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Tumor suppressor FOXO3 participates in the regulation of intestinal inflammation

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Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is characterized by chronic mucosal injury and the infiltration of inflammatory cells. Tumor suppressor FOXO3 regulates gene expression and its translocation to the cytosol leads to the abrogation of its transcriptional function. We have previously shown that bacterial infection regulates FOXO3 in intestinal epithelial cells and increases cytokine levels. As TNFα is a major contributor in intestinal inflammation, the aim of this study was to assess its effect on FOXO3 and FOXO3's contribution to intestinal inflammation *in vitro* and *in vivo*. TNFα induces the translocation of nuclear FOXO3 into the cytosol where it undergoes proteasomal degradation in human intestinal HT-29 cells. Proximally, the PI3K and IKK pathways mediate TNFα-induced FOXO3 phosphorylation. In FOXO3-silenced HT-29 cells, TNFα-induced IL-8 expression is increased ~83%. *In vivo*, Foxo3 is present in the nuclei and cytosol of colonic crypt epithelia. In DSS-induced colonic inflammation, Foxo3's nuclear localization is lost and it is only found in the cytosol. Consistent with a role for Foxo3 in colitis, Foxo3-deficient mice treated with DSS developed more severe colonic inflammation with an increased number of intraepithelial lymphocytes and PMNs infiltrated in the epithelia, than wild-type mice. In summary, TNFα inactivates FOXO3 in intestinal epithelia through the PI3K and IKK pathways and FOXO3 inactivation leads to the upregulation of IL-8 *in vitro*; *in vivo* Foxo3 is in the cytosol of inflamed colonic epithelia and Foxo3 deficiency leads to severe intestinal inflammation.
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In the intestinal tissue of inflammatory bowel disease (IBD) patients with the active disease, many proinflammatory cytokines are increased, which are imperative in maintaining inflammatory responses.[1] One of the cytokines that drives intestinal inflammation is tumor necrosis factor-α (TNFα).[2–4] TNFα-blocking agents significantly reduce intestinal inflammation in the majority of patients with active IBD.[5]

TNFα binds to cell membrane TNFα receptors (TNFR1 and TNFR2),[6–8] stimulating signaling cascades that lead to the activation of nuclear factor kappaB (NF-κB).[9] From TNFRs, the intracellular signaling pathways, that include several receptor adapters, activate the inhibitory kappaB (IκB) kinase (IKK) complex. Activated IKK phosphorylates IκB proteins causing IκB degradation and the release of NF-κB to freely translocate to the nucleus.[10] Nuclear NF-κB regulates genes that are involved in inflammation, cell survival, and proliferation.[11,12]

The tumor suppressor FOXO3 belongs to the family of Forkhead transcriptional factors.[13] FOXO3 is located in the nucleus regulating the expression of specific target genes involved in the regulation of cell cycle progression, metabolic state, and cellular apoptosis.[13,14] Phosphorylation of FOXO3 can occur by upstream pathways that include PI3K and/or IKK;[14,15] phosphorylated FOXO3 translocates to the cytosol and becomes inactive.[16] Cytosolic FOXO3 might attach to 14–3–3 proteins or be degraded in the proteasome.[15–19]

Foxo3-deficient mice develop spontaneous, multisystemic inflammatory syndrome, accompanied by an increased cytokine production, an increased NF-κB activation, and hyperactivation of T cells.[20] In T cells, the overexpressed Foxo3 inhibits NF-κB and cytokine expression.[20] We have demonstrated that bacterial infection inactivates FOXO3 in the intestinal epithelia and that this contributes to an increased cytokine expression;[21] hence, we hypothesize

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that FOXO3 is involved in the regulation of intestinal inflammation. We have shown here that TNFα inactivates FOXO3 in HT-29 cells through the PI3K and IKK pathways, which additionally increase IL-8. In mice, where colonic inflammation was induced with dextran sulfate sodium (DSS), Foxo3 is also inactive. Foxo3-deficient mice, in response to DSS, developed more severe intestinal inflammation compared with the wild-type mice. Altogether, our results suggest that tumor suppressor FOXO3 has an important role in the regulation of intestinal inflammation *in vitro* and *in vivo*.

MATERIALS AND METHODS

Tissue Culture

Human intestinal epithelial HT-29 cells (ATCC, Manassas, VA, USA; passages 10–20) were cultured in complete McCoy's 5A media (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and Penicillin–Streptomycin (Gibco). The cells were kept in an incubator at 37°C, with 5% CO_2 and then they were plated in 6- or 12-well plates. When the monolayers reached confluency of ~70%, cells were serum-starved overnight before the experimental procedures were performed.

Semi-confluent HT-29 monolayers were stimulated with 10 ng/ml TNFα (R&D, Minneapolis, MN, USA) for different lengths of time to study the effect on FOXO3 and IL-8.

Immunofluorescent Staining

For immunofluorescent staining, HT-29 cells were grown on coverslips. After treatment, the cells were washed with PBS and fixed with 3.7% paraformaldehyde. The fixed cells were washed with PBS and permeabilized with 0.2% Triton-X in PBS followed by blocking in 2.5% bovine serum albumin. The cells were incubated with a primary FOXO3 antibody (Upstate, Billerica, MA, USA) for 1 h, followed by washing and incubation with a secondary antibody for 1 h (Alexa Fluor 488 Molecular Probes, Eugene, OR, USA). The coverslips with cells were mounted on microscope slides (Mounting Solution antifade reagent, Molecular Probes). The results were assessed using a Nikon Opti-Photo microscope. The images were captured using a Spot RT-slider camera (Diagnostic Instruments, Sterling Heights, MI, USA) and managed by Image Pro software (Media Cybernetics, San Diego, CA, USA).

Inhibitor Studies

To study different pathways in FOXO3 regulation, several pharmacological inhibitors were applied. To inhibit the IKK pathway, we utilized its specific inhibitor PS1145 (30 μM) (Sigma). To study the role of the PI3K pathway inhibitors, wortmannin (200 nM) (Calbiochem, Gibbstown, NJ, USA) and LY294002, (30 μM) (Calbiochem) were used. The proteasome inhibitor MG132 (20 μM) (Calbiochem) was applied to study FOXO3 proteasome-dependent degradation. The serum-starved HT-29 monolayers were pre-incubated with an inhibitor for 1 h after which the monolayers were treated with TNFα for different lengths of time.

