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Surfactant Protein D Influences Mortality During Abdominal Sepsis by Facilitating *Escherichia coli* Colonization in the Gut

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OBJECTIVES: Determine the role of surfactant protein D (SPD) in sepsis.

DESIGN: Murine in vivo study.

SETTING: Research laboratory at an academic medical center.

PATIENTS: SPD knockout ($SPD^{-/-}$) and wild-type ($SPD^{+/+}$) mice.

INTERVENTIONS: $SPD^{-/-}$ and $SPD^{+/+}$ mice were subjected to cecal ligation and puncture (CLP). After CLP, *Escherichia coli* bacteremia was assessed in both groups. Cecal contents from both groups were cultured to assess for colonization by *E. coli*. To control for parental effects on the microbiome, $SPD^{-/-}$ and $SPD^{+/+}$ mice were bred from heterozygous parents, and levels of *E. coli* in their ceca were measured. Gut segments were harvested from mice, and SPD protein expression was measured by Western blot. $SPD^{-/-}$ mice were gavaged with green fluorescent protein, expressing *E. coli* and recombinant SPD (rSPD).

MEASUREMENTS AND MAIN RESULTS: $SPD^{-/-}$ mice had decreased mortality and decreased *E. coli* bacteremia compared with $SPD^{+/+}$ mice following CLP. At baseline, $SPD^{-/-}$ mice had decreased *E. coli* in their cecal flora. When $SPD^{-/-}$ and $SPD^{+/+}$ mice were bred from heterozygous parents and then separated after weaning, less *E. coli* was cultured from the ceca of $SPD^{-/-}$ mice. *E. coli* gut colonization was increased by gavage of rSPD in $SPD^{-/-}$ mice. The source of enteric SPD in $SPD^{+/+}$ mice was the gallbladder.

CONCLUSIONS: Enteral SPD exacerbates mortality after CLP by facilitating colonization of the mouse gut with *E. coli*.

KEY WORDS: cecal ligation and puncture; critical illness; *Escherichia coli*; sepsis; surfactant protein D

Sepsis is a syndrome caused by a dysregulated inflammatory response to infection, resulting in organ failure, and causes significant morbidity and mortality (1). Current sepsis therapies are limited to early control of infection and supportive care (2, 3). Many investigational agents have failed to improve sepsis outcomes (4). Surfactant protein D (SPD), a member of the collectin family of pattern recognition receptors, plays a key role in innate immunity (5). Accordingly, SPD is protective in preclinical models of infectious disease (6, 7). Although SPD is largely synthesized in the lung, SPD has also been detected in the mouse gastrointestinal tract after synthesis and secretion from the gallbladder, suggesting extrapulmonary functions of SPD (8). However, the role of SPD in nonpulmonary infections remains unknown.

Given the role of SPD in innate immunity, we hypothesized that SPD deficiency would increase mortality following polymicrobial sepsis induced by cecal ligation and puncture (CLP). Unexpectedly, we found that $SPD^{-/-}$ mice had improved survival after CLP. This difference in mortality was driven by

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SPD-dependent differences in colonization of the mouse gut by *Escherichia coli*.

MATERIALS AND METHODS

Animals and Housing

$SPD^{+/+}$ and $SPD^{-/-}$ mice were provided by Drs. Whitsett and Kingma (Cincinnati, OH) (9). Male mice were used for mortality experiments (**Figs. 1** and **2**), except for a confirmatory mortality experiment performed in females (**Supplemental Fig. 1**, http://links.lww.com/CCX/A995). Otherwise, combinations of adult females and males were used. See **Supplemental Table 1** (http://links.lww.com/CCX/A995) for experimental animal ages. Animals were housed separated by genotype (except as noted for cohousing experiments below) in a barrier facility. Mice were maintained on a mixed background (Black Swiss, 129, C57BL/6). Experiments were conducted in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines (10).

