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

Short Title: PPARδ deletion and fish oil alter T cell activation

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Abbreviations:

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CO, corn oil; FO, fish oil; IEpC, intestinal epithelial cell; IBD, intestinal bowel disease; MLN, mesenteric lymph nodes; PPARδ, peroxisome proliferator-activated receptor-delta; PUFA, polyunsaturated fatty acids; DSS, dextran sodium sulfate; AOM, azoxymethane.
ABSTRACT

The ligand activated transcription factor peroxisome proliferator-activated receptor-delta (PPARδ) is highly expressed in colonic epithelial cells, however, the role of PPARδ ligands such as fatty acids in mucosal inflammation and malignant transformation have not been clarified to date. Recent evidence suggests that the anti-inflammatory/chemoprotective properties of fish oil-derived n-3 polyunsaturated fatty acids (PUFA) may be partly mediated by PPARδ. Therefore, we assessed the role of PPARδ in modulating the effects of dietary n-3 PUFA by targeted deletion of intestinal epithelial cell PPARδ (PPARδΔIEpC).

Subsequently we documented changes in colon tumorigenesis and the inflammatory microenvironment, i.e., both local (mesenteric lymph node, MLN) and systemic (spleen) T cell activation. Animals were fed either chemopromotive (corn oil, CO) or chemoprotective (fish oil, FO) diets during the induction of chronic inflammation/carcinogenesis. Tumor incidence was similar in both control and PPARδΔIEpC mice. FO reduced mucosal injury, tumor incidence, colonic STAT3 activation and inflammatory cytokine gene expression, independent of PPARδ genotype. CD8+ T-cell recruitment into MLN was suppressed in PPARδΔIEpC 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 down-regulated in PPARδΔIEpC mice. Lastly, splenic CD62L expression was down-regulated in both PPARδΔIEpC CD4+ and CD8+ T-cells. These data demonstrate that expression of intestinal epithelial cell PPARδ does not influence azoxymethane/dextran sodium sulphate-induced colon tumor incidence. Moreover, we provide new evidence that dietary n-3 PUFA attenuate intestinal inflammation in an intestinal epithelial cell PPARδ-independent manner.
INTRODUCTION

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 approximately 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 (EPA; 20:5\text{\Delta}5,8,11,14,17) and docosahexaenoic acid (DHA; 22:6\text{\Delta}4,7,10,13,16,19), exhibit beneficial effects in both 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 PUFA, thereby conferring resistance to carcinogenic agents (14, 18, 19). Moreover, n-3 PUFA 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 PUFA 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 PUFA have been shown to alter plasma membrane micro-organization (lipid rafts) at the immunological synapse, ultimately suppressing signal transduction and nuclear translocation/activation of transcription factors (28, 55, 56, 98). However, to date, the effect of n-3 PUFAs on mucosal immunoregulation have not been
determined, but are warranted as approximately 50% of IBD subjects utilize self-prescribed oral complementary alternative medicines/diets that include FO (58).

Independently, ligands for peroxisome proliferator-activator receptors (PPARs) PPARα, PPARδ (also referred to as PPARβ or PPARβ/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 PPARδ remain less clear. Although ubiquitously expressed, colonic epithelial PPARδ mRNA and protein expression is amongst the highest compared to other anatomical sites in rodents (27, 34) and PPARδ 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 PPARδ are anti-inflammatory and enhanced inflammation is observed in the absence of PPARδ expression (44). PPARδ anti-inflammatory activity 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 PPARδ activation (92) and PPARδ-null mice exhibit increased sensitivity to dextran sodium sulfate (DSS)-induced colitis wherein clinical symptoms are exacerbated and expression of inflammatory cytokines are increased (44). Additionally, outcomes of a porcine model of IBD suggest that activation of PPARδ may accelerate colonic regeneration and clinical remission (6). The role of PPARδ activation in colon tumorigenesis remains controversial and is reviewed in detail elsewhere (73). PPARδ null human colon cancer cells (HCT116) have a reduced tumorigenicity in a xenograft model (71). Specifically in the colon, in the absence of PPARδ, colon carcinogenesis is exacerbated in both genetic (APCmin mouse) and chemically induced (azoxy methane, AOM) carcinogenesis models (5, 38, 76); whereas other studies indicate
that activation of a functional PPARδ is required to inhibit AOM-induced colon carcinogenesis (65). Interestingly, n-3 PUFA have been identified as ligands for PPARδ (32, 95), yet it is not known whether the beneficial effects of n-3 PUFA on intestinal inflammatory pathologies are mediated through a PPARδ-dependent mechanism.

In the present investigation, PPARδ was selectively deleted from intestinal epithelial cells utilizing a CRE/lox-mediated recombination strategy to disrupt the PPARδ locus. By generating an intestine-specific PPARδ knock-out mouse, the contribution of dietary n-3 PUFA and PPARδ on mucosa-generated immune responses in a chronic intestinal inflammation/carcinogenesis model was assessed. 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 (MHC) class II antigens and the co-stimulatory molecule, CD86 in the inflamed colon (69, 83). Further, 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 to what extent specific changes in the intestinal epithelial cell from the inflamed mucosa are able to impact the activation status of lymphocyte populations both locally and systemically, and whether this is achieved through PPARδ and/or n-3 PUFA-dependent mechanisms.

