n-3 PUFA AND CURCUMIN MODULATE THE RESOLUTION OF MURINE

INTESTINAL INFLAMMATION

A Dissertation

by

QIAN JIA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2011

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Approved by:

Chair of Committee,	Robert S. Chapkin
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ABSTRACT

n-3 PUFA and Curcumin Modulate the Resolution of Murine Intestinal Inflammtion.

(August 2011)

Qian Jia, B.A., Shanxi Teachers University;

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Chair of Advisory Committee: Dr. Robert S. Chapkin

Bioactive food components containing *n-3* polyunsaturated fatty acids (PUFA) and curcumin modulate multiple determinants that link inflammation to cancer initiation and progression. In this dissertation, both transgenic and dietary mouse models were used to elucidate the effect of *n-3* PUFA and curcumin treatment on murine intestinal inflammation.

Specifically, *fat-1* transgenic mice, which convert endogenous *n-6* PUFA to *n-3* PUFA in multiple tissues, exhibited a reduced number of colonic adenocarcinomas per mouse (1.05 ± 0.29 versus 2.12 ± 0.51, $P = 0.033$), elevated apoptosis ($P = 0.03$), and a decrease in *n-6* PUFA–derived eicosanoids compared with wild-type (wt) mice in an azoxymethane (AOM) - dextran sodium sulfate (DSS) model.

Following a 2-week recovery period after 5 days of DSS exposure, colonic inflammation and ulceration scores returned to pretreatment levels only in *fat-1* mice. In addition, *fat-1* vs wt mice exhibited decreased ($P < 0.05$) levels of $CD3^+$, $CD4^+$ T helper, and macrophage cell numbers in the colon. The ability of *n-3* PUFA to favorably

modulate the resolution of intestinal inflammation in *fat-1* mice was linked to an enhancement ($P < 0.05$) in the percentage of colonic lamina propria (cLP) CD4$^+$FoxP3$^+$ cells and a decrease in both splenic and cLP Th17 cells (0.8 vs 1.2 % in spleen, 1.4 vs 1.7 % in colon) (P<0.05) in *fat-1* mice compared to wt. These results suggest that the antitumorigenic effect of *n-3* PUFA may be mediated via its anti-inflammatory properties.

The combined effect of *n-3* PUFA and curcumin on DSS induced colitis was assessed in C57BL/6 mice. Addition of fish oil (FO) and/or curcumin to a corn oil (CO) based diet increased animal mortality compared to CO alone ($P < 0.05$). Consistently, following 1 or 2 cycles of DSS treatment, both dietary FO and curcumin promoted mucosal injury/ulceration compared to CO. However, compared to other diets, FO and curcumin combined feeding enhanced the resolution of chronic inflammation and suppressed ($p<0.05$) a key inflammatory mediator, NF-kB, in colon mucosa. Mucosal microarray analysis revealed that dietary FO and curcumin differentially modulated the expression of genes induced by DSS treatment. These results suggest that dietary lipids and curcumin interact to regulate mucosal homeostasis and the resolution of chronic inflammation in the colon.

DEDICATION

To my parents, my husband, and my son I dedicate my dissertation.

ACKNOWLEDGEMENTS

I would like to thank a lot of people. I would especially like to express my appreciation for the great help from my advisors, Drs. Chapkin and McMurray, for their guidance and support throughout my doctoral research. They encouraged me to develop independent thinking and scientific research skills, and continually trained me with respect to communication and writing skills that drove my achievements during my Ph.D. studies. I am also extremely grateful to my committee members, Drs. Welsh and Tian, for their kind support and encouragement during my graduate study.

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NOMENCLATURE

Ab   Antibody

ANOVA   Analysis of variance

APC   Antigen presenting cell

AOM   Azoxymethane

BrdU   Bromodeoxyuridine (5-bromo-2-deoxyuridine)

CD   Crohn's disease

CO   Corn oil

Cur   Curcumin

COcur   Corn oil + 2% curcumin

DC   Dendritic cells

DHA   Docosahexaenoic acid ($22:6\Delta^{4,7,10,13,16,19}$)

DSS   Dextran sodium sulfate

DTT   Dithiothreitol

EDTA   Ethylenediaminetetraacetic acid tetrasodium salt dehydrate

EPA   Eicosapentaenoic acid ($20:5\Delta^{5,8,11,14,17}$)

FO   Fish oil

FOcur   Fish oil + 2% curcumin

HBSS   Hank's buffered salt solution

IBD   Inflammatory bowel diseases

IHC   Immunohistochemistry

LPL	Lamina propria lymphocytes
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
TNBS	Trinitrobenzene sulphonic acid
Th cell	T helper cells
Treg	Regulatory T cells
TUNEL	Terminal deoxynucleotidyl transferase-mediated nick end labeling.
UC	Ulcerative colitis
Wt	Wild type

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1. INTRODUCTION: LITERATURE REVIEW

1.1 Intestinal inflammation and cancer

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are chronic, relapsing inflammatory conditions of elusive etiology in the intestinal tract (1, 2). Recent research indicates a dysregulated mucosal innate and adaptive immune function and a primary impaired barrier function allowing entry of microorganisms inducing a secondary inflammatory response (2-6). An important mechanistic basis for the catastrophic intestinal phenotype associated with IBD may be uncontrolled lamina propria mononuclear and T cell activation, which can perturb homeostatic regulation of cell death and division in intestinal epithelia resulting in a failure to heal injury to the intestinal mucosa (5-7).

A cytokine imbalance is believed to contribute to the initiation and perpetuation of IBD. Traditionally, the pathogenesis of IBD involves the perturbation of Th1/Th2 subsets. Th1 cytokines such as IL-2 and IFN-γ predominate in CD, while Th2 produced IL-4, IL-5, IL-10 are linked to UC (8). Recently, IL-17 producing Th17 cells were identified as a new subset of T helper cells unrelated to Th1 or Th2 cells (5, 9-11). They have been associated with the development of autoimmunity and inflammatory responses such as IBD in addition to playing an important role in host defense against extracellular pathogens (11-14). In contrast, regulatory T cells (Treg), which are developed in a reciprocal manner related to Th17, play an immunosuppressive role and

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This dissertation follows the style of *The Journal of Immunology*.

prevent colitis (9, 15, 16). The imbalance of regulatory and effector T cell induction is of primary importance in the etiology of the inflammation observed in multiple experimental IBD models (16-21).

It is well-known that colon cancer risk increases in relation to the duration of IBD. One of the possible reasons is that the inflammation predisposes colon tissue to genetic instability (22-25). Specifically, the risk of colorectal cancer in UC subjects is related to the duration of disease, age at diagnosis, and extent of the colitis while an increased risk of cancer in CD is related to the location of disease in the colon (22). However, despite the well-known functional link between inflammation and colon cancer, the pathways regulating initiation of colon cancer in the presence of chronic inflammation remain unclear. Therefore, it is important to identify overlapping regulatory mechanisms underlying inflammation and tumorigenesis in the colon.

1.2 Anti-inflammatory properties of *n-3* PUFA

Dietary long chain *n-3* polyunsaturated fatty acids (PUFA) found in fish oil (FO), specifically, eicosapentaenoic acid (EPA; $20:5^{\Delta5,8,11,14,17}$) and docosahexaenoic acid (DHA, $22:6^{\Delta4,7,10,13,16,19}$), are capable of modulating critical determinants which link inflammation and cancer development and progression (26-29). For example, dietary *n-3* PUFA has been reported to suppress colitis induced colon cancer initiation and progression in rats by altering the molecular portrait of gene expression profiles in the colonic epithelium (25, 27). In contrast, *n-6* PUFA found in corn oil (CO) and other vegetable oils, e.g., linoleic acid ($18:2^{\Delta9,12}$) and arachidonic acid ($20:4^{\Delta5,8,11,14}$), are

considered pro-inflammatory dietary components that enhance the development of colon tumors (30, 31). To date, the effects of *n-3* PUFA on susceptibility to colitis and colon cancer have not been determined and a unifying mechanistic hypothesis addressing how *n-3* PUFA selectively manipulate colonic inflammation is lacking.

Although there is insufficient evidence to support the claim that *n-3* PUFA are able to suppress IBD and colon cancer risk in human, it has been demonstrated that *n-3* PUFA can inhibit NF-κB activation in the intestine (29, 32-34). NF-κB modulates a number of distinct steps in the inflammatory cascade resulting in the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IFN-γ, IL-12, COX-2 and iNOS (35, 36). Indeed, recent studies indicate that NF-κB regulates both intestinal immune cell homeostasis and epithelial cell wound healing and repair (36).

Dietary *n-3* PUFA has been shown to modulate T cell activation by modulating components of intracellular IL-2 signaling pathways and blunting the production of intracellular second messengers of diacylglycerol and ceramide following mitogen stimulation in vitro (37). In addition, dietary *n-3* PUFA also shifts the Th1/Th2 balance in a manner of direct suppressing Th1 development in vitro (38). This may be due to suppression of antigen-driven proliferation in Th1-polarized cells and/or enhancement of activation-induced cell death (37, 38). Recently accumulated data also demonstrate that dietary *n-3* PUFA not only alter the phospholipids and signaling composition of lipid rafts to modulate T cell receptor signaling complexes formation (37, 39), but also suppress mitochondrial translocation to the immunologic synapse and modulate calcium signaling in T cells to create an environment suppressing T cell activation (40).
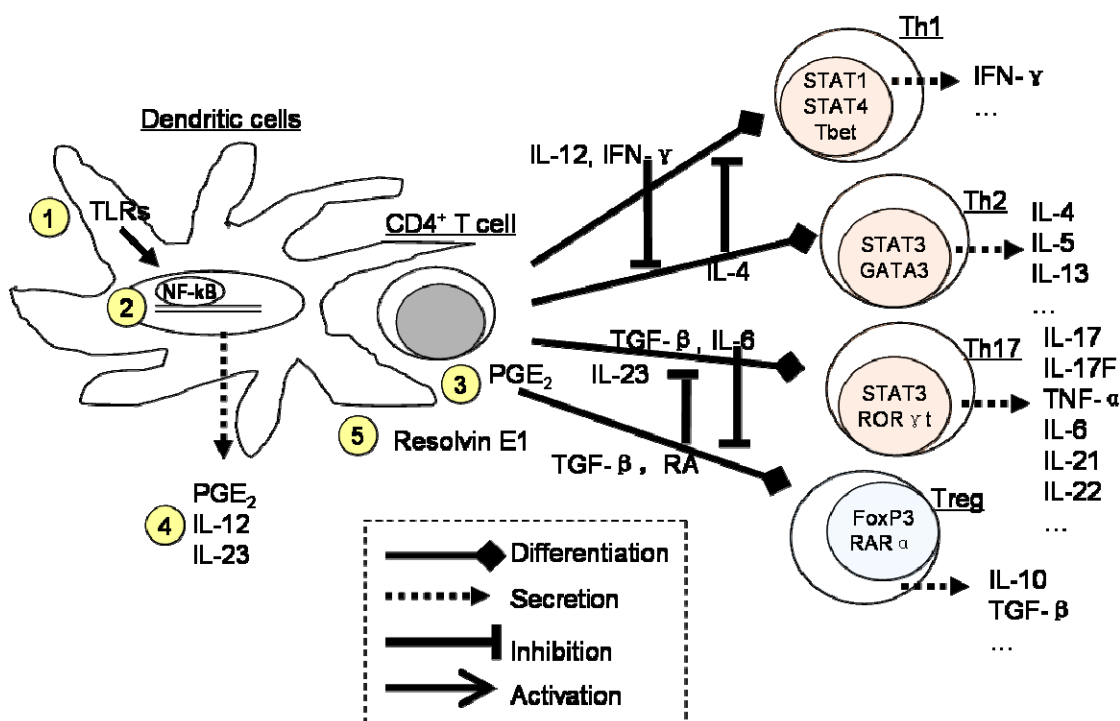
Figure 1. *n-3* PUFA affect T cell polarization in intestine. Intestinal inflammation is controlled by a complex interplay of innate and adaptive immune mechanisms. When the intestinal mucosa is disrupted by genetic mutation or environmental factors, the uncontrolled stimulation of the resultant immune response can trigger IBD. *n-3* PUFA may regulate intestinal immune response by multiple mechanisms: 1, antagonizing Toll like receptor (TLR) pathway by modulating lipid rafts; 2, inhibiting NF-κB activation; 3, inhibiting PGE_2 production directly by inhibiting the biosynthetic COX enzymes, thereby antagonizing its *n-6* PUFA substrates; 4, mediating IL-12 family cytokines production by PGE_2; 5, *n-3* PUFA derived Resolvin E1.

1.3 *n-3* PUFA and T cell polarization

It is well established that inappropriate T cell activation is involved in chronic intestinal inflammation. For example, Crohn's disease has been considered traditionally to be a Th1-mediated chronic disease (8, 41). To date, researchers have implicated IL-23

and Th17 cells in the pathogenesis of experimental colitis and human IBD, particularly CD (11-14, 42-45). Dendritic cells (DC) are potent regulator of Th1/Th17 response in IBD (46). With respect to antigen presenting dendritic cells, prostaglandin E_2 (PGE_2) induces IL-23 and inhibits IL-12p70 production (**Figure 1**), thereby promoting Th17 differentiation (47-49). In contrast, it has also reported that $CD4^+CD25^+$ regulatory T (Treg) cells suppress effector T cell responses in a PGE_2-dependent fashion (50, 51). Interestingly, *n-3* PUFA have been shown to suppress PGE_2 in experimental colitis (29, 52). Although the inhibitory effect of *n-3* PUFA on T cell proliferation has been shown not to be related to eicosanoids or lipid peroxidation (37), Th17 and Treg cell polarization may be altered by *n-3* PUFA via its antagonizing effect on PGE_2. Recently, a beneficial effect of dietary *n-3* fatty acids in experimental autoimmune encephalomyelitis (a model for human multiple sclerosis) has been reported. In this model, DHA affected the ability of bone marrow-derived DC to diminish DC stimulation of $CD4^+$ T cell proliferation and Th1/Th17 differentiation (53).

1.4 Anti-inflammatory properties of curcumin

Curcumin is a natural polyphenol isolated from the dried rhizomes of *Curcuma longa* (turmeric). As the active component in turmeric, curcumin has been widely used in traditional medicine in India and Southeast Asia (54, 55). Moreover, there are an increasing number of reports showing that curcumin ameliorates the degree of inflammation in experimental colitis and human IBD (56-58). These data suggest that select dietary polyphenolics may favorably modulate inflammatory responses in the

colon. The chemoprotective effects of curcumin appear to be mediated in part by NF-κB (57, 59, 60). A randomized, double-blind, multicenter clinical trial of curcumin supplementation has shown that curcumin maintains remission in patients with quiescent UC to prevent relapse (61). Another pilot study with small sample size suggested a favorable effect of curcumin in both CD and UC patients by lowering Crohn's Disease Activity Index (CDAI) scores and sedimentation rates (62).

A previous study has addressed the effect of curcumin on Th1/Th2 balance in a trinitrobenzene sulphonic acid (TNBS) induced chronic colitis mouse model. The intraperitoneal (i.p.) injection of curcumin decreased the expression of Th1 cytokines (e.g., IL-12, TNF-α, IL-2, IFN-γ) and increased Th2 cytokine levels (IL-4 and IL-10) in colon mucosa. Curcumin injection also suppressed the IFN-γ/ IL-4 ratio in splenocytes and serum. These data indicate that curcumin alters the Th1/Th2 balance toward the Th2 pole (63). A previous dietary study from our lab indicated a combination effect of *n-3* PUFA and curcumin to suppress CD4$^+$ T-cell proliferation and IL-2 production in vivo, which, in turn, inhibited Th1 cytokine profiles (64).

Recently, curcumin was reported to suppress PGE$_2$ formation by blocking the expression of COX-2 and microsomal PGE$_2$ synthase-1 and inhibiting the activity of microsomal PGE$_2$ synthase-1 (65). PGE$_2$ is well known as a proinflammatory mediator in colon cancer development since it is capable of enhancing cell proliferation, angiogenesis, cell migration and invasion as well as inhibiting apoptosis, and enhancing tumor growth (66). PGE$_2$ is also important for the healing of ulcers and epithelial injury (67, 68).

1.5 *fat-1* transgenic model

Since mammals cannot produce *n-3* PUFA from the major *n-6* PUFA found in the diet due to the lack of Δ15-desaturase activity, it is necessary to enrich the diet with EPA and/or DHA in order to assess their biological properties *in vivo*. Recently, the *fat-1* gene encoding an *n-3* fatty acid desaturase has been cloned from *C. elegans* and expressed in mammalian cells (69). This enzyme can catalyze the conversion of *n-6* PUFA to *n-3* PUFA by introducing a double bond into fatty acyl chains. The generation of transgenic mice expressing *fat-1* allowed us for the first time to investigate the biological properties of *n-3* PUFA without having to incorporate DHA in the diet (29, 69).

1.6 AOM-DSS model

The dextran sodium sulfate (DSS) method of induced inflammation is a well-accepted preclinical model of mucosal injury and repair that exhibits mucosal inflammation and mimics many phenotypic characteristics relevant to the human disease (70, 71). DSS has the capacity to disrupt the epithelial cell barrier and therefore to promote increased cellular exposure to normal mucosal microflora. DSS alters mucosal barrier function prior to the onset of colitis (70). As a physical agent–induced colitis model, DSS promotes the activation of lymphocytes and the induction of Th1 and/or Th2 responses superimposed on macrophage-induced inflammation (72). In contrast, the pathology of TNBS induced colitis depends on the mouse strain. TNBS elicits a Th1

response in SJL/J mice and, a mixed response in Balb/C mice, i.e., initial by Th1 and later Th2. C57BL/6 mice are resistant to TNBS induced colitis (73).

When combined with azoxymethane, (AOM), at least 50% of C57BL/6 mice develop colonic adenocarcinomas (70, 74). Macroscopically, a dysplasia-invasive adenocarcinoma sequence is observed, resulting in both flat and polypoid tumors. This is analogous to the dysplasia-associated lesion or mass (DALM) seen in patients with ulcerative colitis (22).

1.7 Summary

Dietary *n-3* PUFA and curcumin may play a protective role in IBD and colon cancerogenesis, although the molecular/cellular mechanisms underlying the effect of their action is not fully understood. In this dissertation, the modulating effect of *n-3* PUFA and curcumin on mucosal immunity were further addressed. With respect to immune response, *n-3* PUFA and curcumin act to suppress mediators of the pathology of inflammation in the colon. In the described experiments, we determined how *n-3* PUFA and curcumin differentially modulate acute and chronic colitis in the mouse model.

1.8 Overall hypothesis

n-3 PUFA and curcumin directly modulate mucosal dendritic cell and T cell function, resulting in the preferential resolution of chronic inflammation and a reduction in colon cancer risk.

1.9 Specific aims and hypotheses

1.9.1 Specific aim 1 was to determine the effect of endogenous n-3 PUFA on colonic chronic inflammation and colon cancer.

In association with this, the following sub-hypotheses were proposed:

Hypothesis 1-1 is that *fat-1* mice, which synthesize endogenous *n-3* PUFA, are protected from colitis – associated colon carcinogenesis.

Hypothesis 1-2 is that *fat-1* mice suppress pro-inflammatory eicosanoid production.

Hypothesis 1-3 is that *fat-1* mice are protected from DSS-induced chronic inflammation.

1.9.2 Specific aim 2 was to determine whether n-3 PUFA affect T regulatory (Treg) and Th17 cell subsets in the intestine.

In association with this, the following sub-hypotheses were proposed:

Hypothesis 2-1 is that endogenous *n-3* PUFA modulate T cell polarization, altering lamina propria (LP) Treg and Th17 subsets.

Hypothesis 2-2 is that endogenous *n-3* PUFA affect the balance between dendritic cell-derived IL-23 and IL-12 production by suppressing PGE_2.

1.9.3 Specific aim 3 was to determine the ability of curcumin, a putative inhibitor of NF-κB, to enhance the anti-inflammatory properties of n-3 PUFA.

In association with this, the following sub-hypotheses were proposed:

Hypothesis 3-1 is that dietary *n-3* PUFA and curcumin favorably modulate inflammation and intestinal cytokinetics during discrete stages of DSS-induced colitis.

Hypothesis 3-2 is that dietary *n-3* PUFA and curcumin suppress NF-κB activation in the colonic mucosa.

Hypothesis 3-3 is that dietary *n-3* PUFA and curcumin modulate the restoration of colonic homeostasis after experimental stress-induced inflammation.

2. REDUCED COLITIS-ASSOCIATED COLON CANCER IN *fat-1* MICE*

2.1 Introduction

Human IBD are chronic, relapsing inflammatory conditions of unknown etiology. Genetic, immunological, and environmental factors have been implicated (1, 75). These diseases are characterized by two overlapping clinical phenotypes, i.e., ulcerative colitis (UC) and Crohn's disease (CD). UC primarily involves active disease confined to the colon with inflammation restricted to the mucosa. Chronic inflammation in CD often involves the small intestine along with the colon and other organs (2). CD affects more than 500,000 individuals in the U.S. and represents the second most common chronic inflammatory disorder after rheumatoid arthritis. In addition, the risk of developing colorectal cancer increases ~0.5-1% each year after 7 years in patients with chronic intestinal inflammation (22, 75).

Colorectal cancer continues to pose a serious health problem in the United States. Over a lifetime, a person has a 1 in 18 chance of developing invasive colorectal cancer (76). From a dietary perspective, a growing number of published reports support the contention that bioactive food components containing *n-3* PUFA modulate important determinants which link inflammation to cancer development and progression (26-29, 77). In addition, clinical and experimental data indicate a protective effect of *n-3* PUFA

on colon cancer (30, 78-85). EPA and DHA are typical *n-3* PUFA (found in fish oil), defined according to the position of the first double bond from the methyl end of the molecule which is designated "*n-3*". In contrast, dietary lipids rich in *n-6* PUFA (found in vegetable oils), e.g., linoleic acid ($18:2^{\Delta 9,12}$) and arachidonic acid ($20:4^{\Delta 5,8,11,14}$), enhance the development of colon tumors (30, 31). These effects are exerted at both the initiation and post-initiation stages of carcinogenesis (30, 85-88). Consistent with human clinical trials (79-82, 85), previous data from our lab have shown that the balance between colonic epithelial cell proliferation and apoptosis can be favorably modulated by dietary *n-3* PUFA, conferring resistance to toxic carcinogenic agents (28, 30). This is significant because the typical Western diet contains 10 to 20 times more *n-6* than *n-3* PUFA (89). Unfortunately, to date, a unifying mechanistic hypothesis addressing how *n-3* PUFA selectively suppress colon cancer compared to *n-6* PUFA is lacking.

In this part of the dissertation, *fat-1* mice were exposed to AOM followed by 3 rounds of DSS in order to test the hypothesis that the endogenous production of *n-3* PUFA affords protection against colitis-associated colon carcinogenesis, specifically, (i) tumor formation, (ii) inflammation and injury scores, (iii) specific activity of lymphoid elements and (iv) eicosanoid production.

2.2 Materials and methods

2.2.1 Animals

fat-1 transgenic mice were generated and backcrossed onto a C57BL/6 background as previously described (29). All procedures followed the guideline

Table 1. Fatty acid profiles in *fat-1* and wt mouse tails.

PUFA	Wild type	*fat-1*
n-6 (mol%)		
AA (20:4 n-6)	7.06±1.05 [a]	1.01±0.21 [b]
DTA (22:4 n-6)	1.34±0.31 [a]	0.21±0.11 [b]
DPA (22:5 n-6)	1.25±0.48 [a]	0.14±0.11 [b]
Total n-6 PUFA	9.65	1.36
n-3 (mol%)		
EPA (20:5 n-3)	0.01±0.04 [a]	2.33±0.34 [b]
DPA (22:5 n-3)	0.09±0.13 [a]	2.17±0.30 [b]
DHA (22:6 n-3)	0.52±0.33 [a]	1.85±0.27 [b]
Total n-3 PUFA	0.62	6.34
Ratio n-6/n-3	**29.60** [a]	**0.22** [b]

Values are means ± SEM, n=30 mice per group.
Different letters indicate differences (P<0.05) between the 2 groups. DTA, docosatetraenoic acid; DPA, Docosapentaenoic acid.

approved by Public Health Service Policy and the Institutional Animal Care and Use Committee at Texas A&M University. The colony of *fat-1* mice used for this study was generated by breeding heterozygous mice. The genotype and phenotype of the offspring were characterized using isolated DNA and total lipids from tails (**Table 1**). Specific pathogen-free animals were maintained under barrier conditions and fed a 10% safflower oil diet (Research Diets) ad libitum with a 12 h light/ dark cycle. The diet contained 40 (g/100 g diet) sucrose, 20 casein, 15 corn starch, 0.3 DL-methionine, 3.5 AIN 76A salt

mix, 1.0 AIN 76A mineral mix, 0.2 choline chloride, 5 fiber (cellulose), 10 safflower oil. Safflower oil is enriched in *n-6* PUFA containing 72% of linoleic acid according to the manufacturer.

2.2.2 Colon tumor induction

fat-1 and litter-mate wt (control) mice (10-20 wks old) were injected intraperitoneally with 12.5 mg/kg body weight AOM (Sigma-Aldrich) followed by 3 rounds of DSS treatment. After 1 wk, 2.5% DSS (molecular weight, 36,000 to 50,000; MP Biomedicals) was administered in the drinking water for 5 d, followed by 16 d of tap water. This cycle was repeated twice (5 d of 2.5% DSS followed by a 16 d recovery period and 4 d of 2% DSS), and mice were terminated 12 wk after completion of the final DSS cycle. Subsequently, each colon was resected proximally at the junction between the cecum and distally at the anus, flushed with PBS, and fixed with 4% paraformaldehyde (30). Tumors were sectioned and dysplasia, adenomas and carcinomas were charted and evaluated by a board-certified pathologist in a blinded manner as we have previously described (30, 90).

2.2.3 In situ apoptosis measurement

Apoptosis was measured in paraformaldehyde-fixed, paraffin-embedded tissues using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end (Oncor) labeling (TUNEL) method (85).

2.2.4 Colitis induction and histological scoring

fat-1 and wt mice (8-14 wks) were provided 2.5% DSS in the drinking water for 5 d, after which animals were provided with water only and allowed to recover for either 3 d or 2 wks prior to sacrifice. At each necropsy interval, the entire colon was removed, measured, fixed in 4% paraformaldehyde and paraffin embedded. Sections were stained with hematoxylin and eosin. Histological examination was performed in a blinded manner by a board-certified pathologist, and the degree of inflammation (score, 0–3) and epithelial injury (score, 0–3) on microscopic cross-sections of the colon was graded. The presence of occasional inflammatory cells in the lamina propria was assigned a value of 0; increased numbers of inflammatory cells in the lamina propria as 1; confluence of inflammatory cells, extending into submucosa, as 2; and transmural extension of the infiltrate as 3. For epithelial injury, no mucosal damage was scored as 0; discrete lympho-epithelial lesions were scored as 1; surface mucosal erosion or focal ulceration was scored as 2; and extensive mucosal damage and extension into deeper structures of the bowel wall was scored as 3.

2.2.5 Isolation of colonic lamina propria lymphocytes

Lamina propria lymphocytes were isolated from mouse colon as previously described with some modification (91, 92). Briefly, the colon was flushed clean and incubated in media containing Ca^{2+}, Mg^{2+} free HBSS (Sigma-Aldrich), 5 mM DTT and 30 mM Na_4EDTA at 37 °C, 100 rpm for 15 min. Subsequently, colons were placed on ice and gently scraped using a rubber policeman to remove the intact crypts. Following a

washing step in Ca^{2+}, Mg^{2+} free HBSS, the remaining tissue was cut into small pieces, and incubated in Ca^{2+}, Mg^{2+} free HBSS containing 1 mg/mL type II and type IV collagenase (Worthington) at 37 °C, 100 rpm for 40 min. The liberated lamina propria cells were freely passed through a stainless steel grid (60-mesh) and further purified by density gradient centrifugation in 40-70 % Percoll (Amersham) in PBS. Lymphocytes enriched at the interface between a 40 and 70% Percoll gradient were collected (**Appendix B1**).

2.2.6 Flow cytometry analysis of CD3$^+$ and CD4$^+$ T cells

Flow cytometry was performed as previously described, using 1 million cells per mouse (93). Lamina propria lymphocytes collected from Percoll gradients were preincubated with an FcγR blocking monoclonal mAb (10 µg/mL) (2.4G2, BD Pharmingen) for 5 min at 4 °C. To measure the proportion of CD3$^+$ and CD4$^+$ T-cells, sample contents were stained with both fluorescein isothiocyanate (FITC)-labeled (5 µg/mL) anti-CD3 (145-2C11, hamster IgG1, BD Pharmingen) and phycoerythrin (PE)-labeled anti CD4 (10 µg/mL) (GK1.5, rat IgG2b, BD Pharmingen). Flow cytometric analysis was conducted on a fluorescence-activated cell sorting (FACS)-Calibur flow cytometer (Becton Dickinson Immunocytometry systems) and analyzed using the CELLQUEST analysis program.

2.2.7 Eicosanoid and phospholipid profiles

Colonic mucosal scrapings were collected and immediately snap frozen in liquid nitrogen. Samples were extracted using the method of Yang et al (94). Briefly, aliquots (20 mL) of 1 N citric acid and of 2.5 μL of 10% BHT were added to samples to prevent free radical peroxidation. Prior to extraction, an aliquot of deuterated eicosanoids (PGE_2-d_4, 15-HETE-d_8, 12-HETE-d_8, and 13-HODE-d_4) (100 ng/mL) was added to each sample as internal standards. Eicosanoids were subsequently extracted with 2 mL of hexane:ethyl acetate (1:1, v/v) and vortexed for 2 min. Samples were then centrifuged at 1800 x *g* for 10 min at 4°C. The upper organic layer was collected and the organic phases from three extractions were pooled, and then evaporated to dryness under a stream of nitrogen at room temperature. All extraction procedures were performed under low-light and low-temperature conditions to minimize potential photooxidation or thermal degradation of eicosanoid metabolites. Samples were then reconstituted in methanol: 10 mM ammonium acetate buffer, pH 8.5 (70:30, v/v) prior to LC/MS/MS analysis. The extracted prostaglandins were quantified using a LC/MS/MS Quattro Ultima tandem mass spectrometer (Waters Corp.) equipped with an Agilent HP 1100 binary pump HPLC inlet (Agilent Technologies). The prostaglandins were separated using a 2 x 150 mm Luna 3 m phenyl-hexyl analytical column (Phenomenex). The mobile phase consisted of 10 mM ammonium acetate, pH 8.5, and methanol. The column temperature was maintained at 50°C, and samples were kept at 4°C during the analysis. Individual analytes were detected using electrospray negative ionization and multiple reaction monitoring of the transitions *m/z* 351 → 271 for PGE_2, *m/z* 349 → 269

for PGE$_3$, and *m/z* 355 → 275 for PGE$_2$-d$_4$. Fragmentation of all compounds was performed using argon as the collision gas at a collision cell pressure of 2.10 x 10^{-3} Torr. The identification of each prostaglandin was confirmed by comparison to authentic reference standards. Total phospholipids from scraped colonic mucosa and splenic CD4$^+$ T-cells were analyzed by gas chromatography as previously described (95).

2.2.8 Immunohistochemical detection of macrophages

Colon tissues were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at 5 μm. Analyses were performed as previously described (91) with minor modification. Following peroxidase blocking using 3% H$_2$O$_2$, diluted (1:150) primary macrophage/monocyte F4/80 (CI:A3-1, rat IgG2b, Serotec) antibody was incubated overnight at 4°C. After washing in PBS, sections were incubated with biotin-conjugated secondary antibody (rabbit anti rat IgG, mouse absorbed, Vector) for 1 h at room temperature, followed by the addition of avidin-biotin-peroxidase complex (ABC kit, Vector). Peroxidase activity was visualized using diaminobenzidine (Sigma). Negative controls were prepared by omission of primary antibody. Morphometric analysis of F4/80 positive area was performed using image analysis software (NIS-Elements AR2.30, Nikon). Ten different randomly chosen areas, which together covered representative fields from submucosa, lamina propria and crypts, were taken from each animal. Results are shown as the percentage of positive area.

2.2.9 Statistics

Data are expressed as mean ± SEM. Differences between experimental groups were analyzed using one-way ANOVA (SPSS software package). P-values less than 0.05 were accepted as significant.

2.3 Results

2.3.1 Weight gain

For AOM/DSS treated animals, neither carcinogen nor *fat-1* genotype significantly altered weight gain (starting weight, wt 35.9 ± 3.0 g vs *fat-1* 37.7±2.9; final weight, wt 35.2±3.4 vs *fat-1* 36.8±3.2), n=19, p > 0.05.

2.3.2 Fatty acid profiles in fat-1 and wild type mice

Both *fat-1* transgenic and wt offspring were fed a 10% safflower oil diet enriched in *n-6* PUFA throughout the duration of the study. Colonic mucosa and splenic CD4$^+$ (systemic) T-cell total lipid fatty acid compositional analyses revealed an increase in EPA (20:5 n-3), DPA (22:5 n-3) and DHA (22:6 n-3) in *fat-1* transgenic mice (**Tables 2&3**). In addition, the ratio of *n-6* PUFA (20:4 n-6, 22:4 n-6, and 22:5 n-6) to the long-chain n-3 PUFA (20:5 n-3, 22:5 n-3, and 22:6 n-3) was significantly (P<0.05) suppressed in *fat-1* T-cells and colonic mucosa (2.44 and 2.70), respectively, compared to wt mice (56.06 and 18.24). These data indicate that an appropriate activity of n-3 fatty acid desaturase was present and that relevant cell types, e.g., T-cells and colonocytes, were enriched in *n-3* PUFA.

Table 2. CD4^{+} splenic (systemic) T-cell fatty acid profiles in *fat-1* and wt mouse.

PUFA	Wild type	Fat-1
n-6 (mol%)		
AA (20:4 n-6)	14.10 ± 1.84[a]	12.25 ± 1.41[a]
DTA (22:4 n-6)	1.68 ± 0.29[a]	0.42 ± 0.26[b]
DPA (22:5 n-6)	1.60 ± 0.33[a]	0.70 ± 0.33[a]
Total n-6 PUFA	17.38	13.37
n-3 (mol%)		
EPA (20:5 n-3)	0 ± 0[a]	0.16 ± 0.16[a]
DPA (22:5 n-3)	0 ± 0[a]	1.68 ± 0.85[b]
DHA (22:6 n-3)	0.31 ± 0.31[a]	3.63 ± 0.65[b]
Total n-3 PUFA	0.31	5.47
Ratio n-6/n-3	**56.06**[a]	**2.44**[b]

Values are means ± SEM, n=5 mice per group.
Different letters indicate differences (P<0.05) between the 2 groups.

Table 3. Colonic mucosa fatty acid content in *fat-1* and wt mouse.

PUFA	Wild type	Fat-1
n-6 (mol%)		
AA (20:4 n-6)	14.71 ± 0.25[a]	10.33 ± 0.49[b]
DTA (22:4 n-6)	1.25 ± 0.07[a]	0.85 ± 0.04[b]
DPA (22:5 n-6)	1.61 ± 0.08[a]	0.54 ± 0.02[b]
Total n-6 PUFA	16.96	13.64
n-3 (mol%)		
EPA (20:5 n-3)	0.00 ± 0.00[a]	2.13 ± 0.15[b]
DPA (22:5 n-3)	0.12 ± 0.12[a]	0.54 ± 0.05[b]
DHA (22:6 n-3)	0.81 ± 0.17[a]	2.28 ± 0.12[b]
Total n-3 PUFA	0.93	5.05
Ratio n-6/n-3	**18.24**[a]	**2.70**[b]

Values are means ± SEM, n=4-8 mice per group.
Different letters indicate differences (P<0.05) between the 2 groups.

2.3.3 Suppression of colorectal tumorigenesis in fat-1 mice following azoxymethane (AOM) and dextran sodium sulphate (DSS) treatment

Colitis associated colonic tumors were induced by a single injection of AOM followed by repeated cycles of DSS ingestion using a well established protocol (74). Mice were terminated 12 wk after completion of the final DSS cycle and grossly visible masses/lesions were typed as adenomas, adenocarcinomas and dysplasia (**Figure 2, A-D**). The incidence of colonic tumors (adenocarcinomas) was lower in *fat-1* relative to wt mice, 15 out of 19 (79%) for *fat-1* vs 17 of 17 (100%, p=0.001) for wt mice. *fat-1* mouse tumors on average tended to be smaller (12.53±1.31 vs 14.11±2.02 mm^2, p=0.09) compared to wt mice. In addition, *fat-1* mice (n=19) exhibited a reduced average number of adenocarcinomas (1.05±0.29 vs 2.12±0.51, *p*=0.033) and dysplasia (0.75±0.19 vs 1.12±0.26, *p*=0.13) per mouse compared to wt mice (**Figure 2 E**).

