Immunomodulatory action of dietary fish oil and targeted deletion of intestinal epithelial cell PPARδ in inflammation-induced colon carcinogenesis

Jennifer M. Monk,1,2* Wooky Kim,1,2* Evelyn Callaway,1,2 Harmony F. Turk,1,2 Jennifer E. Foreman,3 Jeffrey M. Peters,3 Weimin He,4 Brad Weeks,5 Robert C. Alaniz,6 David N. McMurray,2,6 and Robert S. Chapkin1,2

1Program in Integrative Nutrition and Complex Diseases, 2Intercollegiate Faculty of Nutrition, and 3Department of Veterinary Pathobiology, Texas A & M University, 4Institute of Biosciences and Technology, and 5Department of Microbial and Molecular Pathogenesis, Texas A & M University System Health Science Center, College Station, Texas; and 6Department of Veterinary and Biomedical Science and Center for Molecular Toxicology and Carcinogenesis, Pennsylvania State University, University Park, Pennsylvania

Submitted 10 August 2011; accepted in final form 20 September 2011

Monk JM, Kim W, Callaway E, Turk HF, Foreman JE, Peters JM, He W, Weeks B, Alaniz RC, McMurray DN, Chapkin RS. Immunomodulatory action of dietary fish oil and targeted deletion of intestinal epithelial cell PPARδ in inflammation-induced colon carcinogenesis. Am J Physiol Gastrointest Liver Physiol 302: G153–G167, 2012. First published September 22, 2011; doi:10.1152/ajpgi.00315.2011.—The ligand-activated transcription factor peroxisome proliferator-activated receptor (PPAR)-δ is highly expressed in colonic epithelial cells; however, the role of PPARδ ligands, such as fatty acids, in mucosal inflammation and malignant transformation has not been clarified. Recent evidence suggests that the anti-inflammatory/chemoprotective properties of fish oil (FO)-derived n-3 polyunsaturated fatty acids (PUFAs) may be partly mediated by PPARδ. Therefore, we assessed the role of PPARδ in modulating the effects of dietary n-3 PUFAs by targeted deletion of intestinal epithelial cell PPARδ (PPARδIEpC). Subsequently, we documented changes in colon tumorigenesis and the inflammatory microenvironment, i.e., local [mesenteric lymph node (MLN)] and systemic (spleen) T cell activation. Animals were fed chemoprotective [corn oil (CO)] or chemoprotective (FO) diets during the induction of inflammatory colon carcinogenesis. Tumor incidence was similar in control and PPARδIEpC mice. FO reduced mucosal injury, tumor incidence, colonic STAT3 activation, and inflammatory cytokine gene expression, independent of PPARδ genotype. CD8+ T cell recruitment into MLNs was suppressed in PPARδIEpC mice. Similarly, FO reduced CD8+ T cell numbers in the MLN. Dietary FO independently modulated MLN CD4+ T cell activation status by decreasing CD44 expression. CD11a expression by MLN CD4+ T cells was downregulated in PPARδIEpC mice. Lastly, splenic CD62L expression was downregulated in PPARδIEpC CD4+ T cells. These data demonstrate that expression of intestinal epithelial cell PPARδ does not influence aoxymethane/dextran sodium sulfate-induced colon tumor incidence. Moreover, we provide new evidence that dietary n-3 PUFAs attenuate intestinal inflammation in an intestinal epithelial cell PPARδ-independent manner.

peroxisome proliferator-activated receptor-δ; chronic inflammation; malignant transformation; T cell

INFLAMMATORY BOWEL DISEASE (IBD), a chronic and recurring immunoinflammatory condition with unknown etiology, manifests as two overlapping phenotypes, i.e., ulcerative colitis and Crohn’s disease (16, 74). In patients with chronic intestinal inflammation, the risk of developing colorectal cancer increases by ~0.5–1% each year, 7 years after diagnosis (50, 77). Although complicating IBD only accounts for 2% of all colorectal cancer cases within the general population, it is considered a serious sequela of the disease, accounting for one in six of all deaths in IBD patients (68). Therefore, IBD patients represent a significant at-risk population for chronic inflammation-associated colorectal cancer development. Despite the functional link between inflammation and colon cancer, the overlapping regulatory pathways that drive inflammation-associated colonic tumor development remain poorly understood.

Long-chain n-3 polyunsaturated fatty acids (PUFAs) found in fish oil (FO), e.g., eicosapentaenoic acid (20:5n-3), and docosahexaenoic acid (22:6n-3), exhibit beneficial effects in IBD and colon carcinogenesis (8), in part due to their potent anti-inflammatory effects (13, 53). Additionally, the balance between colonic epithelial cell proliferation and apoptosis is favorably modulated by dietary n-3 PUFAs, thereby conferring resistance to carcinogenic agents (14, 18, 19). Moreover, n-3 PUFAs have been shown to modulate the important determinants that link inflammation to cancer development and progression (7, 19, 48, 75, 85). From a mechanistic perspective, the cellular incorporation of dietary n-3 PUFAs favorably affects a broad spectrum of physiological processes, including immune function, wound healing, cell membrane structure/function, eicosanoid signaling, macronutrient metabolism, and nuclear receptor activation (16). With respect to T cell function, dietary n-3 PUFAs have been shown to alter plasma membrane microorganization (lipid rafts) at the immunological synapse, ultimately suppressing signal transduction and nuclear translocation/activation of transcription factors (28, 55, 56, 98). However, the effect of n-3 PUFAs on mucosal immunoregulation has not been determined but is warranted, as ~50% of IBD subjects utilize self-prescribed oral complementary alternative medicines/diets that include FO (58).

Individually, ligands for peroxisome proliferator-activated receptors (PPARs) PPARα, PPARδ (also referred to as PPARβ or PPARb/δ), and PPARγ have been shown to inhibit IBD and colon carcinogenesis (16, 20, 73, 88). Ligand-activated PPAR complexes regulate the expression of PPAR-responsive genes and biological functions, including cell proliferation and differentiation, fatty acid metabolism, energy homeostasis, immune responses, and inflammation (2, 12, 36, 73, 103). Among the PPAR family members, functions of PPARα and PPARγ...
have been well characterized, whereas the physiological functions of PPAR\(\delta\) remain less clear. Although PPAR\(\delta\) mRNA and protein are ubiquitously expressed, among anatomic sites in rodents, expression is highest in colonic epithelium (27, 34), and PPAR\(\delta\) plays an important role in the terminal differentiation of colonic epithelial cells (65, 73). Therefore, it is likely that this nuclear receptor plays an important regulatory role within the gastrointestinal tract.

