NOX1/NADPH oxidase expressed in colonic macrophages contributes to the pathogenesis of colonic inflammation in trinitrobenzene sulfonic acid-induced murine colitis

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Abbreviations: NOX1, NADPH oxidase 1; NOX1KO, NADPH oxidase 1-deficient; WT, wild-type; TNBS, trinitrobenzene sulfonic acid; MPO, myeloperoxidase; TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric oxide synthase; IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; ROS, reactive oxygen species; NOX2, NADPH oxidase 2; HE, hematoxylin and eosin; PBS, phosphate buffered saline; qRT-PCR, quantitative real-time polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; Th, T helper; MAP, mitogen-activated protein; NF, nuclear factor.

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ABSTRACT

NOX1/NADPH oxidase, a non-phagocytic isoform of reactive oxygen species-producing enzymes, is highly expressed in the colon, but the physiological and pathophysiological roles of this isoform are not fully understood. The present study investigated the role of NOX1 in the development of colonic inflammation in a trinitrobenzene sulfonic acid (TNBS)-induced murine colitis model. Intrarectal injection of TNBS caused severe colitis accompanied by body weight loss, diarrhea, and increased MPO activity in wild-type (WT) mice. In contrast, the severity of colitis was significantly attenuated in NOX1-deficient (NOX1KO) mice (the inhibitions of macroscopic damage score, body weight loss, diarrhea score, and MPO activity were 73.1%, 36.8%, 83.3%, and 98.4%, respectively). TNBS-induced up-regulation of inflammatory cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-1β), chemokines (CXCL1 and CXLC2), and inducible nitric oxide synthase (iNOS) was also significantly less in NOX1KO than WT mice (the inhibitions were 100.8%, 89.0%, 63.5%, 96.7%, and 97.1%, respectively). Expression of NOX1 mRNA was detected not only in the lamina propria but also in peritoneal macrophages isolated from WT mice. Increased expression of TNF-α, IL-1β, and iNOS in peritoneal macrophages exposed to lipopolysaccharide was significantly attenuated in macrophages isolated from NOX1KO mice (68.1%, 67.0%, and 79.3% inhibition, respectively). These findings suggest that NOX1/NADPH oxidase plays an important role in the pathogenesis of TNBS-induced colonic inflammation via up-regulation of inflammatory cytokines, chemokines, and iNOS. NOX1 in colonic macrophages may become a potential target in pharmacological intervention for inflammatory bowel disease.
Introduction

Inflammatory bowel disease (IBD), including major pathological conditions such as Crohn’s disease (CD) and ulcerative colitis (UC), is characterized by chronic, remitting, and relapsing inflammatory disease in the lower gastrointestinal tract. IBD has become a global disease due to its increasing prevalence worldwide (Molodecky et al., 2012). Although the etiology of IBD is not fully understood, several pathogenic factors such as dysfunction of the epithelial barrier, abnormal immune responses, and various environmental components including microbiota, have been implicated in the disease process (Kaser et al., 2010).

Abnormal immune responses activate and stimulate macrophages and T-lymphocytes to release various inflammatory mediators as well as reactive oxygen and nitrogen species (Keshavarzian et al., 1992; Khor et al., 2011). Numerous studies reveal that reactive oxygen species (ROS) play a crucial role in the development of IBD (McKenzie et al., 1996; Zhu and Li, 2012). These molecules are generally considered to be released from activated macrophages, T-lymphocytes, and neutrophils, recruited to the colonic mucosa, thereby inducing damage to the surrounding tissue (McKenzie et al., 1996; Kruidenier et al., 2003).

NOX is a catalytic subunit of NADPH oxidase, a major source of ROS identified originally in phagocytes. The prototype isoform has been commonly referred to as gp91Phox (NOX2) (Royer-Pokora et al., 1986; Teahan et al., 1987). NOX1 was cloned as the first homolog of NOX2 and is highly expressed in the colon, particularly in epithelial cells (Suh et al., 1999; Banfi et al., 2003; Szanto et al., 2005). Therefore, NOX1 and NOX2 are recognized as non-phagocytic/colonic and phagocytic isoforms of the enzyme, respectively. Although the physiological and pathophysiological roles of NOX1 in the gut are not fully understood, it is suggested to take part in local innate immune and inflammatory responses in colonic epithelia. Recent studies demonstrated an important role for NOX1 in regulating epithelial homeostasis and barrier functions via epithelial cell proliferation and mucin production in goblet cells (Coant et al., 2010; Jones et al., 2013; Leoni et al., 2013; Treton et al., 2014).
In contrast to these beneficial properties, a detrimental role for NOX1-derived ROS has been reported. Specifically, the expression of NOX1 was up-regulated in the inflamed colon during dextran sulfate sodium (DSS)- and tumor necrosis factor (TNF)-α-induced colitis. Inhibition of ROS production by apocynin, an antioxidant and a non-selective NOX inhibitor in leukocytes (Heumuller et al., 2008), reduced the severity of colonic inflammation (Mouzaoui et al., 2014; Ramonaite et al., 2014). We also reported that NOX1-deficient mice exhibited resistance to intestinal mucositis induced by an anti-cancer agent, 5-fluorouracil (Yasuda et al., 2012). Thus, the role for NOX1-derived ROS in the pathogenesis of gut inflammation has been controversial.

