RETRACTED ARTICLE: *Clostridium difficile* toxin B induces colonic inflammation through the TRIM46/DUSP1/MAPKs and NF-κB signalling pathway

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**ABSTRACT**

*Clostridium difficile* (*C. difficile*) infection results in toxin-induced epithelial injury and marked colonic inflammation. Mitogen-activated protein kinase (MAPK) and NF-κB which regulated by MAP kinase phosphatase (MKP), also known as dual specificity phosphatases, (DUSP) are fundamental signalling pathways that mediate multiple cellular processes. However, the regulation of DUSP/MAPKs and NF-κB pathway in *C. difficile*-induced colonic inflammation remains unclear. Here, we report that TcdB significantly inhibits cell viability and induces production of IL-1β and TNF-α and activation of MAPKs and NF-κB. An E3-ubiquitin ligase, TRIM46, ubiquitinates DUSP1, and its knockdown significantly inhibits TcdB-induced activation of MAPKs and NF-κB and production of IL-1β and TNF-α. Moreover, TRIM46 overexpression induced production of IL-1β and TNF-α also reversed by DUSP1 overexpression. We further found that promoter of TRIM46 also demonstrated binding to NF-κBp5δ, leading to regulate TRIM46 expression. In addition, the increased colonic inflammation induced by *C. difficile* administration was inhibited by TRIM46 knockdown in vivo. Taken together, the present study shows that TRIM46, as a new regulator of DUSP1/MAPKs and NF-κB signalling pathway, plays an important role in TcdB-induced colonic inflammation.

**Introduction**

Human intestinal flora plays an important role in protecting the innate and adaptive immune function of intestinal mucosa and in maintaining the integrity of intestinal epithelial cell barrier and nutrient absorption [1,2]. The ecological imbalance of intestinal microbial flora will lead to intestinal swelling and serious inflammatory bowel disease [3]. *Clostridium difficile* (*C. difficile*) is Gram positive, anaerobic, spore forming rods that causes intestinal infection, diarrhea, and intestinal inflammation in individuals following antibiotic treatment. The main virulence factors of *C. difficile* are the toxins A (TcdA) and B (TcdB). TcdA can cause extensive damage of intestinal tissue more potent than TcdB in most animal models [4,5], while TcdB has greater cytotoxicity than TcdA in cell culture lines [6] and is the primary factor responsible for inducing the *in vivo* host innate immune and inflammatory responses [7].

TcdB is a virulence factor with high cytotoxicity that inhibits cell viability and induces apoptosis [8], also causes severe intestinal injury through the release of pro-inflammatory cytokines, including tumour necrosis factor (TNF)-α and interleukin (IL)-1β [9,10]. TNF-α and IL-1β have been reported to activate all three mitogen-activated protein kinases (MAPKs), including p38, JNK1/2 and ERK1/2, and NF-κB signalling in the inflammatory response [11–13], which in turn induce the production of TNF-α and IL-1β. TcdB activates the TNF-α-mediated ERK1/2 signalling with subsequent release of IL-8 from human colonocytes [14]. Inhibition of p38, ERK, or JNK dramatically reduced the production of TNF-α and IL-1β by bone marrow-derived dendritic cells or bone marrow-derived macrophages exposed to TcdB [15]. MAPK phosphatases (MKPs) also known as dual-specificity protein phosphatases (DUSPs) negatively regulate MAPKs activity in mammalian cells [16]. For example, DUSP1 and DUSP4 inactivate all three MAPKs and DUSP2 inactivates both p38 and ERK while DUSP5 and DUSP6 selectively inactivate ERK. Additionally, the correlation between DUSP1 and NF-κB activation was also found in different cell types [17–19]. However, the role of DUSP/MAPK and NF-κB signalling in the regulation of TcdB-induced intestinal inflammation remains unknown.