Ligands for PPAR\(\delta\) are anti-inflammatory, and enhanced inflammation is observed in the absence of PPAR\(\delta\) expression (44). Anti-inflammatory activity of PPAR\(\delta\) may be attributed, at least in part, to its ability to interfere with NF-κB signaling (73). At high ligand concentrations, inhibition of colitis is associated with PPAR\(\delta\) activation (92), and PPAR\(\delta\) null mice exhibit increased sensitivity to dextran sodium sulfate (DSS)-induced colitis, wherein clinical symptoms are exacerbated and expression of inflammatory cytokines is increased (44). Additionally, outcomes of a porcine model of IBD suggest that activation of PPAR\(\delta\) may accelerate colonic regeneration and clinical remission (6). The role of PPAR\(\delta\) activation in colon tumorigenesis remains controversial and is reviewed in detail elsewhere (73). PPAR\(\delta\) null human colon cancer (HCT116) cells have a reduced tumorigenicity in a xenograft model (71). Specifically, in the colon, in the absence of PPAR\(\delta\), colon carcinogenesis is exacerbated in genetic (APC\(^{min}\)) mouse and chemically induced [azoxy methane (AOM)] carcinogenesis models (5, 38, 76), whereas other studies indicate that activation of a functional PPAR\(\delta\) is required to inhibit AOM-induced colon carcinogenesis (65). Interestingly, n-3 PUFAs have been identified as ligands for PPAR\(\delta\) (32, 95); yet, it is not known whether the beneficial effects of n-3 PUFAs on intestinal inflammatory pathologies are mediated through a PPAR\(\delta\)-dependent mechanism.

In the present investigation, PPAR\(\delta\) was selectively deleted from intestinal epithelial cells utilizing a Cre-lox-mediated recombination strategy to disrupt the PPAR\(\delta\) locus. By generating an intestine-specific PPAR\(\delta\) knockout mouse, we were able to assess the contribution of dietary n-3 PUFAs and PPAR\(\delta\) to mucosa-generated immune responses in a chronic intestinal inflammation/carcinogenesis model. Cross talk between lymphocytes and intestinal epithelial cells is an important component of mucosal inflammatory immune responses (17), evidenced by intestinal epithelial cell expression of major histocompatibility complex class II antigens and the costimulatory molecule CD86 in the inflamed colon (69, 83). Furthermore, intestinal epithelial cells can present antigens to lymphocytes in a mixed leukocyte reaction (10, 67), and the processing and presentation of antigens by intestinal epithelial cells may be important for the induction of colonic inflammation (66). Thus it remains possible that intestinal epithelial cells may condition the mucosal environment and, therefore, influence T cell recruitment and activation. What remains unknown is the extent to which specific changes in the intestinal epithelial cell from the inflamed mucosa are able to impact the activation status of lymphocyte populations locally and systemically and whether this is achieved through PPAR\(\delta\)- and/or n-3 PUFA-dependent mechanisms.

In the current study, we determined the impact of chemoprotective dietary FO and intestinal epithelial cell-specific deletion of PPAR\(\delta\) on the colonic inflammatory microenvironment and on local [mesenteric lymph node (MLN)] and systemic (spleen) resident T cell populations. In addition, we assessed T cell activation markers functionally associated with trafficking to inflammatory sites.

**MATERIALS AND METHODS**

*Animals and diets.* All experimental procedures were conducted in accordance with guidelines approved by the US Public Health Service and the Institutional Animal Care and Use Committee at Texas A & M University. C57BL/6 mice with the NH2-terminal portion of the DNA binding domain of PPAR\(\delta\) were targeted by CRE-lox methodology (PPAR\(\delta^{\text{IEpC}}\)) (5) and crossed with Cre DNA recombinase under the control of the villin promoter (villin-Cre mice) (25). Progeny homozygous for the PPAR\(\delta\)-floxed allele, progeny hemizygous for the villin-Cre transgene (PPAR\(\delta^{\text{IEpC-H9254/IEpC}}\), or littermate control [wild-type (PPAR\(\delta^{\text{IEpC/H9254}}\)] mice were generated. Subsequently, PPAR\(\delta^{\text{IEpC-H9254}}\) and PPAR\(\delta^{\text{IEpC/H9254}}\) mice were inbred to produce littermates on the same genetic background. Mice were genotyped prior to recruitment into the study, housed on a 12:12-h light-dark cycle, and fed ad libitum a 5% (wt/wt) corn oil (CO) or 4% FO + 1% CO diet for 2 wk prior to the initiation of the carcinogen and chronic mucosal inflammation (AOM/DSS) regimen. Males and females were equally represented from each genotype (PPAR\(\delta^{\text{IEpC-H9254}}\) and PPAR\(\delta^{\text{IEpC/H9254}}\)) consuming either of the two experimental diets. At the start of the experiment, PPAR\(\delta\) deletion was assessed by PCR analysis of DNA extracted from tails using a Qiagen DNA tissue kit. PCR was performed using the Platinum Taq polymerase kit (GIBCO BRL). The following primers were used: loxP (5’-GGACCGCTCCTCGGCATCCTTTACAG-3’ and 5’-GGCGTGGGGATTTGCTGCTTCA-3’) and Cre recombinase (5’-GCATACCGCTGATGCAACCGAGTG-3’ and 5’-GAACGCATTAGCGCTGTTGGGTACACCG-3’). After completion of the experimental treatment regimen, PPAR\(\delta\) deletion was confirmed in the target tissue (colon) by PCR and immunoblotting.

