Prevention and Epidemiology

Dietary Fish Oil Alters T Lymphocyte Cell Populations and Exacerbates Disease in a Mouse Model of Inflammatory Colitis

Hillary L. Woodworth¹, Sarah J. McCaskey¹, David M. Durianck¹, Jonathan F. Clinthorne¹, Ingeborg M. Langohr², Elizabeth M. Gardner³, and Jenifer I. Fenton¹,³

Abstract

Inflammatory bowel diseases (IBD) increase the risk of developing colorectal cancer. Dietary components that reduce 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 is 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 postinfection. Mice were fed isocaloric diets modified to include corn oil, safflower oil, or DFO (doses ranging from 0.75% to 6.00%) as the fatty acid source for 8 weeks. Mice were gavaged with H. hepaticus; DFO feeding was 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 cell frequency, FoxP3 expression, and altered L-selectin expression during infection. We concluded that DFO-fed mice may be less equipped to mount a successful response to H. 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 such as IBD.

Cancer Res; 70(20); 7960–9. ©2010 AACR.

Introduction

Individuals with inflammatory bowel disease (IBD) have an increased risk of developing colon cancer. Colorectal cancer is responsible for mortality in ~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 that can easily be detected through 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 shown the efficacy of dietary fish oil (FO) consumption in IBD (6–10). Long-chain omega-3 polyunsaturated fatty acids (PUFA), 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 and leukotriene B4. Increased membrane PUFA content also influences the 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 β (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...
Physiologic relevance of the SMAD3
common in human IBD and colon cancer, supports the
reduced CD8+ T lymphocyte populations, diminished CD69
litis and colonic dysplasia, which may be mediated through
pro-inflammatory and immune responses to cancer.

Although n-3 PUFAs have shown beneficial effects in
gastrointestinal inflammation, recent studies using 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 seems
that FO supplementation may generate a depressed immune
environment through alterations in cytokine production,
T-cell proliferation, and T-cell–mediated cytotoxicity (23).
DHA can exclusively suppress T regulatory (Treg) function
mediated cytotoxicity (23).

Unexpectedly, we found that DFO feeding exacerbated co-
litis 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
expression on Treg cells.

Materials and Methods

Mice
SMAD3+/- and SMAD3−/− breeder pairs of 129SvEv back-
ground were generously donated by Dr. Lillian Maggio-Price
(University of Washington, Seattle, WA). 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 microisolator lids in the
Research Containment Facility at Michigan State University
approved by the Association for Assessment and Accredita-
ion of Laboratory Animal Care. 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 to 8 weeks 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 70 g/kg CO (Dyets); control safflower
oil (SF) diets contained 10 g/kg CO and 60 g/kg high oleic
acid SF (Spectrum Naturals). The DHA diets contained vary-
ing amounts of CO and DHA-enriched FO (DFO; 0.75–6%;
Ocean Nutrition Canada) as summarized in Table 1. 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 to 3 weeks and
stored at −20°C. Food was placed in clean cups and replaced
daily to prevent oxidation.

Bacterial culture and infection
Helicobacter hepaticus strain 3B1 (American Type Culture
Collection) was kindly donated by Dr. Vince Young (Univer-
sity of Michigan, Ann Arbor, MI). Isolates were aseptically
streaked onto blood agar and incubated at 36°C for 24 to
48 hours inside GasPak (BD Diagnostic Systems). Mice were
infected as previously described (21). Briefly, bacteria were
resuspended in tryptic soy broth at an optical density of

| Table 1. Fatty acid composition of experimental diets |
|-----------------------------|-------------|-----------------------------|-----------------------------|
| Oil source                  | Fatty acid composition (g/kg diet)* |
| Experimental diets          | Linoleic acid | Oleic acid | EPA | DHA | Total (n-6) | Total (n-3) | Ratio (n-6)/(n-3) |
| CON                          | 25.6         | 12.9       | 0.00 | 0.00 | 25.6       | 2.71§        | 9.44          |
| CO                           | 42.8         | 18.2       | 0.00 | 0.00 | 42.8       | 0.00         |  —           |
| SF                           | 41.5         | 14.5       | 0.00 | 0.00 | 41.5       | 1.13         | 145           |
| 0.75% DFO                    | 32.1         | 13.7       | 0.38 | 4.05 | 32.1       | 8.48         | 7.26          |
| 2.25% DFO                    | 23.0         | 9.75       | 1.13 | 12.2 | 23.0       | 13.3         | 1.73          |
| 3.75% DFO                    | 37.5         | 13.8       | 5.85 | 1.88 | 20.3       | 19.8         | 0.62          |
| 6.00% DFO                    | 6.12         | 2.60       | 3.00 | 32.4 | 6.12       | 35.4         | 0.17          |

