B CELL IMMUNOMODULATION AND FATTY ACID INCORPORTION OF DIETARY FISH OILS IN COLITIS-PRONE, SMAD3-/- MICE

By

Eric Anthony Gurzell

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ABSTRACT

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Over 30 million people report the use of fish oil supplements in the United States, where dietary fish intake remains low. Prescription fish oil is approved to treat high triglycerides; however, there is increasing evidence that consumption of fish oil may be beneficial for other diseases, such as cardiovascular and autoimmune diseases. It is generally accepted that these potential health benefits stem from omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) found in fish oil. n-3 LCPUFAs, notably eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), alter immune function and are proposed to be beneficial against chronic inflammatory conditions; however, observations across multiple models of pathogen-exposed animals suggest deleterious immunosuppression due to fish oil. The majority of research on fish oil and the immune system has focused primarily on T cells, monocytes, and dendritic cells of the immune system. However, the extent to which fish oil alters the function of B cells, a vital component of humoral immunity, remains relatively uninvestigated. Previous research demonstrated that DHA-enriched fish oil (DFO) exacerbated *Helicobacter hepaticus*-induced colitis in SMAD3-/- mice. Given the aberrant response to *H. hepaticus* in DFO-fed SMAD3-/- mice, it was hypothesized that fish oils alter humoral immunity, specifically B cell function. To test this hypothesis, SMAD3-/- mice were fed either control or various dietary fish oils, including non-enriched (e.g., menhaden oil [MO]) and

enriched (e.g., EPA- or DHA-enriched) fish oils, and then assessed for B cell development and function. The results indicate that the n-3 LCPUFA composition of fish oil mediates observed B cell immunological outcomes. All dietary treatments were found to markedly increase n-3 LCPUFAs and decrease n-6 LCPUFAs of red blood cell (RBC) and B cell phospholipid fatty acid profiles. B cells from SMAD3-/- mice fed MO and DFO dietary treatments share a number of mechanistic and immunological outcomes, such as decreased clustering of membrane microdomains concomitant with increased cytokine production in response to *ex vivo* stimulation. In contrast, EPA-enriched fish oil (EFO) had increased microdomain clustering, decreased expression of B cell surface markers, and *ex vivo* function. The n-3 LCPUFA composition of B cells was tightly correlated to RBCs phospholipid fatty acids, a common biomarker of n-3 LCPUFA exposure; however, it is unclear how levels of n-3 LCPUFAs in the blood relate to levels of n-3 LCPUFAs in other tissues, which may be more prognostic of health benefit at the tissue-level. This prompted the characterization of the phospholipid fatty acid profile of blood and gastrointestinal (GI) tissues, of colitis-prone, SMAD3-/- fed increasing amounts of EPA+DHA. Levels of EPA+DHA were chosen to mirror recommended intakes of n-3 LCPUFAs in humans. RBCs were highly correlated to the n-3 and n-6 LCPUFAs of all other GI; however, the absolute levels of n-3 and n-6 LCPUFAs varied considerably between blood and tissue. These findings confirm a novel role for B cell immunomodulation by dietary fish oil and provide further evidence that RBCs serve as a adequate, surrogate biomarker for n-3 LCPUFA incorporation. As an increasing popular dietary supplement, understanding the mechanistic and functional outcomes of fish oil consumption provide a foundation for research of more targeted fish oil therapeutics.

To my Parents,
Alan and Danette Gurzell

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KEY TO ABBREVIATIONS

%en	Percent of energy
AA	Arachidonic acid
ACK	Ammonium-chloride-potassium buffer
Ag	Antigen
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
CD	Crohn's Disease
CHD	Coronary heart disease
CTxB	Cholera toxin subunit B
CON	Control
d	Day(s)
DC	Dendritic cell
DFO	DHA-enriched fish oil
DHA	Docosahexaenoic acid
DRI	Dietary reference intakes
EFO	EPA-enriched fish oil
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
FACS	Fluorescent activated cell sorting
FAME	Fatty acid methyl ester
FBS	Fetal bovine serum

FITC	Fluorescein isothiocyanate
GC	Gas chromatograph
GI	Gastrointestinal
h	Hour(s)
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel disease
IL	Interleukin
LA	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid
LPS	Lipopolysaccharide
MHCII	Major Histocompatability Complex class II
MFI	Mean fluorescent intensity
min	Minute(s)
MO	Menhaden oil
MsLN	Mesenteric lymph nodes
MUFA	Monounsaturated fatty acid
n-3 LCPUFA	n-3 long chain polyunsaturated fatty acid
NBF	Neutral buffered formalin
NEFA	Non-esterified fatty acid
OVA	Ovalbumin
PBS	Phosphate buffered saline
PE	Phycoerythrin
PP	Peyer’s patches

R10	RPMI 1640 media + 10% FBS
RBC	Red blood cell
s	Second(s)
SFA	Saturated fatty acid
sIg_	Soluble immunoglobulin (A, E, G, and M)
Smad3	Mothers against decapentaplegic homolog-3
SPF	Specific pathogen-free
SpIn	Spleen
TGF-β	Transforming growth factor-beta
Th	T helper
TLR	Toll-like receptor
UC	Ulcerative Colitis
wk	Week(s)
wt	Weight

CHAPTER 1: INTRODUCTION, CHAPTER SUMMARIES, AND LITERATURE REVIEW

Introduction

Omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) found in fish oil are believed to be beneficial against inflammation-based pathologies, such as cardiovascular disease [1] and rheumatoid arthritis [2]. Despite the purported benefits of n-3 LCPUFAs, several scientific studies have reported increased severity of adverse outcomes associated with pathogen-exposed animals fed high levels n-3 LCPUFAs. Included in these studies is an observation by Woodworth *et al*. in 2010, reporting that DHA-enriched fish oil (DFO) resulted in exacerbated colitis due to infection with *Helicobacter hepaticus* in a dose-dependent manner [3]. This observation may be explained in part by the amount of n-3 LCPUFAs in the DFO (i.e. specifically DHA at 54% wt/wt), as they are usually present in much lower levels in non-enriched marine fish oil [4]. As more research on EPA and DHA highlight their unique mechanisms of action [5] and differing immunomodulatory effects[6], there is a clear need to distinguish how differing fish oils affect the immune system.

The research of this dissertation expands on the work of Woodworth et al. by further investigating the immunomodulatory effects of dietary fish oils in the SMAD3-/- mouse model. Specifically, early investigation into the exacerbated inflammation and dysplasia of the DFO-fed, *H. hepaticus*-infected SMAD3-/- mice suggested immune-dysfunction. One aim of this research was to identify immunomodulation by dietary fish oils in the absence of *H. hepaticus* infection in the SMAD3-/- mice. By interrogating

immunological changes due to fish oil feeding prior to infection, there is potential for this research to provide a better understanding of the immunological outcomes associated with the aberrant host response to *H. hepaticus* infection.

As a vital component of humoral immunity and integral to gut homeostasis [7], B cells represent a likely candidate whereby immune-dysfunction could lead to exacerbated infection of the gut. Despite research demonstrating that n-3 LCPUFAs may modulate B cell phenotype and function [8-10]; the majority of fish oil immunomodulation has focused on T cells, monocytes, and dendritic cells [6]. Thus, the guiding objective of this research was to determine the effects of dietary n-3 LCPUFAs on B cells in SMAD3-/-. Furthermore, as few studies have investigated whether the n-3 LCPUFA composition of the fish oil is important, a non-enriched fish oil (e.g., menhaden oil [MO]), EPA-enriched fish oil (EFO), and DFO was used. This research will describe the effects of three dietary fish oils with unique fatty acid composition on B cell phenotype and function in SMAD3-/- mice. Additionally, a complete characterization of the phospholipid fatty acid profile of B cells, blood, gastrointestinal tissues, and systemic organs was conducted. These data will provide a deeper understanding of n-3 LCPUFA immunomodulation of B cells, which could be used to direct research of potential n-3 LCPUFA therapeutics towards specific immunomodulatory outcomes.

Chapter Summaries

. The remainder of Chapter 1 will be dedicated to a literature review will address previous work from the Fenton lab and the potential immunomodulatory significance of dietary n-3 LCPUFAs with special emphasis on B cell function.. Collectively, chapter 2-4 and Appendix A represent prepared manuscripts for publication and dissemination in scientific journals.

Building upon previous work using DFO and SMAD3-/- mice, chapter 2 investigates the effect that DFO has on B cells from SMAD3-/- relative to a control (CON) fed diet. Specifically, the experiment evaluated the size of B cell membrane microdomains, as a potential mechanism by which DFO acts, and a number of B cell immunological parameters, including *ex vivo* B cell function and *in vivo* B cell phenotype. This chapter has been published in the *Journal of Leukocyte Biology* and can be found at http://www.ncbi.nlm.nih.gov/pubmed/23180828. The experimental design and methods established in this chapter are further expanded upon in Chapter 3.

Chapter 3 introduces alternative fish oils as a means to interrogate whether the fatty acid composition of dietary fish oils lead to differential immunomodulation of SMAD3-/- B cells. For this experiment, SMAD3-/- mice were fed either CON or one of three dietary fish oils, including MO, EFO, and DFO. B cell parameters that were assessed include: phospholipid fatty acid composition, membrane microdomains, *ex vivo* response to stimulation and functionality, and *in vivo* phenotyping of splenic and bone marrow-derived B cells. This chapter is currently under review at the *Journal of Nutritional Biochemistry.* The contrasting phospholipid fatty acid profile between red

blood cells and B cells observed in chapter 3, led to a line of questioning regarding the use of blood as a surrogate biomarker for n-3 LCPUFA levels in other tissues. While not surprising for humans, there is a dearth of literature in mice surrounding how well blood serves as a surrogate biomarker for the n-3 LCPUFA composition for other tissues.

As such, chapter 4 shifts focus toward the phospholipid fatty acid composition of blood and gastrointestinal tissues from SMAD3-/- that were fed diets modeling recommended intakes of EPA+DHA for humans. The phospholipid fatty acid composition of red blood cells (RBCs) were correlated and compared against plasma, stomach, small intestine, cecal, and colonic tissues. These data serve to confirm that dietary interventions of fish oil aimed toward gut-associated health outcomes are actually being incorporated into the tissue of interest and demonstrate whether the RBC is an adequate, surrogate biomarker for n-3 LCPUFA incorporation. This chapter has been published in *Prostaglandins, Leukotrienes, and Essential Fatty Acids* and can be found at http://www.ncbi.nlm.nih.gov/pubmed/24913088. The EPA+DHA diets established in chapter 4 were used in the same experimental design for Appendix A.

Chapter 5 briefly summarizes the research presented in chapters 2-4. There is a brief discussion on the results of n-3 LCPUFA immunomodulation of B cell and membrane reorganization. Future directions are considered to better understand the mechanistic transition from membrane to immunological consequences. Lastly, the research implications are related back to H. hepaticus-infected SMAD3-/- mice and overall conclusion.

Appendix A continues the characterization of the phospholipid fatty acid composition of systemic organs from SMAD3-/- mice. Specifically, the previously

reported phospholipid fatty acid profile of RBCs were compared and correlated against heart, skeletal muscle, spleen, lung, and adipose tissues. In addition to the n-3 and n-6 LCPUFA comparisons, the effect of EPA+DHA on various saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) was also evaluated. Similar to chapter 4, the data are presented in both raw and analyzed forms, enabling researchers to use the study data as reference values for other studies. This chapter is in preparation as a follow-up study in *Prostaglandins, Leukotrienes, and Essential Fatty Acids*.

Literature Review

Dietary n-3 LCPUFAs and Infection

With widespread consumption of fish oil supplements [11] and the proposed health benefits of n-3 LCPUFA sufficiency [1], there has recently been a call to establish dietary reference intakes (DRI) for n-3 LCPUFAs [12, 13]. These recommendations have been put forth with little attention given toward potentially excessive intakes, despite significant research suggesting that high dose n-3 LCPUFAs are associated with exacerbated, infection-related adverse outcomes [14]. Multiple animal models have demonstrated increased mortality and susceptibility to viral and bacterial infections when given high levels of dietary fish oil [15-19]. **Table 1** outlines a number of studies that observed adverse immunological outcomes due to n-3 LCPUFA intake in animals models of infection [14]. The majority of these studies have used fish oil derived from MO as the exposure of n-3 LCPUFAs.

Table 1 Evidence demonstrating high dietary EPA/DHA induced immune anergic response to pathogens (since 2000).

Model[1]; Pathogen	(DHA+EPA)/100g[2]	Observed Effects	Reference
BALB/c mice; ***Influenza*A/Queensland/6/72**	5.1%	↓ T cell cytotoxicity ↑ Spleen and alveolar lymph proliferation	Byleveld et al. Clin Exp Immunol. 2000 Feb;119(2):287-92. [20]
BALB/c mice; ***Herpes simplex virus type 1 Strain F***	4.4%	↑ Stromal keratitis ↑ Viral infectivity, DTH	Courreges et al. Nutr Res. 2001 Apr; 21 (1-2):229-41 [19]
BALB/c mice; ***L. monocytogenes***	5.0%	↓ Survival ↓ Clearance	Irons et al. J Nutr. 2003 Apr;133(4):1163-9 [21]
BALB/c mice; ***P. brasiliensis***	2.7%	↓ Antifungal Activity (DHA only)	Oarada et al. Biochim Biophys Acta. 2003 Aug 22;1622(3):151-60 [22]
Yorkshire x Landrace piglets; ***Influenza A/Puerto Rico/ 8/34***	0.34%	↓ T cell proliferation ↑ IL-10	Bassaganya-Riera et al. Am J Clin Nutr. 2007 Mar;85(3):824-36. [23]
BALB/c mice; ***L. monocytogenes*** ± cyclophosphamide (CPA)	4.4%[3]	↓ Survival ↑ Bacterial Load (spleen and liver) Fish oil exacerbates CPA effect	Cruz-Chamorro et al. Clin Nutr. 2007 Oct;26(5):631-9 [17]
B6C3F1 mice; ***Reovirus T1L***	4.1%	↓ Early viral clearance	Beli et al. J Nutr. 2008 Apr;138(4):813-9 [24]
Hartley Strain Guinea Pigs; ***M. tuberculosis***	0.80%	N.S. Lung pathology *vs.* control ↓ DTH ↑ Bacterial Load (lung)	McFarland et al. J Nutr. 2008 Nov;138(11):2123-8 [18]
C57BL/6J mice; ***Influenza A/Puerto Rico/ 8/34***	1.0%	↓ Survival, Weight Recovery ↓ Lung Histopathology	Schwerbrock et al. J Nutr. 2009 Aug;139(8):1588-94. [16]
Fat-1 (C57BL/6) mice; ***M. tuberculosis***	~0%[3]	↑ Bacterial Load (spleen and liver) ↓ Pulmonary Inflammation ↓ TNF-α	Bonilla et al. J Infect Dis. 2010 Feb 1;201(3):399-408. [25]
Wistar rats; ***S. enteritidis***	5.8%	↓ DTH ↓ IFN-y, IgG2b	Snel et al. 2010 APMIS. 2010 Aug;118(8):578-84 [26]
129-Smad3^{tm1Par}/J mice; ***H. hepaticus***	0.44% – 3.5%	↓ Survival ↑ Colon Inflammation & dysplasia	Woodworth et al. Cancer Res. 2010 Oct 15;70(20):7960-9 [3]

[1] All animals were 3-10 weeks old at the start of study and fed EPA/DHA enriched diets for 2-8 weeks prior to challenge

[2] Unspecifed fish oil was assumed to be menhaden oil. EPA+DHA was estimated using USDA Nutrient Database

[3] Transgenic (expresses n3 desaturase) mouse capable of endogenously synthesizing n-3 PUFAs from n-6 PUFAs

SMAD3-/- colitis-prone mice and DHA-enriched fish oil

Previous work in the Fenton lab by Woodworth et al. demonstrated that DFO exacerbates pathology in the SMAD3-/- mouse model of infection-induced colitis [3] (Experimental design provided in **Figure 1**). SMAD3 is involved in TGF-β signaling, ultimately regulating gene transcription [27]; however, SMAD3 is functionally redundant with SMAD2 [28] and the SMAD3 knock-out is non-lethal with SMAD3-/- mice remaining free of colitis and tumors for up to 9 months. Integral to the SMAD3-/- model, when infected with *Helicobacter hepaticus,* SMAD3-/- mice develop severe colitis with subsequent adenocarcinoma [29]. This is in contrast to their colitis-resistant heterozygote (SMAD3+/-) and wild-type (SMAD3+/+) counterparts [29]. As there is increasing appreciation for infection-associated cancers [30], the SMAD3-/- mouse model of infection-induced colitis provides a unique opportunity to simultaneously investigate dietary immunomodulation by n-3 LCPUFAs, while investigating the potentially adverse effect that n-3 LCPUFAs have on infection-based pathologies [31].

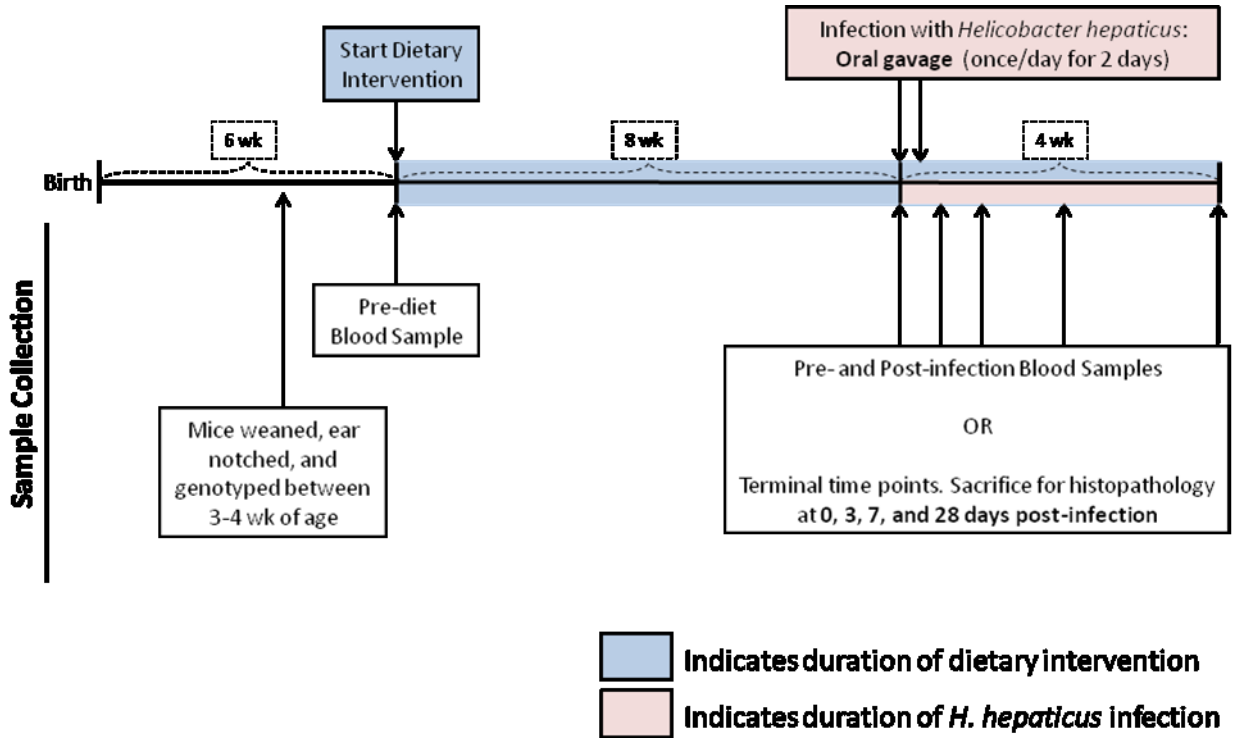


Figure 1 Experimental design for fish oil feeding studies in SMAD3-/- mice infected with *Helicobacter hepaticus*. Mice were genotyped and weaned between 3 – 4 wk of age and placed on standard AIN-93G based pelleted chow (Harlan Teklad; TD.94045) prior to experimental diets. Mice were then continued on an AIN-93G based diet (CON) or fed a diet whereby 6% (by wt) of the dietary fat was replaced with either Menhaden fish oil (MO), EPA-enriched fish oil (EFO), or DHA-enriched fish oil (DFO) for 8 wk. Mice were then infected with *H. hepaticus,* continued on the experimental diets, and sacrificed for tissue collection and further analysis.

Indeed, Woodworth et al. observed that fish oil feeding resulted in increased inflammation and dysplasia in the colon and cecum of fish oil-fed SMAD3-/- mice infected with *H. hepaticus* [3]. Even prior to infection, DFO feeding resulted in decreased CD8+ T cells in the spleen and mesenteric lymph nodes, increased splenic CD4+ T cells, greater frequency of splenic CD4+/CD25+/FoxP3+ regulatory T cells, and decreased L-selectin on T regulatory cells. The authors concluded that DFO-feeding resulted in an immunosuppressed host environment and speculate that diminished expression of L-selectin may have prohibited T regulatory cell migration to the site of infection (i.e. decreasing capacity for immunoregulation). Interestingly, immunohistochemistry staining for T regulatory cells in the colon of DFO-fed SMAD3-/- mice at 4 wk post-infection provide strong evidence that T regulatory cell migration to the site of infection is not impaired (**Figure 2A**). Furthermore, semi-quantitative enumeration of CD4+ (Figure 2B) and FoxP3+ (Figure 2C) stained cells reveal that the highest DFO treatment actually had significantly higher T regulatory cells in the colon compared to controls.

A

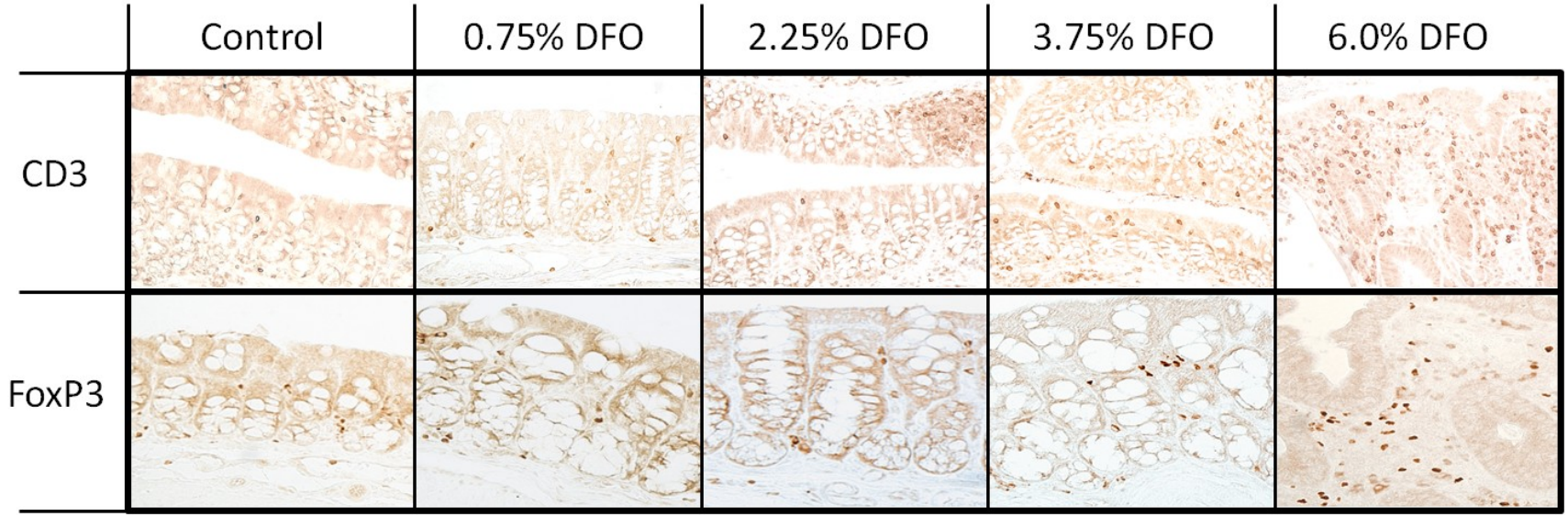
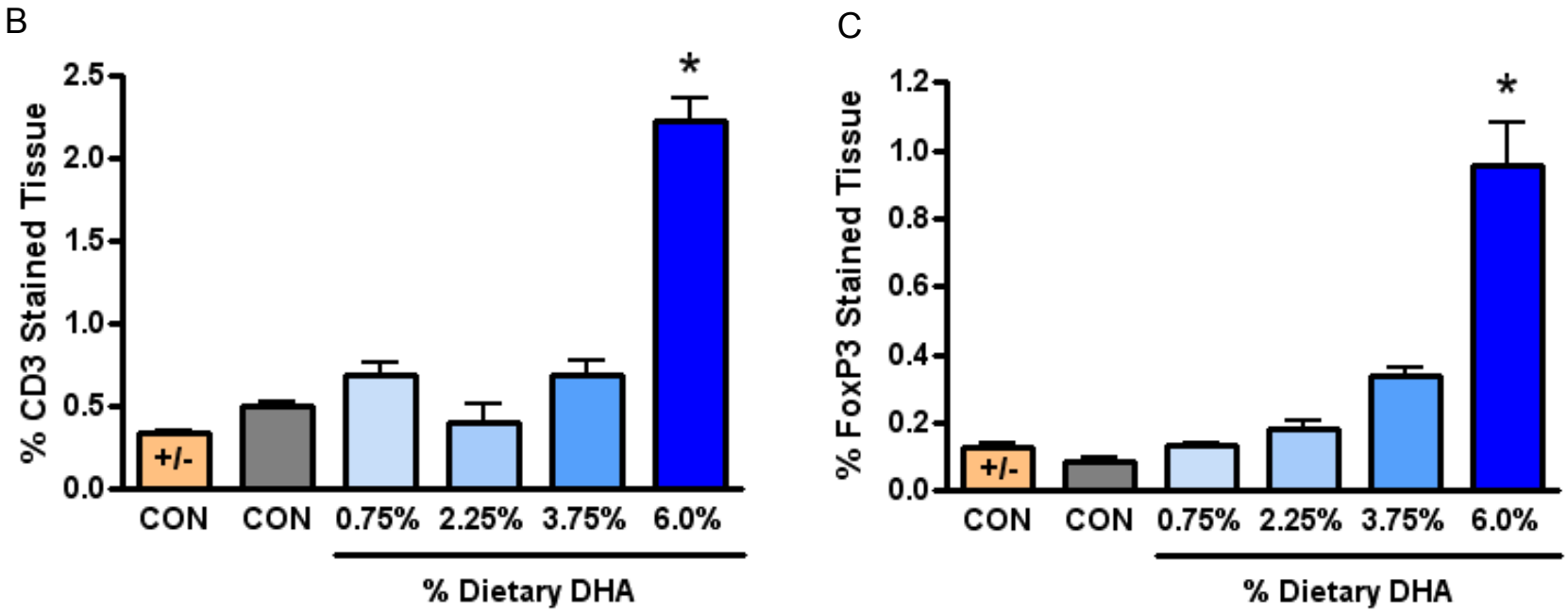


Figure 2 Detection and semi-quantification of CD3+ and Foxp3+ cells by immunohistochemistry in SMAD3-/- mice infected by *H. hepaticus*. SMAD3-/- mice were fed either standard chow (CON) or increasing amounts of DHA-enriched fish oil (DFO) for 8 wk prior to infection with H. hepaticus and for the 4 wk duration of the infection. (A) Representative photos of proximal colon stained for CD3 (Top panels) or FoxP3 (Lower panels) 4 weeks post-infection. Quantification of (B) CD3+ and (C) Foxp3+ staining by immunohistochemistry in SMAD3 -/- mice infected by *H. hepaticus*. Thresholding by intensity provided pixels that were positive for the associated stain, which was taken as a percentage of total tissue assessed. A one-way ANOVA and Tukey's post-hoc analysis was used to assess differences across diets compared to CON fed SMAD3-/- mice. Asterisked data denotes statistical significance: * $P < 0.05$ level.

Figure 2 (cont'd)



Fish Oil and Colitis

When fish oil is administered in other animal models of colitis, results are inconsistent. In models of chemically-induced colitis there appears to be some agreement in the efficacy of fish oil to reduce inflammation [32], however it is unclear if chemically-induced colitis models initiation or recurrence of colitis. Results in other models of colitis that arise out of genetic susceptibility are less consistent. Use of the IL-10-/- mouse model, fish oil at 7% (wt/wt) exacerbated spontaneous colitis and neoplastic development in one study [33], but at 4% (wt/wt) decreased severity of inflammation when non-steroidal anti-inflammatory drugs were used to accelerate colitis [34]. Differences in the pathogenesis of colitis of these animal models are likely to be responsible for the heterogeneous outcomes observed with fish oil treatment. These discrepancies highlight the need to assess dietary n-3 LCPUFAs in multiple models of disease to best understand the immunological outcomes.

Fish Oil and Inflammation

Fish oil supplementation is increasing due to the purported health benefits, notably for the prevention of cardiovascular disease [35, 36] and more recently as a prescription therapy for treating hypertriglyceridemia [37]. These beneficial outcomes are thought to be due to the 'anti-inflammatory' effects of n-3 LCPUFAs found in fish oil, specifically EPA and DHA [38]. Research has shed light on differential mechanisms by which EPA vs. DHA may act and exert differential effects (Reviewed in [39, 40]). Several proposed mechanisms of n-3 LCPUFAs include eicosanoid production [6, 41], generation of pro-resolving mediators such as resolvins [42, 43], altered gene

expression [44, 45], and re-organization of the plasma membrane [46-48]. Furthermore, the composition of fatty acids of the immune cell membrane is capable of altering immunological outcomes [49], such as decreased T cell proliferation and decreased antigen presentation in DCs. With specific focus on humoral immunity regarding the SMAD3-/- mouse model, the effects of dietary n-3 LPCUFA on B cells remain relatively understudied compared to T cell, monocyte, and dendritic cells.

Ontogeny of B Cells

B cells are a vital component of the adaptive immune system and humoral immunity, whose defining hallmark is antibody production [7]. While there are two main sub-sets of B cells, including innate-like B-1 B cells and conventional B-2 B cells, the predominant population of B cells are B-2 and reside in the spleen and lymph nodes [50]. B-2 B cells, from herein referred to simply as B cells, originate from Hematopoietic Stem Cells (HSCs) found in the bone marrow. Progression of HSCs to the earliest, 'committed' B cell [pre-pro-B cell] has been reviewed elsewhere [50], but is most notably associated with acquisition of surface CD127 in Common Lymphoid Progenitors and CD45R (B220 in B cells) in pre-pro-B cells. In addition to survival signals (e.g., via IL-7 signaling), successful V(D)J recombination of Immunoglobulin Heavy Chain and assembly of pre-B Cell Receptor yield pre-B cells. Pre-B cells immediately undergo V(D)J recombination of Immunoglobulin Light Chain and successful recombination and assembly of functional surface IgM (sIgM) yield immature B cells [51]. B cells emigrate from the bone marrow (sIgM-expressing B cells) and continue in transitional, immature states during travel throughout the periphery and eventually mature in the splenic B cell

follicles (conventional acquisition and co-expression of sIgD w/ sIgM). Flow cytometric phenotyping of different stages B cell development in the bone marrow and periphery is described in greater detail chapter 3.

B cells and Membrane incorporation

Despite growing appreciation for B cell contribution to the inflammatory milieu [52], there has been little research into the effect that n-3 LCPUFAs elicit on B cell development/function or the mechanisms involved. Recent studies published by the Shaikh lab suggest that B cell function may be enhanced (e.g., increased cytokine production, upregulation of cell surface markers) after dietary n-3 LCPUFA exposure [53]. One potential mechanism through which n-3 LCPUFAs are thought to act is through reorganization of the lipid membrane [53]. The majority of peripheral B cells are phenotypically mature and have a half-life of approximately 5 weeks [54, 55]; thusly, turnover and generation of peripheral B cells from the bone marrow is a continual process. Traveling as chylomicrons and lipoproteins, dietary lipid, predominantly in the form of triglycerides, is hydrolyzed in the bone marrow to form free fatty acids for use/incorporation into the membrane hematopoietic stem cells. There is significantly limited conversion of α-linolenic acid (ALA) to EPA and DHA [56] when considering the circulating lipid profile. While initial lipid incorporation occurs during growth and division of HSCs in the bone marrow [57], there is a role for CD36 (fatty acid translocase) in circulating B cells [58] and a need for fatty acid uptake to support membrane biogenesis in plasma cells in the periphery. Free fatty acids are transported to and accumulate in the endoplasmic reticulum, where initial processing of fatty acids occur. With specific

interest to this research, arachidonic acid (AA), EPA, and DHA are preferentially packaged into phospholipids and localized at the sn-2 position [59]. It has been shown that that fish oil feeding increases results in increased EPA and DHA content in the plasma membrane of B cells [53], being incorporated into phosphotidyl-ethanolamine, -serine, and -choline fractions, as well as sphingomyelin in both detergent resistant and detergent soluble membrane fractions. Further complicating distribution of n-3 LCPUFAs in the plasma membrane, there is evidence to support differing affinities of EPA and DHA for raft and non-raft domains [60, 61]. Alterations in membrane organization are thought to alter many pleiotropic outcomes, including substrate available for eicosanoid synthesis, gene transcription, and cell signaling through microdomain formations. Considering that many signaling B cell surface markers preferentially localize within lipid rafts, e.g., dimerization of TLR4 and translocation of BCR in mature B cells [62], investigation of the effect that n-3 LCPUFAs have on B cell functional outcomes is warranted.

B cell and n-3 LCPUFAs

Current research foci into the immunomodulatory effects of n-3 LCPUFA include potential effects of T lymphocytes and DCs; however it is less clear the extent to which n-3 LCPUFAs are capable of modulating immunological outcomes of B cells [46]. In vitro studies of n-3 LCPUFA exposed Raji B cells are associated with decreased cytokine production and decreased proinflammatory gene expression [8, 63]. These observations appear in concordance with EL4 B cells [10] and Ba/F3 pro-B cells [64]; however, one study demonstrated differential immunological outcomes when comparing

in vitro vs. *ex vivo*. The authors observed that *ex vivo* LPS-stimulated B cells from fish oil fed mice had increased proinflammatory cytokines and surface expression of CD69 relative to control [10].

The primary goal of this research was to provide mechanistic insight into the immunomodulatory effects of dietary fish oils on *ex vivo* and *in vivo* B cell phenotype and function. Uninfected, healthy SMAD3-/- were utilized as a logical extension from previous findings in the Fenton lab. This research attempts to bridge putative mechanisms with observed immunological outcomes by exploring organization of membrane microdomains across dietary fish oils differing in n-3 LCPUFA composition.

CHAPTER 2: DHA-ENRICHED FISH OIL TARGETS B CELL LIPID MICRODOMAINS AND ENHANCE IN VIVO AND EX VIVO B CELL FUNCTION

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Abstract

Docosahexaenoic acid (DHA) is a long chain n-3 polyunsaturated fatty acid (LCPUFA) in fish oil that generally suppresses T lymphocyte function. However, the effect of fish oil on B cell function remains relatively understudied. Given the important role of B cells in gut immunity and increasing human fish oil supplementation, we sought to determine whether DHA-rich fish oil (DFO) leads to enhanced B cell activation in the SMAD3-/- colitis prone mouse model similar to that observed with C57BL/6 mice. This study tested the hypothesis that DHA from fish oil is incorporated into the B cell membrane to alter lipid microdomain clustering and enhance B cell function. Purified splenic B cells from DFO fed mice displayed increased DHA levels and diminished GM1 microdomain clustering. DFO enhanced LPS-induced B-cell secretion of IL-6 and TNF-α and increased CD40 expression *ex vivo* compared to control. Despite increased MHCII expression in the unstimulated *ex vivo* B cells from DFO fed mice, we observed no difference in *ex vivo* OVA-FITC uptake in B cells from DFO or control mice. *In vivo*, DFO increased lymphoid tissue B cell populations and surface markers of activation

compared to controls. Finally, we investigated whether these *ex vivo* and *in vivo* observations were consistent with systemic changes. Indeed, DFO fed mice had significantly higher plasma IL-5, IL-13, and IL-9 (Th2-biasing cytokines) and cecal IgA compared to control. These results support the hypothesis and an emerging concept that fish oil enhances B cell function *in vivo*.

Introduction

The n-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) found in fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are widely recognized as being significant regulators of inflammation with notable health benefits. Epidemiological studies provide evidence for the nutritional benefit of EPA and DHA in inflammation-associated pathologies, such as cardiovascular disease and rheumatoid arthritis [13]. Despite abundant *in vitro* research on the effects of n-3 LCPUFAs and inflammation, our understanding of dietary n-3 LCPUFAs on *in vivo* immunological outcomes is limited. Furthermore, there is a current gap in the literature on the effects of dietary n-3 LCPUFAs on B cell function.

B cells serve as a vital component of the immune system and the absence of B cells in the immune system results in recurrent infection from childhood on, commonly in the respiratory and gastrointestinal tracts [65]. While secretion of antibodies is a defining hallmark of B cell function, there is a growing appreciation for their contribution to inflammation via innate cytokine production [66-68]. It is increasingly apparent that B cell dysfunction can significantly contribute to the inflammatory milieu and perpetuate

disease. Indeed, Rituximab treatment is clinically used to deplete B cells in chronic inflammatory diseases, showing potentially beneficial outcomes in non-autoantibody inflammatory pathologies, such as rheumatoid arthritis [69], multiple sclerosis [70] and Sjögren's syndrome [70]. Identification of antibody-independent mechanisms through which B cells contribute to inflammatory pathogenesis remains understudied. However, patients with ulcerative colitis and Crohn's disease have aberrant, increased expression of receptors on B cells, such as CD40 and B7, triggering inflammatory pathways [71], highlighting a putative pathway for B cell contribution to inflammatory pathogenesis.

Several proposed mechanisms of n-3 LCPUFAs include eicosanoid production, generation of pro-resolving mediators such as resolvins, altered gene expression, and influencing plasma membrane organization [43, 48]. Altered organization of the lipid membrane is an emerging and complex mechanism by which n-3 LCPUFAs impact immune cell function [72, 73]. Dietary fatty acid intake influences the fatty acid composition of the plasma membrane and can therefore modify lymphocyte function; in part, through the formation of sphingolipid/cholesterol-enriched lipid microdomains [74, 75]. Membrane organization may be of particular importance for signaling, as observed in the immunological synapse [76]. Recent studies have demonstrated that changes in lipid microdomain organization due to fish oil exposure were observed in parallel to altered immunological outcomes with B cells [10]. In contrast to an immunosuppressive phenotype observed in multiple *in vitro* experiments, the *ex vivo* changes in lipid microdomains were associated with an increase in TNF-α, IL-6 and IFN-γ production by B cells when stimulated with lipopolysaccharide (LPS) [53]. These findings highlight the

need to investigate *in vivo* immunological consequences of dietary fish oil consumption on B lymphocytes in several animal model systems.

Given the important role of B cells in gut immunology and increasing human supplementation and consumption of fish oil, we investigated the effect of dietary fish oil on B cells in a colitis-prone model. The natural progression of our work with the SMAD3-/- mouse model has led us to investigate if previous observations with dietary fish oil on B cell function in C57BL/6 mice [53] remain consistent in the SMAD3-/- mouse model. We first determined if dietary fish oil altered lipid microdomain clustering in the SMAD3-/- mouse. Then, we tested the hypothesis that dietary DFO targets both *ex vivo* and *in vivo* B cell function in SMAD3-/- mice.

Materials and Methods

Materials and chemicals

ACK lysing buffer was purchased from Invitrogen (Carlsbad, CA), RPMI medium 1640 was purchased from Sigma Aldrich (St. Louis, MO) and fetal bovine serum (FBS) was purchased from Gibco (Rockland, MD). Boron trifluoride-methanol for fatty acid methylation was purchased from Sigma Aldrich (St. Louis, MO). Standards and the RT®-2560 column for gas chromatography were purchased from Restek (Bellefonte, PA). The following fluorescent antibodies (clone) were purchased from eBiosciences, including B220 (RA3-6B2), MHCII (M5/114.15.2), and CD40 (1C10) (St. Louis, MO). Purified CD16/CD32 (2.4G2) and the following fluorescent antibodies (clone) were purchased from BD Biosciences, including CD11c (HL3), MHCII (2G9), CD8a (53–6.7),

CD11b (M1/70), Gr1 (RB6-8C5), CD80 (16-10A1), and CD86 (GL1) (San Diego, CA). Chicken ovalbumin conjugated to fluorescein (OVA-FITC) used for the antigen uptake assay was purchased from Molecular Probes (Eugene, OR). Cholera toxin subunit-B (CTxB) conjugated to fluorescein and anti-CTxB used for lipid microdomain staining were purchased from Life Technologies (Carlsbad, CA). LPS for the stimulation assay was purchased from Sigma Aldrich (St. Louis, MO).

Murine model

Smad3+/- and *Smad3-/-* breeder pairs (129-Smad3^{tm1Par}/J) were generated in house. Homozygous males and heterozygous females were mated to obtain *Smad3-/-* pups. Genotypes were confirmed by PCR. Mice were housed under SPF conditions in 152.4 cm^2 plastic cages (maximum of 5 adult mice per cage) with microisolator lids in an AAALAC approved facility at Michigan State University. SPF conditions were assured through quarterly serology testing by Charles Rivers (Wilmington, MA) and in-house testing for ectoparasites, endoparasites and fecal *Helicobacter* species (PCR). Full necropsies (including culture and sensitivity) were performed at least yearly on rodent breeding colonies. Animal rooms were maintained at 23.3 ± 2.2°C with a 12-h light-dark cycle. Mice were fed non-purified diets and sterile water *ad libitum*. Animal protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Experimental design and diet composition

Thirty-eight SMAD3-/- mice (age 4-6 weeks) were fed either an AIN-93G based (CON) standard chow (n=18) or a DHA-enriched fish oil (DFO) diet (n=20) of similar

composition to that previously described [3]. **Figure 3** outlines the experimental design. Briefly, the AIN-93G based standard chow was purchased from Harlan Teklad (TD.94045; Haslett, MI). The AIN-93G based pre-mix diet without fat was purchased from Dyets Inc. (Bethlehem, PA). The DHA-enriched fish oil (DHA4E1400 MEG-3^{TM} Fish Oil) used in the treatment diet was generously donated by Ocean Nutrition Canada (Dartmouth, Nova Scotia). Differing only in fat composition, both diets contained 7% fat by weight, providing 17% kcal from fat (0.637 kcal/g). The CON diet contains 7% (wt) soybean oil and the DFO diet contains 1% (wt) corn oil (0.091 kcal/g) and 6% (wt) DHA-enriched fish oil (0.546 kcal/g). The DFO added to the treatment diet consisted of 54% DHA and 20% EPA contributing 3.2% from DHA (0.294 kcal/g) and 1.2% from EPA (0.109 kcal/g) to the total 7% fat diet. Furthermore, the certificate of analysis also indicates that this food grade fish oil is free of contaminants and oxidation. **Table 2** shows the fatty analysis of the prepared diets, which was analyzed with gas chromatography as described below. Animals were fed the experimental diets *ad libitum* for 5 weeks. At the end of the experiment, mice were asphyxiated with CO_2 and exsanguinated through cardiac puncture using a heparin-coated syringe. Blood was collected on ice and centrifuged to obtain a red blood cell fraction and a plasma fraction to assess plasma cytokines.

