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MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS

Fidaxomicin and OP-1118 Inhibit *Clostridium difficile* Toxin A- and B-Mediated Inflammatory Responses via Inhibition of NF-κB Activity

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ABSTRACT *Clostridium difficile* causes diarrhea and colitis by releasing toxin A and toxin B. In the human colon, both toxins cause intestinal inflammation and stimulate tumor necrosis factor alpha (TNF-α) expression via the activation of NF-κB. It is well established that the macrolide antibiotic fidaxomicin is associated with reduced relapses of *C. difficile* infection. We showed that fidaxomicin and its primary metabolite OP-1118 significantly inhibited toxin A-mediated intestinal inflammation in mice *in vivo* and toxin A-induced cell rounding *in vitro*. We aim to determine whether fidaxomicin and OP-1118 possess anti-inflammatory effects against toxin A and toxin B in the human colon and examine the mechanism of this response. We used fresh human colonic explants, NCM460 human colonic epithelial cells, and RAW264.7 mouse macrophages to study the mechanism of the activity of fidaxomicin and OP-1118 against toxin A- and B-mediated cytokine expression and apoptosis. Fidaxomicin and OP-1118 dose-dependently inhibited toxin A- and B-induced TNF-α and interleukin-1β (IL-1β) mRNA expression and histological damage in human colonic explants. Fidaxomicin and OP-1118 inhibited toxin A-mediated NF-κB phosphorylation in human and mouse intestinal mucosae. Fidaxomicin and OP-1118 also inhibited toxin A-mediated NF-κB phosphorylation and TNF-α expression in macrophages, which was reversed by the NF-κB activator phorbol myristate acetate (PMA). Fidaxomicin and OP-1118 prevented toxin A- and B-mediated apoptosis in NCM460 cells, which was reversed by the addition of PMA. PMA reversed the cytoprotective effect of fidaxomicin and OP-1118 in toxin-exposed human colonic explants. Fidaxomicin and OP-1118 inhibit *C. difficile* toxin A- and B-mediated inflammatory responses, NF-κB phosphorylation, and tissue damage in the human colon.

KEYWORDS *Clostridium difficile* infection, antibiotics, signaling

Clostridium difficile infection (CDI) is a gastrointestinal disease associated primarily with the use of antibiotics in a hospital setting (1). With increasing incidence, morbidity, and mortality rates, CDI is a prominent constituent of antibiotic-associated diarrhea and intestinal inflammation (2, 3). *C. difficile*, an anaerobic bacterium, releases toxin A and toxin B, which mediate diarrhea and colitis in animals and humans. Toxin A and toxin B increase proinflammatory cytokine (tumor necrosis factor alpha [TNF-α] and interleukin-1β [IL-1β]) mRNA expression levels and cause histological damage in fresh human colonic explants (4). These toxins also cause epithelial cell apoptosis, as they inactivate GTPases and cause cytoskeleton disruption leading to cell rounding (3,

Received 24 July 2017 **Returned for modification** 15 August 2017 **Accepted** 12 October 2017

Accepted manuscript posted online 16 October 2017

Citation Koon HW, Wang J, Mussatto CC, Ortiz C, Lee EC, Tran DH-N, Chen X, Kelly CP, Pothoulakis C. 2018. Fidaxomicin and OP-1118 inhibit *Clostridium difficile* toxin A- and B-mediated inflammatory responses via inhibition of NF-κB activity. Antimicrob Agents Chemother 62:e01513-17. https://doi.org/10.1128/AAC.01513-17.

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5). Our previous studies also showed that NF-κB activation plays a major role in the proinflammatory effect of *C. difficile* toxin A *in vitro* (6, 7).

Fidaxomicin is a poorly absorbed antimicrobial macrolide (8, 9), which emerged as a new therapeutic agent for CDI. Fidaxomicin has an antibacterial effect against various strains of *C. difficile* by inhibiting the RNA polymerase sigma subunit, which blocks the protein synthesis of bacteria (10). The MIC range of fidaxomicin against *C. difficile* is approximately 0.001 to 1 μg/ml (11). While CDI cure rates with vancomycin, metronidazole, and fidaxomicin are similar, fidaxomicin use is associated with substantially reduced rates of CDI relapse (12–14). Evidence suggests that fidaxomicin exerts its therapeutic effect primarily by preventing bacterial RNA transcription (15) and inhibiting toxin A and toxin B production in CDI (16). Fidaxomicin is hydrolyzed into a less active metabolite, OP-1118, in the intestine (17). The MIC range of OP-1118 against *C. difficile* bacteria is around 0.25 to 2 μg/ml (18).

We recently reported that fidaxomicin and its primary metabolite OP-1118 significantly inhibited *C. difficile* toxin A-mediated enteritis in mice and reduced toxin A-mediated cell rounding in fibroblasts, suggesting that in addition to its antibacterial effects against *C. difficile*, fidaxomicin may also exert potent anti-inflammatory effects against intestinal responses to *C. difficile* toxins (19). The potential anti-inflammatory effects of fidaxomicin and OP-1118 in human colonic tissue, which is the primary target tissue of *C. difficile* and its toxins, however, have never been determined. Also, the mechanism(s) by which fidaxomicin exerts its anti-inflammatory and cytoprotective effects against *C. difficile* toxin A and toxin B remains to be elucidated.

Based on these considerations, this study addressed the hypothesis that fidaxomicin and OP-1118 possess anti-inflammatory and cytoprotective effects against *C. difficile* toxins in the human colon by interfering with NF-κB signaling pathways commonly activated by these toxins.

