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Cyclooxygenase-2 Deficiency Leads to Intestinal Barrier Dysfunction and Increased Mortality during Polymicrobial Sepsis

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Sepsis remains the leading cause of death in critically ill patients, despite modern advances in critical care. Intestinal barrier dysfunction may lead to secondary bacterial translocation and the development of the multiple organ dysfunction syndrome during sepsis. Cyclooxygenase (COX)-2 is highly upregulated in the intestine during sepsis, and we hypothesized that it may be critical in the maintenance of intestinal epithelial barrier function during peritonitis-induced polymicrobial sepsis. COX-2−/− and COX-2+/+ BALB/c mice underwent cecal ligation and puncture (CLP) or sham surgery. Mice chimeric for COX-2 were derived by bone marrow transplantation and underwent CLP. C2BBe1 cells, an intestinal epithelial cell line, were treated with the COX-2 inhibitor NS-398, PGD2, or vehicle and stimulated with cytokines. COX-2−/− mice developed exaggerated bacteremia and increased mortality compared with COX-2+/+ mice following CLP. Mice chimeric for COX-2 exhibited the recipient phenotype, suggesting that epithelial COX-2 expression in the ileum attenuates bacteremia following CLP. Absence of COX-2 significantly increased epithelial permeability of the ileum and reduced expression of the tight junction proteins zonula occludens-1, occludin, and claudin-1 in the ileum following CLP. Furthermore, PGD2 attenuated cytokine-induced hyperpermeability and zonula occludens-1 downregulation in NS-398–treated C2BBe1 cells. Our findings reveal that absence of COX-2 is associated with enhanced intestinal epithelial permeability and leads to exaggerated bacterial translocation and increased mortality during peritonitis-induced sepsis. Taken together, our results suggest that epithelial expression of COX-2 in the ileum is a critical modulator of tight junction protein expression and intestinal barrier function during sepsis. The Journal of Immunology, 2011, 187: 5255–5267.
colonic injury following dextran sulfate sodium, compared with COX-1 deficient and wild-type mice (11). Additionally, COX-2-deficient mice have been reported to develop spontaneous peritonitis (11, 12), further supporting the role of COX-2-derived PGs in preserving mucosal integrity in the GI tract. In the small bowel, COX-1-derived PGs protect against epithelial cell injury following radiation (13, 14); however, the contribution of COX-2-derived prostanooids to mucosal defense in the small intestine is less well characterized.

Increasing evidence suggests that epithelial cell barrier dysfunction may play a significant role in the development of the multiple organ dysfunction syndrome (MODS) during sepsis (15). Furthermore, studies suggest that increased intestinal permeability is common in critically ill patients (16) and that it is independently associated with the development of MODS in patients with critical illness (17). Tight junctions are critical for the maintenance of normal epithelial barrier function and are the key regulators of epithelial paracellular permeability. Tight junctions are located at the most apical region of the paracellular space between adjacent epithelial cells and form a selectively permeable barrier that controls paracellular flux of ions, hydrophilic solutes, and water (18). In the intestine, tight junctions also serve as the principal barrier against the paracellular leak of toxic luminal Abs and translocation of enteric microbial products. Tight junctions are assembled from at least three transmembrane proteins, including occludin, the claudins, and the junctional adhesion molecule, which are anchored to the cytoskeleton via peripher al membrane proteins, such as zonula occludens-1 (ZO-1) (18). ZO-1 plays a key role in the assembly of the tight junction complex, because it has multiple binding domains that allow it to interact with the intracellular domains of the integral membrane proteins occludin and the claudins, as well as bind to actin, thereby anchoring the complex into the perijunctional actomyosin ring (18).

Many extracellular stimuli, including cytokines and bacterial infection, have been shown to disrupt epithelial tight junction integrity and compromise paracellular barrier function (18, 19). In the intestine, this can lead to increased paracellular translocation of normally excluded luminal Abs and enteric bacteria. Furthermore, penetration of luminal microbial products through the paracellular leak pathway can lead to mucosal immune activation, with recruitment of inflammatory cells and secretion of proinflammatory mediators, which further increase barrier permeability and compromise intestinal barrier function. LPS, high mobility group box 1, and several proinflammatory cytokines, including TNF-α, IFN-γ, IL-1β, and IL-6, have been shown to increase transepithelial permeability in intestinal epithelial monolayers (20) and alter expression of tight junction proteins (21) in vitro. In addition, tight junction dysfunction, leading to intestinal barrier disruption and bacterial translocation, has been demonstrated in animal models of endotoxemia (22, 23) and common bile duct ligation (24). We hypothesized that COX-2 plays an essential role in maintaining intestinal epithelial barrier function during sepsis. Although COX-2-derived PGs have a well-recognized protective role in the GI tract (6, 7), the significance of COX-2-derived prostanooids in the intestinal epithelium during sepsis has yet to be elucidated. In this article, we demonstrate that COX-2 deficiency is detrimental in a murine model of peritonitis-induced polymicrobial sepsis, with increased bacterial translocation, exaggerated inflammation, severe hypotension, and increased mortality following cecal ligation and puncture (CLP). Our results suggest that COX-2 induction in the ileal epithelium is critical to the regulation of tight junction proteins and maintenance of intestinal barrier function during polymicrobial sepsis.

Materials and Methods

**Reagents and Abs**

The murine CLP model of polymicrobial sepsis was performed, as described (25, 26). Anesthesia was induced by i.p. administration of 100 mg/kg ketamine HCl and 43 mg/kg xylazine HCl. A 1.5-cm midline abdominal incision was made under sterile conditions, and 50% of the cecum was ligated below the ileocecal valve, thereby preserving intestinal continuity (25, 26). Once ligated, the cecum was punctured once with a 19-gauge (g) needle for mortality experiments or twice with a 23-g needle for everted gut sac experiments to allow for improved survival in COX-2–/– mice. The cecum was repositioned, and the abdominal incision was closed in layers with 6-0 sutures. Sham-operated mice underwent the same procedure, including opening of the peritoneum and exposing the bowel, but without ligation and needle perforation of the cecum. Following abdominal closure, sham and CLP-operated mice were administered 1 ml sterile saline i.p. No antibiotics were administered to the mice after surgery to assess the effect of COX-2 on bacterial levels in blood and organs. Survival rates were determined over an 8-d period, with assessment every 12 h. All mice had ad libitum access to food and water.

