Dietary fish oil alters T lymphocyte cell populations and exacerbates disease in a mouse model of inflammatory colitis

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Running Title: Fish oil exacerbates colitis in SMAD3 mice

Key Words: fish oil, DHA, colitis, SMAD3, T regulatory cells

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ABSTRACT

Inflammatory bowel diseases (IBD) increase the risk of developing colorectal cancer. Dietary components that reduced inflammation are associated with lower cancer risk. The long chain omega-3 fatty acid, docosahexaenoic acid (DHA), is present in fish oil and has potent anti-inflammatory properties. The objective of this study was to determine whether dietary fish oil enriched with DHA (DFO) could reduce experimentally induced colitis and colon cancer risk in a mouse model. When SMAD3-/- mice are exposed to *Helicobacter hepaticus*, mild colitis is observed 4 weeks post infection. Mice were fed isocaloric diets modified to include corn oil, safflower oil, or DFO (doses ranging from 0.75-6.00%) as the fatty acid source for 8 weeks. Mice were gavaged with *H. hepaticus*, DFO feeding continued, and mice were sacrificed 4 weeks after infection. The colon and cecum were collected for histopathology. Spleens and mesenteric lymph nodes were collected and analyzed for T cell populations using flow cytometry. Contrary to expectations, DFO induced severe colitis and adenocarcinoma formation. DFO consumption was associated with decreased CD8+ cell frequency and diminished CD69 expression on CD4+ and CD8+ T cell populations. Mice consuming DFO also exhibited higher FoxP3+ CD25+ CD4+ T regulatory (Treg) cell frequency, FoxP3 expression, and altered L-selectin expression during infection. We concluded DFO-fed mice may be less equipped to mount a successful response to *Helicobacter hepaticus* infection increasing colon cancer risk. These results support the need to establish a tolerable upper limit for DHA intake particularly in the context of chronic inflammatory conditions like IBD.
INTRODUCTION

Individuals with inflammatory bowel disease (IBD) have an increased risk of developing colon cancer. Colorectal cancer is responsible for mortality in approximately 15% of people with ulcerative colitis and Crohn’s disease (1). Despite routine screening procedures and the development of advanced treatments, one third of patients with colon cancer will ultimately experience metastatic disease and most will die of uncontrolled metastasis within a few years of diagnosis (2). In contrast to colon polyps which can be easily detected via colonoscopy, chronic colitis can result in dysplastic lesions that are difficult to identify. Flat colorectal adenoma is associated with accelerated carcinogenesis (3, 4) and poor prognosis (5). Given the negative outcome linked with colitis-associated colon cancer (CACC), prevention strategies aimed at reducing inflammation in individuals with IBD may reduce cancer risk.

Several studies have demonstrated efficacy of dietary fish oil (FO) consumption in IBD (6-10). Long chain omega-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), modulate inflammatory responses through several mechanisms. Increased consumption of dietary DHA and EPA results in increased incorporation of these n-3 fatty acids in immune cell membranes and occurs at the expense of arachidonic acid (11-13). Specifically, EPA can compete with arachidonic acid as a substrate for cyclooxygenase (COX) resulting in inhibition of the production of pro-inflammatory eicosanoids such as prostaglandin E2 (PGE2) and leukotriene B4. Increased membrane PUFA content also influences lipid raft composition and signaling properties of immune cells (14). DHA is capable of influencing membrane fluidity, ion permeability, fatty acid exchange, and resident protein function (14, 15).
Chronic inflammation creates an environment favorable to tumor formation by inducing oxidative stress, inhibiting apoptosis, and stimulating cell proliferation (16). The transforming growth factor beta (TGF-β) receptor is commonly mutated in CACC (17). TGF-β is a cytokine with cytostatic and apoptotic-inducing functions that plays an important role in the control of mammalian cell proliferation and differentiation (18). Binding of the TGF-β receptor activates the SMAD family of transcription factors, notably SMAD2 and SMAD3. The activated SMAD2 and SMAD3 then form a complex with SMAD4, which migrates to the nucleus and induces transcription of a targeted set of genes (19, 20). Price et al. (21) demonstrated that SMAD3 -/- mice develop chronic colitis and cancer in response to gastrointestinal Helicobacter infection. Dysfunctional TGF-β signaling, common in human IBD and colon cancer, supports the physiological relevance of the SMAD3-/- colitis model (22). Hence, we selected this model to study the effects of fish oil (FO) feeding on colitis and CACC outcome.