2.3.4 Omega-3 desaturase expression enhances apoptosis in colonic epithelial cells

Measurements of apoptosis have greater prognostic value to detect dietary effects on colon tumor incidence than do measurements of cell proliferation (30, 85). Therefore, apoptosis was assessed in the colonic epithelium (**Figure 2 F**). The apoptotic index was significantly (p=0.03) higher in *fat-1* compared to wt mice, suggesting that the observed reduction in tumor incidence (described above) may in part be explained by an increase in apoptosis.
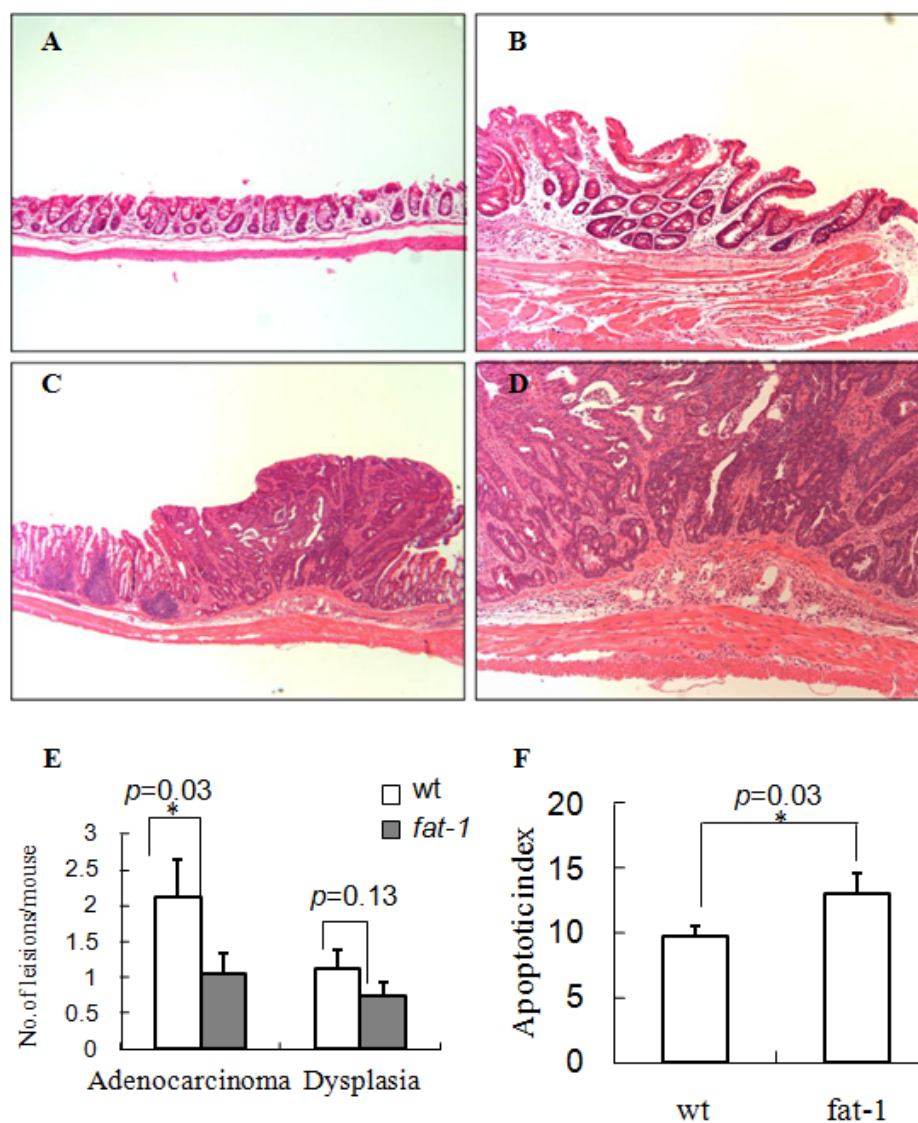
Figure 2. Effect of *fat-1* genotype on colonic adenocarcinomas and apoptosis level. Animals were injected with AOM followed by 3 cycles of DSS in order to induce colitis-associated colon tumors. Animals were terminated 12 wk after completion of the final DSS cycle. Representative histopathological features of hematoxylin and eosin-stained colorectal adenocarcinomas are shown. The lesions were counted on high-resolution photographs of the colons, (A) normal colon, 100x; (B) dysplasia, 100x; (C-D) adenocarcinoma, 40x and 100x. (E) Values represent mean ± SE, n=17-20 mice per treatment. (F) Apoptosis levels in *fat-1* vs wild type mouse colon 38 d after the final DSS treatment. Data are expressed as an apoptotic index, i.e., the total number of apoptotic cells per 100 crypts. Values represent mean ± SEM, n=17-20 mice per treatment.
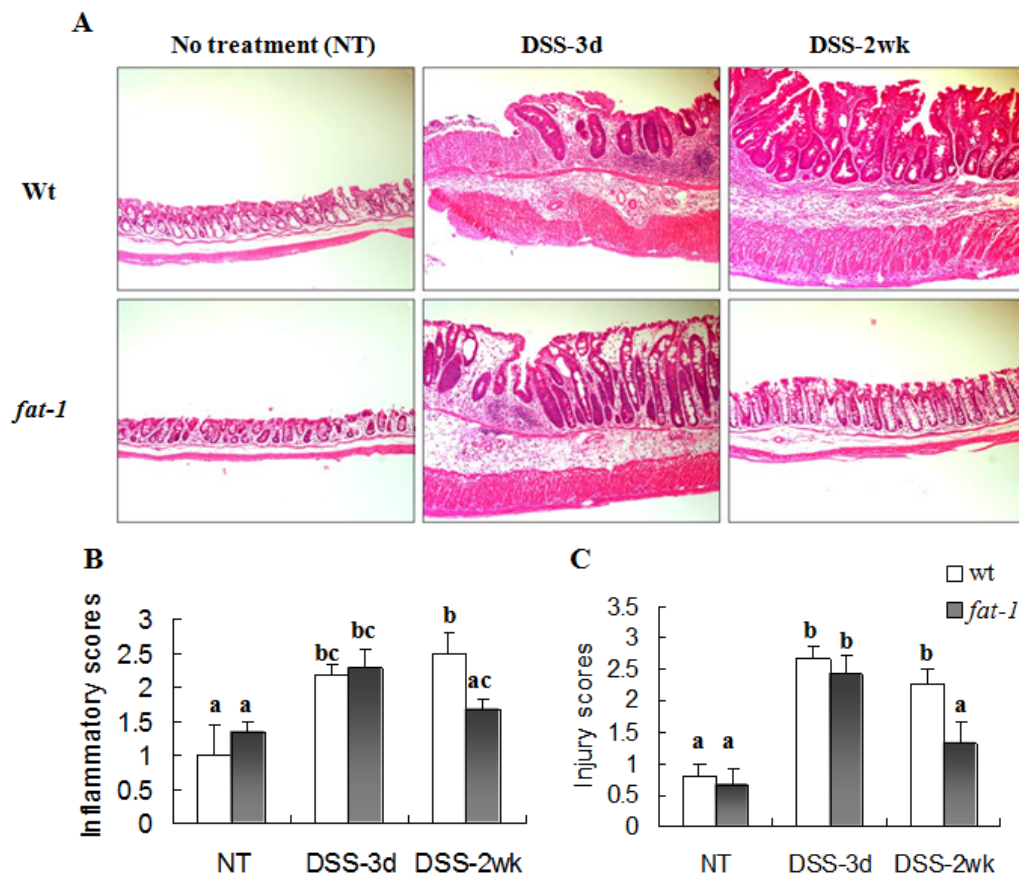
Figure 3. Histological features of colonic inflammation and mucosal injury. To explore the effect of *n-3* PUFA on DSS-induced colonic (A) inflammation and (B) mucosal injury, mice were treated with a single 5 d cycle of DSS followed by either a 3 d or 2 wk recovery period. (A) Representative hematoxylin and eosin-stained colonic tissues from mice with or without 2.5% DSS treatment based on degrees of inflammatory cell infiltration and epithelial injury, 40x. Inflammatory scores (B) and injury scores (C) represent mean ± SEM, n=5-8 mice per treatment. Data not sharing common letters are significantly different, $P < 0.05$.

2.3.5 fat-1 mice are less susceptible to DSS-induced chronic inflammation

In complimentary experiments, we also determined the ability of *n-3* PUFA to modulate susceptibility to DSS-induced colitis. Both acute and chronic colitis was assessed by administering 2.5% DSS in the drinking water for 5 d, followed by a

recovery period of 3 d (acute) or 2 wk (chronic). DSS administration was associated

with a significant (P<0.05) loss of body weight and a reduced colon length (data not

shown). Histological evaluation was subsequently performed in order to access immune

cell infiltration and epithelial injury (**Figure 3 A**). After 3 d of recovery, there was no

effect of *n-3* PUFA with respect to the acute phase inflammatory score or the severity of

injury (**Figures 3 B&C**). In contrast, following a 2 wk recovery period, colonic

inflammation scores returned to pretreatment levels relative to 3 d recovery only in *fat-1*

mice, 2.29±0.29 to 1.67±0.17, *p*=0.03; as compared to wt mice, 2.17±0.17 to 2.50±0.29,

p=0.21. Similar trends were observed with regard to injury scores: 2.43±0.30 to

1.33±0.33 in *fat-1* mice, *p*=0.003; vs 2.67±0.21 to 2.25±0.25, in wt mice, *p*=0.19. Since
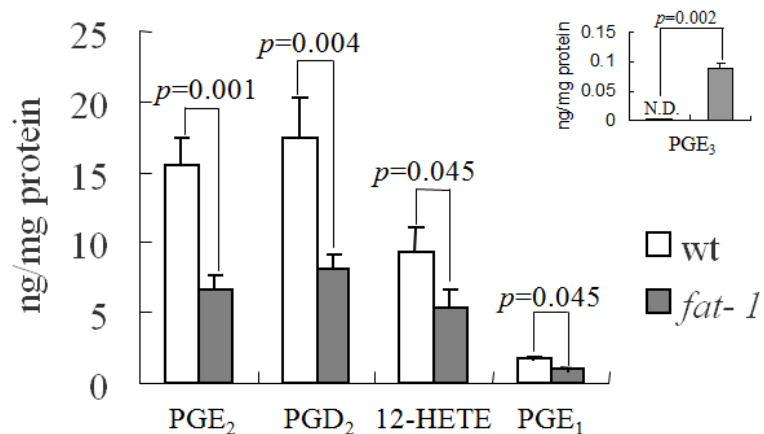
Figure 4. Colonic mucosa eicosanoid profiles of *fat-1* and wild-type mice. Animals were injected with AOM followed by 3 cycles of DSS and terminated 12 wk after completion of the final DSS cycle. Eicosanoids from scraped colonic mucosa were immediately extracted and quantified by LC/MS/MS. *Insert*: Elevated EPA-derived PGE₃ in *fat-1* mice, n.d., not detectable. Values are means ± SEM, n=8 mice per treatment. Levels of n-6-derived eicosanoids were significantly decreased in *fat-1* mice.

DSS treatment followed by a 2 wk recovery period represents a chronic inflammation model in C57BL6 mice (56); these data suggest that *fat-1* mice exhibit an enhanced long-term resolution of inflammatory processes.

2.3.6 Eicosanoid profiles following AOM/DSS exposure

Since arachidonic acid (20:4 n-6) derived 2-series prostaglandins appear to promote cancer development in the colon (66), we also examined key eicosanoids in colonic mucosa following AOM/DSS exposure. Both *n-3* and *n-6* PUFA derived metabolites were assayed, including PGE_2, PGD_2, LTB_4, 15-HETE, 12-HETE, 5-HETE, PGF_2 alpha, PGE_1, PGE_3, 13-HODE, 13,14-dihydro-15-keto-PGE_2, 13, 14-dihydro- 15-keto-PGD_2, 12HHTrE, LTB_5, 5-HEPE, 12-HEPE and 15-HEPE. In general, *n-6* PUFA derived eicosanoids (PGE_2, PGD_2, PGE_1 and 12-HETE) were significantly reduced in *fat-1* mice (**Figure 4**). In contrast, PGE_3, an EPA-derived prostaglandin was elevated in *fat-1* mice. No significant changes were observed with respect to LTB_4, 15-HETE, 5-HETE, PGF_2 alpha, 13-HODE, 13,14-dihydro-15-keto-PGE_2, 13, 14-dihydro- 15-keto-PGD_2, 12HHTrE, LTB_5, 5-HEPE, 12-HEPE and 15-HEPE (data not shown), most of them tend to be suppressed in *fat-1* mice but not significantly. These results indicate that the enhanced incorporation of *n-3* PUFA into *fat-1* mouse colonic mucosa suppressed *n-6* PUFA derived cyclooxygenase and lipoxygenase metabolism.
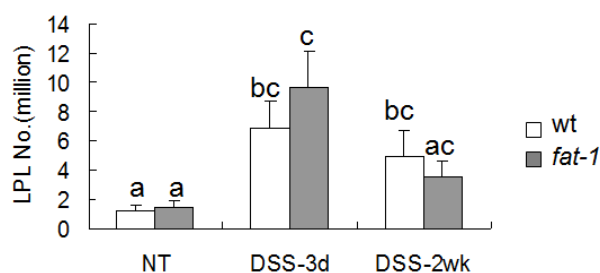
Figure 5. Colonic lamina propria lymphocyte (LPL) yield. NT, no treatment; DSS-3d, 3 d after DSS exposure; DSS-2wk, 2 wk after DSS exposure. Data are expressed as LPL x 10^6 per colon. Values are means ± SEM, n=14-18 mice per group. Different letters indicate differences (P<0.05) between the 2 groups.
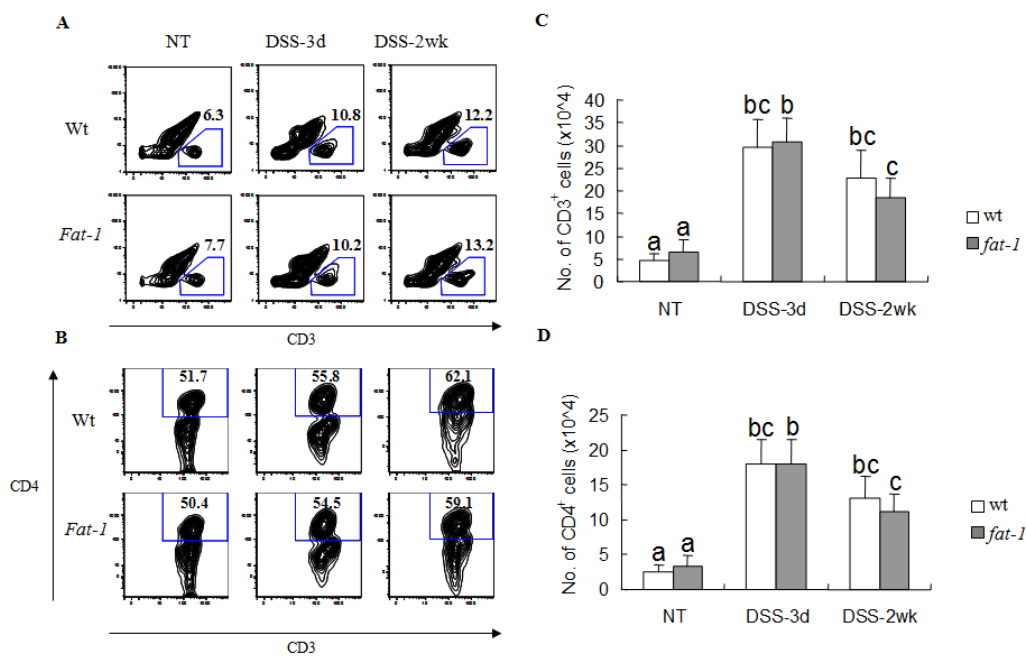
Figure 6. Flow cytometric analysis of CD3^{+} and CD4^{+} colonic lamina propria lymphocytes (LPLs). Mice were exposed to a single 5 d cycle of DSS followed by either a 3 d or 2 wk recovery period and LPLs were isolated from the colon. (A) Plots were gated on CD3^{+} expressing cells. The number in each image indicates the percentage of CD3^{+} cells. (B) Values are means ± SEM, n=5 mice per treatment. (C) Plots were gated on CD4^{+} cells within the CD3^{+} population. The number in each image indicates the percentage of CD4^{+} cells. (D) *fat-1* mice exhibited a decreased number of CD3^{+} and CD4^{+} cells in lamina propria after 2 wk of recovery compared to 3 d. Data not sharing common letters are significantly different, P < 0.05, and are from 2 experiments, n=14-18 per treatment per experiment.

A.

B.

C
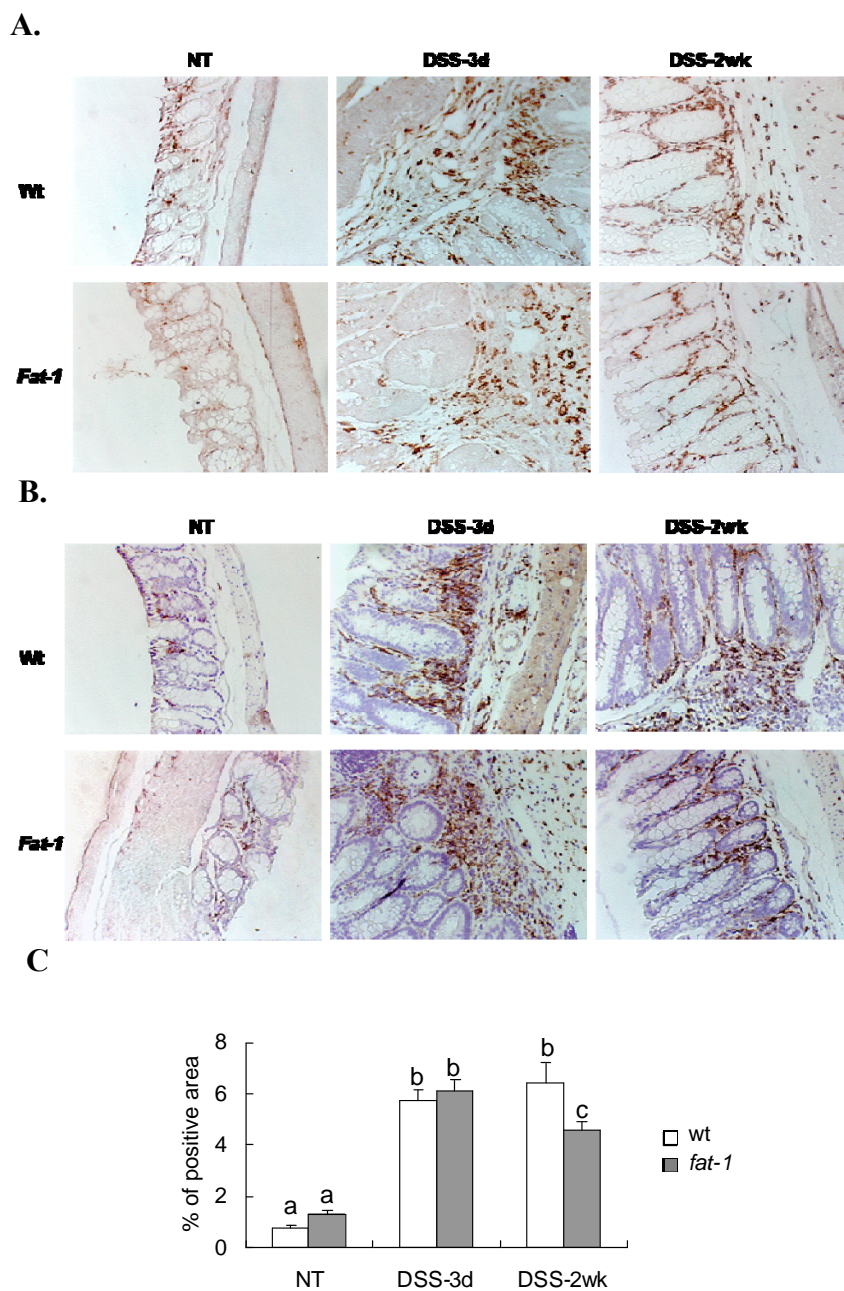
Figure 7. Macrophage accumulation in DSS-treated mouse colon. Representative micrographs from sections probed with F4/80 antibody. A. Immunoreactivity was detected as a brownish product in the absence of counterstain, 200X (refer below for details). B. Immunoreactive sections were counterstained using hematoxylin, 200X. C. Semi-quantitative measurement of macrophage infiltration. NT, no treatment; DSS-3d, 3 d after DSS exposure; DSS-2wk, 2 wk after DSS exposure. Data are expressed as the % of positive area per sample. Values are means ± SE, n=5-8 mice per group. Different letters indicate significant differences (P<0.05) among groups.

2.3.7 Altered infiltration of CD3⁺ and CD4⁺ T-cells in fat-1 mice after DSS exposure

A pathogenic role for CD4⁺ T-cells has been demonstrated in DSS induced inflammatory bowel disease (96, 97). Therefore, in order to contrast and compare local immunological features in *fat-1* and wt mice, colonic lamina propria lymphocytes (LPL) were isolated from mice before and after a single dose of DSS (3 d and 2 wk of recovery period). Notably, there was a dramatic increase in the number of LPL and macrophages in both *fat-1* and wt animals following DSS treatment (**Figures 5 - 7**). Interestingly, only *fat-1* mice exhibited a return to pretreatment levels after 2 wk of recovery. This suggests an inflammation-prone status in intestines from wt mice. For the purpose of examining the specific reactivity of lymphoid elements in the intestine, CD3⁺ T-cells and CD4⁺ T-helper cells from colonic lamina propria were quantified by flow cytometry (**Figure 6**). Comparison of 3 d vs 2 wk recovery time points revealed that *fat-1* mice exhibited decreased CD3⁺ T cells numbers ($x10^5$) per colon (3.08±0.53 to 1.84±0.43, *p*=0.03 in *fat-1* mice, 2.95±0.61 to 2.27±0.61, *p*=0.18 in wt mice) and CD4⁺ T helper cells numbers (1.80±0.35 to 1.12±0.25, *p*=0.048 in *fat-1* mice, 1.78±0.37 to 1.30±0.32, *p*=0.13 in wt mice). These results suggest that the anti-tumorigenic effect of *n-3* PUFA may be mediated in part via its immunosuppressive/anti-inflammatory properties.

2.4 Discussion

Dietary *n-3* PUFA are well known for both their anti-inflammatory and tumor suppressing properties (37, 98). With respect to colon cancer development, we have demonstrated that the chemoprotective effect of fish oil is due to the direct action of *n-3*

PUFA and not to a reduction in the content of *n-6* PUFA (27). Despite compelling data indicating a functional link between inflammation and colon cancer, the effects of *n-3* PUFA on the pathways regulating initiation and maintenance of inflammation during colon cancer development remain poorly understood. Therefore, we examined the phenotype of *fat-1* mice, which synthesize high tissue levels of *n-3* PUFA (69), in order to test the hypothesis that the endogenous production of *n-3* PUFA affords protection against colitis-associated colon carcinogenesis. Our data demonstrate that *n-3* PUFA effectively alter colonic membrane phospholipid composition in the *fat-1* mouse (**Table 3**) resulting in the suppression of inflammation-driven tumor formation (**Figure 2**). These findings support our postulate that *n-3* PUFA have chemoprotective properties. In addition, colonocyte apoptosis was elevated in *fat-1* mice. This is noteworthy because apoptosis is progressively inhibited during colon cancer development (99). It is possible; therefore, that the observed protective effect of *n-3* PUFA is due, in part, to the enhanced deletion of cells though the activation of targeted apoptosis (27, 98).

It is well established that microbially-driven chronic inflammation can lead to colon cancer (37, 100, 101). Conditions which reduce mucosal barrier integrity, e.g., DSS, promote the production of proinflammatory cytokines which act in a paracrine fashion to promote angiogenesis and tumor growth (102). These mediators can, in turn, promote cyclooxygenase-2 related signaling pathways, which are capable of enhancing cell proliferation, angiogenesis, cell migration and invasion, while inhibiting apoptosis (66). Therefore, it is noteworthy that eicosanoid levels in colonic mucosa were significantly suppressed in *fat-1* relative to wild type mice (**Figure 4**). This finding is

consistent with the well documented ability of *n-3* PUFA (EPA and DHA) to supplant arachidonic acid and subsequently antagonize prostaglandin (PGE_2 and PGD_2) and hydroxy fatty acid (12-HETE) biosynthesis (103).

Although the subject of much debate, there is growing evidence that *n-3* PUFA suppress IBD in humans (79-81, 104). Since an inability to maintain an appropriate balance of T-cell subsets is a critical component contributing to the development of IBD (1, 105), and anti-inflammatory therapy is efficacious against neoplastic progression and malignant conversion, we specifically determined the susceptibility of *fat-1* mice to DSS-induced chronic inflammation. Our results demonstrate that *fat-1* mice exhibit an enhanced ability to resolve chronic colitis. Similar effects of *n-3* PUFA were observed in previous acute inflammation experiments using this model (29, 33) as well as the interleukin 10 (IL-10) null mouse colitis model (62), although, a previous report has described contrasting data (106). Of relevance to the immune system in the intestine, we have shown that *n-3* PUFA alter the balance between $CD4^+$ T-helper (Th1 and Th2) subsets by directly suppressing Th1 cell development (93). This is noteworthy, because Th1 cells in part mediate IBD onset and progression (105). Collectively, these observations suggest that *n-3* PUFA dampen the persistent inflammation and immune activation that are associated with DSS-induced mucosal ulceration, thereby suppressing epithelial carcinogenesis.

In conclusion, these data demonstrate that endogenously synthesized *n-3* PUFA suppress colonic (i) chronic inflammation and tissue injury, (ii) specific activity of lymphoid and macrophages elements in the intestine, and (iii) tumor formation. To our

knowledge, this is the first study to show that *n-3* PUFA, which are incorporated into both colonocytes and T-cells, suppress inflammation-driven tumor progression. Further understanding of the effects of fatty acids on the bidirectional interactions between colonocytes and T-cells in the lamina propria will provide insight into the ability of *n-3* PUFA to favorably modulate the inflammation-dysplasia-carcinoma axis.

3. *n-3* PUFA FAVORABLY MODULATE T CELL POLARIZATION DURING

DEXTRAN SODIUM SULPHATE (DSS)-INDUCED EXPERIMENTAL COLITIS

3.1 Introduction

The data from Section 2 have shown that the effects of *n-3* PUFA on susceptibility to colitis and colon cancer rely in part on their ability to modify eicosonoid profiles, enhance epithelial apoptosis and alter innate and adaptive immune responses (98, 107). An important mechanistic basis for the intestinal phenotype associated with IBD involves upregulated lamina propria mononuclear and T cell activation, which can perturb homeostatic regulation of cell death and division in intestinal epithelia resulting in a failure to heal injury to the intestinal mucosa (2). Crohn's disease has been considered traditionally to be a Th1-mediated chronic disease (108). Recent findings have implicated IL-23 and the subset of T cells producing IL-17 (Th17) in the pathogenesis of experimental colitis (42-45). In contrast, regulatory T cells (Treg) play an immunosuppressive role and prevent colitis. The imbalance of regulatory and effector T cell induction is of primary importance in the etiology of the inflammation observed in multiple experimental IBD (17-21).

It is well known that Th1/Th17 differentiation is controlled by IL-12 family cytokines from activated DC and macrophages; both IL-12 and IL-23 have a primary role in the induction of intestinal inflammation in mouse models and humans (5, 109). IL-12 is vital for Th1 differentiation and has the ability to drive dysregulated Th1 response (5, 15). Naive T cells in mice are induced to initially differentiate to Th17 cells

in the presence of TGF-β and IL-6 with contributing roles for TNF-α and IL-1β (110, 111). Interestingly, Th17 differentiation is inhibited by IFN-γ and IL-4, demonstrating that Th17 cells are truly a separate lineage of T cells (112). Th17 cells also produce TNF-α, IL-6, IL-21 and IL-22, but not substantial levels of other typical Th1 or Th2 cytokines (IFN-γ, IL-4 or IL-5) (15, 112).

Dendritic cells (DC) are an important player in the gut immune response, serving as antigen presenting cells and cytokine producers. In vitro studies have demonstrated that prostaglandin E_2 (PGE_2) induces IL-23 and inhibits IL-12p70 production in DC, thereby promoting Th17 differentiation (47-49). In contrast, it has also been reported that $CD4^+CD25^+$ regulatory T (Treg) cells suppress effector T cell responses in a PGE_2-dependent fashion (50, 51). Therefore, any agents which down regulate PGE_2 levels would be expected to suppress Th17 differentiation. This is particularly relevant because *n-3* PUFA have been shown to suppress PGE_2 biosynthesis in experimental colitis-associated neoplasia (**Figure 5**, 52). In addition, data in Section 4 demonstrate that *n-3* PUFA can suppress STAT3 expression and activation in the colon mucosa. This is noteworthy because STAT3 is a key regulator of Th17 differentiation (113-115).

Therefore, we have hypothesized that *n-3* PUFA are capable of altering T cell polarization, thereby favorably modulating chronic inflammation in the intestine. To date, no studies have examined the effect of *n-3* PUFA on mucosal T effector cells. In these studies, *fat-1* mice were exposed to three rounds of DSS to test the hypothesis that the endogenous production of *n-3* PUFA protects against chronic injury/inflammation by modulating T cell polarization. Specifically, we determined how *n-3* PUFA influences

colonic mucosal; (a) chronic injury/inflammation, (b) Th17 and Treg differentiation, (c) PGE_2 production and (d) cytokine production.

3.2 Materials and methods

3.2.1 Animals and diet

fat-1 transgenic mice were generated and backcrossed onto a C57BL/6 background as previously described (69). The colony of *fat-1* mice used for this study was generated by breeding heterozygous mice and the genotype and phenotype of offspring were characterized as previously described (**Figures 1, 2 & 3**). All procedures followed the guidelines approved by Public Health Service Policy and the Institutional Animal Care and Use Committee at Texas A&M University. Specific pathogen-free animals were maintained under barrier conditions and fed a 10% safflower oil diet (Research Diets) ad libitum with a 12 h light/ dark cycle. The diet contained 40 (g/100 g diet) sucrose, 20 casein, 15 corn starch, 0.3 DL-methionine, 3.5 AIN 76A salt mix, 1.0 AIN 76A mineral mix, 0.2 choline chloride, 5 fiber (cellulose), 10 safflower oil.

3.2.2 Colitis induction and histological scoring

To induce chronic inflammation, 2.5% DSS (molecular weight, 36,000 to 50,000; MP Biomedicals) was administered in the drinking water for 5 d, followed by 16 d of tap water. This cycle was repeated twice (5 d of 2.5% DSS followed by a 16 d recovery period and 4 d of 2% DSS), and mice were terminated 10 d after completion of the final DSS cycle. Termination was scheduled to monitor the discrete inflammatory
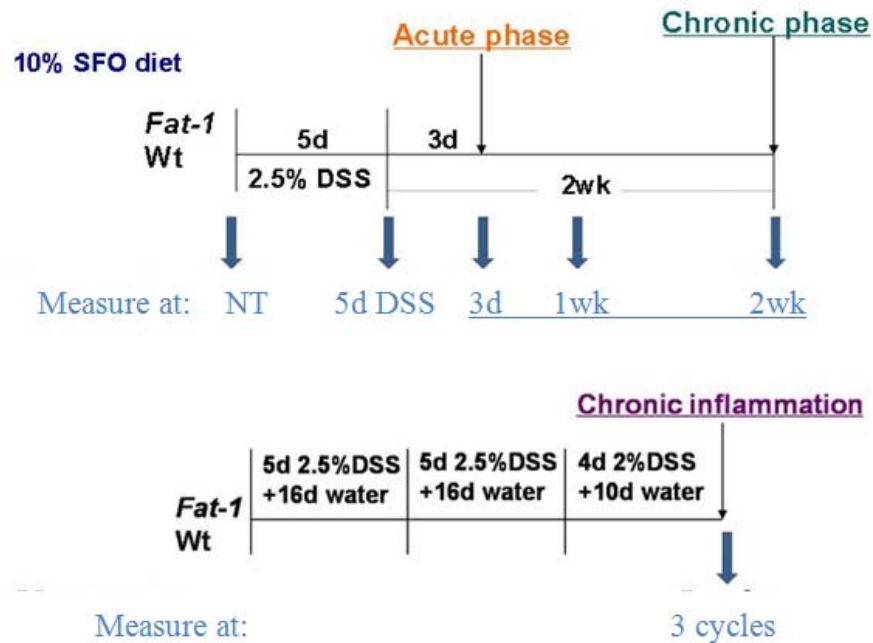
Figure 8. Experimental design for DSS induction of acute and chronic colitis. NT, no DSS treatment; 5d DSS, 5 day of DSS; 3d, 3 day after DSS; 1wk, 1 week after DSS; 2wk, 2 week after DSS; 3 cycles, 3 cycle of DSS treatment.

events following DSS exposure including 5 d of 2.5% DSS, 3 d recovery period followed, 1 wk recovery period followed, 2 wk recovery period followed, and the end of 3 cycles of DSS (**Figure 8**). At necropsy, the entire colon was removed, flushed with PBS, fixed in 4% paraformaldehyde and paraffin embedded. Sections were stained with hematoxylin and eosin. Histological examination was performed in a blinded manner by a board-certified pathologist and the degree of inflammation (score, 0–3) and epithelial injury (score, 0–3) in microscopic cross-sections of the colon was graded as previously described (104). Briefly, the presence of occasional inflammatory cells in the lamina

propria was assigned a value of 0; increased numbers of inflammatory cells in the lamina propria as 1; confluence of inflammatory cells, extending into submucosa, as 2; and transmural extension of the infiltrate as 3. For evaluation of epithelial injury, no mucosal damage was scored as 0; discrete lympho-epithelial lesions were scored as 1; surface mucosal erosion or focal ulceration was scored as 2; and extensive mucosal damage associated with the deeper structures of the bowel wall was scored as 3.

3.2.3 Isolation of splenic lymphocytes and colonic lamina propria lymphocytes (cLPL)

Splenic lymphocytes were initially isolated using Lympholyte M (Cedarlane) according to the manufacturer's instruction. Lamina propria lymphocytes were isolated from mouse colon as previously described with some modification (refer to the Methods section of Section 2) (91). Briefly, the colon was flushed clean and incubated in media containing Ca^{2+}, Mg^{2+} free HBSS (Sigma-Aldrich), 5 mM DTT and 30 mM Na_4EDTA at 37 °C, 100 rpm for 15 min. Subsequently, colons were placed on ice and gently scraped using a rubber policeman to remove intact crypts. Following a washing step in Ca^{2+}, Mg^{2+} free HBSS, the remaining tissue was cut into small pieces, and incubated in Ca^{2+}, Mg^{2+} free HBSS containing 1 mg/mL type II and type IV collagenase (Worthington) at 37 °C, 100 rpm for 1 hour. The digests were further processed in a Medimachine (BD Pharmingen) for 2 min to induce the release of T cells (**Appendix B2**). Liberated lamina propria cells were freely passed through a 30 μm Pre-Separation Filter (Miltenyi Biotec) and further purified by density gradient centrifugation in 40-70 % Percoll (Amersham)

in PBS. Lymphocytes enriched at the interface between a 40 and 70% Percoll gradient were collected.
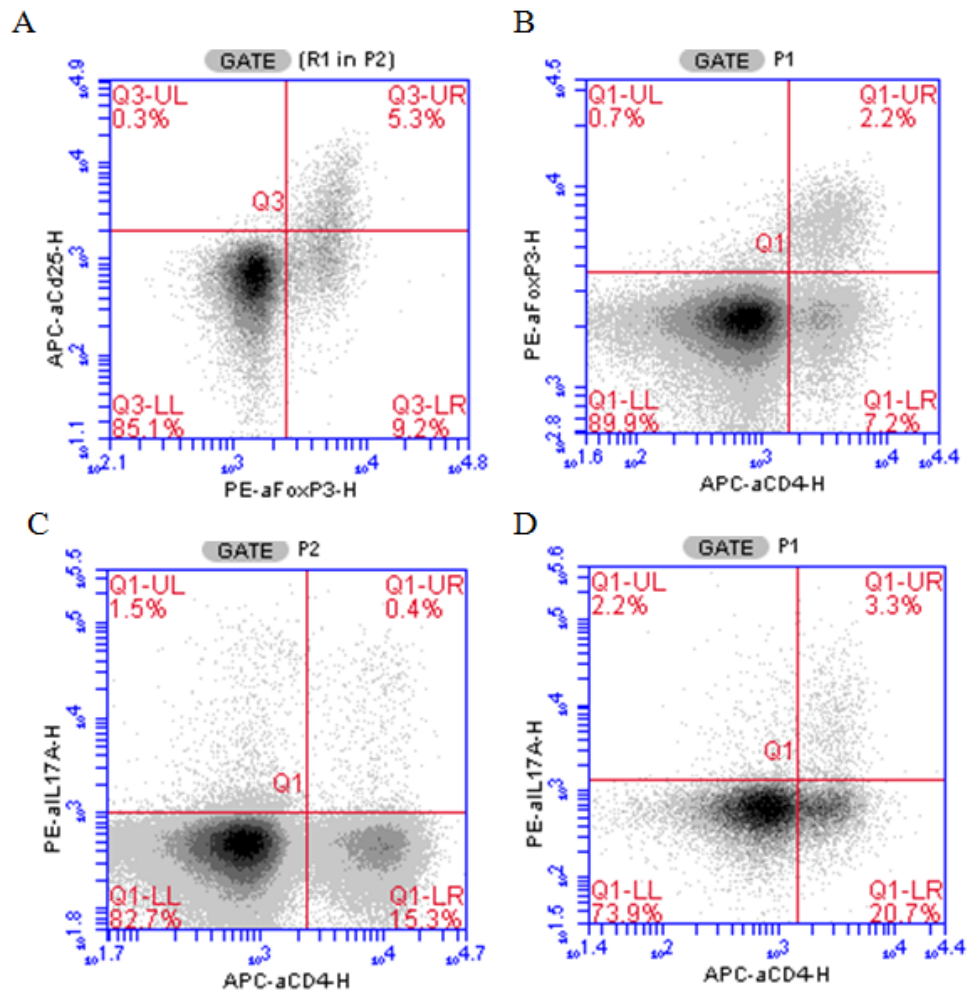
Figure 9. Quantification of splenic and colon lamina propria lymphocyte effector cells. A, splenic Treg cells were detected by flow cytometry using a triple staining procedure consisting of FITC-anti-CD4, APC-anti-CD25, PE-anti-FoxP3. B, colon lamina propria lymphocyte (cLPL) Treg cells were detected by double staining of APC-aCD4 and PE-aFoxP3. C, D, splenic Th17 and LPL Th17 cells were detected by double staining of APC-aCD4 and PE-aIL17A.