In the current study, we determined the impact of chemoprotective dietary FO and IEpC-specific deletion of PPARδ on the colonic inflammatory microenvironment and on both local (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 Public Health Service and the Institutional Animal Care and Use Committee at Texas A&M University. C57BL/6 mice with the N-terminal portion of the DNA binding domain of PPARδ were targeted by CRE-lox methodology (PPARδ\textsuperscript{F/F})\(^5\) and crossed with Cre DNA recombinase under the control of the villin promoter (Villin-Cre mice)\(^25\). Progeny homozygous for the PPARδ-floxed allele, hemizygous for Villin-Cre transgene (PPARδ\textsuperscript{ΔIEpC}) or littermate control mice (PPARδ\textsuperscript{F/F}) were generated. Subsequently, PPARδ\textsuperscript{ΔIEpC} and PPARδ\textsuperscript{F/F} were inbred to produce littermates on the same genetic background. Mice were genotyped prior to recruitment into the study, housed on a 12 h light/dark cycle, and fed ad libitum either a 5% (wt/wt) corn oil (CO) or 4% fish oil (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 (PPARδ\textsuperscript{ΔIEpC} and PPARδ\textsuperscript{F/F}) consuming either of the two experimental diets. At the start of the experiment, PPARδ deletion was assessed by PCR analysis of DNA extracted from tails using a Qiagen DNA tissue kit. PCR was performed using Platinum Taq polymerase kit (Gibco BRL). Primers (loxP) 5'GAGCCGCCTCTCGCCATCCTTTCAG-3', 5'-GGCGTGGGGATTTGCCTGCTTCA-3'; Cre recombinase 5'-GCATTACCGGTCGATGCAACGAGTG-3', 5'-GAACGCTAGAGCCTGTTTTGCACGTTC-3'. Following the completion of the experimental treatment regimen, PPARδ deletion was confirmed in the target tissue (colon) by PCR and immunoblotting.

Colitis and carcinogen induction

Following a 2 wk diet intervention period, mice were injected intraperitoneally with 7.5 mg azoxymethane (AOM, Sigma-Aldrich) per kg body weight. While maintained on the same diets, chronic inflammation was induced by exposure to 3 cycles of 1% (w/w) dextran sodium
sulfate (DSS, MP Biomedicals) in the drinking water (1 cycle = 4 d DSS + 17 day fresh tap water). Animals were euthanized after the completion of the final DSS cycle (Figure 1). At the time of euthanasia, colons were dissected proximally at the junction between the cecum and distally at the anus. Following flushing with PBS, colon tissue was processed by Swiss-rolling the entire colon (n=11-14 mice/experimental group). Colon lesions were mapped and excised and mucosal scrapings were subsequently collected from the remaining non-involved tissue (n=9-13 mice/ experimental group) and snap frozen awaiting further analysis. Tissues were fixed in 4% paraformaldehyde, paraffin embedded, stained with H&E stained and evaluated in a blinded manner by a board-certified pathologist (B.W.). Colon lesions were typed and the degree of colon epithelial injury (score 0-3) on microscopic cross sections of the colon were graded as previously described (53).

RNA isolation and quantitative real-time PCR

RNA was isolated using RNAqueous Total RNA kit (Ambion), treated with DNase inactivation reagent (Ambion) and its integrity assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies), quantified and stored at −80°C. Reverse transcription of 1 μg of sample RNA was performed using M-MLV Reverse Transcriptase (Invitrogen). Expression of PPARδ in tissue-specific knockout mice was determined using mRNA isolated from colonic mucosa, duodenum and kidney. Real-time PCR was performed using AB 7900 (Applied Biosystems, Foster City, CA) and Taqman Probes (Assay-on-Demand, Applied Biosystems) for PPARδ exon boundaries 4-5 (Mm01305435_m1) and PPARδ exon boundaries 7-8, (Mm00803186_g1).

For mucosal cytokine mRNA expression, Taqman gene expression kits (Applied Biosystems) were used for IL-6 (Mm00446190_m1), IL-17A (Mm00439618_m1), IL-17F (Mm00521423_m1), IL-21 (Mm00517640_m1), IL-23 (Mm00518984_m1), IL-23R (Mm00519943_m1), IL-27 (Mm004611664_m1) and IFN-γ (Mm01168134_m1).

Amplification of mRNA (fluorescence) was recorded over 40 cycles and the corresponding
cycle numbers (Ct) were used to calculate mRNA expression according to the calculation:

\[ 2^{(40 - Ct)} \]. Target gene expression was normalized to ribosomal 18S expression (Hs03928990_g1).

**Immunoblotting**

Colonic mucosa was scraped from the underlying smooth muscle and, protein extraction and immunoblotting was conducted as described previously (89). Protein detection utilized primary monoclonal rabbit anti-mouse phospho-Stat3 antibody (Cell Signaling Technology Inc., Danvers, MA), or polyclonal rabbit anti-mouse Stat3 (sc-482, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted in PBS containing 1.5% BSA and 0.1% Tween 20. Membranes were washed with PBS containing 0.1% Tween 20 and incubated with secondary peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry, Gaithersburg, MD) as per manufacturer’s instructions. Bands were developed using Pierce SuperSignal West Femto maximum sensitivity substrate and subsequently scanned with a Fluor-S Max Multilmager system (Bio-Rad, Hercules, CA).

