Impairment of a NIK-SIX Feedback Axis Results in Dysregulation of Intestinal Immune Homeostasis and Promotes Early-onset Fatal Spontaneous Colitis

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ABSTRACT

Background: The negative feedback circuit NIK-SIX could inhibit the systemic inflammation and protect mouse from endotoxic shock. However, the physiological significance of NIK-SIX feedback circuit in the maintenance of intestinal immune homeostasis and prevention of early-onset spontaneous colitis is not known.

Objective: To explore the role of NIK-SIX axis in the maintenance of intestinal immune homeostasis.

Methods: The conditional knockout of NIK encoding gene, Map3k14, in the Cd11c⁺ dendritic cells were generated by crossing Map3k14-flox mice with Cd11c-Cre mice. DSS was used for colitis models. The expression of cytokines in the intestinal immune cells, isolated from Map3k14-cKO mice were detected by qPCR. The siRNA molecules were used for the silencing of SIN-proteins. Then luciferase assays and chromatin immunoprecipitation combined with qPCR were applied for mechanism investigations.

Results: The expression of SIX1 and SIX2 protein in BMDMs from WT were significantly lower than in the Map3k14-cKO mice. In vitro, the NIK⁻⁻ human-derived circulating monocytes also failed to express SIX-proteins under the stimulation of non-canonical NF-κB agonists. The expression of cytokines was significantly decreased in human circulating monocytes with overexpression SIN-proteins. The expression of cytokines in macrophages, DCs and T cells isolated from Map3k14-cKO mice were significantly increased in the DSS-induced models. Higher expression of cytokines was observed in the SIN1⁻⁻ and SIN2⁻⁻ cells including human circulating monocytes, mouse-derived BMDMs, intestinal macrophages and DCs. SIN-proteins directly bound the promoter region of inflammatory genes.

Conclusion: NIK-SIX axis down-regulated inflammatory gene expression and plays a pivotal role in the maintenance of intestinal immune homeostasis.

Keywords: NF-κB inducing kinase, Spontaneous colitis, SIX, Intestinal immune homeostasis

INTRODUCTION

Inflammatory bowel disease (IBD) in children is common in recent years (1). There is a huge negative impact on the physical growth and life quality if the patients suffered from the onset of IBD in childhood (2-4). The incidence of severe ulcerative colitis in children is higher than in adults and ~ one-third of children with colitis require hospitalization and medical management for acute severe ulcerative colitis (4, 5). To date, studies regarding colitis in children are limited, and the differences in bowel physiogenesis and immune homeostasis between adults and children may further limit our understanding of the pathogenesis of colitis in children.

Early-onset spontaneous colitis in mice may better simulate colitis in children. In animal experiments studying the pathogenesis of autoimmune colitis and IBD, the most widespread disease model used is the DSS-related pathological colitis mouse; however, this drug-related colitis model may not reflect the actual alterations of intestinal immune function and genetic deficiency relevant to early-onset spontaneous colitis (6-8). In previous studies, mice with IL-10 deficiency developed autoimmune spontaneous colitis by the age of 3 months (9, 10). Therefore, the Il10-/- model may also not be suitable for investigation regarding the mechanisms of early-onset colitis, which also indirectly suggests the distinctive mechanisms underlying this condition in mice pups compared with the grown ones.

Intestinal microbiota homeostasis and immune balance serve a crucial role in the protection against IBD (11). A previous study showed that a specific deficiency of NF-κB inducing kinase (NIK) in dendritic cells (DCs) resulted in severe bacterial infection and infectious intestinal inflammation due to a comprised host defense system in the bowels (12). NIK is a key rate-limiting protein kinase that contributes to the downstream initiation of the non-canonical NF-κB pathway (13). The NIK could integrate different signals transduced by distinct receptors binding to cellular membranes, such as CD40L, lymphotoxin beta, RANKL, and TWEAK. The receptors mentioned above could lead to the downstream phosphorylation of IkappaB kinase-alpha (IKKa), which was regarded as the first step of the NIK-induced downstream initiation of the unclassical NF-κB pathway (14, 15). And in turn, p100 protein could be activated by the phosphorylation by IKKa, which facilitated the ubiquitination and stepwise degradation of IKKa, eventually primarily inducing the phosphorylation of p52 and secondary appearances of RelB-p52 complexes, initially launching the cascade response of non-classical NF-κB signaling pathway. Therefore, the expression of the Map3k14 gene plays a vital role in the regulation of the non-classical NF-κB signaling pathway and inflammatory response.

