

**IMMUNOSUPPRESSIVE DIETARY N-3 POLYUNSATURATED FATTY ACIDS  
DIFFERENTIALLY MODULATE COSTIMULATORY REGULATION OF  
MURINE CD4<sup>+</sup> T-CELL FUNCTION**

A Dissertation

by

LAN H. LY

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

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Nutrition

**IMMUNOSUPPRESSIVE DIETARY N-3 POLYUNSATURATED FATTY ACIDS  
DIFFERENTIALLY MODULATE COSTIMULATORY REGULATION OF  
MURINE CD4<sup>+</sup> T-CELL FUNCTION**

A Dissertation

by

LAN H. LY

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

DOCTOR OF PHILOSOPHY

Approved as to style and content by:

---

Robert S. Chapkin  
(Chair of Committee)

---

David N. McMurray  
(Member)

---

C. Jane Welsh  
(Member)

---

Vernon L. Tesh  
(Member)

---

Robert S. Chapkin  
(Chair of Nutrition Faculty)

---

John McNeill  
(Head of Department)

December 2004

Major Subject: Nutrition

**ABSTRACT**

Immunosuppressive Dietary n-3 Polyunsaturated Fatty Acids Differentially Modulate Costimulatory Regulation of Murine CD4<sup>+</sup> T-cell Function. (December 2004)

Lan H. Ly, B.S., University of California, San Diego

Chair of Advisory Committee: Dr. Robert S. Chapkin

Consumption of fish oils (FO) enriched with the n-3 polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), is beneficial to a variety of inflammatory disorders due, in part, to the alteration of membrane composition of T-lymphocytes and other immune cells. We previously observed that down-regulation of proliferation and cytokine synthesis by CD4<sup>+</sup> T-cells in mice fed diets rich in n-3 PUFA was dependent on the involvement of CD28, a co-stimulatory molecule necessary for T-cell activation. Since the co-receptor homologues, CD28 and CTLA-4, have opposing effects on T-cell activation, we hypothesized that the balance of costimulatory and downregulatory properties of CD28 and CTLA-4, respectively, would be altered by diet. A significant increase ( $p < 0.05$ ) in CD28 and CTLA-4 surface expression was observed in CD4<sup>+</sup> T-cells post-stimulation with phorbol ester and calcium ionophore (PMA/Iono) or anti-CD3 and anti-CD28 ( $\alpha$ CD3/CD28) antibodies in all diet groups. A significant increase ( $p < 0.01$ ; 20%) in the number of CD28 molecules was observed in n-3 PUFA vs. CO-fed mice after 48 h of *in vitro* CD4<sup>+</sup> T-cell activation, and both CTLA-4 mRNA transcript and protein levels were upregulated by 50% at 72 h post-activation ( $p < 0.01$ ). Treatment with anti-CTLA-4 mAb *in vivo* in *Mycobacterium bovis* (BCG)-vaccinated mice did not alter the suppressive effects of dietary n-3 PUFA on antigen (PPD)-induced lymphocyte proliferation or delayed hypersensitivity reactions.

Ligation of CD28 upregulates IL-10 receptor (IL-10R) expression on CD4<sup>+</sup> T-cells. Therefore, we hypothesized that dietary n-3 PUFA would suppress T-cell function through the effects of IL-10. Surprisingly, the proliferation of purified splenic CD4<sup>+</sup> T-cells activated *in vitro* with  $\alpha$ CD3/CD28 was suppressed by dietary n-3 PUFA in both conventional mice (C57BL/6) and IL-10 gene knockout (IL-10<sup>-/-</sup>) mice. Furthermore, IL-10R cell surface expression was significantly down-regulated on CD4<sup>+</sup> T-cells from both the C57BL/6 and IL-10<sup>-/-</sup> mice fed dietary n-3 PUFA after 72 h of *in vitro* stimulation with  $\alpha$ CD3/CD28. CD4<sup>+</sup> T-cells from C57BL/6 mice fed DHA produced significantly less IFN $\gamma$  and IL-10, while CD4<sup>+</sup> T-cells from IL-10<sup>-/-</sup> mice fed dietary n-3 PUFA produced significantly more IFN $\gamma$  compared to the CO-fed group.

## **ACKNOWLEDGMENTS**

I would like to thank Laurie Davidson, Evelyn Callaway, and Yang-Yi Fan for their assistance and support in the Chapkin lab. Their valuable input and helping hands were critical in the completion of many of my studies. I would also like to thank Drs. Robert Chapkin and David McMurray for their collaborative mentoring efforts.

## TABLE OF CONTENTS

	Page
ABSTRACT .....	iii
ACKNOWLEDGMENTS .....	v
TABLE OF CONTENTS.....	vi
LIST OF FIGURES .....	viii
CHAPTER	
I INTRODUCTION .....	1
II DIETARY N-3 POLYUNSATURATED FATTY ACIDS MODULATE THE BALANCE BETWEEN CD28 AND CTLA-4 EXPRESSION ON MURINE CD4 <sup>+</sup> T-CELLS .....	14
Introduction.....	14
Materials and Methods.....	17
Results.....	23
Discussion.....	39
III DIETARY N-3 POLYUNSATURATED FATTY ACIDS SUPPRESS SPLENIC CD4 <sup>+</sup> T-CELL FUNCTION IN IL-10 <sup>(-/-)</sup> MICE .....	45
Introduction.....	45
Materials and Methods.....	47
Results.....	50
Discussion.....	59
IV SUMMARY AND CONCLUSIONS .....	64
REFERENCES .....	68
APPENDIX A: OTHER DATA .....	97

	Page
APPENDIX B: PROTOCOLS.....	113
VITA.....	135

## LIST OF FIGURES

FIGURE		Page
1	Dietary DHA and EPA down-regulate murine CD4 <sup>+</sup> T-cell Proliferation .....	26
2	Activated CD4 <sup>+</sup> T-cells from mice fed DHA display significantly increased surface expression of CD28.....	28
3	Splenic CD4 <sup>+</sup> T-cells from mice fed dietary n-3 PUFA express significantly increased CTLA-4 protein (total and surface) and mRNA levels following <i>in vitro</i> activation.....	31
4	<i>In vitro</i> effect of CTLA-4 ligation with antibody on CD4 <sup>+</sup> T-cell Proliferation .....	33
5	Anti-CTLA-4 antibody (Pharmingen) decreases CD4 <sup>+</sup> T-cell proliferation in C57BL/6 mice.....	34
6	Anti-CTLA-4 Ab (prepared in-house) enhances whole splenocyte proliferation in C57BL/6 mice.....	36
7	Anti-CTLA-4 mAb treatment <i>in vivo</i> does not alter diet-induced changes in PPD-specific T-cell responses of BCG-immunized mice.....	37
8	Representative gels of IL-10 knockout mouse genotyping.....	49
9	Dietary DHA and EPA down-regulate murine CD4 <sup>+</sup> T-cell proliferation in IL-10 <sup>-/-</sup> mice.....	51
10	Dietary n-3 PUFA suppress IL-10R surface protein expression .....	53
11	Dietary n-3 PUFA differentially modulate CD4 <sup>+</sup> T-cell IFN $\gamma$ and IL-10 production in C57BL/6 mice .....	55
12	Dietary n-3 PUFA enhance IFN $\gamma$ production in CD4 <sup>+</sup> T-cells from IL-10 <sup>-/-</sup> mice .....	56
13	Diagram of results obtained from C57BL/6 mice.....	57
14	Diagram of results obtained from IL-10 <sup>-/-</sup> mice.....	58



## CHAPTER I

### INTRODUCTION

Lipids (oils and fatty acids) are indispensable for the growth and survival of all organisms. They are important structural components of membranes and, in many organisms, play a crucial role in energy storage. During the past three decades, the interest in some specific animal oils, i.e., the long-chain polyunsaturated fatty acids (PUFA) present in fish oils (FO), has increased considerably, due to their beneficial health effects (1-6).

Long-chain PUFA are composed of a long hydrocarbon chain (18 or more carbon atoms) and a terminal carboxylate group having two or more double carbon bonds. They are classified according to the position of the first double bond, as counted from the methyl terminus. A so-called  $\omega$ -3 PUFA has its first double bond at position 3, as counted from the methyl terminus. Other PUFA groups are  $\omega$ -6 and  $\omega$ -9, where the first double bond is located six and nine carbons from the methyl terminus, respectively. As a synonym of  $\omega$ , the symbol  $n$  is often used to classify PUFA (7). The most prominent examples of dietary  $n$ -6 PUFA are linoleic acid (LA; 18:2 $n$ -6),  $\gamma$ -linolenic acid (GLA; 18:3 $n$ -6) and arachidonic acid (AA; 20:4 $n$ -6). Examples of dietary  $n$ -3 PUFA include  $\alpha$ -linolenic acid (ALNA; 18:3 $n$ -3), eicosapentaenoic acid (EPA; 20:5 $n$ -3), and docosahexaenoic acid (DHA; 22:6 $n$ -3). The most abundant  $n$ -3 PUFA ingested with vegetable oils, ALNA, is not efficiently converted to EPA or DHA in humans (8). Both

---

This dissertation follows the style of *Journal of Immunology*.

EPA and DHA are primarily found in marine FO (7). The position of the double bond in the fatty acids strongly affects the properties of its derivatives. For example, eicosanoids derived from AA (n-6) have strong inflammatory properties, whereas those produced from EPA (n-3) are anti-inflammatory (9-12).

Evidence for the health benefits of dietary n-3 PUFA intake was derived, in part, from epidemiological observations of a very low incidence of chronic inflammatory conditions in Eskimos (13). Studies on the role of n-3 PUFA in inflammatory bowel diseases (IBD) started at the end of the 1980s. McCall et al treated 6 patients with active ulcerative colitis (UC) by giving 3-4 g of EPA daily for 12 weeks, which resulted in improvement of clinical symptoms and histological appearance, and a decrease in serum leukotrienes (LTB<sub>4</sub>), an eicosanoid mediator (14). Similarly, other studies continued to report these beneficial anti-inflammatory effects of n-3 PUFA (15, 16), as well as to demonstrate improvement in all disease activity variables (17, 18) and prolonged remission (19, 20). Recent epidemiological evidence suggests that the increasing incidence of Crohn's Diseases (CD) in Japan is strongly correlated with, among other factors, the increasing ratio of n-6: n-3 PUFA intake in the average Japanese diet. Increased dietary intake of n-6 PUFA with lower levels of n-3 PUFA may contribute to the development of CD (21). Although some studies report no benefit (22), it has recently been shown that increasing dietary n-3 PUFA intake in patients with IBD may enhance (by 65%) the absorption and the utilization of saturated fatty acids such as palmitic acid, improving overall nutritional status (6). These data strongly support the efficacy of dietary n-3 PUFA in the treatment of patients with IBD or other chronic inflammatory conditions.

A variety of molecular mechanisms have been proposed to explain how n-3 PUFA can modulate inflammation and immune cell function. Alterations in eicosanoid (e.g., prostaglandins (PG), leukotrienes) synthesis have long been regarded as the primary anti-inflammatory mechanism of n-3 PUFA. Eicosanoids are a group of chemical messengers that act within the immune system to modulate the intensity and duration of inflammatory responses (reviewed in (23-25)). For example, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) has a number of proinflammatory effects including inducing fever, increasing vascular permeability and vasodilation, and enhancing pain and edema caused by other agents such as bradykinin and histamine. These compounds provide a link between PUFA, inflammation, and immune function. Eicosanoids are synthesized from n-6 and n-3 PUFA, in particular AA, di-homogamma linolenic acid (DGLA), and EPA. The fatty acid precursor for eicosanoid synthesis is released from cell membrane phospholipids by the action of phospholipase A<sub>2</sub> activated in response to a cellular stimulus. AA is usually the principal precursor for eicosanoid synthesis due to its dominant presence over EPA and DGLA in the membranes of immune cells. Therefore, the ability to produce these mediators is strongly influenced by the fatty acid (FA) composition of membrane phospholipids. Increased consumption of FO results in increased proportions of EPA and DHA in inflammatory cell phospholipids, partly at the expense of AA (26, 27), thereby leaving less substrate for AA-derived eicosanoids. The reduction in the generation of AA-derived mediators that accompany FO consumption has led to the hypothesis that FO is anti-inflammatory because it affects PG synthesis (12, 28). However, it is now apparent that the ability of long-chain n-3 PUFA to influence production of eicosanoids extends beyond simply decreasing substrate availability. For example, EPA competitively

inhibited the metabolism of AA by cyclooxygenase enzyme-2 (COX-2), thereby reducing the generation of 2-series PG and thromboxanes (TX) (29). Furthermore, n-3 PUFA were able to act as a substrate for COX, giving rise to derivatives that differ in structure from those produced from AA (i.e., 3-series PG). Therefore, the n-3 PUFA-induced suppression in the production of AA-derived eicosanoids may be accompanied by an elevation in the production of n-3 PUFA-derived eicosanoids (27, 30). Consistent with these results, PGE<sub>2</sub> concentrations in the medium taken from lymphocytes cultured in the presence of a range of different fatty acids did not correlate with the inhibitory effects of the fatty acids upon proliferation (31). Moreover, indomethacin, a COX inhibitor, did not reverse the anti-proliferative effects of the fatty acids (32). These observations provide convincing evidence that the immunosuppressive effects of dietary n-3 PUFA are independent of the production of eicosanoids.

In order to maintain their integrity, cells are surrounded by a plasma membrane composed of fatty molecules. Molecules that make up the membrane have two long fatty acid chains with a head group that is H<sub>2</sub>O-soluble by virtue of being electrically charged, making them amphipathic. Thus, membranes are formed because these bipartite molecules, called phospholipids, spontaneously orient themselves to form a double layer, or bilayer, with their fatty chains facing inward and their water-seeking head groups facing outward. The membrane is given rigidity by the interspersions of cholesterol, which helps stabilize the outer membrane of all cells. Carbohydrates, covalently bound either to proteins as constituents of glycoproteins or to lipids as constituents of glycolipids, also help stabilize the conformation of many membrane proteins. Sphingolipids, phospholipids that bear a sphingosine instead of a glycerol backbone, and

cholesterol interact to help cluster proteins into regions called microdomains. These microdomains function as “rafts” or platforms for the partitioning of proteins that are critical for signal transduction (33, 34). By their distinct lipid composition, lipid rafts concentrate lipid-modified signaling proteins within the plane of the plasma membrane to facilitate signal transduction and cellular response. The importance of membrane rafts in immune cells will be discussed further in subsequent paragraphs.

T-cells are lymphocytes which develop in the thymus. They can be divided into two subpopulations,  $CD4^+$  or  $CD8^+$ , both of which express a T-cell receptor (TCR) associated with a complex of transmembrane signaling proteins called CD3 (35). Cell-mediated immunity is the effector function of T-lymphocytes, which serves as an important defense mechanism against many microbes. The  $CD4^+$  T-cells differentiate into helper T-cells (Th), while the  $CD8^+$  T-cells differentiate into cytotoxic T-cells (CTL), each performing very different effector functions in the immune system. Two different subsets of Th-cells activate macrophages (Th1) or promote B-cell antibody production (Th2), whereas CTLs recognize and kill host cells infected with viruses or other intracellular microbes (36). Despite their various effector functions following differentiation, all naïve T-cells require at least two distinct extracellular signals to induce their proliferation and transformation into effector cells. The first signal originates from the ligation of the TCR complex and its co-receptors (e.g., CD4 or CD8) with a specific major histocompatibility complex (MHC)-peptide complex. The second signal is dependent upon the ligation of cell surface molecules that provide essential costimulatory signals which are complementary to the TCR engagement (37).

The immune system has developed powerful mechanisms to prevent unnecessary activation of T-cells and thus either autoimmunity, i.e., a state of responsiveness to self antigens, or hypersensitivity, i.e., the prolonged and intense state of responsiveness to foreign antigens. One such mechanism is the intricate balance between positive and negative costimulatory signals delivered to T-cells after antigenic counter (38). Positive costimulatory signals are pivotal in determining whether recognition of antigen by T-cells leads to full T-cell activation or to anergy/death. CD28 is the principal co-receptor, which is expressed on more than 90% of CD4<sup>+</sup> and 50% of CD8<sup>+</sup> naïve T-cells (39) and provides the second signal. Although it is constitutively expressed prior to T-cell activation, it is well documented that CD28 expression is regulated following TCR engagement with the MHC-peptide complex (40, 41). The intracellular signal cascade generated upon CD28 ligation with CD80 (also known as B7-1) and CD86 (B7-2), which are expressed on activated antigen presenting cells (APC), acts in concert with signals from the TCR complex to fully activate the T-cells. The CD28 costimulatory activity has been demonstrated *in vitro* and *in vivo* by antibody cross-linking and blocking experiments in normal mice and in CD28-deficient mice (42).

In addition, recent findings indicate that regulation of immune responses may be achieved by the expression of inhibitory costimulatory molecules on APC and peripheral tissues that mediate negative costimulatory signals to the T-cell (42, 43). The most prominent inhibitory receptor is cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), a homolog of CD28. Expressed only on activated T-cells, CTLA-4 binds to the same ligands as CD28, albeit with much higher affinity, thereby leading to the termination of the immune response (44). In both human (45) and murine (44) systems, cell surface

expression of CTLA-4 peaks 48 h after activation, returning to background levels by 96 h. Thus, the period of time in which CD28 is transiently downregulated and less responsive to signaling is the time period during which CTLA-4 expression is maximal, suggesting that CTLA-4 may be functionally active at a time when CD28 function has waned. Studies have shown that ligation of CTLA-4 inhibits T-cell activation by reducing IL-2 production and IL-2 receptor expression, and by arresting T-cells at the G1 phase of the cell cycle (44, 46).

An increasing body of evidence indicates that T-cell activation involves the lateral migration of many molecules associated with TCR-mediated signaling. When T-cells are stimulated with planar lipid bilayers or APC possessing antigenic peptide-MHC, a highly ordered macromolecular interface, termed the immunological synapse (IS), forms between the T-cell and the APC surface (47). The formation of an IS correlates with the clustering of glycolipid-enriched microdomains known as lipid rafts which were introduced earlier. By their distinct lipid composition, lipid rafts concentrate lipid-modified proteins within the plane of the plasma membrane. At the exoplasmic face, proteins attached to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor are almost exclusively found in rafts, whereas acylated proteins are found at the cytoplasmic face of rafts. Many proteins involved in signal transduction are modified by acyl moieties to be attached to the plasma membrane and/or concentrated into lipid rafts (48). For example, Src-family kinases such as Lck and Fyn are acylated at the N-terminus by myristoyl (14:0) and palmitoyl (16:0) moieties (reviewed in (49)). The IS is composed of a central core containing the TCR, protein kinase C  $\theta$  (PKC $\theta$ ), Lck, Fyn, and CD28, surrounded by a ring enriched with adhesion molecules (i.e., LFA-1, CD43)

(50-52). As mentioned previously, lipid rafts are enriched with signaling molecules critical for TCR-mediated signaling such as Lck, linker for activation of T-cells (LAT), and PKC $\theta$  and their biological integrity is essential for proper TCR-mediated signaling (53-55).

It has been demonstrated by us (56-58) and others (59, 60) that dietary n-3 PUFA affect raft composition and function on T-cells through an eicosanoid-independent mechanism. Treatment of lipid rafts from Jurkat T-cells with EPA markedly displaced Lck and Fyn content, thereby reducing subsequent downstream signaling events (61). Similarly, we have recently demonstrated that dietary DHA suppressed PKC $\theta$  recruitment to lipid rafts in murine T-lymphocytes (62). In contrast, the GPI-anchored proteins, CD59 and CD48, the ganglioside GM1, and caveolin remain in rafts after dietary n-3 PUFA enrichment (63). Protein displacement from lipid rafts could be due to altered protein acylation or changes in raft lipid composition. These studies illustrate that diet n-3 PUFA-mediated changes in membrane composition, as well as protein acylation, are likely to have a broad impact on lymphocyte signaling pathways (57).

Immediately following stimulation, lymphocytes begin to transcribe genes that were previously silent to synthesize a variety of new proteins. These proteins include secreted cytokines (in T-cells), which stimulate the growth and differentiation of the lymphocytes themselves and of other effector cells, and cytokine receptors, which make lymphocytes more responsive to cytokines. As the principal mediators of communication between lymphocytes, cytokines play a critical role in mediating inflammatory and immune responses.



Although it is important for the immune system to mount a proper response to any foreign invader, it is even more important to inhibit it in a timely manner. An over-exaggerated response is potentially detrimental for the host immune system itself and to other tissues. Several mechanisms are involved in regulating and balancing immune and inflammatory responses. These include cytokines possessing anti-inflammatory, immunosuppressive activities such as interleukin-10 (IL-10) (reviewed in (64)). IL-10 limits and ultimately terminates inflammation by adjusting the intensity of the immune and inflammatory responses to the severity of destruction caused by a pathological condition or pathogen. In addition, IL-10 regulates growth and death of B-cells, natural killer (NK) cells, cytotoxic and helper T-cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells. IL-10 also plays a key role in the differentiation and function of the T-regulatory cell, which may assist prominently in the control of immune responses and tolerance *in vivo* (65).

As a mechanism of maintaining immunologic tolerance to self antigens, IL-10 induces T-cell anergy, a state of immune inactivation characterized by abolished proliferative and cytokine responses. It was recently demonstrated that the unresponsive state of murine T-cells which had been tolerized by IL-10, was reversed by anti-CD3 monoclonal antibody (mAb) stimulation and IL-2, a growth-promoting cytokine, but not by anti-CD28 mAb stimulation (66). IL-10 inhibited tyrosine phosphorylation of CD28, thereby blocking its binding to phosphatidylinositol-3 kinase (PI3K). Furthermore, CD28 stimulation significantly enhanced IL-10 receptor (IL-10R) expression after 24 h *in vitro* activation. Together, these results demonstrate that the suppressive effects of IL-10 involve direct action on the CD28 co-stimulatory pathway. In turn, CD28 ligation

upregulates IL-10R expression, rendering the T-cell more susceptible to IL-10 suppression (67).

The beneficial anti-inflammatory effects of dietary n-3 PUFA can be attributed, in part, to suppressed production of pro-inflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ , and IL-6) (68) and decreased expression of adhesion molecules (e.g. ICAM-1, VCAM-1) as shown in both animal and human studies (69, 70). These effects also occur at the level of altered gene expression. Recent studies have shown that n-3 PUFA can down-regulate the activity of the nuclear transcription factor nuclear factor kappa B (NF $\kappa$ B) (71), which plays a role in inducing a range of inflammatory genes, including COX-2, ICAM-1, VCAM-1, TNF $\alpha$ , IL-1 $\beta$ , and IL-6, in response to inflammatory stimuli (72).

Animal feeding studies have shown that diets enriched with EPA and DHA decreased *ex vivo* production of TNF $\alpha$ , IL-1 $\beta$ , and IL-6 by rodent macrophages (73) and monocytes (74). Feeding fish oil decreased the level of MHC II (75) and IFN $\gamma$  receptor expression (76) on murine peritoneal macrophages and diminished *ex vivo* presentation of antigen (keyhole limpet hemocyanin: KLH) by spleen cells (77). These studies suggest that dietary fish oil might impair the cell-mediated immune response by decreasing the activity of antigen-presenting cells and by decreasing the sensitivity of macrophages to T lymphocyte-derived cytokines.

Experiments conducted by us and others revealed that fish oil decreases NK cell activity, cytotoxic T lymphocyte activity (78), expression of the high affinity IL-2 receptor alpha gene in activated lymphocytes (5), lymphocyte proliferation, and the production of IL-2 (10). Addition of either EPA or DHA to the diet of mice consuming a safflower oil diet (devoid of n-3 PUFA) decreased the delayed-type hypersensitivity

(DTH) response to tuberculin in mice vaccinated with *Mycobacterium bovis* BCG (3).

These results suggest that fish oil may impair cell-mediated immunity, and could play a role in the prevention and/or therapy of chronic inflammatory diseases characterized by a dysregulated CD4<sup>+</sup> T-cell response which drives the production of pro-inflammatory cytokines and eicosanoids.

Animal feeding studies have often used large amounts of FO, i.e., a diet in which FO contributes 20% by weight will mean that DHA plus EPA comprise up to 30% of dietary fatty acids (79). We and others have demonstrated that diets containing relatively low levels of dietary n-3 PUFA [EPA or DHA at a level of 18% of total fatty acids or less (~45% of total calories)] have similar effects to whole FO (3, 9, 10, 80) using a short-term feeding paradigm. The hypothesis that the active constituents of FO, namely DHA and EPA, have similar effects on the immune response has been supported by some studies (81-83) and rejected by others (84-86).

Mice are the mainstay of *in vivo* immunological experimentation and, in many respects, they mirror human biology remarkably well (reviewed in (87)). This conservation of function is reflected in recent reports on the sequencing of both human and mouse genomes, which reveal that, to date, 99% of mouse genes are homologous to human genes (88). We and others have continued to successfully use the mouse model to elucidate the effect of dietary n-3 PUFA on T-cell functions (3, 10, 56, 58, 78, 89). Moreover, the murine model has been used extensively to study T-cell activation (90, 91), intracellular signaling pathways (92-94), and cytokine profiles (95, 96) in murine splenocytes. Although caution needs to be taken in extrapolating data obtained in mice to

humans, mice will continue to be absolutely essential for continued progress in our understanding of the immune response in health and disease (87, 97).

There is mounting evidence that CD4<sup>+</sup> and CD8<sup>+</sup> T-cells are affected differentially by diet in their immunologic responses (98, 99). Many studies report n-3 PUFA-mediated effects on whole splenocyte cultures and few have addressed the role of n-3 PUFA on T-cell subset regulation. Our laboratory has demonstrated that these T-cell subsets are affected differentially by various dietary components. Feeding dietary DHA to mice reduced the proportion of splenic CD8<sup>+</sup> T-cells, but not CD4<sup>+</sup> T-cells (9). Moreover, dietary n-3 PUFA principally modulated the function of CD4<sup>+</sup> T-lymphocyte subsets specifically by suppressing the function of CD4<sup>+</sup> Th1 cells which were polarized *in vitro* with a particular agonist set [phorbol-12-myristate-13-acetate (PMA) and a calcium ionophore, Ionomycin (Iono)] without affecting or even enhancing the function of CD4<sup>+</sup> Th2 cells (10). Enhanced activation induced cell death (AICD) or apoptosis in CD4<sup>+</sup> Th1 polarized cells was also seen in mice fed FO, which agrees with the previous data (100). Thus, we have chosen to further elucidate the effects of dietary n-3 PUFA on purified murine splenic CD4<sup>+</sup> T-cells.

The effect of dietary n-3 PUFA on T-cell function appears to be dependent upon the *in vitro* stimuli used in culture. In earlier studies, it was clearly demonstrated that the selective dietary effects of n-3 PUFA on CD4<sup>+</sup> T-cells was dependent upon ligation of the co-stimulatory molecule, CD28, whose activation is required in addition to the T-cell receptor/CD3 (TCR/CD3) complex to induce a functional response for proper T-cell propagation (4). Ligation of CD28 is known to upregulate CTLA-4 and IL-10R expression, either or both of which may be responsible for suppression of T-cell

proliferation and/or cytokine production (67, 89). Therefore, to determine the precise role CD28 plays in diet-mediated immunosuppression, we examined the influence of dietary n-3 PUFA on CD28, CTLA-4, and IL-10R protein expression on the surface of purified CD4<sup>+</sup> T-cells cultured *in vitro* with antibodies to anti-CD3 and anti-CD28 ( $\alpha$ CD3/ $\alpha$ CD28). In studies where we examined the CD28 receptor protein itself, activation with the PMA/Iono stimuli was used to bypass the membrane receptors. In addition, we proceeded to investigate the role of the suppressive IL-10 cytokine, in the context of CD28, in IL-10 gene knockout mice fed dietary DHA or EPA. Our overall hypothesis was that dietary n-3 PUFA would alter the regulation or function of certain T-cell protein receptors and/or modulate the interplay between CD28, CTLA-4, and IL-10.

**CHAPTER II**  
**DIETARY N-3 POLYUNSATURATED FATTY ACIDS**  
**MODULATE THE BALANCE BETWEEN CD28 AND CTLA-4 EXPRESSION**  
**ON MURINE CD4<sup>+</sup> T-CELLS**

Introduction

Activation of T-lymphocytes is thought to require at least two signals, one delivered by the T-cell receptor (TcR) complex after antigen (Ag) recognition, and one provided on engagement of co-stimulatory receptors, such as CD28. Upon interaction with its ligands B7.1 (CD80) and/or B7.2 (CD86), CD28 transduces a signal which enhances T-cell proliferation and cytokine secretion, and sustains the T-cell response (101-103). Conversely, the CD28 homologue cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) (CD152) inhibits T-cell responses by reducing IL-2 production and IL-2 receptor expression, and by arresting T-cells at the G1 phase of cell cycle (89, 104). Both B7 ligands bind to CD28 and CTLA-4, but they differ in their binding affinity, structure and temporal expression (105). CTLA-4 reportedly binds B7 with a 20-fold higher affinity than CD28 (106, 107) and thereby aids in the termination of immune responses (108-110).

Others have shown that blockage of CTLA-4 signals prolong T-cell activation and expansion both *in vitro* (44) and *in vivo* (111, 112). The importance of CTLA-4 as a regulator of lymphocyte homeostasis was confirmed by the generation of CTLA-4 deficient mice, whose development of a lymphoproliferative disorder and severe autoimmune disease results in death by 4-5 wk of age. When restimulated *in vitro*, T-cells from these mice secrete high levels of IL-4 and IFN $\gamma$ , consistent with a role for

CTLA-4 in the regulation of T-cell differentiation (113, 114). Furthermore, anti-CTLA-4 treatment exacerbates autoimmune disease in a murine model of multiple sclerosis (115). Thus, CTLA-4 is likely to play a critical role in the regulation of many aspects of the T-cell response.

The interplay between CD28 and CTLA-4 has become more evident and more complex. An increasing body of evidence indicates that T-cell activation involves lateral migration of many molecules associated with TcR-mediated signaling to form the immunological synapse (IS). Functional and imaging experiments suggest that membrane microdomains, termed detergent-insoluble glycosphingolipid-enriched domains (or “rafts”), may play an important role in forming the structure of the IS. It has been shown that the recruitment of lipid rafts to the IS is highly dependent on CD28 costimulation (116). Furthermore, CTLA-4 localization to the raft is necessary for CTLA-mediated negative signaling (117). The physiological relevance of lipid raft-associated CTLA-4, however, is currently unknown. Interestingly, cell surface accumulation of CTLA-4 is regulated by its rapid endocytosis (104) suggesting that much of the “bioavailable” CTLA-4 may be intracellular.

Previous results from our laboratory show that T-lymphocytes from mice fed the n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6), found in fish oil (FO) produced significantly less IL-2, but only when cells were activated with  $\alpha$ CD3/CD28 and not when activated with  $\alpha$ CD3/PMA (phorbol ester) (4). Since CD28 ligation is essential for the rearrangement of lipid rafts at the IS (118), dietary n-3 PUFA may alter the ability of CD28 to trigger this event and/or modulate signal-transducing proteins that associate to the rafts upon T-

cell activation. Our laboratory recently demonstrated for the first time that dietary n-3 PUFA differentially modulate T-cell raft and non-raft membrane phospholipid and fatty acyl composition *in vivo* (56). Furthermore, we found that dietary n-3 PUFA feeding suppressed partitioning of the signaling molecule, PKC $\theta$ , into lipid rafts which was associated with a reduction in AP-1 and NF- $\kappa$ B activation (62). These results suggest that by altering the raft membrane composition, diet may influence signaling complexes and modulate T-cell activation, thereby, altering the raft function/dynamics which affect the opposing costimulatory molecules, CD28 and CTLA-4.

On this background, we hypothesized that the balance of costimulatory and downregulatory properties of CD28 and CTLA-4, respectively, would be altered by diet. To investigate this hypothesis, we have determined the influence of dietary DHA and EPA on the regulation of both CD28 and CTLA-4 mRNA and cell surface expression in purified murine CD4<sup>+</sup> T-cells. Surprisingly, dietary n-3 PUFA feeding significantly upregulated both CD28 and CTLA-4 cell surface protein expression. However, the levels of CD28 mRNA expression were markedly reduced, while CTLA-4 transcript levels were enhanced. The role of the CTLA-4 molecule in diet-mediated immunosuppression was studied *in vivo* using anti-CTLA-4 treatment. CTLA-4 blockade *in vivo* in *Mycobacterium bovis* (BCG)-vaccinated mice fed dietary n-3 PUFA did not alter the suppressive effects of diet. Therefore, we demonstrate that dietary n-3 PUFA may downregulate T-cell function by altering co-receptor regulation.



## Materials and Methods

### **Diet and animals**

Female, pathogen-free young (12-14g) C57BL/6 mice purchased from Frederick National Cancer Research Facility (Frederick, MD) were assigned to one of three semi-purified diets: 5% corn oil (CO) (control diet containing no n-3 PUFA), 1% DHA + 4% CO (DHA), or 1% EPA + 4% CO (EPA), for 14 days. Diets were analyzed by gas chromatography prior to feeding, aliquoted, and stored at -80°C. Fresh diet was provided daily to prevent lipid peroxidation. There was no significant difference in food intake between dietary groups and weight gain was similar in all groups (data not shown). The purified diets met National Research Council nutrition requirements and varied only in lipid composition as previously described (3, 10). The vitamin E levels in the diets were approximately equal (mean  $\pm$  SEM=169.2  $\pm$  4.4 mg/kg diet) and exceeded the minimum requirement (22 mg Vitamin E/kg diet). DHA (88.9% as 22:6, n-3) and EPA (94.1% as 20:5, n-3) were obtained in ethyl ester form from Martek Biosciences (Columbia, Maryland) and Laxdale Ltd (United Kingdom), respectively. Corn Oil (57.3% as 18:2, n-6) was obtained from Degussa Bioactives (Champaign, IL) (4).

### **Measurement of fatty acid composition from mouse sera**

Sera were obtained from mice after 14 d of feeding the three experimental diets. Fatty acid methyl esters were extracted using hexane and 0.1 M potassium chloride and analyzed by capillary gas chromatography as previously described (56).

### **Isolation and preparation of splenic lymphocytes**

Mice were killed by CO<sub>2</sub> asphyxiation. Spleens were placed in RPMI-complete medium [RPMI 1640 with 25 mmol/L HEPES (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (FBS; Irvine Scientific) or 2.5% FBS + 2.5% homologous mouse serum (HMS) (58),  $1 \times 10^5$  u/L penicillin and 100 mg/L streptomycin (Irvine Scientific), 2 mmol/L L-glutamine and 10  $\mu$ mol/L 2-mercaptoethanol]. Spleens were dispersed with glass homogenizers and passed through a 149- $\mu$ m wire mesh filter to create single-cell suspensions. Cells were subsequently washed with RPMI-complete medium before T-cell enrichment as previously described (10).

### **CD4<sup>+</sup> T-cell purification**

Total lymphocytes were initially enriched by density gradient centrifugation using Lympholyte-M (Cedarlane, Canada) in accordance with the manufacturer's protocol. The resulting cell fraction from each spleen was incubated with an antibody cocktail provided by the manufacturer, loaded onto a negative-selection mouse CD4 T-cell purification column (R&D Systems, Minneapolis, MN), and incubated for 10 min at room temperature (RT). Non-adherent cells were eluted for purity and viability analysis, proliferation, FACS analysis, and real-time PCR assays. The purity of the CD4 T-cell population was analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, Bedford, MA) using anti-CD4 antibody conjugated to fluorescein isothiocyanate (Pharmingen, San Diego, CA) and determined to be  $90.3 \pm 1.4\%$  (n=3) (10).

### **T-cell proliferation**

Purified CD4<sup>+</sup> T-cells were cultured at  $2 \times 10^5$  cells per well (200  $\mu$ l total) in 96-well round-bottomed microtiter plates (Falcon, Becton-Dickinson). Cells were cultured in the presence of 1  $\mu$ g/ml plate-bound purified hamster anti-mouse CD3 monoclonal antibody (BD Pharmingen) alone, or with 5  $\mu$ g/ml soluble purified hamster anti-mouse CD28 monoclonal antibody (BD Pharmingen). These concentrations were determined by preliminary experiments to induce proliferation without compromising viability (data not shown). For the *in vivo* anti-CTLA-4 antibody treatment study, whole splenocytes or inguinal lymph node cells were stimulated with 2.5  $\mu$ g/ml of the polyclonal T-cell mitogen ConA (Sigma) or 10, 20, or 40  $\mu$ g/ml of mycobacterial purified protein derivative (PPD; Statens Seruminstitut, Copenhagen, Denmark). Cells were incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> in air for 72 or 96 h. For the final 6 h, 1.0  $\mu$ Ci [<sup>3</sup>H]-thymidine/well (New England Nuclear, North Bellerica, MA) was added to the cultures. Cells were harvested on a 96-well cell harvester (Packard Instrument Co.) and cellular thymidine uptake was measured using a liquid scintillation counter (Beckman Coulter). Results are expressed as net disintegrations per minute (DPM).