Protein Extraction

After treatment, the HT-29 monolayers were washed with PBS (Gibco) and protein was extracted by applying cell lysis buffer (Cell Signaling, Danvers, MA, USA), containing a protease inhibitor cocktail (Sigma). Protein concentrations were determined using the Quick Start Bradford Protein Assay kit (Biorad, Hercules, CA, USA) as described by the manufacturer. The protein extracts were stored at −20°C until further processing. The nuclear proteins were extracted using the NE-PER Pierce extraction kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol and stored at −80°C.

Immunoblots

Equal amounts of protein (40 μg) per sample were separated on 10% Sodium Dodecyl Sulfate polyacrylamide (SDS-PAGE) gels. Protein was transferred to nitrocellulose membranes (Biorad). The immunoblots were probed with appropriate antibodies against FOXO3 (Upstate), anti-phospho-Thr32-FOXO3 (Upstate), anti-phospho-Ser644-FOXO3 (generous gift of Dr Hu), and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) all according to the manufacturers' protocols. The secondary antibodies were horseradish peroxidase-conjugated (Chemicon, Temecula, CA, USA). The protein bands were detected by using the ECL Plus western blotting detection system (GE Healthcare, Amersham, UK).

SiRNA Experiments

To study the effects of FOXO3 on TNFα-induced IL-8 in intestinal epithelial cells, we transiently knocked-out FOXO3 by applying siRNA. The HT-29 cells were transfected with two different oligos, designed to silence FOXO3 (30 nM) or a negative control oligo (Invitrogen, Carlsbad, CA, USA). Oligos were introduced into the cells using Lipofectamine RNAiMax (Invitrogen) following the manufacturers protocol. Initially, 48 h post-transfection showed optimal knock down of FOXO3. Therefore, 36 h after transfection with siRNA, the monolayers of HT-29 cells were serum deprived overnight and sequentially treated with TNFα for 4 h. The media was collected and stored at −20°C until it was used for IL-8 quantification. Whole cell lysates were used as a control for knock down of FOXO3 with western blotting.

IL-8 Quantification

To quantify IL-8, the media collected from monolayers was used for IL-8 ELISA (R&D) according to the manufacturer's protocol.

Statistical Analysis

These data were represented as the mean ± s.d.. The student's *t*-test was used to compare these data and the differences were considered significant when the *P*-values were ≤ 0.05.

Animal Studies

For an *in vivo* study, colonic inflammation was induced by introducing 2.5% DSS (MP Biomedicals, LLS, Solon, OH, USA) into the drinking water of C57BL/6J mice (6–8 weeks old) for 5 days, followed by two recovery days. Body weight was monitored during the course of the treatment. Gastrointestinal bleeding was evaluated using Coulter Hemoccult (Fisher, Pittsburgh, PA, USA). After 7 days, the animals were euthanized and the colonic tissue was prepared for histopathological studies.

Furthermore, to study the *in vivo* role of FOXO3 in colonic inflammation, we utilized 4 to 6-week-old Foxo3-deficient mice kindly provided by Dr Stanford Peng (Roche Palo Alto LLC, Palo Alto, CA, USA). The genotypes of the breeds were determined by PCR on tail DNA using primers according to Lin *et al*.[20]

All animals were kept in the Biological Resources Laboratory at the University of Illinois at a Chicago facility and all experimental procedures were performed in compliance with the local protocols and guidelines approved by the local ethical committee.

Histological Analysis

Colonic tissue was formalin-fixed and paraffin-embedded. The tissue sections (5-μm thick) were stained with routine hematoxylin and eosin (H&E) staining. The distribution of Foxo3 in colonic tissue was assessed as we described before.[21] The degree of inflammation was evaluated according to the following criteria: (0) completely uninvolved, no architectural distortion or infiltrates; (1) architectural distortion, increased lamina propria lymphs, no activity; (2) increased lamina propria granulocytes without definite intraepithelial granulocytes (ie, no activity); (3) intraepithelial granulocytes (ie, activity) without crypt abscesses; (4) crypt abscesses in less than 50% of crypts; (5) crypt abscesses in greater than 50% of crypts or erosion/ulceration.

RESULTS

TNFα Regulates FOXO3 in HT-29 Cells

Nuclear FOXO3 is involved in the regulation of transcription; signaling by growth factors and other stimuli results in FOXO3 translocation from the nucleus to the cytosol and terminates FOXO3 gene regulation.[16–18] This process can be modeled in human colonic epithelial HT-29 cells, where FOXO3 is localized in the nucleus but translocated to the cytosol after bacterial infection.[21] To address the effect of cytokines on this process, FOXO3 localization was examined in HT-29 cells treated with TNFα. FOXO3 translocated from the nucleus to the cytosol during the first 30 min of TNFα treatment (Figure 1a). An immunoblot analysis of subcellular fractions confirmed that the amount of nuclear FOXO3 decreases, whereas the amount of cytosolic FOXO3 increases after TNFα stimulation (Figure 1b). This data suggests that FOXO3 becomes inactive after TNFα treatment of the HT-29 cells.

It was shown that cytosolic FOXO3 might degrade by proteasome.[15] Therefore, we examined the fate of cytosolic FOXO3 in HT-29 cells treated with TNFα. Figure 2a shows that TNFα induces FOXO3 degradation in colonic HT-29 cells. To further define the mechanism of TNFα-induced FOXO3 degradation, we used the proteasome inhibitor MG132. The pretreatment of HT-29 cells with MG132 caused a small, statistically insignificant, increase in total FOXO3 and treatment with TNFα of MG132-pretreated HT-29 cells, blocked FOXO3 degradation (Figure 2b). In summary, TNFα induced the degradation of FOXO3 and that the degradation is proteasome mediated in HT-29 cells.