PPARδ protein detection was performed as described previously (34) using a primary antibody for PPARδ (#8099, 0.053 μg/mL in TBST) and secondary antibody, biotinylated anti-rabbit IgG (1:10000) in TBST. Immunoreactive proteins were detected with 125I-labeled streptavidin using phosphorimaging analysis after 72 h of exposure. Hybridization signals for the proteins of interest were normalized to the hybridization signal of a housekeeping gene, lactate dehydrogenase (LDH). The positive control used was a cell lysate from COS1 cells transfected with a mouse PPARδ expression vector as previously described (34).

**Flow cytometry analysis of lymphocytes**

Mesenteric lymph nodes (MLN) and spleens were isolated and placed in sterile RPMI 1640 medium with 25 mmol/L HEPES (Irvine Scientific), supplemented with 5% fetal bovine serum (FBS, Irvine Scientific), 2 mM GlutaMAX (Gibco), penicillin 100 U/mL and
streptomycin 0.1 mg/mL (Gibco), henceforth “complete medium”. Tissues were broken into
single cell suspensions by filtering through a 70 µm mesh cell strainer. Lymphocytes were
subsequently enriched by density gradient centrifugation using Lympholyte-M (Cedarlane
Laboratories). Single cells from MLN or spleens were resuspended in flow cytometry
staining buffer (eBioscience) followed by incubation with anti-CD16/32 mAb as per
manufacturer’s instructions (Fc block, eBiosciences). Subsequently, cells were labeled with
anti-CD3-APC (eBioscience), plus anti-CD4-FITC or anti-CD8a-FITC mAb (eBioscience).
Markers for T cell activation status were assessed by additional staining with either anti-
CD11a-PE, anti-CD44-PE, or anti-CD62L-PE mAb (eBioscience) and analyzed using a C6
flow cytometer (Accuricytometer). Cell characterization and expression status as
determined by mean fluorescence intensity (MFI) of marker proteins (i.e. CD11a, CD44, and
CD62L) was assessed by CFlow Plus software (Accuricytometer). Cell viability was
determined by propidium iodide staining and averaged 99%.

Statistics
The predetermined upper limit of probability for statistical significance throughout this
investigation was $P \leq 0.05$, and analyses were performed using the SAS System for
Windows (version 9.2). Data from all analyses were subjected to a two-way analysis of
variance (ANOVA) and the main effects were diet and genotype unless stated otherwise. If
justified by the resulting probability value (i.e., $P \leq 0.05$), analyses between means were
performed using the Tukey's Studentized Range test. Moreover, if a statistically significant
interaction term emerged from a two-way ANOVA, the permissible preplanned comparisons
(i.e., equal in number to the treatment degrees of freedom) were made using the least-
squares means procedure. Data sets not exhibiting a normal distribution were subjected to
the Kruskal-Wallis test ($\chi^2$ approximation) followed, if justified, by the statistical probability
outcome ($P < 0.05$), by Wilcoxon two-sample testing.
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\textsuperscript{IEC\textsuperscript{Δ}}) and wild type (PPARδ\textsuperscript{F/F}) mice (Figure 2). Deletion of PPARδ exon 4 resulted in the expression of a non-functional PPARδ, which was confirmed by mRNA expression in PPARδ\textsuperscript{IEC} colon and duodenum compared to wild type (PPARδ\textsuperscript{F/F}), whereas kidney expression, another anatomical site known to produce high levels of PPARδ, remained intact (34) (Figure 3). Using primer sets that target the PPARδ exon boundaries 4-5, demonstrated the successful tissue specific deletion of PPARδ in PPARδ\textsuperscript{IEC} mice (Figure 3A), whereas detection of PPARδ exon boundaries 7-8 demonstrated that a non-functional form of PPARδ was still expressed (Figure 3B). Additionally, we confirmed the successful deletion of PPARδ in the colonic mucosa of Cre-homozygous floxed mice (PPARδ\textsuperscript{IEC}) at both mRNA and protein levels at the end of the AOM/DSS treatment regimen (Figures 3C&D). Both PPARδ mRNA and protein were readily detectable in the colonic mucosa of wild type PPARδ\textsuperscript{F/F}, whereas PPARδ expression was undetectable, as expected, in the colonic mucosa of Cre-homozygous floxed mice (PPARδ\textsuperscript{IEC}). 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 indicated that exposure to AOM/DSS did not alter the expression of the 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 subsequently assessed. Dietary FO ameliorated colon injury scores in the middle region of the colon compared to CO-fed animals (P=0.03) (Figure 4A), whereas there was no
difference between dietary groups in the most proximal ($P=0.67$) and distal regions of the colon ($P=0.80$) (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 in PPARδ null (PPARδ<sup>IEC</sup>) as compared to wild type (PPARδ<sup>F/F</sup>) mice. However, an independent effect of genotype within the middle region of the colon was apparent, where the average colon injury score was higher ($P=0.007$) in PPARδ<sup>IEC</sup> as compared to PPARδ<sup>F/F</sup> mice (Figure 4B).

