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Ablation of Peroxiredoxin II Attenuates Experimental Colitis by Increasing FoxO1-Induced Foxp3+ Regulatory T Cells

Hee Yeon Won,*† Eun Jung Jang,*† Kihyun Lee,* Sera Oh,* Hyo Kyung Kim,* Hyun Ae Woo,* Sang Won Kang,* Dae-Yeul Yu,*† Sue-Goo Rhee,* and Eun Sook Hwang*†

Peroxiredoxin (Prx) II is an intracellular antioxidant molecule that eliminates hydrogen peroxide, employing a high substrate-binding affinity. PrxII deficiency increases the levels of intracellular reactive oxygen species in many types of cells, which may increase reactive oxygen species–mediated inflammation. In this study, we investigated the susceptibility of PrxII knockout (KO) mice to experimentally induced colitis and the effects of PrxII on the immune system. Wild-type mice displayed pronounced weight loss, high mortality, and colon shortening after dextran sulfate sodium administration, whereas colonic inflammation was significantly attenuated in PrxII KO mice. Although macrophages were hyperactivated in PrxII KO mice, the amount of IFN-γ and IL-17 produced by CD4+ T cells was substantially reduced. Foxp3+ regulatory T (Treg) cells were elevated, and Foxp3 protein expression was increased in the absence of PrxII in vitro and in vivo. Restoration of PrxII into KO cells suppressed the increased Foxp3 expression. Interestingly, endogenous PrxII was inactivated through hyperoxidation during Treg cell development. Furthermore, PrxII deficiency stabilized FoxO1 expression by reducing mouse double minute 2 homolog expression and subsequently activated FoxO1-mediated Foxp3 gene transcription. PrxII overexpression, in contrast, reduced FoxO1 and Foxp3 expression. More interestingly, adoptive transfer of naive CD4+ T cells from PrxII KO mice into immune-deficient mice attenuated T cell–induced colitis, with a reduction in mouse double minute 2 homolog expression and an increase in FoxO1 and Foxp3 expression. These results suggest that inactivation of PrxII is important for the stability of FoxO1 protein, which subsequently mediates Foxp3+ Treg cell development, thereby attenuating colonic inflammation. The Journal of Immunology, 2013, 191: 4029–4037.

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Prx act as antioxidants by catalyzing the conversion of hydrogen peroxide or alkyl hydroperoxides to water or alcohol, processes mediated by an oxidizing redox-sensitive cysteine residue at the active site (5–8). Prxs are widely expressed in the cytosol, mitochondria, peroxisome, and endoplasmic reticulum (9, 10), and six different mammalian isoforms have been discovered (7). Prxl and PrxII are most abundantly expressed in the cytosol and are involved in fine-tuning intracellular ROS concentrations generated by receptor activation (5). Prxl knockout (KO) mice exhibit increased sensitivity to oxidative DNA damage (11), and PrxII KO mice develop severe hemolytic anemia, because of erythrocye hemolysis induced by oxidative stress (12). In addition, PrxII KO mice are susceptible to LPS-induced inflammation and atherosclerosis due to activated macrophages and increased endothelial adhesion molecules (13, 14). T lymphocytes and CD11c+ dendritic cells of PrxII KO mice are also highly proliferative and are thus involved in the development of allergen-induced airway inflammation (15, 16). This indicates the important role of PrxII in maintaining immune cell homeostasis. However, little is known about the function of PrxII in adaptive immune cells such as CD4+ T cells or in the development of T cell–mediated inflammatory diseases.

Emerging evidence suggests that ROS are generated not only in phagocytes, such as macrophages and dendritic cells, but also in T cells through the stimulation of TCRs (17–19). NOX-mediated ROS generation in TCR-activated T cells activates Fas ligand and...
stimulates activation-induced apoptotic cell death (20–23). However, ROS generation is essential for IL-2 production and the proliferation of T cells (24). Interestingly, increased oxidative stress in GPx1-deficient T cells affects lineage commitment of CD4+ Th cells, reducing Th2 and Th17 development and thereby attenuating allergen-induced airway inflammation (24). Conversely, an abnormal redox balance in NOX2-deficient T cells enhances Th2 and Th17 differentiation and causes severe airway inflammation and spontaneous rheumatoid arthritis (25, 26). This suggests that fine-tuning of redox homeostasis in CD4+ T cells is critical for Th cell development and control of inflammatory disease. Given the importance of redox homeostasis in Th cell differentiation, we investigated the effects of PrxII in T cell–mediated colonic inflammation.

Materials and Methods

Reagents

All cytokines and anti-cytokine Abs for ELISA and flow cytometry analysis were obtained from BD Pharmingen (San Diego, CA). Mouse IL-1β, TNF-α, and IL-10 ELISA Ready-Set-Go ELISA kits were purchased from eBioscience (San Diego, CA). Dextran sulfate sodium (DSS) was purchased from MP Biomedicals (Solon, OH).

