Suppression of Intestinal Inflammation and Inflammation-driven Colon Cancer in Mice by Dietary Sphingomyelin: Importance of Peroxisome Proliferator-activated Receptor γ Expression.

Joseph Cayetano Mazzei

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Eva M. Schmelz (Chair)
Paul C. Roberts
Robert W. Grange

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ABSTRACT

Sphingolipid metabolites play a role in the initiation and perpetuation of inflammatory responses. Since intestinal inflammation is a driving force in the development of colon cancer, in the present study, we investigated the suppression of dextran sodium sulfate (DSS)-induced colitis by dietary sphingomyelin in mice that lack functional peroxisome proliferator-activated receptor γ (PPAR-γ) in intestinal epithelial and immune cells. Dietary spingomyelin decreased colonic inflammation in mice of both genotypes but more efficiently in mice expressing PPAR-γ. Using a real-time polymerase chain reaction array, we detected an up-regulation in genes involved in Th1 (interferon γ) and Th17 (interleukin [IL]-17 and IL-23) responses despite the reduced inflammation. However, the genes involved in Th2 (IL-4, IL-13 and IL-13ra2) and Treg (IL-10rb) anti-inflammatory responses were up-regulated in a PPAR-γ-dependent manner. In order to direct mechanistic studies of how PPAR-γ expression is involved in SM-induced suppression of DSS colitis, we investigated the effect of dietary SM in DSS-treated mice that lack PPAR-γ in the CD4+ T-cells. While the pathogenesis of colitis was independent of PPAR-γ expression in CD4+ T-cells, dietary SM decreased disease activity and colonic inflammation in mice of both genotypes but more efficiently in mice expressing PPAR-γ, indicating both PPAR-γ dependent and independent signaling pathways. In conclusion, in contrast to endogenous sphingolipid metabolites, dietary SM modulated both pro- and anti-inflammatory responses at the early stages of the disease in a partially PPAR-γ dependent manner resulting in a suppression of inflammation that may be critical for the suppression of inflammation-driven colon cancer.

Attribution

Several colleagues aided in the writing and research behind one of my chapters presented as part of this thesis. A brief description of their contributions is included here.

Chapter 3: Suppression of intestinal inflammation and inflammation-driven colon cancer in mice by dietary sphingomyelin: Importance of peroxisome proliferator-activated receptor γ expression.

Eva Schmelz, PhD currently does research on chemopreventative and chemotherapeutic uses of natural and synthetic sphingolipid metabolites at Virginia Tech. Dr. Schmelz was co-author on this paper and helped with histopathology, diet and treatment parameters, and PCR-Array.

Raquel Honticellas, PhD is currently a research immunologist at Virginia Tech. Dr. Honticellas was a co-author on this paper and helped with determination of tumor load, and immune cell isolation.

Josep Bassaganya, PhD is currently a research immunologist at Virginia Tech. Dr. Bassaganya was a co-author on this paper and helped with animal care and development, the determination of tumor load, immune cell isolation, and statistical analysis.

Chapter 4: Suppression of intestinal inflammation and inflammation-driven colon cancer in mice by dietary sphingomyelin: Importance of peroxisome proliferator-activated receptor γ expression in CD4+ T-cells.

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Abbreviations:

Abbreviation	Full Name
UC	Ulcerative Colitis
IBD	Inflammatory Bowel Disease
CD	Crohn's Disease
CRC	Colorectal Cancer
DSS	Dextran Sodium Sulfate
AOM	Azoxymethane
DMH	Dimethylhydrazine
SI	Small Intestine
GI	Gastrointestinal
MLN	Mesentary Lymphnodes
Tregs	Regulatory T Cells
PPAR	Peroxisome Proliferator Activated Receptor
SM	Sphingomyelin
Alk-SMase	Alkaline Sphingomyelinase
NCDase	Neutral Ceramidase
S1P	Sphingosine-1-Phosphate
CK1	Ceramide Kinase-1
C1P	Ceramide-1-Phosphate
NF-kB	Nuclear Factor Kappa B
IFN-γ	Interferon-Gamma
DAI	Disease Activity Index
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
ANOVA	Analysis of Variance
IHC	Immunohistochemistry
$PPAR^{+/+}$	Mouse Group with active PPAR-γ gene
$PPAR^{-/-}$	Mouse Group lacking PPAR-γ in colonic epithelial tissue, macrophages, and T-cells
T-$PPAR^{-/-}$	Mouse Grouplacking PPAR-γ in T cells
C57BL/6	Mouse Group with active PPAR-γ gene

Chapter 1
Introduction

Colon cancer is one of the most prevalent causes of cancer-related deaths among the elderly [1]. Ulcerative Colitis (UC) patients have an increased chance of developing colon cancer of up to 18% after 30 years of UC onset [3]. Dietary patterns, environmental factors, and genetic predisposition are the most common risk factors involved in colorectal cancer formation. Colorectal inflammation has been shown to be directly correlated with the early onset of colon cancer in both mouse and human models. Irritable Bowel Disease (IBD), Ulcerative Colitis (UC), and Crohn's Disease (CD) are characterized by increased inflammation, immune cell infiltration, and immune-mediated destruction of the lining of the gastro-intestinal tract [2]. The severity of bowel inflammation is directly correlated with an increased risk in colorectal cancer formation particularly in younger patients [3]. Therefore, strategies that target intestinal inflammation could prevent colon cancer.

While the exact molecular mechanisms by which inflammation increases the risk of colon cancer are unclear, peroxisome proliferator-activated receptors (PPARs) likely play a role in the suppression of intestinal inflammation. PPARs are a family of nuclear hormone receptors that regulate inflammatory, immune, and metabolic responses in nearly all human tissues. Suppression of inflammation-driven colon cancer with natural compounds is dependent on PPAR-γ expression [4]. In particular, PPAR-γ is highly expressed in intestinal epithelial tissue, T-cells, and macrophages. The activation of PPAR-γ represses Nuclear Factor-κB (NF-κB)-mediated inflammation and regulates anti-inflammatory phenotypes [5, 6, 7]. Furthermore, PPAR-γ is believed to be involved in immune homeostasis via regulation of immune responsiveness, thus, it has potential as a preventative and therapeutic agent against IBD [8].

Dietary factors have a profound effect on the development of colon cancer [9]. Our lab has investigated the effect of sphingolipids on the incidence and severity of early and late stages of colon cancer in rodent models [10, 11, 12, 13]. Orally administered sphingolipids are hydrolyzed to their bioactive metabolites ceramide and sphingosine throughout the intestinal tract [14, 15, 16, 17]. Ceramide and sphingosine are sphingolipid metabolites that help regulate cellular growth, death, differentiation and proliferation, among others [18, 19]. These metabolites are also involved in the cellular inflammatory response, shown in mouse and cell models. Endogenous sphingolipid metabolites have been shown to increase inflammatory responses *in vitro* and *in vivo* but previous studies have implicated some metabolites in an anti-inflammatory response in endothelial cells [20] and immune cells [21]. The diet-derived sphingolipid metabolites could exacerbate intestinal inflammation, and thereby increase colon cancer development. This would limit the use of dietary sphingolipids in colon cancer prevention approaches. This will be tested in the specific Aims:

Specific Aim 1

- Determine the effect of dietary sphingomyelin on DSS-induced colitis, and the dependence on PPAR-γ expression. (Chapter 3)
- Determine changes in pro- and anti-inflammatory gene expression levels in response to dietary sphingomyelin and dependence on PPAR-γ expression (Chapter 3)

Specific Aim 2

- Determine the dependence of an anti-inflammatory effect of dietary sphingomyelin on CD4+ T-cell PPAR-γ expression. (Chapter 4)
- Determine changes in pro- and anti-inflammatory gene expression in response to dietary sphingomyelin and dependence on CD4+ T-cell PPAR-γ expression (Chapter 4).

Hypotheses:

1. In contrast to endogenous sphingolipids, dietary sphingomyelin supplementation will reduce inflammation caused by DSS treatment.
2. Dietary sphingomyelin supplementation will reduce inflammation caused by DSS treatment in a PPAR-γ dependent manner, specifically in a CD4+ T-cell PPAR-γ dependent manner.
3. Both down- and up-regulated genes will be observed in response to the dietary sphingomyelin, creating an overall anti-inflammatory environment. Furthermore, this response to dietary sphingomyelin will be dependent on CD4+ T-cell PPAR-γ expression

The goal of this project was to determine the effect of dietary sphingomyelin (SM) on intestinal inflammation, and identify the genetic markers of the inflammatory response within the colon that respond to sphingomyelin in a PPAR-γ expression dependent manner. Furthermore, this study provides insight as to whether dietary sphingolipids can be used as a viable prevention or treatment option for inflammation-driven colon cancer and whether the anti-inflammatory response to sphingolipid treatment is dependent on PPAR-γ expression. Mice with the loss of PPAR-γ gene in epithelial, T-cells, and macrophages were be used to determine PPAR-γ dependence while orally

administered sphingomyelin was used to prevent inflammation. The studies described here will enhance our understanding of how dietary sphingomyelin affects the intestinal inflammatory milieu, and will direct our further mechanistic studies of how sphingolipids prevent inflammation-driven colon cancer. These studies will be instrumental for the development of genetic markers as molecular targets for colon cancer prevention trials with dietary sphingolipids.

Chapter 2
Background

Sphingolipids

Sphingolipids are a diverse class of lipids derived from the aliphatic amino alcohol-sphingosine. The general structure of a sphingolipid contains a long chain, sphingoid base back bone, an amide-linked long chain fatty acid, and a head group that varies depending on which sphingolipid is produced (Figure 1).

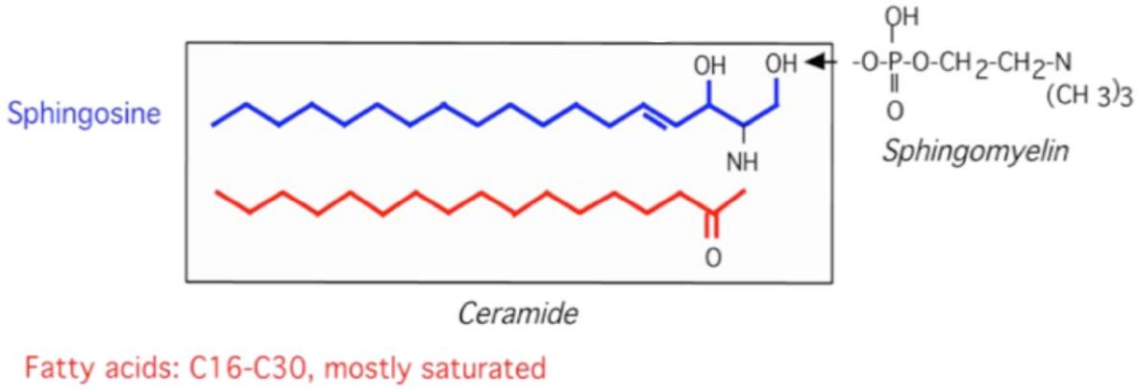

Figure 1. The Sphingosine backbone (blue highlight) is the predominant sphingoid base backbone for all mammalian sphingolipids. Addition of a fatty acid generates ceramide; its composition will deviate by changing the length of the fatty acid (red highlight) and/or head group attached at the C1 position (black arrow).

Sphingolipids are localized in cellular membranes and lipid rich structures such as skin and brain and are ubiquitous throughout the human body. Sphingolipids can be broken down into 3 main components based on their primary structure and attached head group. Sphingosine is a major sphingoid base found in mammalian cells while changes in the number and location of the double bonds. Addition of a fatty acid to the amino group generates ceramide. Ceramide, as shown in figure 1 can contain fatty acids of varying chain lengths. Thus, in contrast to the chemically defined sphingosine, ceramides are a group of sphingolipids with varying sphingoid backbones and fatty acids. Adding a head group to the 1-hydroxy group of ceramides forms more complex sphingolipids. The head group is important to both structural and functional abilities of the particular sphingolipid. There is a large number of variations of components within the sphingolipid family due to changes in the head group and length of the fatty acid chain attached to the sphingosine or other sphingoid bases. The sphingolipid metabolites are involved in membrane structure and fluidity, regulating cellular adhesion and motility, and function as bioactive lipid second messengers. As lipid second messengers, sphingolipid metabolites act as

regulatory factors in cellular functions such as proliferation, differentiation, growth, and programmed cell death. The role that each sphingolipid plays has shown variation among species and it should be noted that they can act as either induce or inhibit proliferation, depending their structure, localization and cellular context. For example, while sphingosine and ceramide are pro-apoptotic, their phosphorylated counterparts, sphingosine-1-phosphate and ceramide-1-phosphate are generally mitogenic but not in every context. Therefore, sphingolipid metabolism must be very strictly regulated considering the variety of functional differences that can occur due to minor changes in cellular sphingolipid structure or profile [18].

Sphingolipids are found in trace amounts in many foods common to the western diet. Sphingomyelin (SM) is the most common sphingolipid found in the average western diet which contains a high proportion of animal products. The amount in foods can vary from micromoles in fruits and vegetables to millimoles in eggs and soybeans (as glucosylceramide) [22]. SM and other complex sphingolipids enter the small intestine (SI) to be hydrolyzed by the alkaline SMase (alk-SMase) into ceramide, and neutral Ceramidase (nCDase) into sphingosine, and free fatty acids as can be seen in figure 2 [19]. SM digestion in the gastrointestinal tract is slow and incomplete, leaving the colon exposed to trace amounts of non-digested SM and

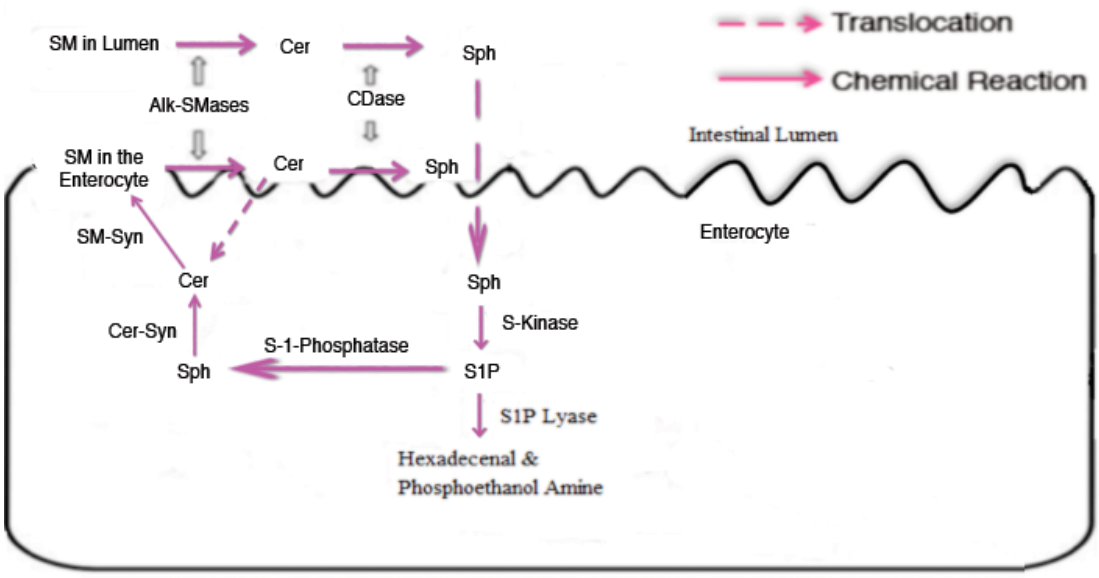

Figure 2. Metabolism of dietary SM in the small intestine of mice [19]. After luminal hydrolysis, sphingosine is taken up by the enterocyte, phosphorylated by sphingosine kinase and either irreversibly degraded to hexadecenal and phosphoethanolamine, or incorporated into complex sphingolipids, here shown as sphingomyelin (SM).

nondegraded ceramide. Sphingosine is readily absorbed in the SI [19]. After absorption in the SI, sphingosine is phosphorylated by sphingosine-kinase-1 into

sphingosine-1-phosphate (S1P). At this point S1P can either be irreversibly degraded into hexadecenal and phosphoethanolamine by S1P lyase or be synthesized back into SM to be incorporated into the enterocyte lipid membrane (figure 2). SM, ceramide and other sphingolipid metabolites that are not absorbed into the mucosal cells move onto the colon.

Once in the colon most sphingolipids will be excreted. Some of the ceramide and sphingosine will be absorbed by the colonic cells; SM may also be taken up by microflora and hydrolyzed into ceramide and sphingosine to be absorbed in trace amounts by the colonic epithelium [12, 23]. While the average diet contains about 0.3-0.4 g/day [22], sphingolipids are synthesized within each cell of the body and are not an essential part of the daily diet.

Sphingolipid functions are dependent on their structure, concentration, sphingolipid profile and cell type in which they are generated. This change in sphingolipid profile greatly affects the response of the cell. Spingolipids are regulating various normal cellular functions but are also directly and indirectly involved in a large number of diseases. Sphingolipids are involved in the generation or perpetuation of inflammation-driven diseases such as cardiovascular disorders, atherosclerosis, neurodegenerative diseases, and cancer formation [24, 25, 26]. While not a causal factor, changes in the sphingolipid profile play a significant role in the development of Alzheimer's disease [24]. Chronic intestinal inflammation, partly regulated by endogenous sphingolipids, is involved in aberrant crypt formation and subsequent colorectal tumorigenesis [12]. Finding a link between intestinal inflammation and dietary sphingolipids in contrast to endogenous sphingolipid metabolites may provide information on the potential and limitations of sphingolipids as therapeutic approach to prevent inflammation-driven colon cancer.

Inflammation

The human body has two types of immune responses to deal with outside pathogens, tissue damage and infections: innate and adaptive. The innate immune response provides immediate protection against infection and attacks all foreign invaders across a wide spectrum. Inflammation is one of the first responses to infection or damage to the organism. Redness, swelling, and heat are all symptoms of the inflammatory response, due to increased blood flow to the damaged area. Damaged cells secrete eicosanoids and cytokines to attract immune cells and stimulate inflammation. Eicosanoids include prostaglandins, regulate fever production and dilation of blood vessels for increased blood flow, and leukotrienes, which recruit leukocytes to the damaged tissue. Cytokines are much more varied in their involvement with inflammation. Cytokines include interleukins involved with white blood cell communication, chemokines that promote chemotaxis, and interferons that help in antiviral defense, such as halting protein synthesis. The adaptive response is triggered by the innate response and works to kill specific pathogens and mount a stronger attack each time that pathogen reemerges. The adaptive immune response remembers and recognizes previously seen pathogens. Since sphingolipids are part of the

cellular membranes, they could have a role in the adaptive immune response but that is beyond the scope of this project. The role of sphingolipids in the innate immune response, particularly the inflammatory pathway, will be the primary focus.

Patients with IBD such as UC and CD have an increased risk of developing colorectal cancer (CRC). This increased risk is thought to be the result of prolonged inflammation within the colon rather than genetic predisposition [27]. CRC patients without chronic inflammation of the colon generally slowly develop precancerous polyps (adenoma), which can be removed via polypectomy. CRC patients that experience chronic inflammation have an early onset of multiple adenomas as markers of colon inflammation; these constitute an increased risk for tumor development which can require removal of the tumor or potentially the entire colon/rectum to prevent subsequent colon cancer metastases [27].

Mouse models have been used to demonstrate the effects of inflammation on the development of colon cancer. There are two widely used models for inflammation-driven colon cancer in mice. The first is a carcinogen treatment such as injecting mice with azoxymethane (AOM) or its metabolite dimethylhydrazine (DMH) to initiate intestinal cancer, specifically in the colon, followed by the induction of inflammation via treatment with dextrane sodium sulfate (DSS). The other prominent model is the IL-10 knockout mouse. This is the model for Crohn's disease since spontaneous enterocolitis or colonic inflammation, is seen in patients with mutations in the IL-10 receptor**.** IL-10 knockout models have been shown to cause a high incidence of colorectal carcinoma mice without any stimuli, 60% after 6 months [28]. Researchers typically choose to expedite the process by using an AOM-DSS stimulus similar to that used in this thesis project [28, 29]. DSS treatment without the IL-10 knockout was the preferred model for this project because of its wide-spread use as an inflammatory model, available data for comparisons and it being more in line with long term damage from environmental sources of inflammation rather than an internal mutation thus better mimicking UC rather than Crohn's Disease.