Colon tumor entities (including both adenocarcinomas and adenomas) were mapped to their specific region within the colon, excised and typed by a board certified pathologist (B.W.). Tumors did not develop in the proximal region of the colon in any of the experimental groups. In the most distal region of the colon, defined as the most distal 2 cm of the colon (46), there was no effect of genotype or diet on the total number of tumors ($P=0.81$ and $P=0.78$, respectively). However, in the middle region of the colon, an independent effect of diet emerged wherein FO reduced the total number of tumor entities compared to CO-fed animals ($P=0.01$), whereas PPARδ deletion had no effect on total tumor number in the middle colon ($P=0.38$) (Figure 4C&D). Moreover the percentage of animals that failed to develop colon tumors was higher (48%) in FO-fed compared to (18%) CO-fed mice.

Therefore, in a chronic inflammation and malignant transformation model (AOM/DSS regimen) intestinal epithelial cell specific deletion of PPARδ affected the gross colonic phenotype by increasing colon injury scores, as seen previously in PPARδ null mice (44), but had no effect on tumor incidence. Interestingly, independent of PPARδ genotype, dietary FO had a beneficial effect on the gross colonic phenotype by reducing both colon injury and tumor incidence. However, these effects were site specific and restricted to the middle region of the colon.

Dietary FO Decreases Mucosal Inflammatory Biomarker Expression

We verified that the AOM/DSS treatment established a subclinical chronic inflammatory microenvironment in the colon by examining STAT3 phosphorylation status, a well-accepted
marker of mucosal inflammation (47). Examination of phosphorylated (i.e., activated) STAT3 relative to total STAT3 expression within the colonic mucosa revealed a significant effect of the inflammation/carcinogen treatment regimen as compared to saline treated controls ($P=0.001$, Figure 5A) on the induction of this critical inflammatory biomarker, whereas PPARδ status had no effect ($P=0.86$, Figure 5A). Within the AOM/DSS treated groups, we confirmed the anti-inflammatory effect of FO feeding ($P=0.002$, Figure 5B) in the context of chronic intestinal inflammation on colonic mucosal STAT3 expression and activation. These findings were independent of intestinal epithelial cell PPARδ expression (genotype $P=0.27$, Figure 5B).

**Dietary FO decreases colonic mucosal mRNA expression of critical inflammatory cytokines**

Administration of the AOM/DSS increased mucosal mRNA expression of several key inflammatory cytokines above the baseline expression levels in saline treated control animals in all groups. As expected, the average mRNA expression level of inflammatory cytokines in AOM/DSS treated mice was increased compared to saline controls for: IL-6 ($P=0.01$, 2.5-fold increase), IFN-γ ($P=0.04$, 3.3-fold increase), IL-17A ($P=0.006$, 28-fold increase), IL-17F ($P=0.0002$, 10-fold increase), IL-21 ($P=0.0003$, 9-fold increase), IL-23 ($P=0.01$, 5.6-fold increase). A distinct difference in the cytokine expression profile emerged between dietary groups. FO feeding consistently decreased the colonic mucosal mRNA expression level of several critical inflammatory cytokines as compared to CO-fed animals, including IL-6 ($P=0.04$), IFN-γ ($P=0.03$), IL-17A ($P=0.03$), IL-17F ($P=0.02$), IL-23 ($P=0.01$) and IL-23R ($P=0.01$) (Figure 6 A-F). 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 PPARδ status) on colonic mucosal inflammatory cytokine gene expression: IL-6 ($P=0.27$), IFN-γ ($P=0.99$), IL-17A ($P=0.12$), IL-
Dietary FO and targeted deletion of intestinal epithelial cells PPARδ alters T cell populations locally and systemically

We measured the effect of dietary FO and/or the intestinal epithelial cell-specific deletion of PPARδ expression on T cell populations in the MLN and spleen. Representative histograms depicting double stained lymphocytes (CD3+CD4+ or CD3+CD8+) from the MLN are presented in Figure 7 A&B, respectively. Similar outcomes were obtained for double positive lymphocyte populations isolated from the spleen (results not shown). The percentage of CD3+, CD4+/CD3+ and CD8+/CD3+ T cells residing in either the MLN or spleen are depicted in Figure 7 C&D, respectively. There was no effect of dietary FO and/or intestinal epithelial cell PPARδ deletion on the percentage of total T cells (CD3+) or within the CD4+ T cell population in either tissue site. However the percentage of CD8+ T cells in the MLN was decreased in FO-fed mice (P=0.04) (Figure 7E). Interestingly, PPARδ-deletion had a similar localized effect, decreasing the percentage of MLN CD8+ T cells relative to wild-type mice (P=0.05), although these effects were not additive (interaction, P=0.60) (Figure 7F). The aforementioned localized effects of FO consumption in the MLN were accompanied by a systemic effect, wherein the percentage of splenic CD8+ T cells was decreased in FO-fed animals relative to those consuming the CO diet (P=0.05) (Figure 7G).

Overall, a suppressive effect of dietary n-3 PUFA was observed on the percentage of CD8+ T cells residing locally (MLN) and systemically (spleen) in both genotypes.