While n-3 PUFAs have demonstrated beneficial effects in gastrointestinal inflammation, recent studies employing animal models and human studies provide controversial results (23). Several studies have found no correlation between n-3 PUFA supplementation and IBD (24-28). In general, it appears that FO supplementation may generate a depressed immune environment via alterations in cytokine production, T cell proliferation, and T cell-mediated cytotoxicity (23). DHA can exclusively suppress Treg function (29). Interestingly, the immunomodulatory effects of DHA+EPA may be beneficial under certain circumstances such as chronic inflammation, but could be deleterious in acute pro-inflammatory and immune responses to cancer.

Unexpectedly, we found that DFO feeding exacerbated colitis and colonic dysplasia, which may be mediated through reduced CD8+ T lymphocyte populations, diminished CD69
expression on CD4+ and CD8+ T cells, and attenuated FoxP3 and L-selectin expression on Treg cells.

**METHODS**

*Mice.* SMAD3+/- and SMAD3-/- breeder pairs of 129SvEv background were generously donated by Dr. Lillian Maggio-Price (University of Washington). Homozygous males and heterozygous females were mated to obtain SMAD3-/- pups. Genotypes were confirmed by PCR. Animals were housed under specific pathogen-free conditions in 60 square inch plastic cages with microinsolator lids in the AAALAC approved Research Containment facility at Michigan State University. Animal protocols were approved by the Michigan State University All University Committee on Animal Care and Use.

*Diets.* SMAD3-/- animals were fed isocaloric experimental diets ad libitum beginning at 6-8 wks of age. The composition was based on the AIN-93G formulation of Reeves (30) and modified as described previously (31). Control corn oil (CO) diets contained 70g/kg corn oil (Dyets, Bethlehem, PA); control safflower oil (SF) diets contained 10g/kg corn oil and 60g/kg high oleic acid safflower oil (Spectrum Naturals, Boulder, CO). The DHA diets contained varying amounts of corn oil and DHA-enriched fish oil (0.75%-6%; Ocean Nutrition Canada, Mulgrave, NS, Canada) as summarized in Table 1. The DHA-enriched fish oil (DFO) contained 540 mg/g DHA and 50 mg/g EPA. The highest dose of DFO at 6% was selected because it was shown to suppress Interleukin-6 (IL-6) expression in previous studies (32). Diets were freshly prepared every 2-3 wks and stored at -20°C. Food was placed in clean cups and replaced daily to prevent oxidation.
**Bacterial culture and infection.** *H. hepaticus* strain 3B1 (ATCC 51488) was kindly donated by Dr. Vince Young (University of Michigan). Isolates were aseptically streaked onto blood agar and incubated at 36°C for 24-48 hrs inside GasPak™ (BD Diagnostic Systems, Sparks, MD). Mice were infected as previously described (21). Briefly, bacteria were resuspended in tryptic soy broth at an optical density $\geq 1.8$. Animals were gavaged with 0.3 mL of fresh bacterial suspension on two consecutive days.

**Experimental Design.** Three separate diet studies were performed. In study 1, male and female mice were fed either Harlan Teklad rodent chow (CON), CO, SF, or 6.00% DFO for 8 wks pre-infection. In study 2, mice received either SF, or DFO (0.75, 2.25, 3.75, or 6.00%; $n=16-20$ mice/treatment). Mice consumed the assigned diet for 8 wks prior to infection and throughout 4 wks post-infection when the experiment was terminated. For study 3, mice were fed either 6.00% DFO or CON and euthanized. See Table 1 for fatty acid composition of the diets.

