standard (such as rat liver extract) on each plate

⇒ Place TLC plate, top side up, into tank. Replace glass cover and heavy object.

- Place the plate's edge in the middle of the bottom of the tank

⇒ Run for about 2h or until solvents reach <0.5cm from the top line

⇒ While the plate is running **prepare leak-proof tubes**

- Will need 7 tubes per sample spotted
- Label the tubes for: Sphingosine, PC, PS, PI, PE, Cardiolipin, and Neutral lipids
- Pour off the Acetone. Dry by inverting tubes in rack
- Rinse tubes and lids with 1ml MeOH (Tracepur grade) and pour off. Dry by inverting in rack
  -Invert and rotate tube to thoroughly rinse
  -*Make sure not to mix up lids (tubes are only guaranteed to be leak-proof w/ the corresponding lids)*

⇒ Add 500ng (10ul of the 50ng/ul stock) of 17:0 free fatty acid. This serves as an internal standard.

-To determine the amount of internal std to add to PL tubes: pmoles/10^6 cells of PL = 2.9

Breakdown by PL:

	pmoles/10^6 cells	MW
PC	1.53	760
PE	0.60	770
PS	0.26	810
PI	0.36	900
Sph	0.14	750
CL	0.14	1500

Calculation: PC = $(1.53 \times 10^{-9})(760) = 1.16 \times 10^{-6}$, Sph = $(0.14 \times 10^{-9})(750) = 0.1 \times 10^{-6}$

Range is ~1 µg-0.1 µg, so add **500ng** internal std.

- Add 3ml 6% HCl/MeOH and invert tube to mix with the ISD

- Keep tubes in dark until ready to use

⇒ Once plate in done running remove from tank and dry for ~5 min

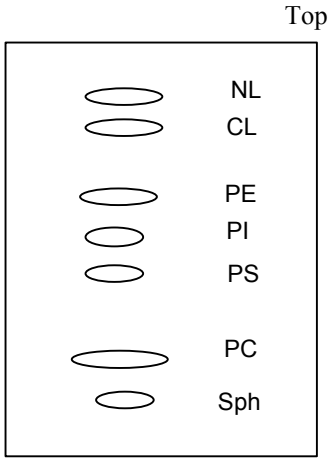
⇒ *In dark* **spray plate** with 0.1% ANS (8-Anilino-1-naphthalene-sulfonic acid) to view bands

- *0.1% ANS is stored at 4°C and is light sensitive*
- Place plate inside 3-sided box to contain ANS
- Spray plate by attaching flask container (containing 0.1% ANS) to N_2
- Spray evenly, but not so much as to soak the plate

⇒ View the plate with UV (if unable to see the bands clearly, spray again)

⇒ Circle the bands with pencil while viewing under UV

⇒ Bands should be located similar to illustration below:



⇒ **Scrape** the circled bands into the appropriate leak-proof tubes

- Use a TLC scraper blade (similar to an Exacto knife)
- Scrape in an upward motion so as not to lose the band
- Scrape over a funnel into tube
- Tap out all remaining pieces of plate caught in funnel into tube

⇒ Flush with N_2 and vortex for 1 min

⇒ Methylate at 76°C for 15h

⇒ **Extract** FAME:

- Add 2ml Hexane and 1ml 0.1M KCl to the leak-proof tubes
- Vortex for 1 min
- Centrifuge for 5 min at 3000rpm at 4°C (Jouan centrifuge) *–use rubber tube inserts to prevent tubes from breaking*
- Transfer upper phase, using Pasteur pipet, to a labeled 4ml glass vial- *be sure to replace black lids with green lids for organic solvents*

- Repeat the 2ml Hexane extract

⇒ Dry down the FAME under N_2

⇒ Redissolve in 25ul CH_2Cl_2, flush with N_2 and vortex

⇒ Run GC to calculate the mass of the phospholipids

Item	**Company**	**Catalog #**
TLC plate	VWR Scientific	EM5721-7
Omnisolv MeOH	EM	MX0480-1
Omnisolv $CHCl_3$	EM	CX1054-1
Tracepur Acetic Acid	EM	AX0077-1
HCl	Aldrich Chem. Co.	25,814-8
Filter paper	Whatman	1001-917
17:0 Free Fatty Acid	Sigma	T-2151
ANS	Aldrich Chem. Co.	13,992-0
Omnisolv Hexane	EM	HX0297-1
Acetone	Fisher	A929-4
Omnisolv CH_2Cl_2	EM	DX0837-1
KCl		

APPENDIX B-7

EXPT. 2: T cell Apoptosis *48h* - Experiment Description

Diets:
1. Olive Oil Ethyl Esters (2% olive oil + 3% corn oil)
2. Safflower Oil Ethyl Esters (2% safflower oil + 3% corn oil)
3. Fish Oil (2% menhaden fish oil + 3% corn oil)

n = 6 mice per diet = 18 mice total

Stimuli:
- Untreated
- αCD3/αCD28 (1ug/ml / 5ug/ml)
- PMA/Ionomycin (1ng/ml / 500nM)
- PMA/ αCD3 (0.5ng/ml / 1ug/ml)
- FasL/αFLAG (50ng/ml / 1ug/ml)
- PMA/Ionomycin (1ng/ml / 500nM) + FasL/αFLAG (50ng/ml / 1ug/ml)
- PMA/ αCD3 (0.5ng/ml / 1ug/ml) + FasL/αFLAG (50ng/ml / 1ug/ml)

7 treatments per mouse
culture 500,000 cells per well (treatment)
so, need 3.5 x 10^6 cells per animal

Purified T cells from column yields ~ 10 X 10^6 cells per animal

Culture 1 treatment per 24-well plate = 7 plates
Will use 18 wells per plate — 1 for each animal
so, will have 126 total wells (samples)

Analysis:
Annexin V/ PI - at 48h for 126 samples

APPENDIX B-8

EXPT. 2 - Diet Composition and Preparation

Diet Composition:

14g/mouse/day for 2 weeks
6 mice per group x 14 days x 14g/m/day
1176g per diet ⇒ make 1400g each diet

3 Groups:
1. 2% Olive Oil + 3% CO
2. 2% Saf + 3% CO
3. 2% FO + 3% CO

Lot # L90325WF *Lot* # L93271YF *Lot* # L95108BB

	g/100g	OO 1.4kg	Saf 1.4kg	FO 1.4kg	Total
Casein	20	280	280	280	840
Sucrose	42	588	588	588	1764
Starch	21.98	307.7	307.7	307.7	923.1
Cellulose	6	84	84	84	252
Mineral	3.5	49	49	49	147
Vitamin	1	14	14	14	42
Met.	0.3	4.2	4.2	4.2	12.6
Chol. Cl	0.2	2.8	2.8	2.8	8.4
Tenox-20A	0.1	1.4	1.4	1.4	4.2
OO	2	28			28
Saf	2		28		28
FO	2			28	28
CO	3	42	42	42	126

Tenox-20A = Source of **TBHQ**
32% Glycerol
30% Corn Oil
20% **TBHQ**
15% Propylene glycol
3% Citric acid
Amount of TBHQ = 0.02g/100g diet

Sources of other dietary ingredients:
- Sucrose: product # 3900, Bioserv, lot # 1979.08, 10kg
- Casein: product # 1100, Bioserv, lot # 1589.01, 10kg
- Choline Chloride: product # 30200, Harlan-Teklad, 500g
- Tenox-20A: B&D Nutritional Ingredients, Inc., lot # 90114, 4oz
- Salt Mix, AIN-76: product # F8505, Harlan-Teklad, lot # 30230, 4kg
- Vitamin Mix, AIN-76A: product # 40077, Harlan-Teklad, Rx 993083, 1kg
- Methionine: product # 1340, Bioserv, lot # 1806.02, 0.5kg
- Cellulose: product # 3425, Bioserv, lot # 2667.01, 10kg
- Corn Starch: product # 160170, Harlan-Teklad, lot # 96420, 25kg
- Corn Oil: product # BLKC40, Traco Labs, Inc., lot # 29025

Vitamin E content of diets

A mouse requires 22mg Vit. E/ kg diet – *NRC, Nutrient Requirements of Laboratory Animals*, 1995

Vitamin Mix:
10g vit E/kg Vitamin mix
= 1% vit E
= 0.01g vit E/100g diet
= 100mg vit E/kg diet

Olive Oil Ethyl Ester:
2.05mg vit E/g OO
= 2050mg vit E/kg OO
since 2% of diet
= 41mg vit E/kg diet

Safflower Oil Ethyl Ester:
1.9mg vit E/g Saf
= 1900mg vit E/kg Saf
since 2% of diet
= 38mg vit E/kg diet

Fish Oil:
1.9mg vit E/g FO
= 1900mg vit E/kg FO
since 2% of diet
= 38mg vit E/kg diet

Corn Oil:
896ppm = 896 mg vit E/kg CO
since 3% of diet
= 26.88mg vit E/kg diet

Total Vitamin E in each diet:

OO diet:
= 2% OO + 3% CO + Vitamin Mix
= 41mg + 26.88mg + 100mg
= 167.88mg vit E/kg diet

Saf diet:
= 2% Saf + 3% CO + Vitamin Mix
= 38mg + 26.88mg + 100mg
= 164.88mg vit E/kg diet

FO diet:
=2% FO + 3% CO + Vitamin Mix
= 38mg + 26.88mg + 100mg
= 164.88mg vit E/kg diet

See *Appendix B-2* for Diet Preparation details

APPENDIX B-9

EXPT. 2 Cell Preparation

*Take out **T cell columns and buffer, Lympholyte M**, and **10% FBS-RPMI** and warm to room temperature.*

Necropsy

⇒ Fill 15ml tubes (one for each animal) with 3ml of 10% FBS-RPMI
⇒ Assemble necessary equipment to take over to LARR:
__labcoat
__sterile scissors and forceps
__gloves
__EtOH
__15ml tubes
__keys, ID card
⇒ Go to LARR, room 202 to get mice
⇒ Get scale and take to necropsy room
⇒ Asphyxiate mice (1 cage at a time) with CO_2, then weigh each individually
⇒ Remove spleens aseptically and place in 15ml tubes

Homogenization

⇒ Have the following equipment ready and in the hood:
__sterile glass homogenizers (1 per spleen)
__10cc syringes
__20 gauge needles
__wire filters
⇒ Take out the bottom portion of the homogenizer from the sterile pouch –be careful to not touch any part of the homogenizer that will come in contact with the spleen
⇒ Pour the spleen and RPMI into the homogenizer
⇒ Homogenize with the top portion of the homogenizer until no spleen is visible (~5-7 strokes)
⇒ Pass homogenate through wire filter and 20 gauge needle to produce a single cell suspension
- Screw a 20 gauge needle into the smaller opening of the filter
- Screw a 10cc syringe into the larger opening of the filter
- Remove the syringe plunger
- Place assembly over 15cc tube
- Pour homogenate into syringe and reinsert plunger to push all cells *gently* through the filter

⇒ Fill tube with RPMI to wash
⇒ Centrifuge at 200g for 5 min
⇒ Aspirate supernatant

RBC Removal

⇒ Resuspend cells in 3ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500g for 15 min with the brake turned OFF

⇒ Carefully remove from centrifuge
⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15ml tube, fill with RPMI and spin at 300g for 5 min
⇒ Aspirate
⇒ Resuspend cells in 2ml of 1x R&D wash buffer (see below)

Column Loading for T Cell Purification

Begin preparing columns during 15 min spin from above

⇒ Dilute 10x R&D wash buffer to 1x using sterile H_2O (Will need 18ml/column)
⇒ Place columns in the R&D column rack
⇒ Remove the TOP cap first – to avoid drawing air into the bottom of the column
⇒ Remove the bottom cap
⇒ Rinse tip with EtOH while column drains into a waste receptacle (a 50ml tube)
⇒ Wash the column with 8ml of 1x wash buffer by applying the buffer to the top of the column
⇒ Replace the waste receptacle with a sterile 15cc tube
⇒ Apply the 2ml cell suspension to the top of the column. The cells will enter the column and displace the wash buffer in the column. This is collected in the 15cc tube.
⇒ Incubate the cells within the column at room temperature for 10 min
⇒ Elute the T cells from the column with 8ml of 1x wash buffer
⇒ Centrifuge the collected T cells at 200g for 5min
⇒ Aspirate and resuspend in 1ml of RPMI for counting

Cell Counting with Trypan Blue

⇒ Add 198ul of Trypan Blue to a 12x75mm tube with cap
⇒ Mix the cells by pipetting
⇒ Add 2ul of cells to 198ul Trypan Blue and resuspend
⇒ Resuspend the cells without forming bubbles and add to one side of a hemocytometer
⇒ Add the next sample to the other side of the hemocytometer
⇒ Using a 10x or 20x magnification look at the entire grid to make sure the cells are evenly distributed
⇒ Count the viable cells in one of the four 16-square grids
⇒ Write down number and repeat
⇒ 1 16-square grid = ___x10^6 cells/ml. Take the average of the two counts
⇒ Culture cells at 2 x 10^6/ml
Calculation:
(___x10^6 cells/ml)(1ml) = (2x10^6 cells/ml)(X) – 1ml

Example:
Count 7x10^6 cells/ml
(7x10^6 cells/ml)(1ml) = (2x10^6 cells/ml)(X)
X = 3.5 – 1ml (volume cells are presently in), so add 2.5ml 10%FBS-RPMI to cell suspension

Cell Culture

⇒ Label 24-well plates (one plate per stimulus)
⇒ Rinse plates containing incubating αCD3 from day before (day before add 300ul 1ug/ml αCD3 to each well of plates using the αCD3 stimulus)
- Forcefully flick out αCD3 solution
- Add 300ul RPMI to each well with a repeator pipet
- Shake briefly and flick out
- Repeat 2 more times

⇒ Add stimuli solutions in the appropriate volumes to the appropriate wells

⇒ Add the cells at the $2x10^6$ cells/ml concentration to the wells with the solutions
⇒ Place in 37°C incubator for 48h incubation

STIMULI CALCULATIONS

Plates 2, 4, 7

αCD3 1ug/ml
-Need 18ml, make 1ug/ml sol'n
(1000ug/ml)(x) = (1ug/ml)(18000ul)
18ul stock into 18,000ul PBS Þ 300ul/well

Plate 2

αCD28 5ug/ml
-Need 6ml, make 10ug/ml sol'n
(1000ug/ml)(x) = (10ug/ml)(6000ul)
60ul stock into 5940ul RPMI Þ 250ul/well

PMA 1st dilution
[1] (1000ug/ml)(x) = (2ug/ml)(4000ul)
8ul stock into 4000ul RPMI