Dietary FO and targeted deletion of intestinal epithelial cell PPARδ alters T cell activation status both locally and systemically

We determined if changes in the intestinal epithelium influences the activation status of T cell populations located in local mucosal (MLN) and systemic (spleen) immunological sites.
For this purpose, surface expression of the T cell activation markers CD11a, CD44 and CD62L were examined in each lymphoid tissue site. Representative histograms for surface markers expressed by the MLN CD4$^+$ and CD8$^+$ T cell populations are presented (Figure 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 are shown in Figure 9A. Intestinal epithelial cell-specific deletion of PPARδ resulted in a decrease in surface expression of CD11a within the CD4$^+$ T cell population of the MLN (Figure 9B). Dietary FO also caused a decrease in CD44 expression within the CD4$^+$ T cell population compared to cells isolated from animals fed the control (CO) diet (Figure 9C). Total CD62L surface expression and bimodal expression (hi versus lo) was unaffected by either dietary FO or intestinal epithelial cell PPARδ deletion. These results are indicative of a reduction in T cell activation. Interestingly, neither FO consumption nor intestinal epithelial cell-specific deletion of PPARδ 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, and are shown in Figure 10. In contrast to the effects observed in the MLN, there was no effect of diet or intestinal epithelial cell PPARδ genotype on the surface expression of CD11a or CD44 on splenic CD4$^+$ T. Moreover, there was no effect of diet on CD62L expression in either the CD4$^+$ and CD8$^+$ T cell populations ($P=0.84$ and $P=0.66$, respectively). Interestingly, the effect of intestinal epithelial cell PPARδ genotype on CD62L expression exhibited a bimodal distribution. Splenic T cells from wild type PPARδ$^{+/+}$ mice exhibited a high surface expression of CD62L (Figure 10) while PPARδ null (PPARδ$^{-/-}$IEC) mice exhibited low expression of CD62L in both the CD4$^+$ ($P=0.05$) and CD8$^+$ ($P=0.02$) T cell populations (Figure 10B&C). Therefore, the effect of intestinal epithelial cell deletion of PPARδ had a differential effect in local and systemic secondary lymphoid organs. MLN exhibited a surface marker expression pattern consistent with reduced T cell activation, whereas splenic T cells exhibited changes in surface marker expression consistent with recent activation.

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Additionally, the combined effect of FO consumption and PPARδ 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 PPARδ 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 PPARδ knock out 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 PUFA are putative natural ligands for PPARδ (32, 95), we also determined if dietary n-3 PUFA consumed at physiologically relevant levels for humans (57) would further impact T cell activation status. The contribution of the epithelium to intestinal pathologies is currently garnering greater appreciation as growing evidence implicates epithelial cell dysfunction as a primary cause of inflammatory pathologies arising in different tissue sites (87).

Dietary FO reduced both colon injury and tumor incidence (adenomas and adenocarcinomas) following exposure to carcinogen and the induction of chronic colonic inflammation (Figure 4 A&C). This confirms a previous report that n-3 PUFA reduce colitis-associated colon tumor formation in a genetic model that produces n-3 PUFA de novo (53), and extends this finding by showing that dietary intervention with n-3 PUFA can also reduce colitis-associated colon tumor formation. In contrast to the effects of n-3 PUFA on colon cancer, the role of PPARδ in colon tumorigenesis remains controversial (73). Some studies
haves 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 (reviewed in (73)). In the present study,
intestinal epithelial cell PPARδ status had no effect on tumor incidence (Figure 4D). 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 approach used to induce colon tumorigenesis (AOM alone versus
AOM/DSS). Interestingly, deletion of PPARδ within the colonic intestinal epithelium
(PPARδ<sup>ΔIEpC</sup>) increased the degree of colon injury in response to the AOM/DSS treatment
regimen (Figure 4 B&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 no evidence that PPARδ protects
against colon tumorigenesis was observed in the present study, the finding that AOM/DSS-
induced colon injury was exacerbated 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 ~4X as likely to die from this
disease as compared to colorectal cancer patients with relatively higher expression of
PPARδ in primary tumors (96). To more definitely determine the role of PPARδ in colon
tumorigenesis, future studies assessing the effect of PPARδ status within specific cell types
present 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 (Figure 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, whose downstream targets ultimately link inflammation and tumorigenesis via 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 pro-oncogenic inflammatory pathways and enhancing the transcription of genes associated with cell-cycle progress, 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 signalling which is associated with anti-apoptotic signalling and c-myc expression (43, 73). However, in the present study there was no effect of intestinal epithelial cell PPARδ deletion on either colon tumor incidence or mucosal STAT3 activation. Conversely, dietary FO suppressed colonic mucosal STAT3 activation and the mRNA expression of IL-6 and IFNγ, which coincided with a reduced incidence of colon tumor entities and epithelial injury in FO-fed mice, thereby 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 pro-inflammatory 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 pro-carcinogenic Th17 response (59, 94). Interestingly, in a chronic colitis model (3 cycles DSS exposure), n-3 PUFA reduced the percentage of Th17 cells (CD4+ IL-17A+) within the inflamed colon lamina propria and reduced mucosal mRNA expression of critical Th17 cell-derived inflammatory cytokines, IL-17 and IL-21 (Monk J.M. et al., submitted for publication).
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 PUFA on Th17 cell function, as dietary FO decreased mucosal mRNA expression of IL-17A and IL-17F (Figure 6 C&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 in a STAT3-dependent mechanism (91), and all three of these mediators were reduced by FO following 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 are also elevated in human colon cancer and IL-23p19−/− mice are resistant to tumor induction (60). Interestingly, dietary FO decreased both IL-23 and IL-23R colonic mucosal gene expression following AOM/DSS exposure (Figure 6E&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 PUFA in a chronic inflammation carcinogenesis model, wherein tumor incidence, colonic injury and inflammatory/pro-tumorigenic mediators were all depressed by dietary FO.

