Defining the Roles of TcdA and TcdB in Localized Gastrointestinal Disease, Systemic Organ Damage, and the Host Response during *Clostridium difficile* Infections

Glen P. Carter, Anjana Chakravorty, Tu Anh Pham Nguyen, Steven Mileto, Fernanda Schreiber, Lucy Li, Pauline Howarth, Simon Clare, Bliss Cunningham, Susan P. Sambol, Adam Cheknis, Iris Figueroa, Stuart Johnson, Dale Gerding, Gordon Dougan, Trevor D. Lawley, Dena Lyras

Department of Microbiology, Monash University, Victoria, Australia; Wellcome Trust Sanger Institute, Hinxton, United Kingdom; Medical Service and Research Service, Hines VA Hospital, Hines, Illinois, USA; Infectious Disease Section, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois, USA

G.P.C. and A.C. contributed equally to the work.

ABSTRACT *Clostridium difficile* is a leading cause of antibiotic-associated diarrhea, a significant animal pathogen, and a worldwide public health burden. Most disease-causing strains secrete two exotoxins, TcdA and TcdB, which are considered to be the primary virulence factors. Understanding the role that these toxins play in disease is essential for the rational design of urgently needed new therapeutics. However, their relative contributions to disease remain contentious. Using three different animal models, we show that TcdB is the primary factor responsible for inducing the in vivo host innate immune and inflammatory responses. Surprisingly, the animal infection model used was found to profoundly influence disease outcomes, a finding which has important ramifications for the validation of new therapeutics and future disease pathogenesis studies. Overall, our results show unequivocally that TcdB is the major virulence factor of *C. difficile* and provide new insights into the host response to *C. difficile* during infection. The results also highlight the critical nature of using appropriate and, when possible, multiple animal infection models when studying bacterial virulence mechanisms.

IMPORTANCE *Clostridium difficile* is a leading cause of antibiotic-associated diarrhea and an important hospital pathogen. TcdA and TcdB are thought to be the primary virulence factors responsible for disease symptoms of *C. difficile* infections (CDI). However, the individual contributions of these toxins to disease remain contentious. Using three different animal models of infection, we show for the first time that TcdB alone causes severe damage to the gut, as well as systemic organ damage, suggesting that this toxin might be responsible for the onset of multiple organ dysfunction syndrome (MODS), a poorly characterized but often fatal complication of *C. difficile* infection (CDI). Finally, we show that TcdB is the primary factor responsible for inducing the in vivo host innate immune and inflammatory responses. Surprisingly, the animal infection model used was found to profoundly influence disease outcomes, a finding which has important ramifications for the validation of new therapeutics and future disease pathogenesis studies. Overall, our results show unequivocally that TcdB is the major virulence factor of *C. difficile* and provide new insights into the host response to *C. difficile* during infection and should guide the rational development of urgently required nonantibiotic therapeutics for the treatment of CDI.
totoxin family. These toxins are potent monoglucosyltransferases that irreversibly modify members of the Rho family of host regulatory proteins, leading to disruption of downstream signaling pathways and cell death (4). Infection with toxigenic *C. difficile* strains therefore causes extensive colonic inflammation and epithelial tissue damage. The net effect is rapid fluid loss into the intestinal lumen, which manifests as diarrhea (4). *C. difficile* isolates that produce TcdB but not TcdA have emerged and continue to be isolated; these isolates cause the full clinical spectrum of CDI despite only producing one of the major toxins (5). Many human and animal strains also produce a third toxin, binary toxin or CDT, encoded by the *cdaA* and *cdaB* genes (6). The role of this toxin during infection and disease remains to be elucidated; however, recent studies suggest that this toxin may play a role in adherence and colonization of *C. difficile* in the host (6).

