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A novel murine infection model for Shiga toxin–producing Escherichia coli

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Enterohemorrhagic E. coli (EHEC) is an important subset of Shiga toxin–producing (Stx-producing) E. coli (STEC), pathogens that have been implicated in outbreaks of food-borne illness and can cause intestinal and systemic disease, including severe renal damage. Upon attachment to intestinal epithelium, EHEC generates “attaching and effacing” (AE) lesions characterized by intimate attachment and actin rearrangement upon host cell binding. Stx produced in the gut transverses the intestinal epithelium, causing vascular damage that leads to systemic disease. Models of EHEC infection in conventional mice do not manifest key features of disease, such as AE lesions, intestinal damage, and systemic illness. In order to develop an infection model that better reflects the pathogenesis of this subset of STEC, we constructed an Stx-producing strain of Citrobacter rodentium, a murine AE pathogen that otherwise lacks Stx. Mice infected with Stx-producing C. rodentium developed AE lesions on the intestinal epithelium and Stx-dependent intestinal inflammatory damage. Further, the mice experienced lethal infection characterized by histopathological and functional kidney damage. The development of a murine model that encompasses AE lesion formation and Stx-mediated tissue damage will provide a new platform upon which to identify EHEC alterations of host epithelium that contribute to systemic disease.

Introduction

Shiga toxin (Stx) is a potent phage-encoded cytoxin, and Stx-producing Escherichia coli (STEC) have been implicated in serious outbreaks of food-borne disease. For example, an E. coli serotype O104 strain lysogenized with an Stx phage was responsible for a recent large outbreak of diarrheal illness with serious and in some instances lethal systemic complications (1). Enterohemorrhagic E. coli (EHEC) represent a subset of STEC, and EHEC, particularly serotype O157:H7, have been responsible for both sporadic cases and epidemic outbreaks of diarrheal illness in the United States as well as worldwide (2, 3). Human EHEC infection can cause severe local disease, such as hemorrhagic colitis, characterized by damage to the intestinal epithelium, acute abdominal pain, and bloody diarrhea (4–7). EHEC infection may also result in life-threatening systemic disease, most notably hemolytic uremic syndrome (HUS), characterized by the triad of hemolytic anemia, thrombocytopenia, and renal failure (6, 8). In fact, HUS is the leading cause of renal failure in children (9, 10).

A characteristic pathogenic feature of EHEC — one that it shares with enteropathogenic E. coli (EPEC), a cause of infantile diarrhea in the developing world, and Citrobacter rodentium, a natural murine pathogen — is the ability to generate attaching and effacing (AE) lesions (2, 11). These histopathological lesions are characterized by intimate adherence of the bacterium to the host cell, effacement of brush border microvilli, and the formation of filamentous actin–rich pedestals beneath bound bacteria (ref. 12; for review, see ref. 2).

The formation of AE lesions requires translocation of effector proteins into the mammalian cell via a type III secretion system (13, 14). A critical type III effector protein is Tir, which localizes in the host plasma membrane and acts as a receptor for the bacterial outer membrane protein intimin, promoting intimate adherence and filamentous actin assembly beneath bound bacteria (15–19). Both Tir and intimin are essential for colonization and virulence in several animal models of EHEC and Citrobacter infection (20–25).

Of the three AE pathogens, only EHEC produces Stx, which is largely responsible for the extensive tissue damage and systemic disease generated by EHEC (6, 26, 27). Given that Stx is encoded by lambdoid prophages, its production is often enhanced upon phage induction (28–30). Stx traverses the intestinal epithelium and targets the endothelium, leading to vascular damage, particularly in the kidney and CNS, tissues that express high levels of the Stx receptor and the glycolipid globotriaosylceramide, or Galβ1,3Galβ1. Glucose once inside eukaryotic cells, Stx inhibits protein synthesis by inactivating ribosomes. The action of Stx on endothelial cells leads to the induction of proinflammatory cytokines, thrombus formation in the vasculature, and renal damage (34–38). Stx1 and Stx2 are the two major serotypes of Stx, and EHEC that express exclusively Stx2 are associated with a greater risk of HUS (39–41). There are several variants of Stx2, of which Stx2c and Stx2dact are particularly important in human disease (42–44). In particular, Stx2dact is proteolytically activated in the intestinal mucus and is associated with STEC that cause toxigenic illness in humans despite the fact that they lack the ability to generate AE lesions (45, 46).

Injection of Stx into mice results in the induction of proinflammatory cytokines, vascular damage, hemolysis, and renal damage (47–50), but in contrast to piglet and rabbit infection models (23, 51–53),...
murine oral EHEC infection models do not recapitulate AE lesion formation, extensive mucosal colonization, or intestinal damage (54–56). For example, in conventional mice, Stx-mediated mortality occurs only after administration of large numbers of EHEC, numbers that diminish in the days following inoculation (55, 56). Disruption of the normal flora by antibiotic pretreatment, or its complete elimination by rearing of the animals in a germ-free environment, sensitizes mice to lethal infection after relatively low-dose oral inoculation (57–59). However, only STEC that produce the mucus-activatable allele Stx2dact consistently cause lethal infection of streptomycin-pretreated mice (58, 59). Furthermore, EHEC infection of altered-flora mice does not result in well-documented AE lesions, nor have bacterial genes that promote AE lesion formation been demonstrated to be required for intestinal colonization or disease (57–61). Indeed, a laboratory strain of C. rodentium produced by mitomycin C treatment, as predicted. Notably, the levels of Stx produced by uninduced samples, cultures of EDL933, EC1720a, and C. rodentium (kstx2dact) treated with mitomycin C produced several orders of magnitude more Stx2, particularly in the supernatant, consistent with lytic induction of the stx-encoding prophage (Figure 1). Strain TV93-0 and C. rodentium (kstx2dact::kanR) did not produce detectable toxin.