### **T-cell activation for flow cytometry and real-time RT-PCR**

Purified CD4<sup>+</sup> T-cells were cultured at  $1-5 \times 10^6$  cells per well (2 ml total volume) in 24-well flat-bottomed microtiter plates (Falcon, Becton-Dickinson). Cells were cultured in the presence of 1 ng/ml PMA with 500 nM Ionomycin (Calbiochem-Novabiochem, San Diego, CA) or 1  $\mu$ g/ml anti-CD3 with 5  $\mu$ g/ml anti-CD28 (BD Pharmingen) at 37°C in an atmosphere of 5% CO<sub>2</sub> in air for the indicated times (4).

### **Immunofluorescence flow cytometry**

For quantitative surface receptor staining,  $10^6$  CD4<sup>+</sup> T-cells from activated and control cultures were first incubated for 10 min at RT with 0.5  $\mu\text{g/ml}$  CD16/32 Ab (BD Pharmingen) to block Fc receptors. The addition of 7-aminoactinomycin D (7-AAD, Sigma) at 1  $\mu\text{g/ml}$  for 10 min allowed for the exclusion of dead cells. Cells were then incubated with anti-CD28 or anti-CTLA-4-phycoerytherin (PE, red) (BD Pharmingen) labeled mAb diluted to 4  $\mu\text{g/ml}$  in 0.5% BSA solution for 30 min. Samples were washed to remove unbound antibody before flow cytometric analysis. The cells were analyzed on a FACSCalibur (Becton-Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer, using CellQuest (Becton-Dickinson) acquisition software. Phycoerythrin fluorescence was collected through the 585/42 nm bandpass filter, and 7-AAD fluorescence was collected through the 670 nm long pass filter. List mode data were acquired on a minimum of 10,000 events defined by light scatter gates. Surface protein expression was quantified using QuantiBrite PE Beads (Becton-Dickinson) conjugated with 4 levels of PE. Data analysis was performed in CellQuest/FlowJo (Treestar, Inc., Ashland, OR), using forward and side light scatter to gate on the lymphocyte population and 7-AAD fluorescence to exclude non-viable cells from analysis. The Calibration Platform in FlowJo was used to convert the fluorescence intensity scale into absolute number of PE molecules. For each sample stained with a mAb, the median fluorescence intensity, expressed as the equivalent number of PE molecules, was determined to be the median antibody binding capacity (ABC) for the cell population. By using a known ratio of PE to antibodies (1:1), PE molecules per cell were then converted to antibodies per cell or antibody binding capacity (ABC) (119).

For intracellular (total) detection of CTLA-4, purified splenic CD4<sup>+</sup> T-cells were first incubated with CD16/32 Ab as described above, fixed in 1% paraformaldehyde at 4°C (Electron Microscopy Sciences) and stained with anti-CTLA-4 mAb at 4°C in the presence of 0.03% saponin. Cells were then extensively washed in 0.03% saponin and 1% BSA solution before analysis by flow cytometry (119).

### **Real-time RT-PCR**

T-cell RNA was isolated using RNAqueous Total RNA kit (Ambion) according to the manufacturer's protocol. Total RNA was quantified using a RiboGreen RNA Quantitation Kit (Molecular Probes). Real time PCR was performed using the ABI 7700 (Applied Biosystems) and Taqman Probes as described previously (120). Probes and primers for CD28 and CTLA-4 mouse genes were designed by Primer Express software, version 1.7. Primer and probe sequences were then checked for sequence homology against known genes using a BLAST search (<http://www.ncbi.nlm.nih.gov/blast>). All data were normalized to 18S rRNA expression.

### ***In vitro* treatment with anti-CTLA-4 mAb**

Mouse splenic CD4<sup>+</sup> T-cells were cultured for cell proliferation as described above with the addition of 100 µg/ml purified mAb anti-CTLA-4 (clone UC10-4F10; BD Pharmingen) or isotype hamster IgG control (BD Pharmingen).

### ***In vivo* treatment with anti-CTLA-4 mAb**

UC10-4F10 (hamster anti-murine CTLA-4) hybridoma cells were obtained from ATCC (HB-304). The high titered supernatant was purified over HiTrap protein G columns (Pharmacia) and acid eluted as per the manufacturer's protocol. Extracts were then dialyzed with 1X PBS overnight at 4°C using Slide-A-Lyzer cassettes (Pierce, Rockford, IL). Antibody was subsequently concentrated using Amicon Ultra Centrifugal Filter Devices (Millipore). The specificity of the Ab was checked by Western blotting using recombinant CTLA-4 protein (BD Pharmingen) and the bioactivity was determined by measuring whole splenocyte proliferation in the presence of purified Ab and anti-CD3 (44). The purified CTLA-4 Ab was stored at -80°C prior to use. Modulation of CTLA-4 function *in vivo* was accomplished by the daily intraperitoneal injection of 100 µg/mouse of purified mAb anti-CTLA-4 in 100 µl PBS or affinity purified hamster IgG (Cappel Research Products, Durham, NC) as a negative control for the 5 d prior to sacrifice.

### **Immunization and footpad testing**

Mice were immunized with approximately  $10^6$  viable *Mycobacterium bovis* (BCG) organisms (Danish 1331; Statens Seruminstitut, Copenhagen, Denmark) by subcutaneous injection 6 wks prior to sacrifice to allow an Ag-specific immune response to develop. Diets were initiated 14 d before the end of the 6 wk vaccination period as previously described (3). Testing with 0.05 ml of purified protein derivative (PPD) containing 100 tuberculin units was performed by intradermal injection into both hind footpads 3 d prior to sacrifice to determine the degree of antigen-specific delayed-type hypersensitivity (DTH). Footpad swelling was quantified with microcalipers and

compared to preinjection footpad thickness in millimeters according to our previously published protocol (3).

### **Statistical analysis**

Data were analyzed using two-way ANOVA for main treatment effects using SuperANOVA statistical software (Berkeley, CA). A difference between means was tested using Duncan's multiple range test. Significant and highly significant differences were defined as  $p < 0.05$  and  $p < 0.01$ , respectively, for all tests.

### Results

#### **Dietary n-3 PUFA suppress splenic CD4<sup>+</sup> T-cell proliferation**

Previous results from our laboratory suggested that reduced IL-2 cytokine production of CD4<sup>+</sup> T-cells in mice fed diets rich in n-3 PUFA was highly dependent on the involvement of the co-stimulatory molecule, CD28 (4). To define the ability of DHA and EPA to modulate CD4<sup>+</sup> T-cell function, CD4<sup>+</sup> T-cells purified from the spleens of mice fed 5% (w/w) corn oil (CO; control diet containing n-6 PUFA), 1% DHA, or 1% EPA were stimulated for 72 h and subsequently pulsed with <sup>3</sup>H-[TdR]. Fig. 1A illustrates that both experimental diets significantly reduced CD4<sup>+</sup> T-cell proliferation when cultured with anti-CD3 and anti-CD28 in the presence of 10% FBS ( $p < 0.01$ ). Due to the

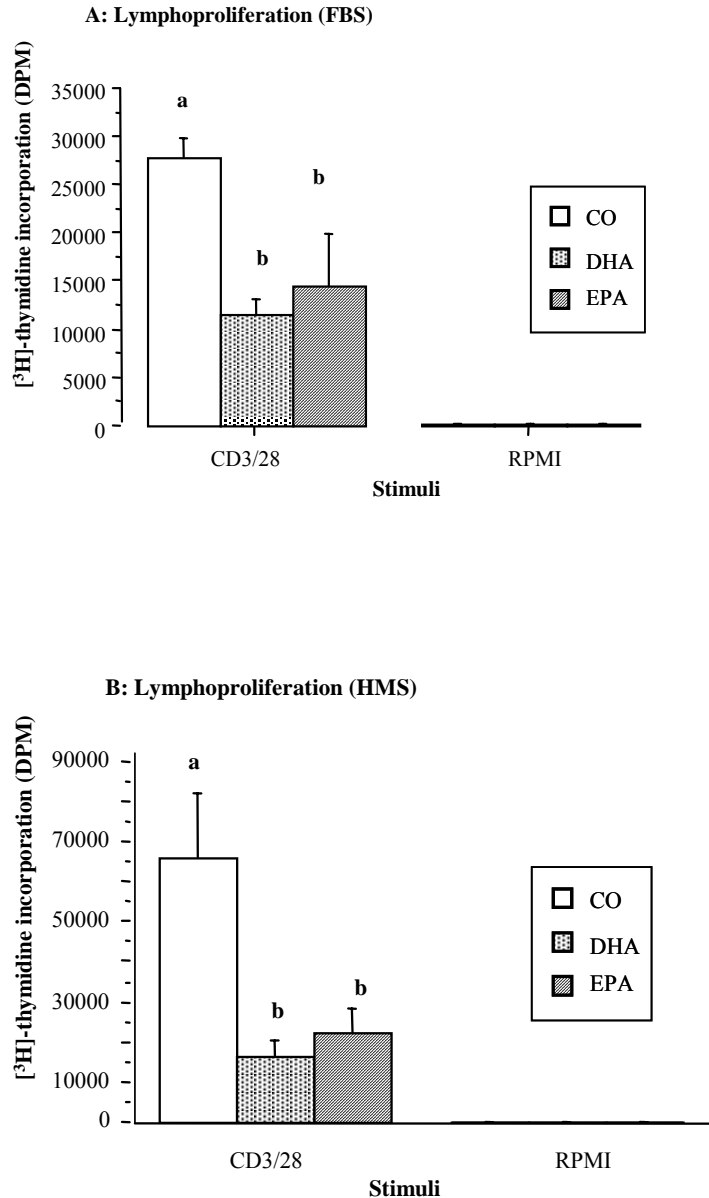
length of time in culture, a parallel set of cultures were maintained in diet-matched homologous mouse serum (HMS) to prevent the loss of cell membrane fatty acids as we and others have previously reported (58, 121, 122). Fig.1B demonstrates that maintaining the T-cell lipid environment *in vitro* by the addition of HMS sustained the *in vivo* modifications induced by diet. The suppressive effect of dietary n-3 PUFA on proliferation was significantly enhanced in the presence of HMS. We recently reported that diet markedly alters the fatty acid composition of sera collected from mice fed control and experimental diets (58). Table 1 shows that FBS was relatively devoid of n-3 PUFA (20:5n-3, 22:5n-3, 22:6n-3) whereas mouse sera from DHA- and EPA-fed mice was highly enriched in 22:6n-3 and 20:5n-3, respectively. Therefore, most of the *in vitro* experiments presented below were performed using HMS.



**TABLE I.** Fatty acid composition of sera used for cell culture.

Fatty Acid	FBS	MS-CO	MS-DHA	MS-EPA
16:0	1.71 ± 0.02	14.20 ± 0.55	11.36 ± 0.21	13.65 ± 0.55
18:0	0.80 ± 0.02	7.93 ± 0.20	2.56 ± 0.42	4.78 ± 0.11
18:1n-9/n-7	1.63 ± 0.05	15.54 ± 0.22	10.24 ± 0.62	10.36 ± 0.42
18:2n-6	0.42 ± 0.01	17.72 ± 0.37	9.96 ± 0.46	7.04 ± 0.56
20:4n-6	0.53 ± 0.01	12.32 ± 0.61	1.72 ± 0.36	1.67 ± 0.15
20:5n-3	tr	tr	2.07 ± 0.28	5.64 ± 0.44
22:5n-3	0.15 ± 0.00	tr	tr	0.49 ± 0.01
22:6n-3	0.17 ± 0.01	2.24 ± 0.24	5.51 ± 0.65	2.40 ± 0.26

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



**FIGURE 1.** Dietary DHA and EPA down-regulate murine CD4<sup>+</sup> T-cell proliferation. Purified splenic CD4<sup>+</sup> T-cells from mice fed CO, DHA, or EPA were activated with antibodies to surface receptors CD3 and CD28 and cellular uptake of [<sup>3</sup>H]-thymidine was measured 72 h post-activation. Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=7. Different letters denote highly significant differences found between diet groups ( $p < 0.01$ ). A: cells cultured in the presence of 10% FBS; B: cells cultured in the presence of 2.5% homologous mouse serum (HMS) + 2.5% FBS.

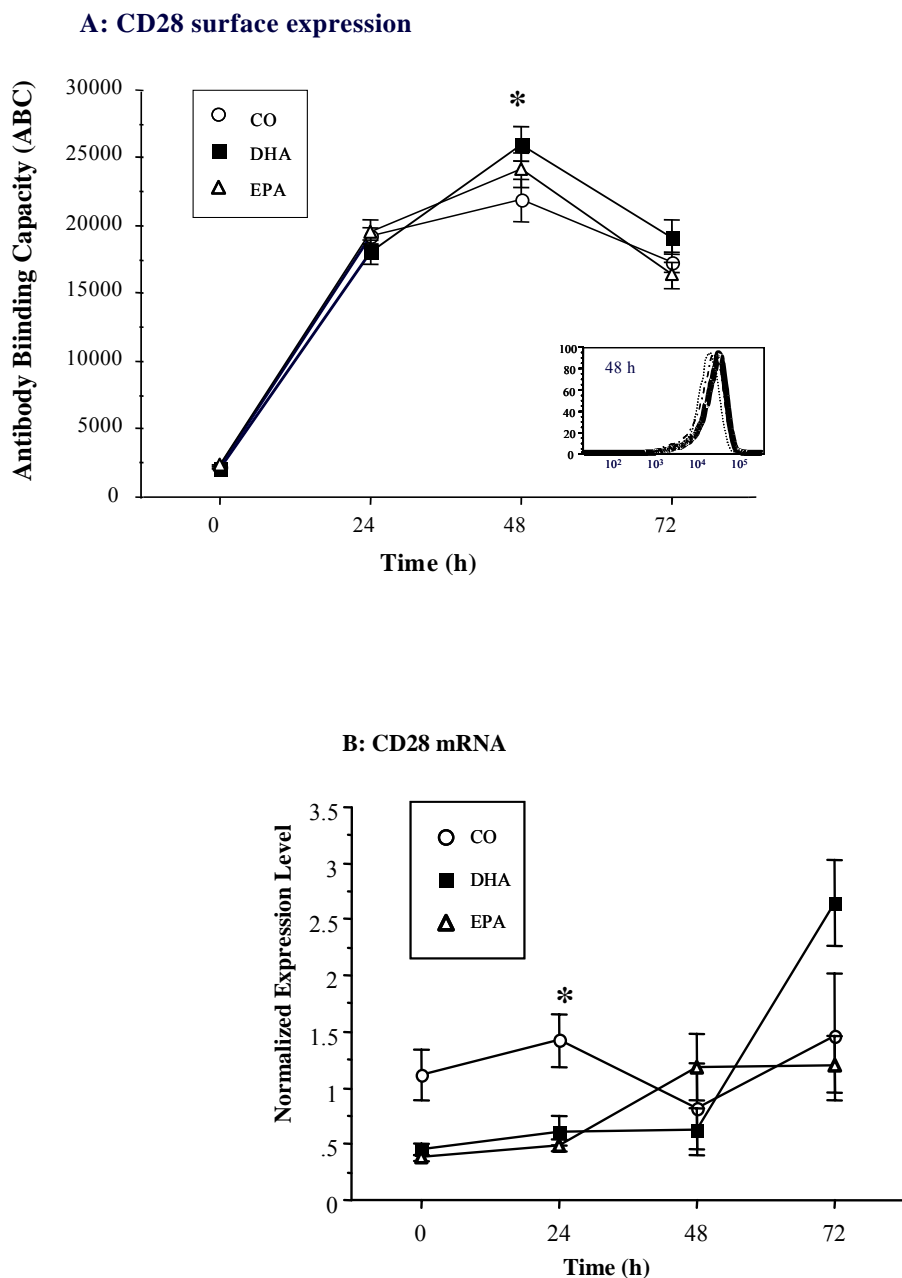
### **Dietary n-3 PUFA enhance surface expression of CD28 on CD4<sup>+</sup> T-cells**

We initiated experiments to define the role of the co-stimulatory receptor CD28 in diet-mediated immunosuppression. As shown in Fig. 2A, activation of CD4<sup>+</sup> T-cells with phorbol ester (PMA) and calcium ionophore (Iono) rapidly upregulated cell surface

protein expression of CD28 with levels peaking at 48 h post-activation. Cells from mice fed DHA had significantly higher levels (20%) of CD28 molecules on a per-cell basis than those fed the CO (control) diet after 48 h of *in vitro* stimulation ( $p < 0.01$ ; Fig. 2A Inset). These results were contrary to our hypothesis that dietary n-3 PUFA would reduce the levels of CD28 protein present on the cell surface, thereby leading to reduced cell proliferation and cytokine production (5, 10). Real-time PCR analysis (Fig. 2B) indicated that dietary n-3 PUFA markedly reduced levels of CD28 mRNA expression at  $t = 0$  and 24 h post-activation with PMA/Iono when compared to the CO diet ( $p < 0.05$ ). Following 72 h activation, all diet groups approached maximum mRNA expression levels with no significant differences found.

#### **Dietary n-3 PUFA enhance both CTLA-4 protein (total and surface) and mRNA transcript levels on CD4<sup>+</sup> T-cells**

It has been reported that cell surface accumulation of CTLA-4 is primarily regulated by its rapid endocytosis (104). Indeed, flow cytometric analyses revealed that the majority of CTLA-4 protein accumulated intracellularly (total) at all time points following CD4<sup>+</sup> T-cell activation with anti-CD3 and anti-CD28 when compared to cell



**FIGURE 2.** Activated CD4<sup>+</sup> T-cells from mice fed DHA display significantly increased surface expression of CD28. **A:** Time course analyses of the expression of CD28 molecules were carried out in CD4<sup>+</sup> T-cells cultured in the presence of 10% FBS from mice fed CO, DHA, or EPA. The y-axis represents the antibody binding capacity (ABC) equivalent sites for CD28 after culture with phorbol ester (PMA) and calcium ionophore (Ionomycin). Values represent means  $\pm$ SEM of cultures from 6-10 mice. \*Highly significant differences were found between DHA and CO-fed mice ( $p < 0.01$ ) *Inset:* Histogram represents the mean fluorescence of anti-CD28-PE. CO (dotted line), DHA (solid line), or EPA (dashed line). **B:** CD28 mRNA quantification of CD4<sup>+</sup> T-cells from mice fed CO, DHA, or EPA was performed by real-time RT-PCR. Values for cycle threshold (Ct) were converted to “expression levels” to allow for fold comparisons between samples, expression level =  $2^{(40-Ct)}$ . Data were normalized to 18S rRNA and expressed as means  $\pm$ SEM,  $n=6-10$ . \*Significant differences were found between control (CO) and experimental diet groups  $p < 0.05$ .

surface expression (Fig. 3A and 3B). This is consistent with previous reports demonstrating that anti-CD28 mAb augments anti-CD3-induced CTLA-4 surface expression (123, 124). *In vitro* culture with PMA/Iono stimuli (used in Fig. 2) for CD28 analysis failed to induce CTLA-4 expression (data not shown).

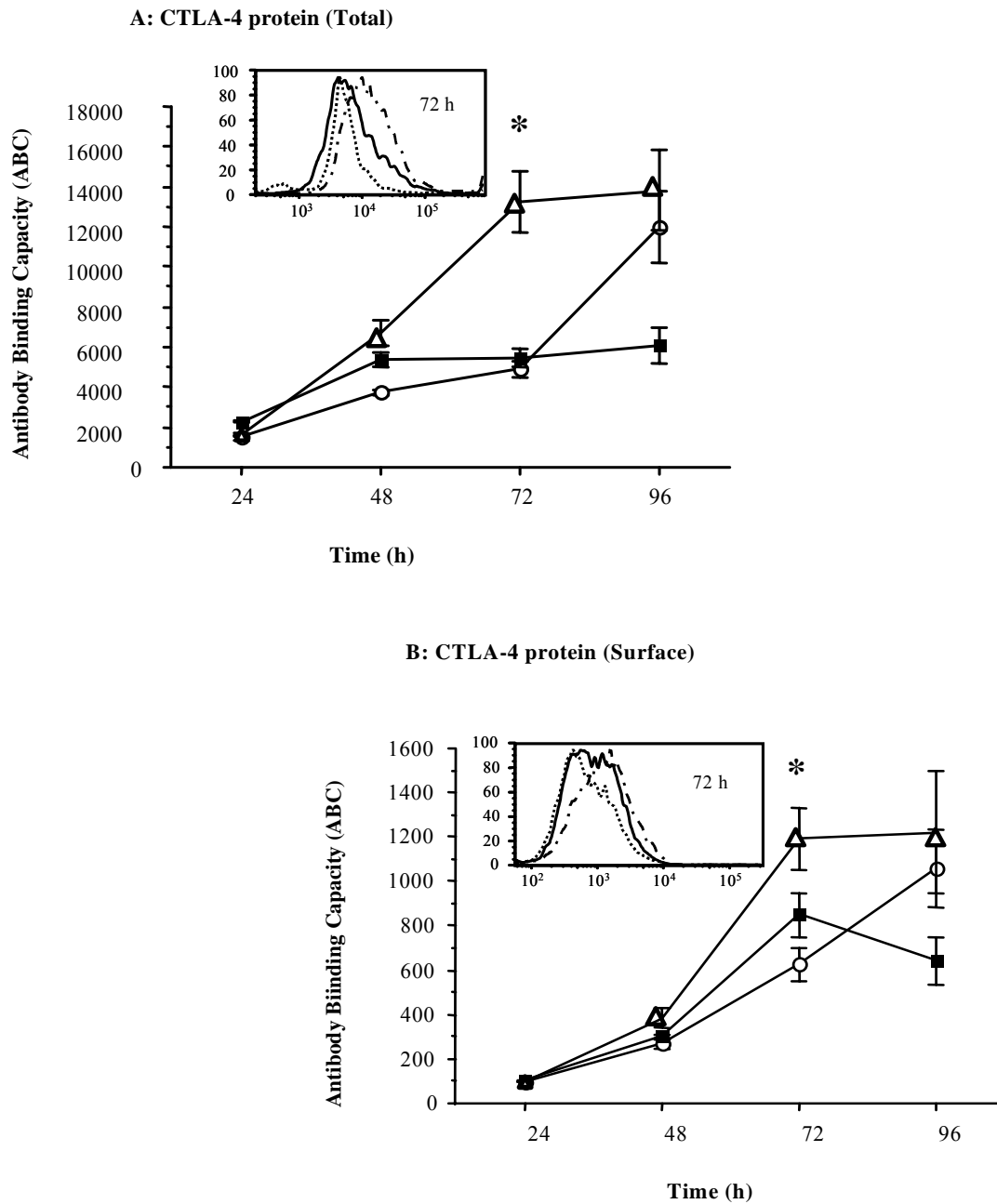
Although the absolute values differed, the kinetics of both total and surface CTLA-4 protein expression were similar for all diet groups. Consistent with other findings, mice fed the CO control diet displayed a continuous increase in CTLA-4 protein expression over time as antibody binding capacity (ABC) plateaued at 96 h post-activation (Fig. 3A and 3B) (104). However, CTLA-4 expression levels on CD4<sup>+</sup> T-cells from mice fed the EPA diet reached significantly higher levels at 72 h of *in vitro* T-cell activation ( $p < 0.01$ ; Fig. 3A and 3B Inset). It is noteworthy that CD4<sup>+</sup> T-cells from mice fed dietary EPA had uniformly higher levels of CTLA-4 molecules than cells from mice fed CO, whereas cells from mice fed dietary DHA were heterogenous, with some time points behaving similarly to CO and others similar to the EPA-fed group. These results demonstrate that dietary n-3 PUFA significantly and differentially enhance the temporal expression of both cell surface-expressed and total CTLA-4.

Quantitative measurement of CTLA-4 mRNA expression showed enhanced transcript levels in mice fed dietary n-3 PUFA relative to CO (Fig. 3C). Dietary DHA significantly enhanced CTLA-4 mRNA levels 72 h post-stimulation ( $p < 0.05$ ). Unlike the CD28 analysis (Fig. 2), there was a direct relationship between CTLA-4 protein and transcript levels in CD4<sup>+</sup> T-cells from mice fed dietary n-3 PUFA.

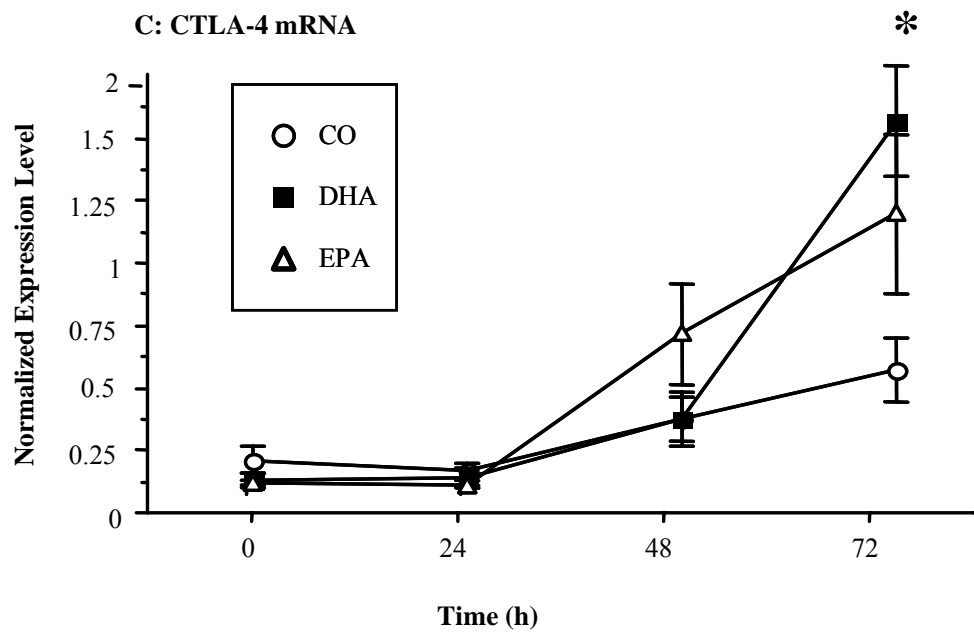
### **Anti-CTLA-4 mAb treatment *in vivo* and *in vitro* does not relieve the suppression of lymphocyte responses in mice fed dietary n-3 PUFA**

Since CTLA-4 has been implicated in down-modulating T-cell activation (89, 106, 125, 126), and is significantly upregulated in CD4<sup>+</sup> T-cells from mice fed dietary n-3 PUFA, we next investigated whether CTLA-4 engagement was involved in diet-mediated immunosuppression. Splenic CD4<sup>+</sup> T-cells from mice fed the experimental or control diets were cultured as described in Fig. 1 with the addition of anti-CTLA-4 (mAb clone UC10-4F10; BD Pharmingen) or IgG control. As expected, dietary n-3 PUFA suppressed lymphocyte proliferation after *in vitro* activation (Fig. 4A). The presence of CTLA-4 mAb in cultures had little effect on the T-cell proliferative responses of mice fed DHA and EPA (Fig. 4A). When all diet groups were combined, the presence of CTLA-4 mAb did, however, inhibit T-cell proliferation (Fig. 4B), although the difference was not statistically significant. These results suggest that anti-CTLA-4 mAb does not enhance T-cell proliferation and may be suppressive when added to cultures suboptimally stimulated with anti-CD3 and anti-CD28 mAbs as previously observed by us (Fig. 5) and by Walunas et al (44). Thus, in our model, *in vitro* administration of mAb anti-CTLA-4 appeared to deliver a positive signal through the CTLA-4 receptor to down-regulate T-cell proliferation rather than block transduction of a negative signal.

To determine whether anti-CTLA-4 treatment *in vivo* could reverse the suppressive effect of diet, splenocytes from *Mycobacterium bovis* (BCG)-vaccinated mice were cultured for 96 h in the presence of HMS with purified protein derivative (PPD) or concanavalin A (ConA) to allow comparisons between antigenic and mitogenic stimulation. Splenocytes taken from the CTLA-4 mAb treated group responded similarly

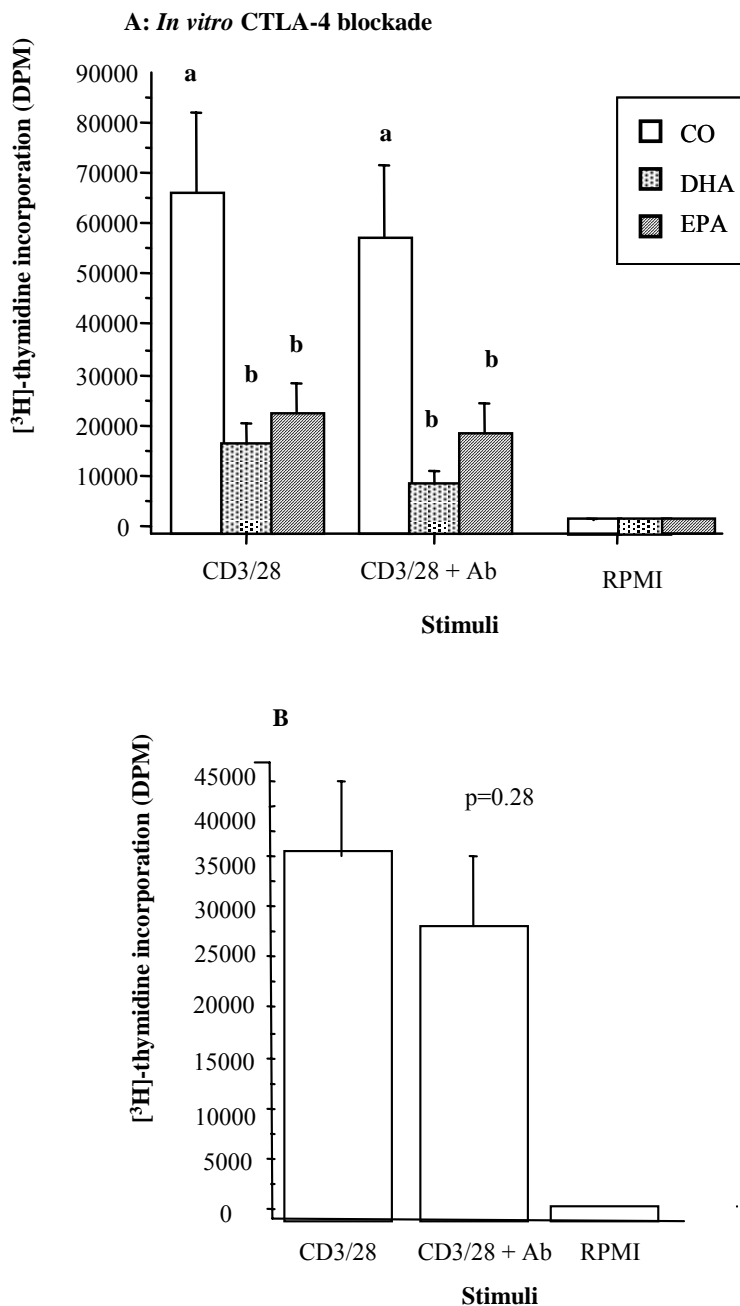


**FIGURE 3.** Splenic CD4<sup>+</sup> T-cells from mice fed dietary n-3 PUFA express significantly increased CTLA-4 protein (total and surface) and mRNA levels following *in vitro* activation. Time course analyses of A: total and B: surface CTLA-4 expression were carried out in CD4<sup>+</sup> T-cells from mice fed CO, DHA, or EPA in the presence of HMS. The y-axis represents the antibody binding capacity (ABC) equivalent sites for CTLA-4 after culture with anti-CD3 and anti-CD28. Values represent means  $\pm$ SEM of cultures from n=6-7 mice. \*Highly significant differences were found between EPA and CO-fed mice ( $p < 0.01$ ). *Inset:* Histogram represents the mean fluorescence of anti-CTLA-4-PE. CO (dotted line), DHA (solid line), or EPA (dashed line).

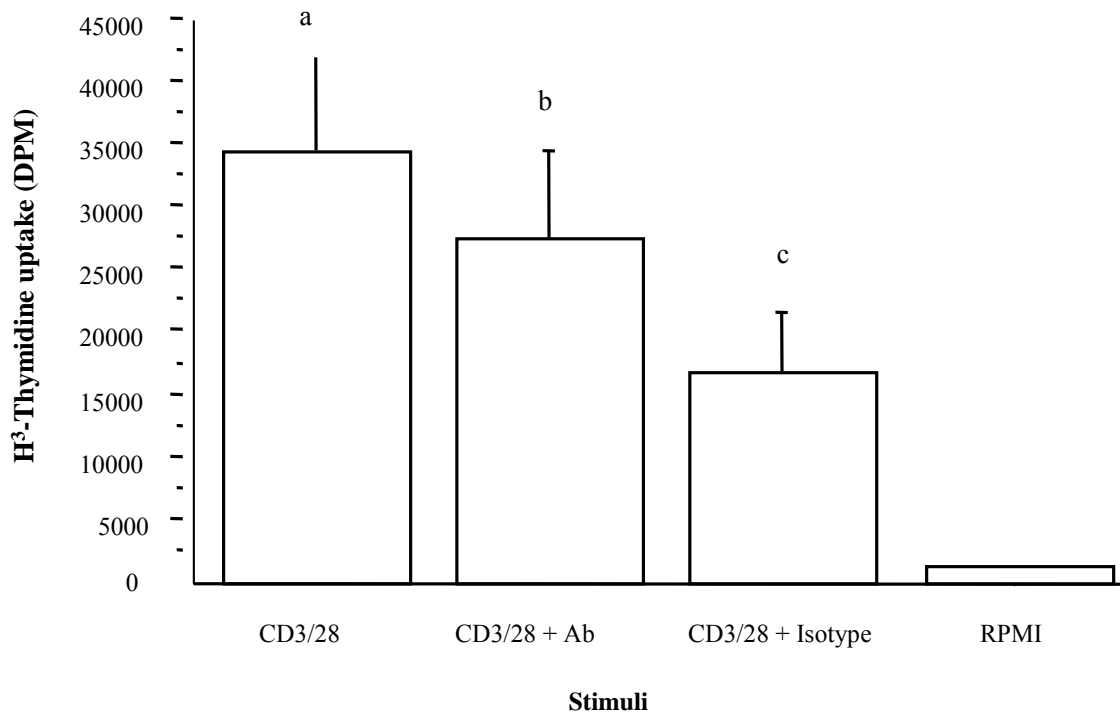


**FIGURE 3. Continued.** C: CTLA-4 mRNA quantification of CD4<sup>+</sup> T-cells from mice fed CO, DHA, or EPA was performed by real-time RT-PCR as described in Fig. 2B. \*Significant differences were found between control (CO) and the experimental diet groups  $p < 0.05$ .