Plate 3

PMA 1ng/ml
-Need 4ml, make 4ng/ml sol'n
[2] (2000ng/ml)(x) = (4ng/ml)(4000ul)
8ul [1] into 4000ul RPMI Þ add to Ionomycin (2000nM sol'n)

Ionomycin 500nM
-Need 4ml, make 2000nM sol'n
(1000uM)(x) = (2uM)(4000ul)
8ul stock into 4000ul RPMI Þ add to PMA (4ng/ml sol'n) Þ add 250ul/well

Plate 4

PMA 0.5ng/ml
-Need 6ml, make 1ng/ml sol'n
[2] (2000ng/ml)(x) = (1ng/ml)(6000ul)
3ul [1] into 6000ul RPMI Þ add 250ul/well

Plate 5

FasL 50ng/ml
-Need 3ml, make 200ng/ml sol'n
(100,000ng/ml)(x) = (200ng/ml)(6000ul)
12ul stock into 6000ul RPMI Þ add 3000ul to aFLAG
******* Use remaining for plate 7***

αFLAG 1ug/ml
-Need 3ml, make 4ug/ml sol'n
(1000ug/ml)(x) = (4ug/ml)(3000ul)
12ul stock into 2988ul RPMI Þ add to FasL Þ add 250ul/well

Plate 5 (cont.**)**

Plate 6

PMA 1ng/ml
-Need 2ml, make 6ng/ml sol'n
(2000ng/ml)(x) = (6ng/ml)(2000ul)
6ul [1] into 2000ul RPMI Þ add to Iono, FasL, and a**FLAG**

Ionomycin 500nM
-Need 2ml, make 3000nM sol'n
(1000uM)(x) = (3uM)(2000ul)
6ul stock into 2000ul RPMI Þ add to PMA, FasL, and a**FLAG**

FasL 50ng/ml
-Need 2ml, make 300ng/ml sol'n
(100,000ng/ml)(x) = (300ng/ml)(2000ul)
6ul stock into 2000ul RPMI Þ add to PMA, Iono, and a**FLAG**

αFLAG 1ug/ml
-Need 2ml, make 8ug/ml sol'n
(1000ug/ml)(x) = (8ug/ml)(2000ul)
16ul stock into 1984ul RPMI Þ add to PMA, Iono and FasL

Plate 7

PMA 0.5ng/ml
-Need 6ml, make 2ng/ml sol'n
(2000ng/ml)(x) = (2ng/ml)(3000ul)
3ul [1] into 3000ul RPMI Þ add to FasL and a**FLAG**

FasL 50ng/ml
Þ add 3ml to PMA and a**FLAG**

αFLAG 1ug/ml
-Need 3ml, make 6ug/ml sol'n
(1000ug/ml)(x) = (6ug/ml)(3000ul)
18ul stock into 2982ul RPMI Þ add to PMA and FasL

APPENDIX B-10

EXPT 3: Whole Splenocyte - Experiment Description

Diets:

1. Safflower Oil Ethyl Esters (2% safflower oil + 3% corn oil)
2. Fish Oil 2% (2% menhaden fish oil + 3% corn oil)
3. Fish Oil 4% (4% menhaden fish oil + 1% corn oil)

n = 6 mice per diet = 18 mice total

Stimuli:

- Untreated
- αCD3/αCD28 (1ug/ml / 5ug/ml)
- PMA/ αCD3 (0.5ng/ml / 1ug/ml)
- ConA (2.5ug/ml)

4 treatments per mouse
culture 5 x 10^5 cells per well (treatment)
so, need 2 x 10^6 cells per animal

Culture 1 treatment per 24-well plate = 4 plates
Will use 18 wells per plate — 1 for each animal
so, will have 72 total wells (samples)

Analysis:

1. a **CD3-PE/AnnexinV-FITC/ 7AAD** - at 24h for 72 samples
   - Gate on T cell population and accessory cell population for separate apoptosis readouts

APPENDIX B-11

EXPT. 3 & 4 - Diet Composition and Preparation

Diet Composition:

EXPT. 3:
14g/mouse/day for 2 weeks
6 mice per group x 14 days x 14g/m/day
1176g per diet

EXPT. 4:
14g/mouse/day for 2 weeks
12 mice per group x 14 days x 14g/m/day
2352g per diet

Total: 3528g per diet
make 3900g each diet

3 Groups:
1. 2% Saf + 3% CO
2. 2% FO + 3% CO
3. 4% FO + 1% CO

Lot # L90325WF *Lot* # L93271YF *Lot* # L95108BB

	g/100g	SAF 3.9kg	FO2% 3.9kg	FO4% 3.9kg	Total
Casein	20	780	780	780	2340
Sucrose	42	1638	1638	1638	4914
Starch	21.98	857.2	857.2	857.2	2571.6
Cellulose	6	234	234	234	702
Mineral	3.5	136.5	136.5	136.5	409.5
Vitamin	1	39	39	39	117
Met.	0.3	11.7	11.7	11.7	35.1
Chol. Cl	0.2	7.8	7.8	7.8	23.4
Tenox-20A	0.1	3.9	3.9	3.9	11.7
SAF	2	78			78
FO 2%	2		78		78
FO 4%	4			156	156
CO	3/1(FO 4%)	117	117	39	273

Tenox-20A = Source of **TBHQ**
32% Glycerol
30% Corn Oil
20% **TBHQ**
15% Propylene glycol
3% Citric acid
Amount of TBHQ = 0.02g/100g diet

Sources of other dietary ingredients:

- Sucrose: product # 3900, Bioserv, lot # 1979.08, 10kg
- Casein: product # 1100, Bioserv, lot # 1589.01, 10kg
- Choline Chloride: product # 30200, Harlan-Teklad, 500g
- Tenox-20A: B&D Nutritional Ingredients, Inc., lot # 90114, 4oz
- Salt Mix, AIN-76: product # F8505, Harlan-Teklad, lot # 30230, 4kg
- Vitamin Mix, AIN-76A: product # 40077, Harlan-Teklad, Rx 993083, 1kg
- Methionine: product # 1340, Bioserv, lot # 1806.02, 0.5kg
- Cellulose: product # 3425, Bioserv, lot # 2667.01, 10kg
- Corn Starch: product # 160170, Harlan-Teklad, lot # 96420, 25kg
- Corn Oil: product # BLKC40, Traco Labs, Inc., lot # 99-017-22

See *Appendix B-2* for diet preparation details

APPENDIX B-12

EXPT. 3 Cell Preparation

*Take out **T cell columns and buffer, Lympholyte M**, and **10% FBS-RPMI** and warm to room temperature.*

Necropsy

⇒ Fill 15ml tubes (one for each animal) with 3ml of 10% FBS-RPMI
⇒ Assemble necessary equipment to take over to LARR:
__labcoat
__sterile scissors and forceps
__gloves
__EtOH
__15ml tubes
__keys, ID card
⇒ Go to LARR, room 202 to get mice
⇒ Get scale and take to necropsy room
⇒ Asphyxiate mice (1 cage at a time) with CO_2, then weigh each individually
⇒ Remove spleens aseptically and place in 15ml tubes

Homogenization

⇒ Have the following equipment ready and in the hood:
__sterile glass homogenizers (1 per spleen)
__10cc syringes
__20 gauge needles
__wire filters
⇒ Take out the bottom portion of the homogenizer from the sterile pouch –be careful to not touch any part of the homogenizer that will come in contact with the spleen
⇒ Pour the spleen and RPMI into the homogenizer
⇒ Homogenize with the top portion of the homogenizer until no spleen is visible (~5-7 strokes)
⇒ Pass homogenate through wire filter and 20 gauge needle to produce a single cell suspension
- Screw a 20 gauge needle into the smaller opening of the filter
- Screw a 10cc syringe into the larger opening of the filter
- Remove the syringe plunger
- Place assembly over 15cc tube
- Pour homogenate into syringe and reinsert plunger to push all cells *gently* through the filter

⇒ Fill tube with RPMI to wash
⇒ Centrifuge at 200g for 5 min
⇒ Aspirate supernatant

RBC Removal

⇒ Resuspend cells in 3ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500g for 15 min with the brake turned OFF

⇒ Carefully remove from centrifuge
⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15ml tube, fill with RPMI and spin at 300g for 5 min
⇒ Aspirate
⇒ Resuspend cells in 1ml of RPMI

Cell Counting with Trypan Blue

⇒ Add 198ul of Trypan Blue to a 12x75mm tube with cap
⇒ Mix the cells by pipetting
⇒ Add 2ul of cells to 198ul Trypan Blue and resuspend
⇒ Resuspend the cells without forming bubbles and add to one side of a hemocytometer
⇒ Add the next sample to the other side of the hemocytometer
⇒ Using a 10x or 20x magnification look at the entire grid to make sure the cells are evenly distributed
⇒ Count the viable cells in one of the four 16-square grids
⇒ Write down number and repeat
⇒ 1 16-square grid = ___x10^6 cells/ml. Take the average of the two counts
⇒ Culture cells at 2 x 10^6/ml
Calculation:
(___x10^6 cells/ml)(1ml) = (2x10^6 cells/ml)(X) – 1ml

Example:
Count 7x10^6 cells/ml
(7x10^6 cells/ml)(1ml) = (2x10^6 cells/ml)(X)
X = 3.5 – 1ml (volume cells are presently in), so add 2.5ml 10%FBS-RPMI to cell suspension

Cell Culture

⇒ Label 24-well plates (one plate per stimulus)
⇒ Rinse plates containing incubating αCD3 from day before (day before add 300ul 1ug/ml αCD3 to each well of plates using the αCD3 stimulus)
- Forcefully flick out αCD3 solution
- Add 300ul RPMI to each well with a repeator pipet
- Shake briefly and flick out
- Repeat 2 more times

⇒ Add stimuli solutions in the appropriate volumes to the appropriate wells
⇒ Add the cells at the 2x10^6 cells/ml concentration to the wells with the solutions
⇒ Place in 37°C incubator for 24h incubation

STIMULI CALCULATIONS

αCD3 1ug/ml
Need 10.8ml for 2 plates x 18 wells
Make 12ml, 1ug/ml sol'n
(1000ug/ml)(x) = (1ug/ml)(12000ul)
12ul stock into 12,000ul PBS Þ 300ul/well - **Plates 2 & 3**

αCD28 5ug/ml
Need 4.5ml for 1 plate x 18 wells
Make 6ml, 10ug/ml sol'n
(1000ug/ml)(x) = (10ug/ml)(6000ul)
60ul stock into 5940ul RPMI Þ 250ul/well – **Plate 2**

PMA 0.5ng/ml
Need 4.5ml for 1 plate x 18 wells
Make 6ml, 1ng/ml sol'n
(2000ug/ml)(x) = (1ng/ml)(6000ul)
3ul stock into 6000ul RPMI Þ 250ul/well – **Plate 3**

ConA 2.5ug/ml
Need 4.5ml for 1 plate x 18 wells
Make 6ml, 5ug/ml sol'n
(10000ug/ml)(x) = (5ug/ml)(6000ul)
3ul stock into 6000ul RPMI Þ 250ul/well – **Plate 4**

APPENDIX B-13

Three color staining for flow cytometry (αCD3-PE/AnnexinV-FITC/7AAD)

Reagents and Equipment:

- αCD3-PE (0.1mg/ml) *Pharmingen #553063*
- dilute to 10ug/ml in Wash Buffer
- FC Block (0.5mg/ml) *Pharmingen #01241D*
- dilute to 5ug/ml in Wash Buffer
- AnnexinV-FITC (200 tests) *Pharmingen #556419*
- 7AAD (100 tests) *Pharmingen #555816*
- AnnexinV Binding Buffer, 10x concentrate (0.1M HEPES, pH 7.4; 1.4M NaCl; 25nM $CaCl_2$) *Pharmingen #556454*
  - dilute to 1x concentrate with sterlie H_2O
- Wash Buffer (pH 7.4):
  - 100ml PBS
  - 0.5g BSA (*Boehringer Mannheim #100030*)
  - 0.1g NaN_3
- 1xPBS, stored at 4°C
- 2.0ml microtubes
- 12x75mm Polystyrene FACS tubes (for the flow cytometer)
- Aluminum foil

Protocol:

1. Remove 24-well plates containing 24h-stimulated splenocytes at a concentration of $2x10^6$ cells/ml in 500ul from incubator.
2. For each well:
   - Resuspend cells in well several times *forcefully* and aspirate using a p1000 pipettor set at **500ul**.
   - Transfer aspirated cells to a 2.0ml tube.
   - Rinse wells by resuspending *forcefully* several times with **500ul** cold 1xPBS and add to the 2.0ml tube.
3. Centrifuge cells at 200xg, 5 min.
4. Aspirate supernatant leaving cell pellets behind.
5. Resuspend cells in **50ul** Wash Buffer
6. Add **10ul** FC Block [5ug/ml] and shake at RT for 10 min.
7. *In the dark* add **10ul** αCD3-PE [10ug/ml] and incubate *in the dark* for 30 min.
8. Wash with 1xPBS.
9. Centrifuge at 200xg, 5 min., asprirate supernatant
10. Resuspend cells in **100ul** 1x Binding Buffer
    - transfer cells to 12x75mm FACS tubes
11. *In the dark* add to each tube:
    5ul AnnexinV-FITC
    20ul 7AAD
12. Wrap tubes in Aluminum foil and incubate *in the dark* for 10-15 min
13. Add **400ul** 1x Binding Buffer to each tube. *No need to mix—samples will be vortexed before loading into the flow cytometer*
14. Cover tubes in Aluminum foil again
15. Promptly take samples to the Vet school's flow cytometer facility

Samples need to be analyzed within 1 hour of staining with Annexin V

APPENDIX B-14

EXPT. 4: AICD - Experiment Description

Diets:

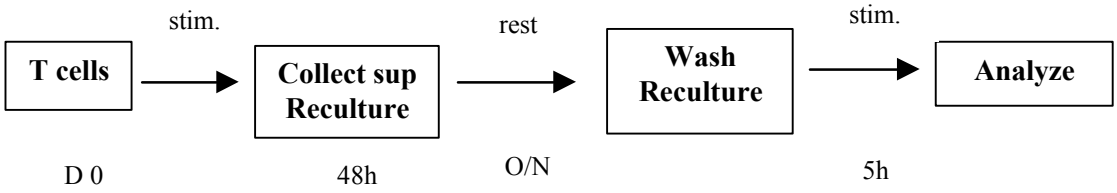
1. Safflower Oil Ethyl Esters (2% safflower oil + 3% corn oil)
2. Fish Oil 2% (2% menhaden fish oil + 3% corn oil)
3. Fish Oil 4% (4% menhaden fish oil + 1% corn oil)

n = 6 mice per diet , pool 2 spleens per n = 36 mice total

Stimuli:

