The Genotoxin Colibactin Exacerbates Lymphopenia and Decreases Survival Rate in Mice Infected With Septicemic Escherichia coli

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Sepsis is a life-threatening infection. Escherichia coli is the first known cause of bacteremia leading to sepsis. Lymphopenia was shown to predict bacteremia better than conventional markers of infection. The pks genomic island, which is harbored by extraintestinal pathogenic E. coli (ExPEC) and encodes the genotoxin colibactin, is epidemiologically associated with bacteremia. To investigate a possible relationship between colibactin and lymphopenia, we examined the effects of transient infection of lymphocytes with bacteria that were and those that were not producing the genotoxin. A mouse model of sepsis was used to compare the virulence of a clinical ExPEC isolate with its isogenic mutant impaired for the production of colibactin. We observed that colibactin induced double-strand breaks in the DNA of infected lymphocytes, leading to cell cycle arrest and to cell death by apoptosis. E. coli producing colibactin induced a more profound lymphopenia in septicemic mice, compared with the isogenic mutant unable to produce colibactin. In a sepsis model in which the mice were treated by rehydration and antibiotics, the production of colibactin by the bacteria was associated with a significantly lower survival rate. In conclusion, we demonstrate that production of colibactin by E. coli exacerbates lymphopenia associated with septicemia and could impair the chances to survive sepsis.

Keywords. E. coli; toxin; lymphopenia; sepsis; colibactin.

Sepsis is a clinical syndrome that complicates severe infection. In addition to exhibiting the cardinal signs of inflammation, patients present marked respiratory and hemodynamic instability [1]. A subset of patients encounters multiple organ dysfunction syndrome, which rapidly leads to death [2]. Another subset recovers but is more prone to developing secondary life-threatening microbial infections [3]. Sepsis is therefore the most common cause of death in many intensive care units [4]. An epidemiological study in North America revealed that the incidence of sepsis correlates with an annual burden of approximately 750 000 cases. The overall mortality rate is approximately 30% and can rise to 40% among elderly people and to 50% among patients displaying a more-severe syndrome (ie, septic shock) [5].

The initial phase of sepsis is thought to result from a cytokine storm caused by the activation of the cells of innate and adaptive immunity and from the systemic release of proinflammatory mediators [5]. This initial hyperinflammatory response is followed by a period of immunosuppression or so-called immunoparalysis [6–8]. During the late prolonged immunosuppressed phase of sepsis, even after successful early therapy patients are susceptible to life-threatening secondary hospital-acquired infections [9] or to reactivation of latent viruses [10]. This immunosuppression is characterized by numerous deficiencies in both the innate and adaptive immune systems. Autopsies of patients with sepsis...
have revealed extensive apoptosis of lymphocytes in the spleen [11, 12], suggesting that the loss of immune effector cells could result in the profound immunosuppression associated with this disorder. These findings were corroborated with animal studies that showed that the immune defect was critical to pathogenesis and mortality [13, 14] and that the prevention of lymphocyte apoptosis improved survival [11, 15, 16].

*Escherichia coli* is a bacterium that colonizes the mammalian gut within few days after birth and becomes the predominant facultative anaerobic bacterium in the adult microbiota. The genetic diversity of *E. coli* strains is substantial. The majority of *E. coli* strains can be assigned to 5 main phylogenetic groups: A, B1, B2, D, and E [17]. We identified a genomic island in *E. coli* strains of the phylogenetic group B2, the *pks* island, that is responsible for the production of a hybrid non-ribosomal peptide/polyketide genotoxin termed colibactin. Colibactin was shown to induce DNA damage and genomic instability both in vitro and in vivo in enterocytes or colonocytes [18, 19]. The *pks* island is highly represented within a highly virulent subset of B2 strains, namely, extraintestinal pathogenic *E. coli* (ExPEC), that exhibit an increased likelihood of causing bacteraemia [20].

The aim of our study was therefore to determine whether colibactin is genotoxic on lymphocytes and to examine the role of the toxin in the virulence of ExPEC.