Other strains of mice are also susceptible to colon cancer formation via the induction of inflammation through injury models. For example, $MSH2^{-/-}$ mice, due to having a defective mismatch repair gene, are susceptible to developing colon cancer but will not do so spontaneously. When orally fed DSS, $MSH2^{-/-}$ mice developed dysplasia or cancer [30]. Furthermore, $APC^{min/+}$ mice which carry a mutant *APC* allele rarely develop colonic tumors but after receiving DSS, there was a direct correlation of the incidence of tumors in the colon to the DSS treatment. Tumors developed in high frequency in areas of previously healed mucosa as well as areas of acute and chronic inflammation [31], suggesting that adenoma development occurs more frequently from damaged or inflamed tissue giving rise to further investigation of injury based models in the colon. In comparison to previous studies our project, utilizing the DSS-induced colorectal

inflammation, serves as relevant injury-based mouse model because of the similarities to UC.

Sphingolipid metabolites have multiple effects on cellular processes. Metabolites such as ceramide are pro-apoptotic and induce inflammation. Previous studies have found a pro-inflammatory response in the gut due to TNF-α and IL-1ß mediated increase in pro-inflammatory cytokines such as IL-6 [32]. In response to these cytokines accumulation in the gut, an increased formation of ceramide either via sphingomyelinase activation or de novo synthesis is initiated, linking ceramide generation to pro-inflammatory effects [32]. Homaidan et al. tested the effects of IL-1 and ceramide on the production of NF-κB in intestinal epithelial cells. They found that treatment of these cells with IL-1 and ceramide produced a significantly increased level of NF-κB in the nucleus of Mode-K cells. This was mediated by the inhibition of IκB-α and IκB-ß, upstream regulators of NF-κB, suggesting that ceramide treatment targets the inhibitors to allow translocation of NF-kB to the nucleus and activation of transcription of pro-inflammatory genes [33]. Several functions of sphingosine are similar to ceramide; it is pro-apoptotic and can act as a pro-inflammatory molecule. Ballou et. al found that sphingosine enhanced IL-1-mediated PGE2 production in human foreskin, specifically fibroblasts, causing the activation of COX-2 and increased expression of TNF-α. Ballou et al. concluded that sphingosine combined with IL-1 could potentially activate COX-2 and TNF-α [34]. These results were only shown *in-vitro* in fibroblasts derived from human foreskin, thus the effects may be tissue specific or lead to potentially different results with intestinal epithelial tissue.

S1P, synthesized by sphingosine kinase, is mostly anti-apoptotic while maintaining pro-inflammatory properties. S1P acts as a chemoattractant for neutrophils and macrophages to aid the inflammation process. S1P also induces COX-2 activity, an important enzyme in the production of eiconsanoid inflammatory mediators. This downstream effect causes the formation of PGE2, a major prostaglandin in the GI tract[35]. Thus, the increase in COX-2 expression and activity can be damaging, particularly to IBD patients. Ceramide-1-Phosphate (C1P) has also shown pro-inflammatory effects, but unlike ceramide, C1P has mitogenic and prosurvival properties. Furthermore, C1P is an important mediator of inflammatory responses, an action that is mediated by stimulation of cytosolic phospholipase A2 [36]. Pettus et al. showed that by down-regulating ceramide-kinase, which decreases C1P, caused a decrease in IL-1β activation. This suggests that C1P is involved in the activation of IL-1β which results in a pro-inflammatory response [37]. Chalfant et al. demonstrated that C1P effectively and specifically stimulated arachidonic acid release and prostanoid synthesis in lung carcinoma cells. The group found that the arachidonic acid stimulation occurred through direct activation of $cPLA_2$ which is associated with increased risk of cardiac disease and atherosclerosis, both are diseases known for being directly involved with chronic inflammation [38]. Together, these studies demonstrate the variety of effects that sphingolipid metabolites have on inflammatory processes, and suggest not only a structure dependency of the

effect but also the importance of the context in which sphingolipid signaling is activated.

Peroxisome Proliferator-activated Receptor γ (PPAR-γ)

PPAR-γ is a member of the PPAR Nuclear Hormone Receptor family. There are 3 subtypes in the PPAR family: PPAR-α, PPAR-γ, PPAR-δ. They are important regulators in physiological processes including lipid homeostasis, inflammation, adipogenesis, reproduction, wound healing, and carcinogenesis [39]. PPAR-γ may be activated by growth factors, fatty acids and other /metabolites, as well as pharmacological drugs used for regulating diabetes. When an activating ligand (i.e., Growth Factor) binds to the cellular membrane it will initiate a signaling cascade that ultimately activates PPAR-γ by binding an RXR tetramer with cis-Retinoic acid, thus starting transcription. Certain ligands such as growth factor, anti-diabetic drugs, and fatty acids are believed to induce an anti-inflammatory response by activating PPAR-γ (Figure 3).

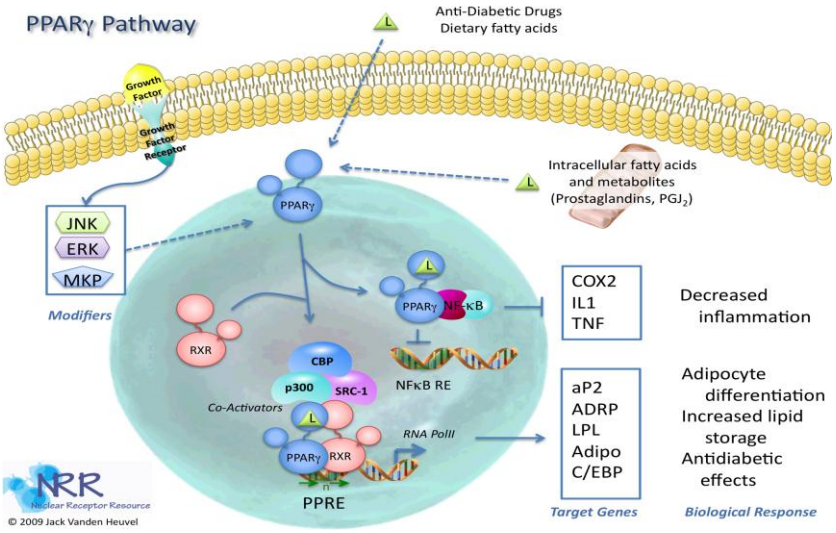

Figure 3. PPAR-γ activation and pathway within a cell. Activation via growth factor causes signaling intermediates like JNK, ERK, and MKP to activate inuclear PPAR-γ. PPAR-γ activation blocks the transcription of several pro-inflammatory markers (Cox-2, IL1, and TNF-α), thus providing an anti-inflammatory response [40].

Since PPAR-γ is ubiquitously expressed, it also has important functions in the cells found in the intestinal tract. As such, it has already been identified as a target for preventative and therapeutic efforts since its activation attenuates inflammatory responses. Bassaganya et al. reported activation of the nuclear receptor PPAR-γ reduced the severity of IBD by suppressing excessive pro-inflammatory responses [41, 42]. While this could be due to changes in the

transcription of several pro-inflammatory genes, it is thought that the activation of PPAR-γ represses NF-κB mediated inflammation and promotes a regulatory, anti-inflammatory phenotype [6, 7, 43.] Furthermore, Mohapatra et al. demonstrated that adequate expression of PPAR-γ in intestinal epithelial cells is required for the regulation of mucosal immune responses and prevention of experimental IBD, possibly by modulation of lysosomal and antigen presentation pathways [44]. Other studies have revealed that PPAR-γ deficient macrophages cause a more severe inflammatory response to DSS-induced colitis than PPAR-γ expressing cells [45]. More research is needed to determine the relevance of PPAR-γ in T-cells as specific mediators of pro- and anti-inflammatory responses in IBD or other forms of colonic inflammation.

Fatty acids are known ligands for activating PPAR-γ; due to structural similarities of fatty acids and sphingoid bases, it is possible that the colonic inflammatory response, part of which is controlled by PPAR-γ, could be activated by sphingolipid metabolites. Sphingolipid metabolites are known to generate or perpetuate the inflammatory responses. Sphingolipid metabolites such as ceramide and sphingosine increase the inflammatory response to tissue damage [19]. Dietary sphingolipids, however, have been shown to suppress colon and breast cancer in several animal models [11, 46, 47]. PPAR-γ activation provides an anti-inflammatory response in the colon; therefore, implementing possible therapies using sphingolipids that target PPAR-γ to prevent diseases such as IBD and CD and, subsequently, CRC, could prove to be beneficial. A reduction in chronic inflammation could also reduce the incidence of colon cancer due to chronic inflammation.

Chapter 3

Suppression of Intestinal Inflammation and Inflammation-driven Colon Cancer in Mice by Dietary Sphingomyelin: Importance of Peroxisome Proliferator-activated Receptor γ Expression.

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Abstract

Inflammation of the gastrointestinal tract increases the risk of developing colon cancer especially in younger adults. Dietary compounds are not only associated with the etiology of inflammation and colon cancer, but also in their prevention. Sphingolipid metabolites have been shown to play a role in the initiation and perpetuation of inflammatory responses. In the present study, we investigated the suppression of dextran sodium sulfate (DSS)-induced colitis and azoxymethane-induced colon cancer by dietary sphingomyelin in mice that lack functional (PPAR-γ) in intestinal epithelial and immune cells. Dietary sphingomyelin decreased disease activity and colonic lesions in mice of both genotypes but more efficiently in mice expressing PPAR-γ. The decreased survival and suppression of tumor formation in the sphingomyelin-fed mice appeared to be independent of PPAR-γ expression in immune and epithelial cells. Using a real-time PCR array, we detected an up-regulation in genes involved in Th1 (interferon γ) and Th17 (interleukin [IL]-17 and IL-23) responses despite the reduced inflammation scores. However, the genes involved in Th2 (IL-4, IL-13 and IL-13ra2) and Treg (IL- 10rb) anti-inflammatory responses were up-regulated in a PPAR-γ-dependent manner. In line with the PPAR-γ dependency of our *in vivo* findings, treatment of RAW macrophages with sphingosine increased the PPAR-γ reporter activity. In conclusion, dietary sphingomyelin modulated inflammatory responses at early stages of disease by activation PPAR-γ, but its anti-carcinogenic effects followed a PPAR-γ-independent pattern.

Introduction

Colorectal cancer is a prevalent cancer among the older population (146,970 were diagnosed and 49,920 died of colorectal cancer in 2009) with a median age at diagnosis of 71 years [1]. The most common risk factors include genetic predisposition and environmental factors such as carcinogens and dietary patterns. Intestinal inflammation (ulcerative colitis and Crohn's disease), however, drastically increase the risk of developing colon cancer especially at early age (< 30 years of age) [84]. The risk of developing colorectal cancer for an ulcerative colitis patient is estimated to be 2% after 10 years, 8% after 20 years, and 18% after 30 years of disease onset [3]. A prevention strategy that targets inflammation could, therefore, be successful to prevent the colon cancer in these high-risk groups.

Dietary components affect the risk of developing colon cancer [85]. In this regard, our studies have shown that dietary sphingolipids suppress early and late stages of carcinogen-induced colon cancer [10, 11, 12, 13,] and suppress tumor formation in Min mice [86, 87]. Orally administered complex sphingolipids are hydrolyzed to ceramide and sphingosine throughout the intestinal tract [14, 15, 16, 17] which are the same bioactive metabolites generated *in vitro* shown to regulate growth, death, differentiation, and motility of cells [18, 19]. Bioactive sphingolipid metabolites have also been implicated in the generation or perpetuation of inflammatory responses in endothelial cells [20], adipose tissue [88], lung [89], and immune cells [21]. A recent report demonstrated an increase of ceramide in colonic lipid extracts from DSS-treated mice *in vivo* [78]. On the other hand, dietary SM did not affect antibody formation, natural killer cell cytotoxicity, or delayed-type hypersensitivity in carcinogen-treated mice [90]. However, a recent study found that orally administered SM lowered DSS-induced inflammation in Jci:ICR mice [82]. This appears to contradict the pro-inflammatory effect of the bioactive sphingolipid metabolites summarized above.

While the exact mechanism for the elevated colon cancer risk by colonic inflammation is still unknown, recent studies have been investigating whether the marked reduction in levels of the nuclear PPAR-γ in colons of patients with ulcerative colitis may play a role in their increased susceptibility to developing colorectal cancer [91]. PPARs belong to the superfamily of nuclear hormone receptors with 48 members identified in the human genome. There are three known PPAR isoforms; α, β/δ or and γ which differ in their tissue distribution and functional activity [92]. PPARs are endogenously controlled molecular switches that regulate inflammation, immunity and metabolism [93, 94] but their main biological function is the sensing of intracellular nutrient concentrations and regulation of gene expression involved in maintaining both metabolic and immune homeostasis. Since PPAR-γ is ubiquitously expressed in immune cells and the gut, it has already been identified as a target for preventive and therapeutic efforts since its activation attenuates inflammatory responses. PPAR-

γ is expressed by CD4+ T cells, and regulates their differentiation into at least four functionally distinct subsets referred to as T helper (Th) 1, Th2, Th17, and induced regulatory T cells (iTreg). Functionally, Th1 and Th17 subsets are linked to increased risk of autoimmune disorders whereas Treg cells can prevent inflammatory and immune mediated diseases [71].

In this regard, we have shown previously that conjugated linoleic acid suppressed inflammation-induced colorectal cancer in a strictly PPAR-γ dependent manner [4]. We have also demonstrated that abscisic acid can suppress experimental IBD in a manner dependent on T cell PPAR-γ [48]. In the present study, we set out to investigate the effect of dietary SM on inflammation-driven colon cancer to clarify the apparent contradiction of the immunological effects of sphingolipid metabolites, and test the dependence of an anti-inflammatory effect on PPAR-γ expression. Our studies show that dietary SM greatly inhibited the tissue damage caused by DSS-induced colitis, enhanced the survival and reduced tumor formation. Interestingly, the anti-inflammatory of SM, but not its anti-carcinogenic effects, were enhanced by PPAR-γ expression in the intestinal epithelial and immune cells.

Methods and Materials

Mice

Since whole body deletion of PPAR-γ is lethal by day E10 [95], tissue-specific PPAR-γ null mice were generated as previously described [96, 97]. PPAR-γ fl/fl mice (carrying PPAR-γ gene flanked with two loxP boxes on exon 2 that are recognized by a transgenic recombinase) were cross-bred with transgenic mice carrying the Cre-gene under control of the MMTV-LTR promoter which express a transgenic recombinase only in epithelial and hematopoietic cells [5]. PPAR-γ fl/fl; Cre^{+} (named PPAR-$\gamma^{-/-}$ throughout the manuscript) express a truncated form of mRNA transcript and do not express PPAR-γ protein in the intestinal epithelial cells, T-cells or macrophages as confirmed by real-time RT-PCR and Western Blotting. These mice are viable without any known problems and have been used in previous studies by our group [5, 59]. Both female and male mice (5 each per group) were used in the present studies. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Virginia Polytechnic Institute and State University and met requirements of the Public Health Service/National Institutes of Health and the Animal Welfare Act.

Diets and Treatments

PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ mice were randomly assigned into either the control or the SM-supplemented diet (10 per group). The mice in all four groups were fed the semi-purified sphingolipid-free AIN76A diet [98] throughout the study. The sphingolipid groups received 1g/kg SM (0.1% by weight) (Avanti, Alabaster, AL) in the diet. Bovine milk SM powder (99% pure) was mixed thoroughly into small batches of diet using a mixer and stored at 4˚C until use. The milk SM contains mostly saturated fatty acids [38] and is stable under these conditions. The amount of SM used does not add significant amounts of caloric value to the diet and has demonstrated to have no side effects in either of our studies or those of other groups; even 1% was tolerated well over 2 generations of rats [83]. Amounts up to 0.1% have been used in our previous studies and have suppressed carcinogen-induced colon cancer by up to 80% [12,13,38]. After 7 days on the experimental diets, the mice were injected with a single dose of azoxymethane (10 mg per kg bodyweight in bicarbonate buffer) to induce colon cancer. Inflammation was induced one week later (day 13) by adding 2.0% DSS (molecular weight 36,000 to 44,000 g/L; ICN Biomedicals, Aurora, OH) to the drinking water for 7 consecutive days. During this time the mice were weighed on a daily basis and examined by blinded observers for clinical signs of disease

associated with colitis (i.e., perianal soiling, rectal bleeding, diarrhea, and piloerection). The disease activity index (DAI) consisted of a scoring for diarrhea and lethargy (0–3), whereas rectal bleeding consisted of a visual observation of blood in feces and the perianal area (0–4). On day 8 of the DSS challenge, the mice were switched to regular drinking water for the duration of the colon cancer study. DAI and weight determinations were continued weekly until the end of the study.

Determination of Tumor Load

After the DAI increased – bleeding indicated the existence of colon tumors- the mice were euthanized by CO_2 asphyxiation 80 days after the carcinogen injection. The colons were removed, opened longitudinally, and the tumor area was measured since the colons appeared macroscopically to be mostly a solid sheet of tumor. In case of identifiable single tumors, the tumors were measured individually and their surface areas were combined. Then parts of the colons were either fixed in formalin or OCT for immunohistochemistry and determination of tumor progression by an experienced veterinarian, used for isolation of immune cells or preserved in RNAlater (Qiagen) using the same parts of the colon of each animal for these analyses.

Immune Cell Isolation from Mesenteric Lymph Nodes (Mln)

MLNs were excised, crushed, and placed into 3 ml of FACS buffer (1xPBS, 5% FBS, and 0.1% Sodium Azide). After the cells were centrifuged, the supernatant was discarded and the cells were washed with 10 ml of FACS buffer and centrifuged at 1200 RPM. The supernatant was discarded and the cells resuspended at $2x10^6$ cells/ ml. MLN cells were then immunophenotyped as described previously [4]. Briefly, MLN cells were seeded into 96-well plates, blocked with FcBlock (BD-Pharmingen), incubated with anti-F4/80-PE-Cy5, anti-CD11b-FITC (eBioscience), and anti-CCR2-PE (R&D systems, Minneapolis, MN) antibodies to assess macrophage infiltration, anti-CD4-FITC, anti-CD25-Pe-Cy5 (BD Pharmingen) and anti-FoxP3 (after permeabilization with *Cytofix-Cytoperm* from BD Pharmingen) to determine T regulatory cells (Tregs) infiltration. Cells were resuspended in PBS for BD LSR II flow cytometer analysis and data analysis was performed with FACS Diva software.

Histopathology

The excised colons were fixed in 10% buffered neutral formalin, embedded in paraffin, and then sectioned (5 µm) and stained with H&E stain for histological examination by RH and JBR who have more than 15 years of experience in histological/histopathological tissue analysis. Tissue slides were examined in a Nikon 80*i* eclipse epifluorescence microscope, equipped with DIC,

color and monochromatic digital cameras. Images were digitally captured using the NIS software (Nikon) and processed in Adobe Photoshop Elements 2.0 (Adobe Systems Inc., San Jose, CA).

Cytokine Real-time Pcr Array

For the initial determination of changes in inflammatory responses that may be associated with the diet and genotype, PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ mice (n=12 per group) were placed on the AIN76A diet with or without the 0.1% SM throughout the study (6 per group). After one week, 2% DSS was added to the drinking water of 3 animals of each group for 7 days during which they were weighed and scored daily. Then, all mice were euthanized by CO_2 asphyxiation followed by secondary thoracotomy, the colons were removed, rinsed and a portion of each colon was fixed in 10% neutral buffered formaldehyde, embedded in paraffin and sectioned for H&E staining for determination of tissue architecture or placed into RNAlater. To extract RNA, the remaining colonic tissue was cut into 15-25mg pieces and rinsed with RNAse free water to remove the RNAlater. Then the tissue was snap-frozen in liquid nitrogen, homogenized, and RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and stored at -80ºC until analysis.