*As reported by the manufacturer.
†CO contained 612 g/kg linoleic acid and 260 g/kg oleic acid.
‡SF contained 140 g/kg linoleic acid and 750 g/kg oleic acid.
§DFO contained 540 g/kg DHA and 50 g/kg EPA.
∥Chow fat sources include soybean oil, wheat, fishmeal, corn, and oats as reported by the manufacturer.
¶As α-linolenic acid.
≥1.8. Animals were gavaged with 0.3 mL of fresh bacterial suspension on 2 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 weeks preinfection. In study 2, mice received either SF or DFO (0.75%, 2.25%, 3.75%, or 6.00%; \( n = 16–20 \) mice per treatment). Mice consumed the assigned diet for 8 weeks before infection and throughout 4 weeks postinfection 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 through cardiac puncture. Tissue was removed, flushed with 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 a pathologist using a blinded scoring 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 weeks of DFO consumption, hepatic lipid fractions were extracted and analyzed by gas chromatography (GC) as previously described (33, 34).

Lymphocyte isolation
Spleens and mesenteric lymph nodes were aseptically removed and placed in ice-cold RPMI 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) and washed twice in RPMI. Mesenteric lymph nodes were passed through a 70-μm filter and washed with RPMI. Cell counts were performed with a hemocytometer by using trypan blue exclusion and resuspended to a concentration of 1 million cells per milliliter of medium.

Flow cytometry
Cells from mesenteric lymph nodes or splenic tissue were resuspended in fluorescence-activated cell sorting buffer (FACS), 0.1% sodium azide, 1% fetal bovine serum, in dPBS blocked with anti-Fc receptor γII/II [CD16/CD32 (clone 2.4G2)] for 10 minutes on ice, and incubated with fluorochrome-conjugated antibodies (E-bioscience or BD Bioscience) 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 minutes. Intracellular staining was performed using FoxP3 staining buffer set as per the manufacturer’s instructions (E-bioscience). Briefly, after surface staining, cells were washed twice in FACS buffer, fixed in 4% paraformaldehyde for 25 minutes, and permeabilized for 30 minutes. Permeabilization was followed by incubation for 30 minutes with the appropriate antibodies diluted in permeabilization diluent. Samples were then acquired on LSR II (BD Bioscience) and analyzed using the FlowJo software (Tree Star, Inc.).

Statistics
Data analysis was performed using GraphPad Prism (GraphPad Software). 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 weeks postinfection, mice consuming 6.00% DFO had the highest degree of inflammation and dysplasia compared with the three control diets (CO, SF, and 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. Because 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 showed a greater number of inflammatory cells as well as epithelial proliferative lesions at 4 weeks postinfection 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 with CON-fed mice (5.71% ± 5.15, n = 5) or SF-fed mice (3.44% ± 1.68, n = 5; *P* < 0.001).

**DFO feeding increased postinfection mortality and body mass loss**

Mice consuming DFO had a higher mortality rate following infection with *H. hepaticus* compared with 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 at 4 weeks, with mortality being observed as early as 1 week postinfection in this group. The cause of mortality in the DFO group was not further investigated as this was an unexpected finding. However, we observed that animals in this group gained less weight from weeks 0 to 4 after infection compared with the CO- or SF-fed counterparts (Fig. 3B). These weight changes were in accordance with survival data through week 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 4 postinfection in mesenteric lymph nodes of DFO-treated animals (*P* < 0.05; Fig. 4A). DFO induced potent reductions in the proportion of CD8+CD3+ cells in both tissues before gavage and throughout infection (Fig. 4A). In addition, DFO-fed animals exhibited increased CD4+CD3+ splenic T lymphocytes before infection and at day 7 (*P* < 0.001; Fig. 4A). However, no differences were noted in the mesenteric lymph nodes. DFO resulted in greatly decreased percentages of CD4+ T cells from the mesenteric lymph nodes expressing CD69 (Fig. 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
FoxP3 + CD25 + CD4+ Treg cells expressed CD62L in the by n-3 PUFAs (36). A reduced percentage of the splenic required for the migration of Treg cells (35) and can be altered examined the expression of L-selectin (CD62L), which is re-