RESULTS

Fidaxomicin and OP-1118 inhibit toxin A- and toxin B-induced TNF-α and IL-1β mRNA expression in human colonic explants. We previously reported that the administration of fidaxomicin or OP-1118 in mouse ileal loops significantly reduced ileal IL-1β expression in response to toxin A injection (19). To assess the potential anti-inflammatory role of fidaxomicin and OP-1118 in the human colon, we treated fresh human colonic explants with various concentrations of fidaxomicin or OP-1118, followed by exposure to toxin A or toxin B. The levels of mRNA expression in explant tissues and protein secretion into conditioned medium were then determined. As shown in Fig. 1, toxin A and toxin B stimulated TNF-α mRNA expression (Fig. 1A). Fidaxomicin and OP-1118 dose-dependently reduced the TNF-α mRNA expression level induced by toxin A and toxin B (Fig. 1B). However, only toxin B significantly induced TNF-α protein secretion into conditioned medium (Fig. 1C and D). Fidaxomicin and OP-1118 dose-dependently reduced TNF-α protein secretion induced by toxin B.

We also observed that toxin A and toxin B stimulated IL-1β mRNA expression (Fig. 2A). Fidaxomicin and OP-1118 dose-dependently reduced IL-1β mRNA expression induced by both toxins (Fig. 2B). Only toxin B significantly induced IL-1β protein secretion into conditioned medium, but both fidaxomicin and OP-1118 dose-dependently reduced IL-1β protein secretion induced by toxin B (Fig. 2C and D).

Fidaxomicin and OP-1118 inhibit toxin A-induced TNF-α expression via NF-κB phosphorylation. Toxin A and toxin B induced mucosal NF-κB phosphorylation in human colonic tissues (Fig. 3A; see also Fig. S1A in the supplemental material), which was reduced by the pretreatment of human colonic explants with fidaxomicin or OP-1118. Similarly, fidaxomicin or OP-1118 also substantially reduced NF-κB phosphorylation in toxin A-treated mouse ileal loops (Fig. 3B and Fig. S1B). Toxin B did not induce NF-κB phosphorylation in mouse ileal loops (data not shown). Based on this evidence, we next determined whether the anti-inflammatory mechanism of fidaxomicin and OP-1118 against toxin A and toxin B involved NF-κB activation.

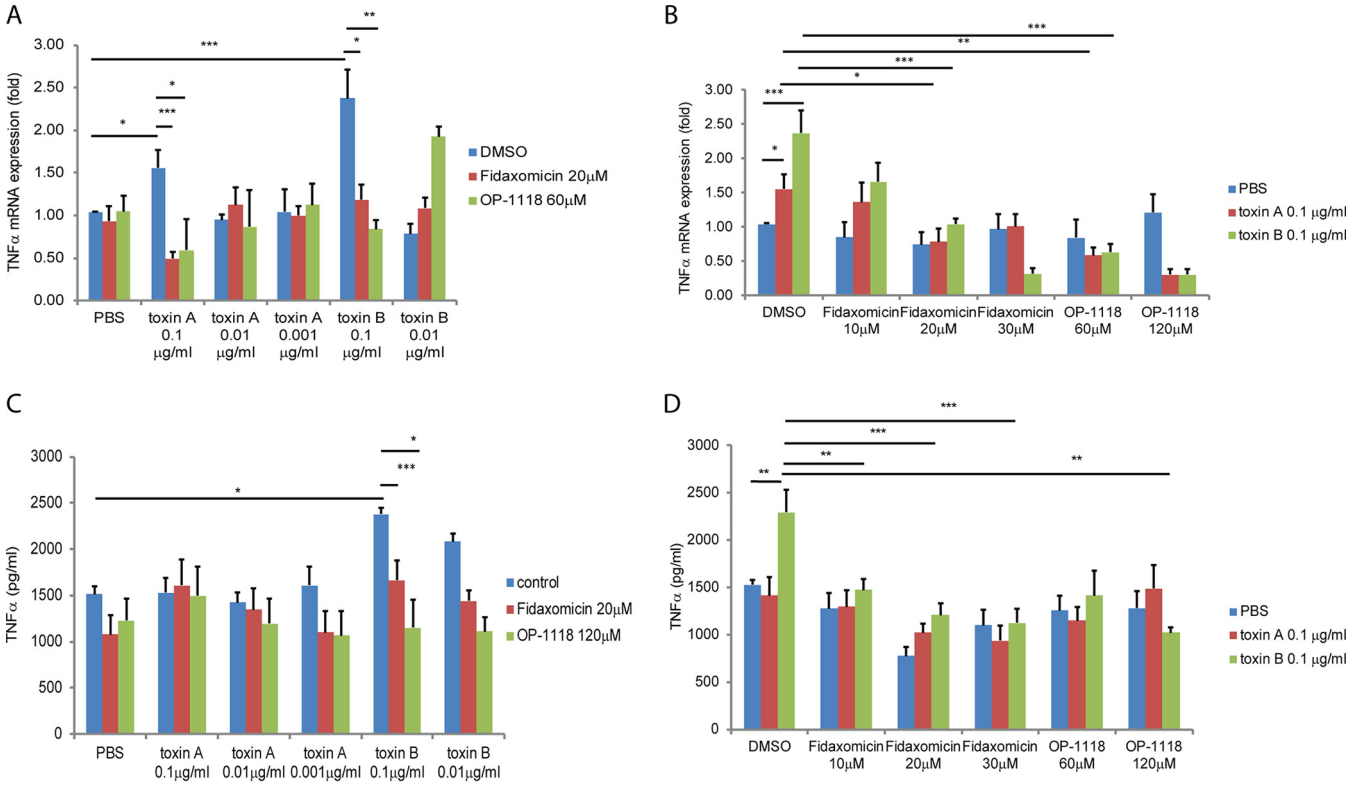

FIG 1 Fidaxomicin and OP-1118 inhibit toxin A- and B-induced TNF-α mRNA expression in human colonic explants. (A) Dose response of toxin A- and B-induced TNF-α mRNA expression. DMSO was used as a solvent for fidaxomicin and OP-1118. (B) Dose response of fidaxomicin and OP-1118 in toxin A- and B-induced TNF-α mRNA expression. (C) Dose response of toxin A- and B-induced TNF-α protein expression. (D) Dose response of fidaxomicin and OP-1118 in toxin A- and B-induced TNF-α protein expression. Each group consisted of 6 fresh human colonic explants. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