**Blood pressure analysis**

Systolic blood pressure (SBP) was measured 24 h before and every 24 h following CLP, using a tail-cuff method, as described (27).

**Histological and cytokine analysis**

Ileums and colons were harvested, fixed in 10% formalin or Methyl Carnoy’s at 4˚C, and embedded in paraffin. Sections were stained with H&E or Alcan blue, and immunostaining was performed with an anti-CD45 Ab (1:1000), anti-Ly-6G Ab (1:200), and anti-COX-2 Ab (1:250) (28, 29). Histologic scoring of H&E-stained ileum sections was performed in a blinded manner by a pathologist. The sections were scored for mucosal injury using the Chiu/Park scoring system (30, 31). Mucosal damage was graded from 0 to 8, according to the following criteria: grade 0, normal mucosal villi; grade 1, development of subepithelial space; grade 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; grade 3, massive epithelial lifting down the sides of villi, possibly with a few denuded tips; grade 4, denuded villi with lamina propria and dilated capillaries exposed; grade 5, digestion and disintegration of the lamina propria, hemorrhage, and ulceration; grade 6, crypt injury; grade 7, mucosal infarction; grade 8, transmural infarction. Quantitative assessment of mucosal damage was performed using FRIDA Software (FRamework for Image Dataset Analysis, http://bui2.win.ad.jhu.edu/frida/) (32, 33). This software provides a pixel
color threshold mask (hue, saturation, and brightness) through which a range of positive immunohistochemistry color signal is specified, and the software quantitates all pixels with the selected range of colors within a field. The same pixel color mask was applied to all samples being analyzed for a given Ab marker. Cytokines were measured in serum by SearchLight multiplex immunoassay (Aushon, Billerica, MA).

Flow cytometry analysis of lamina propria

Twenty-four hours following sham and CLP, ileums were isolated, and cells from the lamina propria were isolated, as described (34), with modification. Ileums were flushed with PBS to remove fecal contents, inverted, and shaken in PBS containing 5% DTT and 0.5 M EDTA for 30 min at 37°C. After removing epithelial cells and fat tissue, the intestines were digested in PBS, cut into small pieces, and incubated in RPMI 1640 containing 5% FBS, 1.5 mg/ml collagenase type II (Life Technologies), and 0.5 mg/ml dispase (Life Technologies) for 1 h at 37°C under constant horizontal shaking (250 rpm). Cells from digested intestinal tissues were isolated by centrifugation, washed three times, and resuspended in PBS containing 2% FBS. The isolated cell suspensions were incubated with the mAb 2A4G2 (10 μg/ml) for 20 min at 4°C to block FcγRs. The cells were stained with a combination of the following directly conjugated fluorescent anti-mouse mAbs for 30 min at 4°C: CD45–PE–Cy7, F4/80–PE, rat IgG2b–PerCP–Cy5.5, rat IgG2b–allophycocyanin–Cy7, rat IgG2a–FITC, rat IgG2a–PE–Cy7, rat IgG2a–PE, and rat IgG2b–PE (eBioscience); Ly-6G–PE, and CD11b–PerCP–Cy5.5 (BD Biosciences); and CD11b–allophycocyanin–Cy7 and F4/80–PerCP–Cy5.5 (BioLegend). The cells were analyzed with a BD Biosciences Canto II flow cytometer and FlowJo software (Tree Star). Live cell and CD45+ cell gating was used to exclude dead and nonhematopoietic cells. Neutrophils were quantified as Ly-6G–Ly-6C– gated on CD45+CD11b+ cells, and macrophages were quantified as F4/80+CD11b+ gated on CD45+ cells.

Bacterial cultures

Serial log_{10} dilutions of whole blood and homogenized tissue were made, and aliquots were cultured on Luria-Bertani (LB) agar plates, as described (25). Mesenteric lymph nodes (MLNs) were isolated and weighed, homogenized in 250 μl PBS as described (35), and aliquots of serial log_{10} dilutions were cultured on LB agar plates. The number of CFU were counted following overnight incubation at 37°C (25).

Bone marrow transplantation

Animals chimeric for COX-2 were derived, as previously described (29, 36). Recipient COX-2−/− or COX-2+/− mice (6–8 wk old) received total body irradiation of 900 cGy ([60Co] source) in two separate doses 4 h apart. One hour following the second dose of irradiation, 2 × 10^6 donor cells were delivered to the recipient animal via tail vein injection. Following bone marrow transplantation (BMT), mice remained in a pathogen-free facility with ad libitum access to food and water. Six weeks following bone marrow transplantation (BMT), mice were heat inactivated and labeled with FITC, as described (25, 38). FITC-labeled C. jejuni (3 × 10^8 CFU) or S. aureus (3 × 10^8 CFU) were injected into the peritoneum of COX-2−/− and COX-2+/− mice, and peritoneal neutrophils were isolated after 24 h, as described (25). Following a 2-h incubation at 37°C, adherent cells were washed and treated with 0.2% trypsin blue to quench extracellular fluorescence. The cells were washed, treated with 5 mM EDTA in PBS, gently scraped, and then centrifuged (25). The pellet was resuspended in 400 μl PBS containing 4% FBS and 0.009% sodium azide. Total cells were counted, and 1 × 10^6 cells were scanned by flow cytometry.

In vivo permeability assay

In vivo intestinal permeability was measured using the FITC-labeled dextran (FD-4) method, as described (39, 40), with modification. Mice were administered 100 μl FD-4 (50 mg/ml) by oral gavage 18 h following CLP and sham. Six hours later, blood was harvested, and serum was isolated. Serial dilutions of FD-4 were made to generate a standard curve, and serum concentrations of FD-4 were determined using a BioTek FLX800 Fluorescence Microplate Reader (BioTek, Winooski, VT), with an excitation wavelength of 490 nm and emission wavelength of 530 nm. Serum concentrations of FD-4 following CLP were normalized to FD-4 concentrations following sham.