We showed that dietary FO decreased the percentage of CD8+ T cells residing in both the MLN and the spleen; an impact that was not apparent within the CD4+ T cell population in either organ site (Figure 7). A similar localized effect within the MLN CD8+ T cell population was observed when PPARδ was deleted from the intestinal epithelial cell (Figure 7). These changes were statistically significant, albeit modest, thus further studies are
required to determine if CD8+ effector functions are similarly affected. Previously, consumption of a 4% FO diet elicited a similar modest change in the percentage of lung CD8+ T following influenza infection whereas the CD4+ T cell population was unaffected (82). Despite decreasing cell numbers in secondary lymphoid organs, FO had no effect on CD8+ T cell expression of activation surface markers, indicating that function was unlikely compromised by n-3 PUFA. In support of this interpretation, n-3 PUFA consumption was shown to have no effect on antigen-driven splenic CD8+ T cell proliferation (49). Expansion and differentiation of CD8+ T cells is 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 PUFA on anti-microbial functions of CD8+ T cells.

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., in/out of lymphoid organs and into sites of immune or inflammatory reactivity) is also dependent upon adhesion molecule expression (78). In this study, activation status of resident T lymphocytes in the MLN and spleen were identified based on the surface expression pattern of three markers functionally associated with trafficking to inflammatory sites (3, 21, 72), namely, CD11a (LFA-1), CD44 and CD62L (L-selectin). Typically CD11a and CD44 expression is low, whereas CD62L expression is high on naïve T cells compared to 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 were 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 (Figure 9). Additionally, within the same lymphocyte population, intestinal epithelial cell PPARδ deletion resulted in decreased CD11a expression (Figure 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 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 inflammatory bowel disease (80, 86). We have previously demonstrated that n-3 PUFA 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 PUFA (13, 15, 16, 53). Previously, n-3 PUFA 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 (Ick and fyn) into lipid rafts at the immunological synapse (31). Interestingly, n-3 PUFA 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 in CD44 localization into lipid rafts. Future studies are required to evaluate the effect of n-3 PUFA and modulation of lipid rafts with respect to the contribution of CD44 and actin remodelling 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 (Figure 9), a finding consistent with reduced T cell activation. Conversely, in the spleen, a bimodal CD62L surface expression pattern emerged in both the CD4+ and CD8+ lymphocyte populations, wherein PPARδΔIEpC mice exhibited low expression and PPARδF/F mice exhibited high expression (Figure 10). Therefore, intestinal epithelial cell PPARδ deletion promoted both 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 (Figure 10), indicating a synergistic effect of both dietary bioactive ingredient (n-3 PUFA) 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 versus 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.
Due to 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 MCH class II and co-stimulatory molecules ((17, 69, 83, 90), and whose expression are up-regulated in response to pro-inflammatory signals (11). Under normal intestinal conditions, intestinal epithelial cells lack classical co-stimulatory molecule expression and interaction with naive CD4+ T cells would likely result in the induction of anergy, a necessary mechanism 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.

In situations 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 occurs (i.e., AOM/DSS model), epithelial cell-derived mediators re-direct 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, what links the mucosal immune compartments together remains 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 demonstrates 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 supports a growing number of studies indicating that bioactive food components can favorably modulate the clinical course of IBD and colorectal cancer.
ACKNOWLEDGEMENTS

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DISCLOSURES

The authors have no conflicts of interest to disclose.


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**FIGURE LEGENDS**

**Figure 1.** Experimental dosing regimen. Wild type (PPAR<sup>F/F</sup>) mice and intestinal epithelial cell (IEpC) PPARδ null (PPARδ<sup>IEpC</sup>) mice consuming either a 5% corn oil diet (CO), 1% CO plus 4% fish oil (FO) diet were acclimated to their experimental diets for 14 d prior to intraperitoneal injection of azoxymethane (AOM, 7.5 mg/kg body weight). Subsequently,
mice were exposed to three cycles of dextran sodium sulphate (DSS, 1% w/w via the
drinking water). Mice were terminated 12 wk after completion of the final DSS cycle.

Figure 2. PPARδ deletion and genotyping strategy. A) DNA gel showing mouse
genotypes which were assessed using two primer sets, even numbered lanes (2-20)
detected LoxP sites at 400 base pairs (bp), marked with an arrow, and odd numbered lanes
(3-21) detected Cre recombinase at 380 bp. B) Primer sets to detect the specific deletion of
PPARδ exon 4 by the Cre recombinase, resulting in a non-functional form of PPARδ (primer
set 1), whereas primer set 2 detecting PPARδ exon 7-8 indicates that the remainder of
PPARδ is still intact.

Figure 3. Confirmation of an intestine-specific PPARδ knockout mouse. RNA was
extracted from scaped colonic mucosa, duodenum and kidney. In panels A-C, bars
represent mean values ± SEM. Exon 4 of PPARδ was deleted in the PPARδΔIEpC mice,
which was confirmed by detecting PPARδ mRNA expression using primer sets detecting
exon boundaries 4-5 (panel A, n=4-6 mice/genotype at each tissue site). Panel B shows
the detection of PPARδ exons 7 and 8 (primer set detecting the exon boundaries 7-8) are
intact (n=4-6 mice/genotype at each tissue site) indicating that a partial, non-functional
protein, was expressed. Confirmation of PPARδ deletion within the colonic mucosa
following the completion of the AOM/DSS treatment regimen panel C) mRNA (n=4-8
mice/experimental group) and panel D) protein expression level (PPARδΔIEpC , n=8 and
PPARF/F n=3 mice). Protein expression was normalized to the housekeeping gene, lactate
dehydrogenase (LDH), and the positive control (+) was a cell lysate from COS1 cells
transfected with a mouse PPARδ expression vector. PPARF/F mice have a band at 52 kDa
(marked with the arrow) that is absent in PPARδΔIEpC mice.