Mice

C57BL/6 wild-type (WT) and PrxII KO mice with a C57BL/6 genetic background (12) were housed and maintained in the animal facility at Ewha Womans University. Animal handling was performed in accordance with the institutional guidelines. All procedures were approved by the Institutional Animal Care and Use Committee (ELAGC-07-1042 and IACUC-2010-8-3).

Development and assessment of DSS-induced colitis

Males at 10–12 wk of age were administered 3% DSS in water for 7 d to induce colitis. Chronic colitis was induced by four cycles of administrating 3% DSS in water for 4 d, followed by normal drinking water for 3 d. The disease activity index, representing the severity of colitis, was calculated by combining scores for percentage of weight loss, stool consistency, and fecal bleeding, as described previously (27). For histopathological examination, mouse colon was fixed in 4% paraformaldehyde (Sigma-Aldrich), embedded in paraffin, and sectioned serially at 5 μm thickness. Sections were stained with H&E stain or periodic acid–Schiff (PAS) and viewed using an Eclipse E200 microscope (Nikon). The histological score was calculated by averaging the epithelium and infiltration scores, as previously described (27).

Bone marrow chimera

Bone marrow (BM) cells were harvested from the femur and tibia of WT and KO males at 8–10 wk of age. Recipient WT mice were sublethally (6.5 Gy) irradiated, and each recipient received 1 × 10^7 BM cells via tail vein injection. Recipients were allowed to reconstitute for an additional 60 d. At day 60, survival was 70% after transplantation of BM. Chimeric mice were then treated with normal water or 3% DSS in drinking water for 8 d. Colonos were collected for examination of colon shortening, histopathology, and lamina propria cell (LPC) isolation.

Immunohistochemistry

Mouse colon was sectioned, incubated with Alex Fluor 647–conjugated anti-mouse CD4 Ab (eBioscience, San Diego, CA), and stained with DAPI (Invitrogen, Carlsbad, CA) and DAPI. Cells were photographed with a fluorescence confocal microscope.

Immunoblot analysis

Single-cell suspensions of mesenteric lymph nodes (mLN) were harvested from the mice. Total protein was extracted from the mLN and cultured cells and analyzed by SDS-PAGE, followed by immunoblot analysis with Ab against retinoic acid–related orphan receptor (ROR) γt, Foxp3 (eBioscience), STAT3, NF-κB p65, JNK, mouse double minute 2 homolog (MDM2), β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), pSTAT3, FoxO1, pJNK, phosphatase and tensin homolog (PTEN) (Cell Signaling Technology, Danvers, MA), PrxII, PrxII-SO2/3 (LabFrontier, Seoul, Korea), or FoxO3 (Millipore, Temecula, CA).

Culture of colonic LPC and ELISA

A piece of colon tissue (1 cm) was removed, cut open longitudinally, and washed in PBS containing penicillin and streptomycin. Single suspensions of LPC were isolated from the mLN and cultured cells by using DSS-PAGE, followed by immunoblot analysis. Single-cell suspensions of mLNs were stained with anti-CD3 Ab (1 μg/ml) for 24 h. Culture supernatants were harvested and incubated on a nitrocellulose membrane (Merck Millipore, Foster City, CA). The specific primer sets used were as follows: IL-21,
Flow cytometry analysis

Single-cell suspensions of spleen, mLN, or regulatory T (Treg) cells were incubated with PE-conjugated anti-F4/80 Ab or an Ab mixture containing allophycocyanin CD4, FITC CD25, and PE Foxp3 Abs for 20 min on ice, according to the manufacturer’s instructions (eBioscience). Cells were then stained with antibodies specific to various markers and analyzed using a flow cytometer. The data were analyzed using flow cytometric software to quantify the expression of different markers on the cell surface.

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FIGURE 2. Protection against inflammatory colitis by PrxII deficiency. WT and KO mice (n = 6–9 for each group) were administered normal water or 3% DSS water for 7 d, and mice were sacrificed at day 7. (A) Representative macroscopic images of colons from vehicle- or DSS-treated WT and KO mice. Colon length was assessed at necropsy on day 7. Data are expressed as the mean ± SEM (n = 6). (B) Representative images of colon tissues stained by H&E and PAS. Scale bar, 1000 μm. (C) The histological score was calculated from six mice, as described in Materials and Methods. (D) LPC from the colon (pooled from three mice) were isolated by Percoll gradient centrifugation and cultured ex vivo for 24 h in complete RPMI 1640 medium. The supernatant was collected and subjected to ELISA for measuring INF-γ, IL-17, IL-6, and IL-1β. The results shown are the mean ± SEM of three independent experiments (n = 9). nd, not detected; ns, not significant; *p < 0.05, **p < 0.005, ***p < 0.0005.