Due to the discrepancy in Treg cells between tissues, we were found in mesenteric lymph nodes (data not shown).

differences in Treg cell percentages between CON and DFO

Figure 3. DFO feeding has a negative impact on survival of 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 with CON (n = 10), CO (n = 18), or SF (n = 38) mice following infection with H. hepaticus (PI, postinfection). B, DFO-fed mice gained less weight compared with SF and CO control animals from 1 to 4 wk postinfection. Only animals that survived to week 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.

response to DFO feeding both before infection (P < 0.05) and at days 3, 7, and 28 postinfection (P < 0.001; Fig. 5A). No differences in Treg cell percentages between CON and DFO were found in mesenteric lymph nodes (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 with CON-fed animals. However, the numbers of Treg cells were significantly reduced in mesenteric lymph nodes of DFO-fed animals at day 28 postinfection (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 mesenteric lymph nodes. An increased number of Treg cells from the CON group did not express CD62L. Cell counts in the mesenteric lymph nodes of CON-fed animals increased significantly at days 7 and 28 postinfection, whereas no changes occurred in the DFO group. Although the percentages of FoxP3+ CD25+ CD4+ T differed only in the spleen and not in mesenteric lymph nodes, the expression of FoxP3 in these cells was altered by DFO feeding. DFO consumption resulted in higher FoxP3 expression at days 0 and 28 postinfection in both spleen and mesenteric lymph nodes (P < 0.01 or P < 0.001), whereas DFO increased FoxP3 expression at day 7 postinfection in spleen alone (P < 0.05; Table 2).

Discussion

This study investigated the effect of dietary DFO on chronic colitis and CACC in SMAD3−/− mice in response to infection with H. hepaticus. The results presented here show 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 (Figs. 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 to Mycobacterium tuberculosis infection through diminished macrophage production of tumor necrosis factor-α, IL-6, and IL-1β (39). A recent study by Schwerbrock and colleagues (40) showed 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 whereas 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 to 12 weeks (42). In contrast, another study reported that FO supplementation at 4% was protective in the IL-10−/− model when colitis development was accelerated with nonsteroidal anti-inflammatory drug treatment (43). These studies are difficult to compare due to differences in genetic background.
and the undefined role of COX-2 inhibition in colitis. Although the majority of studies show 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 FO dose, relative EPA and DHA content, and length of feeding protocol are also other sources of variation. In our study, the most severe colitis and CACC correlated with the 6.00% DFO dose for a feeding period of 8 weeks (Fig. 1). The dietary composition and feeding period are comparable with other FO feeding studies, with the exception that our DFO contained mostly DHA and only little EPA. Interestingly, we observed that low-dose (0.75%) DFO consumption did not ameliorate colitis score compared with 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 with the 6.00% DFO diet (Figs. 1 and 2). Our results indicate that 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 that DHA feeding may further influence Treg cell populations. DFO-fed mice exhibited a high proportion of splenic CD4+ T cells, and a high percentage of this population was 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

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**Figure 4.** DFO alters T lymphocyte populations in uninfected animals (day 0) and at days 3, 7, and 28 postinfection. 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 to 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 postinfection in mesenteric lymph nodes 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 before gavage and throughout infection. DFO-fed animals exhibited increased CD4+CD3+ splenic T lymphocytes before infection and at day 7 (P < 0.001). However, no differences were noted in the mesenteric lymph nodes. Spn, spleen; MsLN, mesenteric lymph nodes. B, DFO resulted in greatly decreased percentages of CD4+ T cells expressing CD69. CD69+ events were gated out of the CD4+ cell population.
found in FoxP3+ CD25+ populations in the mesenteric lymph nodes, FoxP3 mean fluorescence intensity expression was enhanced in both the spleen and mesenteric lymph nodes in DFO-fed mice (Table 2). Indeed, a recent study by Yessoufou and colleagues (46) showed that dietary DHA increases FoxP3 expression in murine Treg cells possibly through inhibition of SMAD7. The authors also noted that upregulation of FoxP3 did not necessarily correlate with the suppressive capacity of Treg cells because Treg cells from DHA-fed animals failed to suppress the proliferation of cocultured CD4+ T effector cells also derived from DHA-fed animals. Hence, DHA may induce a strong Treg phenotype through upregulation of