RNA purity was assured by assessing the A260:A230 ratio and A260:A280 ratio (NanoDrop, Thermo Fisher) and the ribosomal RNA integrity using the Experion™ Nano LabChip (Biorad). In each group, 2ng of RNA per mouse (n=3 in each group) were pooled for cDNA synthesis using the RT^2 First Strand Kit (SABiosciences). Real-time RT-PCR was performed in a RNA microarray format using the Inflammatory Response and Autoimmunity RT^2 Profiler PCR Array (PAMM-3803 SABiosciences) according to the manufacturer's instructions on an ABI 7900HT PCR machine (Applied Biosystems, Foster City, CA). Data analysis was performed using the SABiosciences analyzing tools provided online. Included in the arrays were controls for genomic DNA contamination, reverse transcription and positive PCR controls and 5 housekeeping genes (ß-actin, GAPDH, Hsp90ab1, Hprt1, Gusb). Data presented are the fold differences between the 2^ DCT values for genes that were at least 2-fold up- or down-regulated in the treatment versus the appropriate control group, or the same treatment in different genotypes. Since the array was performed with pooled samples of 3 mice, no statistical analyses of the data were performed.

Statistics

Data were analyzed as 2 x 2 factorial arrangements of treatments. The statistical model was: $Y_{ijk} = \mu + \text{Genotype}_i + \text{Diet}_j + (\text{Genotype} \times \text{Diet})_{ij} + \text{error } A_{ijk}$, in which μ was the general mean, Genotype_i was the main effect of the i_{th} level of the genotypic effect (expression of PPAR-γ by immune and epithelial cells), Diet_j

was the main effect of the j_{th} level of the dietary effect (sphingomyelin vs no supplement), (Genotype $\times$ Diet)$_{ij}$ was the interaction effect between genotype and diet, and error A representing the random error. To determine the statistical significance of the model, analysis of variance (ANOVA) was performed using the general linear model procedure of Statistical Analysis Software (SAS Institute Inc., Cary, NC). When the model was significant, the analysis was followed by Fisher's Least Significant Difference multiple comparisons method. Data were expressed as the means ± standard error of the mean. Statistical significance was assessed at a *P* value of < 0.05.

Results

Effect of dietary sphingomyelin on DSS-induced inflammation

To determine if orally administered SM affect DSS-induced colitis and colitis-driven colon cancer and whether these effects are dependent on the expression of PPAR-γ, mice expressing or lacking PPAR-γ in the intestinal epithelium and immune cells were injected with a single dose of AOM after acclimatization, and treated a week later with DSS for 7 days. The DAI was determined as described under methods and materials. During the acute phase of inflammation, the mice lost weight as was expected but there was no significant difference in the weight of the control mice of either genotype during DSS treatment, recovery or throughout the rest of the study (Table 1).

Table 1. Effect of dietary supplementation with sphingomyelin (SM; 0.1 g/100 g) in PPAR-γ $^{+/+}$ or PPAR-γ $^{-/-}$ mice on body weights (in g) prior to and following the induction of inflammation-induced colorectal cancer.

	PPAR-$\gamma^{+/+}$		PPAR-$\gamma^{-/-}$			ANOVA *P* value		
Day	Control	0.1% SM	Control	0.1% SM	SEM	Diet	Genotype	D × G
D0	21.66	20.41	19.41	20.83	1.203	NS	NS	NS
D7	23.00	21.40	20.82	23.45	1.079	NS	NS	NS
D13	23.67	21.50	21.18	23.90	1.159	NS	NS	0.045
D14	23.19	21.96	21.91	24.00	1.180	NS	NS	NS
D15	23.78	21.85	21.01	23.98	1.142	NS	NS	0.042
D16	24.08	22.00	21.23	24.07	1.109	NS	NS	0.035
D17	23.62	21.65	20.88	24.00	1.077	NS	NS	0.026
D18	23.19	21.17	20.33	23.58	1.049	NS	NS	0.018
D19	22.29	21.01	19.86	23.25	1.048	NS	NS	0.035
D26	18.82^{ab}	18.65^{ab}	16.29^{b}	21.73^{a}	0.926	0.013	NS	0.008
D33	22.55^{ac}	20.95^{bc}	17.54^{b}	24.60^{a}	0.752	0.006	NS	0.0001
D40	24.18^{a}	22.525^{ab}	19.45^{b}	25.18^{a}	0.793	0.04	NS	0.001
D47	24.36^{ab}	22.51^{ab}	21.38^{b}	25.88^{a}	0.836	NS	NS	0.005
D54	24.98^{ab}	23.00^{ab}	21.66^{b}	25.98^{a}	0.815	NS	NS	0.004
D61	24.22	23.70	21.85	25.79	0.827	NS	NS	0.03
D68	25.2	21.88	20.97	25.79	0.981	NS	NS	0.002

[1] Least squares means values (n = 10) in a row for a particular body weight (grams) with different superscripts are significantly different ($P < 0.05$).

[2] On day 7, mice were challenged with azoxymethane (i.p.) and on day 13 mice were administered 2% dextran sodium sulfate in the drinking water for 7 days as described in *Materials and Methods*.

[3] Data were analyzed as a 2 × 2 factorial arrangement (i.e., 2 genotypes and 2 dietary treatments).

In both the PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ control groups fed the AIN76A diet without supplements, the DAI increased on day 15 of the study (after 3 days of

DSS treatment) and was consistently higher in the PPAR-$\gamma^{-/-}$ group throughout the study (significant at $p<0.001$ on day 4 of the DSS treatment (Table 2). The recovery from the DSS challenge was significantly delayed in the PPAR-$\gamma^{-/-}$ control group compared to the PPAR-$\gamma^{+/+}$ group ($p<0.0001$, $p<0.0001$, and $p<0.007$ on day 14, 21, and 28 of study, respectively) (Fig.4 and Table 2). This confirms earlier studies showing an increased DAI after DSS treatment in PPAR-$\gamma^{-/-}$ mice [5] and indicates an anti-inflammatory effect of PPAR-γ.

Table 2. Effect of dietary supplementation with sphingomyelin (SM; 0.1 g/100 g) in PPAR-$\gamma^{+/+}$ or PPAR-$\gamma^{-/-}$ mice on disease activity indices (DAI) following the induction of inflammation-induced colorectal cancer.

	PPAR-$\gamma^{+/+}$		PPAR-$\gamma^{-/-}$			ANOVA *P* value		
Day	Control	0.1% SM	Control	0.1% SM	SEM	Diet	Genotype	D × G
D0	-	-	-	-	-	-	-	-
D7	-	-	-	-	-	-	-	-
D13	0.00	0.00	0.00	0.00	0.000	-	-	-
D14	0.00	0.00	0.00	0.00	0.000	-	-	-
D15	0.10	0.00	0.40	0.00	0.971	0.01	NS	NS
D16	0.85^{ab}	0.11^{b}	1.50^{a}	0.85^{ab}	0.196	0.001	0.001	NS
D17	2.20	1.77	2.60	1.60	0.338	0.045	NS	NS
D18	2.85	2.11	3.20	2.10	0.297	0.004	NS	NS
D19	3.05	2.61	3.20	2.70	0.249	NS	NS	NS
D26	2.00^{ab}	0.00^{c}	2.87^{a}	1.80^{b}	0.200	0.0001	0.0001	0.039
D33	0.00^{b}	0.00^{b}	0.80^{a}	0.10^{b}	0.081	0.001	0.0001	0.001
D40	0.00^{b}	0.00^{b}	0.83^{a}	0.10^{b}	0.125	0.02	0.007	0.029
D47	0.20	0.06	0.08	0.40	0.175	NS	NS	NS
D54	0.40	0.69	0.66	1.20	0.377	NS	NS	NS
D61	0.90	1.06	0.33	1.20	0.318	NS	NS	NS
D68	0.60	1.38	1.50	0.89	0.373	NS	NS	NS

[1] Least squares means values (n = 10) in a row for a particular DAI with different superscripts are significantly different ($P < 0.05$).

[2] On day 7, mice were challenged with azoxymethane (i.p.) and on day 13 mice were administered 2% dextran sodium sulfate in the drinking water for 7 days as described in *Materials and Methods*.

[3] Data were analyzed as a 2 × 2 factorial arrangement (i.e., 2 genotypes and 2 dietary treatments).

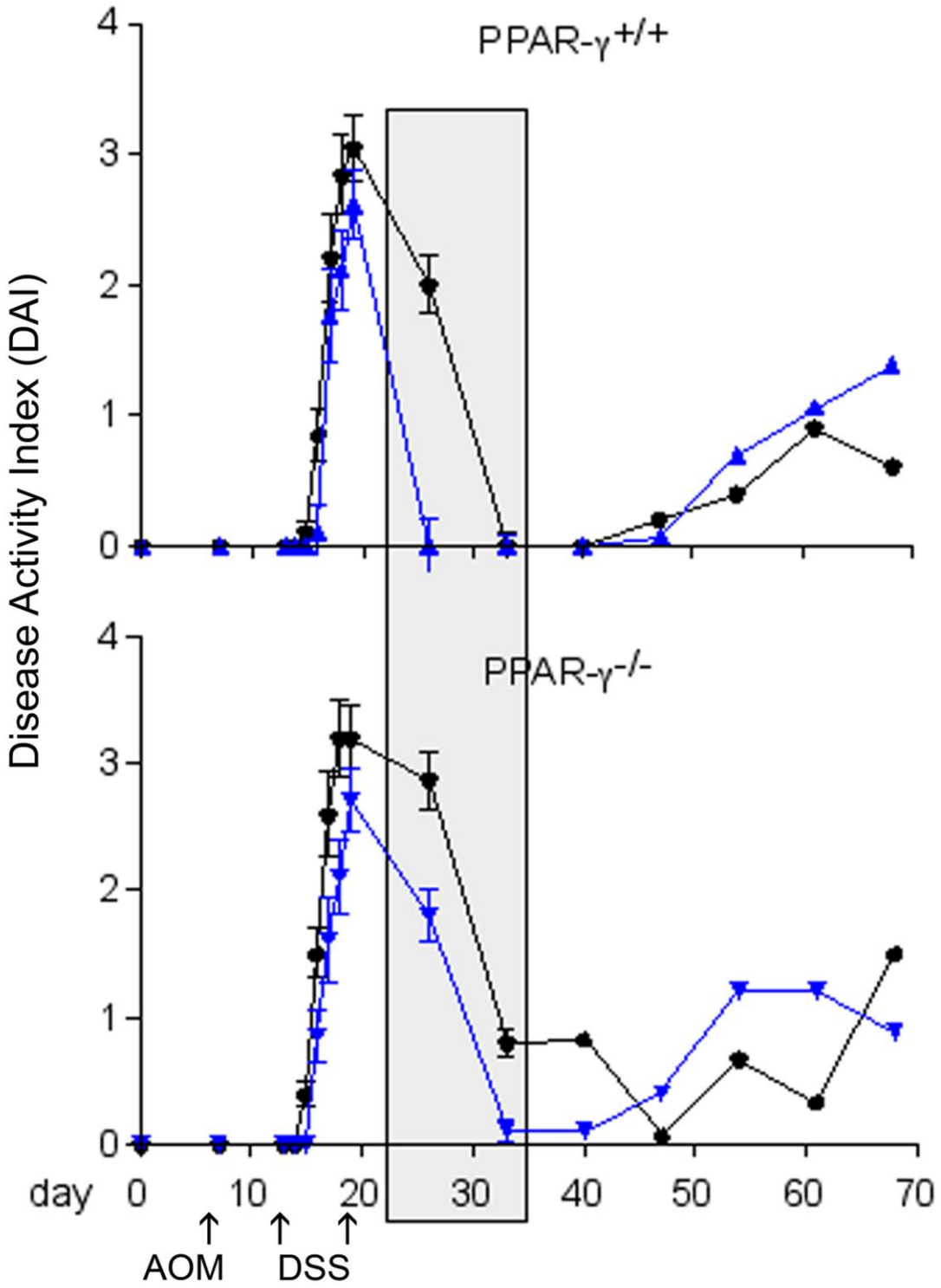

Figure 4. Disease activity index in PPAR-γ-expressing (PPAR-$\gamma^{+/+}$) and tissue-specific knockout (PPAR-$\gamma^{-/-}$) mice lacking PPAR-γ in intestinal epithelial cells, macrophages and T cells. All mice were injected with a single dose of AOM, treated with 2.0% DSS for 7 days and fed either the AIN76A diet alone (circles) or supplemented with 0.1% SM (triangles) (n=10 per group).

SM supplements significantly suppressed the inflammatory response to DSS compared to the controls of both genotypes. While there was no difference in the DIA during acute inflammation between the groups fed SM, the onset of the inflammation was delayed in PPAR-$\gamma^{+/+}$ mice and their recovery was significantly accelerated after the DSS was removed from the drinking water (p<0.039, p<0.001, and p<0.029 for diet x genotype on day 26, 33 and 40 (Fig. 4, Table 2); 7 days after the last day of DSS treatment, the DAI had already returned to zero in the SM-fed PPAR-$\gamma^{+/+}$ mice while PPAR-$\gamma^{+/+}$ mice still showed an average DAI of 1.80. These results suggests both PPAR-γ-dependent and independent pathways of suppression of colonic inflammation and recovery from the DSS colitis by dietary SM. After the recovery from the DSS-induced inflammation, the DAI in all groups increased over the course of the study, indicating the development of colonic tumors which is often associated with intestinal bleeding and blood in the feces. However, the number of mice in the control groups was very small at the end of the study and there were no statistically significant differences in DAI at these time points (Table 2).

Long-term effects of the combined AOM and DSS treatment

The DSS-induced intestinal inflammation is an established model for ulcerative colitis and has been used in many studies to investigate the tumor-promoting effect of intestinal inflammation. Upon removal of DSS from the drinking water mice will recover from the damage; this has been also observed in our previous studies [59]. Surprisingly, in the present study, the control mice showed an overly strong response to the treatment although the DSS concentration was similar or even lower than those used in other reports and several mice did not recover after DSS discontinuation and reached the euthanasia endpoint as defined by the animal welfare guidelines and were sacrificed when found moribund. As shown in Figure 5, only 3 of the PPAR-$\gamma^{+/+}$ and 2 of the PPAR-$\gamma^{-/-}$ control mice survived until the end of the study. In the SM-fed groups, 7 of the PPAR-$\gamma^{+/+}$ and 8 of the PPAR-$\gamma^{-/-}$ survived (p=0.075 and p=0.0051 compared to the appropriate controls, respectively). This increased survival in the SM-fed groups suggests that the suppression of the initial response to the DSS treatment may be critical for the survival of the mice. Also, the choice of basal diet may have affected the severity of the inflammatory response. AIN76A is essentially sphingolipid-free [17] while the lipid source has been changed from corn to soy oil in the AIN93 diets. Soy is a rich source of sphingolipids and even small amounts may have exerted protective effects in previous studies. However, this needs to be investigated in more detail.

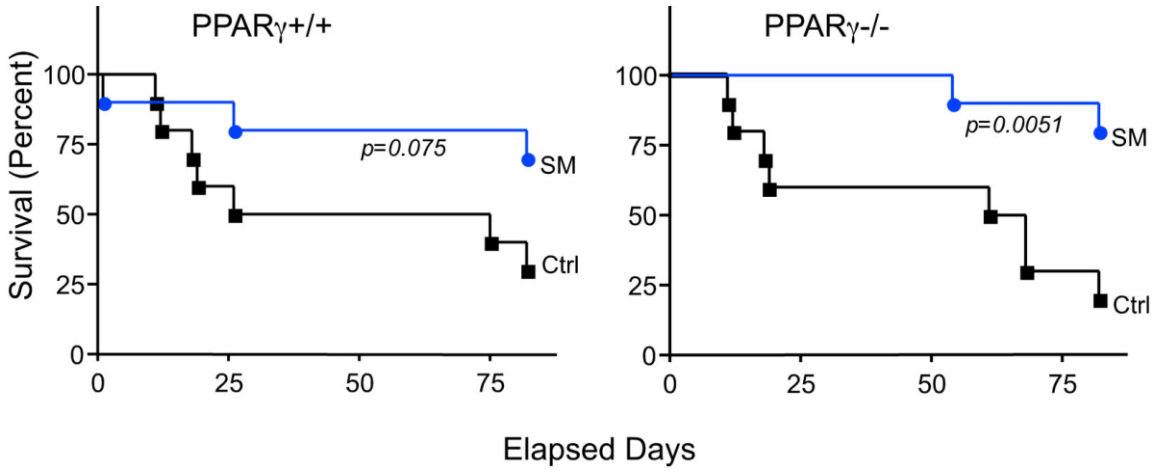

Figure 5. The survival of mice after DSS treatment (2% for 7 days) is enhanced in mice fed SM independent of their genotype.

Hematoxylin and eosin-stained sections of the colons of mice at the end of the study showed a distortion of the columnar shape of the colonic crypts generating a loose tissue architecture (arrows), and a lack of mucin-producing goblet cells in most areas of the colons of PPAR-$\gamma^{-/-}$ mice (arrow heads). In PPAR-$\gamma^{+/+}$ mice, the tissue architecture was better preserved, and more goblet cells were visible (Fig.6, upper panels). However, these tissues were derived from mice that survived the DSS treatment while 7/10 and 8/10 of the PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ mice, respectively, succumbed to the DSS-induced injuries; these colons, therefore, may represent mice with less initial damage to their tissue architecture. These deleterious changes were greatly reduced by the dietary SM in the colons of the mice of either genotype (Fig.6, lower panels). Most of the PPAR-$\gamma^{+/+}$ mice showed the distinct colonic architecture with abundant goblet cells (open arrow heads); this was also seen in the PPAR-$\gamma^{-/-}$ mice but there were also larger areas of less tight crypts that lacked goblet cells despite the SM in the diet in this group.