CLP and Fibrin Clot Sepsis Models

CLP was performed, as described (11–13). Briefly, after induction of anesthesia and analgesia with ketamine (85 mg/kg) and xylazine (34 mg/kg), mice were anesthetized, and a midline laparotomy was performed. The cecum was externalized, ligated, and punctured, after which a small amount of cecal contents were extruded from the puncture holes. The cecum was then replaced in the abdomen, and the abdominal incision was closed in layers. The mice were resuscitated with 1-mL phosphate-buffered saline (PBS) and placed in a warmed cage for postoperative recovery. As our study was focused on bacterial growth, the mice did not receive antibiotics to avoid reducing the sensitivity of blood and stool cultures. Fibrin clots were prepared as described (14, 15) prior to inoculation with *Escherichia coli* isolated from $SPD^{+/+}$ mice stool.

Bacterial Culture From Blood and Peritoneal Fluid

Mice underwent CLP (50% ligation, two 19G-holes) and were sacrificed 24 hours later. Peritoneal lavage was performed with 10-mL PBS, and blood was withdrawn from the right ventricle. Assessment for bacterial load in the peritoneum was measured by culturing samples on Luria-Bertani [LB] agar.

Cecal Culture of *E. coli*

Mice were anesthetized, and ceca were removed under sterile conditions, washed in sterile PBS, weighed, and frozen (−80°C). Frozen samples were transferred to 5 mL of sterile PBS at room temperature, then homogenized (26,000 rotations per minute [RPM], 10 s), plated onto MacConkey agar, and incubated overnight (37°C). Lactose fermenting colonies that were flat and nonmucoid were recultured onto sheep's blood agar, and indole testing was performed. *E. coli* colonies were identified as indole positive, flat, nonmucoid, pink colonies on MacConkey agar (16).

Phagocytosis Assays

For in vivo assays, green fluorescent protein (GFP)-labeled *E. coli* (American Type Culture Collection [ATCC] 25922GFP) were injected (intraperitoneally [IP]) into $SPD^{+/+}$mice. At 1 hour, the peritoneal lavage was performed, and cells were harvested (see **Supplement**, http://links.lww.com/CCX/A995). Using flow cytometry, neutrophils ($CD45^{+}$, $CD11b^{high}$, $Ly6G^{high}$, and $Ly6C^{high}$) and macrophages ($CD45^{+}$ and $F4/80^{high}$) were identified, and phagocytosis was quantified by measuring the positive GFP signal in those populations. For ex vivo assays, mice were pretreated with 1-mL 2% biogel P-100 (Biorad, Hercules, CA) IP, followed 24 hours later by peritoneal lavage. Neutrophils were isolated and incubated with GFP-labeled *E. coli* and then identified by flow cytometry ($Ly6G^{high}$ and $GR1^{+}$), and the amount of phagocytosis was determined as above.

Cohousing Experiments

Littermate $SPD^{-/-}$ mice and $SPD^{+/+}$ mice were bred from SPD heterozygous ($SPD^{+/-}$) parents. The mice were then either cohoused until sacrificed and cultured or separated by genotype after weaning (3–4 wk), and then killed prior to culture of cecal contents.

Immunoblotting

After sacrifice, gut segments were flushed with PBS. Tissue was harvested and flash frozen in liquid nitrogen and stored (−80°C). Frozen tissues were homogenized in radioimmunoprecipitation assay protein lysis buffer, centrifuged at 4°C, 8,000 RPM for 10 minutes, and supernatant was collected. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to nitrocellulose, membranes were incubated with anti-SPD

