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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 Haras,† 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, idiopathic, and relapsing form of inflammatory bowel disease that affects mostly young adults. Although the etiology of CD is not completely understood, there is growing evidence indicating that the dysfunction of mucosal T cells, altered cytokine production, and cellular inflammation play an important role in the pathogenesis of CD (1). The available therapies for CD patients based on immunosuppressive drugs are widely used to inhibit autoreactive immune responses. However, these drugs are not entirely effective and are nonspecific, thus leading to the development of numerous side effects during prolonged treatment, and thereby making them less than ideal for the treatment of CD patients.

Although biological drugs targeting TNF-α have proven to be effective for CD patients, 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
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 Biologics (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 restriction site and an EcoRI restriction site was added into each synthetic gene sequence of human PON-1. The amino acid sequence of G3C9-C284A was based on that of G3C9 (27), with cysteine 284 modified to alanine. The amino acid sequence shown in National Center for Biotechnology Information GenBank (AY499193) used the sequence of G3C9. The GS expression vector, pEE12.4, carries the GS gene, and its product catalyzes the formation of glutamine from glutamate and ammonia (28). The CHO cells were transfected with each expression vector and cultured in glutamine-free CHO medium in the presence of the GS inhibitor methionine sulfoximine (Sigma-Aldrich). The GS-CHO cells overexpressing each protein were seeded in CD-CHO medium supplemented with CHO CD EfficientFeed B (Invitrogen). The supernatants were harvested after 12 d of fermentation and then were directly loaded on the UNOSphere Q (Bio-Rad). After being loaded, the column was washed and proteins were eluted. Eluted proteins were applied on the ceramic hydroxyapatite column type I (Bio-Rad). After being loaded on this column, the proteins were eluted with 50 mM phosphate buffer (pH 7.2). Finally, the eluted proteins were dialyzed against 20 mM Tris-HCl, 2 mM CaCl2 buffer (pH 7.0). The measurement of the enzymatic activity of PON-1 or G3C9-C284A (G3C9) was determined as the paraoxon hydrolysis activity. The method used to assess the paraoxon hydrolysis activity was adapted to a microtiter plate assay method essentially as described in a previous study (29).

SDS-PAGE and two-dimensional electrophoresis

SDS-PAGE (5–20% gels) was used to separate proteins. After electrophoresis, the gels were stained with Coomassie brilliant blue or silver. Two-dimensional electrophoresis was performed as described previously (30), with slight modifications. Briefly, protein samples were mixed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% ampholine [pH 3–10], 20 mM DTT, and 0.0015% bromphenol blue) and loaded onto Immobiline DryStrips (pH 3–10, nonlinear; GE Healthcare). Isoelectric focusing was performed for a total of 30,000 Vh on an IPGPhor apparatus (GE Healthcare). After a standard SDS equilibrating step, the proteins were further separated by SDS-PAGE.

Preparation of CD4 T cells and neutral/Th1/Th17 cell differentiation

Murine splenic naïve CD4 T cells were purified from spleenocytes using the CD4+CD62L+ T Cell Isolation Kit II (Miltenyi Biotec) and autoMACS cell sorting (Miltenyi Biotec). Naïve CD4 T cells were stimulated with 3 μg/ml immobilized anti-TCRB 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-TCRB 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-TCRB 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 (XMGI12; BioLegend), and anti–IL-2 (JES6-1A12; eBioscience) mAbs (32) for Th17 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 (Amsersham Biosciences). After centrifugation, the mononuclear cells in the interface were harvested. Human naïve CD4 T cells were purified from mononuclear cells using a naïve CD4+ T Cell Isolation Kit II (Miltenyi Biotec) and autoMACS cell sorting. The cells were stimulated with 20 μg/ml immobilized anti-CD3 mAb (Jansen 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-technology). Th1 cell differentiation was performed with 20 μg/ml immobilized anti-CD3 mAb for 2 d in the presence of 1 ng/ml TGF-β (PeproTech), 10 U/ml IL-2, 10 ng/ml IL-1β (PeproTech), 10 ng/ml IL-6 (PeproTech), 10 ng/ml IL-23 (PeproTech), 5 μg/ml anti-CD28, and anti–IFN-γ (4S.B3; BioLegend) mAbs (32) for Th17 cell differentiation.

