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Paraoxonase-1 Suppresses Experimental Colitis via the Inhibition of IFN-γ Production from CD4 T Cells

Junji Yamashita,*† Chiaki Iwamura,* Toshihiro Ito,* Masakuni Narita,† Yukio Hara,‡ Tetsuya Sasaki,*† Daisuke Masuda,† Munehisa Takahashi,† Manami Tsuchiya,† Kaori Hada,† Makoto Ishikawa,† Takato Matsuo,† Yoichi Ohno,† Hitoshi Tanaka,† Hideya Maruyama,† Yasumasa Ogawa,† and Toshinori Nakayama*‡

Crohn’s disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract, where excessive Th1 cell responses are observed. We performed experiments to identify immunologically bioactive proteins in human plasma and found that paraoxonase (PON)-1, which has esterase activity and is associated with high-density lipoproteins, inhibited the IFN-γ production by both murine and human differentiating Th1 cells. Trinitrobenzene sulfonic acid–induced colitis was attenuated by the administration of PON-1. The beneficial effects of PON-1 were associated with a reduced ratio of IFN-γ–producing CD4 T cells in the mesenteric lymph nodes and decreased production of T cell–related cytokines in the colon. PON-1 inhibited the TCR-induced activation of ERK-MAPK signaling and the nuclear translocation of NF-κB in CD4 T cells. Interestingly, an excessive CD4 T cell response was observed in PON-1–deficient mice under physiological and pathological conditions. Additionally, the efficacy of PON-1 or G3C9-C284A (G3C9), which shows a higher esterase activity than PON-1, and the efficacy of G3C9 against the colitis was similar to that of an anti–TNF-α mAb, which is a clinically used CD treatment. Moreover, G3C9 more effectively suppressed CD4+CD45RBhigh cell transfer–induced chronic colitis in mice than did PON-1, and the efficacy of G3C9 against the colitis was similar to that of the anti–TNF-α mAb. Therefore, PON-1 (or G3C9) administration may be clinically beneficial for CD patients. The Journal of Immunology, 2013, 191: 949–960.

Although biological drugs targeting TNF-α have proven to be effective for CD patients (2, 3), some patients treated with anti–TNF-α therapies experience adverse events, including serious infections (4, 5), and thus the development of new therapeutic agents is awaited by CD patients.

Effector CD4 T cell–mediated immune responses play an important role in the development and progression of CD. In particular, Th1 cells have long been considered to be responsible for the intestinal inflammation in CD. Several animal models of CD, such as the colitis in IL-2– and IL-10–deficient mice, the colitis in SCID mice transferred CD4+CD45RBhigh cells, and the colitis induced by trinitrobenzene sulfonic acid (TNBS), are characterized by the increased mucosal production of IFN-γ by Th1 cells (6–10). Indeed, IFN-γ deficiency or the administration of a neutralizing anti–IFN-γ Ab prevents colitis and can decrease the severity of disease in murine models of CD (8, 11, 12). Furthermore, the beneficial effects of anti–IL-12 Ab administration in TNBS–induced colitis are thought to result, at least in part, from a reduction of IFN-γ (9). Therefore, IFN-γ–producing CD4 T cells appear to be a good therapeutic target for CD.

Paraoxonase (PON)-1 is a 45-kDa glycoprotein that is expressed in the liver and has been found to be associated with high-density lipoprotein (HDL) particles in the blood. PON-1 was initially identified for its ability to catalyze the hydrolysis of a diverse group of substrates, including aryl esters, lactones, oxidized phospholipids, and organophosphorus compounds (13–16). Studies on the physiological and pathophysiological effects of PON-1 have focused on its reverse cholesterol transport and antioxidant functions, because HDL is thought to protect against atherosclerosis (17, 18). These properties are probably attributable to the enzyme’s ability to protect low-density lipoprotein (19, 20), as well as HDL (21), from oxidation and to decrease the macrophage oxidative status (22). Indeed, studies using PON-1 knockout (KO) mice have

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The online version of this article contains supplemental material. Abbreviations used in this article: ATIII, antithrombin III; CD, Crohn’s disease; CHO, Chinese hamster ovary; DAL, disease activity index; HDL, high-density lipoprotein; HPRT, hypoxanthine phosphoribosyltransferase; i.r., intrarectally; KO, knockout; mLN, mesenteric lymph node; PON, paraoxonase; PON-1, homozygous paraoxonase-1 knockout; TNBS, trinitrobenzene sulfonic acid; WT, wild-type.

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shown increased serum and macrophage oxidative stress (23, 24), whereas PON-1 transgenic mice are characterized by an increased capacity of their HDL to inhibit low-density lipoprotein oxidation (25). However, the role of PON-1 in the immune response and in the pathogenesis of inflammation remains to be elucidated.

In this study, we demonstrate that PON-1 has a suppressive effect on IFN-γ production by murine or human differentiating Th1 cells, and that it attenuates TNBS-induced colitis and CD4+CD45RBhigh cell transfer–mediated chronic colitis in mice. PON-1 could therefore potentially be an effective new therapeutic agent for Th1-dependent colitis in humans.

Materials and Methods

Mice and cell lines

Male C57BL/6 mice (6 wk old) were purchased from Charles River. Female BALB/c or SCID mice (7 wk old) were purchased from SLC. Heterozygous PON-1 KO mice, which were purchased from The Jackson Laboratory, were bred to produce wild-type (WT) controls and homozygous PON-1 KO (PON-1−/−) mice (23, 26). All of the mice used in this study were maintained under specific pathogen-free conditions. All animal care and experimental protocols were conducted in accordance with the guidelines of Chiba University. The CHOK1 cell line and the GS Gene Expression System were provided by Lonza Biologies (Berkshire, U.K.). The CHOK1 cells were cultured in Erlenmeyer flasks and maintained in CD-Chinese hamster ovary (CHO) medium (Invitrogen) according to the manufacturer’s instructions. All experimental procedures were conducted in compliance with the approval of the Ethics Committee for Animal Experiments of Nihon Pharmaceutical.