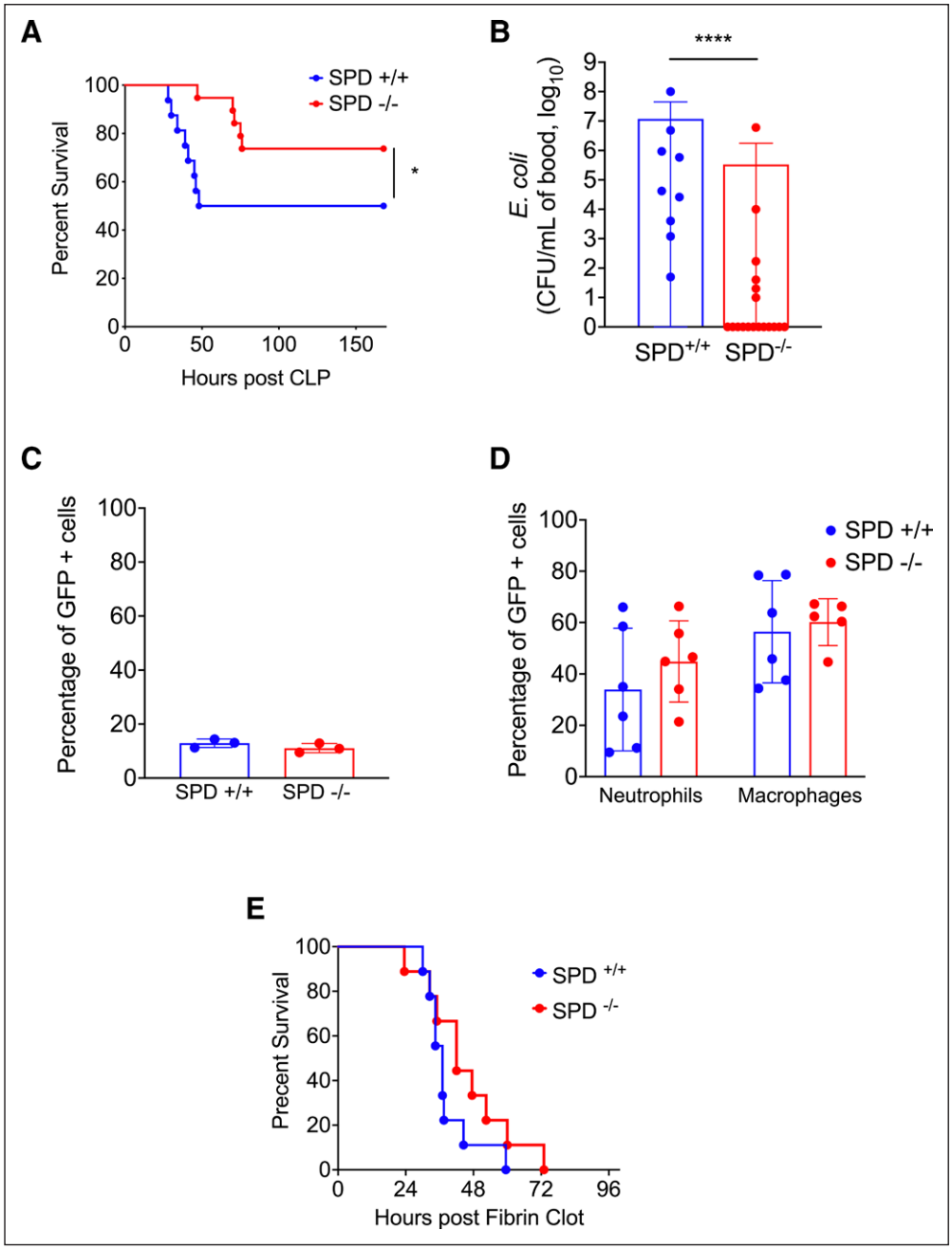

Figure 1. Surfactant protein D (SPD) deficiency reduces *Escherichia coli* bacteremia and mortality after cecal ligation and puncture (CLP) by reducing cecal colonization by *E. coli*. **A**, CLP (100 % ligation, 19-G puncture, two holes) was performed on $SPD^{+/+}$ and $SPD^{-/-}$ mice ($n = 16$/group) (Gehan-Breslow-Wilcoxon, $^{*}p < 0.05$). **B**, Blood *E. coli* load following CLP. *E. coli* colony forming units (CFU) in blood 24 hr after CLP (100% ligation, 19-G puncture, one hole) in $SPD^{+/+}$ ($n = 9$) and $SPD^{-/-}$ ($n = 18$) mice (Mann-Whitney, $^{****}p < 0.0001$). **C**, Ex vivo phagocytosis. Neutrophils were isolated from $SPD^{+/+}$ and $SPD^{-/-}$ mice ($n = 3$/group) and incubated with green fluorescent protein (GFP) expressing *E. coli*. The percentage of GFP+ cells was determined by flow cytometry ($p > 0.05$, Mann-Whitney). **D**, In vivo phagocytosis. $SPD^{+/+}$ mice ($n = 5$–6) and $SPD^{-/-}$ mice ($n = 6$) were injected intraperitoneally with GFP expressing *E. coli*. Neutrophils and macrophages were identified by cell surface markers, and the percentage of GFP+ cells was determined by flow cytometry ($p > 0.05$, Mann-Whitney). **E**, Post *E. Coli* fibrin clot survival of $SPD^{+/+}$ and $SPD^{-/-}$ mice ($n = 9$/group) (Gehan-Breslow-Wilcoxon, $p > 0.05$), where both $SPD^{+/+}$ and $SPD^{-/-}$ mice were exposed to exogenous *E. coli*. *E. coli* for fibrin clot was isolated from $SPD^{+/+}$ cecum.