In the absence of methods to genetically manipulate *C. difficile*, early disease studies involved the intragastric administration of purified toxins to animals. This research suggested that TcdA was the major virulence factor and that TcdB played a less important role in disease (7). This hypothesis was challenged upon the emergence of naturally occurring variant strains that did not produce TcdA but did produce TcdB (TcdA<sup>−</sup> TcdB<sup>+</sup>) that caused fulminating human CDI (5). Two recent infection studies in hamsters attempted to clarify the roles of TcdA and TcdB in disease by using isogenic toxin mutants constructed in the low-virulence clinical isolate 630 (8, 9). The first study found that TcdB alone resulted in disease (9), while the second concluded that both TcdB and TcdA could individually cause severe disease (8). More recently, a third study using isogenic mutants constructed in strain R20291, an epidemic BI/NAP1/027 strain, together with a hamster infection model also concluded that both toxins could cause fulminant disease independently (10). A number of possibilities could explain the discrepancies in the first two studies, such as genetic differences in the *C. difficile* 630-derived strains, differences in animal challenge protocols, or variations in the intestinal microbiota of animals at different research facilities, which can substantially influence infection outcomes (11). As a consequence, it was suggested that the same panel of strains should be virulence tested in multiple laboratories to minimize the impact of experimental variation (12). To address these disparities and comprehensively define the contributions of TcdA and TcdB to disease, we used three independent animal models to study the relative virulence of a new group of isogenic *C. difficile* toxin gene mutants derived from a Canadian epidemic *C. difficile* BI/NAP1/027 isolate (strain M7404) that had not been extensively laboratory passed.

**RESULTS**

**Construction and characterization of tcdA, tcdB, and cdaA toxin gene mutants in a BI/NAP1/027 clinical isolate.** Two independent tcdA (TcdA<sup>−</sup> TcdB<sup>+</sup>) and tcdB (TcdA<sup>+</sup> TcdB<sup>−</sup>) mutants (mutants 1 and 2 for each) and a tcdAB (TcdA<sup>−</sup> TcdB<sup>−</sup>) double mutant were constructed in strain M7404. Like all BI/NAP1/027 isolates, this strain encodes a third toxin, CDT, an ADP-ribosyltransferase binary toxin (13), which was disrupted separately by mutagenesis of the *cdaA* gene (CDT<sup>−</sup>). Before virulence testing, each mutant was genotypically confirmed by PCR (data not shown) and Southern hybridization analysis, which confirmed the specific targetron insertions (see Fig. S1 in the supplemental material). Western immunoblot analyses (see Fig. S2) together with Vero and HT29 cell cytotoxicity and neutralization assays (see Fig. S3 and S4) then confirmed the expected toxin profile of each mutant compared to that of the wild type. No differences in the sporulation frequencies of the mutant and wild-type strains were detected (see Table S1).

**TcdB-producing strains are more virulent than tcdB-deficient isogenic mutants in mouse and hamster infection models.** To maximize the robustness of the infection outcomes, we used three different animal models, performed in three independent laboratories. First, an optimized mouse infection model, based on a previously published model (14) and further developed at Monash University (Australia), in which animals consistently develop severe disease was used. Infection with the wild type (TcdA<sup>+</sup> TcdB<sup>+</sup>) and the TcdA<sup>−</sup> TcdB<sup>−</sup> mutants resulted in marked weight loss (Fig. 1A), with 100% and 95% of mice, respectively, dying within 48 h, rising to 100% for the latter group by 72 h (Fig. 1B). These animals also showed other signs of severe disease, such as shallow and labored breathing, profuse diarrhea, and isolation from littermates (Fig. 1C). In contrast, only moderate weight loss was recorded for TcdA<sup>−</sup> TcdB<sup>−</sup> strain-infected mice (Fig. 1A); significantly more (80%) of these animals survived than did wild-type strain-infected animals (log rank test, *P* < 0.0001) (Fig. 1B), and they showed significantly less signs of physiological distress (Mann-Whitney test, *P* < 0.001) (Fig. 1C). Importantly, a 500-fold-higher infectious dose of the TcdA<sup>−</sup> TcdB<sup>−</sup> strain did not elicit more severe disease outcomes (see Fig. S5A and B in the supplemental material) than the lower dose (Fig. 1A and B). Finally, TcdA<sup>−</sup> TcdB<sup>−</sup> strain-infected mice lost no weight (Fig. 1A), survived the infection (Fig. 1B), and exhibited no other disease signs (Fig. 1C). As with the TcdA<sup>+</sup> TcdB<sup>−</sup> strain-infected mice, the results for survival and physiological distress were significantly different in TcdA<sup>−</sup> TcdB<sup>−</sup>-infected mice than in mice infected with the wild-type strain (*P* < 0.0001 for both survival and distress). Statistical analysis using a Mann-Whitney test subsequently showed that mice infected with TcdB-producing strains had significantly shorter colons than mock-infected mice (*P* < 0.0001 for the wild-type strain, *P* = 0.004 and *P* < 0.001 for TcdA<sup>−</sup> TcdB<sup>−</sup> mutants 1 and 2, and *P* < 0.001 and *P* < 0.001 for CDT<sup>−</sup> mutants 1 and 2) (Fig. 1D), a common feature in mouse models of ulcerative colitis (15). The colon lengths in the TcdA<sup>−</sup> TcdB<sup>−</sup> (P = 0.345 and P = 0.540) and TcdA<sup>−</sup> TcdB<sup>−</sup> (P = 0.862) strain-infected mice were statistically the same as in mock-infected mice (Fig. 1D).