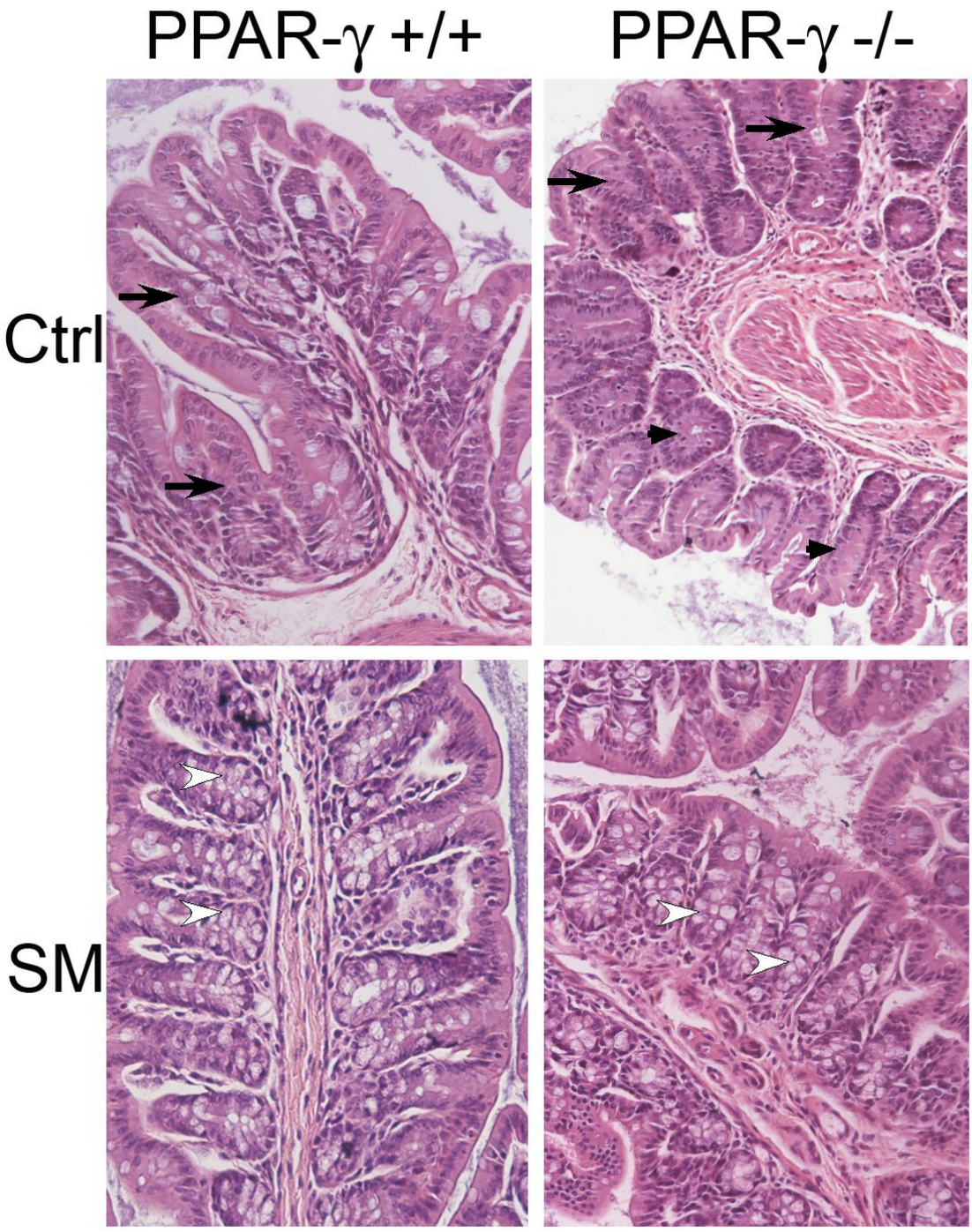

Figure 6. Changes in the colonic architecture, determined by H&E staining in colons from mice treated with a single dose of AOM, 2% DSS for 7 days and maintained on control AIN76A diet alone (upper) or supplemented with 0.1% SM (80 days after the beginning of the study; n=2/17 PPAR-$\gamma^{+/+}$ control/SM fed, n=3/18 PPAR-$\gamma^{-/-}$ control/SM).

Tumor formation

Mice of both genotypes were injected with a single dose of azoxymethane to induce colon tumors. After 80 days, the colons of control mice macroscopically appeared as a solid tumor sheet without discernible single tumors. Therefore, the tumor area rather than tumor number was determined. As shown in Figure 7, the tumor area in the PPAR-$\gamma^{-/-}$ mice was larger than in the PPAR-$\gamma^{+/+}$. There was no effect of the SM supplement in the PPAR-$\gamma^{+/+}$ mice but the tumor area in the PPAR-$\gamma^{-/-}$ mice was significantly smaller in the SM-fed mice (p=0.0251). This confirms our observations that by dietary SM suppressed tumor formation in other models; however, since the number of mice in this study was small, these results need to be confirmed.

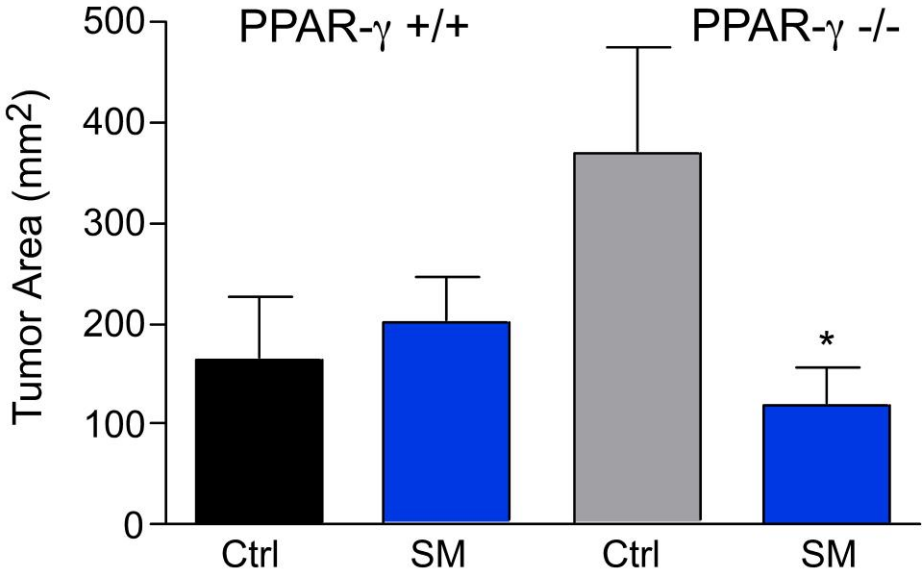

Figure 7. Tumor area PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ mice 80 days after a single AOM injection (□P<.05).

Next, we determined the infiltration of immune cells into the colonic tissue and the progression of the colonic tumors microscopically in H&E stained colonic sections. As shown in Figure 8, there was a substantial lymphoplasmacytic infiltrate in the colonic tissue of control-fed mice of either genotype as determined by histological examination of the H&E stained colonic sections; this was reduced by dietary SM. There was no difference in the number of adenomas per section but fewer areas that had progressed to adenocarcinomas in the PPAR-$\gamma^{+/+}$ mice. Dietary SM had no effect on the number of adenomas but SM-fed mice exhibited fewer adenocarcinomas, confirming the results of our earlier studies in carcinogen-induced colon cancer models and suggesting that the suppression of tumor progression by dietary SM is PPAR-γ independent.

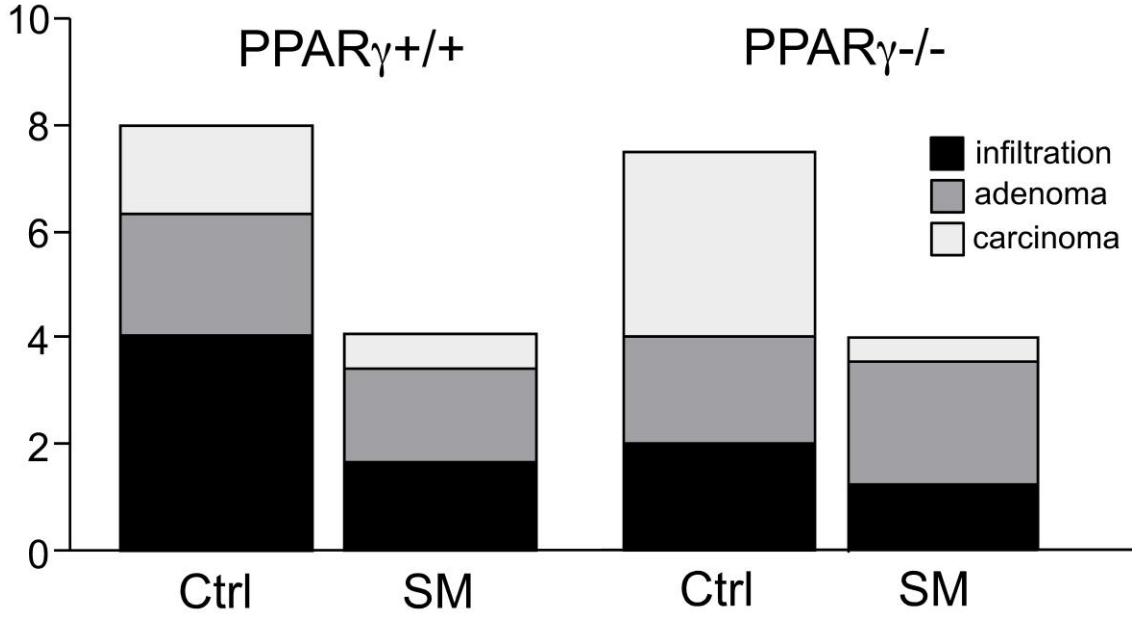

Figure 8. Immune cell infiltration and progression of colon cancer in PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ mice fed AIN76A diet alone or with SM supplements. Means without common letter are different (P<.05).

SM changes immune cell population in mesenteric lymph nodes (MLN)

MLN and spleen were harvested to determine if the effect of SM is restricted locally to the intestinal mucosa and inductive mucosal sites (MLN) or if there is a systemic change in immune cells populating the spleen. While there was no effect on immune cell population in the spleen (data not shown), there was a reduction in the F4/80+ macrophage population in MLN in the SM-fed groups of either genotype, and a trend to reduced number of $CD4^+$ T cells (Fig. 9). These numbers, however, need to be confirmed in a larger group, but suggest that dietary SM can affect immune cell populations in the mesentery lymph nodes.

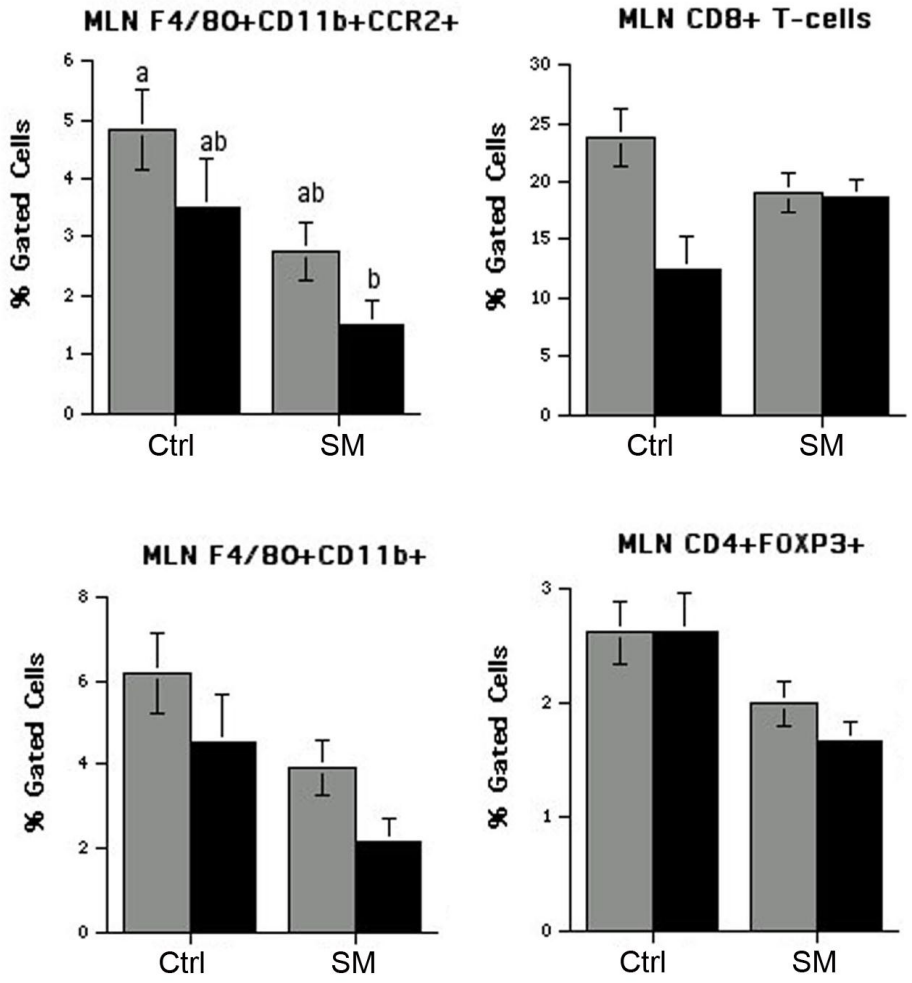

Figure 9. Changes in macrophage (left) and T-cell (right) populations in MLNs of PPAR-$\gamma^{+/+}$ mice (black bars) and PPAR-$\gamma^{-/-}$ mice (grey bars) 80 days after AOM injection. Mice were fed AIN76A diet alone (control) or supplemented with 0.1% SM.

Changes in cytokine and chemokine expression levels by dietary SM

To direct our mechanistic studies of how dietary SM reduced DSS colitis, changes in mRNA expression levels of common pro- and anti-inflammatory and regulatory genes were determined. This is especially important since endogenous sphingolipids have been associated with increased inflammation while dietary SM reduced the severity of DSS-induced inflammation and significantly increased the recovery in an at least partially PPAR-γ dependent manner as shown above. Therefore, mice of both genotypes (n=3 per group) were either treated with DSS or plain water and fed the AIN76A diet alone or supplemented with SM as described under methods. To confirm inflammation after 7 days of treatment, colonic H&E stained sections were evaluated. At the

peak of inflammation, all three PPAR-$\gamma^{+/+}$ mice (Fig. 10, middle panels) showed a greatly compromised colonic tissue architecture when compared to the colons of mice not treated with DSS (upper panels). There was a large influx of immune cells (arrows) and the typical columnar shape of the colonic crypts (arrowheads) was apparent in less than 40% of the observed tissue. In PPAR-$\gamma^{-/-}$ mice, the colonic tissue architecture was completely destroyed; there was a massive influx of immune cells and intact colonic crypts were not found. These changes were attenuated in the SM-fed mice (Fig.10, lower panel). The colons of 2 of 3 PPAR-$\gamma^{+/+}$ mice had recognizable crypt-like colonic architecture in more than 80% of the observed area while this was less in the colons of PPAR-$\gamma^{-/-}$ mice. This confirms our observations that dietary SM reduced the DSS-induced damage to the colonic tissue, and suggests that retaining of some tissue architecture may have been critical for the survival of the SM-fed mice in the study described above. However, a large influx of immune cells, albeit less than in the controls, was also apparent in the SM-fed mice.

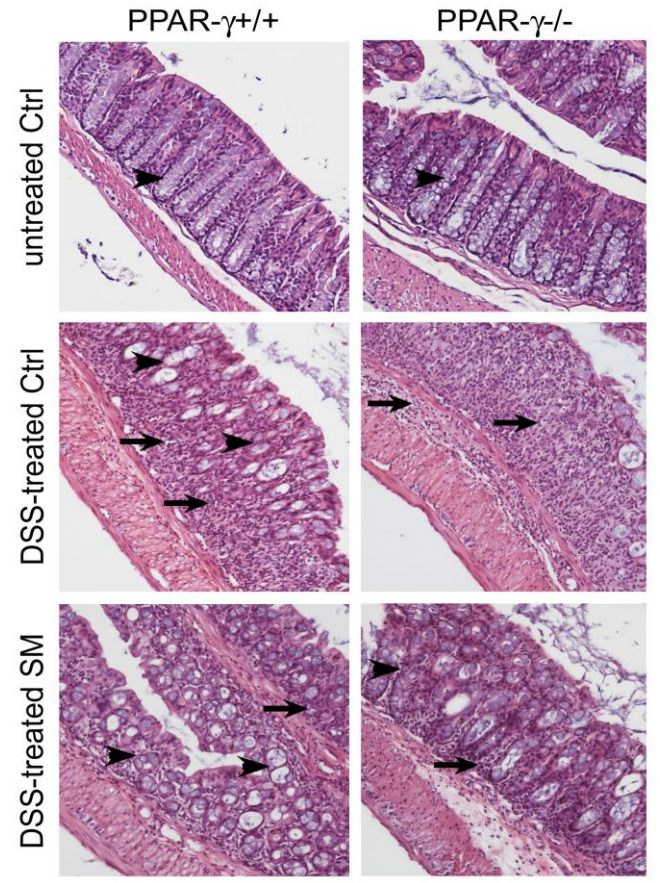

Figure 10. Representative images of H&E-stained colonic sections from untreated PPAR-$\gamma^{+/+}$ or PPAR-$\gamma^{-/-}$ mice (upper), at the peak of intestinal inflammation induced by 2.0% DSS (middle). Mice fed the control AIN76A diet; (lower) representative images from mice fed SM supplements. Arrows: immune cell infiltration; arrowheads: typical colonic columnar tissue architecture.

Since the composition of the inflammatory cells may have been critical for the survival of the SM-fed mice after the DSS challenge, we next began to investigate the pro- and anti-inflammatory gene expression levels in the colons using a real-time RT-PCR array that contained 372 genes. These genes are involved in inflammatory response, autoimmunity, tissue regeneration but some have also been associated with differentiation, proliferation and carcinogenesis and angiogenesis, allowing for the identification of critical signaling events or pathways that are targeted by SM to direct our future mechanistic studies. We first compared gene expression levels in the colons of untreated mice of both genotypes to identify genes specifically regulated by dietary SM and not inherent to the genotype. There were few genes differentially expressed in PPAR-$\gamma^{+/+}$ versus PPAR-$\gamma^{-/-}$ mice (Fig.11, left panel).

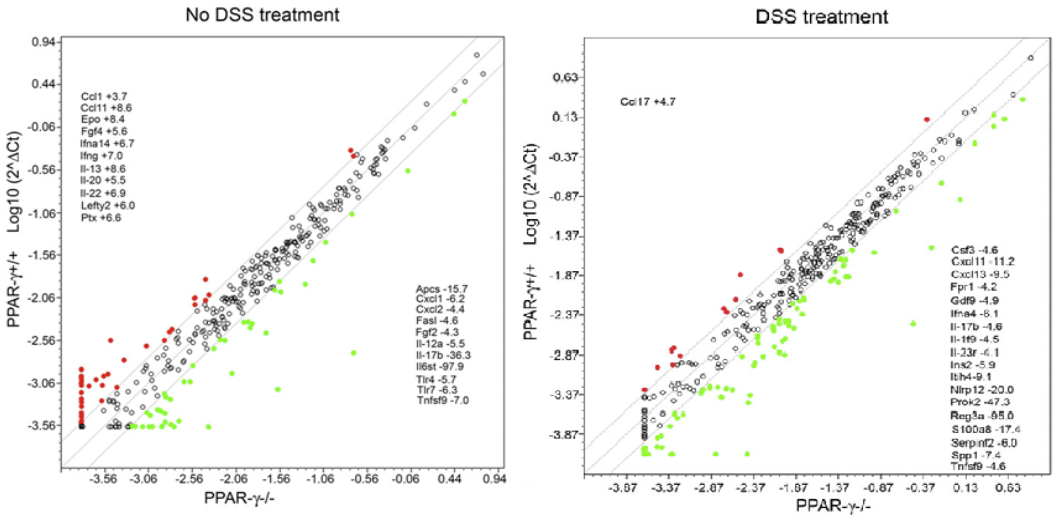

Figure 11. Comparison of pro-inflammatory and anti-inflammatory gene expression levels between PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ mice (left) and after treatment with 2% DSS in the drinking water for 7 days (right) as determined by a real-time PCR array. Green indicates genes down-regulated by more than 2 fold. Red indicates genes up-regulated by more than 2 fold. Black dots indicate no significant changes.

Most of the genes that were more than 2-fold higher expressed in PPAR-$\gamma^{+/+}$ than in PPAR-$\gamma^{-/-}$ mice were cytokines and chemokines; however, these were also the categories of genes with lower expression levels in PPAR-$\gamma^{+/+}$ mice. Cytokine and chemokine expression levels were elevated in both genotypes after DSS treatment (29 genes in PPAR-$\gamma^{+/+}$ and 66 in PPAR-$\gamma^{-/-}$ mice). Most of the inflammatory genes up-regulated in PPAR-$\gamma^{+/+}$ mice were also up-regulated in PPAR-$\gamma^{-/-}$ mice; only a small number of genes were down-regulated by DSS treatment (20 in PPAR-$\gamma^{+/+}$ and 11 in PPAR-$\gamma^{-/-}$ with very little overlap). Comparing the expression levels of these genes after DSS treatment between

the groups, only one gene was higher expressed in the acute inflamed colons of PPAR-$\gamma^{+/+}$ mice by more than 4-fold (Ccl17) while several cyto- and chemokines (Csf3, Cxcl11, Cxcl13, IFNa4) and inflammatory response genes (Prok2, Reg3a, S100a8) showed lower expression levels (Fig.11, right panel). These results demonstrate that PPAR-γ expression in the intestinal epithelium and immune cells greatly affects the inflammatory response to DSS treatment but has a less profound effect in untreated animals.