antibody 2 µg/mL or 10 µg/mL (CatAF6839 R&D systems, Minneapolis, MN) and anti-glyceraldehyde 3-phosphate dehydrogenase antibody (Abcam Cat97051, Cambridge, United Kingdom) or anti-β-actin antibody (Sigma-Aldrich CatA5441, St. Louis, MO) as control (see Supplement, http://links.lww.com/CCX/A995).

GFP-Labeled *E. coli* Colonization

$SPD^{+/+}$ and $SPD^{-/-}$ mice were gavaged with 10 µL/g of ampicillin-resistant GFP-labeled *E. coli* (ATCC 25922GFP, 3.74×10^8 colony forming units/mL) with or without recombinant human surfactant protein D (rSPD) (Cat 1920-SP-050, R&D systems). Twenty-four hours later, mice were sacrificed, and gut segments were harvested under sterile conditions and frozen (−80°C). Cecum and colon were isolated and homogenized in 5 mL of sterile PBS and then cultured on LB agar plates with ampicillin 100 µg/mL selected for growth of ampicillin-resistant GFP-labeled *E. coli*. Fluorescent colonies were identified under UV light and manually counted using a ChemiDoc XRS+ System (Biorad).

Mouse Microbiome Analysis

Cecal contents were collected from three $SPD^{+/+}$ mice and three $SPD^{-/-}$ mice