Disease resulting from infection with the CDT<sup>−</sup> mutants was indistinguishable from disease caused by the wild type for each parameter analyzed (Fig. 1A to D). Note that in all subsequent animal infection experiments, only one TcdA<sup>−</sup> TcdB<sup>−</sup>, TcdA<sup>−</sup> TcdB<sup>−</sup>, or CDT<sup>−</sup> mutant was assessed since the infection phenotypes between the independent mutants using the Monash model were identical.

Strain virulence was next tested at the Sanger Institute (United Kingdom), using a different mouse infection model in which animals typically develop self-limiting intestinal inflammation and rarely progress to severe disease or death (16). Unexpectedly, 40% of TcdA<sup>−</sup> TcdB<sup>−</sup> strain-infected mice succumbed to acute infection within 48 h, which was a significantly higher rate than for mice infected with the wild-type strain (log rank test, *P* = 0.015) (Fig. 1E). In contrast, animals infected with the wild-type, TcdA<sup>−</sup> TcdB<sup>−</sup>, TcdA<sup>−</sup> TcdB<sup>−</sup>, or CDT<sup>−</sup> strain all survived the infection (Fig. 1E).

Finally, the hamster model of CDI developed at Hines VA Hos-
pital and used in earlier studies (9) was used here. In contrast to the mouse infection results, all infected hamsters died irrespective of the infecting strain (Fig. 1F). However, a significant delay (log rank test, \( P = 0.0015 \)) was evident in the time from colonization to death of \( \text{TcdA}^- \text{TcdB}^- \) strain-infected animals (1.33 ± 0.16 days [mean ± standard deviation]) compared to the time to death for wild-type strain-infected animals (0.43 ± 0.16 days). There was no statistical difference in the time to death of \( \text{TcdA}^- \text{TcdB}^- \) (0.8 ± 0.13 days) or \( \text{CDT}^- \) (0.28 ± 0.12 days) strain-infected animals compared to the time to death of wild-type strain-infected animals (Fig. 1F). These results indicate that in the hamster model of infection, only the \( \text{TcdA}^- \text{TcdB}^- \) mutant was attenuated in virulence, which agreed with our previous data using toxin mutants in \( C. \text{difficile} \) strain 630 (9).

To ensure that each strain used in this analysis germinated and colonized the infected hosts with equal efficiency, the colonization efficiencies of the \( \text{tcdA}^- \) (TcdA\(^-\) TcdB\(^-\) mutant 1) and \( \text{tcdB}^- \) (TcdA\(^-\) TcdB\(^-\) mutant 1) mutants in comparison to that of the wild-type M7404 TcdA\(^+\) TcdB\(^+\) strain in the Monash mouse model of CDI and the Hines VA Hospital hamster infection model were determined. No differences were seen in either mice or hamsters, confirming that such differences were not responsible for disease attenuation resulting from infection with the \( \text{TcdA}^- \text{TcdB}^- \) mutants (see Fig. S6A and D in the supplemental material).