Expression and purification of recombinant human PON-1 or G3C9-C284A

Human PON-1 or G3C9-C284A genes, which were optimized for codon use in CHO cells (Invitrogen), were used for our experiments. A HindIII restriction site and an EcoRI restriction site was added into each synthetic gene at the 3′ restriction site and an EcoRI restriction site was added into each synthetic gene at the 3′ restriction site. The expression vectors were constructed in pEE12.4, which carries the GS gene, and its product catalyzes the formation of glutamine from glutamate and ammonia (28). The CHOK1 cells were transfected with each expression vector and expressed differentially in CHO cells (Invitrogen), were used for our experiments. A HindIII restriction site and an EcoRI restriction site was added into each synthetic gene at the 3′ restriction site. The expression vectors were constructed in pEE12.4, which carries the GS gene, and its product catalyzes the formation of glutamine from glutamate and ammonia (28). The CHOK1 cells were transfected with each expression vector and expressed differentially in CHO cells (Invitrogen), were used for our experiments.

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Intracellular cytokine staining

Murine and human CD4 T cells were pretreated with a BD Cytofix/Cytoperm fixation/permeabilization kit (BD Pharmingen) according to the manufacturer’s instructions. Murine CD4 T cells were stained intracellularly with an allophycocyanin-conjugated anti–IFN-γ mAb (XMG1.2; BD Pharmingen), PE-conjugated anti–IL-4 mAb, or PE-conjugated anti–IL-17 mAb (BD Pharmingen). Human CD4 T cells were stained intracellularly with a FITC-conjugated anti–IFN-γ mAb, PE-conjugated anti–IL-4 mAb (BD Pharmingen), FITC-conjugated anti–IL-17 mAb (BD Pharmingen), and PE-conjugated anti–IFN-γ mAb, and the flow cytometric analysis was performed on a FACSCalibur cell sorter (Miltenyi Biotec). Naive CD4 T cell sorting (Miltenyi Biotec). Naive CD4 T cells were immunized with 3 μg/ml immobilized anti-TCRβ mAb (H57-597; BD Pharmingen) in the presence of 25 U/ml IL-2 for neutral culture conditions. The cells were stimulated with 3 μg/ml immobilized anti-TCRβ mAb in the presence of 25 U/ml IL-2, as well as 0.01–1 U/ml IL-12 (BD Pharmingen) and 5 μg/ml anti–IL-4 mAb (11B11; eBioscience) for Th1 cell differentiation (31). The cells were stimulated with 3 μg/ml immobilized anti-TCRβ plus 2 μg/ml soluble anti-CD28 mAbs (37.51; BioLegend) in the presence of 3 ng/ml TGF-β (PeproTech), 10 ng/ml IL-6 (PeproTech), anti–IL-4, anti–IFN-γ (XMG1.2; BioLegend), and anti–IL-2 (JES6-1A12; eBioscience) mAbs (32) for Th1 cell differentiation.

PBMCs from healthy volunteers were collected after obtaining informed consent for preparation of human CD4 T cells. Peripheral blood was diluted with PBS and applied on Ficol-Paque (Amersham Biosciences). After centrifugation, the mononuclear cells in the interface were harvested. Human naive CD4 T cells were purified using a Human naive CD4 T cell Isolation Kit II (Miltenyi Biotec) and autoMACS cell sorting. The cells were stimulated with 20 μg/ml immobilized anti-CD3 mAb (Janssen Pharmaceuticals) for 2 d in the presence of 50 U/ml IL-2 (Shionogi) for neutral culture conditions. The cells were stimulated with 20 μg/ml immobilized anti-CD3 mAb for 2 d in the presence of 50 U/ml IL-2, 1 ng/ml IL-12 (PeproTech), and 5 μg/ml anti–IL-4 mAb (BD Bio-Sciences) for 3 d. The CD4+CD45RBhigh Th1 cell transfer model of chronic colitis was used as described (35, 36). Briefly, splenic CD4 T cells were first purified from

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Human Naive CD4 T cells were purified from splenocytes using the CD4+CD62L+ T Cell Isolation Kit II (Miltenyi Biotec) and autoMACS cell transfer–mediated chronic colitis in mice. PON-1 could therefore potentially be an effective new therapeutic agent for Th1-dependent colitis in humans.
splenocytes from donor BALB/c mice using CD4 (L3T4) MicroBeads (Miltenyi Biotec) and autoMACS cell sorting, then cells with a CD4 (L3T4; BD Pharmingen)-positive and CD45RB hi (16A; BD Pharmingen)-positive phenotype were further purified using a FACS Aria cell sorter (BD Biosciences). The CD45RB hi-positive phenotype was defined as the most brightly stained cells consisting of 30–40% CD4 T cells. Before i.p. injection of CD4+CD45RB hi (5 × 102 cells in 200 μl PBS) into the recipient SCID mice, PON-1 (10 mg/kg, n = 5), G3C9 (10 mg/kg, n = 5), or an equivalent volume of PBS (5 mg/kg, n = 5) was slowly administered via the tail vein. The same dose of PON-1, G3C9, and anti–TNF-α mAb was continuously administered three times per week for 8 wk. The mouse body weight, stool consistency, and rectal bleeding were examined daily during the experiments to assess the disease activity status of the colitis. The DAI score for colitis was determined using a scoring system described in the previous section. Eight weeks after the treatments, blood samples were collected from the heart after euthanizing the mice, and the plasma fraction was then separated by centrifugation of the heparinized sample. These samples were used to measure the enzymatic activity of PON-1. Additionally, the colon samples were isolated and dissected into multiple sections for further analysis.

**Histological and immunohistochemical analyses**

For the histological analysis, colonic tissue specimens from the mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E (Aperio). The specimens were examined using a light microscope at ×80 magnification. The tissue damage was semiquantitatively scored following the previously described microscopic scoring criteria (38). For the immunohistochemical analyses, parafin-embedded colon tissue section was sectioned serially after being defatamined in xylene and rehydrated in ethanol. These sections were incubated with citrate buffer and heated in a microwave oven at 98°C for 30 min before incubation with 0.3% hydrogen peroxide in PBS. The nonspecific binding sites were blocked with normal mouse serum. The specimens were incubated overnight with an anti-CD3 mAb (Dako) at a dilution of 1:100 in 0.5% casein-PBS at 4°C. These sections were incubated with an HRP-conjugated secondary Ab (Fitzgerald Industries International) diluted in PBS supplemented with 0.5% casein. Subsequently, 3,3’-diaminobenzidine (Dojindo Laboratories) was applied for visualization of the CD3+ cells. The specimens were examined using a light microscope at ×80 magnification.