Figure 4. Identification of the colonic phenotype in AOM/DSS treated mice. Wild type
(PPAR$^{\text{F/F}}$) mice and intestinal epithelial cell (IEpC) PPAR$\delta$ null (PPAR$\delta^{\Delta\text{IEpC}}$) mice fed either a 5% corn oil diet (CO, n=11-12/genotype), 1% CO plus 4% fish oil (FO, n=13-14/genotype) diet (n=11-14 mice/treatment group) and terminated 12 wk after completion of the final DSS cycle. Histological scoring of colon epithelial injury (ranked scale 0-3) and the typing of tumor entities (total adenomas and adenocarcinomas) was evaluated in a blinded manner by a board-certified pathologist (B.W). The independent effect of diet (panel A) and genotype (IEpC PPAR$\delta$ status, panel B) on colon injury in the middle region of the colon are shown. Data were analyzed by a Kruskal-Wallis test and bars represent median values. An asterisk (*) denotes statistical significance ($P \leq 0.05$) and for each analysis the resultant $P$-values are shown. The effect of diet and genotype on tumor incidence in the middle region of the colon are shown in panel C and D, respectively. Dot plots identify the distribution of tumor incidence among treatment groups and the solid black line denotes the median value in each group.

**Figure 5.** Ratio of phosphorylated to total STAT3 expression in murine colonic mucosa as assessed by immunoblotting. Within each genotype (PPAR$\delta^{\text{F/F}}$ and PPAR$\delta^{\Delta\text{IEC}}$), mice were fed a CO diet and were treated with AOM/DSS (n=4) or received an equal volume of saline intraperitoneally without DSS (control) in the drinking water (n=2). The ratio of STAT3 protein expression (phosphorylated or activated/total, i.e., pSTAT3/STAT3) was assessed by two-way ANOVA. In all panels, bars represent means, SEMs are shown and statistical significance ($P \leq 0.05$) is denoted with an asterisk (*). Panel A, the effect of AOM/DSS treatment ($P=0.001$) is shown. Panel B, mice from each genotype consumed either the CO (n=4) or the FO (n=4) diet and were treated with AOM/DSS and the effect of diet ($P=0.002$) is shown. Panel C, representative immunoblots from PPAR$\delta^{\text{F/F}}$ mice, (upper panel) phosphorylated-STAT3 and total STAT3 (lower panel). Samples are from AOM/DSS treated FO- PPAR$\delta^{\text{F/F}}$ AOM/DSS (lane 1), AOM/DSS treated CO- PPAR$\delta^{\text{F/F}}$ (lane 2) and saline no DSS treated CO- PPAR$\delta^{\text{F/F}}$ mice (lane 3).
**Figure 6.** Colonic mucosal cytokine mRNA expression. RNA was isolated from colon mucosal scrapings from mice following exposure to carcinogen and 3 subsequent cycles of DSS. mRNA levels were quantified by qRT-PCR and the expression of each gene of interest was normalized to ribosomal 18S expression. Relative expression levels were analyzed for A) IL-6, B) IFN-γ, C) IL-17A, D) IL-17F, E) IL-23 and F) IL-23R. Data were analyzed by two-way ANOVA (main effects: diet and genotype) and significance was at the level of $P < 0.05$. For all data sets $P$-interaction was not significant ($P > 0.05$), however, the outcome from each main effect is shown. Bars represent mean values ± SEM.

**Figure 7.** Characterization of mesenteric lymph node cells by surface staining followed by flow cytometry. Representative plots of either CD3 versus CD4 (panel A) or CD3 versus CD8 (panel B) quadrants from MLN cells of CO fed PPARδF/F mice are shown. Percentages of either CD3⁺, CD4⁺/CD3⁺, or CD8⁺/CD3⁺ T-cells in the MLN (panel C) or spleen (panel D), were calculated by summing the number of events in each quadrant ($n=5-9$ mice per treatment). Within each separate surface marker analysis the resultant $P$-values from a two-way ANOVA are listed in the tables below each graph. Bars represent mean values ± SEM. In all graphs, bars marked with an asterisk (*) denote statistical significance ($P \leq 0.05$). Significant main effects from the two-way analysis conducted on MLN (panels E and F) and spleen (panel G) data.

**Figure 8.** Activation assessment of MLN T-lymphocytes by surface staining. CD3⁺CD4⁺ or CD3⁺CD8⁺ double-positive cells were gated and representative histograms of either CD11a, CD44, or CD62L co-staining are shown.
Figure 9. Quantitative analysis of T lymphocyte activation markers in MLN calculated by mean fluorescence intensity (MFI, panel A). In all graphs, bars represent means and the SEMs are shown. Data were subjected to two-way ANOVA and the corresponding resultant P-values are summarized below the graph (panel A). Significance was determined as $P \leq 0.05$ as denoted with an asterisk (*). Significant main effects are presented in panels B and C.