Conversely, the deletion of the Map3k14 gene in DCs ameliorated colonic inflammation in the Il10-/- mice (12), highlighting the dual properties of NIK in the maintenance of host defense and intestinal immune regulation. Another intriguing study identified the presence of a NIK-SIN signaling axis which serves as a negative feedback mechanism to control inflammation in an endotoxic shock model, suggesting that the SIX families of proteins served a crucial role in the downregulation of NIK and rectification of an aberrant immune response in various pathological conditions (16).

In the present study, the effect of knockout of SIN-proteins in intestinal resident DCs on the occurrence of fatal early-onset spontaneous colitis was determined. It was shown that a NIK-SIN negative feedback loop prevented an excessive inflammatory response and thus protected against the development of severe early-onset spontaneous colitis. Constitutive expression of Map3k14 in intestinal DCs preserved the function of the protein kinase reported recently, which plays a vital role in the activation of the downstream initiation of the non-canonical NF-κB pathway (13).
host defense system in intestinal infection; however, the amplification of inflammatory processes following non-canonical NF-κB signaling was strictly controlled by a NIK-SIN regulatory axis.

**MATERIALS AND METHODS**

**Mice.** Map3k14-flox mice were generated from the Animal Research Center of Nanjing University. The mice with two flox loci flanking Map3k14 were crossed with transgenic mice with a Cre located in promotors of Cd11c to generate Map3k14-cKO and wild-type mice. SIX1−/− mice were generated by the Jackson Laboratory by crossing SIX1-flox with Cd11c-Cre mice to produce SIX1-cKO and control mice. The mice were housed and fed in a specific pathogen-free animal house at 25°C and a humidity of 40%. The sterile food and water were used for feeding. And all animal procedures were contented by the Animal Ethics Committee of our Institute. 28 male mice (all 8 weeks ago) were used for animal experiments and the average weight was 24.2g. The mice were eventually sacrificed after the intraperitoneal injection of amobarbital at a concentration of 100mg/kg weight. And the stopped chest gallery movement is a sign of animal death. To establish the DSS-induced colitis, we applied the 10-week-ago mice with conditional knockout of Map3k14 and the age-matched wild-type controls. The dextran sulfate sodium was administrated by oral liquid at a concentration of 3% and intestinal histopathological analysis was performed after one week. The mice were kept and fed in a standard pathogen-free animal house, and all animal experiments were approved by the Institutional Animal Care and Use Committee of our Institute (Approve NO.2019-129).

**Preparation of intestinal inflammatory cells from the mouse.** The lamina propria mononuclear cells (LPMCs) were regarded as intestinal inflammatory cells and isolated from colonic tissues. Briefly, the colon samples from the mice were resected and then taken from euthanized mice and the residual blood was washed off by phosphate-buffered saline (PBS). The tissue blocks were cut into 1mm³ in Hank’s Balanced Salt Solution containing 5 mM EDTA and 1 mM dithiothreitol and were then repeatedly digested in Hank’s solution containing 0.5 mg/ml collagenase D (Roche Applied Science), 0.5 mg/ml DNase I (Sigma-Aldrich), and 3 mg/ml Dispase II (Roche Applied Science) at 37°C for 30 minutes. The collected supernatants were used for centrifugation, and the obtained cell pellet was then resuspended in 40% Percoll solution according to standard instructions. LPMCs were obtained by density gradient centrifugation. The interphase floated cells were aspirated and then washed with PBS.

**Isolation of bone marrow-derived macrophages and DCs (BMDCs).** BMDCs were generated by cultivating bone marrow cells of wild-type mice and conditional deficient Map3k14 mice in a growth medium supplemented with recombinant GM-CSF (20 ng/ml). Fresh GM-CSF-containing medium was added on days 3 and 6, and the fully differentiated DCs were harvested on day 8 for functional assays. The generated DC population was analyzed by flow cytometry, based on CD11c expression, and further enriched using a CD11c Microbead Isolation kit (Miltenyi Biotec, GmbH). The bone marrow-derived macrophages were enriched directly from mononuclear cells isolated from bone marrow using a CD68 Microbead Isolation kit. The cells were washed and challenged with the non-canonical NF-κB signaling pathway activator, LTαβ2 (50 ng/ml), followed by an overnight incubation with 10 ng/ml IFN-γ (R&D Systems, Inc.). Subsequently, the inflammatory mediators expressed in isolated BMDMs were measured by quantitative (q) PCR.

