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Toll-like receptor 4 regulates intestinal fibrosis via cytokine expression and epithelial-mesenchymal transition

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Abstract

Toll-like receptor 4 regulates intestinal fibrosis via cytokine expression and epithelial-mesenchymal transition

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Intestinal fibrosis induced by chronic and recurrent colitis, which is exacerbated by bowel stenosis, stricture, and obstruction, is challenging to treat.

A dextran sulfate sodium (DSS)-induced murine fibrosis model was the most convenient and popular experimental model but the duration and frequency of DSS administration were not well known. We established an experimental protocol for DSS-induced murine fibrosis model. We compared the severity of fibrosis between one and three cycles of 6-day-DSS treatment. A cycle of DSS sufficiently provoked colonic fibrosis.

Toll-like receptor 4 (TLR4) stimulates innate and acquired immunity in response to specific microbial components, but the role of TLR4 in intestinal fibrosis is largely unknown. We investigated its role in intestinal fibrosis using not only a murine fibrosis model but also human myofibroblasts and intestinal epithelial cells.

Colon fibrosis was induced in TLR4-deficient ($TLR4^{-/-}$) mice and its wild-type counterparts with 3% dextran sulfate sodium. Absence of TLR4 gene attenuated chronic inflammation and colonic macrophages infiltration; intestinal fibrosis and collagen deposition were suppressed. Also, the production of transforming growth factor-β, tumor necrosis factor-α, and interleukin-12p40 was reduced in TLR4-deficient peritoneal macrophages.

TLR4 was silenced in CCD-18Co cells by small interfering RNA (siRNA), and matrix metalloproteinase-1, tissue inhibitor of metalloproteinase, and collagen α1 expression was evaluated. Role of TLR4 in epithelial-mesenchymal transition (EMT) was evaluated in HCT116 cells. Suppression of TLR4 transcription by siRNAs affected myofibroblasts activity, collagen synthesis, and EMT in the human cancer cell line.

Thus, we suggest that TLR4 can be an essential mediator in intestinal chronic

inflammation and fibrosis, indicating that TLR4 signaling is a potential therapeutic target for intestinal fibrosis. And our protocol of DSS administration can help other scientists to provoke intestinal fibrosis by DSS treatment effectively.

Keyword : Toll-Like Receptor 4, Colitis, Fibrosis, Epithelial-Mesenchymal Transition
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Introduction

Intestinal fibrosis is a chronic progressive process involving excessive deposition of extracellular matrix (ECM) components in the intestinal wall. Fibrosis develops as a result of failed wound healing in the mucosa during inflammatory responses. Fibrosis is not the same as chronic inflammation. Unlike inflammation, fibrosis is accompanied by failure of mucosal healing and excessive ECM deposition. Moreover, in the fibrosis process, special reactions such as epithelial-mesenchymal transition (EMT) and endothelial-mesenchymal transition occur (1).

A variety of cells are involved in inflammation and fibrosis processes. Multiple immune cells, including macrophages and neutrophils contribute to tissue injury by releasing oxygen radicals, cathepsin, and matrix metalloproteinase (MMP)(2). As a counterpart, myofibroblasts, activated by inflammation, contract wound area, and produce ECM components. In chronic and recurrent inflammation, the inflammatory cascade is activated for extended time period and the negative feedback, which terminates proliferative and fibrotic response, is not enough to regulate the physiological balance between fibrosis and regeneration.

EMT is a physiological process responsible for not only intestinal fibrosis but also embryogenesis, organ development, and carcinogenesis(3, 4). During EMT, existing epithelial cells lose their polarity and become mesenchymal cells both functionally and morphologically. Interestingly, these cells can possess both epithelial markers such as E-cadherin or cytokeratin 8 and 20, and mesenchymal markers, such as α-smooth muscle actin (α-SMA) or vimentin(5). Thus, fibrosis results in the collapse of normal tissue architecture, permanent scarring, structural change, and organ malfunction(6).

Intestinal fibrosis can occur as a long-term complication of numerous diseases such as inflammatory bowel disease (IBD), radiation enterocolitis, chronic ischemic enterocolitis, collagenous colitis, eosinophilic enteropathy, drug-induced enteropathy, and cystic fibrosis(7). In IBD, it is manifests as complications, including stenosis, stricture, and obstruction. Bowel stenosis and stricture provoke abnormal bowel movement and result in abdominal pain, nausea, vomiting, anorexia, and weight loss in patients with IBD. About 50% of patients with Crohn's disease (CD) suffered from serious intestinal fibrosis that required surgery during a 10-year

disease course(8). Even after surgery, the relapse rate of intestinal fibrosis in patients with CD was too high to ignore. Patients with ulcerative colitis (UC) experience fewer intestinal fibrosis-related complications than patients with CD. The prevalence of stricture in patients with UC was reported various outcomes which were 11.2%(9), 5%(10), or 3.2%(11), respectively. Therefore, the early detection and prevention of intestinal fibrosis are important to improve prognosis of patients with IBD.

Clinically-used anti-inflammatory agents to treat IBD cannot prevent the intestinal fibrosis once excessive ECM deposition process begins(12). Although some studies reported infliximab to be a treatment for small bowel stenosis in patients with CD, the sample size was small and the treatment was far less effective than endoscopic treatment or surgery(13, 14). Moreover, patients with fibrostenotic CD, even subclinical, were more likely to be non-response to infliximab treatment, and they had to eventually undergo surgery(15). Therefore, development of new anti-fibrotic agents for IBD is urgently required.

There were murine experimental models for intestinal fibrosis and dextran sulfate sodium (DSS)-induced fibrosis model was most widely used because of convenience and high reproducibility(16). DSS induces chemical damage toward intestinal epithelial cells and disrupts intestinal mucosal barrier. However, main disadvantage of the DSS-induced fibrosis model is unrevealed relevance of human intestinal fibrosis. DSS-induced fibrosis model is not standardized and the duration and frequency of DSS administration vary from study to study(17, 18). Therefore, we compared the severity of fibrosis between the single cycle of DSS administration and repeated cycles of DSS administration.

Damage-associated molecular patterns (DAMPs) are host-derived molecules released by damaged or dead cells(19, 20). Pathogen-associated molecular patterns (PAMPs) are microbe-derived molecular compounds, including lipopolysaccharide (LPS), flagellins, fungal glucans, bacterial DNA, and double-strand RNA(21). DAMPs and PAMPs provoke inflammatory responses through activating pattern recognition receptors such as Toll-like receptors (TLRs). TLRs consist of TLR1 to TLR9 and induce the activation and maturation of innate and acquired immunity(22). TLR4 is one of the TLRs and can be activated by Gram-negative-derived exogenous LPS and endogenous DAMP ligands(23). TLR4 is expressed on intestinal epithelial cells (IECs), macrophages, and dendritic cells. The TLR4 signaling pathways mainly

consist of myeloid differentiation primary response gene 88 (MyD88)-dependent pathway which stimulates nuclear factor-κB (NF-κB), members of the mitogen-activated protein kinase (MAPK) pathway, interleukin (IL)-1 pathway, and produces multiple pro-inflammatory cytokines(24). In addition, TLR4 establishes MyD88-independent pathway responsible for late activation of NF-κB(25). Although its contribution of intestinal inflammation is well known, the role of TLR4 in intestinal fibrosis remains unclear. Therefore, we investigated the role of TLR4 in intestinal inflammation and thereby the importance in intestinal inflammation as well as fibrosis.

Methods and Materials

Mice

TLR4$^{-/-}$ mice with C57BL/6 background aged 7 to 8 weeks were used. Age- and gender-matched wild-type mice (C57/6NCrljBgi), purchased from Orient (Seongnam, Korea), were used as control. The mice were maintained under specific pathogen-free housing with standard conditions of humidity, temperature, and a light/dark cycle in the Laboratory of Experimental Animal Research of SMG-SNU Boramae Medical Center. Mice were euthanized with isoflurane inhalation either on day or early, when mice exhibited severe body weight loss (25% of their pre-experimental body weight), according to the protocol.