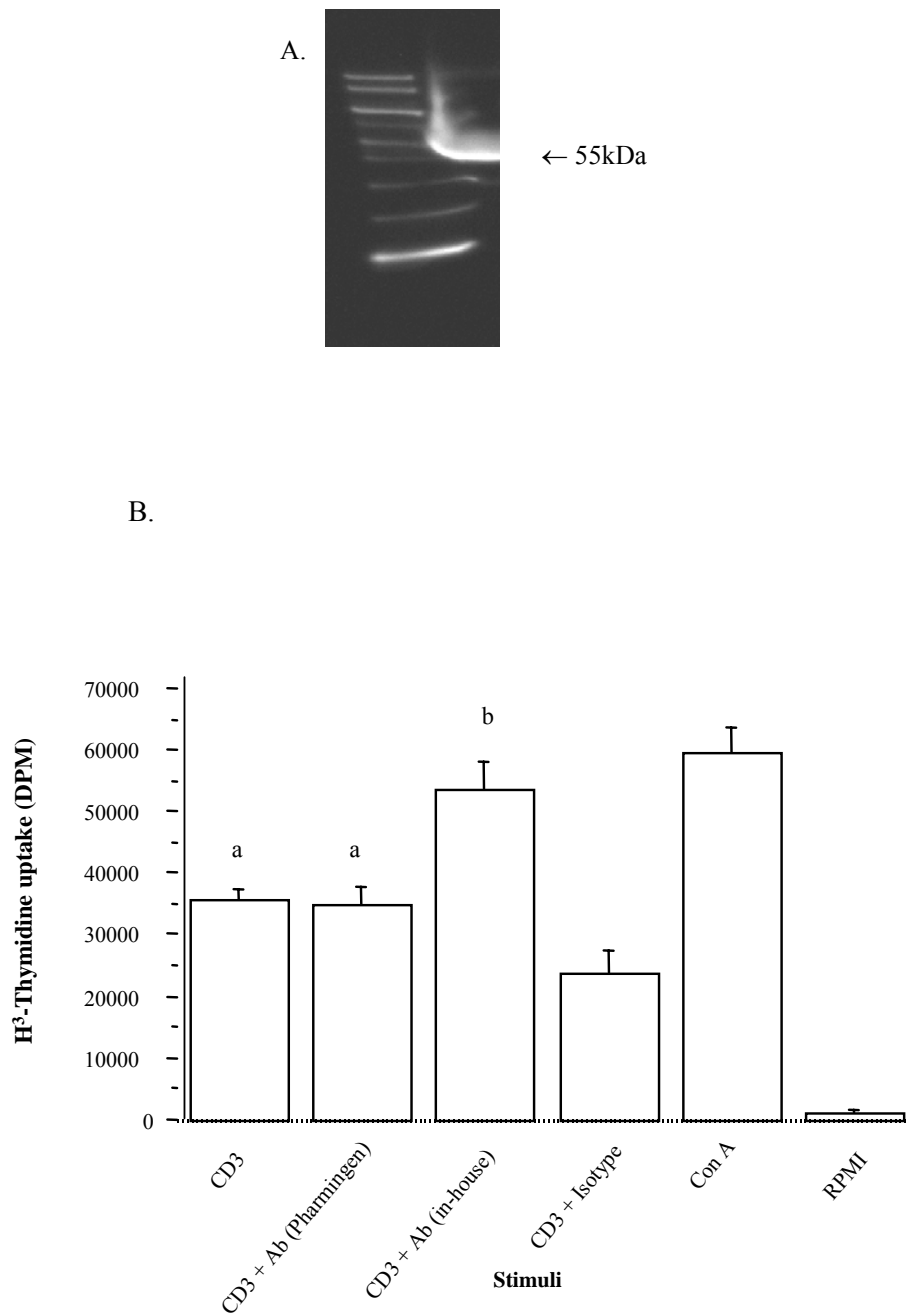
**FIGURE 4.** *In vitro* effect of CTLA-4 ligation with antibody on CD4<sup>+</sup> T-cell proliferation. A: Splenic CD4<sup>+</sup> T-cells from mice fed diets enriched with CO, DHA, or EPA were stimulated with aCD3/aCD28 as described in Fig. 1B in the presence of either 100 mg/ml anti-CTLA-4 mAb (BD Pharmingen) or an isotype-matched control IgG (BD Pharmingen) (data not shown) in the presence of HMS. Cellular uptake of [<sup>3</sup>H]-thymidine was measured 72 h post-stimulation. Results are expressed as the net disintegrations per minute (DPM), n=6. Different letters denote highly significant differences between diet groups (p<0.01). B: Bars represent all diet groups combined for each treatment in Fig. 4A.



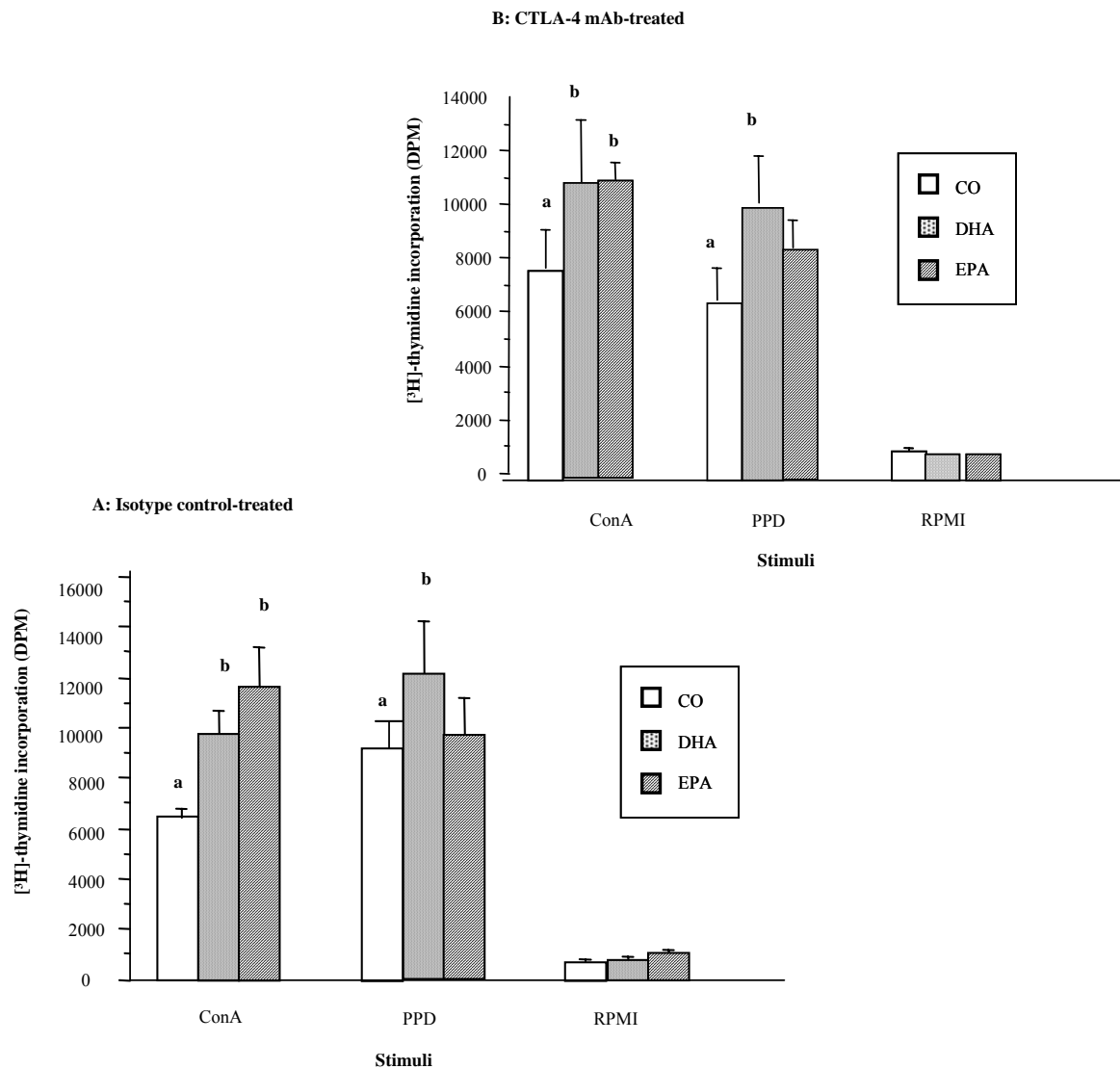
**FIGURE 5.** Anti-CTLA-4 antibody (Pharmlngen) decreases CD4<sup>+</sup>T-cell proliferation in C57BL/6 mice. Purified splenic CD4<sup>+</sup> T-cells were obtained from C57BL/6 mice and cultured with  $\alpha$ CD3/CD28 at 1 and 5  $\mu$ g/ml, respectively, in the absence or presence of 100  $\mu$ g/ml of anti-mouse CTLA-4 Ab (clone 4F10; Pharmlngen) or 100  $\mu$ g/ml of IgG isotype (Iso; Pharmlngen). Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=3 mice.

as those obtained from the isotype control-treated group. Anti-CTLA-4 mAb purified from UC10-4F10 hybridoma cells were tested for specificity (Fig. 6A) and activity (Fig. 6B) prior to injections. Dietary n-3 PUFA (DHA) significantly enhanced the proliferative response of splenocytes to mitogenic and antigenic stimuli from BCG-vaccinated mice treated with either isotype or CTLA-4 mAb (Fig. 7A & 7B).

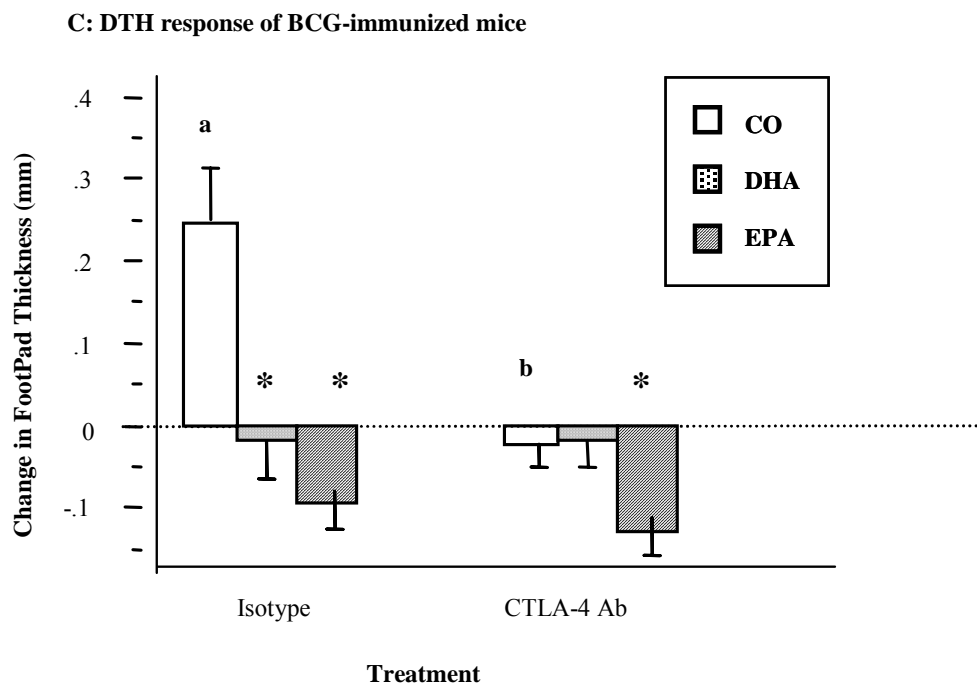
The effect of diet on the *in vivo* antigenic response was evaluated by footpad testing BCG-vaccinated mice treated with CTLA-4 mAb or isotype control with PPD 48 h prior to sacrifice. The difference in footpad thickness was measured with microcalipers before and 48 h after PPD injection. Fig. 7C illustrates that both dietary n-3 PUFA significantly suppressed the delayed-type hypersensitivity (DTH) response to PPD in mice treated with the isotype control. Surprisingly, treatment with the CTLA-4 mAb in the CO-fed group markedly reduced the DTH reaction (Fig. 7C), and further suppressed the response in the footpads of EPA-fed mice.



**FIGURE 6.** Anti-CTLA-4 Ab (prepared in-house) enhances whole splenocyte proliferation in C57BL/6 mice. A: The specificity of the purified anti-CTLA-4 mAb from UC10-4F10 cells was determined by immunoblotting. The transfer blot of 0.25  $\mu$ g of rmCTLA-4 Ab (R&D) was incubated with the purified anti-CTLA-4 Ab (no dilution) as the primary antibody. The rmCTLA-4 protein migrates as a 55kDa protein. B: Whole splenocytes were obtained from C57BL/6 mice and activated with anti-CD3 at 1  $\mu$ g/ml or ConA 2.5  $\mu$ g/ml in the presence or absence of 100 $\mu$ g/ml of anti-CTLA-4 Ab purchased from Pharmingen or prepared in-house from UC10-4F10 cells (refer to page 22) or 100 $\mu$ g/ml of IgG isotype Ab (Pharmingen). Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=3 mice. Letters denote highly significant differences found within the treatment group (p<0.01).



**FIGURE 7.** Anti-CTLA-4 mAb treatment *in vivo* does not alter diet-induced changes in PPD-specific T-cell responses of BCG-immunized mice. Proliferative response of splenocytes from BCG-immunized mice to Concanavalin A (ConA; 2.5  $\mu\text{g}/\text{ml}$ ) and purified protein derivative (PPD; 10  $\mu\text{g}/\text{ml}$ ) in the presence of HMS after 96 h of culture. Results are expressed as the mean  $\pm$  SEM net thymidine uptake (DPM) of  $n=5-6$  mice per diet group. Different letters denote highly significant differences found between diet groups ( $p<0.01$ ). Mice were treated with, A: isotype control (IgG) or, B: anti-CTLA-4 at 100  $\mu\text{g}/\text{mouse}$  daily for 5 days prior to sacrifice.



**FIGURE 7. Continued.** C: DTH response to PPD in the footpads of BCG-immunized mice treated with isotype control or anti-CTLA-4 mAb as described above. Results are expressed as the mean  $\pm$  SEM of the difference in footpad thickness in millimeters (mm) as measured just before and 48 h after injection with PPD,  $n=5-6$  mice per diet group. Different letters denote highly significant differences found between treatment groups ( $p<0.01$ ). \* Highly significant differences were found between CO vs. DHA and/or EPA-fed mice within the treatment group ( $p<0.01$ ).

## Discussion

Successful T-cell activation and clonal expansion, followed by appropriate homeostatic clonal contraction, require a delicate balance of positive and negative regulatory signals. Costimulatory molecules such as CD28 are essential coactivators of proliferation, cytokine production, and cell migration (127-129). To balance these signals, cell surface molecules like CTLA-4 inhibit T-cell responses. We have shown here that dietary n-3 PUFA feeding alters CD28 and CTLA-4 co-receptor expression, apparently disrupting the balance between these two signals to favor reduced T-cell activation.

New data have identified an important link between the co-receptors and the expression of lipid rafts on the surface of T-cells. Lipid rafts segregate proteins and lipids into liquid-ordered domains with distinct biophysical properties (130). Crucial signaling proteins reside in these domains and engagement of the TcR with antigen promotes the entry of receptors into rafts, in which the TcRs are likely to interact with kinases and adaptors (131, 132). Viola et al (118) first showed that ligation of CD28 promotes the cell-surface expression of lipid rafts, whereas Martin et al (133) showed that CTLA-4 potentially inhibits TcR-CD28-mediated raft formation. In this model, costimulatory receptors would function by simply regulating the availability of crucial signal mediators that are required for effective TcR signaling. We and others have previously reported that dietary n-3 PUFA differentially modulate the phospholipid membrane composition of lymphocytes, thereby attenuating T-cell function by altering the recruitment of critical components of the TcR signal transduction machinery (58, 61). Dietary n-3 PUFA feeding reduced the translocation of PKC $\theta$  into lipid rafts, and

inhibited both AP-1 and NF- $\kappa$ B activation, and IL-2 secretion (62). On this background, we hypothesize that dietary n-3 PUFA will also alter the interaction of CD28 and/or CTLA-4 with lipid rafts to influence the development of the immunological synapse (IS) during T-cell-APC-conjugate formation (134). This hypothesis will be the focus of subsequent studies.

Our results indicate that dietary n-3 PUFA feeding upregulated both CD28 and CTLA-4 protein expression levels (Fig. 2 and 3). However, DHA and EPA, the active constituents of fish oil, exhibited somewhat different immunomodulatory properties. DHA significantly enhanced the cell surface expression of CD28 on CD4<sup>+</sup> T-cells (Fig. 2A; t= 48 h post-activation), while dietary EPA upregulated CTLA-4 protein (surface and total) (Fig. 3A and B; t=72 h post-activation). Furthermore, our results indicate that CTLA-4 mRNA levels were significantly increased in only the DHA-fed group (Fig. 3C). Although both experimental diets suppressed CD4<sup>+</sup> T-cell proliferation in a similar manner (Fig. 1), it is clear that EPA and DHA have unique effects on co-stimulatory regulation in our model. Consistent with our findings, a recent human study (135) concluded that EPA and DHA differentially modulate certain immune functions. In that study, the fatty acid composition of plasma phospholipids in neutrophils was differentially altered by supplementation with DHA or EPA. Supplementation with DHA suppressed T-lymphocyte activation, as assessed by CD69 expression, whereas EPA supplementation had no significant effect. The mechanisms responsible for the differential effects of EPA and DHA on T-lymphocyte activation are unclear. EPA and DHA may conceivably have different effects on raft stability because DHA is thought to



adopt a more folded conformation in membranes and has been shown to exclude phospholipase D from lipid rafts at relatively low concentrations (136).

The molecular basis for T-cell inhibition by CTLA-4 has been the subject of much debate. It has been proposed that CTLA-4 may: 1) antagonize CD28 by competing for CD80 or CD86 binding; 2) sequester intracellular enzymes that can bind to both co-receptors; or 3) directly or indirectly reduce TcR signals by association with the immunological synapse (137). The kinetics of CTLA-4 expression were dramatically modified by dietary n-3 PUFA feeding in our study such that the protein levels (surface and total) approached maximum levels 72 h post-activation *in vitro* (EPA >> DHA; Fig. 3A and B). The CO-fed group did not reach maximum CTLA-4 protein expression levels until after 96 h. These results suggest that the down-regulatory CTLA-4 co-receptor may play a key role in diet-mediated immunosuppression (Fig. 1), i.e., dietary n-3 PUFA may rapidly upregulate CTLA-4 protein expression to downregulate overall T-cell function.

Although the cell surface levels of CD28 protein were also significantly enhanced by n-3 PUFA (DHA; Fig. 2) feeding, there was a dissociation with its mRNA expression (Fig. 2B). In contrast, a direct relationship was observed between CTLA-4 transcript and protein levels (Fig. 3). Since dietary n-3 PUFA increased the expression of CD28, which is inconsistent with suppressed T-cell proliferation, it is conceivable that the increased levels of CD28 on the cell surface may not be functional. It is possible that by altering the fatty acid composition of the cellular membrane, diet may inhibit the positive regulatory function of CD28 on the T-cell response. We and others have recently reported that conditions which modify raft structure can disrupt the earliest steps of T-cell activation and the function of those critical signaling components (56, 58, 61).

*In vitro* experiments have shown that the blockade of signals through CTLA-4 augments T-cell expansion, while CTLA-4 cross-linking results in decreased T-cell proliferation due to decreased IL-2 production (138). The experiments presented here suggest that the latter mechanism was operational in our cultures, as the presence of CTLA-4 mAb (4F10) in culture with CD4<sup>+</sup> T-cells activated with  $\alpha$ CD3/ $\alpha$ CD28 certainly did not enhance, and may have reduced, cell proliferation, although the latter effect was not statistically significant (Fig. 4).

The effect of CTLA-4 blockade *in vivo* was first demonstrated in a tumor model in which an enhanced antitumor immunity was observed (139). In other experimental systems, CTLA-4 blockade enhanced the severity of autoimmune diseases by increasing the pool of pathologic T-cells (115, 140). Taken together, these findings indicate that antibody ligation of CTLA-4 in some *in vivo* models leads to an enhanced T-cell response. In contrast, we demonstrate that anti-CTLA-4 treatment *in vivo* may have had an agonist effect. Both dietary n-3 PUFA significantly reduced the DTH response of BCG-immunized mice treated with the IgG isotype control, whereas in mice treated *in vivo* with anti-CTLA-4 mAb, no relief of this diet-mediated suppression was seen. In fact, further suppression of DTH was observed in the EPA-fed group treated with antibody (Fig. 7C;  $p < 0.01$ ). Anti-CTLA-4 mAb also markedly reduced the DTH response of mice fed the control diet (CO;  $p < 0.01$ ) suggesting that the *in vivo* mAb treatment may have had a positive, agonist effect on the lymphocytes.

As previously reported by our laboratory, the enhancing effect of EPA and DHA on mitogen-induced proliferation in the splenocytes of BCG-vaccinated mice may be explained, in part, by alteration in the proportions of functional T-cell subsets (e.g., Th1

& Th2) as well as the relative proportions of activated and/or memory T-cells populating the spleens of mice undergoing a vigorous immune response to an on-going BCG infection (3). It has been reported that CTLA-4 protein expression is much higher in Th2 than in Th1 clones (141). Therefore, it may not be surprising that CTLA-4 ligation *in vivo* with mAb in BCG-vaccinated mice with a predominantly Th1 cell response did not affect the mitogen and antigen-induced proliferative response of splenocytes from mice fed dietary n-3 PUFA (Fig. 7A & 7B). Moreover, the presence of CTLA-4 mAb *in vitro* did not significantly alter [ $H^3$ ]-thymidine uptake of  $\alpha CD3/\alpha CD28$ -stimulated  $CD4^+$  T-cells from naïve mice fed dietary n-3 PUFA (Fig. 4). The presence of anti-CTLA-4 mAb *in vitro* and *in vivo* did, however, have differential effects on lymphocyte proliferation in mice fed dietary n-3 PUFA (Fig. 4 & 7). This may be explained, in part, by the presence of accessory cells in whole splenocyte cultures from mice treated *in vivo* with mAb (Fig. 7) and the lack thereof in the purified  $CD4^+$  T-cell cultures (Fig. 4). Although there is evidence that dietary n-3 PUFA affect accessory cell membrane lipid composition and cytokine production (142-145), we and others have shown that the diet-mediated T-cell immunosuppression is mediated principally through direct effects on T-cells (3, 4, 10, 146).

The suppressive effect of dietary n-3 PUFA can also be attributed to a subpopulation of  $CD4^+$  T-cells known as regulatory T-cells ( $T_{regs}$ ;  $CD4^+ CD25^+$ ), which are immunosuppressive *in vivo* and *in vitro* (147, 148). Thymic-derived  $T_{regs}$  have been shown to regulate autoimmune disease via active suppression of self-reactive T-cells in various models of autoimmunity (149). It has recently been demonstrated that CD28 maintains a stable pool of peripheral  $T_{regs}$  by both supporting their survival and

promoting their self-renewal (150). CD28 engagement promotes survival by regulating IL-2 production by conventional T-cells and CD25 expression on T<sub>regs</sub>. Since CD28 protein expression is upregulated on the surface of CD4<sup>+</sup> T-cells (Fig. 2), from mice fed dietary n-3 PUFA, it would be of interest to determine whether the CD4<sup>+</sup> CD25<sup>+</sup> subpopulation play a role in mediating diet-induced T-cell suppression.

Our data suggest that the enhanced CTLA-4 cell surface protein expressed on the CD4<sup>+</sup> T-cells of mice fed EPA may not be functional, and that factors other than CTLA-4 may be responsible for diet-mediated immunosuppression. It seems likely that the complex interplay between functional CD28 and CTLA-4, and their association (or the lack thereof) with plasma membrane microdomains (“raft”) may be responsible, in part, for the downregulatory effect of diet on T-cell function in this model. Characterization of the co-receptors in relation to lipid rafts following T-cell stimulation will allow a determination of the precise role that altered levels of CD28 and CTLA-4 play in the diet-mediated regulation of T-cell function.

## CHAPTER III

### DIETARY N-3 POLYUNSATURATED FATTY ACIDS

#### SUPPRESS SPLENIC CD4<sup>+</sup> T-CELL FUNCTION IN IL-10<sup>(-/-)</sup> MICE\*

##### Introduction

IL-10 is a major regulatory cytokine of inflammatory responses. It was originally described as a mouse Th2 cell factor, inhibiting cytokine synthesis by Th1 cells (151). However, increasing evidence suggests that IL-10 acts as a general inhibitor of proliferative and cytokine responses of both Th1 and Th2 cells *in vitro* and *in vivo* (152-154). IL-10 is released by mononuclear phagocytes (152, 153), natural killer cells and by both Th1 and Th2 type lymphocytes (154). Its production is tightly regulated, as excess IL-10 leads to the inability to control infectious pathogens, while insufficient IL-10 leads to the pathology secondary to tissue injury. The immunosuppressive potency of IL-10 depends on the timing of IL-10 and IL-10 receptor (IL-10R) expression, and the IL-10 suppressive activity can diminish during immune and inflammatory responses (155, 156).

The co-stimulatory signal induced by complexing CD28 with specific monoclonal antibodies (mAbs) or by interaction with B7 counter-receptors enhances the antigen-dependent T-cell proliferation and cytokine production (102, 157). It has been proven that IL-10 elicits tolerance in T-cells by selective inhibition of the CD28 co-stimulatory pathway and thereby controls suppression and development of antigen specific immunity. IL-10 only inhibits T-cells stimulated by the engagement of low numbers of T-cell

---

\*Reprinted with permission from “Dietary n-3 Polyunsaturated Fatty Acids Suppress Splenic CD4<sup>+</sup> T-cell Function in IL-10<sup>(-/-)</sup> mice” by Ly, L.H., et al, 2004. *Clinical and Experimental Immunology* (accepted). 2004 by Blackwell Publishing.

receptors, i.e., conditions which require CD28 co-stimulation (66, 67). IL-10 inhibited CD28 tyrosine phosphorylation, the initial step of the CD28 signaling pathway, and consequently the phosphatidylinositol 3-kinase p85 binding to CD28 (67). In addition, Akdis et al demonstrated that stimulation of CD45RO<sup>+</sup> memory T-cells from healthy human subjects up-regulated IL-10 receptor (IL-10R) expression, rendering the cells more susceptible to IL-10-mediated suppression. The IL-10-induced selective inhibition of the CD28 co-stimulatory pathway acts as a decisive mechanism in determining whether a T-cell will contribute to an immune response or become anergic.

Previous results from our laboratory show that T-lymphocytes from mice fed the predominant n-3 polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA; 22:6) and eicosapentaenoic acid (EPA; 20:5), found in fish oil (FO) produced significantly less IL-2, but only when cells were activated with  $\alpha$ CD3/CD28 and not when activated with  $\alpha$ CD3/PMA (phorbol ester) (4). Furthermore, dietary n-3 PUFA significantly enhanced the expression of CD28 on the surface of CD4<sup>+</sup> T-cells (Chapter II).

On this background, we hypothesized that the suppressive effects of diet would be mediated by IL-10 and its relationship with CD28. To investigate this hypothesis, we have determined the influence of dietary DHA and EPA on the proliferative response, kinetics of IL-10R expression, and anti-inflammatory cytokine production of purified splenic CD4<sup>+</sup> T-cells from conventional C57BL/6 and IL-10 gene knockout (IL-10<sup>-/-</sup>) mice. Surprisingly, all responses were similar in both mouse groups with the exception of cytokine production. Dietary n-3 PUFA significantly reduced IFN $\gamma$  production in conventional mice while dramatically upregulating extracellular IFN $\gamma$  in IL-10<sup>-/-</sup> mice. Therefore, we conclude that dietary n-3 PUFA suppress CD4<sup>+</sup> T-cell functions through

mechanisms which do not involve IL-10. Furthermore, our results suggest that dietary n-3 PUFA may elicit the normally pro-inflammatory cytokine,  $\text{IFN}\gamma$ , to serve as an immunosuppressive cytokine in IL-10-deficient cells.

## Materials and Methods

### **Diets and animals**

Female, pathogen-free, young (12-14g) C57BL/6 mice were purchased from the Frederick National Cancer Research Facility (Frederick, MD). IL-10<sup>-/-</sup> mouse breeder pairs (129SvEv background) were a generous gift from Dr. Daniel Berg (University of Iowa). The colony is maintained at the Laboratory Animal Resources and Research facility at Texas A&M University where all breeders were genotyped according to protocol (Jackson Laboratory, Bar Harbor, ME) (Fig. 8). Female and male IL-10<sup>-/-</sup> (1-2 months of age) and C57BL/6 mice were assigned to one of three semi-purified diets: 5% corn oil (CO) (control diet containing no n-3 PUFA), 1% DHA + 4% CO (DHA), or 1% EPA + 4% CO (EPA), for 14 days as described on page 17.

### **Isolation and preparation of splenic lymphocytes**

Refer to page 18.

### **CD4<sup>+</sup> T-cell purification**

Refer to page 18.

**T-cell proliferation**

Refer to page 19.

**T-cell activation for flow cytometry**

Refer to page 19.

**Immunofluorescence flow cytometry**

For quantitative surface receptor staining,  $10^6$  CD4<sup>+</sup> T-cells from activated and control cultures were labeled with anti-IL-10R1 (PE, red) (BD Pharmingen) labeled mAb (4 µg/ml) and processed as described previously on page 20.

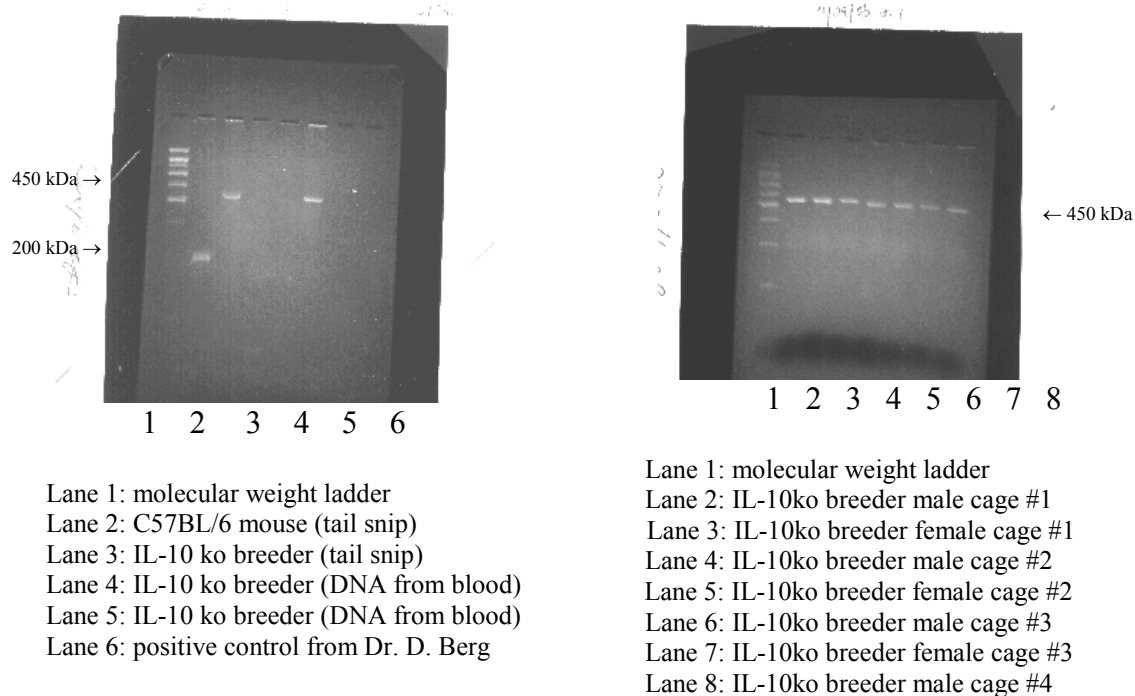
**ELISA analysis**

Activated CD4<sup>+</sup> T-cells from C57BL/6 and IL-10<sup>-/-</sup> mice were analyzed for IFN $\gamma$  and IL-10 production according to manufacturer's protocol (R&D Systems).

**Statistical analysis**

Refer to page 23.





**FIGURE 8.** Representative gels of IL-10 knockout mouse genotyping. The genetic makeup of the IL-10 knockout mouse (129 SvEv) colony breeders were confirmed by DNA isolation from tail snips according to protocol from [www.jax.org](http://www.jax.org) (see Appendix B). Positive wild-type (+/+) and gene knockout (-/-) DNA migrates as a 200 kDa and 450 kDa protein, respectively.

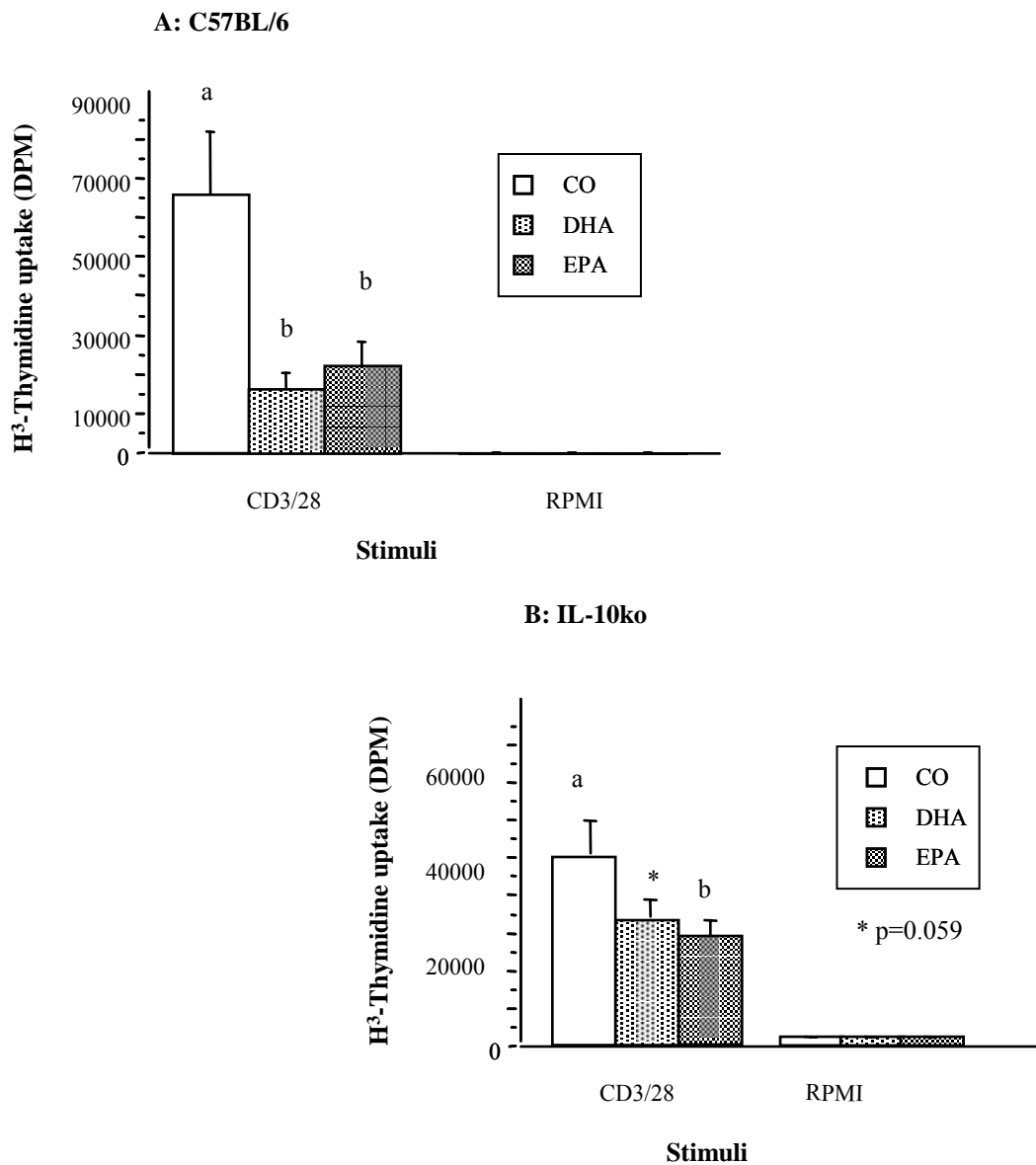
## Results

### **Dietary n-3 PUFA suppress CD4<sup>+</sup> T-cell proliferation in C57BL/6 and IL-10<sup>(-/-)</sup> mice**

To determine the role of IL-10 in diet-mediated immunosuppression, purified splenic CD4<sup>+</sup> T-cells from conventional C57BL/6 and IL-10<sup>(-/-)</sup> mice fed diets containing n-3 polyunsaturated fatty acids (PUFA), DHA or EPA, or corn oil (CO; devoid of n-3 PUFA) for 14 d were activated with antibodies to CD3 and CD28 ( $\alpha$ CD3/CD28) for 72 h. Surprisingly, cell proliferation was significantly suppressed in both the C57BL/6 (Fig. 9A) and IL-10<sup>(-/-)</sup> mice (Fig. 9B) fed dietary n-3 PUFA. Both dietary n-3 DHA and EPA significantly reduced T-cell proliferation in the C57BL/6 mice (Fig. 9A), whereas only EPA had a statistically significant effect on CD4<sup>+</sup> T-cells from the IL-10<sup>(-/-)</sup> mice (Fig. 9B). Therefore, absence of IL-10 does not alter the suppressive effect of dietary n-3 PUFA on polyclonal T-cell activation involving costimulation through CD28. These results were contrary to our hypothesis that dietary n-3 PUFA would not reduce CD4<sup>+</sup> T-cell proliferation in IL-10<sup>(-/-)</sup> mice, thereby demonstrating a role for the IL-10 cytokine in the suppressive effects of diet.

