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J Immunol published online 3 December 2010
http://www.jimmunol.org/content/early/2010/12/01/jimmunol.1000043
Protective Roles of CX3CR1-Mediated Signals in Toxin A-Induced Enteritis through the Induction of Heme Oxygenase-1 Expression

Masanori Inui,*† Yuko Ishida,*† Akihiko Kimura,† Yumi Kuninaka,† Naofumi Mukaida,‡ and Toshikazu Kondo†

The injection of Clostridium difficile toxin A into the ileal loops caused fluid accumulation with the destruction of intestinal epithelial structure and the recruitment of neutrophils and macrophages. Concomitantly, intralereal gene expression of CX3CL1/fractalkine (FKN) and its receptor, CX3CR1, was enhanced. When treated with toxin A in a similar manner, CX3CR1-deficient (CX3CR1−/−) mice exhibited exaggerated fluid accumulation, histopathological alterations, and neutrophil recruitment, but not macrophage infiltration. Mice reconstituted with CX3CR1−/− mouse-derived bone marrow cells exhibited exacerbated toxin A-induced enteritis, indicating that the lack of the CX3CR1 gene for hematopoietic cells aggragated toxin A-induced enteritis. A heme oxygenase-1 (HO-1) inhibitor, tin-protoporphyrin-IX, markedly increased fluid accumulation in toxin A-treated wild-type mice, indicating the protective roles of HO-1 in this situation. HO-1 expression was detected mainly in F4/80-positive cells expressing CX3CR1, and CX3CR1−/− mice failed to increase HO-1 expression after toxin A treatment. Moreover, CX3CL1/FKN induced HO-1 gene expression by isolated lamina propria-derived macrophages or a mouse macrophage cell line, RAW264.7, through the activation of the ERK signal pathway. Thus, CX3CL1/FKN could induce CX3CR1-expressing macrophages to express HO-1, thereby ameliorating toxin A-induced enteritis. The Journal of Immunology, 2011, 186: 000–000.

Clostridium difficile is a Gram-positive anaerobic enteric bacterium that is the major cause of antibiotic-associated pseudomembranous colitis in humans, which is responsible for ~3 million cases of diarrhea and colitis annually in the United States, and has a mortality rate of 1–2.5% (1, 2). The most common risk factor is exposure to antibiotics with broad-spectrum activity such as penicillins, cephalosporins, and clindamycin (3). Exposure to antineoplastic chemotherapy or immunosuppressive agents has less commonly been described as a risk factor (4–6). C. difficile colitis is characterized by diarrhea, epithelial cell destruction, pseudomembrane formation, and increased production of pro-inflammatory mediators (7). C. difficile produces two exotoxins, toxins A and B. Toxin A challenge increases fluid secretion and causes mucosal injury with massive neutrophil recruitment in animals (8, 9).

Toxin A-induced mucosal inflammation is presumed to be initiated by several cytokines and chemokines (10–12); in particular, the chemokines with potent chemotactic activities for neutrophils (10, 11). Toxin A treatment induced the accumulation of macrophages together with enhanced expression of chemokines, which are active for macrophages (13–15). However, the roles of macrophages and the chemokines that can regulate macrophage recruitment remain elusive.

CX3CL1/fractalkine (FKN) is a single CX3C chemokine that characteristically exists in both soluble and membrane-bound forms (16–18). CX3CL1/FKN is presumed to be expressed on the surface of inflamed endothelial cells, epithelial cells, and macrophages and plays an important role in adhesion and migration of inflammatory cells. Moreover, CX3CR1, the unique receptor for CX3CL1/FKN, is predominantly expressed on monocytes/macrophages, NK cells, and CD8+ T cells (16). The CX3CL1/FKN–CX3CR1 interactions can contribute to the development of inflammatory diseases, such as Crohn’s disease, rheumatoid arthritis, and atherosclerosis (19–26). We previously observed that the CX3CL1/FKN–CX3CR1 axis is indispensable for optimal host defense in septic peritonitis (21). Moreover, Kostadinova and colleagues (27) demonstrated the crucial involvement of the CX3CR1–CX3CL1 axis in dextran sulfate sodium-mediated acute colitis in mice. These observations prompted us to examine the pathophysiological roles of the CX3CL1/FKN–CX3CR1 axis in toxin A-induced enteritis using CX3CR1-deficient (CX3CR1−/−) mice.