Induction and evaluation of dextran sulfate sodium (DSS)-induced colon fibrosis

DSS (MP Biochemical, Irvine, CA, USA), a water-soluble, negatively charged sulfated polysaccharide, can cause intestinal inflammation. To examine the effect of DSS administration on intestinal fibrosis, a single DSS group was treated by a single cycle of 6-day 3% DSS and a repeated DSS group by 3 cycles of 6-day 3% DSS. Bodyweight was recorded at least 2 days per week throughout the experiment. Weight loss was calculated as the percent difference between the original weight (day 0) and the weight on any particular day.

Mice were sacrificed on day 60 from the time of the first DSS administration, and the severity of colitis and colonic fibrosis was evaluated. Colon lengths were measured for each mouse. Colon tissue samples were obtained under anesthesia, fixed in 10% buffered formalin, and embedded in paraffin. Sections were stained with H&E. The severity of colitis was scored by two examiners not involving experimental procedures as described previously(26). In brief, three independent parameters were measure: inflammation severity (0, none; 1, mild; 2, moderate; 3, severe), inflammation extent (0, none; 1, mucosa; 2, mucosa and submucosa; 3, transmural), the extent of crypt damage 0, none; 1, damage to the basal one-third portion; 2, damage to the basal two-thirds portion; 3, damage to the entire crypt with surface epithelium intact; 4, erosion). Sum of these scores were quantified as to the percentage of tissue involvement (0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; 4, 76-

100%). To identify intestinal fibrosis, colon tissue samples were stained with Masson's Trichrome (MT). The difference in location and extent of positive area in MT-stained tissues were analyzed under an optical microscope and measured as the percent positive area using ImageJ software (available at http://rsbweb.nih.gov/ij/). The average of three regions from each slide was considered.

To induce colon fibrosis in TLR4$^{-/-}$ mice and their counterparts, 3% DSS dissolved in drinking water was administrated for six days(27). Five mice were randomly assigned to each group. It has already been proven that control group treated with normal water without DSS did not develop chronic fibrotic colitis(28, 29), negative control groups were not assigned due to ethical concerns. Mice were assessed every five days for behavior, water/chow consumption, body weight, stool consistency, and evidence of gross hematochezia. disease activity index (DAI) for assessment of colitis severity was determined as the sum of parameters, consisting of the changes in body weight loss, stool consistency, and presence of rectal bleeding(30). On day 35, colon tissue samples were obtained, fixed in 10% buffered formalin, and embedded in paraffin. Sections were stained with H&E and scored blindly for inflammation severity, inflammation extent, the extent of crypt damage, and the percentage of tissue involvement. After MT staining, the severity of fibrosis was scored based on the area of MT staining. The layer of the colon was divided into the mucosa, muscularis mucosae, submucosa, and muscularis externae, and the severity of collagen deposition was assessed through a relative proportion of MT staining.

Immunohistochemical staining of mouse colon tissues

Immunohistochemistry for F4/80 and α-SMA, markers for macrophages and myofibroblasts, respectively, was performed on colon tissue samples as previously described(31). Thin slices obtained from the formalin-fixed paraffin-embedded colon tissue blocks were stained. Paraffin wax was removed from the samples and the samples were rehydrated. The slides were then immersed three times in a citrate buffer (pH 6.0), maintained just below the boiling temperature for 5 minutes each, and then cooled for 20 min. After washing with Tris-buffered saline (TBS) containing 0.025% Triton X-100, the slides were stained with primary F4/80 antibody (Abcam, Cambridge, MA, USA) or α-SMA antibody (Abcam). The slides were washed with TBS containing 0.025% Triton X-100 and incubated with a

secondary antibody. After developing with a chromogen, the slides were counterstained using hematoxylin. Immunoreactivity of F4/80 was measured as the percent positive area using ImageJ software program. More than five areas encompassing both mucosa and submucosa were evaluated for each slide.

Isolation of murine peritoneal macrophages

We extracted murine peritoneal macrophages as described previously(32-34). Briefly, mice were intraperitoneally injected with 2mL of 4% thioglycolate (Sigma-Aldrich, St. Louis, MO, USA). After four days from the injection, 10ml of Hank's balanced salt solution (Corning Cellgro, Manassas, VA, USA) was intraperitoneally injected at the site and extracted with a syringe. The collected peritoneal fluid was centrifuged, and cells were resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Carlsbad, CA, USA). After culturing for two hours, cells were washed, and the remaining cells were collected as peritoneal macrophages.

To elucidate mechanisms responsible for intestinal fibrosis, isolated peritoneal macrophages were exposed to bacterial endotoxin, LPS. $TLR4^{-/-}$ peritoneal macrophages and their wild-type counterparts were pretreated with sulforaphane and challenged with 10ng/mL LPS (Sigma-Aldrich). After four hours of LPS stimulation, the transcription levels of inflammatory cytokines were measured through real-time reverse transcription-polymerase chain reaction (RT-PCR). Studies using peritoneal macrophages were repeated three times.

Culture and preparation of human cell lines

The human colon myofibroblasts, CCD-18Co cells (Korean Cell Line Bank 21459, Seoul, Korea) were cultured in a Dulbecco's modified Eagle medium (DMEM)/Earle's balanced salt solution (EBSS) medium supplied with 10% fetal bovine serum (FBS), 1mM non-essential amino acids, 1mM sodium pyruvate, and 2mM sodium bicarbonate without antibiotics. HCT116 human IECs (Korean Cell Line Bank 10247, Seoul, Korea) were cultured in the RPMI-1640 medium containing 5% FBS. All cells were incubated at 37 °C in a humidified 5% CO_2 incubator.

TLR4 was silenced by transfecting CCD-18Co cells or HCT116 cells with small interfering RNAs (siRNAs) specific for human TLR4 (Santa Cruz Biotechnology,

Santa Cruz, CA, USA). TLR4 siRNAs were transfected using WelFect-EX™ reagents (Welgene, Daegu, Korea) as per the manufacturer's instructions. Scrambled siRNAs (Santa Cruz Biotechnology) were used as controls. After transfections, CCD-18Co cells were treated with or without 10ng/mL LPS for four hours. HCT116 cells also were treated with or without 10ng/mL LPS after transfections. Studies were repeated three times.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

The role of TLR4 in LPS-treated murine peritoneal macrophages was assessed by evaluating the expression of tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and IL-12p40 by real-time reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from murine peritoneal macrophages, and complementary DNA (cDNA) was synthesized using amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT, Texas, USA) from total RNA isolated from peritoneal macrophages. The primers used were as follows: TNF-α, (5′-CAT CTT CTA AAA ATC GAG TGA CAA-3′ and 5′-TGG GAG TAG ACA AGG TAC AAC CC-3′); IL-12p40, (5′-GGA AGC ACG GCA GCA GAA TA-3′ and 5′-AAC TTG AGG GAG AAG TAG GAA TGG-3′); TGF-β, (5′-TGG AAA TCA ACG GGA TCA G-3′ and 5′-GTC CAG GCT CCA AAT ATA GG-3′). The relative changes in gene expression were calculated by normalizing the expression level of the target gene to the level of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Similarly, the expressions of MMP-1, tissue inhibitor of metalloproteinase (TIMP), and collagen α1 in CCD-18Co cells treated with TLR4 siRNA, and that of hypoxia-inducible factor-1α (HIF-1α) and vimentin in HCT116 cells were also evaluated by real-time RT-PCR. The primers were as follows: MMP-1, (5'- GGT GAT GAA GCA GCC CAG-3' and 3'- CAG TAG AAT GGG AGA GTC-5'); TIMP, (5'- ART CAA CSA GAC CAC CTT ATA CCA-3' and 3'- ASC TGR TCC GTC CAC AAR CA-5'); collagen α1, (5′-GAA CGC GTG TCA TCC CTT GT-3′ and 3′-GAA CGA GGT AGT CTT TCA GCA ACA-5′); HIF-1α, (5'- CAT CTC CAT CTC CTA CCC ACA T-3' and 3'- ACT CCT TTT CCT GCT CTG TTT G-5'); vimentin, (5'- GAA GAG AAC TTT GCC GTT GAA G-3' and 3'- ACG AAG GTG ACG AGC CAT T-5').

Statistical analysis

Data are expressed as mean and standard error of the mean (SEM). Non-parametric Mann-Whitney test was performed to compare values between the groups using SPSS 25 statistical software (SPSS, Chicago, IL, USA). The *P*-values less than 0.05 were considered statistically significant.

Ethical considerations

All procedures were approved by the Animal Care Committee at SMG-SNU Boramae Medical Center (IACUC No.2016-0017). All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health.