3.2.4 Flow cytometry analysis of Treg and Th17 cells

One million splenic and colon lamina propria lymphocytes obtained using the isolation procedure described above were preincubated with an FcγR blocking monoclonal mAb (1 µg/mL) (2.4G2, BD Pharmingen) for 10 min on ice. To measure the proportion of Th17 and Treg cells, surface staining of CD4 and CD25 was conducted by incubation with fluorescein isothiocyanate (FITC)-labeled anti-CD4 (5 µg/mL) (RM 4-5, hamster IgG1, eBioscience) and allophycocyanin (APC)-labeled anti CD25 (2.5 µg/mL) (PC61.5, rat IgG2b, eBioscience) or allophycocyanin (APC)-labeled anti-CD4 (5 µg/mL) (RM 4-5, hamster IgG1, eBioscience) for 30 min at 4 °C. Intracellular staining was performed following cell permeabilization. Specifically, phycoerythrin (PE)-labeled anti-FoxP3 (5 µg/mL) (FJK-16s, rat IgG2a, eBioscience) or phycoerythrin (PE)-labeled anti-IL-17A (5 µg/mL) (TC11-18H10, rat IgG1κ, BD Pharmingen) were incubated for 30 min at 4 °C. Flow cytometric analysis was conducted on an Accuri C6 flow cytometer (Accuri) and analyzed using the C6 analysis program. Splenic Treg cells were detected using anti-CD4, anti-CD25 and anti-FoxP3 staining; cLPL Treg cells were labeled using anti-CD4 and anti-FoxP3 staining; splenic and cLPL Th17 were detected by anti-CD4 and anti-IL-17 staining (**Figure 9, Appendices B3, B4, & B5**).

3.2.5 Enrichment of splenic CD11c$^+$ dendritic cells and cell culture

To obtain sufficient cell numbers, spleens were pooled from 4-5 animals (equal numbers of males and females represented) to obtain final samples sizes comprising 3 and 4 pooled samples in the wild-type and *fat-1* groups, respectively. Splenic

mononuclear cell suspensions were obtained by collagenase digestion (116) followed by

discontinuous centrifugation as described previously (117). The collagenase digestion

solution utilized consisted of 1 g/L of collagenase D (Roche Diagnostics) dissolved in

sterile complete 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". Subsequently the resultant cell population was incubated with

FcγR blocking monoclonal mAb (2.4G2, BD Pharmingen) as per manufacturers'

instructions prior to dendritic cell enrichment based on surface expression of CD11c

using magnetic beads conjugated with anti-CD11c (N418, hamster IgG; Miltenyi Biotec

Aubrun, CA). Cell numbers were assessed using a neubauer chamber and cell viability

post-enrichment column was assessed by trypan blue exclusion and exceeded 87%.

Following enrichment with CD11c microbeads (Miltenyi Biotec), cells were sorted on a

BD FACS Aria II flow cytometer based on a high surface expression of CD11c. Surface

staining for CD11c was conducted initially by FcγR block (2.4G2, BD Pharmingen)

followed by surface staining with phycoerythrin (PE)-conjugated hamster anti-mouse

CD11c (HL3, BD Pharmingen), utilizing a different clone then that recognized by the

CD11c microbeads. Purified CD11c^{hi} and CD11c^{lo} expressing cell populations were

collected at an average purity exceeding 95% (assessed using Accuri C6 flow cytometer)

and viability by trypan blue exclusion always exceeds 96%. Positively sorted CD11c^{hi}

and CD11c^{lo} expressing cells were cultured in 96-well flat bottom Falcon plates (Becton-

Dickinson Labware). Each well contained 2×10^5 viable cells that were cultured for 24 h

at 37°C in sterile complete RPMI. Half of the wells were exposed to lipopolysaccharide (LPS, *E. coli* 055:B5, Sigma Aldrich) to achieve a final concentration of 10 μg/mL in each well. Unstimulated culture wells received an equivalent volume of complete RPMI. Cell culture conditions were repeated in a minimum of two wells and the resultant supernatant from each culture well was pooled, aliquoted for cytokine analysis and stored at -80ºC.

3.2.6 Cytokine analysis of cell culture supernatants

Luminex xMAP technology was utilized to measure in vitro cytokine concentrations using the Bio-Plex 200 System (BioRad) and accompanying software package, Bio-Plex Manager 6.0 (BioRad). IL-1ß, IL-6, IL-10, IL-12p40, IL-17, IFN-γ and TNF-α were simultaneously measured utilizing the Bio-Plex Pro Mouse Cytokine Group I multiplex kit (BioRad). Additionally, Milliplex MAP kits (Millipore) were utilized to individually measure IL-23 (MPXMCYP3-74K), TGF-ß1 (TGFB-64K-01) and IL12p70 (MPXMCYTO-70K). With respect to the analysis of TGF-ß1, culture supernatants were acid activated as per the manufacturers' instructions. For all cytokine assessments, samples were run in duplicate.

3.2.7 Statistics

Data were expressed as mean ± SE. Differences between experimental groups were analyzed using ANOVA from SPSS software package. *p*-values < 0.05 were accepted as significant.

3.3 Results

3.3.1 fat-1 mice are less susceptible to DSS-induced chronic inflammation

Intestinal inflammation was induced by exposing mice to 3 cycles of DSS treatment over 8 weeks as described in the Methods. Histological evaluation was subsequently performed following repeated mucosal injury and healing in order to access immune cell infiltration and epithelial injury at the end of the 8-week protocol (**Figure 10**). *fat-1* mice exhibited a significantly lower inflammatory score (1.25±0.18 vs 1.60±0.16, *P*=0.04) and injury score (1.25±0.22 vs 1.93±0.18, *P*=0.03) in comparison to wt mice. These data are consistent with the enhanced long-term resolution of inflammatory processes and mucosa repair in mice with elevated *n-3* PUFA.
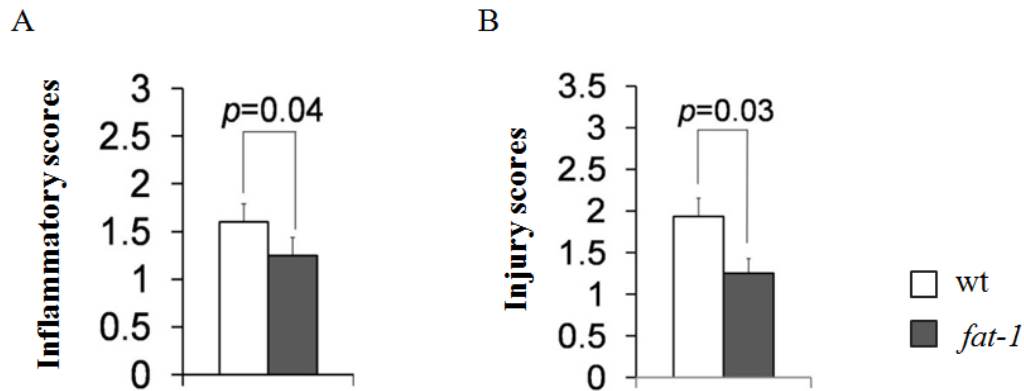
Figure 10. *n-3* PUFA suppress DSS-induced mucosal injury and chronic inflammation. Wt and *fat-1* transgenic (tg) mice were treated with 3 cycles of DSS to induce chronic colitis. A, inflammatory score; B, injury score. n=12-15 mice per treatment.
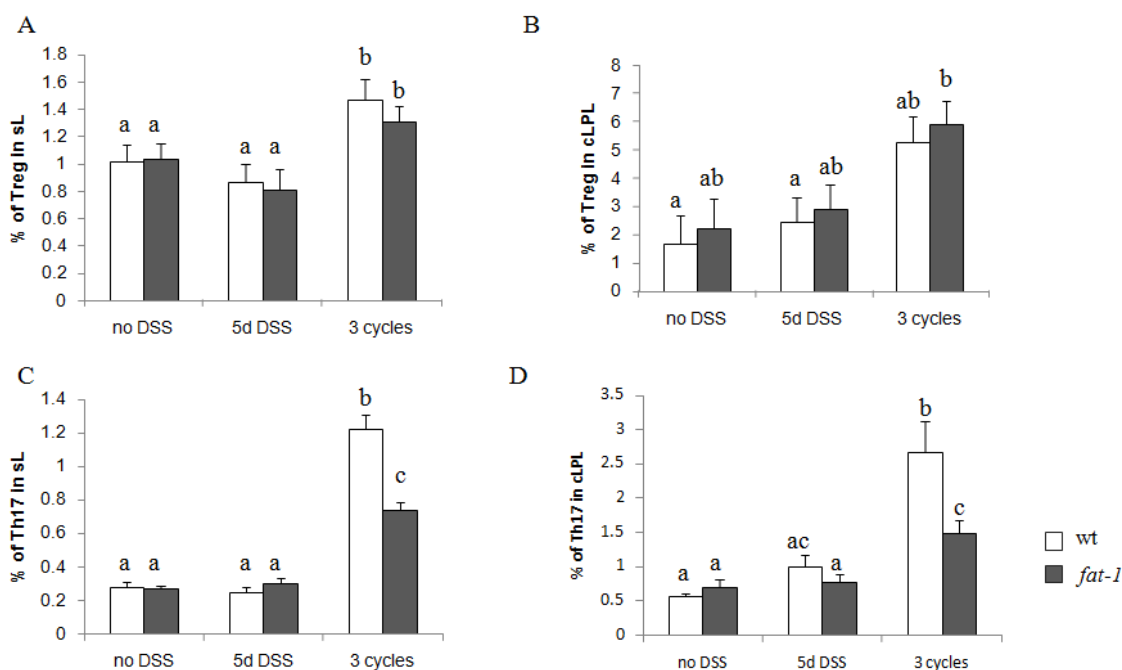
Figure 11. DSS exposure alters Treg and Th17 polarization in *fat-1* and wt mice. Wt and *fat-1* transgenic (tg) mice were treated acutely (5 days) and chronically (3 cycles) with DSS to induce acute and chronic colitis respectively. A, Percentage of $CD4^+CD25^+FoxP3^+$ Treg cells in splenic lymphocytes. B, Percentage of $CD4^+FoxP3^+$ Treg cells in colonic lamina propria (cLP) lymphocytes. C, Percentage of $CD4^+IL17A^+$ Th17 cells in splenic lymphocytes. D, Percentage of $CD4^+IL17A^+$ Th17 cells in cLP lymphocytes. Values not sharing a common letter are significantly different (p<0.05). No DSS, no DSS treatment (n=5 mice); 5 d DSS, 5 days of DSS treatment (n=6-8 mice); 3 cycles, 3 cycles of DSS treatment (n=6-7 mice).

3.3.2 n-3 PUFA alter mucosal Treg and Th17 polarization following DSS exposure

Mice were terminated at 6 time points for the purpose of temporally monitoring discrete inflammatory events following both acute and chronic DSS exposure. The experimental design is shown in **Figure 8**. Both spleen and colon lamina propria lymphocytes were isolated to detect Treg and Th17 effector cell populations by flow cytometry at each of the time points (**Figure 9**). The results demonstrate that DSS

exposure increased both Treg and Th17 portions in colon lamina propria (**Figures 11-13**). Interestingly, *n-3* PUFA differentially affected cells at the acute (5 d DSS) vs chronic (3 cycles) treatment stages. Specifically, Treg and Th17 polarization at the acute inflammation phase following 5 days of DSS exposure was similar in *fat-1* vs wt mice and not changed compared to no treatment animals (**Figure 11 A-D**). In contrast, during chronic inflammation (3 cycles of DSS), *fat-1* mice exhibited a significantly lower percentage of Th17 cells in both spleen and colon lamina propria (**Figures 11 C, D, Figures 12 C, D**). In addition, a combined calculation (pooling the 4 time points after
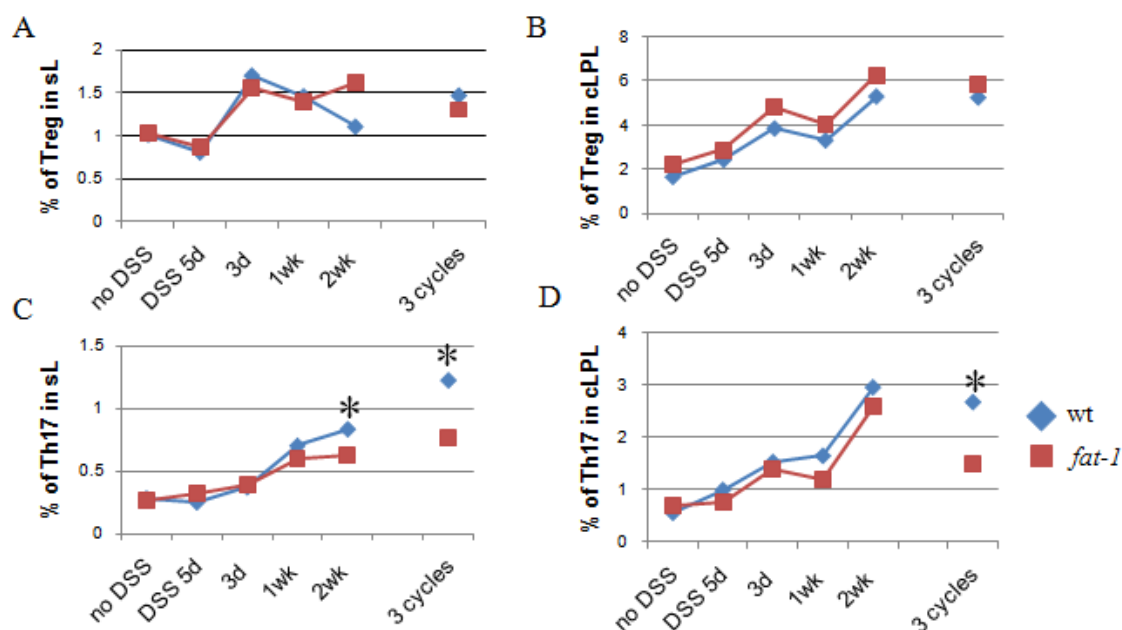
Figure 12. Temporal analysis of DSS effects on Treg and Th17 polarization in *fat-1* and wt mice. A, Percentage of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells in splenic lymphocytes (sL). B, Percentage of CD4$^+$FoxP3$^+$ Treg cells in colonic lamina propria lymphocytes (cLPL). C, Percentage of CD4$^+$IL17A$^+$ Th17 cells in sL. D, Percentage of CD4$^+$IL17A$^+$ Th17 cells in cLPLs. Asterisk indicates significant difference between wt and tg groups at the indicated time point, $p<0.05$.

initial DSS exposure, designated as "with DSS") demonstrated that the percentage of

CD4$^+$FOXP3$^+$ Treg cells in colon lamina propria lymphocytes was higher in *fat-1* as

compared to wt mice (5.74±0.53 vs 4.39±0.59, *P*=0.044) (**Figure 13B**), but the

percentage of Th17 cells in colon lamina propria lymphocytes was down regulated to

pretreatment level only in *fat-1* (**Figure 13D**). A summary of the temporal comparison

between responses of Th17 and Treg cells is shown in **Figure 12**. These data indicate

that both systemic (spleen) and local (colonic lamina propria) Th17 polarization are
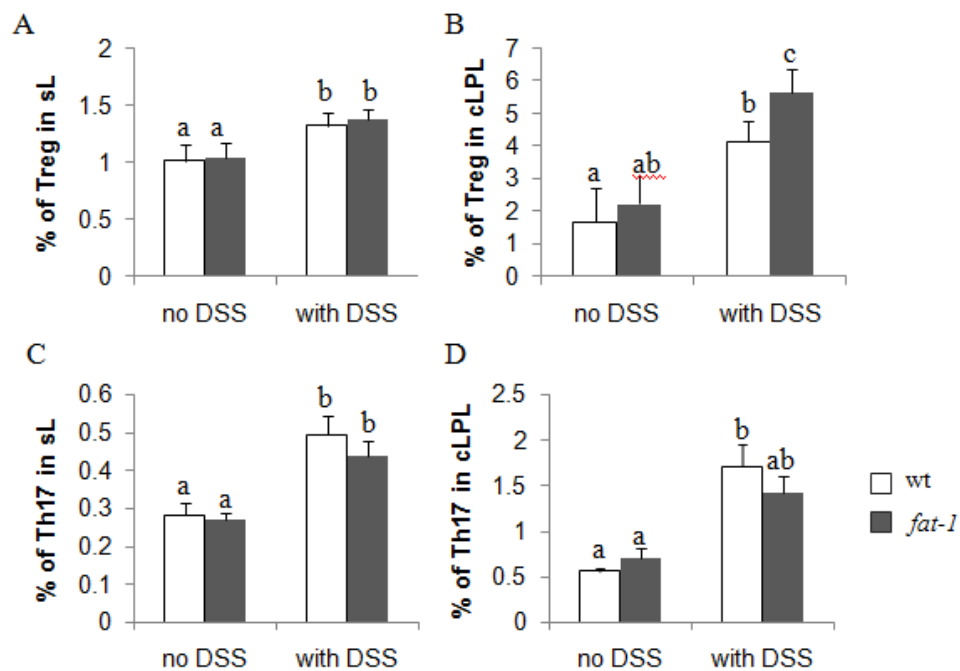
Figure 13. DSS exposure alters Treg and Th17 polarization in spleen and colon. A,
Percentage of CD4$^+$CD25$^+$FoxP3$^+$ Treg cells in splenic lymphocytes (sL). B, Percentage
of CD4$^+$FoxP3$^+$ Treg cells in colon lamina propria lymphocytes (cLPL). C, Percentage
of CD4$^+$IL17A$^+$ Th17 cells in splenic lymphocytes. D, Percentage of CD4$^+$IL17A$^+$ Th17
cells in cLPL. Values not sharing a common letter are significantly different (*p*<0.05).
No DSS (n=4-6 mice), with DSS (n=21-28 mice), includes acute (5 d) and chronic (3
cycles) DSS exposure.

modulated by *n-3* PUFA. These observations are consistent with the reduced inflammatory score observed in the *fat-1* mice (**Figure 10A**), and demonstrate for the first time that *n-3* PUFA favorably alter the Treg and Th17 populations in the colon following DSS exposure.
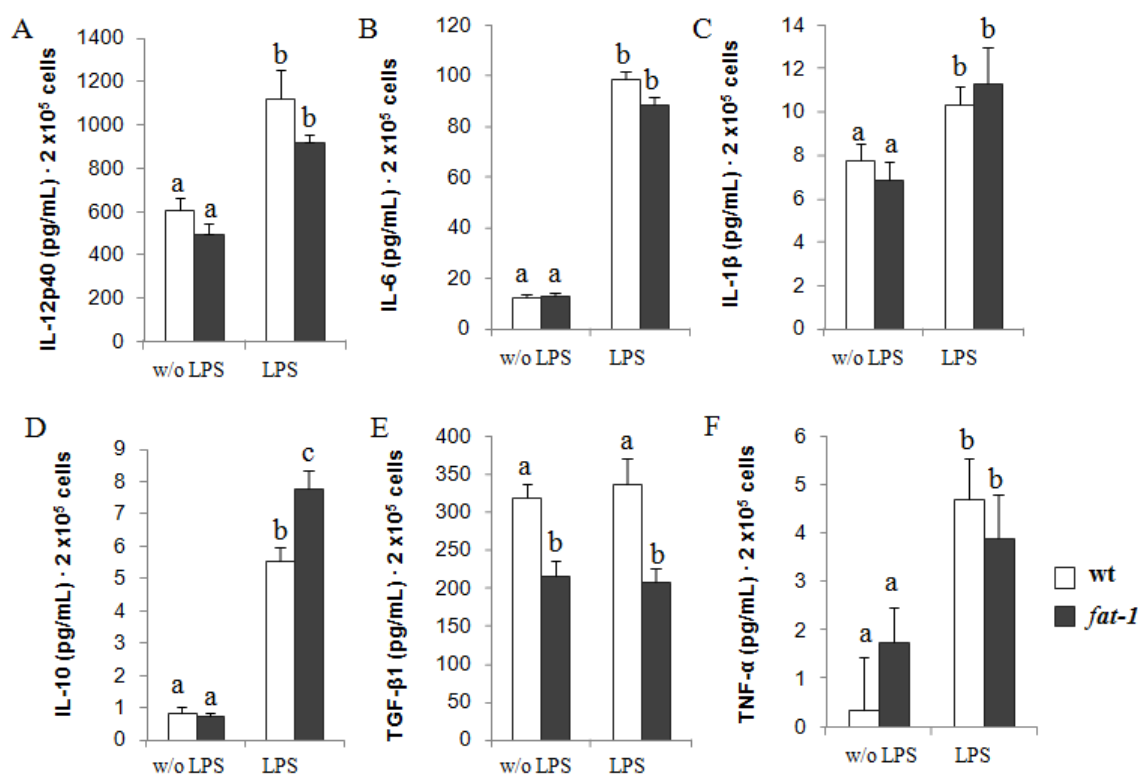
Figure 14.   Effect of DSS and *n-3* PUFA on spleen dendritic cell cytokine production. Wt and *fat-1* transgenic (tg) mice were treated with 3 cycles of DSS to induce chronic colitis. Purified splenic dendritic cells were cultured for 24 h with or without 10 μg/ml LPS. Culture supernatant was analyzed for A, IL-12p40, B, IL-6, C, IL-1β, D, IL-10, E, TGF-β1, F, TNF-α. n=3-4 analysis. Bars labeled with different letters indicate significant difference (p<0.05) between means, w/o LPS; LPS, 10 μg/mL LPS.
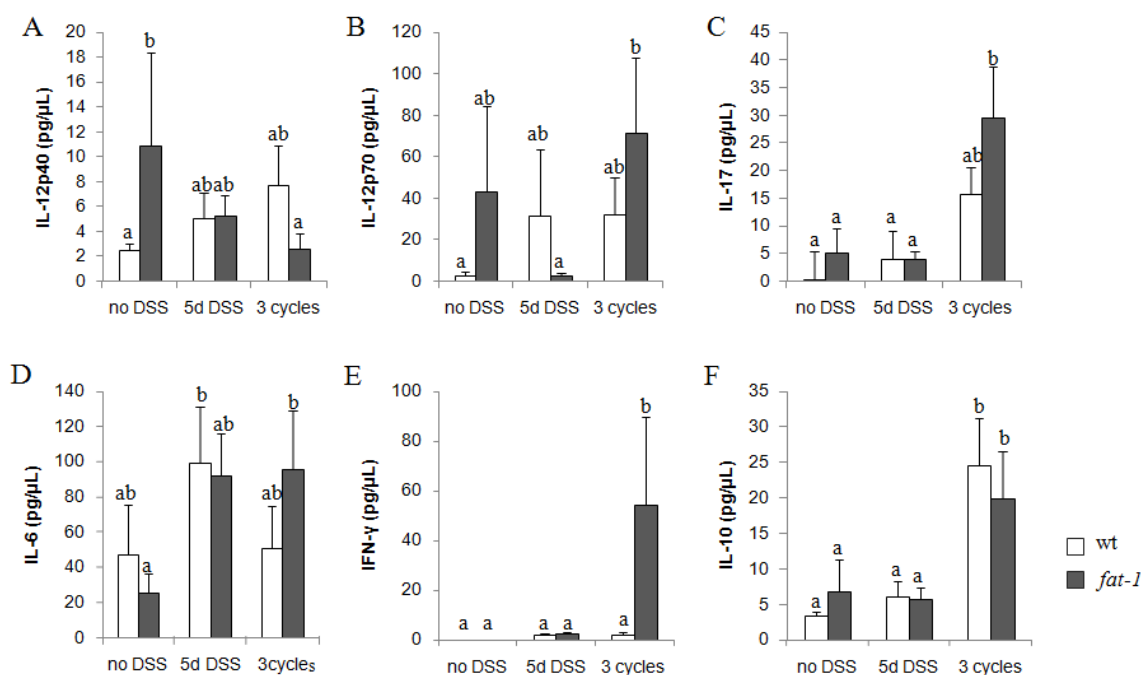
Figure 15. DSS and *n-3* PUFA alter serum cytokine levels. Wt and *fat-1* transgenic (tg) mice were treated for 5 days or 3 cycles of DSS to induce acute and chronic colitis, respectively. A, IL-12p40, B, IL-12p70, C, IL-17, D, IL-6, E, IFN-γ, and F, IL-10. Values not sharing a common letter are significantly different ($p<0.05$). No DSS, no DSS treatment (n=6 mice); 5 d DSS, 5d of DSS treatment (n=10-11 mice); 3 cycles, represents 3 cycles of DSS treatment (n=11 mice).

3.3.3 *n-3* PUFA alter spleen CD11c^{+} dendritic cell cytokine production

Since *n-3* PUFA affect T cell and macrophage infiltration into colon lamina propria (**Figure 6, 7**), we addressed whether dendritic cell function is affected by *n-3* PUFA. For this purpose, splenic CD11c^{+} dendritic cells from both *fat-1* and wt mice were purified by cell sorting (118, 119) and cultured for 24 h for the purpose of measuring cytokine production. Interestingly, no significant difference was observed in IL-12p40 level between wt and *fat-1* (**Figure 14A**), while IL-12p70 and IL-23p19 were

below detectable levels. Following LPS stimulation to induce maximal cytokine production, sorted CD11c$^+$ cells from *fat-1* mice produced higher IL-10 levels as compared to wt mice (**Figure 14E**). This is noteworthy, because IL-10 is considered an anti-inflammatory cytokine capable of suppressing inflammation of the bowel (5). With respect to putative proinflammatory cytokines, TGF-ß levels were suppressed in *fat-1* cultures with or without LPS stimuli (**Figure 14D**). TGF-ß is known as a key regulator for Treg and Th17 polarization although the mechanism remains unclear (5, 120-122). Collectively, these data indicate that *n-3* PUFA alter dendritic cell cytokine production in a manner consistent with the suppression of Th17 cell polarization. With regard to serum cytokine analyses, there was no consistent effect of *n-3* PUFA on systemic cytokine levels (**Figure 15**). Circulating IL-10 levels were elevated following repeated cycles of DSS consistent with previous reports (13).

3.4 Discussion

The gut mucosa has evolved multiple innate and adaptive mechanisms to maintain tolerance in the face of a tremendous number of commensal organisms that impact host metabolism (4, 5, 123). A failure of these mechanisms to prevent bacterial invasion and abnormal intestinal inflammation, however, may contribute to the pathogenesis of inflammatory bowel diseases (4, 123). Treg cells have been identified as central mediators of intestinal inflammation. For example, adoptive co-transfer of CD25$^+$CD45RB^{lo} Treg cells along with naïve CD4$^+$ CD45RB^{hi} T cells to immunodeficient SCID or RAG-deficient mice prevent colitis exhibiting characteristics

of intestinal inflammation resembling human IBD (4, 5). Moreover, an imbalance of regulatory and effector T cell induction is of primary importance in the etiology of the inflammation observed in multiple mouse models of IBD (4, 5, 21, 22). From a dietary perspective, our data indicate that Treg cell differentiation is elevated by *n-3* PUFA following chronic DSS treatment. Therefore, an increase of Treg polarization should be considered as a potential mechanism by which *n-3* PUFA ameliorate intestinal inflammation as exemplified by decreased inflammatory scores (**Figure 10A**). The observed increase of the Treg population following DSS exposure is consistent with a recent report that dietary fish oil fed mice exhibit higher T reg cell frequency in a colitis model of *Helicobacter hepaticus* infection in SMAD3-/- mice (124).

Traditionally, IBD pathogenesis was attributed to the dysregulated induction of Th1 differentiation in response to commensal bacteria in the face of poor control by Tregs. However, the importance of IL-23 in driving pathogenic intestinal inflammation in mice was recently reported (11, 45). IL-23 is an IL-12 family member containing the IL-12p40 chain and a unique IL-12p19 chain produced from activated dendritic cells and macrophages (5, 125). In several animal models of colitis, IL-23 was essential for driving inflammation with increased production of proinflammatory cytokines including IL-6, IFN-γ, IL-1β, IL-17 and TNF-α in the intestine and was shown to play a vital role in the development of Th17 cells (125, 126). Th17 cells produce IL-17A (synonymous with IL-17) and IL-17F as well as TNF-α, IL-6, IL-21 and IL-22, but not substantial levels of other typical Th1 or Th2 cytokines (IFN-γ, IL-4 or IL-5) (126). Our results show that both systemic and local Th17 differentiation is suppressed in fat-1 mice during

the chronic phase of the DSS induced wounding/repair/colitis (**Figures 11 C, D, Figures 12 C, D**). These data demonstrate for the first time that a bioactive food component, *n-3* PUFA, can modulate Th17 polarization in the DSS disease model. Previous data have shown that *n-3* PUFA dampen the persistent inflammation and immune activation that are associated with DSS-induced mucosal ulceration, thereby suppressing epithelial carcinogenesis (**Figure 2-7**). The current data broaden the impact of *n-3* PUFA by documenting its ability to alter T cell polarization.

IL-17 activates NF-κB and MAPK pathways and stimulates the production cytokines (including TNF-α and IL-6) and chemokines (including MCP-1, IL-8, KC and MIP-2) from epithelial cells, endothelial cells, intestinal myofibroblasts and macrophages, leading to inflammation (127, 128). Research from animal models has demonstrated that IL-23 is essential for IBD induction (125, 126). In addition, IL-17 and IL-23 were detected in colonic biopsies from humans with Crohn's disease, and their level could be suppressed with anti-IL-12p40 therapy (129, 130). Moreover, IL-17 levels in the serum were elevated in patients with Crohn's disease and animal models of colitis (13, 129). Although the levels of serum cytokines were not affected by *n-3* PUFA in our study, the profile was similar to previous reports (13, 129, 130). In contrast, cytokine levels in splenic dendritic cell cultures were impacted in *fat-1* mice, resulting in lower levels of TGF-β1. This is noteworthy, since T cells in mice initially differentiate to Th17 cells in the presence of TGF-β and IL-6, with contribution from TNF-α and IL-1β (125, 126). The decreased TGF-β level in *fat-1* mice may contribute to the suppressed Th17 differentiation observed in the intestine.

Th17 and Treg cells modulate the pathology of IBD. We are the first to show that *n-3* PUFA, which are incorporated into both colonocytes and T-cells, enhance the resolution of chronic inflammation, in part, by altering T cell polarization. Dendritic cells, innate antigen presenting immune cells in the gut that are essential for regulating both the innate and adaptive immune responses, were also affected by *n-3* PUFA. Further investigation of the DC-T cell axis in *fat-1* mice will elucidate the mechanism(s) underlying the immunomodulatory ability of *n-3* PUFA.

4. DIETARY FISH OIL AND CURCUMIN COMBINE TO MODULATE COLONIC

CYTOKINETICS AND GENE EXPRESSION IN DSS-TREATED MICE*

4.1 Introduction

Based on current understanding of the pathogenesis of IBD, activated immune cells can destroy the intestinal barrier either directly via cytotoxicity or indirectly through the release of cytokines, reactive oxygen species and other metabolites (131). Although the effects of *n-3* PUFA on susceptibility to colitis and colon cancer have not been adequately determined to date, it has been demonstrated that *n*-3 PUFA can inhibit NF-kB activation (29, 33). This is significant because NF-kB can modulate several steps in the inflammatory cascade by inducing the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IFN-γ, IL-12, COX-2 and iNOS (35, 132), and is required for proliferation/apoptosis homeostatic regulation and protection from acute inflammation in the intestine (7, 36).

Curcumin, a natural polyphenol isolated from the dried rhizomes of *Curcuma longa* (turmeric), has been shown to ameliorate inflammation associated with experimental and human colitis (56-59, 133). These data suggest that select dietary polyphenolics may favorably modulate inflammatory responses in the colon. The chemoprotective effects of curcumin appear to be mediated, in part, through NF-kB

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inhibition (57-60). Therefore, in this study, we evaluated the effect of curcumin supplementation, in the presence or absence of dietary *n-3* PUFA, on NF-kB activation and the resolution of chronic inflammation in the mouse colon.

DSS mouse model has been used as a method to mimic the mucosal injury and repair observed in the human disease (70, 71). Typically, repeated cycles of DSS treatment are used to create the microenvironment of chronic inflammation in the colon (70-72, 134). DSS is directly toxic to crypt cells and the crypt loss may precede the immune response (70). DSS induced colitis in mice represents clinical and histological characteristics of human UC (70-72). The susceptibility to DSS varies between mouse strains by the interplay between the causative factor and genetics (70, 71). In this study, we exposed C57BL/6 mice to either 1 or 2 cycles of DSS treatment to induce acute and chronic inflammation, respectively. We determined how dietary *n-3* PUFA and curcumin influence: (i) mortality, (ii) colonic inflammation and injury scores, (iii) epithelial cytokinetics, e.g., apoptosis and proliferation, (iv) NF-kB activation, and (v) global mucosal gene expression.

4.2 Materials and methods

4.2.1 Animals and diet

Six to eight week old C57BL/6 male mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed in a temperature and humidity controlled facility with a 12 h light/ dark cycle. All procedures followed the guidelines approved by Public Health Service Policy and the Institutional Animal Care and Use Committee at

Table 4. Experimental diet composition.

		g/100g diet		
			CO+ 2% Curcumin	FO+ 2% Curcumin
	Corn oil (CO)	Fish oil (FO)	(COcur)	(FOcur)
Casein	20	20	20	20
Sucrose	42	42	40	40
Corn Starch	22	22	22	22
Cellulose	6	6	6	6
Mineral	3.5	3.5	3.5	3.5
Vitamin Mix	1	1	1	1
Methionine	0.3	0.3	0.3	0.3
Choline Cl	0.2	0.2	0.2	0.2
Corn Oil	5	1	5	1
Fish Oil	-	4	-	4
Curcumin	-	-	2	2
Total (g)	100	100	100	100

C57BL/6 mice were fed the experimental diets throughout the study. All diets contained 5% fat w/w, with or without 2% curcumin. The fish oil diet contained 4% vacuum deodorized menhaden fish oil (FO). Curcumin C3 complex contained ~ 80% curcumin.

Texas A&M University. After one week of acclimatization on standard pelleted diet, animals were randomly grouped (n=15) and fed ad libitum 1 of 4 isocaloric diets (2 x 2 design) (**Table 4**): corn oil (CO) diet containing 5% corn oil, fish oil (FO) diet containing 4% fish oil and 1% corn oil, corn oil plus curcumin diet (CO-curcumin) containing 5% corn oil and 2% curcumin, fish oil plus curcumin diet (FO- curcumin) containing 4% fish oil, 1% corn oil and 2% curcumin. The diets contained 42 (g/100 g diet) sucrose, 20 casein, 22 corn starch, 0.3 DL-methionine, 3.5 AIN 76A salt mix, 1.0 AIN 76A mineral mix, 0.2 choline chloride, 6 fiber (cellulose), 5 fat. The level of curcumin (2%) has been shown to modulate disease pathology in the DSS mouse model

(133), and the relative dose is comparable to levels previously used in a human clinical trial (135). To prevent formation of oxidized lipids, diets were stored at -20°C and provided fresh to animals each day. Corn oil was obtained from Dyets (Bethlehem, PA) and fish oil was obtained from Omega Protein (Houston, TX). Curcumin C3 complex which contains 80% curcumin, 3% bisdemethoxy curcumin and 17% demethoxy curcumin was provided by Sabinsa Corporation (Pason, UT). Heavy metals, including arsenic and lead, were below the limits of detection.

4.2.2 Colitis induction and histological scoring

Animals were fed experimental diets one week prior to DSS (molecular weight, 36,000 to 50,000; MP Biomedicals) treatment (**Figure 16**). To induce chronic inflammation, 2.5% DSS was administered in the drinking water for 5 d, followed by 16 d of tap water. This cycle was repeated with a lower dose of DSS (3 d of 1.5% DSS followed by a 14 d recovery period) after which mice were euthanized humanely. Acute inflammation was induced by 5 d of 2.5% DSS, followed by a 3 d recovery period. At each necropsy interval, the entire colon was removed, flushed with PBS, fixed in 4% paraformaldehyde and paraffin embedded. Sections were stained with hematoxylin and eosin. Histological examination was performed in a blinded manner by a board-certified pathologist and the degree of inflammation (score, 0–3) and epithelial injury (score, 0–3) in microscopic cross-sections of the colon was graded as previously described in Section 2. Briefly, the presence of occasional inflammatory cells in the lamina propria was
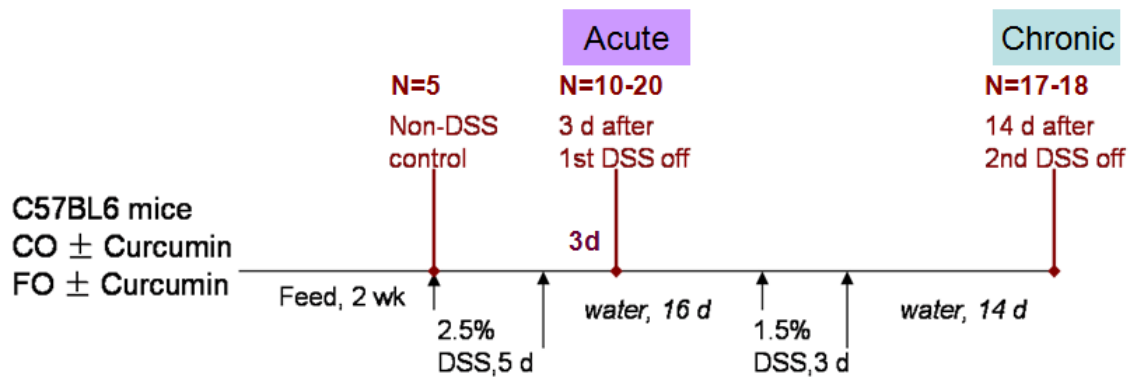
Figure 16. Experimental design involving acute and chronic inflammatory models. C57BL/6 mice were fed experimental diets 2 wk prior to DSS (Dextran Sodium Sulfate) exposure. A group of animals were terminated prior to DSS exposure (non DSS control). For the acute inflammatory model, a second group was terminated after treatment with 2.5% DSS for 5 d followed by a 3 d recovery period. In addition, for the purpose of inducing chronic colitis, mice were exposed to 2 cycles of DSS followed by a 16 d recovery period.

assigned a value of 0; increased numbers of inflammatory cells in the lamina propria as 1; confluence of inflammatory cells, extending into submucosa, as 2; and transmural extension of the infiltrate as 3. For evaluation of epithelial injury, no mucosal damage was scored as 0; discrete lympho-epithelial lesions were scored as 1; surface mucosal erosion or focal ulceration was scored as 2; and extensive mucosal damage associated with the deeper structures of the bowel wall was scored as 3.