1. αCD3/αCD28 (1ug/ml / 5ug/ml)
2. PMA/αCD3 (0.5ng/ml / 1ug/ml)
3. PMA/Iono (1ng/ml / 500nM)

3 treatments per mouse (n)
culture 2 wells of 3 x 10^6 cells per well per treatment at d0
so, need 18 x 10^6 cells per n



Analyses:

1. AnnexinV/PI – for 54 samples
2. Cytokine quantitation via ELISA

APPENDIX B-15

EXPT. 4 AICD – Cell Preparation

DAY 0

*Take out **T cell columns and buffer, Lympholyte M**, and **10% FBS-RPMI** and warm to room temperature.*

Necropsy

⇒ Fill 15ml tubes (one for each pair of animal) with 3ml of 10% FBS-RPMI
⇒ Assemble necessary equipment to take downstairs to basement:
__labcoat
__sterile scissors and forceps
__gloves
__EtOH
__15ml tubes
__keys
⇒ Asphyxiate mice (1 cage at a time) with CO_2, then weigh mice in pairs
⇒ Remove spleens aseptically and place 2 each in 15cc tubes

Homogenization

⇒ Have the following equipment ready and in the hood:
__sterile glass homogenizers (1 per 2 spleens)
__5cc syringes
__20 gauge needles
__wire filters
⇒ Take out the bottom portion of the homogenizer from the sterile pouch –be careful to not touch any part of the homogenizer that will come in contact with the spleen
⇒ Pour the spleen and RPMI into the homogenizer
⇒ Homogenize with the top portion of the homogenizer until no spleen is visible (~5-7 strokes)
⇒ Pass homogenate through wire filter and 20 gauge needle to produce a single cell suspension
- Screw a 20 gauge needle into the smaller opening of the filter
- Screw a 10cc syringe into the larger opening of the filter
- Remove the syringe plunger
- Place assembly over 15cc tube
- Pour homogenate into syringe and reinsert plunger to push all cells *gently* through the filter

⇒ Fill tube with RPMI to wash
⇒ Centrifuge at 200g for 5 min
⇒ Aspirate supernatant

RBC Removal

⇒ Resuspend cells in 3ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500g for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge

⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15cc tube, fill with RPMI and spin at 300g for 5 min
⇒ Aspirate
⇒ Resuspend cells in 2ml of 1x R&D wash buffer (see below)

Column Loading for T Cell Purification

Begin preparing columns during 15 min spin from above

⇒ Dilute 10x R&D wash buffer to 1x using sterile H_2O (Will need 18ml/column)
⇒ Place columns in the R&D column rack
⇒ Remove the TOP cap first – to avoid drawing air into the bottom of the column
⇒ Remove the bottom cap
⇒ Rinse tip with EtOH while column drains into a waste receptacle (a 50cc tube)
⇒ Wash the column with 8ml of 1x wash buffer by applying the buffer to the top of the column
⇒ Replace the waste receptacle with a sterile 15cc tube
⇒ Apply the 2ml cell suspension to the top of the column. The cells will enter the column and displace the wash buffer in the column. This is collected in the 15cc tube.
⇒ Incubate the cells within the column at room temperature for 10 min
⇒ Elute the T cells from the column with 8ml of 1x wash buffer
⇒ Centrifuge the collected T cells at 200g for 5min
⇒ Aspirate and resuspend in 1ml of RPMI for counting

Cell Counting with Trypan Blue

⇒ Add 198ul of Trypan Blue to a 12x75mm tube with cap
⇒ Mix the cells by pipetting
⇒ Add 2ul of cells to 198ul Trypan Blue and resuspend
⇒ Resuspend the cells without forming bubbles and add to one side of a hemocytometer
⇒ Add the next sample to the other side of the hemocytometer
⇒ Using a 10x or 20x magnification look at the entire grid to make sure the cells are evenly distributed
⇒ Count the viable cells in one of the four 16-square grids
⇒ Write down number and repeat
⇒ 1 16-square grid = ___x10^6 cells/ml. Take the average of the two counts
⇒ Culture cells at 3 x 10^6/ml
Calculation:
(___x10^6 cells/ml)(1ml) = (3x10^6 cells/ml)(X) – 1ml

Example:
Count 7x10^6 cells/ml
(18x10^6 cells/ml)(1ml) = (3x10^6 cells/ml)(X)
X = 6 – 1ml (volume cells are presently in), so add 5ml 10%FBS-RPMI to cell suspension

Cell Culture

⇒ Have the following solutions prepared ahead of time (in the AM):
- αCD3/αCD28 (1ug/ml / 5ug/ml)
- αCD3/PMA (1ug/ml / 0.5ng/ml)
- PMA/Iono (1ng/ml / 500nM)

⇒ Label 24-well plates (for large experiments label ahead of time)
⇒ Rinse plates containing incubating αCD3 from day before
- Forcefully flick out αCD3 solution
- Add 300ul RPMI to each well with a repeator pipet
- Shake briefly and flick out

- Repeat 2 more times

⇒ Add 1ml stimuli solutions to the appropriate wells
⇒ Add 1ml cells at $3x10^6$ cells/ml concentration to the wells with the solutions
- Culture 2 wells / sample for each stimulation

⇒ Place in 37°C incubator for 48h incubation

48 HOUR

⇒ Remove 24-well plates from incubator and harvest cells into 15cc tubes (2 wells/tube)
⇒ Centrifuge the collected T cells at 200g for 5min
⇒ Collect 2 x 500ul supernatant from the centrifuged samples and place each one of 500ul aliquots into a sterile microtube
- Store supernatants at -80°C

⇒ Aspirate remaining sup. and resuspend cells in1ml RPMI for counting
⇒ Count cells in 200ul Trypan Blue
⇒ Bring cells to a concentration of $2x10^6$ cells/ml
⇒ Add 1ml cells/well, 1 well per sample to 24-well plates containing 1ml/well RPMI
⇒ Place in 37°C incubator overnight

DAY 3

⇒ Remove 24-well plates from incubator and harvest cells into 15cc tubes
⇒ Centrifuge the collected T cells at 200g for 5min
⇒ Aspirate sup. and resuspend cells in 1ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500g for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge
⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15cc tube, fill with RPMI and spin at 300g for 5 min
⇒ Aspirate sup.
⇒ Resuspend cells in 1ml RPMI for counting
⇒ Count cells in 200ul Trypan Blue
⇒ Bring cells to a concentration of $2x10^6$ cells/ml
⇒ Add 500ul cells/well, 1 well per sample to 24-well plates containing 500ul/well of initial (**Day0**) stimulus
⇒ Place in 37°C incubator 4-5h

STIMULI CALCULATIONS

DAY 0

αCD3 1ug/ml
Will have 72 wells, 500ul/well
Make 40ml, 1ug/ml sol'n
(1000ug/ml)(x) = (1ug/ml)(40000ul)
40ul stock into 40,000ul PBS Þ 500ul/well, Plates 1 & 2 a-b

αCD28 5ug/ml
Will have 36 wells, 1ml/well
Make 40ml, 10ug/ml sol'n
(1000ug/ml)(x) = (10ug/ml)(40000ul)
400ul stock into 39,600ul RPMI Þ 1ml/well, Plates 1 a-b

PMA 0.5ng/ml
Will have 36 wells, 1ml/well
Make 40ml, 1ng/ml sol'n
(2000ng/ml)(x) = (1ng/ml)(40000ul)
20ul stock into 40,000ul RPMI Þ 1ml/well, Plates 2 a-b

PMA 1ng/ml
Will have 36 wells, Comb. w/ Iono = 500ul/well
Make 20ml, 4ng/ml sol'n
(2000ng/ml)(x) = (4ng/ml)(20000ul)
40ul stock

Ionomycin 500nM
Will have 36 wells, Comb. w/ PMA (1ng/ml) = 500ul/well
Make 20ml, 2uM sol'n
(1000uM)(x) = (2uM)(20000ul)
40ul stock

Add both to 20ml RPMI
1ml/well
Plates 3 a-b

DAY 3

αCD3 1ug/ml
Will have 36 wells, 300ul/well
Make 12ml, 1ug/ml sol'n
(1000ug/ml)(x) = (1ug/ml)(12000ul)
12ul stock into 12,000ul PBS Þ 300ul/well, Plates 1 & 2

αCD28 5ug/ml
Will have 18 wells, 500ul/well
Make 10ml, 10ug/ml sol'n
(1000ug/ml)(x) = (10ug/ml)(10000ul)
100ul stock into 9,900ul RPMI Þ 500ul/well, Plate 1

PMA 0.5ng/ml
Will have 18 wells, 500ul/well
Make 10ml, 1ng/ml sol'n
(2000ng/ml)(x) = (1ng/ml)(10000ul)
5ul stock into 10,000ul RPMI Þ 500ul/well, Plate 2

PMA 1ng/ml
Will have 18 wells, Comb. w/ Iono = 250ul/well
Make 5ml, 4ng/ml sol'n
(2000ng/ml)(x) = (4ng/ml)(5000ul)
10ul stock

Ionomycin 500nM
Will have 18 wells, Comb. w/ PMA (1ng/ml) = 250ul/well
Make 5ml, 2uM sol'n
(1000uM)(x) = (2uM)(5000ul)
10ul stock

Add both to 5ml RPMI
500ul/well
Plate 3

APPENDIX B-16

ELISA (*Enzyme-Linked Immunosorbent Assay*)

R&D Systems Quantikine M kit
IL-2: cat # M2000
IL-4: cat # M4000
IL-10: cat # M1000
IFNγ: cat # MIF00

Reagent Preparation

Bring all reagents to room temp before use

Mouse *cytokine* (refers to either IL-2, IL-4, IL-10, or IFNg) Kit Control
Reconstitute with 1.0 ml deionized/distilled water.

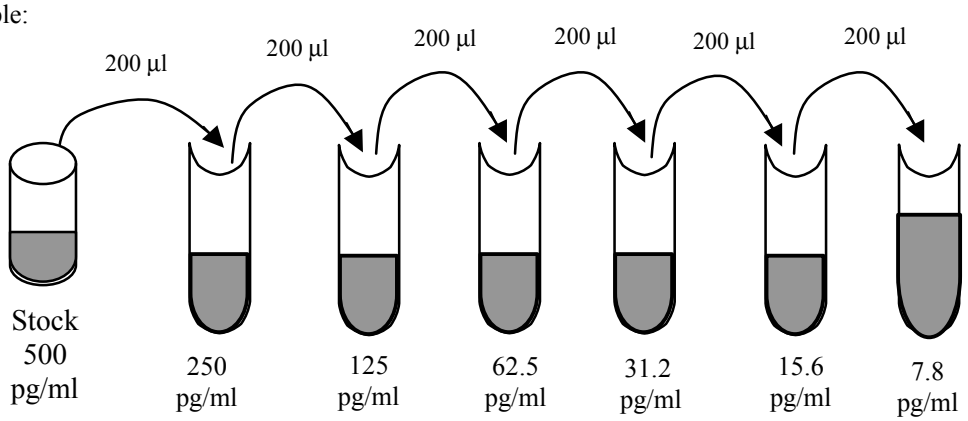
Mouse *cytokine* Conjugate Concentrate
For each plate make 10 ml. Add 450 µl Conjugate Concentrate to 9550 µl Conjugate Diluent.

Wash Buffer
For each plate make 500 ml. Add 20 ml Wash Buffer Concentrate (25X) to 480 ml deionized/distilled water.

Substrate Solution
For each plate make 10 ml. Mix Reagent A and Reagent B together in equal volumes (i.e., 5 ml/5 ml). Protect from light and use within 15 min.

Mouse c*ytokine* Standard
Reconstitute Standard with 2-5 ml (varies with cytokine) of Calibrator Diluent (for cell culture supernatant samples). Allow to sit for 5 min with gentle mixing prior to making dilutions. This reconstitution produces a stock solution.
Standard dilutions:
Use polypropylene tubes
Pipet 200 µl Calibrator diluent into 7 tubes labeled for decreasing dilution series. Use the stock to make a dilution series (see below). Mix thoroughly before each transfer. Use Calibrator diluent for 0 pg/ml Std.

Example:



Assay Protocol (as per Kit instructions):

- Bring kit reagents and 96-well plates to room temp. Thaw supernatant samples on ice.
- Prepare reagents and standard dilutions as previously described. Prepare supernatant samples to predetermined dilutions (dilute with Calibrator Diluent) so that expected absorbances will fall within the Standard ranges.
- Add 50 µl Assay Diluent to each well of the 96-well plate precoated with monoclonal antibody specific for the cytokine of interest.
- Add 50 µl Standards, Control, or Samples to each well. Tap plate gently for 1 min. Cover with the adhesive strip provided. Incubate at room temp for 2h.
- **Wash:** Dump-out plate contents into the sink and wash by adding 400 µl of Wash Buffer to each well using a multipipettor. Dump-out Wash Buffer after gentle shaking and blot plate against a clean paper towel to insure complete content removal. Repeat process 4 more times for a total of 5 washes.
- Add 100 µl Mouse cytokine conjugate to each well. Cover with a new adhesive strip. Incubate at room temp for 2h.
- Repeat wash step as described above.
- Prepare Substrate Solution as described above. Add 100 µl Substrate Solution to each well and cover plate with foil to protect from light. Incubate at room temp for 30 min.
- Add 100 µl Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
- Determine the optical density of each well within 30 min using a spectraphotometer set at 450 nm, with wavelength correction set at 540 nm.
- Generate a standard curve (optical density *vs* the concentration of the Standards) and calculate the Sample concentrations from this curve.