**Tissue fixation and histopathology.** Mice were asphyxiated with CO$_2$ and exsanguinated via cardiac puncture. Tissue was removed, flushed with phosphate buffered saline (PBS) and fixed in 10% formalin overnight and stored in 70% ethanol. Tissues were then processed in paraffin and stained with hematoxylin and eosin (H&E). Longitudinal sections were graded for inflammation and dysplasia by pathologist using a blinded scoring system adapted from Dr. Maggio-Price (21). Cecums and colons were scored on a 1 to 4 scale both for inflammation (1, no inflammation; 2, mild inflammation; 3, moderate inflammation; 4, marked inflammation) and dysplasia (1, no dysplasia; 2, low grade dysplasia; 3, high grade dysplasia; 4, high grade dysplasia with invasion/adenocarcinoma). The two scores for colon and two scores for cecum tissue in each animal were combined such that a score of 4 indicated no inflammation or dysplasia and a score of 16 reflected maximal inflammation and dysplasia.
Tissue Fatty Acid Analysis. To confirm DHA phospholipid incorporation after 8 wks of DFO consumption, hepatic lipid fractions were extracted and analyzed by gas chromatography (GC) as previously described (33, 34).

Lymphocyte isolation. Spleens (Spln) and mesenteric lymph nodes (MsLn) were aseptically removed and placed in ice cold RMPI at the time of necropsy. Spleens were processed with a dounce homogenizer, pelleted, and washed in RPMI. Cells were resuspended in ACK lysing buffer (Invitrogen, Carlsbad, CA) and washed twice in RPMI. MsLn were passed through a 70 µm filter and washed with RPMI. Cell counts were performed with a hemocytometer using trypan blue exclusion and resuspended to a concentration of one million cells per mL of media.

Flow cytometry. MsLn or Spln cells were resuspended in fluorescence-activated cell sorting buffer (FACS; 0.1% sodium azide, 1% fetal bovine serum (FBS), in dPBS blocked with anti-Fc receptor γ II/II [CD16/CD32(clone 2.4G2)] for 10 min on ice, and incubated with fluorochrome-conjugated antibodies (E-bioscience, San Diego, CA or BD Bioscience, San Jose, CA) at concentrations ranging from 1:100 to 1:300 in FACS buffer: CD3 (PerCP-Cy5.5), CD4 (eFluor450), CD8 (PE-Cy7), CD25 (PE), CD62L (APC), and FoxP3 (FITC or Alexa Fluor488) for 30 min. Intracellular staining was performed using FoxP3 staining buffer set as per the manufacturer’s instructions (E-bioscience, San Diego, CA). Briefly, after surface staining, cells were washed twice in FACS buffer, fixed in 4% paraformaldehyde for 25 min, and permeabilized for 30 min. Permeabilization was followed by incubation for 30 min with the appropriate antibodies diluted in permeabilization diluent. Samples were then acquired on a LSR II (BD Bioscience)) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Statistics. Data analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA). All data were represented as mean ± SEM. Two-way ANOVA were performed with
Bonferroni's Multiple Comparison Test to determine differences between two groups within a parameter unless noted otherwise. P values < 0.05 were considered significant.

RESULTS

**DFO-fed mice displayed increased intestinal inflammation and dysplasia following infection with H. hepaticus.** Unexpectedly, we found that 4 wks post-infection mice consuming 6.00% DFO had the highest degree of inflammation and dysplasia compared to the three control diets (CO, SF, and chow (CON) (Fig. 1). The observed exacerbation in colitis was dose-dependent as the 2.25% and 3.75% DFO groups received a score falling between the 6.00% DFO and the three controls; however, the difference in histopathology scores between these was not statistically significant. The 0.75% DFO group was not different from the control groups, however it was significant when compared with the 6.00% DFO. Since no significant differences in colitis scores were found between the three control groups, the chow diet was selected as a control for all remaining experiments.

Representative H&E stains of colon and cecum tissue demonstrated a greater number of inflammatory cells as well as epithelial proliferative lesions at 4 weeks post infection as DFO composition increased (Fig. 2). The control CO and SF-fed mice displayed increased inflammatory cell infiltration and mild hyperplasia in the mucosa of both the cecum and colon. These observations were expected in SMAD3-/- mice in response to infection with *H. hepaticus*. However, DFO fed mice had an even greater severity of inflammation accompanied by dysplastic crypts and mitotic figures (Fig. 2).