Although NOX1 had been recognized as non-phagocytic isoform, it was demonstrated in macrophages and lymphocytes (Lee et al., 2005; Szanto et al., 2005). Thus, the differential role of NOX1 could be explained by expression in both colonic epithelial cells and macrophages/lymphocytes. While most previous studies focused on NOX1 expression in colonic epithelial cells, recent findings suggested that NOX1 expressed in macrophages and lymphocytes may regulate inflammatory responses (Yeligar et al., 2012; Lee et al., 2015). On these grounds, a study was undertaken to investigate the effects of NOX1 deficiency in the trinitrobenzene sulfonic acid (TNBS)-induced murine colitis model. We found that NOX1 in colonic macrophages played a key role in the pathogenesis of TNBS-induced colitis by up-regulating inflammatory mediators. NOX1-mediated responses in macrophages may provide a novel therapeutic target for inhibiting the development of IBD.
Materials and Methods

Animals

All experimental procedures were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” as promulgated by the U.S. National Institutes of Health, and with the approval of the Committee on the Ethics of Animal Research of Kyoto Pharmaceutical University. All studies also complied with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. Male NOX1-deficient (NOX1KO) and their littermate wild-type (WT) mice (Matsuno et al., 2005), weighing 21–26 g, were maintained under standard laboratory conditions (12 h light–dark cycle, temperature 22 ± 1°C). The experiments were carried out using 6–8 mice per group.

Induction of colitis

Experimental colitis was induced by a single rectal injection of 0.1 mL of TNBS (3 mg/mouse in a 50% ethanol solution; Tokyo Kasei, Tokyo, Japan) under ketamine/xylazine anesthesia. Control animals received an equivalent volume of 50% ethanol solution. Disease severity was evaluated daily by measuring body weight and scoring the stool consistency [0, normal; 1, slightly wet and soft; 2, very soft but still formed; 3, diarrhea (wet and unformed); 4, severe diarrhea (watery stool with severe perianal staining)] in accordance with a slightly modified method described previously (Wirtz et al., 2007).

Macroscopic and histological evaluations

On day 4 following TNBS injection, the entire colon was excised and treated with 2% formalin for fixation of the tissue wall. The colon tissues were cut, opened, and macroscopic damage graded [0, no damage; 1, mucosal erythema only; 2, mild edema, slight bleeding or small erosion; 3, moderate edema, ulcerations or erosions, 4, severe ulceration, erosions, edema and tissue necrosis] in accordance with a slightly modified method described previously (Millar et al., 1996). Tissues were then immersed in 10% neutralized formalin.
overnight, embedded in paraffin, cut into 4-µm sections, and stained with hematoxylin and eosin (HE). Histological damage was graded under a light microscope (BX-51, Olympus, Tokyo, Japan) at 200× magnification [0, normal; 1, low leukocyte infiltration; 2, moderate leukocyte infiltration and moderate disruption of epithelium; 3, high leukocyte infiltration, diffuse disruption of epithelium, thickening of the colon wall, moderate goblet cell loss, and focal crypt loss; 4, transmural infiltrations, massive loss of goblet cells, and diffuse loss of crypts] in accordance with a slightly modified method described previously (Gonzalez-Rey et al., 2006). Macroscopic and histological examinations were performed by the same investigator in a blinded manner.

**Determination of myeloperoxidase (MPO) activity**

Excised colon tissues were rinsed in cold phosphate-buffered saline (PBS) and homogenized in 50 mM potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0; Wako, Osaka, Japan). The homogenized samples were subjected to three freeze-thaw cycles and centrifuged at 500 g for 10 min. MPO activity in the supernatant was determined spectrophotometrically at 450 nm using o-dianisidine hydrochloride (Sigma-Aldrich, St. Louis, MO), as described previously (Yasuda et al., 2011). Sample protein content was estimated using a bicinchoninic acid spectrophotometric assay kit (Pierce, Rockford, IL).