4.2.3 In situ apoptosis and proliferation measurement

Intestinal apoptosis was measured in paraformaldehyde-fixed, paraffin-embedded tissues using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end (Oncor, Dallas, TX) labeling (TUNEL) method (34). Cell proliferation was

measured following BrdU injection (Zymed, South San Francisco, CA) as previously described (90).

4.2.4 Assessment of NF-kB activity

NF-kB activation was measured by quantifying p65/Rel A activation as previously described (64). In brief, whole cell protein from snap frozen colonic mucosa scraping was extracted using a Nuclear Extraction Kit (Active Motif, Carlsbad CA) and subsequently incubated with oligonucleotides which comprise the NF-kB consensus DNA-binding site (5'-GGGACTTTCC-3') to detect activated p65/Rel A.

4.2.5 Total RNA isolation

At each necropsy interval, mucosa scrapings from the colon were stored in mirVana™ reagent at -80 ˚C. Total RNA was extracted from each sample using mirVana™ miRNA Isolation Kit (Ambion, Austin TX) according to the manufacturer's instructions. The concentration and quality of the total RNA were assessed using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), respectively.

4.2.6 Codelink mouse whole genome microarray assay

Quadruplicate total RNA samples (including non DSS, corn oil fed control) were processed to generate biotin-labeled cRNA via a modified Eberwine RNA amplification protocol using a CodeLink iExpress Kit (Applied Microarray, Tempe, AZ).

Labeled cRNA was applied to CodeLink Mouse Whole Genome Bioarrays (Applied Microarray, Tempe, AZ), which contain 34,967 unique probe sequences. After incubation, slides were washed, stained, and scanned by Gene Pix. Array images were processed using CodeLink system software version 5.0. The resulting files were imported into zRMicroArray (136) for analysis. After median and log transformation, the gene expression was examined for normality (Shapiro–Wilk test) of the empirical distribution within each experimental group. Only genes that passed the significance test ($p < 0.05$) were considered for subsequent analyses (137).

4.2.7 Immunohistochemistry of pSTAT3

4 μm thick sections were cut from paraffin embedded colon "swiss rolls" for immunohistochemistry of pSTAT3tyr705 (Cell Signaling, Danvers, MA). In brief, tissue sections were deparaffinized and antigen retrieval was performed using EDTA buffer, pH 8. Primary antibody (1:50) was incubated overnight at 4°C followed by addition of Signal Stain Boost IHC Detection Reagent (HRP, Rabbit) (Cell Signaling) for 30 min. Slides were developed using DAB. Staining of pSTAT3 was quantified using a Nikon Cool-SNAP camera and NIS elements software. Four representative stained areas (hot spots) were selected from distal, middle and proximal regions of the colon, corresponding to a total of 12 images from each slide per animal. Stained areas in the mucosa within the intensity threshold were recorded. On average, 12 images per animal were read in a blinded fashion.

4.2.8 Statistics

Data are expressed as mean ± SE. Differences between experimental groups were analyzed using ANOVA from SPSS software package and ANOVA R-package which is embedded in zRMicroArray. One tailed *p*-values < 0.05 were accepted as significant.

4.3 Results

4.3.1 Body weight and colon length

Animal body weight and colon length were recorded at termination points. Body weight dropped significantly following acute DSS treatment but no differences were observed between diets (**Figure 17A**). Similarly, colon length was shortened significantly following both acute and chronic DSS treatment, although no differences between diets were detected (**Figure 17B**).

4.3.2 Dietary FO and curcumin, independently and in combination, enhance acute DSS induced mortality

Animal mortality was determined following both acute (1 cycle) and chronic (2 cycles) DSS exposure. The CO diet was used as a baseline. Therefore, curcumin supplementation to the CO diet (COcur) represents the isolated effect of curcumin. The FO diet represents the effect of fish oil alone, while the FO plus curcumin (FOcur) diet represents the combined effects of FO and curcumin. During the acute inflammatory

A

B
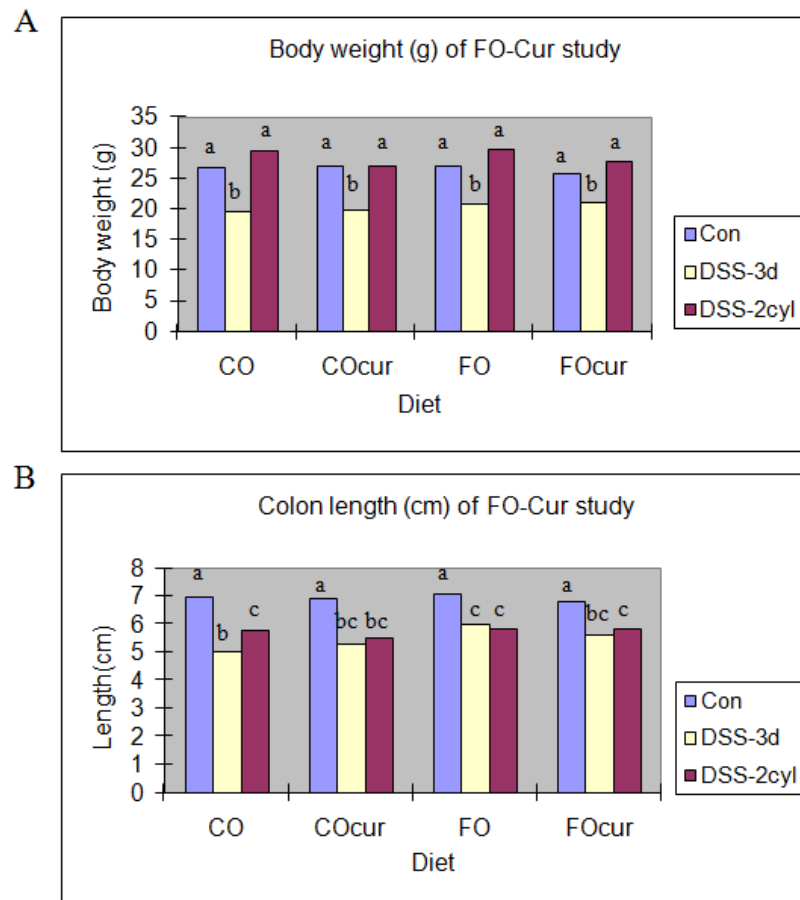
Figure 17. Body weight and colon length. Bars labeled with different letters indicate a significant difference (p<0.05) between means. Con, control/no DSS; DSS-3d, 1 cycle of DSS treatment, 3 day recovery following 5 day DSS exposure; DSS-2cyl, 2 cycles of DSS treatment.

phase (**Figures 18A & C**), FO fed mice exhibited the highest mortality (40%, 17 of 43) compared to CO with the lowest (3%, 1 of 29, *p*=0.0008). Addition of curcumin to CO increased (*p*=0.003) mortality to 37% (13 of 35). FO and curcumin combination treatment also increased (*p*=0.03) mortality to 28%. These results demonstrate that FO or curcumin supplementation increase the mortality rate following acute DSS exposure. Mice surviving acute colitis and exposed to a second round of DSS (chronic phase), died

infrequently (CO, 2 of 17, FO, 1 of 10, COcur, 1 of 11, FOcur, 2 of 14) and exhibited no

effect of diet (**Figures 18B & D**). These data indicate that *n-3* PUFA and curcumin

supplementation exacerbate susceptibility to DSS-induced acute colitis.
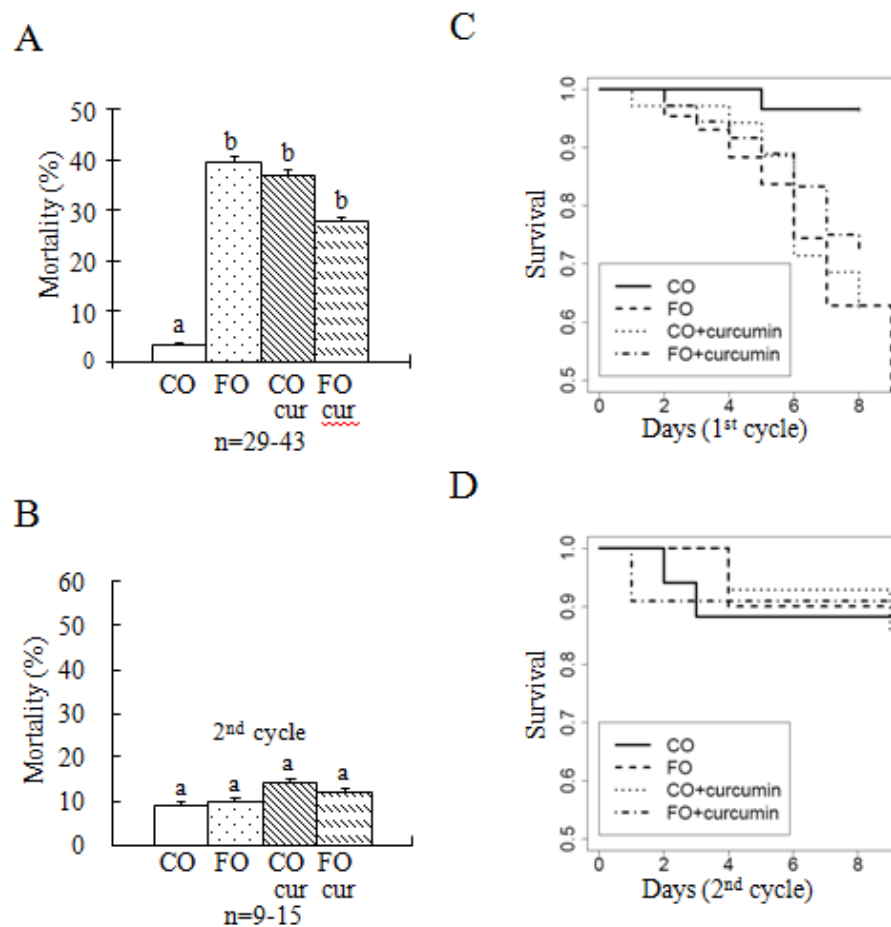
Figure 18. Fish oil and curcumin feeding increase mortality. Animal mortality following DSS treatment was recorded during the (A) acute and (B) chronic inflammatory phases. Kaplan-Meier plots are shown for the (C) acute and (D) chronic experimental phases. CO, 5% corn oil; FO, 4% fish oil + 1% corn oil; COcur, 5% corn oil + 2% curcumin; FOcur, 4% fish oil + 1% corn oil + 2% curcumin. Acute phase represents treatment with DSS for 5 d followed by a 3 d recovery period; chronic phase represents 2 cycles of DSS exposure followed by a 14 d recovery period. Refer to Supplemental Figure 1 for details. Data not sharing common letters are significantly different, $P < 0.05$.

4.3.3 FO and curcumin modulate DSS-induced pathology

To explore the effect of *n-3* PUFA and curcumin on DSS-induced colitis, mice were treated with a single 5 d cycle of DSS followed by a 3 d recovery (acute) or 2 cycles of DSS (chronic) and colonic inflammation and mucosal injury were assessed. Mouse colon sections were H&E stained for histological scoring (**Figure 19A**). Injury and inflammatory scores represent the degree of ulceration and immune cell infiltration, respectively. FO with or without curcumin supplementation and CO with curcumin increased ($p<0.05$) injury scores, compared to control (CO), following both acute and chronic DSS exposure (**Figure 19B**). This is consistent with animal survival data indicating that dietary FO and/or curcumin promoted mucosal injury and mortality during acute phase DSS exposure (**Figure 18A and C**). With respect to the inflammatory score, the addition of curcumin to FO was protective, especially following chronic DSS exposure (**Figure 19C**). These data suggest that *n-3* PUFA and curcumin may affect the pathology of DSS induced colitis by modulating immune cell infiltration and activation in both the acute and chronic phases.

4.3.4 FO and curcumin combination suppresses DSS-induced NF-kB activation

We examined NF-kB activation status following chronic DSS exposure in order to elucidate the mechanism by which *n-3* PUFA and curcumin modulate the inflammatory response in the colon. In comparison to CO control, only the dietary FO and curcumin combination suppressed NF-kB activity in colonic mucosa (**Figure 20**). Together with inflammatory score data (**Figure 19**), these results suggest a mechanistic

link between the enhanced resolution of chronic inflammation and suppression of NF-kB by FO plus curcumin feeding.
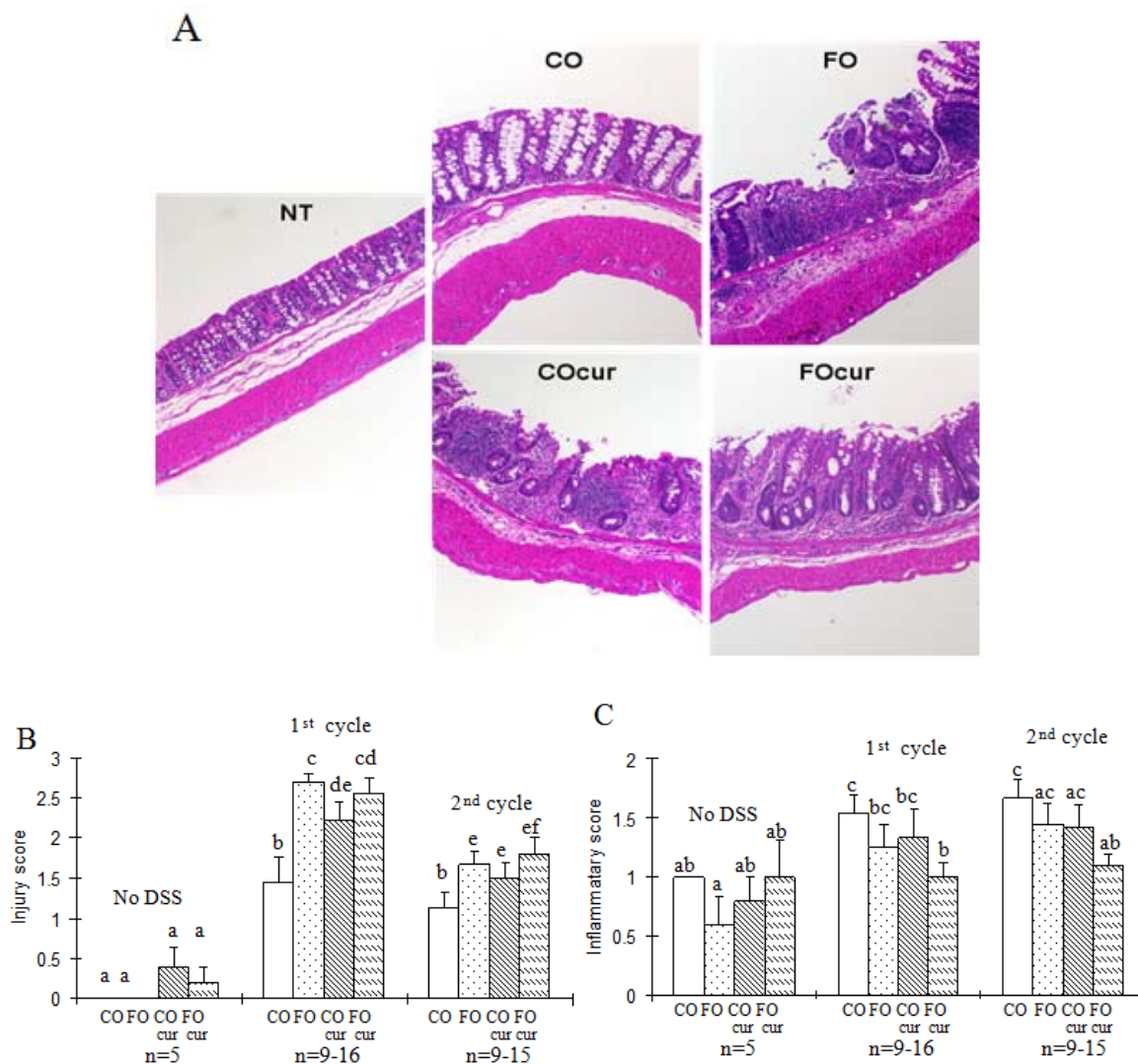
Figure 19. Histological features of colonic inflammation and mucosal injury. Mice were treated with an acute or chronic DSS regimen and colonic inflammation and mucosal injury were assessed. (A) Representative hematoxylin and eosin-stained colonic sections from mice exposed to chronic DSS treatment (100X magnification). Crypts were severely distorted and the epithelium was denuded in FO, FOcur and COcur treatments compared to CO (control) and NT, non-DSS treated animals fed CO. (B) Injury scores and (C) inflammatory scores, data represent mean ± SEM. No DSS, indicates no DSS treatment; 1st cycle, indicates 5d DSS followed by a 3 d recovery period; 2nd cycle, indicates 2 cycles of DSS exposure. Data not sharing common letters are significantly different, $P < 0.05$.
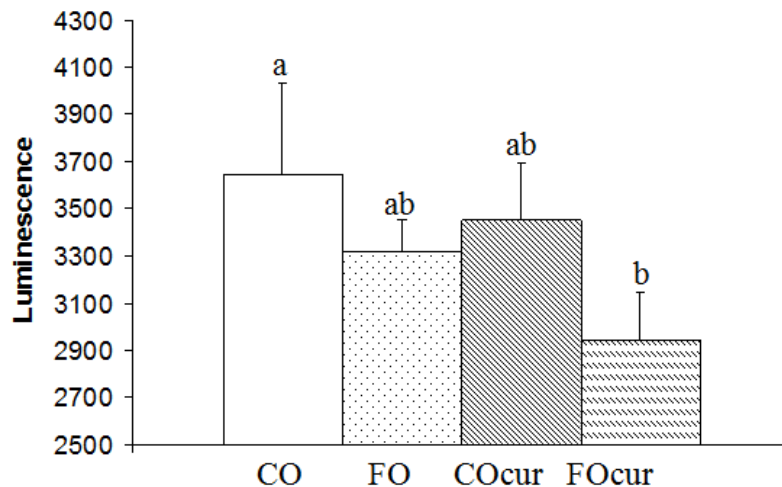
Figure 20. Fish oil and curcumin suppress NF-kB activation in colonic mucosa. Colonic mucosa was isolated following 2 cycles of DSS treatment (chronic inflammatory phase) and p65 NF-kB nuclear activation was determined. Columns represent mean ± SEM (n=7 mice per treatment). Data not sharing common letters are significantly different, P < 0.05. Refer to Figure 18 for legend details.
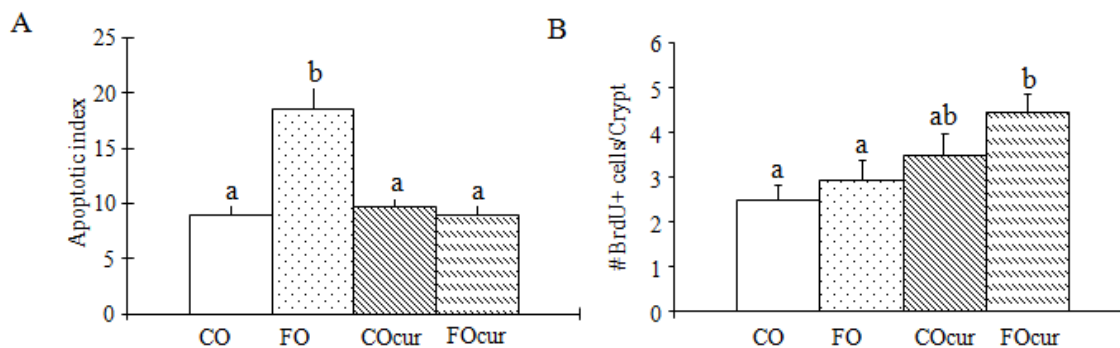
Figure 21. Fish oil and curcumin modulate colonocyte cytokinetics. In situ detection of epithelial apoptosis (A) and proliferation (B) was quantified using TUNEL and BrdU incorporation, respectively. Animals were terminated following 2 cycles of DSS treatment (chronic inflammatory phase). Data are expressed as an apoptotic index (total number of apoptotic cells per 100 crypts). Columns represent mean ± SEM (n = 3–5 mice per treatment, 115-338 crypts per column). Data not sharing common letters are significantly different, P < 0.05

4.3.5 FO and curcumin combination promotes mucosal cell proliferation in the colon

Next, we investigated the ability of FO and curcumin to modulate mucosal cytokinetics following DSS-induced exposure. Epithelial apoptosis was measured by the terminal deoxynucleotidyl transferase (TUNEL) assay. As shown in **Figure 21A**, dietary FO enhanced epithelial apoptosis compared to CO control, which may explain, in part, the elevated injury score associated with FO feeding (**Figure 19B**). Since the initiation of cell proliferation is a prerequisite for mucosal repair (138), we examined epithelial cell proliferation by BrdU incorporation. As shown in **Figure 21B**, feeding FO plus curcumin resulted in an enhanced rate of proliferation relative to CO (control). This suggests that FO plus curcumin diet promotes repair of the colonic epithelium.

4.3.6 Diet and DSS exposure modulate mucosal gene expression

Gene expression in chronic colitis was determined by comparing treated groups (2 cycles of DSS) to CO-fed no-DSS untreated control (CO-con) mice. As shown in **Figure 22**, genes up- or down-regulated by FO, curcumin, and FO plus curcumin combination were contrasted in order to elucidate the biological processes contributing to the tissue injury and inflammation phenotypes. Because dietary FO plus curcumin reduced the inflammatory score and NF-kB activation, and elevated intestinal cell proliferation (**Figures 19-21**), we compared gene expression profiles from mice fed FO plus curcumin with mice fed the CO control diet without DSS treatment (CO-con) (**Figure 22A**). Specifically, genes that were differentially expressed in the CO, CO-curcumin, and FO groups relative to the CO-con following chronic colitis but whose
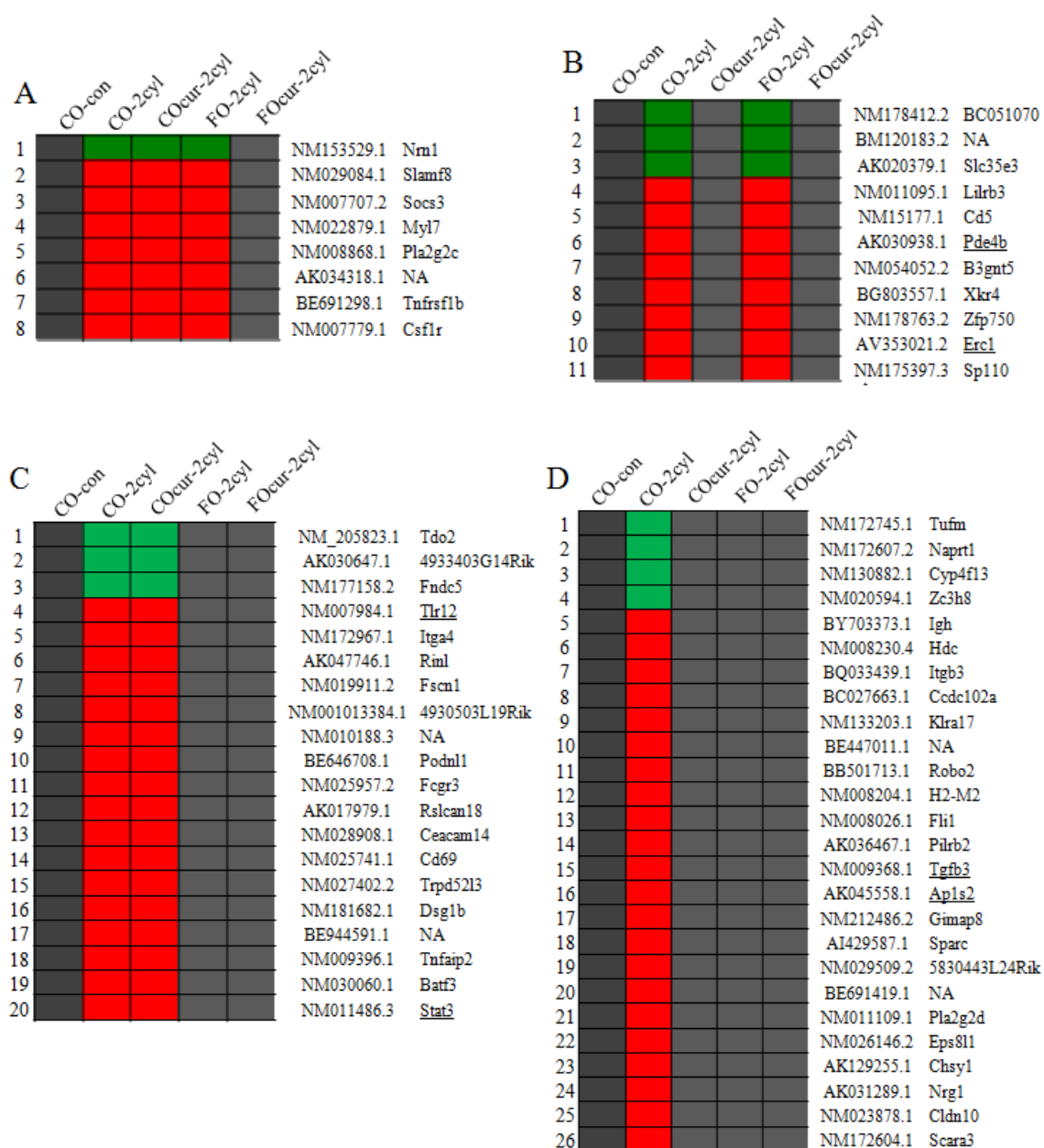
Figure 22. Fish oil and curcumin supplementation differentially regulate intestinal gene expression. Distinctive gene expression signatures are found in colonic mucosa following FO-curcumin dietary treatments and exposure to DSS. Functional annotation of gene sets with similar profiles are compared: (A) expression pattern for genes strictly affected by FO-curcumin combination therapy; i.e., no DSS treatment CO control diet (CO-con) and FO-curcumin-DSS combination vs other, (B) expression pattern for genes strictly affected by curcumin therapy; i.e., CO-con, CO-curcumin-DSS, and FO-curcumin-DSS supplementation vs other, (C) expression pattern for genes strictly affected by FO feeding; i.e.,CO-con, FO-DSS, FO-curcumin-DSS vs other, (D) expression pattern for genes affected by FO or curcumin treatment; i.e., CO-DSS effect, CO-DSS (positive control) vs other. Significantly ($p<0.05$) up-regulated genes are shown in red, and down-regulated genes are shown in green. Black boxes indicate no significant difference ($p>0.05$) compared to CO-con. NF-kB related genes are underlined. n=5 mice for CO-con, n=9-10 for all other groups. All animals were exposed to 2 DSS cycles except for CO-con. NA, gene name not available.

change was blocked by the FO-curcumin diet were identified. Examples include a hypoxic perinecrotic marker of tumor angiogenesis, Neuritin precursor (Nrn1) (139), which was down-regulated by 0.4-0.5 fold in the CO-DSS, FO-DSS, CO-curcumin-DSS group relative to CO-con and FO-curcumin-DSS treatment. In addition, the FO plus curcumin combination prevented the up-regulation of proinflammatory genes (e.g., Lymphocyte antigen 9 (Slamf3), suppressor of cytokine signaling 3 (Socs3), tumor necrosis factor receptor superfamily member 1B precursor (Tnfrsf1b) and macrophage colony-stimulating factor 1 receptor precursor (Csf1r)), expressed in immune cells (140-142). These observations are consistent with the ability of FO plus curcumin to resolve inflammation (**Figure 19**). Up-regulation of secretory phospholipase A_2 precursor (Pla2g2c) and myosin regulatory light chain 2 (Myl7) was also prevented in the FO-curcumin-DSS group.

Genes that were affected by curcumin alone are shown in **Figure 22B.** In this comparison, diets containing curcumin (CO-curcumin-DSS and FO-curcumin-DSS) reduced the expression of ELKS/RAB6-interacting/CAST family member 1 (Erc1) during chronic inflammation to levels similar to the CO-con diet. Erc1 is an essential regulatory subunit of the IKK complex and important for NFkB activation (143), suggesting that curcumin is capable of modulating chronic inflammation via inhibition of NFkB activation in colon mucosa. Curcumin also blocked DSS-induced increase of proinflammatory genes such as T-cell surface glycoprotein CD5 precursor (Cd5), paired Ig like receptor B (Lilrb3), cAMP-specific 3',5'-cyclic phosphodiesterase 4B (Pde4b), nuclear body protein (Sp110) and solute carrier family 35, member E3 (Slc35e3). It is

noteworthy that Pde4b inhibitors exhibit profound anti-inflammatory effects and have utility in the treatment of cancer and the regulation of energy balance (144, 145).

Genes that were selectively affected by fish oil feeding are shown in **Figure 22C.** In this comparison, mice that received FO-DSS and FO-curcumin-DSS treatment produced an expression profile similar to CO-con. Of interest in this panel, two NFkB regulated genes had reduced expression in response to FO, i.e., Toll-like receptor 12 (Tlr12) and signal transducer and activator of transcription 3 (Stat3). An immunohistochemical analysis of pSTAT3 Tyr 705 was performed to confirm the microarray results. Consistent with mRNA data, FO-DSS and FO-curcumin-DSS treatments exhibited suppressed levels of pSTAT3 compared to CO-curcumin-DSS treatment but not CO-DSS (**Figure 23**). Although the function of Tlr12 in colonic inflammation is not known, Stat3 has been reported to be a required regulator for survival of intestinal epithelial cells and development of colitis-associated tumorigenesis (146, 147). FO feeding also reduced the expression of proinflammatory genes including low affinity immunoglobulin gamma Fc region receptor III precursor (Fcgr3), carcinoembryonic antigen-related cell adhesion molecule 14 (Ceacam14), early T-cell activation antigen p60 (Cd69), tumor necrosis factor, alpha-induced protein 2 (Tnfaip2), and basic leucine zipper transcriptional factor ATF-like 3 (Batf3). Interestingly, Batf3 may be required for the differentiation of IL17-producing T helper (TH17) cells (148).

We also examined genes that were uniquely affected by either FO or curcumin treatment, i.e., changes that were exclusive to CO-DSS treatment (**Figure 22D**). Using this comparison, two NFkB target genes, transforming growth factor beta-3 precursor

(Tgfb3) and AP-1 complex subunit sigma-2 (Ap1s2), were selectively up-regulated in CO-DSS mice. This is noteworthy because TGF 3 is a well known proinflammatory marker of IBD (149). Other effector proinflammatory genes which were upregulated include scavenger receptor class A member 3 (Scara3), paired immunoglobulin-like type 2 receptor beta 2 Precursor (Pilrb2), killer cell lectin-like receptor, subfamily A, member 17 (Klra17) (150-152), immunoglobulin heavy chain C gene segment (Igh), and histidine decarboxylase (Hdc). Interestingly, Hdc is modulated by TLR agonists (153). CO plus DSS treatment also increased additional genes that may be involved in mucosa wound repair including pro-neuregulin-1 (Nrg1) (154), chondroitin sulfate synthase 1 (Chsy1) (155), and epidermal growth factor receptor kinase substrate 8-like protein 1 (Eps8l1) (156). Genes specifically down-regulated by CO feeding in the presence of DSS include: elongation factor Tu, mitochondrial Precursor (Tufm), nicotinate phosphoribosyltransferase (Naprt1), cytochrome P450, family 4, subfamily f, polypeptide 13 (Cyp4f13) and Zinc finger CCCH domain-containing protein 8 (Zc3h8).

4.4 Discussion

Dietary *n-3* PUFA and curcumin are receiving substantial attention for their anti-tumorigenic and anti-inflammatory properties (98, 157, 158). In this study, we assessed the combined effects of these two bioactive dietary components on the resolution of both acute and chronic experimental colitis. FO and curcumin, both alone and in combination, unexpectedly enhanced animal mortality and exacerbated colonic mucosa injury following an acute inflammatory episode (**Figure 18A, 18C, Figure 19B**). Wound repair
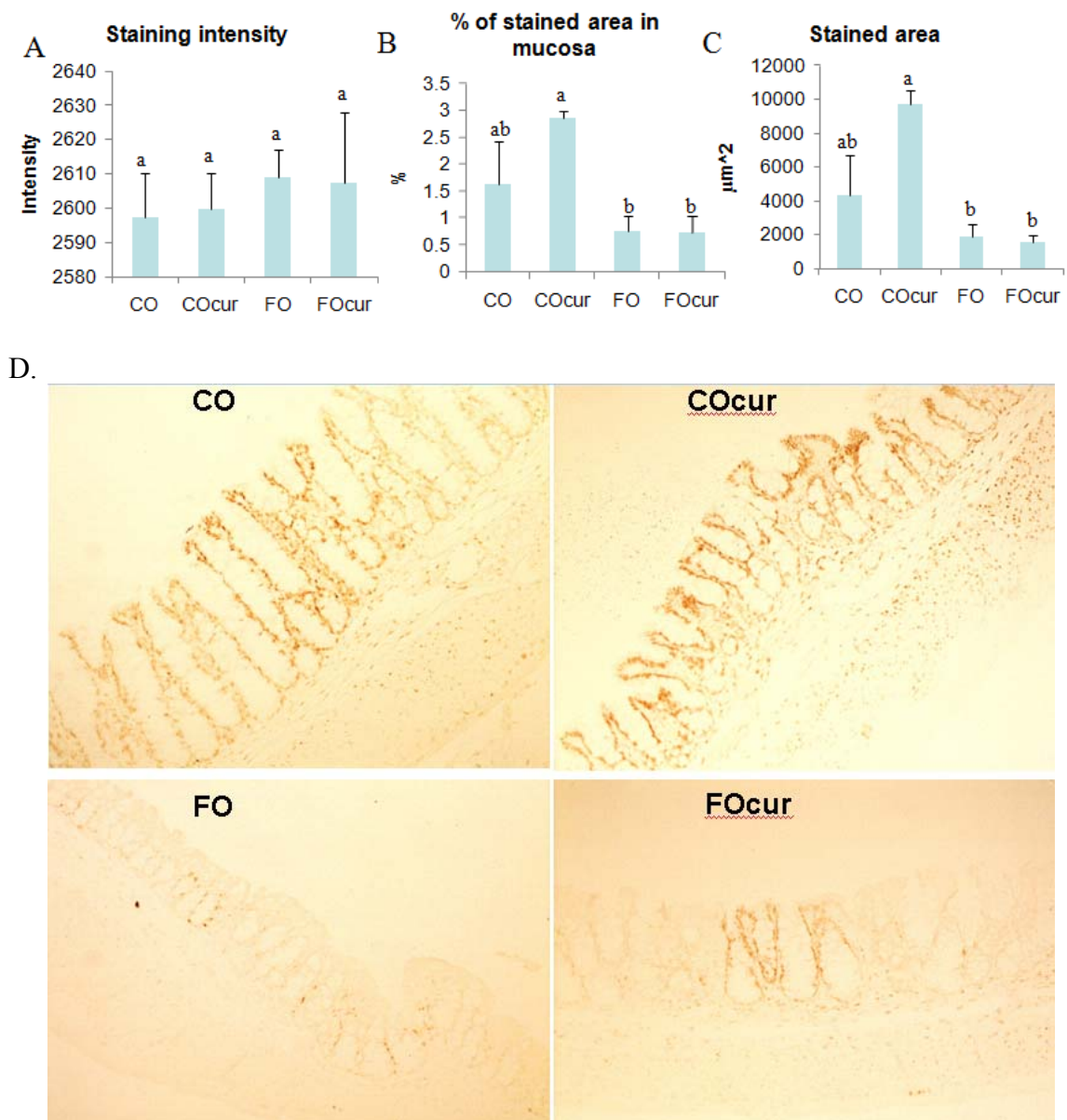
Figure 23. Immunohistochemistry of pSTAT3 Tyr 705. The assay was conducted using colon tissue sections from mice with chronic colitis. Quantification of DAB labeling was determined by staining intensity (A), % of stained area in the mucosa (B), and discrete stained areas (C). Bars labeled with different letters indicate a significant difference ($p < 0.05$) between means. Representative staining is shown in panel (D).

occurs in three overlapping but distinct stages: inflammation, new tissue formation, and remodeling (159). Inflammation occurs immediately after tissue damage and is essential for wound repair. In refractory cases of IBD or experimental chronic inflammation, mucosal repair is disrupted. Repair of damaged intestinal mucosa is regulated by multiple factors at both molecular and cellular levels including innate and adaptive immune cells (138, 160, 161). Although many elements of the acute inflammatory response influence wound repair, particular attention has been paid to growth factors, prostaglandins and cytokines (162, 163). Interestingly, there is a growing body of evidence indicating that *n-3* PUFA (98, 163) and curcumin (64, 88, 164, 165) inhibit these same mediators, which are required for protection from DSS-induced colonic injury. Although there is significant redundancy in the inflammatory response (159), it is possible that FO and curcumin delay epithelial repair mechanisms, which would enhance absorption of bacterial toxins and, therefore, increase mortality.

The second stage of repair of injured intestinal mucosa, new tissue formation, is a process of organized restitution, proliferation and differentiation of mucosal epithelium. Within hours of injury, reepithelialization is initiated and epithelial cell proliferation is stimulated in crypts near the damaged mucosal area (138, 160). Our data show that FO plus curcumin enhanced colonic epithelial proliferation compared to other diets (**Figure 21B**), which suggests that this nutritional combination may promote mucosal repair during chronic inflammation. In contrast, FO alone increased epithelial cell apoptosis (**Figure 21A**). This is noteworthy, because sustained epithelial apoptosis can preclude mucosal healing and decrease animal survival (27), which may explain the elevated

injury scores observed in FO-fed mice during acute phase inflammation. Along these lines, it has been shown that the balance between colonic epithelial cell proliferation and apoptosis can be modulated by dietary *n-3* PUFA, conferring resistance to toxic carcinogenic agents (27, 88).