APPENDIX B-17

EXPT. 5 Part I and II: AICD - Experiment Description

Diets:

1. LFSAF, lowfat safflower oil (2% safflower oil + 3% corn oil)
2. LFFO, lowfat fish oil (4% menhaden fish oil + 1% corn oil)
3. HFSAF, highfat safflower oil (4% safflower oil + 6% corn oil)
4. HFFO, highfat fish oil (9% menhaden fish oil + 1% corn oil)

For each Part: n = 6 mice/diet, 3 spleens/n = 72 mice/Part = 144 mice total

Stimuli:

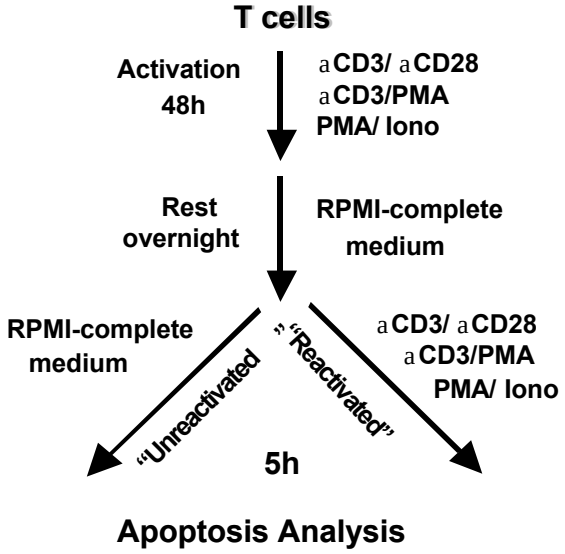
1. αCD3/αCD28 (1ug/ml / 5ug/ml)
2. " unstim. 2º
3. αCD3/PMA (1.0ug/ml / 0.5ng/ml)
4. " unstim. 2º
5. PMA/Iono (1ng/ml / 500nM)
6. " unstim. 2º

***Stagger Parts by one week

6 samples/4 diets = 24 samples x 6 treatments = 144 FACS samples/Part

Analyses:

1. AnnexinV/PI
2. Cytokine quantitation via ELISA (48h. d3)
3. mRNA for FasL and Fas (d3 AM and PM)



Experimental design for EXPT. 5

APPENDIX B-18

EXPT. 5 - Diet Composition and Preparation

14g/mouse/day for 2 weeks for 2 parts
18 mice per group x 14 d x 14g/m/d x 2 parts
7056g per diet ⇒ make 8000g per diet

4 Diet Groups:
1. 2% SAF + 3% CO **SAF LF**
2. 4% FO + 1% CO **FO LF**
3. 4% SAF + 6% CO **SAF HF**
4. 9% FO + 1% CO **FO HF**

	LF			HF			
	g/100g	**SAF 8.0kg**	**FO 8.0kg**	**g/100g**	**SAF 8.0kg**	**FO 8.0kg**	**Total**
Casein	20	1600	1600	20	1600	1600	6400
Sucrose	42	3360	3360	37	2960	2960	12640
Starch	21.9	1752	1752	21.9	1752	1752	7008
Cellulose	6	480	480	6	480	480	1920
Mineral	3.5	280	280	3.5	280	280	1120
Vitamin	1	80	80	1	80	80	320
Met.	0.3	24	24	0.3	24	24	96
Chol. Cl	0.2	16	16	0.2	16	16	64
Tenox-20A*	0.1	8.0	8.0	0.1	8.0	8.0	32
SAF		160			320		480
FO			320			720	1040
CO		240	80		480	80	880

***Tenox-20A** = Source of **TBHQ**
32% Glycerol
30% Corn Oil
20% **TBHQ**
15% Propylene Glycol
3% Citric Acid
Amount of TBHQ = 0.02g/100g diet

Sources of other dietary ingredients:
- Sucrose: product # 3900, Bioserv, lot # 1979.08, 10kg
- Casein: product # 1100, Bioserv, lot # 1589.01, 10kg
- Choline Chloride: product # 30200, Harlan-Teklad, 500g
- Tenox-20A: B&D Nutritional Ingredients, Inc., lot # 90114, 4oz
- Salt Mix, AIN-76: product # F8505, Harlan-Teklad, lot # 30230, 4kg
- Vitamin Mix, AIN-76A: product # 40077, Harlan-Teklad, Rx 993083, 1kg
- Methionine: product # 1340, Bioserv, lot # 1806.02, 0.5kg
- Cellulose: product # 3425, Bioserv, lot # 2667.01, 10kg
- Corn Starch: product # 160170, Harlan-Teklad, lot # 96420, 25kg
- Corn Oil: product # BLKC40, Traco Labs, Inc., lot # 01-010-07

LF *vs* HF diets

A mouse requires 22mg Vit. E/ kg diet – NRC, *Nutrient Requirements of Laboratory Animals*, 1995

LFSAF diet:
= 2% SAF + 3% CO + Vitamin Mix
= 38mg + 22.8mg + 100mg
= 160.8mg vit E/kg diet

LFFO diet:
=4% FO + 1% CO + Vitamin Mix
= 76mg + 7.6mg + 100mg
= 183.6mg vit E/kg diet

HFSAF diet:
=4% SAF + 6% CO + Vitamin Mix
= 76mg + 45.6mg + 100mg
= 221.6mg vit E/kg diet

HFFO diet:
=9% FO + 1% CO + Vitamin Mix
= 171mg + 7.6mg + 100mg
= 278.6mg vit E/kg diet

To equalize to 278.6g Vit E/kg diet need to add 0.006g Vit E/100g diet (6mg/100g)

LF diets
Casein = 20g x 4kcal/g = 80
Sucrose = 42 x 4 = 168
Starch = 21.9 x 4 = 87.6
Lipids = 5 x 9 = 45
380.6 kcal/100g

HF diets
Casein = 20 x 4 = 80
Sucrose = 37 x 4 = 148
Starch = 21.9 x 4 = 87.6
Lipids = 10 x 9 = 90
405.6 kcal/100g

APPENDIX B-19

EXPT. 5 (Parts I and II) AICD – Cell Preparation

DAY 0

*Take out **T cell columns and buffer, Lympholyte M**, and **10% FBS-RPMI** and warm to room temperature.*

Necropsy

⇒ Fill 15ml tubes (one for each pair of animal) with 3ml of 10% FBS-RPMI
⇒ Assemble necessary equipment to take downstairs to basement:
__labcoat
__sterile scissors and forceps
__gloves
__EtOH
__15ml tubes
__keys
⇒ Asphyxiate mice (1 cage at a time) with CO_2, then weigh mice per cage
⇒ Remove spleens aseptically and place 3 each in 15ml tubes

Homogenization

⇒ Have the following equipment ready and in the hood:
__sterile glass homogenizers (1 per 3 spleens)
__5cc syringes
__20 gauge needles
__wire filters
⇒ Take out the bottom portion of the homogenizer from the sterile pouch –be careful to not touch any part of the homogenizer that will come in contact with the spleen
⇒ Pour the spleen and RPMI into the homogenizer
⇒ Homogenize with the top portion of the homogenizer until no spleen is visible (~5-7 strokes)
⇒ Pass homogenate through wire filter and 20 gauge needle to produce a single cell suspension
- Screw a 20 gauge needle into the smaller opening of the filter
- Screw a 10cc syringe into the larger opening of the filter
- Remove the syringe plunger
- Place assembly over 15cc tube
- Pour homogenate into syringe and reinsert plunger to push all cells *gently* through the filter

⇒ Fill tube with RPMI to wash
⇒ Centrifuge at 200g for 5 min
⇒ Aspirate supernatant

RBC Removal

⇒ Resuspend cells in 3ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500g for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge

⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15cc tube, fill with RPMI and spin at 300g for 5 min
⇒ Aspirate
⇒ Resuspend cells in 2ml of 1x R&D wash buffer (see below)

Column Loading for T Cell Purification

Begin preparing columns during 15 min spin from above

⇒ Dilute 10x R&D wash buffer to 1x using sterile H_2O (Will need 18ml/column)
⇒ Place columns in the R&D column rack
⇒ Remove the TOP cap first – to avoid drawing air into the bottom of the column
⇒ Remove the bottom cap
⇒ Rinse tip with EtOH while column drains into a waste receptacle (a 50cc tube)
⇒ Wash the column with 8ml of 1x wash buffer by applying the buffer to the top of the column
⇒ Replace the waste receptacle with a sterile 15cc tube
⇒ Apply the 2ml cell suspension to the top of the column. The cells will enter the column and displace the wash buffer in the column. This is collected in the 15cc tube.
⇒ Incubate the cells within the column at room temperature for 10 min
⇒ Elute the T cells from the column with 8ml of 1x wash buffer
⇒ Centrifuge the collected T cells at 200g for 5min
⇒ Aspirate and resuspend in 1ml of RPMI for counting

Cell Counting with Trypan Blue

⇒ Add 198ul of Trypan Blue to a 12x75mm tube with cap
⇒ Mix the cells by pipetting
⇒ Add 2ul of cells to 198ul Trypan Blue and resuspend
⇒ Resuspend the cells without forming bubbles and add to one side of a hemocytometer
⇒ Add the next sample to the other side of the hemocytometer
⇒ Using a 10x or 20x magnification look at the entire grid to make sure the cells are evenly distributed
⇒ Count the viable cells in one of the four 16-square grids
⇒ Write down number and repeat
⇒ 1 16-square grid = ___x10^6 cells/ml. Take the average of the two counts
⇒ Culture cells at 5 x 10^6/ml
Calculation:
(___x10^6 cells/ml)(1ml) = (5x10^6 cells/ml)(X) – 1ml

Example:
Count 7x10^6 cells/ml
(20x10^6 cells/ml)(1ml) = (5x10^6 cells/ml)(X)
X = 4 – 1ml (volume cells are presently in), so add 3ml 10%FBS-RPMI to cell suspension

Cell Culture

⇒ Have the following solutions prepared ahead of time (in the AM):
- αCD3/αCD28 (1ug/ml / 5ug/ml)
- αCD3/PMA (1ug/ml / 0.5ng/ml)
- PMA/Iono (1ng/ml / 500nM)

⇒ Label 24-well plates (for large experiments label ahead of time)
⇒ Rinse plates containing incubating αCD3 from day before
- Forcefully flick out αCD3 solution
- Add 300ul RPMI to each well with a repeator pipet
- Shake briefly and flick out

- Repeat 2 more times

⇒ Add 1ml stimuli solutions to the appropriate wells
⇒ Add 1ml cells at $5x10^6$ cells/ml concentration to the wells with the solutions
- Culture 2 wells / sample for each stimulation

⇒ Place in 37°C incubator for 48h incubation

48 HOUR

⇒ Remove 24-well plates from incubator and harvest cells into 15cc tubes (2 wells/tube)
⇒ Centrifuge the collected T cells at 200g for 5min
⇒ Collect 2 x 500ul supernatant from the centrifuged samples and place each one of 500ul aliquots into a sterile microtube (**Part I only**)
- Store supernatants at -80°C

⇒ Aspirate remaining sup. and resuspend cells in1ml RPMI for counting
⇒ Count cells in 200ul Trypan Blue
⇒ Bring cells to a concentration of $2x10^6$ cells/ml
⇒ Add 1ml cells/well, 1 well per sample to 24-well plates containing 1ml/well RPMI
⇒ Place in 37°C incubator overnight

DAY 3

⇒ Remove 24-well plates from incubator and harvest cells into 15cc tubes
⇒ Centrifuge the collected T cells at 200g for 5min
⇒ Aspirate sup. and resuspend cells in 1ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500g for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge
⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15cc tube, fill with RPMI and spin at 300g for 5 min
⇒ Aspirate sup.
⇒ Resuspend cells in 1ml RPMI for counting
⇒ Count cells in 200ul Trypan Blue
⇒ Bring cells to a concentration of $2x10^6$ cells/ml
⇒ Add 500ul cells/well, 1 well per sample to 24-well plates containing 500ul/well of initial (**Day0**) stimulus
⇒ Place in 37°C incubator 4-5h
⇒ Save remaining cells for mRNA extraction (**Part II only**)

STIMULI CALCULATIONS

DAY 0

αCD3 1ug/ml
Make 50ml, 1ug/ml sol'n
(1000ug/ml)(x) = (1ug/ml)(50000ul)
50ul stock into 50ml PBS Þ 500ul/well

αCD28 5ug/ml
Make 50ml, 10ug/ml sol'n
(1000ug/ml)(x) = (10ug/ml)(50000ul)
500ul stock into 49.5ml RPMI Þ 1ml/well

PMA 0.5ng/ml
Make 50ml, 1ng/ml sol'n
(2000ng/ml)(x) = (1ng/ml)(50000ul)
25ul stock into 50ml RPMI Þ 1ml/well

PMA 1ng/ml
Make 50ml, 2ng/ml sol'n
(2000ng/ml)(x) = (2ng/ml)(50000ul)
50ul stock

Ionomycin 500nM
Make 50ml, 1000uM sol'n
(1000uM)(x) = (1uM)(50000ul)
50ul stock

Add both to 50ml RPMI 1ml/well

DAY 3

αCD3 1ug/ml
Make 50ml, 1ug/ml sol'n
(1000ug/ml)(x) = (1ug/ml)(50000ul)
50ul stock into 50ml PBS Þ 500ul/well

αCD28 5ug/ml
Make 50ml, 10ug/ml sol'n
(1000ug/ml)(x) = (10ug/ml)(50000ul)
500ul stock into 49.5ml RPMI Þ 500ul/well

PMA 0.5ng/ml
Make 50ml, 1ng/ml sol'n
(2000ng/ml)(x) = (1ng/ml)(50000ul)
25ul stock into 50ml RPMI Þ 500ul/well

PMA 1ng/ml
Make 50ml, 2ng/ml sol'n
(2000ng/ml)(x) = (2ng/ml)(50000ul)
50ul stock

Ionomycin 500nM
Make 50ml, 1000uM sol'n
(1000uM)(x) = (1uM)(50000ul)
50ul stock

Add both to
50ml RPMI
500ul/well

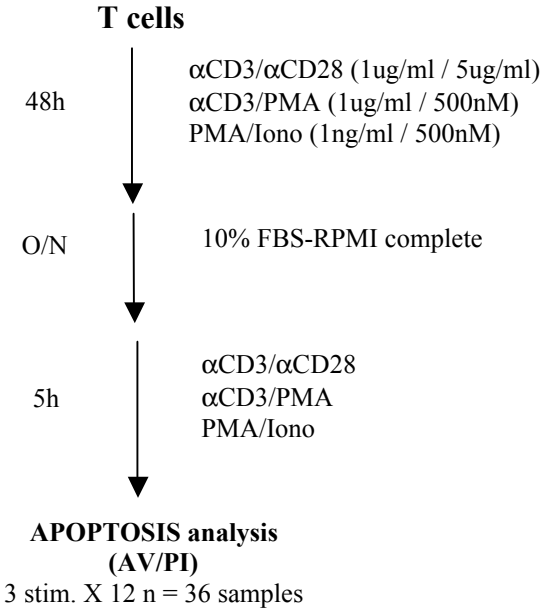
APPENDIX B-20

EXPT. 6: DHA AICD – Experiment Description

Diets:

1. Corn oil (5%)
2. DHA ethyl esters (1% DHA + 4% corn oil)

n = 6 mice per diet, pool 3 spleens per n = 36 mice total



APPENDIX B-21

EXPT. 6 Diet Composition and Preparation

14g/mouse/day for 2 weeks
18 mice per group x 14 d x 14g/m/d _____
3528g per diet ⇒ make 3800g per diet