**Isolation and culture of CD4 T cells from mLNs**

Murine mesenteric CD4 T cells were purified from mLNs using CD4 MicroBeads and autoMACS cell sorting. The CD4 T cells were cultured with 3 μg/ml immobilized anti-TCRβ and 2 μg/ml soluble anti-CD28 mAbs for 48 h. The culture supernatants were harvested, and their levels of secreted IFN-γ or TNF-α were measured by ELISA, and then the CD4 T cells were restimulated with 50 ng/ml PMA and 500 nM ionomycin (Sigma-Aldrich) for 4 h in presence of 2 μM monensin and then were subjected to intracellular cytokine staining.

**Quantitative PCR analysis**

The total RNAs were isolated from tissue samples using an RNeasy Mini Kit (Qiagen), and cDNAs were synthesized using Rever Tra Ace (Toyobo). These cDNA samples were then subjected to a real-time PCR analysis on an ABI Prism 7300 sequence detection system (Applied Biosystems) as described previously (31). The primers and TaqMan probes used for the detection of mouse IL-6 and hyoxanthine phosphoribosyltransferase (HPRT) were purchased from Applied Biosystems. The probes used for the detection of the other genes in this study were purchased from Roche Diagnostics (Basel, Switzerland). The primers purchased from Roche Diagnostics were as follows: TNF-α (forward primer, 5’-ATGACACA-GAAAGGTACGATC-3’; reverse primer, 5’-TACAGGTCTTGATCACTGGAA-3’; forward primer, 5’-AAGGCAGAGAGAGAGGATC-3’; reverse primer, 5’-TCAGCTTGAGGATGAGGC-3’), IL-17A (forward primer, 5’-ATTCCCACTCTAGCTCCAGG-3’; reverse primer, 5’-TTGCTTGTGAGGTC-3’), IL-4 (forward primer, 5’-CTGGC- TCTCCCTTCTTGGGATACT-3’; reverse primer, 5’-TCACCTCCATCCTGGCTG-3’), IL-12A (forward primer, 5’-TCAAGAATCAAACTACGCA-3’; reverse primer, 5’-CCGATATTGACGATGACTG-3’), T-bet (forward primer, 5’-CAACGCGACACAGCAGGAG-3’; reverse primer, 5’-CAACGCGACACAGCAGGAG-3’; reverse primer, 5’-TGCGAGGAGGAGGAGGAGG-3’; reverse primer, 5’-TGGCGAGGAGGAGGAGGAGG-3’), and HPRT (forward primer, 5’-TCCCTCCTAGACGCTTCTTTT-3’; reverse primer, 5’-CCTTCGTTCTACCTGGTTACT-3’). The gene expression was normalized using the HPRT signal.

**Measurements of the cytokine production and proliferation of CD4 T cells**

Murine or human CD4 T cells were stimulated with 3 μg/ml immobilized anti-TCRβ plus 2 μg/ml soluble anti-CD28 mAbs in the presence or absence of PÓN-1. Protein lysates were prepared with a cell lysis kit (Bio-Rad) on samples collected at the indicated time points. The presence of phosphorylated p38MAPK, c-Jun, and IkB-α was detected by a Bio-Plex 3-plex phosphoprotein assay kit (Bio-Rad) and a phosphoprotein testing reagent kit (Bio-Rad), according to the manufacturer’s protocol. The data from the reactions were acquired and analyzed using the Bio-Plex suspension array system.

**Cytokine content of the colon**

Excised colon tissues were weighed and homogenized in lysis buffer (Bio-Rad) on ice. After the homogenates were centrifuged, the cytokine concentration in the supernatant was measured using a Bio-Plex mouse cytokine 4-plex panel (Bio-Rad) according to the manufacturer’s protocol. The data from the reaction were then acquired and analyzed using the Bio-Plex suspension array system. The cytokine content of the colon is shown in picograms per milligram of tissue.

**Immunoblot assay**

Murine splenic naive CD4 T cells were stimulated with 3 μg/ml immobilized anti-TCRβ plus 2 μg/ml soluble anti-CD28 mAbs in the presence or absence of PÓN-1. Nuclear extracts for the detection of NFAT2 (NFATc1) or lamin were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Chemical) on samples collected at the indicated time points. Immunoblotting was performed with an anti-NFATc1 mAb (BD Pharmingen), anti-lamin polyclonal Ab (Santa Cruz Biotechnology), anti–phospho-ERK1/2 mAb (Cell Signaling Technology), or anti-β-actin Ab (Santa Cruz Biotechnology). The protein levels were visualized by ECL (Amersham Pharmacia Biotech) using a HRP-conjugated anti-mouse IgG Ab or anti-rabbit IgG Ab (Amersham Pharmacia Biotech).

**Statistical analysis**

All statistical analyses were performed using the GraphPad Prism software program. The differences were determined by an unpaired two-tailed Student t test or one-way ANOVA with a Dunnett multiple comparison tests, or by Kruskal–Wallis and Steel’s multiple comparison tests. A p value < 0.05 was considered to be statistically significant.

**Results**

**Inhibition of IFN-γ production in murine or human differentiating Th1 cells by PÓN-1**

In the present study, the ethanol precipitation method as reported by Cohn et al. (39) was used to isolate plasma proteins from normal donors to identify new therapeutic agents that can suppress the Th1-dependent inflammatory responses with better safety profiles than the currently used drugs. The 6A, ATIII, or IgG fraction was purified from plasma by a combination of gel filtration chromatography and ethanol precipitation. Fig. 1A shows that the 6A fraction had significantly suppressed IFN-γ production by the murine differentiating Th1 cells. No significant changes were observed when the plasma, ATIII, or IgG fraction was added. Therefore, the study compared the protein contents of the plasma or 6A fraction using SDS-PAGE and silver staining. Several bands indicated by circles showed increased intensity in the 6A fraction.
in comparison with plasma (Fig. 1B). Furthermore, the five spots that increased in the 6A fraction were identified by using two-dimensional electrophoresis and the SWISS-2DPAGE Viewer database (Fig. 1C).