With respect to mechanisms which mediate intestinal cell cytokinetics, it was shown that Toll-like receptor-4 (TLR4)-prostaglandin-dependent signaling directly regulates proliferation and apoptosis in response to acute colitis (162, 166, 167). Although TLR4 activation is beneficial in the short term, chronic signaling may lower the threshold for inflammation-associated colon cancer (168). With respect to diet, DHA and curcumin appear to be pan-inhibitors for various TLR's (169, 170). Additional studies are needed in order to determine if these observations can be validated in vivo. It is also well documented that *n-3* PUFA (EPA and DHA) antagonize arachidonic acid (AA) derived prostaglandins (PGE_2 and PGD_2) in colonic mucosa (34, 171). Curcumin was also reported to suppress PGE_2 formation by blocking expression of COX-2 and microsomal PGE_2 synthase-1 and inhibiting the activity of microsomal PGE_2 synthase-1 (164). This is noteworthy because PGE_2 is capable of enhancing cell proliferation, angiogenesis, cell migration and invasion as well as inhibiting apoptosis, and enhancing tumor growth (66). Recently, it has been demonstrated that PGE_2 is important for the healing of ulcers and epithelial injury (67, 68). Taken together, these data suggest that *n-3* PUFA and curcumin modulate the resolution of inflammation and mucosa repair, in part, by suppressing the TLR4/COX-2/PGE_2 signaling axis.

Our results show that dietary FO plus curcumin combination significantly augmented the resolution of chronic inflammation and suppressed NFkB activity in the colon (**Figures 18C & 19**), suggesting that FO and curcumin act in a combinatory manner. The separate effects of these two bioactive components have been reported previously (33, 59, 172). NFkB is an important intracellular regulator of both the inflammatory response and mucosal integrity via TLR4/COX-2/PGE2 signaling axis which is important for the healing of epithelial injury (68). Recent findings indicate that NFkB can exert either a deleterious or a protective function in the intestine, depending on the stimuli encountered (7, 35, 36, 98). NFkB regulates gene expression of proinflammatory mediators including IL-1β, TNF-α, IL-12p40 and IL-23p19, which contribute to the pathophysiology of chronic intestinal inflammatory diseases (7, 35, 36).

Mucosal microarray analysis revealed that dietary FO, curcumin, and FO plus curcumin combination differentially modulated the expression of genes induced by DSS treatment (**Figure 22**). A regulator of NFkB activation, Erc1, was suppressed at the transcriptional level following supplementation with curcumin (**Figure 22B**). As an essential regulatory subunit of IKK complex (143), Erc1 may be a new target for curcumin which modulates NFkB activation in addition to inhibiting IKK and Akt activation (173). The suppression of NFkB target genes by FO or curcumin is consistent with the critical role of this transcription factor as a key regulator of intestinal inflammation. Interestingly, FO feeding blocked DSS-induced Stat3 up-regulation (**Figure 22C**). Stat3 has been reported recently as a key regulator in IBD and colon cancer (146, 147). Specifically, Stat3 activation in enterocytes is required for cell

survival and its hyperactivation promotes colitis associated tumorigenesis and growth (146, 147). In addition to intestinal epithelial cells, Stat3 is constitutively activated in mucosa immune cell types including dendritic cells, macrophages and T cells (174-176). The Stat3 pathway in $CD4^+$ T cells promotes IL-17-producing Th cell (Th17) development which mediates immune responses in autoimmune disease and IBD induced cancer development (174, 175). Stat3 activation in immune cells promotes an IL-23-mediated procarcinogenic immune response while inhibiting IL-12-dependent Th1 mediated antitumor immunity (176). Our results suggest that Stat3 may be a new target of the antitumorigenic *n-3* PUFA. We are currently investigating whether *n-3* PUFA modulate Th17 polarization in the intestine (see Section 3). Overall, FO and curcumin suppressed the up-regulation of proinflammatory gene expression relative to the CO (control) diet, which is consistent with human studies showing that EPA + DHA intake decrease gene expression of inflammatory pathways, including NFkB signaling (158, 177, 178).

It has been reported that different strains of mice may have differential susceptibility to, and pathogenesis of, DSS induced colitis and colon cancer (179-180). DSS induced chronic colitis in C57BL/6 mice is believed to be a robust model for validating future therapies for treatment of inflammatory bowel disease because of similarities of the pathology compared to symptoms in human UC (70, 181). Additional studis employing other models that mimic human IBD, such as TNBS and adoptive transfer models, are need to corroborate the current data (13, 70, 182, 183). Thus, although different results might be obtained with another mouse strain, we believe that

our findings make an important contribution to the consideration of using fish oil and curcumin as therapeutic agents in chronic IBD.

In conclusion, our data show that dietary FO and curcumin differentially modulate the pathology of DSS-induced chronic colitis in mice. Feeding FO plus curcumin together enhanced the resolution of chronic inflammation but disrupted mucosa repair in part due to the inhibition of NFkB activity in colonic mucosa. FO and curcumin also differentially modulated mucosa cytokinetics which may be attributed to regulation of the TLR4/COX-2/PGE$_2$ and Stat3 signaling pathways in the colon. Collectively, these findings contribute to a better understanding of the ability of *n-3* PUFA and curcumin to modulate the inflammation - mucosa repair - carcinogenesis axis in the colon.

5. SUMMARY AND CONCLUSION

The immune system in the gut contains both innate and adaptive components which maintain the homeostasis between commensal and pathogenic microbiota and the host (184, 185). Inflammatory bowel diseases (IBD) are characterized by chronic relapsing inflammation of the gastrointestinal tract. Although the precise etiology of IBD remains unclear, a suggested key pathogenic role for immune dysregulation in the intestine is the enhanced mucosal T cell responses, especially $CD4^+$ T lymphocytes according to recent research (5-7, 186). Previous concepts of the Th1/Th2 paradigm in chronic inflammatory dominated autoimmune diseases pathology have been revised by the discovery of Th17 cells (186, 187). Particularly, Th17 cells, rather than Th1 cells, have been shown to be the major effector cells in experimental colitis models of IL-10-deficient mice and C3H/HeJBir mice (11, 42). Moreover, there are multiple reports concerning the effects of IL-17 in human IBD (43-45, 188, 189). Therefore, although the Th1/Th17 balance in IBD remains unclear, IBD and Th17 recruitment and activation are intimately linked (186). In contrast, Treg cells negatively regulate immune reactions and play a key role in the maintenance of immunological homeostasis (15, 16, 190). The regulatory function of Treg cells in colonic inflammation/IBD is predominantly exerted by their production of suppressor cytokines, such as IL-10 and/or TGF-ß (191-193). Although the developmental pathway linking the Th17/Treg axis is not fully understood, the imbalance of Treg and effector T cell induction is an important concept in the etiology of the IBD (16-21).

A growing number of researchers have focused on the effect of dietary long chain *n-3* PUFA, such as DHA and EPA, on intestinal inflammation. The epidemiological and clinical data show that *n-3* PUFA can attenuate immune disease including IBD (26, 104, 107, 194). A high consumption of *n-3* PUFA not only lowers the incidence of chronic inflammatory disease, but also results in clinical improvement in patients with IBD (26, 104, 107, 194, 195). The natual polyphenol compound curcumin has been examined with respect to its ability to ameliorate inflammation in experimental colitis and human IBD (56-58). For example, curcumin supplementation has been shown to maintain remission in patients with quiescent UC to prevent relapse (61). The chemoprotective effects of curcumin appear to be mediated by supressing NF-κB activation (57, 59, 60), suppressing PGE_2 formation (65, 66) and altering the Th1/Th2 balance (63, 64)

In this dissertation, the modulatory effect of *n-3* PUFA and curcumin in intestinal immune response was addressed in both endogenous *n-3* PUFA and dietary supplementation models. Endogenous n-3 PUFA effectively altered colonic membrane phospholipid composition in the *fat-1* mouse (**Table 3**) resulting in the suppression of inflammation-driven tumor formation (**Figure 2**). In addition, colonic epithelial apoptosis was elevated in *fat-1* mice. Since apoptosis is progressively inhibited during colon cancer development (99), it is possible that the observed protective effect of *n-3* PUFA is due, in part, to the enhanced deletion of cells though the activation of targeted apoptosis (27, 98). *n-6* PUFA derived eicosanoids were significantly suppressed in the colonic mucosa of *fat-1* relative to wt mice (**Figure 4**). This finding is consistent with

the well documented ability of *n-3* PUFA (EPA and DHA) to supplant arachidonic acid and subsequently antagonize prostaglandin (PGE_2 and PGD_2) and hydroxyl fatty acid (12-HETE) biosynthesis (103). The data also demonstrate that *fat-1* mice exhibit an enhanced ability to resolve chronic colitis by decreasing the CD3+, CD4+ T cell population and macrophages in the colon (**Figures 3, 6, 7**). This is consistent with a previous report that *n-3* PUFA alter the balance between $CD4^+$ T-helper (Th1 and Th2) subsets by directly suppressing Th1 cell development (93). This is noteworthy because Th1 cells, in part, mediate IBD onset and progression (105).

Further, the data indicate that Treg cell differentiation was elevated by *n-3* PUFA following chronic DSS treatment. In contrast, both systemic and local Th17 differentiation was suppressed in *fat-1* mice during the chronic phase of the DSS induced wounding/repair/colitis (**Figures 11 C, D, Figures 12 C, D**). An increase of Treg polarization and decrease of Th17 differentiation maybe a mechanism by which *n-3* PUFA ameliorate intestinal inflammation as exemplified by decreased inflammatory scores (**Figure 10A**). In addition, cytokine levels in splenic dendritic cell cultures were impacted in *fat-1* mice, resulting in lower levels of TGF-ß1 but higher IL-10. Those data indicate a broad impact of *n-3* PUFA on the immune system by documenting its ability to alter T cell polarization and dendritic cell function.

From the study to assess the combined effects of dietary *n-3* PUFA and curcumin on injury-induced experimental colitis, the data show that FO and curcumin, both alone and in combination, unexpectedly enhanced animal mortality and exacerbated colonic mucosa injury following an acute inflammatory episode (**Figure 18A, 18C, Figure 19B**).

However, the addition of curcumin to FO significantly augmented the resolution of chronic inflammation and suppressed NFkB activity in the colon (**Figures 19C & 20**), suggesting that FO and curcumin act in a combinatory manner. This is noteworthy because NFkB is an important intracellular regulator of both the inflammatory response and mucosal integrity via TLR4/COX-2/PGE2 signaling axis which is important for the wound healing of mucosa (68). The data suggest that *n-3* PUFA and curcumin may differentiatly affect the pathology of DSS induced colitis by modulating immune cell infiltration and activation in both acute and chronic phases.

FO plus curcumin enhanced colonic epithelial proliferation compared to other diets (**Figure 21B**) which indicates that this nutritional combination may promote mucosal repair during chronic inflammation. In contrast, FO alone increased epithelial cell apoptosis (**Figure 21A**). Since sustained epithelial apoptosis can preclude mucosal healing and decrease animal survival (27), this may explain the elevated mortality and injury scores observed in FO-fed mice during acute phase inflammation. These data show that the balance between colonic epithelial cell proliferation and apoptosis can be modulated by dietary *n-3* PUFA and curcumin, conferring resistance to toxic carcinogenic agents (27, 88, 90). In addition, mucosal microarray analysis revealed that dietary FO, curcumin, and FO plus curcumin combination differentially modulated the expression of genes induced by DSS treatment (**Figure 22**). Overall, the microarray data suggest that FO and curcumin differentially modulate mucosa cytokinetics, which may be attributed to the regulation of the TLR4/COX-2/PGE_2 and Stat3 signaling pathways in the colon.

In conclusion, these data show that *n-3* PUFA, which are incorporated into both colonocytes and T-cell membranes, suppress inflammation-driven tumor progression. Moreover, the endogenously synthesized *n-3* PUFA (*fat-1* mouse) suppress colonic chronic inflammation and tissue injury, partly mediated by the modulation of specific activity of lymphoid, macrophage and dendriritic cell elements in the intestine. The data also demonstrate for the first time that a bioactive food component, *n-3* PUFA, can modulate Th17 polarization in the DSS disease model. In addition, dietary FO and curcumin differentially modulate the pathology of DSS-induced chronic colitis in mice. Feeding FO plus curcumin together enhanced the resolution of chronic inflammation but disrupted mucosa repair in part due to the inhibition of NFkB activity in colonic mucosa. Collectively, these findings contribute to a better understanding of the ability of *n-3* PUFA and curcumin to modulate the inflammation - mucosa repair - carcinogenesis axis in the colon. Further studies are needed to determine the DC-T cell mechanism(s) underlying the immunomodulatory effects of *n-3* PUFA and curcumin.

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APPENDIX A

SUPPLEMENTARY DATA

Appendix A1. TUNEL and BrdU detection
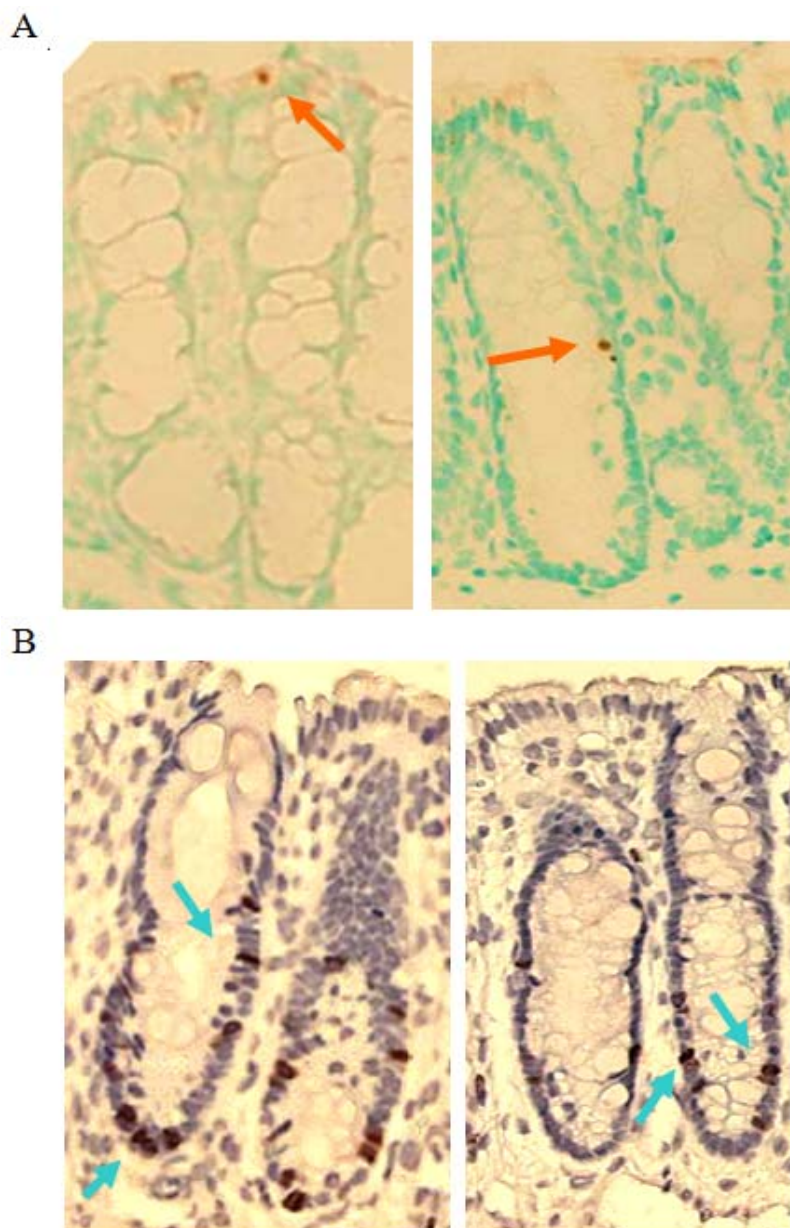
Objective: To measure epithelial apoptosis and proliferation

Method: Refer to Section 4, methods

Results: Apototic cells and proliferative cells were stained respectively. A. apoptotic cells stained by TUNEL assay; B, proliferative cells stained by anti-BrdU.

Appendix A2. *fat-1* Phenotyping
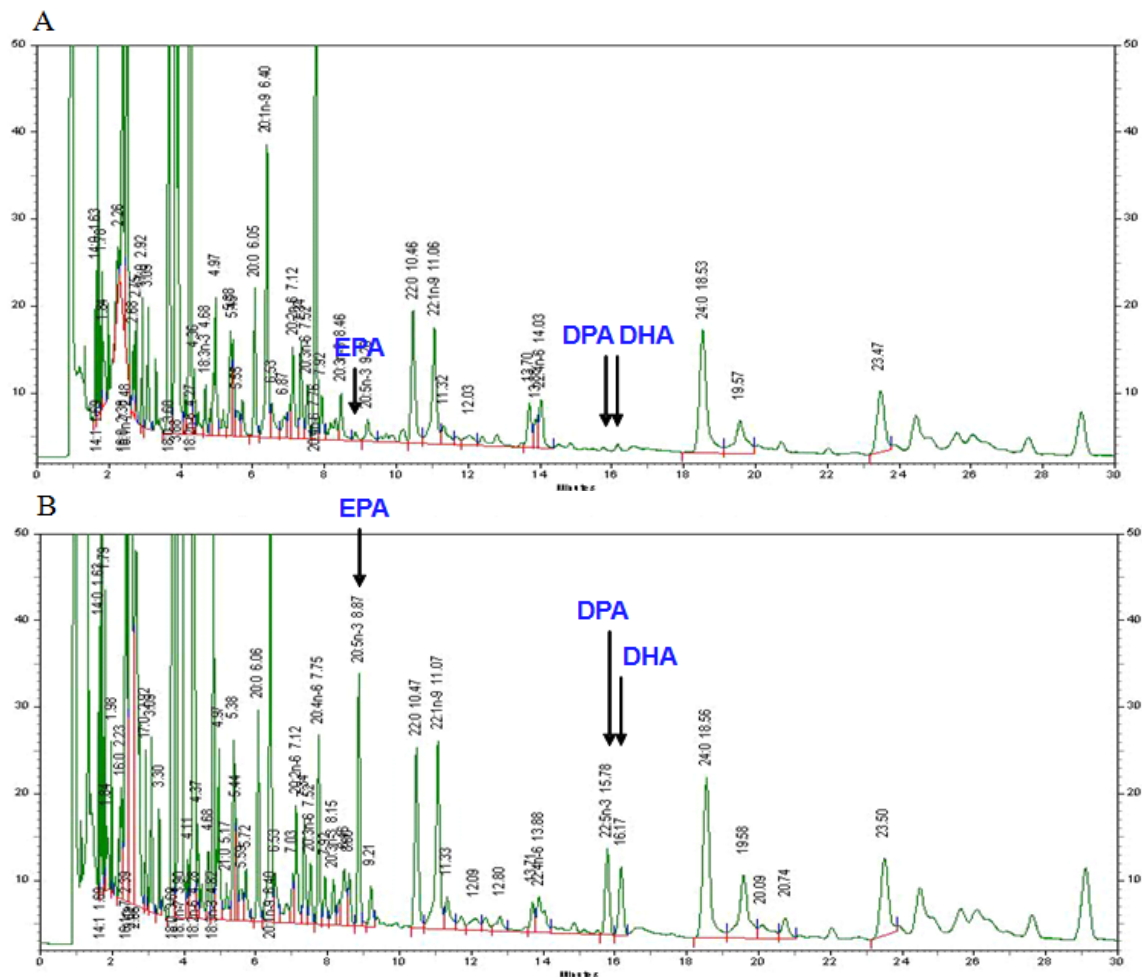
Objective: To characterize the *fat-1* littermates

Method: Refer to Section 2, methods

Results: Lipids were isolated from mice tail and analyzed the contents by gas chromatography. A representative lipids profile of wt mice is short of *n-3* PUFA (A), a typical *fat-1* profile contains enriched *n-3* PUFA (B).

Appendix A3. Lipids profile from *fat-1* mouse tails

ID#	C18:2n-6	C20:4n-6	C22:4n-6	C22:5n-6	Total n-6	C18:3n-3	C20:5n-3	C22:5n-3	C22:6n-3	Total n-3	n-6/ n-3	Pheno type
1181	24.72	0.29	0	0	25.0	1.2	0.49	0.63	0.46	2.8	9.0	wt
1182	23.27	2.94	0.48	0.61	27.3	0	0	0	0	0.0	NA	wt
1183	25.03	4.07	0.66	0.83	30.6	0	0	0	0	0.0	NA	wt
1184	23.27	3.88	0.59	0.75	28.5	0	0	0	0	0.0	NA	wt
1185	23.57	4.42	0.71	0.78	29.5	0	0	0	0	0.0	NA	wt
1186	23.13	4.41	0.75	0.96	29.3	0	0	0	0	0.0	NA	wt
1187	29.36	4.11	0.53	0.73	34.7	0	0	0	0	0.0	NA	wt
1188	29.65	4.77	0.59	0.97	36.0	0	0	0	0.11	0.1	327	wt
1189	27.23	5.45	0.74	0.86	34.3	0	0	0	0	0.0	NA	wt
1190	24.59	6.24	0.9	0.85	32.6	0	0	0	0	0.0	NA	wt
1191	29.67	0.7	0	0	32.6	1.21	1.02	1.07	1.11	4.4	9.0	*fat-1*
1192	27.65	0.65	0	0.12	28.4	1.27	0.98	0.99	1.09	4.3	6.6	*fat-1*
1193	27.68	0.53	0	0	28.2	1.83	1.72	1.17	1.48	6.2	4.6	*fat-1*
1194	28.38	0.52	0	0	28.9	2.22	1.06	0.9	1.14	5.3	5.4	*fat-1*
1195	22.77	5.4	0	0.77	28.9	0	0	0.03	0.16	0.2	152	wt
1196	22.48	5.59	0	0.8	28.9	0	0	0.09	0.25	0.3	85	wt
1197	28.27	0.64	0	0.2	29.1	1.86	1.01	1.42	1.47	5.8	5.1	*fat-1*
1198	23.29	5.11	0	1.06	29.5	0	0	0	0.26	0.3	113	wt
1199	23.79	5.29	0	0.87	30.0	0	0	0	0.18	0.2	166	wt
1200	26.3	0.9	0	0.22	27.4	2.67	0.76	0.79	0.92	5.1	5.3	*fat-1*
1201	26.34	0.47	0	0.25	27.1	1.71	0.96	1.07	1.2	4.9	5.5	*fat-1*
1202	25.62	0.42	0	0.14	26.2	1.67	0.72	0.76	0.99	4.1	6.3	*fat-1*
1203	27.67	0.58	0	0.26	28.5	2.67	0.92	0.83	0.95	5.4	5.3	*fat-1*
1204	24.1	5.11	0	0.95	30.2	0	0	0.2	0.65	0.9	36	wt
1205	31.45	0.51	0	0.28	32.2	1.34	0.81	0.94	1.03	4.1	7.8	*fat-1*
1206	26.39	0.47	0	0.12	27.0	2.76	1.08	1.18	11.5	16.5	1.6	*fat-1*
1207	28.56	0.35	0	0.23	29.1	1.64	0.91	0.73	1.14	4.4	6.6	*fat-1*
1208	25.8	5.49	0	0.62	31.9	0	0	0.25	0.58	0.8	38	wt

Objective: To characterize the *fat-1* littermates' phenotype

Method: Refer to Section 2, methods

Results: Lipids were isolated from mice tail and analyzed the contents by gas chromatography. The table is a sample of lipids content profile and the ratio of total n-6/n-3 pointed out the phenotype of the mice. A high ratio (>20) or NA because total percentage of *n-3* PUFA is 0 represents wt mice. A low ratio (<20) represents *fat-1* mice.

APPENDIX B

EXPERIMENTAL PROTOCOLS

Appendix B1. CD4$^+$ T cells isolation by using Miltenyl beads and columns

Aim:
 To isolate CD4$^+$ T cell from mouse spleen by using Miltenyl beads and column.

Facilities & Instruments:
   Sterile Hoods
   Sterile instruments (i.e. scisors, forceps, etc.)
   Sterile 2.0 ml epi tubes
   Sterile Petri Dishes
   70% EtOH
   keys for basement
   5 cc syringes
   50 ml. and 15ml. conical tubes
   Racks
   Flow tubes (BD#352008)

Materials:
   CD4 (L3T4) Microbeads, mouse (Miltenyl Biotec, # 130-049-201)
   MACS Separation Columns, MS columns (Miltenyl Biotec, # 130-042-201)
   Octo MACS Separator (Miltenyl Biotec, # 008716)
   MACS Separation Buffer (Miltenyl Biotec, # 130-091-221), store at 4°C.
      --Containing phosphate buffered saline (PBS), bovine serum albumin (BSA), EDTA, and 0.09% azide, PH 7.2, sterile-filtered.
   BD Falcon cell strainer, 70 μm nylon mesh (BD, # 35-2350)
   30 μm Pre-Separation Filter (Miltenyl Biotec, # 130-041-407)
   Fc block: Affinity Purified anti-mouse CD16/32 (eBioscience # 140161)
   FITC conjugated rat anti-mouse CD4 (BD, # 553729)

Procedures:
1. Excise spleen from mouse
   1). Sacrifice mice by CO$_2$
   2). Place mice on their right side so that the left side faces you
   3). Apply alcohol to the abdomen area
   4). Grab the skin of the abdomen with forceps and make a small incision
   5). Peel back the skin/fur with fingers to expose the membrane underneath
   6). Grab the membrane with forceps and cut the membrane to expose the organs
   7). Remove the spleen (dark red organ) with forceps
   8). Carefully remove as much fat from the exterior of the spleen as possible
   9). Place spleen(s) in a Petri dish (or 15 mL conical tube) containing ~ 3 mL MACS buffer, transfer to cell culture room.
   * *make sure to use petri dish, not culture dish, otherwise, the cells will stick to the dishes.*

2. Prepare single cell suspension of splenocytes (In sterile hood)

1). Place a Falcon cell strainer (70 μm mesh, BD, # 35-2350), on the dish with MACS buffer and pre-wet it with ~ 5 mL MACS® buffer.

2). Gently press freshly spleen (or cut into several pieces) against the bottom of the strainer with a plunger from a 5 ml syringe. Use up and down motion (not grinding), being careful not to damage cells.

3). When only the spleen connective tissue remains in the strainer, remove the strainer. Remove the cell suspension from the dish to a 15 mL conical tube. (Wash strainer with MACS buffer (5-10 ml) to collect the remaining cells, if don't want lose any cells)

4). Spin down at 300g for 5 min.

5). Remove the supernatant and resuspend the pellet by flicking the tube or ratcheting the tube over a tube rack.

6). Add 10 ml MACS buffer, mix well.

7). Place a 30 μm filter (Miltenyi part # 130-041-407) on top of a 15 ml. conical-bottomed tube and pre-wet with 2 ml. MACS® buffer.

8). Pass the10 ml cells suspension through the 30 μm filter into a 15 ml conical tube. The volume will be 12 ml total. (Save 2 aliquots of this cell suspension after filtration, one (~30ul) for cell count-----Total cell population before purification, another (~200ul) for flow cytometry)

9). Centrifuge the cells at 300g for 5 min.

10). Count the cell number during waiting for centrifuge in a hemacytometer with (1) Trypan Blue to assess percent dead cells (1:10 dilution of cells:stain) and with (2) Crystal Violet stain (1:10 dilution of cells:stain) RBC will be lysed and nuclei of leukocytes will be stained blue) to count white cells.

11). Remove the supernatant and resuspend the pellet by flicking the tube or ratcheting the tube over a tube rack.

3. Magnetic labeling and separation

1). Resuspend cells with 90 µL **cold** MACS buffer per 10^7 total cells.

2). **Shut the light in the hood off before you open the microbeads vial.** Add 10 µL of CD4 (L3T4) Microbeads (Miltenyl Biotec, cat# 130-049-201) per 10^7 total cells. *Make sure you add the beads "directly" into the cell suspension, not on the side of tube, then mix.*

3). Mix well and incubate for 15 min at 4-8 °C (refrigerator, not ice!!) (No shaking needed)

4). Wash cells by adding 1-2 ml of cold MACS buffer per 10^7 cells, (or simply fill up the tube with cold MACS buffer) and centrifuge at 300 x g for 10 min at 4°C. Pipette off supernatant completely. Flick the tube to loose the pellet.

5). Resuspend up to 10^8 cells in 500 µL cold MACS buffer. Pipette up and down to make cells well suspended. *(MS column can hold up to 2 x 10^8 total cells)*

6). Optional*: Place a 30 μm filter (Miltenyi part # 130-041-407) on top of a 15 mL conical tube and pre-wet with 100 µL. MACS® buffer. Pass the 500 µL cells through the 30 μm filter into the 15 ml conical-bottomed tube.

* This step may prevent column block, not affect yield according to JQ + Jenn Monk experience, got ~10% CD4+ cells from splenocytes.

7). Place MS** column at Octo MACS Separator. Place a waste collecting tube/container under the column.

*(**The following buffer volume is for MS column only.)*

8). Rinse MS column with 500 µL cold MACS buffer. Let the buffer slowly drip itself, wait until dry (very quick, less than 1 min).

9). Apply cell suspension onto the column.

10). Collect unlabeled cells which pass through, and wash MS column with 500 µL of cold MACS buffer 3 times. Let the column drip completely dry between washes. Collect the effluent with a sterile *15 mL conical tube. (*The effluent is the unlabelled fraction, does not contain CD4+ T cells. Save ~200ul aliquot for Flow analysis of CD4- population and cell count.)

10). Remove MS column from the separator, and place it on a collection *15 mL conical tube.*

11). Pipette 1 ml. of cold MACS buffer onto the column. Immediately flush out fraction with the magnetically labeled cells by firmly applying the plunger supplied with the column. (The effluent is the CD4+ fraction. Save ~200ul aliquot for Flow analysis of CD4+ population and cell count.)

12). Count the number of negative (the flow thorough) and positive (CD4+ positive selected from the coloum) cells.

13) If need spin down cells for staining and flow cytpmetry, resuspend in PBS or flow staining buffer according manufacture direction of your antibody.

4. Fluorochrome conjugation and flow analysis

Have 100ul of sample (up to 10^6 cells*/per 100 µL PBS or staining buffer)* for flow analysis, *keep on ice.*

1). Block Fc receptors for reducing nonspecific immunofluorescent staining by adding 1 µg Fc Block* (2 µL of 0.5mg/mL stock, BD or eBioscience) per sample, *incubate on ice for 10 min.*

2). (Hide Light) Add 2 µl* of FITC-a-CD4 (BD, cat# 553729, stock 0.5 µg/µL) per 100ul (for labeling CD4+ T cells) in the 3 aliquots (original, negative and positive tubes)

*may vary if using different Ab.

If use APC-aCD4 (eBioscience #17-0042-81), **0.5ug/ 10^6 cells/ 100 ul**

If use FITC-aCD4 (eBioscience #11-0042-81), **0.5ug/ 10^6 cells/ 100 ul**

3). Mix well and incubate at 4-8 °C (refrigerator, not ice!!) for 15 min** (no shaking needed).

**may vary if using different Ab.

4). Wash cells by adding 2 ml of cold PBS, and centrifuge at 300 g for 5 min (better at 4°C). Pipette off supernatant completely.

5). Adjust cell concentration for flow analysis (~10^6 per 500ul) *in PBS or staining buffer. Keep samples on ice if not detected immediately. Please detect viability of your cells with your antibody if concerned to leave cells long time on ice.*

6). Transfer cell suspension into flow tube or epi. tube for flow if needed.

References

1) http://www.miltenyibiotec.com/download/datasheets_en/47/MiltenyiBiotec_DataSheet_CD4-(L3T4)-MicroBeads,-mouse_130-049-201.pdf

2) Allenbach Y, Solly S, Grégoire S, Dubourg O, Salomon B, Butler-Browne G, Musset L, Herson S, Klatzmann D, Benveniste O.2009. Role of regulatory T cells in a new mouse model of experimental autoimmune myositis. *Am J Pathol* 174(3):989-98.

Appendix B2. LPL isolation collegenases and Percoll and CD3, CD4 staining

Aims:

This protocol is employed for purification of lymphocytes from a population of lamina propria cells from C57BL/6J mouse colon and detection of $CD3^+ CD4^+$ T cells by flow cytometer.

You may refer to Protocol B-3 for another option to isolate LPLs. This method yields more cells from a more robust enzymatic digestion of colon lamina propria. Protocol B-3 yields a smaller cell number using a shorter time of enzymatic digestion. This reduces the destruction of suface antigens on cells, which may be critical for certain antigens. You may test one of the methods for your consideration.

Facilities & Instruments:

Sterile instruments (i.e. scissors, forceps, etc.)

70% EtOH

Key for basement

60 x15mm Petri dishes (Falcon, #1007)

150 x15mm Petri dishes (Falcon, #351058)

50 ml and 15ml conical tubes and racks

Water bath (37°C)

Centrifuge (room temperature)

Rubber policeman

Glass plates

Uncoated slides (Fisher Scientific, #12-544-1)

Plastic container

100ml-glass flask

Parafilm

Baffled disposable flasks with caps (Fisher Scientific, #10-041-23)

10ml sterile pipet (BD, # 357551)

Sterile wire filter (~60 μm mesh) or BD Falcon cell strainer, 70 μm nylon mesh (BD, # 35-2350)

Sterile 10 ml syringe (BD, #301604) and 20G needles (BD, #305176)

Tubes for flow cytometry (Falcon, #352054)

Reagents:

HBSS without Calcium and Magnesium (Sigma, # H-6648), store at 2-8°C.

10% BSA, IgG free (Boehringer Manheimer, # 100 018) stock solution in HBSS without Ca and Mg, aliquots in 1.7ml epi tubes, store at -20°C.

200 mM L-Glutamine stock solution (200mM, Gibco, # 25030), store at -20°C.

Dithiothreitol (DTT) (Sigma, #D-0632, MW 154.25), store at 2-8°C.

0.75M EDTA (Sigma # ED 45S) stock solution in HBSS without Ca and Mg (Sigma, # H-6648), aliquots in 15ml tubes, store at -20°C.

Collagenase II (Worthington Biomedical, #LS004176), lyophilized powder, *215U/mg*, store at 2-8°C.

Collagenase IV (Worthington Biomedical, #LS004188), lyophilized powder, *223U/mg*, store at 2-8°C.

Deoxyribonuclease I (DNase I). (Worthington Biomedical, #LS002004), lyophilized powder, *5136U/mg*, store at 2-8°C.

Digesting Solution I (make fresh):

-HBSS without Calcium and Magnesium, with 1mM Glutamine, 0.1% BSA, 30mM EDTA and 5mM DTT

Digesting Solution I (per 100ml)	Final Concentration
-0.5 ml - 200mM Gln	1mM
-1 ml - 10% BSA	0.1%
-4 ml - 0.75M Na_4EDTA	30mM
-0.077 g - DTT	5mM

-Make the solution fresh, calculate the amount of stock solutions needed according to the volume needed, usually 20ml needed per mouse colon.

-Combine Gln, BSA, EDTA, DTT in 80ml HBSS without Ca and Mg

-Adjust pH to 7.4 - 7.5 with HCl / NaOH

-Bring final volume up to 100ml

-Warm to 37°C before use

Digesting Solution II (make fresh):

-HBSS without Calcium and Magnesium, with final concentrations of 125U/ml Collagenase II, 160U/ml Collagenase IV and 5U/ml DNase I. *The Unit for each lot of these enzymes may vary, therefore, pay attention to the calculation, the following calculation is an example.*

Digesting Solution II (per 100ml)	Final Concentration
-60mg Collagenase II (208U/mg)	125U/ml
-72mg Collagenase IV (222 U/mg)	160U/ml
-0.1mg DNase I (5136U/mg)	5U/ml

-Make the solution fresh, calculate the amount of enzymes needed according to the volume needed, usually 20ml needed per mouse colon.

-Combine Collagenase II, Collagenase IV and DNase I in 100ml HBSS without Ca and Mg

-Warm to 37°C before use

RPMI 1640 (Irvine Scientific, #9159, 500ml bottle, added with 5ml Glutamax (100x, Gibco 35050-061) and 5 ml Penicillin-Streptomycin solution (100x, Gibco, 15140-148) before use, refer to **RPMI** in the following text), store at 2-8°C.

HI-FBS (Irvine Scientific, #3003, or Lonza #14-503F, heat inactivated), store in -20°C refrigerator.

RPMI with 10% HI-FBS (Irvine Scientific, #3003, heat inactivated), make 1 day before experiment, store in 2-8°C refrigerator.

RPMI with 5% HI-FBS (Irvine Scientific, #3003, heat inactivated), store at 2-8°C.

Percoll (Amersham Biosciences, #17-0891-02), store at 2-8°C.

10xPBS without Calcium and Magnesium (Gibco, #21600-069)

1xPBS without Calcium and Magnesium (Gibco, #21600-069)
Mouse BD Fc Block^{TM} (BD, # 553124, stock 0.5 μg/μL), store at 2-8°C.
FITC-anti-CD4 (BD, # 553729, stock 0.5 μg/μL), store at 2-8°C.
PE-anti-CD3e (BD, #553063, stock 0.5 μg/μL), store at 2-8°C.
PBS (Gibco, #20012), store at 2-8°C.