The E3-ligase tripartite motif (TRIM) family of proteins plays important role in immune responses through regulating various signalling pathways. Among the TRIM protein family, TRIM8, TRIM22 and TRIM52 have been reported to potentiate NF-κB transcription as well as TNF-α- and IL-1β-induced activation of NF-κB [20–22]. Silence of TRIM45 and TRIM3 elevated the phosphorylation level of p38, ERK1/2 and JNK1/2 in cervical cancer cells [23,24]. However, whether and how other...
TRIMs regulates MAPKs and NF-κB signalling in TcdB-induced intestinal inflammation is not clear. In this study, we determined the molecular mechanism underlying TcdB-induced colonic inflammation. We found that TRIM46 silencing inhibits TcdB-induced the release of TNF-α and IL-1β and activation of MAPKs and NF-κB signalling in FHC human normal colon epithelial cells. Mechanistically, TRIM46 ubiquitinates DUSP1, which in turn induces activation of NF-κB and MAPKs. Animal model bearing the infection of C. difficile and silence of TRIM46 further confirmed the mechanism and implied the promise in the future treatment.

Materials and methods

Cell culture

FHC human normal colon epithelial cells obtained from Cell Bank of China Academy of Sciences (Shanghai, China) were cultured in the DMEM medium (Gibco BRL, Carlsbad, MD) contained with 10% foetal bovine serum (Cambrex Bio Technology, Walkersville, MD) and 100 U/ml penicillin-streptomycin (Solarbio, Beijing, China) at 37 °C.

Cell viability assay

TcdB-induced cytotoxicity in FHC cells was determined using the Cell Counting Kit-8 (CCK-8) (Dojindo Co. Ltd., Kumamoto, Japan) assay. Briefly, FHC cells (3 x 10^3 cells/well) were cultured in a 96-well plate, and cell viability was documented by administration of CCK-8 solution (10 μl per well) in FHC cells treated with 50, 100, 200, 400 and 600 ng/ml of recombinant C. difficile Toxin B/TcdB protein (purity > 95%, endotoxin level < 1.0 EU/μg of protein) purchased from R&D Systems (# 6246-GT; R&D Systems, Inc., Minneapolis, MN) every 24 h. The number of viable cells was determined at the wavelength of 450 nm using a DNM-9602 microplate reader (Proteny Medical Equipment Co., Ltd Nanjing, China).

Cell transfection

The RNAi (RNA interference) sequences targeting position 190–208 (siRNA-1; 5'-GGUGAGGAUAUGCAGACC-3'), position 196–214 (siRNA-2; 5'-GAUAGUCGAGACCUCAACU-3') or 203–221 (siRNA-3; 5'-AGACCUACUUCACUCAAU-3') of the human TRIM46 gene were transfected into FHC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), as per the manufacturer's protocol. TRIM46 and DUSP1 overexpression was constructed by cloning full-length human TRIM46 and DUSP1 into the lentiviral expression vector pLVX-Puro. 293T cells were transfected with the recombinant lentivector using Lipofectamine 2000, as per the manufacturer's protocol. The packaging, purification and titration of lentivirus were performed as previously described [25], and then FHC cells were infected with virus-containing supernatants. Cells transfected with scramble siRNA (siNC) or blank lentivirus (Vector) were used as negative controls. Full-length or mutant DUSP1 cDNA was cloned and inserted into pCMV-Tag 2B vector, and the constructed vectors were named as DUSP1 (WT), DUSP1 (K57R), DUSP1 (K122R) and DUSP1 (K192R). Mutations were introduced by the site-directed mutagenesis using the QuikChange mutagenesis kit (Agilent Technologies, Santa Clara, CA). Myc-tagged TRIM46 sequence was purchased from GENEWIZ, Inc. (Suzhou, China) and cloned into p-DONR221 vector to express myc-TRIM46. For his-ubiquitin (Ub), human ubiquitin was cloned into pcDNA-DEST40 vector with a His tag. All constructs and mutants were confirmed by sequencing. The DUSP1 (WT) or mutant DUSP1 constructs along with myc-TRIM46 and His-Ub constructs were co-transfected into HEK293T cells using the Lipofectamine 2000.