### **Dietary n-3 PUFA suppress IL-10 receptor (IL-10R) protein expression on the surface of CD4<sup>+</sup> T-cells from C57BL/6 and IL-10<sup>(-/-)</sup> mice**

To elucidate the influence of dietary n-3 PUFA on IL-10R expression, kinetic analyses were performed on purified CD4<sup>+</sup> T-cells from conventional and IL-10<sup>(-/-)</sup> mice fed the 3 diets. As expected, activation of CD4<sup>+</sup> T-cells with  $\alpha$ CD3/CD28 upregulated cell surface expression of the IL-10R (Fig. 10). Cells from C57BL/6 mice fed DHA had significantly lower levels (>40%) of IL-10R molecules on a per-cell basis than those fed

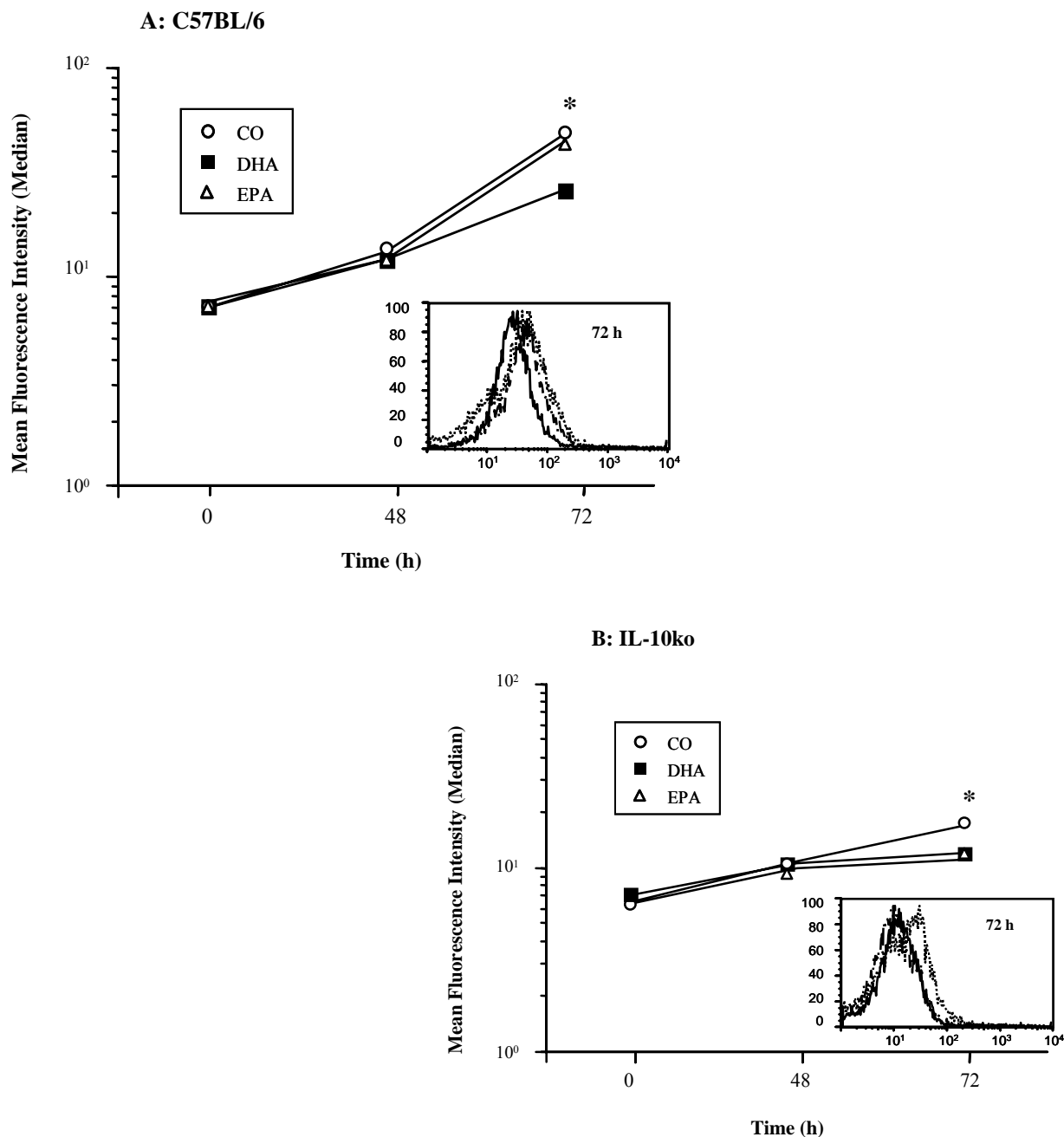


**FIGURE 9.** Dietary DHA and EPA down-regulate murine CD4<sup>+</sup> T-cell proliferation in IL-10<sup>-/-</sup> mice. Purified splenic CD4<sup>+</sup> T-cells from A: C57BL/6 and B: IL-10<sup>-/-</sup> mice fed CO, DHA, or EPA were activated with antibodies to surface receptors CD3 and CD28 and cellular uptake of [<sup>3</sup>H]-thymidine was measured 72 h post-activation. Results are expressed as mean ± SEM of the net disintegrations per minute (DPM), n=5. Different letters denote highly significant differences found between diet groups (p<0.01). All cells were cultured in the presence of 2.5% homologous mouse serum (HMS) + 2.5% FBS.

the CO (control) and EPA diet after 72 h of *in vitro* stimulation ( $p < 0.01$ ; Fig. 10A inset). Similarly, IL-10<sup>(-/-)</sup> mice fed dietary n-3 PUFA displayed markedly reduced levels of IL-10R molecules 72 h post-activation (~35%; Fig. 10B inset). In this instance, both dietary DHA and EPA significantly suppressed the expression of the IL-10R. These results indicate that dietary n-3 PUFA significantly and differentially reduce the temporal expression of cell surface-expressed IL-10R in both conventional and IL-10<sup>(-/-)</sup> mice.

### **Dietary n-3 PUFA differentially modulate IFN $\gamma$ and IL-10 cytokine production in CD4<sup>+</sup> T-cells**

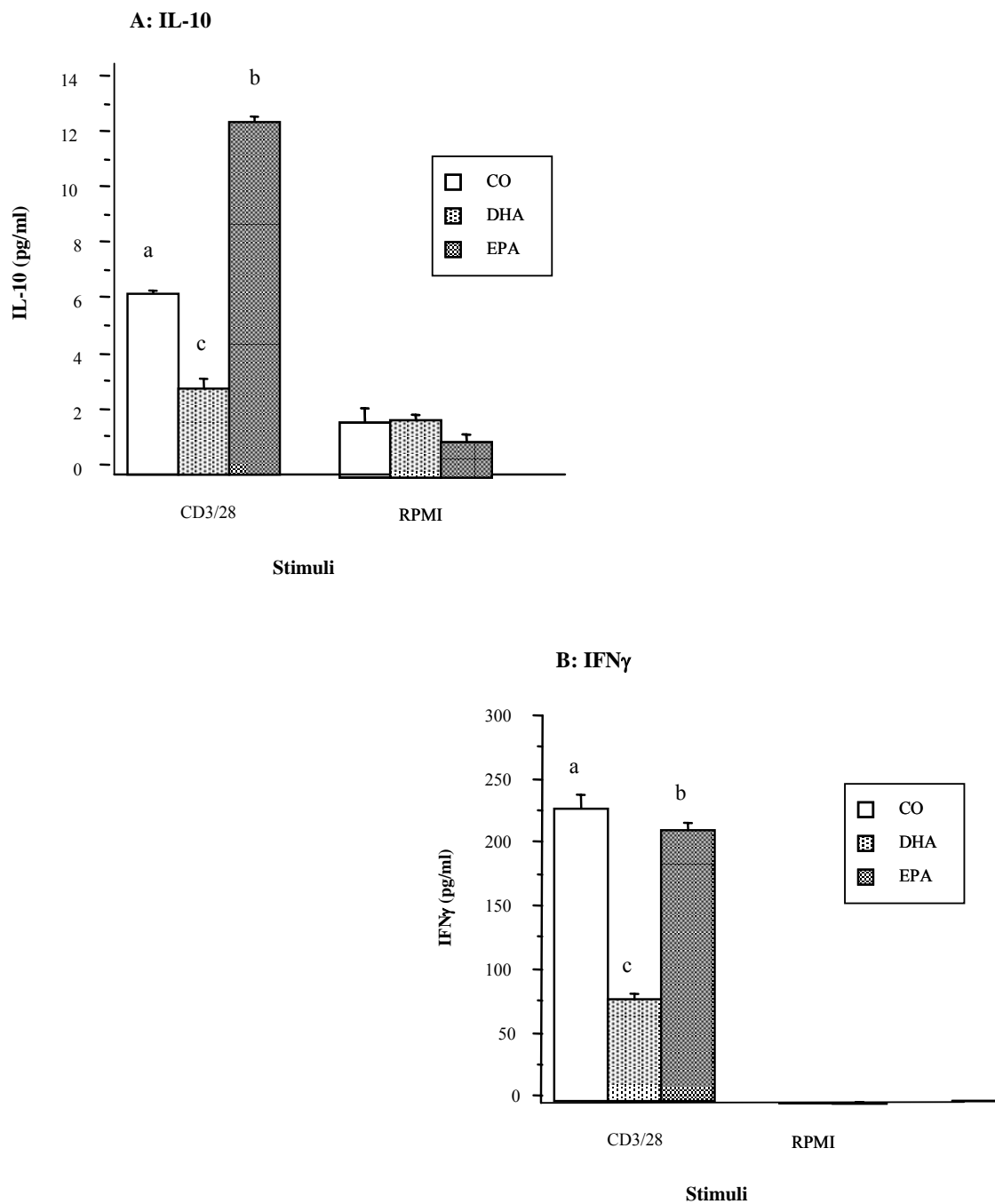
To further investigate the mechanism by which dietary n-3 PUFA suppress CD4<sup>+</sup> T-cell function in conventional and IL-10<sup>(-/-)</sup> mice, the production of the anti-inflammatory cytokine, IL-10, in cell culture supernatants was measured. It has recently been reported that the pro-inflammatory IFN $\gamma$  cytokine possesses anti-inflammatory properties and actively regulates IL-10 activity (158, 159). Thus, it was of interest to measure IFN $\gamma$  production as a potential anti-inflammatory mediator. Purified CD4<sup>+</sup> T-cells were stimulated with  $\alpha$ CD3/CD28 for 48 h and cell supernatants were collected. Quantitative determination of IL-10 concentrations from C57BL/6 mice revealed that the DHA-fed group produced significantly less (>50%) IL-10 than those fed the CO diet, whereas the EPA-fed group secreted dramatically more (2-fold) of the suppressive cytokine (Fig. 11A). These results confirm that the anti-inflammatory cytokine IL-10 does not play a role in the suppression of CD4<sup>+</sup> T-cell proliferation by DHA from C57BL/6 mice (Fig. 9A). On the other hand, the immunosuppressive EPA diet may



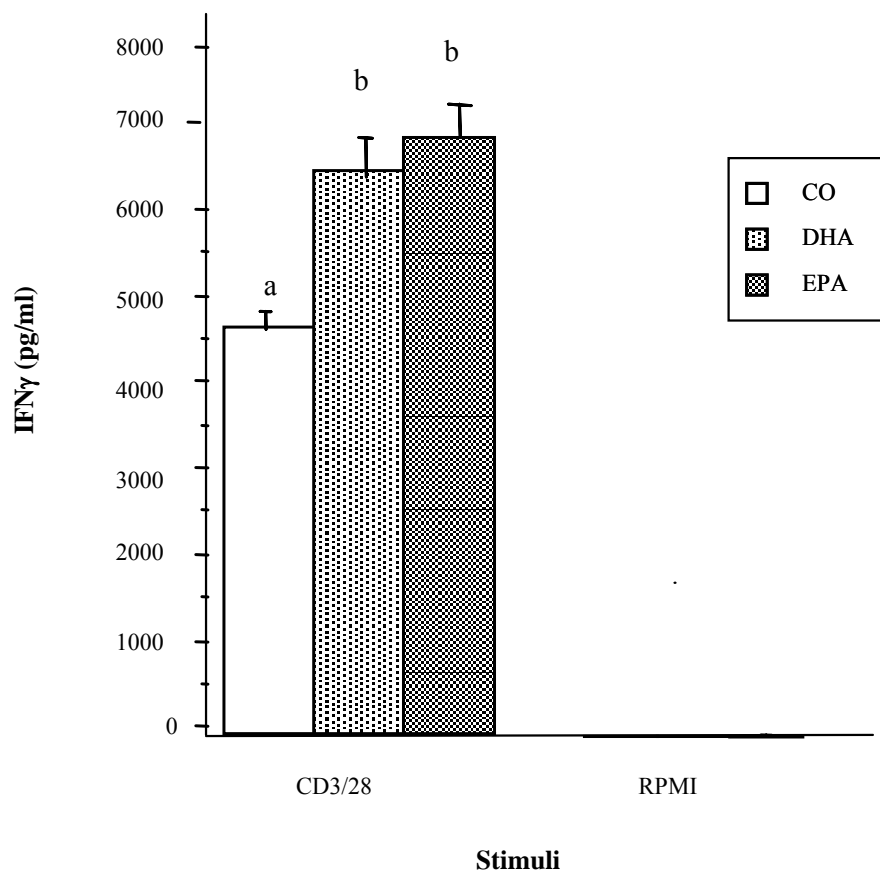
**FIGURE 10.** Dietary n-3 PUFA suppress IL-10R surface protein expression. Time course analyses of the expression of IL-10R molecules were carried out in splenic CD4<sup>+</sup> T-cells cultured in the presence of HMS from A: C57BL/6 and B: IL-10<sup>-/-</sup> mice fed CO, DHA, or EPA. The y-axis represents the antibody binding capacity (ABC) equivalent sites for the IL-10R after culture with  $\alpha$ CD3/CD28. Values represent means  $\pm$ SEM of cultures from n=5 mice. \*Highly significant differences were found between DHA and/or EPA and CO-fed mice ( $p < 0.01$ ) *Inset:* Histogram represents the mean fluorescence of anti-IL10R-PE. CO (dotted line), DHA (solid line), or EPA (dashed line).

utilize IL-10 by upregulating its expression. ELISA analysis of IFN $\gamma$  production in the same CD4<sup>+</sup> T-cell culture supernatants showed that both DHA ( $p < 0.01$ ) and EPA ( $p = 0.01$ ) diets significantly reduced IFN $\gamma$  production (Fig. 11B). This strongly suggests that IFN $\gamma$  does not mediate the suppressive effects of dietary n-3 PUFA on the T-cell response in C57BL/6 mice.

The production of IFN $\gamma$  was also quantitated in cell supernatants from IL-10<sup>(-/-)</sup> mice. Fig. 12 illustrates that dietary n-3 PUFA feeding significantly upregulated IFN $\gamma$  production by CD4<sup>+</sup> T-cells (>40%). Overall, the levels of IFN $\gamma$  in the supernatants of IL-10<sup>(-/-)</sup> CD4<sup>+</sup> T-cells was 20-100 fold higher than those observed under identical culture conditions in CD4<sup>+</sup> T-cells from conventional C57BL/6 mice (Fig. 11B). Our results indicate that T-cells from IL-10<sup>(-/-)</sup> mice may utilize the anti-inflammatory properties of IFN $\gamma$  to down-regulate T-cell function (Fig. 9B). This may occur to compensate for the lack of available IL-10 within the cytokine network.

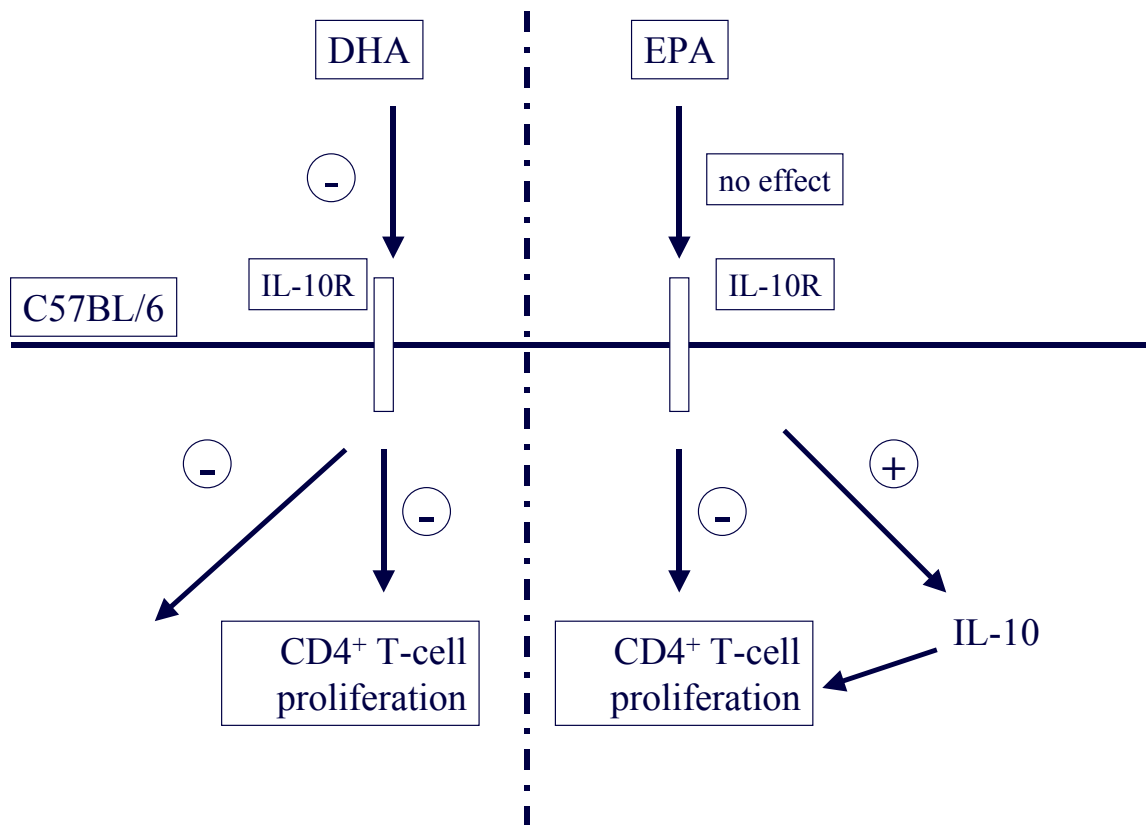


**FIGURE 11.** Dietary n-3 PUFA differentially modulate CD4<sup>+</sup> T-cell IFN $\gamma$  and IL-10 production in C57BL/6 mice. Purified splenic CD4<sup>+</sup> T-cells from mice fed the 3 diets were activated with  $\alpha$ CD3/CD28 in the presence of 2.5% HMS + 2.5% FBS for 48 h. A: IL-10 and B: IFN $\gamma$  in culture supernatant fluids were quantified by ELISA as described in the Materials and Methods. Values from n=5 mice represent the mean  $\pm$  SEM in pg/200,000 cells. Different letters denote highly significant differences found between diet groups ( $p < 0.01$ ).

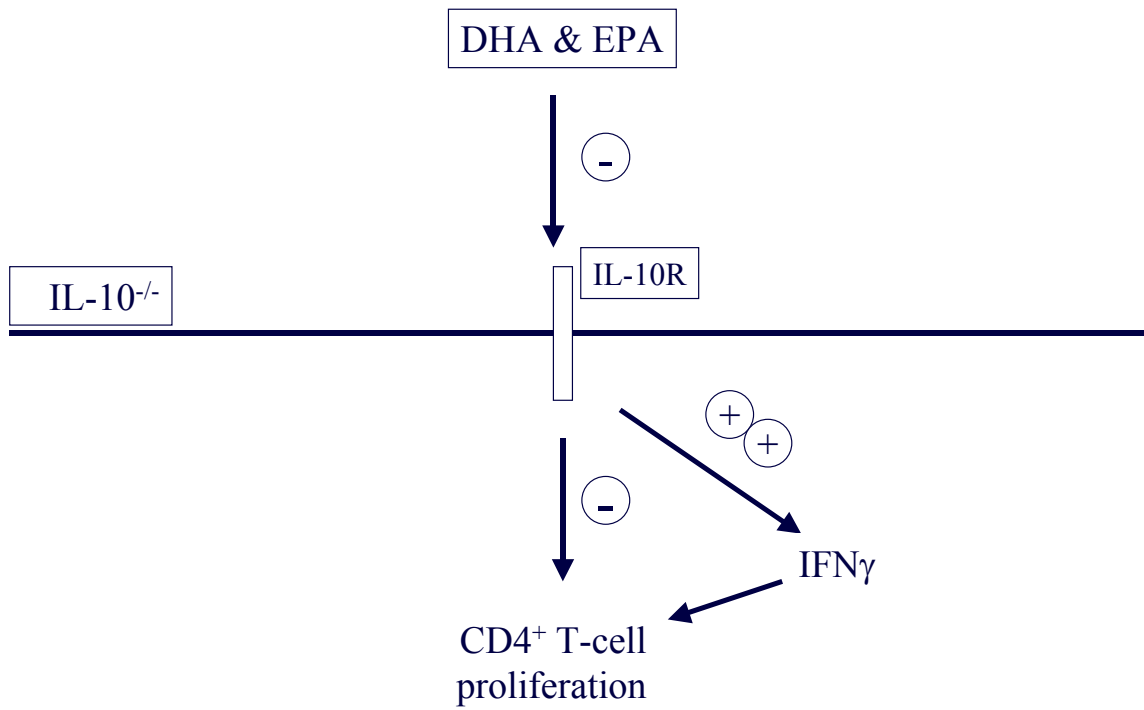


**FIGURE 12.** Dietary n-3 PUFA enhance IFN $\gamma$  production in CD4<sup>+</sup> T-cells from IL-10<sup>-/-</sup> mice. Purified splenic CD4<sup>+</sup> T-cells from mice fed the 3 diets were activated with  $\alpha$ CD3/CD28 in the presence of 2.5% HMS + 2.5% FBS for 48 h. IFN $\gamma$  production in culture supernatant fluids was quantified by ELISA as described in the Materials and Methods. Values from n=5 mice represent the mean  $\pm$  SEM in pg/200,000 cells. Different letters denote highly significant differences found between diet groups (p<0.01).





**FIGURE 13.** Diagram of results obtained from C57BL/6 mice.



**FIGURE 14.** Diagram of results obtained from IL-10<sup>-/-</sup> mice.

Figures 13 and 14 diagram the results of this section obtained in C57BL/6 and IL-10<sup>-/-</sup> mice.

### Discussion

The immunosuppressive role of IL-10 has been demonstrated by several human and mouse studies (160-163). Inflammatory bowel disease and other exaggerated inflammatory responses exhibited by IL-10<sup>-/-</sup> mice indicated that a critical *in vivo* function of IL-10 is to limit inflammatory responses (164-166). Moreover, inhibition of graft-versus-host disease by IL-10 and allograft rejection in human leukocyte antigen-mismatched bone-marrow transplantation in severe combined immunodeficient patients gives further evidence for a key role of this cytokine in the induction and maintenance of anergy (167).

The biological effects of cytokines are mediated through cell surface receptors. These receptors transduce the binding of their cytokines into cytoplasmic signals that eventually trigger a cascade of intracellular responses. The functional receptor complex of IL-10 consists of at least 2 subunits IL-10R1 and IL-10R2, both of which have been characterized and shown to play critical roles in determining whether cells respond to IL-10 (168-172).

Our data in this current study strongly suggest that the anti-inflammatory effects of diets enriched in n-3 PUFA are not likely mediated by IL-10. Fig. 9 and 10 illustrate that dietary n-3 PUFA continued to suppress CD4<sup>+</sup> T-lymphocyte responses in the absence of endogenous IL-10. Conventional C57BL/6 mice fed EPA may utilize the inhibitory IL-10 cytokine to suppress T-cell function (Fig. 9A and 11A), although the IL-

10R expression levels remained unaltered (Fig. 10A). However, in similar mice fed the DHA diet, alternative mechanisms may explain the suppressive effect of diet as both IFN $\gamma$  and IL-10 cytokine production were down-regulated (Fig. 11).

Beside the inhibitory effect of IL-10, other mechanisms acting on co-stimulatory pathways have been demonstrated to render T-cells unresponsive to an antigenic trigger. Blocking of the CD28-B7 interaction by CTLA-4 leads to an inhibition of xenogeneic graft rejection of pancreatic islets in mice (173). It has been shown that CTLA-4 forms a multimolecular complex with TCR $\xi$  and an SH2-containing tyrosine phosphatase (SHP-2), leading to a direct dephosphorylation of TCR $\xi$  and a subsequent inhibition of the TCR signaling pathway (174). We recently demonstrated that dietary n-3 PUFA feeding significantly upregulated both CD28 and CTLA-4 protein expression on the surface of murine CD4<sup>+</sup> T-cells (Chapter II), thereby disrupting the balance between these two signals to favor reduced T-cell activation. These findings are relevant to elucidate the mechanism(s) by which diet reduces T-cell function, without the involvement of IL-10 (Fig. 6 and 7), in conventional mice. Further studies will be needed to determine the extent to which CTLA-4 plays a role in diet-mediated immunosuppression.

Not surprisingly, dietary DHA and EPA exhibited somewhat different immunomodulatory properties. In this study, both EPA and DHA significantly reduced CD4<sup>+</sup> T-cell proliferation in C57BL/6 mice. EPA also suppressed this response significantly in IL-10<sup>-/-</sup> mice (Fig. 9). While proliferation was suppressed to nearly the same level by dietary DHA, the difference was not statistically significant. Measurement of IL-10R expression levels revealed that conventional mice fed DHA had significantly lower levels than the CO-fed group, while both DHA and EPA significantly reduced IL-

10R cell surface molecules in IL-10<sup>(-/-)</sup> mice (Fig. 10; t=72 h post-activation).

Furthermore, our results indicate that the EPA diet reduced IFN $\gamma$  but enhanced IL-10 production, while the DHA diet down-regulated both cytokines in C57BL/6 mice (Fig. 11). Although both experimental diets up-regulated production of IFN $\gamma$  in IL-10<sup>(-/-)</sup> mice in a similar manner (Fig. 12), it is clear that EPA and DHA have unique effects on certain T-lymphocyte responses in our model. Consistent with these current findings, we recently reported that dietary DHA and EPA differentially altered co-stimulatory regulation as DHA significantly enhanced the cell surface expression of CD28, while dietary EPA up-regulated the expression of CTLA-4 (Chapter II). The mechanisms responsible for the differential effects of EPA and DHA on T-lymphocyte responses are unclear. EPA and DHA may conceivably have different effects on membrane raft stability because DHA is thought to adopt a more folded conformation in membranes and has been shown to exclude phospholipase D from lipid rafts (136). We have recently demonstrated an effect of dietary n-3 PUFA on T-cell lipid raft composition in our model (56).

Production of IFN $\gamma$  in response to infection is the hallmark of innate and adaptive immunity (175). IFN $\gamma$  up-regulates a variety of pro-inflammatory mediators such as interleukin (IL)-12, IL-15, TNF- $\alpha$ , iNOS, and caspase-1 (176-179). IL-10 and IFN $\gamma$  have opposing effects during an active phase of an immune or inflammatory response, characterized by high levels of IFN $\gamma$  production and modest IL-10 activity such that pathogens can be effectively cleared (155, 180). These pro-inflammatory characteristics of IFN $\gamma$  contradict certain aspects of its biologic activity. Treatment of rheumatoid arthritis with IFN $\gamma$  in mouse and human studies was associated with a reduction of

leukocyte influx into the synovium, less synovial hyperplasia and erosion, and improved clinical status (181, 182). Similarly, administration of IFN $\gamma$  markedly reduced the incidence of disease in a rat model of insulin-dependent diabetes mellitus (183). In contrast, IFN $\gamma$  has been implicated in the pathophysiology of multiple sclerosis (MS) patients (184). The mechanisms by which the pro-inflammatory IFN $\gamma$  may exert anti-inflammatory properties has recently been reviewed (158). The authors conclude that IFN $\gamma$  re-directs inflammatory responses by inhibiting production of pro-inflammatory IL-1 and IL-8, by up-regulating the production of cytokine antagonists such as IL-1Ra and IL-18BP, inducing expression of the suppressors of cytokine signaling (SOCS), and by inducing apoptosis in leukocytes and local resident cells. The biological triggers responsible for shifting the role of IFN $\gamma$  from pro- to anti-inflammatory in response to immunologic stimuli have not been elucidated. However, these anti-inflammatory properties of the principally pro-inflammatory cytokine may explain, in part, its enhanced production in suppressed T-cells from IL-10<sup>-/-</sup> mice fed dietary n-3 PUFA (Fig. 9B and 12). A clear indication of this compensatory role for IFN $\gamma$  is the overall increase in IFN $\gamma$  production in lymphocytes from IL-10<sup>-/-</sup> vs. C57BL/6 mice (20-100-fold; Fig. 11B and 12). Further experiments will be necessary to examine the anti-inflammatory role of IFN $\gamma$  in IL-10<sup>-/-</sup> mice fed dietary n-3 PUFA.

The dual role of IFN $\gamma$  is also mimicked by the pleiotropic effects of IL-10 (65). Administration of IL-10 to wild-type mice can inhibit antigen-specific immune responses *in vivo* (185). In mice and humans, IL-10 can induce long-term antigen-specific anergy in CD4<sup>+</sup> T-cells (64, 186). In humans, IL-10 has a role in inducing systemic lupus erythematosus (187), yet counteracts psoriasis (188), and Crohn's disease (189).

Intravenous administration of IL-10 to healthy donors has proinflammatory effects through the release of IFN $\gamma$ , IFN $\gamma$ -inducible protein (IP-10) and increased granzyme levels (190). These effects are counteracted by inhibition of the release of monocyte inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), MIP-1 $\beta$  and monocyte chemoattractant protein-1 (MCP-1) (191), TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (155), and T-cell function (192). A switch in cytokine activity that is induced by the absence of an opposing cytokine adds an additional level of complexity to cytokine cross-regulation and cooperation pathways, especially when an immunosuppressive diet is involved.

## CHAPTER IV

### SUMMARY AND CONCLUSIONS

The 20- and 22-carbon n-3 PUFA are unique lipids in that they possess potent immunomodulatory activities. In addition, dietary n-3 PUFA have effects on diverse physiological processes impacting normal health and chronic disease, such as the regulation of plasma lipid levels (193-196), cardiovascular function (197-199), insulin action (200, 201), and neuronal development and visual function (202). Ingestion of n-3 PUFA results in their distribution to virtually every cell in the body affecting membrane composition and function, signaling, and regulation of gene expression (202-204). However, cell-specific lipid metabolism as well as the expression of fatty acid-regulated transcription factors will likely play a role in determining how the cell responds to changes in n-3 PUFA composition.

There is strong evidence for the beneficial effects of dietary FO in patients with chronic rheumatoid arthritis (205-208). Animal studies, including ours, indicate that diets rich in EPA and DHA are anti-inflammatory and immunomodulatory *in vivo*. However, the specific mechanisms by which these effects occur remain unclear. The literature is replete with inconsistencies because many laboratories use different amounts or types of fat in the administered diet, employ different strains of mice, and measure lymphocyte responses using various cell populations and stimuli (reviewed by (209)). In our studies, it is clear that the suppressive effects of n-3 PUFA on T-cell function can, in part, be attributed to alterations in co-stimulatory receptor function and cytokine cross-talk.



Our results suggest that dietary n-3 PUFA disrupts the regulatory interplay between T-cell membrane co-receptors, CD28 and CTLA-4. To compensate for the lack of functional endogenous IL-10, the anti-inflammatory aspects of IFN $\gamma$  may be enhanced in IL-10<sup>-/-</sup> mice fed dietary n-3 PUFA. These data give us a glimpse into the possible mechanisms by which dietary n-3 PUFA exert their immunosuppressive effects. The most important conclusions of this study are summarized here:

- Dietary n-3 PUFA (DHA>EPA) significantly reduced the *in vitro* proliferation of purified CD4<sup>+</sup> T-cell in mice.
- The suppressive effects of dietary n-3 PUFA were amplified by the presence of homologous mouse serum (HMS) *in vitro*.
- The suppressive effects of diet do not appear to involve the CTLA-4 co-receptor, as treatment with CTLA-4 mAb *in vivo* in *Mycobacterium bovis* (BCG)-vaccinated mice did not alter the suppressive effects of dietary n-3 PUFA on antigen (PPD)-induced lymphocyte proliferation or delayed hypersensitivity reactions.
- The downregulatory effects of dietary n-3 PUFA did not appear to involve the suppressive IL-10 cytokine, as the proliferation of purified splenic CD4<sup>+</sup> T-cells in n-3 PUFA fed IL-10<sup>-/-</sup> mice remained suppressed.
- In the absence of IL-10, IFN $\gamma$  may serve as an anti-inflammatory cytokine, T-cell suppressive role in IL-10<sup>-/-</sup> mice fed dietary n-3 PUFA.

To confirm the role of co-stimulatory molecules in n-3 PUFA diet-mediated immunosuppression, studies involving CD28 and CTLA-4 gene knockout mice (lacking a costimulatory signal for T-cell activation or deactivation) would be very helpful. It is

expected that in the absence of these co-receptors, the suppressive effects of n-3 PUFA feeding would diminish. Since CD28 is known to play a pivotal role (129, 210, 211) in the induction of Th2 cytokines, it would be of interest to examine the levels of a prototypic Th2 cytokine (IL-4 or IL-10) in CD28 knockout mice. Although the role of CTLA-4 in Th1 and Th2 cells is debatable (113), recent studies have demonstrated that Th2 clones express higher levels of CTLA-4 than Th1 clones (141). Because surface molecules and raft composition have been shown to behave differently in Th1 and Th2 cells (212, 213), further studies will be needed to delineate the differential effects of dietary n-3 PUFA on both CD4<sup>+</sup> T-cell subsets.

IL-10 production is tightly regulated, as excessive IL-10 impairs the ability to control some infectious pathogens, while insufficient IL-10 leads to pathology secondary to immune-mediated tissue injury. Like many other cytokines, IL-10 binds to its receptor and activates receptor-associated Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2) kinases, leading to the phosphorylation of signal transducers and activators of transcription (STAT) proteins which are required for the many anti-inflammatory effects of IL-10 (64).

One key mechanism that inhibits Jak-STAT signaling is induction of suppressors of cytokine signaling (SOCS) proteins. SOCS are rapidly induced in response to multiple cytokines, including cytokines that activate the Jak-STAT pathway themselves (214, 215). Thus, SOCS proteins play an important role in cross-talk and antagonism among different cytokines. Recently, it was reported that IL-10 signaling on lymphocytes was suppressed by IFN $\gamma$ -induced SOCS1 (159). Since dietary n-3 PUFA feeding suppressed CD4<sup>+</sup> T-cell proliferation in IL-10<sup>-/-</sup> mice and enhanced the production of IFN $\gamma$ , it would

be of interest to investigate the role of SOCS-induced switching of the normally pro-inflammatory IFN $\gamma$  to become anti-inflammatory. These issues are complex and more research will be needed to understand and to elucidate the precise molecular mechanisms by which dietary n-3 PUFA modulate T-cell functions.

## REFERENCES

1. Stulnig, T. M. 2003. Immunomodulation by polyunsaturated fatty acids: mechanisms and effects. *Int Arch Allergy Immunol* 132:310.
2. Calder, P. C. 2003. N-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic. *Lipids* 38:343.
3. Fowler, K. H., R. S. Chapkin, and D. N. McMurray. 1993. Effects of purified dietary n-3 ethyl esters on murine T lymphocyte function. *J Immunol* 151:5186.
4. Arrington, J. L., D. N. McMurray, K. C. Switzer, Y. Y. Fan, and R. S. Chapkin. 2001. Docosahexaenoic acid suppresses function of the CD28 costimulatory membrane receptor in primary murine and Jurkat T cells. *J Nutr* 131:1147.
5. Jolly, C. A., D. N. McMurray, and R. S. Chapkin. 1998. Effect of dietary n-3 fatty acids on interleukin-2 and interleukin-2 receptor alpha expression in activated murine lymphocytes. *Prostaglandins Leukot Essent Fatty Acids* 58:289.
6. French, M. A., A. M. Parrott, E. S. Kielo, R. V. Rajotte, L. C. Wang, A. B. Thomson, and M. T. Clandinin. 1997. Polyunsaturated fat in the diet may improve intestinal function in patients with Crohn's disease. *Biochim Biophys Acta* 1360:262.
7. Jump, D. B. 2002. The biochemistry of n-3 polyunsaturated fatty acids. *J Biol Chem* 277:8755.
8. Zampelas, A., G. Paschos, L. Rallidis, and N. Yiannakouris. 2003. Linoleic acid to alpha-linolenic acid ratio. From clinical trials to inflammatory markers of coronary artery disease. *World Rev Nutr Diet* 92:92.

