BASIC–ALIMENTARY TRACT

Activation of PPAR γ and δ by Conjugated Linoleic Acid Mediates Protection From Experimental Inflammatory Bowel Disease

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Background & Aims: **The molecular targets for the protective actions of conjugated linoleic acid (CLA) on experimental inflammatory bowel disease (IBD) are unknown. We used a loss-of-function approach to investigate whether CLA ameliorated colitis through a peroxisome proliferator–activated receptor γ (PPAR γ)–dependent mechanism.** ***Methods:*** **The expression of PPAR γ, δ, and their target genes in the colon of mice fed control or CLA-supplemented diets was assayed after a 7-day dextran sodium sulfate (DSS) challenge by quantitative real-time polymerase chain reaction (PCR). Additionally, nuclear factor-κ B (NF-κB) p65 activation was quantified in the colon. To determine the involvement of PPAR γ in the mechanism of action of CLA directly, specific deletions of PPAR γ in the colon were performed in mice by using the Cre-lox recombination system. Colonic PPAR γ null mice and wild-type littermates were fed either a CLA-supplemented or a control diet for 42 days and challenged with 2.5% DSS. The therapeutic efficacy of CLA also was examined by using the $CD4^+CD45RB^{hi}$ transfer colitis model.** ***Results:*** **CLA induced PPAR γ and δ, transcriptionally modulated PPAR γ and δ-responsive gene clusters involved in lipid metabolism (uncoupling protein [UCP]1, UCP3, PPAR γ coactivator 1α [PGC-1α], and CD36) and epithelial cell maturation (Gob-4 and Keratin 20). Additionally, CLA repressed tumor necrosis factor α (TNF-α) expression and NF-κB activation while inducing the immunoregulatory cytokine transforming growth factor β 1 (TGF-β_1). Clinically, CLA ameliorated DSS- and $CD4^+$-induced colitis. Loss of the PPAR γ gene in the colon abrogated the beneficial effects of CLA in DSS colitis.** ***Conclusions:*** **Our studies provide molecular evidence in vivo, suggesting that CLA ameliorates colitis through a PPAR γ-dependent mechanism.**

Inflammatory bowel disease (IBD) is a chronic, recurring, immunoinflammatory illness of unknown cause that afflicts over 1 million Americans and is characterized by 2 clinical and histopathologic manifestations—Crohn's disease (CD) and ulcerative colitis (UC). Current treatments for IBD include corticosteroids (i.e., 6-methylprednisolone and budesonide), antibiotics, and immunomodulators (i.e., azathioprine, 6-mercaptopurine, cyclosporine, and methotrexate).[1] Novel therapies include the Food and Drug Administration–approved anti–tumor necrosis factor (TNF)-α (Infliximab; Remicade; Centocor, Inc., Malvern, PA).[1,2] Although IBD therapies have improved, they still are only modestly successful for the long-term management of the disease and result in significant side effects. Hence, exploring novel preventive or therapeutic interventions and their molecular targets remains important.

The use of selective peroxisome proliferator–activated receptor (PPAR) γ agonists in the treatment of IBD has received some attention.[3] PPARs (α, β/δ, and γ) are nuclear receptors that translate nutritional and/or phar-

Abbreviations used in this paper: CLA, conjugated linoleic acid; DSS, dextran sodium sulfate; NF-κB, nuclear factor κ B; PCR, polymerase chain reaction; PGC-1α, PPAR γ coactivator 1α; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; TGF-β1, transforming growth factor-β1; TNF, transforming growth factor; UCP, uncoupling protein.

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macologic stimuli into changes in gene expression.[4] Each receptor is encoded by a discrete set of genes that exhibit unique patterns of expression in vertebrate tissues.[3] Originally, PPARs were identified as components of adipocyte gene expression and differentiation, which regulate lipid homeostasis.[5,6] More recent studies have shown that PPARs also regulate a broader range of biological functions contributing to IBD pathogenesis and/or disease severity[7,8] including inflammation, immunity, and epithelial cell differentiation.[9–14] Synthetic PPAR γ agonists such as thiazolidinediones ameliorated IBD in murine models[15–17] and their therapeutic efficacy is being tested in IBD clinical trials.[3] Results of studies using PPAR γ heterozygous mice revealed their increased susceptibility to develop experimental IBD[18] and allergic encephalomyelitis.[19] However, the literature is devoid of in vivo studies that examine the modulation of PPAR γ transcriptional activities by natural agonists and their health benefits.[4,20,21]

Results from in vitro studies showed that naturally occurring polyunsaturated fatty acids (PUFA) and their metabolites are endogenous PPAR γ ligands.[22] Although many PUFA are good PPAR γ ligands, some of them do not cause transactivation of peroxisome proliferator response elements.[23] Hence, their ligand-binding activities may not be of significance transcriptionally or phenotypically. In contrast, conjugated linoleic acid (CLA) activated PPAR γ in reporter assays,[24,25] and elicited in vivo effects consistent with PPAR γ activation.[26–28] CLA is a mixture of positional and geometric isomers of octadecadienoic acid with conjugated double bonds. Structurally, CLA resembles 13-hydroxyoctadecadienoic acid, which along with 15-hydroxyeicosatetraenoic acid and 15 deoxy-$\Delta^{12,14}$ prostaglandin J_2 were identified as endogenous activators of PPAR γ.[23,29–31] Finally, in line with the current use of synthetic PPAR γ agonists as insulin-sensitizing drugs in the treatment of type 2 diabetes,[32] CLA was found effective in preventing the development of hyperglycemia in the prediabetic Zucker Diabetic Fatty (ZDF) rat.[24]

The importance of PPAR γ in regulating homeostasis in the colon and adipose tissue is supported by the concentrations of PPAR γ messenger RNA (mRNA) in these tissues, which represent the greatest in the body.[33,34] In the colon, the highest concentrations of PPAR γ have been found within the postmitotic, differentiated epithelial cells.[34–36] However, PPAR γ also is expressed by immune cells residing in the colonic mucosa (i.e., lymphocytes and macrophages).[37,38] Previous studies from our laboratory showed that CLA ameliorated tissue damage and enhanced colonic PPAR γ expression in a pig model of bacterial-induced colitis.[39] Others have reported that PPAR γ gene therapy alone or in combination with the thiazolidinedione rosiglitazone ameliorated the inflammatory pathology in a mouse model of DSS colitis.[17] Selective PPAR γ ligands inhibited inflammation through a nuclear factor κ B (NF-κB)–dependent mechanism.[15] We hypothesized that the protective effects of CLA in IBD are mediated, in part, through PPAR γ. To test this hypothesis, we examined the ability of CLA to modulate the expression of PPAR γ target genes and suppress NF-κB p65 activation in a mouse model of DSS colitis. Additionally, the dependence of the protective effect of CLA on the presence of PPAR γ in the colon was investigated by using tissue-specific PPAR γ null mice. Our findings show that the induction and activation of PPAR γ and δ by CLA contributes to the maintenance of intestinal homeostasis and prevention of experimental IBD in mice.

Materials and Methods

Animals

C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Two PPAR $\gamma^{fl/fl}$ (floxed) breeder pairs were obtained from Dr. F. Gonzalez' laboratory at the National Institutes of Health/National Cancer Institute. These mice were generated by flanking the exon 2 of the PPAR γ gene by 2 loxP boxes, which are recognized by a transgenic recombinase.[40] PPAR $\gamma^{fl/fl}$ mice were bred with MMTV-Cre transgenic mice provided by Drs. Cui and Hennighausen (National Institutes of Health/National Institute for Diabetes and Digestive and Kidney Diseases).[41] MMTV-Cre (Cre^+) mice express a transgenic recombinase in epithelial and hematopoietic cells. PPAR γ floxed mice expressing the Cre transgene (e.g., PPAR-γ fl/fl; MMTV-Cre) undergo premature termination of translation after the loss of the exon 2 of PPAR γ owing to the enzymatic activity of the recombinase on genomic DNA.[40] However, it was unknown whether the recombinase would be expressed in the colon and the efficiency of colonic recombination in PPAR $\gamma^{fl/fl}$ Cre^+ mice. All the generations of mice were genotyped before weaning by isolating genomic DNA from tail clippings. The colonic genotype was determined at necropsy. The tail and colonic genotypes of mice was determined by polymerase chain reaction (PCR) analysis as previously described.[42] Briefly, primers for the PPAR γ gene were F (5′-CTC CAA TGT TCT CAA ACT TAC-3′), R1 (5′-GAT GAG TCA TGT AAG TTG ACC-3′), and R2 (5′-GTA TTC TAT GGC TTC CAG TGC-3′), which yielded a 225-bp band from the wild-type allele, a 275-bp band from the floxed allele, or a 400-bp band from the null allele. Primers for the Cre transgene were 5-GGT TCT GAT CTG AGC TCT GAG TG-3′, which binds in the MMTV-LTR, and 5′-CAT CAC TCG TTG CAT CGA CCG G-3′, which binds in the Cre sequence. The MMTV-Cre transgene yielded a 280-bp fragment. The mice were maintained in the animal facilities at Virginia Polytechnic Institute and State University. All exper-

Table 1. Composition of the Experimental Diets

Ingredient	Dietary treatments			
	Linoleic (control diet)	CLA mixture	EPA/DHA mixture	Pure CLA isomers
Casein	200	200	200	200
L-cystine	3	3	3	3
Corn starch	397.486	397.486	397.486	397.486
Maltodextrin	132	132	132	132
Sucrose	100	100	100	100
Cellulose	50	50	50	50
Mineral mix (AIN-93)[a]	35	35	35	35
Vitamin mix (AIN-93)[b]	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
tert-butylhydroquinone[c]	0.014	0.014	0.014	0.014
Soybean oil	60	60	60	60
Linoleic acid	10	—	—	—
EPA/DHA mixture	—	—	10	—
c9, t11/t10, c12 CLA	—	10	—	—
c9, t11 or t10,c12 CLA	—	—	—	10

EPA/DHA, eicosapentaenoic/docosahexaenoic acid.
[a]Supplied per kg of diet: 3 g nicotinic acid, 1.6 g calcium pantotenate, 0.7 g pyridoxine HCl, 0.6 g thiamin HCl, 0.6 g riboflavin, 0.2 g folic acid, 0.02 g D-biotin, 2.5 g vitamin B_{12} (0.1% in mannitol), 15 g DL-α tocopheryl acetate (500 IU/g), 0.8 g vitamin A palmitate (500,000 IU/g), 0.2 g vitamin D_3 (cholecalciferol, 500,000 IU/g), 0.075 g vitamin K (phylloquinone), and 974.705 g sucrose.
[b]Supplied per kg of diet: 357 g calcium carbonate, 196 g potassium phosphate monobasic, 70.78 g potassium citrate, 74 g sodium chloride, 46.6 g potassium sulfate, 24.3 g magnesium oxide, 6.06 g ferric citrate, 1.65 g zinc carbonate, 0.63 g manganous carbonate, 0.31 g cupric carbonate, 0.01 g potassium iodate, 0.01025 g sodium selenate, 0.00795 g ammonium paramolybdate, 1.45 g sodium meta-silicate, 0.275 g chromium potassium sulfate, 0.0174 g lithium chloride, 0.0815 g boric acid, 0.0635 g sodium fluoride, 0.0318 g nickel carbonate, hydroxide, tetrahydrate, 0.0066 g ammonium vanadate, and 220.716 g sucrose.
[c]Antioxidant.

imental protocols were approved by the Institutional Animal Care and Use committee at Virginia Polytechnic Institute and met or exceeded guidelines of the National Institutes of Health.