To address this, we used the well-established RAW264.7 mouse macrophage cell line, which is known to respond to toxin A (6). Our results show that, similar to our findings for mouse ileum and human colon (Fig. 3 and Fig. S1), exposure of macrophages to toxin A induced NF-κB phosphorylation (Fig. 4A and B). Preincubation of cells with fidaxomicin dose-dependently reduced toxin A-induced NF-κB phosphorylation (Fig. 4A and Fig. S1C). Similarly, OP-1118 inhibited toxin A-induced NF-κB phosphorylation in RAW264.7 cells. This inhibition was reversed by the NF-κB activator phorbol myristate acetate (PMA) (Fig. 4B and Fig. S1D). Together, these results suggest that fidaxomicin and its active metabolite OP-1118 inhibit NF-κB activation.

NF-κB activation is involved in the transcription of several proinflammatory cytokines, including TNF-α (20). To examine the functional importance of the anti-NF-κB response exerted by fidaxomicin and OP-1118 in the proinflammatory effects of toxin A, we tested the effects of fidaxomicin and OP-1118 on toxin A-induced TNF-α secretion in RAW264.7 cells. Consistent with previously reported observations (21), toxin A induced TNF-α protein expression in RAW264.7 macrophages, which was significantly reduced by the pretreatment of cells with fidaxomicin or OP-1118 (Fig. 4C and D). Toxin B did not induce TNF-α protein expression in RAW264.7 cells (data not shown). Furthermore, pretreatment of the cells with PMA reversed the inhibitory effects of fidaxomicin and OP-1118 on TNF-α expression in the presence of toxin A (Fig. 4C and D). These findings suggest that fidaxomicin and OP-1118 inhibit toxin A-induced TNF-α expression via NF-κB inhibition.

Fidaxomicin and OP-1118 inhibit toxin A- and B-mediated tissue damage in human colonic explants via NF-κB inhibition. We next studied this protective response in normal human colonic explants. Toxin A and toxin B caused epithelial layer disruption of fresh human colonic explants (Fig. 5), in line with data from our previous reports (4, 22, 23). Incubation of human colonic explants with fidaxomicin or OP-1118