Everted gut sac

Ileums were harvested 24 h following CLP and sham surgeries, and permeability to FD-4 was measured ex vivo by the everted gut sac method, as described (41). Briefly, segments of ileum were ligated at one end, everted over a 18-g Jenco catheter (Smiths, Dublin, OH), and sutured to a 20-g Jenco catheter attached to a 1-ml syringe containing Krebs–Henseleit bicarbonate buffer (KHBB [pH 7.4]). The everted gut sac was distended with 500 μl KHBB and suspended in a beaker containing FD-4 (40 mg/ml) in KHBB. A sample was aspirated from the beaker to measure the initial concentration of FD-4 (mucosal surface), and the sac was incubated in the beaker for 30 min. Following incubation, fluid was aspirated from the inside of the sac (serosal surface), and the length of the gut sac was measured. The serosal and mucosal samples were centrifuged, and the supernatant was diluted with PBS. Fluorescence was measured with an F-2500 fluorescence spectrophotometer (Hitachi, Pleasonton, CA). Permeability is expressed as the mucosal-to-serosal clearance (nl/min/cm²) of FD-4, as described (41).

Cell culture

Human intestinal epithelial cells (C2BBe1) were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 10% FBS, 10,000 U/ml penicillin, 10,000 mg/ml streptomycin, 29.2 mg/ml l-glutamine, 2 mM sodium pyruvate, and 1 × MEM nonessential amino acids in a humidified incubator (21% O₂, 5% CO₂) at 37°C. Cells were grown on collagen-I–coated Biocor tissue culture dishes (BD), and passages 7–10 were used for all experiments.

In vitro epithelial permeability assays

C2BBe1 cells (5 × 10^4 cells/well) were plated in collagen-coated transwell inserts (3 μm pore size) in 24-well plates (COSTAR, Corning, NY), and permeability studies were performed, as described (22, 42), using confluent monolayers 21–24 h after plating. In specific experiments, cells were pretreated with NS-398 at 10 or 50 μM [doses selective for inhibition of COX-2 (43)] or SC-560 (10 or 50 nM) [doses selective for inhibition of COX-1 (44)] in the basolateral media. NS-398 was dissolved in 25% DMSO/75% PBS, whereas SC-560 was dissolved in 50% DMSO/50% PBS; equal volumes of vehicle were administered as controls. In other experiments, cells were pretreated with PGE2 (10 μM) or BW245C (10 μM) in the basolateral media. After 24 h, HEPES-buffered DMEM (pH 6.8) containing FS (200 μg/ml) was added to the apical side of the transwells, and cytokine (CM) (IFN-γ 1000 U/ml, TNF-α 10 ng/ml, IL-1β 1 ng/ml) or vehicle containing NS-398, SC-560, PGE2, or BW245C was added to the basolateral surface. Fluorescence was measured with a fluorescence microplate reader, as above. Permeability is expressed as flux of FS per unit area of membrane divided by the concentration of the probe in the apical compartment (nl/(min·cm²)) as described (21, 22) and is normalized to the respective vehicle.

Transepithelial resistance measurements

C2BBe1 cells (1 × 10^5 cells/well) were plated in collagen-coated transwell inserts (3 μm pore size) in 12-well plates with a surface area of 1.12 cm². Transepithelial resistance (TER) was measured in Ohms (Ω), before and after CM stimulation, with an epithelial volthmometer (World Precision Instruments, Sarasota, FL).

Western blot analysis

Mucosal protein was isolated, as described (24), from ileums of COX-2−/− and COX-2+/− mice. Protein extracts from ileums were analyzed by Western blot with anti-ZO-1 (1:500), anti-occludin (1:2000), anti-claudin-1 (1:1000), anti–COX-2 (1:200), and anti–COX-1 (1:1000) Abs. Equal loading was confirmed with an anti–β-actin Ab (1:5000) or anti-α-tubulin (1:5000) Ab. Densitometry was performed using the Gel Doc XR System with Quantity One-4.6.2 software (Bio-Rad, Hercules, CA) or Image J (National Institutes of Health, Bethesda, MD).

Immunofluorescence confocal microscopy

C2BBe1 cells were treated with NS-398 (50 μM) or vehicle, followed by CM stimulation. After 48 h, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 in PBS, and blocked with 3% BSA in 0.1% Triton X-100 in PBS for 1 h. Cells were incubated with anti-ZO-1

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anti-occludin (1:100), or anti–claudin-1 Abs (1:100) for 1 h. Cells were washed and incubated with secondary Abs (1:200). Cells were washed, counterstained with DAPI, and mounted with VECTASHIELD Mounting Medium. Staining was analyzed using a laser scanning confocal imaging system (Bio-Rad Radiance 2100) attached to an Olympus 1X71 inverted microscope with an oil-immersion 60× objective.

Quantification of cell death
Trypan blue absorbance. Cells were treated with 100 μl 10 mM potassium cyanide (positive controls) or media for 1 h at 37 °C, as described (22). The media were removed, and 100 μl 0.4% trypan blue solution was added to each well and incubated for 15 min at 37 °C (45). The cells were visualized under a microscope, washed, and lysed with 300 μl 0.1% SDS. Absorbance was measured in the SDS/trypsin blue solution at 590 nm, as described (45), and normalized to potassium cyanide-positive control wells. Lactate dehydrogenase assay. Total lactate dehydrogenase (LDH) and LDH release into the basolateral media were measured via a commercial in vitro toxicity assay kit (TOX-7; Sigma).

RT-PCR
Total RNA was extracted from ileums using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was treated with DNase (Invitrogen), followed by reverse transcription to cDNA using the Superscript III qRT-PCR Kit (Invitrogen). Quantitative RT-PCR (qPCR) was performed on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) to determine gene expression for COX-1 (Mm00477214_m1) and COX-2 (Mm00478374_m1) using validated TaqMan gene expression assay primer/probe combinations (Applied Biosystems). All qPCR results were normalized to the expression of the endogenous positive control 18S (Hs99999901_s1). The ΔCt threshold (ΔCt) was calculated as the difference between the Ct for the gene of interest and the respective Ct for 18S.