Dietary Treatments and Induction of Colitis

Mice were fed purified diets that represented a modification of the AIN-93G diet commonly used for the growth, pregnancy, and lactation phases of mice[43] in which the nutritional requirements, including those for PUFA, were met or exceeded (Table 1). The optimal doses of PUFA included in these diets were the result of time-course and dose-titration studies designed to elucidate the optimal anti-inflammatory efficacy of each PUFA performed previously (data not shown). Diets were prepared on a weekly basis and feed was replaced on a daily basis to minimize fatty acid oxidation. Stock fatty acid solutions (e.g., soybean oil, CLA, or (n-3) PUFA) were nitrogen-purged every time that the bottles were opened. The CLA source used in the feeding trials consisted of: (1) a 50:50 mixture of the cis-9, trans-11, trans-10, cis-12 CLA isomers, (2) pure (95%) cis-9, trans-11 CLA, or (3) pure (95%) trans-10, cis-12 CLA (Loders Croklaan BV, Channahon, IL). The source of (n-3) PUFA was a 50:50 mixture of eicosapentaenoic and docosahexaenoic acids. All the experimental diets contained the same amount of energy (isocaloric) and protein (isonitrogenous). Results from the preliminary studies using a pig model indicated that feeding CLA early in life had a greater influence on immune system development and disease activity in later stages of development.[44,45] Hence, we first fed the experimental diets to the dams to facilitate transfer of CLA to the pups through the milk. After weaning, pups were administered the experimental diets supplemented with 1 g/100 g of the treatment oil for 42 days before the induction of colitis and throughout the challenge period equivalent to an optimal therapeutic dosage of 45–80 mg/day/mouse. Colitis was induced by challenging mice with 2.5% dextran sodium sulfate (DSS), 36,000–44,000 mol wt (ICN Biomedicals, Aurora, OH) in the drinking water for 7 days. A preliminary experiment compared the colonic anti-inflammatory efficacy of the CLA mixture, (n-3) PUFA, and soybean oil (control diet). Subsequent studies examined the protective efficacy of the CLA mixture and pure CLA isomers (i.e., c9, t11 CLA; and t10, c12 CLA) against DSS colitis.

$CD4^+CD45RB^{hi}$ Adoptive Transfer

Spleens were removed from 6-week-old, C57BL6/J donor mice fed either control or CLA diets and weaned from dams fed the same diet. Splenocytes were released in a Petri dish containing 7 mL of cold phosphate-buffered saline with 0.1% bovine serum albumin (Sigma, St. Louis, MO) and 2 mmol/L ethylenediaminetetraacetic acid (Sigma) by gently crushing the tissue between the frosted ends of 2 microscope slides. Before the adoptive transfer, splenocytes were immunophenotyped for determining the concentrations of PPAR γ and δ in $CD4^+$ T cells from donors. Splenocytes were enriched in $CD4^+$ T cells to 95% purity by using the I-Mag cell separation system (BD Pharmingen, La Jolla, CA). Cells were incubated with a cocktail of biotinylated antibodies (CD4 T

lymphocyte enrichment set; BD Pharmingen) to anti-mouse $CD8^+$ T cells (CD8a; clone 53-6.7), natural killer cells (CD49b; clone HMα2), B cells ($B220^+$; clone RA3-6B2), erythroid cells (TER-119; clone TER-119), and macrophages ($Mac\text{-}1^+$; clone M1/70), and after a secondary incubation with streptavidin-conjugated iron particles, these subsets were depleted by exposing cells to a magnet. Subsequently, $CD4^+CD45RB^{hi}$ or $CD4^+CD45RB^{low}$ T cells were selected positively by fluorescence-activated cell sorter sorting in an EPICS ALTRA cytometer (Coulter Corp, Miami, FL). All populations were more than 98% pure on reanalysis. A total of 4×10^5 cells were injected intraperitoneally into *Scid* recipients (C57BL6/J background).

Assessment of Colitis

After the DSS challenge or the adoptive transfer, mice were weighed on a daily basis and examined for clinical signs of disease associated with colitis (i.e., perianal soiling, rectal bleeding, diarrhea, and piloerection) by blinded observers. For the DSS challenge, the disease activity indices and rectal bleeding scores were calculated using a modification of a previously published compounded clinical score.[46] Briefly, disease activity index 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). Results from preliminary studies showed a high correlation between results of fecal blood by Hemoccult (SmithKline Diagnostics, Inc., San Jose, CA) and visual observations performed by experienced veterinarians. Mice in the DSS study were euthanized on day 7 of the DSS challenge. For the adoptive transfer experiment, only diarrhea/lethargy and weight loss were examined because rectal bleeding was not observed and mice were euthanized at 8 weeks after the transfer.

Histopathology

Colonic sections from the DSS and adoptive transfer studies were fixed in 10% buffered neutral formalin, later embedded in paraffin, and then sectioned (6 μm) and stained with H&E stain for histologic examination. Tissue slides were examined in an Olympus microscope (Olympus America Inc., Dulles, VA). Images were captured using the FlashBus FBG software (Integral Technologies, Indianapolis, IN) and processed in Adobe Photoshop Elements 2.0 (Adobe Systems Inc., San Jose, CA). Colons were graded with a compounded histologic score including the extent of (1) crypt damage, (2) regeneration, (3) metaplasia/hyperplasia, (4) lamina proprial vascular changes, (5) submucosal changes, and (6) presence of inflammatory infiltrates. The sections were graded with a score of 0–4 for each of the previous categories and data were analyzed as a normalized compounded score.

Quantitative Real-Time Reverse-Transcriptase PCR

Total RNA was isolated from the whole colon of mice using the RNA isolation MiniKit (Qiagen, Valencia, CA) according to the manufacturer's instructions. All RNA samples were checked for quality and quantity on the Agilent 2100 BioAnalyzer system (Agilent Technologies, Palo Alto, CA). Total RNA (1 μg) from each sample was used to generate complementary DNA (cDNA) template using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The total reaction volume was 20 μL. The reaction was incubated as follows in a Tetrad Thermocycler (MJResearch, Waltham, MA): 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, hold at 4°C. cDNA products were diluted 1:10 in diethylpyrocarbonate-treated water. The incubations also were performed on controls with no RNA template and omitting the reverse transcriptase enzyme.

The PCR primer pairs were designed based on previously published sequences (GeneBank) using the Oligo 6 primer design software (Molecular Biology Insights, Cascade, CO). The PCR primer pair sequences, annealing temperatures, accession numbers, and PCR product lengths are shown in Table 2. PCR was performed on the cDNA using Taq DNA polymerase obtained from Invitrogen (Carlsbad, CA) and using previously described conditions.[39,47] Each gene amplicon was purified by using the MiniElute PCR Purification kit (Qiagen). The purified amplicon for each gene was quantified on an agarose gel and also with the GeneQuant Pro spectrophotometer (Amersham Biosciences, Piscataway, NJ). These purified amplicons were used further to optimize the real-time PCR conditions and to generate the standard curves in the real-time PCR assay. Primer concentrations and annealing temperatures were optimized for the iCycler iQ System (Bio-Rad) for each set of primers using the system's gradient protocol. PCR efficiencies were maintained at 100% for each primer set during optimization and also during the real-time PCR of sample cDNA.

mRNA expression of TNF-α, PPARs (α, γ, and δ), CD36, transforming growth factor β 1 (TGF-β1), UCP1, UCP3, Gob-4, keratin 20, lipocalin 2, regenerating gene I, and PPAR γ coactivator 1α (PGC-1α) in the colon was examined by real-time quantitative PCR by using an iCycler iQ System and the iQ SYBR Green Supermix (Bio-Rad). Because SYBR Green I is a general double-stranded DNA intercalating dye, it may result in the detection of nonspecific products and primers/dimers as well as the amplicon of interest. To determine the number of products that have been generated in the PCR and the specificity of the assay, a melting curve analysis was performed on each sample. Specifically, after the final extension step of PCR, the temperature was increased to 95°C and then decreased at a rate of 0.5°C per 10 seconds for 140 cycles. Real-time PCR was used to measure the starting amount of nucleic acid by assaying each unknown cDNA on the same 96-well plate. For each gene, the results are presented as starting quantity of cDNA (picograms) per microgram of colonic RNA.