Results

The duration of DSS administration is not important to induce intestinal fibrosis.

To examine the effect of the frequency and duration of DSS administration, wild-type mice were treated with 3% DSS for 6 days once or three times. As shown in Figure 1A, mice lose their weight after DSS administration during the experiment. The colon length was slightly shorter in the repeated DSS group than in the single DSS group but statistically insignificant (Figure 1B). The transcription levels of TGF-β, TNF-α, and IL-12p40 were not significantly increased in the repeated DSS group as compared to the single DSS group (Figure 1C). When the severity of colitis was evaluated, more severe colitis was discovered in the repeated DSS group, but it was not significantly different from that in the single DSS group (Figure 1D). Also, there was no significant difference in collagen deposition between the two groups (Figure 1E). The results imply that chronic colitis is different from intestinal fibrosis and a single cycle of DSS treatment is sufficient for the development of intestinal fibrosis.

TLR4-deficiency attenuated the severity of chronic inflammation and fibrosis in murine model of colitis

To evaluate the role of TLR4 in chronic colon inflammation and fibrosis, we studied a DSS-induced murine chronic colitis model. We analyzed phenotypic characteristics of the DSS-administered mice, such as body weight and DAI every five days. Wild-type mice showed significantly greater weight reduction and higher DAI as compared to $TLR4^{-/-}$ mice (Figure 2A and 2B); while the weight of wild-type mice rapidly decreased, $TLR4^{-/-}$ mice did not show definite weight loss. Wild-type mice had higher DAI than $TLR4^{-/-}$ mice from day 5 to 20, and both groups recovered to score zero after day 20. The colon samples from wild-type mice showed higher degree of bowel wall edema and fibrotic changes (Figure 2C). Unlike wild-type mice, solid feces were detected in $TLR4^{-/-}$ mice colon. The colon length of wild-type mice was significantly reduced as compared to that of $TLR4^{-/-}$ mice (Figure 2D). These results indicated that TLR4 deletion alleviated colitis and fibrotic changes, suggesting that it may aggravate chronic colon inflammation and fibrosis.

TLR4 induced collagen deposition in colon submucosa along with colonic infiltration of macrophages and myofibroblasts

The severity of colitis and intestinal inflammation was assessed by histopathological analysis using hematoxylin-eosin (H&E) staining (Figure 3A). Histopathological examination revealed severe inflammation and fibrosis in the colon of wild-type mice exposed to DSS. Distorted crypt architecture, multiple inflammatory cells, and extensive edema in the mucosa and submucosa were detected. In contrast, $TLR4^{-/-}$ mice exposed to DSS showed well-preserved mucosal integrity. Additionally, the histological grades between the two groups were markedly different. MT staining of collagen within the colon tissue revealed suppressed collagen deposition in the submucosa of $TLR4^{-/-}$ mice, and showed severe colon fibrosis in wild-type mice (Figure 3B). We separated mucosa, muscularis mucosae, submucosa, and muscularis externae and examined the severity of fibrosis using the Image J program. $TLR4^{-/-}$ mice showed less severe fibrotic change regardless of the colon layer: significantly more severe fibrosis was observed in submucosa in control than in $TLR4^{-/-}$ mice. Next, to determine macrophage and myofibroblast distribution in the colon, we performed immunohistochemical staining using respective markers, F4/80 and α-SMA (Figure 3C and 3D). We noted that a higher number of F4/80- or α-SMA-positive cells infiltrated the lamina propria in wild-type mice as compared to that in $TLR4^{-/-}$ mice.

Intestinal fibrosis might promote the expression of TNF-α, TGF-β, and IL-12p40 in LPS-treated murine peritoneal macrophages

To assess the role of TLR4 and elucidate the mechanisms responsible for intestinal fibrosis, the transcription levels of TNF-α, TGF-β, and IL-12p40 were investigated in LPS-treated peritoneal macrophages from $TLR4^{-/-}$ and wild-type mice. LPS stimulation resulted in reduced transcription levels of TNF-α, TGF-β, and IL-12p40 in peritoneal macrophages from $TLR4^{-/-}$ mice (Figure 4A, 4B, and 4C). The transcription levels of TNF-α and IL-12p40 in macrophages from LPS-stimulated $TLR4^{-/-}$ and wild-type mice were significantly different. Otherwise, TGF-β expression was not shown any statistical significance.

TLR4 facilitated the expression of TIMP and collagen α1 in human colon myofibroblasts

Intestinal inflammation play a key role in fibrotic process(35). However, recent studies suggest that fibrotic mechanisms can be distinct from intestinal inflammation(36). To confirm the role of TLR4 signaling in colon fibroblast, we investigated the effect of TLR4 silencing with or without LPS stimulation on the activation of myofibroblasts. TLR4 knockdown without LPS stimulation in CCD-18Co cells resulted in increased MMP-1 expression as compared to those transfected with control siRNA (Figure 5A). However, transfection with TLR4 siRNA significantly suppressed the expression of TIMP (Figure 5B), and collagen α1 (figure 5C). When stimulating with LPS in CCD-18Co cells, there was no significant difference in the expression of MMP-1 (Figure 4D). However, similar results were shown in TIMP and collagen α1 expression. (Figure 4E, and 4F). This indicated that TLR4 is an essential for TIMP expression and collagen production in myofibroblasts.

TLR4 modulated EMT in human IECs

Previously, HIF-1 and vimentin have been known as mesenchymal markers(37, 38). Therefore, we investigated whether TLR4 signaling facilitated EMT in IECs. TLR4 silencing with or without LPS stimulation in CCD-18Co cells suppressed the expression of HIF-1 and vimentin; especially, vimentin expression was significantly decreased after administration of TLR4 siRNA and LPS (Figure 6).

Discussion

Our current study is the first to demonstrate that the duration of DSS treatment is irrelevant to development of intestinal fibrosis and TLR4 may have an essential function in not only intestinal inflammation but also intestinal fibrosis. There was no difference in the severity of fibrosis between the single DSS group and the repeated DSS group. Based on this result, DSS was treated only 6 days for intestinal fibrosis. The genetic deletion of TLR4 strongly suppressed colon inflammation and fibrosis in DSS-induced fibrosis model. In LPS-stimulated peritoneal macrophages, the expression of TNF-α, TGF-β, and IL-12p40 was decreased in $TLR4^{-/-}$ mice as compared to that in wild-type mice. The silencing of TLR4 through siRNA transfection weakened the activation of human myofibroblasts and collagen synthesis. In addition, TLR4 might regulate EMT in human IECs. To our knowledge, this is the first study to reveal appropriate model of DSS-induced colon fibrosis and demonstrate the role of TLR4 in colon fibrosis.

DSS is a chemical agent provoking intestinal epithelial damage and is considered useful for acute colitis model(16). However, the detailed pathophysiology of DSS-induced fibrosis was undisclosed. Important factors for the development of colonic fibrosis are excessive ECM production, deposition, and tissue remodeling. Multiple epithelial damages induced by DSS might be insufficient or irrelevant to provoke the fibrosis-specific processes.

TLR4, stimulated by gram-negative bacteria-produced LPS or endogenous ligand-produced by damaged host cells, can facilitate inflammatory responses through the maturation of innate immunity(39, 40). Further, TLR4 has a potential function in IBD development; the frequency of TLR4 Asp299Gly polymorphism was higher in patients with UC, and TLR4 expression was increased in the intestine of patients with active UC(41). In the present study, TLR4 regulated not only chronic colitis but also fibrosis. TLR4 exacerbated collagen deposition in colon wall along with macrophage and myofibroblasts infiltration into colon. Based on these, we believe that TLR4 signaling contributes colon fibrosis as well as chronic inflammation, TLR4 signaling pathway is a potential target for the prevention of colon fibrosis in IBD.