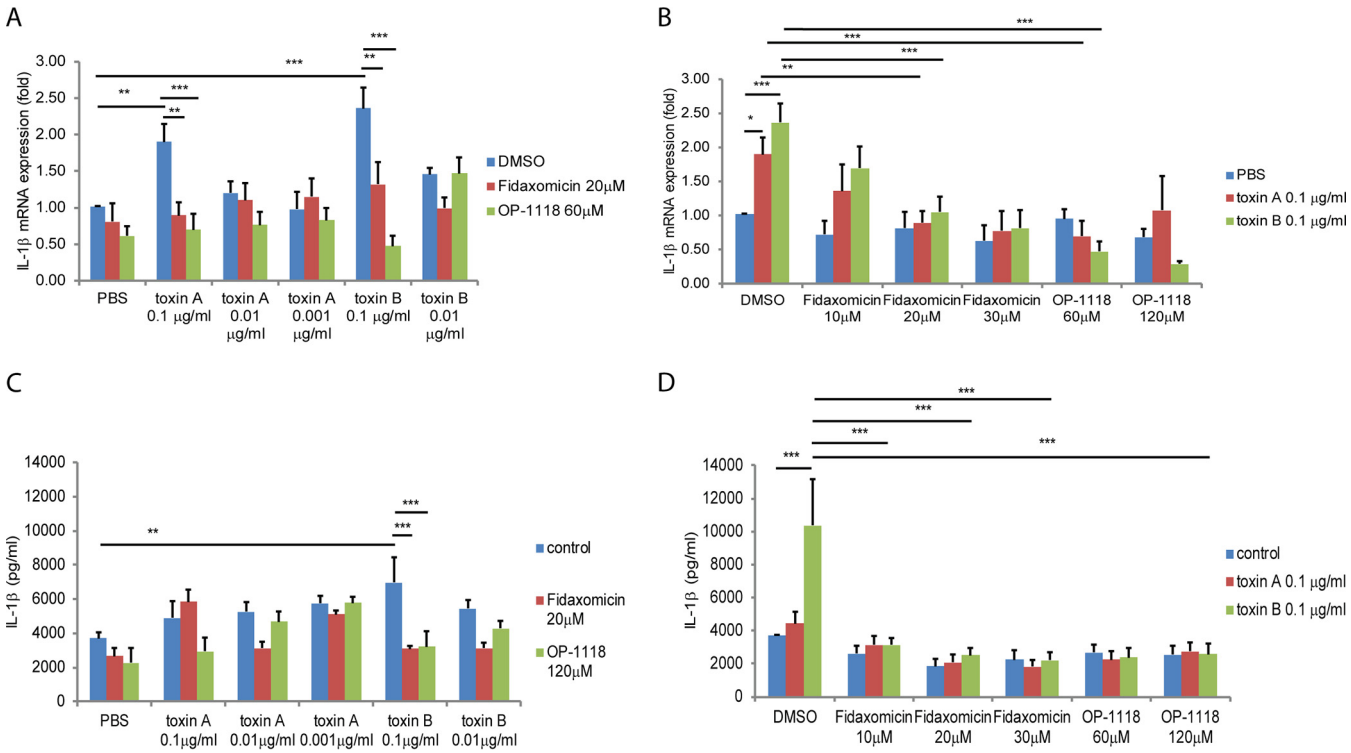

FIG 2 Fidaxomicin and OP-1118 inhibit toxin A- and B-induced IL-1β mRNA expression in human colonic explants. (A) Dose response of toxin A- and B-induced IL-1β mRNA expression. (B) Dose response of fidaxomicin and OP-1118 in toxin A-and B-induced IL-1β mRNA expression. (C) Dose response of toxin A-and B-induced IL-1β protein expression. (D) Dose response of fidaxomicin and OP-1118 in toxin A- and B-induced IL-1β protein expression. Each group consisted of 6 fresh human colonic explants. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

reversed toxin A- and B-mediated tissue damage (Fig. 5B). Exposure to fidaxomicin, OP-1118, or PMA in the absence of toxin A or toxin B did not cause tissue damage (data not shown). However, the addition of PMA reversed all cytoprotective effects of fidaxomicin and OP-1118 against toxin A and toxin B in human colonic explants (Fig. 5 and data not shown).

Fidaxomicin and OP-1118 inhibit toxin A- and B-mediated apoptosis in colonic epithelial cells via NF-κB inhibition. We also determined whether fidaxomicin and OP-1118 protect cells from apoptosis in response to *C. difficile* toxins. As expected (22, 24), both toxin A and toxin B caused apoptosis of human colonic NCM460 epithelial cells as detected by a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (Fig. S2A and S2B). Coincubation of NCM460 cells with fidaxomicin or OP-1118 significantly reduced toxin A- and B-mediated apoptosis (Fig. S2A and S2B). The activation of NF-κB by PMA reversed the antiapoptotic effect of fidaxomicin and OP-1118 on toxin A- and B-exposed cells (Fig. S2A and S2B). In contrast, we did not observe TUNEL-positive cells in fidaxomicin-, OP-1118-, or PMA-treated cells in the absence of toxin exposure (data not shown). This finding suggests that fidaxomicin and OP-1118 inhibit toxin A- and B-induced apoptosis, likely through modulation of the NF-κB pathway.

DISCUSSION

Our recent results using mouse ileal loops indicated that fidaxomicin and its primary metabolite OP-1118 inhibit *C. difficile* toxin A-associated inflammatory responses (19). We showed that both toxins increased TNF-α and IL-1β mRNA expression levels in human colonic explants, as previously described (4), and increased the phosphorylation of the p65 subunit of NF-κB (6). All of these responses were reduced by fidaxomicin and OP-1118 (Fig. 1 to 3). In addition, fidaxomicin and OP-1118 significantly inhibited toxin A- and B-mediated histologic damage in human colonic tissues and apoptosis in human

