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Maternal activation of the EGFR prevents translocation of gut-residing pathogenic Escherichia coli in a model of late-onset neonatal sepsis

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Late-onset sepsis (LOS) is a highly consequential complication of preterm birth and is defined by a positive blood culture obtained after 72 h of age. The causative bacteria can be found in patients’ intestinal tracts days before dissemination, and cohort studies suggest reduced LOS risk in breastfed preterm infants through unknown mechanisms. Reduced concentrations of epidermal growth factor (EGF) of maternal origin within the intestinal tract of mice correlated to the translocation of a gut-resident human pathogen Escherichia coli, which spreads systemically and caused a rapid, fatal disease in pups. Translocation of Escherichia coli was associated with the formation of colonic goblet cell-associated antigen passages (GAPs), which translocate enteric bacteria across the intestinal epithelium. Thus, maternally derived EGF, and potentially other EGFR ligands, prevents dissemination of a gut-resident pathogen by inhibiting goblet cell-mediated bacterial translocation. Through manipulation of maternally derived EGF and alteration of the earliest gut defenses, we have developed an animal model of pathogen dissemination which recapitulates gut-origin neonatal LOS.

Late-onset neonatal sepsis (LOS) can be caused by a variety of pathogens, occurs at least 72 h after birth, accounts for 26% of all deaths in preterm infants, and results in an increased risk of long-term neurocognitive problems (1, 2). LOS has remained an important challenge in preterm infant care (3) due to the continuing reduction in the age of viability of very low birth weight (VLBW, birth weight ≤ 1,500 g) infants, who are at greater risk for bloodstream infections (BSIs), with an incidence of 10% and a mortality rate between 30 to 50% (1). Recent clinical data indicate incidence of LOS may be decreasing, though it remains unclear which interventions have the most impact in reduction of neonatal BSIs and LOS (4). Great effort has been devoted to hygiene related to invasive procedures (5, 6), and there is increasing focus on breastfeeding (7, 8).

In a substantial portion of LOS cases, the causative pathogen can be found to reside in the gut before it disseminates (9–12). It is hypothesized that an immature intestinal barrier enables translocation of such resident gut bacteria, but the mechanisms allowing or inhibiting translocation of the gut microbiota in early life remain unknown. These infections occur during the first 60 d of life, a time in life when exclusive breastfeeding is recommended. Formula-fed preterm infants have greater intestinal permeability compared to breast milk-fed preterm infants (13), and in animal models, formula feeding enables bacterial translocation from the gut to the mesenteric lymph nodes and spleen. Following EGFR disruption or asynchronously cross-fostering, mice developed a rapid fatal response to oral challenge of Escherichia coli isolated from the bloodstream of LOS patients, which were also of gut origin in those neonates. However, other gut-resident commensal Es. coli from infants without disease were not lethal, despite their ability to translocate in this model. E. coli translocation was associated with goblet cell-associated antigen passages (GAPs) formation by colon goblet cells and inhibited by luminal EGF via EGFR activation in goblet cells. Thus maternal

Significance

The gut-originating pathogen Escherichia coli has been associated with a portion of cases of late-onset sepsis (LOS), a leading cause of neonatal mortality. While it remains unclear how E. coli may gain access to the systemic circulation from the intestine, breast milk may protect against bacterial translocation and reduces the risk of LOS. Here we show a mechanism whereby gut-residing E. coli gain systemic access and have developed an animal model replicating this mechanism to explore the protective effects of breast milk in LOS.


EGF, and potentially other EGFR ligands, inhibits translocation of a gut-residing pathogen in offspring by directly acting on goblet cells and inhibiting GAP formation in the offspring’s colon, thereby interdicting gut-resident pathogens from traversing the epithelium and gaining systemic access.