Induction of the Stx-producing prophage often promotes high-level production of Stx by EHEC strains (28–30), so each of the above bacterial strains was treated with mitomycin C to induce lysogenic phage. Compared with uninduced samples, cultures of EDL933, EC1720a, and C. rodentium (kstx2dact) treated with mitomycin C produced several orders of magnitude more Stx2, particularly in the supernatant, consistent with lytic induction of the stx-encoding prophage (Figure 1). Strain TV93-0 and C. rodentium (kstx2dact::kanR) did not produce any Stx upon mitomycin C treatment, as predicted. Notably, the levels of Stx produced by C. rodentium (kstx2dact) were comparable to or higher than the levels generated by the EHEC strains EC1720a and EDL933, suggesting that this strain might produce Stx-mediated disease upon animal infection.

The sequences of stxA (Stx2d activatable A subunit) and stxB (Stx2d activatable B subunit) of C. rodentium (Φ1720a-02) were determined, and although the inferred amino acid sequences aligned well with non-mucus-activatable Stx2d (69), the A subunit contained the C-terminal glutamic acid necessary for activation of Stx2dact (refs. 46, 58, and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI62746DS1). Thus, we tested the capacity of the toxin produced by C. rodentium (kstx2dact) to be activated in the presence of intestinal mucus. Strains B2F1, which encodes the mucus-activatable toxin Stx2dact, and EH250, which encodes the non-activatable Stx2d, were used as positive and negative controls, respectively (46, 69, 70). Culture supernatants from each strain were incubated with murine intestinal mucus or with buffer alone, and the Vero cell cytotoxicity of serially diluted samples was determined. Toxin activity in culture supernatants from E. coli strain B2F1 increased approximately 15-fold upon incubation with murine mucus (Table 1). In contrast, the toxin activity of EH250,
which does not contain mucus-activatable toxin, increased only minimally in the presence of the mucus (Table 1). Toxin activity in the culture supernatant of *C. rodentium* (*λ*stx2dact) increased approximately 18-fold, comparable to the activity of *E. coli* B2F1, upon incubation with mucus, indicating that the Stx produced by *λ*stx2dact is indeed mucus activatable (Table 1).

*C. rodentium* (*λ*stx2dact) causes lethal infection in mice. To determine whether *C. rodentium* (*λ*stx2dact) would provide an Stx-mediated disease model, we infected 5- to 8-week-old C57BL/6 mice with approximately 1 × 10^9 CFU of this strain via oral gavage. As controls, the Stx-deficient derivative *C. rodentium* (*λ*stx2dact::kan^R^) and the parental nonlysogen *C. rodentium* DBS100 were inoculated in parallel. Fecal shedding of *C. rodentium* DBS100 and *C. rodentium* (*λ*stx2dact::kan^R^) reached approximately 3 × 10^9 CFU/gram (Figure 2A), indicating that the production of Stx but starting at post-infection day 6, a time point at which maximal bacterial burden was reached, contained elevated levels of water, as previously observed for non-Stx-producing *C. rodentium* (71). In contrast, water content of feces from mice infected with *C. rodentium* (*λ*stx2dact::kan^R^) had similar water content through day 4 after infection but starting at post-infection day 6, a time point at which maximal bacterial burden was reached, contained elevated levels of water, as previously observed for non-Stx-producing *C. rodentium* (71). In contrast, water content of feces from mice infected with *C. rodentium* (*λ*stx2dact::kan^R^) was significantly elevated (>70%) at 4 days after infection (Figure 2B), indicating that the production of Stx was associated with an early increase in fecal water content. By 8 days after infection, fecal water content declined to normal values, which could be due to the putative increasing dehydration and concomitant water reabsorption that may occur in these mice during later stages of infection (see Figure 2B).

Weight loss is a notable feature in mice after Stx injection or infection in mice with an intact commensal flora with high doses of STEC (47, 48, 54). We found that mock-infected mice, as well as mice infected with *C. rodentium* DBS100 or *C. rodentium* (*λ*stx2dact::kan^R^), maintained body weight throughout infection, indicating that infection with *C. rodentium* strains that do not produce Stx were not associated with weight loss (Figure 2C). In contrast, mice infected with *C. rodentium* (*λ*stx2dact::kan^R^) lost >10% of their starting weight by 6 days after infection and >20% by 8 days after infection.

Finally, the severe weight loss in mice infected with *C. rodentium* (*λ*stx2dact::kan^R^) corresponded temporally with mortality or severe disease warranting euthanasia (Figure 2D). All mock-infected mice or mice

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
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<td>Cr (<em>λ</em>stx2dact)</td>
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<td>5.4–31</td>
<td>4</td>
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<tr>
<td>B2F1</td>
<td>15.2 ± 2.9^A</td>
<td>11.7–21</td>
<td>3</td>
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<tr>
<td>EH250</td>
<td>1.2 ± 0.1</td>
<td>1.1–1.3</td>
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Supernatant from *C. rodentium* (*λ*stx2dact::kan^R^), B2F1 (as a positive control), and EH250 (as a negative control) were tested for Vero cell cytotoxicity in the presence of *C. rodentium* DBS100 or *C. rodentium* (*λ*stx2dact::kan^R^) and the parental nonlysogen *C. rodentium* DBS100 were inoculated in parallel. Fecal shedding of *C. rodentium* DBS100 or *C. rodentium* (*λ*stx2dact::kan^R^) reached approximately 3 × 10^9 CFU/gram of feces at day 6 after infection and remained relatively constant to the end of monitoring, 10 days after infection (Figure 2A). *C. rodentium* (*λ*stx2dact::kan^R^) colonized the feces with similar kinetics, reaching a peak of approximately 10^10 CFU per gram (Figure 2A).

To test whether mice infected with *C. rodentium* (*λ*stx2dact::kan^R^) experience Stx-mediated intestinal disease, we monitored fecal water content. As expected, feces from uninfected mice contained 45%–60% water throughout the course of infection (Figure 2B). Feces from mice infected with *C. rodentium* DBS100 or *C. rodentium* (*λ*stx2dact::kan^R^) had similar water content through day 4 after infection but starting at post-infection day 6, a time point at which maximal bacterial burden was reached, contained elevated levels of water, as previously observed for non-Stx-producing *C. rodentium* (71). In contrast, water content of feces from mice infected with *C. rodentium* (*λ*stx2dact::kan^R^) was significantly elevated (>70%) at 4 days after infection (Figure 2B), indicating that the production of Stx was associated with an early increase in fecal water content. By 8 days after infection, fecal water content declined to normal values, which could be due to the putative increasing dehydration and concomitant water reabsorption that may occur in these mice during later stages of infection (see Figure 2B).