During chronic colitis and tissue injury, macrophages contribute to the

development of intestinal fibrosis(42, 43). M1 macrophages produce pro-inflammatory cytokines such as IL-1β and TNF-α, which start fibrotic processes. M2 macrophages produce pro-fibrotic cytokines such as TGF-β, connective tissue growth factor, platelet-derived growth factor, fibroblast growth factor, and insulin-like growth factor(44). TNF-α from macrophages can facilitate mucosal remodeling and fibrosis(45). TNF-α-stimulated macrophages promote production of TGF-β1 and MMP in myofibroblasts(46). Synergic effect between TNF-α and TGF-β also promote EMT(47). IL-12p40 favors M1 polarization and can induce inflammation and fibrosis(48). However, the role of TLR4 signaling in macrophages remains obscure. In the present study, TLR4 was critical for peritoneal macrophages to activate signaling cascade through TNF-α and IL-12p40 expression. However, there was no significant difference on the expression of TGF-β between peritoneal macrophages from wild-type and $TLR4^{-/-}$ mice. Based on these, we suggest that TLR4 signaling may play a role in colon fibrosis by M1 macrophages rather than M2 macrophages.

The role of TLR4 signaling on the expression of MMPs remains obscure. Previously, TLR4 activation by LPS leads to increased MMP-1 expression in small airway cells and nasal polyp-derived fibroblasts[4,5]. However, a previous study has shown that MMP-2 expression was not affected by LPS-induced TLR4 activation in corneal fibroblasts(49). In our study, knockdown of TLR4 without LPS stimulation resulted in significant suppression of MMP-1 expression in CCD-18Co cells. However, silencing of TLR4 was not associated with the increased expression of MMP-1, suggesting the effect of TLR4 signaling on the expression of MMPs might depend on the cell types or culture environment. Under homeostatic conditions, MMPs are constitutively expressed at a low level and are closely regulated by TIMPs. In damaged tissues, myofibroblasts facilitate TIMP-1 production that inhibits MMPs and may thus cause enhanced deposition of ECM proteins, contributing to intestinal fibrosis(50, 51). Our results demonstrated that silencing of TLR4 was associated with the increased expression of TIMP regardless of LPS stimulation. Based on these results, TLR4 signaling-induced TIMP-1 expression in colon myofibroblasts may play an important role in the process of intestinal fibrosis. More studies are required for demonstrating the role of MMP-1 in intestinal fibrosis.

Studies have reported that mesenchymal stem cells migrate to the damaged

intestine in response to the homing signal generated by chemotactic factors such as TGF-β1(52, 53). However, myofibroblasts may be derived from non-mesenchymal origins, including epithelial cells via EMT. We found reduced number of myofibroblasts in the colon tissues from $TLR4^{-/-}$ mice. Therefore, we further evaluated the effect of TLR4 silencing on the expression of EMT-related genes in HCT116 cells. Our study revealed that TLR4 might promote EMT pathway in HCT116 cells via activation of vimentin. Therefore, we suggest that TLR4 signaling pathway may contribute to EMT, thereby regulating intestinal fibrosis. To our knowledge, this is the first study to demonstrate the role of TLR4 in cell migration associated with intestinal fibrosis.

The present study has some limitations. First, there are murine experiments that are more similar to human intestinal fibrostenosis than the DSS treatment experiment, but only the DSS-induced fibrosis model was performed. SAMP1/Yit mice spontaneously develop chronic terminal ileitis(54). Disease location and perianal disease, and histology resemble human Crohn's disease. However, SAMP1/Yit mice are not commercially available and difficult to breed and induce ileitis. Bacteria-derived components effectively embody human Crohn's disease and fibrostenotic complications(55). This gut microbiota-induced fibrosis model was hard to perform because surgical inoculation of fecal suspension is required. Second, TLR4 was known to be expressed on the cell walls of macrophages, epithelial cells, and myofibroblasts (56-58). However, a difference in TLR4 activation between cell types was not evaluated. In colitis, the mucosal barrier was disrupted, and LPS from enteric bacteria can invade the colon wall layer. TLR4 and LPS interaction in not only intestinal epithelial cells but also intercellular macrophages and myofibroblasts may contribute to colonic fibrosis. Third, MMP-1 and TIMP are enzymes and western blot, enzyme-linked immunospecific assay, and flow cytometry might be appropriate to reflect the protein production(59). However, real-time RT-PCR can measure the exact amount of mRNA product reflecting the expression level of MMP-1 and TIMP. I*n vitro* studies treated scrambled siRNAs for control. If the expression levels of fibrosis or mesenteric markers were evaluated without siRNA treatment, the influence of scrambled siRNAs could be calibrated. Also, the expression of epithelial markers in HCT116 cells treated with TLR4 siRNA can make higher quality results to demonstrate the role of TLR4 in EMT.

The findings of the present study clarify two enigmatic points. DSS-induced fibrosis model was established; a single cycle of DSS is sufficient for murine colonic fibrosis. TLR4 might act as a key mediator in intestinal fibrosis by regulating intestinal epithelial cells, macrophages, and myofibroblasts (Figure 7). Therefore, TLR4 signaling pathway may be a potential treatment target for intestinal fibrosis and help patients with chronic colitis prevent or overcome fibrostenosis.

Bibliography

1. Bettenworth D, Rieder F. Pathogenesis of intestinal fibrosis in inflammatory bowel disease and perspectives for therapeutic implication, Digestive diseases 2017; 35: 25-31.
2. Rieder F, Brenmoehl J, Leeb S, Scholmerich J, Rogler G. Wound healing and fibrosis in intestinal disease, Gut 2007; 56: 130-139.
3. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition, The Journal of clinical investigation 2009; 119: 1420-1428.
4. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis, The Journal of clinical investigation 2003; 112: 1776-1784.
5. Thiery JP, Sleeman JP. Complex networks orchestrate epithelial–mesenchymal transitions, Nature reviews Molecular cell biology 2006; 7: 131-142.
6. Latella G, Di Gregorio J, Flati V, Rieder F, Lawrance IC. Mechanisms of initiation and progression of intestinal fibrosis in IBD, Scandinavian journal of gastroenterology 2015; 50: 53-65.
7. Speca S, Giusti I, Rieder F, Latella G. Cellular and molecular mechanisms of intestinal fibrosis, World journal of gastroenterology: WJG 2012; 18: 3635.
8. Latella G, Rieder F. Intestinal fibrosis: ready to be reversed, Current opinion in gastroenterology 2017; 33: 239-245.
9. De Dombal FT, Watts JM, Watkinson G, Goligher JC. Local complications of ulcerative colitis: stricture, pseudopolyposis, and carcinoma of colon and rectum, Br Med J 1966; 1: 1442-1447.
10. Gumaste V, Sachar DB, Greenstein AJ. Benign and malignant colorectal strictures in ulcerative colitis, Gut 1992; 33: 938-941.
11. Lashner BA, Turner BC, Bostwick DG, Frank PH, Hanauer SB. Dysplasia and cancer complicating strictures in ulcerative colitis, Dig Dis Sci 1990; 35: 349-352.
12. Latella G, Sferra R, Speca S, Vetuschi A, Gaudio E. Can we prevent, reduce or reverse intestinal fibrosis in IBD, Eur Rev Med Pharmacol Sci 2013; 17:

1283-1304.

13. PELLETIER AL, Kalisazan B, Wienckiewicz J, Bouarioua N, SOULÉ JC. Infliximab treatment for symptomatic Crohn's disease strictures, Alimentary pharmacology & therapeutics 2009; 29: 279-285.
14. Holtmann M, Wanitschke R, Helisch A, Bartenstein P, Galle P, Neurath M. Anti-TNF antibodies in the treatment of inflammatory intestinal stenoses in Crohn's disease, Zeitschrift fur Gastroenterologie 2003; 41: 11-17.
15. de Bruyn JR, Becker MA, Steenkamer J, Wildenberg ME, Meijer SL, Buskens CJ et al. Intestinal fibrosis is associated with lack of response to Infliximab therapy in Crohn's disease, PLoS One 2018; 13: e0190999.
16. Rieder F, Kessler S, Sans M, Fiocchi C. Animal models of intestinal fibrosis: new tools for the understanding of pathogenesis and therapy of human disease, American Journal of Physiology-Gastrointestinal and Liver Physiology 2012; 303: G786-G801.
17. Di Gregorio J, Sferra R, Speca S, Vetuschi A, Dubuquoy C, Desreumaux P et al. Role of glycogen synthase kinase-3β and PPAR-γ on epithelial-to-mesenchymal transition in DSS-induced colorectal fibrosis, PLoS One 2017; 12: e0171093.
18. Ding S, Walton KL, Blue RE, MacNaughton K, Magness ST, Lund PK. Mucosal healing and fibrosis after acute or chronic inflammation in wild type FVB-N mice and C57BL6 procollagen α1 (I)-promoter-GFP reporter mice, PLoS One 2012; 7: e42568.
19. Boyapati RK, Rossi AG, Satsangi J, Ho GT. Gut mucosal DAMPs in IBD: from mechanisms to therapeutic implications, Mucosal Immunol 2016; 9: 567-582.
20. Anders H-J, Schaefer L. Beyond tissue injury—damage-associated molecular patterns, Toll-like receptors, and inflammasomes also drive regeneration and fibrosis, Journal of the American Society of Nephrology 2014; 25: 1387-1400.
21. Fiocchi C, Lund PK. Themes in fibrosis and gastrointestinal inflammation: American Physiological Society Bethesda, MD; 2011.
22. Akira S, Takeda K. Toll-like receptor signalling, Nat Rev Immunol 2004; 4: 499-511.