Quantification of Concentration and DNA-Binding Activity of NF-κB

The NF-κB activation was detected and quantified by using the Trans-AM NF-κB p65 enzyme-linked immunosorbent–based assay (Active Motif, Carlsbad, CA). Briefly, frozen

Table 2. Oligonucleotide Sequences for Quantitative Real-Time PCR

Primer	Sequence	Length	Accession number
PPAR αF	5′TGGGGATGAAGAGGGCTGAG3′	143	NM_011144
PPAR αR	5′GGGGACTGCCGTTGTCTGT3′		
PPAR δF	5′ACAGTGACCTGGCGCTCTTC3′	96	U10375
PPAR δR	5′TGGTGTCCTGGATGGCTTCT3′		
PPAR γF	5′CAGGCTTGCTGAACGTGAAG3′	117	NM_011146
PPAR γR	5′GGAGCACCTTGGCGAACA3′		
CD36F	5′CCGGGCCACGTAGAAAACA3′	156	NM_007643
CD36R	5′CCTCCAAACACAGCCAGGAC3′		
UCP1F	5′GCCGGGTTTTGCACCACACT3′	143	NM_009463
UCP1R	5′TTGAAAAAGGCCGTCGGTCC3′		
UCP3F	5′CCGATACATGAACGCTCCC3′	128	AF032902
UCP3R	5′AAGCTCCCAGACGCAGAAAG3′		
TNF-αF	5′CGTGCTCCTCACCCACAC3′	133	M13049
TNF-αR	5′GGGTTCATACCAGGGTTTGA3′		
TGF-β_1F	5′CAACTTCTGTCTGGGACCCT3′	150	NM_011577
TGF-β_1R	5′TAGTAGACGATGGGCAGTGG3′		
PGC-1αF	5′GCCCGGTACAGTGAGTGTTC3′	140	NM_008904
PGC-1αR	5′CTGGGCCGTTTAGTCTTCCT3′		
Regenerating gene IF	5′ACTGTCTCAAGCAGGTGTG3′	116	D14010
Regenerating gene I R	5′TAGGAAAGGAGCAGAGGAAAG3′		
Gob-4F	5′TGGCAGAGCAGTTTGTTCTC3′	149	AB016592
Gob-4R	5′GAGCCGGTTTGAGTATCGTC3′		
Keratin 20F	5′GACCTGTTTGGGGGCAATGG3′	144	AF473907
Keratin 20R	5′CGGCGCGTTGGTTTCGTA3′		
Lipocalin 2F	5′TTTCACCCGCTTTGCCAAGT3′	123	NM_008491
Lipocalin 2R	5′GTCTCTGCGCATCCCAGTCA3′		
β-actinF	5′CCCAGGCATTGCTGACAGG3′	141	X03672
β-actinR	5′TGGAAGGTGGACAGTGAGGC3′		

NOTE. PCR primer pairs were designed for an optimal annealing temperature of 57°C and product length shorter than 157 base pairs.
F, forward; R, reverse.

colons were weighed and diced into smaller pieces using a razor blade in lysis buffer containing dithiothreitol and a protease inhibitor cocktail. No differences in NF-κB activity were detected when using frozen vs. fresh colonic samples in this assay. The tissue was disrupted further by using a dounce ground-glass homogenizer at 4°C. The tissue homogenate was centrifuged for 10 minutes at 10,000 × g at 4°C. The supernatant was used to perform a Bradford-based assay for quantifying the protein concentrations and was stored at −80°C. The 96-well plates were coated with binding buffer containing double-stranded oligonucleotides with the NF-κB consensus site (i.e., 5′-GGGACTTTCC-3′). The whole-cell extracts were added to the wells, incubated for 1 hour at room temperature, and washed 3 times with 200 μL of washing buffer. One hundred microliters of anti–NF-κB p65 primary antibody (1:1000 dilution in binding buffer) were added to all wells, incubated for 1 hour at room temperature, and washed 3 times. One hundred microliters of horseradish peroxidase–conjugated secondary antibody dilution (1:1000) was added to the wells, incubated for 1 hour, and washed 4 times. One hundred microliters of the developing solution was added and incubated for 5–10 minutes at room temperature. When the color became dark blue, 100 μL of stop solution was added. Absorbance was measured at 450 nm by using a μQuant microplate reader (Bio-Tek, Winooski, VT). Recombinant NF-κB p65 protein was used as a standard for estimation of the amounts of activated NF-κB.

Statistical Analyses

Data are expressed as the means ± standard error of the mean. Parametric data were analyzed by using the analysis of variance followed by Scheffe's multiple comparison method as previously described.[47] Nonparametric data were analyzed by using the Mann–Whitney *U* test followed by a Dunn's multiple comparisons test. Analysis of variance was performed by using the general linear model procedure of SAS (SAS Institute Inc., Cary, NC).[48] Statistical significance was assessed at a P value of < 0.05.

Results

CLA Fed in Early Life Protects Against DSS Colitis at Later Stages of Development

In a preliminary study, we sought to investigate the therapeutic efficacy of CLA and (n-3) PUFA in DSS colitis. A challenge with 2.5% DSS in the drinking water[49] for 7 days induced weight loss and colitis-associated clinical disease (Figure 1). Preliminary experiments established an optimal dose of fatty acids of 1 g/100 g of diet (data not shown). Overall, CLA ameliorated the severity of colitis more effectively than (n-3) PUFA or the control diet (Figure 1). The improved weight loss response to DSS observed in CLA-fed mice

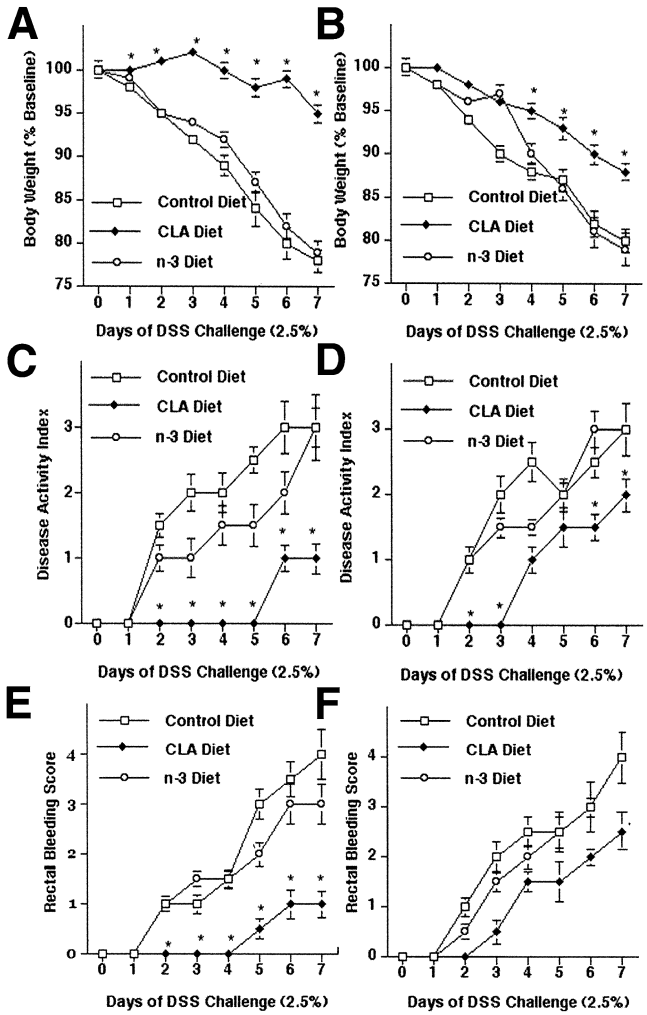

Figure 1. Effects of dietary CLA (1 g/100 g) and an isocaloric control diet on body weight loss, disease activity indices, and rectal bleeding during a 7-day DSS challenge (2.5% wt/vol). (*A, C, E*) Mice that received CLA before weaning through the placenta and milk, and after weaning for 42 days. (*B, D, F*) Mice that received CLA after weaning for 42 days before the DSS challenge. Colitis-associated disease was improved by dietary CLA supplementation in mice receiving CLA at very early stages of development. No significant differences in clinical scores were found between mice fed the control or the CLA diets not challenged with DSS (data not shown). $*P < 0.05$. Data represent mean ± SEM (n = 8 mice per group).

correlated with improved disease activity indices and rectal bleeding scores (Figure 1). In addition, administration of CLA early in life (Figure 1*A*, *C*, and *E*) prevented colitis and colitis-associated disease more effectively than administration of CLA after weaning (Figure 1*B*, *D*, and *F*).

Histopathologic examination of the colons recovered from mice fed the control diet revealed the presence of edema with enlargement of submucosal and lamina proprial spaces, increased mucosal thickness, multifocal, lymphocytic, and polymorphonuclear cell infiltrates, crypt hyperplasia without regeneration, and epithelial erosion (Figure 2*C* and *F*). Conversely, the tissue damage associated with DSS colitis in wild-type mice fed CLA (Figure 2*B* and *E*) was very mild and the overall colonic architecture resembled that of healthy colons from non-DSS controls (Figure 2*A* and *D*).

Identification of the Active CLA Isomer(s)

The source of CLA used in the studies listed earlier was a 50:50 mixture of cis-9, trans-11 and trans-10, cis-12 CLA isomers. To determine which CLA isomer was more effective against IBD, the therapeutic efficacy of the pure CLA isomers and the CLA mixture was examined during a DSS challenge. Weight loss and clinical disease were more accentuated in mice fed the control diet or purified isomer preparations than in mice fed the 50:50 CLA mixture (Figure 3). Hence, the presence of both CLA isomers was necessary for optimal protection against DSS colitis.

Modulation of PPAR γ and δ Transcriptional Activities and NF-κB p65 Activation by CLA

Previously, we showed that dietary CLA supplementation enhanced PPAR γ expression in the colons of pigs after the onset of bacterial-induced colitis.[39] In this study, we sought to investigate the nutritional regulation of PPAR γ, δ, and their target genes. Quantitative real-time PCR analysis showed that the concentrations of PPAR γ mRNA in healthy colons of mice fed CLA not challenged with DSS were greater than in colons of mice fed the control diet (Figure 4*A*). Additionally, the colonic concentrations of PPAR δ were higher in non–DSS-challenged mice fed the control diet than CLA-fed mice (Figure 4*A*). After the 7-day DSS challenge, PPAR γ mRNA expression was maintained in colons of CLA-fed mice, whereas it was shut down in mice fed the control diet (Figure 4*B*). In regard to PPAR δ, the mRNA concentrations were increased slightly in mice fed CLA after the DSS challenge. In contrast, in mice fed the control diet, the DSS challenge decreased PPAR δ mRNA to undetectable concentrations (Figure 4*B*). Similar levels of PPAR α were observed between treatment groups (data not shown).