**Determination of ROS production**

Excised colon tissues were rinsed with cold PBS, homogenized in ice-cold Krebs-HEPES buffer (pH 7.4) containing a cocktail of protease inhibitors (Complete Mini, Roche, Mannheim, Germany), and then centrifuged at 1100 g at 4°C for 15 min. The production of ROS (primarily superoxide) was determined via a chemiluminescence assay using L-012 (Wako, Osaka, Japan) as described previously (Yasuda et al., 2011).

**Determination of the expression of inflammatory mediators**
Excised colon tissues were rinsed with cold PBS and stored in RNAlater (Ambion, Austin, TX) at 4°C until use. Total RNA was extracted using Sepasol RNA-I Super G (Nacalai Tesque, Kyoto, Japan) and reverse transcription was performed using PrimeScript Reverse Transcriptase (Takara, Shiga, Japan). The quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using an ABI 7500 (Applied Biosystems, Foster City, CA) with SYBR Premix ExTaq II (Takara). Specific primer sets for β-actin (MA050368), TNF-α (MA097070), IL-1β (MA025939), CXCL1 (MA104685), CXCL2 (MA152904), IFN-γ (MA025911), IL-4 (MA033674), IL-17A (MA157056), NOX1 (MA147219), NOX2 (MA140075), and inducible nitric oxide synthase (iNOS; MA063888) were obtained from the Perfect Real-Time Supporting System (Takara). The expression level of each mRNA was calculated using the comparative $\Delta\Delta C_T$ method, where signals were normalized to the mean value observed in the control group in WT mice.

Isolation of lamina propria and peritoneal macrophages

Lamina propria mononuclear cells were isolated from the colon of WT and NOX1KO mice in accordance with the slightly modified method described previously (Yoshihara et al., 2006). Briefly, the excised colon was washed with PBS and cut into 2-mm samples, and the epithelium was eliminated by stirring for 20 min in Mg$^{2+}$/Ca$^{2+}$-free Hank’s balanced salt solution containing 1 mM dithiothreitol and 3 mM EDTA at 37°C, and then twice for 30 min in Dulbecco’s modified Eagle’s medium (DMEM) (Wako) containing 2% fetal bovine serum (FBS), 1 mg/mL collagenase IV (Wako), and 0.01 mg/mL DNase I (Roche). The cell pellet was washed and purified to mononuclear cells on a 40%/75% discontinuous Percoll (GE Healthcare, Pittsburgh, PA) gradient with centrifugation at 480 g for 20 min. Mononuclear cells were harvested from the interface.

Peritoneal cells were harvested by washing the peritoneal cavity of WT and NOX1KO mice with ice-cold PBS on day 3 after intraperitoneal injection of 3% (w/v) thioglycolate (2 mL/mouse) (Difco, Detroit, MI).

Lamina propria mononuclear and peritoneal cells were washed with PBS and
centrifuged (600 g at room temperature for 5 min), and the cell pellets resuspended in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were cultured in 24-well microplates and incubated overnight at 37°C in 5% CO₂ with a humidified atmosphere. Non-adherent cells were removed by changing the media. The remaining adherent cells (lamina propria or peritoneal macrophages) were used for the experimental procedures.

**Cell line**

The RAW264.7 cell line was obtained from RIKEN BRC (Ibaraki, Japan). The cells were grown in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂ with a humidified atmosphere.

**Determination of NOX mRNA expression**

Total RNA from macrophages of the lamina propria and the peritoneal cavity, as well as from RAW264.7 cells, was extracted and reverse transcription was performed as described above. PCR was carried out using a thermal cycler (TP-240, Takara) with the Advantage 2 polymerase mixture (Clontech, Mountain View, CA) in 40 cycles with 0.5 min of denaturation at 95°C and 1 min of extension at 68°C. Specific primer sets for NOX1 (MA147219) and NOX2 (MA140075) were used as described above. A portion of the PCR mixture was electrophoresed in a 1.8% agarose gel in TAE buffer (40 mM Tris, 2 mM EDTA, and 20 mM acetic acid; pH 8.1). The gel was stained with ethidium bromide and photographed (BioDoc-It Imaging System, UVP, Upland, CA).

**Determination of TNF-α and IL-1β mRNA expression in peritoneal macrophages**

Peritoneal macrophages isolated from WT and NOX1KO mice were cultured overnight in 24-well microplates with DMEM containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO₂ in a humidified atmosphere. Lipopolysaccharide (LPS) (*Escherichia coli* B4, Sigma-Aldrich; final concentration 10
μg/mL) was added to the medium and total RNA was extracted 4 h later. qRT-PCR was carried out as described above.