In this study, we demonstrated that the lack of CX3CR1 had few effects on macrophage recruitment during toxin A-induced enteritis. However, CX3CL1/FKN-induced macrophages to express heme oxygenase (HO)-1, the molecule that can prevent the development of toxin A-induced colitis, through ERK pathway activation.

Materials and Methods

Reagents and Abs

C. difficile toxin A, recombinant mouse CX3CL1/FKN, and tin-protoporphyrin-IX (SnPP) were purchased from List Biological Laboratories...
(Campbell, CA), R&D Systems (Minneapolis, MN), and Frontier Scientific (Logan, UT), respectively. The following Abs were used in the current study: rabbit anti-CX3CR1 pAbs (Torrey Pines Biolabs, East Orange, NJ); goat anti-CX3CL1/FKN p-Ab; rabbit anti-p38 MAPK p-Ab; rabbit anti-JNK p-Ab (Cell Signaling Technology, Beverly, MA); rabbit anti-p-ERK1/2 p-Ab, mouse anti-p-p38 MAPK mAb, rabbit anti-ERK pAb (Cell Signaling Technology, Beverly, MA); rabbit anti-p-JNK p-Ab (BioVision, Mountain View, CA); FITC-conjugated donkey anti-rabbit IgG p-Ab, FITC-conjugated donkey anti-goat IgG p-Ab, and Cy3-conjugated donkey anti-rat IgG pAbs (Jackson ImmunoResearch Laboratories, West Grove, PA).

**Animals**

Specific pathogen-free 8–10-wk-old male C57BL/6 mice were obtained from Sankyo Laboratories (Tokyo, Japan) and designated as wild-type (WT) mice in this study. CX3CR1<sup>-/-</sup> mice were a generous gift from Drs. P.M. Murphy and J.L. Gao (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (23). Age- and sex-matched CX3CR1<sup>-/-</sup> mice, backcrossed to C57BL/6 mice for 8–10 generations, were used in the following experiments. All mice were housed individually in cages under specific pathogen-free conditions, and all animal experiments were approved by the Committee on Animal Care and Use of Wakayama Medical University (Wakayama, Japan).

**Toxin A-induced enteritis**

Toxin A-induced enteritis was induced as described previously (8). Briefly, mice were anesthetized by i.p. injection of pentobarbital (50 mg/kg body weight), and a laparotomy was performed. Two 4-cm ileal loops were ligated and injected with either 5 mg toxin A in 200 ml PBS or equivalent

### Table 1. Sequences of the primers used for RT-PCR

<table>
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<tr>
<th>Transcript</th>
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<tr>
<td>CX3CR1</td>
<td>(F) 5‘-CCGTCTCTATTGAGCTCTA-3’&lt;sup&gt;r&lt;/sup&gt;&lt;br&gt; (R) 5‘-TGAGTCTCTGAAGCCTCT-3’&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>CX3CL1/FKN</td>
<td>(F) 5‘-ACCTATGCTCATTGACAGG-3’&lt;sup&gt;r&lt;/sup&gt;&lt;br&gt; (R) 5‘-CTGGCAAGCCCTCAGAATCAC-3’&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>HO-1</td>
<td>(F) 5‘-GGAAGAACCTGCTGACCTG-3’&lt;sup&gt;r&lt;/sup&gt;&lt;br&gt; (R) 5‘-GAGGAGATGAACTTGGGACCTC-3’&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-actin</td>
<td>(F) 5‘-ATGCCGTAAAGACCTCTATGCCAAC-3’&lt;sup&gt;r&lt;/sup&gt;&lt;br&gt; (R) 5‘-ATGGAGCCACCGATCCACA-3’&lt;sup&gt;r&lt;/sup&gt;</td>
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F, forward primer; R, reverse primer.