To confirm the presence of DHA in cellular phospholipids, hepatic fatty acids from CON, SF, and DFO-fed animals were extracted and analyzed by GC. As expected, mice consuming 6%
DFO had a higher percentage of DHA-containing phospholipids (25.38% ± 4.90, n=4) compared to CON (5.71% ± 5.15, n=5) or SF fed mice (3.44%±1.68, n=5) (p<0.001).

**DFO feeding increased post-infection mortality and body mass loss.** Mice consuming DFO had a higher mortality rate following infection with *H. hepaticus* compared to the control diets (CON, CO, SF) (Fig. 3A). All mice in the CON and CO groups and 92% of mice on the SF diet survived following infection with *H. hepaticus*. However, only 82% of DFO-fed animals survived 4 weeks, with mortality in this group observed as early as 1 week post infection (PI). The cause of mortality in the DFO group was not further investigated as this was an unexpected finding. However, we did observe that animals in this group gained less weight from wks 0 to 4 after infection compared with CO or SF-fed counterparts (Fig. 3B). These weight changes were in accordance with survival data through wk 4.

**DFO feeding modulated CD4+ and CD8+ T cell populations in SMAD3-/− mice.** The total cell counts in each group did not significantly differ within either tissue (data not shown). CON treatment induced kinetic differences across time in CD3+ cells whereas DFO did not. Note the difference in CD3+ lymphocytes at day 3 PI in MsLn of DFO-treated animals (p<0.05; Figure 4A). DFO induced potent reductions in the proportion of CD8+CD3+ cells in both tissues prior to gavage and throughout infection (figure 4A). In addition, DFO-fed animals exhibited increased CD4+CD3+ splenic T lymphocytes prior to infection and at day 7 (p<0.001; Figure 4A). However, no differences were noted in the MsLn. DFO resulted in greatly decreased percentages of CD4+ T cells from the MsLn expressing CD69 (Figure 4B).

**Regulatory T cell populations and FoxP3 expression were altered by DFO feeding.** The proportion of FoxP3+ CD25+ T cells within the CD4+ T cell population was significantly increased in the spleen in response to DFO feeding both before infection (p<0.05) and at days
3, 7, and 28 PI (P<0.001) (Fig. 5A). No differences in Treg cell percentages between CON and DFO were found in MsLn (data not shown). Due to the discrepancy in Treg cells between tissues, we examined the expression of L-selectin (CD62L) which is required for the migration of Treg cells (35) and can be altered by n-3 PUFAs (36). A reduced percentage of the splenic FoxP3+ CD25+ CD4+ Treg cells expressed CD62L in the DFO group. At day 0, splenic Treg cells from CON and DFO-fed animals displayed no differences in CD62L expression. However at day 28, an increased proportion of Treg cells did not express CD62L compared to CON-fed animals. However, the numbers of Treg cells were significantly reduced in MsLn of DFO-fed animals at day 28 PI (Fig. 5B). Because L-selectin is cleaved from the surface of T lymphocytes upon activation (37), we examined the proportion of CD62L− Treg cells in MsLn. An increased number of Treg cells from the CON group did not express CD62L. Cell counts in the MsLn of CON-fed animals increased significantly at days 7 and 28 PI while no changes occurred in the DFO group. While the percentages of FoxP3+ CD25+ CD4+ T differed only in the spleen and not MsLn, the expression of FoxP3 in these cells was altered by DFO-feeding. DFO consumption resulted in higher FoxP3 expression at days 0 and 28 PI in both spleen and MsLn (p<0.01 or p<0.001) while DFO increased FoxP3 expression at day 7 PI in spleen alone (p<0.05) (Table 2).