**Sequencing of NOX1 mRNA expressed in RAW264.7 cells**

Total RNA was isolated from RAW264.7 cells using the RNeasy Mini Plus Kit and QIAshredder (Qiagen, Germantown, MD). RT-PCR was performed using a *ReverTra Ace* qPCR RT Master Mix with a genomic DNA Remover RT-PCR Kit (Toyobo, Osaka, Japan). The specific primer pairs (5′-ATGGGAACCTGGCTGGTTAACCAC-3′ for N-terminal and 5′-TCAGAACGTTTCTTTGTTGAAGTA-3′ for C-terminal) used for RT-PCR were designed based on the open reading frame of NOX1 (NCBI ID: NM_172203). PCR conditions used for stage-specific RT-PCR analyses consisted of 30 cycles (denatured for 10 sec at 98°C, annealed for 5 sec at 55°C, and extended for 1 min at 72°C) using PrimeSTAR HS DNA Polymerase (Takara). PCR fragments were separated by 1.0% agarose gel electrophoresis, purified with an Illustra GFX PCR DNA and gel band purification kit (GE Healthcare, Pittsburgh, PA), and subcloned to a TA-Cloning pMD20-T vector using a Mighty TA-cloning kit for PrimeSTAR (Takara). pMD20/NOX1 in RAW264.7 cells was sequenced using a PRISM BigDye Terminator Cycle 3.1 sequencing kit and an ABI 3130xl genetic analyzer (Applied Biosystems).

**Statistical analysis**

Data are presented as means ± standard error of the mean (SEM). Statistical analyses were performed with GraphPad Prism 6.0b (GraphPad Software, La Jolla, CA) using a parametric two-way ANOVA followed by Bonferroni’s multiple comparison test, or a non-parametric Kruskal-Wallis test followed by Dunn’s multiple comparison test. P values < 0.05 were regarded as statistically significant.
Results

TNBS-induced body weight loss and diarrhea were alleviated in NOX1KO mice

Intrarectal injection of TNBS (3 mg/mouse in 50% ethanol) induced body weight loss in both WT and NOX1KO mice to the same degree until day 2 following the injection (Fig. 1A). Body weight continued to decrease in WT mice on day 3 and thereafter, down to 78.8 ± 1.9% of the initial body weight. On the other hand, body weight of NOX1KO mice recovered partially until day 4 (88.9 ± 1.6% of the initial body weight), demonstrating a significant difference between genotypes (p < 0.001). Gross physical condition such as posture, coat, and activity were also worsened by intrarectal injection of TNBS, especially from day 3, but the degree was apparently better in NOX1KO mice than WT mice. Intrarectal injection of the control solution (50% ethanol alone) caused a slight decrease in body weight on day 1, but did not affect the subsequent weight gain in either genotype.

The severity of diarrhea induced by TNBS was significantly less in NOX1KO than WT mice (Fig. 1B). The diarrhea scores on day 4 in WT and NOX1KO mice were 3.0 ± 0.4 and 0.5 ± 0.3, respectively (p = 0.004). Intrarectal injection of the control solution induced slight diarrhea on day 1 in both genotypes.

TNBS-induced damage in the colon was alleviated in NOX1KO mice

In the preliminary experiment, we observed that the severity of colitis accompanied by body weight loss and diarrhea reached its maximum on day 4–5 following the intrarectal injection of TNBS. Thus, in the present study, the severity of colitis was evaluated on day 4. In the colon of WT mice on day 4 following the injection of TNBS, there was severe macroscopic damage characterized by severe edema, extensive erosions, and ulcerations (Fig. 2A). The severity of damage to the colon was significantly less in NOX1KO (macroscopic score: 0.9 ± 0.2) compared to WT mice (3.3 ± 0.6) (p = 0.008) (Fig. 2B). Injection of the control solution did not produce any damage in the colon in either genotype.

Histological analyses showed results similar to the macroscopic study. In WT mice,
TNBS injection produced severe histological damage characterized by deep ulcerations with massive transmural infiltration of inflammatory cells, accompanied by the loss of goblet cells and crypts (Fig. 3A). In contrast, minimal loss of goblet and crypts, with slight infiltration of inflammatory cells, was only detected in NOX1KO mice. The severity of histological damage was significantly less in NOX1KO (histological score: 1.9 ± 0.4) compared to WT mice (3.9 ± 0.1) \((p = 0.001)\) (Fig. 3B).