**FIGURE 1.** A and B, Gene expression of CX3CR1 and CX3CL1/FKN in toxin A-stimulated ileum of WT mice. CX3CR1 (A) and CX3CL1/FKN mRNA (B) were quantified by real-time RT-PCR as described in Materials and Methods. All values represent the mean ± SEM (n = 6 animals). CX3CR1 expression in the ileal tissues of WT mice before (C) or after (D) toxin A challenge. The left panel of C shows immunohistochemical images of CX3CR1. Immunofluorescence images of CX3R1 and F4/80 were digitally merged. CX3CL1/FKN expression in the ileal tissues of WT mice before (E) or after (F) toxin A challenge. The left panel of E shows immunohistochemical images of CX3CL1. Arrows indicate CX3CL1-positive endothelial cells. Immunofluorescence images of CX3CL1 and F4/80 were digitally merged. Representative results from six independent experiments are shown (original magnification ×200). *p < 0.05 versus untreated WT mice.

**FIGURE 2.** Toxin A-induced colitis in WT and CX3CR1<sup>-/-</sup> mice. A, At 4 h after toxin A or PBS administration, ileal loops were obtained. Representative macroscopic appearances of ileal loops from six independent experiments are shown. B, At 2 or 4 h after toxin A treatment, fluid accumulation in ileal loops was measured as the weight-to-length ratio. All values represent the mean ± SEM (n = 6 animals). **p < 0.01, CX3CR1<sup>-/-</sup> versus WT. C, The effects of anti-CX3CR1 Abs on fluid accumulation of WT mice treated with toxin A. All values represent the mean ± SEM (n = 6 animals). **p < 0.01, anti-CX3CR1 versus control IgG.
volume of PBS alone, and loops were returned to the abdominal cavity. At
the indicated time intervals, mice were sacrificed, and ileal loops were
removed to measure the length and weight. The obtained tissues were
further processed for histological analysis and protein and RNA extraction.
Fluid secretion was assessed as the loop weight-to-length ratio (mg/cm). In
some experiments, rabbit anti-CX3CR1 Ab (2 μg/mouse), recombinant
mouse CX3CL1/FKN (0.1 or 10 ng/mouse), or SnPP (25 mg/kg) was i.p.
administered at 1 h before toxin A challenge.

**Generation of bone marrow chimeric mice**

The following bone marrow (BM) chimeric mice were prepared: male
CX3CR1−/− BM→female WT mice, male WT BM→female CX3CR1−/−
mice, male WT BM→female WT mice, and male CX3CR1−/− BM→fe-
male CX3CR1−/− mice, as described previously (28). Briefly, recipient
mice were irradiated with 15 Gy using an RX-650 irradiator (Faxitron X-
ray, Wheeling, IL). BM cells were collected from the femurs of donor mice
by aspiration and flushing, and 5×10^6 BM cells in 200 μl sterile PBS

**FIGURE 3.** A, Histopathological observations of the ileum from WT and CX3CR1−/− mice at 4 h after toxin A or PBS treatment (H&E, original
magnification ×200). Representative results from six independent experiments are shown. Histopathological severity, including epithelial damage (B)
and congestion and edema (C), was scored at 4 h after toxin A challenge. All values represent the mean ± SEM (n = 6 animals). *p < 0.05, CX3CR1−/− versus
WT; **p < 0.01.

**FIGURE 4.** Leukocyte recruitment into the ileal tissue of WT and CX3CR1−/− mice. A, Immunohistochemical analysis of neutrophils with anti–Gr-1 Ab in
the ileum of WT and CX3CR1−/− mice at 4 h after toxin A treatment. Representative results from six independent experiments are shown (original magnification ×200). B, MPO activity was measured in the ileal loops of WT and CX3CR1−/− mice at 4 h after toxin A challenge, as described in Materials and Methods. All values represent the mean ± SEM (n = 6 animals). C, Immunohistochemical analysis of macrophages with anti-F4/80 Ab in the ileum of WT and CX3CR1−/− mice at 4 h after toxin A treatment. Representative results from six independent experiments are shown (original magnification ×200). D, Western blotting analysis using anti-F4/80 Ab and anti–β-actin Ab. Representative results from six independent experiments are shown. E, F4/80 to β-actin
ratios obtained by densitometry are shown. All values represent the mean ± SEM (n = 6 animals). **p < 0.01, CX3CR1−/− versus WT.
were i.v. administered to recipient mice. Thereafter, the mice were housed in sterilized microisolator cages and were fed on normal chow and autoclaved hyperchlorinated water for 60 d. To verify successful engraftment and reconstitution of the BM in the transplanted mice, genomic DNA was isolated from peripheral blood and tail tissues of each chimeric mouse 30 d after BM transplantation with a NucleoSpin tissue kit (Macherey-Nagel, Düren, Germany). Then, we performed PCR to detect the Sry gene contained in the Y chromosome (forward primer, 5′-TTGCTCTCAACA-AAA-3′; reverse primer, 5′-AAACTCGCTTCTGTCTGTTTT-3′). The amplified PCR products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. After durable BM engraftment was confirmed, mice were treated with toxin A as described above.