In some cases, PON-1, 6A, antithrombin III (ATIII), the IgG fraction, or a neutrophilic mAb against human PON-1 (H-20; Santa Cruz Biotechnology) were also added into the culture, as indicated. On day 5, the cytokine production was determined by either ELISA or intracellular cytokine staining.

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 and human CD4 T cells were cultured with an allopregocyanin-conjugated anti–IFN-γ mAb (XMGI12; BD Pharmingen), PE-conjugated anti–IL-4 mAb, or PE-conjugated anti–IL-17 mAb (TC11–18H10; BD Pharmingen). Human CD4 T cells were cultured intracellularly with a FITC-conjugated anti–IFN-γ mAb, PE-conjugated anti–IL-4 mAb (BD FastImmune IN–IF–IL–4; BD Pharmingen), FITC-conjugated anti–IL-17 mAb (BL168; BioLegend), or PE-conjugated anti–IFN-γ mAb. The flow cytometric analysis was performed on a FACS Calibur cell sorter (BD Biosciences), and results were analyzed using the FlowJo software program (Tree Star).

In vivo experimental design of TNBS-induced colitis experiments

Experimental colitis was induced by the rectal administration of a TNBS solution in mice as described previously (32). Briefly, mice were anesthetized on day 0, and a silicon catheter was inserted intrarectally (i.r.), 4 cm distal to the anus. TNBS (100 mg/kg) dissolved in 50% ethanol was injected in the colonic lumen via a catheter. Control mice were administered 50% ethanol alone using the same technique. Mice were carefully held in a vertical posture for 10 min after the TNBS injection to ensure that the TNBS was distributed throughout the entire colon and cecum. PON-1 (1 or 10 mg/kg, n = 5), G3C9 (10 mg/kg, n = 5), or an anti–TNF-α mAb (5 mg/kg, n = 5, MP6-XT22; R&D Systems) was slowly administered via the tail vein before the injection of TNBS, and then once a day for 3 d. To assess the therapeutic efficacy of PON-1 (10 mg/kg, n = 5), 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 body weight, stool consistency, and rectal bleeding were examined daily during the experiments to assess the disease activity of the colitis. The disease activity index (DAI) score for colitis was determined using a scoring system described previously (34). Blood was collected from the heart 1 d after the last injection of TNBS in mice as described (35, 36). Briefly, splenic CD4 T cells were first purified from...
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<sup>hi</sup> (16A; BD Pharmingen)-positive phenotype were further purified using a FACSArria cell sorter (BD Biosciences). The CD45RB<sup>hi</sup>-positive phenotype was defined as the most highly stained cells consisting of 30–40% CD4 T cells. Before i.p. injection of CD4<sup>+</sup>CD45RB<sup>hi</sup> cells (5 × 10<sup>6</sup> 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 equal 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 colons and the mLNs were isolated simultaneously 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. 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, paraffin-embedded colon tissue was sectioned serially after being deparaffinized 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<sup>+</sup> cells. The specimens were examined using a light microscope at ×80 magnification.