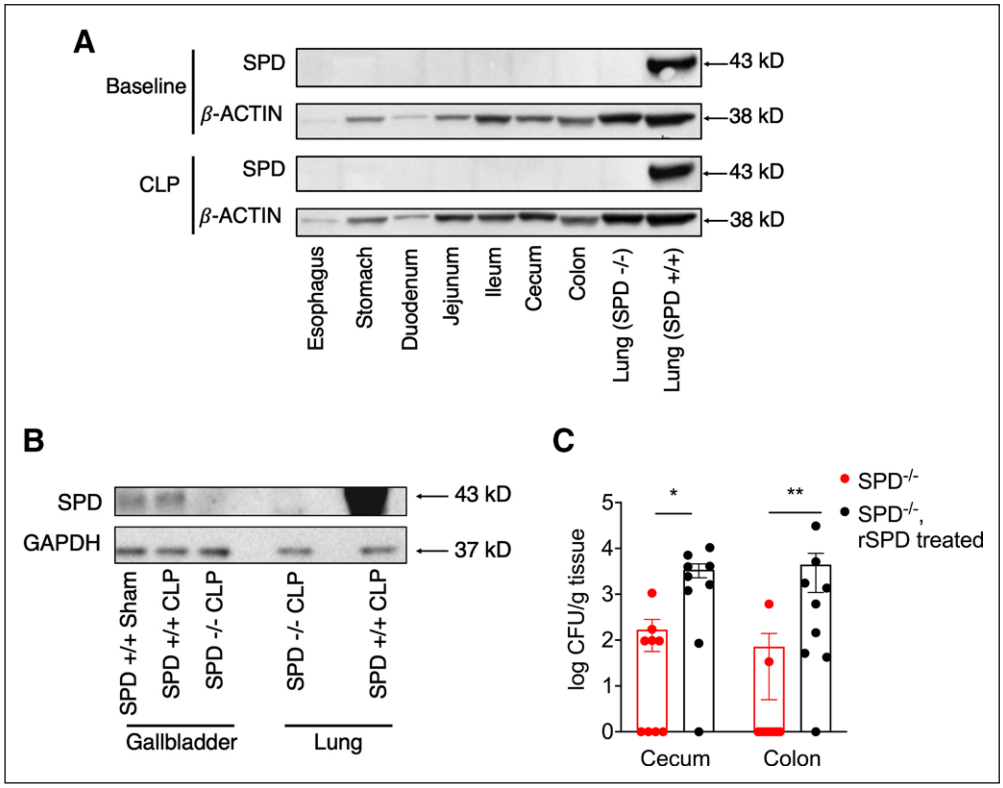

Figure 2. Surfactant protein D (SPD) is synthesized by the gallbladder and promotes colonization of both the cecum and colon with *Escherichia coli*. **A**, SPD$^{+/+}$ gut organs were harvested at baseline or post-cecal ligation and puncture (CLP) with Western blots performed for SPD or β-actin (loading control). *Blots* represent pooled samples, $n = 2$/group. Representative gel shown from three experiments. Lungs from SPD$^{-/-}$ and SPD$^{+/+}$ mice were used as negative and positive controls, respectively. **B**, Gallbladder was isolated from SPD$^{+/+}$ mice after CLP or sham surgery and from SPD$^{-/-}$ mice after CLP with Western blots performed for SPD or glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH; loading control). *Blots* represent pooled samples, $n = 5$–7/group. Lungs from SPD$^{-/-}$ mice and SPD$^{+/+}$ were used as negative and positive controls, respectively. **C**, SPD$^{-/-}$ mice ($n = 9$) were gavaged with recombinant surfactant protein D (rSPD), followed by gavage with green fluorescent protein (GFP)-labeled *E. coli*, and compared with SPD$^{-/-}$ mice gavaged only with GFP-labeled *E. coli* ($n = 9$). After 24 hr, cecum and colon were harvested. GFP-labeled *E. coli* were then detected by culture (Mann-Whitney $^{*}p < 0.05$, $^{**}p < 0.01$). CFU = colony forming units.

and immediately placed in an anaerobic transport system. Contents were incubated and cultured, and colonies were identified based on morphology, Gram stain, or long-chain fatty acid analysis. For isolates unable to be typed by biochemical methods, 16S ribosomal RNA analysis was performed (see Supplement, http://links.lww.com/CCX/A995).

Statistics

Data are reported as mean ± SD. Data were assessed for normality with the Shapiro-Wilk test. Groups were compared using Student *t* test for parametric data or Mann-Whitney *U* test for nonparametric data. Mortality curves were compared using the Gehan-Breslow-Wilcoxon test. Differences were considered significant if *p* value was less than or equal to 0.05.

Study Approval

Animal protocols were approved by the Institutional Animal Care and Use Committee (Institutional Animal Care and Use Committee [IACUC] Protocol 2016N000308) at the Brigham and Women's Hospital in Boston, MA, and the IACUC at the Ohio State University, in Columbus, OH (the Ohio State University IACUC approval 2020A00000037) in agreement with the regulations of the National Institutes of Health Office of Laboratory Animal Welfare, Bethesda, MD.

RESULTS

SPD deficiency reduces *E. coli* bacteremia and mortality after CLP by reducing cecal colonization by *E. coli*. We unexpectedly found SPD$^{-/-}$ mice had increased survival after CLP (Fig. 1*A*; Supplemental Fig. 1, http://links.lww.com/CCX/A995). Following CLP, SPD$^{+/+}$ mice had higher *E. coli* bacteremia than SPD$^{-/-}$ mice (Fig. 1*B*). Immune effector cells from SPD$^{-/-}$ mice did not demonstrate enhanced phagocytosis of *E. coli* compared with SPD$^{+/+}$ mice (Fig. 1, *C* and *D*). To determine whether SPD$^{-/-}$ mice were inherently resistant to infection with *E. coli*, we inoculated SPD$^{+/+}$ and SPD$^{-/-}$ mice using fibrin clot admixed with exogenous *E. coli* implanted into the peritoneal space and found no difference in mortality between SPD$^{+/+}$ and SPD$^{-/-}$ mice (Fig. 1*E*).