ELISA
Ileums were minced in 10 mM sodium phosphate buffer (pH 7.4), as described (46). The supernatants were collected following centrifugation at 9000 × g for 1 min, extracted with acetone, and evaporated to dryness. The supernatants were resuspended in PBS and analyzed for PGD2 (Eli-satech, Aurora, CO) and PGE2 (Cayman) by ELISA.

Statistical analysis
Data are presented as median ± interquartile range, except as indicated. Statistical significance was determined by the nonparametric Mann–Whitney U test or unpaired t test for comparisons between two groups or the nonparametric Kruskal–Wallis one-way ANOVA, followed by Dunn’s post test, for comparisons between more than two groups or for multiple comparisons. Categorical and ordinal variables were compared using the χ2 test for trend. Comparisons of mortality were made by analyzing Kaplan–Meier survival curves, and the log-rank test was used to assess for differences in survival. The number of samples per group (n) is specified in the figure legends. All p values were two-tailed, and statistical significance was accepted at p < 0.05.

Results
COX-2–deficient mice have increased mortality and exaggerated bacteremia following peritonitis-induced polymicrobial sepsis
To examine the role of COX-2 in a polymicrobial model of sepsis, COX-2−/− male BALB/c mice underwent CLP using a 19-g needle and a single cecal puncture. COX-2 expression, but not COX-1 expression, was induced 3-fold in the ileum of COX-2−/− mice at the mRNA level (Fig. 1A, 1B) and >20-fold at the protein level (Fig. 1C, 1D), following CLP. Given the dramatic induction of COX-2 in the ileum following CLP, we hypothesized that COX-2 was playing a critical role in the ileum during peritonitis-induced sepsis. To investigate the significance of COX-2 induction in the ileum on mortality during polymicrobial sepsis, COX-2–deficient (COX-2−/−) and COX-2−/− male BALB/c mice underwent CLP. Absence of COX-2 was detrimental during polymicrobial sepsis, with a significant increase in mortality in COX-2−/− mice fol-

![FIGURE 1](http://www.jimmunol.org/DownloadedFrom/5258.jpg)
following CLP compared with COX-2+/+ mice. COX-2−/− mice had 80% mortality by day 4 following CLP compared with 30% mortality in COX-2−/− mice by day 5 (Fig. 2A). In addition, COX-2−/− mice developed significant hypotension following CLP compared with COX-2+/+ mice. At baseline, there was no significant difference in SBP between COX-2−/− mice (SBP 113 ± 3 mm Hg) and COX-2+/+ mice (SBP 119 ± 1.5 mm Hg); however, COX-2−/− mice developed significant hypotension (SBP 57 ± 4 mm Hg) compared with COX-2+/+ mice (SBP 73 ± 2 mm Hg; p < 0.05) 72 h following CLP.

To investigate the mechanism of increased mortality in COX-2−/− mice during polymicrobial sepsis, blood, MLNs, and organs (lung, liver, and spleen) were cultured 24 h following CLP. COX-2−/− mice demonstrated increased incidence of bacteremia following CLP, in addition to higher numbers of circulating bacteria and translocation to MLNs, compared with COX-2+/+ mice (Fig. 2B, 2C). Similarly, organ cultures from COX-2−/− mice showed significantly higher levels of bacteria in the lung, liver, and spleen following CLP compared with COX-2+/+ mice (Fig. 2D). Increased bacteremia and exaggerated bacterial seeding of organs was also observed in COX-2−/− mice 48 h following CLP (data not shown).

**Increased intestinal inflammation in COX-2−/− mice following peritonitis-induced polymicrobial sepsis**

COX-2−/− deficient mice demonstrated increased ileal inflammation and proinflammatory cytokine production following peritonitis-induced polymicrobial sepsis. Examination of the intestinal tissue revealed no significant difference in necrosis of the cecum between COX-2−/− and COX-2+/+ mice following CLP. Furthermore, although there was marked ileal inflammation and injury in both COX-2−/− and COX-2+/+ mice following CLP (Fig. 3Ac, 3Ad, respectively) compared with sham (Fig. 3Aa, 3Ab, respectively), there was no histologic difference in intestinal mucosal injury between COX-2−/− and COX-2+/+ mice following CLP (Fig. 3E). Similar findings were observed at 24 h following CLP (data not shown). In addition, quantitation of Alcian blue staining demonstrated no difference in goblet cell number per crypt-villus in the ileums of COX-2−/− mice (Fig. 3Ah) following CLP compared with COX-2+/+ mice (Fig. 3Ag, 3C).

However, H&E staining and immunostaining for CD45 and Ly-6G revealed enhanced inflammation in the ileums of COX-2−/− mice (Fig. 3Ad, 3Af, 3Ap) following CLP compared with COX-2+/+ mice (Fig. 3Ac, 3Ak, 3Ao). Although there was no apparent histologic difference in intestinal mucosal integrity following CLP (Fig. 3Ac, 3Ad, 3B), ileums of COX-2−/− mice demonstrated significantly increased numbers of inflammatory cells in the lamina propria and submucosa (Fig. 3Al) compared with COX-2+/+ mice (Fig. 3Ak, 3D). Furthermore, immunostaining for Ly-6G demonstrated an increase in the number of neutrophils in the ileums of COX-2+/+ and COX-2−/− mice following CLP, with a significantly higher influx of neutrophils into the ileums of COX-2−/− mice (Fig. 3Ap) compared with COX-2+/+ mice (Fig. 3Aa, 3E). Similarly, H&E staining and immunostaining for CD45 demonstrated that colons harvested from COX-2−/− mice had increased inflammation compared with COX-2+/+ mice following CLP (Supplemental Fig. 1). In addition, COX-2−/− mice had significantly higher levels of circulating proinflammatory cytokines, including IL-6, TNF-α, and IFN-γ, following CLP compared with COX-2+/+ mice (Supplemental Table I).