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Finally, the severe weight loss in mice infected with *C. rodentium* (*λ*stx2dact::kan^R^) corresponded temporally with mortality or severe disease warranting euthanasia (Figure 2D). All mock-infected mice or mice

### Figure 2

*C. rodentium* (*λ*stx2dact) causes lethal infection in mice. (A) Colonization of 5-week-old C57BL/6 mice by *C. rodentium* (*λ*stx2dact), *C. rodentium* (*λ*stx2dact::kan^R^), and *C. rodentium* (DBS100) was determined by viable stool counts. Shown are the average CFU (±SEM) of 5 mice. (B) Fecal water content of mock-infected 5-week-old mice or mice infected with *C. rodentium* (*λ*stx2dact), *C. rodentium* (*λ*stx2dact::kan^R^), or *C. rodentium* (DBS100). Shown are the averages (±SEM) of groups of 5 mice. Range of average fecal water content of uninfected mice is represented by the vertical bar at 0 days after infection. *P < 0.05 compared with all other groups as determined by 2-way ANOVA followed by Bonferroni post tests. (C) Body weight during infection of 8-week-old mice, expressed as percent change from day 0. Shown are the averages (±SEM) of 5 mice per group. **P < 0.01, ***P < 0.001 compared with all other groups as determined by 2-way ANOVA followed by Bonferroni post tests. (D) Percent survival of groups of five 8-week-old mice that were mock infected or infected with *C. rodentium* (*λ*stx2dact), *C. rodentium* (*λ*stx2dact::kan^R^), or *C. rodentium* (DBS100). For all panels, results are representative of at least 5 independent experiments.
infected with C. rodentium DBS100 or C. rodentium (λstx2dact::kanR) survived. In contrast, mortality was observed in mice infected with C. rodentium (λstx2dact) starting at day 4 after infection, and (in the experiment represented in Figure 2D) no mice survived past day 9. Consistent with the previous correlation between weight loss and serious systemic disease (47, 48, 54), nearly all (~95%) of mice that ~

Consistent with the previous correlation between weight loss and experiment represented in Figure 2D) no mice survived past day 9.

To assess the presence of AE lesions in mice infected with C. rodentium (λstx2dact), we examined colonic tissue by transmission electron microscopy (TEM) at 6 days after infection. Indeed, intimate bacterial attachment, microvillar effacement, and robust actin pedestals were produced on the intestinal epithelium of mice infected with C. rodentium (λstx2dact), in contrast to the normal intestinal architecture observed in mock-infected mice (Figure 3A). As predicted from previous work (11), Stx was not required for the development of AE lesion formation, because mice infected with C. rodentium (λstx2dact::kanR) developed distinct AE lesions beneath bound bacteria (Figure 3A).

To investigate intestinal disease caused by infection with C. rodentium (λstx2dact), the large intestines of mice infected with C. rodentium (λstx2dact) for 7–10 days were examined histologically. In accordance with previous studies, mice infected with C. rodentium DBS100 or C. rodentium (λstx2dact::kanR) showed limited intestinal pathology, with only minimal hyperplasia and inflammatory infiltrates compared with mock-infected mice (data not shown and Figure 3B). C. rodentium infection typically results in colonic hyperplasia only after approximately 14 days of infection [refs. 63, 72] and was not observed here. In contrast, gross evaluation of intestines from mice infected with C. rodentium (λstx2dact) showed severe destruction of the intestinal mucosa, colitis, and acute ischemic injury, with neutrophil infiltration into the muscularis layer and lamina propria and intestinal abscesses (Figure 3B). Also present were areas of necrosis and degenerative changes in the epithelial cells, with crypt withering and an overall loss of goblet cells. These data show that C. rodentium (λstx2dact::kanR) infection is associated with AE lesion formation and severe Stx-mediated intestinal damage.

Stx-mediated systemic induction of proinflammatory cytokines during murine infection with C. rodentium (λstx2dact).

Although Stx is capable of activating complement (73–75), serum levels of complement proteins C3 and Bb in mice infected with C. rodentium (λstx2dact::kanR) were not altered, and sC5b-9 was elevated only transiently at 7 days after infection (data not shown; see Methods).

In contrast, morbidity was detected in mice infected with C. rodentium (λstx2dact;kat) and with the non-Stx-producing C. rodentium (λstx2dact::kanR) (Supplemental Figure 2).

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The kidney is a specific target of Stx-mediated damage during murine infection with *C. rodentium* (*stx2dact*). Although thrombocytopenia and hemolytic anemia are two prominent manifestations of HUS, the platelet and red blood cell blood counts of mice infected with *C. rodentium* (*stx2dact*) were not significantly elevated relative to those of mock-infected mice or mice infected with *C. rodentium* (*stx2dact::kanR*) (data not shown; see Methods). Red blood cells were morphologically normal, and evidence of thrombotic microangiopathy, such as platelet aggregates, excessive clotting, or elevated levels of fibrin D-dimers, was not observed (data not shown).

We also histologically evaluated several extraintestinal tissues for damage upon intestinal infection by *C. rodentium* (*stx2dact*). The lungs and heart of mice infected with *C. rodentium* (*stx2dact*) revealed no pathology (Supplemental Figure 3, A and B). Nasal turbinates express Gb, receptors (79) but appeared normal after *C. rodentium* (*stx2dact*) infection (Supplemental Figure 3C). Although the spleens of mice infected with *C. rodentium* (*stx2dact*) showed reactive germinal center formation and germinal center hyperplasia with large follicles, mice infected with *C. rodentium* (*stx2dact::kanR*) also showed considerable germinal center activity (Supplemental Figure 3D). Finally, examination of the livers of mice infected with *C. rodentium* (*stx2dact*) or *C. rodentium* (*stx2dact::kanR*) revealed foci of neutrophilic and lymphocytic inflammation as well as scattered hepatocellular death (Supplemental Figure 3E). This injury in mice infected with *C. rodentium* (*stx2dact*) was somewhat more severe than in mice infected with *C. rodentium* (*stx2dact::kanR*), consistent with the elevation of serum proinflammatory cytokines. Consistent with the lack of thrombocytopenia, we found no platelet aggregates in any of the tissues (data not shown).