Figure 5. Frequencies of FoxP3+ CD25+ CD4+ Treg cell CD62L expression in spleens and mesenteric lymph nodes at days 3, 7, and 28 postinfection. 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; ***, P < 0.001). A decreased percentage of these Treg cells were CD62L+ in DFO-fed animals beginning at day 3 postinfection (**, P < 0.01; ***, P < 0.001; right). Representative splenic CD62L expression is shown on Treg cells at days 0 and 28 postinfection (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 postinfection in the DFO group (dark line). B, left, FoxP3+ CD25+ CD4+ Treg cell counts from mesenteric lymph nodes were decreased in the DFO group at day 28 postinfection (P < 0.01). The total number of CD62L− Treg cell was also decreased in DFO-fed animals at day 28 postinfection (P < 0.001; right). Note that the cell number in mesenteric lymph nodes increased significantly at days 7 and 28 postinfection in CON (P < 0.05) but not in DFO.
FoxP3 expression; however, 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 and colleagues 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 Th1 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 postinfection (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 mesenteric lymph nodes and Peyer's patches (37), our data suggest that DFO may decrease the ability of Treg cells to migrate to lymph nodes in close proximity to the site of infection. Incorporation of PUFAs into human cell membranes was recently shown to decrease L-selectin expression (49).

It is noteworthy that the percentage of Treg cells in the spleen remained elevated throughout infection. Although the percentage was similar between CON and DFO in mesenteric lymph nodes, we found that the number of Treg cells was significantly decreased at day 28 postinfection. Decreased Treg cells in the mesenteric lymph nodes 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 mesenteric lymph nodes 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 DNA I-like activity, which results in double-stranded DNA breaks and G2 phase cell cycle arrest before 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 toward a Th2 phenotype both through 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 before 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 Concanavalin A. An increased splenic CD4 to 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 postinfection, our data suggest that 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 with control animals, DFO-fed animals may be less equipped to mount a successful response to 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 before and throughout the course of H. hepaticus infection.

These findings support a growing body of literature implicating deleterious effects of high-dose FO consumption and

Table 2. Mean fluorescence intensity of FoxP3 on Treg cells

<table>
<thead>
<tr>
<th>Day postinfection</th>
<th>Spln</th>
<th>MsLN</th>
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<tr>
<td></td>
<td>CON</td>
<td>DFO</td>
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<tr>
<td>0</td>
<td>582.5 ± 33.20</td>
<td>1,374 ± 235.4*</td>
</tr>
<tr>
<td>3</td>
<td>797.2 ± 117.3</td>
<td>1,090 ± 194.0</td>
</tr>
<tr>
<td>7</td>
<td>951.5 ± 133.9</td>
<td>1,399 ± 253.4†</td>
</tr>
<tr>
<td>28</td>
<td>773.0 ± 122.9</td>
<td>1,282 ± 107.0*</td>
</tr>
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NOTE: Values represent the means ± SD from three to six mice in each group. Sample means were significantly different from CON-fed littermates.

Abbreviations: Spln, spleen; MsLN, mesenteric lymph nodes.

*P < 0.001.
†P < 0.05.
‡P < 0.01.
establishing a tolerable upper limit for FO consumption to protect certain immunocompromised sectors of the population who may be at risk for pathogen-associated enteric inflammation and gastrointestinal cancers.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Grant Support**

Michigan Agriculture Experiment Station.

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Received 04/19/2010; revised 06/14/2010; accepted 07/20/2010; published OnlineFirst 08/26/2010.

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_Cancer Res_ 2010;70:7960-7969. Published OnlineFirst August 26, 2010.

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