ELISA analysis of TNF-α and IL-1β

The supernatants of cell culture or peripheral blood were collected at different time points. Concentrations of pro-inflammatory cytokines (TNF-α and IL-1β) were analyzed using human TNF-α and IL-1β ELISA Kits from JRDUN Biotechnology Co., Ltd. (Shanghai, China) and mouse TNF-α and IL-1β ELISA Kits from Shanghai Xin Yu Biotech Co., Ltd. (Shanghai, China) following the instructions of manufacturer's protocol.

RNA isolation and real-time PCR

The total RNA from BGC-823 and MKN-45 cells was extracted using TRIzol reagent (Gibco BRL, Grand Island, NY). Then Reverse Transcription System Kit (Takara Biomedical Technology (Beijing) Co., Ltd, Beijing, China) was used to synthesize the first-strand from 1 μg of total RNA, as per the manufacturer's protocols. Quantitative real-time PCR was performed using SYBR Green real-time PCR Master Mix (Takara, Kusatsu, Japan) on an ABI 7300 instrument (Applied Biosystems, Foster City, CA). The sequences of primer are listed in Table 1. Expression levels are given as ratios to GAPDH, and each assay was performed in triplicate. The relative fold changes in messenger RNA expression were calculated using the 2^-△△Ct method.

Western blotting

Intestinal tissues and FHC cells were lysed by RIPA lysis buffer (Solarbio, Beijing, China). After centrifugation, the supernatants were collected and BCA reagent was used to determine the concentration of protein, while changes in NF-κB and MAPKs. Animal model bearing the infection of C. difficile and silence of TRIM46 further confirmed the mechanism and implied the promise in the future treatment.
Table 1. Real-time PCR primer sequences.

| Gene      | Accession number | Primer sequence (5'-3') |
|-----------|------------------|-------------------------|
| TRIM8     | NM_001345950.1   | F: AACGGCTTACCAGCCTTACC R: CCCGGATTCCTCTCCTCG |
| TRIM11    | NM_145214.2      | F: GGAAGACCTCAGGGAAGGC R: CAGCAAAGGGGAAGAACG |
| TRIM22    | NM_00119573.1    | F: TCTCAAGAGGGGAAGAACG R: CCGGCCAATGTTGTCTGTG |
| TRIM24    | NM_015905.2      | F: TCTTGCTGTGAATGCTTTCTTG R: TCGCTGGTCCTTGCTGATAG |
| TRIM46    | NM_00125660.1    | F: CCCGGATTTCATTCCTTCG R: CAGCAAACGGCGAAGACG |
| TRIM52    | NM_032765.3      | F: CTGTGCCCCACTCCTTATCG R: TCTTGACCACCTCGTTTATGC |
| TRIM65    | NM_001256124.1   | F: TACCGCCAATACATGAAAGTG R: TGTGGCTGTGCTGTGATAG |
| TRIM68    | NM_018073.7      | F: TACTCCGTTGATAAGGCTAG R: GTGTTGTTGGTTCCAATGCTG |
| DUSP1     | NM_004417.3      | F: GCTGGGCGCTCTTCTTCG R: CGCCGTTGCAGCAACACC |
| GAPDH     | NM_001256799.2   | F: AGGCTGTGCTCACATACCTTC |

Cell Signalling Technology, Beverly, MA), DUSP1 (Ab138265; 1:500), DUSP2 (Ab137640; 1:100), DUSP4 (Ab72593; 1:500), DUSP5 (Ab19684; 1:1000), DUSP6 (Ab76310; 1:1000), anti-H3 (ab1791; 1:1000) and TRIM46 (Ab169044; 1:1000; all from Abcam, Cambridge, MA). Then, the membranes were incubated with horseradish peroxidase (HRP)-conjugated IgG (Sigma-Aldrich, St. Louis, MO). After washing, bound antibodies were detected with immobilization western chemiluminescent HRP substrate (Millipore, Temecula, CA) and analyzed using an Odyssey Infra-red Imaging System Scan and software (LI-COR Biosciences, Lincoln, NE).