Kidney damage is a hallmark of HUS, and Stx causes renal pathology in mice after direct injection or upon absorption from intestinal STEC (47, 48, 54, 59). Renal cytokines and chemokines are elevated in murine models of Stx intoxication, so we measured cytokine and chemokine levels in the kidneys of mice infected with *C. rodentium* (*stx2dact*) at 7–10 days after infection. Although the levels of the cytokines IL-6 and G-CSF varied considerably from mouse to mouse, they were significantly increased in the kidneys of mice infected with *C. rodentium* (*stx2dact*) compared with mock-infected controls, as were the chemokines RANTES and KC (Figure 5A). In all cases, the increase in cytokine/chemokine levels was dependent on the production of Stx2dact, because infection by *C. rodentium* (*stx2dact::kanR*) was not associated with any significant elevation in levels (Figure 5A). Furthermore, the increase in renal IL-6 and RANTES was at least in part a reflection of increased transcription of the corresponding genes, because RT-PCR analysis of kidney mRNA revealed elevated levels of *Il6* and *Rantes* mRNA relative to mock-infected mice or mice infected with *C. rodentium* (*stx2dact::kanR*) (data not shown).

To evaluate renal pathology associated with the *C. rodentium* (*stx2dact*) infection model, kidney sections were taken between 7 and 10 days after infection from mice infected with *C. rodentium* (*stx2dact*) and were stained with H&E and PAS. Although renal cytokines were elevated, infection with *C. rodentium* (*stx2dact*) was not associated with a striking renal inflammatory response (Figure 5B and data not shown), nor was CCR1, a chemokine receptor associated with renal inflammation (80), elevated in the kidney (data not shown and see Methods). Instead, consistent with the observation that Stx intoxication of mice results in tubular damage (47–49), kidneys of mice infected with *C. rodentium* (*stx2dact*) showed proximal tubule injury that was not evident in the kidneys of mock-infected mice or mice infected with *C. rodentium* (*stx2dact::kanR*) (Figure 5B). Their proximal tubular epithelium was flattened, with prominent pyknotic nuclear material, loss of luminal brush border, and sloughing of dead cells in the tubular lumen (Figure 5B, arrowheads). Mitotic activity, indicating regenerative repopulation of the proximal tubules, was also observed in some mice (data not shown).

To assess renal function in mice infected with *C. rodentium* (*stx2dact*), we measured blood urea nitrogen (BUN) during the phase of terminal illness (7–10 days after infection) (see Methods). For each mouse euthanized due to severe disease prior to 10 days after infection, 1 mouse each from the mock-infected and *C. rodentium* (*stx2dact::kanR*)–infected groups was euthanized, and they were analyzed in parallel. The mean BUN of mice infected with *C. rodentium* (*stx2dact*) was significantly (*P < 0.01) elevated relative to mock-infected mice, and was also elevated relative to mice infected with *C. rodentium* (*stx2dact::kanR*), although the latter comparison did not reach statistical significance (Figure 5C).

Renal tubular damage, with both leakage of protein and sloughing of cells into the lumen, may result in proteinuria, hematuria, and elevated levels of the renal tubule–specific biomarker kidney injury molecule-1 (Kim-1) (refs. 81–83 and see Methods). Whereas urine of mock-infected mice or mice infected with *C. rodentium* (*stx2dact::kanR*) was not associated with any significant elevation in levels (Figure 5A). Furthermore, the increase in renal IL-6 and RANTES was at least in part a reflection of increased transcription of the corresponding genes, because RT-PCR analysis of kidney mRNA revealed elevated levels of *Il6* and *Rantes* mRNA relative to mock-infected mice or mice infected with *C. rodentium* (*stx2dact::kanR*) (data not shown).
Stx2dact are capable of causing disease in humans (and mice) in the absence of AE lesion formation (45, 58, 59). To test whether murine infection with \textit{C. rodentium} (λstx2dact) provided a disease model that was dependent on AE lesion formation, we determined whether a \textit{C. rodentium} (λstx2dact) defective for Tir production was also defective for colonization and disease. Whereas \textit{C. rodentium} (λstx2dact) and \textit{C. rodentium} (λstx2dact::kanR) were capable of generating actin pedestals indistinguishable from those formed by wild-type \textit{C. rodentium} DBS100 on monolayers of mouse embryonic fibroblasts (MEFs), \textit{C. rodentium} (λstx2dact)Δtir was impaired in cell binding and unable to form pedestals (data not shown). Pedestal formation was restored to this strain by pTir (pEM129), a Tir-expressing plasmid (data not shown; see Supplemental Table 2 and Methods). To confirm that Tir was also required for AE lesion formation during infection, we compared the intestinal epithelia of mice infected with \textit{C. rodentium} (λstx2dact)Δtir and the parent-
Figure 6

Tir is required for colonization and disease upon C. rodentium (λstx₂dact) infection of mice. (A) Colonization of 8-week-old C57BL/6 mice that were mock infected mice or infected with the indicated strain was determined by plating stool for viable counts. Shown are the averages (±SEM) of 5 mice per group. Data are representative of 5 independent experiments. Statistical significance was determined using 2-way ANOVA and Bonferroni post tests and indicates that C. rodentium (λstx₂dact) Δtir colonized mice significantly (*P < 0.05) less efficiently than C. rodentium (λstx₂dact) and C. rodentium (λstx₂dact) Δtir/pTir. (B) Body weight during infection of 8-week-old mice is expressed as percent change from day 0. Shown are the averages (±SEM) of 5 mice per group. Data are representative of 5 independent experiments. (C) Percent survival of mock-infected 8-week-old mice or mice infected with C. rodentium (λstx₂dact), C. rodentium (λstx₂dact) Δtir, or C. rodentium (λstx₂dact) Δtir/pTir. Groups of 5 mice were analyzed. Data are representative of 5 independent experiments. (D) H&E-stained large intestinal sections of mock-infected mice or mice infected with the indicated strain. Scale bars: 50 μm. Asterisks indicate goblet cells that are prominent in normal intestinal tissue. Arrowhead indicates infiltration of inflammatory cells into the muscularis mucosa and muscularis propria layers. (E) H&E-stained micrograph (original magnification, ×600) of kidney sections from mock-infected mice or mice infected with the indicated strain. Scale bars: 50 μm. Arrowhead indicates attenuation and sloughing of epithelial cells within the proximal tubules. Scale bars: 50 μm.