**METHODS**

**Bacterial Strains**

The laboratory K12 *Escherichia coli* strain DH10B hosting the vector bacterial artificial chromosome (BAC vector) or BAC harboring the functional *pks* island (BAC *pks*) were described previously [19]. The O18:K1:H7 ExPEC strain SP15 isolated from newborn meningitis harbors the *pks* island required for colibactin production (SP15 WT) [19]. A chromosomal isogenic mutant of SP15 unable to produce colibactin was generated by disrupting the *clbA* gene by allelic exchange (SP15 Δ*clbA*) as previously described [18]. This isogenic *clbA* mutant was transformed with pMB808 plasmid harboring the wild-type *clbA* gene (SP15 *clbA* + pMB808) to restore colibactin production [19].

**Cell Culture and Bacterial Infection**

Jurkat cells (human, peripheral blood, leukemia, and T cell) were maintained by serial passages in RPMI medium (Invitrogen) supplemented with 10% fetal calf serum (FCS; Eurobio), 50 μM 2-MercaptoEthanol, nonessential amino acids (Invitrogen), and 50 μg/mL gentamicin.

Splenocytes isolated from C57BL/6J mice (Janvier Le Genest Saint Isle, France), were maintained in RPMI supplemented with 10% FCS, 2 mM L-glutamine, penicillin, streptomycin, 10 mM HEPES, 50 μM 2-MercaptoEthanol, nonessential amino acids, 1 mM sodium pyruvate, and 4 μg/mL concanalain A.

For bacterial infections, overnight LB broth cultures of *E. coli* were diluted in interaction medium (RPMI, 5% FCS, 25 mM HEPES), and cell cultures were infected at the appropriate multiplicity of infection (MOI). Four hours after inoculation, cells were washed 3 times with HBSS and incubated in cell culture medium with 200 μg/mL gentamicin until analysis.

**Flow Cytometry and Cell Cycle Analysis**

Fluorescein isothiocyanate (FITC)–conjugated antibody anti-CD8 (53.6.7) and anti-CD4 (GK1.5) and allophycocyanin-conjugated anti-CD45R (RA3–6B2) were purchased from eBioscience (San Diego, CA). Anti-phospho-H2AX was purchased from Cell Signaling Technology. Cells were incubated with antibodies in staining buffer (phosphate-buffered saline [PBS] supplemented with 2.5% FCS) for 20 minutes and then washed. For DNA content analysis, cells were incubated in PBS 70% ethanol for 1 hour at −20°C, washed in PBS, and stained in PBS with 40 μg/mL propidium iodide and 1 μg/mL RNase. Labeled cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson), using FlowJo software (Tree Star, Ashland, OR).

**Western Blot Analysis**

Cells were collected and suspended in Laemmli loading buffer, sonicated to shear DNA, and 5 min heated at 100°C. Proteins were separated on a 4%–12% NuPage gradient gel (Invitrogen), transferred to nitrocellulose membranes, blocked with 10% nonfat milk buffer, and probed with anti-Phospho-H2AX (Cell Signaling Technology) and anti-actin (ICN), followed by horseradish peroxidase–conjugated secondary antibodies and chemiluminescent autoradiography (Lumiglo, Cell Signaling Technology). Protein loading was normalized with anti-actin Western blots.

**Immunofluorescence Microscopy**

For morphological analysis and phosphorylated H2AX detection, Jurkat cells were fixed in 2% formaldehyde, and DNA was labeled with 4′,6-diamidino-2-phenylindole hydrochloride (DAPI). γ-H2AX was stained with rabbit anti-phospho-H2AX antibodies (Cell Signaling Technologies), followed by goat anti-rabbit-FITC antibodies (Zymed). Images were acquired with an Olympus IX70 confocal microscope and Fluoview software FV500.

**Propidium Iodine (PI) Uptake Analysis**

Jurkat T cell lines, splenocytes, CD8+ T-cell, CD4+ T-cell, and B-lymphocyte viability was determined by PI uptake analysis. Eighteen hours later, fluorescence-activated cell-sorter analysis was performed to determine the percentage of cells that died.

**Fluorochrome Inhibitors of Caspases (FLICA) Analysis**

The FLICA Pan-Caspase Detection Kit (Millipore) is a fluorescence-based assay for detection of active caspases in cells undergoing apoptosis. Jurkat cells were incubated 2 hours with FLICA.
at different times (18 hours, 48 hours, and 72 hours) after the end of infection. The percentage of positive cells was determined by flow cytometry.