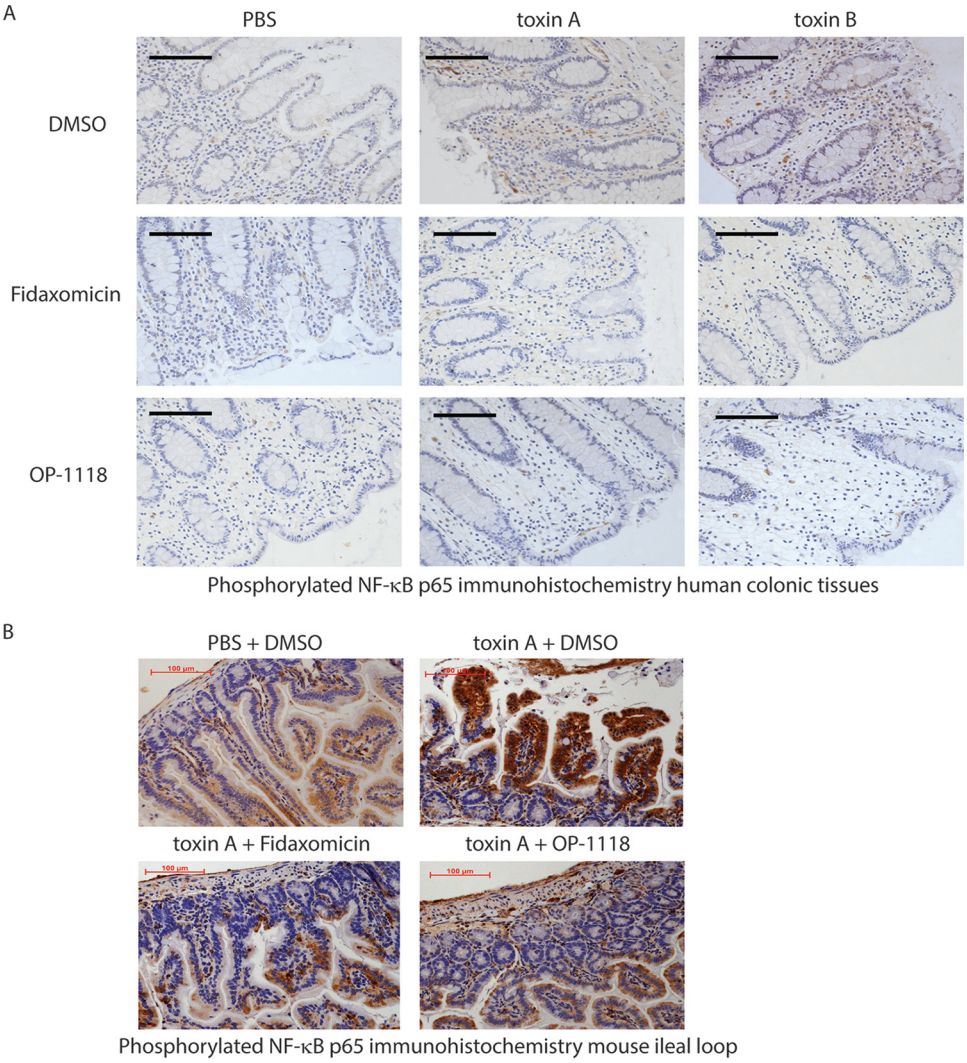

FIG 3 Fidaxomicin and OP-1118 inhibit toxin A- and B-induced NF-κB phosphorylation in human colonic explants and mouse ileal loops. The phosphorylated NF-κB signal in human colonic tissues (A) and mouse ileal loops (B) is shown in brown. (A) Each group consisted of 6 fresh human colonic explants. (B) Each group consisted of 6 mice. Magnification, ×200. Black bars, 100 μm.

colonic epithelial cells, respectively (Fig. 5; see also Fig. S2 in the supplemental material). Together, these results strongly indicate that these anti-inflammatory and antiapoptotic mechanisms may contribute to the therapeutic effects of fidaxomicin on *C. difficile* infection by preserving the integrity of the human colonic mucosa following toxin exposure. These results are also consistent with anti-inflammatory effects of other macrolides on nonintestinal cells and tissues (25–27).

Compared to toxin A, toxin B had a potent effect in inducing increased TNF-α and IL-1β mRNA levels after 4 h of incubation (Fig. 1 to 2). Previous studies indicated that toxin B is more potent than toxin A in inducing epithelial cell damage in the human colon at 5 h (22) and that both toxins are proinflammatory toxins in human intestinal mucosa (28). Data from previous studies by Lyras et al. using toxin B mutants in the *C. difficile* hamster model also underline the importance of toxin B in CDI pathophysiology (29, 30). Overall, our results demonstrate that fidaxomicin exerts anti-inflammatory and cytoprotective effects in the human colon against not only toxin A but also toxin B, which may be relevant for the potential effectiveness of fidaxomicin in CDI caused by hypervirulent toxin A-negative, toxin B-positive *C. difficile* strains such as those of ribotype 017 (31).

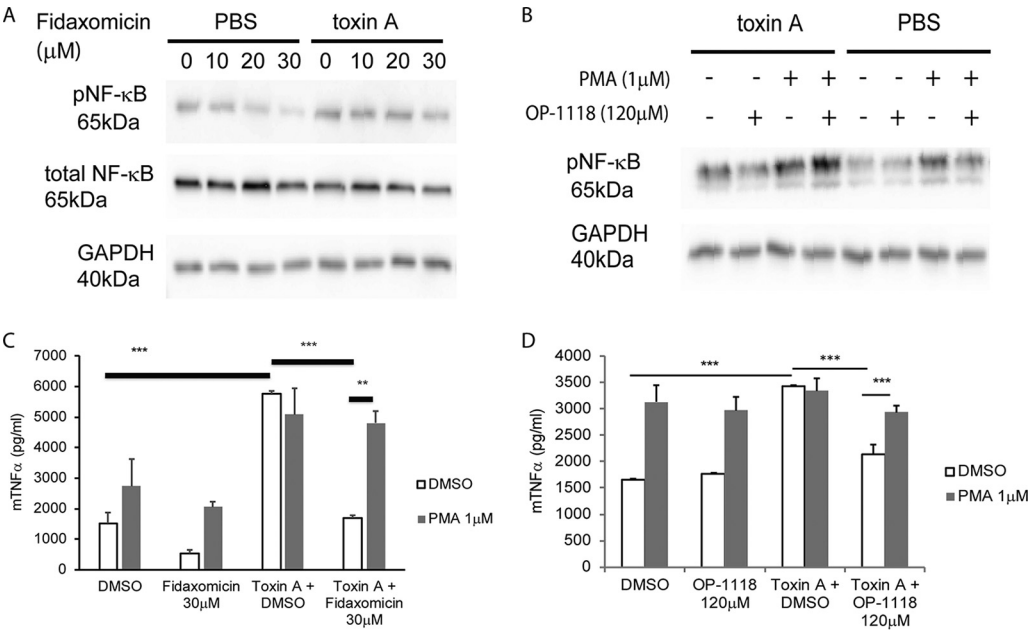

FIG 4 Fidaxomicin inhibits toxin A-mediated TNF-α expression via NF-κB inhibition. (A and B) Western blot showing the protein signals of phosphorylated NF-κB (pNF-κB), total NF-κB, and GAPDH in RAW264.7 macrophages. (C and D) TNF-α levels in conditioned medium of RAW264.7 macrophages. The results are pooled from three separate experiments. **, $P < 0.01$; ***, $P < 0.001$.

Although both toxins induced TNF-α and IL-1β mRNA expression, only toxin B sufficiently induced the protein secretion of TNF-α and IL-1β in human colonic tissues after 4 h of exposure (Fig. 1 and 2). This discrepancy between the levels of mRNA and protein secretion of these cytokines in response to toxin A may be due to the sensitivities of the methods used for their detection (more sensitive reverse transcription-PCR [RT-PCR] for mRNA and a less sensitive protein enzyme-linked immunosorbent assay [ELISA] for protein).