We then examined which proteins could suppress the IFN-\(\gamma\) production by murine differentiating Th1 cells. Among the five proteins tested, PON-1 was the best inhibitor of IFN-\(\gamma\) production by murine differentiating Th1 cells (data not shown). Next, we produced recombinant PON-1 (45 kDa) using the GS Gene Expression System (Fig. 1D), which is one of the most powerful systems for the production of recombinant proteins in mammalian cells (28), because the concentration of PON-1 in human plasma is very low. Finally, we found that recombinant PON-1 showed a potent suppressive effect on IFN-\(\gamma\) production by murine or human differentiating Th1 cells (Fig. 1E). Moreover, the inhibition of IFN-\(\gamma\) production in murine differentiating Th1 cells by PON-1 was almost cancelled by using a neutralizing mAb against human PON-1 (Fig. 1F), whereas no significant effect was observed by the addition of ATIII, a nonspecific protein used as a control (Fig. 1F). Furthermore, the mRNA expression of T-bet, but not STAT4, in the murine differentiating Th1 cells was significantly suppressed by the addition of PON-1 (Fig. 1G).

**FIGURE 1.** The plasma protein PON-1 has the ability to suppress IFN-\(\gamma\) production by murine and human differentiating Th1 cells. (A) Purified murine splenic CD4 T cells were cultured under Th1 culture conditions for 5 d in the presence of human plasma, 6A, ATIII, or IgG (0.5 mg/ml), and the amount of IFN-\(\gamma\) in the culture supernatant was assessed by ELISA. The results are expressed as the means ± SD (n = 5). \(p < 0.05\), in comparison with PBS-treated differentiating Th1 cells. (B) A representative SDS-PAGE gel stained with silver. The circle indicates the increased bands of biologically active proteins in 6A-treated cells compared with those treated with human plasma. (C) A representative two-dimensional electrophoresis pattern of 6A. The circles indicate the spots of biologically active proteins in 6A. (D) A representative SDS-PAGE gel stained with Coomassie brilliant blue showing recombinant PON-1. (E) Purified murine splenic CD4 T cells or human CD4 T cells were cultured under Th1 culture conditions for 5 d in the presence of PON-1 (1.0 mg/ml), and the amount of IFN-\(\gamma\) in the culture supernatant was assessed by ELISA. The results are expressed as the means ± SD (n = 5). \(p < 0.05\), in comparison with PBS-treated murine or human differentiating Th1 cells. (F) Intracellular cytokine staining was performed using anti-cytokine Abs. Purified murine splenic naive CD4 T cells were cultured under Th1 conditions for 5 d in the presence of PON-1. A neutralizing mAb against human PON-1 was used. ATIII was used as a control for PON-1. Then, the CD4 T cells were restimulated with PMA and ionomycin for 4 h in the presence of monensin. The typical percentages of IFN-\(\gamma\) production in Th1 cells of each group are shown in the plots. The mean IFN-\(\gamma\) production in the cells compared with that in the control group ± SD was as follows: saline, 100%; PON-1, 33.6 ± 1.1%; PON-1 plus anti–PON-1 Ab, 96.3 ± 6.0%; ATIII, 103.5 ± 6.2%; n = 3 for each treatment. (G) Results of the quantitative RT-PCR analysis of T-bet and STAT4 mRNA expression in murine differentiating Th1 cells, measured and expressed as a relative ratio to the level of HPRT. The results are expressed as the means ± SD (n = 3). \(p < 0.05\), in comparison with Th1 CD4 T cells.
results indicated that PON-1 is a bioactive protein that suppresses the IFN-γ production by murine or human differentiating Th1 cells by inhibiting T-bet.

**Attenuation of TNBS-induced colitis by PON-1 administration**

We next examined whether the administration of PON-1 could inhibit the colon inflammation induced by rectal injection of TNBS, which generates a murine model of CD with excessive Th1 cell responses. The administration of PON-1 (1 or 10 mg/kg) once a day for 3 d significantly and dose-dependently suppressed the increased DAI score and decreased the length of the colons in the mice injected with TNBS (Fig. 2A, 2B), and these effects were also observed even 7 d after the TNBS injection (data not shown). The study also evaluated the histological changes of the colon 3 d after TNBS injection. The colon of PON-1–treated mice showed a clear and dose-dependent reduction in the severity of ulceration, the loss of goblet cells, and tissue disruption (Fig. 2C), thus indicating a decrease in transmural inflammation.