**Mouse Sepsis Model**
Animal experiments were performed in accordance with the European directive for the protection of animals used for scientific purposes. The protocols were validated by the local ethics committee on animal experimentation, Comité d’éthique Midi Pyrénées pour l’expérimentation animale.

Footpad injections were performed as described previously [21, 22]. Briefly, female, age-matched, 8–9-week-old C57BL/6J mice were used in all experiments. Each mouse was injected subcutaneously in the left hind footpad with PBS or 1 x 10^8 colony-forming units of strains SP15 WT, SP15 ΔclbA, or SP15 ΔclbA + pclbA. When required, mice were euthanized 18 hours after injection. For the mouse sepsis rescue protocol, footpad injections were performed as described above. A total of 14 hours and 24 hours after injection, mice were treated with 0.1 mL of gentamicin injected intraperitoneally (1 mg/mL), together with ringer solution injected subcutaneously (twice 0.5 mL) for rehydration.

**Histologic Analysis**
Eighteen hours after footpad injection, mice were euthanized. Spleen and thymus were collected and frozen rapidly to -80°C. The specimens were embedded in Optimum Cutting Temperature compound. Tissue sections (5–10 μm) were stained with hematoxylin and eosin and examined at ×2.5, ×20, or ×40 magnification by microscopy.

**Quantification of Cytokine Expression**
Spleen samples were frozen in RNAlater (Qiagen). Tissues were homogenized in RLT buffer using the Precellys beads kit (Covaris). Total RNA from tissue homogenate was extracted using the RNeasy Mini Kit (Qiagen) in accordance with the supplier’s protocol and was treated with DNase (Sigma). iScript cDNA synthesis kit (Bio-Rad) was used to convert RNA (1 μg). The amplification reactions were performed in a total volume of 20 μL, using IQ SYBR Green Supermix (Bio-Rad) and a Bio-Rad CFX 97 apparatus. Quantification of tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) expression was performed using pairs of primers (for TNF-α, forward CGTCGTAGCAAACCACCAAG and reverse TTGAAGAGAACCTGGGAGTAGACA; for IL-1β, forward CAACCAACAAGTGATATTCTCCATG and reverse GATCCACACTCTCCAGCTGCA). Gene GAPDH was used as control (forward T TTCACCACTGGAGAGGCC and reverse GCCATGACTGTGGTCATGA).

**Fluorescent TUNEL**
Eighteen hours after footpad injection, mice were euthanized, and cell death was quantified by terminal deoxynucleotidyl transferase–mediated tetramethyl rhodamine-dUTP (TMR-dUTP) nick-end labeling (TUNEL kit, Roche). Frozen sections of spleen were fixed with 2% formaldehyde (pH 7.4) and permeabilized with PBS 0.1%, Triton X 100%–0.1%, sodium citrate. DNA was labeled with TO-PRO-3 (Invitrogen). Apoptotic nuclei presenting DNA nicks (strand breaks) were labeled with TMR-dUTP red. Images were acquired with an Olympus IX70 laser scanning confocal microscope and Fluoview software FV500.

**Statistical Analysis**
Data are presented as a means (±standard errors of the mean) for each group (the numbers of independent experiments are given in the figure legends). Differences for total viable cell yields and percentages were considered to be significant if P values were <.05, as determined by χ² analysis. For in vivo experiments, survival curves were analyzed using the log-rank test adjusted for multiple comparisons by computing the Bonferroni-corrected threshold (P < .05). Statistical significance of differences between experimental groups was tested using 1-way analysis of variance with the Bonferroni multiple-comparisons posttest. Two-sided analyses were used throughout, and P values of <.05 were considered statistically significant.