To further characterize the inflammatory cell infiltration into the ileums of COX-2−/− mice following CLP, flow cytometry of the lamina propria of ileums from COX-2−/− and COX-2+/+ mice following sham and CLP was performed. Absence of COX-2 was associated with an increased percentage of neutrophils in the lamina propria of the ileum following CLP (Fig. 4A), as well as significantly increased numbers of neutrophils in the ileum (Fig. 4B) compared with COX-2+/+ mice following CLP. In addition, COX-2−/− mice had significantly increased numbers of macrophages (Fig. 4C) infiltrating the ileum compared with COX-2+/+ mice following CLP.

**Absence of COX-2 in ileal parenchymal cells correlates with increased circulating bacterial counts and bacterial seeding of vital organs during polymicrobial sepsis**

To investigate the mechanism of increased mortality and bacteremia in COX-2−/− mice following peritonitis-induced polymicrobial sepsis, we performed immunostaining for COX-2 to determine which cell type upregulated COX-2 expression in the ileum following CLP. COX-2 immunostaining demonstrated localization of COX-2 expression to epithelial cells within the crypts of the ileum, as well as to inflammatory cells within the lamina propria of the ileum, of COX-2−/− mice following CLP (Fig. 1F). Because COX-2 was highly expressed in both epithelial cells and inflammatory cells of the ileum following CLP, we generated mice chimeric for COX-2 to determine whether COX-2 expression in
inflammatory cells or parenchymal cells was critical for the development of bacteremia and shock following peritonitis. BMT was used to derive animals chimeric for COX-2 (COX-2\(^{-/-}\) bone marrow-derived cells into COX-2\(^{+/+}\) recipients [COX-2\(^{-/-}\)→ COX-2\(^{+/+}\)] and COX-2\(^{+/+}\) cells into COX-2\(^{-/-}\) recipients [COX-2\(^{+/+}\)→ COX-2\(^{-/-}\)]). Using our current protocol, we previously showed via hematopoietic reconstitution experiments (47) that 90% of circulating bone marrow-derived cells in the recipient originate from donor marrow. Six weeks following BMT, these chimeric mice were subjected to CLP, as described. COX-2\(^{+/+}\)→ COX-2\(^{-/-}\) mice demonstrated higher levels of circulating bacteria following CLP compared with COX-2\(^{-/-}\)→ COX-2\(^{+/+}\) mice (Fig. 5A). Similarly, organ cultures from COX-2\(^{+/+}\)→ COX-2\(^{-/-}\) mice showed significantly higher levels of bacteria in the lung, liver, and spleen compared with COX-2\(^{-/-}\)→ COX-2\(^{+/+}\) mice following CLP (Fig. 5B). To control for the effects of irradiation, nonchimeric irradiated control mice were also generated by BMT (COX-2\(^{+/+}\) donor cells into COX-2\(^{+/+}\) mice [COX-2\(^{+/+}\)→ COX-2\(^{+/+}\)] and COX-2\(^{+/+}\) donor cells into COX-2\(^{-/-}\) mice [COX-2\(^{+/+}\)→ COX-2\(^{-/-}\)]). Following CLP, bacterial levels in the blood, lung, liver, and spleen of these nonchimeric mice (Fig. 5C, 5D) were similar to untransplanted COX-2\(^{+/+}\) and COX-2\(^{-/-}\) mice (Fig. 2B, 2D). Furthermore, bacterial levels in COX-2\(^{-/-}\)→ COX-2\(^{+/+}\) mice were similar to COX-2\(^{+/+}\)→ COX-2\(^{-/-}\) mice following CLP. These findings demonstrated that mice chimeric for COX-2 exhibit the phenotype of the recipient and suggested that COX-2 expression by infiltrating inflammatory cells does not influence the phenotype of the chimeric animal. Furthermore, these results suggest that...
epithelial COX-2 expression in the ileum protects against bacteremia and death from polymicrobial sepsis. COX-2 deficiency does not impair bacterial phagocytosis by peritoneal neutrophils

Previous work demonstrated that PGE2 may impair phagocytosis and bactericidal activity of macrophages (48–51) and that COX-2 deficiency may decrease bacterial burden in murine models of invasive Streptococcus pyogenes (50) and Pseudomonas aeruginosa pneumonia (51). Based on these studies and to further substantiate our BMT findings that COX-2 expression by inflammatory cells does not determine the phenotype of chimeric mice during peritonitis-induced polymicrobial sepsis, we performed in vivo phagocytosis assays using FITC-labeled E. coli or S. aureus in COX-2+/- and COX-2-/ mice. Total peritoneal neutrophil counts were not different between COX-2-/ mice (3.88 ± 1.94 × 10⁴ cells/ml) and COX-2+/- mice (3.77 ± 1.88 × 10⁴ cells/ml) following CLP. Furthermore, COX-2-/ and COX-2+/- mice had no significant difference in the percentage of FITC+ cells with either E. coli or S. aureus (Supplemental Fig. 2). These findings suggested that the mechanism of increased bacteremia and bacterial seeding of organs in COX-2-/ mice is not due to impaired phagocytosis by peritoneal neutrophils during CLP.

Inhibition or deficiency of COX-2 increases intestinal epithelial permeability following peritonitis-induced polymicrobial sepsis

Based on these findings, we next investigated whether COX-2-deficient mice had intestinal epithelial cell dysfunction leading to hyperpermeability of the ileum and enhanced secondary bacterial translocation following CLP. Because our results suggested that inflammatory cell COX-2 expression did not play a role in the development of bacteriaemia after CLP and because COX-2 expression was highly upregulated in the ileal epithelium, we hypothesized that upregulation of COX-2 in the epithelium of the
ileum plays a protective role in intestinal barrier function during CLP. Although histological evaluation of the ileum and colon in COX-2−/− mice undergoing CLP demonstrated preservation of mucosal integrity, this does not preclude epithelial barrier dysfunction resulting in exaggerated paracellular leak of microbial products from the intestinal lumen as the etiology of increased mortality in COX-2−/− mice. To determine whether the absence of COX-2 leads to exaggerated intestinal epithelial barrier dysfunction, intestinal permeability to FD-4 was examined in COX-2−/− and COX-2+/+ mice following CLP. COX-2−/− mice had a >4-fold increase in serum FD-4 concentration following CLP compared with a 1.2-fold increase in COX-2+/+ mice following CLP (Fig. 6A).