**FIG 1** Toxin B is the primary mediator of fulminant \( C. \text{difficile} \) disease. (A to D) Monash mice were infected with \( C. \text{difficile} \) strains. Results for the strains are indicated by different colors as follows: black, wild-type strain M7404 [TcdA\(^+\) B\(^+\) (WT)] (\( n = 19 \)); light blue, tcdB mutant 1 (TcdA\(^+\) B\(^-\)) (\( n = 13 \)); purple, tcdA mutant 2 (TcdA\(^-\) B\(^+\)) (\( n = 10 \)); green, tcdA tcdB double mutant (TcdA\(^-\) B\(^-\)) (\( n = 15 \)); light orange, cdtA mutant 1 (CDT\(^-\)) (\( n = 10 \)); dark orange, cdtA mutant 2 (CDT\(^-\)) (\( n = 10 \)); and gray, mock-infected control (mock) (\( n = 5 \)). Results are shown for weight loss (A), survival (B), activity and appearance (C), and colon length (D) of infected Monash mice. (E) Survival of Sanger mice infected with \( C. \text{difficile} \) strains indicated by different colors as follows: black, wild-type TcdA\(^+\) TcdB\(^+\) strain [TcdA\(^+\) B\(^+\) (WT)] (\( n = 16 \)); light blue, TcdA\(^+\) TcdB\(^-\) mutant 1 (TcdA\(^+\) B\(^-\)) (\( n = 16 \)); pink, TcdA\(^+\) TcdB\(^-\) mutant 1 (TcdA\(^+\) B\(^-\)) (\( n = 13 \)); light orange, cdtA mutant 1 (CDT\(^-\)) (\( n = 15 \)); and gray, mock infected (\( n = 15 \)). (F) Days from infection to death of Hines hamsters infected with \( C. \text{difficile} \) strains indicated by different colors as follows: black, wild-type TcdA\(^+\) TcdB\(^+\) strain [TcdA\(^+\) B\(^+\) (WT)] (\( n = 8 \)); light blue, TcdA\(^+\) TcdB\(^-\) mutant 1 (TcdA\(^+\) B\(^-\)) (\( n = 9 \)); pink, TcdA\(^-\) TcdB\(^+\) mutant 1 (TcdA\(^-\) B\(^+\)) (\( n = 9 \)); and light orange, cdtA mutant 1 (CDT\(^-\)) (\( n = 10 \)). Data represent the mean results ± standard deviations (SD).
Infection with TcdB-producing strains causes severe gut and distal organ damage. Previous studies assessing the roles of TcdA and TcdB in disease did not investigate histological damage arising in the gut as a consequence of CDI. Here, the microoscopic effects of TcdA, TcdB, and CDT on the gut—in colonic and cecal tissues from Monash mice and cecal tissues from Sanger mice, collected 2 and 4 days postinfection, respectively—were examined by histopathology. Similar observations were made for both groups, with the wild type and the TcdA− TcdB+ mutants all causing severe gut damage associated with eroded and often absent crypts, mucosal ulceration, and goblet cell loss (Fig. 2A). Polymorphonuclear cell (PMN) influx into the lamina propria, enterocyte hyperplasia, and severe submucosal edema associated with hemorrhage were also seen (Fig. 2A). In contrast, TcdB-negative strains caused limited tissue damage that was confined to mild edema and PMN influx (Fig. 2A), even when a 500-fold increased dose of the TcdA+ TcdB− strain relative to the dose of the wild type was used (see Fig. S5C to E in the supplemental material). All of the damage was TcdB- or TcdA-induced, since tissues from TcdA− TcdB− strain-infected mice resembled those from mock-infected control mice (Fig. 2A). Independent histological scoring confirmed these observations, with TcdB-producing strains eliciting the greatest injury and strains only producing TcdA causing less damage (Fig. 2B and C). Statistical analysis using a Mann-Whitney test showed significantly less intestinal damage than in wild-type strain-infected mice only in animals infected with the TcdA+ TcdB− mutants (P = 0.0022 and P = 0.0043) and the TcdA− TcdB− mutant (P = 0.0022) in the Monash model of CDI and in the TcdA+ TcdB− mutant-infected mice (P < 0.0001) in the Sanger model of CDI. Histopathological scoring of tissues from CDT− strain-infected mice resembled the results for wild-type strain-infected mice (Fig. 2B and C).