*Colitis and carcinogen induction.* After a 2-wk diet intervention period, mice were injected with AOM (7.5 mg/kg body wt ip; Sigma-Aldrich). While the mice were maintained on the same diets, chronic inflammation was induced by exposure to three cycles of 1% (wt/wt) DSS (MP Biomedicals) in the drinking water (1 cycle = 4 days of DSS + 17 days of fresh tap water). Animals were euthanized after completion of the final DSS cycle (Fig. 1). At the time of euthanasia, colons were dissected at the junction of the cecum (proximally) and the anus (distally). The colon tissue was flushed with PBS, and the entire colon was processed by the Swiss-roll technique (n = 11–14 mice in each experimental group). Colon lesions were mapped and excised, and mucosal scrapings were subsequently collected from the remaining noninvolved tissue (n = 9–13 mice per experimental group) and snap-frozen for further analysis. Tissues were fixed in 4% paraformaldehyde, embedded in paraffin, stained with hematoxylin-eosin, and evaluated in a blinded manner by a board-certified pathologist (B. Weeks). Colon lesions were typed, and the degree of epithelial injury (score 0–3) on microscopic cross sections of the colon was graded as previously described (53).

*RNA isolation and quantitative real-time PCR.* RNA was isolated using the RNAqueous Total RNA kit (Ambion) and treated with DNase inactivation reagent (Ambion); its integrity was assessed using a bioanalyzer (model 2100, Agilent Technologies), and it was quantified and stored at −80°C. Reverse transcription of 1 μg of sample RNA was performed using Maloney’s murine leukemia virus RT (Invitrogen). Expression of PPAR\(\delta\) in tissue-specific knockout mice was determined using mRNA isolated from colonic mucosa, duodenum, and kidney. Real-time PCR was performed using the AB 7900 PCR system (Applied Biosystems, Foster City, CA) and Taqman probes (Assay-on-Demand, Applied Biosystems) for PPAR\(\delta\) exon boundaries 4–5 (Mm01305435_m1) and PPAR\(\delta\) exon boundaries 7–8 (Mm00803186_g1).

For mucosal cytokine mRNA expression, Taqman gene expression kits (Applied Biosystems) were used for IL-6 (Mm00446190_m1),
**RESULTS**

*Generation of intestine-specific PPARδ knockout mice.* PPARδ was specifically deleted from intestinal epithelial cells utilizing the CRE-lox-mediated recombination strategy to disrupt the PPARδ locus, thereby generating PPARδ null (PPARδ<sup>lox/lox</sup>) and wild-type (PPARδ<sup>+/+</sup>) mice (Fig. 2). Deletion of PPARδ exon 4 resulted in expression of a nonfunctional PPARδ, which was confirmed by mRNA expression in colon and duodenum of PPARδ<sup>lox/lox</sup> compared with PPARδ<sup>+/+</sup> mice, whereas expression in the kidney, another anatomical site known to produce high levels of PPARδ, remained unchanged (34) (Fig. 3). Using primer sets that target PPARδ exon boundaries 4–5, we demonstrated the successful tissue-specific deletion of PPARδ in PPARδ<sup>loxIEpC</sup> mice (Fig. 3A), whereas detection of PPARδ exon boundaries 7–8 demonstrated that a nonfunctional form of PPARδ was still expressed (Fig. 3B). Additionally, we confirmed the successful deletion of PPARδ in the colonic mucosa of Cre-homozygous floxed mice (PPARδ<sup>loxIEpC</sup>) at mRNA and protein levels at the end of the AOM/DSS treatment regimen (Figs. 3, C and D). PPARδ mRNA and protein were readily detectable in the colonic mucosa of wild-type (PPARδ<sup>+/+</sup>) mice, whereas PPARδ expression was undetectable, as expected, in the colonic mucosa of Cre-homozygous floxed (PPARδ<sup>loxIEpC</sup>) mice. Cre recombinase has been shown to be transiently expressed in the target tissue in other model systems, highlighting the need to confirm deletion of the floxed gene of interest in the target tissue (81). Our results indicate that exposure to AOM/DSS did not alter expression of Cre recombinase in the target tissue (colon), validating the intestinal epithelial cell-specific deletion of PPARδ.

The effect of targeted intestinal epithelial PPARδ deletion and dietary FO on colon characteristics within the context of a...
carcinogenic and chronic inflammatory pathology was subse-
quently assessed. Colon injury scores in the middle region of
the colon were ameliorated by dietary FO compared with CO
\((P = 0.03); \text{Fig. 4A})\), whereas there was no difference between
dietary groups in the most proximal \((P = 0.67)\) and distal \((P =
0.80)\) regions of the colon (results not shown). Similarly, there
was no difference in the degree of colon injury in the proximal
\((P = 0.12)\) and distal \((P = 0.26)\) regions of the colon between
PPAR\(\delta\) null (PPAR\(\delta\)\(^{\text{ΔEPC}}\)) and wild-type (PPAR\(\delta\)\(^{\text{Δ/Δ}}\)) mice.
However, an independent effect of genotype was apparent
within the middle region of the colon, where the average colon
injury score was higher \((P = 0.007)\) in PPAR\(\delta\)\(^{\text{ΔEPC}}\) than
PPAR\(\delta\)\(^{\text{Δ/Δ}}\) mice (Fig. 4B). Colon tumor entities (including
adenocarcinomas and adenomas) were mapped to their specific
regions within the colon, excised, and typed by a board-certified
pathologist (B.W.). Tumors did not develop in the proximal
region of the colon, whereas tumors did not develop in the distal
region of the colon in any of the experimental groups. In the
context of chronic intestinal inflammation by decreasing
colon mucosal STAT3 activation. These findings were indepen-
dent of intestinal epithelial cell-specific deletion of PPAR\(\delta\) affected
the gross colonic phenotype by increasing colon injury scores,
as seen previously in PPAR\(\delta\) null mice (44), but had no effect on
mRNA expression of several key inflammatory cytokines.
AOM/DSS increased mucosal STAT3 phosphorylation (\(P = 0.007)\); Fig.
5A) on the induction of this critical inflammatory biomarker,
whereas PPAR\(\delta\) status had no effect \((P = 0.86); \text{Fig. 5A})\). Within the
AOM/DSS-treated groups, we confirmed the anti-inflammatory effect
of FO feeding \((P = 0.002); \text{Fig. 5B})\) in the context of chronic intestinal
inflammation by decreasing colonic mucosal STAT3 activation. These findings were indepen-
dent of intestinal epithelial cell PPAR\(\delta\) expression (\(P = 0.27); \text{Fig. 5B})\).
Blots represent results from 8 PPAR\textsuperscript{\textit{δ}/H9254/H9004} including IL-6 (expression level of several critical inflammatory cytokines, emerged between dietary groups. The colonic mucosal mRNA following completion of AOM/DSS treatment regimen. Values are means ± SE. E: mRNA (n = 4–8 mice per experimental group). Values are means ± SE. D: protein expression level.

