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

*J Immunol* 2013; 191:4029-4037; Prepublished online 18 September 2013;
doi: 10.4049/jimmunol.1203247
http://www.jimmunol.org/content/191/8/4029
Ablation of Peroxiredoxin II Attenuates Experimental Colitis by Increasing FoxO1-Induced Foxp3+ Regulatory T Cells

Hee Yeon Won,*1 Eun Jung Jang,*1 Kihyun Lee,* Sera Oh,* Hyo Kyung Kim,* Hyun Ae Woo,* Sang Won Kang,* Dae-Yeul Yu,‡ Sue-Goo Rhee,* and Eun Sook Hwang*1

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 durante 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.

Received for publication November 28, 2012. Accepted for publication August 10, 2013.

This work was supported by National Research Foundation grants funded by the Ministry of Education, Science, and Technology (National Honor Scientist Program to S.-G.R.; Grant 2009-0084879 to E.S.H.) and partly by a Health Technology Research and Development Project funded by Ministry of Health and Welfare Grant A120476.

Address correspondence and reprint requests to Prof. Eun Sook Hwang, College of Pharmacy and Global Top 5 Research Program, Ewha Womans University, Seoul 120-750, Korea; and Korea Research Institute of Bioscience and Biotechnology, Daejeon 605-806, Korea.

1H.Y.W. and E.J.J. contributed equally to this work.

The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; DSS, dextran sulfate sodium; GPx1, glutathione peroxidase 1; KO, knockout; LN, lymph node; LPC, lamina propria cell; MDM2, mouse double minute 2 homolog; MEF, mouse embryonic fibroblast; mM, mesenteric lymph node; NOX, NADPH oxidase; PAS, periodic acid–Schiff; Prx, peroxiredoxin; PTEN, phosphatase and tensin homolog; ROS, reactive oxygen species; Treg, regulatory T; WT, wild-type.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1203247
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 administering 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. Colons were collected for examination of colon shortening, histopathological, and lamina propria cell (LPC) isolation.

Immunohistochrometry

Mouse colon was sectioned, incubated with Alex Fluor 647–conjugated anti-mouse CD4 Ab (eBioscience, San Diego, CA), and stained with DAPI (Roche, Mannheim, Germany). Immunofluorescence was examined using a fluorescence confocal microscope (LSM510; Zeiss). Both WT and KO mouse embryonic fibroblast (MEF) cells were cultured and additionally treated with TGF-β (2 ng/ml) for 4 h. Cells were fixed, labeled with anti-FoxO1 Ab (Cell Signaling Technology, Danvers, MA), and then incubated with Alexa Fluor 555–conjugated anti-rabbit IgG (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 Percoll gradient centrifugation and cultured in RPMI 1640 for 24 h. Supernatants were collected, and cytokine production was assessed using a Multiplex ELISA (eBioscience). Alternatively, single-cell suspensions of mLN were stimulated with anti-CD3 Ab (1 μg/ml) for 24 h. Culture supernatants were harvested and incubated on an Ab-coated ELISA plate (Ready-Set-Go ELISA kit; eBioscience). The levels of IFN-γ, IL-17, IL-6, TNF-α, and IL-18 were measured at 405 nm with an ELISA plate reader (Molecular Devices, Palo Alto, CA). For intracellular cytokine staining, cells were additionally treated with monensin (4 μg/ml) for 3 h and were then stained with allophycocyanin CD4, PE, IL-17, and FITC IFN-γ Abs.

RT-PCR

Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was synthesized by reverse transcription. Real-time PCR was performed using a SYBR Green premix (Invitrogen, Carlsbad, CA). The specific primer sets used were as follows: IL-21,

![FIGURE 1.](http://www.jimmunol.org/DownloadedFrom/107284.png)

**ATTENUATION OF COLITIS IN PRXII DEFICIENCY**
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 H&E and PAS. Scale bar, 1000 μm. 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.
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 naive 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 × 10⁶ 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 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 polybrevine (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 naive 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 × 10⁶ 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 pFoxp3pro and pCMV-β-gal. The pFoxp3pro 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, increased tissue edema, and mucosal injury in WT mice, but not in PrxII KO mice (Fig. 2B). The presence of leukocytes, particularly neutrophils and lymphocytes, was assessed by immunohistochemistry using antibodies specific for CD45, Ly6G, and CD3. CD45+ and Ly6G+ cells were significantly increased in WT mice compared to KO mice (Fig. 2C).
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

---

**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-SO23 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 cytometric 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-SO_2, 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 Treg 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 prominently 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,
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 Foxp3 and FoxO1 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 KO chimeras (Fig. 8H, 8I). The intracellular ROS level was increased induced by DSS treatment were decreased in KO chimeras (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. 8G, 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-expressing 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 development. 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. Retrovirual 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

**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.
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 gene transcription, 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.

Acknowledgments
We thank Drs. Y.D. Yun at Ewha Womans University and H.P. Kim at Yonsei University for providing RAG KO mice and the pFoxp3pro reporter gene, respectively. We also thank Dr. M.A. Bae at the Korean Research Institute of Chemical Technology for providing technical support in generation of bone marrow chimeras.

Disclosures
The authors have no financial conflicts of interest.