DISCUSSION

This study investigated the effect of dietary DFO on chronic colitis and colitis-associated colon cancer (CACC) in SMAD3 −/− mice in response to infection with H. hepaticus. The results presented here demonstrate that DFO feeding increases the severity of colitis and mucinous adenocarcinoma in the SMAD3−/− model. The exaggerated inflammation and carcinogenesis induced by dietary DFO was associated with altered CD8+ T cell populations, CD69+ activation,
FoxP3 expression, and the frequency of FoxP3+ CD25+ CD4+ Treg cells expressing L-selectin (Fig. 4 and 5). These findings implicate that high doses of DHA consumed before and during active colitis in SMAD3-/- animals may promote impaired immune function.

The hyporesponsive environment elicited by FO may play an adverse role in acute bacterial and viral infection. Dietary FO increased mortality and impaired pathogen clearance in response to *Listeria monocytogenes* (38). Similarly, *fat-1* mice with high levels of endogenous n-3 PUFAs are more susceptible *Mycobacterium tuberculosis* infection through diminished macrophage production of TNF-alpha, IL-6, and IL-1β (39). A recent study by Beck *et al.* (40) demonstrated that FO feeding had deleterious effects on the immune response to influenza infection, which was characterized by decreased CD8+ cytotoxic T lymphocytes, reduced neutrophils at the site of infection, and impaired natural killer cell cytotoxicity. Taken together, these observations indicate that while FO supplementation may be beneficial in lowering the risk of some cancers (41), FO may act differently in the etiology of infection-associated cancers.

The present studies clearly support the hypothesis that altered immune dysregulation contributes to exacerbated colitis in SMAD mice. However, FO feeding studies in other well-characterized murine colitis models have produced conflicting results. One investigation reported that FO supplementation at 7% in IL-10 -/- mice increased spontaneous colitis score and colitis-associated neoplasia within 10-12 wks (42). In contrast, another study reported FO supplementation at 4% was protective in the IL-10 -/- model when colitis development was accelerated with non-steroidal anti-inflammatory drug (NSAID) treatment (43). These studies are difficult to compare due to differences in genetic background and the undefined role of COX-2 inhibition in colitis. While the majority of studies demonstrate efficacy of FO in chemically-
induced colitis (44), one study showed exacerbated colitis in response to DFO feeding possibly through modified adiponectin expression (45).

The dose of FO, relative EPA and DHA content, and length of feeding protocol are also another source of variation. In our study, the most severe colitis and CACC correlated with the 6.00% DFO dose for a feeding period of 8 wks (Fig. 1). The dietary composition and feeding period are comparable to other FO feeding studies with the exception that our DFO contained mostly DHA and only little EPA. Interestingly, we observed low dose (0.75%) DFO consumption did not ameliorate colitis score compared to control animals (SF, CO, and CON). Furthermore, animals consuming intermediate doses of DFO (2.25% and 3.75%) experienced exacerbated inflammation and dysplasia that was comparable to the 6.00% DFO diet (Fig. 1 and 2). Our results indicate DFO consumption as low as 2.25% aggravates colitis and accelerates dysplastic tissue transformation.

Due to the potential role of impaired TGF-β signaling in Treg cell response, we hypothesized DHA feeding may further influence Treg cell populations. Not only did DFO-fed mice exhibit a high proportion of splenic CD4+ T cells, but a higher percentage of this population was also FoxP3+ CD25+ (Fig. 5). This suggests that dietary DFO may be inducing a more pronounced splenic Treg cell phenotype both under pathogen-free conditions and during infection. Interestingly, although no difference was found in FoxP3+CD25+ populations in the MsLn, FoxP3 expression (MFI) was enhanced in both spleen and MsLn in DFO-fed mice (Table 2). Indeed a recent study by Yessoufou et al. (46) demonstrated that dietary DHA increases FoxP3 expression in murine Treg cells possibly through inhibition of SMAD7. The authors also noted upregulation of FoxP3 did not necessarily correlate with Treg cell suppressive capacity because Treg cells from DHA-fed animals failed to suppress the proliferation of co-cultured...
CD4+ T effector cells also derived from DHA-fed animals. Hence, DHA may induce a strong Treg phenotype via upregulation of FoxP3 expression but this population fails to initiate suppressive action on effector T cells. Although we did not examine the effect of DFO at doses <6.00% on Treg cells, Yessoufou et al. observed similar effects on FoxP3 expression at roughly 2% FO.