We previously demonstrated that toxin A induces TNF-α expression via an NF-κB-dependent pathway in mouse macrophages (6). Previous results also indicate that both toxin A and toxin B can activate NF-κB-driven cytokine genes in human colonocytes and monocytes (7, 32). Furthermore, toxin A can activate NF-κB in human colonic biopsy specimens via the activation of reactive oxygen species (7). Here we found that fidaxomicin and OP-1118 inhibited NF-κB activation and reduced the expression level of the NF-κB-driven cytokine TNF-α in response to both toxins in human colonic explants as well as in response to toxin A in mouse ileal loops and macrophages. The NF-κB activator PMA was able to reverse the inhibition of toxin A-induced TNF-α expression caused by fidaxomicin and OP-1118, strongly suggesting that the anti-inflammatory effects of this drug and its active metabolite involve the inhibition of the NF-κB system.

It is well accepted that both *C. difficile* toxins trigger apoptosis and apoptosis-related cell death in several target cells, including intestinal epithelial cells (33–35). Our results indicate that in NCM460 human colonic epithelial cells, both toxins increased the number of TUNEL-positive cells, and coincubation of cells with fidaxomicin and OP-1118 can inhibit this effect (Fig. S2). Interestingly, the activation of NF-κB signaling by the addition of PMA reversed the antiapoptotic effect of fidaxomicin and OP-1118 on toxin A- and B-exposed cells (Fig. S2). This finding suggests that fidaxomicin and OP-1118 inhibit toxin A- and B-induced apoptosis in human colonocytes by modulating the NF-κB pathway. Previous results indicated that toxin A-induced apoptosis *in vitro* and *in vivo* (evidenced by increased Fas ligand expression) was linked to NF-κB activation (34). Moreover, NF-κB has been shown to regulate inflammasome expression (36), as the activation of the inflammasome mediates apoptosis (37). Both toxin A and toxin B also mediate cell apoptosis and damage via the inflammasome-dependent

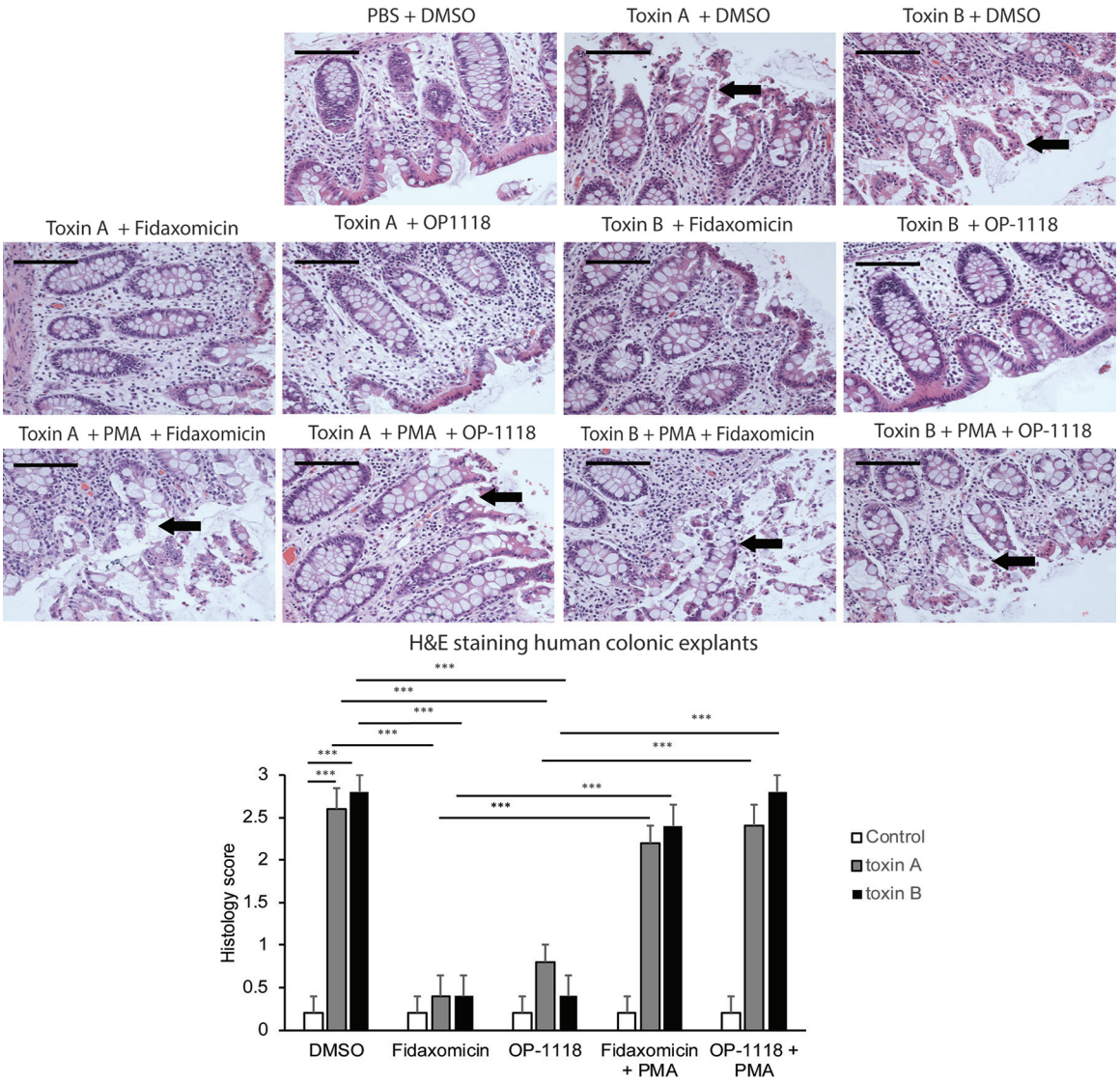

FIG 5 Fidaxomicin inhibits toxin A- and B-mediated epithelial cell damage in fresh human colonic explants via NF-κB inhibition. (Top) The histological structure of the treated human colonic tissues was evaluated by H&E staining at a ×200 magnification. Luminal epithelial cell linings were damaged in human colonic explants exposed to toxin A or toxin B. Arrows indicate the locations of epithelial damage. Black bars, 100 μm. (Bottom) Histology scores for human colonic explants. *C. difficile* toxins caused damage to the colonic mucosal epithelial layer. The data are pooled from 6 patients per group. ***, $P < 0.001$.

