Original Article

Stromal Fibroblasts Produce Interleukin-11 in the Colon of TNBS-treated Mice

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ABSTRACT

Introduction: Interleukin (IL) -11 is a member of the IL-6 cytokine family and is implicated to play a role in pleiotropic functions such as hematopoiesis, bone development, tissue repair, and tumor development. Although the protective function of IL-11 in trinitrobenzene sulfonic acid (TNBS)-induced colitis has been reported, whether *Il11* expression is induced in the colon of TNBS-treated mice is still unclear.

Methods: After inducing TNBS-induced colitis in C57BL/6 mice, *Il6* and *Il11* expressions in the colon were determined using quantitative PCR. Colonic sections were stained with hematoxylin and eosin (H&E) or immunostained with anti-IL-11 antibody along with antibodies against lineage-specific markers. To assess the contribution of the transforming growth factor (TGF)-β signal or mitogen-activated protein kinase/ERK kinase (MEK)/ERK pathway to upregulation of *Il11* expression, we treated mice with TNBS in the presence of neutralizing antibody against TGF-β or a MEK inhibitor, trametinib. Subsequently, *Il11* expression in the colon was determined using qPCR.

Results: TNBS treatment increased *Il11* expression in the colon. Immunohistochemical analysis revealed that IL-11$^+$ cells appeared in the subepithelial tissues of the inflamed colon. IL-11$^+$ cells expressed podoplanin, vimentin, and collagen IV but did not express α-smooth muscle actin, suggesting that these cells were fibroblasts, and not myofibroblasts. Moreover, TNBS administration induced ERK activation in the colon, and the blockade of the MEK/ERK pathway abolished *Il11* expression in the colon.

Conclusions: Stromal fibroblasts produced IL-11 in the colon of TNBS-treated mice in a MEK/ERK-dependent manner.

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Introduction

Crohn's disease is a relapsing inflammatory disease that affects mucosa, sub-mucosa, and muscular and connective tissues of the gastrointestinal tract.[1, 2] Multiple factors, such as genetic factors, host immune system, oxidative stress, and environmental factors, are known to be involved in the development of Crohn's disease.[1, 2] However, the detailed molecular mechanisms of how these factors contribute to Crohn's disease are not fully understood. Trinitrobenzene sulfonic acid (TNBS) -induced colitis is one of the most popular murine models used to investigate such mechanisms.[3–5] TNBS is a contact-sensitizing agent that stimulates a $CD4^+$ T cell-mediated delayed-type hypersensitivity response to 2,4,6-trinitrophenyl hapten-modified self-antigens. Intrarectal administration of TNBS to sensitized mice results in body weight loss and severe epithelial damage of the colon. Moreover, a previous study reported that the administration of interleukin (IL)-11 attenuates TNBS-induced colitis in rats.[6] However, it remains unclear whether *Il11* expression is induced in TNBS-treated mice.

IL-11 is a member of the IL-6 family and plays a role in pleiotropic functions such as inflammation, hematopoiesis, osteogenesis, tissue repair, fertility, and tumor development.[7, 8] The IL-11 receptor (IL-11R) is composed of IL-11R α and gp130 that bind to IL-11 and transmit signals to the nucleus via Janus kinase (JAK) activation.[9, 10] The activation of the signaling pathway induces the expression of various target genes associated with cell proliferation and suppression of apoptosis. We previously reported that reactive oxygen species and an electrophile named 1,2-naphthoquinone induce IL-11 production, thereby promoting the tissue repair of the liver and intestines.[11, 12] Although various types of cells, including stromal cells, hematopoietic cells, and epithelial cells, have been reported to produce IL-11 in response to different stimuli, the cellular sources of IL-11 *in vivo* are not fully understood.[13–16]

In the present study, we found that *Il11* expression was increased in the colon of TNBS-treated mice. Moreover, immunohistochemistry (IHC) revealed that IL-11-producing ($IL\text{-}11^+$) cells expressed vimentin and podoplanin but not express α-smooth muscle actin (α-SMA), suggesting that $IL\text{-}11^+$ cells were stromal fibroblasts and not myofibroblasts. Furthermore, we found that the blockade of the mitogen-activated protein kinase/ERK kinase (MEK)/ERK pathway reduced *Il11* expression in the colon of TNBS-treated mice. Thus, the activation of the MEK/ERK pathway induced IL-11 production in the colon of TNBS-treated mice.