PPAR γ activation results in decreased NF-κB activity and transcriptional regulation of PPAR γ-responsive genes.[38,50] We examined whether the induction of PPAR γ and δ was associated with a transcriptional activity consistent with PPAR γ and δ activation and suppression of NF-κB activity. Specifically, we used quantitative real-time PCR to compare the mRNA expression of genes that are controlled by selective agonists of PPAR δ (i.e., UCP1 and UCP3 induced) or γ (i.e., CD36, PGC-

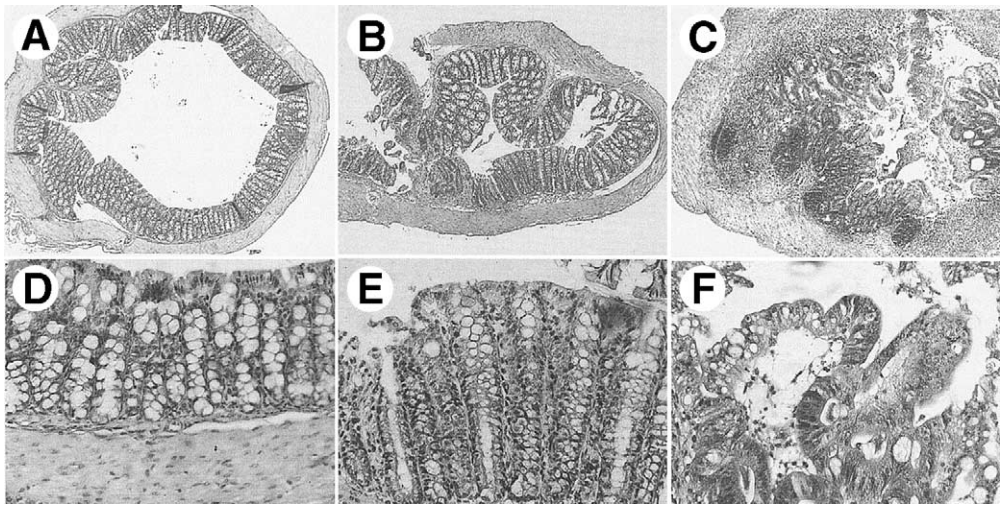

Figure 2. Photomicrographs of H&E-stained slides of paraffin-embedded colonic tissue recovered from (*A*, *D*) non–DSS-challenged mice, (*B*, *E*) mice fed the CLA, or (*C*, *F*) control diets and challenged with 2.5% DSS for 7 days. At a magnification of 40×, a thickening of the colonic mucosa was observed in (*C*) the group fed the control diet, when compared with (*B*) the CLA-fed group or (*A*) the nonchallenged group. (*F*) At a magnification of 400×, the group fed the control diet showed inflammatory cell infiltration, flattening of epithelial cells, and epithelial erosion. (*D*, *E*) The group fed the CLA diet showed a moderate thickening of the colonic mucosa without erosion or infiltration. The epithelial cells maintained their normal columnar shape. Mucus-producing cells were more abundant in colons of CLA-fed mice challenged with DSS or non–DSS-challenged mice. The tissues are representative of 8 mice from each experimental group.

1α, Keratin 20 induced, and Gob-4, TNF-α repressed) in colons of mice fed control or CLA-supplemented diets before and after weaning. We found that CLA induced UCP1, UCP3, CD36, PGC-1α; Keratin 20 and repressed Gob-4 and TNF-α. Regenerating gene I is a gene repressed by synthetic PPAR γ ligands[34] that induces proliferation of pancreatic β and acinar cells.[51] Although CLA caused a decrease in regenerating gene I mRNA expression, this numeric difference was not statistically significant (data not shown). The DSS challenge enhanced NF-κB p65 activation in mice fed the control diet, whereas the concentrations of activated NF-κB p65 in mice fed CLA resembled that of the nonchallenged mice (Figure 5). Overall, the transcriptional profiles were consistent with activation of PPAR γ and δ and the decreased activation of NF-κB p65 in the colon was consistent with activation of PPAR γ.

Although TGF-β1 has not been identified as a PPAR γ or δ target gene, its expression was significantly greater in colons of CLA-fed mice than those fed the control diet (Figure 6*H*). TGF-β1 is an anti-inflammatory cytokine produced by regulatory T cells and epithelial cells.[7,13] A member of the lipocalin family (e.g., neutrophil gelatinase-associated lipocalin) was induced in human epithelial cells by synthetic PPAR γ ligands.[34] Members of this family are characterized by their ability to transport lipid molecules. We examined mouse colonic lipocalin 2 expression, which was induced after the DSS challenge. However, no significant differences in expression of colonic lipocalin 2 were detected when comparing control- and CLA-fed mice (data not shown).

Dependence of the Protective Effect of CLA on the Presence of PPAR γ in the Colon

Whole-body deletion of PPAR γ is lethal by day E10.[52] Therefore, it has not been possible to investigate directly if colonic PPAR γ is required for the maintenance of intestinal homeostasis and prevention of IBD other than by using PPAR γ heterozygotes.[18] To overcome this obstacle, we generated tissue-specific PPAR γ null mice[40,42] by breeding PPAR γ floxed (fl/fl) mice with transgenic mice carrying the Cre-gene under control of the MMTV-LTR promoter.[41] The transgenic recombinase is expressed in epithelial and hemopoietic cells. PPAR $\gamma^{fl/fl}$ Cre^{+} express a truncated form of mRNA transcript and do not express PPAR γ protein.[40] The deletion of exon 2 of the PPAR γ gene in mouse colon was examined by PCR analysis. We showed recombinase activity in the colons of PPAR $\gamma^{fl/fl}$ Cre^{+} mice by detecting the recombination product resulting from the deletion of exon 2 of the PPAR γ gene (Figure 7). Cre^{+} and Cre^{-} littermates fed control of CLA-supplemented diets were challenged with 2.5% DSS in the drinking water for 7 days. The CLA mixture fed early in life did not protect PPAR $\gamma^{fl/fl}$ Cre^{+} mice from DSS colitis, although it protected PPAR $\gamma^{fl/fl}$ Cre^{-} mice (Figure 8).

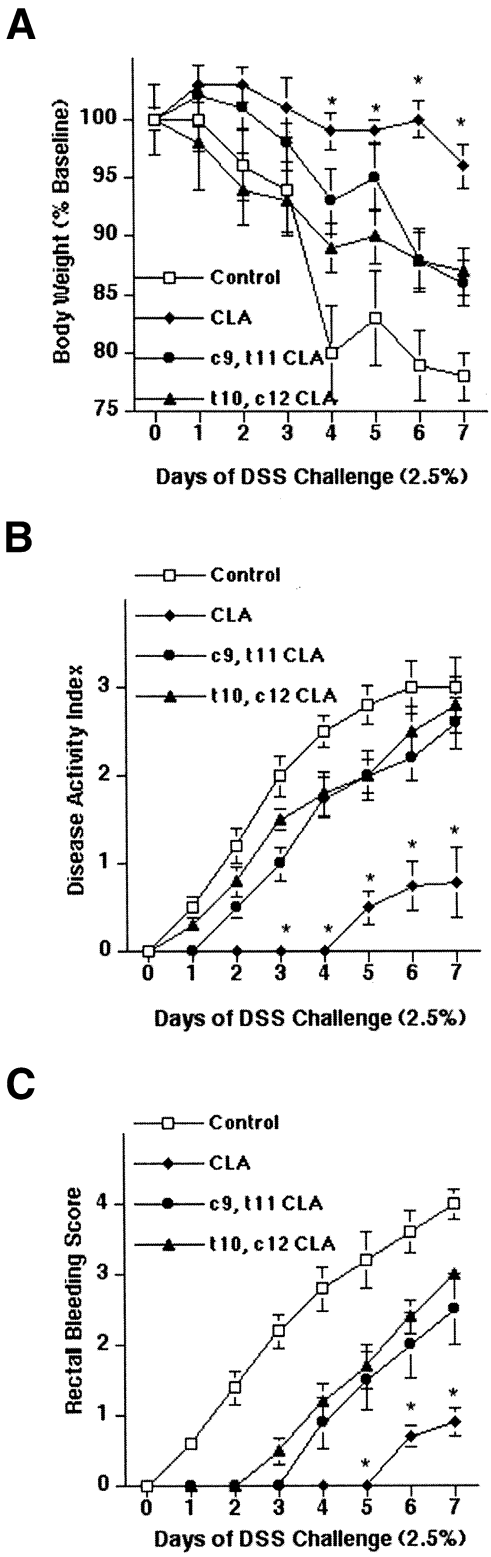

Figure 3. Effects of a CLA mixture (50% cis-9, trans-11 and 50% trans-10, cis-12 CLA), pure cis-9, trans-11 CLA, pure trans-10, cis-12 CLA, or a control diet on colitis-associated disease during a 7-day challenge with DSS (2.5% wt/vol). Optimal clinical protection against DSS colitis was obtained in mice receiving the CLA mixture. $*P < 0.05$. Data represent mean ± SEM (n = 8 mice per group).

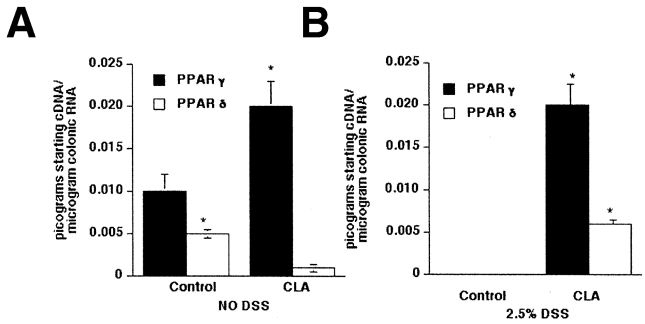

Figure 4. Quantification of mRNA expression of PPAR γ and PPAR δ in (*A*) healthy or (*B*) DSS-challenged colons of mice fed a control or CLA-supplemented (1 g/100 g) diet by using reverse-transcriptase, real-time PCR. No significant differences in expression of the housekeeping gene (i.e., β-actin) were found between treatment groups. $*P < 0.05$. Data represent mean ± SEM (n = 8 mice per group).

Modulation of $CD4^+CD45RB^{hi}$ T-Cell–Induced Colitis by CLA

Tissue injury in the DSS colitis model is caused primarily by activation of macrophages and apoptosis of colonic epithelial cells. However, dysregulated immune responses such as stimulated differentiation of $CD4^+$ T cells toward a T helper 1 phenotype also contribute to the pathogenesis and severity of IBD.[53,54] We aimed to investigate whether oral administration of CLA prevented or ameliorated the colitogenic effects of the $CD4^+CD45RB^{hi}$ T-cell subset. This population was isolated from the spleens of donor mice fed control or CLA-supplemented diets, transferred into immunologically naive recipients (*Scid*), and tested for its colitogenic effects (Figure 9). *Scid* mice transferred with $CD4^+CD45RB^{low}$ T cells were used as negative controls for the colonic immunopathology. Our results indicate that *Scid* mice receiving cells from donors fed the control diet developed severe colitis characterized by increased mucosal thickness (Figure 10*A* and *B*). Lesions were milder in mice that received $CD4^+CD45RB^{hi}$ T cells isolated from CLA-fed donors (Figure 10*E* and *F*). No lesions were observed after the transfer of $CD4^+CD45RB^{low}$ T cells (Figure 10*C* and *D*).