23. Lee KM, Seong SY. Partial role of TLR4 as a receptor responding to damage-associated molecular pattern, Immunol Lett 2009; 125: 31-39.
24. Noreen M, Shah MAA, Mall SM, Choudhary S, Hussain T, Ahmed I et al. TLR4 polymorphisms and disease susceptibility, Inflammation Research 2012; 61: 177-188.
25. Pålsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4, Immunology 2004; 113: 153-162.
26. Koh SJ, Choi Y, Kim BG, Lee KL, Kim DW, Kim JH et al. Matricellular Protein Periostin Mediates Intestinal Inflammation through the Activation of Nuclear Factor kappaB Signaling, PLoS One 2016; 11: e0149652.
27. Kiesler P, Fuss IJ, Strober W. Experimental models of inflammatory bowel diseases, Cellular and molecular gastroenterology and hepatology 2015; 1: 154-170.
28. Melgar S, Karlsson A, Michaëlsson E. Acute colitis induced by dextran sulfate sodium progresses to chronicity in C57BL/6 but not in BALB/c mice: correlation between symptoms and inflammation, American Journal of Physiology-Gastrointestinal and Liver Physiology 2005; 288: G1328-G1338.
29. Suzuki K, Sun X, Nagata M, Kawase T, Yamaguchi H, Sukumaran V et al. Analysis of intestinal fibrosis in chronic colitis in mice induced by dextran sulfate sodium, Pathology international 2011; 61: 228-238.
30. Cooper HS, Murthy S, Shah R, Sedergran D. Clinicopathologic study of dextran sulfate sodium experimental murine colitis, Laboratory investigation; a journal of technical methods and pathology 1993; 69: 238-249.
31. Koh SJ, Kim JM, Kim IK, Kim N, Jung HC, Song IS et al. Fluoxetine inhibits NF-kappaB signaling in intestinal epithelial cells and ameliorates experimental colitis and colitis-associated colon cancer in mice, Am J Physiol Gastrointest Liver Physiol 2011; 301: G9-19.
32. Koh SJ, Kim JW, Kim BG, Lee KL, Kim DW, Kim JS. Matricellular protein periostin promotes colitis-associated colon tumorigenesis in mice, Carcinogenesis 2019; 40: 102-111.

33. Seo JY, Lee C, Hwang SW, Chun J, Im JP, Kim JS. Nimbolide Inhibits Nuclear Factor-capital KA, CyrillicB Pathway in Intestinal Epithelial Cells and Macrophages and Alleviates Experimental Colitis in Mice, Phytother Res 2016; 30: 1605-1614.
34. Lee C, Chun J, Hwang SW, Kang SJ, Im JP, Kim JS. Enalapril inhibits nuclear factor-kappaB signaling in intestinal epithelial cells and peritoneal macrophages and attenuates experimental colitis in mice, Life Sci 2014; 95: 29-39.
35. Rieder F, Brenmoehl J, Leeb S, Schölmerich J, Rogler G. Wound healing and fibrosis in intestinal disease, Gut 2007; 56: 130-139.
36. Wynn TA. Fibrotic disease and the TH 1/TH 2 paradigm, Nature Reviews Immunology 2004; 4: 583-594.
37. Yang M-H, Wu M-Z, Chiou S-H, Chen P-M, Chang S-Y, Liu C-J et al. Direct regulation of TWIST by HIF-1α promotes metastasis, Nature cell biology 2008; 10: 295-305.
38. Evans AJ, Russell RC, Roche O, Burry TN, Fish JE, Chow VW et al. VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail, Molecular and cellular biology 2007; 27: 157-169.
39. Janeway Jr CA, Medzhitov R. Innate immune recognition, Annual review of immunology 2002; 20: 197-216.
40. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity, Cell 2006; 124: 783-801.
41. Franchimont D, Vermeire S, El Housni H, Pierik M, Van Steen K, Gustot T et al. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis, Gut 2004; 53: 987-992.
42. Kühl AA, Erben U, Kredel LI, Siegmund B. Diversity of intestinal macrophages in inflammatory bowel diseases, Frontiers in immunology 2015; 6: 613.
43. Wynn TA, Vannella KM. Macrophages in tissue repair, regeneration, and fibrosis, Immunity 2016; 44: 450-462.
44. Froehlich F, Juillerat P, Mottet C, Pittet V, Felley C, Vader J-P et al.

Fibrostenotic Crohn's disease, Digestion 2007; 76: 113-115.

45. Zhang Y, Lee TC, Guillemin B, Yu M-C, Rom WN. Enhanced IL-1 beta and tumor necrosis factor-alpha release and messenger RNA expression in macrophages from idiopathic pulmonary fibrosis or after asbestos exposure, The Journal of Immunology 1993; 150: 4188-4196.

46. Lenti MV, Di Sabatino A. Intestinal fibrosis, Molecular Aspects of Medicine 2019; 65: 100-109.

47. Bates RC, Mercurio AM. Tumor necrosis factor-α stimulates the epithelial-to-mesenchymal transition of human colonic organoids, Molecular biology of the cell 2003; 14: 1790-1800.

48. Huaux F, Arras M, Tomasi D, Barbarin V, Delos M, Coutelier J-P et al. A profibrotic function of IL-12p40 in experimental pulmonary fibrosis, The Journal of Immunology 2002; 169: 2653-2661.

49. Wong Y, Sethu C, Louafi F, Hossain P. Lipopolysaccharide regulation of toll-like receptor-4 and matrix metalloprotease-9 in human primary corneal fibroblasts, Investigative ophthalmology & visual science 2011; 52: 2796-2803.

50. Darby IA, Laverdet B, Bonté F, Desmoulière A. Fibroblasts and myofibroblasts in wound healing, Clinical, cosmetic and investigational dermatology 2014; 7: 301.

51. Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA. Myofibroblasts and mechano-regulation of connective tissue remodelling, Nature reviews Molecular cell biology 2002; 3: 349-363.

52. Leeb SN, Vogl D, Grossmann J, Falk W, Schölmerich J, Rogler G et al. Autocrine fibronectin-induced migration of human colonic fibroblasts, American Journal of Gastroenterology 2004; 99: 335-340.

53. Leeb SN, Vogl D, Gunckel M, Kiessling S, Falk W, Göke M et al. Reduced migration of fibroblasts in inflammatory bowel disease: role of inflammatory mediators and focal adhesion kinase, Gastroenterology 2003; 125: 1341-1354.

54. Rivera-Nieves J, Bamias G, Vidrich A, Marini M, Pizarro TT, McDuffie MJ et al. Emergence of perianal fistulizing disease in the SAMP1/YitFc mouse, a spontaneous model of chronic ileitis, Gastroenterology 2003; 124: 972-

982.

55. Mourelle M, Salas A, Guarner F, Crespo E, García–Lafuente A, Malagelada JR. Stimulation of transforming growth factor β1 by enteric bacteria in the pathogenesis of rat intestinal fibrosis, Gastroenterology 1998; 114: 519-526.

56. Lu Y-C, Yeh W-C, Ohashi PS. LPS/TLR4 signal transduction pathway, Cytokine 2008; 42: 145-151.

57. Boza P, Ayala P, Vivar R, Humeres C, Cáceres FT, Muñoz C et al. Expression and function of toll-like receptor 4 and inflammasomes in cardiac fibroblasts and myofibroblasts: IL-1β synthesis, secretion, and degradation, Molecular immunology 2016; 74: 96-105.