**Preparation of human-derived monocytes.** The collection of human-derived circulating monocytes was approved by the Medical Ethics Committee of Wuhan Children’s Hospital and informed consents
were subscribed by all the subjects. The circulating monocytes were collected from the children with colitis from May 2019 to January 2020 in the Wuhan Children’s Hospital. In total, 5 children were enrolled in this study, and 5 of them were male. The age of these subjects was 7.72±3.14 years old. The inclusion criteria included the children with mild and moderate colitis and with significant alleviation after standard treatment and exclusion criteria included severe secondary intestinal infection and refractory colitis. Peripheral blood mononuclear cells (PBMCs) were isolated from 5 ml venous blood from 5 individuals and purified using flow cytometry sorting as follows: CD14+CD16-HLA-DR+, CD16+CD14+HLA-DR+, and CD14-CD16+HLA-DR+ cells. The simulated differentiation process of human circulating monocytes to tissue-resident DCs was performed as mentioned above. The human-derived CD14+CD16-HLA-DR+ monocytes were cultured in a growth medium supplemented with recombinant GM-CSF (20 ng/ml) and IL-4 (500U/mL). Fresh medium containing GM-CSF and IL-4 was added on days 3 and 6, and the fully differentiated DCs were harvested on day 8 for functional assays. The generated DC population was analyzed by flow cytometry, based on CD11c expression, and further enriched using a CD11c Microbead Isolation kit (Miltenyi Biotec, GmbH). The DCs differentiated from human-derived monocytes were cultured in RPMI1640 medium supplemented with 10% FBS with LTɑ1β2 at a concentration of 50 ng/ml or transfected with SIX1 overexpression vectors. The levels of inflammatory mediators were measured using qPCR assays. The informed consent for the blood sample collection of the patients and the controls was obtained from the parent or legal guardian of the participant and all procedures conformed to the Declaration of Helsinki. The experiment was approved by the Human Experiment Ethics Committee (Approve NO.2019-101).

**Transfection.** Lentivirus-mediated overexpression vectors were used to enhance the expression of SIX-proteins (including SIX1 and SIX2) in human-derived monocytes and BMDMs from mice according to the Liposome Transfection. Meanwhile, Lentivirus-mediated vectors containing Map3k14-shRNA sequences were used to silence the expression of NIK in the human-derived monocytes. The oligonucleotides used for negative control (NC) were obtained using scrambled shRNA that were incapable of silencing the gene. The Lentivirus vectors used in vitro experiments were obtained from GeneChem (Shanghai, China). The transfection of shRNA and the controls was performed according to the standard instructions. Human-derived monocytes and BMDMs from mice were seeded on 96-well plates (5000 cells/well) and incubated in an incubator. On the second day, the cells were transfected with a lentivirus vector constructed by SIX1-shRNA (CTTCAGTCCCTCCCTGGAA), or NC-shRNA (CGTGAAACCGTAGGTCCGTCC) for 10–12 h at a concentration of 100ng/well of lentivirus vectors and at a concentration of 0.5μL/well of lipofection reagents by using Lipofectamine 3000 (Invitrogen). The overexpression vectors were also delivered into human-derived circulating monocytes. The cells expressing green fluorescent protein were considered as the ones with stable transfection of lentivirus-mediated shRNA. The quantitative real-time assay was used to detect the SIX-proteins or NIK to ensure the knockdown or overexpression after the treatment of lentivirus vectors.

**Chromatin-immunoprecipitation (Ch-IP).** A SIX1 with green fluorescent proteins cell line was constructed by adding GFP-SIX1 gene segments into a given with a feature of blasticidin-resistance vector. 293 cells were transfected by the pSCRPSY vector with blasticidin resistance and then selected by 100 U/ml blasticidins. Ch-IP was performed according to the standard protocol. Briefly, 1.0x10^7 cells were induced using 2% PFA for 30 min at 37°C and 150mM glycine.
was used for the secondary incubation. Cells were then washed and lysed twice. The nuclei were obtained by centrifugation at 12,000 rpm for 10 min and the cell pellets were resolved in RIPA buffer. Nuclei lysates were sonicated using a Bioruptor (Diagenode). Eventually, the terminal products of 6 μg IgG and corresponding primary antibodies were co-incubated in the existence of the protein G beads (Cat. No. 10004D; Invitrogen) for 2 h and blocked by PBS containing 12% BSA for 30 minutes. Finally, the RIPA buffer was used for washing the bound beads at first and then combined with TE buffer twice. The protein-DNA complexes were eluted using 500 μl elution buffer (100 mM NaHCO3, 1% SDS). A total of 20 μl NaCl solution was used to destroy the binding of protein and DNA by heating at 80°C for 6h. DNA was recovered and the qPCR method was for the measurement of gene expression levels. The qPCR methods were used for the measurement of enrichment of proinflammatory cytokines. The fold change of each gene was normalized to the experimental control.