Discussion

Traditional therapies against IBD are modestly successful but result in significant side effects, including immune suppression and enhanced susceptibility to cancer and infectious disease. Because the cause is unknown, the majority of therapies target the symptoms but not the cause of IBD. The present study was designed to identify novel interventions with no side effects that can be applied to the prevention and/or treatment of human enteric inflammatory disorders while maintaining opti-

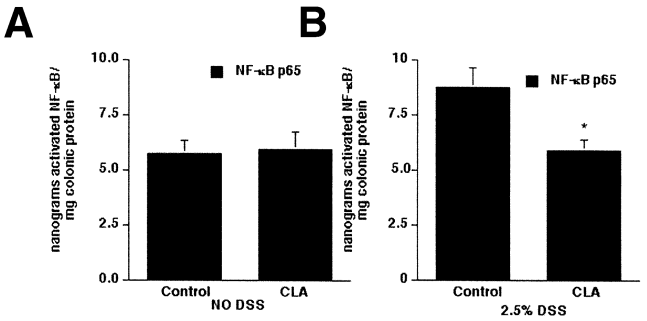

Figure 5. Concentration of activated NF-κB p65 in (A) healthy or (B) DSS-challenged colons was determined by using an enzyme-linked immunosorbent–based assay. The concentrations of activated NF-κB in colons of DSS-challenged mice are consistent with activation of PPAR γ. Recombinant protein was used as a standard for estimation of the amounts of activated NF-κB p65 in vivo. $*P < 0.05$. Data represent mean ± SEM (n = 8 mice per group).

mal levels of immune surveillance. Previous studies have shown that PPAR γ gene therapy enhances PPAR γ expression, resulting in dramatic therapeutic benefits in the DSS colitis model.[17] We found that dietary CLA induced colonic PPAR γ expression and provided protection against disease in a pig model of bacterial-induced colitis.[39] Furthermore, studies in animal models and humans show that CLA suppresses inflammation while enhancing antigen-specific responses against viral and bacterial pathogens.[45,55] Therefore, CLA protects from colitis while maintaining or enhancing immune responsiveness.

Although PPAR γ is recognized as a central inhibitor of intestinal inflammation in DSS colitis,[15,18,56,57] initial studies in mice did not show protection against DSS colitis by CLA. In these studies, CLA treatment was initiated at weaning (21 days of age); earlier studies in pigs administered CLA at earlier stages of development showed protection from disease.[39] By incorporating CLA in the mouse diet very early in life (i.e., supplementing the diets of dams with CLA), the protective efficacy of CLA against DSS colitis increased significantly. This suggests that CLA may regulate molecular events related to cellular differentiation occurring at early stages of development. These early developmental events influence the susceptibility to enteric disease at later stages of development. The greater efficacy of the CLA mixture when compared with pure CLA isomer preparations is consistent with previous results of a human clinical trial that examined the immunomodulatory effects of CLA.[58]

The DSS colitis model resembles human UC because it represents a diffuse change without segmentation confined primarily at the mucosal level. We reported that expression of PPAR γ and δ but not PPAR α was decreased after the onset of DSS colitis.[59] This finding is in line with results of a human study showing that the colonic concentrations of PPAR γ mRNA and protein in UC patients were lower than in healthy controls, whereas UC did not change PPAR α expression.[60] Interestingly, 8% of primary colorectal tumors in humans carried a loss-of-function mutation in one allele of the PPAR γ gene, also suggesting a role of PPAR γ in tumor suppression.[61] The interrelationship between chronic colonic

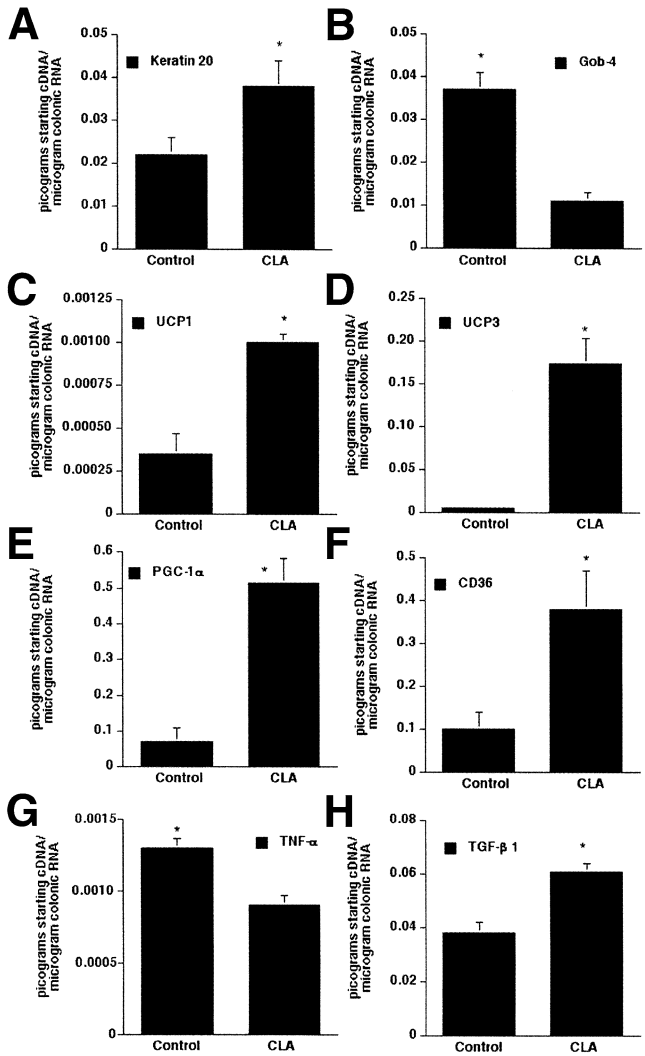

Figure 6. Quantification of mRNA expression of (*A*) Keratin 20, (*B*) Gob-4, (*C*) UCP1, (*D*) UCP3, (*E*) PGC-1α, (*F*) CD36, (*G*) TNF-α, and (*H*) TGF-β1 in colons of mice fed a control or CLA-supplemented (1 g/100 g) diet by using reverse-transcriptase, real-time PCR. No significant differences in expression of the housekeeping gene (i.e., β-actin) were found between treatment groups. Colonic samples used in both assays were collected at the end of a 7-day DSS challenge (2.5% wt/vol). The changes in expression genes involved in lipid metabolism (UCP1, UCP3, and CD36), colonic epithelial cell maturation (Keratin 20 and Gob-4), and inflammation (TNF-α), as well as the co-activator molecule PGC-1α are consistent with activation of PPAR γ and δ. TGF-β1 has not been identified as a PPAR γ target gene but its expression also can be modulated by CLA. $*P < 0.05$. Data represent mean ± SEM (n = 8 mice per group).

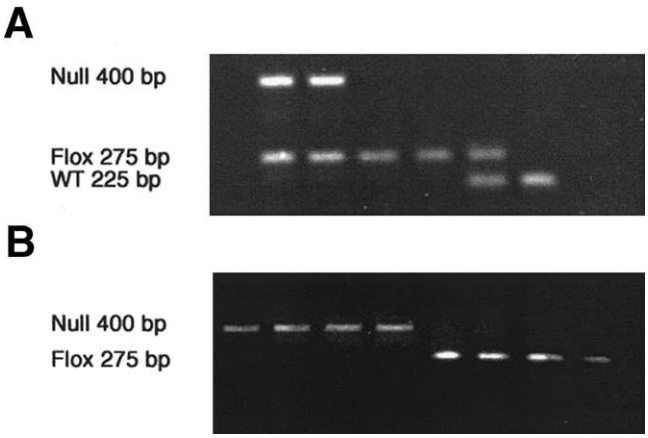

Figure 7. Conditional deletion of the PPAR γ gene in mouse colon. MMTV-Cre–mediated recombination of the PPAR γ gene in the (*A*) tail clippings and the (*B*) colon by PCR analysis. The null band (400 bp) is the recombination product after deletion of exon 2 of the PPAR γ gene (primers F/R2). The flox band (275 bp) is the floxed (fl) allele (primers F/R1). The wild-type (WT) allele of PPAR γ gave a 225-bp band. (*A*) Left to right: depicts fl/fl with recombination in the tail (Cre^+) (*lanes 1–2*), fl/fl without recombination (Cre^-) (*lanes 3–4*), fl/WT (*lane 5*), and WT (*lane 6*). (*B*) Left to right: depicts fl/fl with efficient recombination in the colon (Cre^+) (*lanes 1–4*) and fl/fl without recombination (Cre^-).

pathologies such as IBD and/or colon cancer and expression of PPAR δ has not been examined in humans.

We showed that PPAR γ mRNA expression is induced by CLA in colons of both healthy and DSS-challenged mice. Additionally, the shut down of colonic PPAR γ and δ mRNA expression induced by DSS was prevented by CLA treatment. Additional changes in gene expression caused by CLA treatment provide in vivo evidence of PPAR γ– and δ–dependent transcriptional activities. Specifically, CLA increased the mRNA expression of UCP1, UCP3, CD36, Keratin 20, and PGC-1α significantly, while suppressing Gob-4 and TNF-α. UCP1 and UCP3, 2 genes involved in energy oxidation and uncoupling, are induced by PPAR δ agonists.[62] However, the activation of PPAR δ caused no significant changes on CD36 expression, a gene involved in lipid storage, or Gob-4, a gene expressed by goblet cells and involved in epithelial cell maturation.[63] Keratin 20 is a gene expressed in the most differentiated intestinal epithelial cells, where the greatest concentrations of PPAR γ can be found.[35,36,64,65] Selective PPAR γ agonists modulated the expression of CD36, Gob-4, and Keratin 20, whereas activation of PPAR δ by synthetic agonists induced UCP1 and UCP3 expression.[34,62,66]

PPAR γ–mediated transcriptional regulation involves ligand binding, dimerization with retinoid X receptor α, dissociation from co-repressors and clustering with co-activators, nuclear translocation, and binding to DNA response elements.[28] Both the ligand binding and changes in the composition of co-repressors and co-activators are necessary for effective transcriptional regulation. We found that the potent co-activator of both PPAR γ[67] and δ[62] and regulator of mitochondrial biogenesis,[68] PGC-1α, can be induced by CLA. In contrast, PPAR γ–binding protein, another co-activator of PPAR γ, was induced in mice fed the control diet.[59] The ability of linoleic acid and CLA to modulate co-activator expression (PPAR γ–binding protein and PGC-1α, respectively) differentially may delineate their regulatory effects on gene transcription in vivo. In support of this hypothesis, Wu et al.[69] reported that subtle differences in ligand structure are perceived by PPAR γ and trans-