B-cell purification

Spleens were harvested from SMAD3-/- mice fed either the CON or DFO diet and immediately placed in ice-cold RPMI supplemented with 10% FBS. The spleen was processed using a dounce homogenizer, pelleted, and washed in RPMI as previously described [3]. Cells were briefly resuspended in ACK lysing buffer for red blood cell

lysis, washed twice in RPMI, and passed through 70 μm filters. Cell counts were performed with a hemacytometer using trypan blue exclusion and resuspended to a concentration of 2 x 10^7 cells/mL media. Negative selection was used to isolate a purified B cell population using BD IMag Cell Separation as per the manufacturer's protocol for their B Lymphocyte Enrichment System (BD Biosciences, San Diego, CA). Briefly, biotinylated antibodies against Ter-119, CD4, and CD43 were used to label unwanted cells (non-B cell populations) followed by subsequent incubation with streptavidin-magnetic beads. Lastly, incubation with the supplier's magnet separated the unwanted cells and permitted collection of the remaining B cells. Cells were counted using Trypan blue exclusion on a hemacytometer, resuspended at 1 x 10^6 cells/mL, and stored on ice. The enriched population of cells was >90% B220 positive, confirming the B cell purification (**Figure 4**).

B-cell fatty acid analysis

Lipid composition of purified B-lymphocytes was determined using gas chromatography. Total lipids were extracted from a minimum of 4 x 10^6 purified B-lymphocytes using the Folch method [77]. Fatty acids were first methylated using boron trifluoride-methanol for 90 min at 100°C followed by extraction of the fatty acid methyl esters (FAMEs) into hexane and separated by a 2010 Shimadzu capillary GC (Shimadzu Scientific Instruments, Columbia, MD) with a Restek RT®-2560 column. The retention times of standards were used to identify FAMEs peaks. The areas under the FAME peaks were summed and individual peak areas were expressed as a percentage of total peak area for a given treatment. All experiments were conducted with HPLC

grade organic solvents and using stringent precautions to avoid oxidation as previously described [53].

B-cell lipid microdomain staining

B cell lipid microdomain cross-linking was performed as previously described [9]. Cholera subunit B (CTxB) conjugated to FITC (CTxB-FITC) was diluted as per manufacturer's protocol. An aliquot of 1 x 10^6 purified B cells from each sample was stained with CTxB-FITC for 10 min, washed in PBS, and subsequently stained with anti-CTxB for 15 min to induce cross-linking of GM1 molecules. Samples were washed in PBS prior to fixation for 24 h in 4% formaldehyde. The samples were loaded into VitroTubes™ (VitroCom, Mountain Lakes, NJ) and mounted onto microscope slides for visualization. Using an Olympus IX81 inverted microscope and a 100X objective, fluorescent images were obtained for 10 cells per sample. Laser settings, detector, and image acquisition settings were saved and kept constant between samples. All analyses of lipid microdomains were conducted with blinded samples.

LPS stimulation of purified B-cells

2 x 10^6 purified B cells per sample were stimulated in 2 mL of LPS-containing media overnight. Media for overnight cultures were RPMI 1640 supplemented with 5% FBS, 1% Penicillin/Streptomycin, 2 mM L-glutamine, 500 nM β-mercaptoethanol, and 1 μg/mL LPS. Cells were cultured at 37°C and 5% CO_2 in a sterile incubator for 24 h. Cells were then placed on ice, centrifuged at 300xg and 4°C for 5 min, and supernatants were divided into aliquots and frozen at -80°C.

LPS-stimulated B cells were washed and resuspended in FACS buffer followed by blocking with anti-Fc receptor γII/II for 10 min on ice. The stimulated cells were subsequently stained for B220, MHCII, CD40, CD80, and CD86. All flow cytometric analyses were performed on a FACSCanto II (BD Biosciences, San Diego, CA) and analyzed using FlowJo (TreeStar, Inc, Ashland, OR).

Supernatant cytokine production of IL-6, IFN-γ, and TNF-α were determined using MILLIPLEX^{TM} MAG Mouse Cytokine/Chemokine panel (Millipore, Billerica, MA) as per the manufacturer's protocol. Briefly 25 µL of standards, quality controls, and supernatant samples were loaded onto 96-well plates followed by addition of 25 µL antibody-bound beads and 25 µL of assay diluent and incubated at 4°C overnight on an orbital shaker. The next day, plates were washed and incubated with 25 µL of detection antibody for 1 h at room temperature. Afterward, plates were washed and incubated with streptavidin-phycoerythrin for 30 min at room temperature. Lastly, plates were washed, beads resuspended in sheath fluid, and read on a Luminex 200 system.

Antigen uptake assay

Antigen (Ag) uptake was measured in B cells isolated from the spleen as previously described [78]. FITC labeled-ovalbumin (OVA-FITC) was used as the model Ag. Briefly, 1 x 10^6 B cells were incubated in polystyrene tubes containing 100 µL of 50 µg/mL OVA-FITC in RPMI 1640 supplemented with 10% FBS. At the end of the incubation period, cells were placed on ice and diluted with 2 mL FACS buffer to suspend metabolic processes, including uptake. Cells were washed and resuspended in FACS buffer for further staining. All samples were stained for B220 to confirm purity. All flow cytometric analyses were performed as described above.

In vivo B-cell analysis

For *in vivo* lymphocyte analysis, spleens, mesenteric lymph nodes, and Peyer's patches were removed and placed in ice-cold RPMI at the time of necropsy. Tissues were processed with a dounce homogenizer, pelleted, and washed in RPMI as previously described [3]. Cells were briefly resuspended in ACK lysing buffer, washed twice in RPMI, and passed through 70 μm filters. Cell counts were performed with a hemacytometer using trypan blue exclusion and resuspended to a concentration of 2 x 10^7 cells/mL media. Afterward, aliquots of the cells were washed and resuspended in FACS buffer, 0.1% sodium azide and 1.0% fetal bovine serum in PBS, and blocked with anti-Fc receptor γII/II for 10 min on ice. Cells were subsequently stained for B220, CD11b, CD11c, GR1, CD8a, and MHCII and analyzed with flow cytometry.

Plasma cytokine analysis

Plasma was used to assess systemic cytokine concentrations. Plasma concentrations of IL-5, IL-13, and IL-9 were determined using MILLIPLEX^{TM} MAP Mouse Cytokine/Chemokine panel (Millipore, Billerica, MA) as per the manufacturer's protocol and described above.

Fecal IgA ELISA

Prior to protein extraction, the contents of the cecum from mice fed the CON or DFO diets were snap frozen on dry ice and stored at -80^{o}C. The cecal contents were thawed and immediately placed in PBS containing protease inhibitors (c0mplete mini, Roche) at a concentration of 25% w/v. Samples were vigorously vortexed for 10 min or

until cecal contents were completely resuspended before being spun at 16,000xg for 10 min. The supernatants were collected in separate tubes, centrifuged a second time, and the final supernatant collected. Mouse IgA ELISA Quantitation Set (Bethyl Laboratories, Inc., Montgomery, TX) was used to analyze the cecal content protein for immunoglobulin A. Standards were prepared as per the manufacturer's protocol, samples were diluted 1:750 in sample diluent, and the plate was read at 450 nm on a Synergy HT Multi-mode Microplate reader (Biotek, Winooski, VT).

Statistical analysis

Flow cytometric analyses were reported as either percentage of total lymphocytes when comparing populations or as median fluorescent intensity (MFI) when comparing cell surface marker expression. Using NIH ImageJ software, measurements of lipid microdomain size were obtained as previously described [9]. Briefly, NIH ImageJ was used to analyze particles ≥0.1 μm^2, smaller sizes could not be measured accurately. The Feret's diameter was calculated for each particle and all particles of a given cell were averaged. Subsequently, individual cell averages were then averaged for a given animal (n=10 cells/animal and n=10 animals/group).

All reported values are means ± SEM. Statistical significance was set at $P < 0.05$. Normally distributed data (i.e. data that was assumed Gaussian and passed the Kolmogorov-Smirnov test) were statistically analyzed using a student's t test for significant differences in cell phenotyping, fatty acid analysis, lipid microdomain staining, and cecal IgA quantification between CON and DFO fed mice. A two-way, repeated measures ANOVA was used to assess the differences in antigen uptake between CON and DFO mice over time. Due to non-normal distributions of the data obtained from the

cytokine assay, Mann-Whitney U tests were used to test for significant differences in the plasma cytokines and supernatant cytokines of LPS-stimulated B cells between CON and DFO fed mice. All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc; La Jolla, CA).

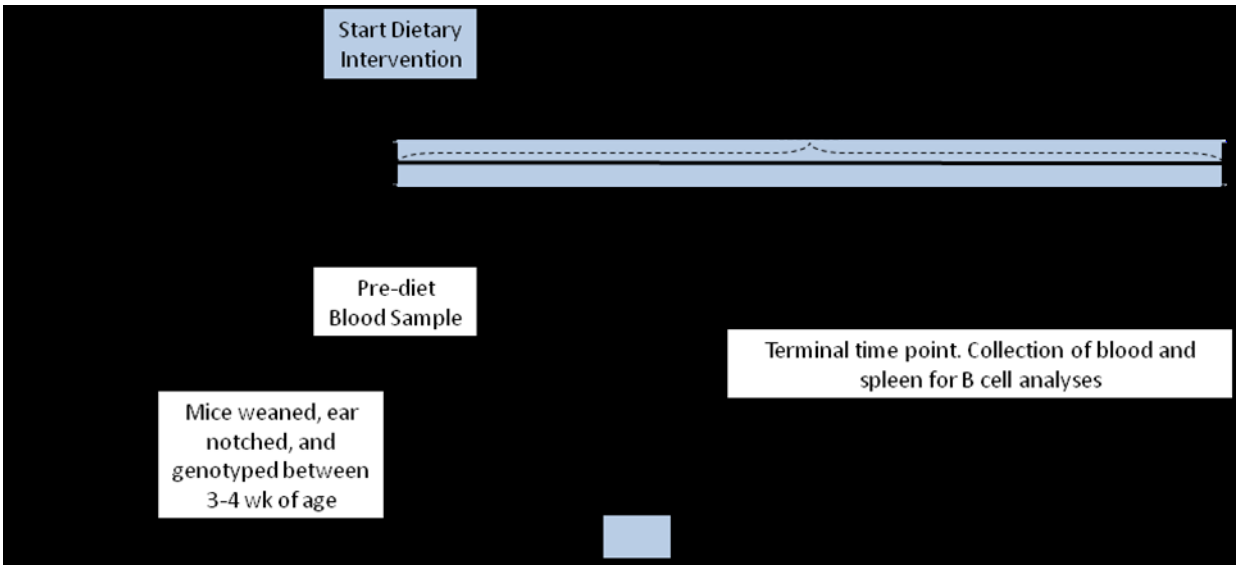


Figure 3 Experimental design for fish oil feeding studies in SMAD3-/- mice. SMAD3-/- mice were bred in-house at a specific-pathogen free facility. Mice were genotyped and weaned between 3 – 4 wk of age and placed on standard AIN-93G based pelleted chow (Harlan Teklad; TD.94045) prior to experimental diets. Mice were then continued on an AIN-93G based diet (CON) or fed a diet whereby 6% (by wt) of the dietary fat was replaced with DHA-enriched fish oil (DFO). After the 5 wk dietary intervention, mice were sacrificed and tissues were collected for further analysis.

Table 2 Fatty acid analysis of experimental diets[1]

Fatty Acid	CON	DFO
14:0	0.11 ± 0.01	0.14 ± 0.02
16:0	4.46 ± 0.18	2.71 ± 0.10
16:1	0.06 ± 0.00	0.48 ± 0.06
18:0	3.87 ± 0.03	1.39 ± 0.04 ***
18:1 *trans*	0.10 ± 0.09	0.01 ± 0.00
18:1 *cis*	23.12 ± 0.12	6.97 ± 0.07 ***
18:2 (n-6)	59.85 ± 0.09	10.89 ± 0.15 ***
18:3 (n-6)	0.49 ± 0.03	0.03 ± 0.01 ***
18:3 (n-3)	7.89 ± 0.03	0.14 ± 0.00 ***
20:4 (n-6)	0.024 ± 0.01	1.18 ± 0.01***
20:5 (n-3)	0.00 ± 0.00	20.52 ± 0.09 ***
22:5 (n-3)	0.00 ± 0.00	5.08 ± 0.13 ***
22:6 (n-3)	0.00 ± 0.00	50.45 ± 0.21 ***
∑SFA	8.44 ± 0.22	4.24 ± 0.15
∑MUFA	23.29 ± 0.15	7.45 ± 0.11 ***
∑PUFA (n-3)	7.90 ± 0.02	76.20 ± 0.39 ***
∑PUFA (n-6)	60.37 ± 0.12	12.10 ± 0.14 ***

[1]Data are mean ± SEM reported as percent total, n = 3. A student's t test was used compare differences between CON and DFO diet fatty acid composition. Asterisks indicate significant difference: *** $P < 0.001$

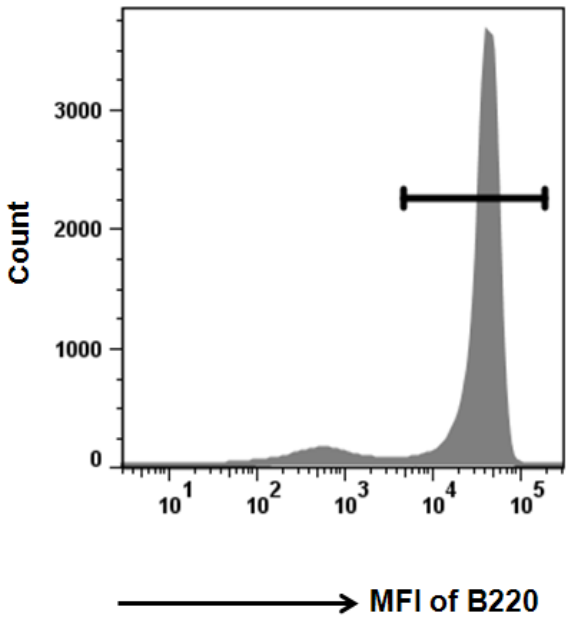


Figure 4 Purity of B cell isolation using negative selection from spleens of SMAD3-/- mice. Spleens of SMAD3-/- mice were dounce homogenized, red blood cells were lysed, and the remaining single cell suspension was stained with biotinylated-CD43, CD4, and Ter-119 antibodies. Cells were then stained with anti-biotin antibodies conjugated to magnetic microbeads and magnetically seperated from the suspension. The remaining unlabeled pool was predominantly B cells (>95% B220+) as confirmed by flow cytometry.

Results

DFO diet alters B cell lipid composition

The aim of this study was to investigate the effect of fish oil on B cell membrane organization, function, and phenotypic outcomes. We first sought to demonstrate that DHA-enriched fish oil (DFO) feeding resulted in alterations in the lipid membrane of B cells, prior to demonstration of altered membrane organization. Total lipids were extracted from purified B cells to confirm incorporation of dietary n-3 LCPUFAs into B cells. After a 5-wk diet regimen, total lipids from purified B cells of DFO fed mice contained significantly less 18:0 (CON: 20.0% vs. DFO: 17.0%) and 20:4(n-6) (CON: 18.2% vs. DFO: 6.8%), but significantly more 20:5(n-3) (CON: 0.2% vs. DFO: 5.5%), 22:5(n-3) (CON: 1.1% vs. DFO: 2.6%), and 22:6(n-3) (CON: 3.7% vs. DFO: 10.2%) compared to the CON fed mice. These changes resulted in an overall significant decrease of total SFAs (CON: 41.8% vs. DFO: 40.4%) and total n-6 PUFAs (CON: 33.3% vs. DFO: 21.1%) and a significant increase of total n-3 PUFAs (CON: 6.2% vs. DFO: 19.6%) in DFO fed mice compared to the CON fed mice (**Table 3**).

DFO diminishes lipid microdomain clustering

Previous studies have shown that exposure of B cells to dietary menhaden fish oil or exposure of T-lymphomas to DHA *in vitro* diminishes lipid microdomain clustering, which makes the domains appear larger in size on a micron scale [9, 53]. Using DFO (>50% DHA /wt), we sought to confirm that dietary exposure to DHA (primarily) targeted lipid microdomain size *ex vivo* concomitantly with altered lipid composition in the 129 background SMAD3-/- mice. We measured changes in lipid microdomain size with the DFO diet relative to the CON diet using confocal microscopy. **Figure 5A** shows

representative fluorescent images of clusters on B cells isolated from CON (left) or DFO (right) fed mice. The DFO diet clearly diminishes microdomain clustering. The clusters on B cells from mice fed the DFO diet were on average 0.275 μm larger (22% larger) compared to CON fed mice (**Figure 5B**).

DFO alters B cell activation, but not antigen uptake

Increased lipid microdomain size was previously observed concomitantly with enhanced ex vivo B cell response to stimulation. To test this in our model, LPS, a TLR-4 agonist and a T-independent antigen, was used to stimulate B cells from DFO fed mice. After 24 h stimulation with 1 μg/mL LPS, we stained for cell surface markers of activation (CD40, CD80, CD86, and MHCII). LPS-stimulated purified B cells from DFO fed mice had significantly increased CD40 expression (16%), but there was no change in CD80, CD86, and MHCII expression (**Figure 6A**). All stimulations resulted in increased MFIs compared to the unstimulated baseline; however, only MHCII was significantly different at baseline ($p<0.05$, data not shown).

Furthermore, we assayed the supernatants from the LPS-stimulated B cells to determine if there were differences in cytokine production. There was a significant increase in IL-6 (50%) and a non-significant ($p=0.052$) increase in TNF-α production (40%) in DFO fed mice compared to CON fed mice. We were unable to detect IFN- γ production (**Figure 6B**).

Antigen uptake is the initial step required by an antigen presenting cell in order to further initiate immunological outcomes via cell signaling. To determine if a DFO diet alters this capacity in B cells, we incubated purified B cells with OVA-FITC. Despite

overall increases of OVA-FITC uptake over time, no significant differences in OVA-FITC uptake between B cells from DFO-fed mice and B cells from CON-fed mice were detected (**Figure 7**).

DFO increases B cell populations and surface markers of activation

The next set of experiments investigate the effect DFO feeding has on *in vivo* systemic immunological outcomes. We examined *in vivo* the percent of B cells in multiple lymphatic tissues and expression of functional surface markers. Use of B220 as a pan-B cell marker revealed significant *in vivo* B cell differences in DFO fed mice compared to CON fed mice. There was a significant increase in $B220^{+}$ $MHCII^{+}$ lymphocytes in the mesenteric lymph nodes (2.0-fold change) and the Peyer's patches (1.18-fold change) of DFO fed mice compared to CON fed mice (**Figure 8**).

In order to further refine B cell populations, we gated on $Gr1^{-}$ $CD8a^{-}$ $B220^{+}$ $MHCII^{+}$ lymphocytes leading to two distinct phenotypes, denoted $B220^{hi}$ and $B220^{lo}$ B cells. The MFI of MHCII on $B220^{hi}$ B cells was 45% higher in the spleen of DFO fed mice compared to CON fed mice (**Figure 9A**). The MFI of MHCII on $B220^{lo}$ B cells was 41% higher in the spleen and 50% higher in the mesenteric lymph nodes of DFO fed mice compared to CON fed mice (**Figure 9B**). The MFI of CD11c on $B220^{hi}$ B cells was 37% higher in the spleen, 56% higher in the mesenteric lymph nodes, and 88% higher in the Peyer's patches of DFO fed mice compared to CON fed mice (**Figure 9C**). The MFI of CD11c on $B220^{lo}$ B cells was 33% higher in the spleen, 66% higher in the mesenteric lymph nodes, and 60% higher in the Peyer's patches of DFO fed mice compared to CON fed mice (**Figure 9D**).

DFO increases plasma cytokines and fecal IgA

The increase in B cells in the lymphoid tissues and the increased expression of MHCII and CD11c prompted further investigation of *in vivo* immunological outcomes. Plasma cytokines were assayed specifically for Th2-biasing cytokines due to their propensity for B cell growth and proliferation [79]. Indeed, the plasma of DFO fed mice had significantly higher levels of IL-5 (237.5%) and IL-13 (273%). IL-9 was only detectable in the plasma of DFO fed, but not the CON fed mice (**Figure 10A**). Plasma IL-1β, IL-2, IFN-γ, and TNF-α were not detectable; IL-6 did not differ between groups (data not shown).

The apparent skewing towards a Th2-response encouraged the investigation of systemic functional endpoints of *in vivo* B cell function. Furthermore, the increased B cell populations and increased surface marker expression in the mesenteric lymph nodes and Peyer's patches indicate that secretory IgA levels, the predominant gut immunoglobulin, might be altered by DFO feeding. Cecal IgA was significantly higher in DFO-fed mice (343%) compared to CON mice after 5 wk of feeding (**Figure 10B**).

Table 3 Dietary DFO alters the acyl chain composition of B cells[1]

Fatty Acid	CON	DFO
14:0	0.1 ± 0.0	0.2 ± 0.1
16:0	21.7 ± 0.5	23.2 ± 0.4
18:0	20.0 ± 0.2	17.0 ± 0.1 ***
18:1 *trans*	0.1 ± 0.0	0.1 ± 0.0
18:1 *cis*	17.3 ± 0.2	16.7 ± 0.5
18:2 (n-6)	15.0 ± 0.2	14.4 ± 0.3
18:3 (n-6)	0.0 ± 0.0	0.1 ± 0.0
18:3 (n-3)	1.2 ± 0.0	1.2 ± 0.1
20:4 (n-6)	18.2 ± 0.3	6.8 ± 0.1 ***
20:5 (n-3)	0.2 ± 0.0	5.5 ± 0.2 ***
22:5 (n-3)	1.1 ± 0.0	2.6 ± 0.1 ***
22:6 (n-3)	3.7 ± 0.1	10.2 ± 0.4 ***
∑SFA	41.8 ± 0.4	40.4 ± 0.4 *
∑MUFA	18.7 ± 0.2	18.7 ± 0.5
∑PUFA (n-3)	6.2 ± 0.1	19.6 ± 0.7 ***
∑PUFA (n-6)	33.3 ± 0.4	21.1 ± 0.2 ***

[1]Data are mean ± SEM reported as percent total, n = 5. A student's t-test was used compare differences between CON and DFO B cell fatty acid composition. Asterisks indicate significant difference: * $P < 0.05$ *** $P < 0.001$.

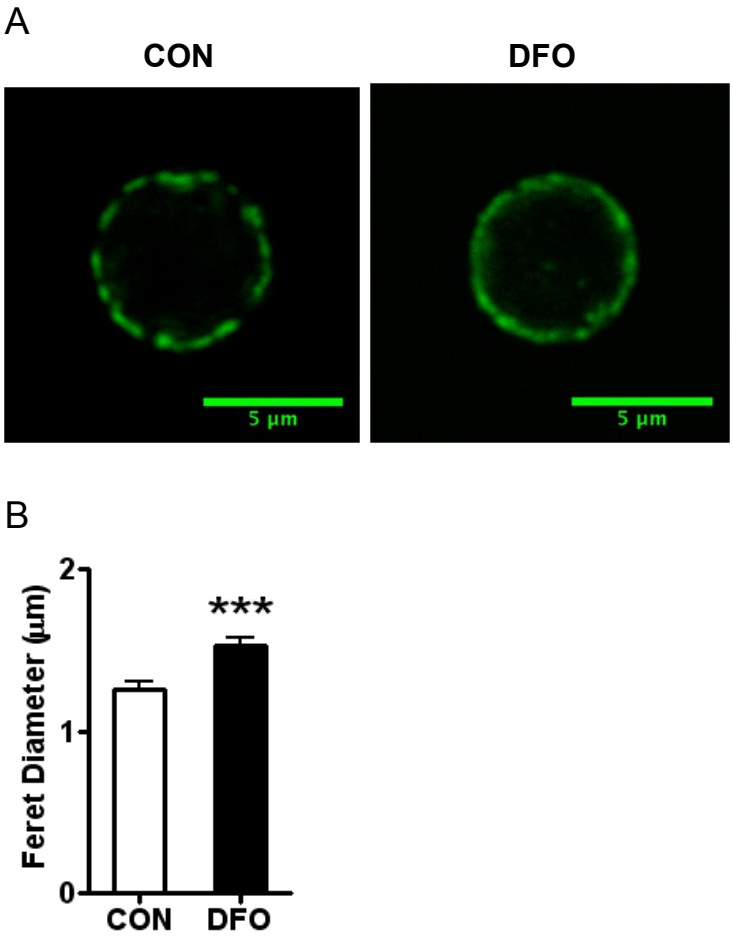


Figure 5 Lipid microdomain organization in B cells from SMAD3-/- mice. (A) Representative fluorescent images of lipid microdomains on purified B cells from SMAD3-/- mice fed either the CON (left) or DFO (right) diet. Cells were stained with cholera toxin subunit-B conjugated to FITC for visualization of clustered GM1 molecules. (B) Lipid microdomain size on a micron scale, measured with the Feret's diameter, of purified B cells from SMAD3-/- fed either the CON (□) or DFO (■) diet. Data are represented as mean ± SEM, n = 10. Asterisked data indicate significant differences between DFO diet compared to CON diet: *** $P < 0.001$

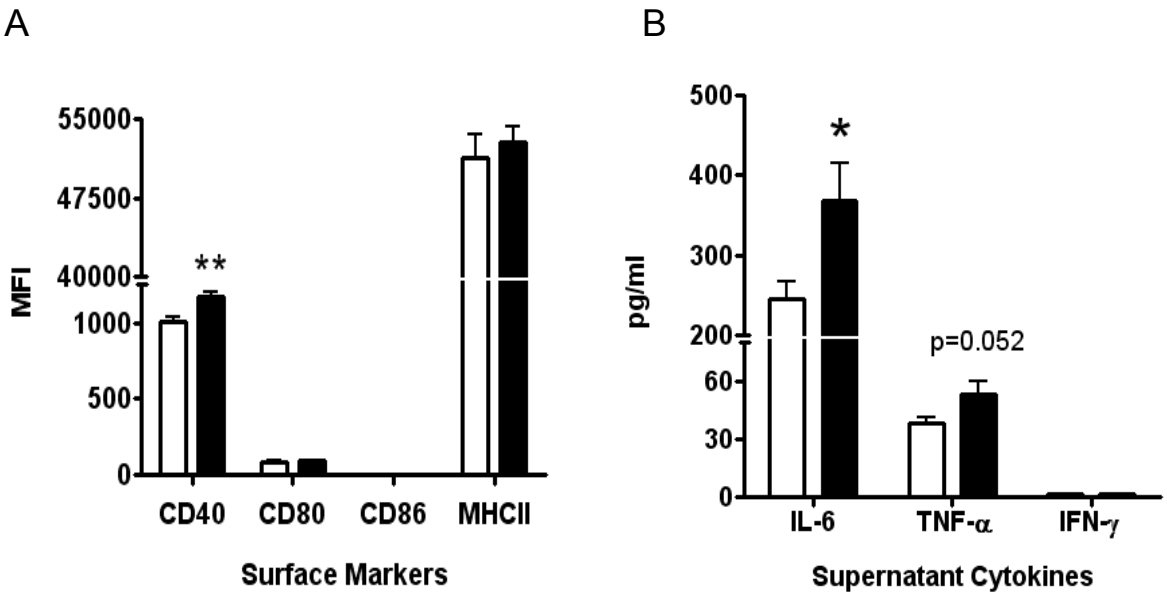


Figure 6 Expression of surface markers associated with cell signaling and cytokine production in LPS-stimulated purified B cells from SMAD3-/- mice. Aliquots of splenic B cells, purified using negative selection and identified above as $B220^{+}$, were cultured for 24 hours in media containing 1 ug/mL LPS. (A) Cell surface marker expression of LPS-stimulated B cells in SMAD3-/- mice fed either the CON (☐) or DFO (■) diet. (B) Cytokine production in the supernatants of LPS-stimulated B cells from SMAD3-/- mice fed either the CON or DFO diet were assayed. Data are represented as mean ± SEM, n = 8-10. Asterisked data indicate significant differences between the DFO diet compared to the CON diet: * $P < 0.05$, ** $P < 0.01$

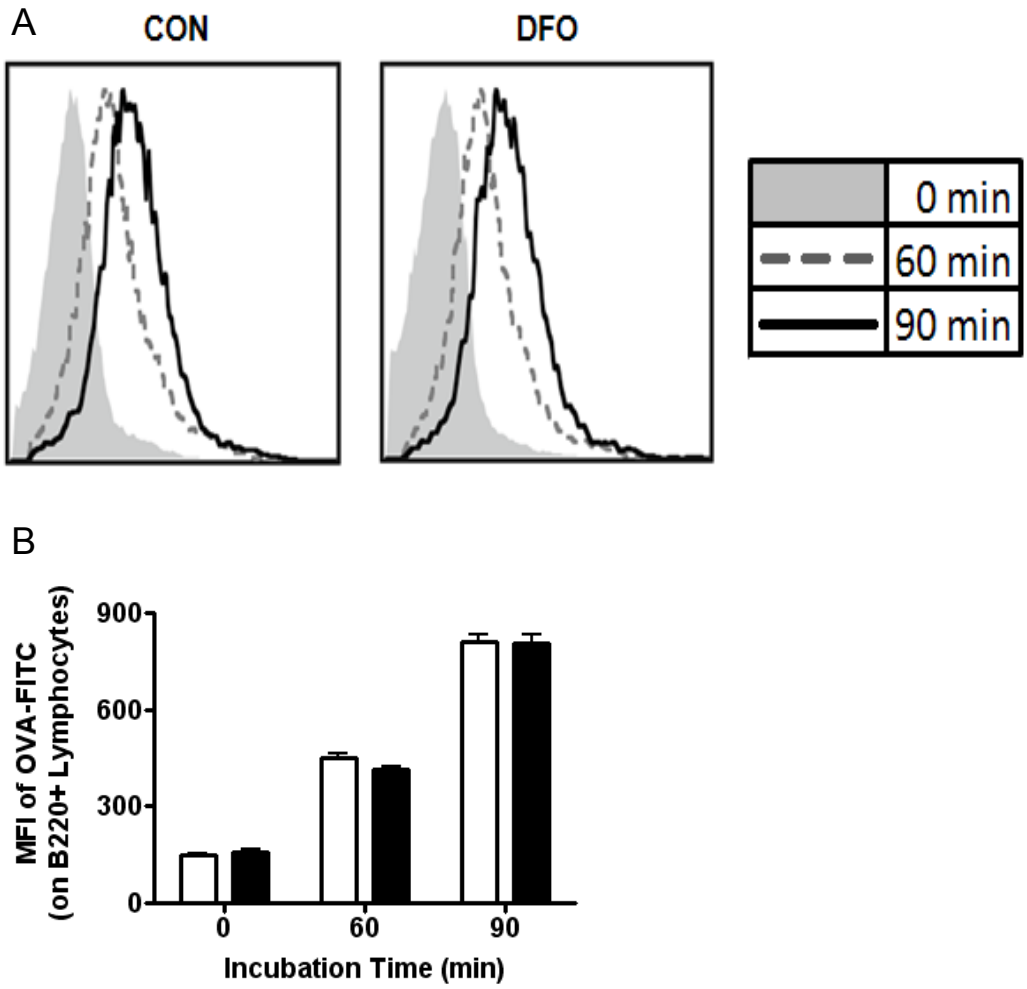


Figure 7 Uptake of ovalbumin (OVA) as a model antigen in purified B cells from SMAD3-/- mice. Aliquots of B cells were resuspended with 50 µg/mL OVA conjugated to FITC (OVA-FITC) at 37°C for 0, 60, and 90 min before being placed on ice. Purified B cells were negatively selected from the spleens of SMAD3-/- fed either the CON (☐) diet or the DFO (■) diet. (A) Representative histograms displaying the change in fluorescence over time with incubation with OVA-FITC in CON (left) and DFO (right) fed SMAD3-/- mice B cells. (B) Change in median fluorescent intensity of OVA-FITC on purified B cells over time. Data are represented as mean ± SEM, n = 10.

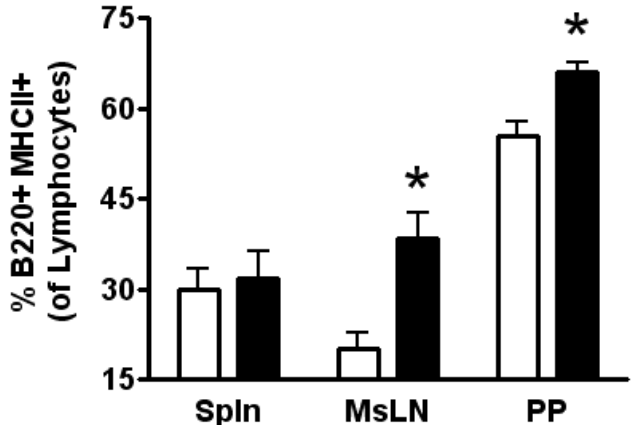


Figure 8 *In vivo* distribution of B220$^+$ MHCII$^+$ lymphocytes in varying tissues of SMAD3-/- mice. Flow cytometry was performed on the spleen (Spln), mesenteric lymph nodes (MsLN), and Peyer's patches (PP) of SMAD3-/- mice fed either the CON (□) diet or the DFO (■) diet. Represented as a percentage of total lymphocytes, B220$^+$ MHCII$^+$ lymphocytes of dounce-homogenized tissues were identified. Data are represented as mean ± SEM, n = 3-5. Asterisked data indicate significant differences: * $P < 0.05$

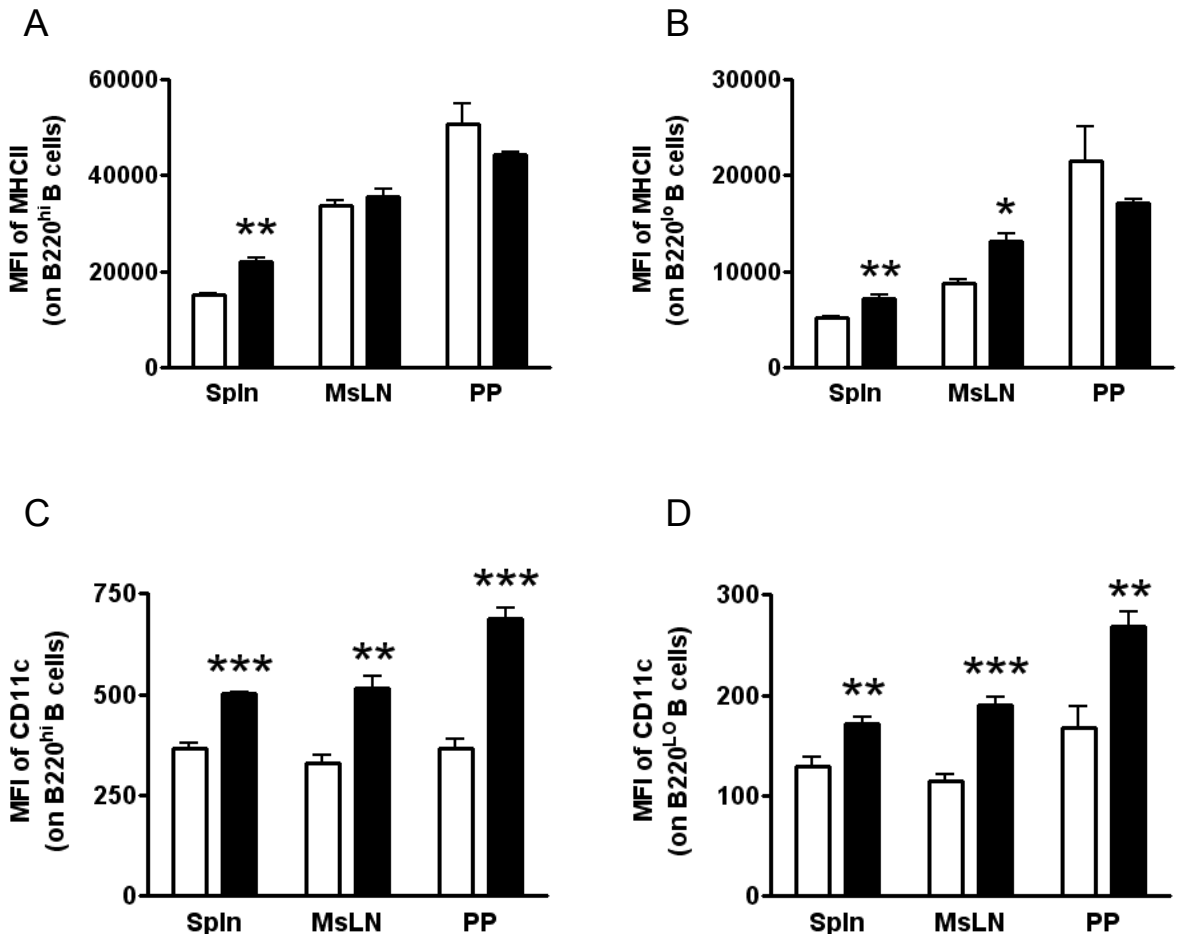


Figure 9 Surface marker expression on B cells from SMAD3-/- mice. An *in vivo* characterization was performed on the spleen (Spln), mesenteric lymph nodes (MsLN), and Peyer's patches (PP) of SMAD3-/- mice fed either the CON (□) diet or the DFO (■) diet. The median fluorescent intensity of MHCII and CD11c was determined on B cells, identified as $CD8a^{-}$ $Gr1^{-}$ $B220^{+}$ $MHCII^{+}$ lymphocytes. (A) Staining for MHCII on $B220^{hi}$ B cells. (B) Staining for MHCII on $B220^{lo}$ B cells (C) Staining for CD11c on $B220^{hi}$ B cells. (D) Staining for CD11c on $B220^{lo}$ B cells. Data are represented as mean ± SEM, n = 3-5. Asterisked data indicate significant differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

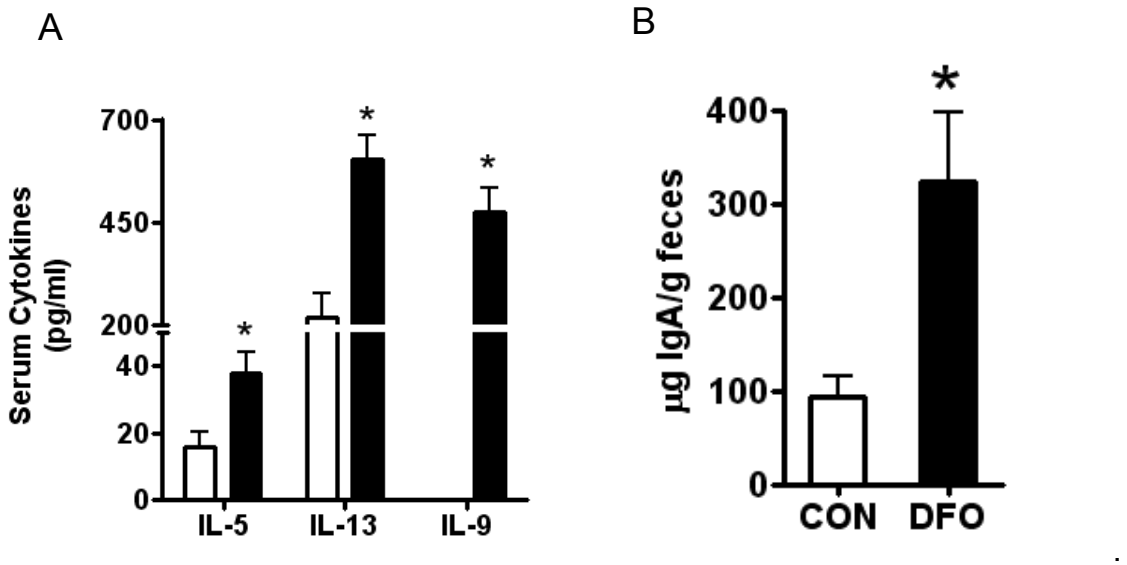


Figure 10 Systemic immunological outcomes of SMAD3-/- mice fed CON or DFO diets. (A) Th2-associated cytokines from the plasma of SMAD3-/- mice fed either the CON (☐) or DFO (■) diet. Plasma samples were assayed using a MILLIPLEX MAP mouse cytokine/chemokine assay. (B) Secretory immunoglobulin A (IgA) in the cecal contents of SMAD3-/- mice fed either the CON (☐) diet or the DFO (■) diet. Cecal contents were homogenized with protease inhibitors and twice-spun at 16,000xg to remove debris. Supernatants were analyzed using ELISA. Data are represented as mean ± SEM, (A) n = 7-8, (B) n = 4-5. Asterisked data indicate significant differences between DFO diet compared to CON diet: * $P < 0.05$

Discussion

The purpose of this study was to investigate the changes that DFO feeding elicits on B cells, an understudied, yet important cell population with respect to inflammatory pathologies. The rationale of the present study was to investigate the generalizability of previous observations suggesting that DHA/fish oil enhance B cell function in C57BL/6 mice, while progressing our laboratory's research on dietary fish oil exposure in SMAD3-/- mice. Our data suggest an overall increase in the activation of B cells in varying lymphoid tissues when exposed to DFO. Consistent with previous studies, splenic B cells from the fish oil fed mice revealed n-3 LCPUFAs targeted B cell lipid microdomain clustering/size, leading to putative changes in membrane order and cell signaling [9, 53]. *Ex vivo* stimulation of B cells from DFO fed mice increased cell surface markers for cell signaling and increased cytokine secretion, despite no change in antigen uptake. *In vivo* characterization of B cells from lymphoid tissues show altered population phenotype and upregulation of select functionally-associated molecules. These population-specific changes were observed in parallel with systemic alterations in cytokine profile and humoral immunity.