9. Jolly, C. A., Y. H. Jiang, R. S. Chapkin, and D. N. McMurray. 1997. Dietary (n-3) polyunsaturated fatty acids suppress murine lymphoproliferation, interleukin-2 secretion, and the formation of diacylglycerol and ceramide. *J Nutr* 127:37.
10. Arrington, J. L., R. S. Chapkin, K. C. Switzer, J. S. Morris, and D. N. McMurray. 2001. Dietary n-3 polyunsaturated fatty acids modulate purified murine T-cell subset activation. *Clin Exp Immunol* 125:499.
11. Gill, I., and R. Valivety. 1997. Polyunsaturated fatty acids, Part 1: Occurrence, biological activities and applications. *Trends Biotechnol* 15:401.
12. Gill, I., and R. Valivety. 1997. Polyunsaturated fatty acids, Part 2: Biotransformations and biotechnological applications. *Trends Biotechnol* 15:470.
13. Kromann, N., and A. Green. 1980. Epidemiological studies in the Upernavik district, Greenland. Incidence of some chronic diseases 1950-1974. *Acta Med Scand* 208:401.
14. McCall, T. B., D. O'Leary, J. Bloomfield, and C. A. O'Morain. 1989. Therapeutic potential of fish oil in the treatment of ulcerative colitis. *Aliment Pharmacol Ther* 3:415.
15. Stenson, W. F., D. Cort, J. Rodgers, R. Burakoff, K. DeSchryver-Kecskemeti, T. L. Gramlich, and W. Beeken. 1992. Dietary supplementation with fish oil in ulcerative colitis. *Ann Intern Med* 116:609.
16. Hawthorne, A. B., T. K. Daneshmend, C. J. Hawkey, A. Belluzzi, S. J. Everitt, G. K. Holmes, C. Malkinson, M. Z. Shaheen, and J. E. Willars. 1992. Treatment of ulcerative colitis with fish oil supplementation: a prospective 12 month randomised controlled trial. *Gut* 33:922.

17. Salomon, P., A. A. Kornbluth, and H. D. Janowitz. 1990. Treatment of ulcerative colitis with fish oil n-3-omega-fatty acid: an open trial. *J Clin Gastroenterol* 12:157.
18. Aslan, A., and G. Triadafilopoulos. 1992. Fish oil fatty acid supplementation in active ulcerative colitis: a double-blind, placebo-controlled, crossover study. *Am J Gastroenterol* 87:432.
19. Loeschke, K., B. Ueberschaer, A. Pietsch, E. Gruber, K. Ewe, B. Wiebecke, W. Heldwein, and R. Lorenz. 1996. n-3 fatty acids only delay early relapse of ulcerative colitis in remission. *Dig Dis Sci* 41:2087.
20. Belluzzi, A., C. Brignola, M. Campieri, A. Pera, S. Boschi, and M. Miglioli. 1996. Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. *N Engl J Med* 334:1557.
21. Shoda, R., K. Matsueda, S. Yamato, and N. Umeda. 1996. Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. *Am J Clin Nutr* 63:741.
22. Belluzzi, A. 2002. N-3 fatty acids for the treatment of inflammatory bowel diseases. *Proc Nutr Soc* 61:391.
23. Lewis, R. A., K. F. Austen, and R. J. Soberman. 1990. Leukotrienes and other products of the 5-lipoxygenase pathway. Biochemistry and relation to pathobiology in human diseases. *N Engl J Med* 323:645.

24. Tilley, S. L., T. M. Coffman, and B. H. Koller. 2001. Mixed messages: modulation of inflammation and immune responses by prostaglandins and thromboxanes. *J Clin Invest* 108:15.
25. Kinsella, J. E., B. Lokesh, S. Broughton, and J. Whelan. 1990. Dietary polyunsaturated fatty acids and eicosanoids: potential effects on the modulation of inflammatory and immune cells: an overview. *Nutrition* 6:24.
26. Gibney, M. J., and B. Hunter. 1993. The effects of short- and long-term supplementation with fish oil on the incorporation of n-3 polyunsaturated fatty acids into cells of the immune system in healthy volunteers. *Eur J Clin Nutr* 47:255.
27. Sperling, R. I., A. I. Benincaso, C. T. Knoell, J. K. Larkin, K. F. Austen, and D. R. Robinson. 1993. Dietary omega-3 polyunsaturated fatty acids inhibit phosphoinositide formation and chemotaxis in neutrophils. *J Clin Invest* 91:651.
28. Endres, S., R. Ghorbani, V. E. Kelley, K. Georgilis, G. Lonnemann, J. W. van der Meer, J. G. Cannon, T. S. Rogers, M. S. Klempner, P. C. Weber, and et al. 1989. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *N Engl J Med* 320:265.
29. Obata, T., T. Nagakura, T. Masaki, K. Maekawa, and K. Yamashita. 1999. Eicosapentaenoic acid inhibits prostaglandin D2 generation by inhibiting cyclooxygenase-2 in cultured human mast cells. *Clin Exp Allergy* 29:1129.
30. Lee, T. H., R. L. Hoover, J. D. Williams, R. I. Sperling, J. Ravalese, 3rd, B. W. Spur, D. R. Robinson, E. J. Corey, R. A. Lewis, and K. F. Austen. 1985. Effect of

- dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. *N Engl J Med* 312:1217.
31. Calder, P. C., S. J. Bevan, and E. A. Newsholme. 1992. The inhibition of T-lymphocyte proliferation by fatty acids is via an eicosanoid-independent mechanism. *Immunology* 75:108.
  32. Santoli, D., P. D. Phillips, T. L. Colt, and R. B. Zurier. 1990. Suppression of interleukin 2-dependent human T cell growth in vitro by prostaglandin E (PGE) and their precursor fatty acids. Evidence for a PGE-independent mechanism of inhibition by the fatty acids. *J Clin Invest* 85:424.
  33. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature* 387:569.
  34. Simons, K., and W. L. Vaz. 2004. Model systems, lipid rafts, and cell membranes. *Annu Rev Biophys Biomol Struct* 33:269.
  35. Norcross, M. A. 1984. A synaptic basis for T-lymphocyte activation. *Ann Immunol (Paris)* 135D:113.
  36. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature* 383:787.
  37. Kersh, G. J., E. N. Kersh, D. H. Fremont, and P. M. Allen. 1998. High- and low-potency ligands with similar affinities for the TCR: the importance of kinetics in TCR signaling. *Immunity* 9:817.
  38. Rothstein, D. M., and M. H. Sayegh. 2003. T-cell costimulatory pathways in allograft rejection and tolerance. *Immunol Rev* 196:85.



39. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu Rev Immunol* 14:233.
40. Vallejo, A. N., J. C. Brandes, C. M. Weyand, and J. J. Goronzy. 1999. Modulation of CD28 expression: distinct regulatory pathways during activation and replicative senescence. *J Immunol* 162:6572.
41. Vallejo, A. N., C. M. Weyand, and J. J. Goronzy. 2001. Functional disruption of the CD28 gene transcriptional initiator in senescent T cells. *J Biol Chem* 276:2565.
42. Chambers, C. A., and J. P. Allison. 1999. Costimulatory regulation of T cell function. *Curr Opin Cell Biol* 11:203.
43. Khoury, S. J., and M. H. Sayegh. 2004. The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. *Immunity* 20:529.
44. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1:405.
45. Linsley, P. S., J. Bradshaw, M. Urnes, L. Grosmaire, and J. A. Ledbetter. 1993. CD28 engagement by B7/BB-1 induces transient down-regulation of CD28 synthesis and prolonged unresponsiveness to CD28 signaling. *J Immunol* 150:3161.
46. Krummel, M. F., and J. P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 182:459.

47. Bromley, S. K., W. R. Burack, K. G. Johnson, K. Somersalo, T. N. Sims, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 2001. The immunological synapse. *Annu Rev Immunol* 19:375.
48. Fullekrug, J., and K. Simons. 2004. Lipid rafts and apical membrane traffic. *Ann NY Acad Sci* 1014:164.
49. Pizzo, P., and A. Viola. 2004. Lipid rafts in lymphocyte activation. *Microbes Infect* 6:686.
50. Monks, C. R., H. Kupfer, I. Tamir, A. Barlow, and A. Kupfer. 1997. Selective modulation of protein kinase C-theta during T-cell activation. *Nature* 385:83.
51. Monks, C. R., B. A. Freiberg, H. Kupfer, N. Sciaky, and A. Kupfer. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395:82.
52. Delon, J., K. Kaibuchi, and R. N. Germain. 2001. Exclusion of CD43 from the immunological synapse is mediated by phosphorylation-regulated relocation of the cytoskeletal adaptor moesin. *Immunity* 15:691.
53. Xavier, R., T. Brennan, Q. Li, C. McCormack, and B. Seed. 1998. Membrane compartmentation is required for efficient T cell activation. *Immunity* 8:723.
54. Zhang, W., R. P. Tribble, and L. E. Samelson. 1998. LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. *Immunity* 9:239.
55. Bi, K., Y. Tanaka, N. Coudronniere, K. Sugie, S. Hong, M. J. van Stipdonk, and A. Altman. 2001. Antigen-induced translocation of PKC-theta to membrane rafts is required for T cell activation. *Nat Immunol* 2:556.

56. Fan, Y. Y., D. N. McMurray, L. H. Ly, and R. S. Chapkin. 2003. Dietary (n-3) polyunsaturated fatty acids remodel mouse T-cell lipid rafts. *J Nutr* 133:1913.
57. Switzer, K. C., McMurray, D. N., and Chapkin, R.S. 2004. Effects of Dietary n-3 Polyunsaturated Fatty Acids on T-cell Membrane Composition and Function: A Unifying Hypothesis. *Lipids Submitted for publication*.
58. Switzer, K. C., Y. Y. Fan, N. Wang, D. N. McMurray, and R. S. Chapkin. 2004. Dietary n-3 polyunsaturated fatty acids promote activation-induced cell death in Th1-polarized murine CD4+ T cells. *J Lipid Res* 45:1482.
59. Webb, Y., L. Hermida-Matsumoto, and M. D. Resh. 2000. Inhibition of protein palmitoylation, raft localization, and T cell signaling by 2-bromopalmitate and polyunsaturated fatty acids. *J Biol Chem* 275:261.
60. Liang, X., A. Nazarian, H. Erdjument-Bromage, W. Bornmann, P. Tempst, and M. D. Resh. 2001. Heterogeneous fatty acylation of Src family kinases with polyunsaturated fatty acids regulates raft localization and signal transduction. *J Biol Chem* 276:30987.
61. Stulnig, T. M., J. Huber, N. Leitinger, E. M. Imre, P. Angelisova, P. Nowotny, and W. Waldhausl. 2001. Polyunsaturated eicosapentaenoic acid displaces proteins from membrane rafts by altering raft lipid composition. *J Biol Chem* 276:37335.
62. Fan, Y. Y., Ly, L. H., Barhoumi, R., McMurray, D. N., and Chapkin, R. S. 2004. Dietary docosahexaenoic acid suppresses T-cell protein kinase C-theta lipid raft recruitment and interleukin-2 production. *J Immunol Submitted for publication*.

63. Stulnig, T. M., M. Berger, T. Sigmund, D. Raederstorff, H. Stockinger, and W. Waldhausl. 1998. Polyunsaturated fatty acids inhibit T cell signal transduction by modification of detergent-insoluble membrane domains. *J Cell Biol* 143:637.
64. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683.
65. Mocellin, S., M. C. Panelli, E. Wang, D. Nagorsen, and F. M. Marincola. 2003. The dual role of IL-10. *Trends Immunol* 24:36.
66. Joss, A., M. Akdis, A. Faith, K. Blaser, and C. A. Akdis. 2000. IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. *Eur J Immunol* 30:1683.
67. Akdis, C. A., and K. Blaser. 2001. Mechanisms of interleukin-10-mediated immune suppression. *Immunology* 103:131.
68. Yaqoob, P., and P. Calder. 1995. Effects of dietary lipid manipulation upon inflammatory mediator production by murine macrophages. *Cell Immunol* 163:120.
69. Sanderson, P., and P. C. Calder. 1998. Dietary fish oil diminishes lymphocyte adhesion to macrophage and endothelial cell monolayers. *Immunology* 94:79.
70. Sanderson, P., and P. C. Calder. 1998. Dietary fish oil appears to prevent the activation of phospholipase C-gamma in lymphocytes. *Biochim Biophys Acta* 1392:300.
71. Xi, S., D. Cohen, and L. H. Chen. 1998. Effects of fish oil on cytokines and immune functions of mice with murine AIDS. *J Lipid Res* 39:1677.

72. Chen, F., V. Castranova, X. Shi, and L. M. Demers. 1999. New insights into the role of nuclear factor-kappaB, a ubiquitous transcription factor in the initiation of diseases. *Clin Chem* 45:7.
73. Wallace, F. A., E. A. Miles, and P. C. Calder. 2000. Activation state alters the effect of dietary fatty acids on pro-inflammatory mediator production by murine macrophages. *Cytokine* 12:1374.
74. Grimm, H., A. Tibell, B. Norrlind, C. Blecher, S. Wilker, and K. Schwemmler. 1994. Immunoregulation by parenteral lipids: impact of the n-3 to n-6 fatty acid ratio. *JPEN J Parenter Enteral Nutr* 18:417.
75. Huang, S. C., M. L. Misfeldt, and K. L. Fritsche. 1992. Dietary fat influences Ia antigen expression and immune cell populations in the murine peritoneum and spleen. *J Nutr* 122:1219.
76. Feng, C., D. H. Keisler, and K. L. Fritsche. 1999. Dietary omega-3 polyunsaturated fatty acids reduce IFN-gamma receptor expression in mice. *J Interferon Cytokine Res* 19:41.
77. Fujikawa, M., N. Yamashita, K. Yamazaki, E. Sugiyama, H. Suzuki, and T. Hamazaki. 1992. Eicosapentaenoic acid inhibits antigen-presenting cell function of murine splenocytes. *Immunology* 75:330.
78. Fritsche, K. L., and P. V. Johnston. 1990. Effect of dietary alpha-linolenic acid on growth, metastasis, fatty acid profile and prostaglandin production of two murine mammary adenocarcinomas. *J Nutr* 120:1601.
79. Puertollano, M. A., E. Puertollano, A. Ruiz-Bravo, M. Jimenez-Valera, M. A. De Pablo, and G. Alvarez De Cienfuegos. 2004. Changes in the immune functions

- and susceptibility to *Listeria monocytogenes* infection in mice fed dietary lipids. *Immunol Cell Biol* 82:370.
80. Peterson, L. D., F. Thies, P. Sanderson, E. A. Newsholme, and P. C. Calder. 1998. Low levels of eicosapentaenoic and docosahexaenoic acids mimic the effects of fish oil upon rat lymphocytes. *Life Sci* 62:2209.
81. Hughes, D. A., and A. C. Pinder. 1997. N-3 polyunsaturated fatty acids modulate the expression of functionally associated molecules on human monocytes and inhibit antigen-presentation in vitro. *Clin Exp Immunol* 110:516.
82. Baldie, G., D. Kaimakamis, and D. Rotondo. 1993. Fatty acid modulation of cytokine release from human monocytic cells. *Biochim Biophys Acta* 1179:125.
83. Endres, S., H. J. Fulle, B. Sinha, D. Stoll, C. A. Dinarello, R. Gerzer, and P. C. Weber. 1991. Cyclic nucleotides differentially regulate the synthesis of tumour necrosis factor-alpha and interleukin-1 beta by human mononuclear cells. *Immunology* 72:56.
84. Thies, F., G. Nebe-von-Caron, J. R. Powell, P. Yaqoob, E. A. Newsholme, and P. C. Calder. 2001. Dietary supplementation with eicosapentaenoic acid, but not with other long-chain n-3 or n-6 polyunsaturated fatty acids, decreases natural killer cell activity in healthy subjects aged >55 y. *Am J Clin Nutr* 73:539.
85. Spika, S., I. Dey, C. Buda, J. Csongor, G. Szegedi, and T. Farkas. 1996. The mechanism of inhibitory effect of eicosapentaenoic acid on phagocytic activity and chemotaxis of human neutrophil granulocytes. *Clin Immunol Immunopathol* 79:224.

86. Khair-el-Din, T. A., S. C. Sicher, M. A. Vazquez, W. J. Wright, and C. Y. Lu. 1995. Docosahexaenoic acid, a major constituent of fetal serum and fish oil diets, inhibits IFN gamma-induced Ia-expression by murine macrophages in vitro. *J Immunol* 154:1296.
87. Haley, P. J. 2003. Species differences in the structure and function of the immune system. *Toxicology* 188:49.
88. Waterston, R. H., K. Lindblad-Toh, E. Birney, J. Rogers, J. F. Abril, P. Agarwal, R. Agarwala, R. Ainscough, M. Alexandersson, P. An, S. E. Antonarakis, J. Attwood, R. Baertsch, J. Bailey, K. Barlow, S. Beck, E. Berry, B. Birren, T. Bloom, P. Bork, M. Botcherby, N. Bray, M. R. Brent, D. G. Brown, S. D. Brown, C. Bult, J. Burton, J. Butler, R. D. Campbell, P. Carninci, S. Cawley, F. Chiaromonte, A. T. Chinwalla, D. M. Church, M. Clamp, C. Clee, F. S. Collins, L. L. Cook, R. R. Copley, A. Coulson, O. Couronne, J. Cuff, V. Curwen, T. Cutts, M. Daly, R. David, J. Davies, K. D. Delehaunty, J. Deri, E. T. Dermitzakis, C. Dewey, N. J. Dickens, M. Diekhans, S. Dodge, I. Dubchak, D. M. Dunn, S. R. Eddy, L. Elnitski, R. D. Emes, P. Eswara, E. Eyraas, A. Felsenfeld, G. A. Fewell, P. Flicek, K. Foley, W. N. Frankel, L. A. Fulton, R. S. Fulton, T. S. Furey, D. Gage, R. A. Gibbs, G. Glusman, S. Gnerre, N. Goldman, L. Goodstadt, D. Grafham, T. A. Graves, E. D. Green, S. Gregory, R. Guigo, M. Guyer, R. C. Hardison, D. Haussler, Y. Hayashizaki, L. W. Hillier, A. Hinrichs, W. Hlavina, T. Holzner, F. Hsu, A. Hua, T. Hubbard, A. Hunt, I. Jackson, D. B. Jaffe, L. S. Johnson, M. Jones, T. A. Jones, A. Joy, M. Kamal, E. K. Karlsson, et al. 2002.

- Initial sequencing and comparative analysis of the mouse genome. *Nature* 420:520.
89. Alegre, M. L., K. A. Frauwirth, and C. B. Thompson. 2001. T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* 1:220.
  90. Call, M. E., and K. W. Wucherpfennig. 2004. Molecular mechanisms for the assembly of the T cell receptor-CD3 complex. *Mol Immunol* 40:1295.
  91. Thomas, S., A. Preda-Pais, S. Casares, and T. D. Brumeanu. 2004. Analysis of lipid rafts in T cells. *Mol Immunol* 41:399.
  92. Krogsaard, M., J. B. Huppa, M. A. Purbhoo, and M. M. Davis. 2003. Linking molecular and cellular events in T-cell activation and synapse formation. *Semin Immunol* 15:307.
  93. Sommers, C. L., L. E. Samelson, and P. E. Love. 2004. LAT: a T lymphocyte adapter protein that couples the antigen receptor to downstream signaling pathways. *Bioessays* 26:61.
  94. Schmitz, M. L., S. Bacher, and O. Dienz. 2003. NF-kappaB activation pathways induced by T cell costimulation. *Faseb J* 17:2187.
  95. Stetson, D. B., D. Voehringer, J. L. Grogan, M. Xu, R. L. Reinhardt, S. Scheu, B. L. Kelly, and R. M. Locksley. 2004. Th2 cells: orchestrating barrier immunity. *Adv Immunol* 83:163.
  96. O'Garra, A., and D. Robinson. 2004. Development and function of T helper 1 cells. *Adv Immunol* 83:133.
  97. Mestas, J., and C. C. Hughes. 2004. Of mice and not men: differences between mouse and human immunology. *J Immunol* 172:2731.



98. Foulds, K. E., L. A. Zenewicz, D. J. Shedlock, J. Jiang, A. E. Troy, and H. Shen. 2002. Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. *J Immunol* 168:1528.
99. Gett, A. V., F. Sallusto, A. Lanzavecchia, and J. Geginat. 2003. T cell fitness determined by signal strength. *Nat Immunol* 4:355.
100. Switzer, K. C., D. N. McMurray, J. S. Morris, and R. S. Chapkin. 2003. (n-3) Polyunsaturated fatty acids promote activation-induced cell death in murine T lymphocytes. *J Nutr* 133:496.
101. Thompson, C. B., T. Lindsten, J. A. Ledbetter, S. L. Kunkel, H. A. Young, S. G. Emerson, J. M. Leiden, and C. H. June. 1989. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc Natl Acad Sci U S A* 86:1333.
102. Linsley, P. S., W. Brady, L. Grosmaire, A. Aruffo, N. K. Damle, and J. A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med* 173:721.
103. Lindstein, T., C. H. June, J. A. Ledbetter, G. Stella, and C. B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science* 244:339.
104. Alegre, M. L., P. J. Noel, B. J. Eisfelder, E. Chuang, M. R. Clark, S. L. Reiner, and C. B. Thompson. 1996. Regulation of surface and intracellular expression of CTLA4 on mouse T cells. *J Immunol* 157:4762.

105. Carreno, B. M., and M. Collins. 2002. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu Rev Immunol* 20:29.
106. Brunner, M. C., C. A. Chambers, F. K. Chan, J. Hanke, A. Winoto, and J. P. Allison. 1999. CTLA-4-Mediated inhibition of early events of T cell proliferation. *J Immunol* 162:5813.
107. Chambers, C. A., and J. P. Allison. 1999. CTLA-4--the costimulatory molecule that doesn't: regulation of T-cell responses by inhibition. *Cold Spring Harb Symp Quant Biol* 64:303.
108. June, C. H., J. A. Bluestone, L. M. Nadler, and C. B. Thompson. 1994. The B7 and CD28 receptor families. *Immunol Today* 15:321.
109. Harper, K., C. Balzano, E. Rouvier, M. G. Mattei, M. F. Luciani, and P. Golstein. 1991. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. *J Immunol* 147:1037.
110. Freeman, G. J., F. Borriello, R. J. Hodes, H. Reiser, K. S. Hathcock, G. Laszlo, A. J. McKnight, J. Kim, L. Du, D. B. Lombard, and et al. 1993. Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. *Science* 262:907.
111. Kearney, E. R., T. L. Walunas, R. W. Karr, P. A. Morton, D. Y. Loh, J. A. Bluestone, and M. K. Jenkins. 1995. Antigen-dependent clonal expansion of a trace population of antigen-specific CD4<sup>+</sup> T cells in vivo is dependent on CD28 costimulation and inhibited by CTLA-4. *J Immunol* 155:1032.

112. Krummel, M. F., T. J. Sullivan, and J. P. Allison. 1996. Superantigen responses and co-stimulation: CD28 and CTLA-4 have opposing effects on T cell expansion in vitro and in vivo. *Int Immunol* 8:519.
113. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3:541.
114. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctl4-4. *Science* 270:985.
115. Karandikar, N. J., C. L. Vanderlugt, T. L. Walunas, S. D. Miller, and J. A. Bluestone. 1996. CTLA-4: a negative regulator of autoimmune disease. *J Exp Med* 184:783.
116. Burack, W. R., K. H. Lee, A. D. Holdorf, M. L. Dustin, and A. S. Shaw. 2002. Cutting edge: quantitative imaging of raft accumulation in the immunological synapse. *J Immunol* 169:2837.
117. Darlington, P. J., M. L. Baroja, T. A. Chau, E. Siu, V. Ling, B. M. Carreno, and J. Madrenas. 2002. Surface cytotoxic T lymphocyte-associated antigen 4 partitions within lipid rafts and relocates to the immunological synapse under conditions of inhibition of T cell activation. *J Exp Med* 195:1337.
118. Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 283:680.

119. Salazar-Fontana, L. I., E. Sanz, I. Merida, A. Zea, A. Sanchez-Atrio, L. Villa, A. C. Martinez, A. de la Hera, and M. Alvarez-Mon. 2001. Cell surface CD28 levels define four CD4<sup>+</sup> T cell subsets: abnormal expression in rheumatoid arthritis. *Clin Immunol* 99:253.
120. Davidson, L. A., J. R. Lupton, E. Miskovsky, A. P. Fields, and R. S. Chapkin. 2003. Quantification of human intestinal gene expression profiles using exfoliated colonocytes: a pilot study. *Biomarkers* 8:51.
121. Yaqoob, P., E. A. Newsholme, and P. C. Calder. 1995. Influence of cell culture conditions on diet-induced changes in lymphocyte fatty acid composition. *Biochim Biophys Acta* 1255:333.
122. Yaqoob, P., E. J. Sherrington, N. M. Jeffery, P. Sanderson, D. J. Harvey, E. A. Newsholme, and P. C. Calder. 1995. Comparison of the effects of a range of dietary lipids upon serum and tissue lipid composition in the rat. *Int J Biochem Cell Biol* 27:297.
123. Damle, N. K., K. Klussman, G. Leytze, S. Myrdal, A. Aruffo, J. A. Ledbetter, and P. S. Linsley. 1994. Costimulation of T lymphocytes with integrin ligands intercellular adhesion molecule-1 or vascular cell adhesion molecule-1 induces functional expression of CTLA-4, a second receptor for B7. *J Immunol* 152:2686.
124. Lindsten, T., K. P. Lee, E. S. Harris, B. Petryniak, N. Craighead, P. J. Reynolds, D. B. Lombard, G. J. Freeman, L. M. Nadler, G. S. Gray, and et al. 1993. Characterization of CTLA-4 structure and expression on human T cells. *J Immunol* 151:3489.

125. Oosterwegel, M. A., R. J. Greenwald, D. A. Mandelbrot, R. B. Lorsbach, and A. H. Sharpe. 1999. CTLA-4 and T cell activation. *Curr Opin Immunol* 11:294.
126. Greenwald, R. J., M. A. Oosterwegel, D. van der Woude, A. Kubal, D. A. Mandelbrot, V. A. Boussiotis, and A. H. Sharpe. 2002. CTLA-4 regulates cell cycle progression during a primary immune response. *Eur J Immunol* 32:366.
127. Dustin, M. L., and A. S. Shaw. 1999. Costimulation: building an immunological synapse. *Science* 283:649.
128. Bluestone, J. A. 1995. New perspectives of CD28-B7-mediated T cell costimulation. *Immunity* 2:555.
129. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261:609.
130. Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat Rev Mol Cell Biol* 1:31.
131. Magee, T., N. Pirinen, J. Adler, S. N. Pagakis, and I. Parmryd. 2002. Lipid rafts: cell surface platforms for T cell signaling. *Biol Res* 35:127.
132. Dykstra, M., A. Cherukuri, H. W. Sohn, S. J. Tzeng, and S. K. Pierce. 2003. Location is everything: lipid rafts and immune cell signaling. *Annu Rev Immunol* 21:457.
133. Martin, M., H. Schneider, A. Azouz, and C. E. Rudd. 2001. Cytotoxic T lymphocyte antigen 4 and CD28 modulate cell surface raft expression in their regulation of T cell function. *J Exp Med* 194:1675.

134. Rudd, C. E., and H. Schneider. 2003. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat Rev Immunol* 3:544.
135. Kew, S., M. D. Mesa, S. Tricon, R. Buckley, A. M. Minihane, and P. Yaqoob. 2004. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on immune cell composition and function in healthy humans. *Am J Clin Nutr* 79:674.
136. Diaz, O., A. Berquand, M. Dubois, S. Di Agostino, C. Sette, S. Bourgoin, M. Lagarde, G. Nemoz, and A. F. Prigent. 2002. The mechanism of docosahexaenoic acid-induced phospholipase D activation in human lymphocytes involves exclusion of the enzyme from lipid rafts. *J Biol Chem* 277:39368.
137. Chikuma, S., and J. A. Bluestone. 2002. CTLA-4: Acting at the synapse. *Mol Intervent* 2:205.
138. Walunas, T. L., and J. A. Bluestone. 1998. CTLA-4 regulates tolerance induction and T cell differentiation in vivo. *J Immunol* 160:3855.
139. Leach, D. R., M. F. Krummel, and J. P. Allison. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271:1734.
140. Ratts, R. B., L. R. Arredondo, P. Bittner, P. J. Perrin, A. E. Lovett-Racke, and M. K. Racke. 1999. The role of CTLA-4 in tolerance induction and T cell differentiation in experimental autoimmune encephalomyelitis: i.p. antigen administration. *Int Immunol* 11:1881.
141. Alegre, M. L., H. Shiels, C. B. Thompson, and T. F. Gajewski. 1998. Expression and function of CTLA-4 in Th1 and Th2 cells. *J Immunol* 161:3347.

142. Chapkin, R. S., C. C. Akoh, and C. C. Miller. 1991. Influence of dietary n-3 fatty acids on macrophage glycerophospholipid molecular species and peptidoleukotriene synthesis. *J Lipid Res* 32:1205.
143. Chapkin, R. S., and S. L. Carmichael. 1990. Effects of dietary n-3 and n-6 polyunsaturated fatty acids on macrophage phospholipid classes and subclasses. *Lipids* 25:827.
144. Lokesh, B. R., H. L. Hsieh, and J. E. Kinsella. 1986. Peritoneal macrophages from mice fed dietary (n-3) polyunsaturated fatty acids secrete low levels of prostaglandins. *J Nutr* 116:2547.
145. Somers, S. D., R. S. Chapkin, and K. L. Erickson. 1989. Alteration of in vitro murine peritoneal macrophage function by dietary enrichment with eicosapentaenoic and docosahexaenoic acids in menhaden fish oil. *Cell Immunol* 123:201.
146. Calder, P. C. 1997. n-3 polyunsaturated fatty acids and cytokine production in health and disease. *Ann Nutr Metab* 41:203.
147. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 182:18.
148. Shevach, E. M. 2002. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol* 2:389.

149. Chatenoud, L., B. Salomon, and J. A. Bluestone. 2001. Suppressor T cells--they're back and critical for regulation of autoimmunity! *Immunol Rev* 182:149.
150. Tang, Q., K. J. Henriksen, E. K. Boden, A. J. Tooley, J. Ye, S. K. Subudhi, X. X. Zheng, T. B. Strom, and J. A. Bluestone. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4+CD25+ regulatory T cells. *J Immunol* 171:3348.
151. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 170:2081.
152. Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 147:3815.
153. de Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. 1991. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 174:1209.
154. Del Prete, G., M. De Carli, F. Almerigogna, M. G. Giudizi, R. Biagiotti, and S. Romagnani. 1993. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* 150:353.
155. Pajkrt, D., L. Camoglio, M. C. Tiel-van Buul, K. de Bruin, D. L. Cutler, M. B. Affrime, G. Rikken, T. van der Poll, J. W. ten Cate, and S. J. van Deventer. 1997. Attenuation of proinflammatory response by recombinant human IL-10 in human endotoxemia: effect of timing of recombinant human IL-10 administration. *J Immunol* 158:3971.



156. Avdiushko, R., D. Hongo, H. Lake-Bullock, A. Kaplan, and D. Cohen. 2001. IL-10 receptor dysfunction in macrophages during chronic inflammation. *J Leukoc Biol* 70:624.
157. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* 356:607.
158. Muhl, H., and J. Pfeilschifter. 2003. Anti-inflammatory properties of pro-inflammatory interferon-gamma. *Int Immunopharmacol* 3:1247.
159. Herrero, C., X. Hu, W. P. Li, S. Samuels, M. N. Sharif, S. Kotenko, and L. B. Ivashkiv. 2003. Reprogramming of IL-10 activity and signaling by IFN-gamma. *J Immunol* 171:5034.
160. Go, N. F., B. E. Castle, R. Barrett, R. Kastelein, W. Dang, T. R. Mosmann, K. W. Moore, and M. Howard. 1990. Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. *J Exp Med* 172:1625.
161. MacNeil, I. A., T. Suda, K. W. Moore, T. R. Mosmann, and A. Zlotnik. 1990. IL-10, a novel growth cofactor for mature and immature T cells. *J Immunol* 145:4167.
162. Rousset, F., E. Garcia, T. Defrance, C. Peronne, N. Vezzio, D. H. Hsu, R. Kastelein, K. W. Moore, and J. Banchereau. 1992. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. *Proc Natl Acad Sci U S A* 89:1890.

163. Chen, W. F., and A. Zlotnik. 1991. IL-10: a novel cytotoxic T cell differentiation factor. *J Immunol* 147:528.
164. Kuhn, R., J. Lohler, D. Rennick, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75:263.
165. Berg, D. J., R. Kuhn, K. Rajewsky, W. Muller, S. Menon, N. Davidson, G. Grunig, and D. Rennick. 1995. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. *J Clin Invest* 96:2339.
166. Berg, D. J., M. W. Leach, R. Kuhn, K. Rajewsky, W. Muller, N. J. Davidson, and D. Rennick. 1995. Interleukin 10 but not interleukin 4 is a natural suppressant of cutaneous inflammatory responses. *J Exp Med* 182:99.
167. Bacchetta, R., M. Bigler, J. L. Touraine, R. Parkman, P. A. Tovo, J. Abrams, R. de Waal Malefyt, J. E. de Vries, and M. G. Roncarolo. 1994. High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med* 179:493.
168. Ho, A. S., Y. Liu, T. A. Khan, D. H. Hsu, J. F. Bazan, and K. W. Moore. 1993. A receptor for interleukin 10 is related to interferon receptors. *Proc Natl Acad Sci U S A* 90:11267.
169. Liu, Y., S. H. Wei, A. S. Ho, R. de Waal Malefyt, and K. W. Moore. 1994. Expression cloning and characterization of a human IL-10 receptor. *J Immunol* 152:1821.

170. Kotenko, S. V., C. D. Krause, L. S. Izotova, B. P. Pollack, W. Wu, and S. Pestka. 1997. Identification and functional characterization of a second chain of the interleukin-10 receptor complex. *Embo J* 16:5894.
171. Spencer, S. D., F. Di Marco, J. Hooley, S. Pitts-Meek, M. Bauer, A. M. Ryan, B. Sordat, V. C. Gibbs, and M. Aguet. 1998. The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. *J Exp Med* 187:571.
172. Ding, Y., L. Qin, D. Zamarin, S. V. Kotenko, S. Pestka, K. W. Moore, and J. S. Bromberg. 2001. Differential IL-10R1 expression plays a critical role in IL-10-mediated immune regulation. *J Immunol* 167:6884.
173. Lenschow, D. J., Y. Zeng, J. R. Thistlethwaite, A. Montag, W. Brady, M. G. Gibson, P. S. Linsley, and J. A. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* 257:789.
174. Lee, K. M., E. Chuang, M. Griffin, R. Khattri, D. K. Hong, W. Zhang, D. Straus, L. E. Samelson, C. B. Thompson, and J. A. Bluestone. 1998. Molecular basis of T cell inactivation by CTLA-4. *Science* 282:2263.
175. Billiau, A. 1996. Interferon-gamma: biology and role in pathogenesis. *Adv Immunol* 62:61.
176. Yoshida, A., Y. Koide, M. Uchijima, and T. O. Yoshida. 1994. IFN-gamma induces IL-12 mRNA expression by a murine macrophage cell line, J774. *Biochem Biophys Res Commun* 198:857.
177. Hayes, M. P., S. L. Freeman, and R. P. Donnelly. 1995. IFN-gamma priming of monocytes enhances LPS-induced TNF production by augmenting both transcription and mRNA stability. *Cytokine* 7:427.

178. Xie, Q. W., and C. Nathan. 1993. Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon-gamma and bacterial lipopolysaccharide. *Trans Assoc Am Physicians* 106:1.
179. Tamura, T., S. Ueda, M. Yoshida, M. Matsuzaki, H. Mohri, and T. Okubo. 1996. Interferon-gamma induces Ice gene expression and enhances cellular susceptibility to apoptosis in the U937 leukemia cell line. *Biochem Biophys Res Commun* 229:21.
180. Hart, P. H., M. J. Ahern, M. D. Smith, and J. J. Finlay-Jones. 1995. Comparison of the suppressive effects of interleukin-10 and interleukin-4 on synovial fluid macrophages and blood monocytes from patients with inflammatory arthritis. *Immunology* 84:536.
181. Wahl, S. M., J. B. Allen, K. Ohura, D. E. Chenoweth, and A. R. Hand. 1991. IFN-gamma inhibits inflammatory cell recruitment and the evolution of bacterial cell wall-induced arthritis. *J Immunol* 146:95.
182. Rockey, D. C., J. J. Maher, W. R. Jarnagin, G. Gabbiani, and S. L. Friedman. 1992. Inhibition of rat hepatic lipocyte activation in culture by interferon-gamma. *Hepatology* 16:776.
183. Nicoletti, F., P. Zaccone, R. Di Marco, G. Magro, S. Grasso, F. Stivala, G. Calori, L. Mughini, P. L. Meroni, and G. Garotta. 1998. Paradoxical antidiabetogenic effect of gamma-interferon in DP-BB rats. *Diabetes* 47:32.
184. Link, H. 1998. The cytokine storm in multiple sclerosis. *Mult Scler* 4:12.
185. Rohrer, J. W., and J. H. Coggin, Jr. 1995. CD8 T cell clones inhibit antitumor T cell function by secreting IL-10. *J Immunol* 155:5719.