FIGURE 3. Impaired IL-17 production and augmented Foxp3+ Treg cells in PrxII KO mice under inflammatory conditions. Acute colitis was induced in WT and KO mice (n = 6) by treating with 3% DSS water for 7 d. WT and KO mice were fed normal water and used as a control, and mLN and LPC were harvested at day 7. (A and B) Single-cell suspensions were isolated from the mLN and were stimulated with plate-bound anti-CD3 for 24 h. The supernatant was used for measuring IFN-γ and IL-17 by ELISA (A). Cells were harvested for preparing total RNA using TRIZol. The relative transcript levels of IL-21 and IL-23R determined by reverse transcription and real-time PCR were normalized to the β-actin level (B). Data are expressed as the mean ± SEM of six mice. ns, not significant; *p < 0.05, **p < 0.005, ***p < 0.0005. (C) Total protein was isolated from the mLN and subjected to immunoblot analysis. (D and E) Single-cell suspensions of mLN cells were incubated with allopregocyanin CD4 and PE IL-6R (D) or allopregocyanin CD4, FITC CD25, and PE Foxp3 (E) and were subsequently analyzed by flow cytometry. Surface IL-6R expression and CD4+CD25+Foxp3+ T cells were assessed by the CellQuest program and presented as the mean ± SEM (n = 5). ns, not significant; **p < 0.005. (F) Colonic LPC were harvested from DSS-treated WT and KO mice (four mice were pooled), stained with allopregocyanin CD4 and PE Foxp3, and analyzed by flow cytometry. Representative flow cytometry plots of three independent experiments are shown.
washed twice with PBS and analyzed by flow cytometry and CellQuest software (BD Biosciences, San Diego, CA).

**In vitro Th cell differentiation**

Naive CD4+ T cells were isolated from the draining lymph nodes (LN) and spleen of WT or KO mice using a naïve CD4+ T cell isolation kit (R&D Systems, Minneapolis, MN). The cells were stimulated with plate-bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (1 μg/ml) for 24 h and cultured in the presence of human IL-2 (10 U/ml; BD Pharmingen). For Th1, Th2, and Th17 cell differentiation, cells were additionally treated with cytokine and anti-cytokines, as follows: IL-12 (2 ng/ml) and anti-IL-4 Ab (5 μg/ml) for Th1; IL-4 (10 ng/ml) and anti–IFN-γ (5 μg/ml) for Th2; and TGF-β (5 ng/ml), IL-6 (5 ng/ml), anti-IL-4 Ab (5 μg/ml), and anti–IFN-γ (5 μg/ml) for Th17 cell development. At day 3, the cells were expanded in the presence of additional IL-2, cultured for an additional 3–4 d, and stimulated for a second stimulation with anti-CD3 (1 μg/ml) for 24 h.

**Development of Foxp3+ Treg cells and retroviral transduction**

Naive CD4+ T cells (2 × 10^6 cells/ml) were stimulated with plate-bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (1 μg/ml) in the presence of TGF-β (5 ng/ml), IL-2 (10 U/ml), anti-IL-4 Ab (5 μg/ml), and anti–IFN-γ (5 μg/ml) for 24 h. For retroviral transduction, Platinum (Plat)-E cells were transfected with mock vector (RV) or PrxII expression vector together with viral genes. Transfected cells were cultured in 10% RPMI 1640 medium for 24 h posttransfection and incubated at 32°C for 48 h. The viral supernatants were concentrated using a polyethylene glycol virus precipitation kit (BioVision, Milpitas, CA) and added to 24-h activated Treg cells in the presence of polybrene (4 μg/ml) overnight. Treg cells were then refreshed with 10% RPMI 1640 medium and cultured under Treg-skewing conditions for an additional 3–4 d.

**T cell transfer model of colitis**

Naive CD4+ T cells were isolated from LN of WT and KO mice using naïve CD4+ T cell isolation kit (R&D systems) with 94% purity, and cells were transferred to 6- to 7-wk-old RAG1 KO mice (0.5 × 10^6 cells/mouse) by i.p. injection. RAG1 WT and RAG1/KO mice were then allowed to develop colitis and sacrificed at 40 d following the adoptive transfer.

**Reporter gene assay**

MEF cells from WT and KO mice were established and maintained in DMEM supplemented with 10% FBS. MEF and highly transfectable 293T cells were transiently transfected with expression vectors for PrxII, NF-κB p65, or FoxO1 together with pFirePro and pCMVβ-gal. The pFirePro reporter gene contains the −500 to +100 region of the Foxp3 gene promoter linked to the luciferase gene (28). As previously described (29), cells were harvested for the reporter gene assay using a luciferase assay kit (Promega, Madison, WI) and a Galacto-Light system (TROPIX, Bedford, MA).