**TNBS-induced changes in MPO activity, ROS production, and the expression of NOX1 mRNA**

Compared to controls, MPO activity was increased markedly in the colon of WT mice on day 4 following the injection of TNBS (0.19 ± 0.02 to 0.57 ± 0.02 \(\mu\)mol H\(_2\)O\(_2\)/min/mg protein) (Fig. 4A). The TNBS-induced increase in MPO activity was totally suppressed in NOX1KO mice on day 4 (0.16 ± 0.01 \(\mu\)mol H\(_2\)O\(_2\)/min/mg protein) \((p = 0.0145)\).

The production of ROS was enhanced significantly in the colon of WT mice on day 4 following the injection of TNBS (2.1 ± 0.4 to 12.0 ± 5.4 \(\times 10^3\) RLU/mg protein) \((p = 0.0358)\) (Fig. 4B). In contrast, there was no TNBS-induced enhancement of ROS production in NOX1KO mice at this time point (3.0 ± 0.4 \(\times 10^3\) RLU/mg protein). Although production of ROS was suppressed in NOX1KO mice treated with TNBS, there was no statistical difference between WT mice \((p = 0.0634)\).

The expression of NOX1 mRNA in the colon was unaffected by TNBS in WT mice (Fig. 4C).

**TNBS-induced up-regulation of inflammatory mediators was suppressed in NOX1KO mice**

In WT mice, administration of TNBS significantly increased mRNA levels of the inflammatory mediators, TNF-\(\alpha\), IL-1\(\beta\), CXCL2, and iNOS on day 4 (4.6 ± 0.9, 35.1 ± 8.2, 270 ± 42, and 7.5 ± 1.5 times those in control animals, respectively) (Fig. 5). TNBS-induced
upregulation of TNF-α, IL-1β, and CXCL2 was significantly suppressed in NOX1KO mice compared with WT mice \( (p < 0.001) \). The expression of CXCL1 appeared to be elevated by TNBS, and this response was also suppressed in NOX1KO mice, but the changes were not statistically significant \( (p = 0.125) \). The expression of IFN-γ, IL-4, and IL-17 was not affected by TNBS in WT and NOX1KO mice.

**Expression of NOX1 in macrophages and RAW264.7 cells**

To assess the expression of NOX1 and NOX2 in macrophages, we isolated peritoneal and lamina propria macrophages from WT and NOX1KO mice. A macrophage cell line, RAW264.7, was also examined. The expression of NOX2 mRNA was readily detected in all types of macrophages (83 bp) (Fig. 6). The expression of NOX1 mRNA was evident in peritoneal and lamina propria macrophages isolated from WT mice, as well as in RAW264.7 cells (184 bp). The absence of NOX1 mRNA was confirmed in peritoneal and lamina propria macrophages of NOX1KO mice.

To further verify the presence of NOX1 mRNA in macrophages, the open reading frame of NOX1 mRNA extracted from RAW264.7 cells was sequenced. The sequence completely corresponded to that reported previously (mouse NOX1 gene; NM_172203) (data not shown).

**LPS-induced up-regulation of inflammatory mediators was alleviated in peritoneal macrophages isolated from NOX1KO mice**

To delineate the cell population responsible for the different responses to TNBS between genotypes, isolated peritoneal macrophages were exposed to LPS, a potent bacterial endotoxin and inflammogen. Consistent with the augmented expression of NOX1 mRNA \( (p < 0.001) \), a marked increase in TNF-α, IL-1β, and iNOS mRNA levels \( (625 \pm 64, 10,096 \pm 1474, \text{ and } 1803 \pm 173 \text{ times those in the control group, respectively}) \) was demonstrated in macrophages isolated from WT mice (Fig. 7). These responses were significantly blunted in cells isolated from NOX1KO mice, by 68.2%, 67.1%, and 79.3%, for TNF-α, IL-1β and
iNOS, respectively (p < 0.001). These findings suggested that NOX1 in macrophages plays a key role in the development of colitis induced by TNBS.
Discussion

The results of the current study demonstrated a crucial role for NOX1/NADPH oxidase in the pathogenesis of TNBS-induced colonic inflammation. While the intrarectal injection of TNBS produced severe macroscopic damage accompanied by body weight loss and diarrhea in WT mice, the severity of damage and clinical symptoms were reduced significantly in NOX1KO mice. Histological analyses in WT mice revealed severe epithelial destruction, ulceration, and the loss of goblet cells and crypts, which was accompanied by a marked infiltration of inflammatory cells at day 4 after TNBS injection. Importantly, all responses were attenuated in NOX1KO mice. The TNBS-induced increase in MPO activity was also suppressed in the colon of NOX1KO mice. These findings strongly suggest that mice deficient in NOX1 are resistant to TNBS-induced colitis and that ROS derived from NOX1/NADPH oxidase take part in the pathogenesis of this disorder.