Dual-luciferase reporter assay

The pGL3-Enhancer Firefly luciferase reporter was co-transfected with pRL-TK Renilla luciferase reporter plasmid (Promega, Madison, WI) using Lipofectamine 2000 reagent according to the manufacturer’s protocol into the cells under the different indicated treatments. Luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s instructions. Relative luciferase activity was calculated as the ratio of Firefly/Renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed as described previously [26]. The chromatin solutions were incubated overnight with NF-kBp65 or control IgG antibody. The immune complex was then captured by protein A/G beads. The purified DNA from the immune complex was subjected to SYBR green real-time PCR. The 3’ untranslated region (3’-UTR) was used as a negative control.

Co-immunoprecipitation and ubiquitination assay

Cell lysates were prepared with Radioimmunoprecipitation assay (RIPA) lysis buffer, incubated with anti-DUSP1 (J0930; Novus Biologicals, LLC., Littleton, CO), anti-TRIM46 (orb164435; Biorbyt LLC., San Francisco, CA), or normal IgG antibody (sc-2027; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then with Protein A/G PLUS-Agarose beads (sc-2003; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h at 4°C. The immunocomplexes were washed three times in lysis buffer and then analyzed by Western blot analysis using anti-DUSP1 (ab138265; Abcam, Cambridge, MA) and anti-ubiquitin (ab7780; Abcam, Cambridge, MA) antibody.

His-ubiquitin pull-down assay

HEK293T cells were cotransfected with the DUSP1 (WT) or mutant DUSP1 constructs along with myc-TRIM46 and His-Ub constructs. After 48 h transfection, cell lysates were incubated with Ni2+-NTA agarose beads (Qiagen, Valencia, CA). The washed complexes were eluted by boiling in SDS sample buffer and separated by SDS-PAGE and the interactions were analyzed by western blotting.

Animal experiments

Three shRNAs targeting mouse TRIM46 (point 691-709 shRNA-1, 5’-GCTACCTCTGTAAACGAT-3’; point 1261-1279 shRNA-2, 5’-GGAGGAAACACGCTCATA-3’; point 1439-1457 shRNA-3, 5’-CCGGCGCTCAGGATCAA-3’) were cloned into the pShuttle-H1 adenovirus vector. HEK293A cells were transfected with the recombinant adenoviral vector using Lipofectamine 2000, as per the manufacturer’s instruction. The packaging, purification and titration of adenovirus were performed as previously described [27]. Forty-eight hour after transfection, cell culture medium containing viral particles was collected. Clostridium difficile strain VPI 10463 obtained from ATCC was cultured in Brain Heart Infusion medium (Becton, Dickinson and Company, Franklin Lakes, NJ) at 37°C in an anaerobic environment (Anaerocult A, Merck, Darmstadt, Germany). Thirty-six male C57/B6J mice aged 8 weeks obtained from Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China) were intragastric administration of saline (0.5 ml) on day 0. Mice received a single intragastric administration of saline (0.5 ml) on day 0 as control. Mice (six per group) were killed on days 1 and 3. At the time of sacrifice, the concentration of TNF-α and IL-1β in the peripheral blood was determined with ELISA, and the colon tissues were collected, fixed and stained with haematoxylin and eosin (H&E) as previously described [28]. The related animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Fudan University.

Statistical analysis

Data are presented as mean±SD of three independent experiments with triplicate items. Samples were evaluated by the SPSS/Win11.0 software (SPSS, Inc., Chicago, IL) using one-way ANOVA followed by Dunnett’s post-hoc test.
When $p < .05$, the difference between groups was statistically
significant.