**Statistical analysis**

Data presented as the mean ± SEM were analyzed using an unpaired Student *t* test or ANOVA. The *p* value < 0.05 was considered statistically significant. Significance was annotated as follows: ns, not significant; *p* < 0.05; **p** < 0.005; ***p*** < 0.0005.

**Results**

**PrxII-deficient mice are resistant to the development of DSS-induced colitis**

Imbalance between antioxidants and pro-oxidants and subsequent ROS dysregulation affects the development of inflammatory diseases (2). Consequently, we investigated the susceptibility of PrxII KO mice to DSS-induced colitis. We first confirmed increased levels of ROS in the mLN, the spleen, and LFC after treatment with DSS. Intracellular ROS generation was higher in the LFC from DSS-treated KO mice than in the LFC from DSS-treated WT mice (Supplemental Fig. 1A). After treatment with DSS, WT mice exhibited significant weight loss, diarrhea, bloody stools, histological injury, and mortality (Fig. 1). PrxII KO mice exhibited substantially less weight loss (Fig. 1A) and lower disease severity scores following DSS treatment (Fig. 1B). Moreover, PrxII KO mice displayed delayed mortality compared with WT mice (Fig. 1C). We next induced chronic colitis in WT and PrxII KO mice and measured body weight every other day during the induction period. WT mice lost ~15–20% of their original body weight by day 9 after DSS administration and gradually regained the lost weight, but no decrease in body weight was observed in PrxII KO mice during this period (Fig. 1D).

**Colonic inflammation is reduced in PrxII KO mice**

Because colitis is accompanied by histological injuries, such as colon shortening, leukocyte infiltration, and loss of the intestinal epithelial barrier and goblet cells, we analyzed colon length and histological phenotypes in WT and KO mice. When untreated, PrxII KO mice had colon lengths similar to WT mice. After treatment with DSS, pronounced colon shortening was observed in WT mice. However, DSS-induced colon shortening was significantly attenuated in PrxII KO mice (Fig. 2A). Furthermore, histological analysis based on H&E and PAS staining showed extensive leukocyte infiltration,

**FIGURE 4.** Suppression of Foxp3+ Treg cell development by PrxII. Naive CD4+ T cells were isolated from WT and KO mice (*n* = 8) and induced to differentiate into effector and Treg cells for additional 4–5 d. After restimulation with anti-CD3, cell supernatants of Th1, Th2, Th17, and Treg cells were harvested and used for measuring IFN-γ (*A*), IL-4 (*B*), IL-17 (*C*), and IL-10 (*D*), respectively. Data are given as the mean ± SEM for four experiments. ns, not significant; **p** < 0.005. (*E*) Developing Treg cells were harvested, stained with FITC CD25 and PE Foxp3, and analyzed by flow cytometry. Representative flow cytometry images are shown. The percentage of Foxp3+ Treg cells was calculated from six mice. Data are given as the mean ± SEM (*n* = 6). (*F*) Total protein was harvested from each developing effector Th and Treg cell 48 h after TCR stimulation and analyzed by immunoblotting. (*G*) Naive CD4+ T cells were isolated from WT and KO mice and labeled with CFSE (5 μM). CFSE-labeled cells were induced to differentiate into Treg cells by treatment with TGF-β (10 ng/ml). Cells were harvested at days 2 and 3 and were analyzed by flow cytometry. The percentage of Treg cells in divisions 1 through 6 was determined by CellQuest software. (*H*) WT and KO Treg cells were generated from naive CD4+ T cells by treatment with TGF-β for 72 h. CFSE-labeled naive CD4+ T cells were separately stimulated with anti-CD3 and anti-CD28 for 24 h and cultured with WT or KO Treg cells at a Treg/T effector ratio of 1:2 for an additional 48 h. Proliferating cells were analyzed by flow cytometry. Representative images of three independent experiments are shown in (G) and (H).
goblet cell loss, and intestinal epithelial disturbances and blunting in DSS-treated WT mice, but these responses were not prominent in PrxII KO mice (Fig. 2B). Histopathological scores confirmed a significant reduction of colonic inflammation in PrxII KO mice compared with that in WT mice (Fig. 2C). Additional analysis of inflammatory cytokines in the colon demonstrated that proinflammatory cytokines such as IFN-γ, IL-17, IL-6, and IL-1β were elevated in WT mice after DSS treatment. PrxII KO mice produced significantly less IFN-γ, IL-17, and IL-1β, but expressed a comparable amount of IL-6, relative to WT mice (Fig. 2D).