Histopathological and immunohistochemical analyses

Resected ileal loops were fixed in 4% formaldehyde buffered with PBS and then embedded with paraffin. Deparaffinized 6-μm-thick sections were stained with H&E solution, and histological changes were graded by an examiner without any prior knowledge of the experimental procedures as described previously (8). In parallel, immunohistochemical analysis with anti-Gr-1 mAb, anti-F4/80/80 mAb, anti-CX3CL1 pAbs, anti-CX3CR1 pAbs, and anti–HO-1 pAbs was conducted as described previously (8).

Double-color immunofluorescence analysis

Double-color immunofluorescence analysis was performed to determine the types of CX3CR1−, CX3CL1−, or HO−1−expressing cells in the ileum of WT mice before and after toxin A challenge, as described previously (29). Briefly, deparaffinized sections were incubated with PBS containing 1% normal donkey serum and 1% BSA to reduce nonspecific reactions. The sections were then incubated with the combination of anti-CX3CR1 and anti-F4/80/80, anti-CX3CL1/FKN and anti-F4/80/80, or anti–HO-1 and anti-F4/80/80 Abs at a concentration of 1 μg/ml at 4˚C overnight. Postincubation of fluorochrome-conjugated secondary Abs, images were observed under a fluorescence microscope.

Myeloperoxidase assay

Myeloperoxidase (MPO) activity in ileal loop segments was determined as described previously (8). Briefly, ileal scrapings were homogenized in 1 ml 50 mM potassium phosphate buffer with 0.5% hexadecyltrimethyl ammonium bromide (Sigma-Aldrich, St. Louis, MO). Samples were then frozen and thawed three times, sonicated for 20 s, and centrifuged at 12,000 × g at 4˚C. MPO activity in the supernatants was determined using a peroxidase assay kit (Sumitomo Bakelite, Osaka, Japan), and results were normalized with milligrams of protein (8).

Isolation of lamina propria macrophages

The lamina propria macrophages were isolated as described previously (30). Peyer’s patches, fatty tissues, and mesentery were removed from the small intestine. The small intestine was opened longitudinally and cut into 5-mm pieces. The pieces were washed gently and shaken twice for 20 min at 37˚C in RPMI 1640 medium supplemented with 10% FCS and 2 mM EDTA. Cell suspensions were passed through a strainer, and the remaining intestinal tissue was washed, then minced and shaken for 20 min at 37˚C in RPMI 1640 medium containing 10% FCS and 2 mg/ml collagenase. Cell suspensions were passed through a strainer, and the remaining cell suspensions were collected and passed through a strainer, then pelleted by centrifugation. This process was repeated two additional times. Collected cells were placed on a 40/75% discontinuous Percoll gradient, and cells at the interface between 40 and 75% were collected. Samples were enriched for CD11b+ cells by positive selection with CD11b microbeads.

Stimulation of isolated lamina propria macrophages or RAW264.7 cells with CX3CL1/FKN

The isolated lamina propria macrophages or RAW264.7 cells were cultured in DMEM medium containing 10% FCS, seeded at 1 × 10⁶ cells/well in six-well plates, and cultured overnight. After cells were stimulated further with various concentrations of recombinant mouse CX3CL1/FKN for 4 h at 37˚C, total RNA and protein were extracted for real-time PCR and Western blotting analysis, respectively. In some experiments, the cells were incubated with PD98059 (Sigma-Aldrich) for 1 h before CX3CL1/FKN stimulation.