tal strain C. rodentium (λstx₂dact) by TEM at 6 days after infection. Whereas infection by C. rodentium (λstx₂dact) resulted in effacement of the intestinal brush-border microvilli and the formation of robust pedestals (Figure 3A), infection by C. rodentium (λstx₂dact) Δtir left the epithelial cytoskeletal architecture indistinguishable from that of mock-infected controls. When tir was introduced in trans on pTir, the intestines of mice infected with C. rodentium (λstx₂dact) Δtir/pTir showed many bound bacteria elevated on actin pedestals. The lack of mucosal attachment by C. rodentium (λstx₂dact) Δtir/pTir was capable of efficiently colonizing mice, with peak bacterial burdens reaching almost 10^10 per gram of stool (Figure 6A). Notably, the kinetics of colonization by C. rodentium (λstx₂dact) Δtir/pTir were somewhat delayed relative to C. rodentium (λstx₂dact), consistent with previous reports that plasmid complementation of a C. rodentium tir mutant is incomplete (20, 84).

The defect of C. rodentium (λstx₂dact) Δtir in intestinal colonization was reflected in all aspects of Stx-mediated disease. Histological analyses of the intestines of mice infected with C. rodentium (λstx₂dact) Δtir were indistinguishable from those of mock-infected mice, in contrast to those of mice infected with C. rodentium (λstx₂dact) Δtir/pTir, which showed areas of necrosis, inflammatory infiltrates, and the loss of goblet cells (Figure 6D). Renal damage was associated only with infection by C. rodentium (λstx₂dact) Δtir/pTir, not with the Tir-deficient mutant (Figure 6E). These histological assessments of disease correlated with weight loss, because mice infected with C. rodentium (λstx₂dact) Δtir/pTir lost on average 18% body weight by 4 days after infection. The kinetics of weight loss by mice infected with the Tir-complemented strain, similar to the kinetics of colo-
Discussion

STEC have been implicated in serious outbreaks of diarrheal disease and systemic damage, including a European outbreak due to a serotype O104 STEC strain (1). An important subclass of STEC is EHEC, which triggers the formation of AE lesions on the intestinal mucosa. Some rabbit and piglet models manifest both AE lesion formation and Stx-mediated disease (21, 23, 52, 53, 85), but no such model has yet been developed in conventional mice. We show here that C. rodentium lysogenized with λStx2dact, which produces a mucus-activatable Stx previously associated with severe disease in mice (59–61), provides a model that features prototypic AE lesions during intestinal colonization and Stx-mediated weight loss and death, recapitulating renal dysfunction observed in human HUS and murine toxin injection models (4, 54).

Systemic Stx-mediated disease in humans is associated with the induction of inflammatory cytokines (77, 86), and patients with a preponderance of proinflammatory cytokines often have more severe disease (87, 88). Indeed, one model of Stx-mediated disease in mice requires the co-administration of LPS and Stx (50). Several means by which inflammatory cytokines may promote systemic disease have been proposed, including induction of the Gb3 glycolipid Stx receptor on host cells (89–91), alteration of fibrinolysis and anticoagulation, or recruitment of inflammatory cells into damaged tissues (77). In response to Stx, cultured monocytes secrete TNF-α and IL-6 (90, 92, 93), and we found that these cytokines were elevated in the serum or kidneys of mice infected with C. rodentium (λStx2dact). This response was dependent on Stx production, because neither TNF-α nor IL-6 was elevated in mice infected with C. rodentium (λStx2dact::kan^R). MCP-1 and KC were also elevated in C. rodentium (λStx2dact::kan^R)-infected mice in an Stx-dependent manner. Notably, MCP-1 and IL-8, a functional human analog of KC, are found at elevated levels in the urine of children with HUS (94).

Renal damage is central to human HUS (9), and the induction of kidney cytokines in the C. rodentium (λStx2dact) model was accompanied by renal compromise. Glomerulonephritis is a prominent feature of human HUS, a manifestation that correlates with the relative abundance of Gb3 receptors on glomerular endothelium (95). In contrast, Gb3 is enriched in the renal tubules in mice (49), and the tubular damage we observed in infected mice likely reflects this localization. Consistent with this hypothesis, tubular damage is also typically observed in EHEC-infected germ-free or streptomycin-treated mice (49, 57–59). Elevated serum creatinine, an indicator of renal dysfunction, has been observed in some other murine models for Stx-mediated disease (47, 48, 59), but we found that serum creatinine was not significantly elevated in mice infected with C. rodentium (λStx2dact) (E. Mallick, unpublished observations).

A possible explanation is that elevated serum creatinine is a late marker of renal injury (82) and affected by many non-renal factors (81, 96). In fact, the renal histopathological findings were reflected in other measures of functional compromise, because mice infected with C. rodentium (λStx2dact), but not C. rodentium (λStx2dact::kan^R), demonstrated elevated BUN, proteinuria, hematuria, and urinary Kim-1, a marker of tubular damage, consistent with other murine models of HUS (47, 48, 54, 57, 59).

Several manifestations of murine Stx-mediated disease and human HUS are absent in the C. rodentium (λStx2dact) murine model. For example, neurological damage is associated with HUS, and cerebral hemorrhage and edema have been reported in EHEC-infected, streptomycin-pretreated mice (97, 98). Although mice infected with C. rodentium (λStx2dact) occasionally displayed tremors and seizures, we did not observe consistent pathological changes in the nasal turbinates, a region of the CNS rich in the Stx receptor (79).