**Luciferase reporter assay.** A total of 1x10^4 293T cells were seeded in each well of a 48-well-plate. The plasmids were transfected into the cells followed by the transfection control LacZ and 5x κB-LUC promotor sequences and incubated for 48 cytokine treatment, after 24 h of transfection, the cytokine treatment was performed with 50 ng/ml LTα1β2 for 24 h. Luciferase activity was measured according to standard instructions (cat. no. E1500; Promega Corporation). Relative luciferase activity was calculated by normalizing it to the LacZ control and the experimental control.

**Western blotting.** Total protein extracts from the colonic tissues and inflammatory cells isolated from colon resected from mouse were prepared using RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40, and 0.1% SDS) containing 1μM protease and phosphatase inhibitors. A total of 30 μg protein was loaded per well on an SDS-gel, resolved using SDS-PAGE, and transferred to membranes. The membranes were incubated overnight at 4°C with primary antibodies, including anti-SIX1 (Cat. No. 16960. Cell Signaling Technology, Inc.), anti-SIX2 (ab111827, Abcam) or anti-GAPDH (ab9485, Abcam). All the primary antibodies were used at a dilution of 1:1,000. Subsequently, membranes were incubated with corresponding secondary antibodies against the primary antibodies described above (Cell Signaling Technology, Inc.), immunoblots were exhibited and a grey value was calculated using a ChemiDoc Touch Imaging System (ChemiDoc; BioRad Laboratories, Inc.).

**Histological evaluation.** The entire colon was resected from the experimental animals, which was then soaked and fixed in PBS containing 4% formaldehyde for 1h at 25°C. Fixed tissues were dehydrated by gradually soaking in a series of decreasing concentrations of alcohol solutions followed by xylene, and then embedded in paraffin. The paraffin-embedded specimens were cut into 5-μm sections, stained with hematoxylin-eosin (H&E), and observed using a digital inverted light microscope (EVOS; Thermo Fisher Scientific, Inc.). The pathological indices associated with inflammation were scored by two independent pathologists in a blinded manner.

**Reverse transcription-qPCR.** Collected colonic tissues or primary cells were frozen in an RNA-later buffer. Total RNA was isolated and the quality control was performed. The purified tracked RNA was transformed into cDNA by reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) , which were used for the analysis of the expression of GAPDH, MCP-1, interferon-γ (IFN-γ), Tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-1 (MCP-1) and CCL8 mRNA were amplified using a LightCycler 480 System II (Roche Applied Science). The primer sequences used were:

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GAPDH forward, 5' - CAACCTTTGTCAAGCTCATTTCC - 3' and
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reverse 5’-GCGTCATGCTATCTTACTCC-3’; IFN-γ forward, 5’-AGTCAGTGCAGTTCA-3’ and reverse 5’-ATTTCCAACCCTCAATCACAGAG-3’; TNF-α forward, 5’-AGCTTCTCATGGCTTCTTT-3’ and reverse 5’-ATCATCTCAGGCTTTT-3’; MCP-1 forward, 5’-GGGATATGTCGCTGCGGCTGCT-3’ and reverse 5’-CGTGGATACG-3’; and CCL8 forward, 5’-GCTCCGCGTACGTCAACAC-3’ and reverse 5’-AGTGATCTCGTCGCG-3.

The expression levels of the genes mentioned above were indicated the relative expression of GAPDH.

Statistical Analysis

Statistical analysis was carried out using SPSS 23.0 and R studio. Statistical significance was analyzed using a two-tailed unpaired t-test and a one-way ANOVA. The differences in the incidence rate of fatal spontaneous early-onset colitis were compared using the Fisher’s exact test. The difference in the mortality rate was compared using the Kaplan-Meier method, with comparisons performed using a log-rank test. P<0.05 was considered to indicate a statistically significant difference.

RESULT

Intestinal inflammatory phenotypes of wild-type and Map3k14-cKO mice under physiological and pathologic conditions.