Recent evidence suggests that Treg cells may promote tumor progression through the production of IL-17 (47). Under poorly regulated pro-inflammatory conditions, Treg may fail to inhibit and may instead contribute to a T helper (Th)-17-driven procarcinogenic process (48). DFO may promote the production of Treg cells with decreased suppressive capacity and simultaneously accelerate adenocarcinoma development during the later stages of infection. The exact mechanism for these observations and their consequences are still unknown. We observed that a significantly decreased proportion of splenic Treg cells in the DFO group expressed L-selectin at days 3, 7, and 28 PI (Fig 5A). This observation is consistent with previous studies showing decreased L-selectin expression in response to DHA (46). Because L-selectin is important in lymphocyte migration to the MsLn and Peyer’s patches, (37) our data suggests that DFO may decrease the ability of Treg cells to migrate to lymph nodes in close proximity to the site of infection. Incorporation of polyunsaturated fatty acids into human cell membranes was recently shown decrease L-selectin expression(49).

It is noteworthy that the percentage of Treg cells in the spleen remained elevated throughout infection. Although percentage was similar between CON and DFO in MsLn, we found that the number of Treg cells was significantly decreased at day 28 PI. Decreased Treg cells in the MsLn at day 28 could contribute to the increase in inflammation and dysplasia observed in DFO-fed animals. Moreover, a decreased number of Treg cells were defined as L-
selectin negative (CD62L- in the MsLn of DFO-fed mice. This indicates that in addition to fewer numbers of Treg cells near the site of infection at day 28, the DFO group had a decreased number of activated Treg cells at day 28 as indicated by the loss of L-selectin (37). 

*H. hepaticus* produces a virulence factor, cytolethal distending toxin (CDT), which is required for extended colonization and inflammation of the murine gut (50). CDT enters mammalian cells and exhibits DNase I-like activity which results in double-stranded DNA breaks and phase G2 cell cycle arrest prior to mitosis (51), induces apoptosis in cultured T cell lines (52) and suppresses the function of macrophages and dendritic cells (53). CD8+ cytotoxic T lymphocytes are essential in eliminating infected and transformed host cells. Thus, CDT promotes pathogenesis by disabling immune response and impairing repair of damaged tissue.

Recent studies reveal that DFO polarizes CD4+ T helper cells towards a T helper type-2 (Th2) phenotype both via inhibition of the Th1 cytokine, INF-γ (54), and direct suppression of IL-2-induced autocrine proliferation of Th1 cells (43). We observed diminished CD8+ T cell populations in DFO-fed mice prior to infection (Fig. 4C). Additionally, DFO feeding was associated with decreased CD69 activation on CD4+ and CD8+ T lymphocytes. A recent study found that DHA supplementation in humans specifically decreased CD69 expression on lymphocytes in response to stimulation with Con A. An increased splenic CD4:CD8 ratio resulting from both decreased CD8+ cell populations and increased CD4+ cell populations could in part be accounted for by the increased predominance of FoxP3 CD25 CD4+ Treg cells in DFO-fed animals. Although we did not characterize these cell populations post-infection, our data suggest DFO feeding creates an immunosuppressed host environment, characterized by reduced numbers of CD8+ cytotoxic T cells, decreased CD69 activation, and unfavorable L-selectin expression on Treg cells. Compared to control animals, DFO-fed animals may be less
equipped to mount a successful response to \textit{H. hepaticus} infection. Further studies are required to assess proliferative capacity, cytokine production, and migratory abilities of adaptive immune cell populations in DFO-fed animals prior to and throughout the course of \textit{H. hepaticus} infection.