Methods

Reagents

TNBS (Sigma-Aldrich) and trametinib (LC Laboratories) were obtained from the indicated sources.[17, 18] In this study, the following antibodies and reagents were obtained from the indicated sources: anti-IL-11 (in house),[19] anti-phospho-ERK (4370, CST), anti-CD45 (13917, CST), anti-podoplanin (127403, BioLegend), anti- α-SMA (ab5694, Abcam), anti-collagen IV (ab6586, Abcam), anti-E-cadherin (560062, BD Biosciences), anti-vimentin (9856, CST), ImmPRESS® VR anti-rabbit IgG HRP polymer detection kit (MP-6401, Vector Laboratories), biotinylated anti-rat IgG antibody (BA-4001, Vector Laboratories), and streptavidin/HRP (E0397, DAKO). Furthermore, Alexa Fluor 594-conjugated donkey anti-rat IgG (A21209), Alexa Fluor 647-conjugated donkey anti-rabbit IgG (A31573), and Alexa Fluor 647-conjugated streptavidin (S21374) were purchased from Invitrogen.

The hybridoma cell line (1D11), which produces neutralizing antibodies against all transforming growth factor (TGF)-β isoforms (β1, β2, and β3), was purchased from ATCC,[20] and anti-TGF-β antibody was produced in house. Control mouse IgGs were then purchased from Sigma-Aldrich (I5381).

Mice

C57BL/6 mice were purchased from Japan-SLC. All animals were housed and maintained under specific pathogen-free conditions in the animal facility at Faculty of Medicine, Toho University.

Ethics statement

The animal study was reviewed and approved by Toho University Animal Care and User Committee (No. 19-51-414).

Induction of TNBS-induced colitis

TNBS-induced colitis was induced according to a standard method with minor modifications[3, 4] Eight- to ten-

week-old C57BL/6 mice were sensitized with 100 μl of 1% TNBS in acetone and olive oil was applied on the skin on day-7. Mice were deprived of food for 24 hours on day-1, and then intrarectally administered with 100 μl of 4% TNBS in 40% ethanol on day 0. To ensure distribution of TNBS within the entire colon and rectum, we held the mice in a vertical position for 1 min after intrarectal instillation.

To neutralize TGF-β in TNBS-treated mice, we intraperitoneally injected mice with anti-TGF-β antibody or control mouse IgGs (5 mg/kg) on day 1 and day 0 after TNBS treatment. To inhibit ERK activation, a MEK inhibitor, trametinib (2 mg/kg) (6 and 24 hours) was administered by gavage into TNBS-treated mice at the indicated times just before sacrificing them.

Quantitative PCR (qPCR) Assays

Total RNAs were extracted from the indicated tissues of mice using Sepasol II Super (Nacalai Tesque), and cDNAs were synthesized employing the Revertra Ace qPCR RT Kit (Toyobo). Quantitative polymerase chain reaction (qPCR) analysis was performed using the 7500 Real-Time PCR detection system with Fast SYBR® Green Master Mix (Thermo Fisher Scientific) with the 7500 SDS software (Thermo Fisher Scientific). The relative quantification of mRNA analyses was performed using the ΔΔCT method with murine *Hprt* acting as an internal control gene. In this study, the following primers were used (forward and reverse primers, respectively): *Foxp3*, 5′-CCCATCCCCAGGAGTCTTG-3′ and 5′-ACCATGACTAGGGGCACTGTA-3′; *Hprt*, 5′-AACAAAGTCTGGCCTGTATCCAA-3′ and 5′-GCAGTACAGCCCCAAAATGG-3′; *Il6*, 5′-GTATGAACAACGATGATGCACTTG-3′ and 5′-ATGGTACTCCAGAAGACCAGAGGA-3′; *Il11*, 5′-CTGCACAGATGAGAGACAAATTCC-3′ and 5′-GAAGCTGCAAAGATCCCAATG-3′; *Tgfb1*, 5′-TTGCTTCAGCTCCACAGAGA-3′ and 5′-TGGTTGTAGAGGGCAAGGAC-3′; *Tgfb2*, 5′-CTTCGACGTGACAGACGCT-3′ and 5′-GCAGGGGCAGTGTAAACTTATT-3′; *Tgfb3*, 5′-CAGGCCAGGGCAGTCAGAG-3′ and 5′-ATTTCCAGCCTAGATCCTGCC-3′.