Previously, it has been shown that the Map3k14 gene in the intestinal epithelial cells, which encodes NIK, functions in the development and maintenance of M cells (12). Physiological expression of Map3k14 is required for protection against DSS-induced colitis. However, another study found that the specific deletion of Map3k14 in DCs ameliorated the inflammatory response in the IL-10-/- mice (14). To further verify the role of NIK in different disease models and cell types, Map3k14-cKO mice were used to establish a DSS-induced model of colitis. The morphological differences in the colon were assessed via routine H&E staining (Figure 1A). There were no significant differences in the body weight, colon length, bleeding scores, and diarrhea scores between the Map3k14-cKO and wild-type mice in the DSS-induced model of colitis (Figures 1B-E). The expressions of inflammatory cytokines were also similar between the Map3k14-cKO and wild-type mice in the DSS-induced model of colitis. These data demonstrate that the expression of Map3k14 in intestinal DCs does not have any influence on intestinal inflammation in drug-induced colitis, which may be attributed to unknown physiological compensatory mechanisms. Intriguingly, mice with conditional knockout of Map3k14 in Cd11c+ DCs developed early-onset spontaneous colitis after 4-5 weeks with a rapid and self-limiting recovery, manifested by temporary weight loss, slight alterations in the histopathology, and increased expression of cytokines in the isolated colonic tissues compared with the wild-type (Figures 2A-C). However, no significant alterations in colon length, bleeding scores, and diarrhea scores were observed in all the subjects 12 weeks after birth (Figures 2D-F). These data suggested that NIK may potentially serve as a pathogenic factor and that its biological effects are likely strictly controlled under physiological conditions. Conversely, inducible overexpression of Map3k14 may have been associated with the occurrence of intestinal immune disequilibrium and spontaneous colitis.

Knockout of Map3k14 in mice results in downregulation of expression of SIX-proteins in tissue-resident and bone marrow-derived macrophages. To verify the inducible expression of SIX-proteins in the presence of expression of Map3k14, the expression levels of SIX-proteins in the macrophages which resided in the colon and those derived from the bone marrow of Map3k14-cKO mice were treated with DSS
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Figure 1. Intestinal inflammation in the DSS-induced mouse model of colitis was similar between the wild-type and Map3k14-cKO mice. (A) Representative images of H&E staining of colonic tissues from wild-type and Map3k14-cKO mice treated with 2% DSS. (B) Colon length of wild-type and Map3k14-cKO mice treated with DSS. (C) Bleeding and (D) diarrhea scores of wild-type and Map3k14-cKO mice treated with DSS. (E) Dynamic changes in body weight in the wild-type and Map3k14-cKO mice for 8 days following treatment with DSS. (F) The expression of inflammatory cytokines measured by RT-PCR in the DSS-induced colitis of wild-type and Map3k14-cKO mice with spontaneous colitis. Data are presented as the mean ± the standard error of the mean. A Student’s t-test or a repeated measures ANOVA were used for statistical analysis. n=10. *P<0.05, **P<0.01.

Figure 2. Self-limiting and early-onset spontaneous colitis in the Map3k14-cKO mice. (A) Representative images of hematoxylin and eosin staining of colonic tissues from wild-type and Map3k14-cKO mice with spontaneous colitis. (B) Dynamic changes in the body weight of wild-type and Map3k14-cKO mice for 10 weeks following birth. (C) The expression of inflammatory cytokines measured by RT-PCR in the wild-type and Map3k14-cKO mice with spontaneous colitis. (D) Colon length, (E) diarrhea scores, and (F) bleeding score of the wild-type and Map3k14-cKO mice. Data are presented as the mean ± the standard error of the mean. A Student’s t-test or a repeated measures ANOVA were used for statistical analysis. n=10. *P<0.05, **P<0.01.
SIX-proteins (SIX1 and SIX2) in the colon significantly decreased in the Map3k14-cKO mice compared with the wild-type mice (Figure 3A). Expression of SIX1 and SIX2 in the tissue-resident macrophages and BMDMs isolated from colonic samples of Map3k14-cKO mice significantly reduced compared with the wild-type mice stimulated with the agonist of the non-canonical NF-κB signaling pathway (Figure 3B). We then isolated the CD14++CD16- classical monocytes to analyze the role of NIK in the regulation of SIX-proteins because the CD14++CD16-classical monocytes could infiltrate into the local tissue and continue to differentiate into the macrophages. The sorting strategy for the classical and non-classical monocytes from PBMCs is shown in Figure 3C. In the purified circulating classical monocytes (CD14++CD16-) from the patients and the controls, SIX1 and SIX2 expression was significantly reduced compared with the wild-type mice (Figure 3D). All experiments were repeated at least five times.