These findings support a growing body of literature implicating deleterious effects of high dose FO consumption and certain disease states. Investigations regarding FO supplementation in human IBD have employed FO doses ranging between 500 mg and 7 g per day (24). Our diets mimicked 1g (0.75%), 3g (2.25%), 5g (3.75%) and 8g (6.00%) per day DFO when calculated as a component of a 2,000 kcal human diet. We observed inflammatory and dysplastic changes at the 3, 5 and 8g equivalent. The most dramatic increase was observed at the 8 g/day dose. Currently, efforts are underway to establish dietary reference intakes for EPA and DHA due to substantial evidence supporting beneficial effects of FO consumption in the prevention of common diseases such as coronary artery disease and cognitive decline (55). Consumer intakes of DHA and EPA continue to increase with growing FO supplement consumption and addition of n-3 PUFAs to foods (i.e. functional foods). Studies from our and other groups advocate establishing a tolerable upper limit for FO consumption to protect certain immune compromised sectors of the population who may be at risk for pathogen-associated enteric inflammation and gastrointestinal cancers.
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Table 1. Fatty acid composition of experimental diets.

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<th>Oil source</th>
<th>Fatty acid composition ¹</th>
<th>²</th>
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<th>Ratio (n-6): (n-3)</th>
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<td>DHA-enriched fish oil</td>
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¹ as reported by manufacturer
² corn oil contained 612g/kg linoleic acid and 260g/kg oleic acid
³ safflower oil contained 140g/kg linoleic acid and 750g/kg oleic acid
⁴ DHA-rich fish oil contained 540g/kg DHA and 50g/kg EPA
⁵ as α-linolenic acid
⁶ chow fat sources include soybean oil, wheat, fishmeal, corn, and oats as reported by manufacturer
Table 2. MFI of FoxP3 on T regulatory cells

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<td>1374 ± 235.4***</td>
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<td>773.0 ± 122.9</td>
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*values represent means (± standard deviation) from 3-6 mice in each group. Sample means were significantly different from CON-fed littermates; *, p<0.05; **, p<0.01; ***, p<0.001
FIGURE LEGENDS

Figure 1. DFO feeding increases severity of inflammation and dysplasia of SMAD3-/- mice in response to infection with *H. hepaticus* (4 wk PI). Each animal received a total of 4 numerical scores (4= lowest, 16= highest). CON=standard Harlan Teklad pellet diet 7913 (n=36), CO=corn oil (n=20), SF=safflower oil (n=26) and DFO=DHA-rich fish oil by percent composition, 0.75%, 2.25%, 3.75%, 6.00% (n=8, 7, 8, 18, respectively). The CON, CO, SF and 0.75% treatments were not significantly different. *=p<0.05 CON vs 2.25%, 3.75%; **=p<0.001 CON vs 6.00%. No statistical differences were found when 2.25% and 3.75% were compared to 6.00%.

Figure 2. H&E stained cecum (left panel) and proximal colon sections from SMAD3-/- mice 4 wks after infection with *H. hepaticus*. CON. The number of inflammatory cells in the lamina propria is slightly increased, consistent with mild inflammation (*). DFO, 0.75%. The cecal mucosa is mildly thickened due to crypt cell hyperplasia (*). The colonic lamina propria is expanded by mild inflammation (*). Note increased numbers of mitotic figures in the crypts denoting increased cell proliferation (circle). DFO, 2.25%. Elongated crypts lined by hyperplastic epithelium with decreased or absent goblet cells cause marked thickening of the cecal mucosa (arrow). Mild reduction in the number of goblet cells and increased mitotic figures (arrow) are also evident in the colonic crypts. In both locations, these proliferative alterations are accompanied by mild inflammation (*). DFO, 3.75%. The cecal mucosa is markedly distorted by tortuous and variably branching dysplastic crypts (arrows), surrounded and separated by moderately increased numbers of inflammatory cells (#). In the colon, crypts are elongated and lined by hyperplastic epithelium with inconspicuous goblet cells. The lamina propria is mildly
inflamed (*). DFO, 6.00%. The tunica muscularis of the cecum is multifocally disrupted by an adenocarcinoma, composed of single or nests of crypts lined by relatively well differentiated epithelium (arrows) and lakes of mucin that extend into and beyond the serosal lining (*). In the colon, the wall is thickened due to moderate inflammation and hyperplastic crypts (arrow) that occasionally herniate into the submucosa (*).