**RESULTS**

**E. coli Strains Producing Colibactin Induce Double-Strand DNA Breaks and Cell Cycle Arrest in T Lymphocytes**
To investigate the genotoxicity of colibactin on T lymphocytes, Jurkat T cells were infected with E. coli producing or not producing colibactin. The Jurkat T cells were exposed for 4 hours to laboratory E. coli strain K12 that was or was not harboring the pks island coding for colibactin (K12 + BAC pks or K12 + BAC vector, respectively). DNA damage, megalocytosis (Figure 1A–C), and cell cycle arrest (Figure 1D)—3 hallmarks of the colibactin effect [19]—were analyzed in infected T cells at the same magnification. Immunofluorescence analysis revealed increased phosphorylation of histone H2AX (γ-H2AX), a marker of DNA double-strand breaks, resulting from infection with colibactin-producing E. coli K12 + BAC pks (Figure 1A). γ-H2AX was further examined by flow cytometry 6 hours, 9 hours, 12 hours, and 18 hours after exposure to the bacteria (Figure 1B). This revealed a strong induction of γ-H2AX in cells infected with colibactin-producing E. coli K12 + BAC pks beginning 12 hours after infection (Figure 1B). This finding was confirmed by Western blotting 18 hours after infection with strain K12 + BAC pks (Figure 1C) but also with the ExPEC strain SP15, which naturally produces colibactin (WT; Figure 1C). Disruption of the clbA gene, required for the synthesis of colibactin, abrogated the induction of γ-H2AX in the infected cells (ΔclbA; Figure 1C).

The ability of colibactin-producing bacteria to induce cell cycle arrest in Jurkat T cells was also investigated 18 hours...
after infection. Infection of Jurkat T cells with an increasing MOI of colibactin-producing *E. coli* resulted in G2 cell cycle arrest (colibactin +; Figure 1D). Cell cycle arrest was not observed following infection with *E. coli* strains unable to produce colibactin (ie, K12 + BAC vector and SP15 ΔclbA; colibactin –; Figure 1D).

These data demonstrated that *E. coli* strains producing colibactin induce double-strand DNA breaks and cell cycle arrest in T lymphocytes, similar to that previously shown in cultured epithelial cells and enterocytes.

### E. coli Strains Producing Colibactin Induce Death by Apoptosis in T Lymphocytes

To further investigate the susceptibility of lymphocytes to colibactin-induced toxicity, we quantified T-cell death by examining membrane integrity. Jurkat T lymphocytes were transiently infected with *E. coli* K12 + BAC pks or + BAC vector, and the ExPEC strain that was or was not producing colibactin (SP15 WT and SP15 ΔclbA, respectively). The cell membrane permeability was determined 72 hours after infection by estimating the PI uptake capacity of infected Jurkat T cells (Figure 2A).
This showed that about 65% of Jurkat T cells died after exposure to bacteria producing colibactin, whereas only 20% of the cells died after contact with bacteria unable to produce colibactin (Figure 2A). To determine whether this cell death involved a caspase-dependent apoptotic pathway, Jurkat T cells were incubated with the FLICA probe 18 hours, 48 hours, and 72 hours after injection. The probe is a membrane-permeant, fluorescent caspase inhibitor that binds activated caspase enzymes and thus stains apoptotic cells. This revealed activation of caspases in 40%–60% of Jurkat T cells 48 hours and 72 hours after infection with *E. coli* producing colibactin (K12 + BAC *pks* and SP15 WT), contrary to the strains not producing the genotoxin (K12 + BAC vector and SP15 ∆*clbA*; Figure 2B). Together, these data showed that *E. coli* producing colibactin induced cell death by apoptosis in T lymphocytes.

**E. coli** Strains Producing Colibactin Induce Cell Death in Primary Lymphocytes

To examine the lymphotoxicity of colibactin on primary lymphocytes, splenocytes were collected from C57BL/6J mice. Collected lymphocytes were exposed to *E. coli* that was or was not producing colibactin. Cell death among the different populations of lymphocytes was determined by estimating the PI uptake capacity of the cells. Noninfected splenocytes were used as a negative control, whereas splenocytes isolated from 10-Gray irradiated mice were used as positive controls (Figure 3A). Analysis of 3 independent experiments showed that 75%–90% of CD4+ and CD8+ T lymphocytes and B220+ B lymphocytes died after exposure to bacteria producing colibactin (Figure 3B). Infection with either *E. coli* K12 + BAC *pks* or SP15 WT resulted in levels of lymphotoxicity equivalent to the lymphotoxicity resulting from total body irradiation (Figure 3B). Infection with different MOIs of colibactin-producing *E. coli* revealed a dose-dependent response of lymphotoxicity (Table 1). In contrast, cell death rates in noninfected splenocytes and splenocytes infected with nongenotoxic bacteria (K12 + BAC vector and SP15 ∆*clbA*) were not significantly different (Table 1). Thus, *E. coli* producing colibactin induced cell death in T lymphocytes cell line, as well as in primary lymphocytes.