Real-time RT-PCR

Total RNA was extracted from the ileal tissue or RAW264.7 cells with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). First-strand cDNA was synthesized from 1 μg total RNA with oligo(dT) primer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). mRNAs were quantified by real-time RT-PCR with SYBR Premix Ex Taq polymerase II (Takara Bio, Shiga, Japan) using the specific primers (Table I, Supplemental Table I).

Western blotting analysis

At the indicated time intervals after toxin A challenge, ileal loops were obtained and homogenized with a lysis buffer (10 mM PBS [pH 7.4] containing 0.01% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) containing Complete Protease Inhibitor Mixture, Phosphatase Inhibitor Cocktails for serine/threonine protein phosphatases, and tyrosine protein phosphatases (P2850 and P5726; Sigma-Aldrich) and were centrifuged to

FIGURE 5. Toxin A-induced enteritis in various BM chimeric mice. Recipient mice were transplanted with BM cells from CX3CR1−/− or WT donors as described in Materials and Methods. Toxin A or PBS was injected into the ileal loops of BM chimeric mice. A, Fluid accumulation in the ileal loops was measured at 4 h after toxin A injection. All values represent the mean ± SEM (n = 6 animals). *p < 0.05. B, Histopathological analyses of the ileum at 4 h after toxin A injection. H&E, original magnification ×200. Representative results from six independent experiments are shown. C, Histopathological severity, including epithelial damage, congestion, and edema, was scored at 4 h after toxin A challenge. All values represent the mean ± SEM (n = 6 animals). *p < 0.05.
obtain lysates. Thereafter, equal amounts of protein (10 μg) were separated by 5% or 15% SDS-PAGE and transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA). Postincubation of the membrane with anti-F4/80 mAb, anti–HO-1 pAbs, or anti–β-actin, immune complexes were detected using an ECL reagent (Millipore), according to the manufacturer’s instructions. In another series of experiments, lamina propria-derived macrophages or RAW264.7 cells were scraped and centrifuged, and the cell pellets were dissolved with SDS sample buffer. Thereafter, Western blotting analysis was performed to detect ERK1/2, p-ERK1/2, p38 MAPK, p-p38 MAPK, JNK, p-JNK, and anti–β-actin, as described previously (21).

Statistical analysis
The means and SEMs were calculated for all parameters determined in this study. Statistical significance was evaluated using ANOVA or Mann–Whitney U test. A p value < 0.05 was accepted as statistically significant.

Results
The expression of CX3CL1/FKN and CX3CR1 in the ileum of WT mice after toxin A challenge

We first analyzed the gene expression of CX3CR1 and its ligand, CX3CL1/FKN, in the ileal tissue after toxin A challenge. Under the present experimental conditions, both CX3CR1 and CX3CL1/FKN mRNA were detected in the ileal tissue of WT mice even before toxin A challenge. Toxin A injection significantly increased mRNA expression of CX3CR1 and CX3CL1/FKN at 4 h (Fig. 1A, 1B), whereas PBS injection failed to do so (data not shown). To determine the type of cells expressing CX3CL1/FKN and CX3CR1, we conducted immunohistochemical and double-color immunofluorescence analyses. CX3CR1 protein was exclusively detected on F4/80-positive cells in the ileum both before and after toxin A treatment (Fig. 1C, 1D). As reported previously (27, 31), in the ileum of untreated WT mice, CX3CL1/FKN was detected on epithelial cells and endothelial cells as well as F4/80-positive macrophages in lamina propria (Fig. 1E). Toxin A treatment disrupted the normal architecture of ileum, and CX3CL1/FKN was detected on F4/80-positive cells (Fig. 1F). In contrast, neither CX3CR1 nor CX3CL1/FKN was detected on Gr-1–positive neutrophils (data not shown). These observations implied that the major cellular source of CX3CL1 was F4/80-positive cells in the toxin A-treated ileum.