Immunohistochemistry

Tissues were fixed in 10% formalin and embedded in paraffin blocks. Paraffin-embedded colonic sections were used for H&E staining, and immunohistochemical and immunofluorescence analyses. For IHC, paraffin-embedded sections were treated with Instant Citrate Buffer Solution (RM-102C, LSI Medicine) or Target Retrieval Solution (S 1699, Dako), as appropriate, to retrieve the antigen. Next, tissue sections were stained with appropriate antibodies, followed by visualization with Alexa-conjugated secondary antibodies or biotin-conjugated secondary antibodies and subsequently with Streptavidin-HRP. The endogenous biotin was blocked using an avidin/biotin blocking kit (SP-2001, Vector).

For fluorescent imaging analyses, tissue sections were preincubated with MaxBlock™ Autofluorescence Reducing Kit (MaxVision Biosciences) according to the manufacturer's instructions. After blocking, tissue sections were stained with the indicated antibodies as described above.

Images were then obtained with an all-in-one microscope (BZ-X700, Keyence) and analyzed with BZ-X Analyzer (Keyence) software. Confocal microscopy was performed using an LSM 880 (Zeiss), and images were processed and analyzed using ZEN software (Zeiss).

Statistical analysis

Statistical significance was determined using the unpaired two-tailed Student's *t*-test or one-way ANOVA with Tukey's post-hoc test. $^*p<0.05$ was considered to be statistically significant. All statistical analyses were performed with GraphPad Prism 8 software (GraphPad Software).

Results

***Il11* expression is increased in TNBS-induced colitis**

To induce TNBS-induced colitis in mice, we sensitized wild-type C57BL/6 mice with TNBS on the dorsal skin, and the sensitized mice were intrarectally administered TNBS 1 week after sensitization (Fig. 1A). TNBS administration induced body weight loss and severe epithelial damage of the colon of mice (Fig. 1B, C). Under these experimental conditions, we found that *Il6* and *Il11* expressions were increased in the colon of mice after TNBS treatment (Fig. 1D). These results indicate that *Il11* expression was increased in TNBS-induced colitis.

IL-11$^+$ cells express a stromal cell marker in the colon of TNBS-treated mice

To characterize IL-11-producing (IL-11$^+$) cells *in vivo*, we stained colonic tissues with anti-mouse IL-11 antibody.[19] We found that a number of IL-11$^+$ cells appeared in subepithelial tissues in the colon of TNBS-treated mice; however, they were not detected in vehicle-treated control mice (Fig. 2A). To further characterize IL-11$^+$ cells in the colon of TNBS-treated mice, we analyzed the expression of lineage-specific markers in IL-11$^+$ cells. IL-11$^+$ cells expressed podoplanin (a stromal cell marker) but not express