C. difficile infection can cause multiple organ dysfunction syndrome (MODS) (17, 18), but the role of toxins is unknown. Organs were therefore collected from Monash CDI model mice and their histopathology examined; 88% of wild-type strain-infected and 80% of TcdA− TcdB+ strain-infected mice had damage to the thymus, sagittal lymph nodes, spleen, or kidneys (Fig. 3A). No organ damage was detected in mice infected with the TcdA+ TcdB− or TcdA− TcdB− strain or mock-infected mice (Fig. 3A). To determine whether an increased amount of TcdA could elicit systemic effects similar to those observed with TcdB-producing strains, mice infected with a 500-fold higher infectious dose of the TcdA− TcdB− strain were also examined; however, no organ damage was detected (see Fig. S5C in the supplemental material). Infection with TcdB-producing strains was particularly devastating to the thymus, as evidenced by a complete loss of medullary and cortical junctions and widespread lymphocyte apoptosis (Fig. 3B). In severe cases, there were signs of lymphoid necrosis (lymphorrhaxis) found outside the secondary follicles and associated with phagocytic macrophages. Similarly, in wild-type strain- and TcdA− TcdB+ strain-infected mice, sagittal lymph nodes showed a reactive phenotype categorized by the presence of lymphocytic apoptosis, areas of lymphoid depletion, and focal regions of macrophage aggregates and multilaminate giant cells, as well as a decrease in the number and size of lymphoid follicles and a marked absence of germinal centers (Fig. 3B). Mild lymphocyte apoptosis was also observed in splenic tissue isolated from mice infected with the wild type and the TcdA− TcdB+ mutant (Fig. 3B), with focal areas of tissue showing loss of red and white pulp morphology, as well as an increase in tingible body macrophages and periarteriolar lymphoid sheaths (PALS). Kidney damage was only seen in wild-type-infected mice, where capular lesions were observed.

Infection with C. difficile toxin gene mutants elicits unique host response signatures. To further explore the effects of TcdA and TcdB on the host, we studied the immune response transcriptional signatures seen in the large intestinal tissues collected from Monash and Sanger wild-type, TcdA− TcdB+, and TcdA+ TcdB− strain- and mock-infected mice (see Table S2 in the supplemental material). Tissues collected from CDT− strain-infected mice were not included in this analysis, since CDT appears to have little effect on virulence under the conditions tested in this study. Hierarchical clustering of samples showed that most TcdA− TcdB− strain-infected tissues displayed expression profiles similar to those of wild-type strain-infected tissues, whereas TcdA+ TcdB− strain-infected samples clustered closely with mock-infected tissues, consistent with the attenuated virulence phenotype of the TcdA+ TcdB− strains (Fig. 4). Infection with TcdB-producing strains (wild-type and TcdA− TcdB+ strains) induced expression changes in a diverse group of host genes involved in the innate immune response, inflammation, and cellular apoptosis. These included s100d8a, ccl2, ccl11, il1b, and ddx2, which encode the S100 calcium binding protein, lipidocalin 2, chemokine ligand 1, interleukin 1β, and dual oxidase 2, respectively. In the absence of TcdB, these genes did not appear to be differentially regulated. Importantly, however, the expression of many of these genes was higher following infection with the wild-type strain than with the TcdA− TcdB+ mutant, suggesting that TcdA, as well as TcdB, is involved in modulating the host innate immune and inflammatory response to C. difficile infection. Many genes involved in cellular metabolism were also differentially regulated in response to CDI, including genes encoding enzymes such as synthases, methylenetetrahydrofolate reductase genes (ugt1a6b, ugt1a6a, ugt1a1, ugt1a7c, ugt1a10, ugtb2, and ugtb3), cytochrome P450 family protein-encoding genes (cyp2c55, cyp2c65, cyp4f14, cyp2d12, cyp2a13, cyp2d22, and cyp2d26), and carboxylesterase genes (ces1f, ces2, ces1d, and ces2e) significantly downregulated. Although unrelated, these protein families play a key role in protecting host cells from damage caused by endogenous and exogenous toxins, as well as being involved in the removal of xenobiotic substances (19–21). Finally,
FIG 2. Toxin B causes severe local histopathological damage. Histopathological analyses of tissues collected from mice infected with the wild-type strain M7404 [TcdA\(^+\) B\(^+\) (WT)], tcdA mutant 1 (TcdA\(^-\) B\(^+\)), tcdB mutant 1 (TcdA\(^+\) B\(^-\)), tcdA tcdB double mutant (TcdA\(^-\) B\(^-\)), or cdtA mutant 1 (CDT\(^-\)) or mock infected with PBS were performed. (A) Representative images of hematoxylin-and-eosin-stained tissues from Monash mice on day 2 postinfection and from Sanger mice on day 4 postinfection are shown. Scale bars are shown (200 \(\mu\)m). (B) Histopathological scoring of damage to tissues from Monash mice infected with the wild-type and mutant C. difficile strains. Scores are shown for tissues from mice infected with the TcdA\(^+\) TcdB\(^+\) (WT) strain (\(n = 12\)), TcdA\(^+\) TcdB\(^-\) mutant 1 (\(n = 6\)), TcdA\(^+\) TcdB\(^-\) mutant 2 (\(n = 5\), TcdA\(^-\) TcdB\(^+\) mutant 1 (\(n = 6\)), TcdA\(^-\) TcdB\(^+\) mutant 2 (\(n = 6\)), TcdA\(^-\) TcdB\(^-\) double mutant (\(n = 6\)), CDT\(^-\) mutant 1 (\(n = 5\), and CDT\(^-\) mutant 2 (\(n = 5\)) and from mock-infected mice (\(n = 6\)). All values are mean results ± SD. (C) Histopathological scoring of damage to tissues in Sanger mice infected with wild-type and mutant C. difficile strains. Scores are shown for tissues from mice infected with the TcdA\(^+\) TcdB\(^+\) (WT) strain (\(n = 12\)), TcdA\(^-\) TcdB\(^-\) mutant 1 (\(n = 12\), TcdA\(^-\) TcdB\(^-\) mutant 1 (\(n = 8\), and CDT\(^-\) mutant 1 (\(n = 12\) and from mock-infected mice (\(n = 12\)). All values are mean results ± SD.
several genes encoding solute carrier (SLC) family proteins (slc37a1, slc26a3, slc17a4, slc39a5, slc30a10, slc26a2, slc40a1, slc16a5, slc9a2, slc02b1, slc16a9, and slc5a6) were also found to be downregulated, predominantly in response to infection with the wild-type strain and the TcdA\(^{+}\)TcdB\(^{-}\)mutant. These proteins play a key role in many central metabolic cellular processes since they are involved in the transport of a variety of substrates, such as sugars, inorganic ions, nucleotides, and amino acids (22). Collectively, these data highlight the profound effect that \textit{C. difficile} infections have on central processes within host epithelial cells, particularly in response to TcdB. Furthermore, unique signatures of the host response to each toxin mutant strain were identified, suggesting that clinical \textit{C. difficile} strains might elicit differential host responses depending on the combination of toxins produced by the infecting strain.