2 Diet Groups:
1. 5% Corn Oil
2. 1% DHA ethyl ester + 4% CO

	g/100g	CO 3.8kg	DHA 3.8kg	TOTAL
Casein	20	760	760	1520
Sucrose	42	1596	1596	3192
Starch	21.9	832.2	832.2	1664.4
Cellulose	6	228	228	456
Mineral	3.5	133	133	266
Vitamin	1	38	38	76
Met.	0.3	11.4	11.4	22.8
Chol. Cl	0.2	7.6	7.6	15.2
Tenox-20A*	0.1	3.8	3.8	7.6
CO		190	152	342
DHA		---	38	38

***Tenox-20A** = Source of **TBHQ**
32% Glycerol
30% Corn Oil
20% **TBHQ**
15% Propylene Glycol
3% Citric Acid
Amount of TBHQ = 0.02g/100g diet

Sources of other dietary ingredients:
- Sucrose: product # 3900, Bioserv, lot # 1979.08, 10kg
- Casein: product # 1100, Bioserv, lot # 1589.01, 10kg
- Choline Chloride: product # 30200, Harlan-Teklad, 500g
- Tenox-20A: B&D Nutritional Ingredients, Inc., lot # 90114, 4oz
- Salt Mix, AIN-76: product # F8505, Harlan-Teklad, lot # 30230, 4kg
- Vitamin Mix, AIN-76A: product # 40077, Harlan-Teklad, Rx 993083, 1kg
- Methionine: product # 1340, Bioserv, lot # 1806.02, 0.5kg
- Cellulose: product # 3425, Bioserv, lot # 2667.01, 10kg
- Corn Starch: product # 160170, Harlan-Teklad, lot # 96420, 25kg
- Corn Oil: product # BLKC40, Traco Labs, Inc., lot # 01-010-07
- 4 oz. Storage containers: # 02-544-216A, Fisher Sci., 300/cs

Vitamin E content of diets

A mouse requires 22mg Vit. E/ kg diet – NRC, *Nutrient Requirements of Laboratory Animals*, 199**5**

Vitamin Mix:
10g vit E/kg Vitamin mix
= 1% vit E
= 0.01g vit E/100g diet
= 100mg vit E/kg diet

Corn Oil (5%):
896ppm = 896 mg vit E/kg CO
since 5% of diet
= 44.80mg vit E/kg diet

Corn Oil (4%):
896ppm = 896 mg vit E/kg CO
since 4% of diet
= 35.84mg vit E/kg diet

DHA Ethyl Ester:
3.0mg vit E/g SAF
= 3000mg vit E/kg DHA
since 1% of diet
= 30mg vit E/kg diet

Total Vitamin E in each diet:

CO diet:
= 5% CO + Vitamin Mix
= 44.80mg + 100mg
= 144.80mg vit E/kg diet

DHA diet:
=1% DHA + 4% CO + Vitamin Mix
= 30mg + 35.84mg + 100mg
= 165.84mg vit E/kg diet

APPENDIX B-22

EXPT. 6 Cell Preparation

DAY 0

*Take out **T cell columns and buffer, Lympholyte M**, and **10% FBS-RPMI** and warm to room temperature.*

Necropsy

⇒ Fill 15ml tubes (one for each set of 3 animals) with 3ml of 10% FBS-RPMI
⇒ Assemble necessary equipment to take downstairs to basement:
__labcoat
__sterile scissors and forceps
__gloves
__EtOH
__15ml tubes
__keys
⇒ Asphyxiate mice (1 cage at a time) with CO_2, then weigh mice in sets of three
⇒ Remove spleens aseptically and place 3 each in 15cc tubes

Homogenization

⇒ Have the following equipment ready and in the hood:
__sterile glass homogenizers (1 per 3 spleens)
__5cc syringes
__20 gauge needles
__wire filters
⇒ Take out the bottom portion of the homogenizer from the sterile pouch –be careful to not touch any part of the homogenizer that will come in contact with the spleen
⇒ Pour the spleens and RPMI into the homogenizer
⇒ Homogenize with the top portion of the homogenizer until no spleen is visible (~5-7 strokes)
⇒ Pass homogenate through wire filter and 20 gauge needle to produce a single cell suspension
- Screw a 20 gauge needle into the smaller opening of the filter
- Screw a 5cc syringe into the larger opening of the filter
- Remove the syringe plunger
- Place assembly over 15cc tube
- Pour homogenate into syringe and reinsert plunger to push all cells *gently* through the filter

⇒ Fill tube with RPMI to wash
⇒ Centrifuge at 200xg for 5 min
⇒ Aspirate supernatant

RBC Removal

⇒ Resuspend cells in 3ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500xg for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge

⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15cc tube, fill with RPMI and spin at 300xg for 5 min
⇒ Aspirate
⇒ Resuspend cells in 1ml of 1x R&D wash buffer (see below)

Column Loading for T Cell Purification

Begin preparing columns during 15 min spin from above

⇒ Dilute 10x R&D wash buffer to 1x using sterile H_2O (Will need 18ml/column)
⇒ Place columns in the R&D column rack
⇒ Remove the TOP cap first – to avoid drawing air into the bottom of the column
⇒ Remove the bottom cap
⇒ Rinse tip with EtOH while column drains into a waste receptacle (a beaker)
⇒ Wash the column with 6ml of 1x wash buffer by applying the buffer to the top of the column
⇒ Replace the waste receptacle with a sterile 50cc tube
⇒ Apply the 1ml cell suspension to the top of the column. The cells will enter the column and displace the wash buffer in the column. This is collected in the 50cc tube.
⇒ Incubate the cells within the column at room temperature for 10 min
⇒ Elute the T cells from the column with 8ml of 1x wash buffer
⇒ Centrifuge the collected T cells at 200xg for 5min
⇒ Aspirate and resuspend in 1ml of RPMI for counting

Cell Counting with Trypan Blue

4. Add 198ul of Trypan Blue to a 12x75mm tube with cap
⇒ Mix the cells by pipetting
⇒ Add 2ul of cells to 198ul Trypan Blue and resuspend
⇒ Resuspend the cells without forming bubbles and add to one side of a hemocytometer
⇒ Add the next sample to the other side of the hemocytometer
⇒ Using a 10x or 20x magnification look at the entire grid to make sure the cells are evenly distributed
⇒ Count the viable cells in one of the four 16-square grids
⇒ Write down number and repeat
⇒ 1 16-square grid = ___x10^6 cells/ml. Take the average of the two counts
⇒ Culture cells at 5 x 10^6/ml
Calculation:
(___x10^6 cells/ml)(1ml) = (5x10^6 cells/ml)(X) – 1ml

Example:
Count 7x10^6 cells/ml
(20x10^6 cells/ml)(1ml) = (5x10^6 cells/ml)(X)
X = 4 – 1ml (volume cells are presently in), so add 3ml 10%FBS-RPMI to cell suspension

Cell Culture

⇒ Have the following solutions prepared ahead of time (in the AM):
- αCD3/αCD28 (1ug/ml / 5ug/ml)
- αCD3/PMA (1ug/ml / 0.5ng/ml)
- PMA/Iono (1ng/ml / 500nM)

⇒ Label 24-well plates (for large experiments label ahead of time)
⇒ Rinse plates containing incubating αCD3 from day before
- *Carefully* aspirate αCD3 solution from each well – tilt plates so as to avoid touching well bottoms with pastuer pipet
- Add 300ul RPMI to each well with a repeator pipet

- Shake briefly and aspirate
- Repeat 2 more times

⇒ Add 1ml stimuli solutions to the appropriate wells
⇒ Add 1ml cells at $5x10^6$ cells/ml concentration to the wells with the solutions
- Culture 2 wells / sample for each stimulation

⇒ Place in 37°C incubator for 48h incubation

48 HOUR

⇒ Remove 24-well plates from incubator and harvest cells into 15cc tubes (2 wells/tube)
⇒ Centrifuge the collected T cells at 200xg for 5min
⇒ Aspirate sup. and resuspend cells in1ml RPMI for counting
⇒ Count cells in 200ul Trypan Blue
⇒ Bring cells to a concentration of $3x10^6$ cells/ml
⇒ Add 1ml cells/well, 2 wells per sample to 24-well plates containing 1ml/well RPMI
⇒ Place in 37°C incubator overnight
⇒ Coat plates with αCD3 (1ug/ml) for Day 3, store @4°C overnight

DAY 3

⇒ Prepare stimulation solutions for reactivation (αCD28, PMA/Iono)
⇒ Remove 24-well plates from incubator and harvest cells into 15cc tubes (2 wells/tube)
⇒ Centrifuge the collected T cells at 200xg for 5min
⇒ Aspirate sup. and resuspend cells in 1ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500xg for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge
⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15cc tube, fill with RPMI and spin at 300xg for 5 min
⇒ Aspirate sup.
⇒ Resuspend cells in 2ml RPMI (should be ~ $2x10^6$ cells/ml)
⇒ Rinse plates containing incubating αCD3 from day before
- *Carefully* aspirate αCD3 solution from each well – tilt plates so as to avoid touching well bottoms with pastuer pipet
- Add 300ul RPMI to each well with a repeator pipet
- Shake briefly and aspirate
- Repeat 2 more times

⇒ Add 500ul cells/well, 1 well per sample to 24-well plates containing 500ul/well of initial (Day0) stimulus
⇒ Place in 37°C incubator 4-5h

STIMULI CALCULATIONS

DAY 0

αCD3 1ug/ml
Make 25ml, 1ug/ml sol'n
(1000ug/ml)(x) = (1ug/ml)(25000ul)
25ul stock into 25ml PBS Þ 500ul/well

αCD28 5ug/ml
Make 25ml, 10ug/ml sol'n
(1000ug/ml)(x) = (10ug/ml)(25000ul)
250ul stock into 24.75ml RPMI Þ 1ml/well

PMA 0.5ng/ml
Make 25ml, 1ng/ml sol'n
(2000ng/ml)(x) = (1ng/ml)(25000ul)
12.5ul stock into 25ml RPMI Þ 1ml/well

PMA 1ng/ml
Make 25ml, 2ng/ml sol'n
(2000ng/ml)(x) = (2ng/ml)(25000ul)
25ul stock

Ionomycin 500nM
Make 25ml, 1000uM sol'n
(1000uM)(x) = (1uM)(25000ul)
25ul stock

Add both to 25ml RPMI 1ml/well

DAY 3

αCD3 1ug/ml
Make 13ml, 1ug/ml sol'n
(1000ug/ml)(x) = (1ug/ml)(13000ul)
13ul stock into 13ml PBS Þ 500ul/well

αCD28 5ug/ml
Make 13ml, 10ug/ml sol'n
(1000ug/ml)(x) = (10ug/ml)(13000ul)
130ul stock into 13ml RPMI Þ 500ul/well

PMA 0.5ng/ml
Make 13ml, 1ng/ml sol'n
(2000ng/ml)(x) = (1ng/ml)(13000ul)
6.5ul stock into 13ml RPMI Þ 500ul/well

PMA 1ng/ml
Make 13ml, 2ng/ml sol'n
(2000ng/ml)(x) = (2ng/ml)(13000ul)
13ul stock

Ionomycin 500nM
Make 13ml, 1000uM sol'n
(1000uM)(x) = (1uM)(13000ul)
13ul stock

Add both to
13ml RPMI
500ul/well

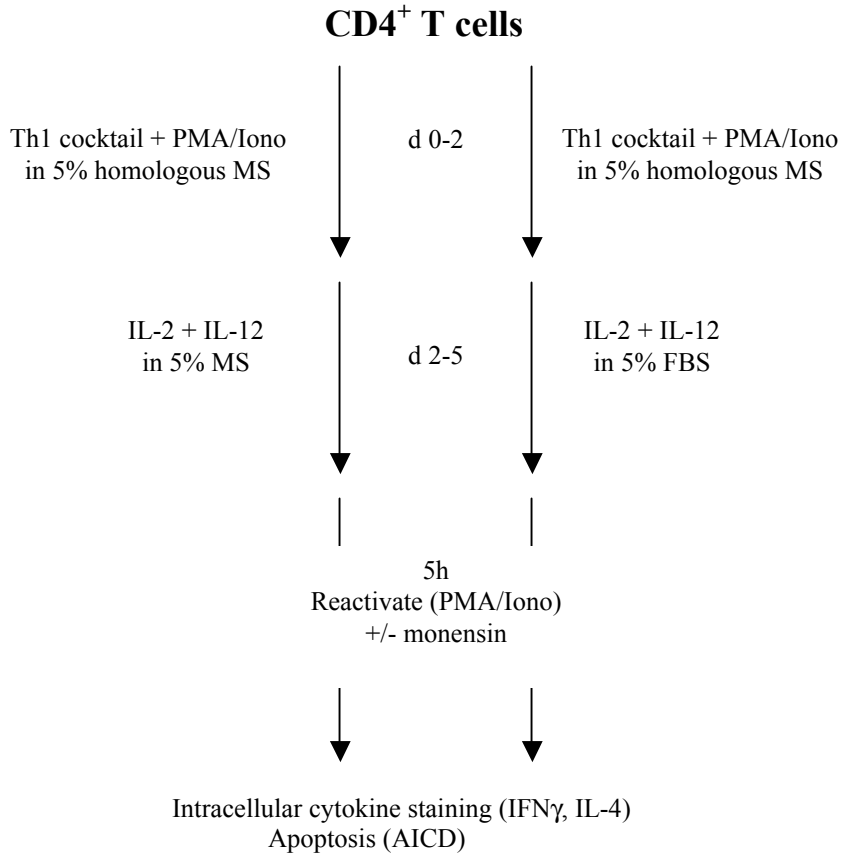
APPENDIX B-23

EXPT. 7 – Th1 AICD – Experimental Description

Diets:

1. 5% Corn oil
2. 4% Fish oil (+ 1% corn oil)

4 mice/n, n = 6/diet group = 24 mice/diet group



n=6 /diet = 12 samples for each analysis X 2 (for MS and FBS) = 48 TOTAL samples

Each n will have 4 outcomes:

1. FBS – AICD
2. FBS – cytokines
3. MS – AICD
4. MS – cytokines

APPENDIX B-24

EXPT. 7 Diet Composition and Preparation

14g/mouse/day for 2 weeks
24 mice per group x 14 d x 14g/m/d _____
4704 g per diet ⇒ make 5000 g per diet

2 Diet Groups:

1. 5% Corn Oil
2. 4% Menhaden fish oil + 1% CO

Use Basal Mix (95/5) from Harlan-Teklad (*see ingredients below*)
* use 950 g/kg diet in conjunction with 50 g of fat source

for 5000 g diet = 4750 g Basal Mix + 250 g fat source

CO Diet CO lot # 856-03		**FO Diet** FO lot # L951088B	
Basal Mix	4750 g	**Basal Mix**	4750 g
CO	250 g	**FO (4%)**	200 g
		CO (1%)	50 g

Basal Mix (95/5) Harlan-Teklad

Ingredient	g/kg
Casein	210.53
DL-methionine	3.16
Sucrose	441.89
Corn Starch	231.58
Cellulose	63.16
Mineral Mix, AIN-76	36.84
Vitamin Mix, AIN-76	10.53
Choline Chloride	2.11
TBHQ	0.21

APPENDIX B-25

EXPT. 7 - Th1 Polarization

Reagents

-- 5% FBS-RPMI
per 500 ml:
- 500 ml RPMI-1640 HEPES (Irvine Scientific #9159)
- 25 ml Heat inactivated FBS (Irvine Scientific #3003)
- 5.5 ml L-Glut (Gibco #3050-061)
- 5.5ml Pen-Strep. (Gibco #15140-148)
- 100 µl [10 µM] 2-ME (Sigma #M-7522)

-- 5% homologous mouse serum (MS)-RPMI
5% (2.5% MS + 2.5% FBS)
per 150 ml:
- 150 ml RPMI-1640 HEPES
- 3.75 ml heat inactivated MS-CO/MS-FO
- 3.75 ml heat inactivated FBS
- 1.5 ml L-Glut
- 1.5 ml Pen-Strep.
- 30 µl [10 µM] 2-ME

-- Lympholyte-M (Cedarlane #CL5031)
-- $CD4^+$ T cell enrichment columns
Mini Columns (can load up to 100 x 10^6 cells) (R&D Systems #MCD43)
Small Columns (can load up to 200 x 10^6 cells) (R&D Systems #MCD4C-1000)
-- Phorbal Myristate Acetate (PMA) (Sigma #P8139)
-- Ionomycin (Calbiochem #407952)
-- recombinant mouse IL-2 (Pharmingen #554592)
-- recombinant mouse IL-12 (R&D Systems #402-ML)
-- rat anti-mouse IL-4 (11B.11) (gift from NCI)

DAY 0

Take out $CD4^+$ T cell columns and buffer, Lympholyte M, and 10% FBS-RPMI and warm to room temperature.