Inflammatory cytokines IFN-γ and IL-17 are reduced in DSS-treated PrxII KO mice

To examine the mechanisms underlying the protective effects of PrxII deficiency in colonic inflammation, we analyzed proinflammatory cytokines in the mLN. DSS stimulation substantially increased IFN-γ and IL-17 production in WT mice, but KO mice produced much less of these cytokines (Fig. 3A). Furthermore, PrxII deficiency attenuated the expression of IL-21 and IL-23R, IL-17-related inflammatory molecules increased by treatment with DSS (Fig. 3B). We then examined signaling molecules that control IFN-γ and IL-17 production in T cells, particularly CD4+ T cells (30). RORγt and phosphorylation of STAT3 (pSTAT3) were increased in WT mice following treatment with DSS, but DSS-treated KO mice showed a significant reduction in RORγt expression and pSTAT3 (Fig. 3C). Consistently, the expression of IL-6R, a molecule upstream of pSTAT3, was lower in KO cells (Fig. 3D). Interestingly, the expression of a transcription factor for Treg cell development, Foxp3, was diminished in mLN cells from DSS-treated WT mice, but was maintained in KO cells (Fig. 3C). Consistent with sustained Foxp3 expression in DSS-treated KO cells, the percentage and absolute number of CD4+CD25+Foxp3+ Treg cells appeared to be higher in KO mice than in WT mice (Fig. 3E, Supplemental Fig. 1B, 1C). Intracellular cytokine staining of activated CD4+ T cells revealed a reduction of IL-17 in KO mice (Supplemental Fig. 1D). Moreover, the frequency and absolute number of Foxp3+ Treg cells were substantially increased in colonic LPC of DSS-treated KO mice (Fig. 3F, Supplemental Fig. 1E).

PrxII deficiency enhances Foxp3+ Treg cell, but not effector Th cell, development

We next investigated the effects of PrxII on the in vitro development of CD4+ T cells into effector Th cells (including Th1, Th2, and Th17 cells) and Treg cells. Naive CD4+ T cells were isolated from the LN and spleen and then subjected to TCR stimulation and differentiation under Th1-, Th2-, Th17-, or Treg-skewing conditions (31, 32). WT and PrxII-deficient CD4+ T cells produced comparable amounts of IFN-γ in differentiated Th1 cells. Differentiation into IL-4– and IL-17–secreting Th2 and Th17 cells was similar between WT and KO mice (Fig. 4A–C). However, the in vitro development of Foxp3+ Treg cells was prominently increased in the absence of PrxII, as evidenced by the enhanced IL-10 production and increased percentage of Foxp3+ Treg cells (Fig. 4D, 4E). Consistent with staining of Foxp3+ cells, immunoblotting analysis showed a significant reduction in PrxII-deficient CD4+ T cells and increased phosphorylation of STAT3 (pSTAT3) compared with WT controls (Fig. 4F).

FIGURE 5. Suppression of Foxp3 expression by the restoration of PrxII. (A) Naive CD4+ T cells were isolated from the LN and spleen of WT mice (n = 4) and induced to differentiate into Treg cells. Total protein was harvested from developing Treg cells at the indicated time points and analyzed by SDS-PAGE and immunoblotting with Abs against Prx-SO2/3 and PrxII. (B–D) Naive CD4+ T cells were isolated from WT and KO mice (n = 4 for each experiment), stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml) for 24 h, and then infected with viruses expressing PrxII or control virus (RV). Cells were cultured under Treg-skewing conditions for an additional 48 h. The cells were harvested, and protein was subjected to immunoblotting analysis (B). Separately, total RNA was isolated from the cells to determine gene transcripts of Foxp3 (C) and TGF-β (D) by real-time PCR. (E) Naive CD4+ T cells were induced to differentiate under Treg-skewing conditions for 48 h and were additionally treated with cell-permeable reduced glutathione (GSH) and N-acetylcysteine (NAC) for 24 h. The cells were then stained for CD25 and Foxp3 and analyzed by flow cytometry. The percentages of CD4+CD25+Foxp3+ Treg cells were calculated by the CellQuest program, and are given as the mean ± SEM (n = 4). (F) Naive CD4+ T cells (n = 3) were activated under Th17-skewing conditions and infected with control (RV) or PrxII-expressing viruses. Cells were then cultured for an additional 48 h and harvested for immunoblot analysis. Representative images of at least three independent experiments are shown in (A), (B), (E), and (F). Data in (C–E) are expressed as the mean ± SEM of four independent experiments. ∗p < 0.05, ∗∗p < 0.005, ∗∗∗p < 0.0005.
analysis revealed that the level of Foxp3 was higher in PrxII-deficient Treg cells than in WT cells (Fig. 4F). We then compared the proliferation of Treg cells and their suppression of effector T cell proliferation. Flow cytomteric analysis of CFSE fluorescence labeling revealed that WT and KO Treg cells proliferated similarly and comparably suppressed the proliferation of effector T cells (Fig. 4G, 4H).