We further investigated the intestinal permeability defect at the level of the ileum, using the everted gut sac method (41). This ex vivo method measures alterations in mucosal barrier function that result in intestinal epithelial hyperpermeability and allows for the measurement of intestinal permeability in the clinically relevant mucosal-to-serosal direction. There was no difference in ileal permeability between COX-2−/− and COX-2+/+ mice following sham surgery; however, following CLP, ileums harvested from COX-2−/− mice had significantly increased epithelial permeability compared with ileums from COX-2+/+ mice and sham controls (Fig. 6B).

To examine the mechanism of exaggerated epithelial barrier dysfunction in COX-2−/− mice during sepsis, we performed in vitro permeability assays in C2BBe1 cells, an intestinal epithelial cell line that forms a polarized monolayer. Selective inhibition of COX-2, but not COX-1, led to enhanced epithelial permeability following CM stimulation (Fig. 7A, 7C). Treatment with NS-398, a selective COX-2 inhibitor, prior to CM stimulation significantly augmented apical-to-basolateral clearance of FS (Fig. 7A) compared with vehicle-treated CM-stimulated cells. Furthermore, selective COX-2 inhibition led to an exaggerated decrease in TER compared with vehicle-treated CM-stimulated cells (Fig. 7B). However, selective inhibition of COX-1 led to similar increases in epithelial permeability at 48 h as vehicle-treated CM-stimulated cells (Fig. 7C). To determine whether the mechanism of enhanced epithelial permeability was due to decreased cell viability, LDH release and trypan blue uptake were measured spectrophotometrically. There was no significant difference in cell death or LDH release between NS-398–treated and vehicle-treated CM-stimulated cells (Supplemental Fig. 3), demonstrating preserved epithelial cell viability.

**Absence of COX-2 leads to reduced expression of tight junction proteins in the ileum following CLP**

To elucidate the mechanism of enhanced epithelial permeability leading to exaggerated intestinal barrier dysfunction in COX-2−/− mice following peritonitis-induced sepsis, we investigated whether the expression of key tight junction proteins was altered in the ileum following CLP. Because tight junctions are essential for sealing the paracellular space between adjacent epithelial cells and maintaining intestinal barrier function (18, 52), we hypothesized that expression of tight junction proteins might be reduced in the ileum of COX-2−/− mice, leading to severe intestinal barrier dysfunction following peritonitis-induced sepsis. Western blot analysis of ileal mucosal protein from COX-2−/− and COX-2+/+ mice revealed a significant reduction in occludin and claudin-1 expression in the ileums of COX-2−/− mice compared with COX-2+/+ mice following sham surgery (Fig. 8A, 8B). Furthermore, following CLP, there was a dramatic reduction in the expression of ZO-1, occludin, and claudin-1 in the ileums of COX-2−/− mice compared with COX-2+/+ mice (Fig. 8A, 8B).

In addition, selective inhibition of COX-2 with NS-398 in CM-stimulated C2BBe1 cells led to significant reduction in immunostaining for ZO-1 (Fig. 8Cf, g), occludin (Fig. 8Ch), and claudin-1 (Fig. 8Cj) by confocal microscopy compared with vehicle-treated CM-stimulated cells (Fig. 8Cb, Cf, Cj). Although vehicle-treated CM-stimulated cells demonstrated ruffling of immunostaining for ZO-1 (Fig. 8Cb), cells treated with NS-398 prior to CM had profound loss of membrane staining for both ZO-1 (Fig. 8Cd) and claudin-1 (Fig. 8Cf). Notably, many cells had disruption of circumferential staining, with the remaining staining distributed in a punctate manner along the cell membrane. Selective inhibition of COX-2 in CM-stimulated C2BBe1 cells also led to significant disruption in membrane staining for occludin (Fig. 8Ch), as well as cytoplasmic staining suggestive of internalization, whereas occludin immunostaining remained relatively intact in vehicle-treated CM-stimulated cells (Fig. 8Cf). These findings suggest that COX-2–derived prostanoids may modulate expression of tight junction proteins in the intestinal epithelium and consequently limit intestinal barrier dysfunction during peritonitis-induced sepsis.

**PGD2 attenuates epithelial hyperpermeability and downregulation of ZO-1 expression induced by selective inhibition of COX-2**

Given the protective role of prostanoids in the GI tract and the importance of COX-2–derived PGD2 in regulating inflammation and epithelial secretion following colonic injury, we investigated whether PGD2 could attenuate intestinal barrier dysfunction following CLP. Although PGE2 was not induced in the ileum fol-
Prostanoids have long been recognized as mediating many of the physiologic derangements of the systemic inflammatory response syndrome. Early investigation into nonselective inhibition of COX with aspirin or nonsteroidal anti-inflammatory drugs showed hemodynamic improvement and improved survival in several animal models of endotoxemia (53). However, although administration of ibuprofen to patients with sepsis improved certain physiologic parameters, it did not improve survival or prevent the development of the acute respiratory distress syndrome (54). More recently, studies from our laboratory demonstrated that COX-2−/− mice had attenuated inflammation and improved survival during endotoxemia (55), whereas work from Reddy et al. (56) showed that pharmacologic inhibition of COX-2 improved early survival following endotoxin injection but not following bacterial peritonitis. In this study, we demonstrated that COX-2 deficiency leads to enhanced intestinal bacterial translocation, increased inflammation, and decreased survival during peritonitis-induced polymicrobial sepsis. Furthermore, we found that COX-2 expression in intestinal epithelial cells, not inflammatory cells, is critical to attenuate bacteraemia and bacterial seeding of organs following CLP. Taken together, these prior studies and our present work reinforce that CLP represents a more clinically relevant model of sepsis than does endotoxemia (57) and further highlight that disruption of the COX-2 pathway has distinct effects following a pure inflammatory stimulus versus during a microbial infection.