**FIGURE 2.** PON-1 administration attenuates TNBS-induced colitis. (A) DAI score of each group in the mice with TNBS-induced colitis. TNBS (100 mg/kg, i.r.) and vehicle (saline, i.v.) or TNBS and PON-1 (1 or 10 mg/kg, i.v.) were injected into C57BL/6 mice. The results are expressed as the means ± SE (n = 5). *p < 0.05, in comparison with the saline-treated mice. (B) Typical colon morphology of each group 3 d after TNBS injection. The mean lengths of the colons ± SE were as follows: Non, 10.4 ± 0.2 cm; TNBS plus saline, 7.9 ± 0.3 cm; TNBS plus PON-1 (1 mg/kg), 8.9 ± 0.4 cm; and TNBS plus PON-1 (10 mg/kg), 9.9 ± 0.2 cm; n = 5 for each group. (C) The left panels show the typical histological pictures of each group (H&E staining). The right graph shows the histological scores of each group. The results are expressed as the means ± SE (n = 5). Colons were collected 3 d after TNBS injection. Original magnification, ×80. *p < 0.05, in comparison with saline-administered mice. (D) Results of the quantitative RT-PCR analysis for IFN-γ, IL-17, TNF-α, IL-6, IL-12, and IL-4 mRNA expression in the colon tissue were obtained 3 d after TNBS injection and are expressed as the relative ratio to that of HPRT. The results are expressed as the means ± SE (n = 3). *p < 0.05, in comparison with saline-administered mice. (E) Colon tissue of each group 3 d after TNBS injection was homogenized and assayed for the expression of IFN-γ, TNF-α, IL-6, and IL-12p40 by a Bio-Plex study. The results are expressed as the means ± SE (n = 5). *p < 0.05, in comparison with saline-administered mice. (F) Intracellular cytokine staining was performed with fluorescently conjugated cytokine Abs. Purified murine mesenteric CD4 T cells 3 d after TNBS injection were cultured with an anti-TCRβ mAb plus a soluble anti-CD28 mAb for 48 h, and the CD4 T cells were restimulated with PMA and ionomycin for 4 h in the presence of monensin. The left panels show the typical profile of IFN-γ– and IL-17–producing cells of CD4 T cells. The right graph shows the relative percentages of the TNBS plus saline group. The results are expressed as the means ± SE (n = 5). *p < 0.05, in comparison with saline-administered mice. (G) Purified murine mesenteric CD4 T cells obtained 3 d after TNBS injection were cultured with an anti-TCRβ mAb plus a soluble anti-CD28 mAb for 48 h, and the amount of TNF-α in the culture supernatant was assessed by ELISA. The results are expressed as the means ± SD. *p < 0.05, in comparison with saline-administered mouse. (H) DAI score of each group of mice with TNBS-induced colitis. TNBS (100 mg/kg, i.r.) and vehicle (saline, i.v.) or TNBS and PON-1 (10 mg/kg, i.v.) were injected into C57BL/6 mice. PON-1 was slowly administered via the tail vein 1 d after the injection of TNBS, and then once a day for 3 d. The results are expressed as the means ± SE (n = 5). *p < 0.05, in comparison with the saline-administered mice.
cytokines that have been reported to be involved in the pathology of CD was also examined in the colon tissue (40–42). The mRNA expression of IFN-γ, TNF-α, IL-6, and IL-12, but not IL-4, in the colon of the mice administered PON-1 was significantly lower than that of the mice administered saline 3 d after TNBS injection, whereas no IL-17 mRNA expression was detected in any of the groups (Fig. 2D). Additionally, the protein expression levels of IFN-γ, TNF-α, and IL-6 observed 3 d after TNBS injection in saline-treated mice were significantly suppressed by the administration of PON-1, and the increase in IL-12p40 tended to be attenuated as well (Fig. 2E). Next, the effect of PON-1 on Th1 or Th17 cells in mLNs was investigated 3 d after TNBS injection. The CD4 T cells were isolated from the mLNs of TNBS-induced colitis model mice, and then the CD4 T cells were stimulated with an anti-TCRβ mAb in vitro. The PON-1–treated mice showed a decreased ratio of IFN-γ–producing mesenteric CD4 T cells in comparison with the saline-treated mice, whereas IL-17–producing mesenteric CD4 T cells were marginally detected in all groups (Fig. 2F). Additionally, the TNF-α production from mLN CD4 T cells was also suppressed in PON-1–treated mice compared with saline-treated mice (Fig. 2G). We also examined whether PON-1 treatment showed therapeutic efficacy during the later phases of the disease when acute colitis was fully established. We started PON-1 treatment 1 d after TNBS administration, when the mice were already losing body weight and showing severe mucosal inflammation. As shown in Fig. 2H, PON-1 treatment reduced the colitis by the next day, and the mice had significantly recovered 3 d after starting the PON-1 treatment. These results indicated that PON-1 administration improved the TNBS-induced inflammation in the colon, possibly via the inhibition of several inflammatory cytokines. Thus, PON-1 has a therapeutic effect even on previously established active colitis. The treatment of normal mice with PON-1 also induced no apparent side effects or histological changes (data not shown).

**Suppression of the activation and function of murine and human CD4 T cells by PON-1**

We next assessed the effects of PON-1 on the activation and function of murine CD4 T cells in vitro to clarify the mechanisms underlying the PON-1–induced inhibition of TNBS-induced colitis. Fig. 3A shows that PON-1 dose-dependently suppressed the production of IFN-γ or IL-4 from murine CD4 cells cultured under neutral conditions. Additionally, the IFN-γ production from murine CD4 cells under Th1 differentiation was clearly and dose-dependently suppressed by the addition of PON-1, although the IL-17–producing murine CD4 cells under Th17 differentiation were marginally suppressed. The [3H]thymidine uptake (Fig. 3B) and propidium iodide cell generation trend to be increased by the addition of PON-1 (Supplemental Fig. 1), and it suppressed the IFN-γ and IL-2 production from murine CD4 T cells after the stimulation with an anti-TCRβ plus anti-CD28 mAb (Fig. 3C). We then examined whether the treatment of murine CD4 T cells with PON-1 induced the signal transduction pathways downstream of the TCR. As shown in Fig. 3D and 3E, the phosphorylation of p38MAPK and ERK1/2 induced by anti-TCRβ plus anti-CD28 mAbs was selectively inhibited by the addition of PON-1. The phosphorylation of c-Jun was detected 3 h after anti-TCRβ plus anti-CD28 mAb stimulation, and this was also inhibited by PON-1. The phosphorylation of c-Jun and IkBα, which indicates the activation of the NF-κB signaling pathway, was also decreased by the addition of PON-1 3 h after stimulation with the anti-TCRβ plus anti-CD28 mAbs. No clear difference was observed in the nuclear translocation of NFATc1 following treatment with the anti-TCRβ plus anti-CD28 mAb (Fig. 3F). Next, the reactivity of PON-1 in human CD4 T cells was investigated. PON-1 dose-dependently suppressed the ratio of IFN-γ–producing cells in human CD4 cells cultured under neutral or Th1 conditions, whereas no obvious effect on IL-17–producing cells was observed following the addition of PON-1 (Fig. 3G). The [3H]thymidine uptake of human CD4 T cells was also significantly increased following the addition of PON-1 (Fig. 3H). Moreover, PON-1 also suppressed the IFN-γ and IL-2 production from human CD4 T cells 2 d after the stimulation with anti-CD3 mAbs (Fig. 3I). These results indicate that PON-1 suppresses the activation and function of CD4 T cells by inhibiting the ERK-MAPK and NF-κB signaling pathways activated by the anti-TCRβ plus anti-CD28 mAbs.

**The CD4 T cell response of PON-1−/− mice in vivo and in vitro**

To assess the physiological role of PON-1, we examined the TNBS-induced colitis using PON-1−/− mice. CD4 and CD8 T cell profiles of the thymus, spleen, mLNs, and peripheral blood was normal in PON-1−/− mice (Supplemental Fig. 2). However, no spontaneous pathological features or histological changes of the colon were observed in PON-1−/− mice maintained under physiological conditions (Fig. 4A–C). Additionally, even when TNBS was injected, no significant differences were observed in the DAI score and length of the colon between PON-1−/− and WT mice (Fig. 4A, 4B). However, the PON-1−/− mice showed an increased severity of transmural inflammation and neutrophil infiltration in the colon in comparison with the colons of the WT mice 3 d after the TNBS injection (Fig. 4C). Additionally, the mRNA expression of IFN-γ and IL-6 in the colon 3 d after the TNBS injection was significantly higher in PON-1−/− mice than in WT mice, although the mRNA expression levels of IL-12 and IL-4 were similar between PON-1−/− and WT mice (Fig. 4D). Moreover, the levels of IFN-γ and TNF-α production from the CD4 T cells in the mLNs were significantly higher in PON-1−/− mice than in WT mice following TNBS injection (Fig. 4E). Furthermore, the IFN-γ production in PON-1−/− mice under IL-12-dependent Th1 conditions was significantly higher than that in WT mice, and it was equivalently inhibited by the addition of PON-1 in both PON-1−/− or WT CD4 T cell cultures (Fig. 4F). These results indicate that IFN-γ– and TNF-α–producing CD4 T cells, as well as the development and progression of TNBS-induced colitis, are affected in the absence of PON-1.