Results
Infants born preterm and fed mother’s own milk (MOM) have a reduced risk of LOS (21, 22). EGF is abundant in breast milk after parturition, decreases throughout lactation, and has been observed to improve intestinal barrier function and reduce bacterial translocation in offspring (20). Therefore, we measured EGF in the stool of VLBW infants (SI Appendix, Table S1) that were fed MOM or formula over the first 60 d of life to assess the contribution of maternal breast milk to luminal EGF concentrations. Stools from the breastfed children contained significantly greater concentrations of EGF, and this concentration diminished over time, reflecting the reduction in concentration of maternal EGF in the milk (17–19) (Fig. 1A and SI Appendix, Fig. S1A). Similar temporal trends of EGFR ligands amphiregulin (AREG), transforming growth factor alpha (TGF-α), and heparin-binding EGF like growth factor (HB-EGF), which have been reported to be present in human breast milk (17, 23, 24), were not observed in the stool. Additionally, there were no differences in the fecal concentrations of AREG, TGF-α, and HB-EGF in the stools of MOM-fed or formula-fed children, which were overall less abundant than EGF in the stool, particularly in the first 4 wk of life (SI Appendix, Fig. S1B–D, compare to Fig. 1A). EGF concentrations in murine milk also diminished over time, reflecting a gradient that could also be measured in the stool (SI Appendix, Fig. S2A) and lumen (25) of pups during early life, a time when neonatal mice immunologically resemble the preterm human infant gut (26, 27).

We next asked if maternal EGF induces a response in the offspring that prevents translocation of gut bacteria via activation of the EGFR. Pups were colonized with *E. coli* by oral gavage of $2 	imes 10^5$...
AnalR or C1nalR could be found in the stool of all mice 3 d after injection. At 24 h, nodes, and spleen were homogenized and plated on MacConkey small intestine and colon lamina propria, mesenteric lymph nodes, and spleen were homogenized and plated on MacConkey agar plates containing nalidixic acid following oral challenge and EGFRi injection. At 24 h, E. coli BSI-AnalR or C1nalR could be found in the stool of all mice 3 d after gavage, but only the MLNs, spleen, and liver of pups treated with epidermal growth factor receptor inhibitor (EGFRi) contained E. coli BSI-AnalR or C1nalR (Fig. 1C). EGFRi-treated mice developed neutrophilia (Fig. 1D) and died following colonization with E. coli BSI-AnalR but not C1nalR (Fig. 1E). Illness was characterized by a lack of weight gain (Fig. 1F), and lethargy. Mice injected i.p. with BSI-A, but not C1, succumbed rapidly, confirming the virulence of BSI-A as a pathogen when introduced directly into the body cavity of mice (SI Appendix, Fig. S3). The mean lethality rate per litter in EGFRi-treated mice exposed to BSI-A was 75% (SI Appendix, Fig. S4). Thus, following inhibition of EGFR in pups, E. coli resides in the gut disseminate and, depending on the microbe, can cause rapid and significant lethality.

To identify where E. coli translocate following colonization, small intestine and colon lamina propria, mesenteric lymph nodes, and spleen were homogenized and plated on MacConkey agar plates containing nalidixic acid following oral challenge and EGFRi injection. At 24 h, E. coli BSI-AnalR were largely found in the lamina propria of the colon, but not small intestine. E. coli BSI-AnalR was recovered from the MLN after 48 h and from the spleen and liver after 72 h (Fig. 2A). These data suggest that E. coli BSI-AnalR translocate via the colon epithelium. Goblet cells, intestinal epithelial cells of the secretary lineage, have been observed to be a portal of entry for pathogens (28, 29) and are present and functional in the neonatal intestinal epithelium following birth (30). Specifically, colon goblet cells can deliver antigens and live bacteria from the intestinal lumen to the colon lamina propria and colon mesenteric lymph node, through the fecal-oral route (28, 29) but not C1nalR (Fig. 2B). Luminal EGFR inhibits colon GAP formation in neonates through EGFR activation specifically on goblet cells (25, 32), suggesting maternal disruption of EGF in ACF mice specifically by systemic disease, but commensal EGFRi treatment. EGFR on the epithelium was restored upon luminal EGF administration (Fig. 2C). Three days postinfection, E. coli BSI-AnalR and C1nalR were found in the stool of both SCF and ACF mice indicating all mice died equally in response to i.p.-injected E. coli BSI-AnalR, suggesting maternal disruption of EGFR in ACF mice specifically increases risk of sepsis due to enteric pathogens, but not systemically introduced pathogens (SI Appendix, Fig. S5).