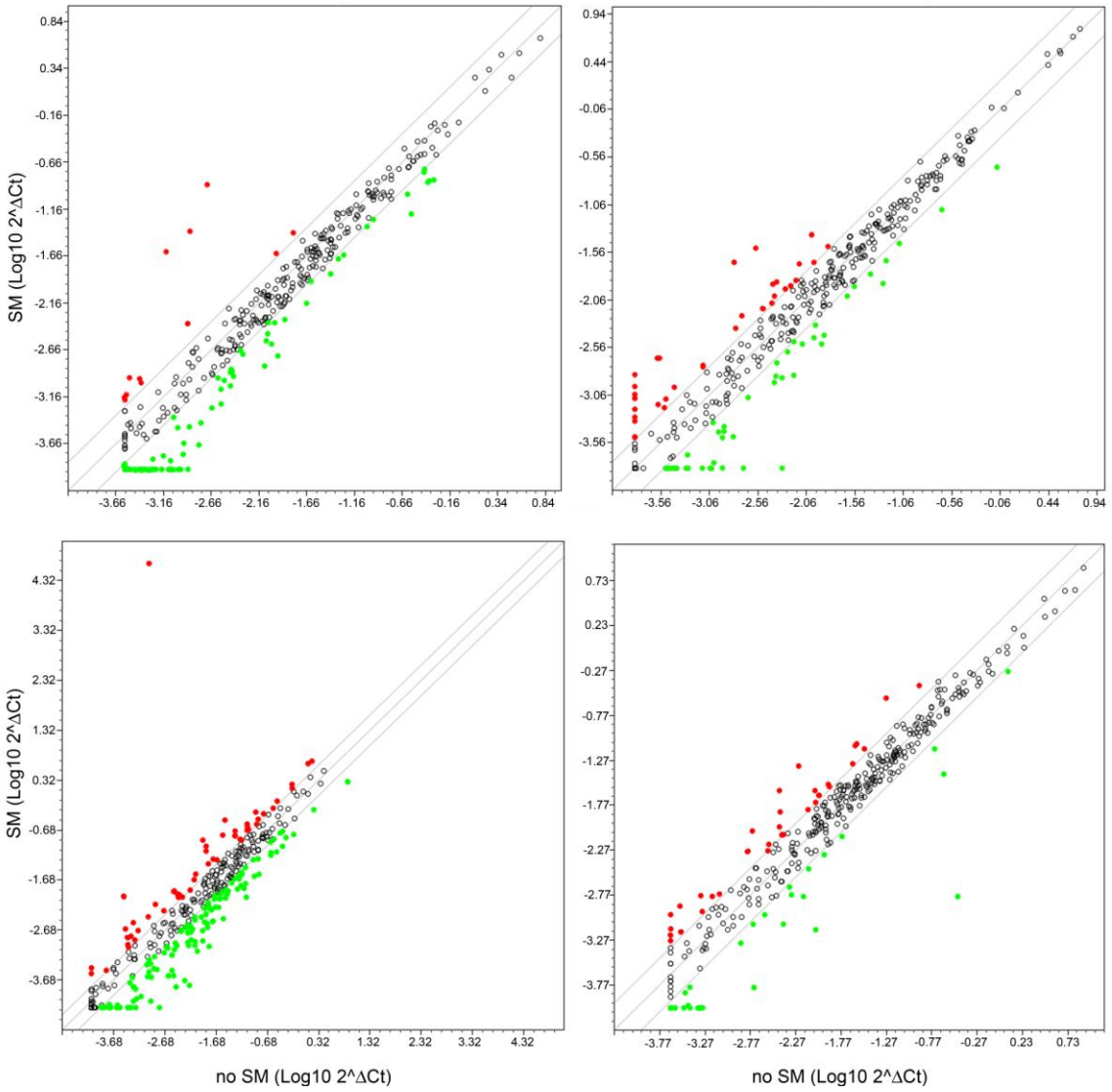

Figure 12. Changes in gene expression levels in the colons of PPAR-$\gamma^{+/+}$ (left) and PPAR-$\gamma^{-/-}$ mice (right) in response to dietary SM. The mice were not treated (upper panels) or treated with 2% DSS for 7 days (lower). Green indicates genes down-regulated by more than 2 fold. Red indicates genes up-regulated by more than 2 fold. Black dots indicate no significant changes.

We next determined which inflammatory genes are targeted by dietary SM and if this is dependent on PPAR-γ expression. Changes in gene expression levels in response to SM supplements were compared in colons from mice either treated with DSS or vehicle alone. As shown in Fig. 12 (upper panels), SM supplements increased several cyto- and chemokines by more than 2-fold in both genotypes. However, more cytokines, cytokine receptors and genes involved in the inflammatory response were down-regulated in PPAR-$\gamma^{+/+}$ mice (genes listed in Table 3). While the categories of genes were similar and several genes were regulated in the same direction as in PPAR-$\gamma^{+/+}$ mice (Table 3, underlined), several genes were changed in the opposite direction in PPAR-$\gamma^{-/-}$ mice (Table 3, in bold).

Table 3: Gene expression changes in PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ mice fed 0.1% SM. Bold genes are those changing in the opposite direction in mice of these genotypes, potentially dependent on PPAR-γ expression. Underlined genes are those changing in the same direction independent of PPAR-γ expression.

Table 3	No DSS treatment			
	PPAR-$\gamma^{+/+}$		PPAR-$\gamma^{-/-}$	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Cytokines	Gdf9, Epo, **Il11**, Il12, Il17, Muc4	Cd70, Cer1, Cmtm1,2, Csf3, Ctf2, Epo, Fasl, **Fgf3**, Fgf4, Fgf5, Fgf6, Fgf8, Gdf2, Gdf3, **Gdf6**, Grem1, Ifna11, Ifna14, IIfna2, **Ifna4**, Ifna9, Ifnab, Ifnb1, **Ifne**, **Ifng**, **Il1f10**, Il1f5, Il1f6, Il1f8, Il1f9, Il12, Il13, Il16, Il17a, Il17c, **Il17d**, Il17f, Il19, Il1b, Il20, Il21, Il22, Il23a, Il31, Il4, Il5, Il6, Il9, Inhba, **Lefty2**, Ltb, Nodal, Nrg1, Prl, Slco1a4, **Spp1**, Thpo, Tnfsf15, Tnfsf9	Cd27, Ebi1, **Fgf3**, **Gdf6**, **Ifna4**, **Ifne**, **Ifng**, Il10, **Il17d**, **Il1f10**, Il24, **Lefty2**, Muc4, **Spp1**	Areg, Clcf1, Csf3, Fasl, Fgf2, Fgf5, Gdf2, Gdf5, Ifna2, Ifna9, **Il11**, Il5, Il17a, Il1a, Ltb, Slurp1, Thpo, Tnf, Tnfsf11, Tnfsf15, Tnfsf8
Cytokine rec.	Il23r, Il6st	Epor, Ifgr1, Il13ra2, Il18r1, Il18rap, Il12rb1, Il12rb, Il1rapl2, Il1rl1, Il1rl2, Il20ra, Il2rb, Il21r, Il3ra, Il5ra, Il7r, Il8ra, Il8rb, Mpl,	Gfra1, Il2ra, Prlr	Il20ra, Il21r, Il21ra, Il22ra2, Il23r
Chemokines	Ccl12, Cxcl2	Ccl28, **Ccl3**, Ccl4, Ccl8, Cxcl15, Cxcl16	Ccl12, **Ccl3**, Cxcl9, Pxmp2	Ccl17, Cxcl1, Cxcl5
Chemokine rec.		Ccr2, **Ccr3**, Ccr5, Ccr9, **Ccrl2**, Cxcr4	Ccrl1, **Ccr3**, **Ccrl2**, Cxcr6	Ccr8, Ccr9, Cxcr5,
Inflamm.respons	Apol7a, Reg3g, Tlr7	Ahsg, Apol8, Bmp7, Fos, Fpr1, Kng1, Mmp25, Nfam1, Nos2, Olr1, Prg2, Prg3, Prok2, Ptgs2, Ptx3	Apol8, C3ar1	Adora1, Ahsg, Cd180, Fos, Ltb4r1, Mmp25, Siglec1, Tnfaip6,
Acute phase		Crp, Fn1,Ins1, Ins2, **Itih4**, **Reg3a**, Sele, Serpinf2	**Itih4**, **Reg3a**, Reg3g, Saa4	Apcs, Crp, F2, Serpina1a

In DSS-treated mice, the response to SM was more pronounced compared to untreated mice (Fig. 12, lower panels; gene list in Table 4). The patterns of genes up-regulated by dietary SM during DSS colitis consisted essentially of: 1) chemokines and chemokine receptors expressed in epithelial cells and involved in cell trafficking to the gut (Ccl19, Ccl11, Ccl20, Cxcl9, Cxcl11), 2) genes involved in CD4+ T cell differentiation and fate, including Th1 (IFN-g), Th17 (IL-17 and IL-23), Th2 (IL-4, IL-13 and IL-13R) and Treg (IL-10R). CD4+ naïve T helper cells respond to antigenic stimulation by differentiating into one of, at least, four known fates: Th1, Th2, Th17, and regulatory T cell (Treg). Each of these phenotypes is characterized by their function as well as the cytokines being secreted. The majority of genes and pathways upregulated by SM (Th1, Th17, gut homing chemokines and chemokine receptors) are immunostimulatory and pro-inflammatory which is in agreement with results from previous studies demonstrating the pro-inflammatory effect of sphingolipid metabolites. However, the up-regulation of genes in the Th2 differentiation pathway (IL-4, IL-13 and IL-13R) and Treg-related genes such as IL-10R is in line with an anti-inflammatory effect and are modulated by SM in a PPAR-γ-dependent manner. Furthermore, dietary SM suppressed the expression of other regulatory molecules such as members of the TGF superfamily that mediate apoptosis (Fasl), inflammatory signaling intermediates such as Myd88, DOCK2, and transcriptional regulators such as Nfe2l1, NfrkB and Stat3 also in a PPAR-γ dependent manner, thereby favoring an anti-inflammatory environment.

Several SM-regulated genes have also been associated with tissue protection or regeneration (Fasl [99], Serpina3n, Spp1 [50], Trap1[51]) or have a potential effect on colon cancer (ErbB2, Slurp1 [52], Lta [53], Ltb [54], NfrkB [55], Serpina1 [56], Tlr5 [57]), suggesting that in addition to modifying the pro-and anti-inflammatory balance towards an anti-inflammatory environment, SM also may affect tumor initiation, and promotion.

Table 4: Gene expression changes in PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ mice treated with 2.0% DSS and supplemented with 0.1% dietary SM. Bold genes are changing in the opposite direction in mice of these genotypes, potentially dependent on PPAR-γ expression. Underlined genes are those genes changing in the same direction suggesting regulation by SM independent of PPAR-γ expression.

Table 4	DSS treatment			
	PPAR-$\gamma^{+/+}$		PPAR-$\gamma^{-/-}$	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Cytokines	Figf, Gdf6, **Gdf9**, Ifng, **Il1f6**, Il1f10, Il1f6, Il1f9, **Il17b**, Il1a, Il1b, Il23b, Il27, Il4, Il7, Mif, Nrg1, Pglyrp1, Tnfsf14,Tnfsf9, Trap1	Bmp2, Bmp3, Cast, Clcf1, Csf1, Csf3, Ctf1, Erbb2, Fasl, Fgf3, Flt3l, Gdf1,Gdf5, Gdf7, Glmn, Grem1, 2, Grn, Ifna2, Ifna9, Ifnk, Il11, Il15, Il16, Il17a, Il7c, Il17d, Il17f, Il19, Il24, Il3, Inha, Inhba, Inhbb, Pdgfb, Pdgfc, Ptn, Scg2, Slco1a4, Spred1, Srgap1, Tnfsf13, **Tnfsf13b**, **Tnfsf4**, Tymp, Vegfa, Vgefb, Yars	Cd70, Fgf7, Ifna2, Ifnk, Il1a, Il1b, Il1f9, Il10, Il6, Inhba, Lefty2, Osm, Slurp1, Tnf, **Tnfsf4**, Tnfsf11, **Tnfsf13b**	Cer1, Cmtm1, Crp, Il17a, Il17b, Il19, Epo, Fgf3, Fgf4,Fgf5, Fgf6, Fgf8, Gdf3, **Gdf9**, Infa11, Ifna14, Ifna4, Ifna9, Ifnb1, Ifne, Il12b, Il13, Il16, Il17a, **Il17b**, Il19, Il1f5, **Il1f6**, Il1f8, Il20, Il21, Il3, Il31, Il5, Il9, Mstn, Nodal, Prl, Slco1a4, Tnfsf15, Tnfsf18, Tymp
Cytokine receptors	Il10rb, Il1r2, Il31ra, Il2ra	Cfra2, Cntfr, Csf2ra, Epor, Ifngr2, Il11ra1, Il12rb1, Il15ra, Il1rl2, Il21r, Il2rb, **Il5ra**, Il17r, Il17rb, Il18r1, Il18rap, Il1rapl2, Il20ra, Il21r, Il22ra1, Il28ra, Il7r, Il9r, Lifr, Ttn	Il1rl1, Il2ra, Il31ra, **Il5ra**	Il6st, Il1rapl2, Il23r, Il8ra, Mpl
Chemokines	Ccl19, Ccl3, Ccl4, Ccl8, Cxcl10,Cxcl11Cxcl3, Cxcl2, Cxcl5, Cxcl9	Ccl1, Ccl17, Ccl22, Ccl24, Ccl25, Cyp26b, Pf4, Ppbp, Pxmp2	Ccl3, Ccl4, Cxcl11, Cxcl2	Ccl24, Cxcl13, Cxcl15,
Chemokine rec.	Cxcr3	Ccr10, Ccr3, Ccr4, Ccr8, Ccr9, Ccrl1, Cx3cr1, Cxcr4, Cxcr5, Cxcr6	Ccr7, Ccrl2	Ccr8, Ccrl1
Inflamm. response	Aif1, Cd14, Cd40, Itgb2, Ly86, Nmi, Pla2g7,Reg3g, S100a8, Spp1, Tacr1	Adora1, C3ar1, Dock2,F11r, Gpr68, Hdac 4,5,9, Hrh1, Lta, Ltb, Ltb4r1, Mmp25, Myd88, Nfam1, Nfatc4, Nfe2l1, Nfrkb,Nfx1, Nos2, Prdx5, Ptafr, Reg3a, Syk, Tlr2, Tlr5, Vsp45	Ptx3, Sele, Tnfaip6	Ahsg, Apol8, Kng1, Prg2, Prg3, Ptpra, Reg3a, Spaca3
Acute phase		F2, Fn1, Lbp, Serpina1a, Serpinf2, Stat3	F8, Saa4	Ins1, Ins2, Itih4, Reg3a, Serpinf2

Effect of sphingolipid metabolite sphingosine on PPAR-γ transcriptional activity of macrophages

To support our findings that SM affects anti-inflammatory events in a PPAR-γ dependent manner, we determined whether sphingolipid metabolites activate PPAR-γ in immune cells we treated RAW 264.7 macrophages with sphingosine, the metabolite also generated in the intestinal tract after oral administration of complex sphingolipids and presumably responsible for the suppression of carcinogen-induced colon cancer [10]. As shown in Figure 13, sphingosine modestly but significantly activated PPAR-γ transcriptional activity in a concentration-dependent manner.

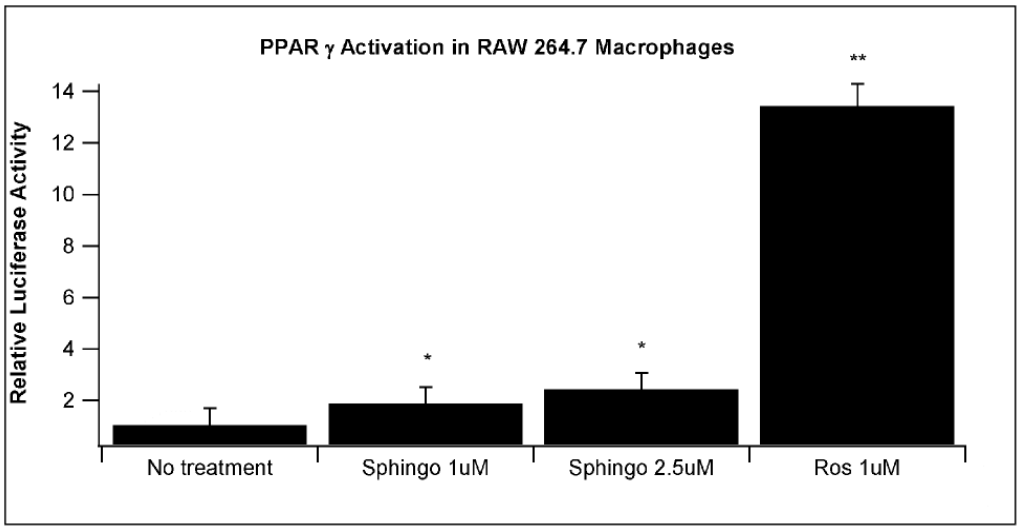

Figure 13: Non-toxic concentrations of sphingosine activate PPAR-γ transcriptional activity in macrophages, determined by luciferase promoter activity assay, compared to the PPAR-γ agonist rosilitazone.
*p<0.05; **p<0.01

Discussion

Endogenous sphingolipid metabolites have been implicated in the induction of inflammatory responses both *in vivo and in vitro*. The present studies show that dietary SM reduced DSS-induced inflammation, accelerated disease recovery, and reduced AOM-induced colon tumors. These findings were associated with an improved preservation of the colonic microscopic architecture during acute inflammation in both PPAR-$\gamma^{+/+}$ and PPAR-$\gamma^{-/-}$ mice, and an increased survival of the mice. Interestingly, the expression of PPAR-γ in immune and epithelial cells enhanced the anti-inflammatory effects of dietary SM while its anti-carcinogenic actions appear to be mostly independent of the mouse genotype.

Complex sphingolipids are digested to the bioactive metabolites ceramide and sphingosine [14,15,17,58]. These metabolites are likely the mediators of the observed tumor suppression since all complex sphingolipids tested (SM, glucosylceramide, lactosylceramide, GM1, GD3) [12,100] showed the same effect independent of the headgroup; additional effects of other molecular structures such as the fatty acid and the choline headgroup, however, cannot be ruled out. Many recent studies have focused on the association of ceramide and the initiation or perpetuation of inflammation. In macrophages, an increase in ceramide levels was associated with a higher Cox-2 expression [21] via NF-kB activation [88]. Exogenous ceramide mimicked these events in murine macrophages and it is thought that a ceramide-mediated suppression of PPAR-γ prevents suppression of NF-kB activity (see recent review [101]). Other studies suggest a key role of ceramide in the increased secretion of pro-inflammatory cytokines via NF-kB activation in adipose tissue-associated macrophages [88], and Cox-2, cPLA2 and 12-LOX in primary astrocytes [64]. Another sphingolipid metabolite, S1P, generated by SK1, is a regulator of cell growth and a potent immune stimulator. The immune suppressor FTY720 activates specifically S1P receptor1 and mediates sequestration of circulating lymphocytes within secondary lymphoid tissues, thereby decreasing CD4+ T-cells in the colonic lamina propria, and homing of circulating lymphocytes into Peyer's patches and MLN [65]. In DSS-induced colitis, FTY720 attenuated infiltration of CD4+ T-cells into the colonic lamina propria, and the disease severity [66].