SPD$^{-/-}$ mice have diminished cecal *E. coli* colonization. We found significantly more *E. coli* in cecal

contents from SPD$^{+/+}$ relative to SPD$^{-/-}$ mice (**Fig. 3*A***), suggesting that decreased mortality and *E. coli* bacteremia in SPD$^{-/-}$ mice following CLP are driven by decreased cecal colonization with *E. coli*. *E. coli* was one of several bacterial species that differed between genotypes (**Supplemental Fig. 2**, http://links.lww.com/CCX/A995). Caging conditions and maternal microbiota impact the gut microbiome (17, 18). To control for these variables, we bred SPD$^{+/+}$ and SPD$^{-/-}$ mice from SPD heterozygous parents and cohoused wild-type and knockout offspring. SPD$^{-/-}$ offspring from heterozygous parents housed with SPD$^{+/+}$ littermates became colonized with similar levels of *E. coli* compared with wild-type littermates (**Fig. 3*B***). However, when heterozygous-bred SPD$^{-/-}$ offspring were separated from SPD$^{+/+}$ littermates after weaning, SPD$^{-/-}$ mice had decreased cecal colonization with *E. coli* compared with SPD$^{+/+}$ mice (**Fig. 3*C***), demonstrating that effects of SPD persist when controlling for maternal microbiome and cage conditions. We next hypothesized that cohousing would eliminate mortality differences observed between SPD$^{-/-}$ and SPD$^{+/+}$ mice. When SPD$^{+/+}$ and SPD$^{-/-}$ offspring from heterozygous parents were cohoused, there were no differences in mortality following CLP (**Fig. 3*D***). SPD$^{-/-}$ offspring from homozygous parents raised in