Blots represent results from 8 PPAR\textsuperscript{\textit{δ}/H9254/H9004} and 3 PPAR\textsuperscript{\textit{δ}/H9254} mice. Protein expression was normalized to the housekeeping gene lactate dehydrogenase (LDH), and positive control (+) was a cell lysate from COS1 cells transfected with a mouse PPAR\textsuperscript{δ} expression vector. PPAR\textsuperscript{\textit{δ}/H9254} mice have a band at 52 kDa (arrow) that is absent in PPAR\textsuperscript{\textit{δ}/H9254} mice.

A distinct difference in the cytokine expression profile emerged between dietary groups. The colonic mucosal mRNA expression level of several critical inflammatory cytokines, including IL-6 (P = 0.04), IFN-\(\gamma\) (P = 0.03), IL-17A (P = 0.03), IL-17F (P = 0.02), IL-23 (P = 0.01), and IL-23R (P = 0.01), was consistently decreased in FO-fed compared with CO-fed animals (Fig. 6). There was no effect of diet on the mucosal mRNA expression of IL-21 (P = 0.31) or IL-27 (P = 0.15) (results not shown). Among the AOM/DSS-treated animals, there was no effect of genotype (i.e., intestinal epithelial cell PPAR\textsuperscript{δ} status) on colonic mucosal inflammatory cytokine gene expression: IL-6 (P = 0.27), IFN-\(\gamma\) (P = 0.99), IL-17A (P = 0.12), IL-17F (P = 0.14), IL-23 (P = 0.37), and IL-23R (P = 0.76) (Fig. 6) and IL-21 (P = 0.31) and IL-27 (P = 0.22) (results not shown).

Dietary FO and targeted deletion of intestinal epithelial cell PPAR\textsuperscript{δ} alters T cell populations locally and systemically. We measured the effect of dietary FO and/or intestinal epithelial cell-specific deletion of PPAR\textsuperscript{δ} expression on T cell populations in the MLN and spleen. Representative histograms depicting double-stained lymphocytes (CD3\textsuperscript{+}/CD4\textsuperscript{+} or CD3\textsuperscript{+}/CD8\textsuperscript{+}) from the MLN are presented in Fig. 7, A and B, respectively. Similar outcomes were obtained for double-positive lymphocyte populations isolated from the spleen (results not shown). The percentage of CD3\textsuperscript{+}, CD4\textsuperscript{+}/CD3\textsuperscript{+}, and CD8\textsuperscript{+}/CD3\textsuperscript{+} T cells residing in the MLN or spleen is depicted in Fig. 7, C and D, respectively. There was no effect of dietary FO and/or intestinal epithelial cell PPAR\textsuperscript{δ} deletion on the percentage of total T cells (CD3\textsuperscript{+}) or within the CD4\textsuperscript{+} T cell population in the MLN or spleen. However, the percentage of CD8\textsuperscript{+} T cells in the MLN was decreased in FO-fed mice (P = 0.04; Fig. 7E). Interestingly, PPAR\textsuperscript{δ} deletion had a similar localized effect, decreasing the percentage of MLN CD8\textsuperscript{+} T cells relative to wild-type mice (P = 0.05), although these effects were not additive (interaction: P = 0.60; Fig. 7F). The aforementioned localized effects of FO consumption in the MLN were accompanied by a systemic effect, wherein the percentage of splenic CD8\textsuperscript{+} T cells was decreased in FO- compared with CO-fed animals (P = 0.05; Fig. 7G). Overall, a suppressive effect of dietary n-3 PUFAs was observed on the percentage of CD8\textsuperscript{+} T cells residing locally (MLN) and systemically (spleen) in both genotypes.

Dietary FO and targeted deletion of intestinal epithelial cell PPAR\textsuperscript{δ} alters T cell activation status locally and systemically. We determined if changes in the intestinal epithelium influence the activation status of T cell populations at local mucosal (MLN) and systemic (spleen) immunological sites. For this

Fig. 3. Confirmation of an intestine-specific PPAR\textsuperscript{δ} knockout mouse. RNA was extracted from scraped colonic mucosa, duodenum, and kidney. A: deletion of exon 4 of PPAR\textsuperscript{δ} in PPAR\textsuperscript{δ\textit{ΔEpC}/H9254} mice, which was confirmed by detection of PPAR\textsuperscript{δ} mRNA expression using primer sets detecting exon boundaries 4–5 (n = 4–6 mice/genotype at each tissue site). B: intact PPAR\textsuperscript{δ} exons 7 and 8 (primer set detecting exon boundaries 7–8), indicating expression of a partial, nonfunctional protein (n = 4–6 mice/genotype at each tissue site). Values are means ± SE. C and D: confirmation of PPAR\textsuperscript{δ} deletion within colonic mucosa following completion of AOM/DSS treatment regimen. C: mRNA (n = 4–8 mice per experimental group). Values are means ± SE. D: protein expression level.
DELETION AND FISH OIL ALTER T CELL ACTIVATION

Fig. 4. Identification of colonic phenotype in AOM/DSS-treated mice. PPARγ/F and PPARγΔIEpC mice were fed CO (n = 11–12 per genotype) or FO (n = 13–14 per genotype) diet (n = 11–14 mice/treatment group) and euthanized 12 wk after completion of the final DSS cycle. Histological scoring (0–3) of colon epithelial injury and typing of tumor entities (total adenomas and adenoacarcinomas) were carried out in a blinded manner by a board-certified pathologist (B. Weeks). A and B: independent effect of diet and genotype (IEpC PPARγ status) on colon injury in the middle region of the colon. Data were analyzed by Kruskal-Wallis test, and bars represent median values. *Statistical significance (P ≤ 0.05). C and D: effect of diet and genotype on tumor incidence in the middle region of the colon. Dot plots identify tumor distribution among treatment groups; solid black line denotes median value in each group.

purposelongation of T cell activation markers CD11a, CD44, and CD62L was examined at each lymphoid tissue site. Representative histograms for surface markers expressed by the MLN CD4+ and CD8+ T cell populations are presented in Fig. 8; similar results were obtained in the spleen (results not shown). The mean fluorescence intensity of each surface marker assessed in the CD4+ and CD8+ T cell populations in the MLN is shown in Fig. 9A. Intestinal epithelial cell-specific deletion of PPARγ resulted in a decrease in surface expression of CD11a within the CD4+ T cell population of the MLN (Fig. 9B). Dietary FO also caused a decrease in CD44 expression within the CD4+ T cell population compared with cells isolated from animals fed the control (CO) diet (Fig. 9C). Total CD62L surface expression and bimodal expression (high vs. low) was unaffected by dietary FO or intestinal epithelial cell PPARγ deletion. These results are indicative of a reduction in T cell activation. Interestingly, neither FO consumption nor intestinal epithelial cell-specific deletion of PPARγ influenced the activation status of CD8+ T cells residing in the MLN.