**The efficacy of PON-1 is similar to that of an anti–TNF-α mAb in TNBS-induced colitis**

Anti–TNF-α mAbs are well-established treatment for CD (36, 42–45). We therefore compared the effects of the mAb and PON-1 in terms of their suppression of TNBS-induced colitis. The anti–TNF-α mAb or PON-1 was administered once a day for 3 d into the mice with TNBS-induced colitis. The anti–TNF-α mAb administration suppressed the severity of colitis in the mice injected with TNBS, and this was accompanied by improvements in the DAI score, colon length and histology, the mRNA expression of inflammatory cytokines, and the presence of IFN-γ–producing mesenteric CD4 T cells, and the administration of PON-1 had an almost equivalent effect (Fig. 5A–E). These findings indicate that the efficacy of PON-1 against TNBS-induced colitis was similar to that of the anti–TNF-α mAb, which is currently the most effective clinically used therapeutic agent. Interestingly, a reduced PON activity in plasma was observed 3 d after TNBS injection in the mice with TNBS-induced colitis, and this change was improved after the administration of PON-1, although the improvement of
Means stimulation, expressed as the means ± SD under neutral, Th1, or Th17 conditions for 5 d in the presence of PON-1, and CD4 T cells were restimulated with PMA and ionomycin for 4 h in presence of PON-1, and CD4 T cells were restimulated with PMA and ionomycin for 4 h in presence of PON-1. The results are expressed as the means ± SD of NFATc1 by dividing the density of lamin. The lower panel shows the arbitrary densitometric units after the stimulation shown the protein expression of NFATc1 by the density of lamin. The lower panel shows arbitrary densitometric units after the stimulation, expressed as the means ± SE (n = 3). (G) Intracellular cytokine staining was performed with fluorescence-conjugated anti-cytokine Abs. Purified murine splenic naive CD4 T cells were cultured under neutral, Th1, or Th17 conditions for 5 d in the presence of PON-1, and CD4 T cells were restimulated with PMA and ionomycin for 4 h in presence of monensin. The typical percentages of IFN-γ and IL-2 in the culture supernatant were assessed by ELISAs. Purified splenic CD4 T cells were stimulated with immobilized anti-TCR plus soluble anti-CD28 mAbs in the presence of PON-1, as assessed by a Western blot analysis. Arbitrary densitometric units were calculated by dividing the density of pERK1/2 by the density of -actin. The lower panel shows the arbitrary densitometric units after the stimulation, expressed as the means ± SE (n = 3). (H) Proliferative response of murine CD4 T cells was determined by 3H-thymidine uptake. Purified splenic CD4 T cells were stimulated with immobilized anti-TCR plus soluble anti-CD28 mAbs for 48 h in the presence of PON-1. The results are expressed as the means ± SD of CD4 T cells compared with that in the control group = 3). *p < 0.05, in comparison with saline-treated CD4 T cells. (D) Phosphorylation of signaling molecules was assessed by the Bio-Plex system. The results are expressed as the means ± SD (n = 5). *p < 0.05, compared with saline-treated CD4 T cells. (G) Intracellular cytokine staining was performed with fluorescence-conjugated anti-cytokine Abs. Purified murine splenic naive CD4 T cells were cultured under neutral, Th1, or Th17 conditions for 5 d in the presence of PON-1, and CD4 T cells were restimulated with PMA and ionomycin for 4 h in presence of monensin. The typical percentages of IFN-γ and IL-2 in the culture supernatant were assessed by ELISAs. Purified splenic CD4 T cells were stimulated with immobilized anti-TCR plus soluble anti-CD28 mAbs for 48 h in the presence or absence of PON-1. The results are expressed as the means ± SD of IFN-γ production in the cells compared with that in the control group = 3). *p < 0.05, in comparison with saline-treated CD4 T cells. (H) Proliferative response of murine CD4 T cells was determined by 3H-thymidine uptake. Purified splenic CD4 T cells were stimulated with immobilized anti-TCR plus soluble anti-CD28 mAbs for 48 h in the presence or absence of PON-1. The results are expressed as the means ± SD (n = 5). *p < 0.05, in comparison with saline-treated CD4 T cells.
PON activity administration of the anti–TNF-α mAb was small (Fig. 5F).

G3C9 suppresses the development of the CD4+CD45RBhigh cell transfer–induced chronic colitis