**ExPEC Producing Colibactin Enhance Sepsis-Associated Lymphopenia**

To address the effects of colibactin lymphotoxicity on the virulence of *E. coli* in vivo, we investigated sepsis resulting from infection of mice with virulent *E. coli* strain SP15, an ExPEC of serotype O18:K1:H7 isolated from neonatal meningitis. The SP15 WT, SP15 ∆*clbA* mutant, and SP15 ∆*clbA* + *pckbA* complemented strains were injected into mice footpads. Mice injected with PBS were used as controls. Monitoring of mouse survival indicated that 70%–80% animals died ≤26 hours following ExPEC injection (Figure 4A). Similar kinetics of mortality were measured in the groups of mice injected with ExPEC that was or was not producing colibactin (Figure 4A), consistent with our previous findings [22].

To examine a possible role for colibactin in histologic alteration of primary and secondary lymphoid tissues before mortality, mice were euthanized 18 hours after injection with PBS, ExPEC SP15 WT, or ∆*clbA*, and the thymus and spleen were collected. Microscopic sections of thymus (Figure 4B) and spleen (Figure 4C) were stained with hematoxylin and eosin and examined. Contrary to thymi, which presented similar aspects (Figure 4B), we observed a lower density of splenocytes in

**Figure 2.** Colibactin induces death by apoptosis in immortalized T lymphocytes. Jurkat cells were transiently infected for 4 hours with colibactin-producing *Escherichia coli* strains that were (K12 + BAC *pks* and ExPEC SP15 WT) or were not (K12 + BAC vector and ExPEC SP15 ∆*clbA*) producing colibactin. A. The cells were collected 72 hours after infection, and propidium iodide was added to evaluate, by flow cytometry, the percentage of cells that died. B. Jurkat T cells were incubated with the fluorochrome inhibitors of caspases (FLICA) probe (which stains active caspases in apoptotic cells) at different times points after the end of infection. The results are representative of 3 independent experiments. The data are presented as means (with standard errors of the mean) for each group. The statistical significance of differences in total viable cell yields was determined by the χ² test. ***P < .001.
mice infected with SP15 WT, compared with mice injected with SP15 ΔclbA or PBS (Figure 4C). The bacterial load in spleens was quantified on LB agar plates and did not differ between the groups injected with the different derivatives of strain SP15 (data not shown).

To determine whether the histologic alteration detected in the spleen tissue was associated with quantitative modifications, splenocytes were quantified in each group of animals. The mice injected with strain SP15 producing colibactin displayed a lower number of splenocytes than those injected with strain SP15 ΔclbA or PBS (Figure 4D).

Two main representative proinflammatory mediators produced in spleen during infection are TNF-α and IL-1β [23]. The expression of genes encoding both cytokines was quantified and was found to be significantly higher 18 hours after infection in the spleens of mice injected with the SP15 strain producing colibactin, compared with the animals infected with strain SP15 ΔclbA impaired for the production of the genotoxin.

**ExPEC Producing Colibactin Enhance Lymphocyte Death In Vivo**

To test whether the increased lymphocyte death associated with colibactin production observed in vitro also occurred in vivo, apoptosis was examined in spleens of infected mice 18 hours after infection. The tissues were labeled using the fluorescent TUNEL method to identify apoptotic nuclei (Figure 5A). This revealed that infection with ExPEC that was or was not producing colibactin had no significant effect on the percentage of TUNEL-positive splenocytes (Figure 5A).

To delineate the population in the spleen that was undergoing death, splenocytes isolated from infected animals were cultured with complete medium and 4 µg/mL concavalin A, a polyclonal lymphocyte activator, and then stained with a combination of fluorescently labeled antibodies (anti-CD4, anti-CD8, and anti-B220) and PI (Figure 5B). The PI uptake analysis demonstrated a marked increase in the frequency of death among splenocytes and, more precisely, among CD4⁺ T cells and B220⁺ lymphocytes in the mice injected with SP15 WT.
or SP15 △clbA + pclbA (Figure 5B). Together, these data indicate that, in this sepsis model, colibactin increased lymphopenia in the spleen.