DFO and the membrane

Lipid-protein organization is emerging as a mechanism through which n-3 LCPUFAs exert their pleiotropic effects. We observed changes in the lipid composition of B cells by feeding DFO to SMAD3-/- mice (**Table 3**) and a change in the distribution of microdomains (**Figure 5**). These data are consistent with recent publications where DHA targeted lipid microdomain clustering in EL4 T lymphomas when treated with DHA *in vitro* [9] and of purified B cells from mice fed a diet containing menhaden fish oil [53].

Furthermore, a recent study showed that DHA was more effective than EPA in increasing lipid microdomain size [60]. These data suggest a commonality in mechanism by which fish oil targets lymphocytes across different murine models; however the immunological outcomes of fish oil on these lymphocytes, specifically B cells, are not yet completely understood. Mechanistically, it is unclear how the change in lipid microdomain clustering promotes B cell activation. We are currently pursuing the underlying mechanisms using a combination of techniques. One possibility is that DHA may be diminishing microdomain clustering by promoting changes in cholesterol lateral distribution and subsequently effecting protein clustering and downstream signaling [80].

Ex vivo studies

Earlier investigations examining the effect of *in vitro* fish oil exposure on Raji B cells demonstrate decreased cytokine production and inflammatory gene expression [8, 63]. More recently, studies using other B cell lines have also demonstrated decreased cytokine production [10] and decreased proinflammatory gene expression through inhibition of TLR4 signaling [64] after *in vitro* DHA exposure. While fish oil is generally considered immunosuppressive, an observation by Rockett et al. highlighted differential outcomes between B cells that were exposed *in vitro* compared to *in vivo* dietary fish oil exposure [10]. Thus, the differences between our study and previous work may be due the use of *in vitro* versus *in vivo* exposure to n-3 fatty acids.

B cells from mice fed DFO, stimulated with LPS, had increased CD40 expression compared to the control (**Figure 6A**). While we observed no change in MHCII, CD80, or

CD86 expression between groups, this is consistent with a recent study with C57BL/6 mice [10]. CD40 is a protein that is expressed on antigen presenting cells and its ligation by CD40L plays a significant role in B cell activation and antibody production. Rockett et al. had observed an increase in B cell CD69 expression with fish oil in response to LPS. Though it is unclear whether the increased expression of CD40 and CD69 are congruent between models, it is interesting that these molecules are both associated with activation and downstream cell signaling.

Our observations of increased IL-6 and TNF-α ($p=0.052$) also remain consistent with the study by Rockett *et al*. (**Figure 6B**) however we were unable to detect IFN-γ in the supernatants from the LPS-stimulated B cells. Taken together with the increased surface marker expression of CD40, it is reasonable to assume that the B cells from the DFO fed mice are more activated and may suggest increased potential immunogenicity.

Despite no difference in MHCII expression after LPS-stimulation between groups, there was a significant increase of MHCII expression on unstimulated B cells from DFO fed mice (data not shown, $p < 0.05$). B cells are capable of receptor-mediated pinocytosis/endocytosis allowing for uptake of soluble proteins [81]. Evidence for B cells to perform phagocytosis is beginning to emerge. [81, 82]. We observed no difference in antigen uptake between the B cells from CON or DFO fed mice (**Figure 7**). This appears consistent with literature suggesting no change in splenic neutrophil and monocytes phagocytosis after DHA supplementation [83], but is inconsistent with previous assertions that changes in the plasma membrane alter phagocytic capacity [84]. Additional work is needed to determine whether antigen processing is altered in B

cells of DFO fed mice and what the functional significance of increased MHCII expression entails.

In vivo studies

The activated B cell phenotype *ex vivo* in response to DFO feeding led us to determine if *in vivo* changes also reflect an activated phenotype. The data revealed significantly increased $B220^+$ $MHCII^+$ cells in the mesenteric lymph nodes and Peyer's patches of DFO fed SMAD3-/- mice (**Figure 8**). Previous work with dietary fish oil feeding shows a decrease in expression of functionally associated proteins in unstimulated mice peritoneum cells [85], human monocytes [86], and rat dendritic cells [87]. Our analyses of the B cells from SMAD3-/- mice fed DFO show increases in expression of MHCII (consistent with *ex vivo* observations) and CD11c on varying lymphoid tissues (**Figure 9**). The contrast to previous literature may be a result of the different type of fish oil used in the present study This finding appears in agreement with data on MHC class I expression on EL4 cells treated with DHA ethyl esters *in vitro* [9]. These observations may suggest that DHA specifically increases expression of MHC molecules on lymphocytes.

Further investigation at the systemic level revealed that mice fed DFO had increased (IL-5 and IL-13) or detectable (IL-9) Th2 cytokines circulating in the plasma prior to any challenge compared to control mice (**Figure 10A**). Despite the functional redundancy among Th2 cytokines [79], these cytokines play a significant role in effector function during a Th2 response with specific respect to B cell function. For example, IL-5 is a required cytokine for certain B cells to elicit maximal Th-cell dependent responses

[88], as well as promotion of IgA secretion [89]. IL-9 can increase innate-like B cell growth [90] and potentiate IgE and IgG1 release from B cells [91]. IL-13 can elicit stimulated cells to increase production of IgM, IgG1, IgG2a, IgG2b, and IgG3 [92], while inducing IgE production and increasing MHCII expression [93].

Consistent with increased B cell numbers in gut-associated lymphoid tissues and the stimulatory effect of IL-5 on B cell function, we observed a significant increase of secretory IgA in the feces of DFO fed mice (**Figure 10B**). The data on antibody production remain inconsistent; a previous study had observed no change in IgG2a or IgA after feeding DFO and challenge with an enteric infection [24], while Ramon et al. very recently reported that administration of DHA-derived pro-resolving mediators of inflammation increased B cell antibody production, specifically IgG and IgM production [94].

Implications

The work presented here, in collaboration with our previous work [10, 53], suggest a novel role for fish oil in targeting humoral immunity. Enhanced humoral immunity could have both beneficial and deleterious implications, depending on the population that is consuming the fish oil. Elucidating the functional and mechanistic effects of fish oil on B cells is critical given that immunocompromised populations may benefit whereas others may not. Our data are of significance since they show that results recently reported for C57BL/6 mice are reproducible in a different genetic background. It is increasingly accepted that these EPA/DHA derived mediators have an impact on enhancing the recruitment of monocytes to sites of inflammation and even

increasing their phagocytic activity [95]. Thus, fish oil-enhanced B cell activation may aid immune responses associated with pathogen clearance, likely upregulating the resolution phase of inflammation and dampening the totality of the inflammatory response. The current study suggests that dietary DFO increases B cell activation, likely benefiting situations where the host is challenged and a humoral response is required, such as a parasitic infection, revealing potential clinical utility of dietary DFO. It would be appropriate to further study the systemic immunoglobulin changes of DFO feeding to confirm the putative changes that the Th2 cytokine profile elicits. These findings highlight the need for additional *in vivo* n-3 LCPUFA research to fully understand the phenotypic, functional, and mechanistic basis for n-3 LCPUFA B-cell immunomodulation.

CHAPTER 3: MARINE FISH OILS ARE NOT EQUIVALENT WITH RESPECT TO B CELL MEMBRANE ORGANIZATION AND ACTIVATION

Data in this chapter is under review at *J. Nutr. Biochem.* by Gurzell et al. (2014)

Abstract

We previously reported that DHA-enriched fish oil (DFO) feeding altered B cell membrane organization and enhanced B cell function. The purpose of this study was to evaluate whether menhaden oil (MO) and EPA-enriched fish oil (EFO) feeding alters B cell function/phenotype as observed with DFO. Mice were fed soybean oil-based AIN-93G (CON) or diets incorporating MO, EFO, or DFO for 5 weeks. We determined the phospholipid fatty acid composition of B cells, assessed membrane microdomain organization, measured *ex vivo* B cell functionality, and phenotyped *in vivo* B cell subsets from spleen and bone marrow. Compared to B cells from CON fed mice, MO and DFO resulted in decreased clustering of membrane microdomains, whereas EFO increased clustering. Despite all fish oil treatments increasing CD40 expression following LPS stimulation, increased cytokine production was observed in MO (IFN-γ) and DFO (IL-6), while EFO resulted in decreased MHCII expression and IL-6 production. MO increased antigen uptake, while EFO decreased antigen uptake. Phenotyping of both splenic and bone marrow B cells from MO, EFO, or DFO treatments reveal consistent alterations of B cell subsets toward decreasingly mature B cell subsets. We conclude that diets high in n-3 LCPUFAs may elicit similar phenotypic outcomes, as observed in the bone marrow and splenic B cell subsets, but different

organizational and functional outcomes. More specifically, these data suggest that the EPA and DHA content of a diet influences immunological outcomes, highlighting the importance of understanding how specific n-3 LCPUFAs modulate B cell development and function.

Introduction

Over 30 million people in the US report using fish oil as a dietary supplement [11]. Fish oil is rich in the n-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs): eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Lovaza™, a prescription fish oil containing 465 mg EPA and 375 mg DHA per 1 g capsule, is currently prescribed for the treatment of hypertriglyceridemia; however, there is evidence that regular consumption of fish may result in cardiovascular benefit [96]. Indeed, fish oil has been investigated as a potential therapeutic for a wide range of illnesses ranging from mental health, such as depression, to physical health, such as inflammatory bowel diseases (IBD). It has been hypothesized that the inflammation-mediating effects of n-3 LCPUFAs may be responsible for their potential health effects.

As potent mediators of inflammation, the immunomodulatory effects of n-3 LCPUFAs on various immune cells have been studied. EPA and DHA alter inflammation through a variety of mechanisms, including production of eicosanoid and specialized proresolving mediators, gene expression, and membrane organization, among others [46]. The bulk of research regarding fish oil immunomodulation has highlighted functional suppression of leukocytes, including T cells and monocytes [6].

However, recent research has demonstrated that feeding n-3 LCPUFAs may significantly increase B cell activation, enhance B cell response to antigen, and increase total B cell number [10, 53, 97-99]. Despite the vital role of B cells in the immune system, there is still little research elucidating the effects of n-3 LCPUFAs on B cells. Specifically, animal models investigating the effects of fish oil on B cell function has thus far inadequately addressed the discrepancy between purified n-3 LCPUFA exposures *in vitro* compared to common dietary fish oil exposures *in vivo*. Indeed, Rockett et al. observed differential outcomes of B cells exposed to n-3 LCPUFAs *in vitro* compared to *in vivo* [10].

Research on fish oil immunomodulation is further confounded by demonstrating differences in the mechanistic and functional outcomes of EPA and DHA on immune cells [6]. Currently, the majority of animal models investigating the effect of n-3 LCPUFAs have used common fish oils (e.g., menhaden fish oil); whereas, therapeutics and dietary supplements have shifted toward EPA- and DHA-enriched formulations despite the lack of knowledge regarding immunomodulatory outcomes from specific mixtures of n-3 LCPUFAs.

We previously reported that feeding DHA-enriched fish oil to colitis-prone SMAD3-/- mice increased B cells *in vivo* and enhanced activation *ex vivo* after stimulation with LPS [97]. These observations are consistent with recently published observations elsewhere using C57BL/6 mice [10, 53]. The plethora of research demonstrating the different mechanisms by which EPA and DHA exert their effects [100] suggests a need to understand the immunological outcomes of dietary exposure to fish oils that are enriched with EPA or DHA. The objective of this study was to

investigate the *in vivo* immunomodulatory effects of different fish oil composition on B cell function expounding on our previous observations using DHA-enriched fish oil in our colitis-prone model. We sought to compare the phenotype and function of B cells isolated from mice fed MO, EFO or DFO diets. To that end, we assessed the phospholipid fatty acid composition and microdomain organization of purified, splenic B cells. We also examined *ex vivo* B cell functionality, including the cytokine response to LPS-stimulation and antigen uptake. In addition, we characterized the effect of these diets on *in vivo* subsets of splenic and bone marrow B cells.

Materials and Methods

Materials and chemicals

ACK lysing buffer was purchased from Invitrogen (Carlsbad, CA, USA), RPMI-medium 1640 was purchased from Sigma-Aldrich (St. Louis, MO, USA), and FBS was purchased from Gibco (Gaithersburg, MD, USA). HPLC-grade water, toluene, and sulfuric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). 2-propanol and butyrated hydroxytoluene were purchased from Sigma-Aldrich. HPLC-grade chloroform and n-hexane were purchased from OmniSolv (Charlotte, NC, USA). Isolute-XL® SPE aminopropyl columns were purchased from Biotage (Charlotte, NC, USA). High purity methanol was purchased from Burdick & Jackson (Morristown, NJ, USA). Standards and the RT®-2560 column for gas chromatography were purchased from Restek (Bellefonte, PA, USA). The following fluorescent antibodies (clone) were purchased from eBioscience (San Diego, CA, USA): B220 (RA3-6B2), MHCII (M5/114.15.2), CD40

(1C10), IgM (11/41), CD23 (B3B4), and CD21/CD35 (4E3). Purified CD16/CD32 (2.4G2) and biotinylated-CD24 (M1/69), as well as the following fluorescent antibodies (clone) / secondary fluorophores were purchased from BD Biosciences (San Diego, CA, USA): CD80 (16-10A1), CD86 (GL1), IgD (11-26c.2a), and Streptavidin PE-Cy7. Chicken ovalbumin conjugated to fluorescein (OVA-FITC) used for the antigen uptake assay was purchased from Molecular Probes (Eugene, OR. USA). Cholera toxin subunit B (CTxB) conjugated to fluorescein and anti-CTxB used for lipid microdomain staining was purchased from Life Technologies (Carlsbad, CA, USA). Lipopolysaccharide (LPS) for the stimulation assay was purchased from Sigma-Aldrich. The EPA-enriched fish oil (EPA4E1400 MEG-3™ fish oil) and DHA-enriched fish oil (DHA4E1400 MEG-3™ fish oil) were generously donated by Ocean Nutrition Canada (Dartmouth, Nova Scotia, Canada). The Menhaden Oil was purchased from Sigma Aldrich (St. Louis, MO, USA). A Certificate of Analysis was provided with each of the fish oils indicating food-grade quality of the fish oil and that it is free of contaminants and oxidation.

Murine model

The colitis-prone SMAD3-/- mouse model was utilized for these studies. The natural progression of our work with the SMAD3-/- mouse model has led us to investigate the effect of dietary fish oil on B cell function. SMAD3+/- and SMAD3-/- breeder pairs (129-Smad3tm1Par/J) were generated in-house. Homozygous males and heterozygous females were mated to obtain SMAD3-/- pups. Genotypes were confirmed by PCR. Mice were housed under SPF conditions in 152.4 cm^2 plastic cages (maximum of five adult mice/cage) with microisolator lids in an Association for

Assessment and Accreditation of Laboratory Animal Care-approved facility at Michigan State University (East Lansing, MI, USA). SPF conditions were assured through quarterly serology testing by Charles River Laboratories International (Wilmington, MA, USA) and in-house testing for ectoparasites, endoparasites, and fecal *Helicobacter* species (PCR). Full necropsies (including culture and sensitivity) were performed at least yearly on rodent-breeding colonies. Animal rooms were maintained at 23.3 ± 2.2°C with a 12-h light-dark cycle. Mice were fed non-purified diets and sterile water *ad libitum*. Animal protocols were approved by the Michigan State University Institutional Animal Care and Use Committee.

Experimental design and diet composition

SMAD3-/- mice (age 4–7 weeks) were fed an AIN-93G soybean oil-based (CON) standard diet or one of three fish oil (MO, EFO, and DFO) substituted diets. The AIN-93G pre-mixed diet without fat was purchased from Dyets (Bethlehem, PA, USA) and formulated as previously described [97]. Differing only in fat composition, all diets contained 7% fat by weight, providing 17% kcal from fat (0.637 kcal/g). The CON diet contains 7% (wt) soybean oil, while the fish oil diets contain 1% (wt) corn oil (0.091 kcal/g) and 6% (wt) of the respective fish oils (0.546 kcal/g). **Table 4** shows the fatty analysis of the prepared diets, which was analyzed with gas chromatography as described below. Animals were fed the experimental diets *ad libitum* for 5 week. *Ex vivo* studies of B cells from mice fed MO, EFO, or DFO were performed on separate experiments with CON fed mice present in each experiment serving as the control. Experiments performed to identify the fatty acid composition of B cells and RBCs

phospholipids, as well as phenotyping of B cell subsets were performed collectively (i.e. CON, MO, EFO, and DFO fed mice all initiated diet and were sacrificed at the same time). At the end of the experiment, mice were asphyxiated with CO_2 and exsanguinated through cardiac puncture using a heparin-coated syringe. Blood was collected on ice and centrifuged to obtain a red blood cell fraction and a plasma fraction to assess plasma cytokines.

B cell purification

Spleens were harvested from SMAD3-/- mice, fed the CON, MO, EFO, and DFO diet and immediately placed in ice-cold RPMI supplemented with 10% FBS. Splenocytes were isolated and B cells were negatively selected for using BD IMag cell separation, per the manufacturer's protocol for their B Lymphocyte Enrichment Set system (BD Biosciences) as previously described [97].

Lipid extraction, phospholipid isolation, and preparation of FAMEs

Lipid extraction was performed using the Rose and Oklander method [101], which utilizes a mixture of 2-propanol:chloroform (11:7 v/v) to minimize heme interferences within erythrocytes [102]. Briefly, under dim lighting, packed erythrocytes (100 – 150 mg) or pelleted B cells (approximately 6 million) were carefully transferred into borosilicate glass screw-capped tubes, immediately mixed with 2 mL of ice-cold HPLC-grade water, tightly capped and allowed to incubate on ice for 20 minutes. Lysates were then combined with 6 mL of 2-propanol (Sigma – Aldrich, St. Louis, MO) containing 100 µg/mL butyrated hydroxytoluene (BHT; Sigma – Aldrich), vortexed, and incubated for 1 h on ice with occasional mixing. Following incubation, 3.8 mL of HPLC-

grade chloroform (OmniSolv, Charlotte, NC) was added and samples where incubated for an additional hour on ice with occasional mixing. Samples were centrifuged, the lower phase extracted, and dried under a gentle stream of nitrogen at 40°C. Phospholipid isolation using solid-phase extraction was performed according to the modified procedures of Agren et al. [103]. Briefly, Isolute-XL® SPE aminopropyl columns (500 mg; Biotage, Charlotte, NC) were conditioned twice with 5 mL acetone:water (7:1 v/v) and activated twice with 4 mL *n*-hexane (high purity solvent, OmniSolv, Charlotte, NC). Dried lipids were dissolved in 2 mL of *n*-hexane:chloroform:acetic acid (100:5:5 v/v/v) containing 100 µg/mL BHT. Lipid samples were then added to individual columns and monitored to prevent them from drying. To remove neutral lipids and non-esterified free fatty acids (NEFA), columns were washed with 2 mL of *n*-hexanes, followed by 5 mL of *n*-hexane:chloroform:ethyl acetate (100:5:5 v/v/v; 100 µg/mL BHT), and 5 mL methanol:chloroform:acetic acid (100:2:2 v/v/v; 100 µg/mL BHT). Fresh collection tubes were placed on ice under the columns and phospholipids were eluted using 2 x 4 mL washes of methanol:chloroform:water (100:5:4 v/v/v; 100 µg/mL BHT) and 1 mL high-purity methanol (Burdick & Jackson, Morristown, NJ) to remove any residual phospholipids [104]. Solvent fractions were dried under a gentle stream of nitrogen at 40°C. Fatty acid methyl esters (FAMEs) were prepared from isolated phospholipids fractions by incubation with acidified methanol, according to the methods of Burdge et al. [105]. Isolated phospholipid FAMEs were resuspended in 1-8 µL/mg tissue of *n*-hexane (100 µg/mL BHT), transferred to a GC autosampler vial, and stored under nitrogen at -80°C until analysis and analyzed as previously described [97].

B cell lipid microdomain staining and ex vivo stimulation

B cell lipid microdomain cross-linking and image acquisition, LPS-stimulation of purified B cells to assess cytokine production and surface marker expression, and OVA-FITC antigen uptake was performed as previously described [97].

Flow cytometry of splenic and bone marrow B cell subsets

Spleens and femurs were harvested from mice fed the CON, MO, EFO, and DFO diet and immediately placed in ice-cold RPMI supplemented with 10% FBS. The spleen was processed using a dounce homogenizer, pelleted, and washed in RPMI, as described previously [97]. Splenocytes were briefly resuspended in ACK lysing buffer for red blood cell lysis, washed twice in RPMI, and passed through 70 μm filters. Cell counts were performed with a hemacytometer using trypan blue exclusion and resuspended to a concentration of 2 x 10^7 cells/mL media. Splenocytes were stained with B220, IgM, IgD, CD23, CD24, and CD21 to identify various B cell subsets. Splenic B cells (B220+) subsets were phenotyped as transitional 1 (T1) B cells (CD23- $CD24^{Bright}$ CD21-), transitional 2 (T2) B cells (CD23- $CD24^{Bright}$ $CD21^{Bright/Dim}$ and CD23+ $CD24^{Bright/Dim}$ $CD21^{Bright}$), follicular (FO) B cells (CD23+/- $CD24^{Dim}$ $CD21^{Dim}$), and marginal zone (MZ) B cells (CD23- $CD24^{Bright/Dim}$ $CD21^{Bright}$).

Femurs were harvested, cleaned, placed in RPMI on ice, and then flushed with RPMI. The single cell suspension from the bone marrow was briefly resuspended in ACK lysing buffer for red blood cell lysis and washed twice in RPMI. Cell counts were performed with a hemacytometer using trypan blue exclusion and resuspended to a

concentration of 2 x 10^7 cells/mL media. Isolated cells from the bone marrow were stained with B220, IgM, IgD, CD24, and CD43 to identify various stages of B cell development. Bone marrow precursor and developmental B cells (B220+) subsets were phenotyped as pre-pro-B cells (IgD- IgM- CD24+ CD43-), pro-B cells (IgD- IgM- CD24+ CD43+), pre-B cells (IgD- IgM- CD24- CD43), immature B cells (IgM+ IgD-), and mature B cells (IgM+ IgD+). All flow cytometric analyses were performed on a FACSCanto II (BD Biosciences) and analyzed using FlowJo (TreeStar, Ashland, OR, USA).

Statistical analysis

All reported values are mean ± SEM. Statistical significance was set at $P < 0.05$. Normally distributed data (i.e., data that were assumed Gaussian and passed the Kolmogorov-Smirnov test) were statistically analyzed using a Student's t-test for significant differences in fatty acid analyses, lipid microdomain scoring, and cell surface marker expression staining between CON and MO, EFO, or DFO fed mice. A Pearson's correlation was used to measure the linear correlation RBC and B cell fatty acids. Individual cells (10 cells/animal) were categorically scored as having either clustered, not clustered, or mixed clustering of lipid microdomains based on a qualitative scoring by a researcher blinded to the treatments as previously described [9]. For the lipid microdomain analysis, cells were scored and controls were aggregated; a one-way ANOVA was used to assess differences across the dietary treatments. A two-way, repeated measures ANOVA was used to assess the differences in antigen uptake between CON and MO, EFO, or DFO fed mice over time. As a result of non-normal distributions of the data obtained from the cytokine assay, Mann-Whitney U tests were

used to test for significant differences in the supernatant cytokines of LPS- stimulated B cells between CON and MO, EFO, of DFO fed mice. A one-way ANOVA with a Dunnett's post-hoc test was used to assess the differences in splenic B cell subsets and bone marrow B cell developmental subsets from fish oil fed mice compared to the CON fed mice. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Table 4 Fatty acid analysis of experimental diets[1]

Fatty Acid	CON	MO	EFO	DFO
16:0	4.46 ± 0.18	18.98 ± 0.80 ***	3.84 ± 1.11	2.71 ± 0.10
16:1	0.06 ± 0.00	9.00 ± 0.86 ***	0.94 ± 0.70	0.48 ± 0.06
18:0	3.87 ± 0.03	4.85 ± 0.27 **	4.05 ± 0.06	1.39 ± 0.04 ***
18:1 *trans*	0.10 ± 0.09	0.05 ± 0.02	0.02 ± 0.00	0.01 ± 0.00
18:1 *cis*	23.12 ± 0.12	13.85 ± 0.34 ***	11.60 ± 0.06 ***	6.97 ± 0.07 ***
18:2 (n-6)	59.85 ± 0.09	13.01 ± 0.28 ***	10.37 ± 0.28 ***	10.89 ± 0.15 ***
18:3 (n-6)	0.49 ± 0.03	0.32 ± 0.01 ***	0.14 ± 0.02 ***	0.03 ± 0.01 ***
18:3 (n-3)	7.89 ± 0.03	1.81 ± 0.06 ***	0.74 ± 0.10 ***	0.14 ± 0.00 ***
20:4 (n-6)	0.024 ± 0.01	1.27 ± 0.06 ***	3.09 ± 0.13 ***	1.18 ± 0.01 ***
20:5 (n-3)	0.00 ± 0.00	16.69 ± 0.87 ***	52.40 ± 2.39 ***	20.52 ± 0.09 ***
22:5 (n-3)	0.00 ± 0.00	2.95 ± 0.18 ***	2.16 ± 0.10 ***	5.08 ± 0.13 ***
22:6 (n-3)	0.00 ± 0.00	14.31 ± 0.85 ***	10.28 ± 0.51 ***	50.45 ± 0.21 ***
∑SFA	8.44 ± 0.22	26.74 ± 1.58 ***	8.25 ± 1.38	4.24 ± 0.15
∑MUFA	23.29 ± 0.15	22.90 ± 0.69	12.56 ± 0.68 ***	7.45 ± 0.11 ***
∑PUFA (n-3)	7.90 ± 0.02	35.75 ± 1.95 ***	65.59 ± 1.69 ***	76.20 ± 0.39 ***
∑PUFA (n-6)	60.37 ± 0.12	14.61 ± 0.31 ***	13.60 ± 0.39 ***	12.10 ± 0.14 ***

[1]Data are mean ± SEM reported as percent total, n = 3. A student's t test was used compare differences in fatty acid composition between CON and DFO, EFO, or MO diets. Asterisks indicate significant difference: * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$

Results

Various fish oils different in EPA and DHA content increase RBC and B cell phospholipid n-3 LCPUFAs

We previously demonstrated that a 5-wk dietary regimen was sufficient to alter total lipid content in purified, splenic B cells and the RBC fraction of SMAD3-/- mice [97]. To further expand on our previous findings using a DHA-enriched fish oil diet (DFO), we included menhaden oil (MO) and EPA-enriched fish oil (EFO) diet in the current study. In this present study, we isolated the phospholipid fraction from the membrane of RBCs and B cells to confirm n-3 LCPUFAs incorporation into the phospholipid fraction of cellular membranes. **Table 4** shows the results from a gas chromatographic analysis of the lipid content of the differing diets.

It has previously been proposed that alterations in the lipid composition of the cellular membrane may contribute to the mechanism through which n-3 LCPUFAs act, i.e. by remodeling of the phospholipid membrane. To distinguish the lipid composition of the cellular membrane from other components containing lipid (e.g., lipoprotein contamination), we utilized a lipid extraction specific for phospholipids. This was an improvement upon our previous methodology because it more appropriately reflects changes in the membrane lipid composition by removing contaminating lipid from lipoproteins and non-membrane associated lipid compartments. The fatty acid composition of phospholipids from RBCs (**Table 5**) and B cells (**Table 6**) from SMAD3-/- mice fed CON, MO, EFO, of DFO diets for 5-wk are provided. Across all diets, the fatty acid profile of B cell phospholipids had increased levels palmitic acid (16:0) compared to

RBCs; whereas there was a compensatory decrease in oleic acid (18:1), linoleic acid (18:2n-6), arachidonic acid (AA; 20:4n-6), and DHA (22:6n-3) in B cell phospholipids.

In RBCs, MO, EFO, and DFO feeding resulted in increases of palmitic acid (16:0), EPA (20:4n-3), n-3 docosapentaenoic acid (DPA (n-3); 22:5n-3), and DHA (24:6n-3), whereas linoleic acid (18:2n-6), α-linolenic acid (18:3n-3), and AA (20:4n-6) decreased compared to CON fed mice. In B cells, MO, EFO, and DFO feeding resulted in increases of EPA, and decreases of AA compared to CON fed mice; only EFO feeding resulted in increased DPA (n-3), while MO and DFO feeding resulted in increased DHA.

The three experimental diets are high in EPA and/or DHA compared to the CON diet (**Figure 11A**). All LCPUFAs demonstrated strong, significant (all $p < 0.0001$) linear correlations between RBCs and B cells, as evidenced by Pearson's r coefficients for EPA ($r = 0.849$, **Figure 11B**), DHA ($r = 0.892$, **Figure 11C**), AA ($r = 0.973$, **Figure 11D**), and EPA+DHA ($r = 0.927$, **Figure 11E**). These data demonstrate that RBC phospholipid fatty acids are a robust biomarker for the phospholipid fatty acid composition of purified B cells.

The diets supplemented with the different fish oils intentionally resulted in stark contrasts in the n-3 LCPUFA composition of the phospholipid fatty acids of B cells between the experimental groups. EPA was highest in the phospholipid fatty acids of B cells from mice fed EFO, followed by MO, and lowest in DFO; whereby DHA in the phospholipid fatty acids of B cells was highest in DFO, middle in MO, and lowest in EFO (**Figure 11B-D**).

Despite differences in individual content of EPA and DHA, we observed that all three experimental diets resulted in an increase in the omega-3 index, defined as the sum percentage of EPA+DHA over total lipid in RBCs [106], ranging between 15-22% in fish oil fed mice compared 5-7% in CON fed mice. The EPA+DHA content of B cells was much lower compared to RBCs, but we observed the same fold change between B cells of CON fed mice (2-4% EPA+DHA) compared to the fish oil treatments (7-10%). These data demonstrate a high correlation between the EPA+DHA content of RBCs and B cells providing evidence that the omega-3 index is useful to predict the EPA+DHA content of purified B cells. While the levels of n-3 LCPUFAs in RBCs have been shown to be highly correlated to other tissues (e.g., cardiac [106]), our observations suggest that the RBC may serve as a surrogate biomarker of n-3 LCPUFA exposure for individual cell populations, including B cells.

DHA-rich oil alters the clustering of lipid microdomains

Changes in the fatty acid composition of the phospholipid membrane may influence membrane organization, as it has previously been shown that dietary fish oils diminished lipid microdomain clustering, as measured by the size of micron scale domains [9, 97]. Here, we assessed the presence or absence of lipid microdomain clustering using a blinded scoring analysis (**Figure 12A-D**). Purified B cells from mice fed MO and DFO diets had decreased clustering compared to B cells from CON and EFO mice, whereas B cells from EFO fed mice had increased clustering compared to CON, MO, and DFO fed mice (all $p < 0.05$, **Figure 12E**). As expected, this was observed in tandem with B cells from MO and DFO fed mice having more cells that

were non-clustered ($p < 0.05$); however, there was no difference the amount of non-clustered lipid microdomains on B cells between CON and EFO fed mice (**Figure 12F**).

n-3 LCPUFAs differentially alter B cell response to stimulation

We previously reported that LPS-stimulated B cells from mice fed DFO diet had increased expression of CD40 and production of IL-6 and TNF-α. As the B cell stimulation was performed on different days for each of the experimental fish oils (with controls for each experiment), all data were normalized to the control response in each experiment to evaluate the specific effect of fish oil feeding. After LPS-stimulation, B cells from mice fed MO and EFO diets had increased CD40 expression compared to CON (**Figure 13A**). B cells from MO mice had greater CD40 expression in response to stimulation than those from EFO or DFO mice (**Figure 13A).** Contrastingly, LPS-stimulated B cells from EFO fed mice had decreased MHCII expression compared to B cells from CON mice and responded unlike B cells from MO and DFO fed mice (**Figure 13B**).

IL-6 production by LPS-stimulated B cells was increased from MO mice; however, it was not statistically significant ($p = 0.11$). There was a statistically significant increase in IL-6 production from DFO mice ($p < 0.05$), whereas, IL-6 production from LPS-stimulated B cells of EFO mice was decreased ($p < 0.05$) (**Figure 13C**). TNF-α production from LPS-stimulated B cells was increased in mice fed MO and DFO; however, these increases were not statistically significant ($p = 0.092$ and $p = 0.052$, respectively). There was no change in TNF-α reduction from LPS-stimulated mice fed

EFO (**Figure 13D**). IFN-γ production from LPS-stimulated B cells was increased in mice fed MO ($p < 0.05$) with no change in EFO or DFO (**Figure 13E**).

MO increases, but EPA-enriched oil decreases antigen uptake

We previously reported that B cells from mice fed DFO had no alteration in antigen uptake using the model antigen, ovalbumin. In the current study, we observed increased antigen uptake in B cells from MO fed mice ($p < 0.05$) by 90 minutes incubation, where as B cells from EFO fed mice had significantly decreased antigen uptake by 90 minutes ($p < 0.001$) (**Figure 14A-D**).

Dietary fish oil decreases mature splenic and bone marrow B cell subsets

Despite having differential effects on splenic B cell function, MO, EFO, and DFO had few effects on splenic B cell subsets (**Figure 15**). There were reduced splenic marginal zone (MZ) B cells in the MO and EFO ($p < 0.05$) fed mice compared to CON fed mice (Figure 5A). There was a slight non-significant reduction in MZ B cells in DFO fed mice (Figure 5A). Fish oil diets did not significantly alter the splenic T1, T2, and mature follicular B cell populations (**Figure 15A**).

Similar to the spleen, MO, EFO, and DFO generally skew bone marrow B cell subsets in the same direction. Mature B cells in the bone marrow were decreased in MO, EFO and DFO fed mice compared to CON fed mice ($p<0.05$; **Figure 15B**). Bone marrow pre-pro-B cells, pro-B cells, pre-B cells and immature B cell populations were not significantly altered by fish oil diets.

Table 5 Fatty acid analysis of phospholipids from red blood cells[1]

Fatty Acid	CON	MO	EFO	DFO
16:0	31.57 ± 0.89	35.89 ± 0.58 **	36.17 ± 0.39 **	37.75 ± 0.80 **
16:1	0.29 ± 0.03	0.74 ± 0.02 **	0.28 ± 0.03	0.35 ± 0.03
18:0	12.72 ± 0.30	11.73 ± 0.06	11.56 ± 0.62	10.17 ± 0.21 **
18:1 *trans*	0.24 ± 0.03	0.20 ± 0.01	0.25 ± 0.01	0.24 ± 0.01
18:1 *cis*	11.04 ± 0.31	11.93 ± 0.33	12.79 ± 0.29 **	1.35 ± 0.34
18:2 (n-6)	15.95 ± 0.73	6.37 ± 0.26 **	3.88 ± 0.21 **	5.13 ± 0.14 **
18:3 (n-6)	0.07 ± 0.00	0.05 ± 0.00 *	0.03 ± 0.00 **	0.02 ± 0.01 **
18:3 (n-3)	0.20 ± 0.01	0.06 ± 0.01 **	0.04 ± 0.01 **	0.03 ± 0.00 **
20:4 (n-6)	11.85 ± 0.28	5.33 ± 0.14 **	5.51 ± 0.33 **	3.95 ± 0.15 **
20:5 (n-3)	0.25 ± 0.03	6.11 ± 0.16 **	9.01 ± 0.35 **	6.36 ± 0.45 **
22:5 (n-3)	0.77 ± 0.03	2.14 ± 0.04 **	3.98 ± 0.28 **	1.71 ± 0.04 **
22:6 (n-3)	5.59 ± 0.31	10.61 ± 0.28 **	8.01 ± 0.38 **	13.52 ± 0.16 **
∑SFA	47.88 ± 1.43	52.39 ± 1.17	52.25 ± 1.38	52.52 ± 1.28
∑MUFA	13.53 ± 0.45	15.01 ± 0.44	15.75 ± 0.40 **	14.57 ± 0.46
∑PUFA (n-3)	6.81 ± 0.38	18.92 ± 0.49 **	21.05 ± 1.02 **	21.62 ± 0.65 **
∑PUFA (n-6)	31.79 ± 1.25	13.68 ± 0.48 **	10.95 ± 0.67 **	10.76 ± 0.38 **

[1]Data are mean ± SEM reported as percent total, n = 4 mice/group. A student's t test was used compare differences in fatty acid composition between CON and MO, EFO, or DFO diets. Asterisks indicate significant difference: * $P < 0.05$ ** $P < 0.01$

Table 6 Fatty acid analysis of phospholipids from purified splenic B cells[1]

Fatty Acid	CON	MO	EFO	DFO
16:0	33.22 ± 0.41	34.00 ± 0.42	31.53 ± 0.85	34.50 ± 0.81
16:1	0.19 ± 0.04	0.36 ± 0.05	0.19 ± 0.02	0.40 ± 0.08
18:0	36.73 ± 1.78	37.81 ± 0.95	36.66 ± 2.47	37.95 ± 2.67
18:1 *trans*	1.52 ± 0.21	1.17 ± 0.32	1.58 ± 0.13	1.27 ± 0.07
18:1 *cis*	6.21 ± 0.11	6.10 ± 0.26	6.04 ± 0.21	5.99 ± 0.31
18:2 (n-6)	3.26 ± 0.21	2.21 ± 0.21 *	3.14 ± 0.36	1.61 ± 0.25 **
18:3 (n-6)	0.04 ± 0.00	0.04 ± 0.01	0.05 ± 0.02	0.07 ± 0.02
18:3 (n-3)	0.06 ± 0.01	0.03 ± 0.01	0.08 ± 0.01	0.11 ± 0.03
20:4 (n-6)	9.51 ± 0.28	3.14 ± 0.20 **	4.35 ± 0.10 **	1.99 ± 0.29 **
20:5 (n-3)	0.37 ± 0.07	3.10 ± 0.14 **	4.87 ± 0.23 **	1.51 ± 0.05 **
22:5 (n-3)	0.72 ± 0.08	1.17 ± 0.17	2.18 ± 0.20 **	0.86 ± 0.05
22:6 (n-3)	2.76 ± 0.41	4.82 ± 0.19 **	3.42 ± 0.41	7.61 ± 0.36 **
∑SFA	71.35 ± 2.28	72.98 ± 1.50	69.79 ± 3.60	74.19 ± 3.76
∑MUFA	10.14 ± 0.72	9.54 ± 0.88	10.48 ± 0.74	10.06 ± 0.87
∑PUFA (n-3)	3.91 ± 0.57	9.12 ± 0.51 **	10.55 ± 0.86 **	10.09 ± 0.49 **
∑PUFA (n-6)	15.83 ± 0.72	8.80 ± 0.66 **	10.38 ± 0.83 **	6.15 ± 0.72 **

[1]Data are mean ± SEM reported as percent total, n = 4 mice/group. A student's t test was used compare differences in fatty acid composition between CON and MO, EFO, or DFO diets. Asterisks indicate significant difference: * $P < 0.05$ ** $P < 0.01$

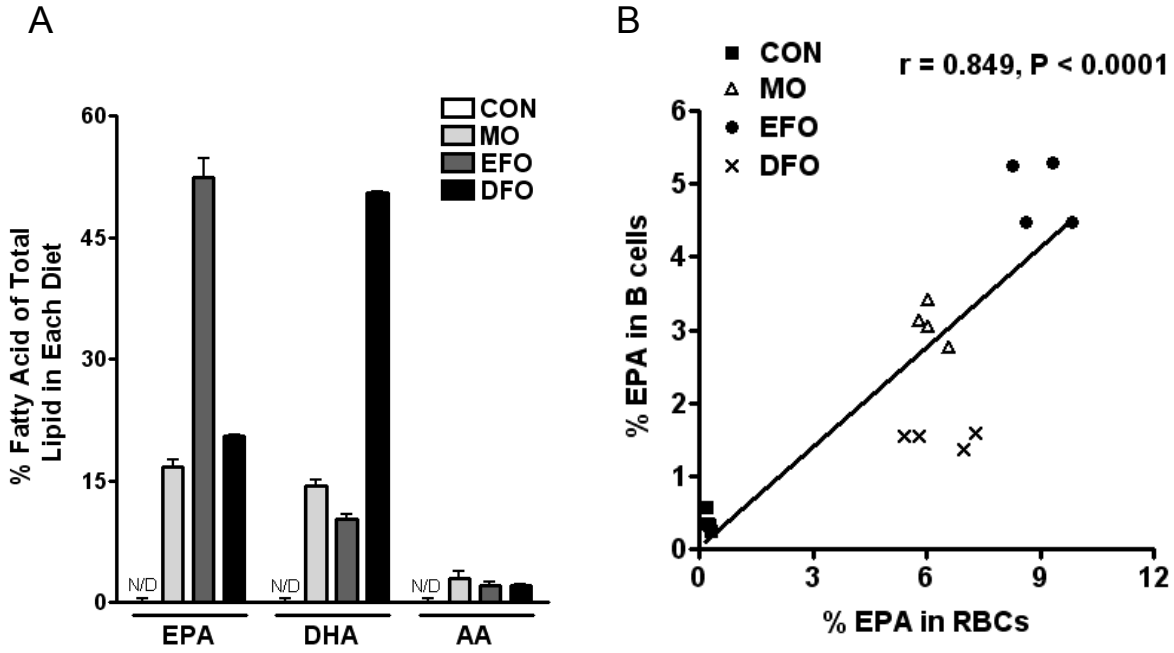
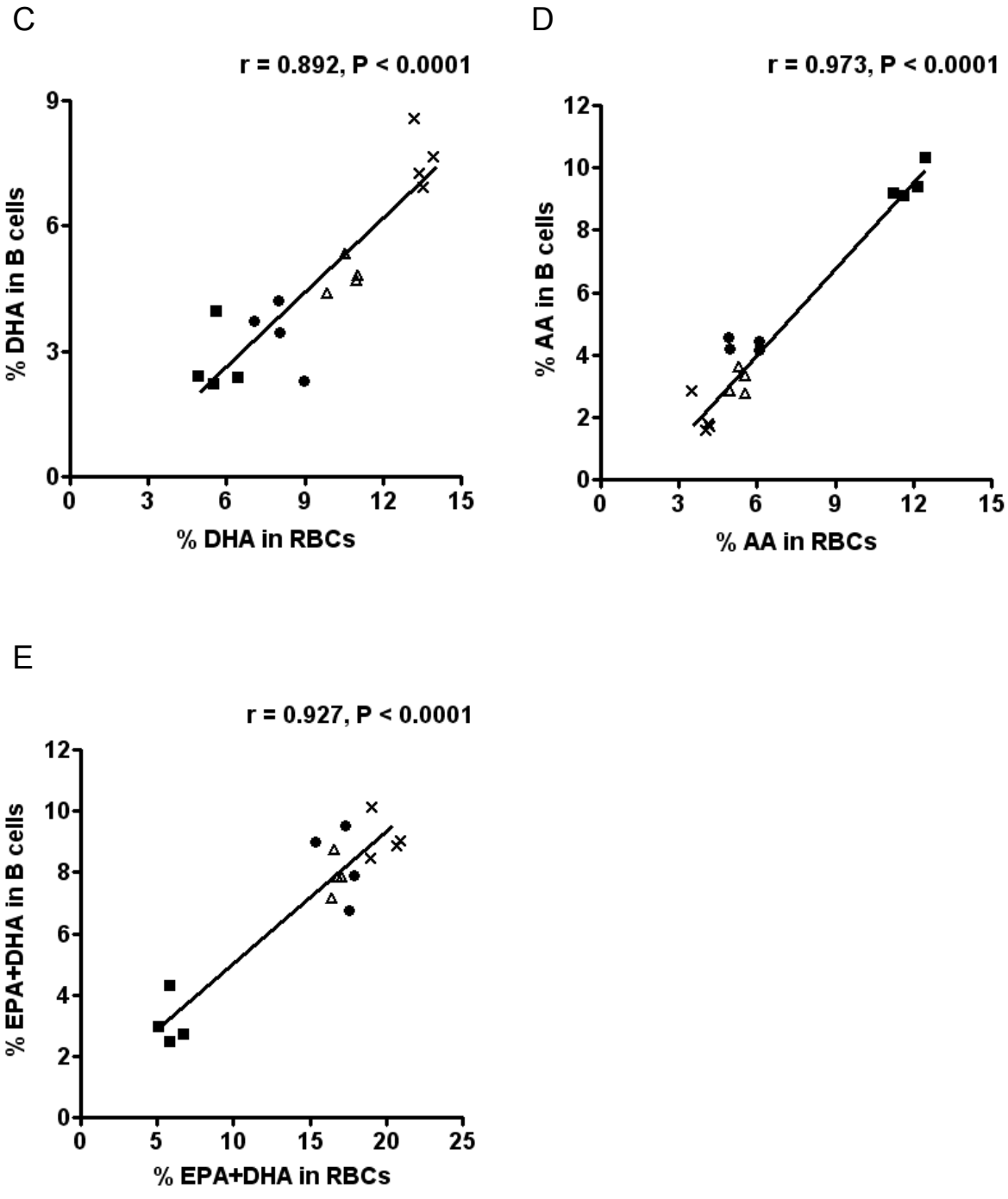


Figure 11 n-3/6 LCPUFA composition of experimental diets and phospholipids from murine red blood cells and B cells. (A) The EPA, DHA, and AA content of the experimental diets (CON, MO, EFO, and DFO) were analyzed by gas chromatography. Triplicates were run on a single batch of each diet. Correlations of the (B) EPA, (C) DHA, (D) AA, and (E) EPA+DHA content between red blood cell phospholipids and B cell phospholipids from mice fed either CON, MO, EFO, or DFO diets was performed. A Pearson's r was used to assess the linear correlation between the two samples from each animal; n = 4 mice/group.