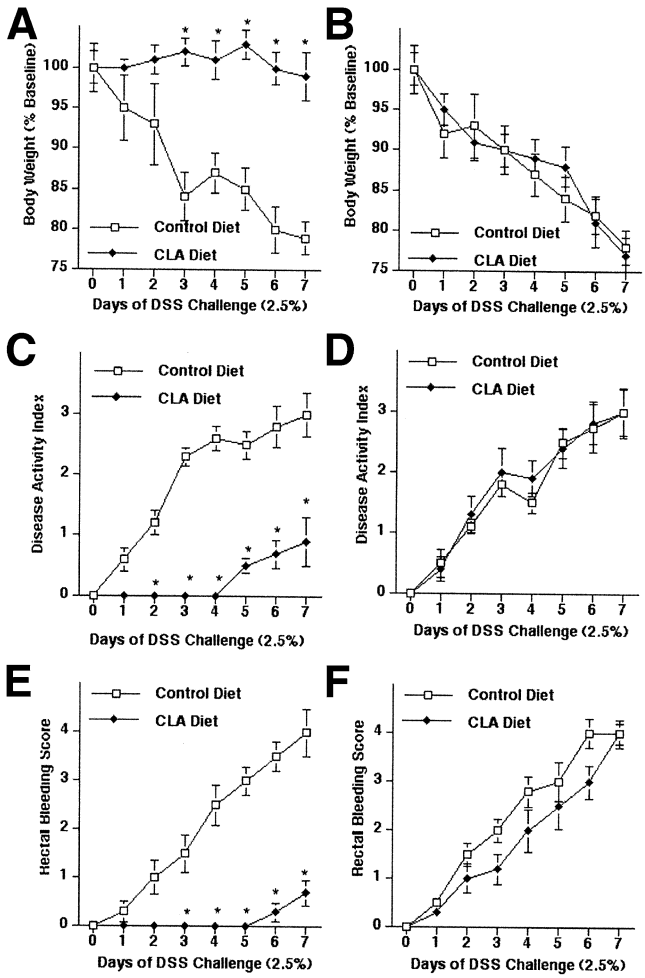

Figure 8. Effects of dietary CLA (1 g/100 g) on protection against colitis in (*A*, *C*, *E*) mice expressing the exon 2 of the PPAR γ gene in the colon (PPAR $\gamma^{fl/fl}$ Cre^-) or (*B*, *D*, *F*) mice lacking the exon 2 of the PPAR γ gene (PPAR $\gamma^{fl/fl}$ Cre^+) owing to MMTV-Cre–mediated recombination. Colitis-associated disease in the group fed the control diet was compared with the CLA-fed group in terms of (*A*, *B*) body weight loss, (*C*, *D*) disease activity index, and (*E*, *F*) rectal bleeding. These measurements were performed throughout a 7-day DSS challenge (2.5% wt/vol). Colitis improvement in CLA-fed mice was observed in PPAR $\gamma^{fl/fl}$ Cre^-but not in PPAR $\gamma^{fl/fl}$ Cre^+ mice. $^*P < 0.05$. Data represent mean ± SEM (n = 8 mice per group).

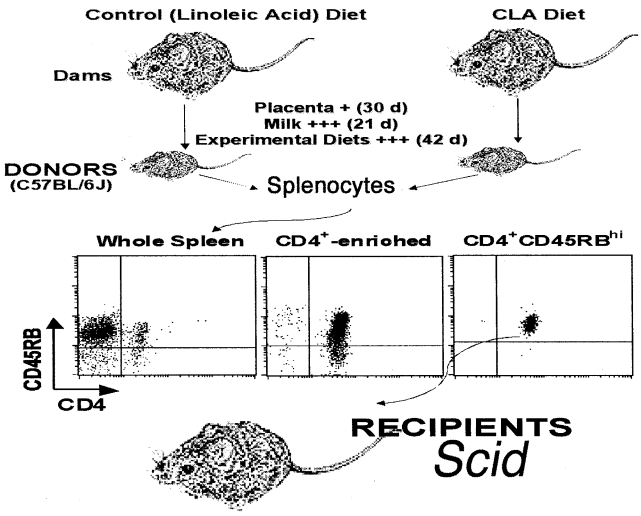

Figure 9. C57BL/6J mice were fed a basal AIN-93G diet in which 14.28% of the soybean oil (i.e., 1 g/100 g diet) was replaced by linoleic acid or the CLA isomeric mixture. The offspring were weaned at 3 weeks of age, given the same experimental diet for 42 additional days, and used as donors in adoptive transfer studies. Splenocytes obtained from donor mice were enriched in $CD4^+$ T cells by magnetic negative selection using the I-Mag cell separation system (BD Pharmingen) and $CD4^+$ $CD45RB^{hi}$ T cells were sorted by a fluorescence-activated cell sorter. *Scid* recipient mice were transferred (intraperitoneally) 4×10^5 $CD4^+$ $CD45RB^{hi}$ T cells obtained from either CLA- or control diet–fed mice. *Scid* mice receiving the same amount of $CD4^+$ $CD45RB^{lo}$ T cells were controls.

lated into a unique display of co-activator–receptor combinations.

TNF-α is a cytokine produced by macrophages, T cells,[70] and epithelial cells[71] that promotes intestinal inflammation. The efficacy of the Food and Drug Administration–approved, anti–TNF-α chimeric monoclonal immunoglobulin G1 antibody in the acute treatment and management of fistulas in CD patients[1] provides a rationale for examining the effect of novel therapies on TNF-α expression. Synthetic PPAR γ and δ agonists such as rosiglitazone and GW0742 repressed several lipopolysaccharide-responsive genes (i.e., inducible nitric oxide synthase, interleukin 12 p40, cyclooxygenase-2), but not TNF-α in vitro.[50] In contrast, the selective PPAR γ agonist pioglitazone repressed TNF-α expression in a rat model of ischemia and reperfusion.[72] Hence, based on the controversial results of studies using synthetic PPAR γ agonists, the suppressive effect of CLA on TNF-α could be interpreted as a PPAR γ–dependent or –independent mechanism. In contrast, more definitive evidence shows that TNF-α expression is not regulated through a PPAR δ–dependent mechanism. Specifically, the expression of TNF-α remained unchanged between wild-type and PPAR $\delta^{-/-}$ mouse macrophages or in RAW 264.7 cells (a mouse macrophage cell line) overexpressing PPAR δ.[73]

In contrast, TGF-β1 has not been identified as a PPAR γ–responsive gene; therefore, the stimulatory effect of CLA on colonic TGF-β1 expression may be PPAR γ–independent. However, PPAR γ and TGF-β1 signaling pathways share a downstream component (i.e., TGF-stimulated clone-22), which is abundant in postmitotic epithelial cells and plays an important role in intestinal epithelial cell growth and differentiation.[13] Further investigations are required to clarify whether TGF-β1 is a PPAR δ–responsive gene.

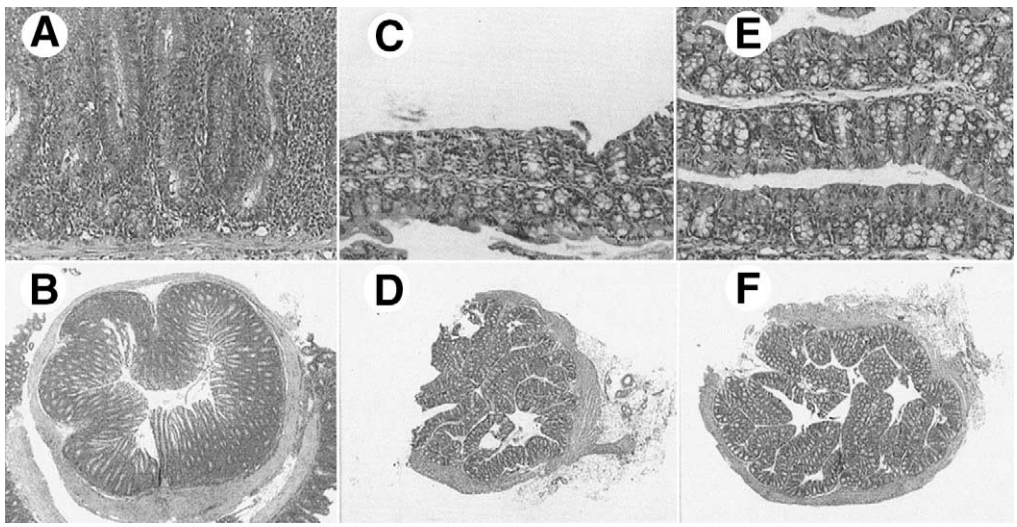

Figure 10. (*A–F*) Representative photomicrographs of the colons of *scid* recipient mice after transfer of $CD4^+$ T cells. (*C*, *D*) The colons in *scid* recipients restored with $CD4^+CD45RB^{low}$ T cells were histologically normal. (*A*, *B*) Conversely, mice receiving 4×10^5 $CD4^+CD45RB^{high}$ T cells from donors fed the control diet developed severe colitis characterized by enlargement of the colonic mucosa and lymphocytic infiltrates. (*E*, *F*) Oral administration of CLA to the donors contributed to attenuation of the development of colitis induced by transfer of $CD4^+CD45RB^{high}$ T cells. (Top) Original magnification: 250×; and (bottom) 40× (n = 8 recipient mice per group).

CLA induced PPAR γ and δ expression in the colon and modulated their ability to induce or repress target genes involved in epithelial cell differentiation, lipid homeostasis, and immunoregulation. Although the role of PPAR γ in IBD has been examined extensively and found to be beneficial, the role of PPAR δ is not understood completely. A recent study shows that activated PPAR δ represses atherogenic inflammation by suppressing the expression of macrophage inflammatory mediators.[73] Lee et al.[73] proposed that PPAR δ may control an inflammatory switch by virtue of its association (proinflammatory) and disassociation (anti-inflammatory) with transcriptional repressors such as BCL-6. In this regard, our data show that the concentrations of PPAR δ in healthy colons of mice fed CLA are lower than in control-fed mice. However, PPAR δ can be induced significantly in colons of CLA-fed mice after an inflammatory stimulus, indicating that PPAR δ may elicit anti-inflammatory actions after an inflammatory challenge. Moreover, PPAR δ activation resulted in increased resistance of epithelial cells to death and improved wound healing after injury,[14] suggesting positive effects of PPAR δ activation on tissue restitution after an acute episode of IBD.

Increased concentrations of activated NF-κB have been detected in the intestinal mucosa of patients with IBD.[60] Therefore, the suppression of NF-κB p65 activity by CLA is of significance to the prevention of human IBD. Synthetic ligands of PPAR γ down-regulate the expression of proinflammatory genes by antagonizing the activity of NF-κB.[38] We have found that the DSS challenge increases NF-κB p65 activation. However, the concentrations of activated NF-κB p65 in the colons of CLA-fed mice were significantly lower than those of mice fed the control diet. The effects of CLA on the NF-κB pathway in vivo are consistent with in vitro results showing that lipopolysaccharide-stimulated RAW 264.7 macrophages cultured in the presence of CLA resulted in diminished DNA-binding affinities of NF-κB.[74] Although in vivo studies directly examining the regulation of NF-κB activation by PPAR δ are not available, selective PPAR δ agonists did not inhibit the nuclear entry or DNA-binding activity of NF-κB p65 in PPAR $\gamma^{-/-}\delta^{+/+}$ macrophages in vitro,[50] suggesting that PPAR δ repressed inflammatory gene expression through a NF-κB–independent mechanism.