**Colibactin Decreases the Survival Rate of Mice Infected With ExPEC**

Next, to investigate the effect of colibactin on the later sepsis phase, we investigated animal survival in a therapeutic protocol where infected mice are treated with antibiotics (Figure 6). The different derivatives of strain SP15 (SP15 WT, SP15 △clbA, or SP15 ΔclbA + pclbA) were injected in mouse footpads. Fourteen hours and 24 hours after injection, the animals were administered gentamicin (100 µg per mouse). Monitoring of animal survival revealed that the mortality of mice injected with colibactin-producing SP15 (SP15 WT and SP15 ΔclbA + pclbA) was significantly higher than that in the SP15 △clbA group (Figure 6). Thus, in a mouse model mimicking the antibiotic response given to septicemic patients, production of colibactin appears to be an aggravating factor, since it decreases the survival rate.

**DISCUSSION**

Postmortem studies of patients who died of sepsis have provided important insights into why septic patients die and highlighted key immunological defects impairing host immunity [2, 24, 25]. Many patients dying from sepsis display profound lymphocyte depletion. Sepsis-induced multiple organ failure is indeed associated with lymphocyte apoptosis in the spleen, in the intestinal lamina propria, and in lymphoid aggregates throughout the body [26]. Therefore, sepsis results in the generation of an immunosuppressive environment [29, 30]. In addition, apoptotic cells themselves are immunosuppressive [31, 32]. Animal studies have also shown that sepsis induces extensive apoptosis [14, 15]. Considered together, these studies provide strong supporting evidence that loss of lymphocytes in sepsis is a central pathogenic event. Our in vitro results demonstrate that the induction of death in lymphocytes by transient *E. coli* infection producing colibactin is a consequence of DNA double-strand breaks and G2 cell cycle arrest. We propose that the immune system could represent one of the primary targets of colibactin in vivo. In vivo, the death of T lymphocytes creates a situation that favors suppression of the immune response of the host cells, thereby affecting the course of the initial infection by facilitating spread, multiplication, and persistence, but may also lead to enhanced susceptibility to infection by secondary pathogens [27].

It is essential that patients with sepsis reconstitute their immune effector cells if they are to eradicate their primary infection and avoid contracting secondary hospital-acquired infections. Several studies have reported the prevention of lymphocyte apoptosis by a variety of compounds that inhibit caspases, which resulted in improved survival in a peritonitis

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**Table 1. The Lymphotoxicity of Colibactin Is Dose Dependent**

<table>
<thead>
<tr>
<th>Treatment of Cells</th>
<th>All Splenocytes</th>
<th>CD4+ T Cells</th>
<th>CD8+ T Cells</th>
<th>B220+ B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>23 ± 1.56</td>
<td>42.08 ± 3.54</td>
<td>49.6 ± 2.73</td>
<td>37.73 ± 6.29</td>
</tr>
<tr>
<td>Irradiated cells</td>
<td>41 ± 0.65</td>
<td>91.8 ± 0.15</td>
<td>94.47 ± 0.93</td>
<td>98.17 ± 1.18</td>
</tr>
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Infectected cells

**BAC pks**

| MOI 1              | 23.7 ± 0.34      | 56.68 ± 5.06  | 64.75 ± 8.87  | 48.1 ± 12.6   |
| MOI 5              | 30.5 ± 4.57      | 77.52 ± 2.75  | 65.1 ± 10.43  | 49.6 ± 5.17   |
| MOI 10             | 41 ± 6.05        | 86.3 ± 3.82   | 81.85 ± 3.2   | 74.83 ± 12.35 |