Several studies indicated the involvement of NADPH oxidase-derived ROS in the pathogenesis of gut inflammation in patients as well as in animal models of IBD (Davies et al., 1992; McKenzie et al., 1996; Zhu and Li, 2012). Apocynin, an antioxidant and inhibitor of phagocytic NADPH oxidase, decreased colonic inflammation in DSS- and TNF-α-induced experimental murine colitis models (Mouzaoui et al., 2014; Ramonaite et al., 2014). Because the main source of ROS during colitis has been considered to be macrophages and neutrophils recruited into the inflamed colonic mucosa, the phagocytic isoform NOX2, rather than the non-phagocytic/colonic isoform NOX1, appears to play an important role in the pathogenesis of IBD (Grisham and Granger, 1988; Maloy and Powrie, 2011). Indeed, gp91phox (NOX2)-deficient mice are less susceptible to acute DSS-induced colitis, possibly due to a reduction of ROS production in the colon (Bao et al., 2011).

On the other hand, NOX1 is highly expressed in colonic epithelial cells, and regulates epithelial homeostasis and barrier functions via local immune responses, proliferation, and mucin production (Coant et al., 2010; Jones et al., 2013; Leoni et al., 2013; Treton et al., 2014). Interestingly, there is no difference in the severity of DSS-induced colitis.
between WT and NOX1KO mice, while epithelial wound repair is impaired significantly in NOX1KO mice (Leoni et al., 2013). These findings suggested that NOX1/NADPH oxidase was involved in the healing process rather than the pathogenesis of colonic inflammation in the DSS-induced colitis model. DSS has a direct effect on the inner mucus layer of the colon, allowing enterobacteria to penetrate. A lack of the inner mucus layer in the colon may be an initial event in the occurrence of inflammation, which allows bacteria to reach the epithelial cells (Johansson et al., 2010). The DSS-induced colitis model is hence associated with mucus and epithelial barrier functions. In contrast, TNBS initiates inflammatory responses via macrophage-mediated recognition and degradation of TNBS-modified mucosal cells and proteins (Grisham et al., 1991). Therefore, the results obtained in our present study may differ from those reported previously using NOX1KO mice (Leoni et al., 2013) due to different pathogenic mechanisms in TNBS- and DSS-induced colitis.

Various inflammatory mediators, as well as NO derived from iNOS, contribute to the pathogenesis of TNBS-induced colitis, and decreased production of the mediators ameliorated the severity of disease (McCafferty et al., 1999; An et al., 2005; van Lierop et al., 2010). We presently observed a marked up-regulation of TNF-α, IL-1β, CXCL1, CXCL2, and iNOS after injecting WT mice with TNBS, which was suppressed in NOX1KO mice. Thus, it is likely that NOX1/NADPH oxidase-derived ROS up-regulate these inflammatory mediators to exacerbate TNBS-induced colitis. Because this colitis model depends on direct immune and inflammatory responses in immune cells, the increased inflammatory mediators in response to TNBS observed in the present study may be primarily attributed to immune and inflammatory cells.

Given that NOX1 is expressed in macrophages and lymphocytes (Lee et al., 2005; Szanto et al., 2005), NOX1 may regulate inflammatory and immune responses in both cell types. Indeed, we confirmed the expression of NOX1 mRNA in both adherent (macrophage rich) and non-adherent cells (lymphocyte rich) isolated from colonic lamina propria (Supplemental figure 1). Nevertheless, in the present study, we failed to detect the upregulation of IFN-γ, IL-17A, or IL-4 in TNBS-induced colitis. Th1 cytokines, such as
IFN-γ, and Th17 cytokines, such as IL-17A, are commonly considered to be enhanced in TNBS-induced colitis models (Alex et al., 2009). These discrepancies may be partly explained by the strain of mice used in this study. BALB/c mice are generally used for TNBS-induced colitis models because C57BL/6 mice are relatively resistant to TNBS (Wirtz et al., 2007). Based on the cytokine profiles determined in this study, however, we presume that macrophage-derived cytokines (TNF-α and IL-1β) and chemokines (CXCL1 and CXCL2) may be more relevant compared to lymphocyte-derived cytokines (IFN-γ, IL-4, and IL-17).

When peritoneal macrophages were exposed to the inflammogen, LPS, the expression of TNF-α, IL-1β, iNOS, and NOX1 mRNA was markedly up-regulated in WT mice. In contrast, LPS-induced expression of these inflammatory mediators was significantly attenuated in peritoneal macrophages isolated from NOX1KO mice. These findings strengthen the view that NOX1/NADPH oxidase expressed in macrophages regulates the expression of inflammatory mediators to aggravate TNBS-induced inflammation.