Overexpression of PrxII selectively suppresses Foxp3 gene expression in T cells

We assessed the expression of PrxII during the development of Treg cells to confirm the inhibitory role of PrxII in Treg cells. PrxII was abundantly expressed in Treg cells, and its expression was unchanged during Treg development. However, PrxII was inactivated by hyperoxidation of its catalytic cysteine to Prx-SO2/3 during Treg cell development, resulting in the accumulation of intracellular ROS in Treg cells (Fig. 5A). Retroviral transduction of PrxII into KO cells substantially suppressed Foxp3 expression, as evidenced by immunoblotting and real-time PCR analyses (Fig. 5A, 5B). TGF-β expression by Treg cells was also decreased by overexpression of PrxII (Fig. 5D). Indeed, ROS scavengers, including N-acetylcysteine and reduced glutathione, suppressed in vitro Foxp3+ Treg cell development (Fig. 5E). Overexpression of PrxII in developing Th17 cells had no effect on RORγt expression (Fig. 5F).

PrxII deficiency enhances stability of FoxO1 protein

Next, we investigated the molecular mechanisms behind the PrxII-mediated suppression of Foxp3 gene transcription. First, using a reporter gene system, we tested whether PrxII controlled Foxp3 promoter activity in WT or KO MEF cells after treatment with TGF-β. We observed that Foxp3 promoter activity was substantially higher in PrxII-deficient MEF cells than in WT cells. Foxp3 promoter activity was suppressed by PrxII overexpression (Fig. 6A). Because transcription factors such as FoxO1, FoxO3, and NF-κB p65 modulate Foxp3 promoter activity (33–37), we examined p65- and FoxO1-mediated Foxp3 promoter activity and the effect of PrxII on this activity in 293T cells. Foxp3 promoter activity was increased markedly by the expression of p65 or FoxO1. The increase in Foxp3 promoter activity induced by p65 was unchanged by PrxII expression, but FoxO1-induced promoter activity was drastically inhibited by PrxII expression (Fig. 6B). In addition, the expression of endogenous FoxO1, but not p65, increased markedly in PrxII-deficient MEF cells after treatment with TGF-β (Fig. 6C). Nuclear and cytoplasmic expression of FoxO1 was enhanced by PrxII deficiency, as demonstrated by immunofluorescence staining of endogenous FoxO1 protein (Fig. 6D). Interestingly, FoxO1 protein stability was decreased by the ectopic overexpression of PrxII and was increased in the absence of PrxII (Fig. 6E, 6F).

PrxII modulates FoxO1 expression in T cells in vitro and in vivo

Because PrxII deficiency modulated endogenous FoxO1 protein expression in MEF cells, we determined whether PrxII modulated the expression of FoxO1 in Treg cell development in vitro and in vivo. The level of endogenous FoxO1 was markedly increased in developing KO Treg cells, and restoration of PrxII expression in PrxII-null Treg cells decreased the level of FoxO1 protein (Fig. 7A). Consistent with the in vitro modulation of FoxO1 and Foxp3 by PrxII in Treg cells, FoxO1 expression in the mLN was significantly higher in DSS-treated KO mice compared with the parallel WT group (Fig. 7B). Furthermore, immunofluorescence staining revealed enhanced expression of FoxO1 in the colon of KO colon after DSS treatment (Fig. 7C). We then assessed the regulatory mechanisms underlying the enhanced protein stability of FoxO1 in PrxII-deficient cells. Examination of ROS-mediated signaling molecules, including pJNK, JNK, and PTEN, revealed no difference between WT and KO Treg cells. However, MDM2 expression was decreased in PrxII-null Treg cells in vitro (Fig. 7D). Accordingly, MDM2 expression was predominantly decreased in the mLN of KO mice, whereas FoxO1 expression was increased (Fig. 7E).

PrxII deficiency attenuates CD4+ T cell–mediated inflammatory colitis in vivo

To confirm the in vivo function of PrxII in CD4+ T cell–mediated colonic inflammation, we isolated naive CD4+ T cells from WT and KO mice and adoptively transferred the cells into immune-deficient RAG KO mice. We then analyzed colitis symptoms in WT/RAG and KO/RAG mice. The generation of ROS was augmented in CD4-gated LPC of RAG/KO mice (Supplemental Fig. 2A). Histological examination of colon tissue verified that colonic inflammation was prominent in WT/RAG but much less pronounced in KO/RAG colons (Fig. 8A). The histological score,
followed by SDS-PAGE and immunoblotting analysis. The relative FoxO1 intensity was quantified by densitometry. (B) Protein extracts were prepared from the mLN of WT and KO mice treated with either normal water or 3% DSS water, followed by SDS-PAGE and immunoblotting analysis. The relative FoxO1 intensity was quantified by densitometry. **p < 0.005, ***p < 0.0005. (C) Colon tissues of DSS-treated WT and KO mice were fixed and stained with Abs against CD4 and FoxO1. Cells were subsequently stained with fluorescence-tagged IgG and observed under a fluorescence microscope (D). Developing Treg cells were generated from naive CD4+ T cells of WT and KO mice. Protein was analyzed by immunoblot analysis. (E) Protein was harvested from mLN of DSS-treated mice and analyzed by SDS-PAGE and immunoblotting with Ab against MDM2, FoxO1, PTEN, and β-actin. Representative images of at least three independent experiments are shown.