Splenic T cell activation status did not mirror the outcomes determined locally in the MLN (Fig. 10). In contrast to the effects observed in the MLN, there was no effect of diet or intestinal epithelial cell PPARγ genotype on the surface expression of CD11a or CD44 on splenic CD4+ T cells. Moreover, there was no effect of diet on CD62L expression in the CD4+ and CD8+ T cell populations (P = 0.84 and P = 0.66, respectively). Interestingly, the effect of intestinal epithelial cell PPARγ genotype on CD62L expression exhibited a bimodal distribution. Splenic T cells from wild-type PPARγ/F mice exhibited a high surface expression of CD62L (Fig. 10), while PPARγ null (PPARγΔIEpC−/−) mice exhibited low expression of CD62L in the CD4+ (P = 0.05) and CD8+ (P = 0.02) T cell populations (Fig. 10, B and C). Therefore, intestinal epithelial cell deletion of PPARγ had a differential effect in local and systemic secondary lymphoid organs. MLN exhibited a surface maker expression pattern consistent with reduced T cell activation, whereas splenic T cells exhibited changes in surface marker expression consistent with recent activation. Additionally, the combined effect of FO consumption and PPARγ deletion in the intestinal epithelial cell decreased the number of CD4+ CD62L+ expressing lymphocytes in the spleen (P = 0.03), indicating that changes in the intestinal epithelium can impact T cell activation status systemically and can be further modified by diet.

DISCUSSION

In the context of a chronic intestinal inflammation/carcinogenesis model, we have examined the impact of dietary FO and targeted deletion of PPARγ within intestinal epithelial cells on the colonic microenvironment and the activation status of resident T cells in local (MLN) and systemic (spleen) secondary lymphoid organs. To our knowledge, this is the first study to utilize an intestinal epithelial cell-specific PPARγ knockout mouse to determine how alterations in the intestinal epithelial cells can impact adaptive immune competence following carcinogen exposure and the induction of chronic intestinal inflammation. This is noteworthy because T cells have been demonstrated to play a pathogenic role in IBD (80, 86). Since n-3 PUFAs are putative natural ligands for PPARγ (32, 95), we also determined if dietary n-3 PUFAs consumed at physiologically relevant levels for humans (57) would further impact T cell activation status. The contribution of the epithelium to intestinal pathologies is garnering greater appreciation as growing evidence implicates epithelial cell dysfunction as a
AOM/DSS treatment (Pn genotype fed CO (immunoblots from PPAR/H9254 by 2-way ANOVA. Values are means activated) to total STAT3 protein expression (pSTAT3/STAT3) was assessed/nDSS (control) in drinking water (DSS (Fig. 4, B and D), which is consistent with a previous report showing that DSS-induced inflammation is exacerbated in PPARδ null mice (44). These findings collectively support a large body of evidence indicating that PPARδ has potent anti-inflammatory activities in multiple models (54). Additionally, while we found no evidence that PPARδ protects against colon tumorigenesis in the present study, exacerbation of AOM/DSS-induced colon injury when expression of PPARδ was deleted from intestinal epithelial cells is consistent with a protective role for PPARδ in colon tumorigenesis. This is in line with a recent retrospective study demonstrating that colorectal cancer patients with relatively low expression of PPARδ in primary tumors were nearly four times more likely to die from this disease than colorectal cancer patients with higher expression of PPARδ in primary tumors (96). To more definitively determine the role of PPARδ in colon tumorigenesis, future studies assessing the effect of PPARδ status within specific cell types in the inflamed colon and/or colon tumor microenvironment are required, as suggested elsewhere (73). While the mechanisms of colon tumorigenesis are complex, data from the present study do not support a role of intestinal epithelial cell PPARδ in this process.

Within the inflamed colonic mucosa, we confirmed that dietary FO decreases the expression of an inflammatory biomarker, phosphorylated (i.e., activated) STAT3 (52), following carcinogen exposure and the induction of chronic inflammation (Fig. 5), whereas intestinal epithelial cell PPARδ status had no effect on colonic STAT3 expression. Within the gastrointestinal tract, STAT3 resides at the nexus of multiple signaling inputs, the downstream targets of which ultimately link inflammation and tumorigenesis by mediating the activity of inflammatory cytokines and cancer-promoting inflammatory responses (51). Interestingly, STAT3 is constitutively activated in diverse types of cancer and plays a procarcinogenic role by promoting prooncogenic inflammatory pathways and enhancing the transcription of genes associated with cell cycle progression, cell survival, angiogenesis, and immune evasion (99–101). Moreover, STAT3 is often overexpressed in colon cancer, and the anti-inflammatory effects of PPARδ are believed to be mediated, at least in part, via inhibition of STAT3 and its downstream signaling, which is associated with anti-apoptotic signaling and c-myc expression (43, 73). However, in the present study, there was no effect of intestinal epithelial cell PPARδ deletion on colon tumor incidence or mucosal STAT3 activation. Conversely, dietary FO suppressed colonic mucosal STAT3 activation and the mRNA expression of IL-6 and IFN-γ, which coincided with a reduced incidence of colon tumor entities and epithelial injury in FO-fed mice, thereby

primary cause of inflammatory pathologies arising in different tissues (87).