Given the efficacy of PON-1 in the TNBS-induced colitis model, we sought to examine whether PON-1 treatment could also suppress colitis in a different model. The CD4+CD45RBhigh cell transfer model of chronic colitis is an experimental animal model for CD (35). Using this model, we administered PON-1 (10 mg/kg) to the mice with colitis three times per week for 8 wk. However, PON-1 administration did not suppress the chronic colitis in these mice. Therefore, we generated G3C9, which has higher esterase activity than PON-1 (enzymatic activity: PON-1, 938 U/mg protein versus G3C9, 4436 U/mg protein). We had previously observed that G3C9 administration clearly attenuated TNBS-induced colitis (Supplemental Fig. 3). Moreover, the administration of G3C9 (10 mg/kg) three times per week for 8 wk significantly suppressed the DAI score and loss of colon length to a level similar to that observed following the administration of the anti–TNF-α mAb (Fig. 6A, 6B). As was expected based on the previous studies, no significant suppression was observed in the PON-1–treated mice (Fig. 6A, 6B). Additionally, consistent with the prevention of a decreased colon length, the histological findings such as ulceration, the loss of goblets cells, presence of crypt abscesses, mucosal erosion and ulceration, and submucosal spread to transmural involvement throughout the colon were also improved by the administration of either G3C9 or the anti–TNF-α mAb, but not PON-1 (Fig. 6C). The immunohistochemical analyses revealed that there was massive infiltration of CD3+ T cells in the colonic lamina propria 8 wk after the injection of CD4+CD45RBhigh T cells, and this was inhibited by the administration of either G3C9 or an anti–TNF-α mAb, but not PON-1 (Fig. 6C). We then examined the mRNA expression of cytokines in the colon 8 wk...
after the injection of CD4+CD45RBhigh T cells. The mRNA expression levels of IL-6 and IL-12 in the G3C9- and anti–TNF-α mAb–treated mice, but not the PON-1–treated mice, were reduced compared with those in the saline-treated mice (Fig. 6D). IFN-γ was also significantly decreased in the G3C9-treated mice. Next, we investigated the influence of all three treatments on the Th1 or Th17 cells in the mLNs 8 wk after the injection of CD4+CD45RBhigh T cells. As shown in Fig. 6E, the generation of IFN-γ–producing mesenteric CD4 T cells appeared to be reduced in both the G3C9 or anti–TNF-α mAb–treated groups compared with the PON-1–treated group, whereas the generation of IL-17–producing mesenteric CD4 T cells tended to increase in G3C9-treated group. It is also of interest that the PON activity in the plasma of the G3C9-treated mice, but not the PON-1–administered mice, was recovered to the normal level (Fig. 6F). These results indicate that the esterase activity plays an important role in the protection of mice against CD4+CD45RBhigh cell transfer–induced colitis, and the efficacy of G3C9 in this model was similar to that of the anti–TNF-α mAb.

Discussion

In this study, we provide clear evidence indicating that PON-1, which is an HDL-associated lipo-lactonase expressed in human plasma, has a suppressive effect on TNBS-induced colitis. Additionally, the administration of G3C9, which shows higher esterase activity than PON-1, attenuated the CD4+CD45RBhigh cell transfer–induced chronic colitis. CD4 T cells from PON-1−/− mice showed higher IFN-γ and TNF-α production than did WT mice under both normal and TNBS-induced colitis conditions. Furthermore, PON-1 exerted a potent suppressive effect on both
murine and human CD4 T cells in vitro and ex vivo studies. Therefore, PON-1 may represent a novel and relatively safe therapeutic agent for CD4 T cell–related colitis in humans.

Activated Th1 cells and the subsequent uncontrolled production of inflammatory cytokines play a critical role in the pathogenesis of CD. The immune pathogenesis of TNBS-induced colitis is associated with increased levels of inflammatory cytokines, including IFN-γ (46–48). Additionally, some inflammatory cytokines, such as IL-17 (32, 49) and IL-6 (36, 50), play a crucial role in the induction of CD in patients and experimental colitis in mice. PON-1 inhibited the IFN-γ production from both murine and human CD4 T cells (Fig. 3). The PON-1–treated colitis mice exhibited lower expression of IFN-γ in mesenteric CD4 T cells, and PON-1 also suppressed the mRNA expression of IFN-γ in the colon tissue (Fig. 2D–F). Interestingly, the mRNA expression of all inflammatory cytokines tested was suppressed to within the normal levels in the colon tissue obtained from PON-1–treated mice after TNBS injection (Fig. 2D–F). Therefore, the inhibition of TNBS-induced colitis by PON-1 treatment was accompanied by reductions in the expression of several inflammatory cytokines, such as IFN-γ, that have been reported to be involved in the pathology of CD.

PON-1 inhibited the phosphorylation of p38MAPK, ERK1/2, and c-Jun, a member of the AP-1 transcription factor family, and suppressed the nuclear translocation of NF-κB in TCR-stimulated CD4 T cells (Fig. 3D, 3E). Therefore, this could be the mechanism by which PON-1 inhibits the activation of and IFN-γ production by CD4 T cells. In fact, IκB degradation and

FIGURE 6. Attenuation of chronic colitis in the CD4+CD45RBhigh transfer model by the administration of G3C9 or an anti–TNF-α mAb, but not by PON-1. (A) DAI score of each group in CD4+CD45RBhigh cell transfer–induced colitis in SCID mice. CD4+CD45RBhigh T cells (5 × 10^5 cells i.p.) and vehicle (saline, i.v.), CD4+CD45RBhigh T cells and PON-1 (10 mg/kg, i.v.), CD4+CD45RBhigh T cells and G3C9 (10 mg/kg, i.v.), or CD4+CD45RBhigh T cells and anti–TNF-α mAb (5 mg/kg, i.v.) were injected into the recipient SCID mice. The results are expressed as the means ± SE (n = 5). *p < 0.05, compared with saline-administered mice. (B) Typical colon morphology of each group 8 wk after the transfer of CD4+CD45RBhigh T cells. The mean colon lengths ± SE were as follows: Non, 14.8 ± 0.2 cm; TNBS plus saline, 11.4 ± 0.5 cm; TNBS plus PON-1, 11.6 ± 0.4 cm; TNBS plus G3C9, 13.7 ± 0.6 cm; and TNBS plus anti–TNF-α mAb, 13.8 ± 0.3 cm; n = 5 per group. (C) The left panels show the typical histological pictures of each group (upper panels, H&E staining; lower panels, an Ab specific for CD3 staining). The right graph shows the histological scores of each group. The results are expressed as the means ± SE (n = 3). *p < 0.05, in comparison with saline-administered mice. Colons were collected 8 wk after the transfer of CD4+CD45RBhigh T cells. Original magnification, ×80. (D) Results of the quantitative RT-PCR analysis of the IFN-γ, IL-17, TNF-α, IL-6, IL-12, and IL-4 mRNA expression in the colon tissues was measured 8 wk after the transfer of CD4+CD45RBhigh T cells and was expressed as a ratio to that of HPRT. The results are expressed as the means ± SE (n = 3). *p < 0.05, compared with saline-administered mice. (E) Percentages of IFN-γ– and IL-17–producing cells in CD4 T cells. Purified mouse mesenteric CD4 T cells collected 8 wk after the transfer of CD4+CD45RBhigh T cells were cultured with PMA and ionomycin for 4 h in the presence of monensin. The left panels show the typical profile of IFN-γ– and IL-17–producing cells of CD4 T cell in each group. The right graph shows the relative percentages of CD4+CD45RBhigh plus saline group. The results are expressed as the means ± SE (n = 5). *p < 0.05, in comparison with saline-administered mice. (F) Plasma PON activity of each group in the SCID mice with CD4+CD45RBhigh cell transfer–induced colitis. The plasma was collected 8 wk after the transfer of CD4+CD45RBhigh T cells. The results are expressed as the means ± SE (n = 5). *p < 0.05, compared with saline-administered mice.
nuclear accumulation of NF-κB subunits is a central feature of CD in human patients (51, 52), as well as in experimental mouse models (53). Additionally, inhibition of NF-κB and ERK/MAPK signaling attenuates the inflammatory response in TNBS-induced colitis (54). The activation of these nuclear transcription factors is essential for the activation of T cells and the transcriptional upregulation of the various cytokine genes (55–57). Taken together, these findings indicate that PON-1 administration attenuated CD by suppressing the TCR/CD28-mediated signaling pathways and their subsequent production of cytokines, such as IFN-γ. Therefore, CD4 T cells appear to be a major target of the inhibitory effects of PON-1 against TNBS-induced colitis.