Exaggerated toxin A-induced enteritis in the absence of CX3CR1

To explore the pathophysiological roles of the CX3CL1/FKN–CX3CR1 axis in toxin A-induced enteritis, we induced toxin A-induced enteritis in WT and CX3CR1−/− mice. PBS injection failed to increase fluid accumulation in both WT and CX3CR1−/− mice until 4 h posttreatment (data not shown). In contrast, toxin A treatment increased fluid accumulation to a larger extent in CX3CR1−/− than WT mice (Fig. 2A, 2B). Likewise, combined treatment of toxin A and anti-CX3CR1 Ab enhanced fluid accumulation in WT mice compared with the treatment with toxin A and control Abs (Fig. 2C). Moreover, histopathological alterations such as epithelial damage, congestion, and edema were more evident in CX3CR1−/− mice than in WT mice (Fig. 3A–C). Collectively, these observations indicate that the absence of CX3CR1 exaggerated toxin A–induced colitis. Toxin A–induced neutrophil infiltration was more evident in CX3CR1−/− mice than WT mice, as evidenced by immunohistochemical analysis using anti–Gr-1 mAb and MPO assay (Fig. 4A, 4B). In contrast, toxin A induced intraileal macrophage infiltration to similar extents in WT and CX3CR1−/− mice, as evidenced by immunohistochemical analysis and Western blotting analysis using anti-F4/80 mAb (Fig. 4C, 4D). These observations indicate that the lack of the CX3CL1/FKN–CX3CR1 axis exaggerated toxin A-
induced neutrophil infiltration, with few effects on macrophage infiltration. Consistently, CX3CR1<sup>−/−</sup> mice exhibited exaggerated mRNA expression of neutrophil-tropic chemokines such as CXCL1/KC, CXCL2/MIP-2, and CCL3/MIP-1α, but not a macrophage-tropic chemokine, CCL2/MCP-1 (Supplemental Fig. 1).

**Essential involvement of BM-derived cells in toxin A-induced enteritis**

CX3CR1 can be expressed by nonhematopoietic cells such as endothelial cells in addition to hematopoietic cells such as F4/80-positive macrophages. To evaluate the roles of BM-derived cells in toxin A-induced enteritis, we generated BM chimeric mice between WT and CX3CR1<sup>−/−</sup> mice to distinguish between WT and CX3CR1<sup>−/−</sup> macrophages. To evaluate the roles of BM-derived cells in toxin A-induced enteritis, we generated BM chimeric mice between WT and CX3CR1<sup>−/−</sup> mice and injected toxin A into them. Irrespective of the recipient mice, the absence of CX3CR1 in BM cells significantly increased the fluid accumulation at 4 h after toxin A challenge (Fig. 5A). Consistently, histopathological damage was more evident in mice bearing CX3CR1<sup>−/−</sup> BM cells than those bearing WT BM cells (Fig. 5B, 5C). Collectively, these observations implied that BM-derived CX3CR1-expressing cells might have a protective role in toxin A-induced enteritis.

**Attenuated expression of macrophage-derived HO-1 in CX3CR1<sup>−/−</sup> mice treated with toxin A**

HO-1 is expressed by macrophages and possesses potent cytoprotective properties in various types of tissue injury including several colitis models (32, 33). This notion was further supported by the observation that SnPP, an HO-1 inhibitor, markedly increased fluid accumulation in WT mice when it was i.p. injected at 1 h before toxin A administration (Fig. 6A). We next examined the gene expression of HO-1 in the ileum of WT and CX3CR1<sup>−/−</sup> mice after toxin A treatment. Although toxin A challenge enhanced the gene expression of HO-1 in the ileum of WT and CX3CR1<sup>−/−</sup> mice, the increment was significantly attenuated in CX3CR1<sup>−/−</sup> mice compared with that of WT mice (Fig. 6B). Similar observations were made for HO-1 protein expression in the ileum of WT and CX3CR1<sup>−/−</sup> mice (Fig. 6C, 6D). Moreover, a double-color immunofluorescence analysis detected HO-1 protein on F4/80-positive macrophages (Fig. 6E) but not neutrophils (data not shown) in the course of toxin A-induced enteritis. These observations implied that the lack of CX3CR1 could attenuate toxin A-induced increase in HO-1 expression.