TNFα Regulates FOXO3 in HT-29 Cells via PI3K and IKK

The translocation of FOXO3 from the nucleus to the cytosol occurs after its phosphorylation.[16] The PI3K pathway mediates the phosphorylation of FOXO3 in many cell types.[16] We have previously reported that LPS-induced FOXO3 phosphorylation in intestinal epithelial cells is through the PI3K pathway.[21] Therefore, we assessed the role of PI3K in the phosphorylation of FOXO3 in intestinal epithelial cells treated with TNFα. We used an antibody that recognizes FOXO3 phosphorylation at Thr32, which is known to be phosphorylated by the PI3K pathway.[16] TNFα increased FOXO3 phosphorylation at Thr32 by 3.1 ± 0.8-fold in the first 30 min of treatment (Figure 3). The pharmacological PI3K inhibitors, wortmannin or LY294002, were used to assess the contribution of the PI3K pathway in TNFα-induced FOXO3 phosphorylation. The inhibition of the PI3K pathway with these inhibitors blocked the phosphorylation of Akt, which is responsible for the phosphorylation of FOXO3[13] (data not shown). Both inhibitors blocked TNFα-induced FOXO3 phosphorylation in HT-29 cells (Figure 3 shows inhibition with LY294002). Also, the inhibition of PI3K-blocked TNFα-induced degradation of Foxo3 (data not shown). This data shows that TNFα-induced FOXO3 phosphorylation and degradation in HT-29 cells is controlled proximally by the PI3K pathway.

Hu reported that FOXO3 might be phosphorylated by IKK in breast cancer cells.[15] To examine the contribution of IKK in TNFα-dependent FOXO3 inactivation we employed an antibody that recognizes FOXO3 phosphorylation at Ser644, which is only phosphorylated by IKK.[15] The TNFα treatment of HT-29 cells increased FOXO3 Ser644 phosphorylation 2.7 ± 0.2-fold within 30 min (Figure 4a). To further examine the role of the IKK pathway in TNFα-induced FOXO3 regulation in HT-29 cells, we used a specific IKK-inhibitor, PS1145.[22,23] We confirmed the specificity of IKK inhibition by PS1145 by showing the attenuation of p65 phosphorylation[24] (data not shown). Pretreatment