**DISCUSSION**

Over the last 30 years, most studies defining the roles played by the \textit{C. difficile} major toxins in disease utilized purified TcdA or TcdB and their direct administration to animals or application to cell lines, which provided invaluable insights into the mechanism of action of each of the toxins. It has only been in the last decade, however, that genetic manipulation technology has allowed toxin gene mutants to be constructed, facilitating hamster infection studies that have allowed the roles of the toxins to be more accurately defined (8, 9, 23). These infection studies all suggested that TcdB plays a more important role in disease than suggested by the earlier work involving purified toxins (8–10, 24). The study presented here has further defined the roles of TcdA and TcdB and has also assessed the role of CDT binary toxin in CDI, using three different animal models of disease. This work comprehensively shows that both TcdA and TcdB play a role in disease pathogenesis but that TcdB is the major virulence factor and causes severe host damage and disease in all animal models. In contrast, the CDT binary toxin appears to play a minor role in CDI under the experimental conditions used in this study, although other studies have suggested that this toxin can enhance colonization (25) or cause tissue damage in a small number of animals in a hamster CDI model (10). It is clear from this and other studies that CDT is not a major virulence factor, with a minority of virulent strains producing this toxin (13). Furthermore, a previous study has shown that TcdA- and TcdB-negative but CDT-positive strains are avirulent in the hamster infection model (26). The design of future experiments to clarify the role of CDT during infection will need to be carefully considered, since they will need to capture the subtle and synergistic effects that this toxin is likely to have in vivo.
to be exerting on the host. At this time, however, the role of CDT in *C. difficile* infection and disease pathogenesis remains undefined.