Figure 3. Silencing of Map3k14 results in reduced expression of SIX-proteins. (A) Western blotting analysis showed significantly reduced protein expression levels of SIX1 and SIX2 in the Map3k14-cKO mice compared with the wild-type mice, and (B) in the colonic resident macrophages and BMDMs of the Map3k14-cKO mice compared with the wild-type mice in DSS-induced model of colitis. (C) The sorting strategy for the subtypes of monocytes from human PBMCs. The CD14++CD16-, CD14+CD16+ intermediate, CD14lowCD16++ monocytes were isolated from CD16+CD14+ monocytes according to the flow sorting. CD14++CD16- classical monocyte was isolated by flow cytometry sorting to perform further experiments. (D) The representative figures and semi-quantitative analysis of expression of SIX-proteins measured by western blot assays in the patients and the controls. (E) Western blotting showed significantly reduced expression of SIX1 and SIX2 in the human circulating monocytes with NF-κB inducing kinase expression knocked down. All experiments were repeated at least five times. *P<0.05, **P<0.01. BMDM.
healthy controls. And we examined the expression of SIX-proteins in the patients and the healthy controls and then found the significantly lower expression of SIX-1 and SIX-2 in the CD14++CD16- classical monocytes in the patients compared with the healthy controls (Figure 3D). The expression of Map3k14 silenced and the expression levels of SIX1 and SIX2 were determined. The results observed in animal models were also replicated in the DCs differentiating from human circulating monocytes in the vitro experiments. The expression of SIX1 and SIX2 significantly decreased following the silencing of Map3k14 in differentiated DCs from the patients (Figure 3E). These data demonstrated that the expression of Map3k14 regulated the expression of SIX-proteins, suggesting the presence of a NIK-SIX signaling pathway in human and murine monocytes/macrophages.

**SIX-proteins downregulate the expression levels of cytokines in inflammatory cells in-vivo and in-vitro.**

To determine the biological effects of SIX-proteins on the intestinal inflammatory response, adenovirus overexpression vectors were used to overexpress the SIX-proteins in human-derived circulating monocytes. Overexpression of SIX1 resulted in significantly reduced expression of inflammatory mediators, including IL-1β, IFN-γ, TNF-α, MCP-1, and CCL8 when stimulated with the agonist of the non-canonical NF-κB signaling pathway (Figure 4A). Subsequently, the expression levels of pro-inflammatory cytokines in the colonic tissues of mice were measured and the results showed that similar expression levels of the inflammatory mediators in the primary DCs and macrophages in colonic tissues of Map3k14-cKO mice were observed compared with the wild-type (Figure 4B). The concentrations of various inflammatory cytokines in the culture supernatant from primary DCs and macrophages are shown in Figure 4C. We found a similar concentration of various inflammatory cytokines in the primary DCs and macrophages from Map3k14-cKO mice and the WT controls, consistent with the results of quantitative Real-time PCR assays mentioned above (Figure 4C). The expression levels of pro-inflammatory cytokines in the colonic tissues in the DSS-induced experimental colitis model were assessed, and it was found out

**Figure 4.** SIX-proteins significantly inhibit the expression of inflammatory mediators. (A) Overexpression of SIX1 reduced the mRNA expression levels of IFN-γ, TNF-α, MCP-1, and CCL8 in human circulating monocytes under stimulation of LTα1β2. (C) Similar mRNA expression levels of IFN-γ, TNF-α, MCP-1, and CCL8 were observed in the primary intestinal DCs and macrophages isolated from wild-type and Map3k14-cKO mice under physiological conditions. (D) mRNA expression levels of IFN-γ, TNF-α, MCP-1, and CCL8 in colonic tissues of wild-type and Map3k14-cKO mice with DSS-induced colitis. (E) mRNA expression levels of IFN-γ, TNF-α, MCP-1 and CCL8 in colonic resident macrophages, DCs and T cells from wild-type and Map3k14-cKO mice with DSS-induced colitis. A Student’s t-test or Bonferroni correction were used for statistical analysis. n=10. *P<0.05, **P<0.01. IFN-γ, interferon-γ; TNF-α, tumor-necrosis factor-α; MCP-1, macrophage inflammatory protein-1; DCs, dendritic cells.
that their expression levels in the Map3k14-cKO significantly increased compared with the wild-type mice (Figure 4D). Subsequently, DCs, macrophages and T cells isolated from the colonic tissues of Map3k14-cKO mice following treatment with the agonist of the non-canonical NF-κB signaling pathway, and the expression levels of the inflammatory mediators were assessed. The expression levels of IL-1b, IFN-γ, IL-8, TNF-α, MCP-1, and CCL12 significantly increased in the colonic macrophages and DCs, but not in the colonic T cells in the Map3k14-cKO mice when compared with the wild-type (Figure 4E). These data suggest that the appropriate expression levels of Map3k14 serve an important role in the maintenance of intestinal immune homeostasis; thus, both the overexpression and deficiency of Map3k14 may result in an imbalance of immune homeostasis in the colon.