**Results**

**TcdB inhibited cell viability and induced production of IL-1β and TNF-α in FHC cells**

To determine the TcdB-induced cytotoxicity in FHC cells, cell viability was detected by CCK-8 assay. Figure 1(A) demonstrates that treatment of FHC cells with different concentrations of TcdB ranging from 50 to 600 ng/ml for 48 and 72 h markedly suppressed cell viability in a dose-dependent manner. However, only 600 ng/ml of TcdB significantly inhibited the cell viability at 24 h. We found that the viability of FHC cells was worse as the treatment time increased from 24 to 72 h. Furthermore, to investigate the effect of TcdB on colonic inflammation, the release of pro-inflammatory cytokine TNF-α and IL-1β from FHC cells was measured by ELISA. As shown in Figure 1(B,C), treatment of FHC cells with different concentrations of TcdB ranging from 50 to 600 ng/ml for 24 h significantly increased the production of TNF-α and IL-1β in a dose-dependent manner.

**Effect of TcdB on the DUSP/MAPKs and NF-κB signalling pathway in FHC cells**

Given the important role of TNF-α- and IL-1β-induced activation of MAPKs and NF-κB in the inflammatory response [11–13], the expression of ERK1/2, JNK1/2 and p38 and their phosphorylation levels as well as the NF-κBp65 levels in nucleus and cytoplasm fractions were also measured by western blotting. We found that treatment of FHC cells with 200 ng/ml TcdB for 1 h led to a noticeable increase in the phosphorylation levels of ERK1/2, JNK1/2, p38 and NF-κBp65 as well as NF-κBp65 nuclear translocation, but had no effect on the expression of ERK1/2, JNK1/2 and p38 in FHC cells (Figure 2(A,B)). DUSPs including DUSP1, DUSP2, DUSP4, DUSP5 and DUSP6 that act as negative regulators of MAPK and NF-κB activity were also measured in FHC cells with 200 ng/ml TcdB treatment for 24 h. We found that TcdB significantly inhibited the protein expression of DUSP1, DUSP2, DUSP5 and DUSP6 by 67.1%, 65.7%, 61.5% and 28.1%, respectively, compared with control (Figure 2(C)). These data suggest that TcdB may activate MAPKs and NF-κB signalling through regulation of the DUSP protein expressions. DUSP1 that can inactivate all three MAPKs and NF-κB and demonstrated the lowest expression in response to TcdB is, thus, investigated in our following study.
Since TcdB did not change the mRNA expression of DUSP1 in FHC cells (data not shown), we speculate that TcdB inhibits DUSP1 expression through post-transcriptional regulation. As expected, treatment of FHC cells with TcdB at concentrations ranging from 100 to 400 significantly induced DUSP1 ubiquitination in a dose-dependent manner (Figure 2(D)). Meanwhile, TRIM family proteins that act as E3-ubiquitin ligases, including TRIM8, TRIM11, TRIM22, TRIM24, TRIM46, TRIM52, TRIM65 and TRIM68, have been found to be associated with inflammatory response \[20–22,29–33\] and are therefore examined in response to TcdB. As shown in Figure 2(E), FHC cells with 200 ng/ml TcdB treatment for 24 h markedly decreased the mRNA expression of TRIM22 by 18.6% and increased the mRNA expression of TRIM8, TRIM11, TRIM24, TRIM46, TRIM52 and TRIM65 by 20.8%, 48.7%, 24.6%, 200.9%, 50.1% and 103.8%, respectively, compared with control.