Procedures:
 * **1 day before the starting:**
____*bake the glass flasks needed at 180°C oven for 2 hours*
____*aliquot the reagents if needed (HBSS, RPMI, PBS, etc. each colon need 70 ml HBSS, 20ml RPMI with 10% HI-FBS, 10ml RPMI with 5% HI-FBS, 50 ml PBS)*
 ** **1h before the starting:**
____*prepare 90% percoll (9 part percoll + 1 part 10 x PBS, 5 ml needed for each colon)*
 *** **30mins before the starting:**
____*warm water bath to 37°C*
____*warm bottle PBS (at least 400ml) to room temperature*
____*warm bottle Ca, Mg free-HBSS (at least 160ml) to room temperature*
____*warm fresh make Digestion Solution I to 37°C in water bath, ~30ml for 2 mice colon.*
____*warm fresh make Digestion Solution II to 37°C by water bath, ~15ml for 2 mice colon.*
____*put 150x15mm and 60x15mm Petri dishes from the sterile bags in UV hood.*
____*warm RPMI to room temperature*
____*warm RPMI (with 5% HI-FBS) to 37°C*
____*chill PBS (Gibco, #20012) at 4°C, this is for flow detection, 5 ml for each flow sample*

Conduct the work on the bench top.
1. **Remove epithelial compartments from intestines** (modified by Satya's protocol)
   1). Sacrifice 3* mice by CO_2, apply alcohol to the abdomen area, grab the skin of the abdomen with forceps and make a small incision, peel back the skin/fur with fingers to expose the membrane underneath, grab the membrane with forceps and cut the membrane to expose the organs, move all the contents of the abdomen away from the ventral side of the animal. The colon is in the back. Resect colon proximally from the colon-cecum junction and distally from the rectum.
   : Each colon from C57BL/6 mouse yields ~1 million LPL, use 2-3 practice mice for practice, then you could have 2-4 samples for flow cytometry, or use your own plan for your situation.
   2). Open the colon longitudinally, remove the feces, grab the tissue with a forcep, wash within ~60ml room temperature warm PBS in plastic container for 3 times in 3 different containers.

3). Place the 3 colons in a 100 ml flat bottom glass flask with 45~60 ml of pre-warmed Digesting Solution I (3 colon can be in the same flask), seal with parafilm. *If you have a single colon, incubate with 20 ml Digesting Solution I.*

4). Incubate the flask in the shaking water bath at 37°C at speed 100 rpm for 15 minutes.

5). Pour contents of the flask into a 150x15mm Petri dish (on ice). Gently scrape the mucosal side (the side you can see the ladder look of the proximal colon) of the colon with a rubber policeman. Leave the scraped parts in the dish, transfer the remaining colon tissue into a plastic container with ~40ml room temperature warm Ca, Mg free-HBSS.

6). Grab the tissue with a forcep, wash it in Ca, Mg free-HBSS for 2 times in different containers.

2. Digest lamina propria parts and make single cell suspension

1). Cut the tissue by mince the tissue in a petri dish for 3 minute to small pieces, transfer the pieces into a new 50ml glass flask (baffled) with ~20 ml of pre-warmed Digesting Solution II. Incubate the flask at 37°C in a shaker water bath for 1 hour at speed 100 rpm till the tissue pieces look less and thin.

At this time, turn on UV of hood in cell culture room

2). Stop the incubation from water bath, add 20ml 37°C warmed RPMI (with 10% HI-FBS) into each flask, pipette up and down 1-2 min to separate the cells by using a 10ml sterile pipet (don't use glass pipet). The remaining tissue in the solution should be less and almost translucent. If not, repeat the digestion with ~20ml new fresh Digesting Solution II to incubate for 20 minutes.

-Work the following steps in the hood/cell culture room

3). Transfer all the cells solution into a new 50 ml conical tube. Spin down at 200g, 5min, ROOM TEMPERATURE.

4). **Resuspend cells into 10ml room temperature warm RPMI (with 5% HI-FBS). Connect a 10ml syringe with a sterile wire filter, connect the 20G needle with the filter, pour the cell suspension into the syringe, press it by plunger and pass these liberated lamina propria cells freely through the wire filter, harvest the cells in a new 15ml conical tube.

**This step could also be done by passing the cell suspension though a 30 μm filter (Miltenyi part # 130-041-407) placed on top of a 15 ml conical tube.*

5). Spin down cells at 200g, 5min, room temperature.

3. Isolate lymphocytes from lamina propria (by using Percoll (Amersham Biosciences, #17-0891-02))

1). Resuspend cells in 3ml room temperature-warm PBS (1xPBS). Aliquot 10ul for counting (2X dilution, 10ul cells+10ul Typan blue), count cell numbers.

The number of total cells from the lamina propria is ____x10^6 from 3 mice colons. (One C57BL/6 colon yields 20-50 x 10^6 LP cells).

2). Prepare Percoll gradient: First, make a 90% Percoll solution by diluting 9 parts Percoll with one part 10x PBS. Next, prepare 40% and 70% Percoll solutions by diluting

the 90% Percoll solution with 1x PBS to the appropriate concentration. It does not matter if you add 90% Percoll or 1x PBS first into the 50 ml tube. Gently invert the tube up and down to mix the solution evenly.

-90% Percoll solution can be prepares ahead of time, but the 70% and 40% solutions should be made fresh.

-for 40% and 70% Percoll solutions

90% Percoll	1xPBS	
4.44 ml	*5.56ml*	*for 10ml 40% Percoll*
7.78ml	*2.22ml*	*for 10ml 70% Percoll*

3). Make the 70% and 40% Percoll gradient using the following steps:

-Transfer 3 mL of 40% Percoll in a 15 mL conical tube.

-Lay down 3 mL of 70% Percoll beneath the 40% Percoll using a 2 mL pipette. Push the pipette slowly and gently, pay attention NOT to mix the two layers!

- Overlay the cell suspension gently using Laurie's 1ml pipette, you may load 1~2x10^7 cells per tube.

Centrifuge at 2500 rpm for 20 minutes with the <u>break off</u> at room temperature (Use the Jouan centrifuge in R321).

4). Collect the interface between 70 and 40% Percoll by using a 1ml pipette, which are lymphocytes, and transfer to fresh 15ml conical tubes. Fill the conical tube to brim with room temperature-warm PBS (1xPBS) in order to wash away Percoll. Centrifuge at 200g, 5min in room temperature.

You may also want to collect the top and bottom layer for checking the population when you practice.

5). Remove the supernatant, resuspend cells with 400ul room temperature PBS (Gibco, #20012), and count cells by hemocytometer.

4. Count cell numbers

Count the cell number in a hemacytometer with (1) Trypan Blue to assess percent dead cells (10ul cells+10ul dye+80ul PBS) and with (2) Crystal Violet stain (nuclei of leukocytes will be stained blue) to count white cells (10ul cells+90ul dye). The number of total lymphocytes from the lamina propria is:

_____x10^6 by trypan blue staining; _____x10^6 by crystal violet staining.

Notice that this is a population enriched with lymphocytes and other type of cells. You may detect your target using specific markers or further purify certain cell types.

The yield of LPLs per mouse is ~ 0.5-1 x10^6 cells, viability is more than 80% by Typan blue.

5. Fluorochrome conjugation and flow analysis

_____*bring FITC-anti-CD4, PE-anti-CD3e and Fc Block from lab refrigerator to cell culture room's refrigerator, protected from light.*

_____*pre-chill the centrifuge in Room 321 (the one behind Laurie's bench) to 4°C.*

1). Adjust the cell suspension into 10^6/100ul PBS. (If the total number is less than 4x10^6, keep the 400ul volume). Transfer the cell suspensions from conical tubes into 4 flow-tubes, 100ul each. Set samples as following:

A. Unstained control
B. Staining with FITC-anti-CD4 only
C. Staining with PE-anti-CD3e only
D. Staining with both FITC-anti-CD4 and PE-anti-CD3e

2). (Hide Light) Add 2 µl (1µg) of Mouse BD Fc Block[TM] (BD, cat# 553124, stock 0.5 µg/µL) per 10^6 cells (for blocking FcRs) in tubes B-D.

3). Mix well (by pipetting up and down) and incubate at 4 °C (refrigerator, not on ice!!) for 5 min. (no shaking needed)

4). (Hide Light) Add 2 µl (1µg) of each mAb (stock 0.5 µg/µL) or both per 10^6 cells in tubes B-D, for tube D, add FITC conjugated Ab firstsince it's less bright then PE second)

5). Mix well (by pipetting up and down) and incubate at 4 °C (refrigerator, not on ice!!) for 5 min. (no shaking needed)

6). Wash cells by adding 2 ml ice cold PBS into each tube, and centrifuge at 300 x g for 5 min at 4°C. Pipette off supernatant completely.

7). Re-suspend cells into 400ul ice cold PBS for flow analysis

References:

1). Sund M, Xu L, Rhaman A, Qian BF, Hammarstrom ML, Danielsson A. 2005. Reduced susceptibility to dextran sulphate sodium-induced colitis in the interleukin-2 heterozygous (IL-2^{+/-}) mouse. *Immunology* 114:554-64.

2). Totsuka T, Kanai T, Iiyama R, Uraushihara K, Yamazaki M, Okamoto R, Hibi T, Tezuka K, Azuma M, Akiba H, Yagita H, Okumura K, Watanabe M. 2003. Ameliorating effect of anti-inducible costimulator monoclonal antibody in a murine model of chronic colitis. *GASTROENTEROLOGY* 124:410–421.

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4). Boone DL, Dassopoulos T, Chai S, Chien M, Lodolce J, Ma A.2003. Fas is not essential for lamina propria T lymphocyte homeostasis. *Am J Pgysiol Gastrointest Liver Physiol* 285: G382-388.

5). http://www.bcm.edu/labs/goodell/protocols/Goodell_Musclestemcells.pdf

Representative results: Figure 6, p25.

Appendix B3. LPL isolation by collegenases, Percoll and Medimachine

Aim:
To prepare LPL from mouse colon for Treg and Th17 detection.

Materials
1. Medimachine (BD 340587)
2. Medicons (non-sterile, 50um, BD 340592)
3. HBSS (sigma H-6648, Ca, Mg free)
4. 10% BSA, IgG free (Boehringer Manheimer 100018) in HBSS, aliquoted and stored in -20°C.
5. 200mM Glutamine (Gibco #25030), aliquoted and stored in -20°C.
6. Dithiothreitol (DTT) (Sigma D-0632, MW 154.25), stored in 4-8°C.
7. 0.75M EDTA (Sigma ED4SS) in HBSS, aliquoted and stored in -20°C.
8. Collagenase II (Worthington Biochem LS004176), stored in 4-8°C. Units of enzyme activity vary with different lot.
9. Collagenase IV (Worthington Biochem LS004188), stored in 4-8°C. Units of enzyme activity vary with different lot.
10. DNase I (Worthington Biochem LS002004), stored in 4-8°C.
11. 100mm Petri dish (BD 351029)
12. RPMI 1640 (Irvine Scientific, #9159, 500ml bottle, added with 5ml Glutamax (100x, Gibco 35050-061) and 5 ml Penicillin-Streptomycin solution (100x, Gibco, 15140-148) before use, refer to RPMI in the following text), store at 2-8°C.
13. HI-FBS (Irvine Scientific, #3003, or Lonza #14-503F, heat inactivated), store in -20°C refrigerator.
14. RPMI with 10% HI-FBS (Irvine Scientific, #3003, heat inactivated), make 1 day before experiment, store at 2-8°C.
15. 30 μm MACS Pre-separation Filter (Miltenyi Biotec, cat# 130-041-407)

Solution preparation
1. Digesting Solution I: HBSS (Ca and Mg free) with 1mM Glutamine, 0.1% BSA, 30mM EDTA and 5mM DTT, 20ml needed for each mouse colon

Digesting Solution I (per 100ml)	Final Conc
-0.5 ml - 200mM Gln	1mM
-1 ml - 10% BSA	0.1%
-4 ml - 0.75M Na4EDTA	30mM
-0.077 g – DTT	5mM

-Make the solution fresh
-Combine Gln, BSA, EDTA, DTT in 80ml HBSS without Ca and Mg
      -Adjust pH to 7.4 - 7.5 with HCl / NaOH
      -Bring final volume up to 100ml (or other designated volume)
-Warm to 37°C before use

2. Digesting Solution II: HBSS (Ca and Mg free) with 200U/ml Collagenase II, 200U/ml Collagenase IV and 5U/ml DNase I. 20ml needed for each mouse colon. *The Unit for each lot of these enzymes may vary, therefore, pay attention to the calculation; the following calculation is an example.*

Digesting Solution II (per 100ml)   Final Conc
     -__mg Collagenase II   200U/ml
     -__mg Collagenase IV   200U/ml
     -0.1mg 5136U/mg DNase I   5U/ml
-Make the solution fresh
-Combine Collagenase II, Collagenase IV and DNase I in ___ml HBSS without Ca and Mg
-Warm to 37°C before use

Procedures
    a. Removal of epithelium
        i. Sacrifice mice by CO_2.
        ii. Dissect colon and cut longitudinally.
        iii. Rinse with 1x PBS three times.
        iv. Incubate in 20 mL digestion solution I in 37 °C water bath at speed 100 rpm for 15 min, label the flask with your animal ID.
        v. Transfer the tissue/solution into a 100 mm petri dish on ice.
        vi. Gently scrape the mucosa by rubber policeman.
        vii. Wash the remaining tissue wit HBSS three times.
        viii. Place the tissue on a 100 mm petri dish lid.
    b. Digestion by collagenases
        i. Dissect the tissue with scissors thoroughly **(** ** **continually cut it for 3min, as small as possible, critical for digestion)**
        ii. Transfer dissected tissues with 10 mL of digestion solution II into a baffled flask, label the flask with your animal ID.
        iii. Wash the lid with another 10 mL digestion solution II and add into the same flask.
        iv. Incubate in 37°C water bath at speed 100 rpm for 1h.
        v. Pipette up/down every 10 min **(** ** **critical for digestion)**.
        vi. After incubation, add 10 mL 10% FBS/RPMI to stop collagenase activities.
        vii. Transfer suspension into a 50 mL conical tube.
        viii. Centrifuge at 3000rpm for 5 min at room temperature.
        ix. Transfer the supernatant into another 50 mL conical tube (keep the supernatant).
    c. Mechanical isolation by Medimachine
        i. Resuspend the pallets in 1 mL of HBSS and transfer to a Medicon.
        ii. Assemble Medicon into Medimachine.
        iii. Run Medimachine for 2 min at room temperature.

iv. Put a 30 μm MACS Pre-separation Filter on top of a 15mL conical tube, wet the membrane by 1mL HBSS. Lid the cap of the medican, such the cell suspension from the side hole by a pippte, let the cell suspension go through the filter into a 15 mL conical tube.

v. Wash the cube by 1ml HBSS twice, collect suspension from the side hole and go through the filter into the same 15ml conical tube. If the filter is blocked by tissue piece, use a new one (remember you also need to prewet the filter by 1mL HBSS), carefully remove the unfiltered cell suspension from the blocked filter and let it go through the new filter. Also collect those on the bottom of the filter if possible.

vi. Centrifuge both 15 mL AND 50 mL (from step b-ix.) at 300x g (or 1500rpm) for 5 min at room temperature.

vii. Aspirate supernatant and mix both pellets in 1 mL RPMI.

d. LPL enrichment by Percoll

Percoll preparation: -90% Percoll solution can be prepares ahead of time, but the 70% and 40% solutions should be made fresh.

90% Percoll: 9 part Percoll + 1 part 10 x PBS

-for 40% and 70% Percoll solutions

90% Percoll	1xPBS	
11.1 ml	13.9 ml	for 25 ml 40% Percoll
19.45 ml	5.55 ml	for 25 ml 70% Percoll

i. Transfer 3 mL of 40% Percoll in a 15 mL conical tube.

ii. Lay down 3 mL of 70% Percoll beneath the 40% Percoll using **2 mL pipette**.

** Pay attention NOT to mix the two layers!

iii. Lay cell suspension on top of 40% Percoll gradient **by using Laurie's 1ml pipette and tips with the sharpe end cut (Laurie's pipette is gentle and the open end tips can easily collect the interface).**

iv. Centrifuge at 2500 rpm for 20 min **with the break off at room temperature** by using centrifuge behind Laurie's bench in 321.

v. Transfer the cells between 40% and 70% Percoll layers into a new 15 mL conical tube.

vi. Wash cells by filling the tube with 1x PBS up to 10 mL. Leave a 20ul aliquot for counting cell number.

vii. Centrifuge at 300x g for 5 min. Count cell numbers during the time.

viii. Aspirate the supernatant and resuspend cells in 500 uL staining buffer. Proceed with staining for flow

References

1). Sund M, Xu L, Rhaman A, Qian BF, Hammarstrom ML, Danielsson A. 2005. Reduced susceptibility to dextran sulphate sodium-induced colitis in the interleukin-2 heterozygous (IL-2$^{+/-}$) mouse. *Immunology* 114:554-64.

2). http://www.bcm.edu/labs/goodell/protocols/Goodell_Musclestemcells.pdf

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Appendix B4. Th17 flow detection by eBioscience reagents (splenocytes and LPLs)

Aim:
  This protocol is to determine the Th17 population size in lamina propria of colon (local) and spleen (systemic) in C57BL/6 mice. For primary experiment to set up the methodology, will just use splenocytes and polarize the cells for maximum staining.

Rationale & Markers:
2 color staining for splenocytes and LPL

Cell	Marker	Type	Ab	Clone	Company
Th17	CD4	Surface	APC-anti-CD4	RM4-5	eBioscience#17-0042
	IL-17	Intracellular	PE-anti-IL-17	TC11-18H10	BD #559502

Materials:
--- 6 well or 24 well tissue culture plate
--- 70 µm Cell Strainer (BD#352350, Fisher# 08-771-2)
--- 30 µm MACS Pre-separation Filter (Miltenyi Biotec, cat# 130-041-407)
--- auto MACS Running Buffer (MACS Separation Buffer, Miltenyi Biotec, cat# 130-091-221).
--- Lympholyte M (Cedarlane # CL5030)
--- Alexa Fluora 647 a-IL17A (eBioscience # 12-7177, stock 0.2mg/mL)
--- Alexa Fluora Rat IgG2a isotype control (eBioscience # 51-4321, stock 0.2mg/mL)
--- PE rat anti mouse a-IL17 (BD #559502, stock 0.2mg/mL)
--- PE Rat IgG1, κ Isotype Control (BD #554685, stock 0.2mg/mL)
--- neutralizing a-IL2 (eBioscience # 16-7022, , stock 1mg/mL)
--- FITC a-CD4 (clone RM4-5) (eBioscience # 11-0041, stock 0.2mg/mL)
--- APC a-CD4 (clone RM4-5) (eBioscience # 17-0042, stock 0.2mg/mL)
--- Brefeldin A (eBioscience # 00-4506, 1000X)
--- human rTGF-β (eBioscience # 14-8348, 2.5 ug/mL stock)
--- murine rIL-6 (eBioscience # 14-8061, 50 ug/mL stock)
--- anti mouse IL2 (eBioscience # 16-7042, stock 1mg/mL)
--- PMA (Sigma # P1585), dilute in DMSO, stock 0.1mg/ml in -20°C
--- Ionomycin (Sigma # I0634) , dilute in DMSO, stock 2mg/ml in -20°C
--- Fc Block^{TM} (BD Pharmingen # 553124, stock 0.5 µg/µL)
--- anti-CD3 mAb (clone 145-2c11) (BD Pharmingen # 553057)
--- anti-CD28 mAb (clone 37.51) (BD Pharmingen # 553294)
--- Foxp3 Staining Buffer Set (eBioscience # 00-5523 or from kit #88-8111). Containing Fixation/Permeabilization Concentrate, Fixation /Permeabilization Diluent, and 10X Permeabilization Buffer.
--- Flow Cytometry Staining Buffer (eBioscience # 004222), refer to sBuffer in the following text

---   RPMI 1640 (Irvine Scientific #9159), 500ml bottle, added with 5ml Glutamax (100x, Gibco 35050-061) and 5 ml Penicillin-Streptomycin solution (100x, Gibco, 15140-148) before use, refer to RPMI in the following text), store at 2-8°C.

  HI-FBS (Irvine Scientific #3003), store in -20°C freezer.

  Complete RPMI (cRPMI) = RPMI 1640 with 10% HI-FBS + 5 ml of Penicillin-Streptomycin (100x, Gibco #15140-148) + 5 ml of Glutamax (100x, Gibco #35050-061), store in 2-8°C refrigerator.

  *** add 2-ME (2-beta mercaptoethanol, Sigma # M7522) just before use

Procedure:
Part 1: Isolate splenocytes
Keep MACS buffer cold (on ice), but perform the rest of procedure at room temperature except when indicated.

1. Remove the spleen from a mouse as followed. Sacrifice mice by CO2
    1) Place mice on their right side so that the left side faces you
    2) Apply alcohol to the abdomen area
    3) Grab the skin of the abdomen with forceps and make a small incision
    4) Peel back the skin/fur with fingers to expose the membrane underneath
    5) Grab the membrane with forceps and cut the membrane to expose the organs
    6) Remove the spleen (dark red organ) with forceps
    7) Carefully remove as much fat from the exterior of the spleen as possible
    8) Place a spleen in a 15ml conical tube containing 3ml MACS buffer, transfer to cell culture room.

Perform the following steps under sterile hood.

2. Prepare single cell suspension from spleen (In sterile hood)
    1) Place a Falcon cell strainer (70 μm mesh, BD, # 35-2350), on the 100mm petri dish, and pre-wet with 5mL MACS® buffer.
    2) Transfer the spleen into cell strainer.
    3) Gently press spleen against the bottom of the strainer with a plunger from a 5 ml syringe. Use up and down motion (not grinding), being careful not to damage cells.
    4) When only the spleen connective tissue remains in the strainer, remove the plunger.
    5) Transfer the cell suspension from the dish to the original 15mL conical tube.
    6) Wash strainer with 5ml MACS buffer, and transfer to the same tube to collect the remaining cells.
    7) Spin down at 300xg for 5 min.
    8) Remove the supernatant and resuspend the pellet by flicking the tube or ratcheting the tube over a tube rack.
    9) Place a 30 μm filter (Miltenyi part # 130-041-407) on top of a new 15 mL conical tube

10) Take 10mL MACS buffer and and pre-wet 30um filter with 2 mL buffer.
11) Resuspend cells with the rest 8mL MACS buffer.
12) Pass the cells through the 30 μm filter into a 15 ml conical tube.
13) Remove 10ul to a coulter counter vial for counting.
14) Centrifuge the cells at 300x g for 5-10 min.
15) Remove the supernatant and resuspend the pellet by flicking the tube or ratcheting the tube over a tube rack.
16) Count the cell number during waiting for centrifuge by Coulter counter (prepare counting fluid 24h before experiment) with 4um cut-off (or hemacytometer as an alternative option).
   a. Power on Coulter counter.
   b. Replace Coulter clenz solution with isotone fluid containg vial.
   c. Thouch "Function" → "Flush"
   d. Flush again with a new isotone fluid.
   e. Touch "Set up" → Size: 4um
   f. Touch "Output" → result: concentration, dilution factor: 1000
   g. Replace the vial with a cell-containing vial.
   h. Touch "Start"
   i. After cell counting the concentration will be displayed, for example "1.523 E7", which means 1.523×10^7 or 15.23×10^6 cells/ml (**concentration!!**)
   j. Since cells are suspended in 10mL, multiply by 10 to calculate the **cell number**.

3.   Resuspend cells at the density <u>of 20 x 10^6 cells / ml of RMPI medium</u> (serum free).

4.   Add an **equal amount** of Lympholyte-M by layering it beneath the suspended cells.⇒ insert the pipet tip into the bottom of the conical tube before dispensing the Lympholyte-M. ⇒ this step must be done slowly and carefully in order to see 2 distinct bands. *Maximumly, typically 1 spleen in 5 mL RPMI then layer 5 ml Lympholyte M beneath.*

5.   Centrifuge the cells at 500 x g for 15 min. at room temperature ⇒ deactivate centrifuge brake at this step (**acc/dcc = 0**) ⇒ when samples are done spinning, you will see 2 distinct layers (a white one on the top and a clear, pink one on the bottom) and an interface band separating them. Your goal is to remove this interface band. The bottom clear layer contains the unwanted red blood cells, plasma, etc.
     You may prepare the EXPERIMENTAL medium according to part 3 during this time.

6.   Right after the centrifugation is done, carefully remove the interface band between the medium and Lympholyte-M layer with a 2 mL size pipette and dispense it into a new conical tube (should see a red pellet)

7.   Fill the conical tube with RPMI to wash and centrifuge the cells at 300g for 5-10 min at room temperature.

8. Resuspend cells at the density of 5 x10^6 cells/3 mL EXPERIMENTAL medium (refer to part 3) with stimuli (see below) for culture use.

Part 2: LPL isolation, refer to LPL isolation protocol (Appendix A3)

Part 3: Culture with stimuli (For polarization only, skip this step for direct detection of Th17)

In day -1: Anti-CD3 pre-coat

  Dilute anti-CD3 mAb (BD Pharmingen cat# 553057, stock 1mg/ml) 1000 times in sterile PBS to make 1ug/mL working solution (Mix 1ul anti-CD3 stock + 1ml PBS). Transfer 1ml diluted solution into aCD3/28 stimulation-designated wells in 6-well plates. Tape the plate and incubated at 4 °C refrigerator overnight or 37°C for 2h.

In day 0: In vitro culture with stimuli

   Prepare complete medium just before use,
    -*Add 10ul Stock 2-ME (14.3 M) → 14.3ml medium, then 10mM solution*
    -*Add 10ul 10mM solution → 10ml medium, then 10uM final complete medium.*

   Aspirate the aCD3 solution from 6-well plate. Stimulation is followed by recipe from eBioscience. Prepare the EXPERIMENTAL medium (with 2-ME added) with **eBioscience cytokines** of Th17 stimulation cocktail including 10 ug/ml anti-CD28 (100x dilution of 1mg/ml stock), 1 ng/ml recombinant human TGF-β1 (2500x dilution of 2.5 ug/mL stock), 20 ng/ml recombinant murine IL-6 (2500x dilution of 50 ug/mL stock), 1 ug/ml of anti-mouse IL-2 (1000x dilution of 1 mg/mL stock). Resuspend splenocytes in the EXPERIMENTAL medium 5 million / 3 ml and culture in 6 wells 3 ml per well. Tape the plate then place in 37 °C CO$_2$ incubator for 3 d.

   Note: have 2 well cultures with anti-CD28 only as a basal control.

  If you only have 1 million cells from 1 colon, then use 24-well plate for culture with 1ml volume.

On day 3:

Part 4: P/I stimulation

  (Optional, if you need to remove dead cells, if not, skip this and go to next paragraph)
Harvest cells in 15 ml conical tubes from 6-well plates, wash wells with 3 ml RPMI and collect into the same conical tubes. Spin down the cultures then resuspend in 3 ml RPMI. Add 3 ml Lympholyte-M by laying it beneath the suspended cells (insert pipet into the bottom of conical tube, dispense Lympholyte-M slowly and carefully to see 2 layers). Centrifuge at 500g for 15 min with break off. Remove interface into a new 15ml conical rube, fill RPMI, centrifuge at 300g for 5 min with break on. Aspirate and resuspend cells in complete RPMI with PMA (50 ng/mL), Ionomycin (1ug/mL) and Brefeldin A (1:1000 dilution), then incubate 3ml/ well (6-well plate*) in the P/I stimulation-designated wells at 37°C incubator for 5h.

If you don't need to remove dead cells, just add PMA (1:2000, final 50 ng/mL), Ionomycin (1:2000, final1ug/mL) and Brefeldin A (1:1000 dilution) in each cell. Then incubate the well at 37°C incubator for 5h.

Note: have 1 well culture (after 3d culture with full stimuli) with Brefeldin A **only as a no P/I control.**

Part 5: Harvest cells

Harvest cells from 6 well plates by pipettes into 15 ml conical tubes (wash by PBS 3-6 ml to collect all cells) and spin down at 300g for 5 min and decant supernatant gently by aspirator. Resuspend the pellets in PBS or sBuffer of 10 million cells / mL, aliquot 100 uL per tube, now cells are ready for staining.

Part 6: Staining
  Sample sets:
  Unstained
  APC anti- CD4 (RM4-5)
  APC-CD4 + PE-anti-IL17 (TC11-18H10)
  APC-CD4 + PE Rat IgG1 isotype control
  Samples are 100uL of 10 x10^6 spelenocytes or LPLs / mL cold sBuffer in 15mL conical tubes, label the liquid level in the conical tubes by a sharpe pen.

1). Fc block (BD Pharmingen # 553124, stock 0.5 μg/μL)
  Block Fc receptors for reducing nonspecific immunofluorescent staining by adding 2μL Fc Block stock per sample, incubate on ice for 10 min.

  Tips: Protect cells from light throughout the following staining, shut down the light for hood whenever you have antibody in hood
  Take an aliquot of the antibody you need for this time from the stock vial to avoid cross-contamination
  Aspirate your supernant just after centrifugation to avoid disturb the pallet, then flick the tube bottom on the hood to loose the pallet
2). Surface staining
  Add 0.5 μg/test of APC-anti-CD4 antibody in sample for surface staining *(New vial of Ab needs titration beforehand)*. Mix gently. Incubate in dark at 4°C for 30 minutes. Wash in 2 mL cold PBS or sBuffer. Centrifuge cells for 400g 5 minutes at 4°C or room temperature. Aspirate supernatant, leave about (no more than) 100 ul volume.

3). Fix and permeabilize Cells

> Prepare working dilution
>   Dilute the Fixation/Permeabilization Concentrate (1 part) into the Fixation/Permeabilization Diluent (3 parts) to the desired volume of <u>Fixation/Permeabilization working solution</u>. Use it for no more than 1 day.
>   1 ml / sample x _____ samples (including controls) = _____ ml needed
>   Dilute the 10 x stock Permeabilization Buffer in distilled water to a <u>1x Permeabilization solution</u> prior to use. Make fresh before each experiment.
>   8 ml / sample x _____ samples (including controls) = _____ ml needed

You may want to have a small alquot (10uL) of sample for before fixation detection of CD4 staining.

Resuspend cell pellet with pulse vortex and add 1 ml of freshly prepared Fixation/Permeabilization working solution (eBioscience #00-5523) to each sample. Pulse vortex 5 times. Incubate at 4°C for 35 minutes in the dark.

Wash by adding 2 ml 1X Permeabilization Buffer (made from 10X Permeabilization Buffer, eBioscience #00-5523). Centrifuge cells 5 minutes (300-400xg) at 4°C or room temperature. Aspirate supernatant, leave about (no more than) 100 ul volume.

[OPTIONAL] Block with 2% (2 μl) normal rat serum in 1X Permeabilization Buffer, in approximately 100 μl volume, at 4°C for 15 minutes.

4). Stain intracellular cytokines

(Light sensitive, always dim light)

  Without washing after blocking step, add 0.5 μg/test of diluted PE-anti-IL17A antibody *(New vial of Ab needs titration beforehand)* or isotype control in 1X Permeabilization Buffer, pulse votex t times to mix well, and incubate at 4°C for at least 30 minutes in the dark.

  Wash cells with 2 ml 1X Permeabilization Buffer. Centrifuge cells 5 minutes (300-400xg) at 4°C or room temperature. Aspirate supernatant.

5). Flow cytometry

  Resuspend in appropriate volume (~200μL) sBuffer, Transfer cell suspension into 12 x 75 Polystyrene tubes and analyze on cytometer. Please note that due to the fixation and permeabilization procedure, the FSC/SSC distribution of the cell population will be different than live cells. Therefore the gate and voltages will need to be modified.

References
1). Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126(6):1121-33.
2).http://www.bdbiosciences.com/external_files/pm/doc/tds/brm/live/web_enabled/2329 5A_559502.pdf
3). http://www.ebioscience.com/ebioscience/specs/antibody_51/51-7177.htm

Representative results: Figure 9, p. 36.

A

B

C

Cytokines + PMA/ Ionomycin
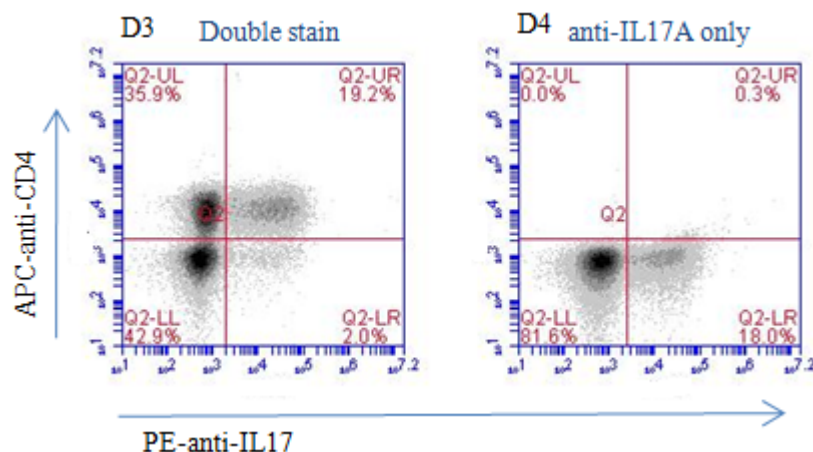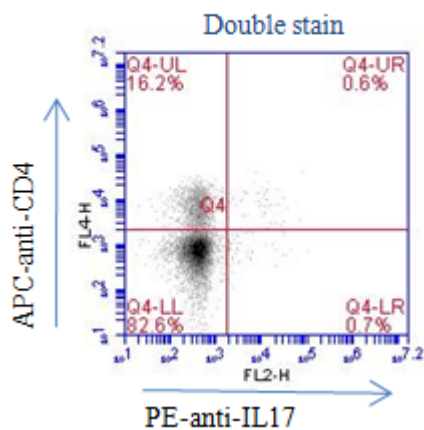
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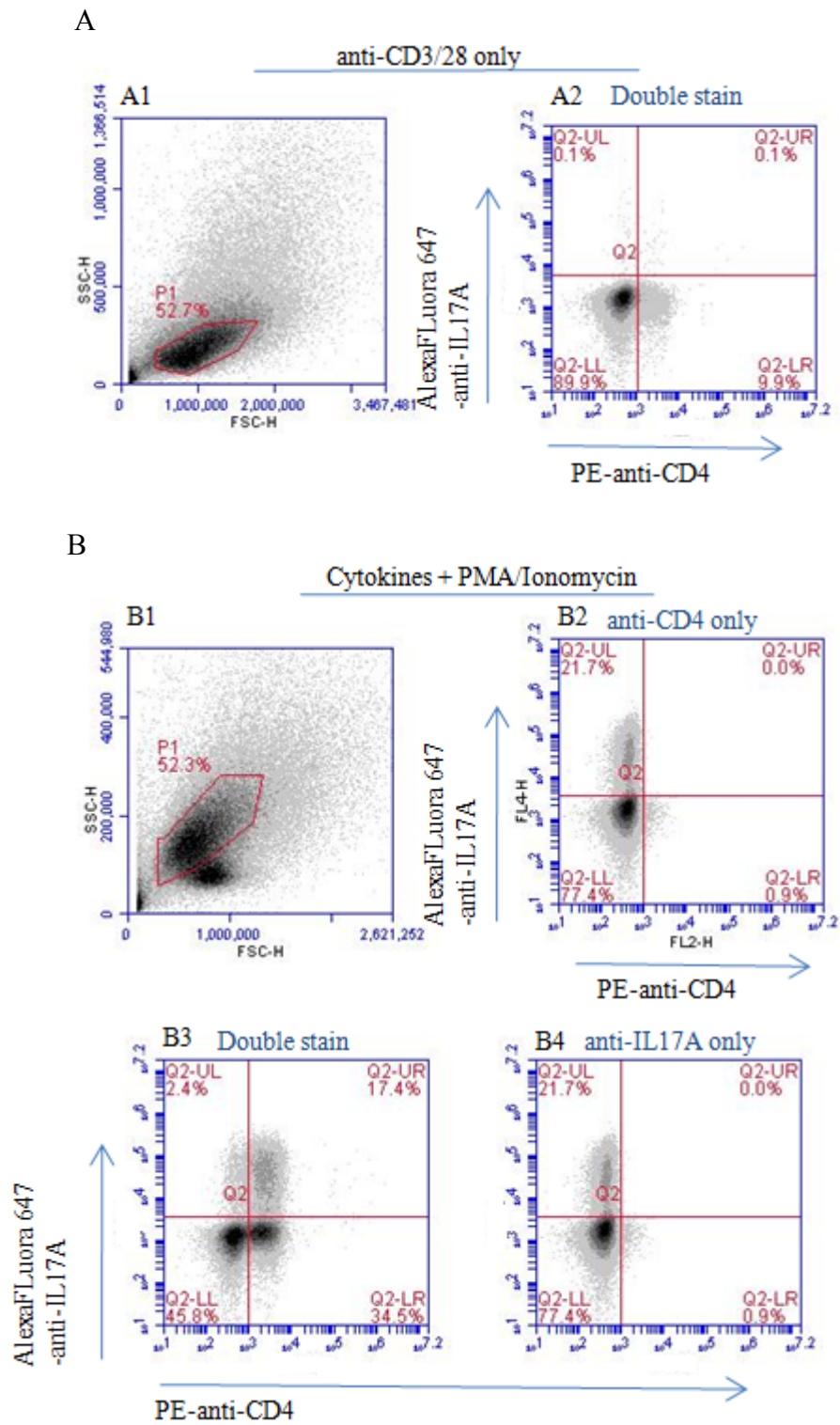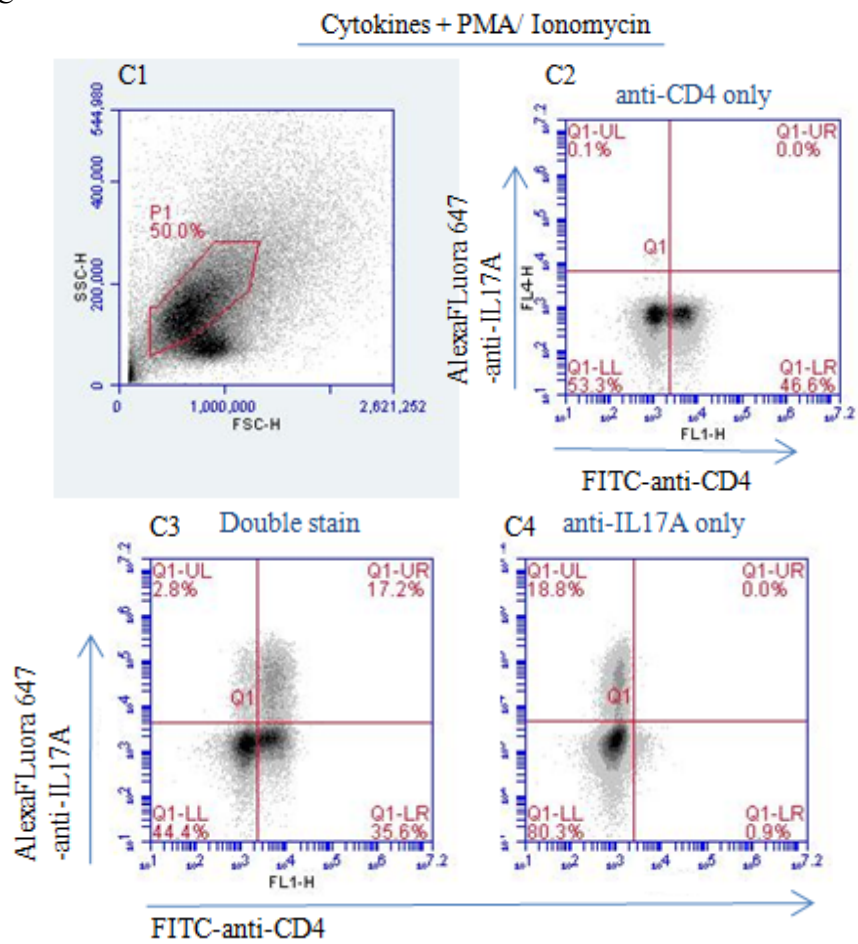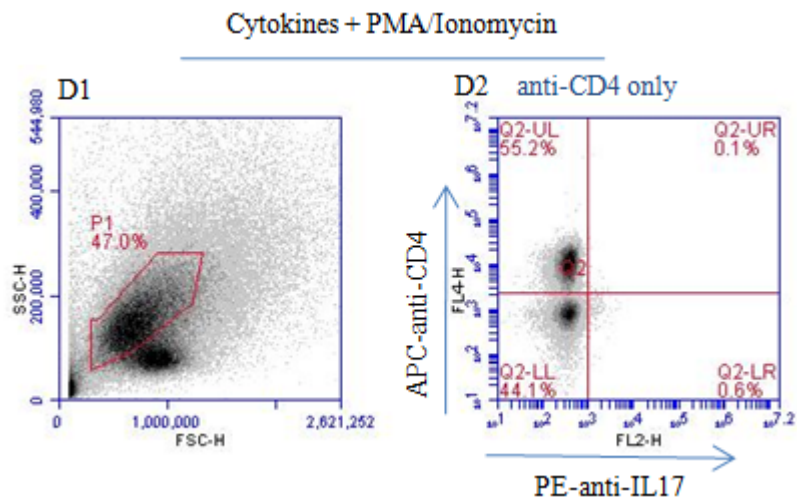
Cytokines + PMA/Ionomycin

Th17 polarization of splenocytes from C57/BL6 mouse. Th17 cells were detected by double staining of anti-CD4 and anti-IL-17. **A**. Th17 detection in splenocytes cultured with anti-CD3 and anti-CD28; **B-D,** Th17 detection in splenocytes stimulated by eBioscence cytokines plus PMI/Ionomycin as described in the ptotocol; **E**, Th17 detection in splenocytes stimulated by eBioscence cytokines (see details in the protocol and next paragraph) without PMI/Ionomycin.