Figure 11 (cont'd)



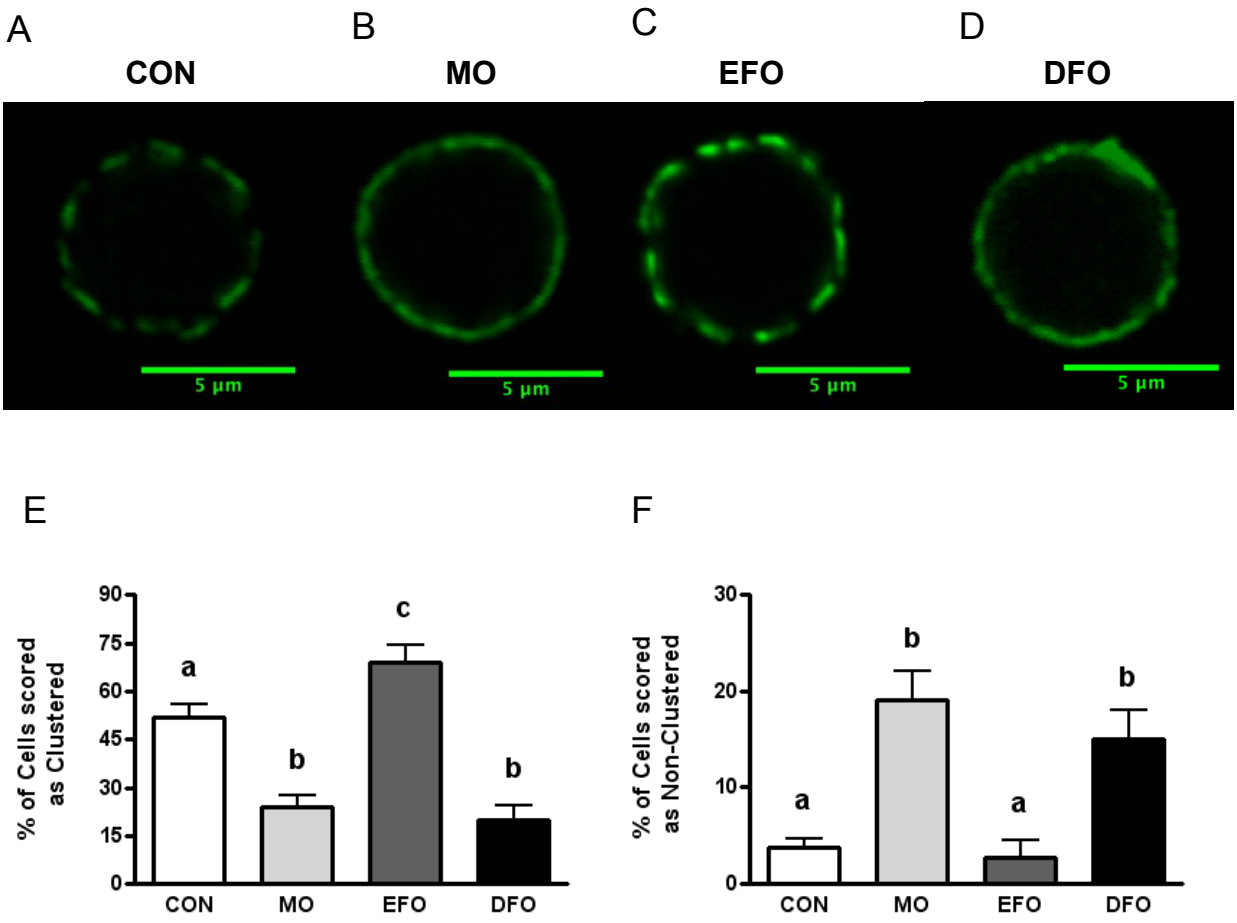


Figure 12 Clustering of lipid microdomains on murine B cells. (A-D) Representative fluorescent images of lipid microdomains on purified, splenic B cells from SMAD3-/- mice fed CON, MO, EFO, or DFO diets. Cholera toxin subunit B conjugated to FITC was used to visualize GM1, an extensively used reporter of lipid rafts. Blinded scoring analysis of fluorescent lipid microdomains on cells as (E) clustered or (F) non-clustered. Data are represented as mean ± SEM, whereby 10 cells were scored per animal and n = 10-15 mice/group. Different letters denote statistically significant differences at the $P < 0.05$ level.

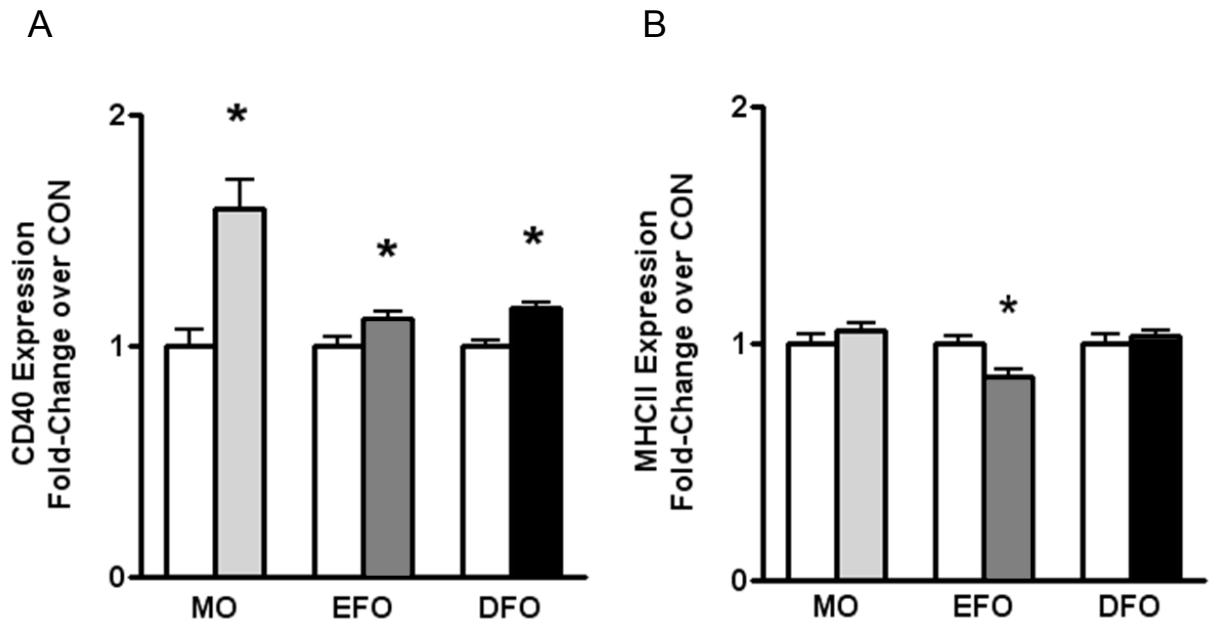
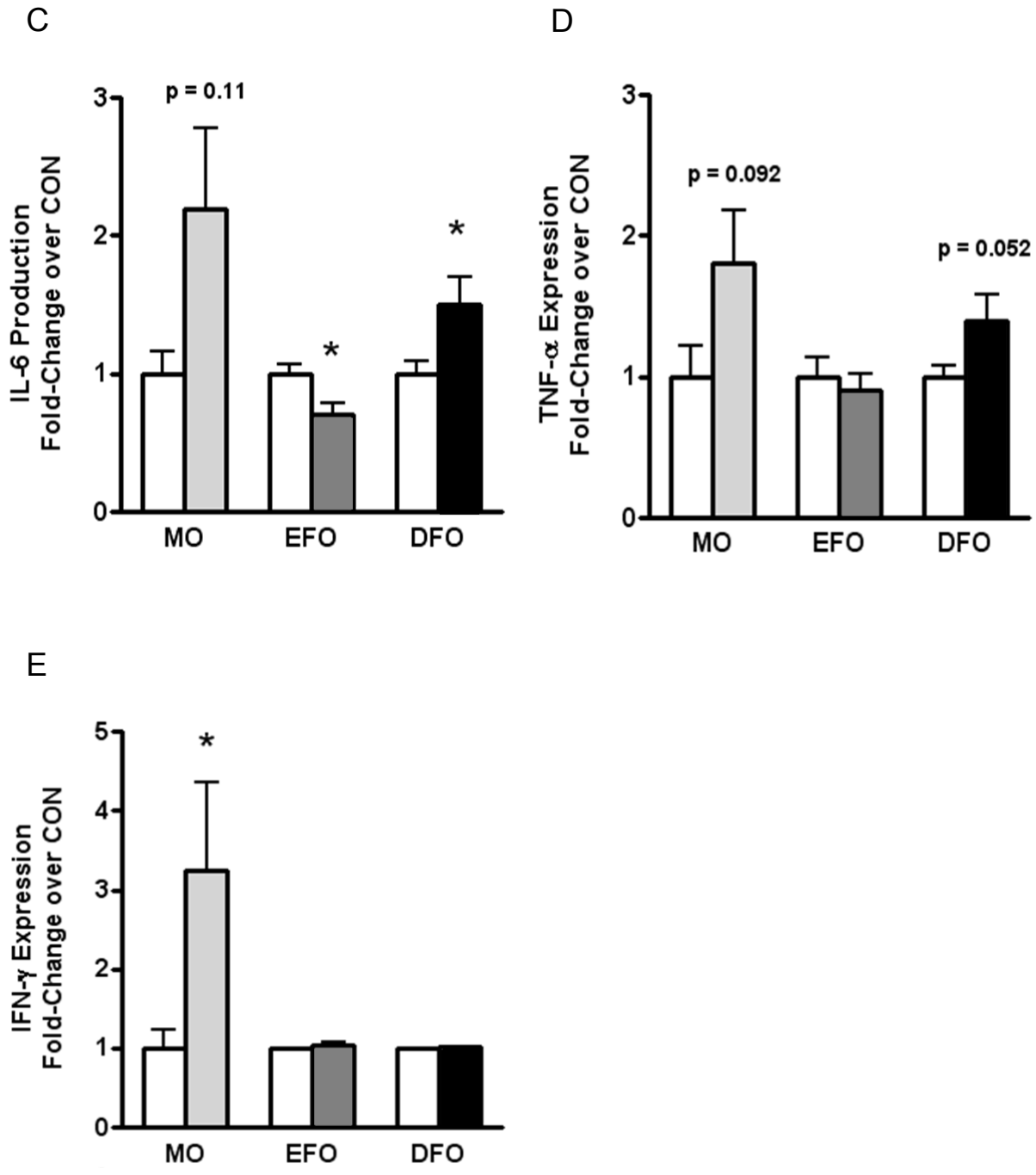


Figure 13 Expression of B cell surface markers and secreted cytokines in LPS-stimulated murine B cells. Purified, splenic B cells from SMAD3-/- mice fed CON, MO, EFO, or DFO diets were cultured for 24 h in the presence of 1µg/mL LPS. Flow cytometry on LPS-stimulated B cells was used to assess expression of B cell surface markers: (A) CD40 and (B) MHCII expression as a fold-change over CON. Flow-based multiplex assay was used to quantify secreted cytokines in the supernatants of LPS-stimulated B cells, including: (C) IL-6, (D) TNF-α, and (E) IFN-γ as a fold-change of cytokine production over CON. Data are represented as mean ± SEM; n = 5-15 mice/group. Each of the fish oil treatments were performed separately and therefore have their own controls. Different letters denote significant difference between treatment fold-changes in response to stimulation. Asterisks indicate significant differences compared to the CON diet: * $P < 0.05$

Figure 13 (cont'd)



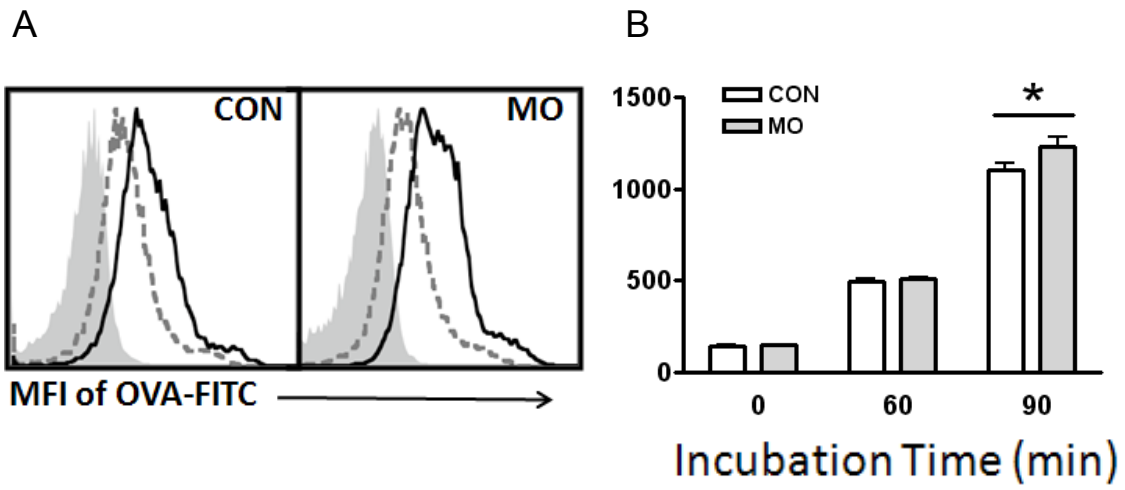
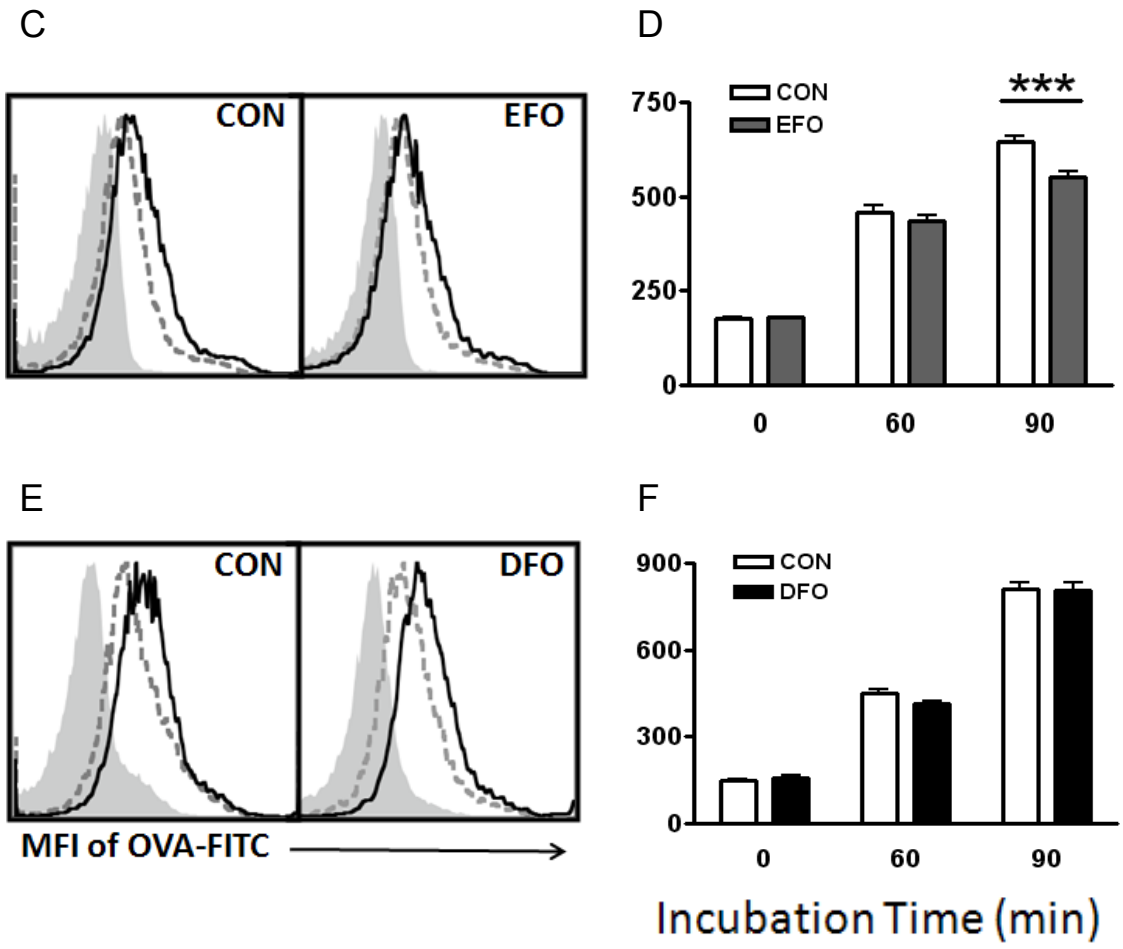


Figure 14 Antigen uptake in murine B cells. Uptake of ovalbumin conjugated to FITC (OVA-FITC) was used as a model antigen to assess B cell antigen uptake. Purified, splenic B cells from SMAD3-/- mice fed CON, MO, EFO, and DFO diets were incubated at 37°C for 0, 60, and 90 min. (A+C+E) Representative histograms demonstrate an increase in the MFI of OVA-FITC from 0 min (filled, light grey) to 60 min (dashed, dark grey) to 90 min (solid black). (B+D+F) Change over time of OVA-FITC MFI on purified B cells. Data are represented as mean ± SEM; n = 10-15 mice/group. Each of the fish oil treatments were performed separately and therefore have their own controls. Asterisks indicate significant differences compared to the CON diet: * $P < 0.05$ *** $P < 0.001$

Figure 14 (cont'd)



A

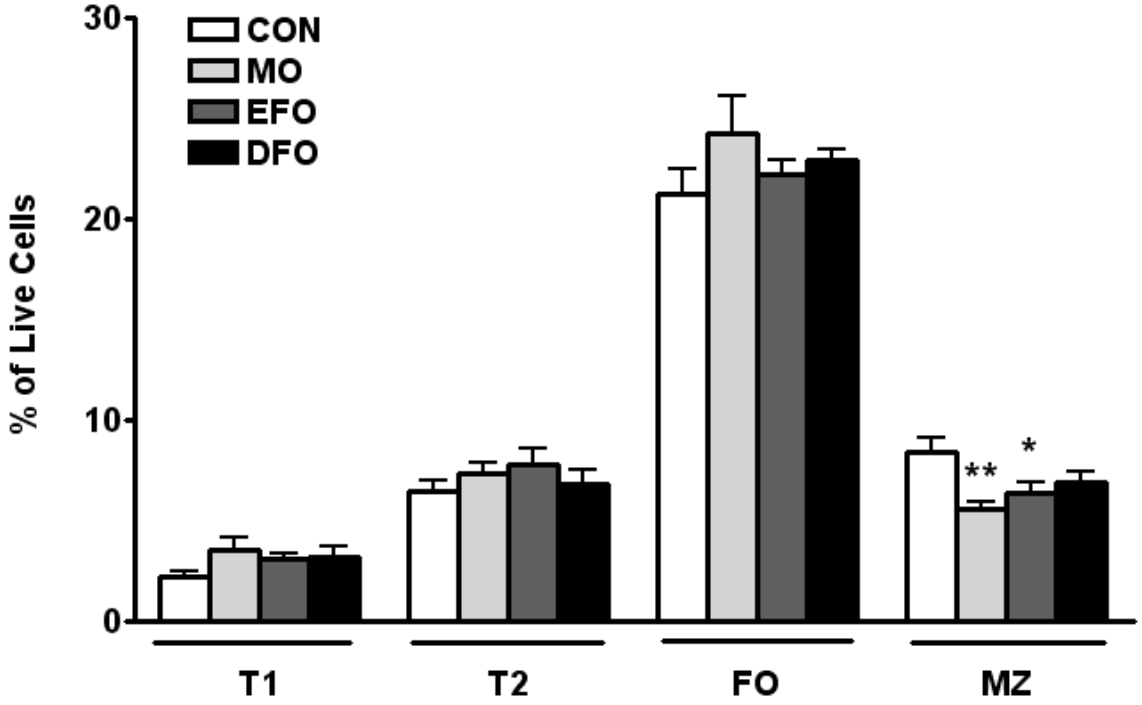
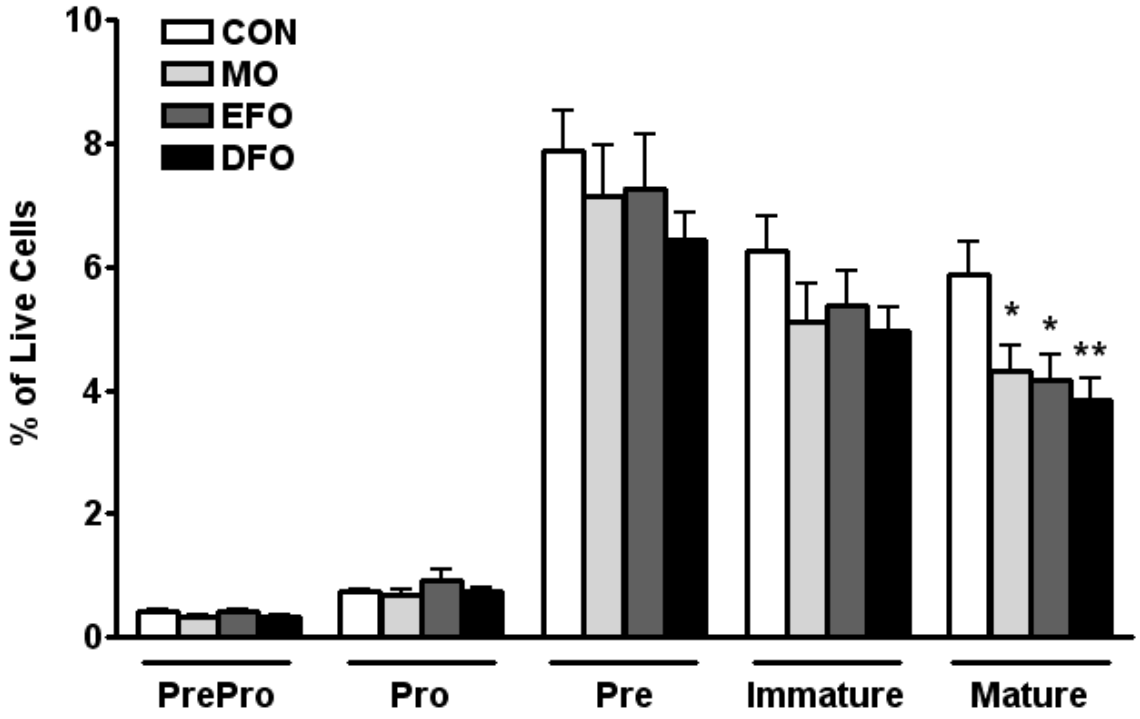


Figure 15 *In vivo* B cell subset phenotyping of spleen and bone marrow. Flow cytometric phenotyping of B cell subsets was performed on spleen and bone marrow tissues in SMAD3-/- mice fed CON, MO, EFO, or DFO diets. (A) Splenic B cells (B220+) subsets were phenotyped as T1 B cells (CD23- $CD24^{Bright}$ CD21-), T2 B cells (CD23- $CD24^{Bright}$ $CD21^{Bright/Dim}$ and CD23+ $CD24^{Bright/Dim}$ $CD21^{Bright}$), follicular B cells (CD23+/- $CD24^{Dim}$ $CD21^{Dim}$), and marginal zone B cells (CD23- $CD24^{Bright/Dim}$ $CD21^{Bright}$). (B) Bone marrow precursor and developmental B cells (B220+) subsets were phenotyped as pre-pro-B cells (IgD- IgM- CD24+ CD43-), pro-B cells (IgD- IgM- CD24+ CD43+), pre-B cells (IgD- IgM- CD24- CD43+), immature B cells (IgM+ IgD-), and mature B cells (IgM+ IgD+). Data are represented as mean ± SEM; n = 10 mice/group. Asterisks indicate significant differences compared to the CON diet: * $P < 0.05$ ** $P < 0.01$

Figure 15 (cont'd)

B



Discussion

In the current study, we observed that MO and DFO treatments elicit similar outcomes on B cell microdomain organization and function, whereas EFO treatment often resulted in opposite outcomes. The effects of MO and DFO include decreased microdomain clustering, increased activation and cytokine secretion in response to *ex vivo* stimulation, and increased antigen uptake (in the case of MO). Contrastingly, we observed that EFO increased microdomain clustering, but resulted in decreased activation, antigen uptake, and cytokine secretion following *ex vivo* stimulation compared to CON fed mice. The different fish oils influenced bone marrow and splenic B cell subsets in a similar direction. These observations highlight the crucial need to understand the differential immunological outcomes of fish oils varying in n-3 LCPUFA composition and the potential for dramatically different effects on immune function depending on the composition of the fish oil.

The lipid membrane is increasingly recognized as a critical platform through which lipid-protein organization and interactions occur. The amount of saturated fatty acid (SFAs) in B cell phospholipids (70-75%) was notably higher than RBCs (47-53%), while RBCs had higher MUFA, n-3 PUFA, and n-6 PUFA. Despite these differences, both RBCs and B cell fatty acid profiles responded similarly to the experimental fish oil diets, with increasing n-3 PUFA and decreasing n-6 PUFA. We previously reported that DFO diminished the clustering micron-scale lipid microdomains on B cells [97], which agreed with the work of others using MO [9]. Using a blinded, semi-quantitative scoring method, B cells from mice fed control (CON) or fish oil diets were assessed as having clustered, non-clustered, or mixed clustering/non-clustering lipid microdomains. B cells

from mice fed DFO and MO diets were found to have significantly decreased clustered microdomains and increased non-clustered microdomains (**Figure 12**). Contrasting the observed changes in the microdomain clustering of B cells from MO and DFO fed mice, EFO feeding resulted in increased clustering, suggesting EPA and DHA may differentially contribute to organization of membrane microdomains. Our observations corroborate *in vitro* studies demonstrating that DHA, but not EPA, is capable of increasing lipid raft size and decreasing clustering on cultured EL4 cells [9]. These observations may be explained by more recent biophysical studies on the molecular organization of EPA and DHA in artificial rafts, demonstrating that DHA has a greater affinity for lipid microdomains than EPA [107]. Surprisingly, despite the highly disordered nature of acyl-chain DHA, Teague et al. found that DHA was capable of adapting to more highly ordered environments, becoming more ordered itself, even suggesting potential interaction with cholesterol [61]. These data provide evidence to support an appearance of increasing microdomain size due to DHA.

The observations by us and others [60] that EPA and DHA may have differential effects on membrane organization, led us to hypothesize that fish oil composition will have differential effects on B cell function. Previous research on fish oil exposure using B cell lines *in vitro* resulted in decreased cytokine production and decreased inflammatory gene expression [8, 10, 63] and is suggestive that n-3 LCPUFAs may dampen B cell immunological responses. However, our previous observations that dietary DFO increased B cell response to stimulation and increased IgA secretion in the gut are in agreement with others' observations that dietary MO increases B cell activation and response to stimulation, suggesting that dietary n-3 LCPUFAs may

increase activation of humoral immunity [10, 98]. We observed that MO elicited a similar immunological phenotype in B cells as DFO, including a significant increase in CD40 expression and increased IL-6, TNF-α, and IFN-γ in response to *ex vivo* LPS stimulation. While EFO also led to increase in CD40, the other immunomodulatory effects of EFO appear contradictory to MO and DFO, such as decreased MHCII expression and decreased IL-6 upon LPS stimulation. We are unaware of any *ex vivo* stimulation studies that compare the functional consequences of B cells after feeding different compositions of n-3 LCPUFAs, specifically diets rich in EPA or DHA.

B cells are capable of receptor-mediated pinocytosis/endocytosis and antigen presentation [81, 82] making antigen uptake an additional parameter of B cell function. We utilized ovalbumin conjugated to FITC (OVA-FITC) as the model antigen. We observed a significant decrease in OVA-FITC uptake in B cells from EFO fed mice but an increase in OVA-FITC in B cells from MO fed mice compared to CON. Interestingly, the decrease in MHCII on LPS-stimulated B cells from EFO fed mice appears congruent with our antigen uptake data. These data support the hypothesis that changes in the plasma membrane alter phagocytic capacity [84]. Turk et al. corroborate differential outcomes of feeding EPA vs. DHA in mice [108]. While both EPA- and DHA-fed mice inhibited wound healing, the EPA-fed mice had increased mortality compared to corn oil or DHA-fed mice following colitis-induction using dextran sodium sulfate [108]. Taken together, these data highlight differential B cell immunomodulation depending on the fatty acid composition of fish oil.

Next we tested the hypothesis that fish oil composition alters B cell development in spleen and bone marrow. Regardless of dietary fish oil, all mature B cells were

reduced compared to control diet, which is in agreement with recent data [98]. It is possible that dietary fish oil induces development of B cells and emigration of B cells from the bone marrow, or increased hematopoiesis of non-B cell populations. We observed skewed hematopoiesis with an increased percentage of granulocytes (Ly6C^{dim} CD31-) and reduced percentage of lymphocytes (Ly6C- CD31+) in bone marrow of fish oil fed mice (data not shown). Total bone marrow cellularity was slightly increased in fish oil fed mice resulting in increased granulocyte numbers but no significant difference in total bone marrow B220+ B cell numbers (data not shown). Therefore, even though fish oil appears to have skewed hematopoiesis from lymphopoiesis to granulopoiesis B cell development remained intact.

It is interesting to speculate that fish oil feeding is inducing emigration of transitional B cells from the bone marrow. Specifically, early transitional B cells, T1 B were increased in the spleen by MO, as well as EFO and DFO (**Figure 15A**). Our spleen T1 B cell data supports a recent publication by our collaborator [98]. Teague et al. observed an increase in T1 B cells with an MO diet in the absence of antigen. In conjunction with sufficient B cell development in the bone marrow, increased T1 B cells indicates either increased emigration of transitional B cells from the bone marrow or proliferation of T1 B cells in the spleen. However, we observed a decrease in marginal zone (MZ) B cells in mice fed MO, EFO, and DFO. Our MZ B cell data contrasts Teague et al.'s observation that MZ B cells were increased in MO fed mice. T2 B cells are heterogeneous, some of these cells may seed the spleen directly from the bone marrow, give rise to mature follicular (FO) or MZ B cells, and are cycling *in vivo* [109]. We did not observe a difference in T2 B cells with fish oil feeding, but reduced MZ B

cells could be the result of altered developmental program of T2 B cells from MZ to FO B cells.

Cells at the T2 B cell stage make the decision to become resident MZ B cells or circulating FO B cells based on signal strength of the B cell receptor and other signaling cascades. Hoek et al 2006 showed diacylglycerol responses were specific to spleen B cell subsets where diacylglycerol, IP3, PIP2, and phosphorylated PKC were constitutively expressed at higher levels in T2 B cells compared to T1 B cells [110]. Therefore, altered membrane composition and organization may alter the B cell subset cell survival and development. These observations are tangentially similar to a recent report by Monk et al., whereby they demonstrated in T cells that Th17 polarization was modified by fish oil; furthermore, they observed the same effect on development regardless of whether EPA alone, DHA alone, or EPA+DHA was provided in the diet [111]. Fish oil feeding did not increase FO B cells in the spleen. Therefore, fish oil feeding may induce T2 to mature B cell development and subsequent homing of mature B cells to other peripheral tissues or apoptosis of T2 B cells resulting in reduced MZ B cells and no difference in FO B cells in the spleen. As cells undergoing cell cycle and robust responses to B cell receptor stimulation [109, 112], T2 B cells may provide a target for acute dietary intervention such as fish oil feeding. The differential effects of DFO, EFO, and MO on functional B cell outcomes was not due to differences in B cell developmental phenotype in the spleen as all fish oil diets affected B cell development in the same manner. Lipid microdomain reorganization and phospholipid content changes with fish oil may alter the kinetics of B cell development. Specific n-3 LCPUFAs may differentially alter signaling cascades in B cells resulting in our observed

differences in functional outcomes B cells. This is an area of active and future research for our laboratories.

Fish oil supplementation continues to increase in popularity for purported health benefits. However, the omega-3 fatty acid composition of these supplements varies widely in EPA and DHA content. Fish oil supplements sold for therapeutic are increasingly enriched in EPA, DHA, or both. Yet, the effect of increasing consumption of one or the other on immune function is poorly understood. Findings from this study highlight the differential immunomodulatory outcomes of fish oils differing in fatty acid content on the activation of B cells and their subsequent function. These data and future similar research are critical when considering the clinical immunologic outcomes resulting from fish oil supplementation.

CHAPTER 4: IS THE OMEGA-3 INDEX A VALID MARKER OF INTESTINAL MEMBRANE PHOSPHOLIPID EPA+DHA CONTENT?

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Abstract

Despite numerous studies investigating n-3 long chain polyunsaturated fatty acid (LCPUFA) supplementation and inflammatory bowel diseases (IBD), the extent to which dietary n-3 LCPUFAs incorporate in gastrointestinal (GI) tissues and correlate to the omega-3 index is unknown. In this study, mice were fed three diets with increasing percent of energy (%en) derived from eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA). Dietary levels reflected recommended intakes of fish/fish oil by the American Heart Association. We analyzed the FA composition of phospholipids extracted from red blood cells (RBCs), plasma, and GI tissues. We observed that the 0.1%en EPA+DHA diet was sufficient to significantly increase the omega-3 index (RBC EPA+DHA) after 5 week feeding. The baseline EPA levels were 0.2 – 0.6% across all tissues increasing to 1.6 – 4.3% in the highest EPA+DHA diet; these changes resulted in absolute increases of 1.4 – 3.9% EPA across tissues. The baseline DHA levels were 2.2 – 5.9% across all tissues increasing to 5.8 – 10.5% in the highest EPA+DHA diet; these changes resulted in absolute increases of 3.2 – 5.7% DHA across tissues. These increases in EPA and DHA across all tissues resulted in strong ($r > 0.91$) and significant

($p < 0.001$) linear correlations between the omega-3 index and plasma/GI tissue EPA+DHA content, suggesting that the omega-3 index reflects the relative amounts of EPA+DHA in GI tissues. These data demonstrate that the GI tissues are highly responsive to dietary LCPUFA supplementation and that the omega-3 index can serve as a valid biomarker for assessing dietary EPA+DHA incorporation into GI tissues.

Introduction

Besides the purported cardiovascular benefits of omega-3 (n-3) long chain polyunsaturated fatty acids (LCPUFAs) found in fish, n-3 LCPUFA supplementation has been widely used in the treatment of inflammatory bowel diseases (IBD). Despite numerous clinical trials investigating the effect of n-3 LCPUFA supplementation on gastrointestinal (GI) diseases, no clear and consistent effect of fish oil on ulcerative colitis (UC) has been observed [113]. Pre-clinical diets high in fish and seafood may even increase the risk of developing UC and Crohn's Disease (CD) [114]. Furthermore, in animal models of colitis, treatment with fish oil has shown both beneficial [32, 34] and adverse [3, 108, 115, 116] health outcomes. Aside from disparate models of colitis induction, inconsistent results may be due to varying dose, type, and duration of fish oil exposure. Currently, there is no widely utilized biomarker of GI incorporation of n-3 LCPUFAs, further limiting comparability between studies.

Studies investigating the effect of n-3 LCPUFAs on cardiovascular health outcomes use the n-3 LCPUFA levels of red blood cells (RBCs) as a biomarker of dietary n-3 LCPUFA intake. The omega-3 index, defined as the sum of

eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) in RBCs expressed as a percentage of total fatty acids [117], is a standard for measuring n-3 LCPUFA status *in vivo*. RBCs are longer-lived than platelets and lipoproteins and thus their fatty acid composition is more stable and reflective of chronic n-3 status (much like hemoglobin A1c is a better marker of glycemic status than plasma glucose) [118]. The omega-3 index was originally suggested as an inverse marker of risk for death from coronary heart disease (CHD) and has many characteristics of a risk factor [119]. In a human fish oil supplementation study identifying surrogate tissues for the assessment of n-3 LCPUFA in cardiac tissue, RBCs, plasma, and cheek cells responded to supplementation of 1g EPA+DHA for 6 months with increasing incorporation of n-3 LCPUFAs [106]. The authors observed that the levels of n-3 LCPUFAs in RBCs remain highly correlated to the levels of n-3 LCPUFAs found in cardiac tissue both before and after supplementation with fish oil, despite significant differences in the absolute n-3 LCPUFA levels between the tissues. The baseline EPA+DHA in both the RBCs (4.7%) and cardiac tissue (1.7%) of patients increased on average by ~100% (RBC 9.0%; cardiac tissue 2.7%) demonstrating comparable responses to long-term supplementation. Thus, the n-3 LCPUFA levels in RBCs are not only reflective of dietary n-3 LCPUFA intake, but also serve as a surrogate biomarker of n-3 LCPUFA levels in cardiac tissue [119].

It is unclear, however, whether the omega-3 index adequately reflects the n-3 LCPUFA levels in tissues other than the heart. With inconsistent outcomes among studies assessing the effects of fish oil supplementation on gut health, a surrogate biomarker of n-3 LCPUFA incorporation for GI tissues may help identify discrepancies

that stem from differences in study design (dose, duration, type of fish oil, etc). Therefore, the aim of our research was to investigate whether the fatty acid composition of RBCs (omega-3 index) reflects that of GI tissues. We supplemented the diets of colitis-prone mice with different concentrations of n-3 LCPUFAs (by percent energy in the diet, %en). The doses of EPA+DHA in the murine diets were based upon recommendations by the American Heart Association (AHA) for consumption of fish or fish oil supplementation in humans [120]. These doses translated to 0.1%en, 0.675%en, and 1.8%en from dietary EPA+DHA. Therefore, for someone consuming a 2000 kcal diet, whereby 30% of energy is derived from fat, these diets equate to 250 mg, 1500 mg, and 4000 mg EPA+DHA per day. We assessed the acyl chain composition of phospholipid fatty acids from RBCs, plasma, and several GI tissues (stomach, small intestine, cecum, and colon) to determine whether the RBC is a valid surrogate biomarker for plasma and GI FA composition across increasing intakes of n-3 LCPUFAs.