**Effect of TRIM46 on TcdB-mediated inflammatory response and DUSP1/MAPKs and NF-κB signalling**

Given the role of TcdB in the regulation of TRIMs expression, we indicate that TRIMs may involve in TcdB-mediated DUSP1/MAPKs signalling and inflammatory response. TRIM46 that shows the highest expression in response to TcdB is introduced and silenced by siRNA in FHC cells. FHC cells transfected with siRNAs targeting human TRIM46 showed marked decrease in the mRNA and protein expression of TRIM46 compared with the siRNA-NC group (Figure 3(A,B)).
Moreover, the increased release of TNF-α and IL-1β induced by 200 ng/ml TcdB was markedly reversed by siRNA-1 and siRNA-2 transfection, respectively (Figure 3(C,D)). The results indicate that TRIM46 knockdown inhibits TcdB-induced inflammatory response in FHC cells.

Next, we measured DUSP1/MAPKS and NF-κB signalling in response to TcdB in the presence of TRIM46 knockdown. After treatment of FHC cells with 200 ng/ml TcdB, the decreased DUSP1 expression and increased phosphorylation levels of p38, ERK1/2, JNK1/2 and NF-κBp65 and the NF-κBp65 nuclear translocation were significantly reversed by siRNA-1 and siRNA-2 transfection, respectively (Figure 4(A–G)). These results suggest that TRIM46 knockdown inhibits TcdB-induced inflammatory response in FHC cells.

**Effect of DUSP1 overexpression on TRIM46-mediated release of TNF-α and IL-1β**

To investigate the involvement of DUSP1 in the regulation of TRIM46-mediated inflammatory response, FHC cells were transduced with pLVX-Puro-TRIM46 and pLVX-Puro-DUSP1 lentivirus. Overexpression of TRIM46 and DUSP1 was significant increase in the mRNA and protein expression of TRIM46 and DUSP1, respectively (Figure 5(A–D)). In addition, after FHC cells were transduced with pLVX-Puro-TRIM46, the decreased DUSP1 protein expression and increased release of TNF-α and IL-1β were significantly inhibited by DUSP1 overexpression (Figure 5(E–G)). These findings indicate that DUSP1 inhibits TRIM46-induced inflammatory response in FHC cells.

Since TRIM46 overexpression did not change the mRNA expression of DUSP1 in FHC cells (Figure 5(E)), we speculate that TRIM46 inhibits DUSP1 expression through post-transcriptional regulation. Our co-immunoprecipitation analysis showed an interaction between TRIM46 and DUSP1 (Figure 5(H)). Moreover, TRIM46 overexpression induced ubiquitination of DUSP1 (Figure 5(I)) and pull down results indicated that the DUSP1 K57 is required for TRIM46 induced ubiquitination of DUSP1 (Figure 5(J)).

**NF-κB as a transcription factor involves in TcdB-induced TRIM46 and DUSP1 expression**

To further investigate the role of NF-κB signalling in TcdB-induced regulation of TRIM46/DUSP1, PDTC, an inhibitor of
NF-κB, was used. As shown in Figure 6(A), TcdB-induced increased TRIM46 and decreased DUSP1 expression was significantly inhibited by PDTC in FHC cells. Since NF-κB is a transcription factor, we, therefore, investigated whether it is capable of regulating the TRIM46 transcription. Predictably, TcdB treatment markedly increased the activation of TRIM46 promoter, which was reversed by PDTC stimulation (Figure 6(B)). Promoter of TRIM46 (−43 to −32) was predicted binding to NF-κBp65 using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), a virtual laboratory for the identification of putative transcription factor binding sites (TFBS) in DNA sequences from a species or groups of species of interest, and our ChIP assay further indicated that NF-κBp65 was directly bound to the TRIM46 promoter (−674 to +51) (Figure 6(C)).