**BAC vector**

| MOI 10             | 19.1 ± 2.68      | 43.36 ± 2.68  | 51.45 ± 2.19  | 39.8 ± 7.73   |

| ExPEC WT           |                  |              |              |               |
| MOI 1              | 26.5 ± 2.64      | 49.55 ± 2.2  | 43.73 ± 2.1  | 56.9 ± 13.25  |
| MOI 5              | 32.8 ± 6.86      | 67.83 ± 7.6  | 71.2 ± 6.97  | 72.45 ± 8.25  |
| MOI 10             | 43.3 ± 8.3       | 79.18 ± 8.9  | 80.2 ± 8.4   | 83.25 ± 3.9   |

| ExPEC ΔclbA        |                  |              |              |               |
| MOI 10             | 21.6 ± 5.17      | 49.93 ± 4.39 | 47.6 ± 2.35  | 45.3 ± 7.95   |

Data are from 3 independent experiments. Cells were infected at different MOIs with bacteria that were (K12 + BAC pks or SP15 WT) or were not (K12 + BAC vector or SP15 △clbA) producing colibactin.

Abbreviations: MOI, multiplicity of infection; SEM, standard error of the mean.

* P < .05 for differences in total viable cell yields, by the $\chi^2$ test.

* P < .01 for differences in total viable cell yields, by the $\chi^2$ test.

* P < .001 for differences in total viable cell yields, by the $\chi^2$ test.
Figure 4. Effect of colibactin in a mouse model of sepsis. A, Mice underwent footpad injection with phosphate-buffered saline (PBS) or $10^8$ colony-forming units of strains SP15 WT, SP15 ΔclbA, and SP15 ΔclbA+pclbA. Mouse survival was evaluated in the different groups. B and C, Mice used for acute studies were euthanized 18 hours after injection to examine the thymus and spleen (B). Hematoxylin and eosin staining of microscopic sections of thymus and spleen (C) isolated from mice infected with ExPEC that were or were not producing colibactin. The results shown are representative of 2 independent experiments with 5 mice each. D, The number of splenocytes in the spleen of mice euthanized 18 hours after infection was evaluated after erythrocyte depletion. E, Quantification of the expression of 2 proinflammatory cytokines, tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), in the spleen, by quantitative reverse-transcription polymerase chain reaction.
animal model of sepsis [6, 11, 16]. Recovery from lymphopenia relies on the expansion of peripheral T cells, which results in the restoration of their numbers. This process is called lymphopenia-induced homeostatic proliferation (HP). Because HP can result in the generation of memory and effector T cells, the possibility exists that this process could lead to the generation of self-reactive clones that mediate autoimmunity or to primed memory cells that would be protective upon rechallenge with the same organisms [28]. Therefore, it would be of interest to compare the repertoire of T lymphocytes after sepsis induced by an E. coli strain that does or does not produce colibactin.

Moreover, understanding the mechanisms of T-cell recovery following septic insult will help in the design of rational interventions that could accelerate reconstitution of the immune system and reduce the number of deaths in intensive care units.

Johnson et al reported that the E. coli colibactin synthesis genes were significantly associated with an especially high-virulence subset of bacteremic E. coli strains that exhibit elevated virulence scores and an increased likelihood of causing bacteremia [20]. Here, we observed that the production of colibactin was not associated with increased mortality in the classical untreated sepsis mouse model. This was consistent with our recent study that revealed that the bacterial siderophores, rather than colibactin, are crucial for the mice survival in this model [22]. Nevertheless, we demonstrate here that the production of colibactin by the septicemic E. coli strain decreases the survival rate among mice in a sepsis model including antibiotic treatment aiming at rescuing the animals. Therefore, this work provides the first experimental evidence that colibactin is a virulence factor for E. coli.

In vitro, a single, short infection of various mammalian cells with live E. coli producing colibactin induced anaphase bridges, micronuclei, and chromosome aberrations [19]. Aneuploidy, tetraploidy, anaphase bridges, and ring chromosomes persisted in dividing cells, indicating occurrence of breakage-fusion-bridge cycles. Infected cells also exhibited a significant increase in gene mutation frequency, indicating impaired reparability of colibactin-induced double-strand breaks and genomic instability [18]. Our present results suggest that insulted lymphocytes
that survive the initial genotoxic exposure could acquire geno-
mictasticity. We thus speculate that the presence of the col-
iibactin-encoding pks island may constitute a predisposing
factor for the development of lymphoma.

Notes

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Conflicts of Interest. Conflicts that the editors consider relevant to the con-
tent of the manuscript have been disclosed.

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