Not only NOX1, but also the phagocytic isoform, NOX2, was expressed in macrophages of lamina propria and the peritoneal cavity, as well as in RAW264.7 cells. However, the absence of NOX1 did not affect the expression of NOX2 in peritoneal macrophages isolated from NOX1KO mice. Although expression of NOX1 in RAW264.7 and peritoneal macrophages was reported previously (Yeligar et al., 2012; Lee et al., 2015), we confirmed the coding sequence of NOX1 mRNA expressed in RAW264.7 to be identical to that of the mouse NOX1 gene. Accordingly, NOX1, ubiquitously expressed in macrophages, may regulate inflammatory responses during TNBS-induced colitis.

Macrophages display phenotypic plasticity in response to the microenvironment (Biswas et al., 2012; Liu et al., 2014). Two principle phenotypes have been identified: M1, which are classically activated pro-inflammatory macrophages, having a central role in host defense against infection; and M2, which are alternatively activated macrophages associated with responses to anti-inflammatory reactions and tissue remodeling. M1 macrophages express several pro-inflammatory mediators including TNF-α, IL-1β, IL-6, and iNOS, while
M2 macrophages express IL-10 and arginase 1 (Gordon and Martinez, 2010; Biswas et al., 2012). Although it is unclear whether NOX1 is expressed in M1, M2, or both phenotypes, the present results suggest that NOX1 regulates M1 macrophage responses.

It should be noted that NOX1 mRNA was up-regulated in peritoneal macrophages exposed to LPS, whereas the expression of NOX1 was unchanged in the inflamed colon during TNBS-induced colitis. A partial explanation for these findings is that the up-regulation of NOX1 in colonic macrophages was masked due to its abundant basal expression in colonic epithelial cells (Coant et al., 2010; Jones et al., 2013; Leoni et al., 2013; Treton et al., 2014). The increased production of ROS demonstrated in the inflamed colon seemed to derive from NOX2 in inflammatory cells mobilized into the tissue. Immunohistological assessment of NOX1 in macrophages during TNBS-induced colitis was difficult because presently available antibodies only detect NOX1 expressed in colonic epithelial cells but not in other tissues with low expression levels (Matsumoto et al., 2014). Further studies are needed to confirm the expression of NOX1 in macrophages associated with the development of colonic inflammation.

Because the levels of NOX1 were lower than NOX2 in macrophages, NOX1-derived ROS may act as signaling molecules to induce inflammatory responses via redox-sensitive pathways mediated by mitogen-activated protein (MAP) kinases (Torres and Forman, 2003; Mouzaoui et al., 2014) and nuclear factor (NF)-κB (Otani, 2004). Indeed, it was reported that NOX1-derived ROS modulate MAP kinase (Lassegue et al., 2001; Kim et al., 2007) and NF-κB (San Martin et al., 2007; Lee et al., 2015), leading to the induction of various inflammatory mediators. Activation of these redox-dependent pathways downstream of NOX1/NADPH oxidase may elicit the production of inflammatory mediators during TNBS-induced colitis. Further investigation is required to clarify the molecular mechanisms underlying NOX1-induced inflammatory responses.

Overall, the results of the present study suggest that NOX1/NADPH oxidase plays a crucial role in the development of colonic inflammation in the TNBS-induced colitis model. NOX1 expressed in colonic macrophages takes part in the disease process by up-regulating
various inflammatory mediators. Previous histopathological and immunological studies demonstrated that TNBS and DSS elicit CD- and UC-like colitis, respectively (Strober et al., 2002; Alex et al., 2009). NOX1/NADPH oxidase may thus take part in the pathogenesis of human CD, rather than UC. In this context, our findings in mice deficient in NOX1 provide a rationale for preventive and therapeutic effects of NOX1 inhibition. NOX1/NADPH oxidase in macrophages may be a novel and promising target for pharmacological intervention of IBD.

Authorship Contributions
Participated in research design: Yokota, Iwata, Kato.
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Figure legends

**Fig. 1.** Changes in body weight and diarrhea scores induced by TNBS in WT and NOX1KO mice. Animals were given TNBS (3 mg/mouse in 50% ethanol) intrarectally. Controls received an equal volume of 50% ethanol intrarectally. (A) Body weight, monitored daily, is expressed as a percentage of the initial body weight. (B) The severity of diarrhea was scored daily using the five-grade scale (0 to 4) described in Methods. Data are presented as means ± SEM for 8 mice. Significantly different at P < 0.05; *from WT mice; #from control.