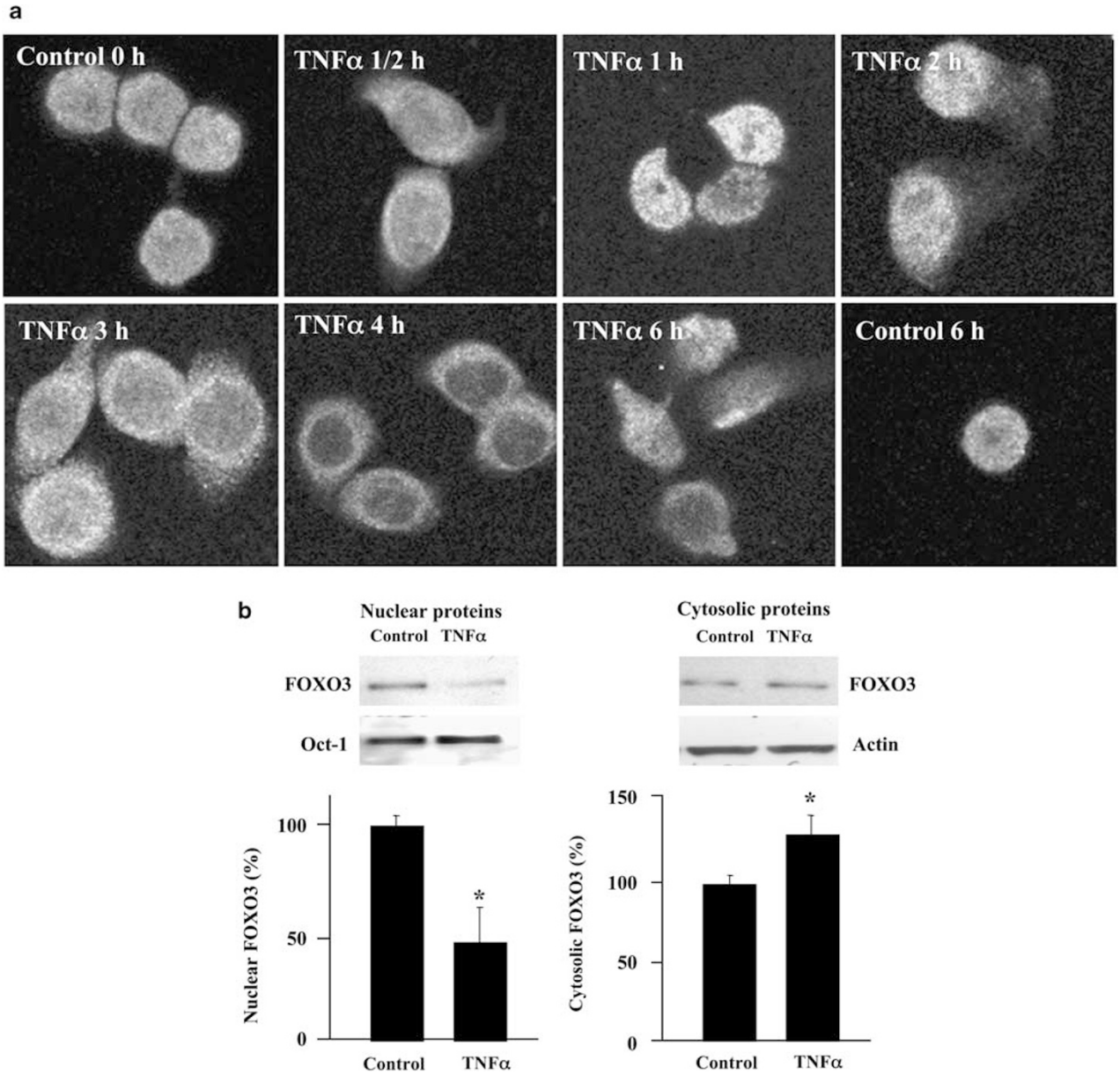

Figure 1 TNFα induces FOXO3 translocation in HT-29 cells. (**a**) HT-29 cells, control and treated with TNFα were fixed, immunofluorescent stained for FOXO3 and images were taken with matched exposures. In control cells, FOXO3 is localized in the nucleus. During TNFα treatment, nuclear FOXO3 translocates to the cytosol, suggesting its inactivation (× 60 magnification). This experiment was repeated three independent times. (**b**) Nuclear (8 μg) and cytosolic (40 μg) proteins from HT-29 cells, control and TNFα treated for 3 h, were separated on SDS-PAGE and immunoblotted for FOXO3. Immunoblots reveal decreased nuclear and increased cytosolic amounts of FOXO3 after TNFα treatment. Immunoblots were reprobed with antibodies against Oct-1 for nuclear extracts and actin for cytosolic extracts as a control. Densitometric analysis shows a significant differences (*) between groups ($P < 0.05$).

of HT-29 cells with PS1145 almost completely blocked TNFα-induced FOXO3 degradation (Figure 4b). These data suggest that IKK participates in the regulation of FOXO3 in HT-29 cells treated with TNFα. The inhibition of IKK did not change PI3K-dependent phosphorylation of FOXO3 (Figure 4c), suggesting that FOXO3 regulation by PI3K is independent of IKK in HT-29 cells treated with TNFα.

FOXO3 is Involved in the Regulation of TNFα-Induced IL-8

IL-8 is a proinflammatory chemokine that is a strong chemoattractant for neutrophils and lymphocytes.[25–27] In the tissue of IBD patients, IL-8 is significantly upregulated [27–30] and the level of IL-8 is in direct proportion to the degree of inflammation.[30] Cultured intestinal epithelial cells secrete IL-8 following TNFα treatment.[31,32] Under the conditions used in this study, HT-29 cells increased IL-8 secretion

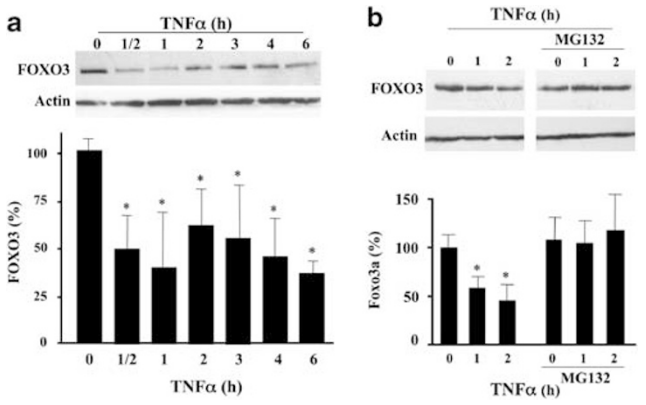

Figure 2 TNFα treatment of HT-29 cells induces degradation of FOXO3 by proteasome. (**a**) The total proteins from HT-29 cells, control and treated with TNFα for various time points, were separated on SDS-PAGE and immunoblotted for FOXO3 and actin. (**b**) The HT-29 monolayers were pre-incubated with proteasome inhibitor MG132 for 1 h and then treated with TNFα for various time points. Protein was separated on SDS-PAGE and immunoblotted for FOXO3 and actin. Each experiment was performed in triplicate and the densitometric analysis shows significant (*) degradation of FOXO3 during the course of TNFα treatment compared with untreated cells and protection with MG132 ($P<0.05$).

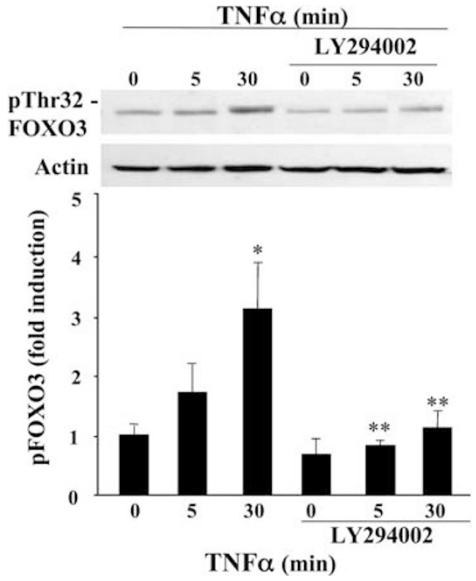

Figure 3 TNFα-induced FOXO3 phosphorylation is PI3K dependent. The HT-29 cells; with or without pretreatment with PI3K inhibitor (LY294002) were incubated with TNFα. Protein was separated on SDS-PAGE and immunoprobed with an antibody against phosphorylated FOXO3 at the Thr32 PI3K-dependent site and actin. The immunoblot is representative of three independent experiments (three samples were used per experimental group). Densitometric analysis shows a significant increase (*) in phosphorylated FOXO3 after TNFα treatment, which is attenuated in the presence of LY294002 (**) ($P<0.05$).

20-fold within 6 h of TNFα treatment (control: 23 ± 2 pg/ml; TNFα: 456 ± 13 pg/ml) (Figure 5a). To assess the contribution of FOXO3 to TNFα-induced IL-8 expression in intestinal HT-29 cells, we performed siRNA experiments. The efficiency of FOXO3 silencing by siRNA in HT-29 cells was ~90% (Figure 5b). The silencing of FOXO3 increased the basal level of IL-8 in HT-29 monolayers insignificantly. IL-8 was increased on average 83% in monolayers with silenced FOXO3 compared with IL-8 in monolayers with negative siRNA control treated with TNFα for 4 h (TNFα (−) control: 304 ± 18 pg/ml; TNFα siRNA: 544 ± 110 pg/ml) (Figure 5c). This data is consistent with our previously published data where an inactive FOXO3 contributes to the upregulation of IL-8 during bacterial infection.[21]

Cytosolic FOXO3 is Associated with Colonic Inflammation

To further examine the role of FOXO3 in *in vivo* intestinal inflammation, we used a mouse model. Mouse colonic inflammation was stimulated by introducing 2.5% DSS in drinking water. DSS treatment elevates cytokines and triggers the infiltration of inflammatory cells in the colon.[33,34] Strong nuclear staining of Foxo3 was detected in mouse colonic epithelial cells, and slight cytosolic staining was found in crypt cells.[21] In DSS-treated mice, Foxo3 was detected primarily in the cytosol of the colonic epithelia with no nuclear staining (Figure 6a and b), suggesting that Foxo3 is not active in the inflamed colonic epithelia. These findings correlate with *in vitro* data where Foxo3 translocation into the cytosol was detected. The degradation of Foxo3 *in vivo* was not apparent; we hypothesize that in intestinal tissue Foxo3 may also degrade but at this particular time point of DSS treatment degradation was not noticeable.