Materials and Methods

Dietary Treatment

Experimental diets contained increasing amounts of EPA+DHA-enriched fish oil. The pharmaceutical fish oil, Lovaza™, contains 465 mg EPA and 375 mg DHA per 1-g fish oil capsule; therefore, we created an EPA+DHA-enriched fish oil that was not predominantly either EPA or DHA. An EPA+DHA-enriched oil was made by combining appropriate volumes of EPA-enriched and DHA-enriched fish oils (Ocean Nutrition

Canada, Dartmouth, Nova Scotia, Canada) until a 1:1 ratio of EPA:DHA was established. The oils were free of toxins, heavy metals, and oxidation products. The composition of the diets is detailed in **Table 7** and the fatty acid composition in **Table 8**. Using standard AIN-93G as the base diet, all diets contained 7% fat by weight, providing a total of 16.7% Kcal of energy from fat. The control diet contained 7% weight-by-weight (wt/wt) soybean oil, while the experimental diets substituted a portion of the soybean oil with the EPA+DHA-enriched fish oil. The control diet modeled no intake of EPA or DHA. The following recommendations by the AHA for human intakes of fish and fish oil were converted to murine diets on a %en basis. For example, individuals without documented heart disease or dyslipidemia, 250 mg EPA+DHA approximates the n-3 LCPUFA content from the current AHA recommendation to consume at least 2 servings of fish a week. The medium n-3 LCPUFA treatment models the 1000-1500 mg EPA+DHA recommendation from the AHA for patients with documented heart disease, whereas the high n-3 LCPUFA intake models a ~4000 mg EPA+DHA prescription (Lovaza™) for patients with high triglycerides. Therefore, the human doses that were chosen for this study were 250 mg, 1500 mg, and 4000 mg EPA+DHA and were based on a standard 2000 kcal human diet with 30%energy (%en) from fat. These doses translate to 0.1%en, 0.675%en, and 1.8%en from dietary EPA+DHA. To verify accuracy of the EPA:DHA ratio and increasing n-3 LCPUFA content of the diets, the fatty acid composition of the diet was measured by gas chromatography (**Table 8**). Mice were fed the experimental diets *ad libitum* for 5 wk. Diets were kept frozen and changed daily.

Experimental Design

All animals were housed under specific pathogen-free (SPF) conditions in 60-square-inch plastic microisolator cages in the Research Containment Facility at Michigan State University, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal procedures were previously approved by the Michigan State University All-University Committee on Animal Care and Use. A total of twenty-four male and female, 5-wk-old SMAD3-/- mice (n = 6/treatment) were fed either control or one of the three EPA+DHA diets for 5 wk. One of the goals of our lab is to investigate the immunomodulatory effects of fish oil feeding in the SMAD3-/- mouse model of infection-induced colitis. In the absence of infection, SMAD3-/- mice are otherwise healthy and remain free of colitis. To keep consistent with previous studies, we used this mouse model under the assumption that knockout of SMAD3, a transcription factor for TGF-β signaling, does not affect fatty acid uptake and membrane remodeling. After dietary treatment, mice were asphyxiated with CO_2 and exsanguinated through cardiac puncture using a heparin-coated syringe. Tissues were promptly removed, rinsed in cold phosphate buffered saline and placed into sterile microcentrifuge tubes before freezing on dry ice. To obtain red blood cell (RBC) and plasma fractions, whole blood was centrifuged at 13,000 x g for 10 min at 4°C and collected by aspiration into separate tubes. All samples were then transferred under dim lighting into opaque containers for -80°C storage until lipids were extracted.

Extraction of Tissue and Erythrocyte Lipids

Lipid extraction was performed using a modified version of the Rose and Oklander method [101], which utilizes a mixture of 2-propanol:chloroform (11:7 v/v) to

minimize heme interferences within erythrocytes or heme-contaminated tissues [102]. To ensure uniform sampling from each tissue, lipid extraction was preceded by pulverizing frozen tissue (100-200 mg tissue/sample) using a mortar/pestle on dry ice. Under dim lighting, frozen powdered tissue, packed erythrocytes and plasma fractions were transferred into individual 16 x 100 mm Teflon-lined screw-capped glass tubes and immediately resuspended in 2 mL of ice-cold HPLC-grade water (J.T. Baker, Phillipsburg, NJ). In the same vial, samples were homogenized for 30 s using a polytron homogenizer (Kinematica GmbH, Bohemia, NY), tightly capped, and incubated on ice for 15 min. Tissues were then combined with 6 mL of 2-propanol (Sigma – Aldrich, St. Louis, MO) containing 100 µg/mL butyrated hydroxytoluene (BHT; Sigma – Aldrich), vortexed and incubated for 1 h on ice with occasional mixing. Following incubation, 3.8 mL of HPLC-grade chloroform (OmniSolv, Charlotte, NC) was added and samples where incubated for an additional hour on ice with occasional mixing. Phases were divided by centrifugation at 1,800 x g for 30 min (4°C) in a swinging bucket rotor. The lower phase was placed into a new 16 x 100 mm glass tube, while the remaining upper aqueous phase and interfacial protein disc were re-extracted with 3.8 mL of chloroform for 1 h on ice with occasional vortexing. Following centrifugation, the lower phases were combined and dried under a gentle stream of nitrogen at 40°C prior to solid-phase extraction.

Solid-Phase Extraction of Phospholipids

Under minimal lighting, phospholipid isolation using solid-phase extraction was performed according to the modified procedures of Agren *et al.* [103]. Isolute-XL® SPE

aminopropyl columns (500 mg; Biotage, Charlotte, NC) were conditioned twice with 5 mL acetone:water (7:1 v/v) and activated twice with 4 mL *n*-hexane (high purity solvent, OmniSolv, Charlotte, NC). Dried lipids were dissolved in 2 mL of *n*-hexane:chloroform:acetic acid (100:5:5 v/v/v) containing 100 µg/mL BHT. Lipid samples were then added to individual columns monitored to prevent them from drying. To remove neutral lipids and non-esterified free fatty acids (NEFA), columns containing a sample were washed with 2 mL of *n*-hexanes, followed by 5 mL of *n*-hexane:chloroform:ethyl acetate (100:5:5 v/v/v; 100 µg/mL BHT), and 5 mL methanol:chloroform:acetic acid (100:2:2 v/v/v; 100 µg/mL BHT). Collection tubes were placed on ice under the columns and phospholipids were eluted twice with 4 mL of methanol:chloroform:water (100:5:4 v/v/v; 100 µg/mL BHT) and 1 mL high purity methanol (Burdick & Jackson, Morristown, NJ) to remove any residual phospholipids [104]. The collection tubes were brought to volume with chloroform, centrifuged at 2,000 x g for 15 min (4°C) to remove any residual water before completely drying solvent fractions under a gentle stream of nitrogen at 40°C.

Preparation of Fatty Acid Methyl Esters

Fatty acid methyl esters (FAMEs) were prepared from isolated phospholipids fractions by incubation with acidified methanol, according to the methods of Burdge et al. [105]. Dried phospholipids were mixed in 1 mL of toluene (J.T. Baker, Phillipsburg, NJ) by vortexing for 15 s. A 3 mL-aliquot of high purity methanol containing 2% sulfuric acid (J.T. Baker) was then added, gently mixed and incubated under minimal lighting at 55°C for 16 h. The reaction mixture was cooled and neutralized with 3 mL of a solution

containing 250 mM potassium bicarbonate and 500 mM potassium carbonate dissolved in HPLC grade water. Phospholipid FAMEs were isolated by the addition of 5 mL high purity *n*-hexane (containing 100 µg/mL BHT) and the separation of phases by centrifugation at 2,000 x g for 15 min (10°C). The upper *n*-hexane layer was removed, and dried under a gentle stream of nitrogen at room temperature. Isolated phospholipid FAMEs were resuspended in 1-8 µL/mg tissue of *n*-hexane (100 µg/mL BHT), transferred to a GC autosampler vial, and stored under nitrogen at -80°C until analysis.

Fatty Acid Methyl Ester Analysis, Identification, and Quantification

As described previously [117], tissue and erythrocyte fatty acid compositions were analyzed using the HS-Omega-3 Index® methodology at OmegaQuant Analytics, LLC) (Sioux Falls, SD). Phospholipid fatty acid methyl esters were measured by gas chromatography using a GC2010 Gas Chromatograph (Shimadzu, Columbia, MD) equipped with a SP2560, 100-m column (Supelco, Bellefonte, PA) using hydrogen as carrier gas. Fatty acids were identified by comparison with a standard mixture of fatty acids characteristic of erythrocytes. Phospholipid fatty acids were calculated as a percentage of total identified fatty acids after response factor correction. The omega-3 index is erythrocyte EPA plus DHA expressed as weight percentage of total fatty acids [121, 122]. The coefficient of variation for the omega-3 index was < 5%.

Statistical analysis

Significant differences in fatty acid levels between diets for a given tissue were determined using a one-way ANOVA followed by a Tukey *post-hoc* test. Significant

differences in fatty acid levels between tissues for a given diet were determined using a one-way ANOVA followed by a Tukey *post-hoc* test. Within each treatment the data were normally distributed. Pearson correlation coefficients were calculated between RBC vs. plasma and RBC vs. GI tissues for specific fatty acids. Differences with *P* values of < 0.05 were considered statistically significant. All statistical analyses were conducted using the GraphPad Prism 4 software (GraphPad, San Diego, CA).

Table 7 Diet composition

Ingredient	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
	g ingredient / kg diet			
AIN-93G Base[1]	919.986	919.986	919.986	919.986
Soybean Oil	70.0	69.28	65.68	58.48
Enriched Fish Oil[2]	0.0	0.72	4.32	11.52
t-Butyl hydroquinone	0.014	0.014	0.014	0.014
Vitamin Mix	10.0	10.0	10.0	10.0

[1]Products purchased from Dyets, Inc., Bethlehem, PA.

[2]A fish oil enriched for EPA and DHA, where EPA+DHA comprises 65.5% of all fatty acids in the oil (32.75% EPA:32.75% DHA)

Table 8 Fatty acid composition of diets[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
	% of fatty acid of total lipid in the diet			
16:0	3.61 ± 0.03	3.74 ± 011	3.37 ± 0.05	3.39 ± 0.12
16:1	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00	0.07 ± 0.00
18:0	2.70 ± 0.03	2.73 ± 0.06	2.62 ± 0.05	2.77 ± 0.12
18:1 *trans*	0.08 ± 0.01	0.07 ± 0.00	0.07 ± 0.01	0.09 ± 0.01
18:1 *cis*	22.11 ± 0.10	21.60 ± 0.54	21.79 ± 0.09	21.15 ± 0.19
18:2 (n-6)	60.74 ± 0.01	59.13 ± 1.46	58.55 ± 0.23	54.50 ± 0.45
18:3 (n-6)	0.37 ± 0.00	0.36 ± 0.01	0.35 ± 0.00	0.32 ± 0.01
18:3 (n-3)	9.20 ± 0.06	9.00 ± 0.20	8.84 ± 0.14	7.97 ± 0.11
20:4 (n-6)	0.00 ± 0.00	0.01 ± 0.00	0.07 ± 0.01	0.17 ± 0.01
20:5 (n-3)	0.00 ± 0.01	0.03 ± 0.00	1.45 ± 0.09	3.88 ± 0.16
22:5 (n-3)	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.01	0.38 ± 0.02
22:6 (n-3)	0.01 ± 0.00	0.03 ± 0.00	1.44 ± 0.03	3.48 ± 0.14
∑SFA	6.80 ± 0.07	7.12 ± 0.32	6.49 ± 0.11	6.74 ± 0.15
∑MUFA	44.76 ± 0.23	43.72 ± 1.10	44.36 ± 0.23	43.62 ± 0.25
∑PUFA (n-3)	9.21 ± 0.06	9.06 ± 0.20	11.88 ± 0.26	15.70 ± 0.39
∑PUFA (n-6)	61.57 ± 0.02	59.93 ± 1.49	59.51 ± 0.25	55.70 ± 0.30

[1]Data are mean ± SEM reported as percent total fat in the diet, n = 3 technical replications / group.

Results

Diets and the Omega-3 Index

Our first aim was to determine the range of the omega-3 index in mice fed diets reflective of current recommendations for human consumption of fish and/or fish oil supplementation. The omega-3 index increased in a dose-response fashion from 4.5% in mice fed the 0.0%en EPA+DHA diet to ~11% in mice fed the 1.8%en EPA+DHA diet. As the %en from EPA+DHA increased in the diet, the omega-3 index, plasma EPA+DHA, and GI tissue EPA+DHA increased ($p < 0.05$) (**Table 9**). Within the control diet, the omega-3 index and the EPA+DHA content of the small intestine, cecum and colon did not significantly differ. However, the baseline plasma EPA+DHA (6.13%) was significantly higher than the omega-3 index (5.22%), whereas the stomach EPA+DHA (2.34%) was significantly lower. Within the 1.8%en EPA+DHA diet, the EPA+DHA content of the plasma, small intestine, and colon were significantly higher (13.14 – 14.07%), whereas the stomach remained significantly lower (7.34%) compared to the cecum (11.54%) and omega-3 index (11.25%).

To determine whether the changes in the omega-3 index (**Figure 16A**) reflect changes in the plasma and gastrointestinal tract tissues after 5 weeks of feeding, we calculated the Pearson correlation coefficient to test for linear correlations between the omega-3 index and the EPA+DHA content of the other tissues (**Figure 16B-F**). Indeed, strong ($r > 0.91$) and significant ($p < 0.001$) correlations were observed between the omega-3 index and the EPA+DHA content for all other tissues.

LCPUFA Correlations between RBC and plasma or GI tissues

The primary n-3 and n-6 LCPUFAs to change (i.e. the fatty acids that changed the most drastically) were EPA, DHA, and arachidonic acid (AA), whereas minor, albeit significant, changes were observed in other LCPUFAs. The data for all fatty acids from the phospholipids of RBCs, plasma, stomach, small intestine, cecum, and colon across all dietary regimens are provided in **Tables 10-15**. We were next interested in whether any individual n-3 or n-6 LCPUFAs were as strongly correlated between RBCs and plasma and RBCs and GI tissues. RBC EPA was strongly correlated with both plasma EPA and GI tissue EPA, as was the omega-3 index was to plasma/GI EPA+DHA content with correlation coefficients all > 0.91 (**Table 16**). However, RBC DHA was strongly correlated with all other tissue DHA, but was less well correlated to plasma DHA (r = 0.752) and stomach (r = 0.846). RBC AA was also strongly correlated with all other tissue AA, but the correlation was less strong, with all correlations coefficients between 0.81 and 0.89.

EPA, DHA, and AA fold increases

We then calculated the fold-change of EPA, DHA, and AA for each mouse on the experimental diets containing EPA+DHA-enriched fish oil over the respective amount of LCPUFA in the tissues of mice in the control group. Using the fold-change over the control group as an approximation of the expected relative rate of enrichment after five weeks of feeding a control animal at the respective dose, we evaluated n-3 LCPUFA uptake across tissues.

We observed that the RBCs, plasma, stomach, and small intestine took up significantly more EPA than the colon and cecum, regardless of the diet; e.g., the 1.8%en EPA+DHA diet, the RBCs, plasma, stomach, and small intestine contained

approximately 9 – 11 times more EPA than their control counterparts, compared to approximately 4 – 6 times more within the colon and cecum. When correlating the EPA fold-change of RBCs to the fold change of EPA in other tissues, we observed highly significant correlations between tissues over the ranges of doses provided (plasma data not shown, slope ~1.0, $r > 0.70$, and $p < 0.01$) (**Figure 17A-D**). This correlation determines whether the saturation of RBC EPA increases in a similar fashion to that of plasma and GI EPA saturation. Differences in the saturation of tissues may be due to the baseline absolute levels; at baseline, similar differences in the absolute levels of EPA between tissues from mice on the 0.0%en EPA+DHA diet (0.2 – 0.6%) remain apparent after increases in EPA in mice fed the 1.8%en EPA+DHA diet (1.6 – 4.3%) (**Figure 17E-F**).

The fold-change increases of DHA across all tissues were considerably smaller compared to the fold-changes of EPA, resulting in approximately 1.6 – 2.7 times as much DHA in tissues from mice on the 1.8%en EPA+DHA diets compared to those on the control diet. Still, there were significant differences in the fold-change of DHA between tissues within any given diet. For the mice on the 1.8%en EPA+DHA diet, the fold-change of DHA in RBCs (1.6) and plasma (1.7) was considerably lower than the stomach (2.6) and small intestine (2.3). Comparably the fold-changes of DHA in the cecum (2.2) and colon (2.0) were in-between the aforementioned tissues, often with insignificant comparisons. When correlating the DHA fold-change of RBCs to that of other tissues, we observed highly significant correlations between tissues over the ranges of doses provided (plasma data not shown, slope ~1.0, $r > 0.70$, and $p < 0.01$) (**Figure 18A-D**). This correlation determines whether the saturation of RBC DHA

increases in a similar fashion to that of plasma/GI DHA saturation. Differences in the saturation of tissues may be due to the baseline absolute levels; at baseline, similar differences in the absolute levels of DHA between tissues from mice on the 0.0%en EPA+DHA diet (2.2 – 5.9) remain apparent after increases in DHA in mice fed the 1.8%en EPA+DHA diet (5.8 – 10.5%) (**Figure 18E-F**).

Consistent with the literature and despite the increasing amount of AA in the EPA+DHA-enriched fish oil diets, AA was displaced from phospholipid fatty acids as the dose of EPA+DHA increased. Notably, the 0.1%en and 0.675%en EPA+DHA diets did not induce the same displacement of AA when comparing between tissues. However, for all tissues from mice on the 1.8%en EPA+DHA diet, AA was 0.50 – 0.63 times less than the control diet with no significant differences observed between tissues. When correlating the AA fold-change of RBCs to that of other tissues, we observed highly, significant correlations between tissues over the ranges of doses provided (plasma data not shown, slope ~1.0, $r > 0.70$, and $p < 0.01$) (**Figure 19A-D**). This correlation determines whether the displacement of RBC AA increases in a similar fashion to that of plasma/GI AA displacement. Differences in the displacement of tissue AA may be due to the baseline absolute levels; at baseline, similar differences in the absolute levels of AA between tissues from mice on the 0.0%en EPA+DHA diet (6.9 – 14.1%) remain apparent after increases in AA in mice fed the 1.8%en EPA+DHA diet (3.9 – 8.5%) (**Figure 19E-F**).

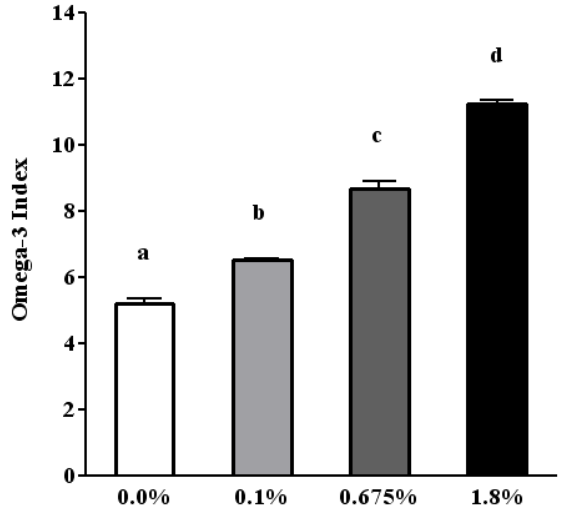
Table 9 Level of EPA+DHA in blood and GI tract tissues of SMAD3-/- mice fed diets with increasing %en from EPA+DHA[1]

	Omega-3 Index[2] / EPA+DHA					
%en EPA +DHA	**RBC**	**Plasma**	**Stomach**	**Small Intestine**	**Cecum**	**Colon**
0.0%	$5.22 \pm 0.16^{a\ A}$	$6.13 \pm 0.42^{a\ B}$	$2.34 \pm 0.18^{a\ C}$	$4.64 \pm 0.28^{a\ A}$	$4.76 \pm 0.28^{a\ AB}$	$5.48 \pm 0.07^{a\ A}$
0.1%	$6.52 \pm 0.06^{b\ A}$	$7.31 \pm 0.32^{a\ B}$	$3.53 \pm 0.06^{ab\ C}$	$6.61 \pm 0.20^{b\ AB}$	$6.11 \pm 0.18^{b\ AB}$	$6.95 \pm 0.18^{b\ A}$
0.675%	$8.69 \pm 0.22^{c\ AC}$	$10.44 \pm 0.66^{b\ C}$	$4.70 \pm 0.17^{b\ B}$	$9.79 \pm 0.67^{c\ AC}$	$8.02 \pm 0.35^{c\ AC}$	$9.60 \pm 0.31^{c\ A}$
1.8%	$11.25 \pm 0.13^{d\ A}$	$13.21 \pm 0.96^{c\ AC}$	$7.34 \pm 0.59^{c\ B}$	$14.07 \pm 0.39^{d\ C}$	$11.54 \pm 0.21^{d\ AC}$	$13.14 \pm 0.24^{d\ A}$

[1] Data are reported as mean ± SEM expressed as the percent of total fatty acid isolated from membrane phospholipids; n = 6 mice/group. Individual one-way ANOVAs with Tukey's *post-hoc* tests were used to assess significant differences across the different %en EPA+DHA doses within a single tissue (lower case letters) or across the different tissues within a single %en EPA+DHA dose (upper case letters). Differences between lower case letters or differences between upper case letters imply significant differences at the $P < 0.05$ level.

[2] The omega-3 index is the sum of EPA + DHA in red blood cells (RBC) expressed as a percentage of all other fatty acids and extended here to other tissues.

A



B

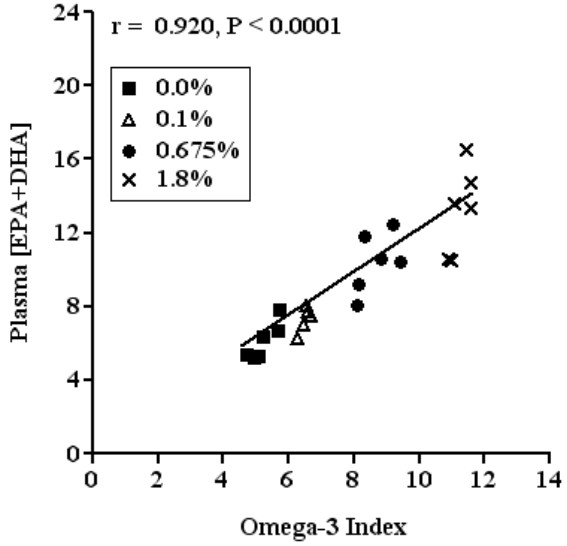


Figure 16 Correlations between the omega-3 index of red blood cell and the EPA+DHA of plasma or gastrointestinal tract tissues from mice fed diets with increasing EPA+DHA. (A) The omega-3 index from mice fed AIN-93G diet with either 0.0%en EPA+DHA, 0.1%en EPA+DHA, 0.675%en EPA+DHA, or 1.8%en EPA+DHA (n = 6/group). A one-way ANOVA and a Tukey's *post-hoc* were used to assess differences in the tissue-specific EPA+DHA compared to each other. Differing letters denote statistical significance at $P < 0.05$. (B-E) The omega-3 index in RBC phospholipids correlated to the EPA+DHA of plasma (B), stomach (C), small intestine (D), cecum (E), and colon (F) phospholipids. A Pearson correlation was used to test for linear correlation between the aforementioned tissues (n=24).

Figure 16 (cont'd)

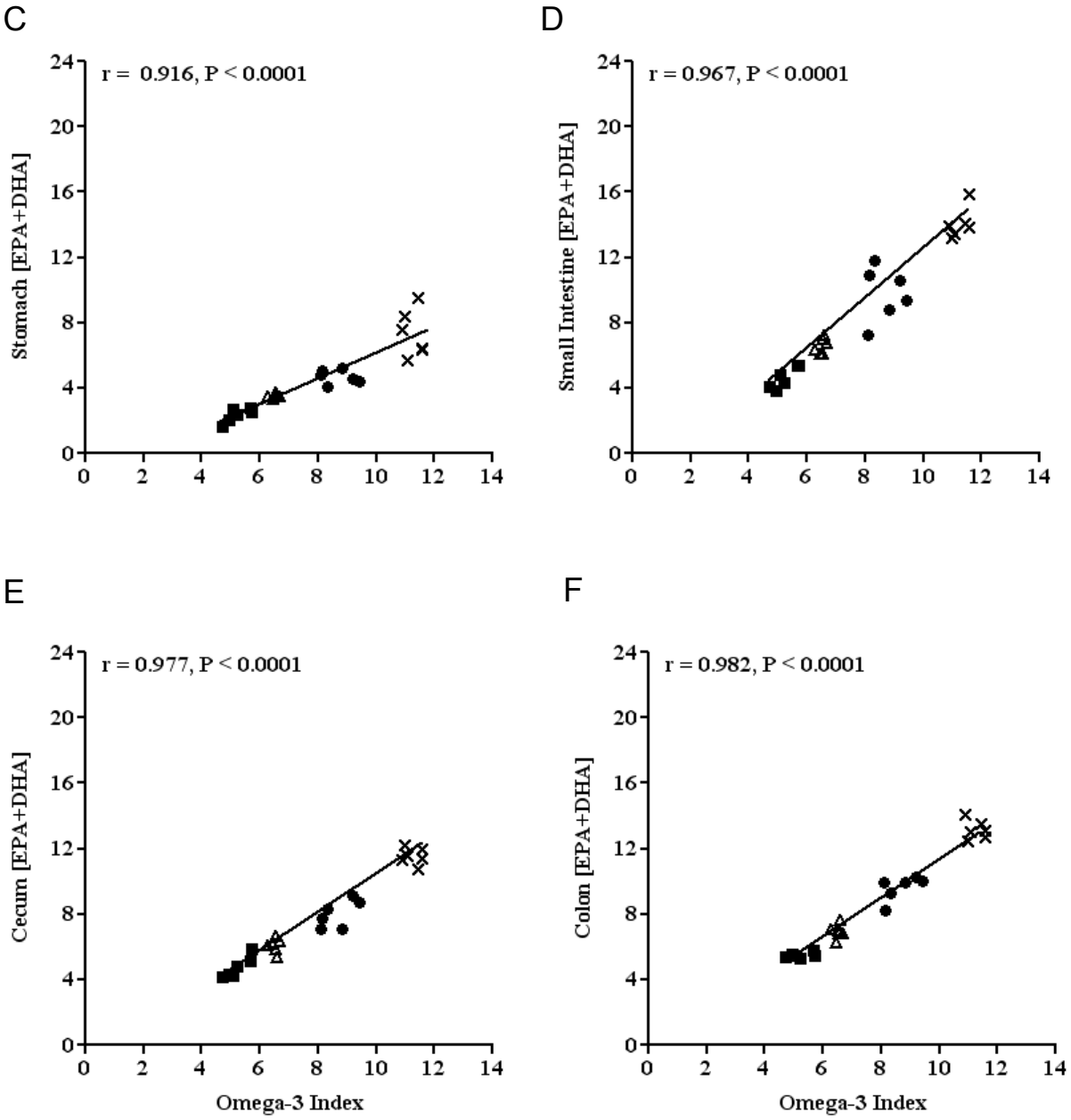


Table 10 Fatty acid composition of red blood cells[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
	% of fatty acid in the red blood cells			
16:0	33.83 ± 0.29	33.71 ± 0.20	34.73 ± 0.54	36.90 ± 0.71
16:1(n-7) *trans*	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.00
16:1 (n-7)	0.31 ± 0.02	0.29 ± 0.01	0.28 ± 0.04	0.29 ± 0.01
18:0	11.28 ± 0.35	11.33 ± 0.19	11.11 ± 0.43	10.91 ± 0.28
18:1 *trans*	0.57 ± 0.28	0.20 ± 0.03	0.28 ± 0.08	0.26 ± 0.02
18:1 *cis*	10.83 ± 0.23	10.88 ± 0.04	10.76 ± 0.06	10.98 ± 0.22
18:2 (n-6) *trans*	0.45 ± 0.16	0.20 ± 0.03	0.24 ± 0.05	0.21 ± 0.04
18:2 (n-6)	14.01 ± 0.38	14.36 ± 0.38	14.90 ± 0.52	12.55 ± 0.47
20.0	0.46 ± 0.01	0.42 ± 0.01	0.42 ± 0.00	0.42 ± 0.02
18:3 (n-6)	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.04 ± 0.00
20:1 (n-9)	0.55 ± 0.12	0.34 ± 0.01	0.33 ± 0.02	0.31 ± 0.02
18:3 (n-3)	0.18 ± 0.01	0.18 ± 0.00	0.18 ± 0.01	0.13 ± 0.01
20:2 (n-6)	0.50 ± 0.08	0.38 ± 0.01	0.39 ± 0.01	0.32 ± 0.02
22:0	1.27 ± 0.02	1.27 ± 0.02	1.24 ± 0.01	1.27 ± 0.04
20:3 (n-6)	1.04 ± 0.04	0.95 ± 0.01	0.93 ± 0.01	0.71 ± 0.02
20:4 (n-6)	11.34 ± 0.32	11.24 ± 0.14	8.03 ± 0.21	5.88 ± 0.11
24:0	2.52 ± 0.07	2.49 ± 0.06	2.55 ± 0.08	2.64 ± 0.10
20:5 (n-3)	0.26 ± 0.04	0.43 ± 0.01	1.55 ± 0.06	3.08 ± 0.10
24:1 (n-9)	2.08 ± 0.06	1.96 ± 0.03	1.99 ± 0.01	2.08 ± 0.04
22:4 (n-6)	1.92 ± 0.04	1.78 ± 0.02	1.20 ± 0.03	0.95 ± 0.03
22:5 (n-6)	0.72 ± 0.07	0.48 ± 0.03	0.36 ± 0.02	0.35 ± 0.03
22:5 (n-3)	0.72 ± 0.03	0.82 ± 0.02	1.21 ± 0.09	1.44 ± 0.04
22:6 (n-3)	4.97 ± 0.16	6.09 ± 0.06	7.14 ± 0.21	8.18 ± 0.08
EPA+DHA	5.22 ± 0.16	6.52 ± 0.06	8.69 ± 0.22	11.25 ± 0.13
EPA+DPA+DHA	5.94 ± 0.15	7.34 ± 0.06	9.90 ± 0.30	12.69 ± 0.13
∑SFA	49.36 ± 0.62	49.22 ± 0.36	50.04 ± 0.19	52.15 ± 0.92
∑MUFA	14.47 ± 0.44	13.80 ± 0.09	13.77 ± 0.12	14.04 ± 0.27
∑PUFA (n-3)	6.12 ± 0.14	7.52 ± 0.06	10.08 ± 0.29	12.82 ± 0.14
∑PUFA (n-6)	30.05 ± 0.31	29.46 ± 0.29	26.11 ± 0.45	21.00 ± 0.57

[1]Data are mean ± SEM reported as percent total, n = 6.

Table 11 Fatty acid composition of plasma[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
		% of fatty acid in the plasma		
16:0	28.98 ± 0.78	29.39 ± 0.45	28.00 ± 0.54	30.62 ± 0.95
16:1(n-7) *trans*	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.15 ± 0.01
16:1 (n-7)	0.28 ± 0.01	0.23 ± 0.02	0.28 ± 0.05	0.31 ± 0.03
18:0	16.12 ± 0.56	16.60 ± 0.91	15.30 ± 0.61	14.62 ± 0.43
18:1 *trans*	0.60 ± 0.27	0.59 ± 0.34	1.09 ± 0.79	1.13 ± 0.65
18:1 *cis*	6.36 ± 0.17	5.79 ± 0.24	6.23 ± 0.45	5.81 ± 0.25
18:2 (n-6) *trans*	0.54 ± 0.22	0.47 ± 0.22	0.60 ± 0.34	0.99 ± 0.59
18:2 (n-6)	28.57 ± 1.20	27.89 ± 0.99	26.90 ± 1.34	22.92 ± 1.45
20.0	0.36 ± 0.04	0.23 ± 0.01	0.26 ± 0.02	0.30 ± 0.03
18:3 (n-6)	0.10 ± 0.00	0.09 ± 0.00	0.09 ± 0.01	0.07 ± 0.01
20:1 (n-9)	0.60 ± 0.19	0.43 ± 0.19	0.56 ± 0.26	0.93 ± 0.54
18:3 (n-3)	0.26 ± 0.01	0.29 ± 0.02	0.25 ± 0.03	0.17 ± 0.03
20:2 (n-6)	0.56 ± 0.11	0.45 ± 0.09	0.53 ± 0.10	0.75 ± 0.30
22:0	0.47 ± 0.03	0.38 ± 0.01	0.39 ± 0.02	0.49 ± 0.03
20:3 (n-6)	1.22 ± 0.04	1.12 ± 0.08	1.47 ± 0.13	1.17 ± 0.05
20:4 (n-6)	6.94 ± 0.44	7.00 ± 0.27	5.58 ± 0.31	3.93 ± 0.25
24:0	0.27 ± 0.01	0.25 ± 0.01	0.32 ± 0.02	0.34 ± 0.02
20:5 (n-3)	0.26 ± 0.02	0.52 ± 0.01	1.61 ± 0.07	2.72 ± 0.38
24:1 (n-9)	0.82 ± 0.05	0.85 ± 0.05	1.00 ± 0.04	1.33 ± 0.09
22:4 (n-6)	0.17 ± 0.02	0.13 ± 0.01	0.08 ± 0.01	0.05 ± 0.00
22:5 (n-6)	0.18 ± 0.02	0.06 ± 0.01	0.05 ± 0.00	0.06 ± 0.00
22:5 (n-3)	0.31 ± 0.02	0.29 ± 0.02	0.45 ± 0.05	0.66 ± 0.05
22:6 (n-3)	5.88 ± 0.40	6.79 ± 0.28	8.83 ± 0.63	10.49 ± 1.04
EPA+DHA	6.13 ± 0.42	7.31 ± 0.29	10.44 ± 0.66	13.21 ± 0.96
EPA+DPA+DHA	6.44 ± 0.41	7.60 ± 0.31	10.88 ± 0.71	13.87 ± 1.01
∑SFA	46.19 ± 1.44	46.86 ± 1.02	44.26 ± 1.05	46.37 ± 1.02
∑MUFA	8.82 ± 0.70	8.04 ± 0.33	9.32 ± 0.88	9.67 ± 1.03
∑PUFA (n-3)	6.70 ± 0.46	7.89 ± 0.31	11.13 ± 0.70	14.04 ± 1.00
∑PUFA (n-6)	38.29 ± 2.04	37.22 ± 1.01	35.29 ± 1.20	29.92 ± 0.84

[1]Data are mean ± SEM reported as percent total, n = 6.

Table 12 Fatty acid composition of stomach[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
		% of fatty acid in the stomach		
16:0	27.63 ± 1.05	26.38 ± 2.03	29.12 ± 1.12	28.74 ± 0.77
16:1(n-7) *trans*	0.10 ± 0.01	0.83 ± 0.50	0.09 ± 0.01	0.26 ± 0.16
16:1 (n-7)	0.82 ± 0.10	0.99 ± 0.18	0.97 ± 0.22	0.86 ± 0.08
18:0	12.54 ± 0.45	12.91 ± 0.73	13.42 ± 0.65	13.75 ± 0.52
18:1 *trans*	0.45 ± 0.06	1.52 ± 0.77	0.34 ± 0.06	0.65 ± 0.26
18:1 *cis*	16.67 ± 0.76	14.61 ± 0.82	15.01 ± 0.56	15.30 ± 0.69
18:2 (n-6) *trans*	0.30 ± 0.11	1.28 ± 0.71	0.42 ± 0.20	0.57 ± 0.26
18:2 (n-6)	17.75 ± 1.04	15.88 ± 0.72	16.99 ± 0.56	15.56 ± 0.55
20.0	1.06 ± 0.07	1.09 ± 0.05	1.06 ± 0.03	1.19 ± 0.09
18:3 (n-6)	0.15 ± 0.01	0.16 ± 0.01	0.14 ± 0.02	0.12 ± 0.01
20:1 (n-9)	0.58 ± 0.06	1.29 ± 0.61	0.45 ± 0.02	0.83 ± 0.27
18:3 (n-3)	0.40 ± 0.10	0.34 ± 0.13	0.22 ± 0.04	0.15 ± 0.02
20:2 (n-6)	0.67 ± 0.03	0.88 ± 0.24	0.60 ± 0.02	0.69 ± 0.16
22:0	1.55 ± 0.12	1.69 ± 0.08	1.63 ± 0.08	1.83 ± 0.13
20:3 (n-6)	0.69 ± 0.06	0.87 ± 0.08	0.90 ± 0.11	0.81± 0.06
20:4 (n-6)	12.06 ± 0.53	9.42 ± 0.16	7.34 ± 0.14	6.07 ± 0.17
24:0	2.20 ± 0.22	2.32 ± 0.21	2.41 ± 0.11	2.64± 0.16
20:5 (n-3)	0.16 ± 0.02	0.32 ± 0.02	0.81 ± 0.08	1.56 ± 0.12
24:1 (n-9)	1.31 ± 0.12	1.27 ± 0.07	1.23 ± 0.03	1.27 ± 0.03
22:4 (n-6)	1.23 ± 0.11	1.20 ± 0.08	0.66 ± 0.04	0.44 ± 0.02
22:5 (n-6)	0.38 ± 0.04	0.25 ± 0.02	0.20 ± 0.02	0.16 ± 0.01
22:5 (n-3)	0.43 ± 0.04	0.59 ± 0.03	0.80 ± 0.08	1.14 ± 0.08
22:6 (n-3)	2.18 ± 0.17	3.21 ± 0.04	3.89 ± 0.13	5.78 ± 0.55
EPA+DHA	2.34 ± 0.18	3.53 ± 0.06	4.70 ± 0.17	7.34 ± 0.59
EPA+DPA+DHA	2.76 ± 0.21	4.13 ± 0.08	5.51 ± 0.24	8.48 ± 0.65
∑SFA	44.98 ± 1.60	44.39 ± 2.82	47.73 ± 1.65	48.16 ± 1.50
∑MUFA	19.93 ± 0.81	20.52 ± 2.59	18.09 ± 0.58	19.17 ± 1.22
∑PUFA (n-3)	3.16 ± 0.13	4.46 ± 0.16	5.72 ± 0.23	8.63 ± 0.64
∑PUFA (n-6)	33.23 ± 0.91	29.95 ± 0.75	27.25 ± 0.59	24.41 ± 0.51

[1]Data are mean ± SEM reported as percent total, n = 6.

T**able 13** Fatty acid composition of small intestine[1]

Fatty Acid	**0.0%en EPA+DHA**	**0.1%en EPA+DHA**	**0.675%en EPA+DHA**	**1.8%en EPA+DHA**
		% of fatty acid in the small intestine		
16:0	20.26 ± 0.47	21.43 ± 0.27	23.70 ± 0.87	23.26 ± 0.44
16:1(n-7) *trans*	0.13 ± 0.01	0.13 ± 0.01	0.14 ± 0.02	0.12 ± 0.02
16:1 (n-7)	0.29 ± 0.07	0.44 ± 0.10	1.25 ± 0.43	0.70 ± 0.23
18:0	21.94 ± 0.41	20.62 ± 0.75	16.83 ± 0.71	19.87 ± 0.94
18:1 *trans*	0.41 ± 0.08	0.53 ± 0.11	0.60 ± 0.16	0.51 ± 0.10
18:1 *cis*	7.41 ± 0.08	8.29 ± 0.58	10.63 ± 1.15	8.70 ± 1.07
18:2 (n-6) *trans*	0.21 ± 0.03	0.31 ± 0.09	0.32 ± 0.09	0.25 ± 0.04
18:2 (n-6)	26.80 ± 1.30	20.90 ± 0.97	15.76 ± 1.01	17.81 ± 1.28
20.0	0.49 ± 0.02	0.60 ± 0.05	0.83 ± 0.06	0.69 ± 0.07
18:3 (n-6)	0.16 ± 0.01	0.17 ± 0.00	0.14 ± 0.01	0.13 ± 0.01
20:1 (n-9)	0.33 ± 0.02	0.42 ± 0.08	0.38 ± 0.03	0.35 ± 0.08
18:3 (n-3)	0.32 ± 0.02	0.27 ± 0.04	0.35 ± 0.08	0.20 ± 0.04
20:2 (n-6)	0.41 ± 0.01	0.42 ± 0.03	0.55 ± 0.05	0.35 ± 0.01
22:0	0.50 ± 0.03	0.70 ± 0.07	0.98 ± 0.06	0.78 ± 0.08
20:3 (n-6)	1.59 ± 0.08	1.79 ± 0.11	1.72 ± 0.22	1.36 ± 0.07
20:4 (n-6)	12.78 ± 0.10	10.61 ± 0.83	10.40 ± 0.81	7.03 ± 0.55
24:0	0.28 ± 0.02	0.41 ± 0.04	0.63 ± 0.03	0.49 ± 0.06
20:5 (n-3)	0.39 ± 0.02	0.68 ± 0.02	1.40 ± 0.14	4.26 ± 0.22
24:1 (n-9)	0.35 ± 0.03	0.54 ± 0.07	0.87 ± 0.06	0.67 ± 0.10
22:4 (n-6)	1.39 ± 0.09	1.72 ± 0.14	1.37 ± 0.08	0.53 ± 0.05
22:5 (n-6)	0.25 ± 0.02	0.22 ± 0.01	0.23 ± 0.02	0.17 ± 0.02
22:5 (n-3)	0.76 ± 0.02	1.17 ± 0.08	2.00 ± 0.16	2.62 ± 0.10
22:6 (n-3)	4.25 ± 0.27	5.94 ± 0.20	8.21 ± 0.48	9.98 ± 0.25
EPA+DHA	4.64 ± 0.28	6.61 ± 0.22	9.79 ± 0.67	14.07 ± 0.39
EPA+DPA+DHA	5.40 ± 0.27	7.78 ± 0.29	12.12 ± 0.80	16.35 ± 0.47
∑SFA	43.46 ± 0.35	43.75 ± 0.66	42.98 ± 0.91	45.10 ± 0.62
∑MUFA	8.92 ± 0.30	10.35 ± 0.86	13.88 ± 1.70	11.06 ± 1.44
∑PUFA (n-3)	5.72 ± 0.29	8.05 ± 0.27	11.97 ± 0.68	17.06 ± 0.50
∑PUFA (n-6)	43.59 ± 1.17	36.16 ± 0.93	30.49 ± 1.28	27.62 ± 1.02

[1]Data are mean ± SEM reported as percent total, n = 6.