E. coli BSI-AnalR was recovered from the colon lamina propria and, to a significantly lesser extent, the small intestine lamina propria 24 h after gavage of ACF mice, (Fig. 3A) similar to the observations in EGFRi-treated mice (Fig. 2A). Gaps formed in the colonies of ACF pups, but not SCF pups, and this formation was inhibited by luminal EGF (Fig. 3B). Additionally, the epithelium of ACF mice contained significantly decreased phosphorylated EGFR, indicating EGFR is less activated in ACF mice. Phosphorylation of EGFR on the epithelium was restored upon luminal EGF administration (Fig. 3C). To confirm that luminal EGF was sufficient to protect from gut-origin sepsis in ACF mice, ACF mice were infected with E. coli BSI-AnalR and gavaged with either a single dose of recombinant murine EGF at the time of infection, or daily administration of EGF throughout the course of the infection model. ACF mice given daily EGF had reduced bacterial translocation to the MLN, spleen, and liver even though colonization was not affected (Fig. 5E). Finally, daily EGF significantly protected ACF mice.
following BSI-A<sup>AB</sup>R infection (Fig. 5F). In contrast, daily administration of EGF to EGFRi-treated mice or EGFR<sup>AB</sup>R to mice failed to inhibit GAP formation, prevent bacterial translocation, or protect from disease (SI Appendix, Fig. S6), indicating the necessity of EGFR ligands to be actively sensed by the offspring to provide protection from translocating bacteria. Taken together, these data suggest disruption of maternally derived EGF, and potentially other EGFR ligands, reduces EGFR activation, GAP formation by GCs, and bacterial translocation via colon GAPs. Additionally, we found dissemination of pathogenic <i>E. coli</i> but not commensal <i>E. coli</i> resulted in a fatal sepsis-like disease in pups following EGFR ligand or EGFR disruption.

**Discussion**

The many postulated beneficial effects of breast milk, particularly MOM, include reducing intestinal permeability (13) and preventing bacterial translocation (14–16). These effects plausibly contribute to the partial reduction of the risk of LOS among breastfed preterm infants (21, 22). EGF and other EGFR ligands, components of breast milk, can contribute to each of these functions through direct ligation of the EGFR on goblet cells in the colon of offspring. Breast milk contains EGF and other EGFR ligands, such as AREG, TGF-α, and HB-EGF (17, 23, 24), though at reduced concentrations compared to EGF (18, 19). We found no significant difference in the concentration of AREG, TGF-α, and HB-EGF in the stool between MOM-fed and formula-fed children, suggesting maternally derived EGF is the predominant EGFR ligand biologically available throughout the entirety of the GI tract, including the lower large intestine. We recognize that other EGFR ligands may also be affected following ACF and EGFR inhibition and that in some systems EGFR ligands can have complementary and overlapping roles (33). However, the
relatively higher levels of EGF in breastmilk, and the ability of enteral recombinant EGF in mice to restore the intestinal barrier function, prevent bacterial translocation, and avert disseminated bacterial infection after enteral challenge with a pathogenic E. coli strongly suggest that EGF is a protective element in maternal milk.

Other breast milk constituents might confer protection from enteric pathogens including maternal immunoglobulin A (IgA) (34, 35) and oligosaccharides (36), both of which are also found in higher concentrations in breast milk soon after parturition compared to later in lactation and may also be disrupted by ACF. The individual contributions of these maternal factors can be explored in the ACF model as IgA may contribute to protection from invasive enteric pathogens through directly binding and preventing adherence and evasion (37, 38), and oligosaccharides support the maturation of the normal infant microbiota that may provide colonization resistance (39, 40).

Other models of enteric infections have demonstrated invasive organisms associate with goblet cells (28, 41). We extend this finding to GAP formation by goblet cells in the colon consistent with our previous work showing bacterial translocation through the colon occurs when bacteria pass transcellularly through goblet cells when forming GAPs (31). EGFR activation inhibits...
analyses. The association between strains and outcomes should be
bloodstream isolates from infants are underrepresented in these
studies of extraintestinal pathogenic
in vivo experimental data to infer mechanisms. There are many
specimen sets from cases and controls and extensive in vitro and
bloodstream isolates and in the controls is potentially interesting,
strains that were not present in at least one of the nonpathogenic
location from the intestine. Initial screens for virulence loci
specific bacterial factors determine virulence following trans-
translates from LOS cases seen in this set of experiments suggest
EGF did not appear to have an effect on bacterial habitation in
increase or death in the pups, despite similar translocation and
from the bloodstream of LOS cases resulted in noticeable dis-
biactive factors present in human milk.
deavor to replace formula with a more complete diet offering
donor milk banks when MOM is unavailable is a laudable en-
provides protection from bacterial translocation and blood-
formation in early life (25), suggesting a role for maternally
derived EGFR ligands is to limit bacterial translocation and
protect the offspring from potentially invasive enteric pathogens.
EGF did not appear to have an effect on bacterial habituation in
the lumen, as all pups were colonized similarly by evidence of E.
colii measured in stool. Rather we find direct sensing of EGFR
ligands by the EGFR on goblet cells in the colon of the offspring
provides protection from bacterial translocation and blood-
stream infection. The recent initiative to provide neonatal inten-
tive care unit (NICU) patients with human milk through
donor milk banks when MOM is unavailable is a laudable en-
edeavor to replace formula with a more complete diet offering
nutrition and protection to the neonate. However, our work
raises the possibility that human milk from donors close to
parturition may offer superior protection compared to human
milk expressed later in lactation, due to temporal availability of
bioactive factors present in human milk.