Foxo3 Deficiency Leads to Increased Inflammation in the DDS Model

To further assess the role of Foxo3 in intestinal inflammation *in vivo*, the Foxo3-deficient mice were employed. Although Lin reported that these animals displayed spontaneous inflammation in several organs,[20] the intestinal tissue did not show signs of inflammation over a period of 2 months (Figure 7c). Wild-type and Foxo3-deficient mice from the same colony, treated with DSS, did not show significant differences in weight loss (WT: 21 ± 4 g, KO: 21 ± 2 g); however, there was increased blood in the stool in Foxo3-deficient mice (Figure 7a) suggesting that the inflammatory process might be more active. A histological examination showed that the injury was significantly less severe and the recovery was enhanced in the wild-type mice relative to Foxo3-deficient mice. Although mild active inflammation was present in the wild-type mice, severe inflammation was present in the Foxo3-deficient mice (Figure 7b). The ulceration was minimal in wild-type mice, involved small areas, and was accompanied by evidence of mucosal healing (Figure 7c). In contrast, there were broad areas of mucosal ulceration with only limited healing present in Foxo3-deficient mice. In Foxo3-deficient mice the lamina propria was expanded with a mixed population of lymphocytes and polymorphonuclear neutrophils (PMNs), especially in

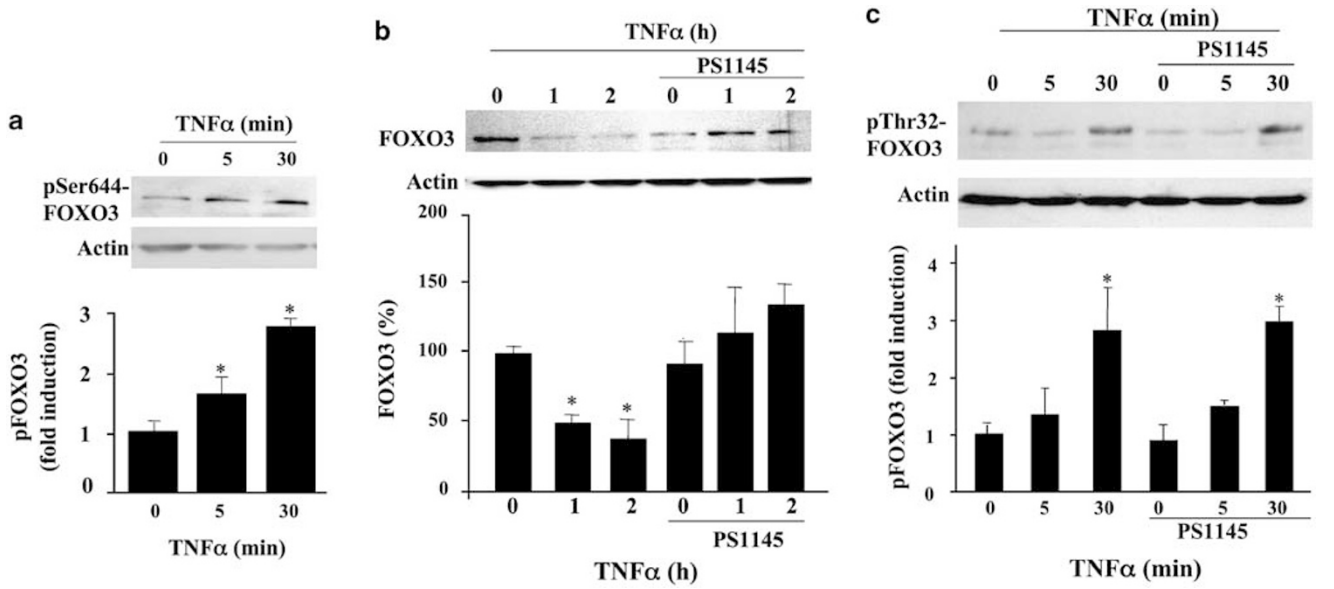

Figure 4 TNFα-induced inactivation of FOXO3 is controlled by IKK. (**a**) Total proteins from untreated and TNFα-treated cells were separated on SDS-PAGE and immunoprobed with an antibody against phosphorylated FOXO3 at the Ser644 IKK-dependent position. Immunoblots were also re-probed with an antibody against actin. (**b**) The HT-29 monolayers were pre-treated with the IKK inhibitor, PS1145, and induced with TNFα for various time points. Protein was separated on SDS-PAGE and immunoprobed with an antibody against total FOXO3 and actin. The graphs represent the densitometric analysis showing a significant decrease of FOXO3 (*) after TNFα treatment ($n=3$, $P<0.05$) and protection of degradation with the IKK inhibitor. (**c**) Protein from the monolayers pretreated with PS1145 and TNFα was separated and immunoprobed against phosphorylated FOXO3 at Thr32 PI3K-dependent site and actin. The densitometric analysis shows a significant increase (*) in phosphorylated FOXO3 after TNFα treatment, which was not attenuated in the presence of PS1145.