This study addressed the physiological role of PON-1 in the protection against TNBS-induced colitis using PON−1/− mice (Fig. 4). The PON−1/− mice showed increased serum and macrophage oxidative stress (23, 24). Therefore, the dysfunction of antioxidant activity in PON−1/− mice may induce a malfunction of CD4 T cells, leading to the enhancement of IFN-γ and TNF-α production from mesenteric CD4 T cells and the full extent of TNBS-induced colitis. However, CD4 T cells from naive PON−1/− mice showed higher IFN-γ production than those from WT mice, indicating that the function of CD4 T cells was altered in PON−1/− mice even without any experimental inflammatory stimuli (Fig. 4E). Additionally, the upregulated CD4 T cell function, such as the increased IFN-γ production observed in PON−1/− CD4 T cells, was improved by the addition of PON-1 (Fig. 4F). Therefore, CD4 T cells appear to be the major target for the inhibitory effect of PON-1 in the TNBS-induced colitis model. Alternatively, the development and progression of TNBS-induced colitis in PON−1/− mice was similar to that in WT mice (Fig. 4A, 4B), although the PON−1/− mice showed an increased severity and cytokine production in the colon compared with the colon of WT mice (Fig. 4C, 4D). PON-2 and PON-3 are also members of the PON gene family, which were both suggested to possess antioxidant properties (58, 59). Additionally, superoxide dismutase protects against TNBS-induced colitis by reducing oxidative stress and inflammation (60). Therefore, it is possible that PON-2 and PON-3 contribute to the protection against TNBS-induced colitis, and they may be sufficient to at least partially compensate for the enhanced TNBS-induced colitis caused by the absence of PON-1. Therefore, a combination of PON-1, together with PON-2 and PON-3, may be necessary to achieve a full protective effect against the excessive CD4 T cell response and TNBS-induced colitis.

The use of an Ab specific for TNF-α has already proven to be a highly effective treatment in CD patients (2, 3) and holds some promise for curing CD. Therefore, this study compared the efficacy of PON-1 and the anti–TNF-α mAb in terms of their suppression of TNBS-induced colitis (Fig. 5). The results demonstrated that the efficacy of PON-1 on TNBS-induced colitis was similar to that of the anti–TNF-α mAb. Because PON-1 is a component of normal human plasma, the side effects induced by the administration of PON-1 might be marginal in comparison with those of the anti–TNF-α mAb. In fact, our preliminary preclinical study in mice suggested that no major side effects are observed (unpublished observation). Another important advantage using PON-1 is that it can suppress the cytokine production from CD4 T cells without interfering with their proliferation (Fig. 3B, 3C, 3H, 3I). Therefore, a combination therapy employing PON-1 with a low dose of the anti–TNF-α mAb may prevent the severe side effects, such as serious infections, while providing more effective treatment for CD patients.

To evaluate the effects of PON-1 on chronic colitis, we administered PON-1 to SCID mice transferred CD4+CD45RBhigh cells (Fig. 6). We found that the administration of PON-1 failed to suppress colitis in this model. However, the administration of G3C9, which shows higher esterase activity than PON-1, suppressed the symptoms. In previous studies of PON-1 transgenic mice, PON-1 appeared to exert its antiatherogenic effects through its antioxidative functions in a dose-dependent manner (25). Therefore, the higher antioxidative activity of G3C9 may be associated with its greater prevention of CD4+CD45RBhigh cell transfer–induced chronic colitis compared with PON-1. Furthermore, reduced PON activity in the plasma was observed after the injection of TNBS, and this change and the severity of colitis after the TNBS injection were improved in all groups following G3C9 treatment (Fig. 5F). Reduced PON activity was also observed in the plasma of the chronic colitis model, and this change was improved by administration of G3C9 but not by treatment with PON-1 (Fig. 6F). These results indicate that the loss of PON activity is closely associated with the severity of the CD4+ CD45RBhigh cell transfer–induced chronic colitis. Previous reports also demonstrated a reduction of the PON-1 enzymatic activity in CD patients and in ulcerative colitis patients (61, 62). Therefore, the PON activity may be inversely correlated with the disease activity, and it could be a good disease marker of CD.

In summary, this study showed that PON-1 protected mice from TNBS-induced colitis, and that G3C9 protected against CD4+ CD45RBhigh cell transfer–induced chronic colitis by suppressing the expression of several inflammatory cytokines from mesenteric CD4 T cells, which was accompanied by a reduced expression of several inflammatory cytokines in the colon, including IFN-γ. The present study may provide a new perspective regarding the treatment of CD T cell–dependent colitis in humans, and it suggests that PON-1 may be a novel target for the disease.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental figure 1. PON-1 treatment did not induce apoptosis in CD4 T cells after the activation.
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Supplemental figure 2. CD4 and CD8 T cell profile in PON-1−/− mice. Flow cytometric analysis of leukocytes from the thymus, spleen, mesenteric lymph node, and peripheral blood in PON-1−/− or Wild-type mice was performed to determine the expression profile of CD4 and CD8.
Supplemental figure 3. G3C9 administration attenuated the TNBS-induced colitis. The DAI score of each group in the mice with TNBS-induced colitis. TNBS (100 mg/kg, i.r.) and vehicle (saline, i.v.) or TNBS and G3C9 (10 mg/kg, i.v.) were injected into C57BL/6 mice. The results were expressed as the means±SE (n=5). *P < 0.05, in comparison to the saline-treated mice.