186. Cavaillon, J. M. 2001. Pro- versus anti-inflammatory cytokines: myth or reality. *Cell Mol Biol (Noisy-le-grand)* 47:695.
187. Llorente, L., Y. Richaud-Patin, C. Garcia-Padilla, E. Claret, J. Jakez-Ocampo, M. H. Cardiel, J. Alcocer-Varela, L. Grangeot-Keros, D. Alarcon-Segovia, J. Wijdenes, P. Galanaud, and D. Emilie. 2000. Clinical and biologic effects of anti-interleukin-10 monoclonal antibody administration in systemic lupus erythematosus. *Arthritis Rheum* 43:1790.
188. Asadullah, K., W. Sterry, K. Stephanek, D. Jasulaitis, M. Leupold, H. Audring, H. D. Volk, and W. D. Docke. 1998. IL-10 is a key cytokine in psoriasis. Proof of principle by IL-10 therapy: a new therapeutic approach. *J Clin Invest* 101:783.
189. Schreiber, S., R. N. Fedorak, O. H. Nielsen, G. Wild, C. N. Williams, S. Nikolaus, M. Jacyna, B. A. Lashner, A. Gangl, P. Rutgeerts, K. Isaacs, S. J. van Deventer, J. C. Koningsberger, M. Cohard, A. LeBeaut, and S. B. Hanauer. 2000. Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. *Gastroenterology* 119:1461.
190. Lauw, F. N., D. Pajkrt, C. E. Hack, M. Kurimoto, S. J. van Deventer, and T. van der Poll. 2000. Proinflammatory effects of IL-10 during human endotoxemia. *J Immunol* 165:2783.
191. Olszyna, D. P., D. Pajkrt, F. N. Lauw, S. J. van Deventer, and T. van Der Poll. 2000. Interleukin 10 inhibits the release of CC chemokines during human endotoxemia. *J Infect Dis* 181:613.
192. Chernoff, A. E., E. V. Granowitz, L. Shapiro, E. Vannier, G. Lonnemann, J. B. Angel, J. S. Kennedy, A. R. Rabson, S. M. Wolff, and C. A. Dinarello. 1995. A

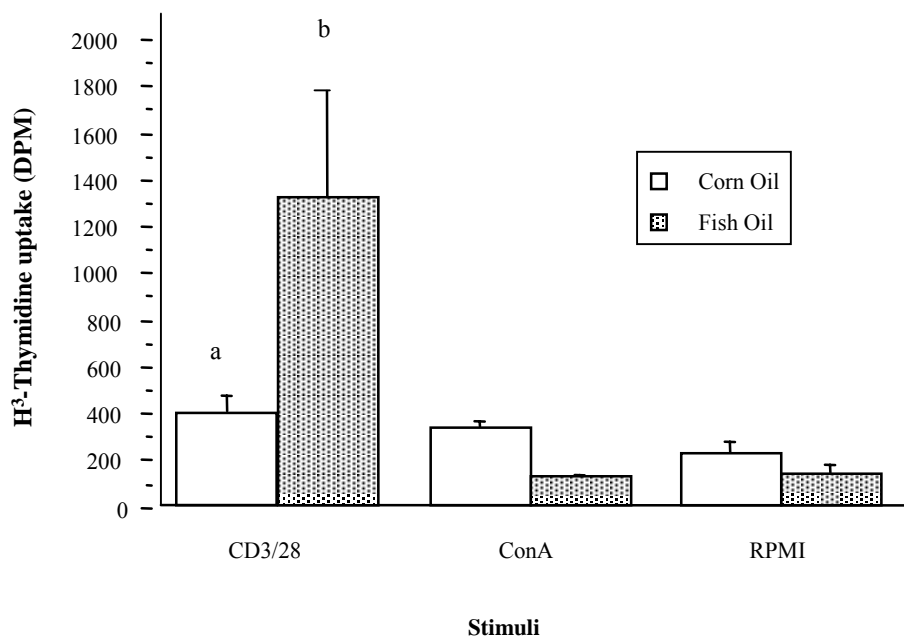
- randomized, controlled trial of IL-10 in humans. Inhibition of inflammatory cytokine production and immune responses. *J Immunol* 154:5492.
193. Harris, W. S. 1997. n-3 fatty acids and serum lipoproteins: human studies. *Am J Clin Nutr* 65:1645S.
194. Rambjor, G. S., A. I. Walen, S. L. Windsor, and W. S. Harris. 1996. Eicosapentaenoic acid is primarily responsible for hypotriglyceridemic effect of fish oil in humans. *Lipids* 31 Suppl:S45.
195. Harris, W. S., B. E. Hustvedt, E. Hagen, M. H. Green, G. Lu, and C. A. Drevon. 1997. N-3 fatty acids and chylomicron metabolism in the rat. *J Lipid Res* 38:503.
196. Mori, T. A., V. Burke, I. B. Puddey, G. F. Watts, D. N. O'Neal, J. D. Best, and L. J. Beilin. 2000. Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. *Am J Clin Nutr* 71:1085.
197. Nordoy, A. 1999. Dietary fatty acids and coronary heart disease. *Lipids* 34 Suppl:S19.
198. Sellmayer, A., N. Hrboticky, and P. C. Weber. 1999. Lipids in vascular function. *Lipids* 34 Suppl:S13.
199. Leaf, A. 2001. Diet and sudden cardiac death. *J Nutr Health Aging* 5:173.
200. Storlien, L. H., A. D. Kriketos, G. D. Calvert, L. A. Baur, and A. B. Jenkins. 1997. Fatty acids, triglycerides and syndromes of insulin resistance. *Prostaglandins Leukot Essent Fatty Acids* 57:379.

201. Storlien, L. H., A. J. Hulbert, and P. L. Else. 1998. Polyunsaturated fatty acids, membrane function and metabolic diseases such as diabetes and obesity. *Curr Opin Clin Nutr Metab Care* 1:559.
202. Salem, N., Jr., B. Litman, H. Y. Kim, and K. Gawrisch. 2001. Mechanisms of action of docosahexaenoic acid in the nervous system. *Lipids* 36:945.
203. Jump, D. B., and S. D. Clarke. 1999. Regulation of gene expression by dietary fat. *Annu Rev Nutr* 19:63.
204. Duplus, E., M. Glorian, and C. Forest. 2000. Fatty acid regulation of gene transcription. *J Biol Chem* 275:30749.
205. James, M. J., and L. G. Cleland. 1997. Dietary n-3 fatty acids and therapy for rheumatoid arthritis. *Semin Arthritis Rheum* 27:85.
206. Leslie, C. A., W. A. Gonnerman, M. D. Ullman, K. C. Hayes, C. Franzblau, and E. S. Cathcart. 1985. Dietary fish oil modulates macrophage fatty acids and decreases arthritis susceptibility in mice. *J Exp Med* 162:1336.
207. Linos, A., E. Kaklamanis, A. Kontomerkos, Y. Koumantaki, S. Gazi, G. Vaiopoulos, G. C. Tsokos, and P. Kaklamanis. 1991. The effect of olive oil and fish consumption on rheumatoid arthritis--a case control study. *Scand J Rheumatol* 20:419.
208. Watson, J., M. L. Byars, P. McGill, and A. W. Kelman. 1993. Cytokine and prostaglandin production by monocytes of volunteers and rheumatoid arthritis patients treated with dietary supplements of blackcurrant seed oil. *Br J Rheumatol* 32:1055.

209. Calder, P. C. 2001. omega 3 polyunsaturated fatty acids, inflammation and immunity. *World Rev Nutr Diet* 88:109.
210. Seder, R. A., R. N. Germain, P. S. Linsley, and W. E. Paul. 1994. CD28-mediated costimulation of interleukin 2 (IL-2) production plays a critical role in T cell priming for IL-4 and interferon gamma production. *J Exp Med* 179:299.
211. King, C. L., R. J. Stupi, N. Craighead, C. H. June, and G. Thyphronitis. 1995. CD28 activation promotes Th2 subset differentiation by human CD4+ cells. *Eur J Immunol* 25:587.
212. Zhang, X., T. Brunner, L. Carter, R. W. Dutton, P. Rogers, L. Bradley, T. Sato, J. C. Reed, D. Green, and S. L. Swain. 1997. Unequal death in T helper cell (Th)1 and Th2 effectors: Th1, but not Th2, effectors undergo rapid Fas/FasL-mediated apoptosis. *J Exp Med* 185:1837.
213. Varadhachary, A. S., S. N. Perdow, C. Hu, M. Ramanarayanan, and P. Salgame. 1997. Differential ability of T cell subsets to undergo activation-induced cell death. *Proc Natl Acad Sci U S A* 94:5778.
214. Yasukawa, H., A. Sasaki, and A. Yoshimura. 2000. Negative regulation of cytokine signaling pathways. *Annu Rev Immunol* 18:143.
215. Ding, Y., D. Chen, A. Tarcsafalvi, R. Su, L. Qin, and J. S. Bromberg. 2003. Suppressor of cytokine signaling 1 inhibits IL-10-mediated immune responses. *J Immunol* 170:1383.



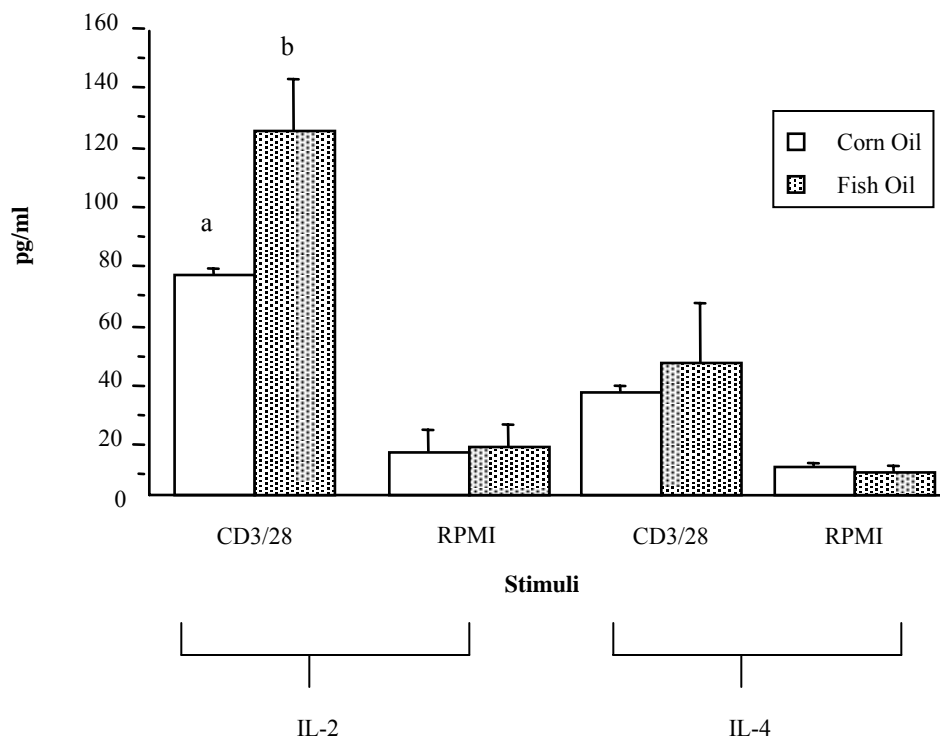
**APPENDIX A****OTHER DATA**



**FIGURE A-1.** Dietary fish oil enhances proliferation of whole Peyer's Patches in C57BL/6 mice.

Objective: To determine the effect of 4% dietary fish oil (FO) + 1% corn oil (CO) vs. 5% CO on the proliferation of whole Peyer's Patches (PP) in C57BL/6 mice.

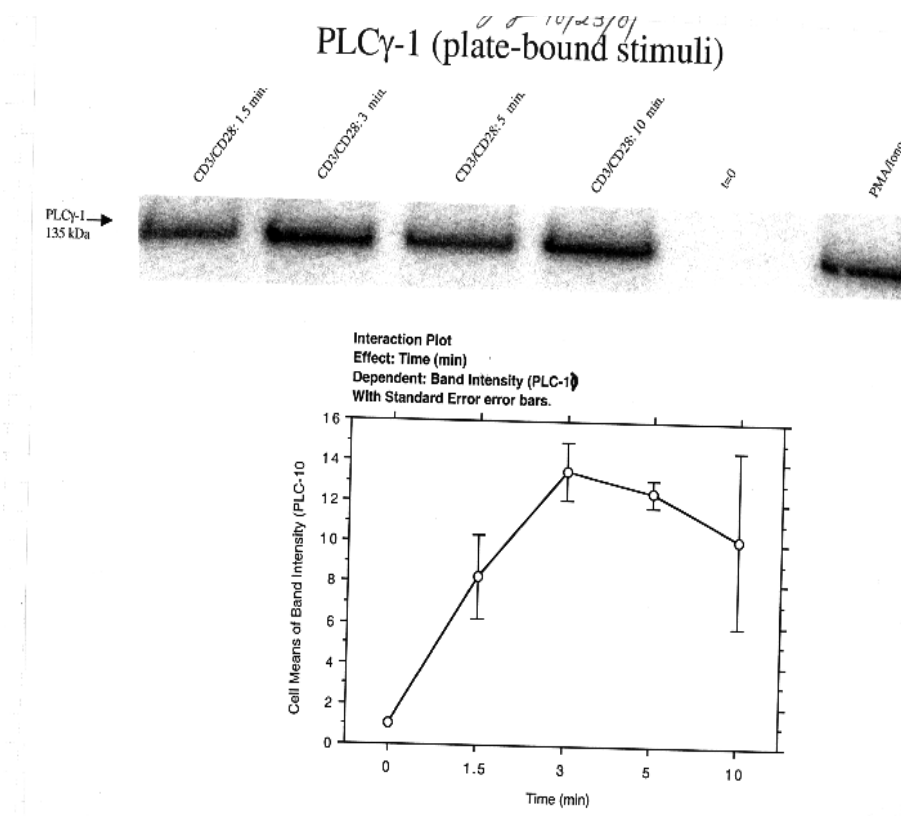
Methodology: Peyer's patches were excised from the small intestines and placed in a petri dish containing complete tissue culture (TC) medium (10% heat-inactivated FBS (Irvine Scientific), 1% L-glutamine (Gibco), 1% Penicillin-Streptomycin (Gibco), 10  $\mu$ M  $\beta$ -mercaptoethanol (Sigma), and 50  $\mu$ g/ml gentamicin sulfate (Gibco)). The PP were then teased apart using 2 curved end forceps over a wire filter (Fisher) and transferred to fresh complete TC medium with gentamicin. A syringe barrel was used to push remainder of matter through the mesh. Using a pasteur pipet, the suspension was passed over the wire filter once again before washing once by centrifugation at 200 x g for 5 minutes at RT. Cells were resuspended in 1 ml complete TC medium containing gentamicin and were passed through a sterile Nylon wool fiber (scrubbed and combed from Polysciences) column (~1 inch of cotton wool was packed loosely into the end of a pasteur pipet) to remove adherent cells. The column was then rinsed with 5 ml of the complete TC medium containing gentamicin. Viable cell numbers were determined by trypan blue exclusion and counting on a hemocytometer. Cells were cultured as described on page 19. Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=2-3. Different letters denote highly significant differences found between diet groups (p<0.01). All cells were cultured in the presence of 10% FBS.



**FIGURE A-2.** Dietary fish oil enhances interleukin-2 (IL-2) production of CD3<sup>+</sup> T-cells from C57BL/6 mice.

**Objective:** To determine the effect of 4% dietary fish oil (FO) + 1% corn oil (CO) vs. 5% CO on the IL-2 production of CD3<sup>+</sup> T-cells from C57BL/6 mice.

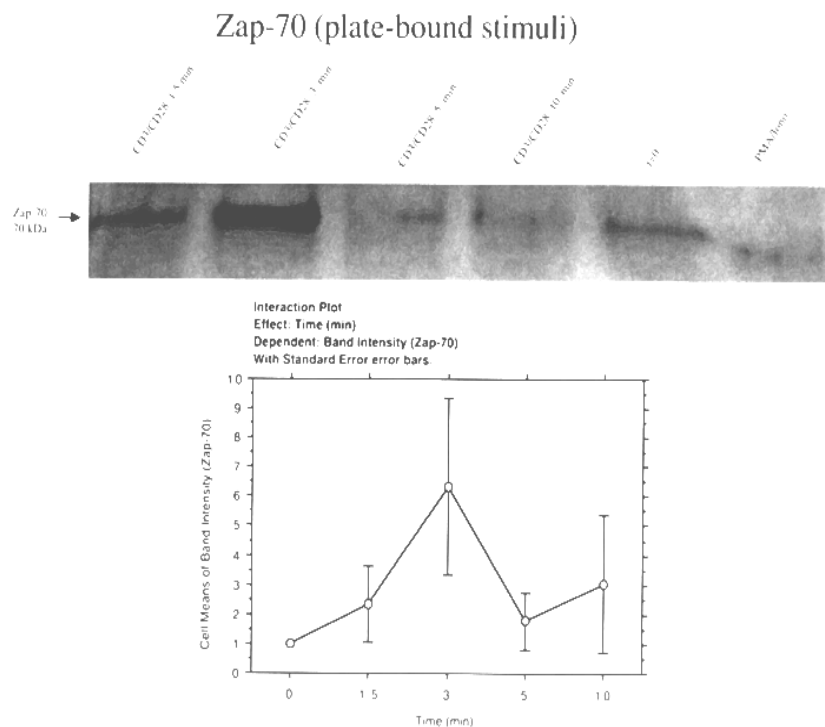
**Methodology:** Mouse CD3<sup>+</sup> T-cells were purified, cultured, and analyzed by ELISA for IL-2 production as described previously (4). Values from n=5 mice represent the mean  $\pm$  SEM in pg/200,000 cells. Different letters denote highly significant differences found between diet groups ( $p < 0.01$ ). All cells were cultured in the presence of 10% FBS.



**FIGURE A-3.** Analysis of the protein expression of PLC $\gamma$ -1 in activated CD3<sup>+</sup> T-cells from chow-fed mice.

**Objective:** To validate the assay procedures to be used to determine the influence of dietary n-3 PUFA on the protein expression of PLC $\gamma$ -1.

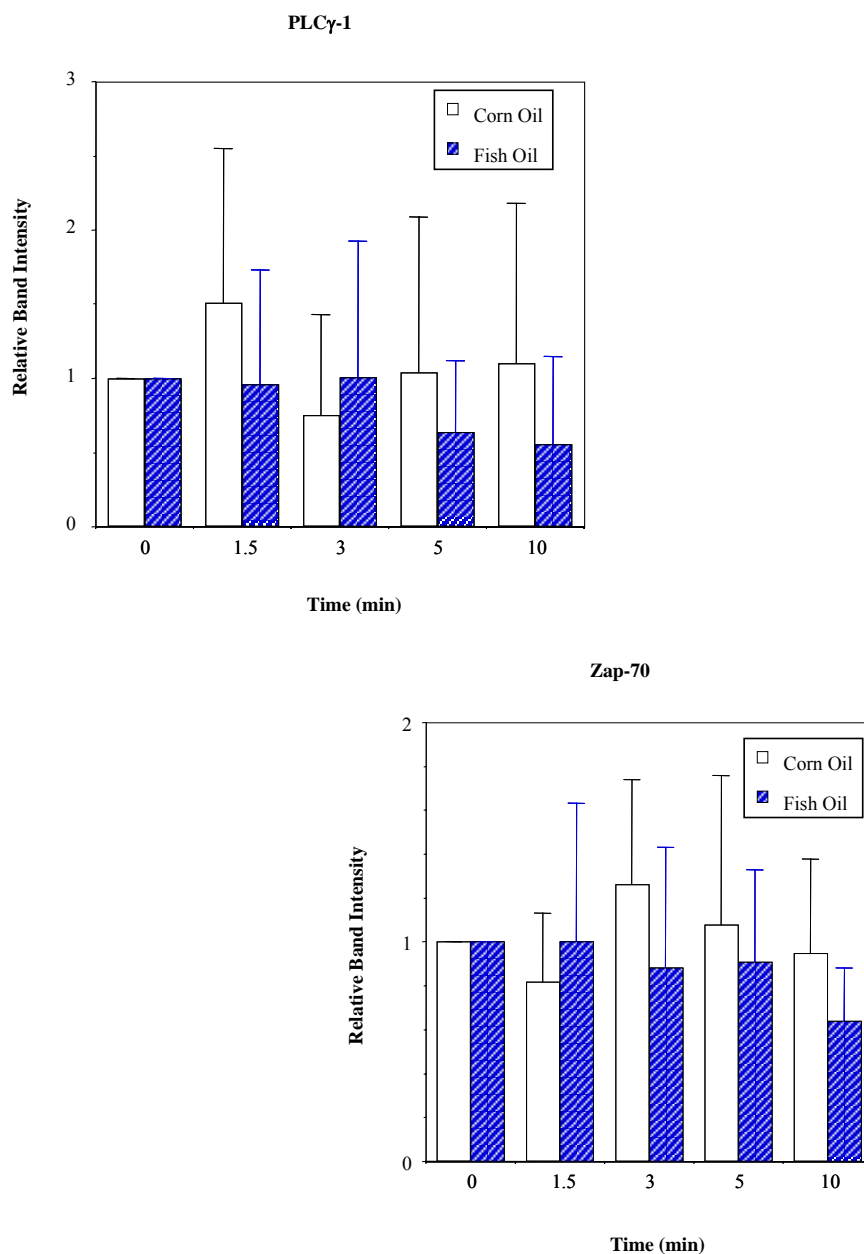
**Methodology:**  $30 \times 10^6$  purified CD3<sup>+</sup> T-cells from 3-4 chow-fed C57BL/6 mice were activated with  $\alpha$ CD3/CD28 for the specified time (0-10 min). Cells were then gently lysed and analyzed for protein content. 200  $\mu$ g of cell lysate protein was incubated with 20  $\mu$ l of anti-phosphotyrosine (4G10) agarose conjugate overnight at 4°C. Samples were eluted using spin filters (Cytosignal) according to the manufacturer's protocol and analyzed by western blots using anti-mouse PLC $\gamma$ -1 antibodies (1:1000; Upstate). Results are expressed as mean  $\pm$  SEM of the relative band intensity after normalizing to t=0 min, n=3 pooled cultures.



**FIGURE A-4.** Analysis of the protein expression of Zap-70 in activated CD3<sup>+</sup> T-cells from chow-fed mice.

**Objective:** To validate the assay procedures to be used to determine the influence of dietary n-3 PUFA on the protein expression of Zap-70.

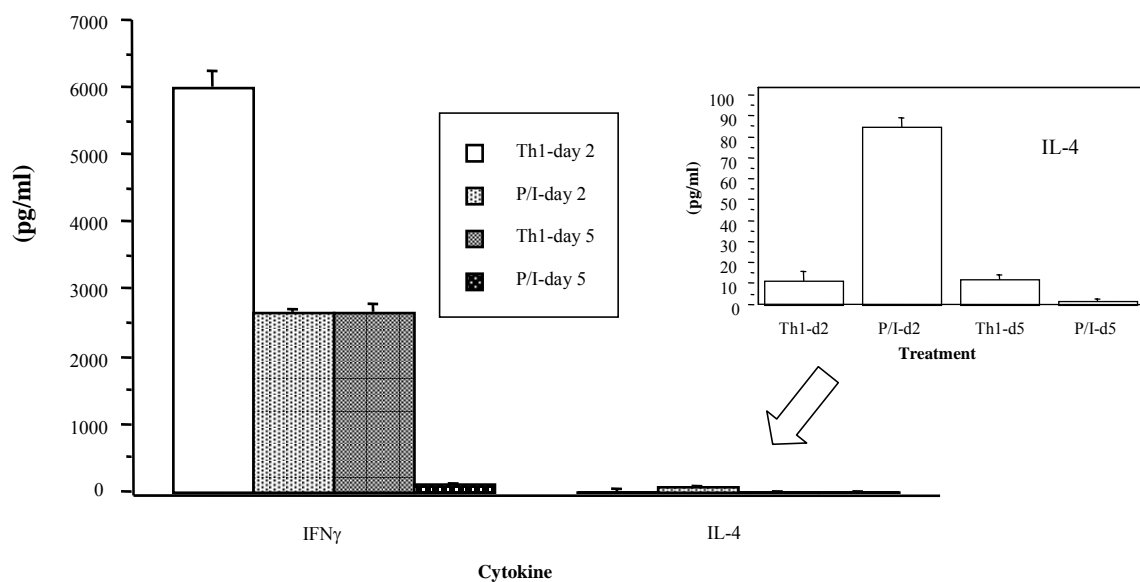
**Methodology:**  $30 \times 10^6$  purified CD3<sup>+</sup> T-cells from 3-4 chow-fed C57BL/6 mice were activated with  $\alpha$ CD3/CD28 for the specified time (0-10 min). Cells were then gently lysed and analyzed for protein content. 200  $\mu$ g of cell lysate protein was incubated with 20  $\mu$ l of anti-phosphotyrosine (4G10) agarose conjugate overnight at 4°C. Samples were eluted using spin filters (Cytosignal) according to the manufacturer's protocol and analyzed by western blots using anti-mouse Zap-70 antibodies (1:1000; Upstate). Results are expressed as mean  $\pm$  SEM of the relative band intensity after normalizing to t=0 min, n=3 pooled cultures.



**FIGURE A-5.** Dietary fish oil (FO) does not alter protein expression of Zap-70 or PLC $\gamma$ -1 in activated murine CD3<sup>+</sup> T-cells.

**Objective:** To determine the influence of 4% dietary FO on the protein expression of phosphorylated Zap-70 and PLC $\gamma$ -1 in activated CD3<sup>+</sup> T-cells from C57BL/6 mice.

**Methodology:**  $30 \times 10^6$  purified CD3<sup>+</sup> T-cells from 3-4 C57BL/6 mice fed the 5% corn oil (CO) or 4% FO + 1% CO diets were activated with  $\alpha$ CD3/CD28 for the specified time (min). Cells were then gently lysed and analyzed for protein content. 200  $\mu$ g of cell lysate protein was incubated with 20  $\mu$ l of anti-phosphotyrosine (4G10) agarose conjugate overnight at 4°C. Samples were eluted using spin filters (Cytosignal) according to the manufacturer's protocol and analyzed by western blots using anti-mouse PLC $\gamma$ -1 or anti-mouse Zap-70 antibodies (1:1000; Upstate). Results are expressed as mean  $\pm$  SEM of the relative band intensity after normalizing to t=0 min, n=5 pooled cultures.



#### Real-time PCR analysis

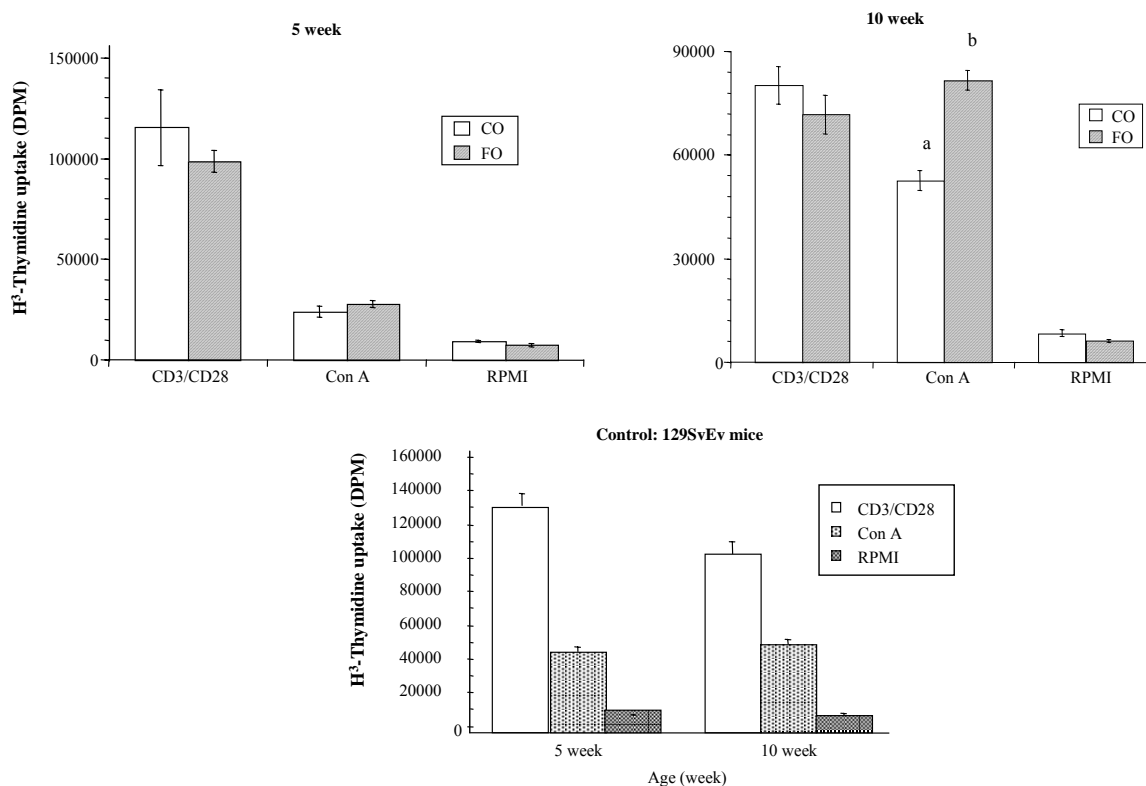
##### Threshold cycle values ( $C_T$ ) at day 5

T-bet	GATA-3
Th1 19.53	Th1 18.99
P/I 21.34	P/I 20.66

**FIGURE A-6.** Th1-polarized CD4<sup>+</sup> T-cells upregulate IFN $\gamma$  production.

**Objective:** To validate the Th1 polarization protocol on purified CD4<sup>+</sup> T-cells from C57BL/6 mice.

**Methodology:** Splenic CD4<sup>+</sup> T-cells from C57BL/6 mice were activated as previously described (58). Values from n=5 mice represent the mean  $\pm$  SEM in pg/200,000 cells. T-cell RNA was obtained from similar cultures at day 5 post-activation as described on page 21 using probes and primers designed for T-bet and GATA-3 mouse genes.

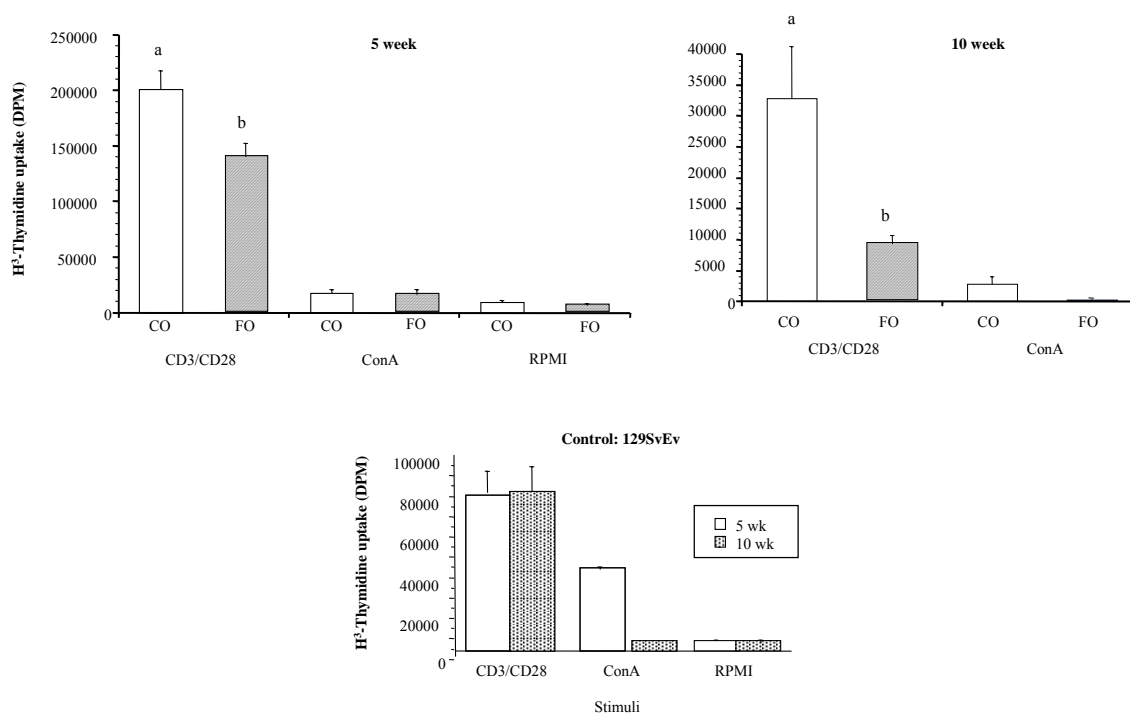


**FIGURE A-7.** Dietary fish oil (FO) does not suppress whole splenocyte proliferation in IL-10<sup>(-/-)</sup> mice.

**Objective:** To determine the effect of dietary FO on whole splenocyte proliferation in IL-10<sup>(-/-)</sup> mice.

**Methodology:** Whole splenocytes were obtained from IL-10<sup>(-/-)</sup> (129 SvEv) or 129 SvEv (control) mice fed the 5% corn oil (CO) or 4% FO + 1% CO diets for 5 or 10 weeks from their date of arrival. Cells were cultured as described on page 19. Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=7 mice. Different letters denote highly significant differences found between diet groups (p<0.01). All cells were cultured in the presence of 10% FBS.

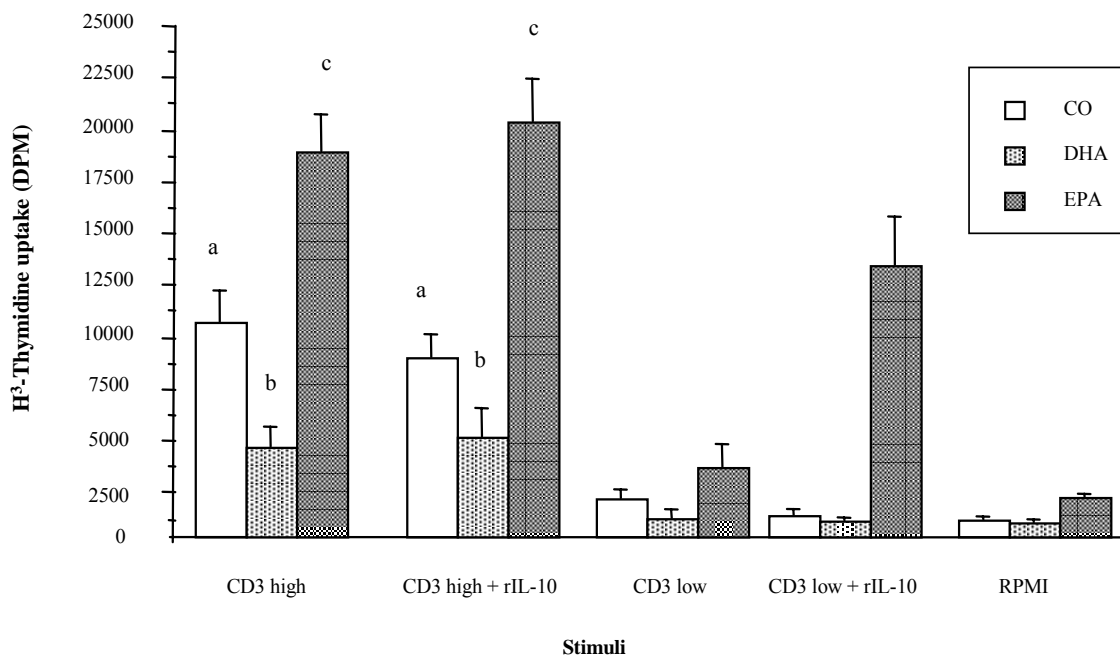




**FIGURE A-8.** Dietary fish oil (FO) reduces whole Peyer's patches proliferation in IL-10<sup>(-/-)</sup> mice.

**Objective:** To determine the effect of dietary FO on whole Peyer's patch proliferation in IL-10<sup>(-/-)</sup> mice.

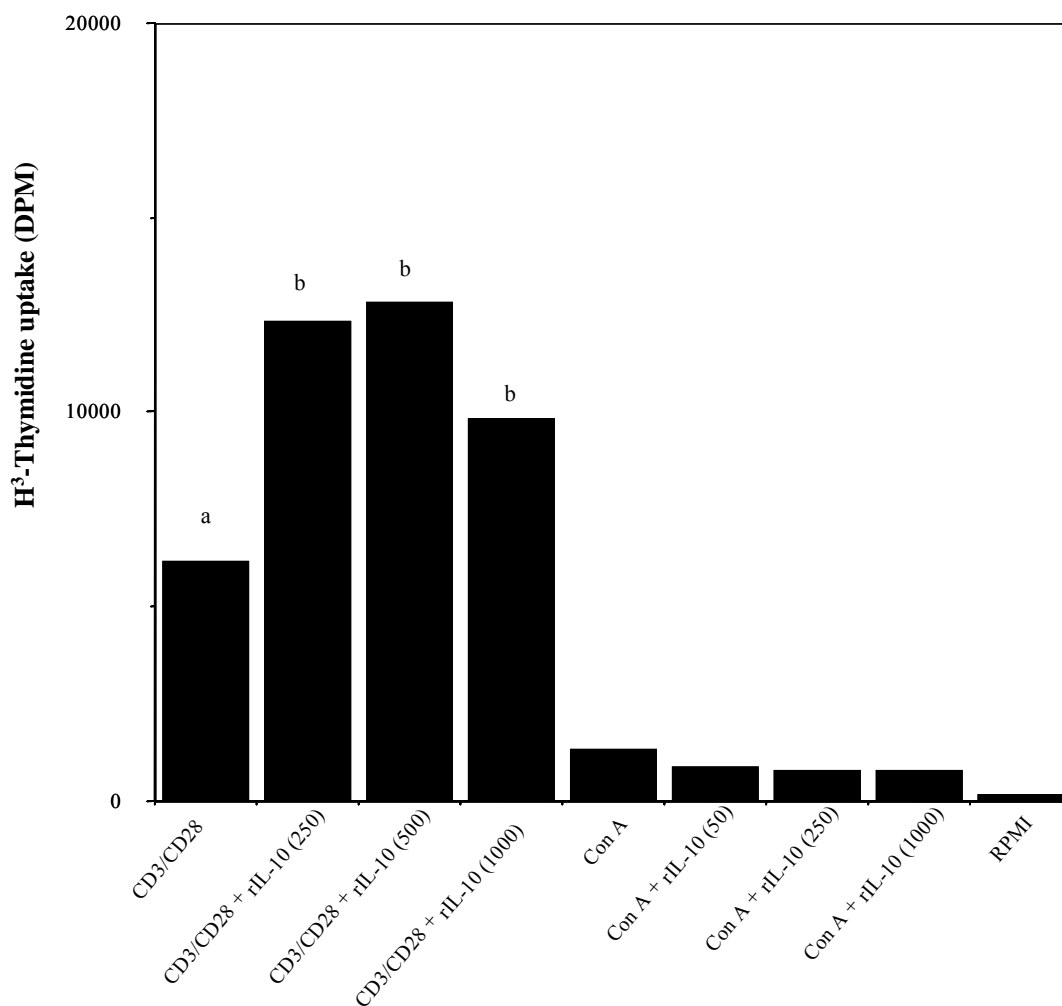
**Methodology:** Whole Peyer's patches were obtained (see A-1) from IL-10<sup>(-/-)</sup> (129 SvEv) or 129 SvEv (control) mice fed the 5% CO or 4% FO + 1% CO diets for 5 or 10 weeks from their date of arrival. Cells were cultured as described on page 19. Results are expressed as mean ± SEM of the net disintegrations per minute (DPM), n=2-3 pooled cultures. Different letters denote highly significant differences found between diet groups (p<0.01). All cells were cultured in the presence of 10% FBS.