58. Schmausser B, Andrulis M, Endrich S, Lee S, Josenhans C, MÜLLER-HERMELINK HK et al. Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in Helicobacter pylori infection, Clinical & Experimental Immunology 2004; 136: 521-526.

59. Bartlett RS, Jetté ME, King SN, Schaser A, Thibeault SL. Fundamental approaches in molecular biology for communication sciences and disorders, Journal of Speech, Language, and Hearing Research 2012.

Figures

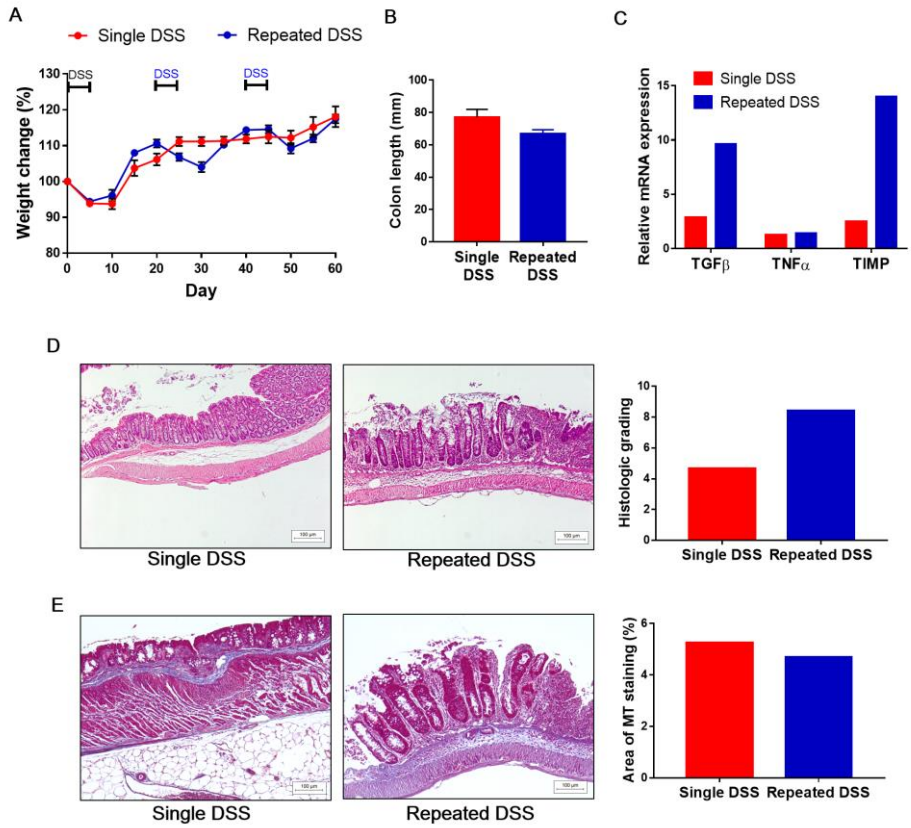

Figure 1. Comparison of one or three cycles of 3% DSS treatment and 3 cycles. (A) Both groups were treated with DSS in 1st cycle. Repeated DSS group was treated with DSS and single DSS group with normal water in 2nd and 3rd cycles. Mice suffered weight loss during DSS treatment but they were recovered after stopping DSS. (B) The repeated DSS group has shorter colon than the single DSS group but it was statistically insignificant. (C) The expression of transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interleukin (IL)-12p40 was increased in the repeated DSS group as compared to the single DSS group, but insignificant. (D) In hematoxylin-eosin (H&E), more severe colitis was developed in the repeated DSS group than the single DSS group. (E) In Masson's Trichrome (MT) staining, collagen deposition was more prominent in the single DSS group than the repeated DSS group.

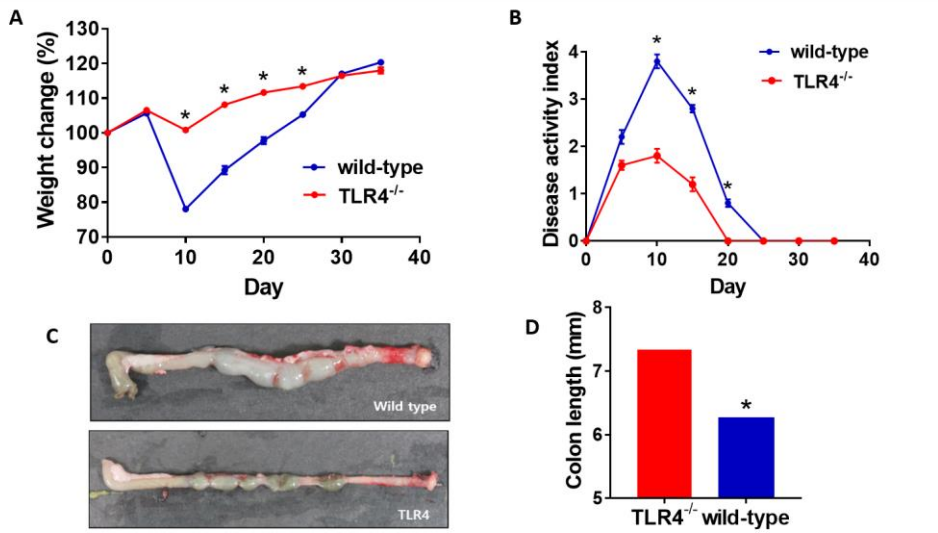

Figure 2. Comparison of clinical indices, gross appearance, and histologic grading in toll-like receptor 4-deficient ($TLR4^{-/-}$) (n = 5) and wild-type mice (n = 5). (A) Wild-type mice experienced extensive weight loss compared to $TLR4^{-/-}$ mice during post dextran sulfate sodium treatment. (B) Disease activity index from day 5 to day 20 was higher in wild-type as compared to $TLR4^{-/-}$ mice. (C) Gross appearance of extracted colons showed bowel edema and fibrotic change in wild-type mice. However, in $TLR4^{-/-}$ mice, bowel edema was not marked. (D) Colon from $TLR4^{-/-}$ mice was longer than that from wild-type mice. Asterisks indicate significant differences (*P*-values < 0.05) between $TLR4^{-/-}$ and wild-type mice.