A1, cells stimulated with anti-CD3 and anti-CD28 only, FSC/SSC histogram to show the postion of lymphocytes; **A2**, cells stimulated with anti-CD3 and anti-CD28 only, histogram represents PE-anti-CD4 (BD, clone RM4-5) and AlexaFluora 647-anti-IL17A (eBioscience, clone eBio17B7); **B1**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, FSC/SSC histogram to show the postion of lymphocytes; **B2**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, histogram by PE-anti-CD4 (BD, clone RM4-5) only; **B3**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, histogram by PE-anti-CD4 (BD, clone RM4-5) and AlexaFluora 647-anti-IL17A (eBioscience, clone eBio17B7); **B4**, cells stimulated with eBioscience cytokines

and PMA/ Ionomycin, histogram by AlexaFluora 647-anti-IL17A (eBioscience, clone eBio17B7) only; **C1**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, FSC/SSC histogram to show the postion of lymphocytes; **C2**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, histogram by FITC-anti-CD4 (BD, clone RM4-5) only; **C3**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, histogram by FITC-anti-CD4 (BD, clone RM4-5) and AlexaFluora 647-anti-IL17A (eBioscience, clone eBio17B7); **C4**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, histogram by AlexaFluora 647-anti-IL17A (eBioscience, clone eBio17B7) only; **D1**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, FSC/SSC histogram to show the postion of lymphocytes; **D2**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, histogram by APC-anti-CD4 (eBioscience, clone RM4-5) only; **D3**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, histogram represents APC-anti-CD4 (eBioscience, clone RM4-5) and PE-anti-IL17A (BD, clone TC11-18H10); **D4**, cells stimulated with eBioscience cytokines and PMA/ Ionomycin, histogram by PE-anti-IL17A (BD, clone TC11-18H10) only; **E**, cells stimulated with eBioscience cytokines without PMA/ Ionomycin, histogram by APC-anti-CD4 (eBioscience, clone RM4-5) and PE-anti-IL17A (BD, clone TC11-18H10).

The portion of Th17 cells in spelnocytes is quite low as shown in panel A. By polarizing the cells in vitro culture with **eBioscence cytokines**, in details, anti-CD3 and anti-CD28, 1 ng/ml recombinant human TGF-β1, 20 ng/ml recombinant murine IL-6, 1 ug/ml of anti-mouse IL-2 in the medium for 72 hours, stimulated by PMA (50 ng/mL) and Ionomycin (1ug/mL) for last 5 hours, Th17 cells portion rised up to >17% (**B-D**). Multiple fluorochromes conjugated antibodies were tested and the best detection is APC-anti-CD4 (eBiosceince, clone RM4-5) and PE-anti-IL17A (BD, clone TC11-18H10). The polarization was not successful without the PMI/Ionomycin stimuli for the last 5 hour (**E**). The results of polarization are comparable to eBioscience report available at http://www.ebioscience.com/ebioscience/specs/antibody_53/53-7177.htm.
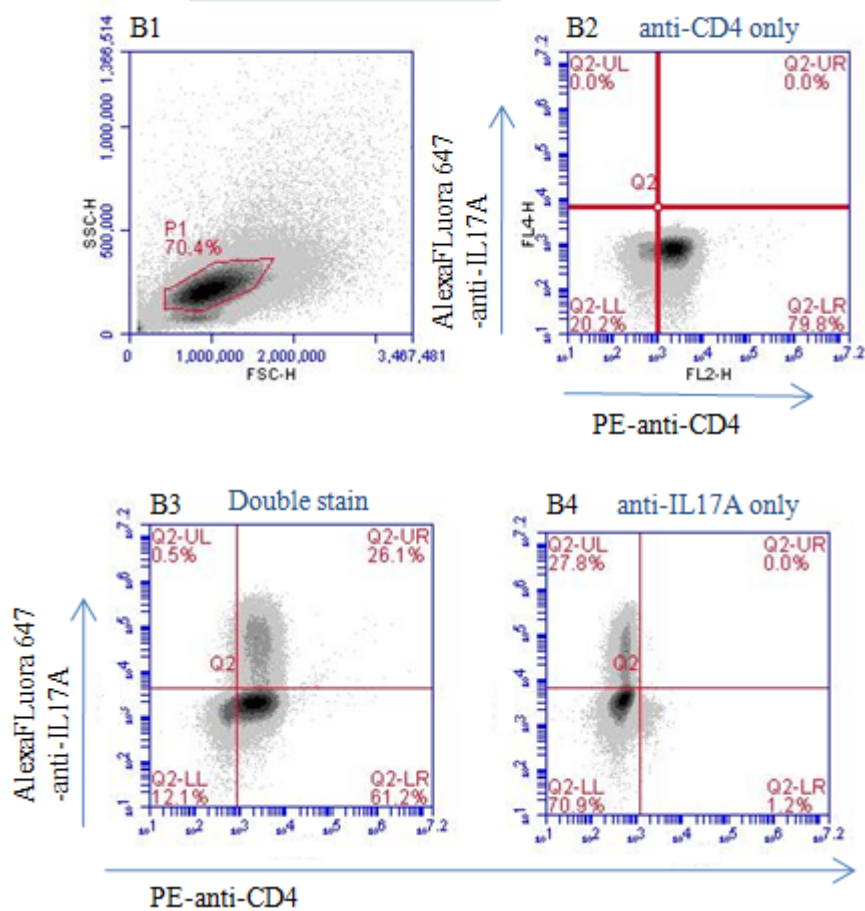
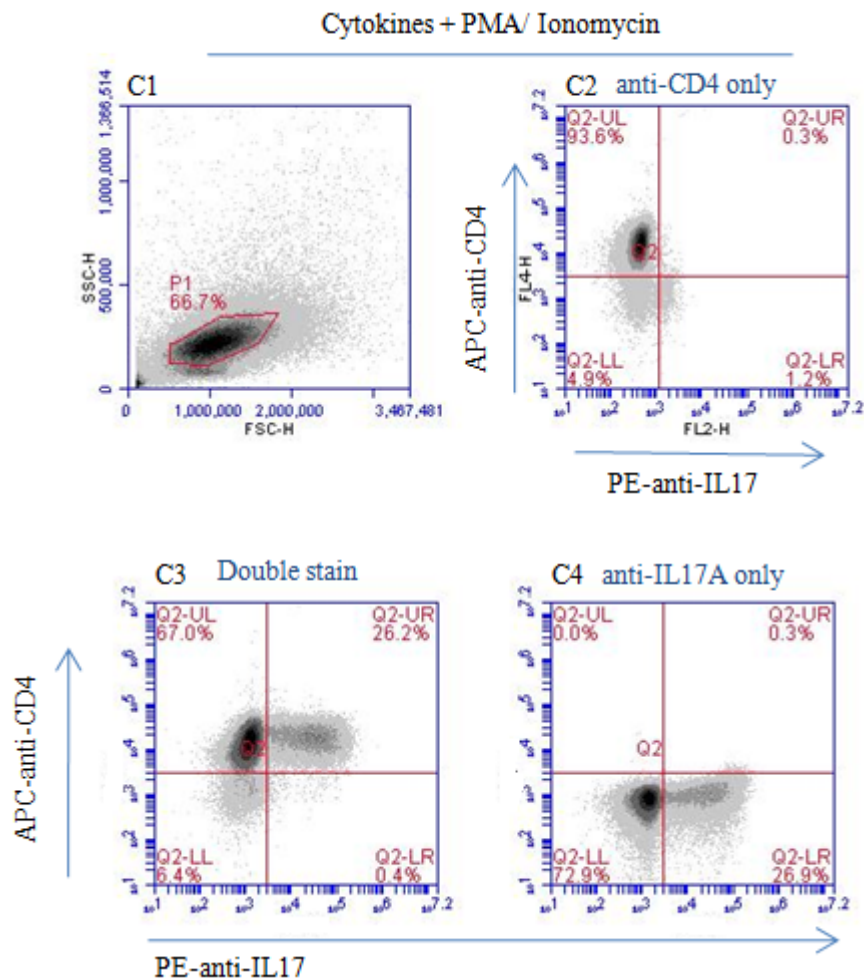A — anti-CD3/28 only

B — Cytokines + PMA/ Ionomycin

C
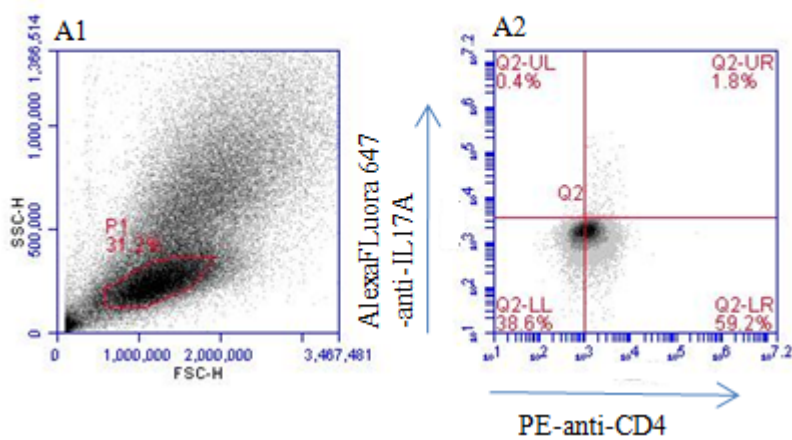
Cytokines + PMA/ Ionomycin

Th17 polarization of CD4$^+$ splenocytes from C57/BL6 mouse. CD4$^+$ splenocytes were purified from mouse spleen according to method from **Appendix B1** and cultured with Th17 polarization stimulis of multiple cytokines. Th17 cells were detected by double staining of anti-CD4 and anti-IL-17. **A**. Th17 detection in CD4$^+$ splenocytes cultured with anti-CD3 and anti-CD28 only; **B** and **C**, Th17 detection in CD4$^+$ splenocytes stimulated by multiple cytokines (including a-CD3/CD28, TGF-β1, IL-6, IL2, anti-IL4, anti-IFNγ) as described in *Ivanov 2006 cell 126:1121* plus PMI/Ionomycin. Th17 cells portion were detected by double staing of PE-anti-CD4 (BD, clone RM4-5) and AlexaFluora647-anti-IL17 (eBioscience, clone eBio17B7) (**B**), or APC-ani-CD4 (eBioscience, clone RM4-5) and PE-anti-IL17 (BD, clone TC11-18H10) (**C**).
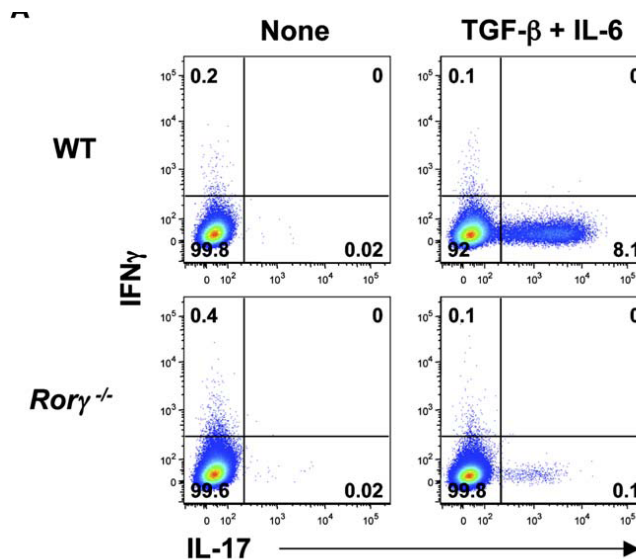
A1, cells stimulated with anti-CD3 and anti-CD28 only, FSC/SSC histogram shows the postion of lymphocytes; **A2**, cells stimulated with anti-CD3 and anti-CD28 only, histogram represents PE-anti-CD4   and AlexaFluora 647-anti-IL17A; **B1**, cells

stimulated with Ivanov cytokines and PMA/ Ionomycin, FSC/SSC histogram to show the postion of lymphocytes; **B2**, cells stimulated with Ivanov cytokines and PMA/ Ionomycin, histogram by PE-anti-CD4 only; **B3**, cells stimulated with Ivanov cytokines and PMA/ Ionomycin, histogram represents PE-anti-CD4 and AlexaFluora647-anti-IL17A; **B4**, cells stimulated with Ivanov cytokines and PMA/ Ionomycin, histogram by AlexaFluora647-anti-IL17A only; **C1**, cells stimulated with Ivanov cytokines and PMA/ Ionomycin, FSC/SSC histogram to show the postion of lymphocytes; **C2**, cells stimulated with Ivanov cytokines and PMA/ Ionomycin, histogram by APC-anti-CD4 only; **C3**, cells stimulated with Ivanov cytokines and PMA/ Ionomycin, histogram represents APC-anti-CD4 and PE-anti-IL17A; **C4**, cells stimulated with Ivanov cytokines and PMA/ Ionomycin, histogram represents PE-anti-IL17A only.

The portion of Th17 cells in CD4$^+$ spelnocytes cultured with anti-CD3 + anti-CD28 is quite low, 1.8% (**A**). By polarizing the cells in vitro culture with plate bound anti-CD3 (5 mg/ml) and soluble anti-CD28 (1 mg/ml) and supplemented with 40 U/ml mouse IL-2 (Roche), 10 mg/ml anti-IL-4 (BD), 10 mg/ml anti-IFN-γ (BD), 20 ng/ml IL-6 (eBioscience), and 5 ng/ml TGF-b (Preprotech) for 72 hours, stimulated by PMA (50 ng/mL) and Ionomycin (1ug/mL) for last 5 hours, Th17 cells portion increased up to >26% (**B** and **C**).

According to the resport in *Ivanov 2006 cell 126:1121*, they got 8.1% Th17 cells following polarization. I got a higher percentage than that. It may be due to the age of animal used or different reagents sources.

Appendix B5. Quantification of Treg cells by intracellular staining (splenocytes)

Aim:
 This protocol is to determine Treg population in spleen (systemic) in mouse.

Rationale & Markers:
Triple color staining for splenocytes

Cell	Marker	Type	Antibody	Clone	Source
	CD4	Surface	FITC-anti-CD4	RM4-5	eBioscience#11-0042
Treg	CD25	Surface	APC-anti-CD25	PC61	eBioscience#17-0251
	FoxP3	Intracellular	PE-anti-Foxp3	FJK-16s	eBioscience#88-8811

Reagents:
 RPMI 1640 (Irvine Scientific #9159), 500ml bottle, added with 5ml Glutamax (100x, Gibco 35050-061) and 5 ml Penicillin-Streptomycin solution (100x, Gibco, 15140-148) before use, refer to RPMI in the following text), store at 2-8°C.
 HI-FBS (Irvine Scientific #3003), store at -20°C.
 Complete RPMI (cRPMI) = RPMI 1640 with 5% FBS + 5 ml of Penicillin-Streptomycin (100x, Gibco #15140-148) + 5 ml of Glutamax L-glutamine (100x, Gibco #35050-061), store at 2-8°C.
 Mouse Regulatory T Cell Staining Kit (eBioscience # 88-8811)
   -Antibodies and controls, Store at 4°C in the dark.:
     FITC anti-mouse CD4 (RM 4-5) (eBioscience # 110042, stock 0.2mg/mL)
     APC anti-mouse CD25 (PC61.5) (eBioscience # 170251, stock 0.2mg/mL)
     PE anti-mouse Foxp3 (FJK16s) (eBioscience # 125773, IgG_{2a}, stock 0.2mg/mL)
     PE Rat IgG_{2a} isotype control (eBioscience # 124321, stock 0.2mg/mL)
     Fc block: Affinity Purified anti-mouse CD16/32 (eBioscience # 14016, stock 0.5mg/mL 1)
   -Buffers:
     Flow Cytometry Staining Buffer (eBioscience # 004222), refer to sBuffer in the following text
     Foxp3 Staining Buffer Set (eBioscience # 00-5523 or from kit #88-8111). Containing Fixation/Permeabilization Concentrate, Fixation /Permeabilization Diluent, and 10X Permeabilization Buffer.
       Fixation/Permeabilization Diluent (eBioscience # 005223)
       Fixation/Permeabilization Concentrate (eBioscience # 005123)
         (This is a 4 x stock solution that must be diluted prior to use with the Fixation/Permeabilization Diluent. Dilute 1 part Fixation/Permeabilization Concentrate with 3 parts Fixation/Permeabilization Diluent. Use within 4 months of receipt.)
       Permeabilization Buffer (10 x) (eBioscience cat# 008333)
         (Dilute to 1 x with deionized/distilled water for use and store at 4°C)
 DPBS (Gibco #14040)

Procedure:
Part 1: Isolate splenocytes
Keep MACS buffer cold (on ice), but perform the rest of procedure at room temperature except when indicated.

1. Remove the spleen from a mouse as followed. Sacrifice mice by CO_2
   1) Place mice on their right side so that the left side faces you
   2) Apply alcohol to the abdomen area
   3) Grab the skin of the abdomen with forceps and make a small incision
   4) Peel back the skin/fur with fingers to expose the membrane underneath
   5) Grab the membrane with forceps and cut the membrane to expose the organs
   6) Remove the spleen (dark red organ) with forceps
   7) Carefully remove as much fat from the exterior of the spleen as possible
   8) Place a spleen in a 15ml conical tube containing 3ml MACS buffer, transfer to cell culture room.

Perform the following steps under sterile hood.

2. Prepare single cell suspension from spleen (In sterile hood)
   9) Place a Falcon cell strainer (70 μm mesh, BD, # 35-2350), on the 100mm petri dish, and pre-wet with 5mL MACS® buffer.
   10) Transfer the spleen into cell strainer.
   11) Gently press spleen against the bottom of the strainer with a plunger from a 5 ml syringe. Use up and down motion (not grinding), being careful not to damage cells.
   12) When only the spleen connective tissue remains in the strainer, remove the plunger.
   13) Transfer the cell suspension from the dish to the original 15mL conical tube.
   14) Wash strainer with 5ml MACS buffer, and transfer to the same tube to collect the remaining cells.
   15) Spin down at 300xg for 5 min.
   16) Remove the supernatant and resuspend the pellet by flicking the tube or ratcheting the tube over a tube rack.
   17) Place a 30 μm filter (Miltenyi part # 130-041-407) on top of a new 15 mL conical tube
   18) Take 10mL MACS buffer and and pre-wet 30um filter with 2 mL buffer.
   19) Resuspend cells with the rest 8mL MACS buffer.
   20) Pass the cells through the 30 μm filter into a 15 ml conical tube.
   21) Remove 50ul for counting (10ul for coulter counter).
   22) Centrifuge the cells at 300x g for 5-10 min.

23) Remove the supernatant and resuspend the pellet by flicking the tube or ratcheting the tube over a tube rack.
24) Count the cell number during waiting for centrifuge by Coulter counter (prepare counting fluid 24h before experiment) with 4um cut-off (or by hemacytometer as an alternative option).
   a) Power on Coulter counter.
   b) Replace Coulter clenz solution with isotone fluid containg vial.
   c) Thouch "Function" → "Flush"
   d) Flush again with a new isotone fluid.
   e) Touch "Set up" → Size: 4um
   f) Touch "Output" → result: concentration, dilution factor: 1000
   g) Replace the vial with a cell-containing vial.
   h) Touch "Start"
   i) After cell counting the concentration will be displayed, for example "1.523 E7", which means 1.523×10^7 or 15.23×10^6 cells/ml (**concentration**)
   j) Since cells are suspended in 10mL, multiply by 10 to calculate the **cell number**.

3.   Resuspend cells at the density <u>of 20×10^6 cells / ml of RPMI medium</u> (serum free).

4.   Add an **equal amount*** of Lympholyte-M by layering it beneath the suspended cells. ⇒ insert the pipet tip into the bottom of the conical tube before dispensing the Lympholyte-M. ⇒ this step must be done slowly and carefully in order to see 2 distinct bands.
   * 3ml of cell suspension + 3ml Lympholyte M for this study

5.   Centrifuge the cells at 500 g for 15 min. at room temperature ⇒ deactivate centrifuge brake at this step (<u>**acc/dcc = 0)**</u> ⇒ when samples are done spinning, you will see 2 distinct layers (a white one on the top and a clear, pink one on the bottom) and an interface band separating them. Your goal is to remove this interface band. The bottom clear layer contains the unwanted red blood cells, plasma, etc.

6.   Carefully remove the interface band between the medium and Lympholyte-M layer with a 2 mL size pipette and dispense it into a new conical tube (should see a red pellet)

7.   Fill the conical tube with RPMI to wash and centrifuge the cells at 300g for 5 min at room temperature.

8.   Resuspend cells at the density of 10×10^6 cells / mL in RPMI or cold staining buffer (sBuffer) for use.

9.   Aliquot 100μL into 15 ml tubes.

Part 2: Staining
 Sample sets--splenocytes:
 Unstained
 FITC anti-mouse CD4 (RM4-5)

*FITC-CD4 + APC anti-mouse CD25 (PC61.5)
PE anti-mouse/rat Foxp3 (FJK-16s)
FITC-CD4 + PE-Foxp3
FITC CD4, APC CD25 and PE Foxp3 (each sample)
*FITC CD4, APC CD25 and PE Rat IgG2a isotype control
* better to have 2 of these controls from 2 randomized picked sample when doing real experiment, if you are doing primary experiment to test the method or reagents, have unfixed unstained and CD4 staining as well.

Samples are 100uL of 10 x10^6 cells / mL cold sBuffer in 15mL conical tubes, label the liquid level in the conical tubes.

1). Fc block

Block Fc receptors for reducing nonspecific immunofluorescent staining by adding 1 µg Fc Block* (2 µL of 0.5mg/mL stock) per sample, *incubate on ice for 10 min.*

> * make n x 2 =____µL of Fc Block + n x 3 =____µL of staining buffer → add 5µL
>
> into each sample.

Tips: Protect cells from light throughout the following staining, shut down the light for hood whenever you have antibody in hood
Take an aliquot of the antibody you need for this time from the stock vial to avoid cross-contamination
Aspirate your supernant just after centrifugation to avoid disturb the pallet, then flick the tube bottom on the hood to loose the pallet
For the tubes need both CD4 and CD25 staining, both antibodies are added in the same time and incubated together!!!

2). Surface staining

Add 0.5 µg/test of FITC-a-CD4* (0.5mg/mL stock) first and/or 0.25 µg/test of APC-a-CD25** (0.2mg/mL stock) antibody in sample (for 1x10^6 cells in 100uL volume, recommended by the company, may need to optimize) for surface staining. Mix gently. *Incubate in dark at 4°C for 30 minutes.* Wash in 2 mL cold sBuffer. Centrifuge cells for 5 minutes (300-400xg) at 4°C (or room temperature). Aspirate supernatant, leave about (no more than) 100µL volume.

> * make n x 1 =____µL of a-CD4 + n x 4 =____µL of staining buffer → add 5µL
>
> into each sample.
>
> ** make n x 1.25 =____µL of a-CD25 + n x 3.75 =____µL of staining buffer →
>
> add 5ul into each sample.

Before fixation, take ~10µL from CD4, CD4+CD25 and 1 of the sample to have a quick run of flow to make sure surface staining is working well.

3). Fix and permeabilize Cells

Prepare working dilution

Dilute the Fixation/Permeabilization Concentrate (1 part) into the Fixation /Permeabilization Diluent (3 parts) to the desired volume of <u>Fixation /Permeabilization working solution</u>. Use it for no more than 1 day.

1 ml / sample x _____ samples (including controls) = _____ ml needed =

¼ x ____ ml of Concentrate + ¾ x _____ ml of diluent

Dilute the 10 x stock Permeabilization Buffer in <u>distilled water</u> to a <u>1x Permeabilization solution</u> prior to use. Make fresh before each experiment.

4 ml / sample x _____ samples (including controls) = _____ ml needed

Resuspend cell pellet **with pulse vortex (5 times) at maximum speed** and add **1 ml** of **freshly** prepared Fixation/Permeabilization working solution to each sample. **Pulse vortex (5 times) at maximum speed again**. *Incubate at 4°C refrigerator for 35 minutes in the dark.* Wash cells with 2mL 1x Permeabilization Buffer. Centrifuge at room temperature, 300xg for 5 min and decant supernatant by aspirator, leave about 100µL volume.

4). [OPTIONAL] Block with 1 µg/test Fc block in 1 Permeabilization Buffer **(cells will be always in Perm Buffer since then to keep permeablization),** in approximately 100 µL volume, at 4°C for 15 minutes.

5). Intracellular staining

Resuspend cell pellet **with pulse vortex (5 times) at maximum speed**, add 0.5* µg/test anti-Foxp3 Ab (0.2mg/mL stock) or **0.2*** µg/test IgG$_{2a}$ isotype control in 1 X Permeabilization Buffer and incubate at 4°C refrigerator for at least 30 minutes in the dark.

* make n x 2.5 = ____ µL of anti-Foxp3 + n x 2.5 = ____ µL of staining buffer →

add 5ul into each sample.

Wash cells with 2 ml 1X Permeabilization Buffer. Centrifuge at 300xg for 5 min and decant supernatant.

6). Flow cytometry

Resuspend in 200µL staining Buffer and transfer into 12 x 75 Polystyrene tubes and analyze on cytometer, **count 200, 000 events**.

References:
1). Reardon C, Wang A, McKay DM. 2008. Transient local depletion of Foxp3+ regulatory T cells during recovery from colitis via Fas/Fas ligand-induced death. *J Immunol* 180:8316-26.
2). http://www.ebioscience.com/ebioscience/specs/antibody_88/88-8111.htm
3). Patton DT, Garden OA, Pearce WP, Clough LE, Monk CR, Leung E, Rowan WC, Sancho S, Walker LS, Vanhaesebroeck B, Okkenhaug K. 2006. Cutting edge: the
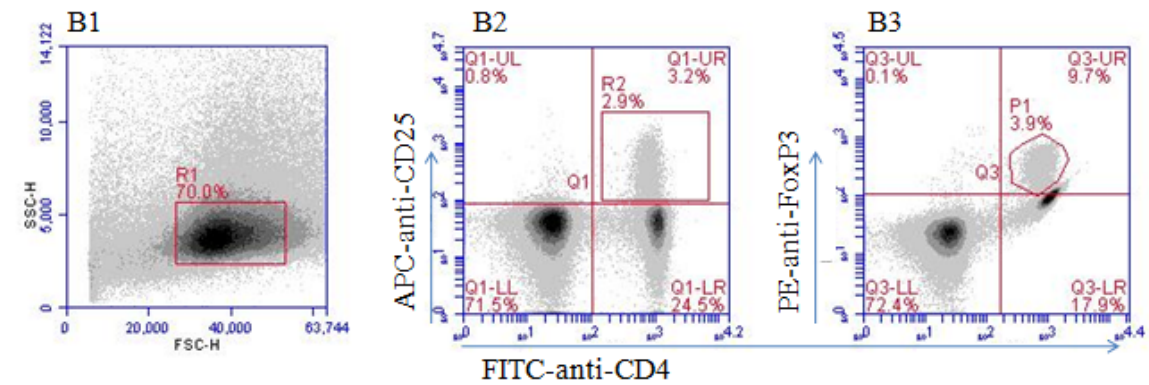
phosphoinositide 3-kinase p110 delta is critical for the function of CD4+CD25+Foxp3+ regulatory T cells. *J Immunol* 177(10):6598-602.
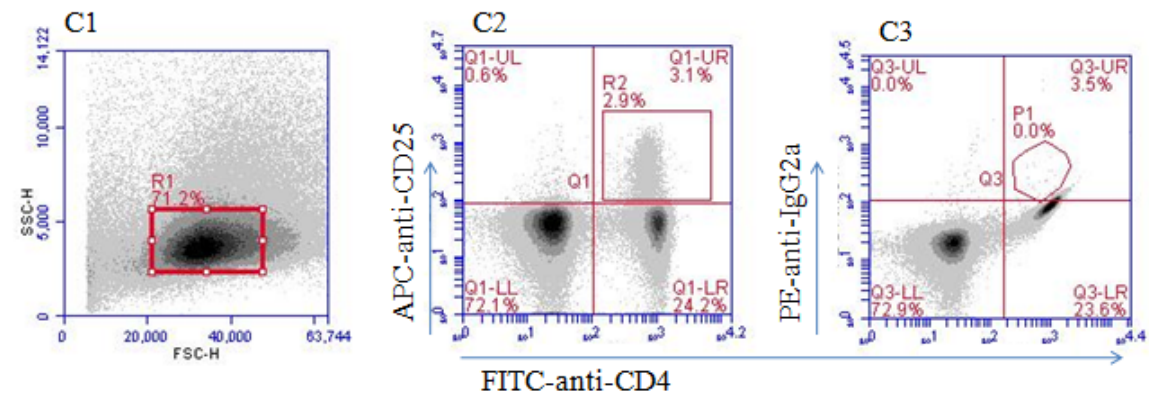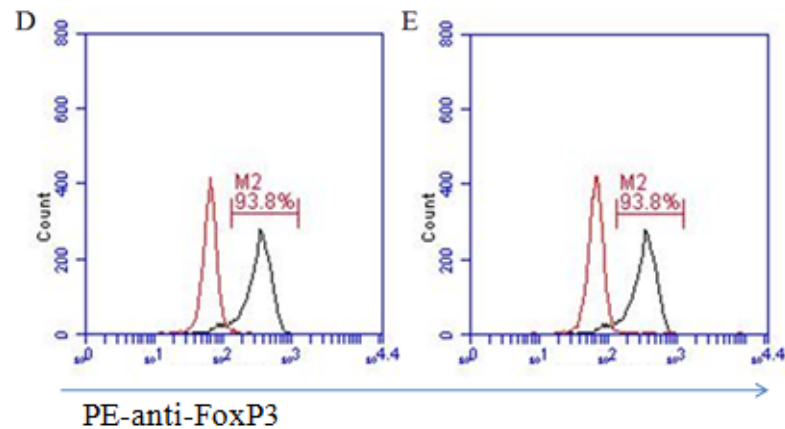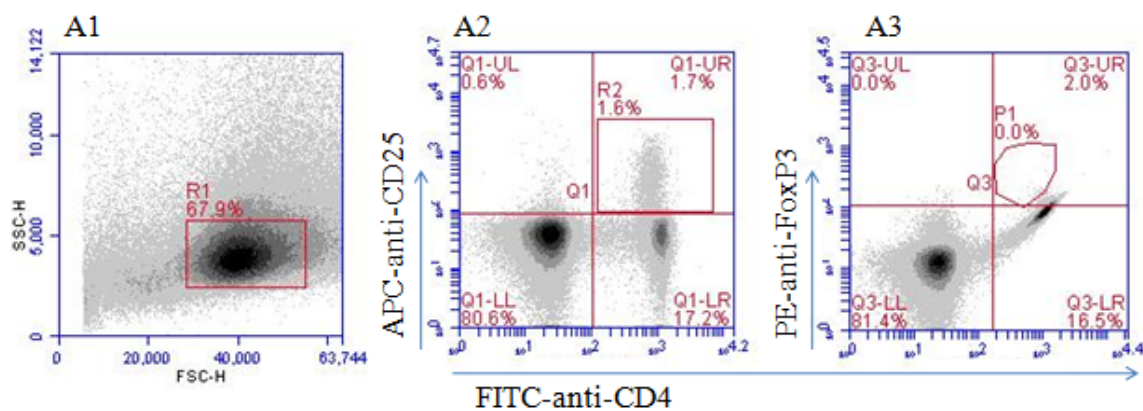
Representative results:

PE-anti-FoxP3
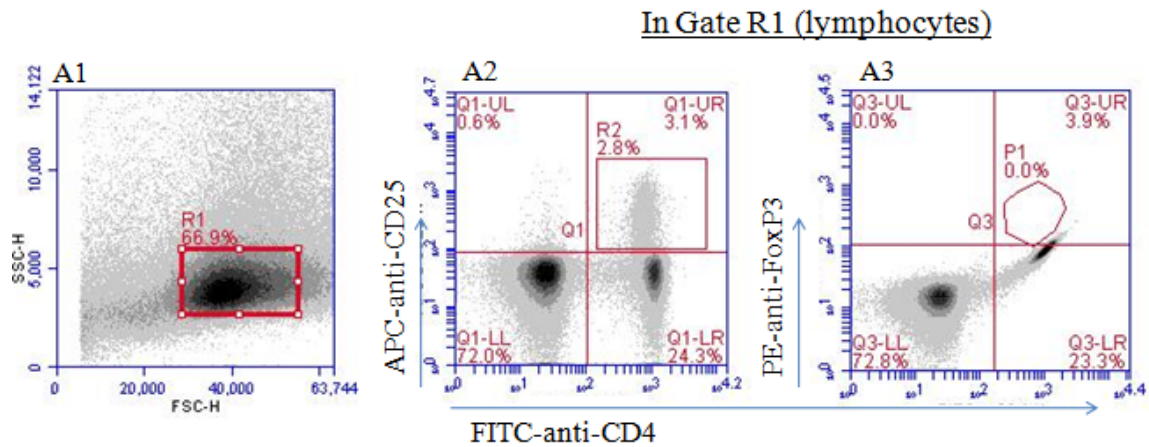
Flow cytometry of splenic Treg cells from Balb/C mice. Treg cells were detected in splenocytes isolated from 5-7 wk old BalB/C mice. The left row of **A1-C1** showed that the lymphocytes population was selected from the FSC/SSC, and then the portion of CD4$^+$CD25$^+$ was observed in lymphocytes as shown in the middle row of **A2-C2**. The right row **A3-C3** showed the portion of CD4$^+$FoxP3$^+$ in lymphocytes. **A**. splenocytes stained with 0.5 ug FITC-anti-CD4 (eBioscience, clone RM4-5) and 0.25 ug APC-anti-CD25 (eBioscience, clone PC61.5); **B**, splenocytes stained with 0.5 ug FITC-anti-CD4 and 0.25 ug APC-anti-CD25 and 0.5 ug PE-anti-FoxP3 (eBioscience, clone FJK16s); **C**, splenocytes stained with 0.5 ug FITC-anti-CD4 and 0.25 ug APC-anti-CD25 and 0.2ug PE-IgG2a; **D**, an overlap of sample **A** and **B** to show the percentage of FoxP3 staining in CD4$^+$CD25$^+$ cells is 93.8%. Left curve refers sample **A**, right curve refers to sample **B**. **E**, an overlap of sample **C** and **B** to show the percentage of FoxP3 staining in CD4$^+$CD25$^+$ cells is 93.8%. Left curve refers sample **C**, right curve refers to sample **B**.