Dietary FO reduced colon injury and tumor incidence (adenomas and adenocarcinomas) following exposure to carcinogen and the induction of chronic colonic inflammation (Fig. 4, A and C). This confirms a previous report that n-3 PUFAs reduce colitis-associated colon tumor formation in a genetic model that produces n-3 PUFAs de novo (53) and extends this finding by showing that dietary intervention with n-3 PUFAs can also reduce colitis-associated colon tumor formation. In contrast to the effects of n-3 PUFAs on colon cancer, the role of PPARδ in colon tumorigenesis remains controversial (73). Some studies have shown that PPARδ protects against colon tumorigenesis, some studies have shown that PPARδ promotes colon tumorigenesis, and other studies have shown that PPARδ had no influence on APC-dependent colon tumorigenesis (for review see Ref. 73). In the present study, intestinal epithelial cell PPARδ status had no effect on tumor incidence (Fig. 4 D). This is in contrast to a previous study where AOM-induced colon tumorigenesis was mitigated in mice when PPARδ expression was selectively deleted in intestinal epithelial cells (104). The reason for this difference cannot be explained from our results but could be due in part to the differences in the approach used to induce colon tumorigenesis (AOM alone vs. AOM/DSS). Interestingly, deletion of PPARδ within the colonic intestinal epithelium (PPARδIEpC) increased the degree of colon injury in response to the AOM/DSS treatment regimen (Fig. 4, B and D), which is consistent with a previous report that FO-fed PPARδIEpC mice (lane 1), AOM/DSS-treated FO-fed PPARδIEpC mice (lane 2), and saline-treated (no DSS treatment) CO-fed PPARδIEpC mice (lane 3).

![Graph](http://ajpgi.physiology.org/)

Fig. 5. Ratio of phosphorylated to total STAT3 expression in murine colonic mucosa as assessed by immunoblotting. Within each genotype (PPARδIEpC and PPARδIEpC), mice were fed 5% corn oil (CO) diet and treated with AOM/DSS (n = 4) or received an equal volume of saline intraperitoneally without DSS (control) in drinking water (n = 2). Ratio of phosphorylated (i.e., activated) to total STAT3 protein expression (pSTAT3/STAT3) was assessed by 2-way ANOVA. Values are means ± SE. *P < 0.05. A: effect of AOM/DSS treatment (P = 0.0002). B: effect of AOM/DSS in mice from each genotype fed CO (n = 4) or FO (n = 4) diet (P = 0.01). C: representative immunoblots from PPARδIEpC mice (top) and phosphorylated and total STAT3 (bottom). Samples are from AOM/DSS-treated FO-fed PPARδIEpC mice (lane 1), AOM/DSS-treated CO-fed PPARδIEpC mice (lane 2), and saline-treated (no DSS treatment) CO-fed PPARδIEpC mice (lane 3).
providing insight into a potential mechanism underlying the chemopreventive actions of FO (7, 19, 48, 53, 75, 85).

In T cells, STAT3 expression is essential for colitogenic activity and has a critical role in the differentiation of Th17 cells (22), which are characterized by their potent proinflammatory activities. Moreover, the IL-23/Th17 cell pathway is strongly involved in the pathogenesis of colitis (24, 26, 45, 97), and it is suggested that STAT3 promotes a procarcinogenic Th17 response (59, 94). Interestingly, in a chronic colitis model (3 cycles of DSS), n-3 PUFAs reduced the percentage of Th17 cells (CD4+ IL-17A+IL-17F+) within the inflamed colon lamina propria and reduced mucosal mRNA expression of critical Th17 cell-derived inflammatory cytokines, IL-17 and IL-21 (J. M. Monk et al., unpublished observations). Although the percentage of Th17 cells was not assessed in the present study, the outcome from mucosal mRNA expression supports an inhibitory effect of n-3 PUFAs on Th17 cell function, as dietary FO decreased mucosal mRNA expression of IL-17A and IL-17F (Fig. 6, C and D), cytokines predominantly produced by Th17 cells. The role of Th17 cells and IL-17 in the tumor microenvironment is unclear (64), although overexpression of IL-17 in tumors leads to increased angiogenesis and tumor growth (70), and IL-17−/− and IL-17R−/− mice exhibit reduced tumor growth (40, 91). Furthermore, the tumorigenic effects of IL-17 are mediated, at least in part, by IL-6 via a STAT3-dependent mechanism (91), and all three of these mediators were reduced by FO in the colon following AOM/DSS exposure.

IL-23 drives chronic intestinal inflammation by inducing inflammatory cytokine production and by promoting pathogenic Th1 and Th17 responses in the intestine (63). In addition to promoting tumor incidence and growth (64), IL-23 promotes the maintenance of differentiated Th17 cells and is required for providing Th17 cells with a pathogenic phenotype (79). IL-17 and IL-23 expression is also elevated in human colon cancer, and IL-23p19−/− mice are resistant to tumor induction (60). Interestingly, dietary FO decreased IL-23 and IL-23R colonic mucosal gene expression following AOM/DSS exposure (Fig. 6, E and F). Therefore, IL-23 represents an important molecular link between chronic intestinal inflammation and carcinogenesis, which may be beneficially augmented by dietary FO. Collectively, the aforementioned findings demonstrate a new and previously unappreciated beneficial role of n-3 PUFAs in a chronic inflammation carcinogenesis model, wherein tumor
incidence, colonic injury, and inflammatory/protumorigenic mediators were depressed by dietary FO.

We showed that dietary FO decreased the percentage of CD8<sup>+</sup>/H<sub>11001</sub>T cells residing in the MLN and spleen, an impact that was not apparent within the CD4<sup>+</sup>/H<sub>11001</sub>T cell population at either organ site (Fig. 7). A similar localized effect within the MLN CD8<sup>+</sup>/H<sub>11001</sub>T cell population was observed when PPAR<sub>H9254</sub> was deleted from the intestinal epithelial cell (Fig. 7). These changes were statistically significant, albeit modest; thus further studies are required to determine if CD8<sup>+</sup>/H<sub>11001</sub> effector functions are similarly affected. Previously, consumption of a 4% FO diet elicited a similar modest change in the percentage of lung CD8<sup>+</sup>/H<sub>11001</sub>T cells following influenza infection, whereas the CD4<sup>+</sup>/H<sub>11001</sub>T cell population was unaffected (82). Despite decreasing cell numbers in secondary lymphoid organs, FO had no effect on CD8<sup>+</sup>/H<sub>11001</sub>T cell expression of activation surface markers, indicating that function was unlikely compromised by n-3 PUFAs. In support of this interpretation, n-3 PUFA consumption was shown to have no effect on antigen-driven splenic CD8<sup>+</sup>/H<sub>11001</sub>T cell proliferation (49). Expansion and differentiation of CD8<sup>+</sup>/H<sub>11001</sub>T cells are critical for host defense against viral and intracellular bacterial infections, and during DSS-induced intestinal inflammation, the mucosa is structurally dysregulated, thereby reducing epithelial barrier integrity and increasing the exposure of the underlying mucosa to luminal bacteria and antigens. Future studies are required to assess the impact of n-3 PUFAs on antimicrobial functions of CD8<sup>+</sup>/H<sub>11001</sub>T cells.