**In vivo study of inflammation-induced effects of TcdB and therapeutic potential of TRIM46 knockdown**

To study the role of TRIM46 in the context of TcdB-induced colonic inflammation, we constructed mouse model bearing the infection of *C. difficile* with or without intravenous injection of pShuttle-H1-TRIM46-shRNA-mix adenovirus vector. After the mice were killed after 1 and 3 days, the pathological change of the colonic tissues, the expression of TRIM46 and DUSP1 in colonic tissues and the content of TNF-α and IL-1β in the peripheral blood were measured respectively. Compared to the colonic tissues from control mice which showed a normal configuration of the gland without oedema and inflammatory cell infiltration, *C. difficile* infection resulted in unclear colon mucosa tissue with obvious submucosal oedema and swelling, and the presence of neutrophils. However, mice that intravenous injection of pShuttle-H1-TRIM46-shRNA-mix adenovirus vector showed alleviated inflammatory cell infiltration, submucosal oedema and swelling (Figure 7(A)). Moreover, *C. difficile* infection also increased TRIM46 expression and release of TNF-α and IL-1β and decreased DUSP1 expression, which were inhibited by intravenous injection of pShuttle-H1-TRIM46-shRNA-mix adenovirus vector (Figure 7(B–F)). Collectively, our in vivo studies confirm that *C. difficile* induces colonic inflammation through targeting TRIM46.

Figure 4. Effect of TRIM46 knockdown on TcdB-induced DUAP1/MAPKs and NF-κB signalling in FHC cells. FHC cells were treated with 200 ng/ml of TcdB with or without TRIM46 siRNA transfection, and the levels of DUSP1 (A, B), p-p38, p38 (A, C), p-JNK1/2, JNK1/2 (A, D), p-ERK1/2, ERK1/2 (A, E), and cytoplasmic, nuclear, phosphorylated and total of NF-κBp65 (A, F, G) were measured by western blotting. ⋆⋆⋆p < 0.001 compared with siRNA-NC or control. ###p < 0.001 compared with siRNA-NC plus TcdB.
Discussion

The main pathogenesis of *C. difficile* infection includes imbalance in the intestinal flora and impaired host immunity as well as *C. difficile* toxin, which is the main pathogenic factor. Most pathogenic *C. difficile* strains produce two major toxins, toxin A and toxin B, which directly affect intestinal epithelial cells, causing the release of cytokines, cell apoptosis or necrosis, and thus leading to inflammation and the intestinal tract [9,34]. It is worthy to note that TcdB plays more important roles than TcdA in the pathogenesis of *C. difficile* infection [15]. Therefore, we investigated the role of TcdB in intestinal inflammation and the possible mechanism involved. Our results showed that TRIM46 silencing inhibits TcdB-induced the release of TNF-α and IL-1β and the activation of MAPKs and NF-κB in FHC cells. Mechanistically, TRIM46 ubiquitinates DUSP1, which in turn induces activation of NF-κB and MAPKs signalling pathways. Animal model bearing infection of *C. difficile* demonstrated alleviated colonic inflammation after silence of TRIM46. Accordingly, we speculated that up-regulated TRIM46 may ubiquitinates DUSP1 which inhibits NF-κB and MAPKs activation and reduces release of TNF-α and IL-1β and the inflammatory response (Figure 7(G)).