COX-2 is inducible in intestinal macrophages (58) and neutrophils (59) and is upregulated in intestinal epithelial cells following cytokine stimulation (60–62), in response to invasive bacteria (63), and in inflammatory colitis (64). However, the relative contribution and significance of COX-2–derived prostanooid production by inflammatory versus epithelial cells in the ileum during sepsis was heretofore unknown. Using mice chimeric for COX-2, we demonstrated that COX-2 expression in parenchymal cells, not inflammatory cells, influences the development of bacteraemia and bacterial seeding of organs during peritonitis-induced sepsis. Furthermore, we showed both in vivo and ex vivo that COX-2 null mice had exaggerated intestinal epithelial cell permeability following CLP, and we demonstrated in vitro that selective COX-2 inhibition in intestinal epithelial cells significantly enhanced cytokine-induced transepithelial hyperpermeability. Interestingly, we found no significant difference in phagocytosis...
by peritoneal neutrophils between mice deficient in COX-2 compared with COX-2+/+ mice. Although previous studies showed that the absence of COX-2 may enhance bacterial clearance by alveolar and bone marrow-derived macrophages following intratracheal and i.v. administration of bacteria (50, 51), our findings suggest that intestinal epithelial expression of COX-2 plays a dominant role in attenuating bacterial translocation in a peritonitis-induced sepsis model. Although COX-2−/− mice had increased recruitment of inflammatory cells to the intestine and higher levels of circulating proinflammatory cytokines following CLP, our results suggest that this enhanced inflammatory response is triggered by exaggerated bacterial translocation from the disruption of epithelial barrier function. In light of these findings, we propose that an initial breakdown in barrier function in COX-2 null mice leads to enhanced bacterial translocation, followed by recruitment of inflammatory cells and exaggerated production of proinflammatory cytokines and chemokines, which, in turn, leads to further disruption in epithelial

**FIGURE 8.** Absence of COX-2 leads to reduced expression of tight junction proteins in the ileum following CLP. A and B, Ileums were harvested from COX-2+/+ and COX-2−/− mice 48 h following sham or CLP, and total protein was isolated from ileal mucosa. Western blot analysis was performed for ZO-1, occludin, and claudin-1. Bar graphs represent protein levels (normalized for β-actin), as determined by densitometry, and averaged for two to four mice/condition. Protein levels are expressed as the percentage of the levels in COX-2+/+ mice following sham. Data are mean ± SD. *p < 0.05, as indicated. **p < 0.05, COX-2+/+ mice after CLP versus sham control, unpaired t test. C, C2BBe1 cells were treated with NS-398 or vehicle and stimulated with CM in collagen-coated eight-well chamber slides. After 48 h, cells were fixed, permeabilized, and immunostained for ZO-1, occludin, and claudin-1. Confocal fluorescent microscopy was performed, and photographs are representative of three independent experiments.
barrier integrity and ongoing bacterial translocation. However, because COX-2–deficient mice developed more severe hypotension following CLP, and increased intestinal permeability has been observed in animal models of hemorrhagic shock (65), we cannot completely exclude the possibility that hypotension may have contributed to enhanced bacterial translocation and increased mortality in COX-2–/– mice following CLP. Overall, our findings suggest that epithelial COX-2 expression in the ileum protects against bacteremia and death from polymicrobial sepsis.

Although PGs have long been recognized as providing mucosal protection in the upper GI tract (66), the significance of COX-2–derived prostanoids in the lower GI tract has been less clear. In the colon, both COX-1 and COX-2 seem to be critical following intestinal injury. Although both isoforms were protective during intestinal mucosal injury in a murine model of dextran sulfate sodium colitis (11), COX-2–derived PGD2 attenuated colonic injury and inflammation following trinitrobenzene sulfonic acid (46). In addition, both PGE2 and PGD2 play key roles in regulating epithelial chloride secretion (67) in the colon. The role of COX-2–derived prostanoids in the small intestine is less well characterized; however, COX-1 seems to regulate epithelial cell homeostasis following radiation injury (13, 14). Our findings now implicate COX-2 as a key modulator of epithelial tight junction integrity and intestinal barrier function in the small bowel during peritonitis-induced sepsis.

Tight junctions are critical in regulating paracellular permeability between epithelial cells, and disruption of their integrity may lead to increased intestinal permeability and translocation of microbial products into the bloodstream during sepsis. COX-2 is upregulated in the epithelium of the ileum by proinflammatory stimuli, and our data provide evidence that COX-2–derived prostanoids play a protective role in maintaining intestinal epithelial barrier integrity during intra-abdominal sepsis. Our study demonstrated that absence of COX-2–derived prostanoids in the ileum leads to a dramatic reduction in the expression of key tight junction proteins, resulting in enhanced epithelial permeability of the ileum during peritonitis-induced sepsis. Our findings suggest that, in addition to attenuating inflammation, PGD2 plays a critical role in preserving intestinal barrier function by regulating epithelial expression of tight junction proteins in the small intestine during peritonitis-induced polymicrobial sepsis.

PGD2 is the major eicosanoid released by mucosal mast cells during allergic inflammation (68); however, PGD2 is also produced by intestinal epithelial cells (69). Although PGD2 is highly expressed in the intestine compared with other murine tissues, and PGD2 synthase has one of the highest activities in intestinal tissue (70), the importance of PGD2 expression in the intestine is incompletely understood. The biological effects of PGD2 are mediated via binding to one of two G protein-coupled receptors,
DP1 and DP2, or via its metabolite 15-deoxy-Δ12,14-PGJ2, a ligand for peroxisome proliferator-activated receptor-γ. DP1 modulates dendritic cell function (71), and DP2 enhances allergic inflammation via eosinophils, basophils, and Th2 lymphocytes (72). Interestingly, the mouse ileum has the most abundant expression of the DP1 receptor compared with other murine tissues (73), and the human DP1 receptor is detectable only in the retina and small intestine (74). Furthermore, in situ hybridization demonstrated that the DP1 receptor localizes to epithelial cells and goblet cells in the small intestine (75). Signal transduction through the DP1 receptor primarily leads to Gs-coupled activation of adenylate cyclase and subsequent increases in cAMP, but it can also lead to increases in intracellular calcium concentration (74). The findings of our study suggest a new mechanism by which COX-2–derived PGD2 or an agonist of the DP1 receptor attenuates cytokine-induced intestinal hyperpermeability in intestinal epithelial cells.