**FIGURE 7.** Increased expression of FoxO1 and decreased expression of MDM2 in the absence of PrxII. (A) Naive CD4+ T cells were obtained from WT and KO mice and stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (1 μg/ml). Cells were induced to differentiate into Treg cells for 48 h and harvested for protein analysis by SDS-PAGE and subsequent immunoblotting. After 24 h of activation, Treg cells were infected with control virus or virus expressing PrxII. Cells were cultured under Treg-skewing conditions for an additional 48 h. Total protein was harvested and analyzed by immunoblotting. (B) Protein extracts were prepared from the mLN of WT and KO mice treated with either normal water or 3% DSS water, followed by SDS-PAGE and immunoblotting analysis. The relative FoxO1 intensity was quantified by densitometry. **p < 0.005, ***p < 0.0005. (C) Colon tissues of DSS-treated WT and KO mice were fixed and stained with Abs against CD4 and FoxO1. Cells were subsequently stained with fluorescence-tagged IgG and observed under a fluorescence microscope (D). Developing Treg cells were generated from naive CD4+ T cells of WT and KO mice. Protein was analyzed by immunoblot analysis. (E) Protein was harvested from mLN of DSS-treated mice and analyzed by SDS-PAGE and immunoblotting with Ab against MDM2, FoxO1, PTEN, and β-actin. Representative images of at least three independent experiments are shown.

based on the average of the epithelium and infiltration scores, was significantly attenuated in KO/RAG mice compared with WT/RAG group (Fig. 8B). The percentage of Foxp3+ Treg cells increased in the mLN of RAG/KO mice (Fig. 8C). Immunoblot analysis confirmed that RAG/KO mice expressed higher levels of FoxO1 and Foxp3 and lower levels of MDM2 when compared with RAG/WT mice (Fig. 8D). Colonic CD4+ LPC of RAG/KO mice also expressed a greater amount of Foxp3 but produced less IFN-γ (Fig. 8E, 8F). Intracellular cytokine staining confirmed the reduced production of inflammatory cytokines in the mLN and LPC of RAG/KO mice (Supplemental Fig. 2B). To exclude the effects of epithelial cells in DSS-induced colitis, we generated BM chimeras of WT and KO mice and administered normal water or 3% DSS water for 8 d. Colon shortening and the colonic inflammation induced by DSS treatment were significantly attenuated in the absence of PrxII (Supplemental Fig. 2C, Fig. 8G). Consistent with previous findings, the increased levels of IFN-γ and TNF-α induced by DSS treatment were decreased in KO chimeras (Fig. 8H, Supplemental Fig. 2D). The intracellular ROS level was increased by treatment with DSS and was greater in KO chimeras than in WT chimeras (Supplemental Fig. 2E).

**Discussion**

In the current study, we report that PrxII-deficient mice are resistant to DSS-induced colitis. Stabilization of FoxO1 expression and sustained FoxO1-mediated Foxp3 expression in T cells may mediate the effects of PrxII deficiency and thereby attenuate the colonic inflammation in vivo.