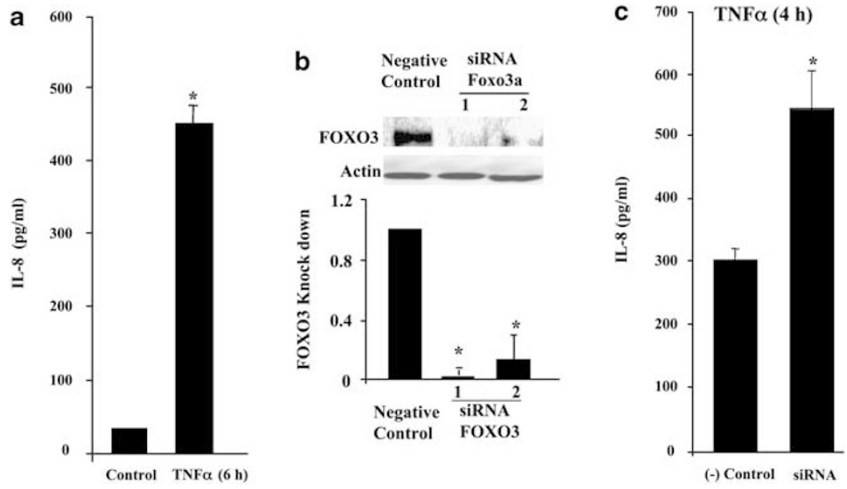

Figure 5 FOXO3 is involved in the regulation of TNFα-induced IL-8 expression. (**a**). The monolayers were treated with TNFα for a period of 6 h and media was collected for IL-8 quantification. (**b**) Representative immunoblot of three independent experiments showing efficiency of FOXO3 knock down. The densitometric analysis shows significant (*) knock down of FOXO3 after 48 h, $n=3$, $P<0.05$. (**c**) The monolayers with silent FOXO3 were treated with TNFα for 4 h and media was collected for IL-8 quantification. The graph represents the average IL-8 ratio of three independent experiments and the asterisk represents a significant difference ($n=4$, $P<0.05$).

areas close to ulcerations. In the intestinal epithelia, the number of lymphocytes and PMNs was increased 2- and 5-fold, respectively (lymphocytes: WT 4.1 ± 1.4; KO 7.6 ± 3.2, PMN: WT 0.9 ± 1.1; KO 6.4 ± 2.7) (Figure 7d and e). These data show that Foxo3 deficiency results in severe disease in response to DSS.

DISCUSSION

In the pathogenesis of inflammatory diseases, cytokines have an important role in maintaining the inflammatory response. The regulation of cytokines is better understood today; however, further elucidation of the mechanisms of cytokine regulation is needed to develop more specific and effective

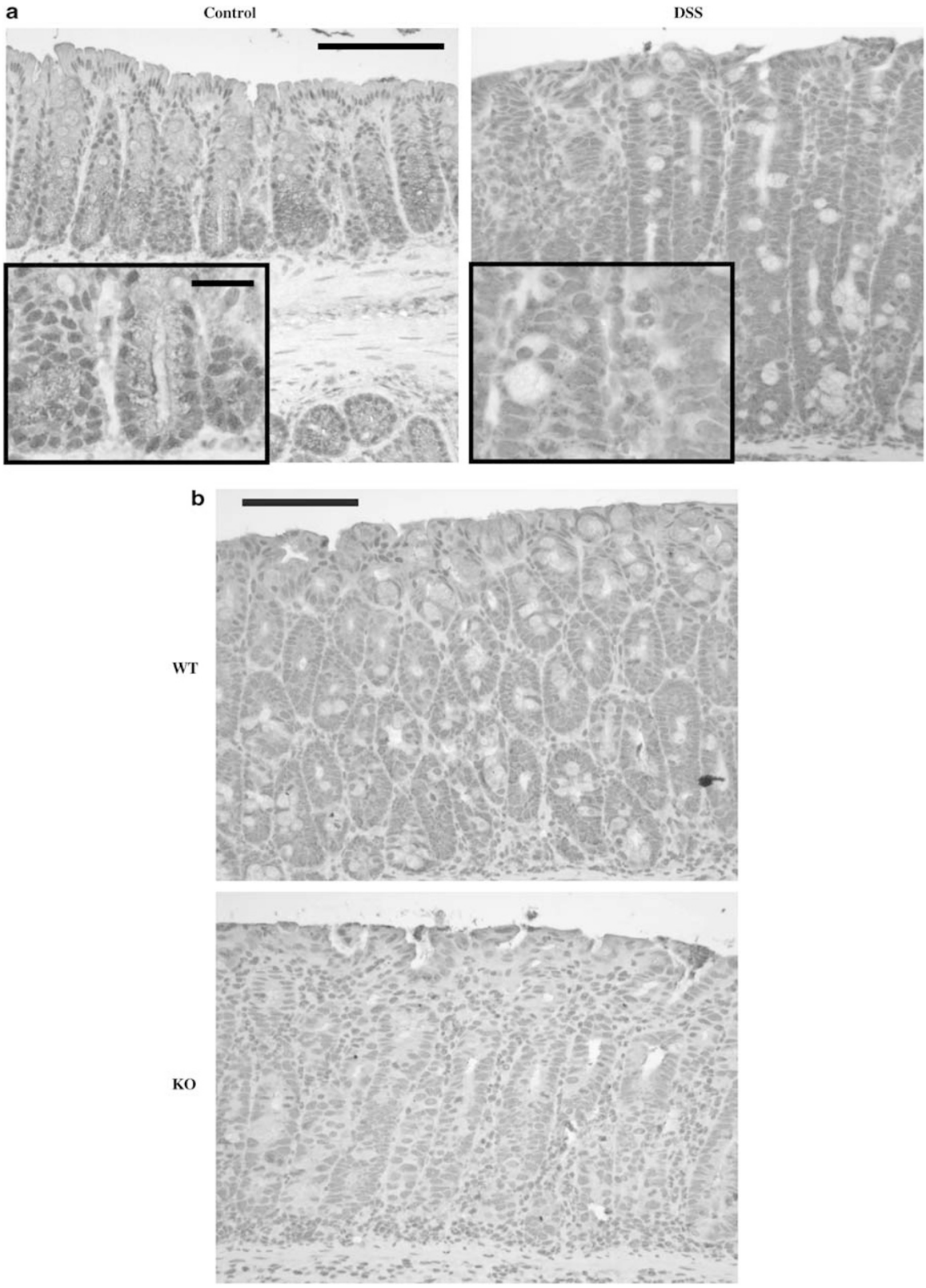

Figure 6 Foxo3 status in colonic epithelia of mice with DSS-induced inflammation. (**a**) Colonic tissue from C57BL/J6 mice, control and treated with DSS were immunohistostained for Foxo3. Immunohistostaining revealed cytoplasmic Foxo3 localization in inflamed colonic epithelia. (**b**) Colonic tissue from Foxo3-deficient mice is immunohistostained for Foxo3 as a control ($\times$ 20 magnification: bar 100 μm; inset $\times$ 63 magnification: bar 40 μm).