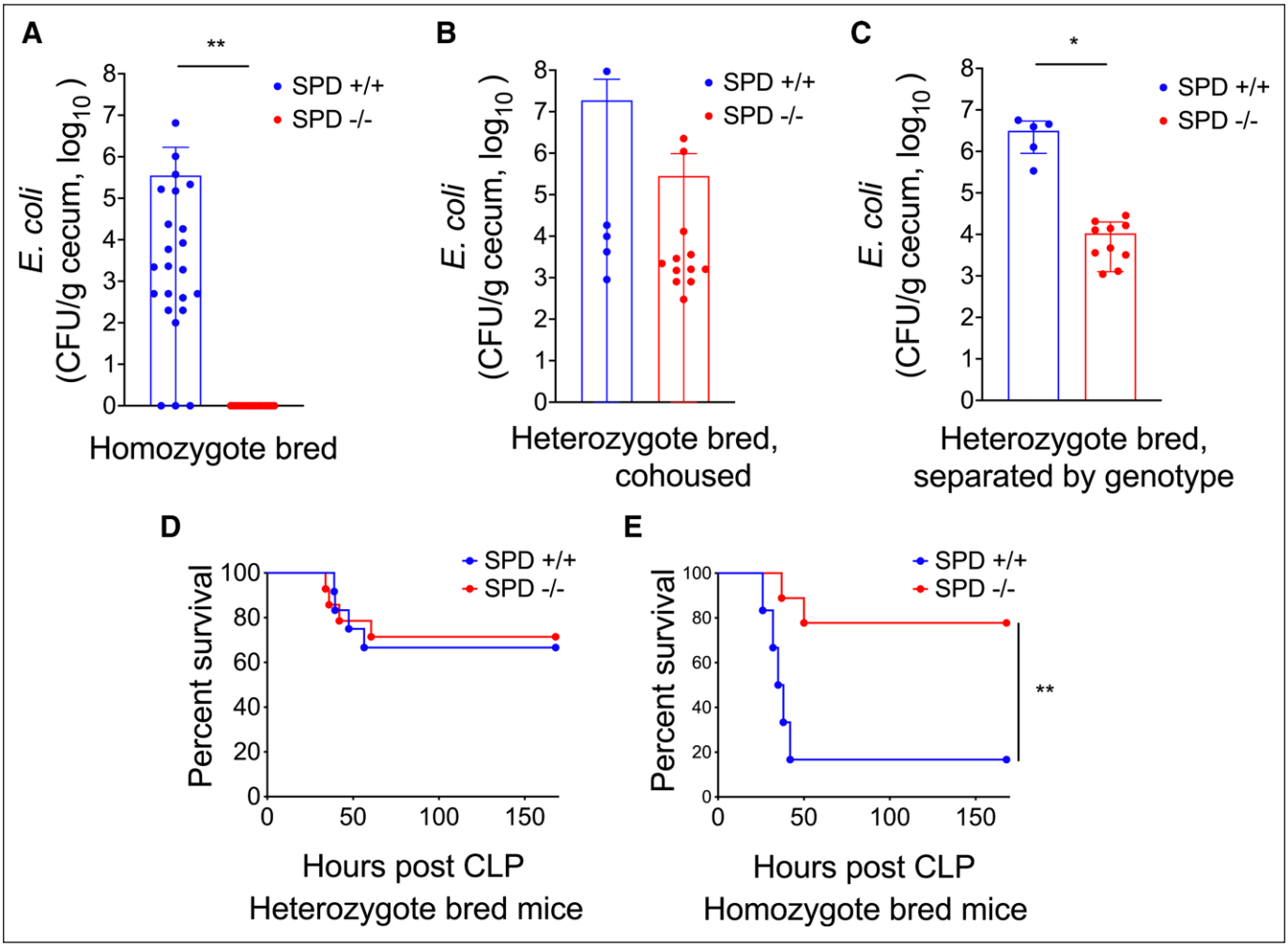

Figure 3. Surfactant protein D (SPD)$^{-/-}$ mice have diminished cecal *Escherichia coli* colonization. **A**, *E. coli* cultured from cecal culture of homozygote bred SPD$^{+/+}$ mice ($n = 24$) and SPD$^{-/-}$ mice ($n = 15$) (**$p < 0.0001$, Mann-Whitney). **B**, *E. coli* cultured from cecal culture of heterozygote bred, cohoused SPD$^{+/+}$ ($n = 5$) mice and SPD$^{-/-}$ mice ($n = 12$) ($p > 0.05$, Mann-Whitney). **C**, *E. coli* cultured from cecal culture of heterozygote bred, SPD$^{+/+}$ mice ($n = 5$) and SPD$^{-/-}$ mice ($n = 10$) after 7 wk of separation, by genotype (*$p < 0.05$, Mann-Whitney). **D**, Post-cecal ligation and puncture (CLP) survival in heterozygote bred, cohoused SPD$^{+/+}$ mice ($n = 12$) and SPD$^{-/-}$ mice ($n = 14$), where SPD$^{-/-}$ mice had exposure to *E. coli* from the feces of SPD$^{+/+}$ mice ($p =$ ns, Gehan-Breslow-Wilcoxon). **E**, Post-CLP survival of homozygote bred SPD$^{+/+}$ ($n = 6$) and SPD$^{-/-}$ ($n = 9$) mice (Gehan-Breslow-Wilcoxon, **$p < 0.01$) for concurrent recapitulation of the findings in Figure 1*A*. CFU = colony forming units.

separate cages from SPD$^{+/+}$ mice again demonstrated improved survival after CLP (**Fig. 3*E***).