**CX3CL1–CX3CR1 axis upregulated HO-1 gene expression in macrophages through ERK activation**

In the next series, we examined the effects of CX3CL1 on HO-1 gene expression in lamina propria-derived macrophages. CX3CL1/FKN (1 or 10 ng/ml) enhanced the gene expression of HO-1 in lamina propria-derived macrophages (Fig. 7A). This increase was abrogated by preincubation of CX3CL1/FKN with anti-CX3CL1/FKN Ab (data not shown). Potential involvement of ERK, p38 MAPK, and JNK in HO-1 expression (34) prompted us to investigate the effects of CX3CL1/FKN on these signal pathways. CX3CL1/FKN enhanced phosphorylation of ERK but not p38 and JNK in lamina propria-derived macrophages (Fig. 7B–E). Furthermore, PD98059, an ERK inhibitor, significantly attenuated CX3CL1/FKN-induced HO-1 expression in lamina propria-derived macrophages (Fig. 7A), but the inhibition of p38 MAPK or JNK signal pathways had few effects on HO-1 gene expression by these macrophages (data not shown). Similar observations were made using a mouse macrophage cell line, RAW264.7 (Supplemental Fig. 2). Collectively, the CX3CL1/FKN–CX3CR1 interactions can activate the ERK signaling pathway and eventually enhance the expression of HO-1, the enzyme that has a protective role against toxin A-induced colitis.

**Exogenous CX3CL1/FKN treatment attenuated toxin A-induced enteritis**

Finally, we examined the effects of exogenous CX3CL1/FKN on toxin A-induced enteritis. CX3CL1/FKN significantly reduced fluid accumulation when it was i.p. injected into WT mice at 1 h before toxin A treatment (Fig. 8). These observations indicate that...
the administration of exogenous CX3CL1/FKN can be effective for the prevention of toxin A-induced enteritis.

Discussion
Toxin A-induced colitis model recapitulates the changes observed in antibiotic-associated pseudomembranous colitis in humans and is frequently used to explore the pathophysiological mechanism of pseudomembranous colitis (8). In this model, toxin A challenge to the ileal loops causes severe mucosal damage with massive neutrophil infiltration, suggesting that neutrophils have a pathogenic role in toxin A-induced enteritis (8). This assumption is supported by the observation that the depletion of neutrophils suppressed toxin A-induced enteritis (35). Neutrophil infiltration is regulated by a distinct set of chemokine receptors expressed by mouse neutrophils, such as CXCRI and CCR2. CXCL1/KC and CXCL2/MIP-2 bind CXCRI, whereas CCL3/MIP-1α binds CCR1. Several lines of evidence demonstrated that the expression of these neutrophil-tropic chemokines was locally enhanced in toxin A-induced enteritis (10, 11, 36–38). Moreover, immunoneutralization of CXCL2/MIP-2 or genetic disruption of CCR1, a receptor for MIP-1α, directly reduced neutrophil recruitment, with subsequent tissue destruction of the ileum after toxin A challenge (10, 11), indicating the pathogenic roles of these chemokines.

Our observation that the expression of both CX3CL1/FKN and CX3CR1 was upregulated in the ileum after toxin A challenge prompted us to explore the pathophysiological roles of the CX3CL1/FKN–CX3CR1 axis in toxin A-induced enteritis. Yang and colleagues (39) reported that the CX3CL1/FKN–CX3CR1 axis could regulate neutrophil recruitment through the upregulation of ICAM-1 expression on the endothelium. Hence, we assumed that the absence of CX3CR1 impaired neutrophil recruitment after toxin A challenge, resulting in attenuated toxin A-induced enteritis. However, CX3CR1+/− mice exhibited exacerbated toxin A-induced enteritis. Moreover, WT mice reconstituted with CX3CR1+/− BM exhibited exaggerated toxin A-induced enteritis. These observations indicate that the absence of CX3CL1/FKN–CX3CR1 axis on hematopoietic cells could accelerate toxin A-induced enteritis.