**FIGURE A-9.** The presence of exogenous rIL-10 does not alter CD4<sup>+</sup> T-cell proliferation in C57BL/6 mice fed dietary n-3 PUFA.

Objective: To determine if the presence of rIL-10 will enhance the suppressive effect of diet.

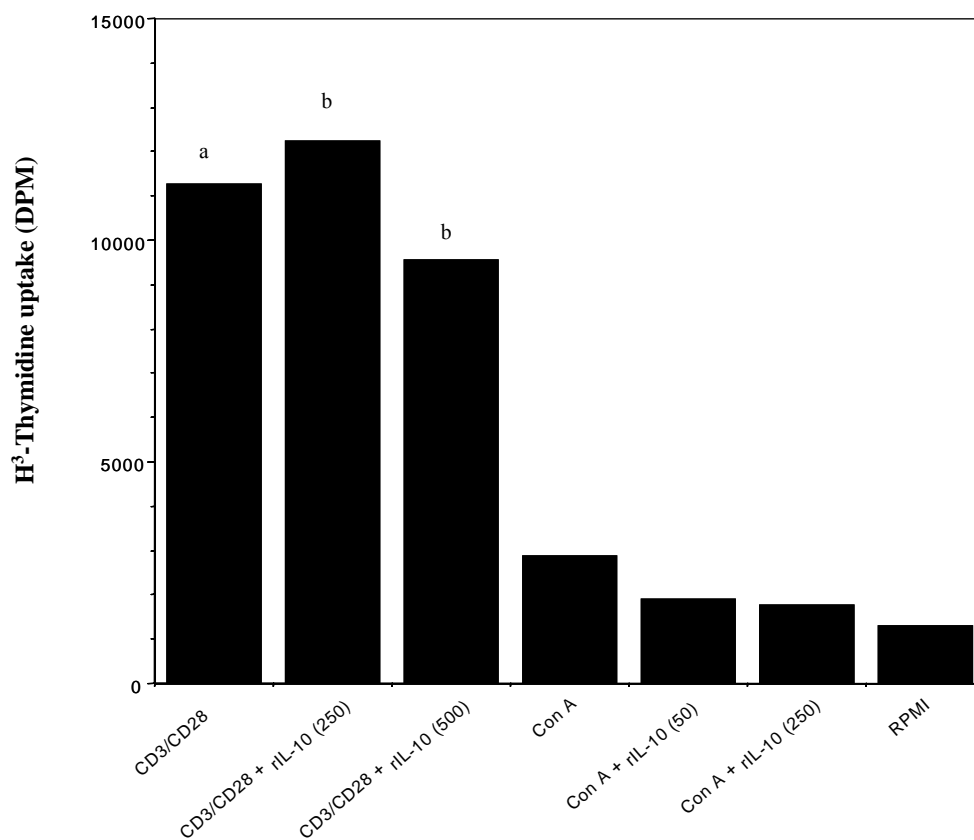
Methodology: Splenic CD4<sup>+</sup> T-cells from C57BL/6 mice fed dietary n-3 PUFA (1% ethyl ester + 4% corn oil (CO)) were cultured with  $\alpha$ CD3 antibody at 0.5  $\mu$ g/ml (CD3 low) or 1  $\mu$ g/ml (CD3 high) in the presence or absence of 50 ng/ml of mouse rIL-10 (R&D Systems) for 72 h. Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=7 mice. Different letters denote highly significant differences found between diet groups (p<0.01). All cells were cultured in the presence of 10% FBS.



**FIGURE A-10.** Exogenous rIL-10 enhances CD4<sup>+</sup> T-cell proliferation in IL-10<sup>(-/-)</sup> mice.

**Objective:** To determine the concentration at which recombinant mouse IL-10 (rIL-10) suppresses CD4<sup>+</sup> T-cell proliferation in IL-10<sup>(-/-)</sup> mice.

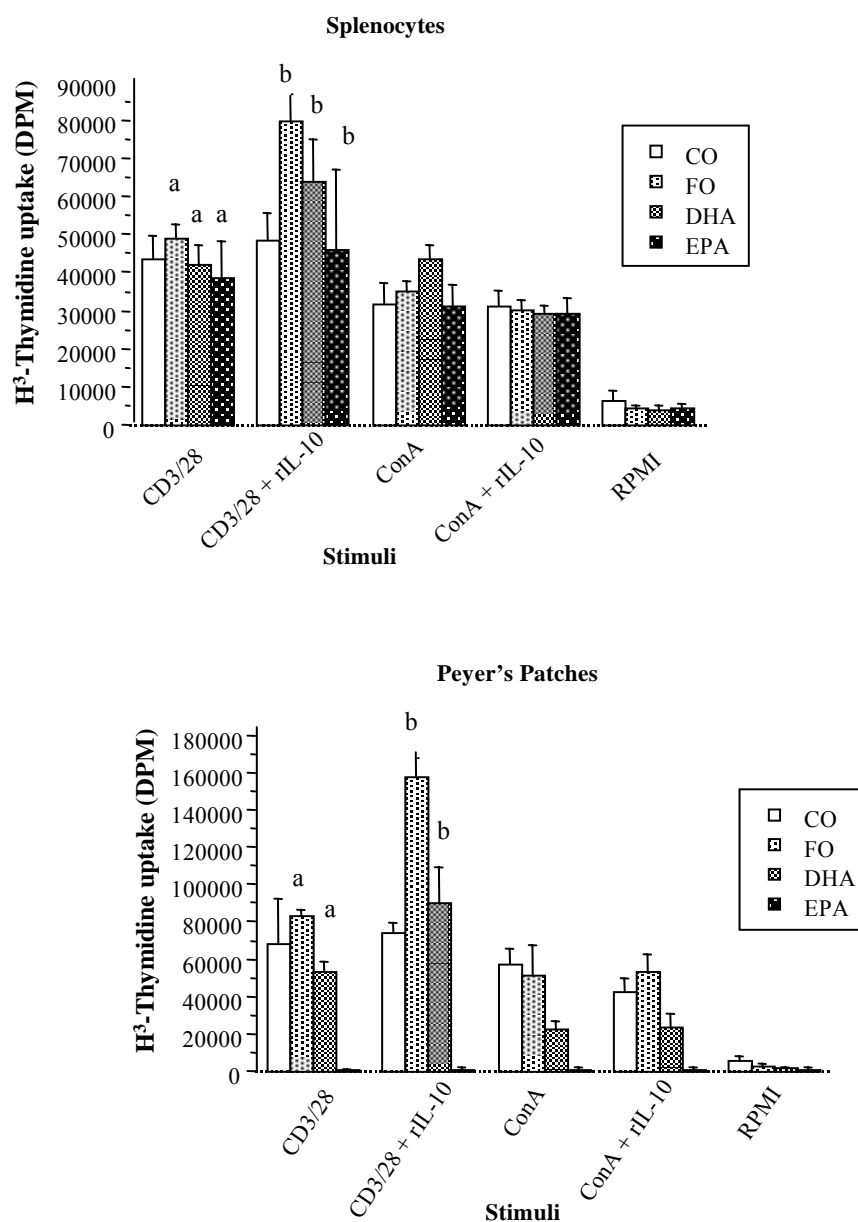
**Methodology:** Purified splenic CD4<sup>+</sup> T-cells from IL-10<sup>(-/-)</sup> mice were stimulated with  $\alpha$ CD3/CD28 (1 and 5  $\mu$ g/ml, respectively) or ConA (2.5  $\mu$ g/ml) with the specified concentrations of r IL-10 (ng/ml; R&D systems) for 72 h. Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=4 mice. Letters denote highly significant differences found within the treatment group (p<0.01).



**FIGURE A-11.** Exogenous rIL-10 enhances proliferative responses of whole Peyer's patches in IL-10<sup>(-/-)</sup> mice.

Objective: To determine the concentration at which recombinant mouse IL-10 (rIL-10) suppresses whole Peyer's patches proliferation in IL-10<sup>(-/-)</sup> mice.

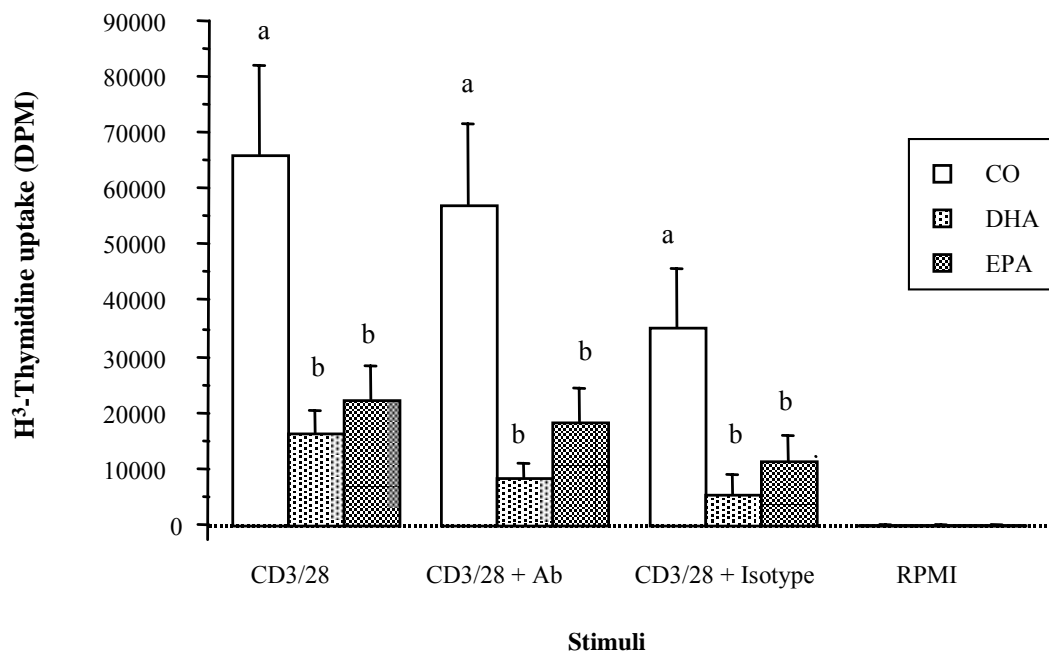
Methodology: Whole Peyer's patches were obtained (see A-1) from IL-10<sup>(-/-)</sup> mice and stimulated with  $\alpha$ CD3/CD28 (1 and 5  $\mu$ g/ml, respectively) or ConA (2.5  $\mu$ g/ml) with the specified concentrations of r IL-10 (ng/ml; R&D systems) for 72 h. Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=2-3 pooled cultures. Letters denote significant differences found within the treatment group (p<0.05).



**FIGURE A-12.** Exogenous rIL-10 enhances cell proliferation in whole splenocyte and Peyer's patches isolated from IL-10<sup>-/-</sup> mice fed dietary n-3 PUFA.

**Objective:** To determine if the dietary effect of n-3 PUFA on the proliferative responses of whole splenocyte and Peyer's patches from IL-10<sup>-/-</sup> mice can be reversed with the addition of rIL-10.

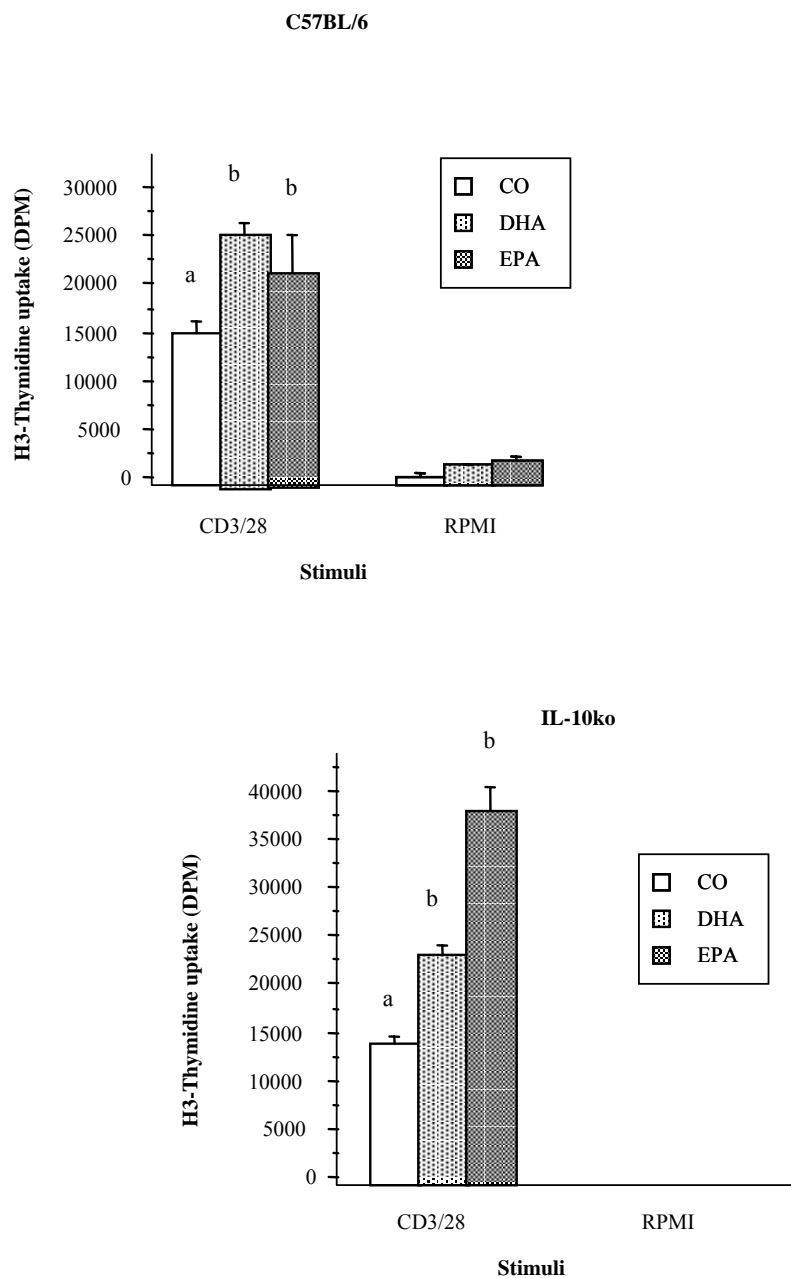
**Methodology:** Whole splenocytes and Peyer's patches (see A-1) from IL-10<sup>-/-</sup> mice fed dietary n-3 PUFA (1% ethyl ester + 4% corn oil (CO) or 4% fish oil (FO) + 1% CO) for 14 days were stimulated with  $\alpha$ CD3/CD28 (1 and 5  $\mu$ g/ml, respectively) or ConA (2.5  $\mu$ g/ml) in the presence or absence of 100 ng/ml rIL-10 (R&D systems) for 72 h. Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=6-13 mice. Letters denote highly significant differences found within the diet groups (p<0.01). All cells were cultured in the presence of 10% FBS.



**FIGURE A-13.** Anti-CTLA-4 Ab (Pharmingen) does not alter CD4<sup>+</sup> T-cell proliferation in C57BL/6 mice fed dietary n-3 PUFA.

Objective: To determine the effect of anti-CTLA-4 (Ab) on CD4<sup>+</sup> T-cell proliferation in C57BL/6 mice fed dietary n-3 PUFA.

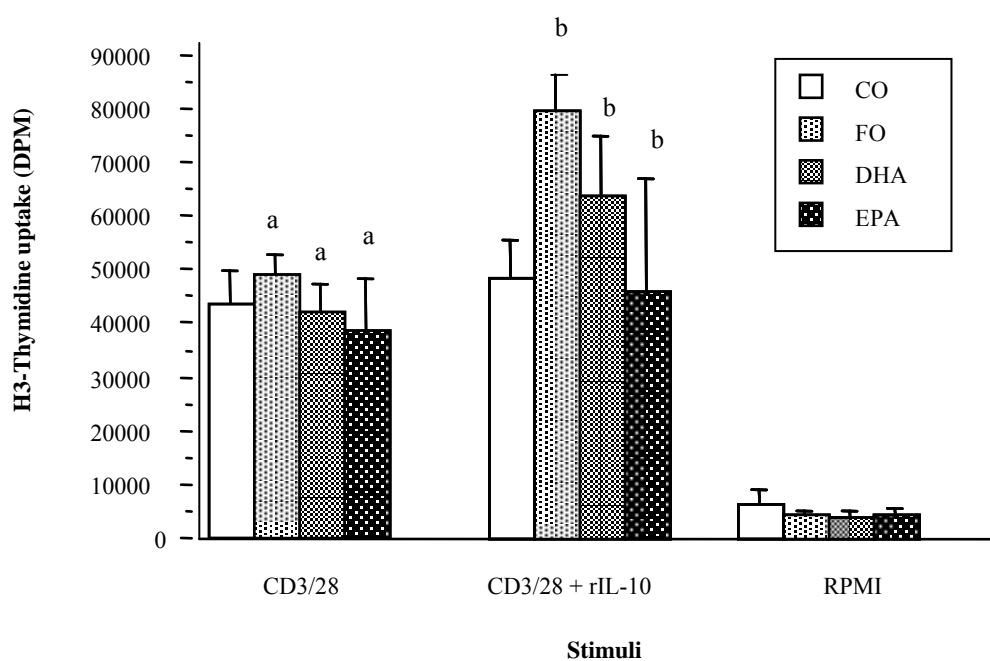
Methodology: Purified splenic CD4<sup>+</sup> T-cells were obtained from C57BL/6 mice fed dietary n-3 PUFA (1% ethyl ester + 4% corn oil (CO)) for 14 days and cultured with  $\alpha$ CD3/CD28 at 1 and 5  $\mu$ g/ml, respectively, in the absence or presence of 100  $\mu$ g/ml of anti-mouse CTLA-4 Ab (clone 4F10; Pharmingen) or 100  $\mu$ g/ml of IgG isotype (Iso; Pharmingen). Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=7 mice. Letters denote highly significant differences found between diet groups within the treatments (p<0.01). All cells were cultured in the presence of 2.5% FBS + 2.5% homologous mouse serum (HMS).



**FIGURE A-14.** Dietary n-3 PUFA enhance whole splenocyte proliferation in both C57BL/6 and IL-10<sup>(-/-)</sup> mice.

**Objective:** To determine the effect of dietary n-3 PUFA on the proliferative responses of whole splenocytes from C57BL/6 and IL-10<sup>(-/-)</sup> mice.

**Methodology:** Whole splenocytes were obtained from C57BL/6 and IL-10<sup>(-/-)</sup> mice fed dietary n-3 PUFA for 14 days. Cells were activated with  $\alpha$ CD3/CD28 at 1 and 5  $\mu$ g/ml, respectively, for 72 h in the presence of 2.5% FBS + 2.5% homologous mouse serum (HMS). Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=7 mice. Letters denote highly significant differences found within the between diet groups (p<0.01).



**FIGURE A-15.** Exogenous rIL-10 enhances whole splenocyte proliferation in IL-10<sup>(-/-)</sup> mice fed dietary n-3 PUFA.

Objective: To determine the effect of recombinant mouse IL-10 (rIL-10) on whole splenocyte proliferation in IL-10<sup>(-/-)</sup> mice fed dietary n-3 PUFA.

Methodology: Whole splenocytes were obtained from IL-10<sup>(-/-)</sup> mice fed dietary n-3 PUFA for 14 days. Cells were activated with  $\alpha$ CD3/CD28 at 1 and 5  $\mu$ g/ml, respectively, for 72 h in the presence of 10% FBS. Results are expressed as mean  $\pm$  SEM of the net disintegrations per minute (DPM), n=7 mice. Letters denote highly significant differences found within the between diet groups (p<0.01).



**APPENDIX B**  
**PROTOCOLS**

## Isolating Peyer's Patches

(from McGee, *et al Immunology* 1998 (64): 697-702 and Chapkin Lab)

### Materials:

- \_\_\_ forceps and scissors
  - \_\_\_ PBS
  - \_\_\_ small weigh boats (to collect and rinse intestines)
  - \_\_\_ Complete PP media
  - \_\_\_ 10% heat-inactivated FBS (Irvine Scientific), 1% L-glutamine (Gibco), 1% Penicillin-Streptomycin (Gibco), 10  $\mu$ M  $\beta$ -mercaptoethanol (Sigma), and 50  $\mu$ g/ml gentamicin sulfate (Gibco).
  - \_\_\_ 5 cc syringe
  - \_\_\_ 20 G needle
  - \_\_\_ glass homogenizer
  - \_\_\_ wire filter
  - \_\_\_ nylon wool fiber column
- Pack ~1 inch of cotton wool (scrubbed and combed from Polysciences, removes adherent cells) loosely into the end of a pasteur pipet. Sterilize

- \_\_\_ 1. Excise Peyer's Patches (PP) (small, white nodules) from small intestines
  - $\Rightarrow$  it may help to split open the small intestine and rinse out the feces in PBS to expose the PP. However, most of the time the PP are noticeable without having to do this
- \_\_\_ 2. Transfer PP to a 15 ml. conical tube containing 5 ml. complete PP media
- \_\_\_ 3. Pipette PP solution into a glass-in-glass homogenizer and homogenize until completely broken up (~5-7 strokes)
- \_\_\_ 4. Assemble a 5 cc syringe, wire filter, and 20 gauge needle-unit for each PP and place on top of a newly labeled 15 ml. conical tube
- \_\_\_ 5. Remove the syringe plungers and transfer the PP suspension into the assembled unit
- \_\_\_ 6. *Slowly* reinsert the plunger to filter the suspension into the conical tube
  - $\Rightarrow$  *cells are fragile, so be gentle with this step*
- \_\_\_ 7. Set up the nylon wool fiber column by placing a clothespin around the center of the column and place it on an R&D T-cell column rack (the clothespin will help the column to stabilize on the rack)
- \_\_\_ 8. Place a new 15 ml. conical tube directly underneath the column to collect the eluent
- \_\_\_ 9. Pipette the PP solution at the top of the column and allow to flow through
  - $\Rightarrow$  a bubble may develop at the top and may interfere with the flow. Keep another sterile pasteur pipet handy to eliminate any bubbles
- \_\_\_ 10. Wash the column with 5ml. complete PP media
- \_\_\_ 11. Spin cells down at 300 x g for 5 min. at RT
- \_\_\_ 12. Aspirate the supernatant and resuspend in 1ml. complete PP media
- \_\_\_ 13. Count cells on hemacytometer and adjust concentration if necessary

## Purifying CTLA-4 Antibody from UC10-4F10 cells

(Lan Ly from Chapkin Lab (May 2004))

### Growing UC10-4F10 cells (as per protocol from ATCC)

#### Materials:

- UC10-4F10 cells from ATCC (HB-304)
  - Culture medium:
    - Dulbecco's modified Eagle's medium (high glucose) (550ml)
    - add 55ml FBS, 11ml L-Glut, 5.5ml Na-Pyruvate, and 1.9 $\mu$ l 2-mercaptoethanol
    - store at 4C and pre-warm to 37C before use
  - Freezing media (Specialty Media; S-002-D)
- 
1. Thaw frozen cells gently by rapid agitation in 37C water bath
  2. Add contents of ampule to ~10 ml culture medium (that has been warmed to 37C).
  3. Spin at 300 x g for 5 min at RT
  4. Aspirate supernatant and resuspend cells gently in 30 ml culture medium
  5. Plate cells in T-75 flask for 2-3 days (medium will turn yellowish, but pH will remain at ~7-8)
    - $\Rightarrow$  although this is a non-adherent cell line, ~50% of the cells will adhere to the flask as per manufacturer
  6. After 2-3 days, place contents of flask into 50 ml conical tube and spin down
  7. Collect supernatant and freeze at -80C
  8. Resuspend cells in 30 ml culture medium and count on hemacytometer
  9. Maintain cultures at cell concentrations between  $10^5$  to  $10^6$  viable cells/ml
  10. To freeze down cells, pellet cells as described above and aspirate or collect supernatant
  11. Resuspend the cells in culture freezing medium at a concentration of  $10^7$  to  $10^8$  as per manufacturer's protocol
  12. Freeze 1 ml of cells/ vial in appropriate cryogenic vials.
  13. Freeze gently and slowly to -80C using cryofreeze container

### Purifying Supernatants (as per manufacturer's protocols)

#### Materials:

- HiTrap Protein G HP affinity columns, 5 ml (Amersham, 17-0405-01)
- Make 20mM NaHPO<sub>4</sub> solution (pH 7):
  - Add 1.38 g NaHPO<sub>4</sub> (monobasic, pH 4.2, S-369) to 0.5 L H<sub>2</sub>O
  - Add 2.68 g NaHPO<sub>4</sub> (dibasic, pH 9.2, S-373) to 0.5 L H<sub>2</sub>O
  - Combine above solutions in 1L bottle and pH to ~7.0
  - Store at RT
- 5 ml syringe (luer-lok)
- Make 0.1 M Glycine solution (pH 2.7):

- Add 3.75 g Glycine to H<sub>2</sub>O
- Adjust pH to ~2.7 and bring up to 0.5 L
- Store at RT
- \_\_\_ Make 1 M Tris-HCl (pH 9)
  - Add 15.76 g Tris to H<sub>2</sub>O
  - Adjust pH to ~9.0 and bring up to 0.1 L
- Store at RT
- \_\_\_ 20% EtOH

- \_\_\_ 1. Thaw collected supernatants and place on ice
- \_\_\_ 2. Prepare collection tubes (15 ml conical tubes) by adding 0.5 ml 1M Tris-HCl, pH 9
- \_\_\_ 3. To wash the column, fill the syringe with NaHPO<sub>4</sub> buffer and connect the column to the syringe using the provided adaptor
- \_\_\_ 4. Remove the twist-off end
- \_\_\_ 5. Slowly “pump” the solution through column drop-by-drop at a rate of ~5 ml/min
  - ⇒ Important!! To avoid introducing air into the column, twist off the syringe, remove plunger, reconnect it to the column, and fill with buffer
- \_\_\_ 6. Wash column with ~5 column volumes
- \_\_\_ 7. Apply the thawed supernatants, 5 ml at a time. The column has a binding capacity of ~ 25 mg human IgG/ml gel. Therefore, ~800 ml of supernatant can be loaded onto the column before eluting.
- \_\_\_ 8. Wash with ~5-6 column volumes of NaHPO<sub>4</sub> buffer or until no material appears in the effluent
- \_\_\_ 9. Elute with ~2-5 column volumes of Glycine buffer (pH 2.7) and collect in the prepared 15 ml conical tubes.
- \_\_\_ 10. The eluted samples can be frozen at –80C until dialysis
- \_\_\_ 11. To continue loading more samples onto the HiTrap column, wash with 5 column volumes of NaHPO<sub>4</sub> buffer and proceed with steps 7-10.
- \_\_\_ 12. To store the column, wash with 3 column volumes of 20% EtOH, close up both ends of column using the provided adaptors, and store at 4C
  - ⇒ column can be used numerous times

#### Dialyzing, “Concentrating,” and Sterilizing Ab

##### Materials:

- \_\_\_ Slide-A-Lyzer Dialysis Cassettes, MWCO 10, 000, 3-12 ml (Pierce, 66810)
- \_\_\_ 1XPBS (cooled to 4C) (~5 L or more)
- \_\_\_ 20 ml syringe
- \_\_\_ 18G needle
- \_\_\_ Amicon Ultra-15 Centrifugal Filter Device (MilliPore, PL-100, 100, 000 MWCO)
- \_\_\_ Acrodisc Syringe Filter, low protein binding, 0.2µm HT Tuffryn Membran (Gelman Labs, 4192)

- \_\_\_ 1. Thaw samples and place on ice

- \_\_\_ 2. Remove Slide-A-Lyzer cassette from its pouch and slip the longest side into the groove of a buoy
- \_\_\_ 3. Immerse the cassette in 1XPBS buffer for ~30 sec
- \_\_\_ 4. Attach syringe with needle and fill with sample
- \_\_\_ 5. Add sample to cassette through a syringe port at the top of the cassette
  - ⇒ be careful not to burst the membrane. Draw back the syringe to remove the air in the pouch and then continue inject the sample
- \_\_\_ 6. Remove any air from the pouch using the syringe
- \_\_\_ 7. Place membrane into 1XPBS buffer and dialyze overnight at 4C with constant gentle stirring
  - ⇒ As per Laurie, ~ 120 ml of sample (~10 cassettes) can be dialyzed in a 5 L container of 1XPBS (final Glycine concentration of sample should be < 0.0006M)
- \_\_\_ 8. Remove sample from cassette by inserting the needle into the bottom syringe port and slowly withdrawing the sample
- \_\_\_ 9. Place sample in the Amicon Filter Unit (up to 15 ml)
- \_\_\_ 10. Spin at 4000 x g for ~7 min at RT (use Juoan)
  - ⇒ volume will be ~0.5 ml at this stage
- \_\_\_ 11. Recover the concentrated sample by using a side-to-side sweeping motion with a pipetter to ensure total recovery
- \_\_\_ 12. Under a sterile hood, sterilize the sample using a sterile 5 ml syringe attached with a Acrodisc Syringe Filter (low protein binding).
- \_\_\_ 13. Aliquot into sterile 0.65 ml eppendorf tubes and store at -80C.
- \_\_\_ 14. Perform protein assay using Coomassie Blue

## Immunoblot Stripping Protocol

(from Nicole Murray, nmurray@utmb.edu)

### Solutions:

#### **1 M Tris:**

121.4 g. Tris base  
 -add ~500ml. ddH<sub>2</sub>O and adjust pH to 6.7 using HCl  
 -bring up to volume (1L) using ddH<sub>2</sub>O  
 -store at 4°C

#### **Final Concentration:**

1M

#### **Stripping Buffer:**

To make 1L:

6.99 ml. 2-ME (14.3 M) 100 mM

100 ml. 20% SDS 2%

62.5 ml. 1M Tris (pH 6.7) 62.5 mM

-add ~200 ml. ddH<sub>2</sub>O and adjust pH to 6.7

-bring up to volume (1L) using ddH<sub>2</sub>O

-store at 4°C (solution will precipitate, but should become soluble at RT)

*To strip membrane of its 1° and 2° antibodies:*

- \_\_\_ 1. Pour stripping buffer in covered dish and pre-warm to 50°C using H<sub>2</sub>O bath on “belly dancer”
- \_\_\_ 2. Place membrane in pre-warmed stripping buffer for 30 min. with gentle rocking
  - ⇒ place “donut” on top of dish so that it is stable in the H<sub>2</sub>O bath
- \_\_\_ 3. Once 30 min. are up, wash membrane vigorously in 1XPBS/Tween solution for 10 min. at RT twice
- \_\_\_ 4. . To ensure that the membrane has been properly stripped, block membrane in 4% nonfat dry milk/1X PBS/0.1%Tween for 1 hr. at RT
- \_\_\_ 5. Incubate membrane in the secondary antibody that will be used for the next 1° antibody for at least 1 hr. at RT
- \_\_\_ 6. Wash membrane vigorously in 1XPBS/Tween for 15 min. at RT twice.
- \_\_\_ 7. Add substrate (Supersignal WestFemto) to membrane on acetate sheet for 5 min.
- \_\_\_ 8. Image blot on Fluor-S Imager to ensure that no signal is detected.
- \_\_\_ 9. Repeat steps 1-10 if signal is still detected.
- \_\_\_ 10. Once blot is properly stripped, proceed with Western Blot protocol as if you have just completed the membrane transfer (i.e, block membrane in milk→ incubate with 1° antibody overnight, etc.)