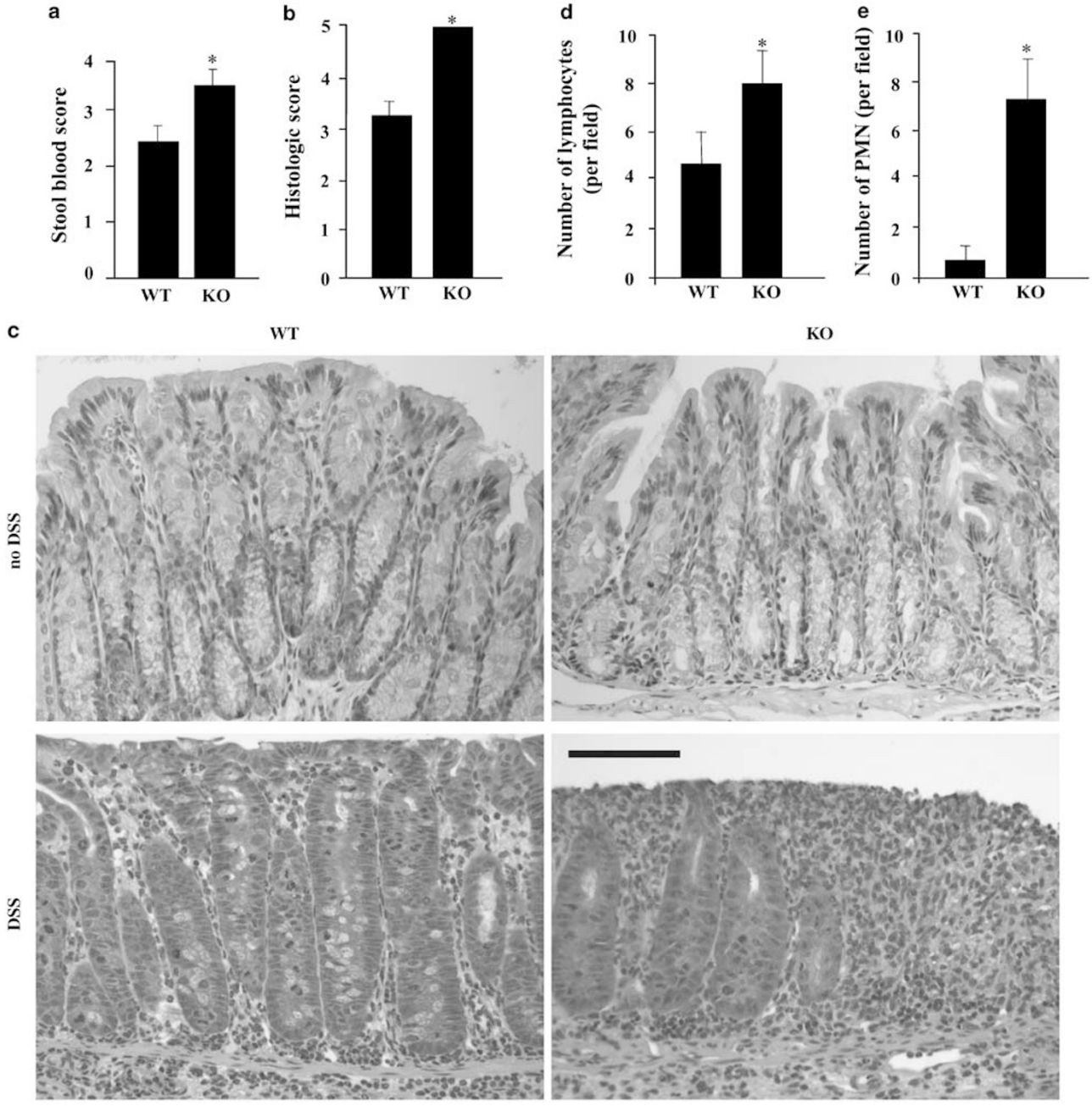

Figure 7 Foxo3 deficiency leads to severe intestinal inflammation. Foxo3 knockout (KO) and wild-type (WT) mice ($n = 7$ from each group) were treated with 2.5% DSS for 5 days and left 2 days to recover. (**a**) Measuring blood in stool revealed that KO mice have more bleeding than WT mice ($P < 0.05$). (**b**) Graph represents inflammation scoring index between KO and WT mice ($P < 0.05$). (**c**) H&E staining revealed mild inflammation in WT and severe inflammation in KO colon ($\times 20$ magnification: bar 100 μm). (**d** and **e**) Graphs represent the number of lymphocytes and PMNs in colonic epithelia enumerated in five different fields. Asterisk represents significant differences between WT and KO mice ($P < 0.05$).

therapies. Here, we have shown that TNFα induces FOXO3 phosphorylation, translocation, and degradation in HT-29 cells. TNFα-mediated FOXO3 degradation is proteasome dependent. Proximally, TNFα used the PI3K and IKK pathways to regulate FOXO3 in HT-29 cells. In addition, our *in vitro* data revealed that silenced FOXO3 leads to dramatically increased TNFα-induced IL-8 expression in HT-29 cells, which is in agreement with our previously published data.[21] *In vivo*, in the mouse DSS colitis model, Foxo3 is localized primarily in the cytosol of the intestinal epithelia, suggesting that Foxo3 is inactive. Also, Foxo3-deficient mice developed a more severe intestinal inflammation compared

with wild-type mice in response to the DSS treatment. Altogether, our results suggest that tumor suppressor FOXO3 regulates intestinal inflammation *in vitro* and *in vivo*.