Fig. 7. Characterization of mesenteric lymph node (MLN) cells by surface staining followed by flow cytometry. A and B: representative plots of CD3 vs. CD4 and CD3 vs. CD8 quadrants from MLN cells of CO-fed PPAR<sub>δFF</sub> mice. C and D: percentages of CD3<sup>+</sup>, CD4<sup>+</sup>/CD3<sup>+</sup>, and CD8<sup>+</sup>/CD3<sup>+</sup> T cells in MLN and spleen calculated by summing the number of events in each quadrant (n = 5–9 mice per treatment). Within each separate surface marker analysis, resultant P values from a 2-way ANOVA are listed in tables below each graph. Values are means ± SE. *Statistical significance (P ≤ 0.05). E–G: significant main effects from 2-way analysis conducted on MLN and spleen data.
Adhesion molecules facilitate the interaction between T lymphocytes and either antigen-presenting cells (APCs) or the vascular endothelium, and efficient cell-mediated immune responses require appropriate surface expression of these molecules. Furthermore, the trafficking of lymphocytes between body compartments (i.e., into/out of lymphoid organs and into sites of immune or inflammatory reactivity) is also dependent on adhesion molecule expression (78). In this study, activation status of resident T lymphocytes in the MLN and spleen was identified on the basis of the surface expression pattern of three adhesion molecules: CD11a, CD44, and CD62L.

**Fig. 8.** Activation assessment of MLN T lymphocytes by surface staining. CD3⁺CD4⁺ or CD3⁺CD8⁺ double-positive cells were gated, and representative histograms of CD11a, CD44, and CD62L costaining are shown. PE, phycoerythrin.

**Fig. 9.** A: quantitative analysis of T lymphocyte activation markers in MLN calculated by mean fluorescence intensity (MFI). Values are means ± SE. *Statistical significance (P ≤ 0.05). P values (main effects) were determined by 2-way ANOVA. B and C: significant main effects.

<table>
<thead>
<tr>
<th></th>
<th>CD3⁺CD4⁺</th>
<th>CD3⁺CD8⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>0.74</td>
<td>0.03*</td>
</tr>
<tr>
<td>Genotype</td>
<td>0.05*</td>
<td>0.16</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.86</td>
<td>0.49</td>
</tr>
</tbody>
</table>

- **A**: MLN
- **B**: P = 0.05
- **C**: P = 0.03
markers functionally associated with trafficking to inflammatory sites (3, 21, 72): CD11a (LFA-1), CD44, and CD62L (L-selectin). Typically, CD11a and CD44 expression is low, whereas CD62L expression is high on naive T cells compared with antigen-experienced T cells (effector and memory), which express high levels of CD11a and CD44 and reduced levels of CD62L (3, 9, 21, 49). Therefore, the expression pattern of these T cell activation markers was specifically chosen to detect antigen-experienced T lymphocytes as seen previously (9, 21, 23). CD62L initiates lymphocyte homing to lymph nodes (61, 93). Interaction between T cells and APCs is facilitated by CD11a (4, 72, 84), thereby promoting T cell activation (1, 4, 72). CD44 is involved in the recruitment of leukocytes to inflammatory sites (21) and plays a role in signaling to downstream target genes involved in orchestrating inflammatory responses (30); thus its expression is elevated within inflamed tissues (39).

With respect to lymphocyte activation status, FO consumption decreased CD44 expression on MLN CD4^+ T cells (Fig. 9). Additionally, within the same lymphocyte population, intestinal epithelial cell PPARδ deletion resulted in decreased CD11a expression (Fig. 9). Both of these modest, but significant, changes in surface marker expression are consistent with reduced T cell activation in the MLN. Moreover, the effect of FO appears to be localized to the MLN, which is in close proximity to and drains the inflamed colon, as no impact of FO on T cell activation status was apparent within the spleen. This finding extends previous reports of FO reducing CD44 expression in human monocytes (62) and rat lymphocytes (78) to that in the mouse within the context of a chronic inflammation/malignant transformation model. Reducing CD4^+ T cell activation may represent an additional mechanism through which FO is able to minimize the effects of pathogenic CD4^+ T cells in DSS-induced IBD (80, 86). We previously demonstrated that n-3 PUFAs directly suppress CD4^+ Th1 cell development (102) and CD4^+ T cell numbers within the lamina propria following exposure to AOM/DSS (53). CD44 expression is important for the recruitment of leukocytes to inflammatory sites (21), and CD44 signaling is involved in the generation of inflammatory responses (30); therefore, the reduced expression in MLN CD4^+ T cells from FO-fed animals is consistent with the general anti-inflammatory biological actions of n-3 PUFAs (13, 15, 16, 53). Previously, n-3 PUFAs have been shown to impair T cell activation at the immunological synapse (28, 29,
55, 56, 98) by altering the phospholipid and signaling protein composition of lipid rafts, i.e., specialized plasma membrane microdomains important for T cell-receptor signaling pathways (15, 35, 37). CD44 has been shown to mediate the cytoskeletal rearrangements that are required for the initiation of T cell activation (31). Therefore, engagement of CD44 on the T cell surface helps stabilize the immunological synapse by initiating F-actin bundle formation, which is accompanied by a redistribution of CD44 and the associated tyrosine kinases (lck and fyn) into lipid rafts at the immunological synapse (31). Interestingly, n-3 PUFA s have been shown to decrease key signaling proteins and F-actin recruitment into lipid rafts at the immunological synapse (55). Therefore, the aforementioned impairment in key aspects of T cell activation by FO may also include a disruption of CD44 localization into lipid rafts. Future studies are required to evaluate the effect of n-3 PUFA s and modulation of lipid rafts with respect to the contribution of CD44 and actin remodeling in T cells at the immunological synapse.