In addition, contrary to what has been observed in human disease (6, 8) and Stx administration models (47–49, 88, 99–101), mice infected with C. rodentium (λStx2dact) did not display renal platelet aggregation, red cell congestion, or fibrin deposition and did not show evidence of thrombocytopenia or anemia. Given the hypothesis that erythrocytes are ruptured upon passage through damaged renal glomeruli (102), it is possible that the lack of glomerulonephritis in our model contributes to the lack of observable anemia. We also found no hematological evidence of thrombotic disease, such as prolonged clotting time or fibrin D-dimer formation. Following systemic administration of Stx, mice that are deficient in ADAMTS13, a protease that cleaves the hemostatic von Willebrand factor, demonstrate wide strain-specific variation in the degree of microvascular thrombosis, thrombocytopenia, and hemolytic anemia (88, 101). It is possible that infection by C. rodentium (λStx2dact) might trigger more overt microangiopathy and thus more closely resemble human HUS, in murine strains different from the C57BL/6 strain utilized here.

Given that a central goal of this study was to test whether C. rodentium (λStx2dact) could recapitulate EHEC toxin–mediated disease, an important finding was the requirement for Tir in promoting Stx-mediated disease in this model. Colonization by a Tir-deficient derivative of C. rodentium (λStx2dact) was many orders of magnitude lower than colonization by the parental strain. A Tir-encoding plasmid complemented the mutant for the ability to colonize mice and cause disease. The kinetics of colonization, weight loss, and death were somewhat slower than with C. rodentium (λStx2dact), suggesting that, as observed in previous studies (20, 84), plasmid-based complementation of a Tir-deficient C. rodentium mutant may result in only partial restoration of the wild-type phenotype.

Interestingly, in spite of the reports that the Stx receptor Gb3 is expressed on cells in the distal colon of the mouse (32, 103), the streptomycin pretreatment and gnotobiotic mouse models of EHEC infection do not typically result in obvious intestinal damage (refs. 57, 59, and A. Melton-Celsa, unpublished observations). Mild inflammatory cell infiltration, goblet cell depletion, and intestinal epithelial necrosis have been reported in a few studies (97, 104–106). In contrast, we found here dramatic intestinal damage in mice infected with C. rodentium (λStx2dact); indeed, these pathological changes were similar to those commonly observed in human infection by EHEC, which in some cases can mimic inflammatory bowel disease or ischemic colitis (107–110). It is tempting to speculate that AE lesion formation, a prominent feature of this murine infection model, is critical to facilitating Stx-mediated intestinal epithelial damage. The development of a murine model that encompasses both AE lesion formation and severe Stx-mediated tissue damage provides an opportunity to more fully explore the relationship between the pathogen-induced alteration in host cytoskeletal structure and subsequent local and systemic disease.
Methods

Plasmids, bacterial strains, and growth conditions. Bacterial strains and plasmids used in this study are listed in Supplemental Tables 1 and 2. All bacteria were cultured in Luria-Bertani broth (LB) (Miller) at 37°C, unless otherwise stated. Antibiotics were used at the following concentrations: kanamycin (25 μg/ml), zeocin (75 μg/ml), and chloramphenicol (10 μg/ml).

Alignment of Φ1720a-02 and Φ1720a-02 from Gobiobius CE1720. Sequencing of the newly identified Φ1720a-02 from CE1720a, a strain originally isolated from retail ground beef (66) (provided by Kari Gobiobius, Food Science Australia, Tingalpa, Queensland, Australia) was performed using the panhandle PCR technique as previously described (111, 112). The adaptor sequences used were F-Adaptor 1 (Supplemental Table 3) and R-Adaptor 2 (Supplemental Table 3). Primers F-AP1 and R-AP2 (Supplemental Table 3) were used for the PCR reaction and sequencing of the PCR reaction, respectively. Reactions for restriction digest and ligation of adaptors to genomic Φ1720a-02 DNA were as follows: 1 μg genomic Φ1720a-02 DNA, 10 pmol F-Adaptor 1, 10 pmol R-Adaptor 2, 2 U blunt-end restriction enzyme EcoRV, PvuII, Rsal, SspI, XmnI, DraI, HindII, Hpal, Scal, 2.5 U T4 DNA ligase, 2 μl 20 mM ATP, 4 μl of x10 One-Phor-All buffer PLUS (Amersham), and up to 20 μl water. Reactions were then incubated at 20°C for 16 hours. Next, enzymes were deactivated by heating at 68°C for 10 minutes, and reactions were ethanol-precipitated and resuspended in 50 μl sterile water. The panhandle PCR reaction was then performed using 1 μl of the ethanol-purified template DNA, 2 μl Taq 10X reaction buffer containing 20 mM MgCl2, 1 μl 10 mM dNTP mix, 10 pmol F-AP1 primer, 10 pmol gene-specific primer, 1 U Taq polymerase, and up to 20 μl water. The PCR cycle parameters were as follows: (a) 95°C for 1 minute, (b) 95°C for 30 seconds, (c) 68°C for 7 minutes; steps b and c were repeated 25 times. PCR reactions were then cleaned up using a QIAGEN spin kit and sequenced at the Tufts Core Sequencing Facility.

To confirm the nucleotide and protein sequences of StxA (Stx2d activatable A subunit) and StxB (Stx2d activatable B subunit) from the newly identified Φ1720a-02 (katX2dact), fragments of both genes, the predicted promoter region, and the region upstream of stx2a were amplified by PCR and TA cloned into JM109 high efficiency competent cells (Promega). The following primers (see Supplemental Table 3 for sequences) were used to clone each fragment: F-Oli218+R-Oli219 (593 bp), F-Oli224+R-Oli210 (1.490 kb), R-Oli220-F-Oli221 (1.647 kb), F-Oli222-R-Oli220 (~2 kb), R-Oli223+R-Oli222 (~1.159 kb), R-Oli224+R-Oli223 (1.3 kb), F-Oli221+R-Oli210 (918 bp), F-Oli224+R-Oli219 (1.2 kb), R-Oli219+R-Oli226 (~2.1 kb), and R-Oli228+R-Oli219 (~2 kb). The clones were screened by colony PCR and restriction digestion. Correct clones were sequenced at the Tufts Core Sequencing Facility. Fragments were aligned using BioEdit software (http://www.mbio.ncsu.edu/ BIOEDIT/bioedit.html) and/or MacVector. Nucleotide sequences of stxA (Stx2d activatable A subunit) and stxB (Stx2d activatable B subunit) were submitted to GenBank and were assigned the following accession numbers: JN194203 and JN194204.