FOXO3 is localized in the nucleus-controlling gene expression both directly and indirectly.[13] In certain tumor cells, the inactivated cytosolic FOXO3 contributes to the pathogenesis and development of tumors by modulating proliferation and apoptosis.[35–37] Our *in vivo* data revealed that Foxo3 is localized in the cytosol in the inflamed colon and that Foxo3 deficiency additionally promotes the inflammation. We previously demonstrated that FOXO3 knock down leads to the reduced inhibitory IκBα in intestinal epithelial cells.[21] Therefore, we concluded that the inactive cytosolic FOXO3 further promotes intestinal inflammation. Kristof *et al*[38] have shown that LPS controls FOXO3 in human lung epithelial cells, thus regulating iNOS and contributing to inflammation. In macrophages, HIV infection keeps FOXO3 in the nucleus, which further contributes to the apoptosis of infected cells.[39] Thus FOXO3 has very different, but important, roles in the regulation of cellular events depending on the cell type and pathological conditions.

FOXO3 is controlled proximally by the PI3K and IKK pathways.[13,15] Our data show that TNFα regulates FOXO3 by both PI3K and IKK pathways in human intestinal HT-29 cells. Previously, we have shown that bacterial infection regulates FOXO3 via PI3K, whereas the role of IKK was insignificant.[21] Thus it appears that the proximal pathways differ depending on the stimulus. At this moment, the exact relationship between the PI3K and IKK pathways, with regard to TNFα-dependent FOXO3 regulation in intestinal epithelial cells, is unclear. The inhibition of IKK did not affect PI3K-dependent FOXO3 phosphorylation, whereas we were unable to clarify how inhibition of PI3K affects IKK-dependent FOXO3 phosphorylation. We speculate that the PI3K and IKK pathways regulate FOXO3 independently. Sudheerkumar *et al*[40] have demonstrated that TNFα can independently activate IKK and PI3K pathways in human glioma cells. Agarwal *et al*[41] showed that simultaneous activation of the IKK and PI3K pathways regulate both NF-κB and β-catenin to facilitate tumor progression. We hypothesize that TNFα facilitates the inflammation of the intestinal epithelia by activating the PI3K and IKK pathways that regulate both NF-κB and FOXO3. We speculate that TNFα activates two separate but interrelated signaling pathways and that both are necessary for the full inactivation of FOXO3, which further promotes inflammation. One set of signals activates the IKK complex to phosphorylate FOXO3 and to initially liberate NF-κB and drive the immediate cytokine expression. The second set of signals uses the PI3K mechanism to additionally phosphorylate FOXO3 and keep it out of the nucleus so that the *de novo* IκBα synthesis is repressed and the transcription of pro-inflammatory genes is uninterrupted.

Foxo3 deficiency in mice leads to spontaneous T-cell activation, cytokine production and a mild lymphoproliferation. In Foxo3-deficient mice, this leads to an autoimmune syndrome with spontaneous inflammation in multiple organs, while having no reported intestinal changes.[20] The intestinal epithelia have an important role in separating the unsterile from the sterile environment and thus, the infiltration of immune cells is tightly controlled. Intestinal inflammation might occur after the intestinal epithelia 'sense' the environmental changes and send signals to summon immune cells to infiltrate the tissue. Therefore, we speculate that the role of Foxo3 in the intestinal tissue is specific. In an inflamed colon, Foxo3 is distributed in the cytosol of the intestinal epithelia, suggesting that Foxo3 is inactive. We hypothesize that the localization of FOXO3 will correlate with the severity of inflammation in the DSS model and that with mild inflammation, Foxo3 will be distributed between the cytosol and the nucleus. In cultured intestinal cells TNFα induces cytosolic FOXO3 degradation, which was not apparent in the intestinal epithelia of the colon. The intestinal epithelial cells in tissue permanently proliferate and differentiate, which is critical for normal growth, development, and disease prevention. FOXO3 also regulates the proliferation of these cells, thus we hypothesize that FOXO3 remains in the cytosol without significant degradation and is ready to go back to the nucleus to regulate other functions.

Foxo3-deficient mice develop more severe inflammatory responses to DSS compared with wild-type mice. We show *in vitro* that FOXO3 deficiency leads to the attenuation of inhibitory IκBα,[21] directly linking FOXO3 with inflammation. A similar role of Foxo3 was observed in T cells. In mouse T cells deficient in Foxo3, NF-κB activation is unrestrained and there are diminished levels of IκBs.[20] It is possible that for colonic inflammation in the DSS model, lymphocytes deficient in Foxo3 are in part responsible. However, the fact that Foxo3-deficient lymphocytes alone do not spontaneously infiltrate colonic tissue suggest that Foxo3 has the primary role in intestinal epithelia. In B cells and PMNs, Foxo3 has less of an effect on the NF-κB pathway, but it does regulate cell survival and proliferation.[42,43] Foxo3-deficient mice were resistant to induced arthritis due to increased apoptosis of the Foxo3-deficient PMNs.[44] On the contrary, our data showed that in colonic epithelia PMN accumulation and crypt abscesses are increased in Foxo3-deficient mice. We proposed two possible scenarios: (a) infiltrated Foxo3-deficient PMNs in the intestinal tissue are resistant to apoptosis; and (b) due to the large number of PMNs infiltrated in the colon the apoptotic nature on Foxo3-deficient PMN cells is not enough to eliminate them. Yet the role of Foxo3 in infiltrated inflammatory cells in the colon is still unclear as well as FOXO3's role in the healing of inflamed intestinal tissue. We need to further address these questions.

In summary, these data indicate that FOXO3 has an important role in controlling and facilitating intestinal inflammation. Furthermore, FOXO3 should be considered as a potential therapeutic target to treat IBD.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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