Necropsy

⇒ Fill 15ml tubes (one per 4 animals) with 3ml of RPMI
⇒ Assemble necessary equipment to take down to basement:
__scissors and forceps
__gloves
__EtOH
__15ml tubes
__keys
⇒ Asphyxiate mice (1 cage at a time) with CO_2
⇒ Remove spleens aseptically, removing as much fat as possible, and place in 15ml tubes (4 spleens/tube)

Homogenization

⇒ Have the following equipment ready and in the hood:
__sterile glass homogenizers
__5cc syringes
__20 gauge needles
__wire mesh filters

⇒ Take out the bottom portion of the homogenizer from the sterile pouch –be careful to not touch any part of the homogenizer that will come in contact with the spleen
⇒ Pour the spleen and RPMI into the homogenizer
⇒ Homogenize with the top portion of the homogenizer until no spleen is visible (~5-7 strokes)
⇒ Pass homogenate through wire filter and 20 gauge needle to produce a single cell suspension
- Screw a 20 gauge needle into the smaller opening of the filter
- Screw a 5cc syringe into the larger opening of the filter
- Remove the syringe plunger
- Place assembly over 15ml tube
- Pour homogenate into syringe and reinsert plunger to push all cells *gently* through the filter

⇒ Fill tube with RPMI to wash
⇒ Centrifuge at 200xg for 5 min
⇒ Aspirate supernatant

RBC Removal

⇒ Resuspend cells in 3ml RPMI
⇒ Add an equal amount of Lympholyte M
- Using a 2ml pipet, insert the tip into the bottom of the tube
- Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the
- bottom of the tube
- Should have two distinct layers

⇒ Careful not to knock the tubes, centrifuge at 500xg for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge
⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15ml tube, fill with RPMI and spin at 300xg for 5 min
⇒ Resuspend cells in 1ml of 1x R&D column wash buffer (see below)

Column Loading for $CD4^+$ T Cell Enrichment

Begin preparing columns during 15 min spin from above

⇒ Dilute 10x R&D column wash buffer to 1x using sterile H_2O (Will need 50ml/column)
⇒ Place columns in the R&D column rack
⇒ Remove the TOP cap first – to avoid drawing air into the bottom of the column
⇒ Remove the bottom cap
⇒ Rinse tip with EtOH while column drains into a waste receptacle
⇒ Wash the column with 10ml of column wash buffer by applying the buffer to the top of the column
⇒ Replace the waste receptacle with a sterile 50ml tube
⇒ Add contents of 1 vial of monoclonal antibody cocktail (0.5ml) to each tube of cells
⇒ Incubate at room 15 min
⇒ Wash cells 2x with 10ml column wash buffer, 300xg for 5 min
⇒ Resuspend cells in 2ml column wash buffer
⇒ Apply the 2ml cell suspension to the top of the column. The cells will enter the column and displace the wash buffer in the column. This is collected in the 50ml tube.
⇒ Incubate the cells within the column at room temperature for 10 min
⇒ Elute the T cells from the column with 10ml of buffer
⇒ Centrifuge the collected $CD4^+$ T cells at 200xg for 5min
⇒ Resuspend in 1ml of 10% FBS-RPMI for counting

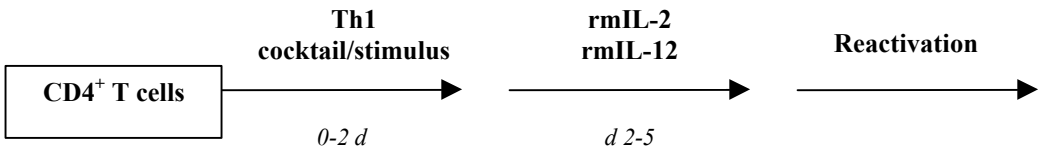
Cell Counting with Trypan Blue

⇒ Add 198µl of Trypan Blue to a 12x75mm tube with cap

⇒ Mix the cells by pipetting
⇒ Add 2? l of cells to 198µl of Trypan Blue and resuspend
⇒ Resuspend the cells without forming bubbles and add to one side of a hemocytometer
⇒ Add the next sample to the other side of the hemocytometer
⇒ Using a 10x or 20x magnification look at the entire grid to make sure the cells are evenly distributed
⇒ Count the viable cells in one of the four 16-square grids
⇒ Write down number and repeat
⇒ 1 16-square grid = ___x10^6 cells/ml. Take the average of the two counts
⇒ Culture cells at 5 X 10^6 cells/ml
Calculation:
(___ x 10^6 cells/ml)(1ml) = (5 x 10^6 cells/ml)(X) – 1ml

Example:
Counted 10x10^6 cells/ml
(10 x 10^6 cells/ml)(1ml) = (5 x 10^6 cells/ml)(X)-1ml
X = 2ml –1ml (volume cells are presently in), so add 1ml 5%FBS/MS to cell suspension

Cell Culture for Th1 Polarization



⇒ Prepare solution containing *Th1 cocktail* plus desired stimulus (ex. PMA/Iono)

Th1 Cocktail
10 µg/ml αIL-4
20 ng/ml rmIL-2
5 ng/ml rmIL-12

⇒ Label 24-well plates (for large experiments label ahead of time)
⇒ Add 1ml/well **Th1 Cocktail/stimulus solution** to the appropriate wells
⇒ Add 1ml of cells at [5 x 10^6 cells/ml] to the wells with the solution
⇒ Incubate at 37°C for 2 d

DAY 2 and DAY 3

⇒ Remove 24-well plates from incubator, pooling cells as needed
⇒ Harvest cells and wash once with RPMI at 200xg
⇒ Resuspend cells in 5%FBS/MS at volume equal to number of wells that will be cultured
(i.e. if culturing 2 wells, then resuspend in 2ml)
⇒ Prepare **rmIL-2 + rmIL-12 solution**
20ng/ml rmIL-2
5ng/ml rmIL-12

⇒ Add 1ml **rmIL-2 + rmIL-12 solution** per well and 1ml cells per well to 24-well plates
⇒ Incubate at 37°C until D3/D5

DAY 5

⇒ Remove 24-well plates from incubator, pooling cells as needed
⇒ Harvest cells and wash once with RPMI at 200xg
⇒ Resuspend cells in 3ml RPMI
⇒ Add an equal amount of Lympholyte M *(see procedure from above)*
⇒ Careful not to knock the tubes, centrifuge at 500xg for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge
⇒ Pipet off the lymphocyte layer
⇒ Place lymphocytes into a new 15ml tube, fill with RPMI and spin at 300xg for 5 min
⇒ Resuspend cells in 1ml of 5%FBS/MS for counting with Trypan Blue *(see above)*
⇒ Bring cell suspension to [2 x 10^6 cell/ml]
⇒ Reactivate 500µl cells/well in 500µl/well of initial D0 stimulus, i.e. PMA/Iono (w/o cytokines)***
⇒ Incubate at 37°C for 4-5h

***For cells designated for intracellular cytokine staining, add Golgistop (monensin) to PMA/Iono (see Intracellular Cytokine Staining protocol) to cultures at D5 reactivation

STIMULI CALCULATIONS

DAY 0

**Make 14 ml of 3 solutions of PMA/Iono + Th1 Cocktail (1 in each medium: 5% FBS, 5%MS-CO, 5%MS-FO)

PMA/Iono:

PMA (1 ng/ml)
(2000 ng/ml)(X) = (2 ng/ml)(14000 µl)
14 ml stock

Ionomycin (500 nM)
(1000 µM)(X) = (1 µM)(14000 µl)
14 ml stock

Th1 Cocktail:

rhIL-2 (20 ng/ml)
(20,000 ng/ml)(X) = (40 ng/ml)(14000 µl)
28 ml stock

rhIL-12 (5 ng/ml)
(50,000 ng/ml)(X) = (10 ng/ml)(14000 µl)
2.8 ml stock

αIL-4 (10 µg/ml)
(17,000 µg/ml)(X) = (20 µg/ml)(14000 µl)
16.5 ml stock

Add all to 14 ml
5% FBS
5% MS-CO
5% MS-FO

DAY 2 & 3

**Make 24 ml of rmIL-2 + rmIL-12 solution (in 5% FBS)

rhIL-2 (20 ng/ml)
(20,000 ng/ml)(X) = (40 ng/ml)(24000 µl)
48 ml stock

rhIL-12 (5 ng/ml)
(50,000 ng/ml)(X) = (10 ng/ml)(24000 µl)
4.8 ml stock

} Add both to 24 ml 5% FBS

**Make 14 ml of 2 solutions of rmIL-2 + rmIL-12 (1 in each medium: 5% MS-CO and 5% MS-FO)

rhIL-2 (20 ng/ml)
(20,000 ng/ml)(X) = (40 ng/ml)(14000 µl)
28 ml stock

rhIL-12 (5 ng/ml)
(50,000 ng/ml)(X) = (10 ng/ml)(14000 µl)
2.8 ml stock

} Add both to 14 ml 5% MS-CO and 5% MS-FO

APPENDIX B-26

Intracellular Cytokine Staining

Reagents and Equipment:

- FC Block (0.5 mg/ml) *Pharmingen #553142*
  - dilute to 5 µg/ml in Staining Buffer
- αIFN? -FITC (0.5 mg/ml) *Pharmingen #554411*
  -- dilute to 2.4 µg/ml in 1x Perm/Wash solution
- αIL-4-PE (0.2 mg/ml) *Pharmingen #554435*
  --dilute to 0.3 µg/ml in 1x Perm/Wash solution
- IgG_1 –FITC (0.5 mg/ml) *Pharmingen #554684*
  -- dilute to 2.4 µg/ml in 1x Perm/Wash solution
- IgG_1 –PE (0.2 mg/ml) *Pharmingen #554685*
  -- dilute to 0.3 µg/ml in 1x Perm/Wash solution
- BD Cytofix/Cytoperm Kit with Golgistop *Pharmingen #554715*
  Contents:
  --Cytofix/cytoperm solution
  --Perm/Wash solution, 10x concentrate (*dilute 1:10 in dH_2O prior to use)*
  --Golgistop (*monensin*)
- Staining Buffer (pH 7.4-7.6), filtered with 0.2µM pore membrane:
  - PBS without Mg^{2+} or Ca^{2+}
  - 1% heat inactivated FBS
  - 0.09% NaN_3
- 2.0ml microtubes
- 12x75mm Polystyrene FACS tubes (for the flow cytometer)
- Aluminum foil

Protocol:

Reactivation of cells with Golgistop (monensin)

In order to induce measurable accumulation of intracellular cytokines, cells must be reactivated in the presence of a golgi trafficking inhibitor such as monensin or brefeldin-A

See also Th1 Polarization protocol for reactivation

DAY 5

- Remove 24-well plates from incubator
- Harvest cells and wash once with RPMI at 200xg
- Resuspend cells in 3ml RPMI
- Add an equal amount of Lympholyte M *(see procedure from Th1 Polarization protocol)*
- Careful not to knock the tubes, centrifuge at 500xg for 15 min with the brake turned OFF
- Carefully remove from centrifuge
- Pipet off the lymphocyte layer
- Place lymphocytes into a new 15ml tube, fill with RPMI and spin at 300xg for 5 min
- Aspirate
- Resuspend cells in 1ml of 10%FBS-RPMI for counting with Trypan Blue
- Bring cell suspension to [2 x 10^6 cell/ml]
- Prepare Reactivation solution containing initial D0 stimulus (without cytokines) and Golgistop. Use 4µl Golgistop per 6ml cell culture

Since we dilute Reactivation solution 1:1 with cell suspension in each well prepare 8ml Golgistop/6ml Reactivation solution
NOTE: Golgistop is toxic so keep in cell culture ≤ 12h

- Add 500µl/well of Reactivation/Golgistop solution and 500µl/well cells
- Incubate at 37°C for 4-5h

Staining - *4-5h post-reactivation*

NOTE: Keep samples on ice following harvesting

- Harvest cells from 24-well plates and add to 2.0ml microtubes (one well/tube)
- Centrifuge cells at 200xg, 5 min @ 4°C. Aspirate supernatant leaving cell pellets behind.
- Resuspend cells in **100ml** Fc Block (5µg/ml). Incubate on ice 15 min
- Centrifuge cells at 200xg, 5 min @ 4°C. Aspirate supernatant leaving cell pellets behind.
- Resuspend cells in **250ml** Cytofix/Cytoperm solution. Incubate on ice 20 min
- Wash 2x in 1x Perm/Wash solution (1ml/tube)

COMPLETE FOLLOWING IN DARK

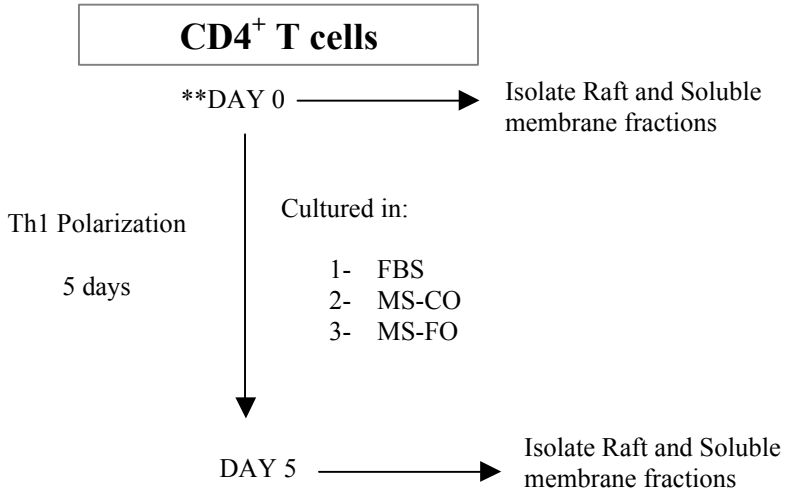
- Resuspend fixed and permeabilized cells in **100ml** of appropriate fluorochrome-conjugated anti-cytokine Ab solution or appropriate negative control. Incubate on ice 30 min
- Wash 2x in 1x Perm/Wash solution (1ml/tube)
- Resuspend cells in **500ml** Staining Buffer
- Store @ 4°C, covered in Al foil until analysis
- Immediately prior to Flow cytometric analysis, transfer stained cells to 12x75mm polystyrene FACS tubes.