DSS-induced colitis in mice is characterized by massive infiltration of inflammatory cells, such as neutrophils, macrophages, and CD4+ T cells, within the colonic walls, which destroys epithelial cells and shortens the colon length. These are also key features of human inflammatory bowel diseases, such as ulcerative colitis and Crohn’s disease (38–40). Macrophages and T cells are major inflammation mediators that produce inflammatory chemokines and cytokines (such as MIP-1α, MCP-1, TNF-α, IL-6, IL-1β, IFN-γ, IL-12, and IL-17), which contribute to pathogenesis (32, 41–43). PrxII KO mice show increased expression of TNF-α and IL-6 in LPS-stimulated macrophages; thus, they are susceptible to LPS-induced inflammation (13). Furthermore, PrxII deficiency increases the expression of VCAM-1 and ICAM-1 and accelerates the infiltration of MCP-1-expressing immune cells, thus exaggerating atherosclerosis in apolipoprotein E-deficient mice (14). Based on previous findings, we expected PrxII KO mice to be more susceptible to colitis development than WT mice. Instead, PrxII KO mice were resistant to DSS-induced colitis. To identify the principal mechanisms underlying this resistance, we evaluated the contributions of macrophages and CD4+ T cells to colitis inflammation. PrxII KO mice revealed significant increases in mature macrophages expressing F4/80 surface marker and chemokines such as MIP-1α and MCP-1 (Supplemental Fig. 3A, 3B). Furthermore, the anti-inflammatory M2 macrophage subset expressing arginase-1 and Ym-1 (44) was substantially increased in KO mice, suggesting a suppressive role for this population in colonic inflammation. However, the levels of anti-inflammatory cytokine IL-10 were comparable in WT and KO mice, both in LPS-stimulated macrophages and in BM-derived M2-polarized macrophages (Supplemental Fig. 3C, 3D). Thus, it is not clear whether PrxII-deficient macrophages contribute to the attenuation of colonic inflammation. Additionally, whether PrxII influences the development of distinct macrophage subsets and whether MCP-1 in PrxII-deficient macrophages helps protect the colon against inflammation remain to be determined.

We also demonstrated that PrxII deficiency enhanced the differentiation of naive CD4+ T cells into Foxp3+ Treg cells but had no significant effect on the differentiation of Th1, Th2, or Th17 cells in vitro. Retroviral transduction of PrxII into Treg cells suppressed Foxp3 expression, whereas PrxII overexpression had no effect on RORγt expression in Th17 cells. The increased Foxp3 expression in Treg cells in PrxII KO mice was due to the activation of FoxO1 expression. Indeed, CD4+ T cells from the PrxII-deficient colon were strongly positive for FoxO1 expression after
DSS treatment. FoxO1 expression is regulated by posttranslational modification, including phosphorylation, acetylation, and ubiquitination (45). In response to oxidative stress, FoxO1 is phosphorylated by the activated JNK, which induces FoxO1 nuclear localization and attenuates FoxO1 protein degradation (46). The lack of PrxII increased the level of FoxO1 in the nucleus and cytoplasm, but had no significant effect on the nuclear localization of FoxO1. Instead, PrxII deficiency decreased the expression of MDM2, an E3 ubiquitin ligase that promotes the ubiquitination and subsequent proteasomal degradation of FoxO1 (47). The reduced level of MDM2 in Treg cells led to the accumulation of FoxO1 protein and promoted FoxO1-induced Foxp3 expression, thereby accelerating Foxp3+ Treg cell development. Our results suggest a selective role for PrxII in the control of FoxO1 expression in Treg cells and colonic inflammation.

In conclusion, PrxII deficiency selectively enhances Foxp3+ Treg cell generation through the regulation of FoxO1 protein stability, and thus attenuates colonic inflammation in vivo, suggesting PrxII as a target for controlling inflammatory diseases.

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Disclosures
The authors have no financial conflicts of interest.

References

FIGURE 8. Attenuation of colonic inflammation by PrxII-deficient T cells. (A–F) RAG/WT and RAG/KO mice were generated by injecting naive CD4+ T cells (0.5 × 10^6 cells/mouse) from WT and KO mice into RAG1 KO mice i.p. (n = 15 for each group). Mice were euthanized on day 40 after adoptive transfer. Colon tissues (n = 6) were fixed and stained by H&E (scale bars, 2000, 1000, and 200 μm) (A). The histological score, calculated as described in Materials and Methods, is given as the mean ± SEM of eight mice for each group (B). Single-cell suspensions were isolated from the mLN (n = 4); stained with allophycocyanin CD4, FITC CD25, and PE Foxp3 Ab; and analyzed by flow cytometry (C). Total protein was harvested from the mLN (n = 3 for each group) and subjected to immunoblotting analysis (D). LPC were isolated from colon (two colons pooled) and stained with allophycocyanin CD4 and PE Foxp3. The mean fluorescence intensity of Foxp3 was determined in CD4-gated LPC by flow cytometry. Data are given as the mean ± SEM of six mice (E). Colonic LPC were harvested and cultured for 24 h. Cell supernatants were collected and analyzed by a Multiplex assay system. Data are expressed as the mean ± SEM of six mice (F). (G and H) BM chimeras (n = 12 for each) were generated by transferring BM cells from WT and KO mice into irradiated recipient mice and maintained for 60 d for recovery. Mice were administered either normal water or 3% DSS water for 7 d. Colon tissues were stained by H&E (scale bar, 100 μm) (n = 4) (G). Colonic LPC were isolated from two mice and cultured for 24 h. Cell supernatants were used to measure IFN-γ using a Multiplex system. Data are represented as the mean ± SEM of six mice (H). Representative images of three independent experiments were shown in (A), (C), (D), and (G). *p < 0.05.