Based on our gene expression analyses, we observed that SM induced a pro-inflammatory response that was paralleled by an anti-inflammatory or counter-regulatory immune response characterized by up-regulation of genes associated with anti-inflammatory phenotypes of $CD4^+$ T cells (i.e., Th2 and Treg) as well as suppression of inflammatory chemokines and their receptors. Recent evidence supports the existence of cross-talk between these pathways and demonstrates specificity and plasticity in CD4+ T cell fate determination [67,68]. Indeed, our data provide evidence that dietary SM supplements trigger the expression of pro-inflammatory and immunostimulatory genes consistent with Th1 and Th17 effector and pro-inflammatory responses while at the same time

up-regulates genes involved in a counter-regulatory immune response that may inhibit pro-inflammatory genes. More specifically, dietary SM up-regulated the expression of genes involved in the differentiation of CD4+ T cells toward Th1 (IFN-γ) and Th17 (IL-17 and IL-23) effector phenotypes involved in cellular responses against pathogens and autoimmunity such as inflammatory bowel disease (IBD), rheumatoid arthritis, and multiple sclerosis [68,69]. This predominant Th1 and Th17-related gene expression pattern observed in SM-fed mice was consistent with increased expression of chemokines involved in leukocyte recruitment into the colonic mucosa (i.e., Ccl19, Ccl11, Ccl20, Cxcl9, Cxcl11). Surprisingly, this inflammatory gene expression pattern was associated with decreased colonic inflammatory lesions, increased survival and improved clinical disease. This apparent contradiction may be explained by the existence of a regulatory response that may have paralleled the effector/pro-inflammatory gene expression pattern described above. The parallel induction of effector and regulatory responses is typical in mucosal sites during inflammation or infection [60,70]. More specifically, SM-fed mice had increased expression of Th2 genes involved in humoral immune responses (IL-4, IL-13 and IL-13R) and a Treg-related gene (IL-10R) involved anti-inflammatory responses. Interestingly, the up-regulation of Th2 and Treg genes was modulated by dietary SM in a PPAR-γ -dependent manner. The better understanding of the potential regulatory actions of dietary components of $CD4^+$ T-cell differentiation *in vivo* is important and further investigations of the effects of sphingolipids in CD4+ T-cell differentiation networks and immune function are currently ongoing in our laboratory.

Dietary SM administration decreased the number of macrophages in the MLN and lymphoplasmacytic infiltration in colons of mice with inflammation-induced colorectal cancer. These immunological changes were associated with a faster recovery from DSS-induced inflammation PPAR-$\gamma^{+/+}$ but not in PPAR-$\gamma^{-/-}$ mice. Macrophages, T cells and intestinal epithelial cells are the primary contributors to intestinal inflammation during inflammatory bowel disease and express PPAR-γ. We hypothesized that the anti-inflammatory effects of SM were mediated through a PPAR-γ -dependent mechanism. We provide *in vitro* evidence demonstrating that SM increased PPAR-γ reporter activity in RAW 264.7 macrophages. These *in vitro* findings are in line with the PPAR-γ-dependent induction of Th2- and Treg-related genes in colons of SM-fed mice with DSS colitis. This pattern is likely to be associated with an increased presence of M2 alternatively activated macrophages, a cell type that is highly responsive to M2 fate determination following activation of PPAR-γ and d [6,72,102]. In this regard, we found a decrease in F4/80CD11bCCR2+ macrophages in MLN of SM-fed mice, a cell phenotype that is pro-inflammatory but also responsive to PPAR-γ [103].

The model of inflammation-induced colorectal cancer utilized followed a biphasic response pattern in which the first peak corresponded to acute inflammation and a second peak that coincided with colonic tumorigenesis. Even though the effect of dietary SM on the first inflammatory peak follows a pattern that depends partly on PPAR-γ expression in epithelial and immune cells, the

improvement in tumor area as well as adenoma and carcinoma formation observed in SM-fed mice at the end of the study appears to be independent of PPAR-γ expression in immune and epithelial cells. However, several genes that may promote cancer (ErbB2, Ltb, NfrkB) or exert anti-tumor effects (Gdf9; [104]) were down- or up-regulated, respectively, by dietary SM only in PPAR-γ expressing mice, suggesting that PPAR-γ may also play a protective role in the suppression of colon cancer by dietary SM. Our results also suggest that in addition to the immune cells, epithelial cells and, perhaps, their interactions may be targets of dietary SM in the suppression of inflammation. These findings will direct our further mechanistic studies.

Conclusion

The results from these studies demonstrate the ability of dietary SM to suppress colonic inflammation and inflammation-driven colorectal cancer, confirming our hypothesis that dietary SM in contrast to endogenous sphingolipids elicit an anti-inflammatory effect. The observed changes in both pro- and anti-inflammatory genes during acute inflammation generated an overall anti-inflammatory and tissue-preserving environment and were at least partially dependent on PPAR-γ expression, and suggest an importance of PPAR-γ expression in T cells. Thus, further studies are needed to dissect the stage-specific PPAR-γ-dependent and independent effects of SM and to further characterize the immune modulatory actions of dietary SM in CD4+ T cells.

Chapter 4

Importance of peroxisome proliferator-activated receptor γ expression in CD4+ T cells for an anti-inflammatory effect of dietary sphingomyelin.

Abstract

Sphingolipid metabolites have been shown to play a role in the initiation and perpetuation of inflammatory responses. However, dietary sphingomyelin reduced intestinal inflammation in a partially PPAR-γ dependent manner. In the present study, we investigated the suppression of DSS-induced colitis by dietary SM in mice that lack functional PPAR-γ specifically in CD4+ T-cells. Dietary SM significantly decreased disease activity and colonic inflammation in mice of both genotypes but more efficiently in mice expressing PPAR-γ, indicating that the anti-inflammatory SM effect is dependent of PPAR-γ expression. Using a real-time polymerase chain reaction array, we confirmed the anti-inflammatory effects of dietary SM, shown in DAI and histology scores. Furthermore, real-time polymerase chain reaction array showed effects of SM to be partially dependent on PPAR-γ expression in the T-cells. In order to direct mechanistic studies of how PPAR-γ expression is involved in the reduction seen by dietary SM on DSS colitis, we next ran a quantitative confirmatory real-time polymerase chain reaction on 7 genes believed to be involved in this response (IL-6, IL5rα, IL-17c. IL-1β, TNF-α, Spp1, and Gdf5), IL-6 and TNF-α were confirmed. In conclusion, dietary SM modulated inflammatory responses at the early stages of the disease by activating PPAR-γ, but its anti-inflammatory effects followed a PPAR-γ independent pattern. Furthermore, we concluded IL-6 and TNF-α are actively involved in this response in a PPAR-γ dependent manner.

Introduction

Data from chapter 3 (specific aim 1) showed the suppression of DSS-colitis by dietary SM, at least partially dependent on PPAR-γ expression. The genes in the TH2 differentiation pathways (IL-4, IL-13, and IL13R) and Treg-related genes such as IL-10R were up-regulated only in PPAR-γ expressing mice [31]. These genes are involved in the differentiation of CD4+ T-Cells, particularly their cellular responses against pathogens and autoimmunity such as IBD. A study conducted by the Bassaganya lab investigated the mechanism by which the deletion of PPAR-γ from T-cells changes the colonic gene expression and the severity of experimental IBD. They found that PPAR-γ deficiency in the T-cells accelerated the onset of the disease as well as up-regulated several inflammatory cytokines such as IL-6 and IL-1B [43]. Furthermore, studies have shown PPAR-γ expression to be important in the function of CD4+ regulatory T-cells in activation of Treg related genes [59, 60, 61]. With this information, we developed the following study using mice with the PPAR-γ gene deletion specifically in T-cells in order to reveal more specific pathways regulated by PPAR-γ and SM, regarding the anti-inflammatory response seen in chapter 3. A better understanding of the potential regulatory actions of dietary components on CD4+ T-cells and, specifically on their PPAR-γ expression in vivo would be important towards finding therapies for intestinal inflammation in order to suppress inflammation-driven colon cancer. Based on the data from chapter 3, **we hypothesize that PPAR-γ expression in T-cells is critical for an anti-inflammatory response of mice to SM treatments in DSS-induced colitis**. The identification of genes that change in response to SM in DSS colitis in PPAR-γ dependent and independent manner will direct our future mechanistic studies and support the establishment of these changes as molecular markers for SM efficacy in future colon cancer prevention trials.

Methods and Materials

Mice

Since whole body deletion of PPAR-γ is lethal by day E10 [95], tissue-specific PPAR-γ null mice were generated as previously described [96,97]. PPAR-γ fl/fl mice (carrying PPAR-γ gene flanked with two loxP boxes on exon 2 that are recognized by a transgenic recombinase) were cross-bred with transgenic mice carrying the Cre-gene under control a promoter which express a transgenic recombinase only in T-cells [5]. PPAR-γ fl/fl; Cre^{+} (named T-PPAR-$\gamma^{-/-}$ throughout the manuscript) express a truncated form of mRNA transcript and do not express PPAR-γ protein in the T-cells as confirmed by real-time RT-PCR and Western Blotting. These mice are viable without any known problems. Both female and male mice were used in the present studies. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Virginia Polytechnic Institute and State University and met requirements of the Public Health Service/National Institutes of Health and the Animal Welfare Act.

Diets and Treatments

T-PPAR-$\gamma^{+/+}$ and T-PPAR-$\gamma^{-/-}$ mice were randomly assigned into either the control (18 mice) or the SM-supplemented diet (19 mice). The mice in all four groups were fed the semi-purified sphingolipid-free AIN76A diet [98] throughout the study. The sphingolipid groups received 1g/kg SM, (0.1% by weight) (Avanti, Alabaster, AL) in the diet. The SM was mixed thoroughly into small batches of diet using a mixer and stored at 4˚C until use. The milk SM contains mostly saturated fatty acids [38] and is stable under these conditions. The amount of SM used does not add significant amounts of caloric value to the diet and has demonstrated to have no side effects in either of our studies or those of other groups; even 1% was tolerated well over 2 generations of rats [83]. Amounts up to 0.1% have been used in our previous studies and have suppressed carcinogen-induced colon cancer by up to 80% [12,13,38]. After 7 days on the experimental diets, inflammation was induced for one week (day 14-21) by adding 2.0% DSS (molecular weight 36,000 to 44,000 g/L; ICN Biomedicals, Aurora, OH) to the drinking water. During this time the mice were weighed on a daily basis and examined by blinded observers for clinical signs of disease associated with colitis (i.e., perianal soiling, rectal bleeding, diarrhea, and piloerection). The DAI consisted of a scoring for diarrhea and lethargy (0–3), whereas rectal bleeding consisted of a visual observation of blood in feces and the perianal area (0–4). On day 21 of the study, the mice were euthanized via CO_2 asphyxiation and colons were removed.

Table 5. DAI scores and symptoms.

DAI Score	Symptoms
0-3	Perianal soiling, diarrhea, piloerection, swelling, redness, visible inflammation
0-4	Perianal soiling, diarrhea, piloerection, swelling, visible inflammation AND Rectal bleeding visualized in the feces and/or perianal area.

Table 6. Experimental time line starting at day 0 and ending on day 21. Day 0-7: mice acclimated to surroundings. Day 7: SM supplementation began and was maintained until day 21. Day 14: DSS treatment began as well as DAI scoring, both maintained until day 21.

Table 7. Illustrates the 4 groups of mice, their genotype, supplementation, diet, treatment, and number of mice in each group.

Group	Genotype	Supplement	Diet	Treatment	N
1	T- PPAR-$\gamma^{-/-}$	SM	AIN76A	DSS	9
2	T- PPAR-$\gamma^{+/+}$	SM	AIN76A	DSS	10
3	T- PPAR-$\gamma^{-/-}$	None	AIN76A	DSS	8
4	T- PPAR-$\gamma^{+/+}$	None	AIN76A	DSS	10

Histopathology

The excised colons were fixed in 10% buffered neutral formalin, embedded in paraffin, and then sectioned (5 µm) and stained with H&E stain for histological examination. Tissue slides were examined in a Nikon 80*i* eclipse epifluorescence microscope, equipped with DIC, color and monochromatic digital cameras. Images were digitally captured using the NIS software (Nikon) and processed in Adobe Photoshop Elements 2.0 (Adobe Systems Inc., San Jose, CA).

Cytokine Real-time PCR array

For the initial determination of changes in inflammatory responses that may be associated with the diet and genotype, T-PPAR-$\gamma^{+/+}$ (n= 20) and T-PPAR-$\gamma^{-/-}$ (C=17) mice were placed on the AIN76A diet with or without the 0.1% SM throughout the study (n= 10 for each genotype). After one week, 2% DSS was added to the drinking water of the animals of each DSS treated group for 7 days during which they were weighed and scored daily (n=8 for T-PPAR-$\gamma^{-/-}$ group and n=9 for T-PPAR-$\gamma^{+/+}$ group). Then, all mice were euthanized by CO_2 asphyxiation followed by secondary thoracotomy, the colons were removed, rinsed and a portion of each colon was fixed in 10% neutral buffered formaldehyde, embedded in paraffin and sectioned for H&E staining for determination of tissue architecture or placed into RNAlater. To extract RNA, the remaining colonic tissue was cut into 15-25mg pieces and rinsed with RNAse free water to remove the RNAlater. Then the tissue was snap-frozen in liquid nitrogen, homogenized, and RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and stored at -80ºC until analysis.

RNA purity was assured by assessing the A260:A230 ratio and A260:A280 ratio (NanoDrop, Thermo Fisher) and the ribosomal RNA integrity using the Experion™ Nano LabChip (Biorad). In each group, 2ng of RNA per mouse (n=3 in each group) were pooled for cDNA synthesis using the RT^2 First Strand Kit (SABiosciences). Real-time RT-PCR was performed in a RNA microarray format using the Inflammatory Response and Autoimmunity RT^2 Profiler PCR Array (PAMM-3803 SABiosciences) according to the manufacturer's instructions on an ABI 7900HT PCR machine (Applied Biosystems, Foster City, CA). Data analysis was performed using the SABiosciences analyzing tools provided online. Included in the arrays were controls for genomic DNA contamination, reverse transcription and positive PCR controls and 5 housekeeping genes (ß-actin, GAPDH, Hsp90ab1, Hprt1, Gusb). Data presented are the fold differences between the 2^ DCT values for genes that were at least 2-fold up- or down-regulated in the treatment versus the appropriate control

group, or the same treatment in different genotypes. The array was performed with pooled samples of equal amounts of mRNA from all mice in the group.

Quantitative Real-Time PCR

In order to confirm results from the PAMM 3803 PCR Array, qRT-PCR was performed on 7 specific genes thought to be critically involved in the response caused by the SM treatment that was dependent on PPAR- γ expression in $CD4^+$ T-Cells. The following genes were designed by Beacon Designer (Biorad) based on the data analysis of the PCR array: IL-6, IL-17C, SPP1, GDF5, IL-5Rα, TNF-α, IL-1ß. RNA extracted from original excised colonic tissue was used to synthesize cDNA with a RT2 First Strand Kit (Promega), using L19 as the housekeeping gene. cDNA was then put into a master mix and loaded into a 384 well PCR plate. The mastermix contained: 7.5ul 2x mastermix, (Bioline) 0.25 ul reference dye (abcam), 1.2uM primer mix 0.6uM reverse + 0.6uM forward (Integrated Device Technology) 0.05 purified H_2O, and 10ng of cDNA concentration of 10ng/ul. The plate was then read according to the manufacturer's instructions on an ABI 7900HT PCR machine (Applied Biosystems, Foster City, CA). Data analysis was done independently, normalizing to the L19 housekeeping gene and reporting ΔCT-values.

Statistics

To determine the statistical significance of the model, ANOVA with a Turkey test was performed using the instat® software (Graphpad, Inc.). Data were expressed as the means ± standard error of the mean. Statistical significance was assessed at a *P* value of < 0.05.

Results

Effect of dietary sphingomyelin on DSS-induced inflammation

DSS treatment of mice expressing (T-PPAR-$\gamma^{+/+}$) or lacking PPAR-γ (T-PPAR-$\gamma^{-/-}$) in CD4+ T-cells caused physiological damage indicated by the increased DAI without a difference between the genotypes as shown in Figure 14. SM treatment significantly reduced the DAI in the C57BL/6 mice by 89% ($p<0.05$) while the reduction in the T-PPAR$^{-/-}$ mice was only 44% ($p<0.05$). This is significantly less than the response to SM in C57BL/6 mice ($p<0.05$).

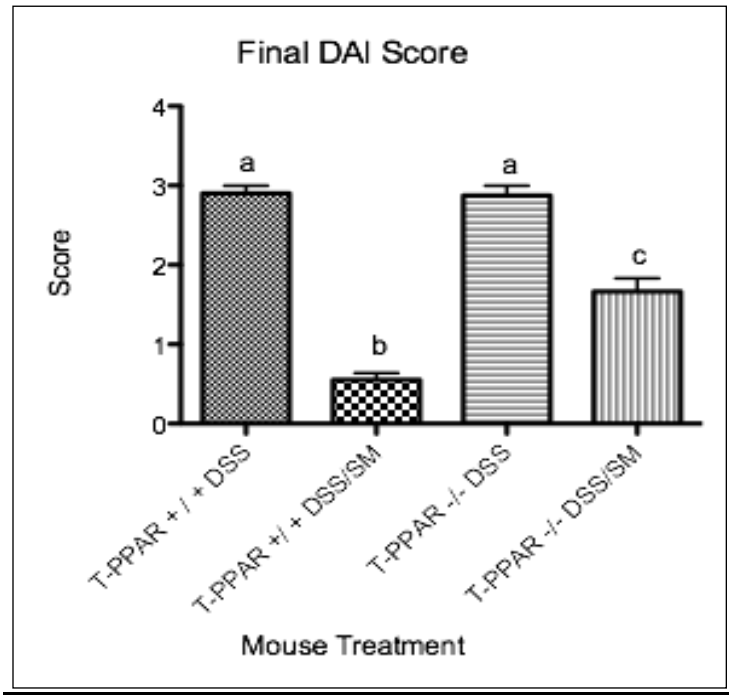

Figure 14. Final DAI scores at day 7 for each mouse group. Letters indicate statistically significant differences by treatment and/or genotype ($P < 0.05$)

These results suggest that the initial inflammatory response to the DSS treatment is not dependent on PPAR-γ expression, or the expression of PPAR-γ cannot rescue the intestinal tissue from DSS-induced inflammation; however, the anti-inflammatory and tissue-preserving effect of SM is at least partially dependent on PPAR-γ expression in the T-cells.

The treatment of DSS increases the DAI in a time-dependent manner, as shown in figure 15. The addition of SM to the diet caused a significant reduction in the DAI score by reducing both severity of the colitis and postponing the onset of the colitis. This was significantly more pronounced in the control mice, indicating that T-cell PPAR-γ expression is required for the anti-inflammatory effect of SM. As can be seen in figure 15, T-cell PPAR-γ expression alone does not have an effect on the onset of experimental colitis but is critical for the protective effect of SM treatment.

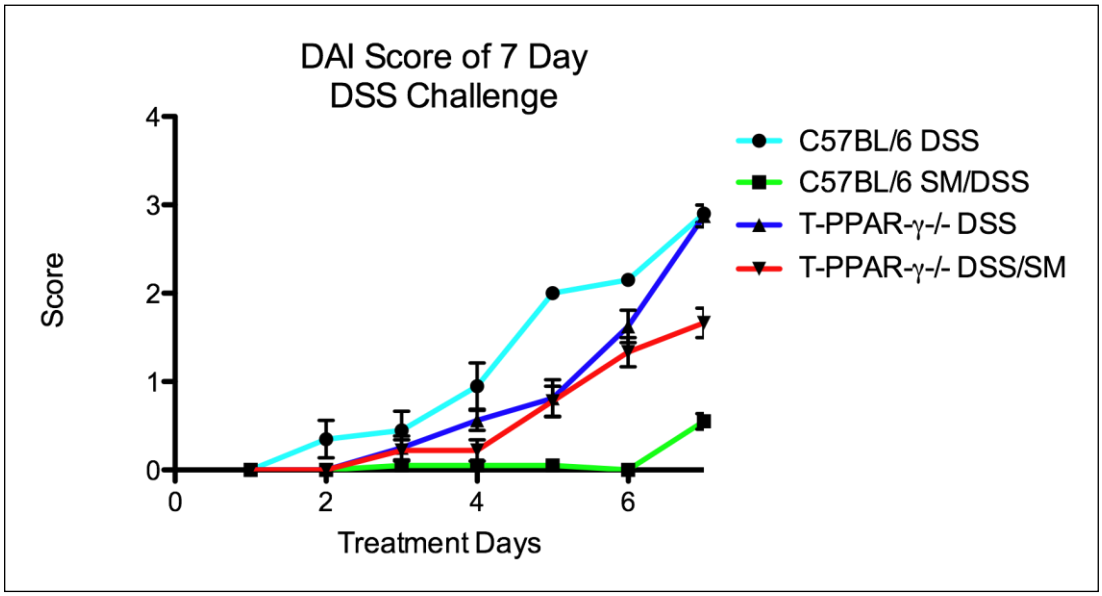

Figure 15. Time line of DAI score in response to DSS treatment from day 14 to day 21. Colors represent different supplementations and/or genotypes. Error bars have been added in order to show variation among DAI scores in mice.