Isolation of Φ1720aΔRz::cat and Φ1720aΔRz::kan. Recombinant Stx bacteriophages Φ1720aΔRz::cat and Φ1720aΔRz:: kan were isolated from the newly modified genetically manipulated EHEC strains JB-13-06 and JB-13-28 (Supplemental Table 1), respectively, as previously described (66, 70). Briefly, EHEC isolates were treated with mitomycin C (0.5 μg/ml) to induce the bacteriophage lytic cycle and production of phage. The cells were treated with 0.5 ml chloroform per 3 ml broth culture and were filtered through a 0.45-μm-pore-size filter to ensure a cell-free lysate.

Construction of a plasmid expressing C. rodentium (katX2d) and C. rodentium (katX2d::kan). The genetically engineered Stx2dact bacteriophages Φ1720a-02ΔRz::cat, which has a chloramphenicol resistance cassette inserted into Rz, and Φ1720a-02ΔRz::cat (katX2d::kan), which has a chloramphenicol resistance cassette inserted into Rz and a kanamycin resistance cassette inserted into Stx2dactAB, were isolated from genetically modified manipulated EHEC strains EC1720aΔRz::cat and EC1720aΔRz:: kan stx2d::kan, respectively. C. rodentium DBS100 (11) was provided with cell surface receptor for bacteriophage Stx2d by transformation of plasmid pTROY9, which contains the E. coli lamB gene and a tetracycline resistance marker (116, 117). The lambda phage receptor was put into DBS100, creating DBS100/pTROY9 and induced by growing overnight at 37°C in lambda broth with 0.2% maltose and 10 mM MgSO4. This strain was then lysogenized with the antibiotic-marked bacteriophages as described previously (118), resulting in DBS770 [C. rodentium (katX2d) of DBS771 [C. rodentium (katX2d::kan)].

Generation of C. rodentium (katX2d::kan) ΔKat. The C. rodentium (katX2d::kan) for deletion mutant was made as previously described (119) using a slightly modified version of the 1-step PCR-based gene activation protocol (120).

Construction of a plasmid expressing C. rodentium Tir. Plasmid DNA was isolated using the QIAGEN Miniprep procedure. Oligonucleotides were from Invitrogen. The C. rodentium complementation vector, pEM129 (pTir), was created using a modified version of pMB84, pEM123, containing the additional unique restriction sites MluI and SacII between KpnI and HindIII.
and HindIII. C. rodentium tir — cesT was then amplified using F-MluI-Cr-tirU and R-SacII-Cr-tir-crl and ligated into MluI and SacII site of pEM123 to create pEM129. Sequencing was performed at the DNA Sequencing Core Facility at Tufts Medical School.

**ELISA quantification of Stx.** Overnight bacterial cultures with antibiotic were diluted into a 5-ml culture with new medium and antibiotic. The cultures were grown to an OD₆₀₀ of 0.5–0.7. Mitomycin C (5 μl of 0.5 ng/ml) was added to cultures, and they were grown overnight with agitation. Cultures were spun at 17,800 × g for 5 minutes at room temperature. Supernatants were collected for analysis. To prepare pellets for ELISA, pellets were washed with 1 M Tris pH 8 on ice, frozen at −70°C for 30 minutes, suspended in 1 ml ice-cold 1 M Tris pH 8, and sonicated (20-second bursts, 3 times) and prepared for Stx2 ELISAs. 96-well plates (Corning MaxiSorp) were coated with 4D1 Stx2 antibody (a gift from Chleste Thorpe, Tufts University) at 1:1,000 in coating buffer, pH 9.6, overnight at 4°C. The plate was then blocked with 2% BSA in PBS or coating buffer for 1 hour at room temperature. Plates were washed 5 times with PBS/Tween 20 (0.05%). Samples of bacterial supernatants or lysed pellets were added to wells, then incubated 1 hour at room temperature. The primary antibody, rabbit anti-Stx2 203aCT69 (a gift from Chleste Thorpe), was added to the wells for 30 minutes, followed by secondary antibody, anti-rabbit IgG Fe AP conjugate (Promega), for 30 minutes. Freshly prepared para-nitrophenyl phosphate (Sigma-Aldrich) was used as substrate. ODs were read at 405 nm.

**Mucus activation assays.** Activation assays were performed as previously described (46, 58). Briefly, culture supernatants, were incubated with intestinal mucus from the small intestine of a mouse at a final concentration of 1 mg/ml for 2 hours at 37°C. As a control, a separate sample of each preparation was treated with HEPES buffer and incubated in the same manner. Following incubation with mucus or HEPES, protease inhibitor was added and samples were frozen at −20°C until ready to assay. The cytotoxicity of the samples was then determined on vero cells (as described in refs. 46, 58). The level ofactivation was determined by dividing the cytotoxicity of the mucus-treated sample by the cytotoxicity of the HEPES-treated sample. Data shown are the average ± SEM of at least 2 activation assays.

**Analysis of pedestal formation by C. rodentium on cultured cells.** Filamentous actin staining (FAS) assays using C. rodentium were done as previously described (119). Briefly, a single colony from each strain was grown in 1 ml medium (with or without antibiotic) for 8 hours. Cultures were diluted 1:500 into 5 ml DMEM supplemented with 0.1 M HEPES (pH 7.0) (with or without antibiotic) and incubated at 37°C without agitation with 5% CO₂ for 12–15 hours. Cell monolayers were prepared by splitting 95%–100% confluent MEFs into 24-well culture plates containing sterile glass coverslips, followed by overnight growth at 37°C with 5% CO₂. Prior to seeding onto culture plates, MEFs were maintained in MEF cell culture medium (DMEM [high glucose] plus 10% fetal bovine serum [FBS]) for 20 minutes at 4°C. Protein concentration of the sample was determined on Vero cells (as described in refs. 46, 58). The level of activation was determined by dividing the cytotoxicity of the mucus-treated sample by the cytotoxicity of the HEPES-treated sample. Data shown are the average ± SEM of at least 2 activation assays.