Figure 10. Quantitative analysis of T lymphocyte activation markers in the spleen calculated by mean fluorescence intensity (MFI, panel A). In all graphs, bars represent means and SEMs are shown. The resultant P-values from a two-way ANOVA are summarized below the graph (panel A). Significance was determined as $P \leq 0.05$ as denoted with an asterisk (*) and significant main effects are presented in panel B.
Figure 1

- Start Diets
- AOM
- Terminate

Wk -2  |  Wk 0  |  Wk 1  |  Wk 4  |  Wk 7  |  Wk 9  |  Wk 13
--- | --- | --- | --- | --- | --- | ---
14 d  | 7 d  | 4 d 1% DSS + 17 d water | 4 d 1% DSS + 17 d water | 4 d 1% DSS + 10 d water | 28 d  |
Figure 2


PPARδ

floxflox    floxflox    floxflox    flox/cre    flox/cre    flox/flox    flox/cre    flox/flox    flox/cre    flox/cre

B) 5' Exon 2  Exon 3 Exon 4 Exon 5 Exon 6 Exon 7 Exon 8 Eco RV

Encodes DNA-binding domain

Primer set 1 will indicate if cre cut out Exon 4 of PPARδ, creating non-functional protein

Primer set 2 will indicate if the rest of PPARδ is still present

Cre recombinase

PPARδ Exon 4

loxp  loxp

loxp  loxp

loxp  loxp

PPARδ Exon 4
Figure 4

(A) *P*=0.025

Colon Injury Score

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(B) *P*=0.007

Colon Injury Score

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(C) *P*=0.001

Tumor Entities/mouse

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(D) *P*=0.38

Tumor Entities/mouse

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Figure 5

A

Treatment: $P=0.0002$
Genotype: $P=0.86$
Interaction: $P=0.98$

Saline

F/F  ΔIEpC

AOM/DSS

F/F  ΔIEpC

B

Diet: $P=0.01$
Genotype: $P=0.27$
Interaction: $P=0.38$

CO  FO

F/F  ΔIEpC

C

p-STAT3 (79 kDa)

STAT3

1  2  3
Figure 6

A  IL-6

B  IFN-γ

C  IL-17A

D  IL-17F

E  IL-23

F  IL-23R

mRNA Expression Relative to Ribosomal 18 S
Figure 7

A. MLN

C. MLN

B. CD8-FITC

D. Spleen

E. Diet: $P=0.04$

F. Genotype: $P=0.05$

G. Diet: $P=0.003$

Main effects ($P$-values)

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Main effects ($P$-values)

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Figure 8

Counts of CD3+CD4+ and CD3+CD8+ cells for different markers (CD11a-PE, CD44-PE, CD62L-PE) for different genetic backgrounds (flox-CO, flox-FO, flox,Cre-CO, flox,Cre-FO, PPARδF/F-CO, PPARδF/F-FO, PPARδΔIEpC-CO, PPARδΔIEpC-FO).
**Figure 9**

**A** MLN

![Bar chart showing mean fluorescence intensity (MFI) for CD11a, CD44, and CD62L for different genotypes and conditions.](image)

**Main effects (P-values)**

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<td><strong>Interaction</strong></td>
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<td></td>
</tr>
<tr>
<td>CD11a</td>
<td>0.86</td>
<td>0.83</td>
</tr>
<tr>
<td>CD44</td>
<td>0.49</td>
<td>0.39</td>
</tr>
<tr>
<td>CD62L</td>
<td>0.99</td>
<td>0.22</td>
</tr>
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</table>

**B** $P=0.05$

![Bar chart showing mean fluorescence intensity (MFI) for CD4+CD11a+ and CD4+CD44+ for different genotypes and conditions.](image)

**C** $P=0.03$

![Bar chart showing mean fluorescence intensity (MFI) for CD4+CD44+ for different conditions.](image)
Figure 10

A Spleen

**Main effects (P-values)**

<table>
<thead>
<tr>
<th></th>
<th>CD3+ CD4+</th>
<th>CD3+ CD8+</th>
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</thead>
<tbody>
<tr>
<td>Diet</td>
<td>0.96</td>
<td>0.51</td>
</tr>
<tr>
<td>Genotype</td>
<td>0.53</td>
<td>0.33</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.53</td>
<td>0.52</td>
</tr>
</tbody>
</table>

**CD4+ CD62L+ MFI**

<table>
<thead>
<tr>
<th></th>
<th>CD11a</th>
<th>CD44</th>
<th>CD62L</th>
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<tbody>
<tr>
<td>Diet</td>
<td>0.63</td>
<td>0.72</td>
<td>0.05*</td>
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<tr>
<td>Genotype</td>
<td>0.46</td>
<td>0.38</td>
<td>0.02*</td>
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<tr>
<td>Interaction</td>
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<td>0.95</td>
<td>0.94</td>
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</table>

C $P=0.05$

<table>
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<tr>
<th></th>
<th>PPARδ^{F/F}</th>
<th>PPARδ^{ΔIEpC}</th>
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<tr>
<td>CD4+ CD62L+ MFI</td>
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<td>4000</td>
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D $P=0.02$

<table>
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<th>PPARδ^{F/F}</th>
<th>PPARδ^{ΔIEpC}</th>
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<tbody>
<tr>
<td>CD8+ CD62L+ MFI</td>
<td>8000</td>
<td>6000</td>
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