**Figure 3.** DFO-feeding has a negative impact on survival in SMAD3-/- mice in response to infection with *H. hepaticus*. A, Kaplan-Meier plot displaying higher mortality in DFO-fed mice at all doses (0.75%, 1.25%, 3.75%, 6.00%; n=56) compared to CON (n=10) CO (n=18) or SF (n=38) mice following infection with *H. hepaticus*. B, DFO-fed mice gain less weight compared to SF and CO control animals from 1 through 4 wks post-infection. Only animals who survived to wk 4 were included. Statistical differences were calculated using Bonferroni's Multiple Comparison Test. SF (n=31), CO (n=18), or DFO (n=45). Significant differences are shown (***p<0.01). There was no difference between the CO and SF groups. DFO was significantly different from CO but not SF.

**Figure 4.** DFO alters T lymphocyte populations in uninfected animals (day 0) and at d 3, 7 and 28 post infection (PI). Live cell suspensions from harvested tissues were stained with fluorochrome-conjugated antibodies and analyzed by flow cytometry. Statistical differences were examined both across time (indicated by a or b) and at each time point between diet treatments (indicated by asterisks), n=5-6 animals per group. A, CON treatment induced kinetic differences across time in CD3+ cells whereas DFO did not. Note the difference in CD3+ lymphocytes at day 3 PI in MsLn of CON versus DFO-fed mice (p<0.05). CD3+ cells were
gated out of all live cells. DFO induced potent reductions in the proportion of CD8+CD3+ cells in both tissues prior to gavage and throughout infection. DFO-fed animals exhibited increased CD4+CD3+ splenic T lymphocytes prior to infection and at day 7 (p<0.001). However, no differences were noted in the MsLn. B, DFO resulted in greatly decreased percentages of CD4+ T cells from the MsLn expressing CD69. CD69+ events were gated out of the CD4+ cell population.

**Figure 5.** Frequencies of FoxP3+ CD25+ CD4+ Treg cells CD62L expression in spln and MsLn at day and 3, 7, and 28 days PI. A, *Left*, Splenic percentages of double positive FoxP3/CD25+ cells remained significantly elevated both at baseline and throughout the course of infection in DFO-fed animals (*p<0.05 and ***p<0.001). A decreased percentage of these Treg cells were CD62L+ in DFO-fed animals beginning at day 3 PI (**p<0.01 and ***p<0.001) (right). Representative splenic CD62L expression is shown on Treg cells at 0 and 28 days PI (gray line=CON, dark line=DFO). No differences in the distribution between CD62L+ and CD62L− were noted at day 0. A greater proportion of Treg cells were CD62L− at day 28 PI in the DFO group (dark line). B, *Left*, FoxP3+ CD25+ CD4+ Treg cell counts from MsLn were decreased in the DFO group at 28 days PI (p<0.01). The total number of CD62L− Treg cell was also decreased in DFO-fed animals at 28 days PI (p<0.001) (right). Note the cell number in MsLn increased significantly at days 7 and 28 PI in CON (p<0.05) but not DFO.
Woodworth, HL
Figure 2
Figure 4

**A**

Spn

<table>
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<th>CD3+</th>
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MsLN

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**B**

CD69+

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**Days PI**

- CON: White bars
- DFO: Black bars

Legend:
- a, b, ab: Statistical significance
- **: p < 0.05
- ***: p < 0.001

Note: The figure illustrates the percentage of live cells and CD3+ cells in Spn and MsLN over different days post-infection (PI). The results show significant differences between CON and DFO treatments at various time points.
Woodworth, HL

Figure 5

A

**FoxP3/CD25+**

![Graph showing FoxP3/CD25+ cells at different days PI](image)

**CD62L+**

![Graph showing CD62L+ cells at different days PI](image)

B

**MsLN**

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**CD62L**

![Graph showing CD62L cells at different days PI](image)
# Dietary fish oil alters T lymphocyte cell populations and exacerbates disease in a mouse model of inflammatory colitis


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