**Tissue collection and histology.** At necropsy, distal colon (−0.5 cm) and one kidney were snap frozen in liquid nitrogen and stored at −80°C until RNA or protein isolation. Remaining colon, cecum, longitudinally divided kidneys, spleen, heart, lung, liver, and nasal turbinates were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Five-micrometer tissue sections were cut and stained with H&E and/or PAS. For CCR1 staining of renal tissues by immunofluorescence, sections were stained with rabbit anti-mouse CCR1 primary antibody (ProSci Inc.) followed by staining with secondary goat anti-rabbit conjugated Alexa488 (Invitrogen). For immunohistochemistry, sections were stained with rabbit anti- mouse CCR1 primary antibody (ProSci Inc.) followed by the Envision+ Dual link system HRP (Dako). Tissue sections were evaluated and scored in a blinded manner.

**Tissue protein isolation and cytokine analysis.** Tissues were homogenized and protein was extracted using the Bio-Plex Cell Lysis Kit (Bio-Rad) following the manufacturer’s instructions. Briefly, 200–500 μl lysis solution was added to sample. Sample was ground on ice using disposable Kontes tubes and disposable pestle and frozen at −80°C. Sample was then thawed and sonicated (duty cycle 40, output 6, pulse 18 times). Sample was then centrifuged at 4,500 × g for 20 minutes at 4°C. Protein concentration of the sample was determined by BCA assay (Thermo Scientific), and protein concentration was adjusted to 2 μg/ml with lysis solution. Cytokine analysis was performed by the UMass Mouse Phenotyping Core Facility using the cytokine mouse 23-plex Bio-Plex kit (Bio-Rad) and Bio-Plex 200 Lumienx machine. For analysis of CCR1 by ELISA, kidneys were taken from mice between 3 and 7 days after infection, snap frozen, and stored at −80°C until further processing. Kidneys were then thawed on ice, resuspended in PBS, homogenized using a Tissueemiser (Fisher Scientific), and centrifuged at 4°C. The supernatant was assayed via ELISA (Chemokine C-C-Motif Receptor 1 [CCR1] ELISA, Antibodies-Online.com) according to the manufacturer’s protocols.
Bb, sCSb-9, C3, and D-dimer ELISAs. For Bb, sCSb-9, and C3 analyses, blood was drawn via cardiac bleed at 6–8 days after infection from mock-infected mice or mice infected with the designated C. rodentium strain. Blood was allowed to clot at 30 minutes at room temperature and was then spun at 10,000 g for 15 minutes at 4°C, and serum was collected. Serum was spun at 10,000 g for an additional 15 minutes at 4°C, and the remaining serum was collected and frozen at −80°C until analysis. ELISA was performed using a Mouse D-Dimer (D2D) ELISA kit (K-Assay, Kamiya Biomedical Co.).

Serum chemistry and cytokine analysis. Blood was collected via cardiac puncture at necropsy and allowed to clot on ice for 30 minutes. Blood was spun at 10,000 g for 15 minutes at 4°C, and serum was collected. Serum was spun at 10,000 g for an additional 15 minutes at 4°C, and the remaining serum was collected and frozen at −80°C until analysis. The UMass Mouse Phenotyping Core Facility determined serum BUN and creatinine using a Cobas analyzer and serum cytokine concentrations using a mouse 23-plex Bio-plex kit (Bio-Rad) with a Bioplex 200 Luminex analyzer. Additional serum chemistry tests including the comprehensive metabolic profile (i.e., electrolytes, creatinine, BUN, AST, globulin, ALT, total protein, alkaline phosphatase, CPK, and albumin) were performed at IDEXX laboratories (North Grafton).

Complete blood cell count analysis. For complete blood count and hematological analysis, at 3–7 days after infection 500 μl murine whole blood was collected by cardiac puncture into an EDTA-containing Eppendorf tube and mixed thoroughly. Tests were performed at the UMass Animal Facility Laboratory and at IDEXX Laboratories. Red blood cell and platelet counts were also measured daily for up to 10 days after infection by flow cytometry in mock-infected mice and mice infected with C. rodentium (kstx2dact) as previously described (121, 122).

TEM. Mouse intestinal tissue samples were taken at various time points after infection and fixed in 2.5% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.2. Samples were processed and analyzed at the UMass Electron Microscopy core facility using standard procedures. Briefly, fixed samples were moved into fresh 2.5% glutaraldehyde in 0.05 M sodium phosphate buffer and left overnight at 4°C. The samples were then rinsed twice in the same fixation buffer and post-fixed with 1% osmium tetroxide for 1 hour at room temperature. Samples were then washed twice with DHB0 for 20 minutes at 4°C and then dehydrated through a graded ethanol series of 20% increments, before 2 changes in 100% ethanol. Subsequently, samples were infiltrated first with 2 changes of 100% propylene oxide and then with a 1:1 mix of propylene oxide/ SPI-Pon 812 resin. The following day, 3 changes of fresh 100% SPI-Pon 812 resin were done before the samples were polymerized at 68°C in plastic capsules. The samples were then reoriented, and thin sections were placed on copper support grids and stained with lead citrate and uranyl acetate. Sections were examined using the FEI Tecnai 12 BT with 80-kV accelerating voltage, and images were captured using a Gatan TEM CCD camera.

Statistical analysis. Data were analyzed using GraphPad Prism software. Comparison of multiple groups was performed using 1-way ANOVA with Tukey’s multiple comparison post-tests or 2-way ANOVA with Bonferroni’s post-tests. Statistical significance of differences between 2 groups was evaluated using 2-tailed unpaired t tests. In all tests, P values below 0.05 were considered statistically significant. In all graphs, data represent the mean ± SEM, unless otherwise indicated.

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