APPENDIX B-27

EXPT. 8 – Th1 Raft – Experimental Description

Diets:
1 - 5% Corn oil
2 - 4% FO (+ 1% corn oil)

10 mice/n, n = 3/diet group = 30 mice/diet group; 60 mice total



**Take 1/2 cells for DAY 0 (~25 x 10^6). Divide remaining 1/2 between FBS and MS cultures

Analyses: For Raft and Soluble fractions
- FA composition following TLC separation of PL
- cholesterol

DAY 0 analyses = CO & FO, n=3, 6 samples (of rafts and soluble)

DAY 5 analyses =

1 – FBS (for CO-fed mice) n=3
2 – FBS (for FO-fed mice) n=3
3 – MS-CO n=3
4 – MS-FO n=3

} 12 samples (of rafts and soluble)

APPENDIX B-28

EXPT. 8 Diet Composition and Preparation

14g/mouse/day for 2 weeks
30 mice per group x 14 d x 14g/m/d
5880 g per diet ⇒ make 6100 g per diet

2 Diet Groups:
1. 5% Corn Oil
2. 4% Menhaden fish oil + 1% CO

Use Basal Mix (95/5) from Harlan-Teklad (*see ingredients below*)
* use 950 g/kg diet in conjunction with 50 g of fat source

for 6100 g diet = 5795 g Basal Mix + 305 g fat source

CO Diet CO lot # 856-AR		**FO Diet** FO lot # L97029BB	
Basal Mix	5795 g	**Basal Mix**	5795 g
CO	305 g	**FO (4%)**	244 g
		CO (1%)	61 g

Basal Mix (95/5) Harlan-Teklad

Ingredient	g/kg
Casein	210.53
DL-methionine	3.16
Sucrose	441.89
Corn Starch	231.58
Cellulose	63.16
Mineral Mix, AIN-76	36.84
Vitamin Mix, AIN-76	10.53
Choline Chloride	2.11
TBHQ	0.21

APPENDIX B-29

EXPT. 8 - Th1 Raft Analysis

Reagents

-- 5% FBS-RPMI
per 500 ml:
- 500 ml RPMI-1640 HEPES (Irvine Scientific #9159)
- 25 ml Heat inactivated FBS (Irvine Scientific #3003)
- 5.5 ml L-Glut (Gibco #3050-061)
- 5.5ml Pen-Strep. (Gibco #15140-148)
- 100 µl [10 µM] 2-ME (Sigma #M-7522)

-- 5% homologous mouse serum (MS)-RPMI
5% (2.5% MS + 2.5% FBS)
per 150 ml:
- 150 ml RPMI-1640 HEPES
- 3.75 ml heat inactivated MS-CO/MS-FO
- 3.75 ml heat inactivated FBS
- 1.5 ml L-Glut
- 1.5 ml Pen-Strep.
- 30 µl [10 µM] 2-ME

-- Lympholyte-M (Cedarlane #CL5031)
-- CD4+ T cell enrichment columns
Mini Columns (can load up to 100 x 10^6 cells) (R&D Systems #MCD43)
Small Columns (can load up to 200 x 10^6 cells) (R&D Systems #MCD4C-1000)
-- Phorbal Myristate Acetate (PMA) (Sigma #P8139)
-- Ionomycin (Calbiochem #407952)
-- recombinant mouse IL-2 (Pharmingen #554592)
-- recombinant mouse IL-12 (R&D Systems #402-ML)
-- rat anti-mouse IL-4 (11B.11) (gift from NCI)

DAY 0

*Take out **CD4$^+$ T cell columns and buffer, Lympholyte M**, and **10% FBS-RPMI** and warm to room temperature.*

Necropsy

⇒ Fill 15ml tubes (one per 4 animals) with 3ml of RPMI
⇒ Assemble necessary equipment to take down to basement:
__scissors and forceps
__gloves
__EtOH
__15ml tubes
__keys
⇒ Asphyxiate mice (1 cage at a time) with CO_2
⇒ Remove spleens aseptically, removing as much fat as possible, and place in 15ml tubes (4 spleens/tube)

Homogenization

⇒ Have the following equipment ready and in the hood:
__sterile glass homogenizers
__5cc syringes
__20 gauge needles

__wire mesh filters

⇒ Take out the bottom portion of the homogenizer from the sterile pouch –be careful to not touch any part of the homogenizer that will come in contact with the spleen
⇒ Pour the spleen and RPMI into the homogenizer
⇒ Homogenize with the top portion of the homogenizer until no spleen is visible (~5-7 strokes)
⇒ Pass homogenate through wire filter and 20 gauge needle to produce a single cell suspension
  - ❑ Screw a 20 gauge needle into the smaller opening of the filter
  - ❑ Screw a 5cc syringe into the larger opening of the filter
  - ❑ Remove the syringe plunger
  - ❑ Place assembly over 15ml tube
  - ❑ Pour homogenate into syringe and reinsert plunger to push all cells *gently* through the filter
⇒ Fill tube with RPMI to wash
⇒ Centrifuge at 200xg for 5 min
⇒ Aspirate supernatant

RBC Removal

⇒ Resuspend cells in 6ml RPMI. Pool all 3 tubes/n, then split into 2 tubes (3ml/tube)
⇒ Add an equal amount of Lympholyte M
  - ❑ Using a 2ml pipet, insert the tip into the bottom of the tube
  - ❑ Layer Lympholyte M underneath the cell suspension by *slowly* adding it to the bottom of the tube
  - ❑ Should have two distinct layers
⇒ Careful not to knock the tubes, centrifuge at 500xg for 15 min with the brake turned OFF
⇒ Carefully remove from centrifuge
⇒ Pipet off the lymphocyte layer (layer at the interface between the two layers) by using a *slow* swirling motion with the pipet
⇒ Place lymphocytes into a new 15ml tube, fill with RPMI and spin at 300xg for 5 min
⇒ Resuspend cells in 1ml of 1x R&D column wash buffer (see below)

Column Loading for $CD4^+$ T Cell Enrichment

Begin preparing columns during 15 min spin from above

⇒ Dilute 10x R&D column wash buffer to 1x using sterile H_2O (Will need 50ml/column)
⇒ Place columns in the R&D column rack
⇒ Remove the TOP cap first – to avoid drawing air into the bottom of the column
⇒ Remove the bottom cap
⇒ Rinse tip with EtOH while column drains into a waste receptacle
⇒ Wash the column with 10ml of column wash buffer by applying the buffer to the top of the column
⇒ Replace the waste receptacle with a sterile 50ml tube
⇒ Add contents of 1 vial of monoclonal antibody cocktail (0.5ml) to each tube of cells
⇒ Incubate at room 15 min
⇒ Wash cells 2x with 10ml column wash buffer, 300xg for 5 min
⇒ Resuspend cells in 2ml column wash buffer
⇒ Apply the 2ml cell suspension to the top of the column. The cells will enter the column and displace the wash buffer in the column. This is collected in the 50ml tube.
⇒ Incubate the cells within the column at room temperature for 10 min
⇒ Elute the T cells from the column with 10ml of buffer
⇒ Centrifuge the collected $CD4^+$ T cells at 200xg for 5min
⇒ Resuspend in 1ml of 10% FBS-RPMI for counting (combine duplicate tubes for ea. n = 6 tubes)

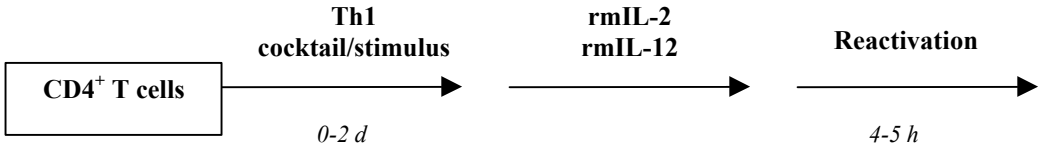
Cell Counting with Trypan Blue

⇒ Add 198µl of Trypan Blue to a 12x75mm tube with cap
⇒ Mix the cells by pipetting
⇒ Add 2µl of cells to 198µl of Trypan Blue and resuspend
⇒ Resuspend the cells without forming bubbles and add to one side of a hemocytometer
⇒ Add the next sample to the other side of the hemocytometer
⇒ Using a 10x or 20x magnification look at the entire grid to make sure the cells are evenly distributed
⇒ Count the viable cells in one of the four 16-square grids
⇒ Write down number and repeat
⇒ 1 16-square grid = ___x10^6 cells/ml. Take the average of the two counts
⇒ Resuspend cells at 5 X 10^6 cells/ml. **Remove 1/2 of cells from each sample for *day 0* raft isolation (see T cell Raft Isolation protocol)

Calculation:
(___ x 10^6 cells/ml)(1ml) = (5 x 10^6 cells/ml)(X) – 1ml

Example:
Counted 10x10^6 cells/ml
(10 x 10^6 cells/ml)(1ml) = (5 x 10^6 cells/ml)(X)-1ml
X = 2ml –1ml (volume cells are presently in), so add 1ml 10%FBS-RPMI to cell suspension

Cell Culture for Th1 Polarization



⇒ Prepare solution containing *Th1 cocktail* plus desired stimulus (ex. PMA/Iono)

Th1 Cocktail
10µg/ml αIL-4
20ng/ml rmIL-2
5ng/ml rmIL-12

⇒ Label 24-well plates (for large experiments label ahead of time)
⇒ Add 1ml/well **Th1 Cocktail/stimulus solution** to the appropriate wells
⇒ Add 1ml of cells at [5 x 10^6 cells/ml] to the wells with the solution
⇒ Incubate at 37°C for 2 d

DAY 2 and DAY 3

⇒ Remove 24-well plates from incubator
⇒ Harvest cells, pooling cells as needed, and wash once with RPMI at 200xg
⇒ Resuspend cells in 5%FBS/MS at volume equal to number of wells that will be cultured
(i.e. if culturing 2 wells, then resuspend in 2ml)
⇒ Prepare **rmIL-2 + rmIL-12 solution**
20ng/ml rmIL-2
5ng/ml rmIL-12

⇒ Add 1ml **rmIL-2 + rmIL-12 solution** per well and 1ml cells per well to 24-well plates
⇒ Incubate at 37°C until D3/D5

DAY 5

⇒ Remove 24-well plates from incubator
⇒ Harvest cells, pooling cells as needed, and wash once with RPMI at 200xg
⇒ Proceed with raft isolation (see T cell Raft Isolation protocol) *count cells before isolation*

STIMULI CALCULATIONS

DAY 0

**Make 3 solutions of PMA/Iono + Th1 Cocktail (20 ml in 5% FBS, 10 ml in 5% MS-CO and MS-FO)

5% FBS solution

PMA/Iono:

PMA (1 ng/ml)
(2000 ng/ml)(X) = (2 ng/ml)(20000 µl)
20 ml stock

Ionomycin (500 nM)
(1000 µM)(X) = (1 µM)(20000 µl)
20 ml stock

Th1 Cocktail:

rhIL-2 (20 ng/ml)
(20,000 ng/ml)(X) = (40 ng/ml)(20000 µl)
40 ml stock

rhIL-12 (5 ng/ml)
(50,000 ng/ml)(X) = (10 ng/ml)(20000 µl)
4 ml stock

αIL-4 (10 µg/ml)
(17,000 µg/ml)(X) = (20 µg/ml)(20000 µl)
23.5 ml stock

Add all to 20 ml 5% FBS

5% MS solution

PMA/Iono:

PMA (1 ng/ml)
(2000 ng/ml)(X) = (2 ng/ml)(10000 µl)
10 ml stock

Ionomycin (500 nM)
(1000 µM)(X) = (1 µM)(10000 µl)
10 ml stock

Th1 Cocktail:

rhIL-2 (20 ng/ml)
(20,000 ng/ml)(X) = (40 ng/ml)(10000 µl)
20 ml stock

rhIL-12 (5 ng/ml)
(50,000 ng/ml)(X) = (10 ng/ml)(10000 µl)
2 ml stock

αIL-4 (10 µg/ml)
(17,000 µg/ml)(X) = (20 µg/ml)(10000 µl)
11.8 ml stock

} Add all to 10 ml 5% MS-CO and 5% MS-FO

DAY 2 & 3

**Make 20 ml of rmIL-2 + rmIL-12 solution (in 5% FBS)

rhIL-2 (20 ng/ml)
(20,000 ng/ml)(X) = (40 ng/ml)(20000 µl)
40 ml stock

rhIL-12 (5 ng/ml)
(50,000 ng/ml)(X) = (10 ng/ml)(20000 µl)
4 ml stock

} Add both to 20 ml 5% FBS

**Make 10 ml of 2 solutions of rmIL-2 + rmIL-12 (1 in each medium: 5% MS-CO and 5% MS-FO)

rhIL-2 (20 ng/ml)
(20,000 ng/ml)(X) = (40 ng/ml)(10000 µl)
20 ml stock

rhIL-12 (5 ng/ml)
(50,000 ng/ml)(X) = (10 ng/ml)(10000 µl)
2 ml stock

} Add both to 10 ml 5% MS-CO and 5% MS-FO

APPENDIX B-30

T cell Raft Isolation *adapted from YY Fan*

Reagent preparation:

Use volumetric flasks for all solutions, make fresh

Lysis Buffer (100ml)	Final conc.
584.4 mg NaCl (MW: 58.44)	100 mM
83.2 mg EDTA (MW: 416.2)	2 mM
28.8 ml AEBSF (stock: 500mM)	144 µM
100 ml Na_3VO_4 (stock: 200mM)	0.2 mM
12.5 ml NaF (stock: 200 mM)	50 µM
595.8 mg Hepes (MW: 238.3)	25 mM

pH to 6.9

-- bring up to volume with dd H_2O

Brij-58 Buffer (5 ml)

Add 0.05 g Brij-58 (Fluka, cat. # 16004) to 5 ml **Lysis Buffer**

Put tube in beaker of slightly warm H_2O to help dissolve Brij-58

85% Sucrose (25 ml)

21.25 g sucrose

-- bring up to volume (25 ml) with **Lysis Buffer**

*Stir solution on slightly warm hot plate ***takes a long time to dissolve!*

35% Sucrose (25 ml)

8.75 g sucrose

-- bring up to volume (25 ml) with **Lysis Buffer**

Stir solution without heat

5% Sucrose (25 ml)

1.25 g sucrose

-- bring up to volume (25 ml) with **Lysis Buffer**

Stir solution without heat

Transfer the following volumes of solutions to 1.7 ml epi-tubes, then add protease inhibitor

Put all tubes on ice

Tube	vol./1 sample	vol. Protease Inhibitor Cocktail
1	500 µl Brij-58 buffer	20 µl
2	500 µl 85% Sucrose	20 µl
3	1.2 ml 35% Sucrose	48 µl
4	500 µl 5% Sucrose	20 µl

Perform everything on ice!