Table 14 Fatty acid composition of cecum[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
		% of fatty acid in the cecum		
16:0	22.23 ± 0.37	23.14 ± 0.49	23.01 ±0.66	23.69 ±0.47
16:1(n-7) *trans*	0.13 ± 0.01	0.11 ± 0.01	0.17 ± 0.04	0.09 ± 0.01
16:1 (n-7)	2.21 ± 0.28	1.77 ± 0.22	1.62 ± 0.32	2.92 ± 0.51
18:0	14.16 ± 0.38	15.57 ± 0.42	16.21 ± 0.68	14.07 ± 1.19
18:1 *trans*	0.87 ± 0.23	0.44 ± 0.05	1.03 ± 0.53	0.50 ± 0.10
18:1 *cis*	18.18 ± 0.49	15.93 ± 0.67	12.84 ± 0.84	17.99 ± 1.37
18:2 (n-6) *trans*	0.72 ± 0.20	0.45 ± 0.07	0.77 ± 0.38	0.50 ± 0.06
18:2 (n-6)	13.32 ± 0.91	11.08 ± 0.49	10.42 ± 0.99	14.62 ±1.34
20.0	0.76 ± 0.04	1.01 ± 0.14	1.15 ± 0.07	0.79 ± 0.07
18:3 (n-6)	0.20 ± 0.01	0.15 ± 0.02	0.12 ± 0.02	0.11 ± 0.01
20:1 (n-9)	0.72 ± 0.15	0.43 ± 0.03	0.67 ±0.24	0.56 ± 0.05
18:3 (n-3)	0.63 ± 0.08	0.40 ± 0.05	0.40 ± 0.11	0.68 ± 0.16
20:2 (n-6)	0.62 ± 0.02	0.67 ± 0.04	0.74 ± 0.03	0.48 ± 0.03
22:0	0.73 ± 0.04	0.97 ± 0.13	1.14 ± 0.05	0.76 ± 0.07
20:3 (n-6)	1.52 ± 0.04	1.58 ± 0.13	1.41 ± 0.11	1.38 ± 0.18
20:4 (n-6)	14.19 ± 0.31	12.22 ± 0.07	10.91 ± 0.36	7.13 ± 0.77
24:0	0.51 ± 0.04	0.65 ± 0.08	0.75 ± 0.02	0.55 ± 0.04
20:5 (n-3)	0.60 ± 0.02	0.70 ± 0.09	1.02 ± 0.09	2.29 ± 0.13
24:1 (n-9)	0.84 ± 0.08	1.15 ± 0.22	1.33 ± 0.09	0.79 ± 0.07
22:4 (n-6)	2.47 ± 0.10	2.74 ± 0.11	2.08 ± 0.12	0.77 ± 0.07
22:5 (n-6)	0.61 ± 0.04	0.49 ± 0.03	0.46 ± 0.04	0.23 ± 0.03
22:5 (n-3)	1.21 ± 0.05	1.43 ± 0.04	1.97 ± 0.13	2.65 ± 0.07
22:6 (n-3)	4.16 ± 0.29	5.41 ± 0.13	7.01 ± 0.28	9.25 ± 0.15
EPA+DHA	4.76 ± 0.28	6.11 ± 0.18	8.02 ± 0.35	11.54 ± 0.21
EPA+DPA+DHA	5.96 ± 0.26	7.54 ± 0.20	10.00 ± 0.45	14.18 ± 0.27
∑SFA	38.39 ± 0.74	41.33 ± 1.00	42.26 ± 0.85	39.86 ± 1.50
∑MUFA	22.94 ± 0.62	19.83 ± 0.62	17.66 ± 0.74	22.85 ± 1.71
∑PUFA (n-3)	6.59 ± 0.20	7.94 ± 0.16	10.39 ± 0.45	14.86 ± 0.17
∑PUFA (n-6)	33.66 ± 0.76	29.39 ± 0.48	26.91 ± 0.64	25.21 ± 0.68

[1]Data are mean ± SEM reported as percent total, n = 6.

Table 15 Fatty acid composition of colon[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
		% of fatty acid in the colon		
16:0	24.87 ± 0.75	24.28 ± 0.63	23.94 ± 0.43	23.70 ± 0.27
16:1(n-7) *trans*	0.09 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.07 ± 0.00
16:1 (n-7)	1.61 ± 0.16	1.53 ± 0.08	1.67 ± 0.32	1.55 ± 0.13
18:0	15.63 ± 0.52	15.60 ± 0.32	16.42 ± 0.39	15.79 ± 0.39
18:1 *trans*	0.58 ± 0.17	0.56 ± 0.49	0.49 ± 0.10	0.49 ± 0.16
18:1 *cis*	14.50 ± 0.34	14.46 ± 0.51	13.09 ± 0.51	14.32 ± 0.48
18:2 (n-6) *trans*	0.62 ± 0.14	0.43 ± 0.08	0.42 ± 0.06	0.42 ± 0.18
18:2 (n-6)	9.65 ± 0.45	10.35 ± 0.71	9.35 ± 0.55	10.47 ± 0.51
20.0	1.01 ± 0.06	1.07 ± 0.09	1.32 ± 0.05	1.03 ± 0.07
18:3 (n-6)	0.28 ± 0.02	0.25 ± 0.02	0.21 ± 0.02	0.17 ± 0.02
20:1 (n-9)	0.35 ± 0.03	0.33 ± 0.01	0.30 ± 0.01	0.27 ± 0.03
18:3 (n-3)	0.24 ± 0.03	0.25 ± 0.04	0.28 ± 0.09	0.22 ± 0.02
20:2 (n-6)	0.63 ± 0.05	0.58 ± 0.05	0.65 ± 0.06	0.55 ± 0.09
22:0	1.00 ± 0.05	1.08 ± 0.09	1.34 ± 0.04	1.06 ± 0.05
20:3 (n-6)	3.88 ± 0.28	3.18 ± 0.24	2.82 ± 0.32	2.61 ± 0.13
20:4 (n-6)	13.51 ± 0.32	13.02 ± 0.28	11.58 ± 0.48	8.50 ± 0.27
24:0	0.65 ± 0.06	0.96 ± 0.23	0.88 ± 0.06	0.78 ± 0.04
20:5 (n-3)	0.55 ± 0.02	0.65 ± 0.03	1.10 ± 0.11	3.15 ± 0.32
24:1 (n-9)	0.92 ± 0.07	1.03 ± 0.12	1.30 ± 0.08	1.05 ± 0.08
22:4 (n-6)	2.57 ± 0.10	2.62 ± 0.08	2.14 ± 0.12	1.13 ± 0.06
22:5 (n-6)	0.51 ± 0.05	0.35 ± 0.03	0.26 ± 0.02	0.15 ± 0.01
22:5 (n-3)	1.03 ± 0.04	1.16 ± 0.06	1.96 ± 0.06	2.48 ± 0.08
22:6 (n-3)	4.93 ± 0.08	6.30 ± 0.19	8.51 ± 0.24	9.99 ± 0.22
EPA+DHA	5.48 ± 0.07	6.95 ±0.18	9.60 ± 0.31	13.14 ± 0.24
EPA+DPA+DHA	6.51 ± 0.08	8.11 ± 0.18	11.57 ± 0.35	15.62 ± 0.24
∑SFA	43.15± 1.14	42.99 ± 1.00	43.89 ± 0.46	42.35 ± 0.52
∑MUFA	18.05 ± 0.52	18.00 ± 0.38	16.93 ± 0.86	17.76 ± 0.48
∑PUFA (n-3)	6.75 ± 0.07	8.36 ± 0.15	11.84 ± 0.27	15.84 ± 0.23
∑PUFA (n-6)	31.65 ± 0.51	30.77 ± 1.00	27.44 ± 0.50	23.99 ± 0.23

[1]Data are mean ± SEM reported as percent total, n = 6.

Table 16 Pearson Correlation Coefficients for n-3 or n-6 LCPUFAs between red blood cells and specified tissues in SMAD3-/- fed diets with increasing %en from EPA+DHA

	Pearson Correlation Coefficients[1] against Red Blood Cell			
	EPA	**DHA**	**AA**	**EPA+DHA**
Plasma	0.912	0.752	0.894	0.920
Stomach	0.969	0.846	0.867	0.916
Small Intestine	0.963	0.947	0.811	0.967
Cecum	0.915	0.948	0.885	0.977
Colon	0.910	0.936	0.877	0.982

[1] All Pearson correlation coefficients assessed the linear correlation between red blood cells vs. plasma or GI tissue for the fatty acids specified. All Pearson's r coefficients were statistically significant at the $P < 0.0001$.

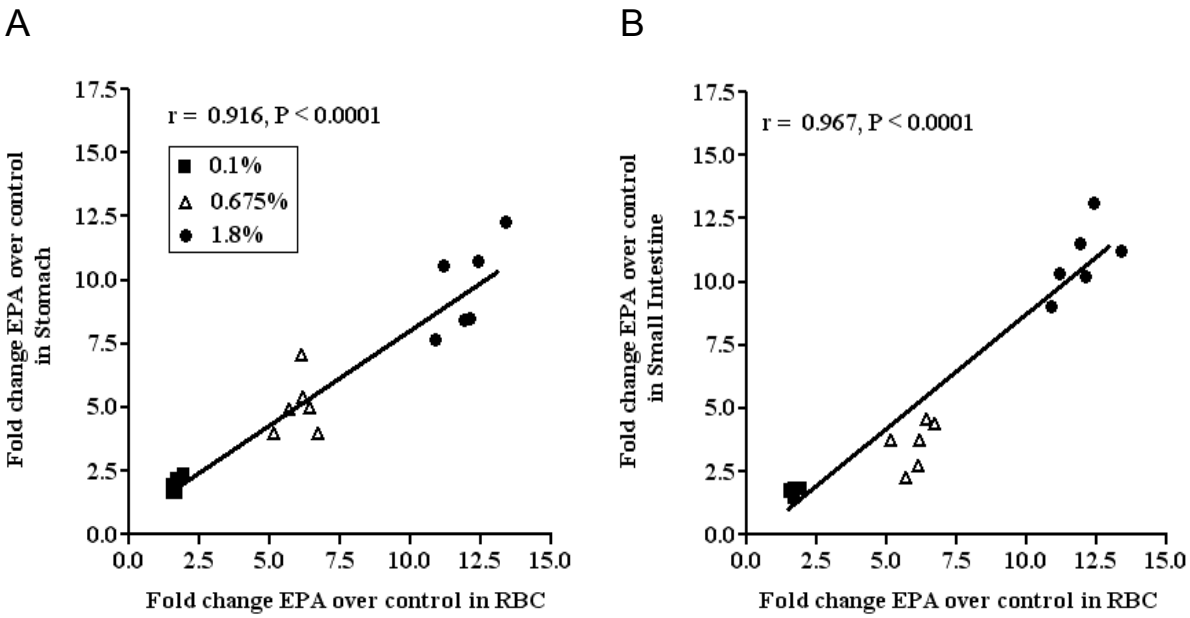
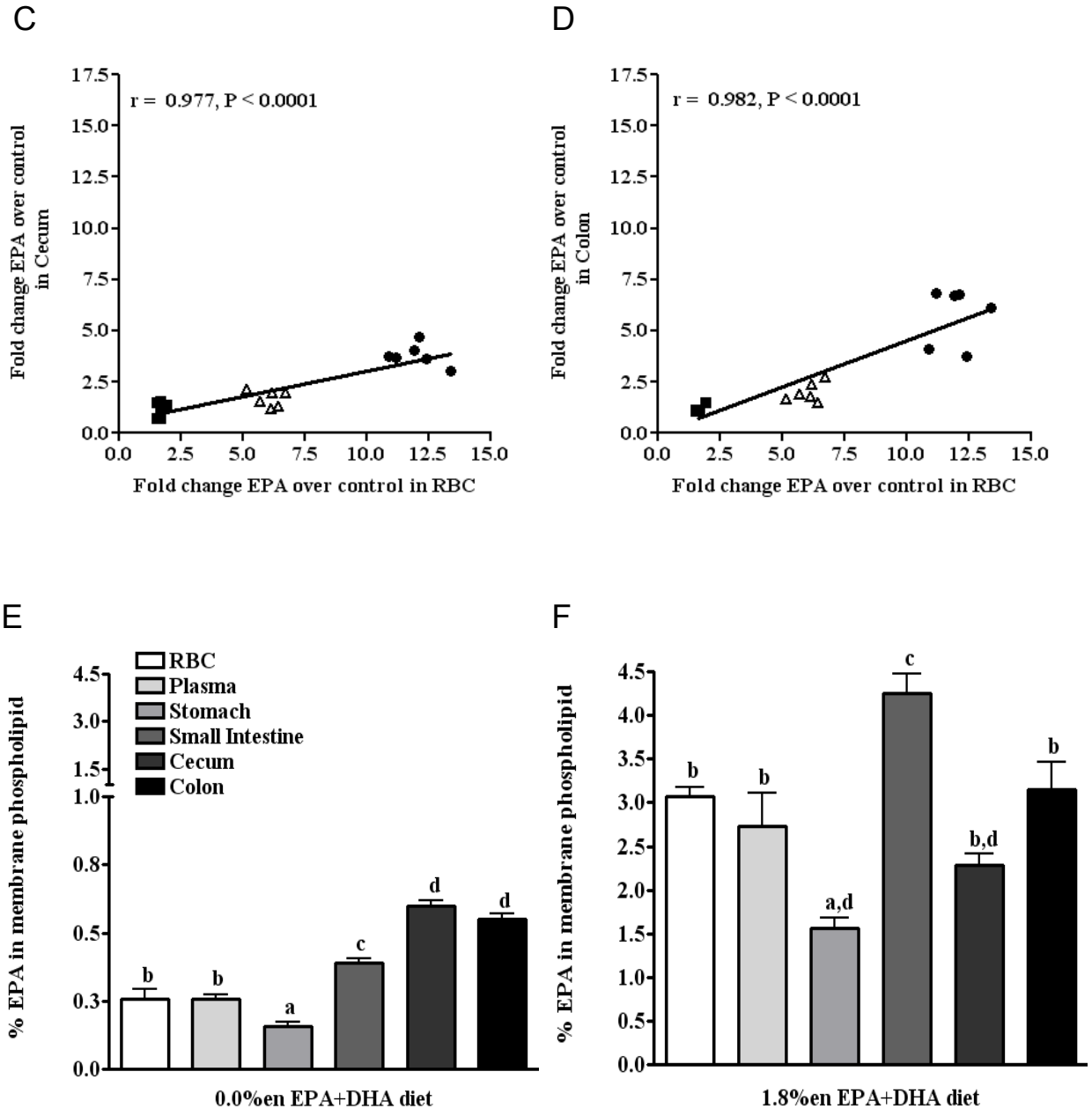


Figure 17 Comparison of EPA content in phospholipid fatty acids from blood and gastrointestinal tissues mice fed diets with increasing EPA+DHA. (A-D) Fold Change in EPA content over the control group in phospholipid fatty acids from gastrointestinal tissues correlated to EPA content in phospholipid fatty acids from red blood cells: (A) Stomach, (B) Small Intestine (C) Cecum, and (D) Colon. Data from the tissues of mice fed experimental diets containing EPA+DHA-enriched fish oil were normalized to the 0.0%en EPA+DHA diet group to assess fold changes in EPA content of phospholipids. A Pearson correlation was used to test linear correlation between gastrointestinal tissue EPA fold change and RBC EPA fold change (n = 18). (E-F) The EPA content from phospholipid fatty acids across blood and gastrointestinal tissues from mice fed either the control (E) or 1.8%en EPA+DHA diet (F) (n = 6/group). A one-way ANOVA and a Tukey's *post-hoc* were used to assess differences in EPA content comparing all tissues to each other. Differing letters denote statistical significance at $P < 0.05$.

Figure 17 (cont'd)



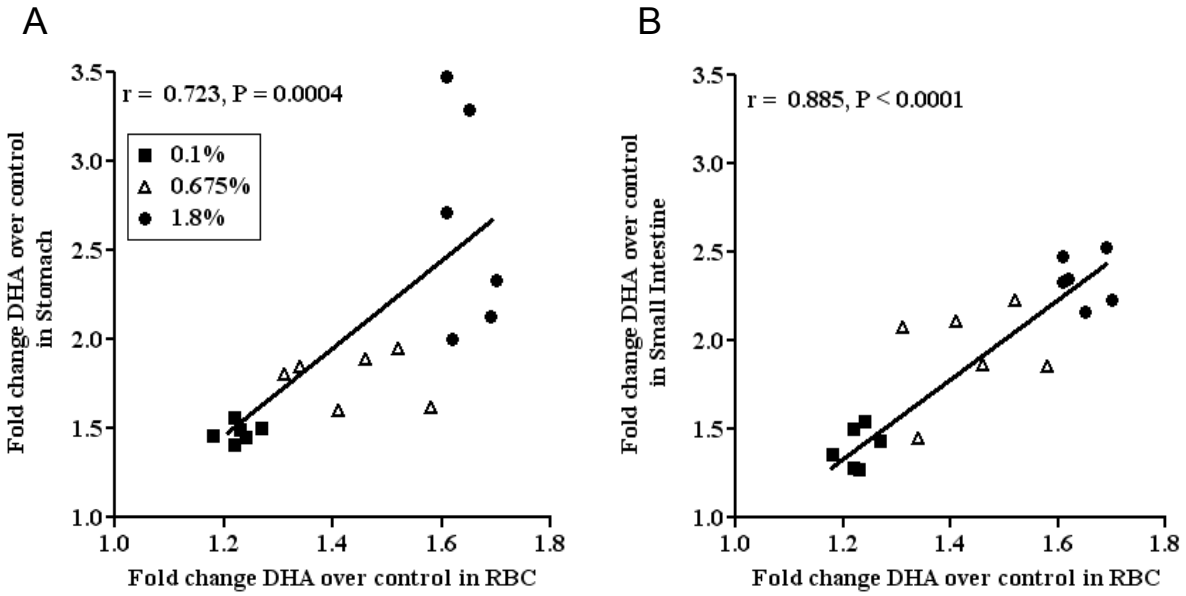
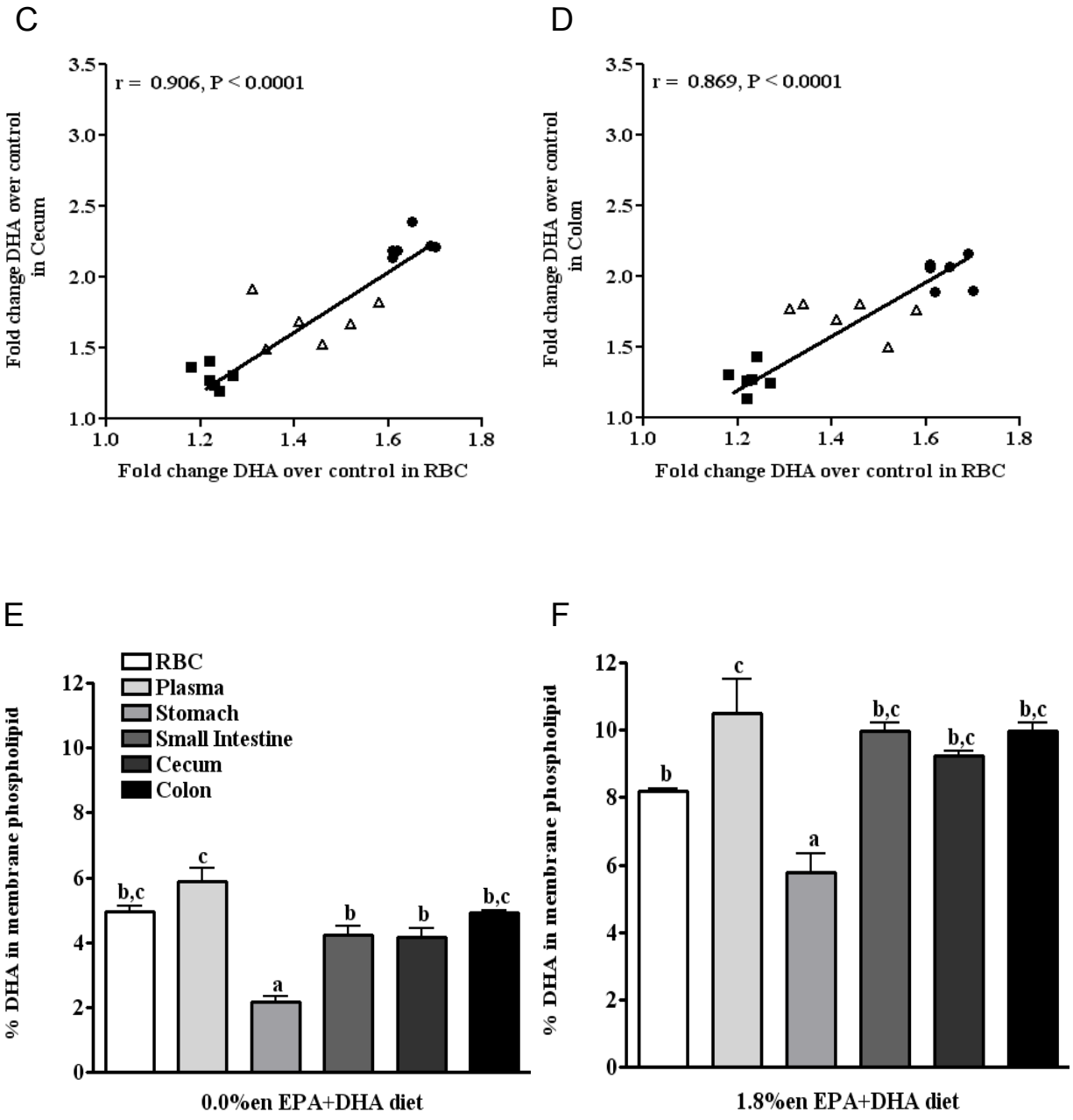


Figure 18 Comparison of DHA content in phospholipid fatty acids from blood and gastrointestinal tissues from mice fed diets with increasing EPA+DHA. (A-D) Fold Change in DHA content over the control group in phospholipid fatty acids from gastrointestinal tissues correlated to DHA content in phospholipid fatty acids from red blood cells: (A) Stomach, (B) Small Intestine (C) Cecum, and (D) Colon. Data from the tissues of mice fed experimental diets containing EPA+DHA-enriched fish oil were normalized to the control group to assess fold changes in DHA content of phospholipids. A Pearson correlation was used to test linear correlation between gastrointestinal tissue DHA fold change and RBC DHA fold change (n = 18). (E-F) The DHA content from phospholipid fatty acids across blood and gastrointestinal tissues from mice fed either the control (E) or 1.8%en EPA+DHA diet (F) (n = 6/group). A one-way ANOVA and a Tukey's *post-hoc* were used to assess differences in DHA content comparing all tissues to each other. Differing letters denote statistical significance at $P < 0.05$.

Figure 18 (cont'd)



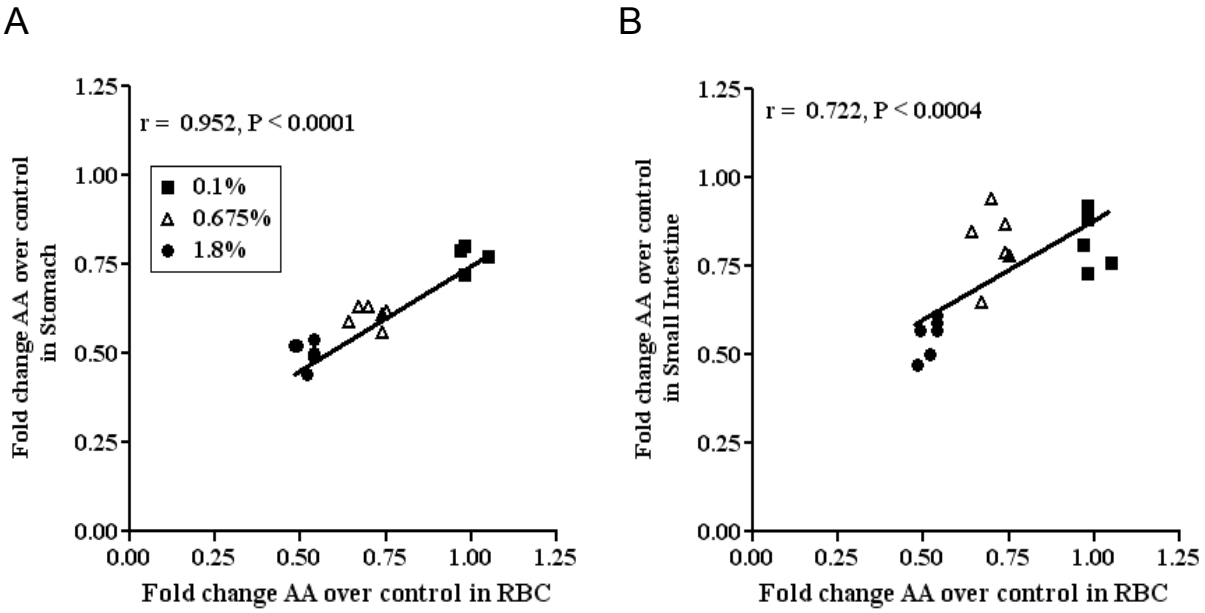
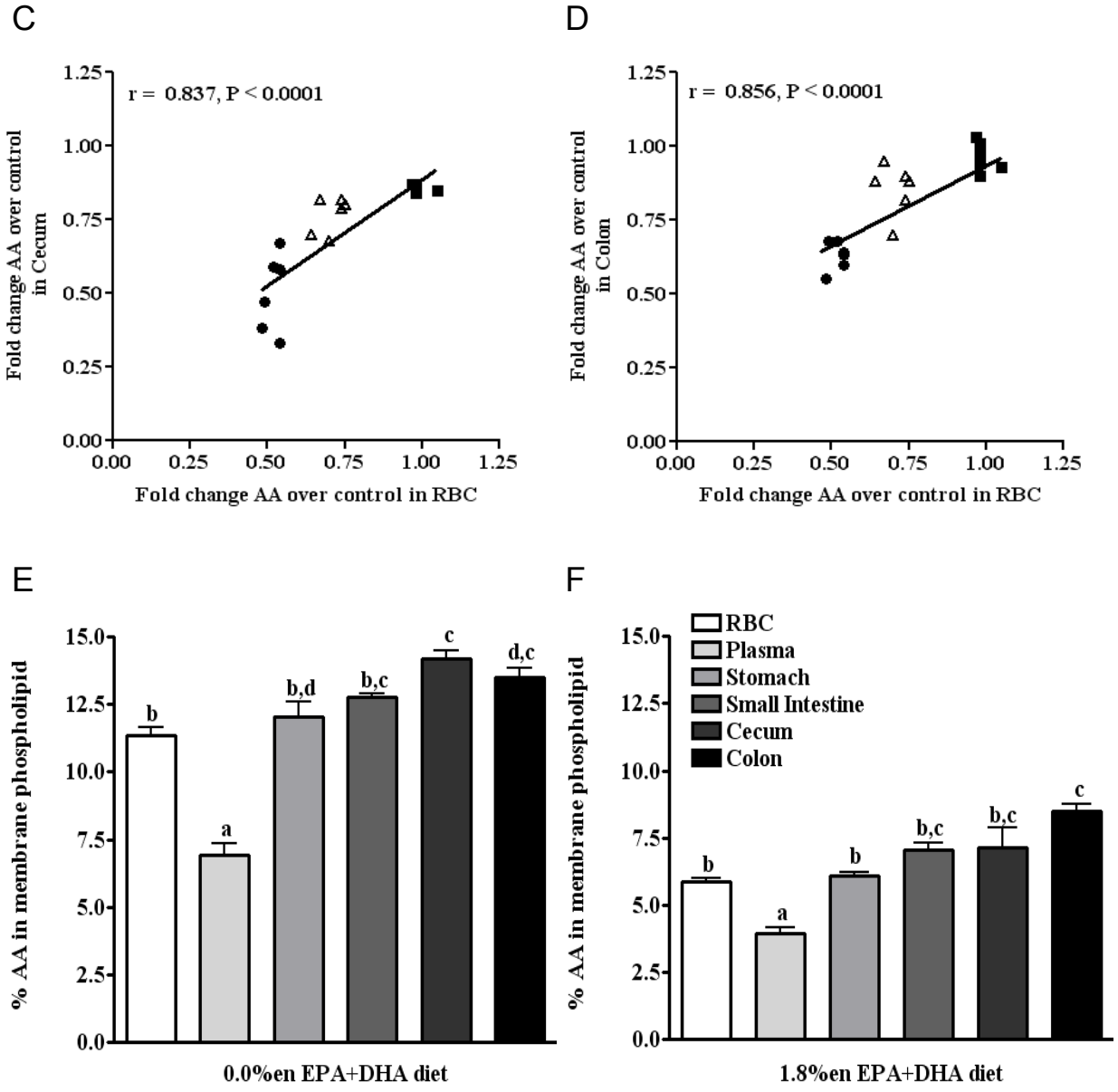


Figure 19 Comparison of AA content in phospholipid fatty acids from blood and gastrointestinal tissues from mice fed diets with increasing EPA+DHA. (A-D) Fold Change in AA content over the control group in phospholipid fatty acids from gastrointestinal tissues correlated to AA content in phospholipid fatty acids from red blood cells: (A) Stomach, (B) Small Intestine (C) Cecum, and (D) Colon. Data from the tissues of mice fed experimental diets containing EPA+DHA-enriched fish oil were normalized to the control group to assess fold changes in AA content of phospholipids. A Pearson correlation was used to test linear correlation between gastrointestinal tissue AA fold change and RBC AA fold change (n = 18). (E-F) The AA content from phospholipid fatty acids across blood and gastrointestinal tissues from mice fed either the control (E) or 1.8%en EPA+DHA diet (F) (n = 6/group). A one-way ANOVA and a Tukey's *post-hoc* were used to assess differences in AA content comparing all tissues to each other. Differing letters denote statistical significance at $P < 0.05$.

Figure 19 (cont'd)



Discussion

We found that, regardless of the organ, GI tissue EPA+DHA and EPA levels were very highly correlated with the RBC levels, whereas DHA and AA levels were also significantly correlated but somewhat less strongly across all tissues. Accordingly, the omega-3 index may be used as a valid surrogate for the relative enrichment of GI tissue EPA+DHA content. We observed that small amount of dietary EPA+DHA (at 0.1%en EPA+DHA) resulted in increased n-3 LCPUFA levels in blood and GI tissues. These data demonstrate the capacity of RBC fatty acids to act as a surrogate biomarker for assessing n-3 LCPUFA incorporation in GI tissues. While the omega-3 index adequately predicts the relative enrichment of EPA+DHA in GI tissues, we observed that the omega-3 index does not reflect the absolute EPA+DHA in GI tissues. In addition, we observed significant disparities in the levels of incorporation between tissues for the primary n-3 LCPUFAs, EPA and DHA, suggesting that rate of enrichment and saturation may be tissue dependent. These findings indicate that the RBC does not mirror the absolute levels of n-3 and n-6 LCPUFAs in GI tissue, but the RBC does reflect the relative enrichment of n-3 and n-6 LCPUFAs in GI tissues.

The clinical utility of the omega-3 index was demonstrated in previous studies showing that the omega-3 index may be a predictor of actual risk for CHD [119]. This may be due in part due to the fact that the omega-3 index is reflective of the cardiac tissue EPA+DHA [119]. Meta-analyses of clinical trials on fish oil supplementation as a potential therapeutic for various GI diseases, including IBD, have been conducted, but are often inconclusive [113]. Given the often disparate exposure of n-3 LCPUFAs (e.g., type of fish oil, duration, etc.), it is important to know the extent to which n-3 LCPUFAs

are actually incorporated into the GI tissues of interest. Our data suggest that the omega-3 index will, at least in mice, provide a valid estimate of GI tissue EPA and DHA levels.

Previous work by Arnold *et al.* examined the fatty acid composition of various tissues of Zucker rats fed a diet comparable in fat composition to the AIN-93G or a fish oil supplemented AIN-93G (where 4.4%en was derived from EPA+DHA) for three weeks [123]. In the animals fed the control diets containing 0.0%en from EPA+DHA in both studies, the omega-3 index for the Zucker rats' were 1.6% in RBCs and 1.8% in plasma, compared to our study where the omega-3 index was 5.2% in RBCs and 6.1% in plasma. Consistent with having comparatively lower levels of n-3 LCPUFAs in tissues of animals fed their control diets, they observed higher AA in the RBCs (26.6%) and plasma (10.5%), than we had observed in our control diet (RBCs, 11.3%; plasma, 6.3%). While we had observed decreases in n-6 LCPUFAs and increases in n-3 LCPUFAs with increasing dietary EPA+DHA, the absolute levels of n-3/6 LCPUFAs of either RBCs or plasma remained considerably different between the studies. The high %en EPA+DHA groups resulted dramatic reduction of AA in the Arnold *et al.* study (RBC, 17.1%; plasma, 3.1%) and the data presented here (RBC, 5.8%; plasma, 3.9%). Unsurprisingly, the high %en EPA+DHA groups also had significant increases in the omega-3 index in the Arnold *et al.* study (RBC, 12.2%; plasma, 7.6%) and the data presented here (RBC, 11.2%; plasma, 13.2%). It is worth noting that the fatty acid levels of the Zucker rats in the Arnold *et al.* study increased in EPA content to a greater degree, despite having similar levels between the studies in the 0.0%en EPA+DHA diets. This difference, as well as others, may be due to the fact that Arnold *et al.* had

extracted total tissue lipid, whereas we had isolated the phospholipid fraction of all tissues. Despite the differences in absolute levels of n-3/6 LCPUFAs between studies, the similar type of supplementation (~1:1 EPA:DHA) in the Arnold *et al.* study provides complimentary evidence to support that RBCs and plasma undergo similar changes in tissue-extracted lipid/phospholipid after inclusion of EPA+DHA in the diet in a relatively short period of time.

We had identified at least one other study that reported the fatty acid composition of membrane phospholipids from the small intestine of mice fed standard AIN-93G-based diet [124] . The diets were supplemented with oleic acid, AA, EPA, or AA+EPA for 8 wks. Consistent with the reported values in their control group, the absolute levels of most major fatty acids (16:0, 18:0, 18:1, 18:2n-6, 20:5n-3, 22:6n-3) reported were similar to the levels obtained in our control group. AA was the only fatty acid that was considerably higher when comparing their findings (16.6%) to ours (12.8%). Because they had used only EPA for the n-3 LCPUFA treatment, direct comparison to our EPA+DHA treatments is not possible. Together these data demonstrate consistency of phospholipid fatty acid analysis of tissues across mouse models.

In the present study, we investigated the effect of increasing EPA+DHA content of soybean oil-based AIN-93G; however, others have investigated the lipid composition of various tissues from mice fed diets with non-standard lipid mixtures [108, 125, 126]. At least two studies that have reported the fatty acid composition of colonic mucosa where the lipid in the diet was primarily low in alpha- linolenic (ALA; 18:3n-3), such as canola or corn oil, have observed low levels of DHA (1.3% [127], 1.6% [108]. This contrasts our reported values for mice fed the 0.0%en diet, whereby the DHA content in

the membrane of colonic tissue was found to be ~5.0% of all phospholipids. The higher level of DHA observed in our study may be due to the use of soybean oil, in contrast to the use of corn oil in their diets. In mice eating diets devoid of fish oil, the level of EPA present in the colon was reported to be low by both us and Turk et al., regardless of base oil composition. Their observations corroborate ours in separate mouse models increasing generalizability of colonic fatty acid composition across murine models.

The use of a non-human model to measure LCPUFA incorporation into tissue is a potential limitation to this study. However, testing the effects of escalating levels of dietary omega-3 on solid organ fatty acid composition in humans is virtually impossible. Therefore, in the present study we assessed the phospholipid fatty acid composition of blood and GI tissues from mice to determine how well blood correlates with GI tissues. There are a number of studies that confirm a strong relationship between intake and the omega-3 index in humans suggesting a high degree of comparability, allowing for reasonable extrapolation of our findings. Specifically, the RBC [106, 128], and plasma [106] LCPUFA levels of healthy humans and those found in mice fed AIN-93G display strong similarities, with a common theme of slightly lower DHA in human RBC and plasma (3.1 – 4.2% and 2.5%, respectively) compared to mice (4.9% and 5.8%, respectively). The 1.8%en EPA+DHA fed mice had approximately 8% DHA in RBCs, which parallels similarly to recent human supplementation studies that found DHA reaching levels of 7.0 – 7.5% in RBCs [129, 130]. Furthermore, increasing research in humans demonstrate that the RBC may serve as a surrogate biomarker for the level of LCPUFAs in certain tissues, such as cardiac [106, 129, 131] and retina or optic nerves [128], but not others, such as the brain [132]. Indeed, in this mouse model we also

observe a strong correlation between RBC and cardiac tissue n-3 LCPUFAs (data not shown).

A number of human studies have investigated whether the fatty acid composition of GI tissues in individuals with IBD differ compared to healthy controls, providing insight into the levels of n-3 and n-6 LCPUFAs found in human colonic mucosa [133-135]. Comparison between mouse and human studies reveal that the levels of n-3/6 LCPUFAs in RBC and plasma are generally in agreement between species, with slightly decreased levels of DHA in humans (2.4 – 3.5%) compared to mice (4.9%) and slightly decreased AA in humans (9.0 – 9.8%) compared to mice (13.5%). In humans consuming either a typical diet (baseline, pre-intervention) or a healthy/mediterranean diet, the colonic n-3 fatty acid % (unspecified n-3 FAs) ranged from 4.4 – 5.1% [136] similar to percentages observed in our mouse study. In another human study examining the effect of EPA supplementation in subjects with a history of adenomas, colonic EPA+DHA ranged from 2.7 – 3.4% of total fatty acids at baseline and supplementation increased it to 5.1%. This increase correlated with significantly reduced proliferation and increased mucosal apoptosis [134]. Finally, in a study comparing mucosal n-3 and n-6 fatty acid in the colorectal adenoma-carcinoma sequence, the EPA+DHA ranged from 2.9 – 3.5% of total fatty acids [137]. Considering that the phospholipid fraction of cell membranes contain the majority of fatty acids in most tissues (i.e. non-adipose, non-steroid synthesizing tissues), these data highlight the similarity in fatty acid composition of various GI tissues between mice and humans. Taken together, the similarities observed between mouse and human fatty acid profiles provide evidence to potentially expand utility of RBC in predicting tissue-level incorporation of n-3/6 LCPUFAs. We

believe that it is not unreasonable to extrapolate our general findings from the mouse model to humans recognizing that the precise dose-response curves may differ in these two mammalian species but the overall features are likely to be similar.

With additional studies addressing the role of n-3 LCPUFAs on tissue-specific health outcomes in humans (e.g., CHD, IBD), there is a need to define an easily accessible and valid biomarker of n-3 LCPUFA status in the tissue(s) of interest. Based on our findings, the omega-3 index appears to serve that function, at least for GI tissues. The extent to which the omega-3 index can serve as a reflection of other tissues (e.g., lung, bone, skeletal or smooth muscle, liver, kidney, brain, etc.) remains to be determined.

CHAPTER 5: CONCLUSIONS

With increasing consumption of fish oil-based dietary supplements and a lack of regulatory oversight, there is a need to better understand the health implications associated with n-3 LCPUFA intake. Much of the research on fish oil demonstrate that immunomodulation by increasing n-3 LCPUFA intake is associated with an anti-inflammatory outcome. Indeed, in animal models of high n-3 LCPUFA intake, research on pathogen-exposed animals warns of exacerbated infection, while models of autoimmunity and chronic inflammation highlight potential benefit. However, there is emerging evidence that contrasts the belief that all outcomes of n-3 LCPUFAs are anti-inflammatory.

The research presented within this dissertation serves to highlight two underrepresented notions regarding fish oil immunomodulation: 1) that the 'immunosuppressive' effect of fish oil is not ubiquitous, specifically in regards to B cells, and 2) that the composition of fish oil (i.e. the varying amounts of each n-3 LCPUFA) matters with respect to the observed outcome.

It is increasingly recognized that EPA and DHA exert their immunomodulatory effects by both shared and differential mechanisms of action. Both EPA and DHA can modulate gene expression (e.g., as a ligand for PPAR-γ), serve as substrate for specialized proresolving mediators (e.g., Resolvin E- and D- series), and alter membrane organization. In the experiments presented herein, membrane reorganization and immunomodulatory effects of fish oil were dependent on the composition of the n-3 LCPUFAs in the diet. B cells from EFO-fed mice had a more

punctate microdomain clustering, observed in tandem with decreased B cell activation and function. In contrast, B cells from MO- and DFO-fed mice had less microdomain clustering, observed in tandem with increased B cell activation and function. There are a number of considerations that may help explain this observed duality between the effects from EFO vs. MO and DFO dietary treatments.

While all fish oil treatments significantly increased EPA in B cell phospholipids, the EFO diet was unable to significantly increase the amount of DHA. The n-3 LCPUFA composition of the EFO treatment was 52% EPA and 10% DHA, which resulted in significant increases of EPA, but not DHA in B cell phospholipid fatty acids. Even though the n-3 LCPUFA composition of MO was only marginally higher in DHA (at 14%) compared to the EFO, both MO and DFO significantly increased *both* EPA and DHA in B cell phospholipid fatty acids. Biophysical studies demonstrate that EPA and DHA have different affinities for lipid microdomains, which may explain the differential clustering observed. These physical differences observed at the membrane level, may also lead to differences observed in functionality. For example, basal expression of inactive TLR-4 is observed primarily in non-raft domains; however, upon activation, TLR-4 dimerizes within membrane microdomains. It is possible that alterations in the fluidity and organization of the plasma membrane may hasten or impair propagation of LPS-stimulated TLR-4 inflammatory signaling cascades. As such, decreased clustering (i.e. as observed in MO and DFO treatments) may have resulted in increased propensity for TLR-4 dimerization within microdomains.