While all E. coli strains spread systemically, only those isolated
from the bloodstream of LOS cases resulted in noticeable dis-
ease or death in the pups, despite similar translocation and
bacterial load in extraintestinal tissue. We have previously shown
gut-resident commensal bacteria translocating via GAPs circu-
late systemically without causing overt disease (31). Combined
with the previous observation, the variation among E. coli iso-
lates from LOS cases seen in this set of experiments suggest
specific bacterial factors determine virulence following trans-
location from the intestine. Initial screens for virulence loci
found no common factors present in both of the pathogenic
strains that were not present in at least one of the nonpathogenic
strains. Our identification of several virulence loci in the
bloodstream isolates and in the controls is potentially interesting,
but the assignment of a virulence locus in E. coli obligates large
specimen sets from cases and controls and extensive in vitro and
in vivo experimental data to infer mechanisms. There are many
studies of extraintestinal pathogenic E. coli virulence loci, but
bloodstream isolates from infants are underrepresented in these
analyses. The association between strains and outcomes should be

considered to be provisional, as one of five bloodstream isolates
did not cause disease in mice. An intensive survey of pathogenicity
of neonatal wild-type E. coli would be informative. Also, even
though this study is limited to E. coli organisms, LOS can be
caused by multiple different genera, including Enterococci, Strept-
tococci, and other Enterobacteriaceae species (3). Though the
generalizability of GAP translocation as a portal of entry of spe-
cific pathogens is not established by our data, we have previously
shown both Gram-negative and Gram-positive bacteria can
translocate via GAPs (31). Further work will determine the variety
of bacteria that can utilize goblet cells to translocate in early life
following EGFR disruption and should pursue additional and
potentially complementary mechanisms, such as toll-like receptor
(TLR) activation in the gut mucosa. Compared to models of
neonatal sepsis utilizing i.p. injection of lipopolysaccharide (LPS),
or cecal contents, ACF more faithfully recapitulates the enteric
route of entry that has been associated with late-onset sepsis cases
(9–12, 42). Combining the previous studies with the observation
that the enteric route of entry has been shown to contribute to
virulence and disease when compared to i.p. injection (43), ACF
and colonization offer a minimally invasive model of dissemina-
tion without pharmacological manipulation or physical breach of
the intestinal or skin barriers.

In summary, our model of enterally acquired bloodstream
infections following translocation of a gut-residing pathogen
demonstrates that maternally derived EGF, and potentially other
EGFR ligands, can prevent this process. GAPs, which formed in
response to decreased or disrupted EGFR signaling, were
exploited by enteric bacteria. Depending on the bacteria, trans-
location resulted in the rapid sepsis-like death of offspring in this
model of LOS. This model has profound relevance and applica-
tion to the study of LOS and the development of therapies for
the prevention and treatment of LOS unique to the neonatal
phase of life.
Materials and Methods