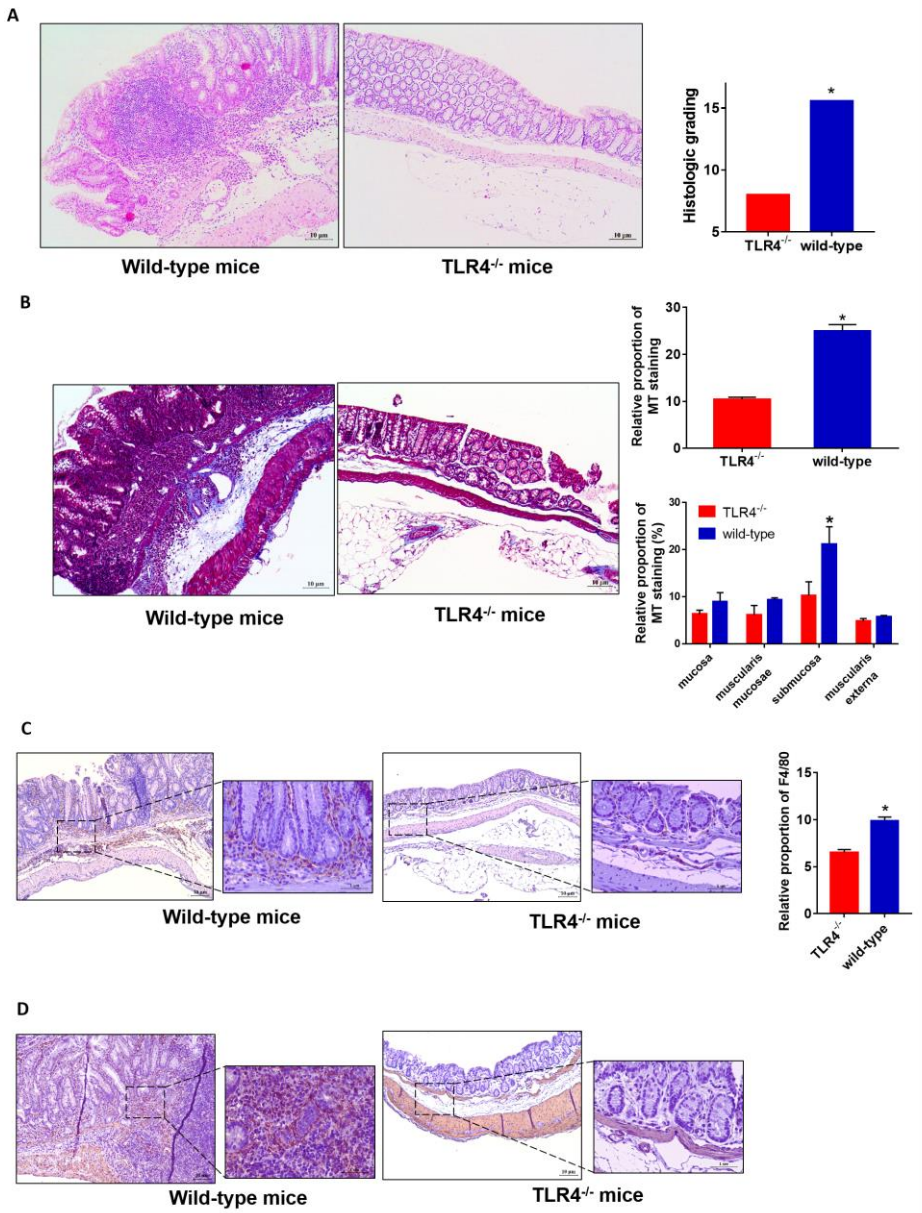

Figure 3. Representative H&E, MT staining, and immunohistochemistry. (A) H&E result revealed prominent mucosal disruption and infiltration of inflammatory cells in wild-type colon. (magnification: ×100). Histological grading, determined by H&E staining, was higher in wild-type than TLR4$^{-/-}$ mice. (B) MT staining of colon tissue samples revealed more severe fibrosis in wild-type than in TLR4$^{-/-}$ mice (magnification: ×100). The proportion of MT-positive area, analyzed using ImageJ program, was significantly lower in TLR4$^{-/-}$ than in wild-type colon tissue. (C) Immunohistochemistry detected increased migration of F4/80-positive cells in wild-

type, as compared to TLR4$^{-/-}$ mice (magnification: ×100 and ×400). The proportion of F4/80-positive region was markedly lower in TLR4$^{-/-}$ as compared with wild-type mice. (D) The proportion of smooth muscle actin (SMA)-positive cells was higher in distorted mucosal structure of wild-type than TLR4$^{-/-}$ mice (magnification: ×100 and ×400). Statistically significant results: * *P*-values < 0.05.

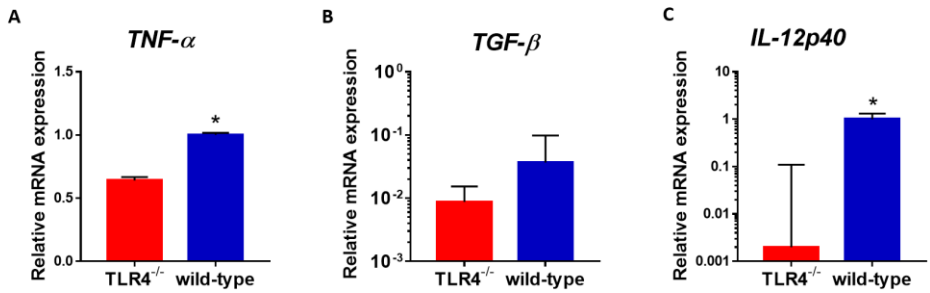

Figure 4. Comparison between the expression levels of inflammatory cytokines in peritoneal macrophages obtained from TLR4$^{-/-}$ mice (n = 3) and wild-type mice (n = 3) after lipopolysaccharides (LPS) stimulation (10ng/mL for four hours). The transcription levels of TNF-α, TGF-β, and IL-12p40, and were measured by real-time reverse transcription-polymerase chain reaction (RT-PCR). Asterisks indicate significant differences (*P*-values < 0.05) between two groups.

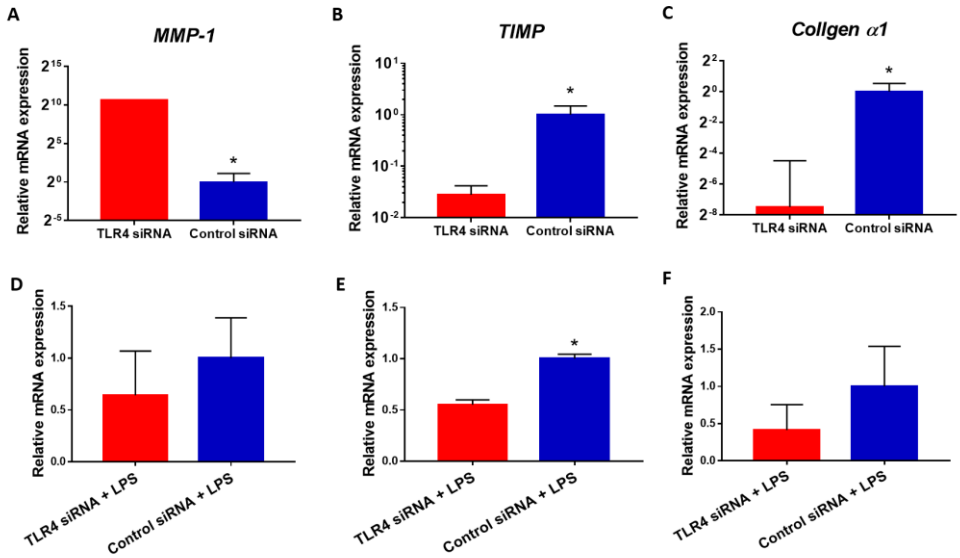

Figure 5. The effect of TLR4 silencing on gene expression in human colon myofibroblasts. Transfection with TLR4 small interfering RNA (siRNA) (n = 5) or control siRNA (n = 5) with or without LPS (10ng/mL for four hours) stimulation was performed in CCD-18Co cells. The transcription levels of matrix metalloproteinase-1 (MMP-1), tissue inhibitor of metalloproteinase (TIMP), and collagen α1 were evaluated by real-time RT-PCR. The expressions of TIMP and collagen α1 were decreased when TLR4 siRNA was treated regardless LPS stimulation. The expression of MMP-1 increased when TLR4 siRNA was treated without LPS, but it decreased when LPS was administrated. Statistically significant results: * *P*-values < 0.05.