Genetic approaches using murine models in which the PPAR γ gene has been disrupted provide the most definitive information regarding the effect of PPAR γ on IBD[3] and the dependence of the protective effect of drugs, nutrients, and nutraceuticals on the presence of PPAR γ. Because whole-body PPAR $\gamma^{-/-}$ mice are not viable owing to defects in placental vascularization and extensive myocardial thinning, we developed tissue-specific PPAR $\gamma^{-/-}$ mice lacking the exon 2 of the PPAR γ gene in epithelial and hematopoietic cells. In this model of tissue-specific recombination, the most abundant cell types in the colon, including epithelial cells, T and B cells, macrophages, and neutrophils, lack the PPAR γ gene. Our findings indicate that the loss of the PPAR γ gene in the colon abrogated the beneficial effects of CLA in DSS colitis, suggesting that the protective effects of CLA are mediated, in part, through a PPAR γ–dependent mechanism. In this regard, the colons of tissue-specific PPAR $\gamma^{-/-}$ mice resembled those of DSS-challenged mice fed the control diet, in which PPAR γ expression was shut down completely and clinical disease was more severe. The role of PPAR δ on IBD remains largely unknown. Further studies are required to determine the role of PPAR δ on intestinal homeostasis and to identify clinically important synergisms between PPAR γ and δ.

In contrast to DSS colitis, which targets primarily macrophages and epithelial cells,[7] the pathogenesis of the adoptive transfer (i.e., $CD4^+CD45RB^{hi}$) model of colitis favors dysregulated $CD4^+$ T-cell responses.[75] In this separate colitis model, chronic intestinal inflammation was caused by polarization of naive $CD4^+$ T cells into a T helper 1 phenotype in response to stimulation with antigens of the resident intestinal microflora. The adoptive transfer model resembles human CD because T helper 1 polarization also is enhanced in the colonic mucosa of patients with CD.[76,77] We found that CLA fed to donor mice protected recipient mice from $CD4^+$ T-cell–induced colitis. Further, the protective effects of CLA on $CD4^+$ T-cell–induced colitis were consistent with results from a previous study showing the ability of CLA to ameliorate bacterially induced colitis in pigs.[39] Our previous model resembled $CD4^+$ T-cell–induced IBD because a factor of bacterial origin triggered the onset of enteric disease by stimulating polarization of $CD4^+$ T cells toward a T helper 1 phenotype.[39,78] We hypothesize that PPAR γ and δ contribute to the maintenance of intestinal homeostasis through a mechanism of intrinsic regulation of $CD4^+$ T-cell function. Future studies will investigate the molecular regulatory mechanisms by which PPAR γ and δ modulate $CD4^+$ T-cell function and differentiation at the intestinal mucosal level.

Katayama et al.[17] envisioned PPAR γ gene therapy as an adjunct intervention to enhance the limited therapeutic efficacy of synthetic PPAR γ ligands in established colitis. However, they reported that PPAR γ gene therapy alone also was effective in suppressing inflammation

and attributed this finding to the action of endogenous agonists. PUFA and their metabolites (i.e., hydroxy derivatives of fatty acids, prostaglandins, and so forth)[29–31] were identified as endogenous PPAR γ agonists. Previously, we showed that the endogenous concentrations of CLA in particular, and the overall fatty acid metabolome, could be regulated nutritionally by supplementing diets with this compound.[39] This therapeutic intervention induced PPAR γ and δ expression as well as PPAR γ and δ target genes involved in lipid metabolism, repressed PPAR γ target pro-inflammatory genes, and induced or repressed target genes involved in epithelial cell maturation. All of which resulted in attenuated disease severity and inflammatory lesions during experimental IBD. Moreover, we provide molecular evidence in vivo showing that the improved enteric disease phenotype observed in CLA-fed mice is mediated through PPAR γ. Finally, our data suggest that CLA also contributed to stabilize immune homeostasis in vivo by regulating $CD4^+$ T-cell function. In conclusion, our experimental approaches have confirmed that PPAR γ is a molecular target for therapeutic interventions against IBD, identified PPAR δ as a potential target, and characterized important cellular and molecular mechanisms by which a natural PPAR γ agonist ameliorates IBD.

References

1. Lichtenstein GR, Abreu M, Present D. Recent advances in the treatment of Crohn's colitis. Killingworth, CT: The Center for Health Care Education, LLC, 2003.
2. Camilleri M. GI clinical research 2002-2003: the year in review. Clin Gastroenterol Hepatol 2003;1:415–420.
3. Wu GD. Is there a role for PPARγ in IBD? Yes, no, maybe. Gastroenterology 2003;124:1265–1315.
4. Schoonjans K, Staels B, Auwerx J. Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. J Lipid Res 1996;37: 907–925.
5. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 1994;8:1224–1234.
6. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 1994;79:1147–1156.
7. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. Annu Rev Immunol 2002;20:495–549.
8. Di Sabatino A, Ciccocioppo R, Luinetti O, Ricevuti L, Morera R, Cifone MG, Solcia E, Corazza GR. Increased enterocyte apoptosis in inflamed areas of Crohn's disease. Dis Colon Rectum 2003; 46:1498–1507.
9. Jones DC, Ding X, Daynes RA. Nuclear receptor peroxisome proliferator-activated receptor alpha (PPARalpha) is expressed in resting murine lymphocytes. The PPARalpha in T and B lymphocytes is both transactivation and transrepression competent. J Biol Chem 2002;277:6838–6845.
10. Cunard R, DiCampli D, Archer DC, Stevenson JL, Ricote M, Glass CK, Kelly CJ. WY14,643, a PPAR alpha ligand, has profound effects on immune responses in vivo. J Immunol 2002;169: 6806–6812.
11. Wang YL, Frauwirth KA, Rangwala SM, Lazar MA, Thompson CB. Thiazolidinedione activation of peroxisome proliferator-activated receptor gamma can enhance mitochondrial potential and promote cell survival. J Biol Chem 2002;277:31781–31788.
12. Natarajan C, Bright JJ. Peroxisome proliferator-activated receptor-gamma agonists inhibit experimental allergic encephalomyelitis by blocking IL-12 production, IL-12 signaling and Th1 differentiation. Genes Immun 2002;3:59–70.
13. Gupta RA, Sarraf P, Brockman JA, Shappell SB, Raftery LA, Willson TM, DuBois RN. Peroxisome proliferator-activated receptor gamma and transforming growth factor-beta pathways inhibit intestinal epithelial cell growth by regulating levels of TSC-22. J Biol Chem 2003;278:7431–7438.
14. Tan NS, Michalik L, Noy N, Yasmin R, Pacot C, Heim M, Fluhmann B, Desvergne B, Wahli W. Critical roles of PPAR beta/delta in keratinocyte response to inflammation. Genes Dev 2001;15: 3263–3277.
15. Su CG, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, Flanigan A, Murthy S, Lazar MA, Wu GD. A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response. J Clin Invest 1999;104:383–389.
16. Saubermann LJ, Nakajima A, Wada K, Zhao S, Terauchi Y, Kadowaki T, Aburatani H, Matsuhashi N, Nagai R, Blumberg RS. Peroxisome proliferator-activated receptor gamma agonist ligands stimulate a Th2 cytokine response and prevent acute colitis. Inflamm Bowel Dis 2002;8:330–339.
17. Katayama K, Wada K, Nakajima A, Mizuguchi H, Hayakawa T, Nakagawa S, Kadowaki T, Nagai R, Kamisaki Y, Blumberg RS, Mayumi T. A novel PPARgamma gene therapy to control inflammation associated with inflammatory bowel disease in a murine model. Gastroenterology 2003;124:1315–1324.
18. Desreumaux P, Dubuquoy L, Nutten S, Peuchmaur M, Englaro W, Schoonjans K, Derijard B, Desvergne B, Wahli W, Chambon P, Leibowitz MD, Colombel JF, Auwerx J. Attenuation of colon inflammation through activators of the retinoid X receptor (RXR)/peroxisome proliferator-activated receptor gamma (PPARgamma) heterodimer. A basis for new therapeutic strategies. J Exp Med 2001;193:827–838.
19. Bright JJ, Natarajan C, Muthian G, Barak Y, Evans RM. Peroxisome proliferator-activated receptor-gamma-deficient heterozygous mice develop an exacerbated neural antigen-induced Th1 response and experimental allergic encephalomyelitis. J Immunol 2003;171:5743–5750.
20. Ren B, Thelen A, Jump DB. Peroxisome proliferator-activated receptor alpha inhibits hepatic S14 gene transcription. Evidence against the peroxisome proliferator-activated receptor alpha as the mediator of polyunsaturated fatty acid regulation of s14 gene transcription. J Biol Chem 1996;271:17167–17173.
21. Gearing KL, Gottlicher M, Widmark E, Banner CD, Tollet P, Stromstedt M, Rafter JJ, Berge RK, Gustafsson JA. Fatty acid activation of the peroxisome proliferator activated receptor, a member of the nuclear receptor gene superfamily. J Nutr 1994;124:1284S–1288S.
22. Hwang D. Fatty acids and immune responses—a new perspective in searching for clues to mechanism. Annu Rev Nutr 2000; 20:431–456.
23. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A 1997;94:4318–4323.
24. Houseknecht KL, Vanden Heuvel JP, Moya-Camarena SY, Portocarrero CP, Peck LW, Nickel KP, Belury MA. Dietary conjugated linoleic acid normalizes impaired glucose tolerance in the Zucker diabetic fatty fa/fa rat. Biochem Biophys Res Commun 1998; 244:678–682.