Mice. All mice were bred for 10 or more generations on the C57BL/6 background. Math1PGRCh-labeled mice (44) were purchased from The Jackson Laboratory and bred and maintained in-house. EGFRf/f mice (45) were a gift of Dr. David Threadgill, University of North Carolina, Chapel Hill, NC. To genetically delete EGFR in GCs, mice were bred to generate EGFRfl/flMath1PGRCh and EGFRfl/littermates. These mice were injected i.p. with mifepristone (Caymen Chemical Company) (10 mg/kg), which was dissolved at a concentration of 2 mg/mL in sunflower oil, on DOL2, 4, and 6. Cohoused littermates were used when possible as experimental groups and controls to minimize differences in the gut microbiota. Animal procedures and protocols were performed in accordance with the Institutional Animal Care and Use Committee at Washington University School of Medicine. In some experiments, mice were injected i.p. with 50 µg/kg typhostin AG1478 (EGFR) (Sigma Aldrich) on DOL3 to inhibit EGFR activation. EGFRi was initially diluted in dimethyl sulfoxide (DMSO) to a concentration of 3.3 mg/mL, then further diluted in phosphate buffered saline (PBS) to a concentration of 33 µg/mL. Mice were injected with 16.6 µL, per gram bodyweight on DOL3 (25). In some experiments, mice were gavaged with murine EGF (Caymen Chemical Company) (10 mg/kg), which was dissolved at a concentration of 10 µg/mL nalidixic acid to identify and fail to gain weight. In survival experiments, mice were monitored twice daily for signs of disease and lack of weight gain. In some mice, 24 h following gavage, small intestine and colon lamina propria were isolated to analyze bacterial translocation. In some mice, 3 d following gavage, spleen, MLN, and liver were isolated to analyze bacterial translocation. Litters of four or more were all colonized with the same E. coli strain to avoid cross-contamination and repeated at least three times to control for litter variability. In EGFRi experiments, litters of eight or more were all colonized with the same E. coli strain and then injected with EGFRi or PBS within the same litter to control for litter variability. EGFRfl/flMath1PGRCh and EGFRfl/littermates within a single litter were all colonized with the same E. coli strain and treated with mifepristone.

Nalidixic Acid-Resistant E. coli Strains. E. coli strains BSI-A, BSI-C, C1, and C2 were grown in 10 mL Luria-Bertani (LB) broth containing 4 µg/mL nalidixic acid (Acros Organics, Thermo Fisher Scientific). Isolates were plated on MacConkey agar plates containing 4 µg/mL nalidixic acid. The process was repeated using increasing amounts of nalidixic acid until E. coli strains grew in media containing 20 µg/mL nalidixic acid, and the MLST types were confirmed to remain unchanged. In all experiments, E. coli was grown by inoculating 10 mL of LB containing 4 µg/mL nalidixic acid with a frozen chip of E. coli glycerol stock (stock made from 10^9 CFUs in 1.5 mL PBS/glycerol in a 1:1 ratio). Cultures were grown shaking, at a 45° slant, at 37 °C to an optical density (OD) of 0.30 for a concentration of 10^6 CFUs/mL. Cultures were pelleted, washed in PBS, and resuspended to a concentration of 10^6 CFUs/mL in PBS for further use.

Virulence Factor Screen. Primers for extraintestinal pathogenic (ExPEC) virulence factors were retrieved from the literature and used for in silico PCR with the command line tool primer search from EMBOSS (48). Only assemblies with matches for both primers for each gene were reported. In addition, assemblies were analyzed against the VirulenceFinder database and hits against ExPEC virulence genes are reported (49).

Stool Samples. Frozen stool previously collected was identified from patients 1) having either an exclusive human milk (HOM) or formula-fed diet during the first 60 d of life, 2) negative for necrotizing enterocolitis or sepsis, and 3) having at least 10 stool specimens available from birth through day of life 60. Five breastfed patients and five formula-fed patients were included in this study, with at least 10 time points per individual between DOL10 and DOL60. Patient metadata are available in SI Appendix, Table S1.

Quantification of EGFR Ligands from Stool. Frozen stool specimens were resuspended in PBS, homogenized, and analyzed by enzyme-linked immunosorbent assay (ELISA) for human EGF (R&D Systems), human amphiuregulin (AREG) (R&D Systems), human TGF-α (R&D Systems), and human heparin-binding EGF (HB-EGF) (R&D Systems), per the manufacturer’s protocol. For mice, stool was collected in PBS, homogenized, and analyzed by ELISA for murine EGF (R&D Systems), per manufacturer’s protocol.

Asynchronous Cross-Fostering. At first sign of pregnancy, male breeders were removed from the cages of female breeders. On DOL1, pups were placed in a new cage with a dam that had delivered pups 10 d prior and was still actively nursing (asynchronous cross-fostering, ACF), or a dam that had delivered pups on the same birthdate as pups (asynchronous cross-fosterring 5CF). All pups, bedding, and dam’s nostrils were wiped with imitation vanillin extract to increase litter acceptance by dam. Mice were monitored for litter acceptance and maternal care, and weighed daily.