In summary, our results reveal a novel role for COX-2–derived PGD2 in mediating protection against intestinal barrier dysfunction during polymicrobial sepsis. Epithelial tight junction dysfunction may result in loss of intestinal barrier function and lead to translocation of luminal microbial products into the bloodstream during sepsis. Furthermore, maintenance of epithelial integrity may be critical to prevent the development of acute respiratory distress syndrome and MODS during sepsis. Our findings have significant implications for our understanding of sepsis and suggest that strategies that modulate epithelial cell expression of COX-2 and PGD2 secretion may yield innovative therapeutic approaches to combat this frequently fatal disease process.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. COX-2 deficient mice have increased colonic inflammation following peritonitis-induced polymicrobial sepsis. COX-2+/+ and COX-2–/– mice underwent CLP with a 19-g needle (1 hole). Colons were harvested 48 h following CLP. Representative H&E staining and immunostaining for CD45 in COX-2+/+ (top) and COX-2–/– (bottom) mice following sham surgery (left) and CLP (right). Arrow indicates inflammatory infiltrate and arrowheads indicate cells staining positive for CD45 in colonic villi of COX-2–/– mice following CLP. Scale bar is 100 μm. Magnification 100×.
Supplemental Figure 2. COX-2 deficiency does not impair bacterial phagocytosis by peritoneal neutrophils. FITC-labeled *E. coli* or *S. aureus* was injected into the peritoneum of COX-2^+/+^ (n=4-5 per group) and COX-2^-/-^ mice (n=4-5 per group) in two independent experiments. Neutrophils were isolated after 24 h and phagocytosis assessed by flow cytometry. There was no difference in phagocytosis of either *E. coli* or *S. aureus* between COX-2^+/+^ and COX-2^-/-^ mice. Data represent median ± IQR from two independent experiments.
Supplemental Figure 3. Selective inhibition of COX-2 does not lead to decreased cell viability in cytomix-stimulated intestinal epithelial cells. C2BBe1 cells were treated with vehicle or NS-398 and stimulated with cytomix (CM: IFN-γ 1000 U/ml, TNF-α 10 ng/ml, IL-1β 1 ng/ml) in collagen-coated transwells. (A) Following 48 h, cells were incubated with 0.4% trypan blue, lysed with 1% SDS, and absorbance measured at 590 nm. Results were normalized to a positive control (KCN). (B) The amount of LDH released into the basolateral media was measured via a commercial in vitro toxicology assay kit (TOX7, Sigma). Data represent median ± IQR for at least two independent experiments performed in triplicate.
**Supplemental Table I.** Circulating cytokine levels in COX-2⁺/⁺ and COX-2⁻/⁻ mice following CLP.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>COX-2⁺/⁺</th>
<th>sham (n=6)</th>
<th>12 h (n=4)</th>
<th>24 h (n=4)</th>
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<tbody>
<tr>
<td>IFN-γ</td>
<td>ND</td>
<td>11.6 (7.8, 11.6)</td>
<td>9.2 (3.3, 14.2)</td>
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<tr>
<td>IL-6</td>
<td>56 (11.7, 193)</td>
<td>2417 (2402, 4803)</td>
<td>1316 (661, 3304)</td>
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<tr>
<td>KC</td>
<td>98 (62, 151)</td>
<td>13841 (186, 36101)</td>
<td>43133 (15878, 109249)</td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>174 (146, 306)</td>
<td>278 (145, 333)</td>
<td>50 (46, 96)</td>
<td></td>
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<tr>
<td>IL-1β</td>
<td>3.1 (3.1, 60)</td>
<td>3.1 (1.6, 3.1)</td>
<td>3.1 (3.1, 3.1)</td>
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</tr>
<tr>
<td>IL-10</td>
<td>1.6 (1.3, 6.3)</td>
<td>68 (34, 234)</td>
<td>12 (4.8, 25.2)</td>
<td></td>
</tr>
<tr>
<td>IL-12p40</td>
<td>3.4 (0.4, 14)</td>
<td>107 (18, 190)</td>
<td>48 (15, 60)</td>
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<tr>
<td>MIP2 (CXCL2)</td>
<td>ND</td>
<td>659 (537, 826)</td>
<td>99.7 (72, 336)</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>ND</td>
<td>8.4 (3.1, 14.7)</td>
<td>7.3 (3.3, 15)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>COX-2⁻/⁻</th>
<th>sham (n=6)</th>
<th>12 h (n=6)</th>
<th>24 h (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>ND</td>
<td>23.1 (8.5, 33.9)</td>
<td>33.8 (21.8, 187.9) * ‡</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>86 (11, 326)</td>
<td>4588 (1375, 9460)</td>
<td>8170 (4931, 531774) * ‡</td>
<td></td>
</tr>
<tr>
<td>KC</td>
<td>129 (108, 247)</td>
<td>20854 (424, 50227)</td>
<td>21703 (11094, 23399) * ‡</td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>231 (141, 356)</td>
<td>143 (67, 297)</td>
<td>143 (131, 253)</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>ND</td>
<td>4 (3.1, 5.1)</td>
<td>42 (3.1, 77)</td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>1.6 (1.4, 10.5)</td>
<td>276 (103, 333)</td>
<td>78 (64, 7196) * ‡</td>
<td></td>
</tr>
<tr>
<td>IL-12p40</td>
<td>3.4 (1.9, 16.8)</td>
<td>874 (437, 1487)</td>
<td>1184 (196, 3474) * ‡</td>
<td></td>
</tr>
<tr>
<td>MIP2 (CXCL2)</td>
<td>ND</td>
<td>1262 (486, 7300)</td>
<td>1146 (655, 34042) * ‡</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>ND</td>
<td>54 (41, 106) * ‡</td>
<td>44.4 (35, 215) * ‡</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05 vs. COX-2⁺/⁺ CLP by the Mann-Whitney test
‡ p < 0.05 vs. sham by the Mann-Whitney test
ND – not detected
Data are presented as median (IQR)