25. Moya-Camarena SY, Van den Heuvel JP, Belury MA. Conjugated linoleic acid activates peroxisome proliferator-activated receptor alpha and beta subtypes but does not induce hepatic peroxisome proliferation in Sprague-Dawley rats. Biochim Biophys Acta 1999; 1436:331–342.
26. Yu Y, Correll PH, Vanden Heuvel JP. Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPAR gamma-dependent mechanism. Biochim Biophys Acta 2002;1581:89–99.
27. Yang M, Cook ME. Dietary conjugated linoleic acid decreased cachexia, macrophage tumor necrosis factor-alpha production, and modifies splenocyte cytokines production. Exp Biol Med (Maywood) 2003;228:51–58.
28. Bassaganya-Riera J, Hontecillas R, Beitz DC. Colonic anti-inflammatory mechanisms of conjugated linoleic acid. Clin Nutr 2002; 21:451–459.
29. Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ, Sternbach DD, Lehmann JM, Wisely GB, Willson TM, Kliewer SA, Milburn MV. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol Cell 1999;3: 397–403.
30. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell 1995;83:803–812.
31. Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. Cell 1998;93:229–240.
32. Schoonjans K, Martin G, Staels B, Auwerx J. Peroxisome proliferator-activated receptors, orphans with ligands and functions. Curr Opin Lipidol 1997;8:159–166.
33. Fajas L, Auboeuf D, Raspe E, Schoonjans K, Lefebvre AM, Saladin R, Najib J, Laville M, Fruchart JC, Deeb S, Vidal-Puig A, Flier J, Briggs MR, Staels B, Vidal H, Auwerx J. The organization, promoter analysis, and expression of the human PPARgamma gene. J Biol Chem 1997;272:18779–18789.
34. Gupta RA, Brockman JA, Sarraf P, Willson TM, DuBois RN. Target genes of peroxisome proliferator-activated receptor gamma in colorectal cancer cells. J Biol Chem 2001;276:29681–29687.
35. Mansen A, Guardiola-Diaz H, Rafter J, Branting C, Gustafsson JA. Expression of the peroxisome proliferator-activated receptor (PPAR) in the mouse colonic mucosa. Biochem Biophys Res Commun 1996;222:844–851.
36. Lefebvre M, Paulweber B, Fajas L, Woods J, McCrary C, Colombel JF, Najib J, Fruchart JC, Datz C, Vidal H, Desreumaux P, Auwerx J. Peroxisome proliferator-activated receptor gamma is induced during differentiation of colon epithelium cells. J Endocrinol 1999; 162:331–340.
37. Clark RB, Bishop-Bailey D, Estrada-Hernandez T, Hla T, Puddington L, Padula SJ. The nuclear receptor PPAR gamma and immunoregulation: PPAR gamma mediates inhibition of helper T cell responses. J Immunol 2000;164:1364–1371.
38. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature 1998;391:79–82.
39. Hontecillas R, Wannemeulher MJ, Zimmerman DR, Hutto DL, Wilson JH, Ahn DU, Bassaganya-Riera J. Nutritional regulation of porcine bacterial-induced colitis by conjugated linoleic acid. J Nutr 2002;132:2019–2027.
40. Akiyama TE, Sakai S, Lambert G, Nicol CJ, Matsusue K, Pimprale S, Lee YH, Ricote M, Glass CK, Brewer HB Jr, Gonzalez FJ. Conditional disruption of the peroxisome proliferator-activated receptor gamma gene in mice results in lowered expression of ABCA1, ABCG1, and apoE in macrophages and reduced cholesterol efflux. Mol Cell Biol 2002;22:2607–2619.
41. Wagner KU, McAllister K, Ward T, Davis B, Wiseman R, Hennighausen L. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res 2001;10:545–553.
42. Cui Y, Miyoshi K, Claudio E, Siebenlist UK, Gonzalez FJ, Flaws J, Wagner KU, Hennighausen L. Loss of the peroxisome proliferation-activated receptor gamma (PPARgamma) does not affect mammary development and propensity for tumor formation but leads to reduced fertility. J Biol Chem 2002;277:17830–17835.
43. Reeves PG, Nielsen FH, Fahey GC Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939–1951.
44. Bassaganya-Riera J, Hontecillas R, Zimmerman DR, Wannemuehler MJ. Dietary conjugated linoleic acid modulates phenotype and effector functions of porcine cd8(+) lymphocytes. J Nutr 2001;131:2370–2377.
45. Bassaganya-Riera J, Hontecillas R, Zimmerman DR, Wannemuehler MJ. Long-term influence of lipid nutrition on the induction of CD8(+) responses to viral or bacterial antigens. Vaccine 2002;20:1435–1444.
46. Saubermann LJ, Beck P, De Jong YP, Pitman RS, Ryan MS, Kim HS, Exley M, Snapper S, Balk SP, Hagen SJ, Kanauchi O, Motoki K, Sakai T, Terhorst C, Koezuka Y, Podolsky DK, Blumberg RS. Activation of natural killer T cells by alpha-galactosylceramide in the presence of CD1d provides protection against colitis in mice. Gastroenterology 2000;119:119–128.
47. Bassaganya-Riera J, Pogranichniy RM, Jobgen SC, Halbur PG, Yoon KJ, O'Shea M, Mohede I, Hontecillas R. Conjugated linoleic acid ameliorates viral infectivity in a pig model of virally induced immunosuppression. J Nutr 2003;133:3204–3214.
48. Statistical Analysis Software Institute, Inc. SAS/STAT user's guide (release 6.0.3). Cary, NC: SAS Institute Inc., 1988.
49. Cooper HS, Murthy SN, Shah RS, Sedergran DJ. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest 1993;69:238–249.
50. Welch JS, Ricote M, Akiyama TE, Gonzalez FJ, Glass CK. PPARgamma and PPAR-delta negatively regulate specific subsets of lipopolysaccharide and IFN-gamma target genes in macrophages. PNAS 2003;100:6712–6717.
51. Okamoto H. New aspects to the functioning and regeneration of pancreatic beta-cells. Cyclic ADP-ribose and Reg gene. Adv Exp Med Biol 1997;426:301–311.
52. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, Evans RM. PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell 1999;4:585–595.
53. Powrie F, Leach MW, Mauze S, Caddle LB, Coffman RL. Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. Int Immunol 1993;5:1461–1471.
54. Asseman C, Read S, Powrie F. Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4+ regulatory T cells and IL-10. J Immunol 2003;171:971–978.
55. O'Shea M, Bassaganya-Riera J, Mohede I. The immunomodulatory properties of conjugated linoleic acid. Am J Clin Nutr 2004; 79:1199S–1206S.
56. Nakajima A, Wada K, Miki H, Kubota N, Nakajima N, Terauchi Y, Ohnishi S, Saubermann LJ, Kadowaki T, Blumberg RS, Nagai R, Matsuhashi N. Endogenous PPAR gamma mediates anti-inflammatory activity in murine ischemia-reperfusion injury. Gastroenterology 2001;120:460–469.
57. Nakajima A, Wada K, Katayama K, Saubermann L, Osawa E, Nagase H, Ueno N, Matsuhashi N, Aburatani H. Gene expression profile after peroxisome proliferator activator receptor-gamma ligand administration in dextran sodium sulfate mice. J Gastroenterol 2002;37(Suppl 14):62–66.

58. Albers R, Van Der Wielen RP, Brink EJ, Hendriks HF, Dorovska-Taran VN, Mohede IC. Effects of cis-9, trans-11 and trans-10, cis-12 conjugated linoleic acid (CLA) isomers on immune function in healthy men. Eur J Clin Nutr 2003;57:595–603.
59. Hontecillas R, Jobgen SC, Wannemeulher MJ, Bassaganya-Riera J. Differential regulation of colonic PPAR expression by dietary fatty acids. FASEB J 2003;17:A1161.
60. Dubuquoy L, Jansson EE, Deeb S, Rakotobe S, Karoui M, Colombel JF, Auwerx J, Pettersson S, Desreumaux P. Impaired expression of peroxisome proliferator-activated receptor gamma in ulcerative colitis. Gastroenterology 2003;124:1265–1276.
61. Sarraf P, Mueller E, Smith WM, Wright HM, Kum JB, Aaltonen LA, de la Chapelle A, Spiegelman BM, Eng C. Loss-of-function mutations in PPAR gamma associated with human colon cancer. Mol Cell 1999;3:799–804.
62. Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H, Evans RM. Peroxisome-proliferator-activated receptor delta activates fat metabolism to prevent obesity. Cell 2003;113:159–170.
63. Komiya T, Tanigawa Y, Hirohashi S. Cloning of the gene gob-4, which is expressed in intestinal goblet cells in mice. Biochim Biophys Acta 1999;1444:434–438.
64. Moll R, Zimbelmann R, Goldschmidt MD, Keith M, Laufer J, Kasper M, Koch PJ, Franke WW. The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. Differentiation 1993;53:75–93.
65. Calnek D, Quaroni A. Differential localization by in situ hybridization of distinct keratin mRNA species during intestinal epithelial cell development and differentiation. Differentiation 1993;53: 95–104.
66. Berger J, Moller DE. The mechanisms of action of PPARs. Annu Rev Med 2002;53:409–435.
67. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 1998;92:829–839.
68. Meirhaeghe A, Crowley V, Lenaghan C, Lelliott C, Green K, Stewart A, Hart K, Schinner S, Sethi JK, Yeo G, Brand MD, Cortright RN, O'Rahilly S, Montague C, Vidal-Puig AJ. Characterization of the human, mouse and rat PGC1 beta (peroxisome-proliferator-activated receptor-gamma co-activator 1 beta) gene in vitro and in vivo. Biochem J 2003;373:155–165.
69. Wu Y, Chin WW, Wang Y, Burris TP. Ligand and coactivator identity determines the requirement of the charge clamp for coactivation of the peroxisome proliferator-activated receptor gamma. J Biol Chem 2003;278:8637–8644.
70. Armstrong AM, Gardiner KR, Kirk SJ, Halliday MI, Rowlands BJ. Tumour necrosis factor and inflammatory bowel disease. Br J Surg 1997;84:1051–1058.
71. La Ferla K, Seegert D, Schreiber S. Activation of NF-kappaB in intestinal epithelial cells by E. coli strains isolated from the colonic mucosa of IBD patients. Int J Colorectal Dis 2004;19: 334–342.
72. Konturek PC, Brzozowski T, Kania J, Kukharsky V, Bazela K, Kwiecien S, Harsch I, Konturek SJ, Hahn EG. Pioglitazone, a specific ligand of the peroxisome proliferator-activated receptor gamma reduces gastric mucosal injury induced by ischaemia/reperfusion in rat. Scand J Gastroenterol 2003;38:468–476.
73. Lee CH, Chawla A, Urbiztondo N, Liao D, Boisvert WA, Evans RM. Transcriptional repression of atherogenic inflammation: modulation by PPARdelta. Science 2003;302:453–457.
74. Cheng WL, Lii CK, Chen HW, Lin TH, Liu KL. Contribution of conjugated linoleic acid to the suppression of inflammatory responses through the regulation of the NF-kappaB pathway. J Agric Food Chem 2004;52:71–78.
75. Annacker O, Powrie F. Homeostasis of intestinal immune regulation. Microbes Infect 2002;4:567–574.
76. Fuss IJ, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol 1996;157:1261–1270.
77. Breese E, Braegger CP, Corrigan CJ, Walker-Smith JA, MacDonald TT. Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. Immunology 1993; 78:127–131.
78. Hontecillas R, Bassaganya-Riera J. Differential requirements for proliferation of CD4(+) and gammadelta(+) T cells to spirochetal antigens. Cell Immunol 2003;224:38–46.

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