**Fig. 2.** Macroscopic analyses of the colon in the TNBS-induced colitis model. (A) Representative photographs of the colon at 4 days following vehicle or TNBS injection. (B) Macroscopic damage was scored using the five-grade scale (0 to 4) described in Methods. Data are presented as means ± SEM for 8 mice. Significantly different at P < 0.05; *from WT mice; #from control.

**Fig. 3.** Histological analyses of the colon in the TNBS-induced colitis model. (A) Representative photographs of HE stained sections obtained from the colon at day 4 after TNBS injection (×100). (B) Histological damage was scored using the five-grade scale (0 to 4) described in Methods. Data are presented as means ± SEM for 8 mice. Significantly different at P < 0.05; *from WT mice; #from control. Scale bar: 50 µm. Black arrows: ulceration. White arrows: loss of goblet cells and crypts. White arrow heads: infiltration of inflammatory cells.

**Fig. 4.** TNBS-induced changes in MPO activity, ROS production, and NOX1 mRNA expression in the colon. (A) MPO activity, (B) ROS production (RLU, relative luminescence units), and (C) NOX1 mRNA expression were determined after 4 days. The expression level of NOX1 mRNA was standardized to that of β-actin mRNA and normalized to the mean value in WT control mice. Data are presented as means ± SEM (control in WT mice: n = 8;
control in NOX1KO mice: n = 6; TNBS in WT mice: n = 8; TNBS in NOX1KO mice: n = 8). Significantly different at P < 0.05; *from WT mice; #from control (ethanol alone).

**Fig. 5.** TNBS-induced changes in the expression of various inflammatory mediators in the colon. Levels of (A) TNF-α, (B) IL-1β, (C) CXCL1, (D) CXCL2, (E) IFN-γ, (F) IL-4, (G) IL-17, and (H) iNOS mRNAs were determined by qRT-PCR after 4 days. mRNA levels were standardized to that of β-actin and normalized to the mean value in WT control mice. Data are presented as means ± SEM for control in WT mice (n = 8), control in NOX1KO mice (n = 6), TNBS in WT mice (n = 8), and TNBS in NOX1KO mice (n = 8). Significantly different at P < 0.05; *from WT mice; #from control.

**Fig. 6.** Expression of NOX1 and NOX2 mRNAs in peritoneal macrophages (PM), lamina propria macrophages (LPM), and RAW264.7 cells. Representative photographs of RT-PCR analyses are shown. PM and LPM were isolated from WT and NOX1KO mice.

**Fig. 7.** Changes in the expression of inflammatory mediators, and NOX1 mRNA, induced by lipopolysaccharide (LPS) in peritoneal macrophages. Peritoneal macrophages isolated from WT and NOX1KO mice were exposed to LPS (0.1 µg/mL). Levels of (A) TNF-α, (B) IL-1β, (C) iNOS, and (D) NOX1 mRNAs were determined by qRT-PCR after 4 hours. mRNA levels were standardized to that of β-actin and normalized to the mean value in WT control mice. Data are presented as the means ± SEM for 6 mice. Significantly different at P < 0.05; *from WT mice; #from control (no LPS).
Fig. 1

Panel A: Body weight (%) over days for WT and NOX1KO mice. 

Panel B: Diarrhea score over days for WT and NOX1KO mice in control and TNBS conditions.
Fig. 2
Fig. 3
**Fig. 4**

A. MPO activity (μmol H₂O₂/min/mg protein)

B. ROS production (x10³ RLU/mg protein)

C. NOX1 mRNA expression (times to normal)
Fig. 5
Fig. 6
**Fig. 7**

(A) TNF-α mRNA expression (fold to normal) in WT and NOX1KO mice control and LPS-treated groups.

(B) IL-1β mRNA expression (fold to normal) in WT and NOX1KO mice control and LPS-treated groups.

(C) iNOS mRNA expression (fold to normal) in WT and NOX1KO mice control and LPS-treated groups.

(D) NOX1 mRNA expression (fold to normal) in WT and NOX1KO mice control and LPS-treated groups.
Supplemental Data for
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NOX1/NADPH oxidase expressed in colonic macrophages contributes to the pathogenesis of colonic inflammation in trinitrobenzene sulfonic acid-induced murine colitis

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Supplemental figure 1. Expression of NOX1 mRNA in adherent and non-adherent cells. Representative photographs of RT-PCR analyses are shown. Adherent (macrophage rich) and non-adherent cells (lymphocyte rich) were isolated from colonic lamina propria of WT and NOX1KO mice.