This experiment provides a strong platform to better understand how alterations in membrane composition effects receptor-mediated signaling. Specifically, to

interrogate the difference between EPA and DHA in relation to membrane reorganization, future studies should consider the use of EFO and DFO treatments. It is imperative to use rate-limiting steps (e.g., EPA $\rightarrow\rightarrow$ DHA) to control for endogenous conversion of fatty acids. While conversion of EPA to downstream products is slow, retroconversion of DHA to EPA is not; therefore, in an *in vivo* model it may be impossible to eliminate the effect of increased membrane EPA when providing a source of DHA. Evaluating colocalization of membrane microdomains with receptors that localize into membrane microdomains (e.g., TLR-4, MHCII) may provide insight into whether varying phospholipid fatty acid compositions of the membrane alter recruitment of receptors.

The impetus of this research was to better understand how DFO exacerbated H. hepaticus infection in SMAD3-/- mice. Interestingly, preliminary evidence suggests that MO and EFO also exacerbate infection, despite the immunological differences observed in B cells between EFO vs. MO and DFO fed mice. It is reasonable to assume that there are many different 'players' (e.g., varying immune cells, epithelial cells) in the host response to the pathogen whose function is also altered by fish oil. When considering the phospholipid fatty acid profile of total tissues, such as the stomach or colon, it was found that very small amounts of EPA+DHA were necessary to significantly increase n-3 LCPUFA and decrease n-6 LCPUFA. While it is likely that the totality of fish immunomodulation results in an overall dampened inflammatory response, these data suggest a novel role for the enhancement of humoral immunity by dietary fish oil. Future studies should evaluate whether n-3 LCPUFAs are capable of improving humoral immunity, against the backdrop of potential immunosuppression.

APPENDIX

APPENDIX: RED BLOOD CELL PHOSPHOLIPIDS ARE A VALID BIOMARKER FOR THE PUFA COMPOSITION OF SYSTEMIC ORGANS

This chapter is in preparation for submission to *PLEFA* by Gurzell et al. (2014)

Abstract

Hundreds of clinical trials examining the use of omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) on various health outcomes have been conducted, and these remain one of the most widely used nutritional supplements. More recently studies have begun to utilize the omega-3 index, defined as the sum of EPA+DHA in red blood cells (RBCs), as both a biomarker of n-3 LCPUFA exposure and a potential risk factor for coronary heart disease (CHD). Considerably less research has been done to evaluate whether RBC phospholipid fatty acids reflect the phospholipid fatty acid composition of other systemic tissues across increasing intakes of n-3 LCPUFAs. We fed mice diets containing increasing amounts of EPA+DHA, equivalent to current recommendations by the American Heart Association on a percent of energy basis, and analyzed the phospholipid fatty acid composition of various tissues in relation to RBCs. We observed that RBCs, heart, muscle, spleen, lung, and adipose tissues all response to dietary supplementation with EPA+DHA with increasing n-3 LCPUFA and decreasing n-6 LCPUFA levels. Furthermore, the n-3 LCPUFA profiles of all measured tissues had strong ($r > 0.7$) and significant ($p < 0.001$) correlations to RBCs. Interestingly, we also observed changes in saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) levels across various tissues in response to

increased EPA+DHA intakes despite there being no change in dietary SFA and MUFA. Specifically, there were increases in RBC SFA and spleen MUFA and decreases in heart MUFA. These demonstrate that the RBC, including the omega-3 index, is a valid, surrogate biomarker for the relative levels of n-3 and n-6 LCPUFAs in phospholipids of tissues.

Introduction

There is increasing evidence to suggest that dietary supplementation with n-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) found in fish oil not only have cardiovascular benefit [138], but may also have potential benefit for other pathologies, including dyslipidemia [139], chronic inflammation [2], and autoimmunity [140, 141]. Over 30 million Americans report consumption of fish oil as a dietary supplement for some purported health benefit [11]; however, FDA-approved fish oil-based pharmaceuticals, including Lovaza™, Vascepa™, and Epanova™, are currently only indicated to lower very high serum triglyceride levels [142]. Despite widespread fish oil supplementation and extensive epidemiologic research on the health effects of n-3 LCPUFAs, results are often inconsistent for various health outcomes, such as Ulcerative Colitis [113, 143]. One potential contributor to this problem is the lack of a standardized and universally accepted biomarker of n-3 LCPUFA exposure. Thus, many studies rely on dietary intake surveys which can be both imprecise and inaccurate.

One biomarker that has been relatively widely used is the red blood cell (RBC) content of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA). This metric,

known as the omega-3 index, has been shown to be a surrogate biomarker for cardiac tissue EPA+DHA [129, 144], and further, has been proposed as a risk factor for coronary heart disease (CHD) [117]. The high degree of correlation between the omega-3 index and cardiac EPA+DHA has led to a growing use of the omega-3 index as a biomarker for n-3 LCPUFA exposure in clinical trials [121, 145-147]. Give that many of the proposed mechanisms by which n-3 LCPUFAs exert their immunomodulatory effects at the cellular level, including gene regulation [6], the production of eicosanoids and pro-resolution molecules [2, 42], and alteration of membrane organization [74, 80], it is important to identify the extent to which circulating RBCs reflect levels of n-3 LCPUFAs in non-cardiac tissues. There is limited evidence suggesting that the omega-3 index does correlate well with the EPA+DHA content of other tissues in humans [129, 144, 148] and mice [149].

The primary aim of this study is to identify whether RBC n-3 LCPUFAs adequately reflect the levels of n-3 LCPUFAs in various tissues of mice. Previously, we reported on the fatty acid profile of RBC phospholipids and gastrointestinal tissue PL of mice consuming increasing intakes of EPA+DHA [149]; herein, we report on the correlations between RBC fatty acids and those of phospholipids from heart, muscle, spleen, lung, and adipose tissue. The experimental design has been previously reported [149]. In brief, mice were fed diets with increasing percent energy derived from EPA+DHA (%en EPA+DHA). The experimental diets were formulated to model a 2000 kcal human intake with either no fish or fish oil (0.0%en), two servings of fish a week (0.1%en), 1-2 g fish oil supplementation a day (0.675%en), or 4 g prescription fish oil a day (1.8%en). We assessed the acyl chain composition of phospholipids from RBCs,

heart, muscle, spleen, lung, and adipose tissues to determine whether RBC phospholipid fatty acids (e.g., omega-3 index) are an adequate biomarker of phospholipid fatty acids from various systemic tissues across increasing intakes of EPA+DHA.

Materials and Methods

Dietary Treatment and Experimental Design

The dietary treatment and experimental design employed here has been previously described in greater detail [149]. Briefly, twenty-four SMAD3-/- mice (n = 6/group) were fed *ad libitum* standard AIN-93G diet containing soybean oil (control) or one of three treatment diets containing increasing amounts of EPA and DHA. The experimental diets were calculated from the percent energy (%en) a human consuming 2000kcal would receive from EPA+DHA following the 2002 American Heart Association recommendations [120]. The experimental diets provided 0.0%en (control), 0.1%en, 0.675%en, and 1.8%en from EPA+DHA. Mice were fed the diets for 5 wk prior to sacrifice. Tissues were collected, rinsed in cold ddH_2O to remove contaminating pools of blood, and stored in -80^oC until lipid extraction. In the current study, the fatty acid composition of phospholipids from heart, skeletal muscle (total skeletal muscle of the left hind limb), spleen, lung, and adipose (mesenteric adipose with removal of lymph nodes) tissues were analyzed and compared with that of RBCs previously reported [149].

Phospholipid Isolation and Analysis of FAMEs

An expanded methods section for the phospholipid extraction and analysis was previously described [149]. Briefly, total lipid was extracted using a modified Rose and Oklander extraction [101], phospholipids were isolated using Isolute-XL® SPE aminopropyl columns (500mg; Biotage, Charlotte, NC) from a procedure modified by Agren et al. [103], and FAMEs generated using acidified methanol described by Burdge et al. [105]. Gas chromatography of phospholipid FAMEs were analyzed at OmegaQuant Analytics, LLC (Sioux Falls, SD). Gas chromatography was performed using a GC2010 Gas Chromatograph (Shimadzu, Columbia, MD) equipped with a SP2560, 100-m column (Supelco, Bellefonte, PA) using hydrogen as carrier gas. Referenced against a standard of fatty acids characteristic of erythrocytes, phospholipid fatty acids were identified and calculated as a percentage of total identified fatty acids after response factor correction.

Statistical Analysis

To evaluate whether RBC n-3 and n-6 fatty acids correlate with those of other tissues, Pearson's correlation coefficients were calculated for EPA+DHA (the omega-3 index), EPA alone, DHA alone, and arachidonic acid (AA) alone using data for all dietary treatments. The data (mean ± SEM) for the phospholipid fatty acid composition for all tissues and dietary treatments are provided in Supplementary Tables 1–5; the comparative statistics (i.e. comparing diet and/or dose) performed and presented within this manuscript specifically address the control (0.0%en EPA+DHA) and the highest dose treatment (1.8%en EPA+DHA) diets. A one-way ANOVA was used to test for

statistically significant differences across EPA+DHA, EPA alone, DHA alone, and AA alone between tissues with a given diet. A student's t-test was used to for statistical significant differences across EPA, docosapentaenoic acid (n-3) (DPA (n-3)), DHA, total saturated fatty acid (SFA), monounsaturated fatty acids (MUFA), total n-3 PUFAs, and total n-6 PUFAs within a given tissue. Statistical significance was set at the $P < 0.05$ level; all statistical analyses were conducted using GraphPad Prism 4 software (GraphPad, San Diego, CA).

Results

Correlated and absolute levels of n-3 / n-6 LCPUFAs

All data on the phospholipid fatty acid profile for the systemic tissues of mice fed the experimental diets are presented in **Supplementary Tables 17–21**. While the correlation data includes all four experimental diets, the statistical comparisons across tissue or diet focused on the lowest control diet (0.0%en EPA+DHA) and the highest treatment diet (1.8%en EPA+DHA). We first correlated the omega-3 index with the EPA+DHA content of heart, muscle, spleen, lung, and adipose tissues. We observed strong ($r > 0.82$) and significant ($p < 0.0001$) correlations for all tissues (**Figure 20A-E**). For mice fed the control diet, the omega-3 index was numerically similar to the EPA+DHA levels in the spleen and lung, it was higher than that in adipose tissue, and lower than that of the heart and leg muscle (**Figure 20F**). In the mice fed the highest level of EPA+DHA (the 1.8%en diet), the EPA+DHA content of RBCs was numerically similar to that of spleen and adipose tissue ($p > 0.05$), but considerably higher than that of lung, heart and leg muscle.

We next evaluated how strongly RBC EPA and DHA each individually correlated with their levels in other tissues. RBC EPA was strongly ($r > 0.92$) and significantly ($p < 0.0001$) correlated EPA levels in all other tissues studied (**Figure 21A-E**). While the RBC EPA reflects the absolute levels of EPA in muscle and spleen in the control animals, it mirrored spleen and adipose tissue on the high omega-3 diet ($p > 0.05$) (**Figure 21F**). RBC DHA was strongly ($r > 0.74$) and significantly ($p < 0.0001$) correlated with DHA levels in all tissues studied (**Figure 22A-E**). RBC DHA reflects the absolute levels of DHA in the spleen, lung, and adipose tissues in both the control and high

omega-3 groups ($p > 0.05$); however, heart and muscle had significantly higher levels of DHA than all other tissues, with the former having significantly more than the latter (**Figure 22F**).

Arachidonic acid (AA) decreased across increasing intakes of EPA+DHA diets. We observed strong ($r > 0.85$) and significant ($p < 0.0001$) correlations between RBC AA and AA in all other tissues (**Figure 23A-E**). The absolute level of RBC AA was significantly different than that of all other tissue in the control animals ($p < 0.05$), and was only similar to that of adipose tissue in the high omega-3 group.

n-3 LCPUFA composition across tissues

In order to better understand the n-3 LCPUFA composition for each tissue in response to increased EPA+DHA intake, we evaluated whether each n-3 LCPUFA increased between mice fed the 0.0%en EPA+DHA and the 1.8%en EPA+DHA diets. Mice fed the 1.8%en EPA+DHA had higher levels of EPA, DPA (n-3), and DHA compared to mice fed the 0.0%en EPA+DHA diet across all tissues, except for the heart (**Figure 24A-F**). Interestingly, the heart tissue of mice fed the 1.8%en EPA+DHA diet had increased EPA and DHA, but no change in the amount of DPA (n-3) compared to mice fed the 0.0%en EPA+DHA diet. In all tissues and across all diets, DHA was the predominant n-3 LCPUFA; specifically, increases in DHA constituted the majority increase in total n-3 LCPUFAs of the heart and muscle, whereas changes in EPA and DPA (n-3) remained marginal in comparison (**Figure 24B-C**).

Change in saturated and unsaturated fatty acids across tissues

Interestingly, in addition to the altering PUFA profile, mice fed increasing amounts of EPA+DHA, showed changes in the SFA and MUFA composition of certain tissues (**Figure 25A-F**). Mice fed EPA+DHA had increased SFA in RBCs compared to control fed mice (**Figure 25A**). Furthermore, mice fed EPA+DHA had decreased MUFAs in the heart (**Figure 25B**) but increased MUFAs in the spleen (**Figure 25D**).

We observed that major (i.e. greater than 5% for any given tissue), non-LCPUFAs varied significantly between tissues and across dietary treatment. Specifically, we found that for palmitic acid, RBC levels were higher than those in heart, muscle, and adipose tissue, were similar to splenic levels, , and were lower than lung levels in both the control and high omega-3 groups (**Figure 26A**). Both RBC and splenic palmitic acid levels increased with high omega-3 feeding relative to control. RBC stearic acid was not statistically different from that in muscle, spleen, lung, or adipose tissue, but remained significantly lower than that in heart tissue across control or EPA+DHA diets (**Figure 26B**). Levels of stearic acid in the heart decreased, whereas levels in adipose tissue increased when mice were fed EPA+DHA. RBC levels of oleic acid were higher than heart, muscle, spleen, and lung tissues, but lower than adipose tissues (**Figure 26C**). RBC oleic was no different from muscle oleic in the control group, while splenic levels of oleic were no different than RBC levels of oleic in the EPA+DHA group. Heart and muscle levels of oleic acid decreased, while splenic oleic acid increased when mice were fed EPA+DHA. Similarly, RBC linoleic acid was higher than heart, muscle, spleen, and lung tissues, but lower than adipose tissues (**Figure 26D**). RBC linoleic acid was no different than splenic linoleic acid in the EPA+DHA group. RBC,

heart, and muscle levels of linoleic acid were lower, while splenic levels of linoleic acid were higher when mice were fed EPA+DHA.

Table 17 Fatty acid composition of the heart[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
	% of fatty acid in the heart			
16:0	17.63 ± 1.02	18.72 ± 1.36	19.57 ± 1.14	17.80 ± 0.83
16:1(n-7) *trans*	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.08 ± 0.01
16:1 (n-7)	0.10 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	0.11 ± 0.02
18:0	25.64 ± 1.05	23.80 ± 1.66	26.01 ± 1.84	22.19 ± 0.84
18:1 *trans*	0.15 ± 0.02	0.16 ± 0.02	0.17 ± 0.01	0.17 ± 0.01
18:1 *cis*	6.93 ± 0.18	6.57 ± 0.29	5.24 ± 0.20	5.25 ± 0.16
18:2 (n-6) *trans*	0.14 ± 0.02	0.13 ± 0.02	0.11 ± 0.00	0.11 ± 0.00
18:2 (n-6)	6.86 ± 0.30	5.84 ± 0.40	4.28 ± 0.27	3.71 ± 0.28
20.0	0.58 ± 0.05	0.64 ± 0.09	0.60 ± 0.05	0.71 ± 0.19
18:3 (n-6)	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
20:1 (n-9)	0.18 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.14 ± 0.01
18:3 (n-3)	0.16 ± 0.05	0.12 ± 0.02	0.08 ± 0.01	0.12 ± 0.03
20:2 (n-6)	0.24 ± 0.01	0.21 ± 0.02	0.16 ± 0.01	0.13 ± 0.01
22:0	0.42 ± 0.03	0.45 ± 0.07	0.43 ± 0.03	0.51 ± 0.15
20:3 (n-6)	0.26 ± 0.01	0.22 ± 0.02	0.17 ± 0.01	0.15 ± 0.01
20:4 (n-6)	6.53 ± 0.30	5.53 ± 0.35	3.07 ± 0.20	2.17 ± 0.12
24:0	0.12 ± 0.01	0.13 ± 0.02	0.12 ± 0.01	0.13 ± 0.05
20:5 (n-3)	0.05 ± 0.00	0.08 ± 0.01	0.21 ± 0.01	0.48 ± 0.03
24:1 (n-9)	0.13 ± 0.01	0.13 ± 0.02	0.11 ± 0.01	0.20 ± 0.05
22:4 (n-6)	0.52 ± 0.03	0.32 ± 0.02	0.12 ± 0.01	0.07 ± 0.00
22:5 (n-6)	2.32 ± 0.29	1.02 ± 0.06	0.49 ± 0.03	0.52 ± 0.02
22:5 (n-3)	1.57 ± 0.08	1.46 ± 0.09	1.35 ± 0.09	1.47 ± 0.04
22:6 (n-3)	30.08 ± 1.23	34.13 ± 1.78	37.53 ± 2.23	44.43 ± 1.32
EPA+DHA	30.12 ± 1.23	34.21 ± 1.79	37.75 ± 2.24	44.90 ± 1.32
EPA+DPA+DHA	31.69 ± 1.27	35.68 ± 1.85	39.10 ± 2.30	46.37 ± 1.35
∑SFA	44.38 ± 1.75	43.74 ± 2.93	46.71 ± 3.00	41.35 ± 1.77
∑MUFA	7.55 ± 0.20	7.17 ± 0.31	5.78 ± 0.22	5.95 ± 0.20
∑PUFA (n-3)	31.85 ± 1.28	35.79 ± 1.87	39.18 ± 2.30	46.49 ± 1.34
∑PUFA (n-6)	16.89 ± 0.74	13.29 ± 0.82	8.41 ± 0.48	6.88 ± 0.30

[1]Data are mean ± SEM reported as percent total, n = 6.

Table 18 Fatty acid composition of skeletal muscle[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
	% of fatty acid in skeletal muscle			
16:0	26.24 ± 1.23	27.30 ± 0.77	30.24 ± 1.19	29.24 ± 1.42
16:1(n-7) *trans*	0.15 ± 0.00	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
16:1 (n-7)	1.45 ± 0.12	1.45 ± 0.15	1.67 ± 0.20	1.32 ± 0.19
18:0	10.07 ± 0.59	10.34 ± 0.62	10.06 ± 0.85	9.24 ± 0.58
18:1 *trans*	0.13 ± 0.01	0.17 ± 0.03	0.09 ± 0.02	0.26 ± 0.05
18:1 *cis*	8.35 ± 0.40	9.15 ± 0.56	8.03 ± 0.79	6.63 ± 0.57
18:2 (n-6) *trans*	0.13 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.22 ± 0.06
18:2 (n-6)	9.94 ± 0.31	9.73 ± 0.79	9.04 ± 0.79	5.59 ± 0.54
20.0	0.14 ± 0.01	0.17 ± 0.02	0.15 ± 0.01	0.15 ± 0.01
18:3 (n-6)	0.06 ± 0.00	0.05 ± 0.01	0.05 ± 0.00	0.03 ± 0.00
20:1 (n-9)	0.15 ± 0.01	0.18 ± 0.01	0.14 ± 0.01	0.19 ± 0.03
18:3 (n-3)	0.74 ± 0.08	0.75 ± 0.07	0.71 ± 0.02	0.41 ± 0.04
20:2 (n-6)	0.36 ± 0.01	0.31 ± 0.02	0.27 ± 0.01	0.16 ± 0.02
22:0	0.17 ± 0.02	0.20 ± 0.03	0.14 ± 0.02	0.15 ± 0.02
20:3 (n-6)	0.74 ± 0.04	0.55 ± 0.03	0.41 ± 0.03	0.25 ± 0.02
20:4 (n-6)	13.26 ± 0.49	10.65 ± 0.51	6.24 ± 0.28	4.16 ± 0.24
24:0	0.17 ± 0.03	0.26 ± 0.06	0.11 ± 0.02	0.12 ± 0.04
20:5 (n-3)	0.19 ± 0.01	0.26 ± 0.01	0.63 ± 0.08	0.99 ± 0.03
24:1 (n-9)	0.24 ± 0.03	0.29 ± 0.03	0.20 ± 0.02	0.23 ± 0.02
22:4 (n-6)	1.48 ± 0.10	1.06 ± 0.06	0.43 ± 0.03	0.24 ± 0.02
22:5 (n-6)	4.28 ± 0.52	2.60 ± 0.13	1.43 ± 0.10	1.29 ± 0.07
22:5 (n-3)	2.69 ± 0.18	2.59 ± 0.12	3.08 ± 0.23	3.20 ± 0.07
22:6 (n-3)	18.87 ± 0.82	20.75 ± 0.84	26.28 ± 1.41	35.78 ± 1.28
EPA+DHA	19.05 ± 0.82	21.01 ± 0.84	26.91 ± 1.38	36.77 ± 1.30
EPA+DPA+DHA	21.74 ± 0.90	23.60 ± 0.93	29.99 ± 1.60	39.97 ± 1.35
∑SFA	36.79 ± 0.91	38.28 ± 1.28	40.70 ± 1.84	38.90 ± 1.94
∑MUFA	10.47 ± 0.48	11.38 ± 0.68	10.26 ± 0.96	8.78 ± 0.71
∑PUFA (n-3)	22.48 ± 0.90	24.35 ± 0.88	30.70 ± 1.60	40.38 ± 1.33
∑PUFA (n-6)	30.26 ± 0.78	25.11 ± 0.78	18.02 ± 0.82	11.93 ± 0.66

[1]Data are mean ± SEM reported as percent total, n = 6.

Table 19 Fatty acid composition of the spleen[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
	% of fatty acid in the spleen			
16:0	31.60 ± 0.52	31.98 ± 0.38	32.19 ± 0.63	34.35 ± 0.77
16:1(n-7) *trans*	0.12 ± 0.01	0.11 ± 0.00	0.10 ± 0.01	0.08 ± 0.01
16:1 (n-7)	0.58 ± 0.01	0.62 ± 0.04	0.81 ± 0.11	0.86 ± 0.11
18:0	13.84 ± 0.25	13.62 ± 0.10	13.69 ± 0.60	13.30 ± 0.20
18:1 *trans*	0.50 ± 0.07	0.72 ± 0.25	0.24 ± 0.08	0.15 ± 0.02
18:1 *cis*	7.54 ± 0.10	7.66 ± 0.08	8.00 ± 0.34	8.64 ± 0.22
18:2 (n-6) *trans*	0.32 ± 0.06	0.50 ± 0.18	0.37 ± 0.06	0.31 ± 0.04
18:2 (n-6)	9.58 ± 0.36	9.21 ± 0.18	10.95 ± 0.46	10.68 ± 0.30
20.0	0.58 ± 0.02	0.55 ± 0.02	0.55 ± 0.03	0.58 ± 0.05
18:3 (n-6)	0.16 ± 0.01	0.15 ± 0.01	0.09 ± 0.02	0.04 ± 0.00
20:1 (n-9)	0.34 ± 0.01	0.37 ± 0.08	0.28 ± 0.02	0.28 ± 0.01
18:3 (n-3)	0.18 ± 0.01	0.17 ± 0.01	0.21 ± 0.03	0.15 ± 0.01
20:2 (n-6)	1.24 ± 0.08	1.22 ± 0.07	1.07 ± 0.04	0.85 ± 0.02
22:0	0.72 ± 0.03	0.74 ± 0.05	0.72 ± 0.02	0.69 ± 0.05
20:3 (n-6)	0.88 ± 0.02	0.88 ± 0.01	1.00 ± 0.01	0.98 ± 0.02
20:4 (n-6)	17.98 ± 0.31	16.92 ± 0.20	12.70 ± 0.34	9.68 ± 0.13
24:0	0.59 ± 0.05	0.60 ± 0.09	0.52 ± 0.05	0.50 ± 0.03
20:5 (n-3)	0.21 ± 0.02	0.39 ± 0.01	1.31 ± 0.08	2.86 ± 0.18
24:1 (n-9)	1.26 ± 0.06	1.22 ± 0.05	1.20 ± 0.03	1.11 ± 0.07
22:4 (n-6)	3.13 ± 0.06	2.45 ± 0.04	1.21 ± 0.04	0.58 ± 0.01
22:5 (n-6)	0.41 ± 0.05	0.19 ± 0.00	0.13 ± 0.01	0.15 ± 0.01
22:5 (n-3)	1.89 ± 0.08	2.20 ± 0.03	3.43 ± 0.12	3.92 ± 0.19
22:6 (n-3)	6.38 ± 0.18	7.55 ± 0.07	9.23 ± 0.20	9.73 ± 0.19
EPA+DHA	6.59 ± 0.17	7.93 ± 0.08	10.54 ± 0.25	12.60 ± 0.31
EPA+DPA+DHA	8.49 ± 0.13	10.14 ± 0.07	13.97 ± 0.35	16.52 ± 0.50
∑SFA	47.34 ± 0.75	47.48 ± 0.41	47.66 ± 1.09	49.42 ± 0.98
∑MUFA	10.34 ± 0.12	10.70 ± 0.29	10.63 ± 0.40	11.12 ± 0.18
∑PUFA (n-3)	8.67 ± 0.13	10.30 ± 0.07	14.17 ± 0.34	16.67 ± 0.50
∑PUFA (n-6)	33.70 ± 0.61	31.52 ± 0.26	27.53 ± 0.60	23.27 ± 0.29

[1]Data are mean ± SEM reported as percent total, n = 6.

Table 20 Fatty acid composition of the lung[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
		% of fatty acid in the lung		
16:0	53.85 ± 1.16	50.64 ± 0.83	52.29 ± 0.28	52.54 ± 1.25
16:1(n-7) *trans*	0.13 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.20 ± 0.08
16:1 (n-7)	2.21 ± 0.12	2.23 ± 0.04	2.28 ± 0.06	2.38 ± 0.12
18:0	8.67 ± 0.20	8.82 ± 0.22	8.40 ± 0.11	8.10 ± 0.16
18:1 *trans*	0.20 ± 0.01	0.43 ± 0.18	0.26 ± 0.03	0.31 ± 0.09
18:1 *cis*	7.46 ± 0.14	7.60 ± 0.18	7.20 ± 0.04	7.44 ± 0.28
18:2 (n-6) *trans*	0.09 ± 0.01	0.19 ± 0.05	0.15 ± 0.03	0.24 ± 0.07
18:2 (n-6)	6.09 ± 0.27	6.60 ± 0.17	6.63 ± 0.19	6.31 ± 0.19
20.0	0.51 ± 0.03	0.52 ± 0.02	0.50 ± 0.04	0.45 ± 0.03
18:3 (n-6)	0.12 ± 0.01	0.13 ± 0.00	0.11 ± 0.01	0.08 ± 0.01
20:1 (n-9)	0.28 ± 0.03	0.33 ± 0.04	0.23 ± 0.01	0.24 ± 0.03
18:3 (n-3)	0.11 ± 0.01	0.10 ± 0.00	0.10 ± 0.01	0.09 ± 0.01
20:2 (n-6)	0.40 ± 0.02	0.43 ± 0.01	0.38 ± 0.01	0.32 ± 0.02
22:0	0.76 ± 0.03	0.78 ± 0.04	0.81 ± 0.06	0.71 ± 0.04
20:3 (n-6)	0.58 ± 0.03	0.67 ± 0.02	0.67 ± 0.02	0.54 ± 0.02
20:4 (n-6)	8.79 ± 0.08	8.33 ± 0.27	6.20 ± 0.27	3.72 ± 0.20
24:0	0.87 ± 0.05	0.93 ± 0.05	1.02 ± 0.07	0.90 ± 0.04
20:5 (n-3)	0.13 ± 0.01	0.27 ± 0.04	0.64 ± 0.03	1.38 ± 0.11
24:1 (n-9)	1.17 ± 0.09	1.31 ± 0.06	1.35 ± 0.12	1.26 ± 0.08
22:4 (n-6)	2.18 ± 0.13	2.01 ± 0.06	1.06 ± 0.10	0.46 ± 0.02
22:5 (n-6)	0.42 ± 0.04	0.27 ± 0.02	0.16 ± 0.01	0.13 ± 0.01
22:5 (n-3)	0.99 ± 0.06	1.41 ± 0.03	2.08 ± 0.15	2.87 ± 0.11
22:6 (n-3)	4.15 ± 0.30	5.83 ± 0.17	7.58 ± 0.33	9.43 ± 0.29
EPA+DHA	4.28 ± 0.31	6.10 ± 0.16	8.22 ± 0.35	10.81 ± 0.38
EPA+DPA+DHA	5.27 ± 0.33	7.52 ± 0.18	10.30 ± 0.47	13.68 ± 0.48
∑SFA	64.66 ± 1.20	61.71 ± 0.73	63.01 ± 0.31	62.71 ± 1.27
∑MUFA	11.45 ± 0.32	12.04 ± 0.17	11.44 ± 0.16	11.84 ± 0.46
∑PUFA (n-3)	5.38 ± 0.33	7.61 ± 0.19	10.40 ± 0.47	13.77 ± 0.48
∑PUFA (n-6)	18.67 ± 0.44	18.64 ± 0.44	15.38 ± 0.44	11.80 ± 0.42

[1]Data are mean ± SEM reported as percent total, n = 6.

Table 21 Fatty acid composition of adipose tissue[1]

Fatty Acid	0.0%en EPA+DHA	0.1%en EPA+DHA	0.675%en EPA+DHA	1.8%en EPA+DHA
	% of fatty acid in adipose tissue			
16:0	24.55 ± 1.50	28.12 ± 1.54	27.92 ± 0.41	24.82 ± 1.18
16:1(n-7) *trans*	0.18 ± 0.08	0.09 ± 0.00	0.08 ± 0.01	0.12 ± 0.01
16:1 (n-7)	2.99 ± 0.32	1.71 ± 0.48	2.37 ± 0.45	2.50 ± 0.44
18:0	9.59 ± 0.89	12.00 ± 0.51	10.18 ± 0.44	13.72 ± 1.26
18:1 *trans*	0.57 ± 0.20	0.36 ± 0.06	0.23 ± 0.03	0.92 ± 0.31
18:1 *cis*	15.14 ± 1.53	13.10 ± 1.89	13.46 ± 1.20	14.10 ± 1.14
18:2 (n-6) *trans*	0.42 ± 0.11	0.24 ± 0.04	0.26 ± 0.04	0.64 ± 0.21
18:2 (n-6)	23.49 ± 1.11	20.30 ± 0.71	24.49 ± 0.64	20.75 ± 0.89
20.0	0.58 ± 0.04	0.51 ± 0.06	0.35 ± 0.02	0.60 ± 0.09
18:3 (n-6)	0.15 ± 0.01	0.14 ± 0.01	0.13 ± 0.00	0.10 ± 0.01
20:1 (n-9)	0.52 ± 0.04	0.39 ± 0.03	0.39 ± 0.03	0.71 ± 0.17
18:3 (n-3)	1.41 ± 0.11	0.43 ± 0.09	0.56 ± 0.12	0.54 ± 0.08
20:2 (n-6)	0.40 ± 0.07	0.51 ± 0.03	0.47 ± 0.03	0.41 ± 0.07
22:0	0.68 ± 0.07	0.60 ± 0.05	0.41 ± 0.02	0.71 ± 0.13
20:3 (n-6)	0.43 ± 0.05	0.59 ± 0.03	0.66 ± 0.07	0.74 ± 0.04
20:4 (n-6)	14.45 ± 0.67	13.49 ± 1.09	9.46 ± 0.79	5.84 ± 0.57
24:0	0.48 ± 0.08	0.67 ± 0.08	0.56 ± 0.05	0.57 ± 0.06
20:5 (n-3)	0.13 ± 0.01	0.47 ± 0.06	1.40 ± 0.09	2.59 ± 0.36
24:1 (n-9)	0.55 ± 0.06	0.56 ± 0.04	0.40 ± 0.03	0.85 ± 0.14
22:4 (n-6)	0.79 ± 0.10	0.72 ± 0.15	0.16 ± 0.02	0.25 ± 0.05
22:5 (n-6)	0.25 ± 0.04	0.23 ± 0.02	0.09 ± 0.02	0.12 ± 0.01
22:5 (n-3)	0.38 ± 0.05	0.83 ± 0.06	0.91 ± 0.04	1.51 ± 0.19
22:6 (n-3)	2.20 ± 0.23	3.94 ± 0.18	5.04 ± 0.50	6.90 ± 0.40
EPA+DHA	2.33 ± 0.24	4.41 ± 0.22	6.44 ± 0.59	9.49 ± 0.69
EPA+DPA+DHA	2.71 ± 0.27	5.24 ± 0.27	7.35 ± 0.62	11.00 ± 0.71
∑SFA	35.88 ± 1.98	41.91 ± 1.86	39.42 ± 0.84	40.42 ± 1.06
∑MUFA	19.96 ± 1.66	16.21 ± 2.42	16.94 ± 1.60	19.19 ± 1.81
∑PUFA (n-3)	4.12 ± 0.30	5.67 ± 0.25	7.90 ± 0.51	11.54 ± 0.66
∑PUFA (n-6)	40.37 ± 1.74	36.21 ± 1.15	35.72 ± 0.41	28.85 ± 1.14

[1]Data are mean ± SEM reported as percent total, n = 6.

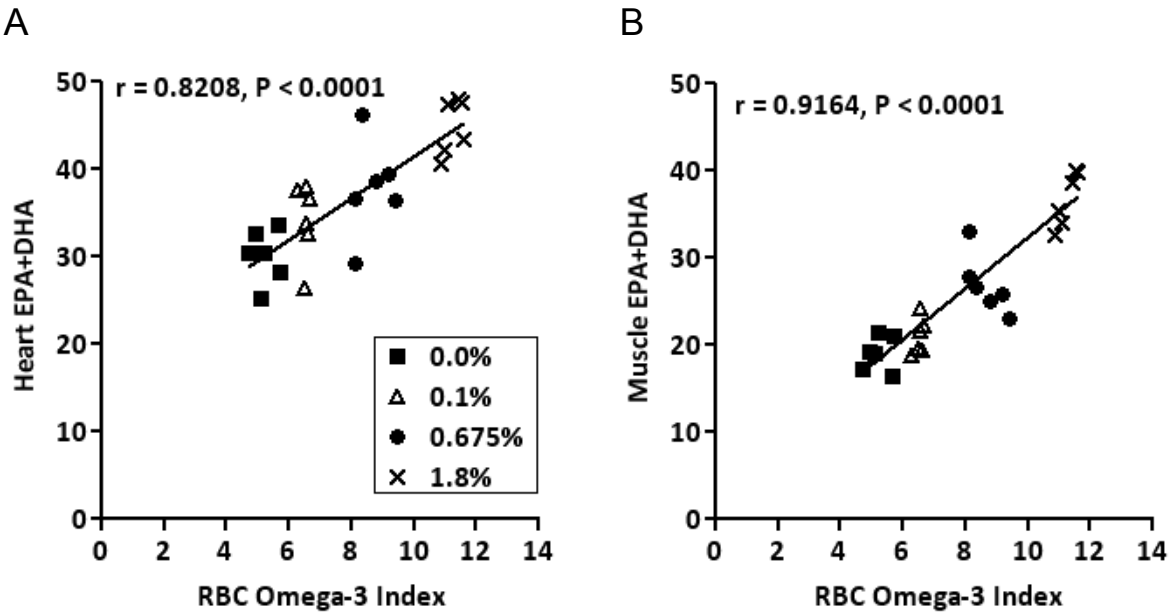
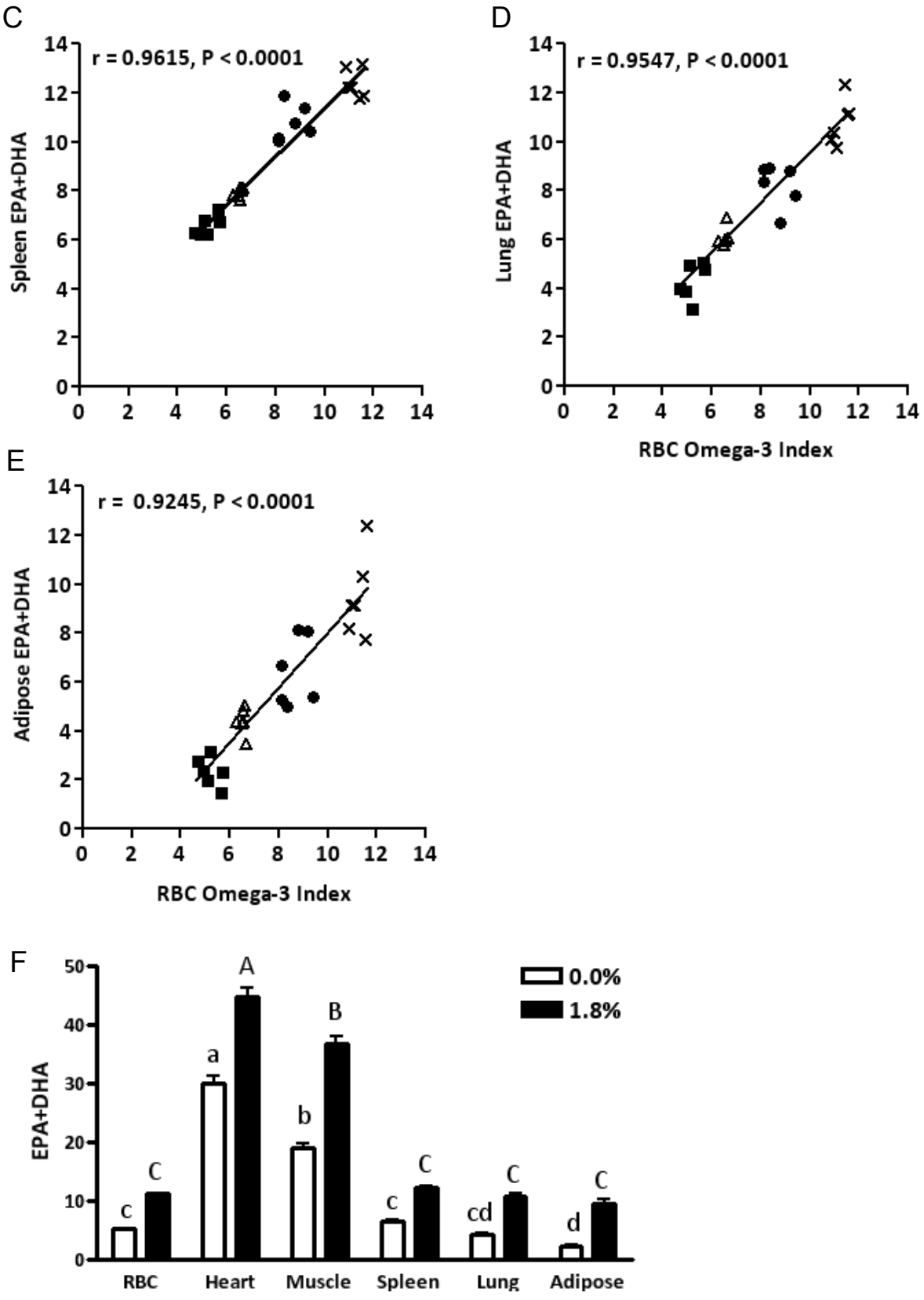


Figure 20 Correlation of EPA+DHA between red blood cells and various systemic tissues of mice fed increasing amounts of EPA and DHA. SMAD3-/- mice were fed diets containing 0.0%en, 0.1%en, 0.675%en, or 1.8%en from EPA+DHA. Gas chromatography was used to analyze the fatty acid composition of isolated phospholipids. A Pearson's correlation coefficient was calculated for EPA+DHA between red blood cells and heart (A), muscle (B), spleen (C), lung (D), and adipose tissues (E). A one-way ANOVA was used to assess differences in EPA+DHA content between tissues within a specified diet (F). Differing letters denote statistical significance at the $P < 0.05$ level; lower letters are used across the 0.0%en EPA+DHA diet and upper case letters are used across the 1.8%en EPA+DHA diet.