release of IL-1β (38). Gerhard et al., however, presented evidence suggesting that toxin A-induced apoptosis (evidenced by caspase activation) depends on the glucosylation of Rho GTPases (39), consistent with previous observations that perturbations of the actin cytoskeleton can mediate apoptosis (40, 41). Although the details of these complex associations need further investigation, the inhibitory effects of fidaxomicin and OP-1118 on the NF-κB pathway and on cytoskeletal disruption may be associated with the suppression of the inflammasome and subsequent apoptosis.

Certain patients with CDI develop systemic infections, which require concomitant antibiotic treatment. However, these concomitant antibiotics often compromise the cure rate for vancomycin against CDI. Fidaxomicin has a higher cure rate than does vancomycin in patients with concomitant antibiotic treatment (42). Fidaxomicin-treated patients also have a lower relapse rate than do vancomycin-treated patients in this concomitant antibiotic-exposed patient cohort (42). We speculate that the anti-inflammatory effect of fidaxomicin may be important for CDI because certain anti-inflammatory drugs (such as aspirin and statin) may reduce the risk of CDI (43, 44). The

pleiotropic mechanisms of fidaxomicin may confer advantages over vancomycin for the treatment of CDI.

In summary, fidaxomicin and its active metabolite OP-1118 significantly reduce *C. difficile* toxin A- and B-mediated proinflammatory cytokine expression and histologic damage in human colonic tissues. These protective effects provide novel insights into the therapeutic mechanism of this antibiotic for CDI.

MATERIALS AND METHODS

***C. difficile* culture and toxin purification.** *C. difficile* strain VPI 10463 (ATCC 43255) was cultured in Difco cooked meat medium (catalog number 226730; BD, Fisher Scientific) at 37°C under anaerobic conditions, and toxin A and toxin B were purified as previously reported (45). The purity of the purified toxin A and toxin B preparations was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Coomassie blue staining, as described previously (45). The cytotoxicity of toxin A and toxin B was determined by cell rounding in 3T3 fibroblasts, as described previously (19).

Human colonic explants. Fresh human colonic explants were obtained from the UCLA surgical pathology department. Institutional Review Board (IRB) approval was waived since no patient-identifiable information was obtained. We used fresh human colonic explants collected from noncancerous regions of colon cancer patients, as previously described (4). The fresh colonic explants were cut into 3- by 3-mm pieces. The explants were placed into RPMI 1640 medium and treated with dimethyl sulfoxide (DMSO) (0.8%), fidaxomicin (10 to 30 μM), or OP-1118 (60 to 120 μM) for 30 min, followed by phosphate-buffered saline (PBS) (1 μl/ml), toxin A (0.001 to 0.1 μg/ml), or toxin B (0.01 to 0.1 μg/ml) for 1 or 4 h as indicated. Some groups were pretreated with DMSO (1 μl/ml) or PMA (1 μM) for 30 min before exposure to fidaxomicin or OP-1118.

Histology scoring. Fresh human colonic explants were treated with DMSO (1 μl/ml) or PMA (1 μM) for 30 min, followed by fidaxomicin (30 μM), OP-1118 (120 μM), or DMSO (0.8%) for 30 min. Tissues were then incubated with PBS (1 μl/ml), toxin A (1 μg/ml), or toxin B (1 μg/ml) for 4 h, followed by formalin fixation overnight. The fixed tissues were sectioned and paraffin embedded at the UCLA tissue processing core laboratory (TPCL).

Some of the tissue sections were stained with hematoxylin and eosin (H&E). The stained slides were analyzed by two independent observers in a blind manner. Epithelial damage was quantified by observing at least 20 different fields of H&E-stained intestinal sections from each group. A histology score of 0 to 3 was assigned, as previously described (46).

Mouse ileal loops. Paraffin-embedded sections of mouse ileal loop tissues were obtained from a previous study (19). Ileal loops of mice were pretreated with fidaxomicin (20 μM), OP-1118 (120 μM), or DMSO (0.8%) for 30 min, followed by exposure to *C. difficile* toxin A (10 μg per ileal loop) or PBS alone (200 μl per ileal loop) for 4 h. The animal study was approved by the UCLA Institutional Animal Research Committee (approval number 2007-116).

Phosphorylated NF-κB immunohistochemistry. Phosphorylated NF-κB immunohistochemistry of human colonic and mouse ileal tissues was performed by the UCLA TPCL. Paraffin-embedded sections were cut at a 4-μm thickness, and paraffin was removed with xylene and rehydrated through graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. Heat-induced antigen retrieval (HIER) was carried out for all sections with 0.01 M citrate buffer (pH 6) by using a Biocare decloaker at 95°C for 25 min. After treatment with blocking buffer (2% bovine serum albumin [BSA]) for 1 h, the slides were then incubated overnight at 4°C with rabbit polyclonal antibody to phosphorylated NF-κB p65 in 2% BSA at a 1:100 dilution (catalog number ab86299; Abcam). The signal was detected by using the rabbit horseradish peroxidase EnVision kit (catalog number K4003; DakoCytomation). This secondary antibody kit was directly applied to the slides, without dilution. All sections were visualized with the diaminobenzidine reaction and counterstained with hematoxylin.