SPD is synthesized by the gallbladder and promotes colonization of both the cecum and colon with *E. coli*. Given that SPD has been detected in the gut (8, 19) and can bind *E. coli* (5), we hypothesized that gut SPD expression was driving differences in *E. coli* colonization between SPD$^{+/+}$ and SPD$^{-/-}$ mice. The gallbladder was the only gut tissue in which we detected expression of SPD (Fig. 2*A*). SPD expression in the gallbladder was not significantly changed after CLP (Fig. 2*B*). SPD$^{-/-}$ mice pretreated with enteral rSPD prior to gavage with GFP-labeled *E. coli* had significantly more *E. coli* retained in the cecum and colon compared with SPD$^{-/-}$ mice that did not receive rSPD (Fig. 2*C*). Together, these data suggest that SPD plays a critical role in colonizing the mouse gut with *E. coli*.

DISCUSSION

Our study demonstrated that mice lacking SPD unexpectedly have improved survival after CLP mediated by decreased gut colonization by *E. coli*. Deficiency of SPD has been implicated in the development of obesity (20), emphysema (21), and pulmonary infections (7); however, this is the first study to our knowledge to investigate the role of SPD in polymicrobial sepsis from an abdominal source. Gut microbiota are an important factor impacting sepsis outcomes (22). *E. coli* bacteremia is an important cause of morbidity and mortality, accounting for 27% of bacteremia and an estimated case fatality rate of 12% (23). Hematopoietic stem cell transplant patients with *E. coli* or *Klebsiella pneumoniae* blood stream infections were more likely to have those bacteria identified in their gut microbiota, suggesting an intestinal source (24). Several studies have noted that species like *E. coli*, which predominate in the absence of anaerobes, are those most likely to translocate and cause bacteremia (24–28).

Our work builds substantially upon Sarashina-Kida et al (8), who described that gallbladder-derived SPD influences gut microbiota in a noninfectious colitis model. However, they did not describe how SPD deficiency affects gut colonization with *E. coli* that we report here. It is possible that their use of untargeted 16S sequencing without standard culture techniques did not permit the detection of *E. coli*. Use of bacterial culture allowed us to detect scarce, but rapidly proliferating organisms that are potentially pathogenic.

CLP is the most frequently used animal model of sepsis and is considered to be among the most relevant because infection results from the release of live endogenous bacteria (12, 29). We and others have demonstrated the central role of the gut microbiota in driving the pathogenesis of CLP (18). However, assessment of the gut microbiome at the time of CLP is not typically performed (12). Gut microbial colonization of animals varies widely based on the vendor supplying the animals, the housing facility, and diet, which makes comparing findings between studies difficult (18). Thus, there is increasing recognition that the microbiome is a critical and potentially confounding variable, even in noninfectious mouse models (30). Future CLP studies should consider the mouse microbiome as a critical variable given its outsized role in driving mortality. Studies like ours, which used littermate controls housed under identical conditions, may decrease potential confounding.

Alterations in gut microbiota have been associated with increased risk of sepsis (31, 32). In addition to driving colonization of the gut with potentially pathogenic organisms, SPD may also increase susceptibility to sepsis via modulation of the microbiome. Our study raises the intriguing possibility that modulation of the interaction between SPD and the gut microbiome could represent a novel therapeutic approach in sepsis.

Our study has several limitations. The nature of the interaction between SPD and *E. coli* is not yet clear. SPD causes agglutination of *E. coli* via interactions with bacterial saccharides; however, it is unknown if this is also the mechanism by which *E. coli* is retained in the gut (33). Several bacteria, including *E. coli*, more effectively colonize mice when pretreated with streptomycin (34–37), and SPD may similarly allow for *E. coli* colonization via disruption of other bacteria that occupy and compete for niches within the gut microbiota. Biological sex is an important modulator of the microbiome (38). Although we showed directionally similar effects in mortality of female SPD$^{+/+}$ versus SPD$^{-/-}$ mice, we did not examine for sex-related differences in the microbiome between male and female mice. Finally, although our study suggests important extrapulmonary effects of SPD in a preclinical model of sepsis, the importance of the relationship between *E. coli* and SPD in humans remains unclear.

CONCLUSIONS

We demonstrated that absence of SPD confers protection from experimental sepsis by regulating gut colonization with *E. coli*. Our work demonstrates a novel role for SPD in the pathogenesis of sepsis.

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