E. coli Infection. On DOLs, mice were gavaged with 20 µL PBS containing 10^9 CFU/mL (equivalent to 2 x 10^6 CFU) E. coli. Stool was collected to monitor colonization. Mice were monitored for disease, including lack of weight gain, lethargy, pallor, lack of nursing, and death. In some experiments, moribund mice were euthanized to confirm translocation of enteric bacteria to extraintestinal organs and compare the MLST of recovered colonies to those of the challenge E. coli. Moribund was recognized as lethargic pups excluded from the nest that remained motionless when weighed on scale and failed to gain weight. In survival experiments, mice were monitored twice daily for signs of disease and lack of weight gain. In some mice, 24 h following gavage, small intestine and colon lamina propria were isolated to analyze bacterial translocation. In some mice, 3 d following gavage, spleen, MLN, and liver were isolated to analyze bacterial translocation. Litters of four or more were all colonized with the same E. coli strain to avoid cross-contamination and repeated at least three times to control for litter variability. In EGFRi experiments, litters of eight or more were all colonized with the same E. coli strain and then injected with EGFRi or PBS within the same litter to control for litter variability. EGFRfl/flMath1PGRCh and EGFRfl/littermates within a single litter were all colonized with the same E. coli strain and treated with mifepristone.

Bacterial Quantification in Organs and Stools. Small intestines or colons were harvested, rinsed with PBS, and Peyer’s patches or colonic patches were removed and discarded. Epithelial cell populations were released by incubating the intestines for 15 min in a 37 °C rotating incubator in 20 mL Hank’s balanced salt solution (HBSS) media (BioWhittaker) containing 5 mM EDTA and gentamycin (50 µg/mL) as previously described (50). Following removal of epithelium, isolated lamina propria was cut into pieces. We recovered splenic, MLN, and lamina propria cells as previously described (50). Lamina propria tissues, spleen, MLN, liver, or stool were homogenized in 500 µL PBS with 200 mg 0.1 mm diameter zirconium silica beads (BioSpec), and homogenized Bullet Blender Tissue Lysis Master Mixer (Next Advance) vortexed on bead beater. Supernatant was plated on MacConkey agar containing 20 mg/mL nalidixic acid to identify nalidixic acid-resistant E. coli, and identity of E. coli strain was confirmed through MLST identification.

Extraction of Milk from Lactating Dams. Milk was extracted from lactating dams every third day beginning on postpartum day 5 as described (51).

GAP Quantification, Goblet Cell Staining, and Bacteria Visualization. With mice under anesthesia, tetramethylrhodamine-labeled dextran 10,000 molecular weight (MW) (10 µg/mL) (Thermo Fisher Scientific) was injected intraluminally into the colon. Thirty minutes later, colon sections were fixed and in 10% buffered formalin for 30 min before blocking in optimal
cutting temperature (OCT) compound and freezing as previously described (32, 52). Sections were cut and counterstained with DAPI. The number of CFUs per gram was quantified by immunofluorescence microscopy as previously described (32, 52). Specimens were blinded for analysis, and CFUs were identified as dextran-filled columns measuring ∼20 μm (height) × 5 μm (diameter) traversing the epithelium and containing a DAPI+ nucleus. Colon CFUs were enumerated within the crypts as CFUs per crypt, which were identified as a ring of DAPI+ nuclei. Colon GAPs were identified as dextran-filled columns measuring ∼15 μm (diameter) traversing the epithelium and containing two-way ANOVA), linear regression analysis, or Mann-Whitney U test was performed using GraphPad Prism (GraphPad Software Inc.).

Flow Cytometry and Sorting of Goblet Cells. CFUs (106) E. coli BSI-L were labeled with Vybrant RubyRed (Thermo Fisher Scientific) per the manufacturer's directions and resuspended into a concentration of 105 CFUs/mL. CFUs (105, 100 μL) were intrarectally administered into the colon. Three hours later, CFUs were removed and fixed in 10% buffered formalin for 15 min before blocking in OCT and freezing as previously described (32, 52). Sections were cut and stained with CK-18 (Abcam) and DAPI. Immunohistochemistry was performed as previously described (52).

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