Figure 20 (cont'd)



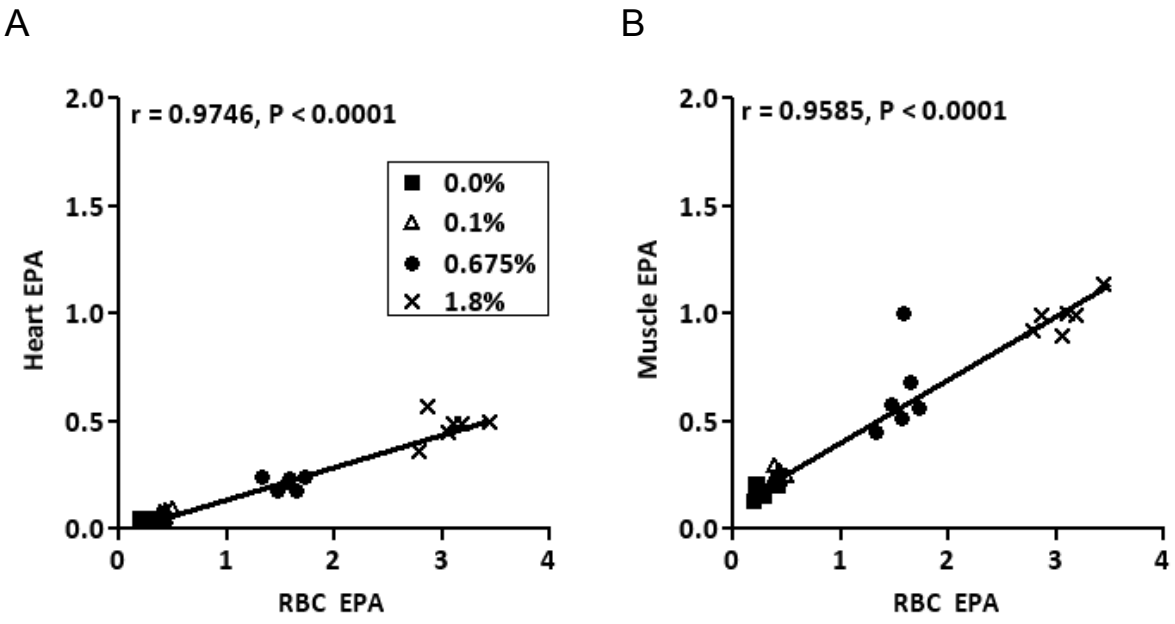
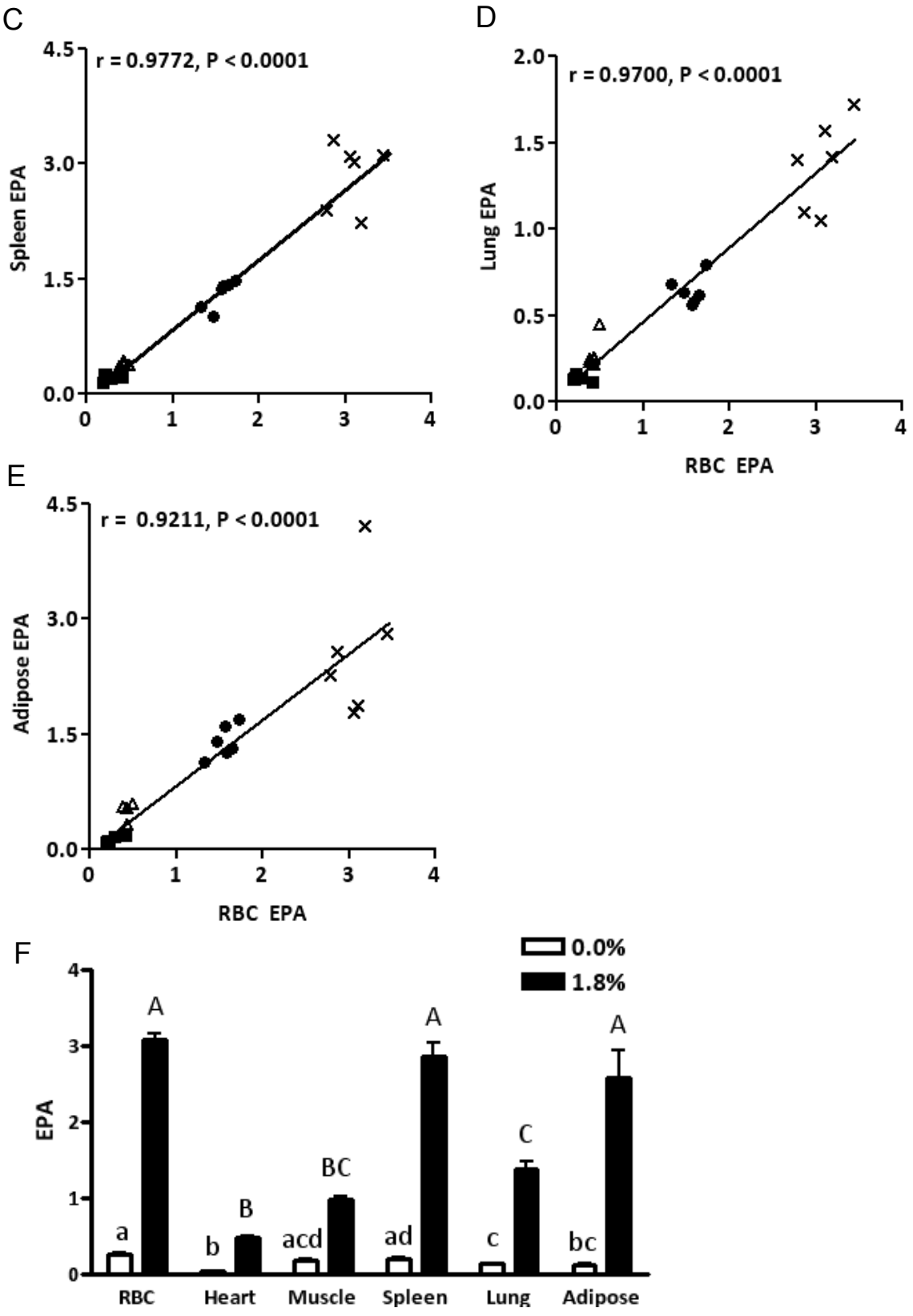


Figure 21 Correlation of EPA between red blood cells and various systemic tissues of mice fed increasing amounts of EPA and DHA. SMAD3-/- mice were fed diets containing 0.0%en, 0.1%en, 0.675%en, or 1.8%en from EPA+DHA. Gas chromatography was used to analyze the fatty acid composition of isolated phospholipids. A Pearson's correlation coefficient was calculated for EPA between red blood cells and heart (A), muscle (B), spleen (C), lung (D), and adipose tissues (E). A one-way ANOVA was used to assess differences in EPA content between tissues within a specified diet (F). Differing letters denote statistical significance at the $P < 0.05$ level; lower letters are used across the 0.0%en EPA+DHA diet and upper case letters are used across the 1.8%en EPA+DHA diet.

Figure 21 (cont'd)



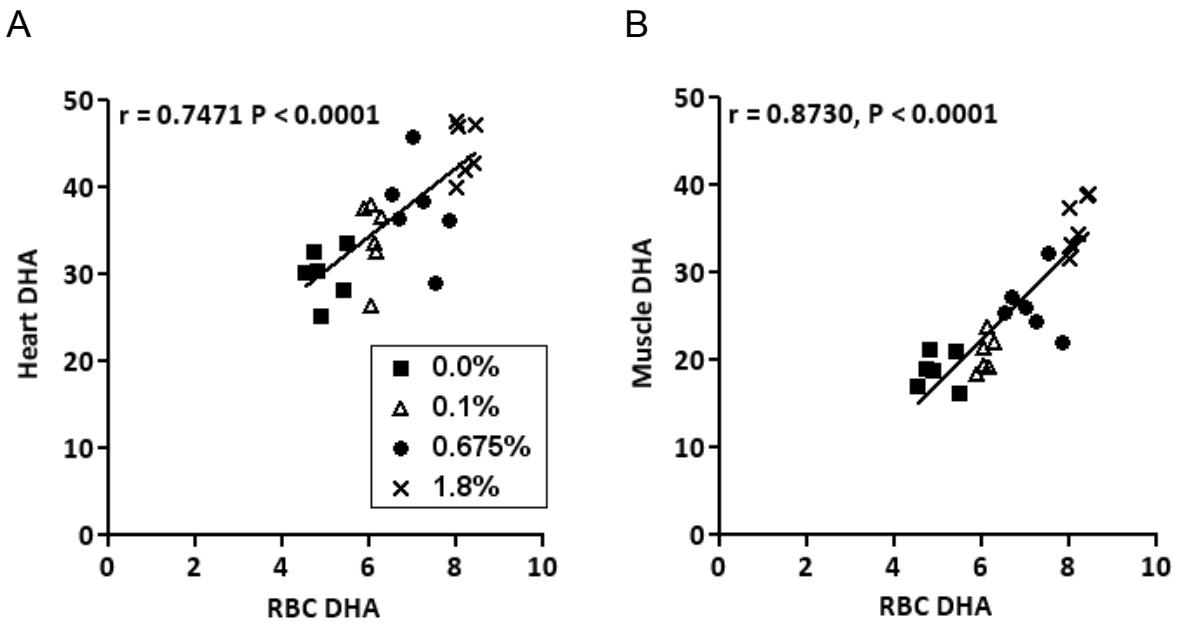
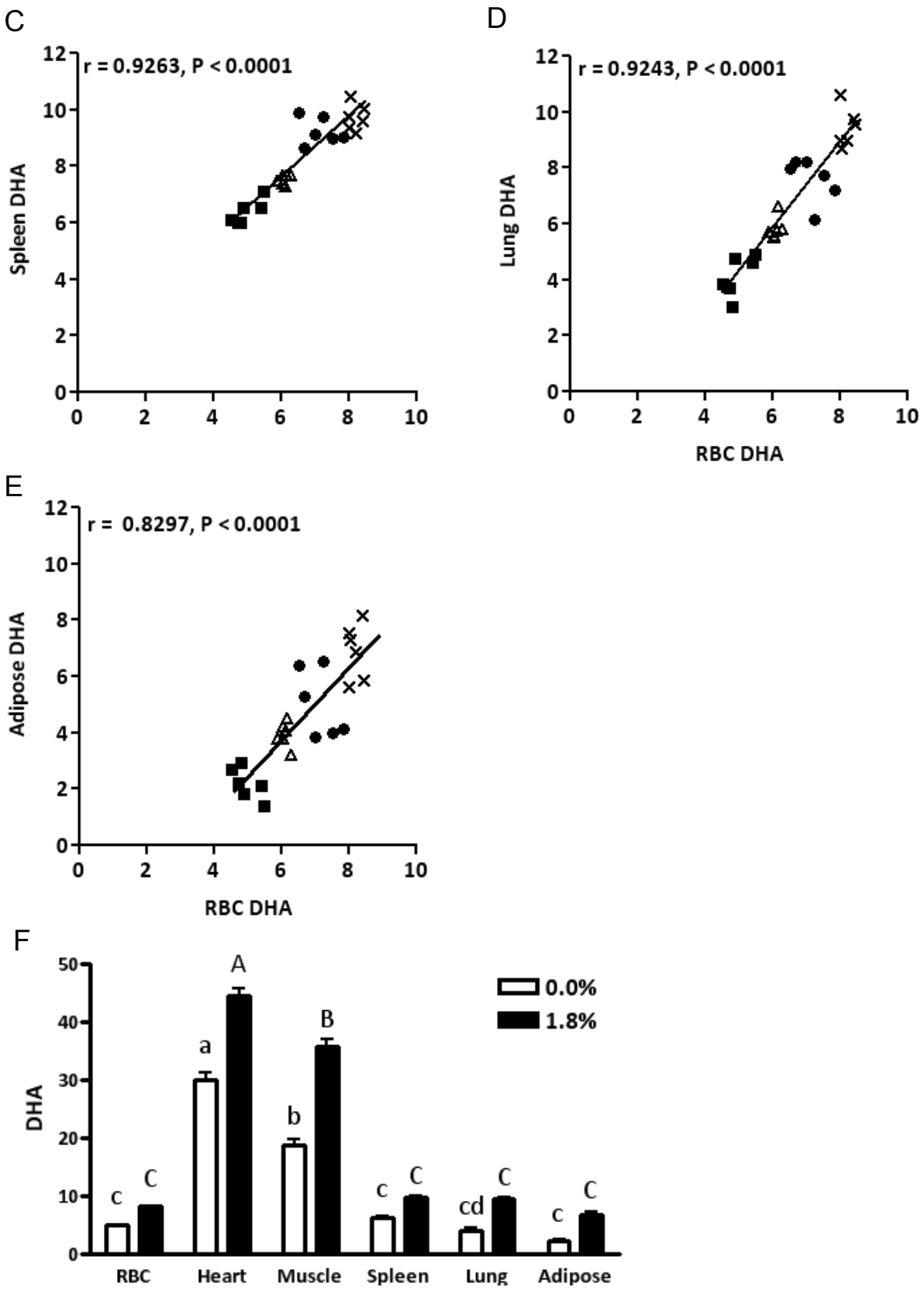


Figure 22 Correlation of DHA between red blood cells and various systemic tissues of mice fed increasing amounts of EPA and DHA. SMAD3-/- mice were fed diets containing 0.0%en, 0.1%en, 0.675%en, or 1.8%en from EPA+DHA. Gas chromatography was used to analyze the fatty acid composition of isolated phospholipids. A Pearson's correlation coefficient was calculated for DHA between red blood cells and heart (A), muscle (B), spleen (C), lung (D), and adipose tissues (E). A one-way ANOVA was used to assess differences in DHA content between tissues within a specified diet (F). Differing letters denote statistical significance at the $P < 0.05$ level; lower letters are used across the 0.0%en EPA+DHA diet and upper case letters are used across the 1.8%en EPA+DHA diet.

Figure 22 (cont'd)



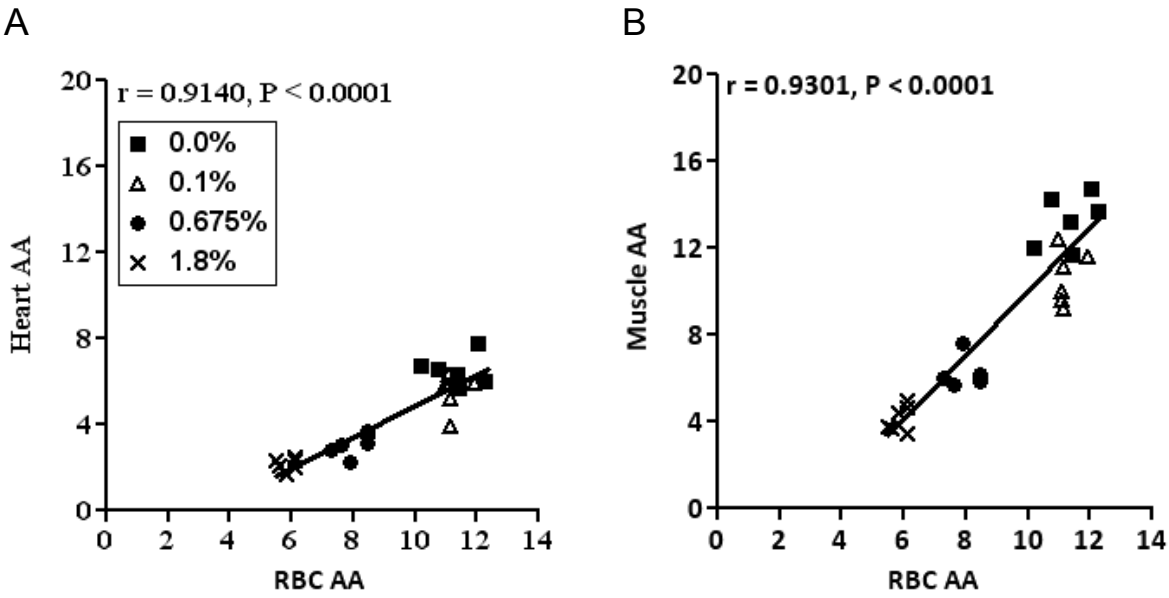
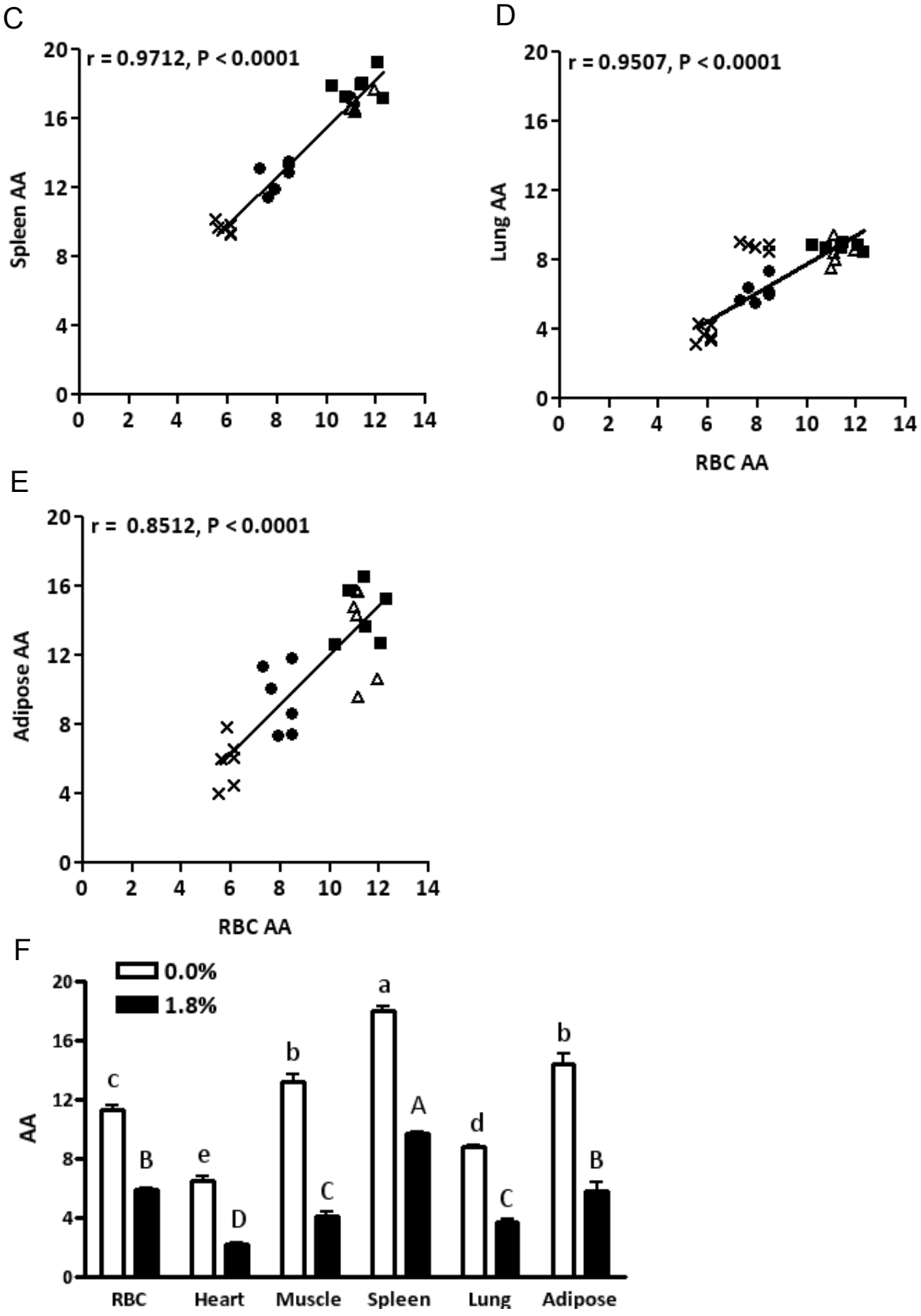


Figure 23 Correlation of AA between red blood cells and various systemic tissues of mice fed increasing amounts of EPA and DHA. SMAD3-/- mice were fed diets containing 0.0%en, 0.1%en, 0.675%en, or 1.8%en from EPA+DHA. Gas chromatography was used to analyze the fatty acid composition of isolated phospholipids. A Pearson's correlation coefficient was calculated for AA between red blood cells and heart (A), muscle (B), spleen (C), lung (D), and adipose tissues (E). A one-way ANOVA was used to assess differences in AA content between tissues within a specified diet (F). Differing letters denote statistical significance at the $P < 0.05$ level; lower letters are used across the 0.0%en EPA+DHA diet and upper case letters are used across the 1.8%en EPA+DHA diet.

Figure 23 (cont'd)



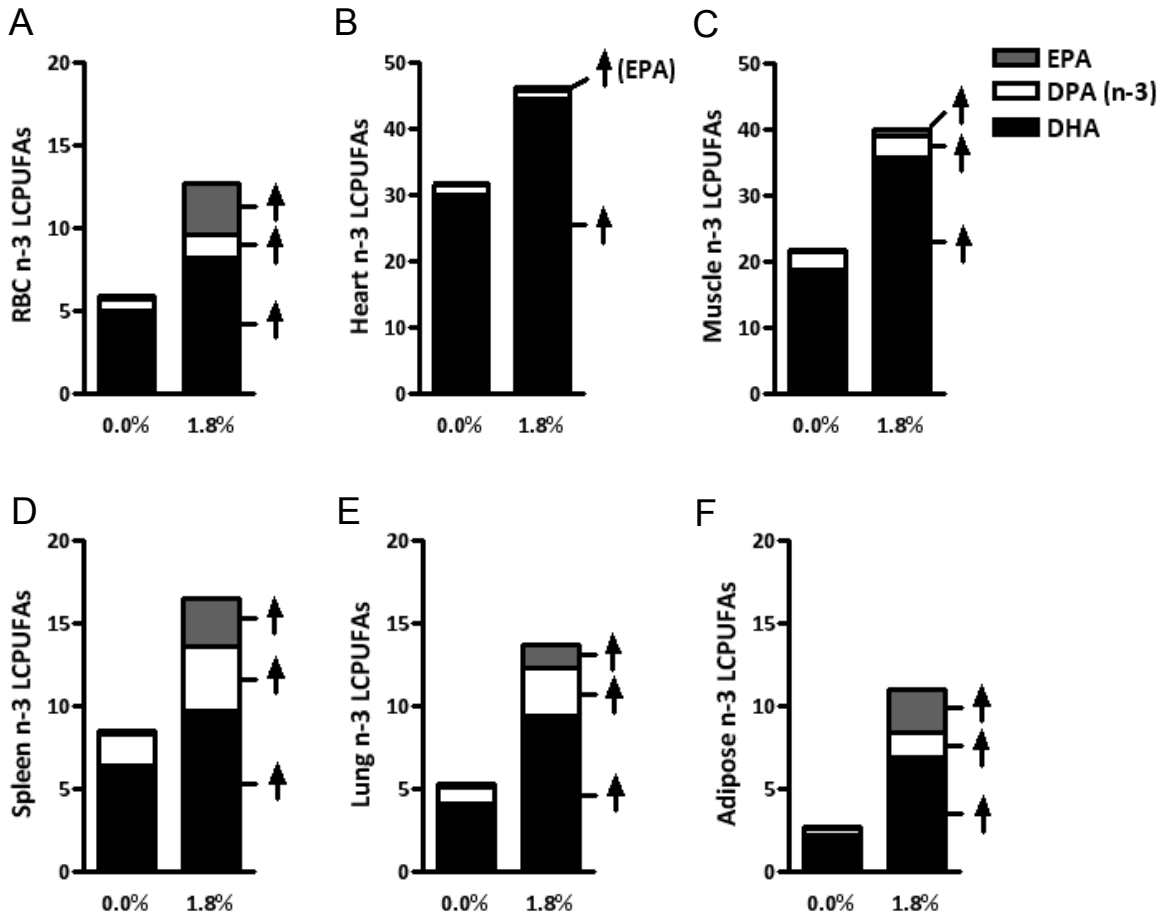


Figure 24 Composition of n-3 LCPUFAs of various systemic tissue mice fed increasing EPA and DHA. Comparison of the n-3 LCPUFA composition between SMAD3-/- fed either 0.0%en or 1.8%en EPA+DHA diets for RBC (A), heart (B), muscle (C), spleen (D), lung (E), and adipose tissues (F). A student's t-test was used to compare the content of eicosapentaenoic acid [EPA; grey], docosapentaenoic acid [DPA (n-3); white], and docosahexaenoic acid [DHA; black] in the phospholipids of various tissues between diets. A student's t-test was used to assess differences between diet within a given tissue; statistical significance is denoted by an arrow indicating an increase or decrease at the $P < 0.05$ level.

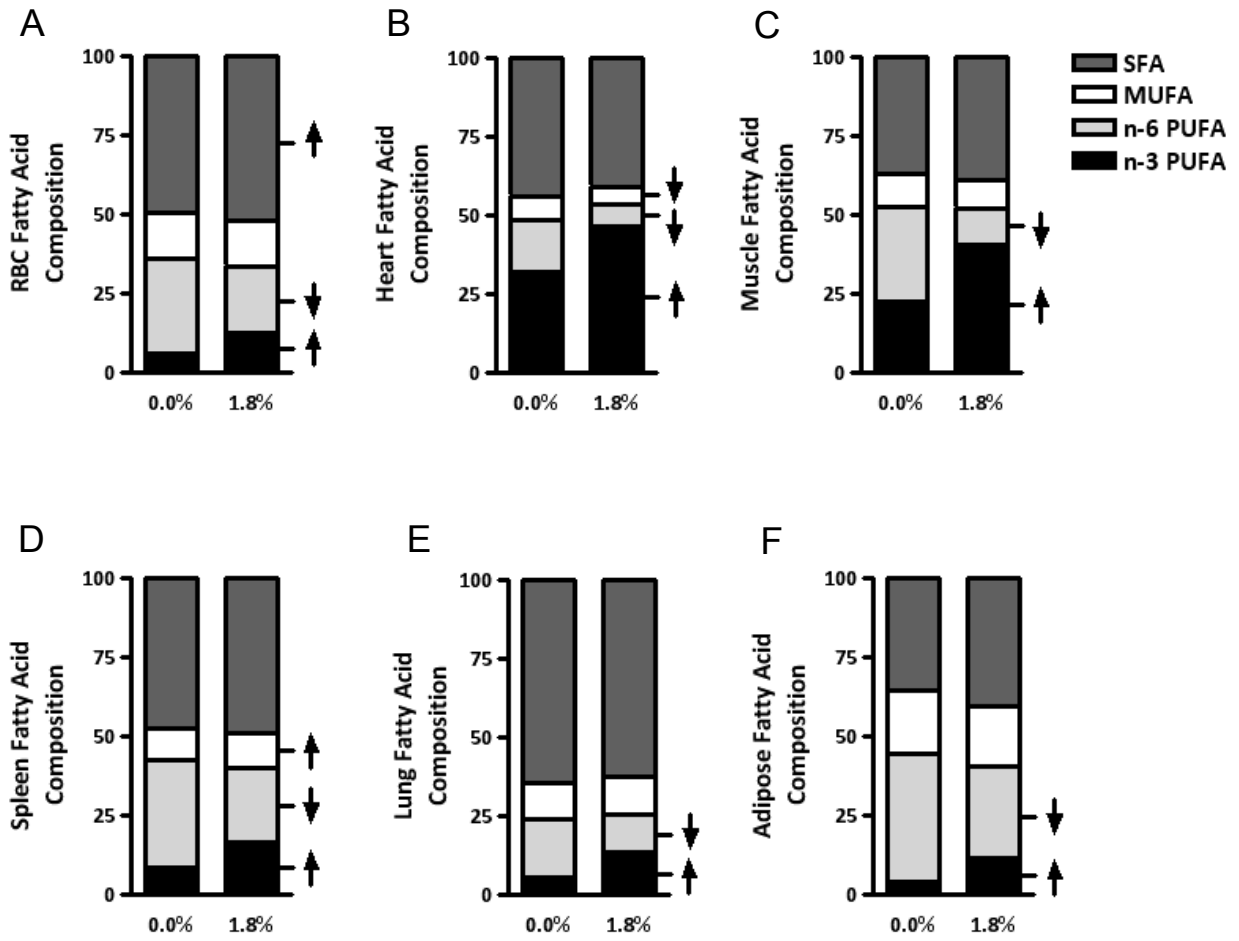


Figure 25 Saturated and unsaturated fatty acid profile of various systemic tissue mice fed increasing EPA and DHA. Comparison of the saturated and unsaturated fatty acid profile between SMAD3-/- fed either 0.0%en or 1.8%en EPA+DHA diets for RBC (A), heart (B), muscle (C), spleen (D), lung (E), and adipose tissues (F). A student's t-test was used to compare the content of saturated fatty acids [SFA; dark grey], monounsaturated fatty acids [MUFA; white], n-6 polyunsaturated fatty acids [n-6 PUFA; light grey], and n-3 polyunsaturated fatty acids [n-3 PUFA; black] in the phospholipids of various tissues between diets. A student's t-test was used to assess differences between diet within a given tissue; statistical significance is denoted by an arrow indicating an increase or decrease at the $P < 0.05$ level.

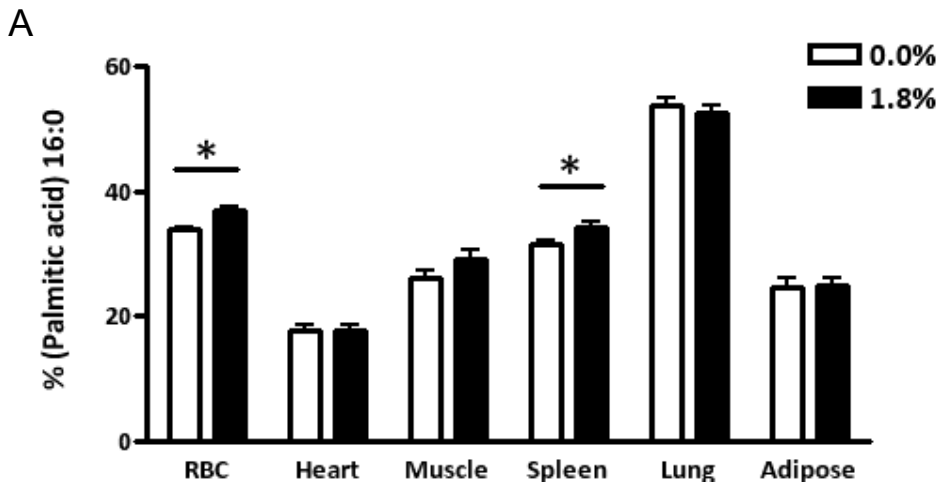
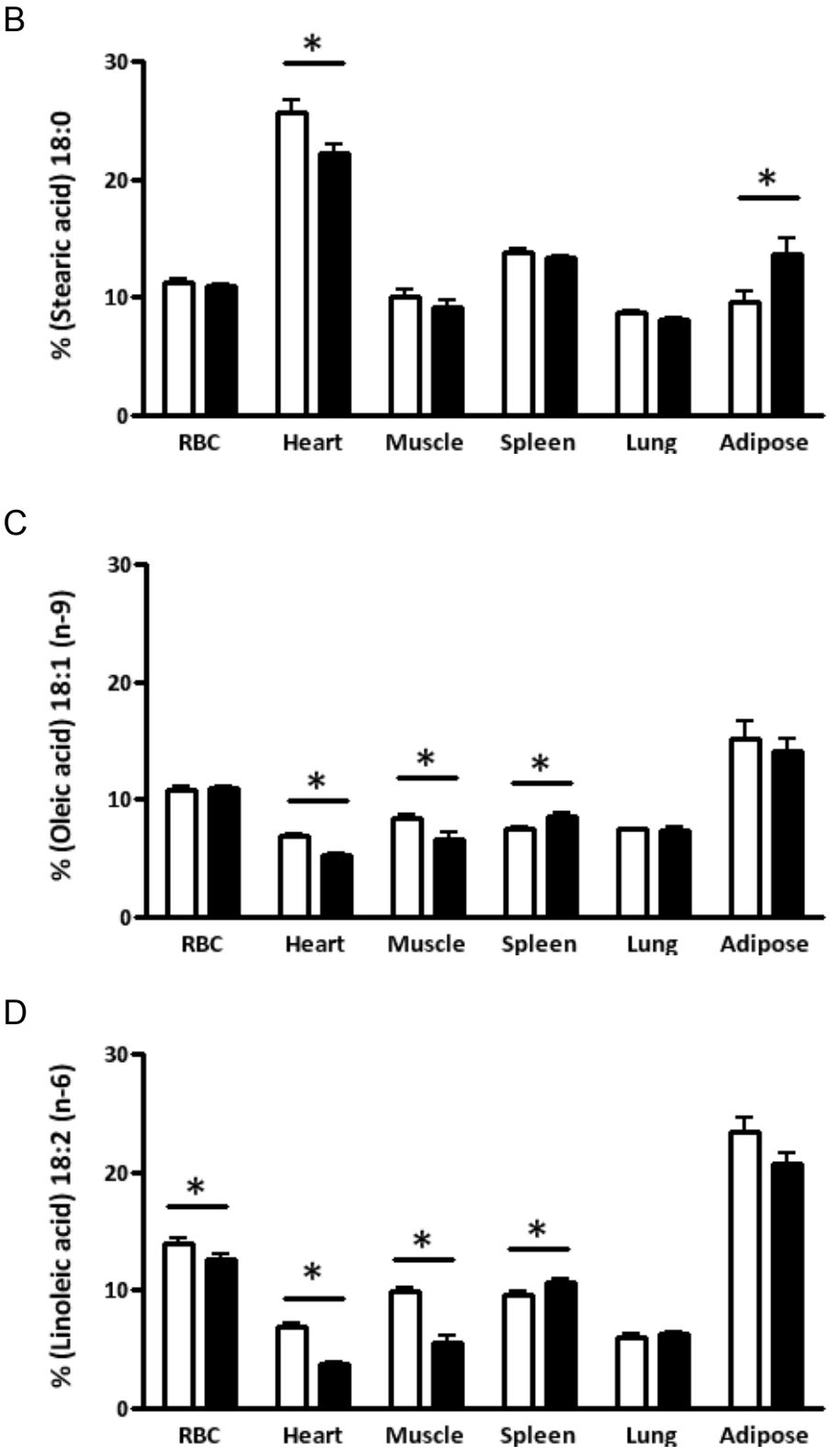


Figure 26 Differences in predominant, non-LCPUFA fatty acids between systemic tissues in mice and changes due to increasing intake of EPA and DHA. Comparison of non-LCPUFA fatty acids that majorly compose the phospholipid membrane between SMAD3-/- mice fed either 0.0%en or 1.8%en EPA+DHA diets across RBC, heart, muscle, spleen, lung, and adipose tissues. The major non-LCPUFA fatty acids (i.e. composing > 5% of phospholipid fatty acids) were palmitic acid (A), stearic acid (B), oleic acid (C), and linoleic acid (D). A one-way ANOVA was used to test for differences across tissues within a given a diet. Differing letters denote statistical significance at the $P < 0.05$ level, whereby lower case letters were used across tissues the 0.0%en diet and upper case letters were used across tissues the 1.8%en. A student's t-test was used to assess differences between diet within a given tissue; asterisked data denotes statistical significance due to diet within a tissue at the $P < 0.05$ level.

Figure 26 (cont'd)



Discussion

We found that levels of n-3 and n-6 LCPUFAs in RBCs adequately reflect the relative levels of LCPUFAs in heart, muscle, spleen, lung, and adipose tissue in response to dietary supplementation with EPA+DHA in mice. We observed strong and significant correlations between RBCs and all other tissues for EPA+DHA, EPA, DHA, DPA (n-3), and AA. Interestingly, EPA alone consistently exhibited the highest degree of correlation ($r > 0.92$) across tissues, followed by EPA+DHA, DPA (n-3), AA, and lastly DHA, which was the least correlated between RBCs and all other tissue. While all tissues responded to EPA+DHA supplementation with increasing n-3 LCPUFAs and decreasing n-6 LCPUFAs, we also observed changes in SFA and MUFA levels across various tissues. We found that dietary supplementation with EPA+DHA also resulted in increases in RBC SFA and spleen MUFA, but decreased heart MUFA. Unsurprisingly, palmitic, stearic, oleic, and linoleic acids were the predominant fatty acids in the phospholipids of the various tissues; however, depending on the tissue, we observed that EPA+DHA supplementation altered the levels of these fatty acids. It is readily apparent that the fatty acid composition of each tissue is unique to that tissue. Therefore, it is not unexpected that RBCs do not reflect the *absolute* levels of most SFAs, MUFAs, n-3 PUFAs, or n-6 PUFAs across most tissues. They do, however, reflect the *relative* level of n-3 and n-6 LCPUFAs of the tissues examined here.

There has been an expansive amount of research on the effects of n-3 LCPUFAs and the mechanisms by which they act. However, surprisingly little attention has been given to the amount of n-3 LCPUFAs in various tissues where they are thought to act. Given the breadth of health outcomes researched in the context murine fish oil

supplementation [6, 14, 140, 150], there is a need to better understand the phospholipid levels of n-3 LCPUFAs within in each tissue and whether an easily accessible tissue (e.g., RBCs) can serve as a surrogate biomarker for tissues available only postmortem. We have previously reported on the phospholipid fatty acid profile of RBCs, plasma, stomach, small intestine, cecum, and colonic tissues in mice fed increasing intakes of EPA+DHA [149]. These data identified that RBCs serve as an adequate biomarker for the *relative* levels of n-3 LCPUFAs in the gastrointestinal tract (e.g., low RBC EPA ≈ low stomach EPA), but, as seen here, they do not serve as an adequate biomarker for the *absolute* levels of n-3 LCPUFAs in the gastrointestinal tract (e.g., if RBC EPA was 3.1%, stomach EPA was 1.6%). Of note, we observed that the omega-3 index, as an increasingly used biomarker of n-3 LCPUFA exposure for cardiac tissue, was highly correlated to all tissues studied, providing further evidence to expand the use of the omega-3 index as a biomarker for additional tissues.

During this study, we made a number of interesting observations regarding the tissue specificity of fatty acid patterns. First, heart and muscle phospholipids are especially enriched in DHA. In the control group (i.e., with no EPA+DHA in the diet), the phospholipid fatty acids of heart and muscle contained 30% and 19% DHA, respectively, compared to 5% in RBCs. These data confirm earlier findings by Couture and Hulbert who reported levels of >20% and >15% DHA, respectively [151]. Additionally, Pepe and McLennan report that the phospholipid fatty acids of Hooded-Wistar rat hearts had 15% DHA at baseline and 27 % DHA after feeding fish oil [152]. Others have reported lower levels of DHA in total heart fatty acids of mice 9.7-13.1%

[153] fed standard diets, but this difference may be due to their analysis of total tissue fatty acids as opposed to just the phospholipid fraction.

Heart and muscle DHA levels increased from 30% and 20% (control) to 44% and 36%, respectively, in the mice fed the highest omega-3 diet, whereas RBC DHA levels went from 5% to 8%. Despite being fed EPA and DHA at a 1:1 ratio, neither EPA nor DPA (n-3) increased by more than 1.0% in either the heart or muscle tissues of mice fed the 1.8%en EPA+DHA diet compared to control. While increases in heart and muscle DHA marginalized any increases in EPA and DPA (n-3), RBCs, spleen, lung, and adipose tissues all had considerable and significant increases in EPA and DPA (n-3). Our observations are paralleled by Arnold et al., who observed that feeding Zucker rats a diet supplemented with EPA and DHA at a 4:3 ratio led to dramatic, significant increases in the DHA content of total lipid fatty acid from the left ventricle [2.9% -> 25%], but not EPA or DPA (n-3) [123]. In a similar study by Owen et al. that also further highlights the need to analyze phospholipid fatty acids, the DHA content of myocardial phospholipid fatty acids increased from 10.9% to 39.1% DHA, whereas EPA increased from 0.0% to 0.7% [154]. These data provide further evidence for the selective enrichment of DHA in cardiac tissue and, possibly, skeletal muscle phospholipids.

In contrast, DHA levels in human cardiac tissue are far lower than those in mice (e.g., 1.5 – 2.3% of total lipid fatty acids [144] and 4.8% – 8.5% in the phospholipids [129]). While supplementation of humans with fish oil did not change levels of DPA (n-3), fish oil supplementation led to increases in EPA in cardiac phospholipids fatty acids from 0.5% to 3.0% [129]. human skeletal muscle biopsies contain levels of DHA around 2.3% – 2.5% and 1.8% – 4.2% in phospholipid fatty acids [155]. Furthermore,

supplementation of humans with fish oil increases levels of EPA and DPA (n-3) in skeletal muscle fatty acids from 0.9% to 5.3% and 1.2% to 2.3%, respectively [155]. Taken together, these data suggest that the dramatic, selective enrichment of DHA in rodent heart and skeletal muscle phospholipids may not be shared with humans. Considering the number of studies investigating the mechanistic effects of n-3 LCPUFAs in rodent myocardial tissue [156], it is prudent to keep this discrepancy in mind.

Another point of interest was the considerable variation between the SFA, MUFA, and n-3 / n-6 PUFA composition between tissues. We previously reported that the levels of SFA in phospholipids from RBCs, plasma, and the tissues of the gastrointestinal tract ranged from 40-50% of total lipid [149]; in the current study, we observed levels of SFA as low as 35% in muscle and adipose and as high as 65% in the lung. Specifically in the lung, the remaining composition was approximately 11.5% MUFA and 23.5% PUFA (PUFA composition dependent on dietary treatment). Of all the tissues analyzed, the unusual SFA, MUFA, and PUFA composition of the lung looks most like the fatty acid composition of phospholipids from purified B cells from the same mice strain [97]. We had initially hypothesized that spleen phospholipids would look most like the B cell phospholipids given the high percent of spleen cellularity being derived from B cells. This contrasting observation may be explained by the multiple cell types that each tissue is made up of. It is possible that the spleen's phospholipid fatty acid profile more resembles RBCs and endothelial cells if they were more dominant than other cell types, because we were unable to isolate each cell type from each. If the lung's cellularity is

heavily derived from circulating lymphocytes, such as SFA-rich neutrophils [157], that may help explain the lung's resemblance to the phospholipid fatty acid profile of B cells.

Lastly, while it is well documented that n-3 LCPUFAs typically displace n-6 LCPUFAs (specifically DHA displacing AA), we observed slight alterations in levels of major , non-PUFA fatty acids (>5.0% of the membrane fatty acids) in response to omega-3 supplementation. Specifically, palmitic, stearic, oleic, and linoleic acids were affected. Kew et al. had previous reported that monocyte and neutrophil immune function were positively correlated with the levels of stearic and negatively correlated with the levels of palmitic acid [84]. These changes may be compensatory to balance the increased incorporation of n-3 LCPUFAs and displacement of n-6 LCPUFAs; regardless, as research has focused on n-3 LCPUFAs and n-6 LCPUFAs, it is prudent to consider alterations in non-PUFA fatty acids as a result of dietary supplementation with EPA+DHA. While the observation by Arnold et al. that EPA+DHA supplementation decreased linoleic acid in rat heart total lipid aligns with our observation, they also report that EPA+DHA increased linoleic in RBCs, which contrasts with our observation [123]; they had not reported on palmitic, stearic, or oleic acids. Consistent with our findings, Owen et al. observed decreased oleic and linoleic acids in rat myocardial phospholipid [with fish oil feeding?]; however, they did not observe any change in palmitic or stearic acids [154].

These data collectively demonstrate the need to analyze phospholipid fatty acids when considering tissue incorporation of lipid, but they also provide additional evidence that alternative tissues, such as RBCs, serve as adequate biomarkers of relative phospholipid fatty acid levels. In order to expand generalizability, future studies on the

mechanisms of action and the health effects of n-3 LCPUFA supplementation should report fatty acid levels. RBC phospholipid EPA+DHA content (the omega-3 index) reflects the relative n-3 LCPUFA level for solid organs in mice and presumably in humans as well.

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