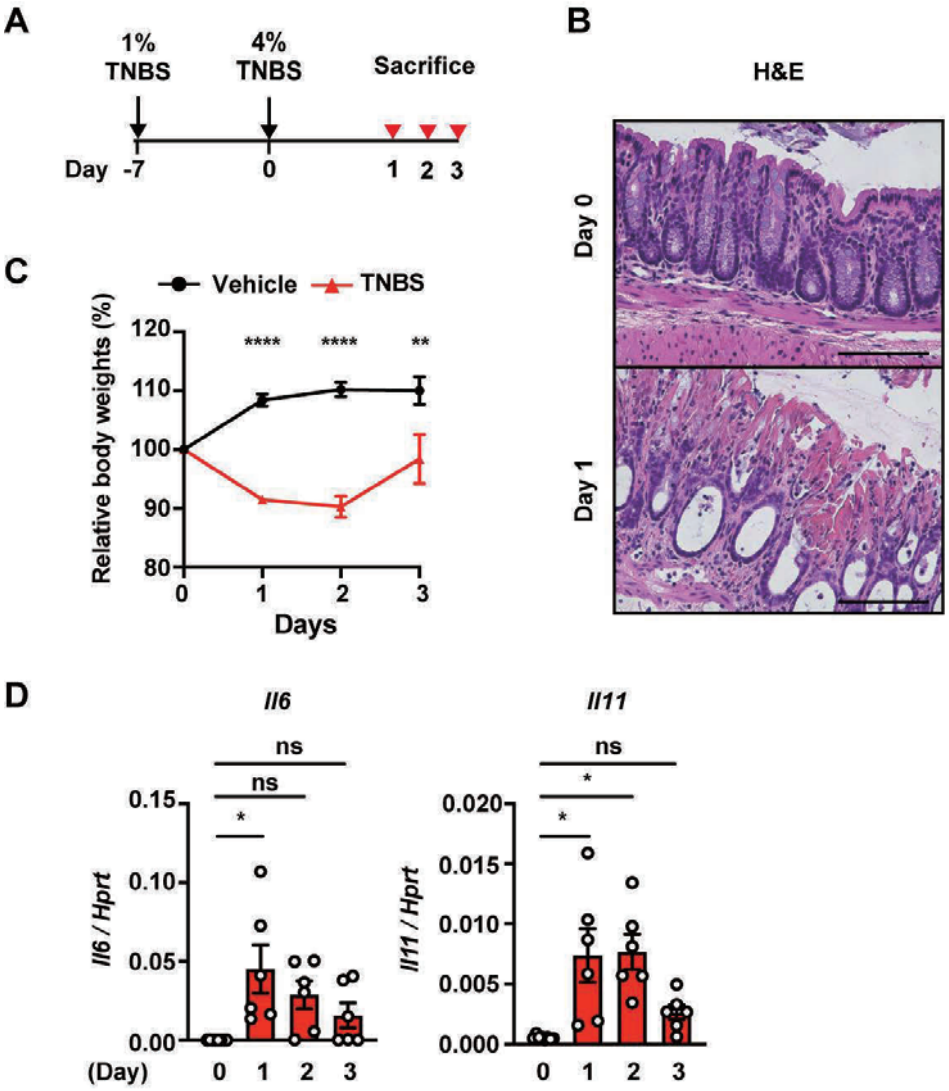

Fig. 1 *Il11* expression is elevated in the colon of TNBS-treated mice

(A) Protocol for induction of TNBS-induced colitis in mice. Mice were sensitized with 1% TNBS on day-7 and intrarectally administered with 4% TNBS on day 0 following one-day starvation. Mice were sacrificed on the indicated days after intrarectal administration.

(B) Colonic sections were stained with hematoxylin & eosin (H&E). Representative images of the colon of mice on day 0 or day 1 after rectal challenge with TNBS (n = 6-7 mice). Scale = 100 μm.

(C) The average body weight is shown as a percentage relative to the initial value following challenge with ethanol or TNBS. Results are mean ± SEM (n = 10 mice). Statistical significance was determined by the two-tailed unpaired Student's *t*-test. ** $p < 0.01$, **** $p < 0.0001$.

(D) *Il11* and *Il6* mRNA expressions in the colon of TNBS-treated mice, determined by qPCR, at the indicated days after TNBS challenge. Results are mean ± SE (n = 6-8 mice). Statistical significance was determined by one-way ANOVA with Tukey's post-hoc test. * $p < 0.05$; ns, not significant.

CD45 (a hematopoietic cell marker) or E-cadherin (an epithelial cell marker) (Fig. 2B-D), which suggests that IL-11$^+$ cells were stromal cells.

IL-11$^+$ cells are stromal fibroblasts but not myofibroblasts in TNBS-induced colitis

Stromal cells in the colon comprise endothelial cells, fibroblasts, and α-SMA-positive myofibroblasts. Stromal

cells express several types of collagen.[21, 22] To further characterize IL-11$^+$ cells, we examined the expression of stromal cell markers. IL-11$^+$ cells expressed vimentin (a fibroblast marker) and collagen IV but did not express α-SMA (a myofibroblast marker) or CD31 (an endothelial cell marker) (Fig. 3A-D), suggesting that IL-11$^+$ cells were fibroblasts and not myofibroblasts or endothelial cells.

Blockade of the MEK/ERK pathway downregulates *Il11* expression in the colon of TNBS-treated mice

Previous studies have reported that TGF-β stimulates IL-11 production in human subepithelial myofibroblasts *in vitro*.[23, 24] To investigate the molecular mechanisms underlying *Il11* expression in TNBS-induced colitis, we examined *Tgfb* mRNA expression in the colon of TNBS-treated mice. We found that TNBS-treatment increased the expression of *Tgfb1* mRNA and not of *Tgfb2* or *Tgfb3* mRNA (Fig. 4A). The administration of neutralizing antibodies against TGF-β did not inhibit the increase in *Il11* expression; however, it downregulated *Foxp3* expression (Fig. 4B). These results suggested that the TGF-β signal was not involved in TNBS-induced IL-11 production.