Within the MLN intestinal epithelial cell, PPARδ deletion resulted in a modest decrease in CD4+ T cell expression of CD11a (Fig. 9), a finding consistent with reduced T cell activation. Conversely, in the spleen, a bimodal CD62L surface expression pattern emerged in the CD4+ and CD8+ lymphocyte populations, wherein PPARδAΔEpc mice exhibited low expression and PPARδ−/− mice exhibited high expression (Fig. 10). Therefore, intestinal epithelial cell PPARδ deletion promoted CD4+ and CD8+ T cell activation in the spleen. Lastly, combined FO consumption and intestinal epithelial cell PPARδ deletion resulted in decreased splenic CD4+ T cell expression of CD62L (Fig. 10), indicating a synergistic effect of dietary bioactive ingredient (n-3 PUFA s) and intestinal epithelial cell PPARδ status.

To our knowledge, we are the first to demonstrate that specific changes in the intestinal epithelial cell during chronic inflammation can impact T cell activation status and that this process can be further modulated by diet. Collectively, these findings demonstrate that, in a chronic intestinal inflammation/carcinogenesis model, alterations in the intestinal epithelial cell (via PPARδ deletion) can differentially impact T lymphocyte activation status in local vs. systemic secondary lymphoid organs, i.e., a modest depressive localized effect in the draining MLN and an enhanced systematic effect in the spleen. The data support the interpretation that intestinal epithelial cells have an active role in intestinal inflammatory processes and have the capacity to impact adaptive immune outcomes beyond the intestine. These findings provide a basis for future research initiatives directed toward identifying the specific contribution of intestinal epithelial cells to inflammatory pathologies.

Because of their centralized position in the intestinal mucosa, intestinal epithelial cells are a critical component of the mucosal immune system. Antigen presentation in the gut is not limited to classical APCs, as intestinal epithelial cells have been demonstrated to present luminal antigen directly to T cells in a polarized fashion with apical antigen uptake and basolateral antigen presentation to mucosal lymphocytes (41, 42) via major histocompatibility complex class II and costimulatory molecules (17, 69, 83, 90), the expression of which is upregulated in response to proinflammatory signals (11). Under normal intestinal conditions, intestinal epithelial cells lack classical costimulatory molecule expression, and interaction with naive CD4+ T cells would likely result in the induction of anergy, a mechanism necessary to support the state of mucosal immune hyporesponsiveness given the load of foreign antigen encountered in the intestine. Under conditions of intestinal inflammation, intestinal epithelial cells have the capacity to provide the second stimulus required to present antigen to naive T cells, thereby potentially contributing to exaggerated T cell activation often attributable to intestinal inflammatory pathologies.

When stress to the epithelium is relatively mild, epithelial cells secrete cytokines and other mediators that directly influence T cell responses and can elicit a range of functional immune outcomes that are essential for host protection and the limitation of immunopathology. When epithelial damage and dysregulation occur (i.e., AOM/DSS model), epithelial cell-derived mediators redirect the nature of T cell-mediated responses toward inflammatory (type 1) responses that are associated with disease (87). Further evidence suggests that the mucosal immune system is a system-wide organ, wherein studies have demonstrated that stimulation in one mucosal compartment can lead to changes in distal areas (33). However, the elements that link the mucosal immune compartments remain undetermined (33). Our findings that genetic and dietary perturbations in the intestinal epithelium can ultimately and differentially impact T cell activation status in local and systemic secondary lymphoid organs demonstrate a previously unidentified role of the intestinal epithelial cell in chronic intestinal inflammation. In addition, it is now evident that the mucosal epithelium can ultimately impact immune cell function in anatomically distant sites. The immunosuppressive effects of dietary FO within the chronically inflamed colon following carcinogen exposure on mucosal inflammatory responses and T cell activation support a growing number of studies indicating that bioactive food components can favorably modulate the clinical course of IBD and colorectal cancer.

ACKNOWLEDGMENTS

PPARδ−/− mice were generously provided by Dr. Ronald Evans (The Salk Institute). Villin-Cre mice were kindly provided by Dr. Sylvie Robine (Institut Curie).

GRANTS

This study was supported by National Institutes of Health Grants DK-071707 and CA-59034, US Department of Agriculture Vegetable Fruit Improvement Center Grant 2009-34402-19831, and Natural Sciences and Engineering Research Council of Canada Postdoctoral Fellowship PDF-388466-2010 (to J. M. Monk).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES

1. Abraham C, Griffith J, Miller J. The dependence for leukocyte function-associated antigen-1/ICAM-1 interactions in T cell activation cannot


93. Wirth TC, Badovinac VP, Zhao L, Dailey MO, Harty JT. Differen-
tiation of central memory CD8 T cells is independent of CD62L-
DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM,
Sears CL. A human colonic commensal promotes colon tumorogenesis
via activation of T helper type 17 T cell responses. Nat Med 15:
95. Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG,
Brown PJ, Sternbach DD, Lehmann JM, Wisely GB, Willson TM,
Kliewer SA, Milburn MV. Molecular recognition of fatty acids by
function and prognostic significance of peroxisome proliferator-activated
B, Kleinschek MA, Owyang A, Mattson J, Blumenschein W, Murphy
E, Sathe M, Cua DJ, Kastelein RA, Rennick D. IL-23 is essential for
T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. J
98. Yog R, Barhoumi R, McMurray DN, Chapkin RS. n-3 polysatu-
rated fatty acids suppress mitochondrial translocation to the immunologic
synapse and modulate calcium signaling in T cells. J Immunol 184:
99. Yu H, Jove R. The STATs of cancer—new molecular targets come of
100. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity:
101. Yu QT, Saruta M, Avanesyan A, Fleschner PR, Banham AH, Papadaki
KA. Expression and functional characterization of FOXP3+ CD4+
regulatory T cells in ulcerative colitis. Inflamm Bowel Dis 13: 191–199,
2007.
RS. Dietary fish oil inhibits antigen-specific murine Th1 cell develop-
103. Zhang X, Young HA. PPAR and immune system—what do we know?
104. Zuo X, Peng Z, Moussalli MJ, Morris JS, Broaddus RR, Fischer SM,
Shureiqi I. Targeted genetic disruption of peroxisome proliferator-
activated receptor-δ and colonic tumorigenesis. J Nutl Cancer Inst 101: