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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 EGF 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 explore the protective effects of breast milk in LOS.

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 (MLNs) and the liver (14–16). Epidermal growth factor (EGF) is a growth factor that is maternally supplied with high concentrations in colostrum and decreases in concentration in breast milk throughout lactation (17–19), and reduces bacterial translocation in formula-fed animals (20).

Here we disrupt EGFR receptor (EGFR) signaling in neonatal mice and use asynchronously cross-fostered pups to reduce high luminal EGF concentrations originating from maternal milk perinatally, both of which result in 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 *E. 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.


Competing interest statement: K.A.K. and R.D.N. are inventors on U.S. Nonprovisional Application Serial No. 15/880,658 Compositions And Methods For Modulation Of Dietary Pharmaceuticals. This article is a PNAS Direct Submission. This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Data deposition: The data reported in this paper have been deposited in the GenBank database, https://www.ncbi.nlm.nih.gov/genbank/ (accession nos. 203059/ATNW00000000/ATNW96639, 203060/ATNW00000000/ATNW96957, GCA_008091795.1, PRUNA602838/SAHMN19092311/AADAA000000000000).

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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 in breast milk of EGF (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. S1 B–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 $2 \times 10^5$...
AnalR or C1nalR could be found in the stool of all mice 3 d after inoculation (SI Appendix, Table S2), a commensal E. coli isolated from the intestinal tract of an infant without a bloodstream infection and injected intraperitoneally (i.p.) with vehicle or an inhibitor of EGFR activation, tyrphostin AG1478, on day of life (DOL) 5. Both E. coli strains were found in the stool of the pups for at least 7 d following gavage confirming colonization (Fig. 1B): no naldixic acid-resistant bacteria were found in the stool of the dam or pups prior to inoculation (SI Appendix, Fig. S2B). E. coli BSI-A\textsuperscript{naldR} or C1\textsuperscript{naldR} 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-A\textsuperscript{naldR} or C1\textsuperscript{naldR} (Fig. 1C). EGFRi-treated mice developed neutrophilia (Fig. 1D) and died following colonization with E. coli BSI-A\textsuperscript{naldR} but not C1\textsuperscript{naldR} (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 resident 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 naldixic acid following oral challenge and EGFRi injection. At 24 h, E. coli BSI-A\textsuperscript{naldR} were largely found in the lamina propria of the colon, but not small intestine. E. coli BSI-A\textsuperscript{naldR} 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-A\textsuperscript{naldR} translocate via the colon epithelium. Goblet cells, intestinal epithelial cells of the secretory 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, but not C1\textsuperscript{naldR}, translocated to the MLN and colon in DOL 5 EGFRi-treated pups (31). Luminal EGF inhibits colon GAP formation in neonates through EGFR activation specifically on goblet cells (25, 32), and accordingly GAP formation occurred in the colon of pups following treatment with EGFRi (Fig. 2B). Using immunohistochemistry and flow cytometry, we observed that fluorescently labeled E. coli BSI-A associated with cytokeratin (CK)-18\textsuperscript{+} goblet cells in the colon of mice following EGFRi treatment but not with intestinal epithelial cells (IECs) of EGFRi-treated mice or goblet cells of vehicle-treated mice (Fig. 2 C–E). Live BSI-A\textsuperscript{naldR} could be isolated from fluorescence-activated cell (FACS)-sorted goblet cells (GCs), but not IECs, of EGFRi-treated mice (Fig. 2F), confirming that translocation of live E. coli occurs via GCs and correlates with GAP formation following EGFRi treatment.

We expanded our testing to additional strains of E. coli isolated from preterm infants. The naldixic acid-resistant mutants of E. coli BSI-C, a bloodstream isolate from a sepsis patient (9), and E. coli C2, a gut commensal not known to cause disease that was isolated from the stool of an infant, translocated similarly following colonization and EGFRi administration (Fig. 2G). Only BSI-A\textsuperscript{naldR} and BSI-C\textsuperscript{naldR} caused morbidity in pups that had been administered EGFRi (Fig. 2H). Furthermore, three of four other E. coli bloodstream isolates from sepsis patients were lethal in pups following EGFRi treatment (Fig. 2I and SI Appendix, Table S2), suggesting that E. coli from bloodstream infections result in systemic disease, but commensal E. coli do not, even though they both disseminate. Initial screen for recognized E. coli virulence factors using whole genome sequences from BSI-A, BSI-C, C1, and C2 revealed no common virulence factors present in both of the pathogenic strains that were not present in at least one of the nonpathogenic strains. Bloodstream isolates BSI-A and BSI-C and control isolates C1 and C2 have four, seven, four, and three putative virulence loci, respectively. Only four loci were shared by more than one isolate: hlyE, which encodes flagellar filament hlyE, which encodes a pore-forming hemolysin, and iss and traT, which encode proteins that confer resistance to serum complement (SI Appendix, Table S3).

Deletion of EGFR from goblet cells using an inducible Math1-driven Cre recombinase resulted in GAP formation on DOL5 in EGFR\textsuperscript{iMath1PGC}cre mice (Fig. 3A). E. coli BSI-A\textsuperscript{naldR} and C1\textsuperscript{naldR} could be found in the stool of all mice 3 d after gavage indicating colonization, but only disseminated to the MLN, spleen, and liver of EGFR\textsuperscript{iMath1PGC}cre mice, replicating translocation of bacteria following EGFR inhibition (Fig. 3B). Again, mice quickly succumbed following infection with E. coli BSI-A\textsuperscript{naldR} but not C1\textsuperscript{naldR} following translocation (Fig. 3C). Thus, sensing by EGFR on goblet cells regulates bacterial translocation across the colon epithelium.

The concentration of EGF in murine milk decreases throughout lactation (SI Appendix, Fig. S2A). To model the exposure to low EGF concentrations seen in formula-fed children, we asymptotically cross-fostered newborn mice housed 1 d old, with dams that delivered 10 d earlier (Fig. 4A). Asynchronously cross-fostered (ACF), but not synchronously cross-fostered (SCF) pups (1 d old pups cross-fostered to a different dam having delivered a litter on the same day, Fig. 4A), had reduced concentrations of EGF in their stool, suggesting that fecal EGF quantities in pups reflect the EGF concentration in their source of maternal milk (Fig. 4B).

to evaluate if ACF enables bacterial translocation of gut-resident bacteria and potentiates sepsis, mice were ACF on DOL1 and colonized with C1\textsuperscript{naldR} or BSI-A\textsuperscript{naldR} on DOL5. Three days postinfection, E. coli BSI-A\textsuperscript{naldR} and C1\textsuperscript{naldR} were found in the stool of both SCF and ACF mice indicating all mice were colonized (Fig. 4C), but were only found in the MLN, spleen, and liver of ACF mice. This is similar to the results in the EGFRi-treated mice (Fig. 4D), and supports that ACF results in bacterial translocation and systemic dissemination. Additionally, ACF mice quickly succumbed following colonization with E. coli BSI-A\textsuperscript{naldR} but not C1\textsuperscript{naldR} (Fig. 4E). Both SCF and ACF pups cross-fostered to dams delivered a litter on the same day, had reduced concentrations of EGF in their stool, suggesting that fecal EGF quantities in pups reflect the EGF concentration in their source of maternal milk (Fig. 4B).

E. coli BSI-A\textsuperscript{naldR} 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. 5A) similar to the observations in EGFRi-treated mice (Fig. 2A). GAPs formed in the colons of ACF pups, but not SCF pups, and this formation was inhibited by luminal EGF (Fig. 5B). Additionally, the epithelium of ACF pups contained significantly increased phosphorylated EGFR, indicating EGFR is less activated in ACF mice. Phosphorylation of EGFR on the epithelium was restored upon luminal EGF administration (Fig. 5C) suggesting reduced activation of epithelial EGFR is consistent with reduced luminal EGF in ACF mice. Additionally, E. coli BSI-A\textsuperscript{naldR} was found within GCs, but not IECs, of the ACF mice, and the presence of E. coli BSI-A\textsuperscript{naldR} in GCs was significantly diminished following EGF administration (Fig. 5D). To confirm that luminal EGF was sufficient to protect from gut-origin sepsis in ACF mice, ACF mice were infected with E. coli BSI-A\textsuperscript{naldR} and gavaged with either a single dose of recombinant murine EGF at the time of infection, or daily administrations 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-AnalR infection (Fig. 5F). In contrast, daily administration of EGF to EGFRi-treated mice or EGFRf/fMath1PGRCre 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 E. coli but not commensal E. coli resulting 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

Fig. 2. Translocation of E. coli is associated with colon GAP formation. Conventionally reared mice were gavaged with 2 × 10⁵ CFUs of E. coli and injected intraperitoneally with EGFRi or vehicle. (A) CFUs per organ (small intestine lamina propria [SI LP], colon lamina propria [Colon LP], mesenteric lymph node [MLN], and spleen), following gavage of 2 × 10⁵ CFUs of BSI-AnalR in EGFRi-treated mice. (B) GAPs per colon crypt in conventionally reared mice treated with EGFRi or vehicle. (C) Immunohistochemistry image of colon epithelium from a vehicle (PBS, Left) or EGFRi-treated (Right) mouse following gavage of CFSE-labeled E. coli BSI-A (green) stained with CK-18 (red), and DAPI (blue), Inset of single CK-18+ goblet cell containing E. coli. (Scale bar: 10 μm.) (D) Gating strategy for colon CD45⁺ epithelial cells showing UEA-I⁻ CK18⁺ goblet cells (GCs) and UEA-I⁺ CK18⁻ intestinal epithelial cells (IECs) containing Vybrant RubyRed-labeled E. coli BSI-A. (F) Percentage of GCs or IECs containing E. coli BSI-A. (F) CFUs of BSI-A in IECs and GCs FACs-sorted based on the gating strategy in D. (G) CFUs in stool, mesenteric lymph node (MLN), spleen, and liver 3 d following gavage of 2 × 10⁵ CFUs of E. coli C1nalR, ZnalR, BSI-CanalR, BSI-CunalR in EGFRi-treated mice. (H) Survival of EGFRi-treated mice following gavage of 2 × 10⁵ CFUs of E. coli strains isolated from patient’s bloodstream (SI Appendix, Table S2). (I) Survival of EGFRi-treated mice following gavage of 2 × 10⁵ CFUs of E. coli strains isolated from patient’s bloodstream (SI Appendix, Table S2). n = 5 mice per group in A and G. n = 3 mice per group in B, E, and F. Representative image and flow plots of three independent mice shown in C and D. n = 5 mice per group in H. Individual data points plotted in A, B, and E–G with mean and SD plotted per group. Statistics used: Mann–Whitney (B), one-way ANOVA (E and F), Kaplan–Meier (H), * denotes statistical significance, P < 0.05 or less.
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 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*, *Streptococci*, and other *Enterobacteriaceae* species (3). Though the generalizability of GAP translocation as a portal of entry of specific 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 represents a physiologic route for bacterial translocation. ACF and colonization offer a minimally invasive model of dissemination 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 EGFR, and potentially other EGFR ligands, can prevent this process. GAPs, which formed in GAPs, which formed 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. EGFR did not appear to have an effect on bacterial habitation in the lumen, as all pups were colonized similarly by evidence of *E. coli* 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 bloodstream infection. The recent initiative to provide neonatal intensive care unit (NICU) patients with human milk through donor milk banks when MOM is unavailable is a laudable endeavor 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 disease 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 circulate systemically without causing overt disease (31). Combined with the previous observation, the variation among *E. coli* isolates from LOS cases seen in this set of experiments suggest specific bacterial factors determine virulence following translocation 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*, *Streptococci*, and other *Enterobacteriaceae* species (3). Though the generalizability of GAP translocation as a portal of entry of specific 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 represents a physiologic route for bacterial translocation. ACF and colonization offer a minimally invasive model of dissemination 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 EGFR, 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, translocation resulted in the rapid sepsis-like death of offspring in this model of LOS. This model has profound relevance and application 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. Math1PGRCre 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 generate heterozygous EGFRf/f (Math1PGRCre and EGFRf/f) littermates. These mice were injected i.p. with mifepristone (Cayman 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 500 μg/kg tyrphostin AG1478 (EGFRi) (Sigma Aldrich) on DOL5 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 DOLS (25). In some experiments, mice were gavaged with 20 μL of 50 μM EGFRi.

Human Studies. Bloodstream and fecal isolates of E. coli, and infant stools, were obtained from a prospective case-cohort study of the role of gut microbial populations in necrotizing enterocolitis in infants hospitalized in the NICU at St. Louis Children’s Hospital and the Children’s Hospital of Oklahomahoma University, and a study of the post-discharge microbiota in infants who had been hospitalized at St. Louis Children’s Hospital. Both site’s institutional review board approved this study; written informed consent was obtained from parents before enrollment. Details of the cohorts and methodology of stool collection are provided in the SI Appendix. Some of the E. coli strains used in this study have been previously published (9, 46). Pairs of E. coli strains BSI-A, BSI-B, BSI-C, BSI-D, BSI-E, BSI-F, BSI-G, and BSI-H (Table S2) were isolated from the bloodstream of NICU patients. Strains BSI-A and BSI-I were identified in the patient’s stool prior to onset of clinical deterioration and positive blood culture diagnosing LOS (9), and we did not seek cognate E. coli in the stools from the other patients from whom we isolated bloodstream E. coli. Commensal E. coli species (E. coli, C1, C2, SI Appendix, Table S2) were isolated from the stool of discharged pediatric patients, and not known to cause disease. DNA was isolated from single colonies and sequenced following PCR amplification of the adk, fumC, gyrB, kdr, mdr, purA, and recA genes for multilocus sequence type (MLST) identification (Geneve9) (SI Appendix, Table S2). Full genome sequences of BSI-A (9), BSI-C (9), C1 (46), and C2 (47) are publicly available. PRJNA/GenBank/Sequence Read Archive (SRA) accession nos. BSI-A (ST69): 203059/ATNVW000000000/5R769309 and BSI-C (ST70): 203060/ATNVW000000005R769057; C1 (ST35): accession no. GCA_00081795_1; C2: PRJNA606283/5R769309/231/JAAADAA0000000000000.

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 (MOM) 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 DOL0 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 immunoabsorbent assay (ELISA) for human EGF (R&D Systems), human amphiregulin (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 ELSA 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-fostering SCF). 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 10^6 CFU/mL (equivalent to 2 × 10^7 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, liver, and colon 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. EGFRf/f Math1PGRCre and EGFRf/f 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 the most nalidixic acid, and the MLST types were confirmed to remain unchanged. In all experiments, E. coli was grown by inoculating 10 μL of LB containing 4 μg/mL nalidixic acid with a frozen chip of E. coli glycerol stock (stock made from 10^8 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 10^5 CFUs/mL. Cultures were pelleted, washed in PBS, and resuspended to a concentration of 10^5 CFUs/mL in PBS for further use.

Blood Collection and Flow Cytometry. On DOLS, mice were gavaged with 20 μL PBS containing 10^5 CFUs of E. coli and injected i.p. with EGFRi or vehicle. Forty-eight hours following gavage, whole blood was collected via cheek bleed. Two drops (10 μL) were collected directly into a microcentrifuge tube containing 90 μL of 50 mM ethylenediaminetetraacetic acid (EDTA). Neutrophils in whole blood were identified using anti-Ly6G* (clone RB6-8C5, eBioscience). Flow cytometry was collected and analyzed on an Attune NxT flow cytometer (Invitrogen).