The colons from these mice were processed for histology and gene analysis to confirm the DAI scores are associated with colonic inflammation, as well as determine the molecular response of the colonic tissue to the DSS challenge. Figure 16 shows the H&E stained colons after termination of the study on day 7. Comparison in colonic architecture between the T-PPAR-$\gamma^{+/+}$ and T-PPAR-$\gamma^{-/-}$ after DSS treatment mice shows that the architecture is better preserved in the control mice, expressing PPAR-γ in the T-cells (arrow heads, top panels). The tissue architecture is also better preserved in the SM treated mice (arrowheads, bottom panels). DSS treatment caused a severe influx of immune cells without appreciable differences between the genotype. SM reduced the influx of immune cells (lower panels, arrows) in both genotypes but more so in the T-PPAR-$\gamma^{+/+}$ mice (figure 16, arrow, bottom panels). The decrease in immune cell influx in the SM treated T-PPAR-$\gamma^{+/+}$ mice suggests that lack of PPAR-γ expression in the T-cells may prevent the T-cell PPAR-γ mediated anti-inflammatory response [62]. Furthermore, by reducing the inflammatory response (figure 14 & 15) the dietary SM helps retain proper columnar architecture within

the colonic epithelia. This correlates well with the significant reduction of DAI by SM in the T-PPAR-$\gamma^{+/+}$ mice, and less effect in the T-PPAR-γ mice.

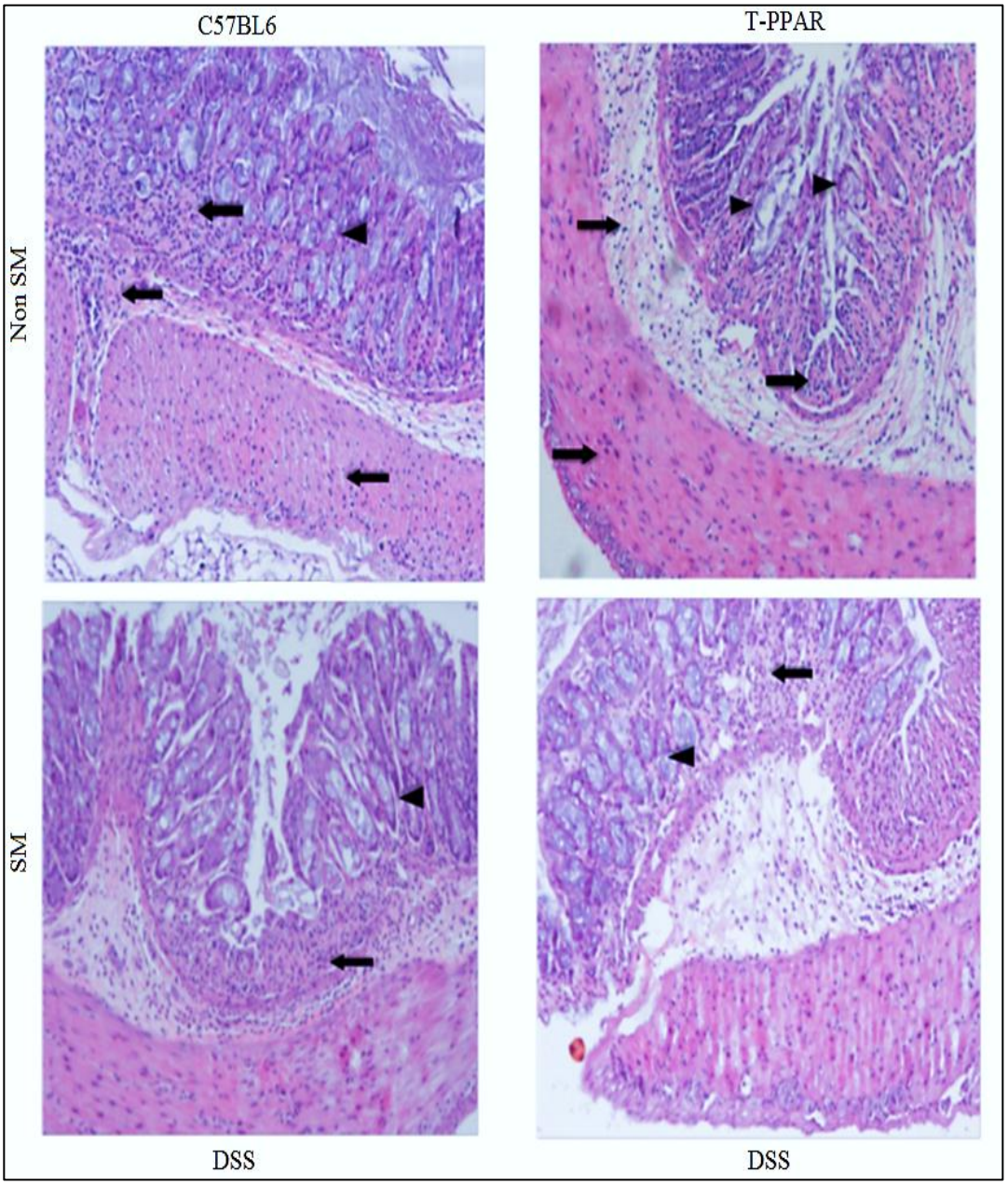

Figure 16. Representative images of H&E-stained colonic sections from treated C57BL6 mice and T-PPAR-$\gamma^{-/-}$ mice, at the peak of intestinal inflammation induced by 2.0% DSS (upper panels). Untreated C57BL6 and T-PPAR-$\gamma^{-/-}$ mice treated with 2.0% DSS and fed the 0.1% SM supplementation (bottom). **Arrows:** immune cell infiltration; **Arrowheads:** colonic columnar tissue

Changes in cytokine and chemokine expression levels by dietary SM

Shown in figure 17 are the differentially expressed genes in the DSS-treated T-PPAR-$\gamma^{+/+}$ and T-PPAR-$\gamma^{-/-}$ mice to determine which genes were involved in the inflammatory response that was dependent on T-cell PPAR-γ expression. Figure 17 reveals that after DSS treatment, a majority of the genes were not differentially expressed; however, 59 genes were down-regulated while 15 genes were up-regulated in the T-PPAR-$\gamma^{-/-}$ mice, suggesting that the lack of PPAR-γ expression in the T-cells suppresses the inflammatory response to DSS-induced colitis. The specific genes identified here were categorized as shown in Table 5A.

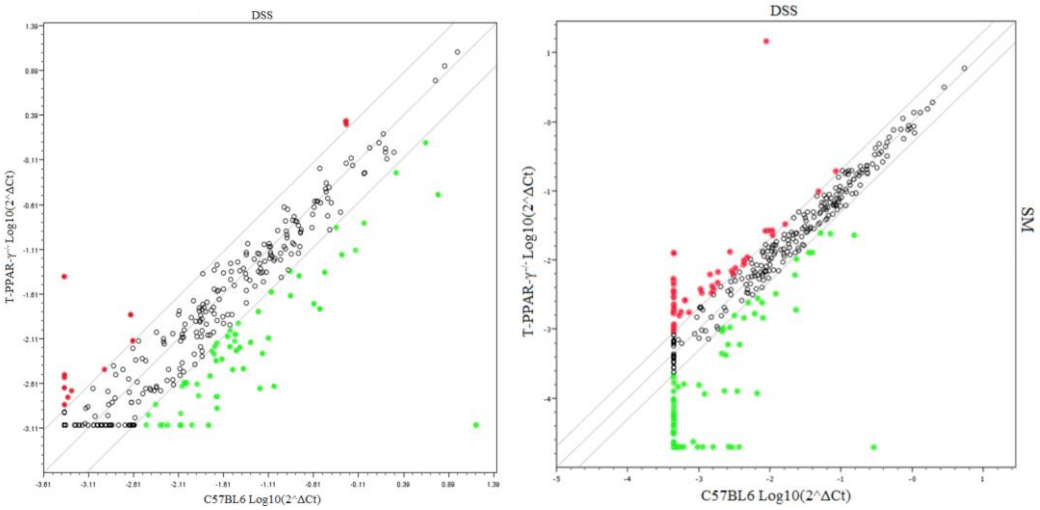

Figure 17. Left Comparison of gene expression levels in the colons of C57BL6 and T-PPAR-$\gamma^{-/-}$ mice treated with DSS; the C57BL6 colons are set as the control. **Right.** Comparison of C57BL6 mice treated with DSS/SM vs. T-PPAR-$\gamma^{-/-}$ mice treated with DSS/SM. Fold differences > 2 in green indicate decreased level of expression and red indicate increased level of expression.

While few genes were higher expressed in the T-PPAR-$\gamma^{-/-}$ mice, 16 cytokines, and 11 chemokines show decreased levels compared to the T-PPAR-$\gamma^{+/+}$ mice. Furthermore, 32 inflammatory response genes show lower levels of expression. These gene changes suggest a potentially less severe inflammatory reaction in the T-PPAR-$\gamma^{-/-}$ mice; this response is surprising since there was no apparent difference between the genotypes in the DAI (figure 14). However, the total number of genes that are differentially expressed is only about 10% of all genes included in the array, and may have not been sufficient to cause drastic difference in disease severity.

After feeding SM to the DSS-treated mice, 66 genes were detected with decreased expression and 31 genes with increased expression levels when compared to the T-PPAR-$\gamma^{+/+}$ mice treated with DSS and SM (table 8B). These include several cytokines with increased expression, induced by the SM supplements. There are, however, more cytokines (23) and inflammatory response genes (21) with a decreased expression level. These gene lists do not overlap between the comparisons, and several of these genes are more highly expressed in DSS-treated T-PPAR-$\gamma^{-/-}$ mice than in the T-PPAR-$\gamma^{+/+}$ mice are actually lower expressed in SM-treated T-PPAR-$\gamma^{-/-}$ mice, i.e., IFNa4, Xcr1, indicating a stronger effect in mice lacking T-cell PPAR-γ, and suggest a PPAR-γ-independent effect of the SM. Other genes are less expressed in DSS-treated T-PPAR-$\gamma^{-/-}$ but comparably higher after feeding SM, i.e., Inhba, Tlr6, Saa4. These comparisons show that dependent on their genotype, mice exhibit different gene expression profiles in response to DSS treatment, and exposure to dietary SM.

Table 8. A) Gene expression changes in T-PPAR-$\gamma^{+/+}$ mice treated with DSS vs. T-PPAR-$\gamma^{-/-}$ mice treated with DSS. **B)** Gene expression changes T-PPAR-$\gamma^{+/+}$ mice treated with DSS and SM vs. T-PPAR-$\gamma^{-/-}$ mice treated with DSS and SM. Bold genes are those expressed in the opposite direction when compared across the two sets of comparisons in figure 17, these genes are believed to be differentially expressed dependently on dietary SM. Underlined genes are those expressed in the same direction when compared across the two sets of comparisons in figure 17, these genes are believed to be differentially expressed independently on dietary SM, thus showing potential in being dependent on T-cell PPAR-γ expression.

Table 8	T-PPAR-$\gamma^{+/+}$ vs. T-PPAR-$\gamma^{-/-}$			
	Table 8A DSS / **No SM**		*Table 8B* DSS / **SM**	
	Higher Expressed	Lower Expressed	Higher Expressed	Lower Expressed
Cytokines	Gdf1, **Infa4**, Il1f8	Csf3, Fgf10, Fgf7, Flt31, Il1a, Il1b, Il22, Il6, **Inhba**, Ltb, Nrg1, Osm, Spp1, Tnf, Tnfsf14, Tnfsf9	Ctf1, Fasl, Gdf5, Ik, Il17d, **Inhba**, Inhbb, Prl, Ptn, Thpo, Tnfsf1, Tnfsf13, Tnfsf13b, Tnfsf4	Cd40lg, Clcf1, Cmtm1, Cmtm2a, Fgf3, Fgf4, Fgf6, Fgf8, Gdf7, Ifna2, **Ifna4**, Ifna9, Ifne, Il12b, Il17c, Il17f, Il19, Il21, IL22, Il23a, Il24, Il27, Il4
Cytokine receptors	Epor, Il18rl, Il22ra	Csf3, sf3r, Gfra2, Ghr, Il11, Il1r2, Il2rg, Il17r, Il8rb	Il20ra, Prl	Cntfr, Il12b, Il12rb1, Il12rb2, Il13ra2, Il1r12, Il21
Chemokine		Ccl12, Ccl25, Ccl3, Ccl4, Cxcl5, Cxcl10, Cxcl16, Cxcl2, Cxcl9, Pf4	Ccl17, Ppbp	Ccl, Ccl19, Ccl22, Cxcl1, Cxcl10, Cxcl11, Cxcl9, Cyp26b1
Chemokine rec.	Ccr10, **Xcr1**	Ccr1, Ccr2, Ccr3, Ccr5, Ccr6, Ccr12, Il8rb	Ccr10, Ccr4	Ccr6, Ccr8, Cxc5, **Xcr1**
Inflamm. response	Hrh1, Il1f8, Nfrkb, **Xcr1**	Ccl2, Ccl25 Ccl3, Ccl4, Ccr1, Ccr2, Ccr3, Cd14, Cd40lg, Cxcl1, Cxcl2, Cxcl5, Cxcl9, Fpr1, Il1a, Il1b, Il22, Il8rb, Nos2, Prok2, Ptafr, Ptgs2, Ptx3, Reg3g, S100a8, Scube1, Spp1, Tlr4, **Tlr6**, Tlr7, Tnf	Ccl1, Ccr4, Dock2, Il17d, Nfam1, Prg2, **Tlr6**	Adora1, Ccl1, Ccl9, Ccl22, Cd40lg, Crp, Cxcl1, Cxcl10 Cxcl11, Cxcl9, Ifna2, Il17c, Il17f, Mmp25, Nfate4, Nlrp12, Nos2, Prg3, Prok2, Tlr9, **Xcr1**
Acute phase	Serpina1a	Il22, Il6, Reg3g, **Saa4**, Serpina3n	**Saa4**	Apcs, Crp, Il22, Ino1
Cytokine metab.	Cd4, Il1b, **Inhba**, Tlr4	Il6	**Inhba**, Inhbb, **Tlr6**	Il12b, Il17f, Il19, Il21, Il27, Il4, Nfrp12, Prg3

In order to direct our future mechanistic studies of signaling pathways involved in the suppression of intestinal inflammation that may contribute to a suppression of colon cancer observed previously [31], we then began to identify the genes that respond to SM in the diet that are either dependent or independent of the expression of PPAR-γ expression in the T-cells. First, we compared the expression profiles of either T-PPAR-$\gamma^{+/+}$ or T-PPAR-$\gamma^{-/-}$ mice, treated with DSS, with and without SM in the diet. As shown in Fig. 18 (left panel), 56 genes are down-regulated and 21 genes are up-regulated due to treatment of dietary SM. These genes are listed in table 9. SM in the diet of DSS-treated C57BL/6 mice increased the expression of several genes (table 9A), mostly cytokines and cytokine receptors.

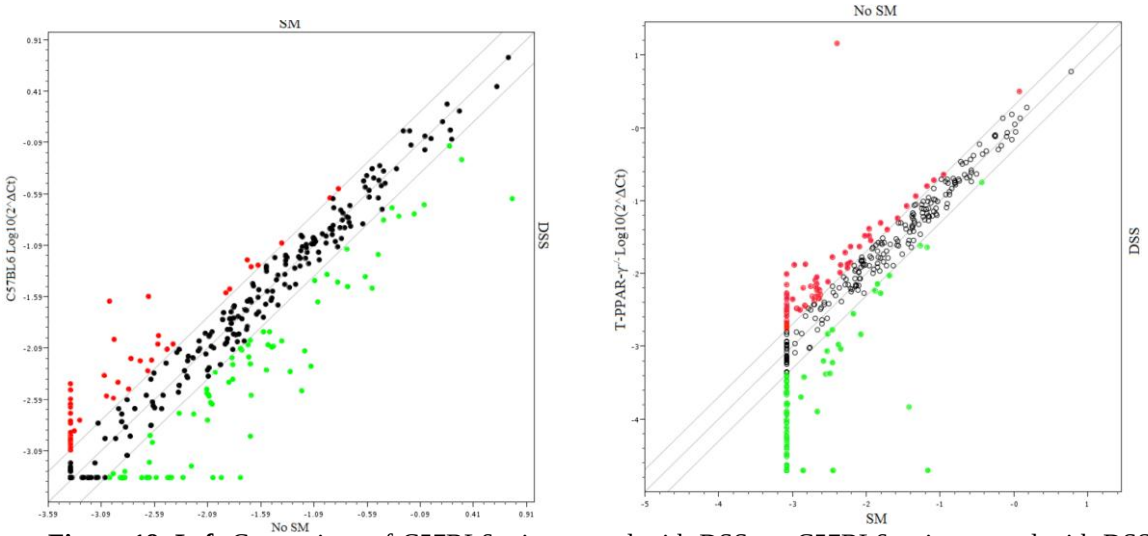

Figure 18. Left Comparison of C57BL6 mice treated with DSS vs. C57BL6 mice treated with DSS and SM. Fold differences > 2 are colored, green indicating a down-regulation and red indicating an up-regulation. **Right** Comparison of T-PPAR-$\gamma^{-/-}$ mice treated with DSS vs. T-PPAR-$\gamma^{-/-}$ mice treated with DSS and SM. Fold differences > 2 are colored green to indicate a down-regulation and red to indicate an up-regulation.

There was a larger number of down-regulated genes which were mostly cytokines and genes involved in the inflammatory response. There was some overlap when we compared the gene profiles between DSS-treated T-PPAR-γ mice with or without SM supplements (Fig. 18, right panel). Several genes changed in the same direction by SM, indicating a T-PPAR-γ independent effect (Table 9, underlined). For example, Bmp2, Ik, and Pxmp2 were higher expressed in mice of either genotype fed SM, while Ccl2, Prok2 and Sele were down-regulated. In total, 29 were up-regulated but 101 genes were down-regulated by SM. Importantly, these comparisons allow for the identification of genes that are changed in the opposite direction, indicating a dependence of the

effect to the expression of T-cell PPAR-γ. As shown in Table 9, genes in bold change in the opposite directions in T-PPAR-$\gamma^{+/+}$ and T-PPAR-γ mice. **Table 9. A)** Gene expression changes in T-PPAR-$\gamma^{+/+}$ mice treated with DSS and SM. **B)** Gene expression changes in T-PPAR-$\gamma^{-/-}$ mice treated with DSS and SM. Bold genes are those expressed in the opposite direction when compared across the two sets of comparisons in figure 18, these genes are believed to be differentially expressed dependently on T-cell PPAR-γ expression. Underlined genes are those expressed in the same direction when compared across the two sets of comparisons in figure 18, these genes are believed to be differentially expressed independently on T-cell PPAR-γ expression.