Images were taken with a Zeiss AX10 microscope in a blind manner. The stained slides were analyzed by two independent observers in a blind manner. Four different locations per tissue section were observed and scored. The quantitative intensity of the phosphorylated NF-κB signal was observed and scored as 0 for normal, 1 for mild, 2 for moderate, or 3 for strong intensity, as described previously (47).

Cell culture. Mouse RAW264.7 macrophages were purchased from the ATCC and cultured in T75 flasks containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen), as described previously (6). Human colonic NCM460 epithelial cells were cultured in M3D medium (Incell) containing 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen), as described previously (48).

TNF-α and IL-1β ELISAs. The levels of proinflammatory mediators, including human TNF-α (catalog number DY210; R&D Systems), human IL-1β (catalog number DY201; R&D Systems), and mouse TNF-α (catalog number DY410; R&D Systems), were measured according to the manufacturer's instructions. RAW264.7 macrophages in 12-well plates (1×10^5 cells/well; 1 ml/well) were pretreated with DMSO (1 μl/ml) and PMA (1 μM) for 30 min, followed by the addition of DMSO (0.8%), fidaxomicin (30 μM), or OP-1118 (120 μM) for 30 min, before exposure to toxin A (0.1 μg/ml; 1 μl/well) for 4 h. Conditioned media were then collected for mouse TNF-α ELISA measurements on 96-well plates (100 μl/well in triplicate).

Quantitative real-time RT-PCR. Total RNA was isolated by using an RNeasy kit (catalog number 74106; Qiagen, CA) and reverse transcribed into cDNA by using a Superscript III kit (catalog number 11752; Invitrogen, Carlsbad, CA). Quantitative PCRs were run in a Bio-Rad CFX384 PCR detector. The mRNA expression level was determined by using cataloged primers (Invitrogen) for human TNF-α

(Hs00174128_m1), IL-1β (Hs01555410_m1), and 18S (Hs99999901_s1). Results were expressed as relative fold differences normalized to the 18S signal.

Western blot analyses. Mouse RAW264.7 macrophages in 12-well plates (1×10^5 cells/well; 1 ml/well) were treated with DMSO (1 μl/ml) or PMA (1 μM) for 30 min, followed by various concentrations of fidaxomicin (10 to 30 μM), OP-1118 (120 μM), or DMSO (0.8%) for 30 min. Cells were then incubated with PBS (1 μl/ml), toxin A (1 μg/ml), or toxin B (1 μg/ml) for 1 h.

Treated cells were lysed in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitors (catalog number sc-24948; Santa Cruz Biotechnology). The protein concentrations were measured by using a bicinchoninic acid assay (BCA) method (catalog number 23225; ThermoFisher Scientific). The lysates were normalized to 20 μg protein per lane and added with blue loading buffer (catalog number 7722; Cell Signaling) to form a 1× loading mixture.

Equal amounts of cell extracts were fractioned by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred onto 0.45-μm nitrocellulose membranes (400 mA for 1.5 h; Bio-Rad). Membranes were blocked in 5% nonfat milk in Tris-buffered saline–Tween 20 (TBST) (50 mM Tris [pH 7.5], 0.15 M NaCl, 0.05% Tween 20) and then incubated with antibodies (phosphorylated NF-κB [catalog number ab86299; Abcam], total NF-κB [catalog number sc-372; Santa Cruz Biotechnology], and glyceraldehyde-3-phosphate dehydrogenase [GAPDH] [catalog number sc-25778; Santa Cruz Biotechnology]) at 1:500 ratios for 16 h at 4°C. The horseradish peroxidase-labeled antibody signal was detected by chemiluminescence using the LAS4000 luminescent-image analyzer (Fujifilm, Tokyo, Japan). The protein signal was quantified by using Multi Gauge software (Fujifilm).

TUNEL apoptosis detection. Human NCM460 colonic epithelial cells (10^5 cells/well; 1 ml/well) were cultured in chambered cell culture slides (catalog number 354104; Corning). Cells were treated with DMSO (1 μl/ml) or PMA (1 μM) for 30 min, followed by fidaxomicin (30 μM), OP-1118 (120 μM), or DMSO (0.8%) for 30 min. Cells were then incubated with PBS (1 μl/ml), toxin A (1 μg/ml), or toxin B (1 μg/ml) for 8 h.

The TPCL assisted with TUNEL staining. The relative severity of apoptosis was observed and scored based on TUNEL-positive signal as follows: 0 for normal, 1 for mild, 2 for moderate, and 3 for strong. Four different locations per well were observed and scored.

Statistical analyses. Quantitative results were expressed as means ± standard errors of the means. Results were analyzed by using the Prism 5 statistics software program (GraphPad). Ordinary two-way analysis of variance (ANOVA) (for examining effects of drug treatment and toxin treatment), followed by Bonferroni posttests, was used for intergroup comparisons unless indicated otherwise. Statistical significance is indicated in the figure legends. Error bars represent standard errors of the means.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01513-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

This study was supported by Merck, Inc. (C.P.). H.W.K. was also supported by NIH grant R03 DK103964. Support to C.P. was also provided by NIH/NIAID grant UO-1 AI124290, the Blinder Research Foundation for Crohn's Disease, and the Eli and Edythe Broad Chair. C.P.K. was supported by NIH grant R01 AI095256, and X.C. was supported by a career development award from the Crohn's and Colitis Foundation of America, Inc.

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