References
lase, and glutathione peroxidase via post-translational modification. Antioxid. Redox Signal. 7: 619–626.


Supporting figure legends

Supporting Figure 1. Analyses of mLN cells and colonic LPC of WT and KO mice after treatment with DSS. WT and KO mice (n = 4 each) were administered normal water (−DSS) or 3% DSS-water (+DSS) for 7 days. (A) Single cell suspensions of mLN, spleen, and colonic LPC were stained with DCFDA (10 μM) at 37°C for 20 min, followed by flow cytometric analysis. The mean fluorescence intensity (MFI) was determined by CellQuest software. (B) Cells from the mLN and spleen were stained with APC-CD4 and PE-Foxp3, and Foxp3 expression in CD4+ T cells was measured using a FACS Calibur flow cytometry (BD Biosciences). (C) Cells were harvested from the mLN for APC-CD4 and PE-Foxp3 staining. The absolute numbers of CD4+ T cells and Foxp3+ Treg cells were calculated from the total cell numbers of mLN and the percentage of CD4+ and CD4+Foxp3+ Treg cells. Data are given as the mean ± SEM of 4 mice. (D) Cells were harvested from mLN and stimulated with anti-CD3 (1 μg/mL) overnight. Cells were then stained with PE-IL-17 and FITC-IFN-γ Ab and analyzed by flow cytometry. (E) Colonic LP cells were isolated and cell numbers were counted using trypane blue staining as well as staining with APC-CD4 and PE-Foxp3. Data are given as the mean ± SEM of 4 mice. Representative images of at least 3 independent experiments are shown in A, B, and D.

Supporting Figure 2. Analyses of mLN and colonic LP cells of RAG and BM chimeric mice generated with WT and KO cells. (A–B) Naïve CD4+ T cells of WT and KO mice were stained with DCFDA, followed by flow cytometry. Naïve CD4+ T cells of WT and KO mice were adoptively transferred to RAG1 KO mice (n = 4 each). Single cells from the mLN and colonic LPC of RAG/WT and RAG/KO mice were stained with DCFDA. Representative histograms of 4 mice are shown (A). Cells were isolated from mLN and colonic LPC and
stained with APC-CD4. Cells were stimulated with anti-CD3 overnight, stained with PE-IL-17 and FITC-IFN-γ, and analyzed by flow cytometry (B). (C–E) BM cells were isolated from WT and KO mice and intravenously injected into irradiated WT recipients. Reconstituted WT and KO BM chimeras (n = 12) were maintained for 60 days and administered normal or 3% DSS-water for 7 days. The colon length was measured in all mice (C). Colonic LPC were harvested and cultured for 24 h. Cell supernatants were used for measuring TNF-α with a Multiplex assay kit (D). Data in C and D are expressed as the mean ± SEM of 6 mice. *, P < 0.05. Single cell suspensions of the mLN were stained with DCFDA and analyzed by flow cytometry. Representative images of 3 independent experiments are shown.

**Supporting Figure 3. Activated macrophages in PrxII KO mice.** WT and PrxII KO mice were administered 3% DSS in water for 7 days and single cell suspensions of spleen were collected. (A) Cells were incubated with anti-F4/80 Ab for 20 min on ice and analyzed by flow cytometry and the CellQuest program. (B) Spleen cells were stimulated with LPS (100 ng/mL; Sigma-Aldrich, St Louis, MO) for 24 h and cell pellets were lysed in TRIzol reagent for total RNA preparation, followed by reverse transcription and real time-PCR for MIP-1α and MCP1. (C) Total RNA was prepared from the mLN of WT and KO mice treated with normal water or 3% DSS-water and subjected to RT and real time-PCR for measuring Arg-1, Ym-1, and IL-10. (D) BM cells were isolated and stimulated with M-CSF (100 ng/mL; PeproTech, Rocky Hill, NJ) for 9 days for M2 polarization. Total RNA isolated from the cells was subjected to reverse transcription and analyzed by real time-PCR (ABI Prism 7700, Applied Biosystems, Foster City, CA). The primer sets were as follows: MIP-1α, 5′-tgttctgctgccaagtagccacatc-3′ and 5′-aacagttgtaaactgggagga-3′; MCP1, 5′-ctctctctcctgctgttca-3′ and 5′-ccagctactcatacgggtgata-3′; Arginase-1 (Arg-1), 5′-acagtctggcagtgaaga-3′ and 5′-gagagttcctgagagaatc-3′; Ym-1, 5′-catgagccagactggagc-
Supporting Figure 4. Development of colonic inflammation in PrxI or Gpx1 KO mice. WT, PrxI KO, and GPx1 KO mice (n = 8) were fed 3% DSS-water for 7 days. (A) Body weight and disease activity score were determined after DSS treatment. (B) Mice were sacrificed at day 7, and colon length was measured. Data in A and B are expressed as the mean ± SEM of 6 mice. ns, not significant; **, P < 0.005. (C) Colon tissues of WT and PrxI KO mice were sectioned and subsequently stained with PAS. (D) Total proteins were harvested from the mLN (n = 4 for each) and subjected to SDS-PAGE and immunoblotting analysis.
Won HY supporting Figure 1
Won HY supporting Figure 2
Won HY supporting Figure 3
Won HY supporting Figure 4