After the 1<sup>st</sup> time, steps 4-9 can be omitted once you are convinced that this process effectively eliminates all non-specific signals

\*\* Note: all antibodies are different so do not assume that this protocol will be effective for all antibodies

## **H<sup>3</sup>-Thymidine Uptake**

(from Chapkin Lab, Lan Ly)

### Materials:

- \_\_\_ sterile 96 well-round bottom plates
- \_\_\_ sterile 1XPBS
- \_\_\_ Complete RPMI mixture
  - RPMI 1640 Irvine Scientific, cat #9159
  - 2.5% homologous sterile filtered mouse serum
  - 2.5% heat inactivated FBS (Irvine Scientific)
  - [10 $\mu$ M] 2-ME
    - $\Rightarrow$  stock 2-ME is 14.3M
    - 1) add 3.5 $\mu$ l. stock 2-ME to 1 ml. RPMI 1640
    - 2) add 100 $\mu$ l. of this solution to the RPMI mixture
- \* for Peyer's Patches, add 50ug/ml Gentamicin
- \_\_\_ Falcon *polypropylene* tubes
- \_\_\_ Multi-petter
- \_\_\_ sterile solution basins

### ***Plate $\alpha$ CD3 24 hours before day of experiment:***

- \_\_\_ 0. Contact Shannon Sedberry (sedberry@tamu.edu) to let her know what day(s) you will be using the harvester
- \_\_\_ 1. In a sterile hood, dilute  $\alpha$ CD3 (Pharmingen: 553057) (stock: 1000ug/ml) to 1ug/ml in sterile 1X PBS
- \_\_\_ 2. Designate and label wells of a 96-well plate (CD3/28, Con A, RPMI, etc)
- \_\_\_ 3. Mix  $\alpha$ CD 3 solution(s) once again and using a multi-petter and a sterile solution basin, pipette 50 $\mu$ l. of  $\alpha$ CD 3 into each well
- \_\_\_ 4. Tape lid to plate and place at 4°C overnight

### ***Day of Experiment:***

\*Before proceeding with T-cell purification, prepare all stimuli solutions 1<sup>st</sup>

- \_\_\_ 1. Dilute  $\alpha$ CD28 (Pharmingen: 553294) (stock: 1000ug/ml) to 10ug/ml in complete RPMI mixture with homologous mouse serum
  - $\Rightarrow$  final concentration of CD28 in plate well will be 5ug/ml because you will dilute this 1:2 later
- pipette solution up and down several times to ensure proper mixing and allow to sit for a few minutes

- \_\_\_ 2. Place all stimuli solutions in 4°C and proceed with T-cell purification and adjust final concentration to  $2 \times 10^6$  T-cells/ml
- 

- \_\_\_ 1. Obtain  $2 \times 10^6$  T-cells/ml. in Complete RPMI mixture with homologous mouse serum and place at RT
- \_\_\_ 2. Aspirate CD3 antibody from the 96-well plate
- \_\_\_ 3. Using a multipipettor and a sterile solution basin, pipette 100ul. Of CD28 solution (and/or other stimuli) into appropriate wells
- \_\_\_ 4. Thoroughly mix the T-cells by pipetting up and down and using a multi-pette and a sterile solution basin, add 100μl. of the T-cells to the labeled wells
- \_\_\_ 5. Tape the lid to the plate and place in 37°C incubator for 72 hours

*Make sure there is a sufficient amount of H<sub>2</sub>O/ BacDown in the bottom of the incubator*

-Make sure and check cells under microscope before and after 3-day incubation to check for proper proliferation

### **Day 3**

- \_\_\_ 1. Remove plates from 37°C incubator and check cells under microscope to ensure T-cells have proliferated.
  - ⇒ also, observe color of media
- \_\_\_ 2. Pipette 4 ml. of complete RPMI into a 15 ml. conical tube (this will be enough for 1 plate)
- \_\_\_ 3. Make a “plate map” by numbering each well of each plate
  - ⇒ this will be used to help keep track of the filter discs
- \_\_\_ 4. Bring plates, 4 ml. RPMI and multipipettor over to McMurray Lab in Med. School
- \_\_\_ 5. In their tissue culture room, there is a refrigerator that will contain a conical tube of 100μCi H<sup>3</sup> Thymidine solution
  - ⇒ if solution is not there or not made up, consult Shannon
- \_\_\_ 6. In the radioactive hood, dilute the 100μCi H<sup>3</sup> Thymidine 1:5 to 20μCi
  - ⇒ 1 ml. of H<sup>3</sup> Thymidine + 4 ml. complete RPMI



- ⇒ final concentration in wells will be  $4\mu\text{Ci H}^3$
- \_\_\_ 7. Using a multipepeter and the radioactive solution basin, add  $50\mu\text{l.}$  of  $20\mu\text{Ci H}^3$  Thymidine to all wells of each plate containing cells
  - \_\_\_ 8. Allow plates to incubate in  $37^\circ\text{C}$  incubator for  $5\frac{1}{2}$ -6 hours
  - \_\_\_ 9. After incubation, bring plate(s) downstairs (2<sup>nd</sup> floor) where cell harvester is
  - \_\_\_ 10. Turn on vacuum pump located below the Packard cell harvester
  - \_\_\_ 11. Cut a filter paper (in drawer below cell harvester) to approximately the size of the 96-well plate
  - \_\_\_ 12. Place the filter in the top compartment of the harvester
  - \_\_\_ 13. Run  $\text{H}_2\text{O}$  through the harvester using COLD and HOT vacuum to ensure all probes are aspirating and dispensing well ( $\sim 3\text{x}$ )
  - \_\_\_ 14. Place the sample plate directly underneath the probes
  - \_\_\_ 15. Suck up contents of plate using HOT vacuum and was plate with  $\text{H}_2\text{O}$  at least 7X
  - \_\_\_ 16. Remove top compartment with vacuum on to help dry the filter
  - \_\_\_ 17. Once filter looks dry, remove the template with the filter still on it
  - \_\_\_ 18. To clean the harvester, fill the bottom platform with EtOH and run EtOH through the harvester.  
    ⇒ make sure to turn off pump when finished
  - \_\_\_ 19. Label scint vials with the designated numbers of each filter disc (see plate map)
  - \_\_\_ 20. Using forceps, gently remove the perforated discs of filter paper and place them in the appropriate scint vial  
    ⇒ remember the orientation of the plate and how it corresponds to the perforated discs (it will be reversed)
  - \_\_\_ 20. Then fill each scint vial with 10 ml. scintillation fluid
  - \_\_\_ 21. Bring scint vials over to the beta/gammer counter at Kleberg rm. 423
  - \_\_\_ 22. Load scint vials in rack from left to right and place a red STOP rack behind the last rack
  - \_\_\_ 23. Use Counter User #1 and AutoCount  
    ⇒ Vials are counted for  $\sim 1$  min. each

## **CD4<sup>+</sup> T-CELL ISOLATION**

*(From the Chapkin Lab, Jennifer Arrington)*

### I. NECROPSY

Materials:

- sterile instruments (i.e. scissors, forceps, etc.)
- EtOH
- a labeled 15 ml. conical tube for each mouse group filled with 3 ml. complete RPMI (10% FBS, 1% L-Glutamine, 1% Pen-Strep, and 10 $\mu$ M 2-ME)  
      $\Rightarrow$  500ml. RPMI + 55 ml. FBS + 5.5 ml. L-Glut + 5.5 ml. P/S + 100 $\mu$ l. of 3.5 $\mu$ l. 2-ME diluted in 1 ml. RPMI
- keys for basement

- 1. Sacrifice mice (\_\_\_\_\_ ) by CO<sub>2</sub>
- 2. Place mice on their right side so that the left side faces you
- 3. Apply alcohol to the abdomen area
- 4. Grab the skin of the abdomen with forceps and make a small incision
- 5. With fingers, peel back the skin/fur to expose the membrane underneath
- 6. Grab the membrane with forceps and cut the membrane to expose the organs
- 7. Remove the spleen (dark red organ) with forceps
- 8. Carefully remove as much fat from the exterior of the spleen as possible
- 9. Place spleen(s) in a conical tube containing complete RPMI

### II. CELL PREPARATION

Materials:

- sterile glass-in glass homogenizers
- sterile wire filter
- 10 cc syringes
- 20 gauge needles
- RPMI mixture
- 15 ml. conical tubes

- 1. Transfer each spleen and RPMI solution into a glass-in-glass homogenizer
- 2. Homogenize spleens until completely broken up (~5-7 strokes)
- 3. Assemble a 10 cc syringe, wire filter, and 20 gauge needle-unit for each spleen and place on top of a newly labeled 15 ml. conical tube
- 4. Remove the syringe plungers and transfer the appropriate spleen suspension into the assembled units

- \_\_\_ 5. *Slowly* reinsert the plunger to filter the suspension into the corresponding conical tube  
     ⇒ *cells are fragile, so be gentle with this step*
- \_\_\_ 6. Fill each sample tube with RPMI for washing and centrifuge at 200 x g (rcf=200, rpm=1096) for 5 min. at RT
- \_\_\_ 7. Meanwhile, soak all used equipment in soapy H<sub>2</sub>O
- \_\_\_ 8. Once samples are done spinning, carefully aspirate the RPMI leaving only 0.5 ml. in each sample tube  
     ⇒ the lymphocytes are now ready to be isolated from the red blood cells, plasma, etc.

### III. REMOVE RED BLOOD CELLS

#### Materials:

- \_\_\_ Lympholyte-M (lot # \_\_\_\_\_) cooled to RT
  - \_\_\_ R&D antibody cocktail in CD4<sup>+</sup> purification kit
  - \_\_\_ RPMI mixture
  - \_\_\_ 1 X column wash buffer (diluted from 10X) (lot # \_\_\_\_\_)
- \_\_\_ 1. Add RPMI to the washed cells (add 2.0 ml RPMI for 30-40 x 10<sup>6</sup> cells or 3 ml RPMI for 60-80 x 10<sup>6</sup> cells, as per Chris Jolly) and resuspend  
     ⇒ normally, one spleen yields 30-40 x 10<sup>6</sup> cells
  - \_\_\_ 2. Add an equal amount of Lympholyte-M by layering it beneath the suspended cells  
     ⇒ insert the pipet tip into the bottom of the conical tube before dispensing the Lympholyte-M.  
     ⇒ this step must be done slowly and carefully in order to see 2 distinct bands
  - \_\_\_ 3. Centrifuge the cells at 500 x g (rcf=500) for 15 min. at RT  
     ⇒ deactivate centrifuge brake at this step (acc/dcc = 0); the centrifuge tends to stop too fast and this will disturb the layer produced by Lympholyte-M  
     ⇒ meanwhile, begin T-cell column preparation  
     ⇒ be careful not to disturb sample layers when removing the samples from the centrifuge  
     ⇒ when samples are done spinning, you will see 2 distinct layers (a white one on the top and a clear, pink one on the bottom) and an interface band separating them. Your goal is to remove this interface band. The bottom clear layer contains the unwanted red blood cells, plasma, etc.
  - \_\_\_ 4. Carefully remove the interface band between the medium and Lympholyte-M layer with a pipette and dispense it into a new conical tube (should see a red pellet)
  - \_\_\_ 5. Fill the conical tube with RPMI to wash and centrifuge the cells at 300 x g for 5 min. at RT
  - \_\_\_ 6. Aspirate off the RPMI and resuspend the cells in 2 ml. 1X R&D wash buffer
  - \_\_\_ 7. Add 1 vial of monoclonal antibody cocktail (1 ml) to suspension and mix gently

- \_\_\_ 8. Allow cells to incubate at RT for 15 min.
- \_\_\_ 9. Wash cells in 10 ml. 1X column buffer and spin at 300 x g for 10 min.
- \_\_\_ 10. Decant supernatant and repeat step 9. Decant.
- \_\_\_ 11. Resuspend final cell pellet in 2 ml. 1X column buffer

#### IV. T-CELL PURIFICATION

##### Materials:

- \_\_\_ R&D CD4<sup>+</sup> T-cell columns (Cat# MCD43; 2spleens minimum on each column) (lot# \_\_\_\_\_) at RT
- \_\_\_ R&D column rack
- \_\_\_ 70% EtOH
- \_\_\_ sterile 15 ml conical tubes
- \_\_\_ waste receptacle
- \_\_\_ timer(s)
- \_\_\_ 1 X column wash buffer (diluted from 10X) (lot# \_\_\_\_\_) (make 75ml. per column)
- \_\_\_ RPMI mixture

- \_\_\_ 1. Place the T-cell column(s) in the R&D column rack and place a waste receptacle directly beneath the column(s)
- \_\_\_ 2. To prepare the columns, remove the top cap of the column FIRST to avoid drawing air into the bottom of the column and then remove the bottom cap
- \_\_\_ 3. Allow the fluid within the column to drain until it reaches the level of the top white filter
  - ⇒ column will stop dripping once it reaches the top filter
  - ⇒ meanwhile, rinse the outside of the column tip with 70% EtOH
- \_\_\_ 4. Wash column with 10ml. of 1X column wash buffer
- \_\_\_ 5. Replace the waste receptacle with a newly labeled 15 ml conical tube
- \_\_\_ 6. Apply the 2 ml cell suspension to the top of the column
  - ⇒ the cells will enter the column and displace the wash buffer in the column, which is collected in the sterile conical tube
- \_\_\_ 7. Allow the cells to sit within the column for 10 min. at RT
- \_\_\_ 8. Apply 10ml. of 1X column wash buffer to the column to elute the T-cells
- \_\_\_ 9. Once all the T-cells have been collected (the column will stop dripping),
  - centrifuge them at 300 x g for 5 min. at RT
- \_\_\_ 10. Aspirate off the supernatant and resuspend in 1 ml. complete RPMI
  - ⇒ the cells are now ready to be counted. You will determine the percentage of live vs. dead cells and obtain a percentage of viability

### V. TRYPAN BLUE EXCLUSION or COULTER COUNTER

Materials:

- Trypan blue
- small sample tubes
- hemacytometer
- cell counter

- 1. Add 190 $\mu$ l. of Trypan Blue to the empty sample tubes
- 2. Swirl the suspended T-cells and remove 10 $\mu$ l. and add them to a sample tube with Trypan Blue
- 3. Mix this mixture well by pipetting up and down several times
- 4. Remove 10 $\mu$ l. of this mixture and plate in a hemacytometer  
 $\Rightarrow$  do this step immediately after the cells have mixed with the Trypan Blue because Trypan Blue kills cells after long exposure
- 5. Count the viable white cells (dead cells appear blue) in at least 4 squares of the grid  
 #1\_\_\_\_\_ #2\_\_\_\_\_ #3\_\_\_\_\_ #4\_\_\_\_\_ Total\_\_\_\_\_
- 6. The # of cells will be the average of the 4 squares : Avg. \_\_\_\_\_  
 $\Rightarrow$  (Avg. # cells) x 10,000 x (dilution factor=20)= # cells/ml. = \_\_\_\_\_

\*\*\*From the cell count obtained prior to T-cell purification procedure, this cell count (in step #6) should be ~30% less) as per Jennifer Arrington.

\*\*\*This CD4<sup>+</sup> T-cell purification yields ~4-5x 10<sup>6</sup> cells/spleen

## WESTERN BLOT

Solutions:

**Pyronin 5X Sample Buffer:**

Materials:

- 40% glycerol (vol)
- 0.31 M Tris Base, pH 6.8 (Roche 604 203)
- 25%  $\beta$ -mercaptoethanol (wt) (Sigma M-6250)
- 20 mM EDTA (Sigma ED4SS)
- 12% SDS (wt) (Fisher BP166-100)
- 0.1% pyronin Y (wt) (Bio-Rad 16+0425)

1. Add 1.5 g. of Tris base in 10 ml. of H<sub>2</sub>O  
Actual pH \_\_\_\_\_
2. Add 4.8 g. of SDS and bring final volume up to 15 ml. with H<sub>2</sub>O
3. Heat gently to dissolve or leave at RT overnight
4. Add 8.9 ml. of  $\beta$ -mercaptoethanol (stock =1.2 g/ml)
5. Add 16 ml. of glycerol
6. Add 0.37 g. of Na<sub>4</sub>EDTA
7. Add 0.04 g. or less of pyronin and let stir at RT to dissolve
8. Aliquot solutions into eppendorf tubes. Keep one at RT for current uses and store rest at -20°C

**Running Buffer: (Tricine SDS Running Buffer)**

Dilute the 10X Tricine SDS running buffer (Novex, #LC1675) to 1X using H<sub>2</sub>O. Store at 4°C

**Transfer Buffer: (Tris-Glycine Transfer Buffer)**

To make 1L:

1. Add 140 ml. of MeOH to 100 ml. of 10X Tris-Glycine (Fisher, cat #BP1306-1 or GeneMATE, cat# 5560)
2. Bring up to volume with H<sub>2</sub>O. Store at 4°C

**PBS/Tween: (0.1% (wt) Tween-20 in 1X PBS)**

1. Dissolve 1 bottle of Dulbecco's Phosphate -Buffered Saline (Gibco, #21600-069) in 1L. of H<sub>2</sub>O
2. Add 10 g. of Tween-20 (Fisher, #BP337-500)  
⇒ weigh the PBS/Tween on the scale while adding the Tween-20
3. Dilute the 10X PBS/Tween using H<sub>2</sub>O to make a 1X solution

Materials:

- \_\_\_ 10-0.6 ml. eppendorf tubes
- \_\_\_ H<sub>2</sub>O
- \_\_\_ Standard (\_\_\_\_\_)
- \_\_\_ 4-12% Tris-Glycine gel (Novex, EC-6025)
- \_\_\_ Electrophoresis unit
- \_\_\_ 2 small staining trays
- \_\_\_ 1 large staining tray
- \_\_\_ wet transfer membrane (Millipore Immobilon-P Transfer, #IPVH00010)
- \_\_\_ Transfer cassette
- \_\_\_ stir bar
- \_\_\_ stir plate
- \_\_\_ 6 mm filter paper (Midwest Scientific, #6MW10X11)
- \_\_\_ non-fat dry milk
- \_\_\_ PBS/Tween
- \_\_\_ test tube

- \_\_\_ transfer unit
- \_\_\_ antibodies (\_\_\_\_\_)
- \_\_\_ chemiluminescent super signal reagent A and reagent B (Super Signal Substrate West Femto Maximum Sensitivity Substrate, Pierce, #99030170)

### *Day 1*

#### I. GEL LOADING

- \_\_\_ 1. Calculate the amounts needed on the Western worksheet for sample, water, and pyronin 5X
- \_\_\_ 2. Pipette the samples, water, and dye into 0.6 ml. eppendorf tubes labeled 1-10
  - ⇒ mix by gentle inversion followed by a quick spin in a minifuge
- \_\_\_ 3. Heat samples at 98°C in a Thermal Reactor for 5-10 min.
  - ⇒ place samples in middle of heat rack; the outside positions tend to heat up unevenly
- \_\_\_ 4. Quick spin on the minifuge to get all the condensation to the bottom of the tube
- \_\_\_ 5. Place the 4-12% Tris-Glycine gel onto the electrophoresis unit:
  - ⇒ mark the position of the wells with a sharpie pen and label them 1-10
  - ⇒ take off the tape and remove the comb from the precast gel
  - ⇒ align the 3<sup>rd</sup> grid onto the gasket of the unit
  - ⇒ use 2 clamps to fasten the gel onto the unit (if only 1 gel is used, put an alumina plate to block the other side of the unit)
- \_\_\_ 6. Transfer the unit into 4°C cold room and pour running buffer into the unit
  - ⇒ make sure to fill the space between the gels
- \_\_\_ 7. Load samples into the corresponding wells
  - ⇒ make sure there is no bubble in the pipette tip when dispensing the sample into the gel well
- \_\_\_ 8. Place the lid on the electrophoresis unit and connect the electrodes (red to red and black to black)
- \_\_\_ 9. Run gel at 30-60 mAmps for ~1 hour
  - ⇒ set voltage higher than the mAmp setting
  - ⇒ check the gel the 1<sup>st</sup> 5 min. and then 20 min. thereafter
  - ⇒ if 2 gels are running together, set the current between 60-120 mAmp
- \_\_\_ 10. Once the dye approaches the bottom of the gel, turn off the power supply and take the unit out of the cold room to sit beside the sink

#### II. BLOT

- \_\_\_ 1. Pour transfer buffer into a clean staining tray
- \_\_\_ 2. Remove the 2 clamps to loosen the gel from the unit; place the gel on the bench (large side down):
  - use a spatula to unseal the plates; carefully remove the gel
- \_\_\_ 3. Make a cut above the gel just above the 1<sup>st</sup> lane; cut and remove the area below the bottom dye band

- \_\_\_ 4. Gently transfer the gel from the cast (grab the gel from the bottom area) to the staining tray containing the transfer buffer
  - ⇒ place the gel the same side up as taken from the cast
- \_\_\_ 5. Wet the transfer membrane with MeOH for a few seconds in a separate staining tray
- \_\_\_ 6. Transfer the membrane into the staining tray containing the transfer buffer
- \_\_\_ 7. Place the transfer cassette into a big staining tray (gray side up): open the cassette and place the thick sponge on the bottom (black colored grid) and then the thin sponge on the top (gray colored grid)
- \_\_\_ 8. Pour the transfer buffer to cover the thick sponge
  - ⇒ pour a little extra in to cover the gel and membrane later
- \_\_\_ 9. Wet a 6 mm. filter paper with transfer buffer; place the paper onto the thick sponge
- \_\_\_ 10. Place the gel onto the filter (same side up; lane #1 is the right side)
- \_\_\_ 11. Place the wet membrane onto the gel and use the side of a glass test tube to roll out any bubbles
- \_\_\_ 12. Wet another 6 mm. filter paper in the transfer buffer and place it on the membrane as well; again, roll out any bubbles with a glass test tube
- \_\_\_ 13. Place the sponge onto the filter paper and close the transfer cassette on both sides
- \_\_\_ 14. Immediately, put the cassette into the transfer unit (black side facing the labeled “black side”)
- \_\_\_ 15. Fill the unit with transfer buffer with a stir bar
  - ⇒ transfer the unit to the 4°C cold room
  - ⇒ release any air bubbles in the cassette by gently dropping the unit on the counter
- \_\_\_ 16. Place the unit on a stir plate and run at 400 mA for 90 min.
- \_\_\_ 17. Towards the end of the transfer, prepare 4% nonfat dry milk/1X PBS/0.1% Tween in a staining tray (1.2 g. milk in 30 ml. PBS/Tween)
- \_\_\_ 18. After transfer has completed, take the unit out of the cold room and place it near the sink
- \_\_\_ 19. Remove the transfer cassette from the unit using the hook attached to the outside of the unit
- \_\_\_ 20. Place the cassette in a big staining tray (gray side up). Open the cassette, remove the thick sponge and filter paper; use a pencil to label lanes #1-10 on the membrane; cut a corner above lane #1

**FAST GREEN STAIN** (optional):

- a. place blot in fast green stain solution and swirl for a few seconds until protein bands are seen
- b. then dump solution back into container (solution is re-usable)
- c. add Destain solution to blot and swirl briefly. Repeat.
- d. leave blot in Destain solution and image
  - ⇒ use large lens (set at 2.7 at top aperture to let light in)
  - ⇒ place blot in bottom of machine (make sure tray is not in machine)
  - ⇒ select Quality One→File→Fluor-S → Select→ Densitometry→ Photograph
  - ⇒ with both lens and main door open, Position and Focus the blot without holding down the SHIFT key
  - ⇒ after blot is positioned and focused, turn top aperture from 2.7 to 22
  - ⇒ close both doors and Acquire image for 10 sec.
  - ⇒ save image on disk and print
- e. rinse blot briefly in PBS (until blot no longer smells like MeOH)



- f. Proceed to step 21 as usual  
 ⇒ milk will turn green, so replace solution ½ way through blocking
- \_\_\_ 21. Transfer the membrane into a staining tray containing 4% nonfat dry milk/1X PBS/0.1%Tween  
 ⇒ make sure membrane is protein side up
- \_\_\_ 22. Gently shake for at least 1 hour at RT
- \_\_\_ 23. Towards the end of the hour, prepare 20 ml. of 4% nonfat dry milk/1X PBS/0.1%Tween (0.8 g. milk in 20 ml. PBS/Tween) and place in a staining tray
- \_\_\_ 24. Before adding the transfer membrane, add \_\_\_ μl. of primary antibody (\_\_\_\_\_) to the tray and swirl to mix
- \_\_\_ 25. Transfer the transfer membrane to the antibody mixture and shake gently in the cold room overnight

### **Day 2**

- \_\_\_ 1. Remove the tray from the cold room and transfer the membrane into another tray containing PBS/Tween for a few seconds
- \_\_\_ 2. Pour off the PBS/Tween and add new PBS/Tween. Shake vigorously for 10 min.
- \_\_\_ 3. Repeat step 2.
- \_\_\_ 4. Towards the end of the last 10 minutes, prepare 30 ml. of 4% nonfat dry milk/1X PBS/0.1%Tween (1.2 g. milk in 30 ml. PBS/Tween) and pour into a staining tray
- \_\_\_ 5. Before adding the transfer membrane to the nonfat milk mixture, add \_\_\_ μl. of secondary antibody (\_\_\_\_\_) into the tray and swirl to mix
- \_\_\_ 6. Gently shake for at least 1 hour
- \_\_\_ 7. Transfer the membrane into a tray containing PBS/Tween for a few seconds
- \_\_\_ 8. Pour off the PBS/Tween and add new PBS/Tween and shake vigorously for 15 min.
- \_\_\_ 9. Repeat step 8
- \_\_\_ 10. Drain the membrane and place it between an acetate sheet
- \_\_\_ 11. Mix 0.5 ml. of chemiluminescent super signal reagent A and 0.5 ml. of reagent B in an eppendorf tube
- \_\_\_ 12. Lift the top acetate sheet and add the super signal reagent mix to the top of the membrane (position lane#1 on left side)
- \_\_\_ 13. Gently lower the acetate sheet back to cover the membrane/reagent mix. Allow it to incubate for 15 min.
- \_\_\_ 14. Cut out a new acetate sheet and place the membrane between the new sheet  
 ⇒ pipette up the supersignal from the wet sheet and add a drop or two to the dry sheet so that the membrane does not dry out too much
- \_\_\_ 15. Visualize the membrane on Fluor-S machine:
- a. Open the top compartment and raise the bottom shield

- b. Pull out the tray under the shield and place the membrane in the center (right side up)
- c. Click on Quality One icon and select File→Fluor-S
- d. Position and focus the membrane so that the image will be centered
- e. From bar menu, select Blot→Chemilluminent→High Resolution
- f. Close the top compartment and lower the shield
- g. Enter an appropriate time session (usually 480 seconds) and Retrieve

---

*Note: An immunoblot is a 3-step process used to separate proteins and then identify a specific protein of interest. The 1<sup>st</sup> step separates the protein mixture on a polyacrylamide gel. The proteins are then blotted onto a nitrocellulose membrane, which binds proteins tenaciously. The membrane is soaked in a 1<sup>o</sup>Ab solution specific for the protein of interest. The membrane is then developed in a solution containing a 2<sup>o</sup>Ab that binds to the 1<sup>o</sup>Ab-coated protein to form a “sandwich” of Ab molecules. The 2<sup>nd</sup> Ab is covalently linked to an alkaline phosphatase, which catalyzes a chromogenic reaction. Finally, a substrate is added and a deep purple precipitate forms, marking the band containing the desired protein.*

## FLOW CYTOMETRY FOR CELL SURFACE RECEPTOR EXPRESSION

*(from the Chapkin Lab, Lan Ly)*

\_\_ arrange procedure with Dr. Roger Smith (rosmith@cvm.tamu.edu) 845-3293; Vet Research Bldg, Room 114)

Materials:

\_\_ **mouse  $\alpha$ CD16/CD32** (Pharmingen, cat #01241D, 0.5mg/ml)

⇒ use 1  $\mu$ l. of stock solution/  $1 \times 10^6$  cells

\_\_ **7-AAD viability probe** (Pharmingen, cat #555816)

⇒ use 20  $\mu$ l of stock solution/  $1 \times 10^6$  cells

\_\_ **Wash Buffer** (1 XPBS + 0.5% BSA + 0.1% NaN<sub>3</sub>, pH 7.4) (for BSA, use fraction-free #100030 or 100018 (different size) from Boehringer Mannheim)

0.5 g. BSA (sprinkle on top and wait for it to dissolve, do not try to stir)

0.1 g. NaN<sub>3</sub>

Bring up to volume with PBS (100 ml)

Store at 4°C

\_\_ **ACK lysis buffer** (*from Handbook of Flow Cytometry Methods, pg. 145*)

1. To make a 10X stock solution:

8.2 g. NH<sub>4</sub>Cl

0.84 g. NaHCO<sub>3</sub>

0.37 g. EDTA

Bring up to volume with dH<sub>2</sub>O (100 ml. )

Store at 4°C for up to 6 months.

2. To make working 1X lyse solution:

Dilute 1:10 (10 ml. stock + 90 ml. dH<sub>2</sub>O)

\_\_ **Fluorescent IL-10R Ab**, Pharmingen (PE) #559914); **CD28-PE**, Pharmingen (#553297), **CTLA-4-PE**, Pharmingen (#553720)

a. dilute cell Ab 1:10 (→ 20 $\mu$ g/ml) in wash buffer (PBS + 0.5% BSA + 0.1% NaN<sub>3</sub>)

\_\_ Fluorescent Isotype control (PE) (Pharmingen #554685)

\_\_ **Quantibrite Beads (PE)** Becton-Dickinson # 340495

\_\_ 1. Resuspend cells =  $2 \times 10^6$ /ml. in RPMI

\_\_ 2. Place 500 $\mu$ l. (or  $1 \times 10^6$  cells) into each of pre-labeled 1.65 ml. tubes

(total of 30 tubes per culture time (3 diets x 5 mice/group x 2 mouse species)

⇒ make sure to include controls

- Only 7-AAD
- Only PE
- Isotype control

\_\_ 3. Add 1 $\mu$ l. of 0.5 mg/ml of  $\alpha$ CD16/CD32 to the cells. Gently vortex

⇒ this process blocks the Fc receptors, which prevents nonspecific binding of the fluorescent Ab to the cells. This eliminates false positive signals

\_\_ 4. Allow the cells to sit for 10 min. at RT

⇒ meanwhile, spin IL-10R and Isotype Ab tube at 16,000 x g for 20 min. @4°C to pellet aggregates

\_\_ 5. Add 20 $\mu$ l. of 7-AAD to each sample. Vortex gently and allow to incubate in dark at RT for 10 min.

⇒ these samples can no longer be fixed with paraformaldehyde

- \_\_\_ 6. Dilute fluorescent IL-10R Ab (stock: 200 $\mu$ g/ml) in wash buffer:
- 1:10 (20 $\mu$ g/ml)  $\Rightarrow$  360 $\mu$ l. Ab + 3240 $\mu$ l. wash buffer x2 (for a total of 7200 $\mu$ l.)
- \_\_\_ 7. Dilute fluorescent Isotype control Ab (stock: 200 $\mu$ g/ml) in wash buffer
- 1:10 (20 $\mu$ g/ml)  $\Rightarrow$  30 $\mu$ l. Ab + 270 $\mu$ l. Wash buffer x2 (for a total of 600 $\mu$ l)
- \_\_\_ 8. Add 200 $\mu$ l. of diluted fluorescent IL-10R or Isotype control Ab to appropriate tube except "control".  
Gently vortex (Final Ab conc. 4 $\mu$ g)
- \_\_\_ 9. Allow cells to incubate in the dark (wrapped in aluminum foil) for 30 min. at RT  
 $\Rightarrow$  meanwhile, prepare 1X ACK lysis buffer from 10X
- \_\_\_ 10. Spin cells at 300 x g for 5 min. at RT. Remove all but 100  $\mu$ l. of supernatant
- \_\_\_ 11. Add 1ml. of 1X ACK lysis buffer to lyse RBCs. Gently vortex and allow to incubate at RT, in the dark, for 10 min.
- \_\_\_ 12. Spin cells down at 300 x g for 5 min. at RT. Decant.
- \_\_\_ 13. Fill tubes with wash buffer and repeat step 12.
- \_\_\_ 14. Fill tubes with 300  $\mu$ l. wash buffer
- \_\_\_ 15 Prepare PE beads by adding 0.5 ml. wash buffer to tube and gently vortex:
- Values for lot # \_\_\_\_\_ :
- | <b>Bead level</b> | <b>Mean # of PE molecules/bead</b> |
|-------------------|------------------------------------|
| Low               | _____                              |
| Med-Low           | _____                              |
| Med-High          | _____                              |
| High              | _____                              |
- \_\_\_ 16. Wrap samples in foil and submit to Dr. Roger Smith for analysis
- bring zip disk to copy results file

---

*Notes: The flow cytometer will give the percentage of cells conjugated to FITC in each tube and thus, the percentage of each cell type.*

*FACS gates (the cells that the flow cytometer actually counts for analysis) are based on the light scattering properties which are inherent in specific cell types. Gates are set by the technician. A cell that binds a labeled Ab will fluoresce whereas one that does not, will not. A histogram will show that the non-T-cells produce a peak shifted to the left while the labeled T-cells produce a peak shifted to the right.*

## IL-10 ko Mouse Genotyping

(Chapkin Lab, Lan Ly)

### Materials:

- \_\_\_ Silver nitrate applicator (Grafc0 #1590)
- \_\_\_ DNeasy Kit (Qiagen # 69504)
- \_\_\_ PCR primers (IL10T1.4 (87), IL10AS (86), and NEO5 (88))
- \_\_\_ Platinum PCR Supermix (Invitrogen #11306-016)
- \_\_\_ Strip-Ease PCR tubes (Robbins Scientific #1044-01-9)
- \_\_\_ 0.5X TBE Buffer (for EtBr gels)
- \_\_\_ 4% NuSieve 3:1 Plus agarose gel (1X TBE Buffer + EtBr) (Reliant # 54927)
- \_\_\_ Scalpel, forceps
- \_\_\_ microcentrifuge tubes
- \_\_\_ LN<sub>2</sub>

### Tail snips

- \_\_\_ 1. Using a scalpel, snip ~0.5cm. of the mouse tail
- \_\_\_ 2. Place the tail into a labelled microcentrifuge tube and flash freeze in LN<sub>2</sub>  
⇒ sample can be stored at -80°C for later analysis
- \_\_\_ 3. Seal the mouse's tail by applying silver nitrate (hold for ~20 sec until no bleeding has stopped)

### DNA isolation

- \_\_\_ 1. Pre-heat a shaking water bath (Belly Dancer) to 55°C (use thermometer)
- \_\_\_ 2. Allow samples to come to room temperature
- \_\_\_ 3. Follow the DNeasy protocol for rodent tails from the DNeasy Qiagen kit  
⇒ make sure to keep everything RNase Free!
- \_\_\_ 4. DNA samples can be stored at -80°C or proceed with PCR

### PCR (keep everything RNase Free) (perform in PCR hood)

- \_\_\_ 1. Label PCR tubes for each sample (make sure to include positive and negative controls)
- \_\_\_ 2. For 50µl. reaction, pipet 45µl of Platinum Supermix into each tube
- \_\_\_ 3. Add 2µl. DNA sample to appropriate tubes
- \_\_\_ 4. For wild-type samples, pipet...
  - ❖ 0.4µl 86 primer (5'-GTG GGT GCA GTT GTC TTC CCG-3') (24 bp; IL-10T 2.2 anti-sense IL-10 primer)
  - ❖ 0.8µl. 87 primer (5'-GCC TTC AGT ATA AAA GGG GGA CC-3') (23 bp; IL-10T 1.4 anti-sense IL-10 primer)
- \_\_\_ 5. For knock-out samples, add...
  - ❖ 0.8µl 87 primer
  - ❖ 0.4µl 88 primer (5'-CCT GCG TGC AAT CCA TCT TG-3') (20 bp; IL-10 KO NEO 5 anti-sense strand)
- \_\_\_ 6. Cap PCR tubes tightly using the capping tool
- \_\_\_ 7. Briefly spin tubes down
- \_\_\_ 8. Run PCR reaction (GeneAmp PCR System)
  - ❖ Program: User→ Lan→Tails (~20min)
  - ❖ Once 20min is over for this program, immediately start Tails2 (~1hr)
  - ❖ Samples can be stored at 4°C

### Southern Blot (keep everything RNase Free!)

- \_\_\_ 1. For each sample, pipet 10µl sample + 1.1µl dye (10 X TAE) in a microcentrifuge tube
  - ❖ Include a ladder (Hyperladder IV (Bioline, 100-1000bp): 2µl ladder + 6µl H<sub>2</sub>O + 2µl 5X loading buffer)
- \_\_\_ 2. Spin tubes down briefly

- \_\_\_ 3. Heat samples to 65°C for 5min in heating block
  - ⇒ in the meantime, set up gel
  - Position 4% NuSieve EtBr agarose gel in gel unit (stored in walk-in freezer)
  - Cover gel with 0.5X TBE buffer
- \_\_\_ 4. Spin tubes down briefly
- \_\_\_ 5. Carefully pipet samples into gel wells
- \_\_\_ 6. Set power source at 200V (for lid, red plug is at bottom) and run gel for ~40min.
- \_\_\_ 7. Take gel out of plastic mold and image on Transilluminator (UV lamp)

## VITA

Lan H. Ly  
 10770 Wallingford Rd.  
 San Diego, CA 92126  
 Home Phone (858) 566-0413  
 E-mail address: lly@mad.scientist.com

### EDUCATION

December 2004 Ph.D Nutrition, Texas A&M University  
 Spring 2000 B.S. Biochemistry, University of California, San Diego (UCSD)

### EMPLOYMENT

1997-1999 Research Technician I, Amylin Pharmaceuticals, San Diego  
 1999-1999 Research Technician I, Cytovia, Inc., San Diego  
 1999-2000 Research Technician I, Ancile Pharmaceuticals, San Diego

### **GRADUATE PROJECT AND ACTIVITIES**

2003-2004: Student Representative on Texas Human Nutrition Conference  
 Planning committee (Texas A&M University)  
 2001-2004: Chair of American Society for Nutritional Sciences (ASNS)  
 Student Research Interest Section (RIS)  
 Fall 2002: Texas A&M University Teaching Assistant, Fundamentals of  
 Nutrition (NUTR 202)

### **PUBLICATIONS**

Fan, Y.Y, McMurray, D.N., **Ly, L.H.** and Chapkin, R.S. (2003). Dietary n-3 polyunsaturated fatty acids remodel mouse T-cell lipid rafts. *J. Nutr.* 133, 1913-1920.

Fan, Y.Y, **Ly, L.H.**, Barhoumi, R, McMurray, D.N, and Chapkin, R.S. (2004) Dietary docosahexaenoic acid suppresses T-cell protein kinase C- $\theta$  lipid raft recruitment and interleukin-2 production. *J. Immunol* (accepted).

**Ly, L.H.**, Switzer, K.S., Smith, R., Chapkin, R.S., and McMurray, D.N. (2004). Dietary n-3 polyunsaturated fatty acids modulate costimulatory regulation of murine CD4<sup>+</sup> T-cells. *J. Immunol.* (submitted).

**Ly, L.H.**, Chapkin, R.S. and McMurray, D.N. (2004). Dietary n-3 polyunsaturated fatty acids suppress splenic CD4<sup>+</sup> T-cells in IL-10 ko mice. *Clin. Exp. Immunol.*(accepted).