Although both of TcdA and TcdB induced toxic in the cells, TcdB displayed a higher cytotoxicity than TcdA [6]. In this study, treatment of FHC cells with different concentrations of TcdB ranging from 50 to 600 ng/ml for 48 and 72 h resulted in cell viability inhibition, suggesting its cytotoxicity. However, the concentrations of TcdB ranging from 50 to 400 ng/ml for 24 h had no cytotoxicity. These results were partly consistent with the previous study that TcdB at concentrations ranging from 100 to 400 ng/ml for 48 and 72 h inhibited cell proliferation while high concentrations of TcdB (200 and 400 ng/ml) also inhibited cell proliferation for 24 h [8]. A significant increase of proinflammatory cytokines TNF-α and IL-1β in patients with *C. difficile* infection has been reported. The release of TNF-α and IL-1β that has been found to be important mediators of intestinal inflammation was significantly increased by TcdB at concentrations ranging from 50 to 600 ng/ml. These findings were in agreement with those published previously [15,35].
MAPKs are recognized as important pathways in the regulation of inflammatory response, especially in the occurrence and development of intestinal inflammation. Host-derived TNF-α and IL-1β can activate MAPK pathways in intestinal epithelial cells [36], which in turn activate the expression of proinflammatory cytokines including themselves. Moreover, NF-κB is a dimeric transcription factor that activates the expression of many genes involved in the inflammatory process, e.g. the cytokines IL-1β and TNF-α, two NF-κB targeted genes [37,38]. In this study, we found that TcdB could induce activation of MAPKs and NF-κB, which was consistent with the previous studies [14,15,39]. Moreover, DUSP1 showed the most decreased protein expression in response to TcdB compared with other indicated DUSPs. In addition to inhibiting MAPKs activation, DUSP1 also inversely correlates with NF-κB activity and expression [17], and, thus, supporting the therapeutic value of DUSP1/NF-κB in inflammatory processes [18]. Taken together, these findings may provide the molecular basis in the context of TcdB-induced MAPKs and NF-κB activation and the release of TNF-α and IL-1β.

Given the role of TcdB in the ubiquitination of DUSP1, the E3-ubiquitin ligases TRIM family proteins that are associated with inflammatory response were increased by TcdB in FHC cells, with the most increased expression of TRIM46. TRIM46 was associated with gout, a common form of inflammatory arthritis caused by hyperuricaemia [31]. TcdB activated NF-κB and MAPKs signalling pathways and induced release of TNF-α and IL-1β were inhibited by TRIM46 silencing, suggesting its important role in TcdB-induced colonic inflammation. Additionally, TRIM46 overexpression induced release of TNF-α and IL-1β was inhibited by DUSP1 overexpression. Mechanically, TRIM46 interacts with DUSP1 and inhibits DUSP1 protein expression through ubiquitination at K57 site. Additionally, NF-κBp65 was directly bound to the TRIM46 promoter and thus regulated the expression of TRIM46 and downstream DUSP1. Therefore, our data indicate that TcdB/TRIM46 significantly promoted inflammation in FHC cells by increasing the activity of NF-κB, which was attributed to enhancement of expression levels of TNF-α and IL-1β, representing a target amenable of therapeutic intervention. However, the mechanism by which TRIM46 activates NF-κB remains to be fully understood. It is likely that inhibition of DUSP1-induced blockade of MAPK signalling may contribute to TRIM46’s actions since MAPK signalling has been reported to regulate NF-κB [17,18]. These, together, prompted us to hypothesize that TRIM46 may promote TcdB-induced TNF-α and IL-1β-associated inflammation in FHC cells through activating MAPKs via ubiquitination of DUSP1 and through activating a positive feedback loop with the NF-κBp65.

In vivo, deletion of TRIM46 significantly reduced the C. difficile infection-induced intestinal injury and release of IL-1β and TNF-α and decreased DUSP1 expression; an effect that was mimicked by TRIM46 in FHC cells in vitro. In agreement with our findings in vivo, a previous study reported that...
persistent of *C. difficile* infection exhibited significant signs of chronic intestinal disease and also markedly increased pro-inflammatory factor expression in caecal tissue of mice, similar to the human immune response [40,41]. Pre-treatment with the IL-1 receptor antagonist anakinra significantly reduced *C. difficile* toxin-induced intestinal injury and inflammation in mice [9]. Eventually, our *in vivo* studies confirmed the potential role of TRIM46/DUSP1 in the context of colonic inflammation caused by TcdB.

In conclusion, we proved that TcdB significantly inhibited FHC cell viability and induced the production of IL-1β and TNF-α and the activation of MAPK and NF-κB signalling by increasing TRIM46 expression. TRIM46 inhibited DUSP1 protein expression through ubiquitination. These findings would help inform the targeted drug development against TcdB-induced colonic inflammation in the future.

**Disclosure statement**

The authors declare that they have no competing interests.

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