SIX-proteins inhibit non-canonical NF-κB signaling and transcription activity of downstream cytokines via binding to promoter regions. To further determine the mechanisms underlying the inhibitory effects of SIX-proteins on the inflammatory response, luciferase reporter assays were used to assess the inhibitory effects on transcription regulation of SIX-proteins using the transcription binding elements of NF-κB. The overexpression lentivirus vectors contained sequences coding SIX1 and SIX2 proteins were co-transfected with luciferase reporter genes driven by a 5xκB binding site, and the luciferase activity was evaluated 24 h after administration of TNF and treated with the agonist of the non-canonical NF-κB signaling pathway, LTα1β2. The vectors containing coding sequences of SIX1 and SIX2 proteins significantly decreased the luciferase activity of 5xκB-LUC compared with the empty vectors in 293T cells (Figures 5A-B). Subsequently, ChIP-qPCR was used to verify whether SIX1 or SIX2 mRNA could bind and occupy the promoter regions of inflammatory cytokines. The results showed that SIX1 could bind to the promoter regions of IL-1b and IL-8 and inhibit their transcription activities (Figure 5C), but SIX2 did not directly bind to the promoter regions of these cytokines (Figure 5D), suggesting that the anti-inflammatory mechanisms of SIX1 and SIX2 are distinct, and that SIX2 promote transcription inhibition depend on the regulation of its trans-activation function.

The specific knockout of SIX1 in mice results in severe early-onset spontaneous colitis. To determine the role of SIX-proteins in the development of early-onset colitis in

![Figure 5](image_url)
vivo, a mouse line with SIX1 expression knocked out specifically in CD11c+ DCs was established. The incidence of early-onset fatal spontaneous colitis and mortality in the SIX1-cKO was found to be significantly higher compared with the wild-type mice (Figures 6A and B). More notable pathological characteristics were observed in the colon and significantly higher expression levels of inflammatory cytokines were observed in the SIX1-cKO compared with the wild-type mice (Figures 6C and D). Pathological alterations in colon length, bleeding scores, and diarrhea scores were observed in the mice with early-onset colitis (Figure 6E). These data demonstrate that the genetic deficiency of SIX1 resulted in intestinal immune disruption in vivo, leading to fatal spontaneous colitis in the young mice.

DISCUSSION

Previous studies have shown that a cell's intrinsic immune defense was dependent on the activation of the non-canonical NF-κB kinase NIK (encoded by MAP3K14) rather than the canonical NF-κB kinase-associated signaling pathway. In addition, overexpression of Map3k14, but not Map3k7, potently inhibited bacterial infection, and NIK inhibited single-stranded RNA viral infection (17-20). The activation of non-canonical NF-κB signaling and subsequent inflammatory cascade is vital for a host’s defense against pathogenic microorganisms and the subsequent inflammatory response in mammals, and the regulation of NIK is the rate-limiting step in the non-canonical NF-κB signaling pathway (12). The mechanisms of inhibition of the
non-canonical NF-κB signaling pathway have been extensively studied. Liu et al (17) found that the sine oculis homeobox homolog family of transcription factors SIX1 and SIX2 served as core inhibitory elements of the non-canonical NF-κB signaling pathway and repressed the downstream excessive inflammatory response. The SIX-proteins may be reactivated, even after developmental silencing via NIK-mediated suppression of the ubiquitin-proteasome. Subsequently, SIX-proteins target the promoter regions of inflammatory cytokines and directly restrain the RelA and RelB trans-activation function (17). Therefore, the SIN-proteins may prevent an excessive inflammatory response following the activation of the non-canonical NF-κB signaling pathway.