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

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-TCR<sup>+</sup> 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 tissues samples using an RNaseasy Mini Kit (Qiagen), and cDNAs were synthesized using ReverTra 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 hypoxanthine 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′-ATGAGCACAAAAGCATTGAC-3′; reverse primer, 5′-TACAAGCCTTGCAGCTTCAATG-3′), IFN-γ (forward primer, 5′-ATCCGAGAAGGAGACTGCAAA-3′; reverse primer, 5′-TCTAGAACCTCAAGAGATGCTGAGTA-3′), IL-17A (forward primer, 5′-CAAGGACACTGTGATGCTGCTGTA-3′; reverse primer, 5′-GCCGTAGCCTTGGAGGATGTG-3′), IL-4 (forward primer, 5′-CTCCGC-TCTTCTTTCTGCGAATG-3′; reverse primer, 5′-CACCATCCCATCCGTCG-5′), IL-12A (forward primer, 5′-TCAGAATCAACAACTCAGC-A-3′; reverse primer, 5′-GCCGATATGTTAGAACAGCAGCTG-3′), T-bet (forward primer, 5′-CAACAAGACCCCCATAGCACA-3′; reverse primer, 5′-CACCCATTATGTTAGAACAGCAGCTG-3′), Sup (forward primer, 5′-GCCGACCTGTCGTTAGCTGCT-3′; reverse primer, 5′-GGGCTATTCTCAGGCAT-G-3′), and HPRT (forward primer, 5′-TCCCTCCTCAGCCTTCTTTT-3′; reverse primer, 5′-CGCTGTCATCATCCTGGAATC-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-TCR<sup>+</sup> plus 2 μg/ml soluble anti-CD28 mAbs or 20 μg/ml immobilized anti-CD3 mAb for 48 h in the presence or absence of PON-1, respectively. The culture supernatants were harvested, and the IL-2 and IFN-γ secretion were measured by specific ELISAs. To assess the proliferation of murine or human CD4 T cells, these cells were stimulated with 3 μg/ml immobilized anti-TCR<sup>+</sup> plus 2 μg/ml soluble anti-CD28 mAbs or 20 μg/ml immobilized anti-CD3 mAb for 40 h in the presence or absence of PON-1, respectively. [3H]thymidine (37 kBq/well) was added to the culture for the last 6 h, and the incorporated radioactivity was then measured on a β-plate (37).

**Phosphoprotein assay**

Murine spleen naive CD4 T cells were stimulated with 3 μg/ml immobilized anti-TCR<sup>+</sup> plus 2 μg/ml soluble anti-CD28 mAbs in the presence or absence of PON-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 IκB-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 spleen naive CD4 T cells were stimulated with 3 μg/ml immobilized anti-TCR<sup>+</sup> plus 2 μg/ml soluble anti-CD28 mAbs in the presence or absence of PON-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 analyses**