*** pre-cool Beckman Bench Optima MAX-E ultracentrifuge and TLS-55 swing-bucket rotor to 4 degrees C

1- Obtain T cells, transfer cells to 2 ml epi-tubes

2- Spin cells at 200 x g for 5 min., 4 degrees C

3- Aspirate supernatant and resuspend cell pellet in 300 µl **Brij-58 Buffer**

4- Pass cells through a 27-G needle once and incubate on ice 30 min.
pass cells Quickly through needle -- hold onto needle so it doesn't come off of syringe

5- Add 330 µl **85% Sucrose** to the lysate, mix well by pipetting up and down a few times
*** final sucrose concentration at this point is 45% (* 330 µl x 85%)/624 ml)

6- Transfer the mixture to the bottom of a Polyallomer clear centrifuge tube (Beckman cat. # 347357), put sample on ice

7- Carefully layer 1 ml **35% Sucrose** to the top of the above mixture
should see a definitive interface band if layered properly

8- Carefully layer 300 µl **5% Sucrose** to the top of the tube
should see a definitive interface band if layered properly

9- Balance the tubes with less than 0.01 g difference between paired tubes
use 5% Sucrose for balancing

10- Put the centrifuge tubes into the rotor

11- Spin samples in ultracentrifuge at 200,000 x g (~48,340 RPM) overnight, Accl/Decl = 9 (~16h, adjust time so that as soon as the samples stop spinning you are ready to collect fractions)

The following morning:

Keep everything on ice
Use low-retention (siliconized) tips for collecting fractions

12- RAFT fraction = collect **0.6 ml** from the top and transfer to a 25 ml glass screw-top tube
INTERMEDIATE fraction = continually collect **0.4 ml** and transfer to a 25 ml glass screw-top tube
SOLUBLE fraction = continually collect **1 ml** and transfer to a 25 ml glass screw-top tube

13- Proceed to lipid extraction

Lipid Extraction

1- Add **400** m**l** 0.1 M KCl to the RAFT tubes and **600** m**l** 0.1 M KCl to the INTERMEDIATE tubes (to bring-up the aqueous solution to 1 ml)

2- Add 5 ml Folch (MeOH:$CHCl_3$, 1:2, v/v) to each tube

3- Vortex for 1 min.

4- Use glass pastuer pipet to transfer the LOWER phase to a 12 ml glass screw-top tube

5- Double extract the 25 ml sample with 5 ml Folch

6- Dry the lipid extract down with N_2

7- Redissolve the lipids in exactly 2 ml Folch. Transfer to 4 ml glass dram vials with green lids, flush with N_2 and store at -20 degrees C

APPENDIX B-31

Thin Layer Chromatography (TLC)
Quantitating fatty acid mass of total phospholipids of T cells

After lipid extraction of the samples:

⇒ Prepare 12ml glass screw-top leak-proof vials (will need 7/sample)
- Put 1ml Acetone into each vial
- Mark Acetone level and tightly screw a black vinyl lid on
- Place at 80°C for 1h
- Use only vials that didn't evaporate

⇒ Pre-activate TLC plate by heating at 110°C for 1h

⇒ Remove extracts from -20°C and place on ice

⇒ Dry the samples under N_2

⇒ Redissolve the sample in 25ul FOLCH (MeOH/$CHCl_3$, 1:2, v:v) and place on ice
- Use a 25ul Wiretrol to add the FOLCH, then vortex thoroughly (make sure to vortex along sides of vial)
- Flush with N_2

⇒ Make-up TLC system solvents
($CHCl_3$/MeOH/Acetic Acid/H_2O, 90 : 8 : 1 : 0.8, v/v)
- Make ~100ml per TLC tank
- Use HPLC-grade H_2O and Tracepur Acetic Acid, MeOH and $CHCl_3$
- *Use glass pipets to add all solvents*

⇒ Pour solvent mixture into a TLC tank

⇒ Gently place 1/3 sheet of filter paper into tank width-side up to equilibrate the solvents

⇒ Place glass lid onto the top of tank and a heavy object (e.g. the top from the N_2 tank) on top of the lid to prevent evaporation

⇒ Allow solvents to equilibrate for at least 30 min

⇒ **Spot** the TLC plate after it has cooled from the pre-activation
- Lie plate silica-side up
- Using the extra wiretrol needle scrape a line across the plate 1/2" from the top
- Place the TLC "plate grid" on top of the plate and mark evenly spaced lines for the number of lanes needed. Scrape lines from top to bottom of the plate. (make sure to leave ~1/2" on both sides of the plate)

- Put "plate grid" back onto plate and use the shadow of the edge of the grid to spot the sample (make sure the shadow falls ~1" from the bottom of the plate)
- Vortex sample well and aspirate sample into the 25ul Wiretrol
- Spot the sample in one lane by releasing droplets, one-after-another, onto shadow-line
  -Spot in the center of the lane, staying away from the edges
  -Be sure not to touch the plate with tip of the Wiretrol
  -If needed, respot over previous spot once dry
- After spotting add 25ul additional FOLCH to sample, vortex and repot in same lane
- Wipe Wiretrol needle with FOLCH between each sample
- Include a standard (such as rat liver extract) on each plate

⇒ Place TLC plate, top side up, into tank. Replace glass cover and heavy object.

- Place the plate's edge in the middle of the bottom of the tank

⇒ Run for about 2h or until solvents reach <0.5cm from the top line

⇒ While the plate is running **prepare leak-proof tubes**

- Will need 7 tubes per sample spotted
- Label the tubes for: Chol. ester, TG, DG, FFA, MG, PL, 17:0 ISD
- Pour off the Acetone. Dry by inverting tubes in rack
- Rinse tubes and lids with 1ml MeOH (Tracepur grade) and pour off. Dry by inverting in rack
  -Invert and rotate tube to thoroughly rinse
  -*Make sure not to mix up lids (tubes are only guaranteed to be leak-proof w/ the corresponding lids)*
- Add 50ng (10ul of the 5ng/ul stock) of 17:0 free fatty acid. This serves as an internal standard.
- Add 3ml 6% HCl/MeOH and invert tube to mix with the ISD
- Keep tubes in dark until ready to use

⇒ Once plate in done running remove from tank and dry for ~5 min

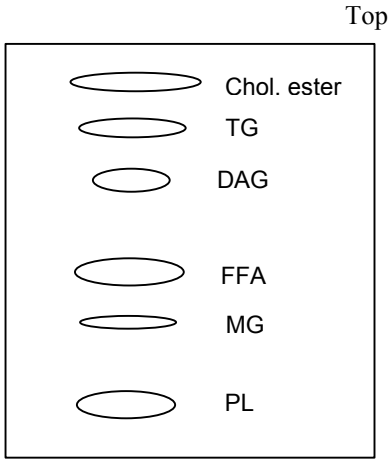
⇒ *In dark* **spray plate** with 0.1% ANS (8-Anilino-1-naphthalene-sulfonic acid) to view bands

- *To make: 200ml H_20 + 0.2g ANS*
- *0.1% ANS is stored at 4°C and is light sensitive*
- Place plate inside 3-sided box to contain ANS
- Spray plate by attaching flask container (containing 0.1% ANS) to N_2
- Spray evenly, but not so much as to soak the plate

⇒ View the plate with UV (if unable to see the bands clearly, spray again)

⇒ Circle the bands with pencil while viewing under UV

⇒ Bands should be located similar to illustration below:



⇒ **Scrape** the circled bands into the appropriate leak-proof tubes

- Use a TLC scraper blade (similar to an Exacto knife)
- Scrape in an upward motion so as not to lose the band
- Scrape over a funnel into tube
- Tap out all remaining pieces of plate caught in funnel into tube

⇒ Flush with N_2 and vortex for 1 min

⇒ Methylate at 76°C for 15h

⇒ **Extract** FAME:

- Add 2ml Hexane and 1ml 0.1M KCl to the leak-proof tubes
- Vortex for 1 min
- Centrifuge for 5 min at 3000rpm at 4°C (Jouan centrifuge) –*use rubber tube inserts to prevent tubes from breaking*
- Transfer upper phase, using Pasteur pipet, to a labeled 4ml glass vial- *be sure to replace black lids with green lids for organic solvents*
- Repeat the 2ml Hexane extract

⇒ Dry down the FAME under N_2

⇒ Redissolve in 25ul CH_2Cl_2, flush with N_2 and vortex

⇒ Run GC to calculate the mass of the phospholipids

APPENDIX B-32

Cholesterol Assay
Amplex Red Cholesterol Assay Kit
(Molecular Probes #A-12216)

Stock Solutions:

20 mM Amplex Red reagent (Component A):

Add 200 µl of DMSO (supplied in kit) to one vial of Amplex Red reagent (1 mg)

Aliquot 40 µl/tube, store at -20°C protected from light

1X Reaction Buffer:

Dilute 5X Reaction Buffer stock solution (Component E) to 1X using dd-H_2O

Make fresh for each assay

200 U/ml horseradish peroxidase (HRP) (Component C):

Add 1 ml of 1X Reaction Buffer to the vial of HRP

Aliquot 125 µl/tube, store at -20°C

20 mM H_2O_2 working solution:

Mix 1.15 µl of 3% H_2O_2 stock (Component D) into 488.5 µl of dd-H_2O

Make fresh for each assay

200 U/ml Cholesterol Oxidase (Component F):

Add 250 µl of 1X Reaction Buffer to the vial of cholesterol oxidase

Aliquot ~32 µl/tube, store at -20°C

200 U/ml Cholesterol Esterase (Component G):

Add 250 µl of 1X Reaction Buffer to the vial of cholesterol esterase

Aliquot ~32 µl/tube, store at -20°C

Protocol:

1. **Prepare cholesterol standards:**

   Dilute 2 mg/ml of cholesterol reference standard (Component H) with 1X Reaction Buffer to produce cholesterol concentrations of 0-8 µg/ml

   *** Prepare Triplicates of each standard

   *** Add 248 µl of 1X Reaction Buffer to 2 µl of 2 mg/ml cholesterol reference standard to make 16 mg/ml Std. Dilute (S1)

	20 mg/ml Std. Dilute	ml of 1X Reaction Buffer	Conc. [mg/ml]
S1	120 µl S1	0 µl	16
S2	60 µl S1	60 µl	8
S3	60 µl S2	60 µl	4
S4	60 µl S3	60 µl	2
S5	60 µl S4	60 µl	1
S6	60 µl S5	60 µl	0.5
S7	0 µl	120 µl	0

2. Take 25 to 50 µl of lipid extract in Folch to be analyzed and transfer to a 2 ml glass vial. Dry down under N_2 and redissolve in 120 µl of 1X Reaction Buffer.
3. Prepare 300 µM Amplex Red reagent mix containing 2 U/ml HRP, 2 U/ml cholesterol oxidase and 2 U/ml cholesterol esterase

   For **10** wells:

7.5 ml	Component **A**	Amplex Red reagent
5.0 ml	Component **C**	HRP
5 ml	Component **F**	Cholesterol oxidase
0.5 ml	Component **G**	Cholesterol esterase
482 ml		1X Reaction Buffer

4. Add 50 µl of sample or standard to a 96-well plate. Run standards in triplicate and samples in duplicate.
5. Add 50 µl of the 300 µM Amplex Red mix to each well.
6. Cover plate with foil to protect from light and incubate at 37°C for at least 30 min.
7. Measure the fluorescence using excitation in the range of 530-560 nm and emission detection at ~ 590 nm

Using Lupton Fluorometer

- Turn on Bio-Tek machine and start-up Bio-Tek KC4 program from desktop.
- Select from protocol menu: YY-Fluor.prt
- Click on Settings button, and then click on Options button
  - Check "Automatic sensitivity adjustment" and "Scale to high well"
  - Scale to wells with highest readings (usually highest set of wells on standard curve)
  - High value should be 50,000
  - Starting sensitivity should be 35
  - For Amplex Red Cholesterol settings:
    - Excitation = 530/25
    - Emission = 590/35
    - Optics position = Top
- To export to Excel:
  - Select Data, export from menu
  - Highlight "M530/590"
  - Click on "Add" button
  - Save as txt file
  - Import into Excel as txt file as semicolon delimited

VITA

KIRSTEN COLLETTE SWITZER
4215 Sunset, Houston, TX 77005
switzer_k@yahoo.com

EDUCATION

B.S., Biological Sciences, August 1995
University of Nebraska, Lincoln

Ph.D., Nutrition, August 2004
Texas A&M University

AWARDS

American Society of Nutritional Sciences/ Procter & Gamble Graduate Student Research Award finalist, at annual meeting of the Federation of American Societies for Experimental Biology, New Orleans, LA, April 2002.

Texas A&M University Institute of Food Science and Engineering Graduate Tuition Scholarship, 2002.

Travel Award, Annual Meeting of the Federation of American Societies for Experimental Biology, Orlando, Florida, April 2001 (Best poster presentation for a graduate student in the Nutritional Immunology Research Interest Section).

PUBLICATIONS

Switzer, K.C., McMurray, D.N., Chapkin, R.S. (2004) Effects of Dietary n-3 Polyunsaturated Fatty Acids on T-Cell Membrane Composition and Function: A Unifying Hypothesis. Lipids (submitted).

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. (in press).

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.