Table 9. A) Gene expression changes in T-PPAR-$\gamma^{+/+}$ mice treated with DSS and SM. **B)** Gene expression changes in T-PPAR-$\gamma^{-/-}$ mice treated with DSS and SM. Bold genes are those expressed in the opposite direction when compared across the two sets of comparisons in figure 18, these genes are believed to be differentially expressed dependently on T-cell PPAR-γ expression. Underlined genes are those expressed in the same direction when compared across the two sets of comparisons in figure 18, these genes are believed to be differentially expressed independently on T-cell PPAR-γ expression.

Table 9	DSS vs. DSS/SM			
	Table 8A T-PPAR-γ+/+		*Table 8B* T-PPAR-$\gamma^{-/-}$	
	Higher Expressed	Lower Expressed	Higher Expressed	Lower Expressed
Cytokine	Bmp2, **Cd40lg**, **Clcf1**, Ik, Il12b, Il1f8, Muc4, Srgap1, Tnfsf18	Csf2, Csf3, Fasl, Flt31, Gdf5, Il17a, Il1a, Il1b, Il4, **Inhba**, Ltb, Osm, **Ptn**, Spp1, Tnf, Tnfsf11, Tnfsf13, Tnfsf13b, Tnfsf9	Bmp2, Fgf10, Fgf7, Glmn, Ik, **Inhba**, Inhbb, Ntf3, Prl, **Ptn**, Siva1, Srgap1, Tnfsf4, Vegfa	**Cd40lg**, **Clcf1**, Cmtm1, Cmtm2a, Csf2, Ctf2, Fgf3, Fgf4, Fgf5, Fgf6, Fgf8, Gdf1, Gdf2, Gdf7, Ifna11, Ifna2, Ifna4, Ifna9, Infab, Ifnb1, Ifne, Ifng, Il17c, Il17f, Il19, Il1f10, Il1f5, Il1f6, Il2, Il20, Il21, Il22, Il23a, Il24, Il27, Il4, Lefty2, Mstn, Nodal, Tnfsf8
Cytokine receptors	Cntf, Csf2ra, Il12b, Il22ra2, Il23r, Il28ra	**Ccr12**, Csf3, Il1r2, Il20ra	Csf3r, Gfra2, Il5ra, Il7r, Prl	Csf2, Epor, Il12rb1, Il12rb2, Il13ra2, Ilrap12, Il1r12, Ilrb, Il8ra, Il9r, Mpl
Chemokines	Pxmp2	Ccl2, Ccl22, Ccl3, Ccl4, Cxcl1, Cxcl10, Cxcl2, Cxcl5, Cxcl9	Ccl17, Cxcl16, Pxmp2	Ccl1, Ccl19, Ccl22, Ccl24, Cxcl11, Cxcl15, Cyp26b1
Chemokine rec.	**Cxcr5**, **Xcr1**	Ccr5, **Ccr12**	**Ccr12**	Ccr8, **Cxcr5**, Il8ra, **Xcr1**
Inflam. respons	Adora1, **Cd40lg**, Il1f8, Tlr9, **Xcr1**	Ccl2, Ccl22, Ccl3, Ccl4, Cxcl1, Cxcl10, Cxcl2, Cxcl5, Cxcl9, Dock2, **Fpr1**, Il17a, Il1a, Il1b, Prg2, Prok2, Ptx3, S100a8, Sele, Spp1, Tlr4, **Tlr6**, Tlr7, Tlr8, Tnf	Apoa2, Ccl17, **Fprl**, Olr1, **Tlr6**	Ahsg, Apol8, Ccl1, Ccl19, Ccl22, Ccl24, **Cd40lg**, Crp, Cxcl11, Cxcl15, Ifna2, Il17c, Il17f, Il1f10, Il1f5, Il1f6, Il20, Il22, Il8ra, Kng1, Ltb4rl, Mmp25, Nfatc4, Nlrp12, Prg3, Prok2, Sele, Sftpd, **Xcr1**
Acute phase		Itih4, **Saa4**, Serpina3n	**Saa4**	Ahsg, Apcs, Crp, Il22, Ins1, Ins2, Itih4, Serpina1a, Serpinf2
Cytokine metab.	**Il12b**, **Irf4**	Il1b, Il4, **Inhba**, Tlr4, **Tlr6**	Apoa2, Glmn, **Inhba**, Inhbb, **Tlr6**	Cd28, Ifng, Il17f, Il19, Il21, Il27, Il4, Nlrp12, Prg3, Sftpd

Identification of genes that respond to SM in a PPAR-γ dependent manner

In order to gain a better understanding of the genes involved in the mechanistic response to SM and PPAR-γ we ran confirmatory real-time polymerase chain reaction. Each PAMM 3803 PCR-array had 372 genes involved in the inflammatory and immunostimulatory systems. The 372 genes were narrowed down by comparing PCR-Array results from chapter 3 and chapter 4, in order to determine which genes are potentially involved in the mechanistic response to the SM treatment with a dependence on T-Cell PPAR-γ expression in mice with DSS-induced colitis.

Step 1

- Wild type mice are compared to wild type mice treated with DSS
- Wild type mice are compared to PPAR-$\gamma^{-/-}$ mice treated with DSS
- Wild type mice are compared to T-cell PPAR-$\gamma^{-/-}$ mice treated with DSS

The genes that were differentially expressed, in the same direction, in all 3 comparisons, were taken out and put into the list seen in table 10. This initial comparison was done in order to determine which genes respond to DSS treatment, regardless of the genotype.

Table 10. Genes that are differentially expressed, in the same direction, by DSS treatment in all 3 mouse genotypes. Red indicates the genes that are higher expressed in all 3 genotypes due to DSS treatment and green indicates genes that are lower expressed in all 3 genotypes dues to DSS treatment.

Genes up -regulated by DSS in all 3 genotypes	Up-regulated Cont.	Genes down -regulated by DSS in all 3 genotypes
AIF1	IL-1β	IL22RA2
APCS	IL1R1	IL5RA
CCL11	IL1R2	LTA
CCL2	IL6	SLURP1
CCL3	PLA2G7	TNFSF13
CCL4	REG3A	
CCL7	S100A8	
CCR7	SCUBE1	
CEBPB	SELE	
CSF3	SPP1	
CXCL11	TACR1	
CXCL13	TLR7	
FIGF	TNF-α	
GDF5	TNFAIP6	
IL12A	TNFSF14	
IL17C		

Step 2

- Wild type mice treated with DSS were set as control group
    a. Control group was compared to wild type mice treated with DSS and supplemented with SM
    b. Control group was compared to PPAR-$\gamma^{-/-}$ mice treated with DSS and supplemented with SM
    c. Control group was compared to T-cell PPAR-$\gamma^{-/-}$ mice treated with DSS and supplemented with SM

Comparison a. was made in order to determine which genes from table 9 are differentially expressed due to SM supplementation in the presence of PPAR-γ. Comparison b. was made in order to determine which genes from table 9 are differentially expressed due to SM supplementation without the presence of PPAR-γ in the epithelial tissue, macrophages, and T-cells. Comparison c. was made to determine which genes from table are differentially expressed due to SM supplementation with PPAR-γ expression in the T-cells. For example TNF-α, shown in table 11, had a decreased expression due to SM supplementation in the presence of PPAR-γ (comparison a.). When the PPAR-γ expression is lacking in the epithelial tissue, macrophages, and T-cells then the response to SM supplementation produced an increase in TNF-α expression (comparison b.). Finally, with the presence of PPAR-γ null in the T-cells there is no differential response to the SM supplementation (comparison c.).

Table 11. Gene expression changes in the gene list from table 9 after being SM supplementation in each of the genotypes (Wild type mice treated with DSS set as the control group). Red indicates the genes that are higher expressed due to SM supplementation and green indicates genes that are lower expressed due to SM supplementation. N/A indicates no change in gene expression due to the comparison.

Control	**Group 1**	**Group 2**	**Group 3**
DSS	*DSS/SM*	*DSS/SM*	*DSS/SM*
C57BL/6	*C57BL/6*	*PPAR-$\gamma^{-/-}$*	*T-PPAR-$\gamma^{-/-}$*
AIF1	N/A	N/A	N/A
APCS	APCS	N/A	APCS
CCL11	N/A	N/A	N/A
CCL2	CCL2	N/A	N/A
CCL3	CCL3	CCL3	N/A
CCL4	CCL4	CCL4	N/A
CCL7	CCL7	N/A	N/A
CCR7	N/A	CCR7	N/A
CEBPB	N/A	N/A	N/A
CSF3	CSF3	N/A	N/A
CXCL11	CXCL11	CXCL11	CXCL11
CXCL13	N/A	CXCL13	N/A
FIGF	N/A	N/A	N/A
GDF5	GDF5	N/A	GDF5
IL12A	IL12A	N/A	N/A

IL17C	N/A	N/A	IL17C
IL-1β	IL-1β	IL-1β	N/A
IL1R1	N/A	N/A	N/A
IL1R2	IL1R2	N/A	N/A
IL6	N/A	IL6	IL6
PLA2G7	N/A	N/A	N/A
REG3A	REG3A	REG3A	N/A
S100A8	S100A8	N/A	N/A
SCUBE1	N/A	N/A	SCUBE1
SELE	SELE	SELE	SELE
SPP1	SPP1	N/A	SPP1
TACR1	N/A	N/A	TACR1
TLR7	TLR7	N/A	N/A
TNFα	TNFα	TNFα	N/A
TNFAIP6	N/A	TNFAIP6	N/A
TNFSF14	N/A	N/A	N/A
IL22RA2	IL22RA2	N/A	N/A
IL5RA	N/A	IL5RA	IL5RA
LTA	N/A	N/A	N/A
SLURP1	SLURP1	SLURP1	N/A
TNFSF13	TNFSF13	N/A	N/A

These differences due to PPAR-γ expression in response to the dietary SM show that TNF-α is potentially dependent on the expression of PPAR-γ in its response to SM supplementation after DSS-treated mice. Using the results obtained from the PCR-array the final gene list was narrowed down to 7 genes (Table 12). Based on the results from chapter 3 and 4 the genes in table 12 showed the most potential in being involved in the anti-inflammatory environment created by the dietary SM in a T-cell PPAR-γ dependent manner, after DSS treatment.

Table 12. Final list of genes determined to have most potential in responding to SM supplementation in a T-cell PPAR-γ dependent manner. Red genes are those that were over expressed due to lack of PPAR-γ expression. Green genes are the genes that were under expressed due to lack of PPAR-γ expression. A brief description of gene involvement in the inflammatory response is given.

Isolated Genes	Description
IL6	Pro-Inflammatory cytokine and secreted by T-Cells.
Il-17C	Pro-Inflammatory cytokine and stimulates production of TNF-α, IL6, and IL-1β
Spp1	Inhibits production of Th2 thus giving rise to Th1 T-cell differentiation.
IL-1β	Pro-Inflammatory cytokine produced by activated macrophages.
Gdf5	Regulator of cell growth and differentiation.
Il5rα	Cytokine receptor that only acts on IL5 and is expressed by B-cells.
TNF-α	Pro-inflammatory cytokine involved in systemic inflammation, specifically acute phase response.

To confirm these results and for statistical analysis, quantitative PCR on individual samples (N=8-10) was performed. As shown in figure 19, differences in the response to SM in DSS treated mice followed (IL-6, IL-1β) or did not follow (Spp1, TNF-α, IL-17c) the analysis of the PCR array. The reasons for this discrepancy need to be investingated in more detail.

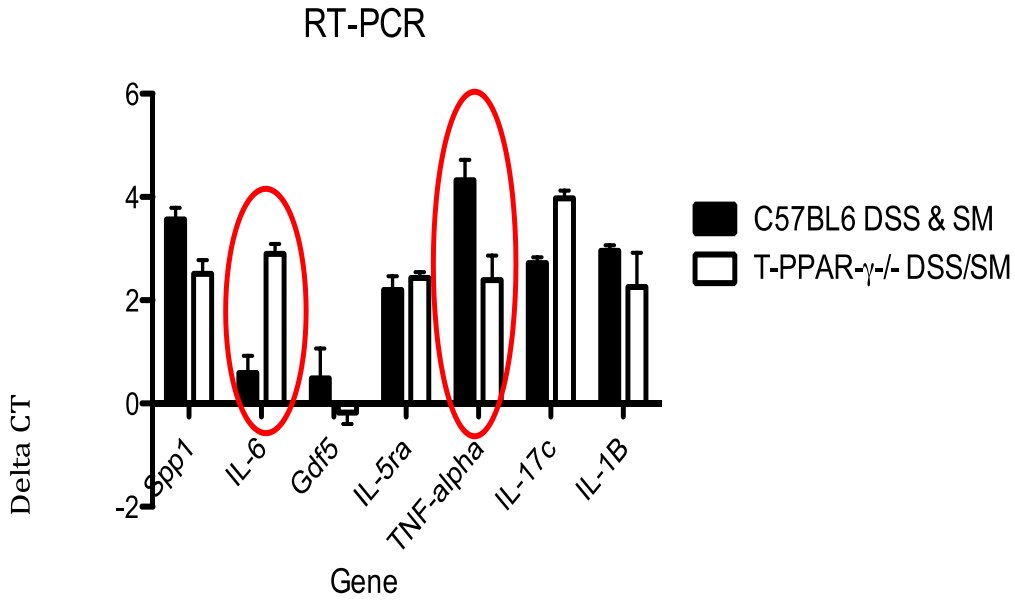

Figure 19. Gene expression changes due to difference in T-cell PPAR-γ expression in mice supplemented with SM and treated with DSS. Black bars indicate wild type mice and white bars indicate mice without PPAR-γ expression in the t-cells. Expression levels measured using Delta Ct, significance indicated by red circle ($p < 0.05$).

Only IL-6 and TNF-α were statistically significantly differently expressed after SM supplementation of DSS treated mice due to the T-cell T-PPAR-γ expression. While IL-6 is reduced by dietary SM in T- PPAR-$\gamma^{+/+}$ mice, indicating a dependece on T- PPAR-γ expression, TNF-α expression levels are lower. Both are pro-inflammatory cytokines but can also increase tumor growth [106]. IL-17c is lower in SM treated T- PPAR-$\gamma^{+/+}$ mice as was seen in the study above while Spp1 was higher. However, these different responses were not significant. Interestingly, growth differentiation factor 5 (GDF5) was only up-regulated in T-PPAR-$\gamma^{+/+}$ mice. The association of these and other inflammatory gene products on tissue integrity and tumor formation in this model requires further analysis.

Discussion

Endogenous sphingolipid metabolites have been implicated in the induction of inflammatory responses both *in vitro* and *in vivo*. We have shown that dietary administration of SM suppressed DSS-induced inflammation and subsequent tumor formation in mice that was partially dependent on PPAR-γ expression in the intestinal epithelium, T-cells and macrophages. The present studies investigated the reliance of exogenous SM on PPAR-γ expression in CD4+ T-cells to suppress DSS-induced intestinal inflammation. We confirmed that dietary SM reduced DSS-induced inflammation and disease severity. These findings were associated with an improved preservation of the colonic microscopic architecture during acute inflammation in both T-PPAR-γ$^{-/-}$ and C57BL6 mice. Importantly, the anti-inflammatory effect of SM was determined by the expression of PPAR-γ in T cells since T-PPAR-γ mice did not respond as strongly, suggesting that PPAR-γ expression in T-cells is at least partially responsible for the anti-inflammatory effect of dietary SM. However, our results also indicate effects of dietary SM that were independent of PPAR-γ expression in the T-cells. If these effects require PPAR-γ expression in other intestinal tissues, or are completely independent of PPAR-γ will be subject to further investigation.

These results confirm earlier studies done by Furuya et al. who also found anti-inflammatory effects induced by dietary SM [83]. Contradictory to our results, Fischbeck et al. recently reported a lack of suppression of DSS-induced colitis in mice treated with SM. Moreover, they reported that SM administered per gavage actually increased intestinal inflammation. This may be due to the amount administered at a single timepoint in contrast to slower uptake via the diet. The composition of the egg SM may also affect the intestine since it differs in its fatty acid lipid profile from the milk SM we used in our studies: it contains about 87% C16 fatty acids while milk SM contains more C22, 23, and 24. This difference in fatty acid lipid profile has been shown to affect lipid absorption [81]. Also, earlier studies have shown that C16 ceramide remains as the major ceramide species in the intestine [38] that may influence cell survival or apoptosis, and inflammation. Together, the administration of a single bolus and the accumulation of C16 ceramide may lead to the opposite effects than milk ceramides in lower concentrations. The inhibition of ceramide or S1P generation has also been shown to suppress DSS-induced inflammation [77, 78, 79, 80] confirming that exogenous sphingolipids and intestinal sphingolipid metabolites can effectively suppress inflammation.

Macrophages, T-cells and intestinal epithelial cells play integral roles in intestinal inflammation during IBD as well as express PPAR-γ. We hypothesized that the anti-inflammatory effects of SM were mediated through a PPAR-γ-dependent mechanism. The results from chapter 3 suggest that the expression

of PPAR-γ in the T-cells is necessary for the anti-inflammatory response to dietary SM.

Previous studies have demonstrated that PPAR-γ has the innate ability to down-regulate many of the cells involved in the innate immune system, specifically those involved in the inflammatory response [73] [74]. The exact mechanism which this occurs has yet to be determined but is consistent with the results in chapter 3 and Saubermann et al. which found that PPAR-γ agonist ligands stimulate a Th2 cytokine response and prevent acute colitis [75]. Aim 2 results showed that the T-cell PPAR-γ expression did not, by itself, significantly reduce the inflammatory response caused by the DSS challenge. Furthermore, our data confirm that the presence of PPAR-γ in T-cells is not critical for the induction of inflammation by DSS as reported by Axelsson et al [105]. Dietary SM treatment significantly decreased acute inflammation, but it did so in a partially PPAR-γ dependent manner. Other studies have suggested that SM plays a critical role in the activation and function of CD4+ T-Cells both *in vivo* and *in vitro* [76]. Results from chapter 4 are consistent with the results reported by Dong et al. that the effects of PPAR-γ seemed to be more pronounced when dietary SM was included in the diet rather than by itself [76]. The changes caused by SM supplementation were more pronounced in the mice with the active PPAR-γ gene within the T-cells. Overall the SM supplementation produced an anti-inflammatory environment in all genotypes, but it was more pronounced in mice expressing the PPAR-γ gene.

Results from quantitative real-time polymerase chain reaction showed that IL-6 and TNF-α were both dependent on PPAR-γ expression in response to SM supplementation after DSS-induced colitis. TNF is directly regulated by NF-kB, which is inhibited by the activation of intra-cellular PPAR-γ. IL-6 is not directly effected by PPAR-γ expression but it is a down stream of IL-1β which is regulated by NF-kB. Our results showed that both TNF-α and IL-6 responded significantly to dietary SM when PPAR-γ was expressed within the T-cells. The interaction of these SM-induced changes in gene products, their effect on tissue architecture, wound healing and cancer initiation and progression need to be investigated in greater detail.

Conclusion

In summary, the results from the studies in chapter 3 and 4 reveal an anti-inflammatory response of mice to dietary SM treatment in contrast to endogenous sphingolipid metabolites that in general promote and perpetuate inflammation. This was associated with a significantly enhanced survival, and reduced tumor formation after combined DSS and AOM treatment, suggesting that suppression of the intial inflammation is critical for inhibiting inflammation-driven colon cancer. This indicates that the use of dietary SM in the prevention of colon cancer is not imited to patients with IBD but may be beneficial to suppress the initial inflammation that promotes colon cancer development. The SM effects are partially dependent on PPAR-γ expression in the intestinal epithelium and immune cells to create an overall anti-inflammatory environment but more so rely on PPAR-γ expression in CD4+ T cells. The present studies have identified genes that are responding to dietary SM both in PPAR-γ dependent and independent manner. Future studies need to confirm that this results in changes of the gene products, and identify critical signaling pathways that are targeted by SM to suppress inflammation. These changes have the potential to be used as biomarkers to predict the efficacy of dietary SM in future clinical trials.

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Appendix:

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