A1, unfixed splenocytes, FSC/SSC histogram showing the postion of lymphocytes; **A2**, unfixed splenocytes, staining using FITC-anti-CD4 and APC-anti-CD25; **A3**, unfixed splenocytes, staining using FITC-anti-CD4 and APC-anti-CD25, showing no staining of PE-anti-FoxP3; **B1**, fixed splenocytes, FSC/SSC histogram showing the postion of lymphocytes; **B2**, fixed splenocytes, staining using FITC-anti-CD4 and APC-anti-CD25; **B3**, fixed splenocytes, staining using FITC-anti-CD4 and PE-anti-FoxP3; **C1**, fixed splenocytes, FSC/SSC histogram showing the postion of lymphocytes; **C2**, fixed splenocytes, staining using FITC-anti-CD4 and APC-anti-CD25; **C3**, fixed splenocytes, staining using FITC-anti-CD4 and PE-anti-IgG2a.
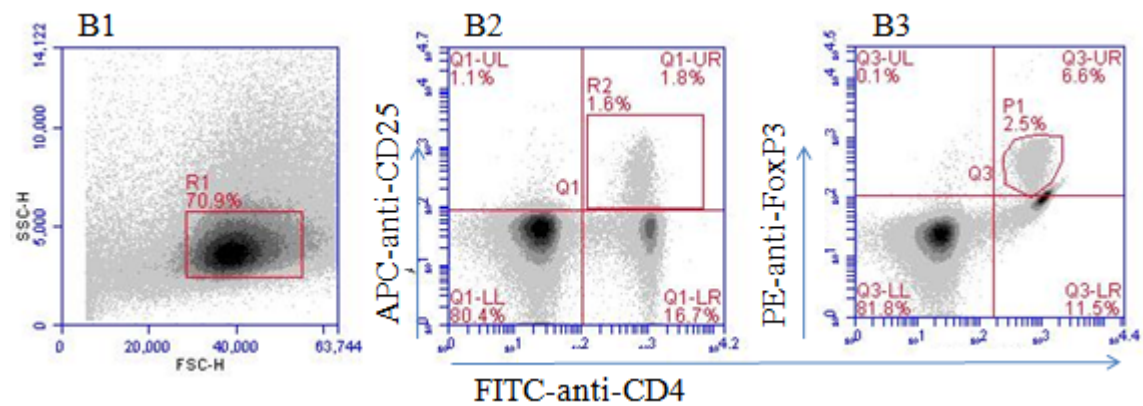
This test was conducted in 5-7 wk old Balb/C mice. The results showed that the percentage of CD4$^+$CD25$^+$ cells is 3.1% in splenocytes (**A**), the percentage of CD4$^+$ FoxP3$^+$ cells is 3.9% (**B**), the percentage of FoxP3$^+$ cells in CD4$^+$CD25$^+$ cells is 93.8% (**D, E**). The results are comparable to eBiosecience data available at: http://www.ebioscience.com/ebioscience/specs/antibody_88/88-8111.htm.
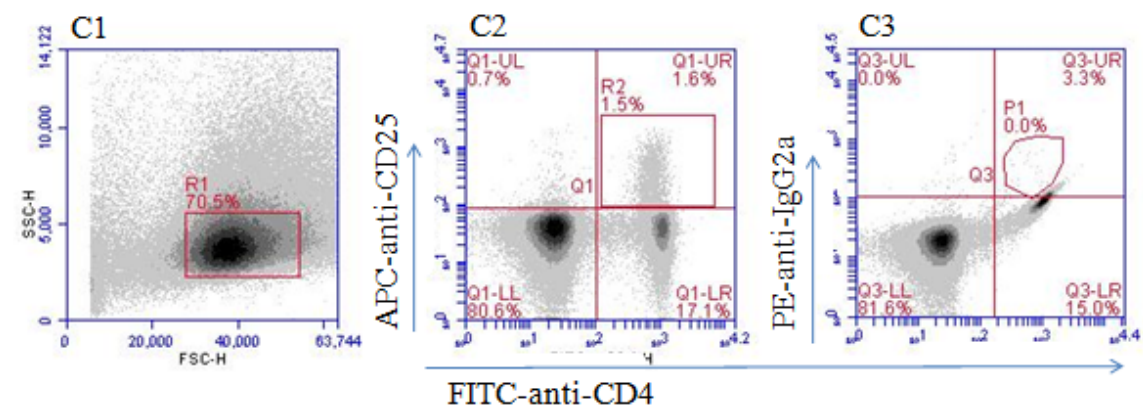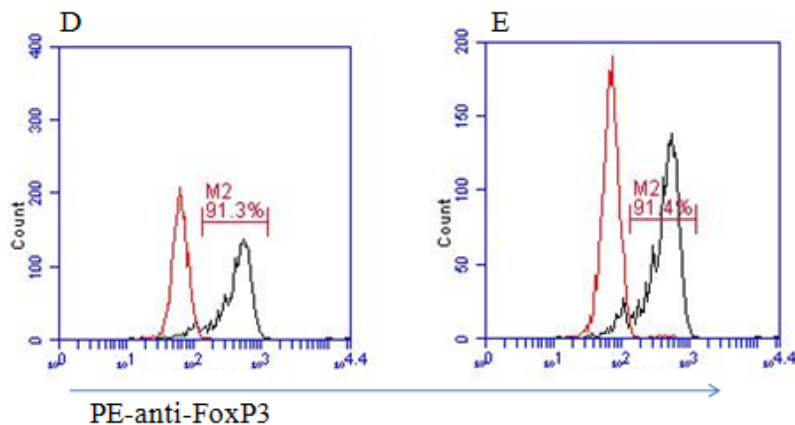
Flow cytometry of splenic Treg cells from C57BL/6 mice. Treg cells were detected in splenocytes isolated from 5-7 wk old C57/BL6 mice. The left row of **A1-C1** showed that the lymphocytes population was selected from the FSC/SSC, and then the portion of CD4$^+$CD25$^+$ was observed in lymphocytes as shown in the middle row of **A2-C2**. The right row **A3-C3** showed the portion of CD4$^+$FoxP3$^+$ in lymphocytes. **A**. splenocytes stained with 0.5 ug FITC-anti-CD4 (eBioscience, clone RM4-5) and 0.25 ug APC-anti-CD25 (eBioscience, clone PC61.5); **B**, splenocytes stained with 0.5 ug FITC-anti-CD4 and 0.25 ug APC-anti-CD25 and 0.5 ug PE-anti-FoxP3 (eBioscience, clone FJK16s); **C**, splenocytes stained with 0.5 ug FITC-anti-CD4 and 0.25 ug APC-anti-CD25 and 0.2ug PE-IgG2a; **D**, an overlap of sample **A** and **B** to show the percentage of FoxP3 staining in CD4$^+$CD25$^+$ cells is 91.3%. Left curve refers sample **A**, right curve refers to sample **B**. **E**, an overlap of sample **C** and **B** to show the percentage of FoxP3 staining in CD4$^+$CD25$^+$ cells is 91.4%. Left curve refers sample **C**, right curve refers to sample **B**.

A1, unfixed splenocytes, FSC/SSC histogram showing the postion of lymphocytes; **A2**, unfixed splenocytes, staining using FITC-anti-CD4 and APC-anti-CD25; **A3**, unfixed splenocytes, staining using FITC-anti-CD4 and APC-anti-CD25, showing no staining of PE-anti-FoxP3; **B1**, fixed splenocytes, FSC/SSC histogram showing the postion of lymphocytes; **B2**, fixed splenocytes, staining using FITC-anti-CD4 and APC-anti-CD25; **B3**, fixed splenocytes, staining using FITC-anti-CD4 and PE-anti-FoxP3; **C1**, fixed splenocytes, FSC/SSC histogram showing the postion of lymphocytes; **C2**, fixed splenocytes, staining using FITC-anti-CD4 and APC-anti-CD25; **C3**, fixed splenocytes, staining using FITC-anti-CD4 and PE-anti-IgG2a.

This test was conducted from 5-7 wk old C57/BL6 mice. The results showed that the percentage of CD4$^+$CD25$^+$ cells is 1.7% in splenocytes (**A**), the percentage of CD4$^+$FoxP3$^+$ cells is 2.5% (**B**), the percentage of FoxP3$^+$ cells in CD4$^+$CD25$^+$ cells is 91.3% (**D, E**).

Appendix B6. Quantification of Treg cells by intracellular staining (LPL)

Aim:
 This protocol is to determine Treg population in lamina propria of colon (local).

Rationale & Markers:
2 color staining for LPL

Cell	Marker	Type	Ab	Clone	Source
Treg	CD4	Surface	a-CD4-APC	RM4-5	eBioscience#17-0042
	FoxP3	Intracellular	a-Foxp3-PE	FJK-16s	eBioscience#88-8811

Reagents:
 RPMI 1640 (Irvine Scientific #9159), 500ml bottle, added with 5ml Glutamax (100x, Gibco 35050-061) and 5 ml Penicillin-Streptomycin solution (100x, Gibco, 15140-148) before use, refer to RPMI in the following text), store at 2-8°C.
 HI-FBS (Irvine Scientific #3003), store at -20°C.
 Complete RPMI (cRPMI) = RPMI 1640 with 5% FBS + 5 ml of Penicillin-Streptomycin (100x, Gibco #15140-148) + 5 ml of Glutamax L-glutamine (100x, Gibco #35050-061), store at 2-8°C.
 Mouse Regulatory T Cell Staining Kit (eBioscience # 88-8811)
  -Antibodies and controls, Store at 4°C in the dark.
   PE anti-mouse Foxp3 (clone FJK16s) (eBioscience # 125773, IgG$_{2a}$, stock 0.2mg/mL)
   PE Rat IgG$_{2a}$ isotype control (eBioscience # 124321, stock 0.2mg/mL)
   Fc block: Affinity Purified anti-mouse CD16/32 (eBioscience # 140161, stock 0.5mg/mL)
  -Buffers:
   Flow Cytometry Staining Buffer (eBioscience # 004222), refer as sBuffer in the text
   Fixation/Permeabilization Diluent (eBioscience # 005223)
   Fixation/Permeabilization Concentrate (eBioscience # 005123)
    (This is a 4 x stock solution that must be diluted prior to use with the Fixation/Permeabilization Diluent. Dilute 1 part Fixation/Permeabilization Concentrate with 3 parts Fixation/Permeabilization Diluent. Use within 4 months of receipt)
   Permeabilization Buffer (10 x) (eBioscience cat# 008333)
    (Dilute to 1 x with deionized/distilled water for use and store at 4°C)

Procedure:
Part 1: Isolate LPL (see protocol in Appendix B3, P123)
Part 2: Staining
 Sample sets--LPL:
 Unstained

APC anti-mouse CD4 (RM4-5)
APC-CD4 + PE-Foxp3 (FJK-16s)
PE anti-mouse/rat Foxp3 (FJK-16s)
APC-CD4 + PE Rat IgG2a isotype control

Samples are 100uL of 10 x10^6 LPLs / mL cold sBuffer in 15mL conical tubes, label the liquid level in the conical tubes, if you are doing primary experiment to test the method or reagents, have unfixed unstained and CD4 staining as well.

1). Fc block

Block Fc receptors for reducing nonspecific immunofluorescent staining by adding 1 µg Fc Block* (2 µL of 0.5mg/mL stock) per sample, *incubate on ice for 10 min.*

> * make n x 2 =____µL of Fc Block + n x 3 =____µL of staining buffer → add 5µL
>
> into each sample.

Tips: Protect cells from light throughout the following staining, shut down the light for hood whenever you have antibody in hood

Take an aliquot of the antibody you need for this time from the stock vial to avoid cross-contamination

Aspirate your supernant just after centrifugation to avoid disturb the pallet, then flick the tube bottom on the hood to loose the pallet

2). Surface staining

Add 0.5 µg/test of APC-a-CD4* (#17-0042, 0.2mg/mL stock) antibody in sample (for 1x10^6 cells in 100uL volume, recommended by the company, may need to optimize) for surface staining. Mix gently. *Incubate in dark at 4°C refrigerator for 30 minutes.* Wash in 2 mL cold sBuffer. Centrifuge cells for 5 minutes (300-400xg) at 4°C (or room temperature). Aspirate supernatant, leave about (no more than) 100 ul volume.

> * make n x 2.5 =____µL of APC-a-CD4 + n x 2.5 =____µL of staining buffer →
>
> add 5µL into each sample.

Before fixation, take ~10µL from CD4, and 1 of the sample to have a quick run of flow to make sure surface staining is working well.

3). Fix and permeabilize Cells

> Prepare working dilution
>
> Dilute the Fixation/Permeabilization Concentrate (1 part) into the Fixation /Permeabilization Diluent (3 parts) to the desired volume of Fixation /Permeabilization working solution. Use it for no more than 1 day.
>
> 1 ml / sample x _____ samples (including controls) = _____ml needed =
>
> ¼ x ____ml of Concentrate + ¾ x _____ml of diluent
>
> Dilute the 10 x stock Permeabilization Buffer in distilled water to a 1x Permeabilization solution prior to use. Make fresh before each experiment.
>
> 4 ml / sample x _____ samples (including controls) = _____ml needed

Resuspend cell pellet **with pulse vortex (5 times)** and add **1 ml** of **freshly** prepared Fixation/Permeabilization working solution to each sample. **Pulse vortex (5 times) again**. *Incubate at 4°C refrigerator for 35 minutes in the dark.* Wash cells with 2mL 1x Permeabilization Buffer. Centrifuge at room temperature, 300xg for 5 min and decant supernatant by aspirator, leave about 100 ul volume.

4). [OPTIONAL] Block with 1-2 μg/test Fc block in 1 Permeabilization Buffer **(cells will be always in Perm Buffer since then to keep permeablization),** in approximately 100μL volume, at 4°C for 15 minutes.

5). Intracellular staining

Resuspend cell pellet **with pulse vortex (5 times)**, add 0.5* μg/test PE anti-Foxp3 Ab (0.2mg/mL stock) or **0.2*** μg/test PE anti-IgG$_{2a}$ isotype control in 1 X Permeabilization Buffer and incubate at 4°C refrigerator for at least 30 minutes in the dark.

> * make n x 2.5 =____μL of PE anti-Foxp3 + n x 2.5 =____μL of staining buffer →
>
> add 5μL into each sample.

Wash cells with 2 ml 1X Permeabilization Buffer. Centrifuge at room temperature, 300xg for 5 min and decant supernatant.
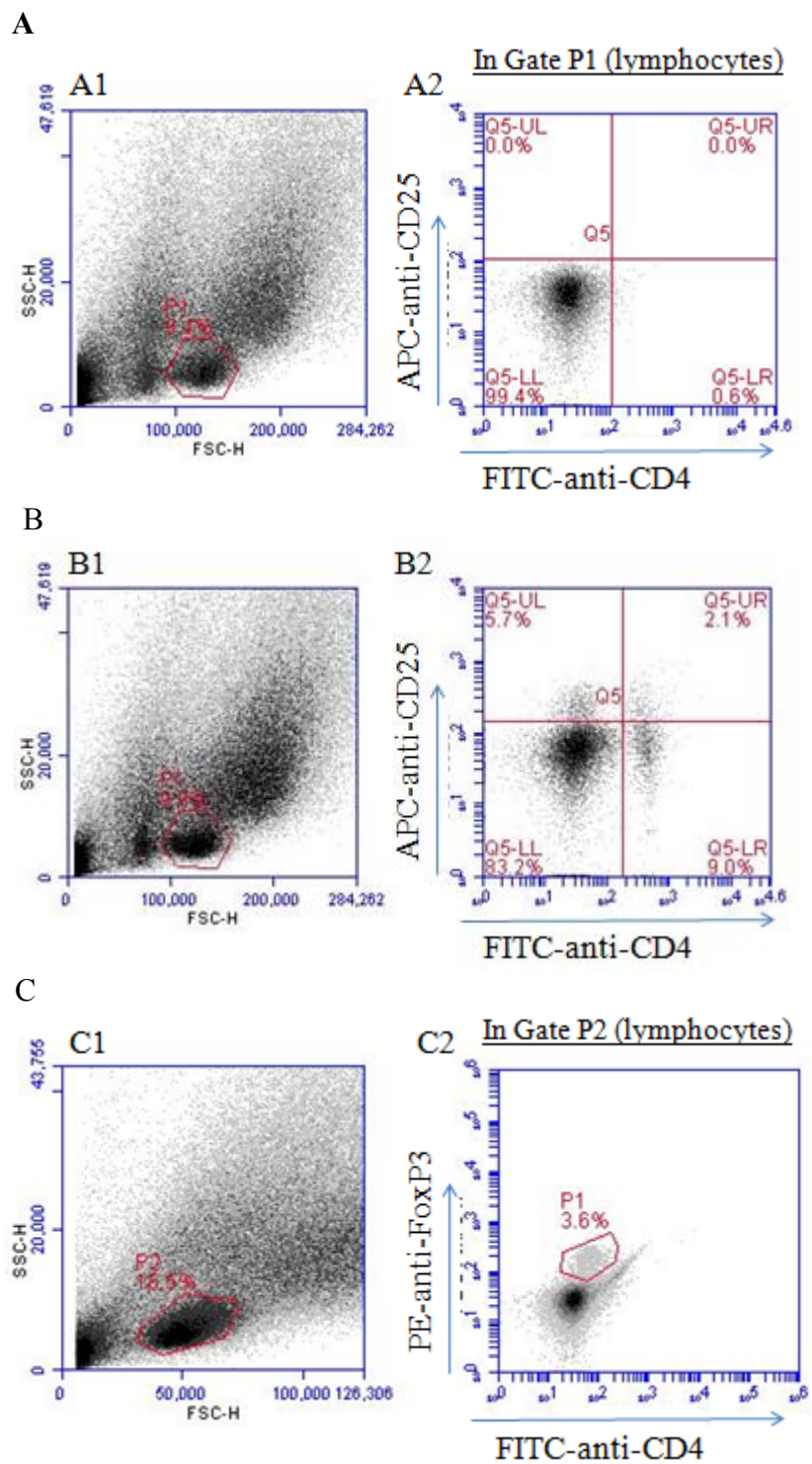
6). Flow cytometry

Resuspend in 200μL staining Buffer and transfer into 12 x 75 Polystyrene tubes and analyze on cytometer, **count 200, 000 events**.

References:

1). Reardon C, Wang A, McKay DM. 2008. Transient local depletion of Foxp3+ regulatory T cells during recovery from colitis via Fas/Fas ligand-induced death. *J Immunol* 180:8316-26.

2). http://www.ebioscience.com/ebioscience/specs/antibody_88/88-8111.htm

3). Makita S, Kanai T, Nemoto Y, Totsuka T, Okamoto R, Tsuchiya K, Yamamoto M, Kiyono H, Watanabe M. 2007. Intestinal lamina propria retaining CD4+CD25+ regulatory T cells is a suppressive site of intestinal inflammation. *J Immunol* 178(8):4937-46.

4). Hontecillas R, Bassaganya-Riera J. 2007. Peroxisome proliferator-activated receptor gamma is required for regulatory CD4+ T cell-mediated protection against colitis. *J Immunol* 178(5):2940-9.

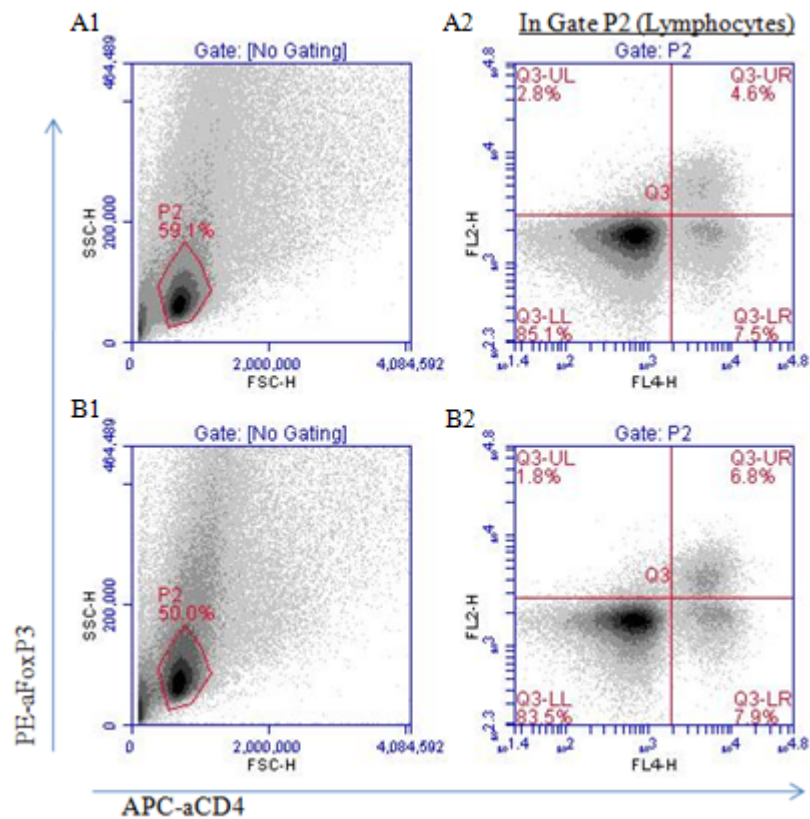**Representative results:**

A

B

C

Flow cytometry of LPL Treg cells from C57/BL6 mice-triple staining. Treg cells were tested in LPL from 5-7 wk old C57 mouse by triple staining of anti-CD4, anti-

CD25, and anti-FoxP3. **A**. unstained LPL; **B**, LPL stained with 0.5 ug FITC-anti-CD4 (eBioscience, clone RM4-5) and 0.25 ug APC-anti-CD25 (eBioscience, clone C61.5); **C**, LPL stained with 0.5 ug FITC-anti-CD4, 0.25 ug APC-anti-CD25 and 0.5 ug PE-anti-FoxP3 (eBioscience, clone FJK16s). This test was following protocol from Appendix B5, test once.

A1, unfixed LPLs, FSC/SSC histogram showing the postion of lymphocytes; **A2**, unfixed LPLs, unstained; **B1**, unfixed LPLs, FSC/SSC histogram showing the postion of lymphocytes; **B2**, unfixed LPLs, staining using FITC-anti-CD4 and APC-anti-CD25; **C1**, fixed LPLs, FSC/SSC histogram showing the postion of lymphocytes; **C2**, fixed LPLs, staining using FITC-anti-CD4 and PE-anti-IgG2a.

According to the results, there are 2.1% of $CD4^+CD25^+$ Treg in colon LPL (**B**), the percentage of $CD4^+$ $FoxP3^+$ cells in colon LPL is 3.6% (**C**). The results are comparable to previous reports [1, 4]

Flow cytometry of LPL Treg cells from C57/BL6 mice-double staining. Treg cells were detected in LPL from wt and *fat-1* mice following 3 cycles of DSS treatment by double staining of anti-CD4 and anti-FoxP3. **A**, representative histograms from a wt mouse; **B**, representative histograms from a *fat-1* mouse.

A1, fixed LPLs, FSC/SSC histogram showing the postion of lymphocytes; **A2**, fixed LPLs, staining using FITC-anti-CD4 and PE-anti-FoxP3; **B1**, fixed LPLs, FSC/SSC histogram showing the postion of lymphocytes; **B2**, fixed LPLs, staining using FITC-anti-CD4 and E-anti-FoxP3.

Appendix B7. IHC of of infiltrated macrophages
(Modified from Dr. Ouyang's protocol)

Aim:
  In situ detection of macrophages in mouse colonic swiss roll paraffin embedded sections

Facilities:
   glass jars
   coverslips
   PAP PEN
   Superfrost Plus slides (Fisher, #12-550-15)
   humid chamber.

Materials:
   PBS, Ca, Mg-free (Gibco, # 14190)
   30% H_2O_2 (Sigma, #H1009)
   BSA IgG free podwer (Roche #03116956001)
   Primary antibodies: Purified antibodies
      -rat anti-mouse F4/80, for Macrophages (IgG2b, clone CI: A3-1, Serotec # MCA497)
   Normal rabbit serum (Vector #S-5000, Lot #T0919)
   Secondary antibodies:
    -Biotinylated rabbit anti rat IgG (H+L) ** affinity purified, mouse absorbed (Vector #BA-4001, Lot #T0109)
   DAB (3, 3'-diaminobenzidine tetrahydrochloride, Sigma #D5637)
   Hematoxylin (optional) (Sigma # 51260)
   Mounting media (Sigma # C0487 or any one available in lab)
<u>Tissue Process:</u>
   4% PFA or $ZnCl_2$ fixed mouse colon, paraffin embedded, 5 μm sections

Staining Procedure:
_____ 1). <u>Deparaffinate.</u> Leave slides in oven at ~60 (usually less than 65) °C to melt the paraffin, since the samples are embedded with low melt (at 58-60°C) paraffin, until the paraffin is melted and turned to be fluid, ~10 min.
 * For fat1 samples, use 62 °C
_____ 2). <u>Rehydrate</u> sections:
                 _____ _____ _____ Xyline, 3x, 5min
                 _____ _____ _____100% EtOH, 3x, 5min
                 _____ 95% EtOH, 1x, 5min
                 _____ 85% EtOH, 1x, 5min
                 _____ 70% EtOH, 1x, 5min
                 _____ _____ _____d H_2O, 3x, 2min

_____ 3). <u>Circle.</u> Let slides air dry for a few minutes. Carefully draw boundaries of sections using the PAP PEN and let it dry for a few minutes.

_____ 4). <u>Block endogenous peroxidase.</u> Immerge slides into a jar with 3% H_2O_2 in d H_2O, 10 min.

_____ _____ _____ _____ 5). <u>Wash</u> in d H_2O, 3x, 2min, then sit in PBS, **NEVER LET IT DRY AGAIN.**

_____ 6). <u>Block by BSA.</u> （optional）Put slides in humidified chamber, cover sections by 3% (1-5% is fine) BSA (IgG free podwer, 90mg for 3 ml PBS) in PBS to block the background, at least 30 min (30 min to 2 hour) at room temperature.

_____ _____ _____ 7). <u>Wash</u> （followed step 6 if needed）in PBS, 3x, 2min

_____ 8). <u>Block by normal serum.</u> Put slides in humidified chamber, cover sections by ~2% serum* (1:50, 100μL for 5 ml PBS, leave the extra for 1° Ab) in PBS to block the background, at least 1 hour (could be much longer for better blocking) at room temperature.

 * The serum is from the same specie that secondary Ab is from.

 * For F4/80, it's normal rabbit serum

_____ 9). <u>1° Ab.</u> Remove extra serum, cover slides with ~100μL primary Ab**, overnight at 4°C in the humidified chamber. Have the serum blocking solution as negative control.

 ** For rat anti mouse F4/80, dilute 1:100~1:200 in serum blocking solution

————————————————1st day ends————————————————

_____ _____ _____ 10). <u>Rinse and Wash</u> in PBS, 1x rinse, 2x10min wash.

_____ 11). <u>2° Ab.</u> Cover slides with 2° Ab diluted in PBS, incubate at least 1 hour at room temperature

 * For Vector #BA-4001, ~1:67 dilutiion.

_____ _____ _____ 12). <u>Rinse and Wash</u> in PBS, 1x rinse, 2x10min wash.

_____ 13). <u>3° Ab.</u> Cover slides with Vectastain Elite ABC Reagent, incubate at least 1 hour at room temperature.

 * For Vector #PK-6100, add exactly 2 drops of reagent A (gray label) to 5 ml of PBS, then add exactly 2 drops of reagent B (gray label) to the same mixture, mix immediately, and allow the reagent to **stand for about 30 min** before use.

_____ _____ _____ 14). <u>Rinse and Wash</u> in PBS, 1x rinse, 2x10min wash.

_____ 15). <u>DAB stain:</u> make 50 μM DAB (80 mg DAB podwer in 1.6 ml DMSO, vortex well, protect from light for 10 min to resolve, then add in 200 ml PBS with 25 μl 3% H_2O_2) in a jar, immerge the slides in it for staining, check under microscope at 8 min, then add 2 min each time to get more staining until get the clear color, usually ~10 min.

 * For fat1 sample, incubate ~12 min

_____ _____ _____ 16). <u>Rinse and Wash</u> in d H_2O, 1x rinse, 2x5min wash.

_____ 17). <u>Counterstain:</u> (optional, don't do this for quantifying F4/80 staining with DAB, but do this for capturing representative picture) Dip the slides in hemotoxin jar, then rinse by tap water. If the stain is too strong, rinse in 0.01% HCl in d H_2O to dilute it.

_____ 18). <u>Dehydrate:</u> bake in 50-60 °C oven overnight to dry the slides.

————————————————2nd day ends————————————————

_____ 19). <u>Wash out:</u> Put the slides (on rack) immediately in fresh xylene for washing out the pap pens, leave in xylene 5-10 min until pap pen circle disappear.

_____ 20). <u>Mount:</u> mount the slides

Quantify Procedure:
1). Turn on Nikon microscope and computer according to the direction on the wall of Kleg 221B, log in computer via 'Chapkin lab'. (For IHC slide, the side with cover-slide is facing down on the microscope slide holder)
2). Turn on software, 'NIS Elements' on desktop.
3). Focus: in the top tool panel, click the green triangle, then click 'OPEN (for single cubes)', then click 'Focus RGB', then adjust microscope to focus well.
4). Capture: in the top tool panel, click 'Acquire', choose 'capture multichannel', select 'multichannel set up', then click 'Load', choose 'RGB'. Now the set up is ready to capture color image. In the right panel, find 'Format for capture', choose '12-bit-binning 2x2'. Also choose auto exposure in right panel. Then click the capture button (a sign with 3 overlapped color squares) in left panel.
5). Save pictures: Save the raw picture first, then click 'X' in keyboard to get 24 bit picture and save again. 10 pictures of high staining areas will be taken from each slide, 10 areas together should cover representative areas from submucosal layer, muscularis to the crypts, lamina propria, outside aggregates and follicles. Try to fill the whole picture area with tissue, this is convenient for quantifying.
6). Quantify: Open the raw picture in the software, click '1:1' to get it the same size as real. In right panel named 'LUTs', scale to adjust brightness and contrast to see it well. In the bottom panel, click 'object count', will see a green cross, click it then click in the target staining (exp. Brown dots with DAB staining) in the picture to set the positive staining, this 'marking' steps could be repeated until all staining in the area are counted (judged by eyes).
7). Read the % of area fraction on the right of the 'object count' panel, this is the % of positive staining in the area. The average from the 10 areas will be the reading of this sample.

Refrences:

1). Berg DJ, Zhang J, Weinstock JV, Ismail HF, Earle KA, Alila H, Pamukcu R, Moore S, Lynch RG. 2002. Rapid development of colitis in NSAID-treated IL-10-deficient mice. *Gastroenterology* 123(5):1527-42.
2). http://www.abdserotec.com/catalog/datasheet-MCA497.html

Prepresentative Results: Figure 7, p26.

Appendix B8. IHC of pSTAT3

Aim:
  In situ detection of Phospho-Stat3 (Tyr705) in mice colon swiss roll paraffin section

Antibody and Staining Kit:
1. Phospho-Stat3 (Tyr705) (D3A7) XP™ Rabbit mAb (Cell Signal #9145), store in minus 20C, no aliquot necessary, should be good for 2 years.
2. SignalStain® Antibody Diluent (Cell Signal #8112)
3. Vectastain ABC Kit (Vector Laboratories #PK-6101) --- use the goat serum from the kit
4. SignalStain® Boost IHC detection reagent, HRP, Rabbit (Cell Signal #8114)
5. DAB stock solution from TACS 2 TdT-DAB in situ apoptosis detection kit (Trevigen #4810-30-K)

Reagents and Solutions:
1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH$_2$O)
4. Hematoxylin (optional)
5. Wash Buffer:
1X TBS/0.1% Tween-20 (1X TBST): To prepare 1 L add 100 ml 10X TBS to 900 ml dH$_2$O. Add 1 ml Tween-20 and mix.
10X Tris Buffered Saline (TBS): To prepare 1 L add 24.2 g Trizma® base (C$_4$H$_{11}$NO$_3$) and 80 g sodium chloride (NaCl) to 1 L dH$_2$O. Adjust pH to 7.6 with concentrated HCl.
6. **Antibody Diluent:**
  SignalStain® Antibody Diluent #8112
7. **Antigen Unmasking:**
EDTA: 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C$_{10}$H$_{14}$N$_2$O$_8$Na$_2$•2H$_2$O) to 1 L dH$_2$O. Adjust pH to 8.0.
10x EDTA: 10 mM EDTA: To prepare 1 L add 3.72 g EDTA (C$_{10}$H$_{14}$N$_2$O$_8$Na$_2$•2H$_2$O) to 1 L dH$_2$O. Adjust pH to 8.0.
8. **3% Hydrogen Peroxide:** To prepare, add 50μL 30% H$_2$O$_2$ to 450 ul dH$_2$O.
9. **Blocking Solution:** TBST/5% normal goat serum: to 500ul 1X TBST add 25μL normal goat serum.
10. SignalStain® Boost IHC detection reagent #8114
  Warm up to room temperature before use.

Procedure:
A. Deparaffinization/Rehydration
NOTE: Do not allow slides to dry at any time during this procedure.
1. **Deparaffinize/hydrate sections:**
a. Incubate sections in three washes of xylene for 5 minutes each
b. Incubate sections in two washes of 100% ethanol for 10 minutes each .

Heat EDTA now.

c. Incubate sections in two washes of 95% ethanol for 10 minutes each

2. Wash sections twice in dH$_2$O for 5 minutes each

B. Antigen Unmasking

  For EDTA: Put the slides into a metal rack, then put the rack into a metal bowl with 1.2L (for the bowl by Laurie) of 1 mM EDTA pH 8.0 (preheated by the heater to sub boiling, which is a status with some small bubbles, but not large bubbles) followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.

C. Staining

1. Wash sections in dH$_2$O three times for 5 minutes each

2. Incubate sections in 3% hydrogen peroxide for 10 minutes

3. Wash sections in dH$_2$O twice for 5 minutes each.

4. Optional (not needed for Tecan): Circle the tissue by using PapPen (Zymed), let it dry for 5 min till complete dry

5. Transfer slides to Tecan

Tecan Program set:

PROGRAM START Name: pSTAT3_IHC, Comment:

  1 WASH   Temp. °C: 23.0, First: Yes, Ch.: 1, Runs: 1, Wash time: 0:01:00, Soak time: 0:00:00

  2 SAMPLE INJECTION Temp. °C: 23.0, Agitation: Yes, BCR: No

      (blocking solution)

  3 HYBRIDIZATION   Temp. °C: 23.0, Agitation Frequency: High, High Viscosity Mode: Yes, Time: 1:00:00

  4 WASH   Temp. °C: 23.0, First: No, Ch.: 1, Runs: 1, Wash time: 0:01:00, Soak time: 0:00:00

  5 SAMPLE INJECTION Temp. °C: 23.0, Agitation: Yes, BCR: No

      (primary antibody)

  6 HYBRIDIZATION   Temp. °C: 4.0, Agitation Frequency: High, High Viscosity Mode: Yes, Time: 16:00:00

  7 WASH   Temp. °C: 23.0, First: No, Ch.: 1, Runs: 2, Wash time: 0:01:00, Soak time: 0:00:30

  8 SLIDE DRYING   Temp. °C: 23.0, Time: 0:02:30, Final Manifold Cleaning: Yes, Ch.: 5

PROGRAM END

6. Remove slides from Tecan.

7. Add 100-400μL DAB*** or suitable substrate to each section and monitor staining closely---12min

 *** Prepare DAB by Laurie's recipe: 2.5μL DAB stock from the Trevigen kit + 5μL 30% H$_2$O$_2$ + 500μL PBS, make within 30min of use.

8. As soon as the sections develop, immerse slides in dH$_2$O.

9. If desired, counterstain sections in hematoxylin per manufacturer's instructions.

10. Wash sections in dH$_2$O two times for 5 minutes each

11. Dehydrate sections:

a. Incubate sections in 95% ethanol two times for 5 minutes each

b. Repeat in 100% ethanol, incubating sections two times for 5 minutes each.
c. Repeat in xylene, incubating sections three times for 5 minutes each to wash out the pappen..
15. Mount coverslips.

Quantification:
DAB staining of pSTAT3 will be quantified by Nikon Cool-SNAP camera and NIS elements software. 4 representative staining areas (hot spots) will be selected from distal, middle and proximal of the colon, so the total will be 12 images from one slide/animal. Only mucosa (but not muscle) will be circled for the quantification. Quantification will be done in the same condition as mentioned in the protocol for all the slides. The staining area, intensity, % of the tissue will be recorded. The average or summary of the 12 images will be a reading of one animal.

Use Roper black and white camera.

<u>Black = 0 and white = 4095 so the scale is reversed. More intensity has a lower value.</u>

Open NIS elements AR 3.1
Check "enable multi camera", has both Nikon and Roper camera selected, then Allow
Select Plan APO 10 X 0.45 objective (the one with yellow ring) (left)
Select "BF-Laurie Mono" on top panel (top right)
Click DIA to open the shutter (top or left), **choose 3 volts**
Choose the one for eye (left or in microscope control panel), check the microscope to find the tissue.
Choose L100 for black and white camera
Click the green triangle (top) to view live image

Want to keep conditions as uniform as possible:
        Set uniform room lighting. Keep light knob on scope at the same setting
        Turn power unit on shelf all the way up to have uniform power every time
Filters on top of scope – push ND and NCB to right.
Under camera settings, select the following:
        Format for live – use no binning
        Format for capture – no binning (binning loses resolution but maximizes signal)
          Conversion gain - low
          Manual exposure – 126 ms for IHC of pSTAT3 (It's up to you to set up the exposure time according to the staining status of your slides)

For DAB stained pSTAT3 slides: DIA: 3 volts. Manual exposure – 126 ms. Intensity Threshold - 0-2703

To quantify the intensity of the image:

Right click—select Analysis controls--- ROI statistics (or Ctrl-Alt-R)
In the top menu, Binary – Define threshold – click and drag to adjust the cover to select most of the staining but not the background (save this for use of all the slides) –OK

References
1). http://www.cellsignal.com/pdf/9145.pdf
2). http://www.cellsignal.com/support/protocols/IHC-Paraffin-SignalStain.html
3). Salcedo R, Worschech A, Cardone M, Jones Y, Gyulai Z, Dai RM, Wang E, Ma W, Haines D, O'hUigin C, Marincola FM, Trinchieri G. 2010. MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J Exp Med* 207(8):1625-36

Representative results: Figure 23, p68.

VITA

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