All statistical analyses were performed using the GraphPad Prism software program. The differences were determined by an unpaired two-tailed Student’s t test or one-way ANOVA with a Dunnett multiple comparison test, 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 PON-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-γ production by murine differentiating Th1 cells. Among the five proteins tested, PON-1 was the best inhibitor of IFN-γ 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-γ production by murine or human differentiating Th1 cells (Fig. 1E). Moreover, the inhibition of IFN-γ 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). These
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 goblets 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) 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 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 colons 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 tend 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 inhibited 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 IκBα, 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
FIGURE 3. PON-1 suppresses the activation and function of murine and human CD4 T cells in vitro. (A) Intracellular cytokine staining was performed with fluorescently 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 results are expressed as the mean IFN-γ production in the cells compared with that in the control group ± SD under neutral conditions was as follows: saline, 100%; 0.04 mg/ml, 58.9 ± 3.4%; 0.2 mg/ml, 46.0 ± 3.2%; and 1.0 mg/ml, 37.6 ± 2.3%. The mean IL-4 production in the cells compared with that in the control group ± SD under neutral conditions was as follows: saline, 100%; 0.04 mg/ml, 97.5 ± 11.1%; 0.2 mg/ml, 55.2 ± 4.2%; and 1.0 mg/ml, 24.8 ± 1.5%. The mean IFN-γ production in the cells compared with that in the control group ± SD under Th1 conditions was: saline, 100%; 0.04 mg/ml, 94.6 ± 3.4%; 0.2 mg/ml, 94.9 ± 1.9%; and 1.0 mg/ml, 79.5 ± 1.5%; n = 3 for each group. (B) Proliferative response of murine CD4 T cells was determined by [3H]thymidine uptake. Purified splenic CD4 T cells were cultured under neutral, Th1, or Th17 conditions for 5 d in the presence of PMA and ionomycin for 4 h in presence of monensin. The results are expressed as the mean IFN-γ production in the cells compared with that in the control group ± SD (n = 5). *p < 0.05, in comparison with saline-treated CD4 T cells. (C) Proliferative response of human CD4 T cells was determined by [3H]thymidine uptake. Purified human CD4 T cells were stimulated with the immobilized anti-CD3 mAb for 48 h in the presence or absence of PON-1, and CD4 T cells were restimulated with PMA and ionomycin for 4 h in presence of monensin. The results are expressed as the mean IFN-γ production in the cells compared with that in the control group ± SD (n = 5). *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. (E) Phosphorylation of signaling molecules was assessed by the Bio-Plex system. The results are expressed as the means ± SD (n = 5). *p < 0.05, in comparison with saline-treated CD4 T cells. (F) 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 of monensin. The results are expressed as the means ± SD under neutral conditions was as follows: saline, 100%; 0.04 mg/ml, 92.5 ± 11.1%; 0.2 mg/ml, 90.5 ± 20.2%; and 1.0 mg/ml, 79.7 ± 6.3%. The mean IFN-γ production in the cells compared with that in the control group ± SD under Th1 conditions was as follows: saline, 100%; 0.04 mg/ml, 94.3 ± 0.7%; 0.2 mg/ml, 90.5 ± 20.2%; and 1.0 mg/ml, 79.7 ± 6.3%. The mean IFN-γ production in the cells compared with that in the control group ± SD under Th17 conditions was as follows: saline, 100%; 0.04 mg/ml, 91.5 ± 5.5%; 0.2 mg/ml, 66.0 ± 12.3%; and 1.0 mg/ml, 35.9 ± 9.2%. The mean IL-17 production in the cells compared with that in the control group ± SD under Th17 conditions was: saline, 100%; 0.04 mg/ml, 94.6 ± 3.4%; 0.2 mg/ml, 94.9 ± 1.9%; and 1.0 mg/ml, 79.5 ± 1.5%; n = 3 for each group. (H) Proliferative response of human CD4 T cells was determined by [3H]thymidine uptake. Purified human CD4 T cells were stimulated with an immobilized anti-CD3 mAb 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. (I) Proliferative response of human CD4 T cells was determined by [3H]thymidine uptake. Purified human CD4 T cells were stimulated with an immobilized anti-CD3 mAb 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.
G3C9 suppresses the development of the CD4⁺CD45RB<sup>high</sup> 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⁺CD45RB<sup>high</sup> 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). We then examined the mRNA expression of cytokines in the colon 8 wk post-injection. The quantitative RT-PCR analysis revealed that IFN-γ, IL-17, TNF-α, IL-6, IL-12, and IL-4 mRNA expression in the colon tissue was measured 3 d after TNBS injection and is expressed as a relative ratio to that of HPRT. The results are expressed as the means ± SE (n = 3). *p < 0.05, in comparison with WT mice. (E) Purified murine mesenteric CD4 T cells 3 d after TNBS injection were cultured in the presence of immobilized anti-TCRβ plus soluble anti-CD28 mAbs for 48 h, and the amounts of IFN-γ and TNF-α in the culture supernatant were assessed by ELISA. The results are expressed as the means ± SD (n = 5). *p < 0.05, in comparison with WT mice. (F) Purified splenic CD4 T cells from PON-1<sup>−/−</sup> or WT mice were cultured under Th1 conditions for 5 d in the presence or absence of PON-1 (1.0 mg/ml), and the amount of IFN-γ in the culture supernatant was assessed by ELISA. The results are expressed as the means ± SD (n = 5). *p < 0.05, in comparison with WT CD4 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+ cell transfer–induced chronic colitis. CD4 T cells from PON-12/2 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...
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. 4E). 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 transfected 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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References
PARAOXONASE-1 INHIBITS COLITIS