We previously reported that oxidative stress induces *Il11* expression in a MEK/ERK-dependent manner.[11] To test whether this pathway is involved in the induction of *Il11* expression in TNBS-induced colitis, we first examined the appearance of phospho-ERK (pERK) -positive cells (a hallmark of ERK activation) in the colon of TNBS-treated mice. The number of pERK-positive cells increased in both epithelial and stromal cells in the colon of TNBS-treated mice compared to those in vehicle-treated mice (Fig. 4C, left and middle panels). However, the administration of a MEK inhibitor, trametinib, abolished pERK-positive cells in the colon of TNBS-treated mice (Fig. 4C, right panels). Note that TNBS-induced increase in *Il11* expression was inhibited in the colon of trametinib-treated mice compared to that of vehicle-treated_mice (Fig. 4D). However, TNBS-induced body weight loss was not attenuated in trametinib-treated mice compared to that of vehicle-treated mice (Fig. 4E), suggesting that the decrease in *Il11* expression was not caused by the attenuation of TNBS-induced colitis. To summarize, *Il11* expression might be induced by the MEK-ERK pathway in TNBS-induced colitis.

Discussion

In the present study, we found that IL-11 expression was induced in the colon of TNBS-treated mice. IL-11$^+$ cells expressed podoplanin, vimentin, and collagen IV and not α-SMA, suggesting that IL-11$^+$ cells were stromal fibroblasts and not myofibroblasts. Moreover, the blockade of the MEK/ERK pathway diminished *Il11* expression in the colon of TNBS-treated mice, suggesting that the MEK/ERK pathway contributed to *Il11* expression in TNBS-induced colitis.

Previous studies have reported that TGF-β induces the production of IL-11 by myofibroblasts, [23, 24] and is also involved in the conversion of fibroblasts to myofibroblasts.[25, 26] Moreover, α-SMA$^+$ smooth muscle cells produce IL-11 under certain conditions.[27] These results suggest that an intimate relationship between myofibroblasts and TGF-β-induced IL-11 production. In sharp contrast, the present study revealed that IL-11$^+$ cells expressed vimentin but did not express α-SMA, suggesting that IL-11$^+$ cells were fibroblasts and not myofibroblasts at least under our experimental conditions. In this respect, we have very recently generated *Il11*-enhanced green fluorescence protein (*Egfp*) reporter mice and are currently investigating surface markers of IL-11$^+$ (EGFP$^+$) cells in the colon of acute murine colitis and colitis-associated colorectal cancer models. Hence the detailed analysis of IL-11$^+$ cells using *Il11-Egfp* reporter mice may provide better understanding of IL-11$^+$ cells under various pathological conditions.

The neutralization of the TGF-β signal significantly decreased the expression of *Foxp3*, a target gene induced by TGF-β in the colon of TNBS-treated mice,[28, 29] whereas the expression of *Il11* was not reduced. These findings suggest that the TGF-β signal did not appear to contribute to *Il11* induction in TNBS-induced colitis, at least under our experimental conditions. In contrast, the blockade of the MEK/ERK pathway decreased *Il11* expression in the colon of TNBS-treated mice. Upon TNBS treatment, pERK-positive stromal cells were observed in the subepithelial tissues of the inflamed colon. Moreover, trametinib treatment significantly decreased the numbers of pERK-positive cells along with downregulation of *Il11* expression. The priming and subsequent induction of pathogenic T cells are required for inducing TNBS-induced colitis.[30] Thus, we cannot formally exclude the possibility that blockade of the MEK/ERK pathway might attenuate TNBS-induced colitis and decrease in *Il11* expression by suppressing activation of pathogenic T cells. However, trametinib did not attenuate body weight loss in TNBS-treated mice, suggesting that the downregulation of *Il11* expression was not attributed to the attenuation of colitis.

Thus, *Il11* expression might be directly regulated by the MEK/ERK pathway in TNBS-induced colitis.

Although the numbers of pERK-positive epithelial cells increased after TNBS treatment, immunohistochemical analysis revealed that epithelial cells were not positive for IL-11 in the colon. These results suggest that the MEK/ERK pathway-dependent IL-11 production might be cell type-specific. Consistent with these results, hepatocytes, but not Kupffer cells produce IL-11 in acetaminophen-induced liver injury.[11] The mechanisms underlying cell type-specific regulation of IL-11 production will be investigated in the future.

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Author contributions: W.T., T.N., and H.N. designed the study; W. T., T.N., M.K., and H.N. performed the experiments and analyzed the data; Y.D. and H.Y. provided critical reagents; T.M. and C.N. supervised the experiments; W.T., T.N., and H.N. wrote the manuscript.

Conflicts of interest: None declared.

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