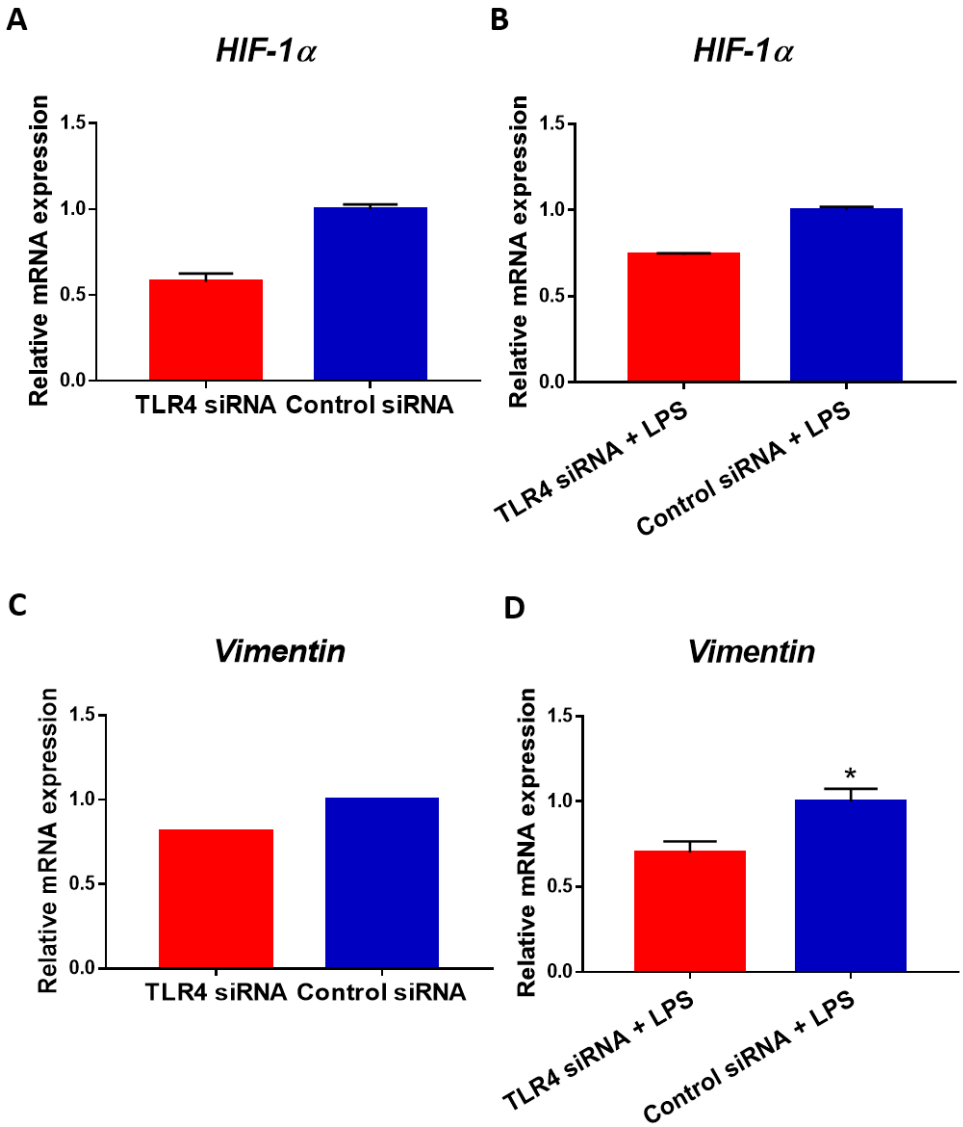

Figure 6. The effect of TLR4 silencing on epithelial-mesenchymal transition in HCT116 cells. HCT116 cells were transfected with TLR4 siRNA (n = 5) or control siRNA (n = 5). After TLR4 silencing, LPS (10ng/mL for four hours) was treated or not. The gene expression levels of all mesenchymal markers was decreased by TLR4 siRNA; especially, the expression levels of vimentin were significantly suppressed when LPS was treated. Statistically significant results: * *P*-values < 0.05.

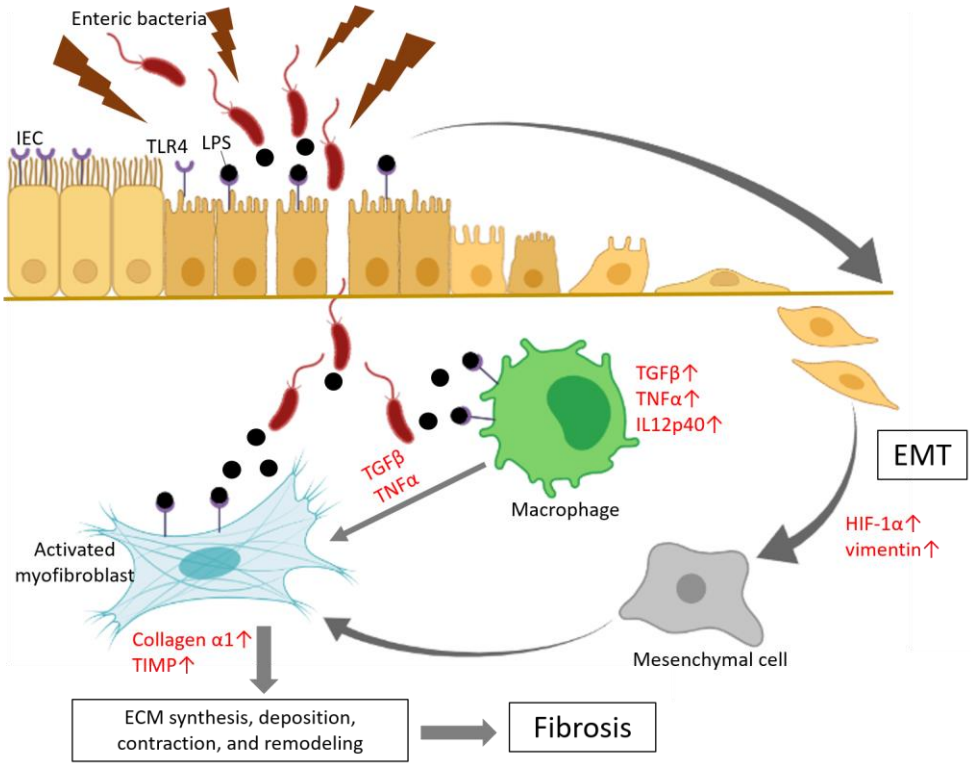

Figure 7. The role of TLR4 in intestinal fibrosis. When bowel injury occurs in the condition of inflammation, intestinal epithelial cells (IECs) are damaged and mucosal permeability is high enough to bacteria and bacteria-derived molecules penetrate mucosal barrier. Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, binds to TLR4 which is expressed on the surface of IECs, macrophages, and myofibroblasts. Interaction between TLR4 and LPS activates macrophages with increased expression of tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and interleukin (IL)-12p40. Macrophages actives myofibroblasts. Epithelial cells lose their structure and function, and change into mesenchymal cells, accompanied by increased mesenchymal markers, such as hypoxia-inducible factor-1α (HIF-1α) and vimentin. Activated myofibroblasts facilitate collagen synthesis and suppress proteolysis by TIMP. After then, intestinal fibrosis was induced through extracellular matrix (ECM) synthesis, deposition, tissue contraction, and remodeling done by myofibroblasts.

초 록

장 섬유화는 만성적이고 반복적인 장염으로 인해 발생하며, 협착과 폐쇄를 유발할 수 있으나 그 치료가 어려운 실정이다. 톨-유사 수용체 4 (TLR4)는 특정 미생물의 구성성분에 반응하여 선천 및 획득면역을 자극할 수 있지만 장 섬유화에서 TLR4의 기능은 거의 알려져 있지 않다. 그래서 우리는 마우스 섬유화 모델과 인간 근섬유아세포, 장 상피세포를 이용하여 장 섬유화에서 TLR4의 역할에 대해 연구하였다.

TLR4에 대한 본격적인 연구에 앞서 DSS-유도 마우스 장 섬유화 모델에 대한 연구부터 하였다. 마우스를 이용한 장 섬유화 실험모델에서 DSS를 많이 사용하지만 DSS-유도 장 섬유화 모델에서 적절한 DSS의 처리 기간은 아직 잘 확립되지 않은 실정이다. 그 결과 6일간의 3% DSS 투약만으로도 충분히 장 섬유화가 발생하였다.

TLR4-결핍 ($TLR4^{-/-}$) 마우스와 야생형 마우스에 3% dextran sodium sulfate를 처리하여 장 섬유화를 유발하였다. 야생형 마우스에 비해 $TLR4^{-/-}$ 마우스에서 만성 염증과 장으로의 대식세포 유입이 감소하였고 장 섬유화와 콜라겐 축적 또한 억제되었다. 또한 $TLR4^{-/-}$ 마우스의 복강 내 대식세포에서 종양 괴사 인자-α, 인터루킨-12p40, 형질전환 생장 인자-β의 발현이 감소되어 있었다. 작은 간섭 리보핵산 (small interfering RNA, siRNA)을 처리하여 CCD-18Co 세포에서 TLR4의 발현을 억제한 후 기질 금속단백질 가수분해효소-1 (matrix metalloproteinase-1, MMP-1), 금속단백 분해효소 조직억제제 (tissue inhibitor of metalloproteinase, TIMP) 콜라겐 α1의 발현을 평가하였다. TLR4 siRNA가 처리된 경우 기질 금속단백질 MMP-1와 콜라겐 α1의 발현은 감소하고 TIMP의 발현은 증가하였다. 그리고 HCT 116 세포에 TLR4 siRNA를 처리하자 상피간엽이행이 억제되었다.

이 연구를 통해 TLR4가 장의 만성 염증과 섬유화에 필수적인 물질임을 밝혔으며 TLR4는 향후 장 섬유화에 대한 치료 표적이 될 수 있을 것이다. 또한 우리가 정립한 DSS-유도 마우스 장 섬유화 모델은 다른 연구에서 장 섬유화를 유발하는데 도움이 될 것이다.

주요어 : 톨-유사 수용체4, 장염, 섬유화, 상피간엽이행
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