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 gentamicin (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 pieces, 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 Grinder (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 3rd 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 mg/mL) (Thermo Fisher Scientific) was injected intravenously into the colon. Thirty minutes later, colon were removed and fixed in 10% buffered formalin for 30 min before blocking in optimal
cutting temperature (OCT) compound and freezing as previously described (32, 32). Sections were cut and counterstained with DAPI. The number of GFP 2 microscopes using AxioVision software (Carl Zeiss). In some experiments, 10^6 CFUs of E. coli B/AI were labeled with carboxyfluorescein succinimidy ester (CFSE) as per the manufacturer’s directions (CellTrace CFSE, Thermo Fisher Scientific) and resuspended into a concentration of 10^6 CFUs/mL. One hundred microliters (10^5 CFUs) was intrarectally administered per crypt, which were identified as a ring of DAPI-positive epithelial nuclei. Colon GAPs were enumerated within the crypts as GAPs per crypt, which were identified as a ring of DAPI-positive epithelial nuclei. Images were analyzed by fluorescent wide-field microscopy that was performed with a Nikon A1 microscope using Axiovision software (Carl Zeiss). In some experiments, 10^6 CFUs of E. coli B/AI were labeled with carboxyfluorescein succinimidy ester (CFSE) as per the manufacturer’s directions (CellTrace CFSE, Thermo Fisher Scientific) and resuspended into a concentration of 10^6 CFUs/mL. One hundred microliters (10^5 CFUs) was intrarectally administered into the colon. Three hours later, colon GAPs were removed and fixed in buffered formalin for 15 min before blocking in OCT and freezing as previously described (32, 32). Sections were cut and stained with CK-18 (Abcam) and DAPI. Immunohistochemistry was performed as previously described (32).

Flow Cytometry and Sorting of Goblet Cells. CFUs (10^6) E. coli B/AI were labeled with Vybrant RubyRed (Thermo Fisher Scientific) per the manufacturer’s directions and resuspended into a concentration of 10^6 CFUs/mL. CFUs (10^5; 100 μL) were intrarectally administered per crypt, which were identified as a ring of DAPI-positive epithelial nuclei. Colon GAPs were enumerated within the crypts as GAPs per crypt, which were identified as a ring of DAPI-positive epithelial nuclei. Images were analyzed by fluorescent wide-field microscopy that was performed with a Nikon A1 microscope using Axiovision software (Carl Zeiss). In some experiments, 10^6 CFUs of E. coli B/AI were labeled with carboxyfluorescein succinimidy ester (CFSE) as per the manufacturer’s directions (CellTrace CFSE, Thermo Fisher Scientific) and resuspended into a concentration of 10^6 CFUs/mL. One hundred microliters (10^5 CFUs) was intrarectally administered into the colon. Three hours later, colon GAPs were removed and fixed in buffered formalin for 15 min before blocking in OCT and freezing as previously described (32, 32). Sections were cut and stained with CK-18 (Abcam) and DAPI. Immunohistochemistry was performed as previously described (32).

6. A. R. Jones, C. Kuchel, S. Jacobs, L. W. Doyle, Reduction in late-onset sepsis on refeeding by gentamicin (50 mg/L) to kill extracellular bacteria. Goblet cells were identified as CK-18–fluorescein isothiocyanate (FITC)–CD45-allophycocyanin (APC)® while IECs were identified as CK-18–FITC–CD45–APC (CK-18 antibody, monoclonal C-04, Abcam; CD45 antibody, monoclonal 30-F11, Thermo Fisher Scientific). Cells were either stained by flow cytometry on an Attune nXt (Innovo) or FACS isolated on a Synergy (Sony) sorter. FACS-isolated cells were homogenized and plated on MacConkey agar containing 20 mg/mL of nalidixic acid.

Statistical Analysis. Data analysis using Kaplan–Meier survival, one-way ANOVA (nonparametric Kruskal–Wallis), two-way ANOVA (repeated measure two-way ANOVA), linear regression analysis, or Mann–Whitney U test was performed using GraphPad Prism (GraphPad Software Inc.).

Data Availability. Whole genome sequences for BSI-A, BSI-C, BSI-D, and C2 are publicly available: Bioproject accession number PRJNA6GenBank/SRA run accession nos. BSI-A (ST69): 203059/ATNW00000000/SRR769039 and BSI-C (ST70): 13060/ATNW00000000/SRR769057, C1) ST35 (accession no. GCA_008081795.1; C2: PRJNA602838/SAMN13909231/AAADA00000000. All materials, data, and associated protocols are available upon request from the corresponding author, K.A.K.

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