NIK is a pivotal governor of DC activation and its function as antigen presentation cells and immunoregulator and is essential in the control of IgA secretion and intestinal microbiota homeostasis (12). DCs promoted IgA secretion through a signaling pathway that requires NIK and subsequent noncanonical NF-κB pathway. The activation of NIK signaling accelerates TLR-stimulated IL-23 secretion and, thereby sustained the polarization of Th17 cells and ILC3s, both of which are major sources of IL-17. IL-17 could reversely promote the production of plgR to facilitate IgA secretion, which is vital for the establishment of intestinal microbiota homeostasis and the function of host defense. DC-specific NIK deletion leads to the relative abundance of Enterococcus spp. and SFB C. Savagelle and ultimately resulted in severe C. rodentium infection, which is characterized by notable body weight loss, significant colon shortening, and colon inflammation (12). DC-specific NIK is essential for the maintenance of intestinal immune homeostasis. IL-23A is a vital target gene that was under the control of the noncanonical NF-κB pathway in DCs (21). IL-23, and its subsequent product IL-17, have contradictory effects on the regulation of bowel immune homeostasis (22, 23). Though IL-17 and IL-23 cytokines strengthen the host’s defense against microbial pathogens and form the complement to control the infections, they are also important contributors to the deterioration of colon inflammation and are related to the incident onset of human IBD. In the present study, it was shown that specific deficiency of Map3k14 in DCs was associated with self-limiting spontaneous early-onset colitis, in agreement with the previous studies, and thus may contribute to the disruption of intestinal microbiota and the reduced response of the host’s defense system.

It has been extensively shown that SIX1 and SIX2 are vital common transcription factors that could determine the differentiation fate and trajectory of various progenitor cell identities in the developing process, and are hypothesized to be at low-expression levels in adults (24-26). In recent years, the anti-inflammatory effects of SIX1 have gained increasing interest (27-29). Yang et al found that knockdown of the Six1 gene significantly suppressed the inflammatory NF-kB pathway in the inflammatory pathological tissues including the lung and liver. They showed that the downregulation of SIX1 effectively inhibited airway inflammation and reversed airway remodeling, suggesting that Six1 indicated a potential therapeutic target for the treatment of allergy-induced asthma (27). As the SIX-proteins are inhibitory components of the non-canonical NF-κB signaling pathway and may provide significant protection in mice against endotoxic shock and substantial inflammatory damage, the role of SIX-proteins in early-onset inflammatory diseases should be further studied. The genetic deficiency and dysfunction of this gene may contribute to the occurrence of early-onset autoimmune inflammatory disease. Thus, whether knockout of expression of SIX-proteins resulted in the development of fatal spontaneous colitis in mice was studied in the present study.

NIK serves a crucial role in the maintenance of intestinal microbiota and the host defense system; however, the pathological aberrations of NIK and non-canonical NF-κB signaling
can result in inflammatory organ damage and inflammatory diseases (12, 16, 30). The NIK-SIX axis, which serves as a negative feedback mechanism regulating the inflammatory response, may contribute to the balance of immune defense and immune regulation (16). In the present study, it was hypothesized that SIX1 may act as an inhibitory element in the protection against spontaneous colitis. The expression levels of Map3k14 did not affect intestinal inflammation in the DSS-induced colitis mice. However, the specific deficiency of Map3k14 in DCs may result in unexpected self-limiting spontaneous colitis, and this might be explained by the disruption of the intestinal flora and the compromised colonic host defense system.

In summary, the present study showed that NIK induced the expression of SIX-proteins and that the SIX-proteins significantly reduced the production of inflammatory mediators in BDMDs or resident DCs and macrophages. Additionally, the SIX1−/− mice developed fatal spontaneous colitis at a young age, in contrast to Il10−/− mice, in which spontaneous colitis occurred 3 months after birth. The model used in the present study may thus better represent early-onset colitis, and the results of the present study suggested that the dysfunction of SIX-proteins may underlie the pathogenesis of acute severe early-onset colitis.

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AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

AUTHORS' CONTRIBUTION

JQC contributed to study conception and design, data collection and analysis, and manuscript writing. YXZ and JQC contributed to the study conception and design and the critical revision and review of the manuscript. JQC and YXH analyzed the data and reviewed the manuscript. YXZ and MH performed the experiments. JQC and YXH analyzed and interpreted the data and contributed to the final manuscript preparation. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present study was approved by the Clinical Research Ethics Committee of Wuhan Children's Hospital, Tongji Medical College, and the Huazhong University of Science Technology.

Conflict of Interest: None declared.

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