Toxin A-induced neutrophil infiltration was augmented in CX3CR1−/− mice compared with that in WT mice. However, CX3CR1 was expressed by F4/80-positive macrophages but not Gr−1-positive neutrophils (data not shown). Moreover, the expression of neutrophil-tropic chemokines such as CXCL1, CXCL2, and CCL3 was increased in CX3CR1−/− mice compared with that of WT mice (Supplemental Fig. 1). Thus, enhanced neutrophil infiltration in CX3CR1−/− mice may be ascribed to enhanced expression of these neutrophil-tropic chemokines, arising from CX3CR1 deficiency.

Gene disruption or immunoneutralization of CX3CR1 diminished macrophage recruitment in atherosclerosis, wound healing, and renal diseases (23–25, 40, 41), but the lack of CX3CR1 gene had few effects on macrophage infiltration in other conditions including thioglycolate-induced or cecal ligation and puncture-induced peritonitis (21, 42, 43). Likewise, there was no significant difference in macrophage recruitment or cecal ligation and puncture-induced peritonitis (21, 42, 43).

Likewise, there was no significant difference in macrophage infiltration in other conditions including thioglycolate-induced or cecal ligation and puncture-induced peritonitis (21, 42, 43). Moreover, HO-1, also known as heat shock protein 32, is a rate-limiting enzyme in the degradation of heme into biliverdin, carbon monoxide, and iron (50, 51) and can attenuate tissue injury caused by various inflammatory stimuli (33–35, 52–56). We observed that an HO-1 inhibitor, SnPP, aggravated toxin A-induced enteritis, as evidenced by an increased fluid accumulation in WT mice. Thus, HO-1 also has a protective role in toxin A-induced colitis. In the ileum of untreated WT mice, macrophages in lamina propria expressed HO-1 (data not shown), and, in the course of toxin A-induced enteritis, macrophages but not neutrophils were the cellular source of HO-1, consistent with a previous report (57). Moreover, HO-1 expression was significantly suppressed at both mRNA and protein levels in CX3CR1−/− mice compared with that in WT mice. Thus, the differences in toxin A susceptibility between WT and CX3CR1−/− mice can be ascribed to the differences in macrophage-derived HO-1 expression.

Our in vitro study demonstrated that CX3CL1/FKN could up-regulate HO-1 gene expression in lamina propria-derived macrophages and a mouse macrophage cell line, RAW 264.7, in a dose-dependent manner. CX3CR1 shares signaling pathways with other chemokine receptors, including intracellular calcium mobilization (18), MAPK activation (58), and PI3K activation (59). Moreover, CX3CL1/FKN can activate NF-κB in human intestinal epithelial cells (60) and aortic smooth muscle cells (61). Furthermore, activation of MAPKs can induce HO-1 expression in diverse cells in response to various stimuli (35). Likewise, exogenous CX3CL1/FKN activated ERK but not p38 MAPK and JNK, and PD98059, an ERK inhibitor, significantly suppressed CX3CL1/FKN-induced HO-1 gene expression in lamina propria-derived macrophages and RAW 264.7 cells. Thus, the CX3CL1/FKN–CX3CR1 axis could induce HO-1 expression in macrophages through ERK activation. Moreover, toxin A activated MAPKs, such as ERK, p38, and JNK, resulting in a marginal induction of HO-1 in macrophages without enhancing CX3CR1 expression (Supplemental Fig. 3A, 3B). Toxin A-induced HO-1 expression in macrophages was further enhanced by CX3CL1 treatment (Supplemental Fig. 3C). Collectively, the CX3CL1–CX3CR1 axis was essential for the HO-1 induction in macrophages during toxin A-induced enteritis.

Our results show that CX3CR1 deficiency in mice enhanced their susceptibility to toxin A-induced enteritis. Given our observation that CX3CL1/FKN has protective effects against murine toxin A-induced colitis, manipulation of CX3CR1-mediated signals may prove to be beneficial for the prevention of toxin A-induced enteritis along with conventional treatment.

Acknowledgments
We thank Drs. Philip M. Murphy and Ji-Liang Gao (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) for providing the CX3CR1−/− mice. We also thank Mariko Kawa-guchi for excellent assistance in the preparation of this manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