Although many of the recent infection studies have unquestionably clarified the role of TcdB in CDI and showed that TcdB function does not depend on the presence of TcdA, the role of TcdA itself is less apparent (8–10, 24). Here, we clearly show in all three infection models that TcdA plays a less important role than TcdB in CDI caused by BI/NAP1/027 *C. difficile* isolates. Histopathological analysis of cecal and colonic tissues collected from infected mice showed that TcdB was responsible for the majority of intestinal damage arising during infection, with TcdA causing more superficial and localized damage. This finding contrasts with previous suggestions that TcdA is the primary cause of damage to the colonic niche during the course of a *C. difficile* infection (8), although it agrees with other research that showed that TcdB is a more potent enterotoxin than TcdA in human intestinal explants (27). Unexpectedly, this work has also identified a role for TcdA in modulating the severe effects of TcdB in the Sanger murine CDI model. Using this model, mice infected with the TcdA\(^+\) TcdB\(^+\) strain succumbed to infection, while those infected with the wild-type or the TcdA\(^-\) TcdB\(^-\) mutant strain did not. This observation aligns with previous clinical observations suggesting that naturally occurring TcdA\(^-\) TcdB\(^+\) strains are capable of causing more severe disease than some TcdA\(^+\) TcdB\(^+\) strains (5). Furthermore, a recent study showed that the administration of TcdA neutralizing antibodies in piglets with CDI resulted in more severe disease than in untreated animals (28). While further work is needed to determine the mechanism by which TcdA modulates the effects of TcdB, the simplest explanation may be one of competition: TcdA intoxication of cells may prevent TcdB from acting on the same cells, thereby limiting the pool of cells on which TcdB can act and consequently reducing disease severity. Regardless, these observations have important implications for the development of next-generation, nonantibiotic, toxin-directed CDI therapeutics, because they suggest that these approaches should focus on both TcdA and TcdB or TcdB alone since targeting only TcdA may return adverse clinical outcomes. The increasing isolation of clinical and veterinary TcdA\(^-\) TcdB\(^+\) variants also reinforces the need for the development of TcdB-based therapies.

The severity of disease caused by TcdB-producing strains may result from the extraintestinal organ damage that is caused by infection with these strains. These systemic effects were absent in mice challenged with *C. difficile* strains that did not produce TcdB. These observations align with previous findings showing that TcdB is cardiotoxic in a zebrafish embryo model of intoxication (29) and a study showing that the administration of TcdB-specific antibodies lessened the systemic effects of CDI in a piglet infection.
model (28). Furthermore, another study showed that both TcdA and TcdB could disseminate from the gut during infection with a strain that produced both toxins, leading to systemic toxemia (18). Our results suggest that TcdB is the factor responsible for systemic dissemination and damage and may therefore be responsible for the onset of MODS, a severe complication of CDI that is associated with high mortality rates. Although this study cannot discern whether extraintestinal damage is directly mediated by TcdB or is a consequence of toxin-induced “leaky gut” that compromises gut integrity and allows the systemic dispersal of microbial components (30), including other toxins, it is clear that TcdB alone is associated with systemic and severe disease.

The systemic effects of infection with TcdB-producing C. difficile strains extended to many organs, including lymphoid tissues and, in particular, the thymus. Thymic injury during infection with other pathogens has been described (14–16) and can be associated with poor outcomes postrecovery (31). Thymic atrophy associated with infectious diseases can result in temporary lymphocyte depletion and altered circulating T-cell populations, which is of little consequence in healthy individuals where the thymus and immune functions are restored rapidly following infection. However, age-related thymic involution can result in an inability to restore immune function following infection (31). CDI effects on the thymus may influence immune function postinfection in a similar way, resulting in an increased risk of disease relapse or secondary infections, such as funguria or vancomycin-resistant Enterococcus (VRE) infections, which are associated with CDI patients (32, 33). This finding is particularly relevant to elderly patients, a group at significant risk for CDI (34) who have already undergone thymic atrophy (31).

Transcriptomic analysis of mice infected with the wild-type and mutant strains revealed that TcdB is the major factor inducing host innate immune and proinflammatory responses. These responses were generally stronger in mice infected with the wild-type strain than in those infected with the TcdA–TcdB− derivative, suggesting that TcdA also plays a role in upregulating these responses, which agrees with similar studies using purified toxins (35, 36). Of note, TcdB was recently shown to induce cellular apoptosis via NADPH oxidase-mediated production of reactive superoxide species, and a reduction in reactive oxygen species (ROS) activity protected colonic explants from TcdB-induced damage (37). Although upregulation of NADPH oxidase genes was not detected in our study, one of the most upregulated pathways following infection was found to be Duox2, which produces ROS in a way similar to NADPH oxidase. Duox2 is predominantly expressed in gastrointestinal epithelial cells, where it plays an important role in host defense against invading pathogens, such as Listeria monocytogenes, Salmonella enterica serovar Typhimurium, and Helicobacter spp., through the inducible production of hydrogen peroxide and ROS (38). As with NADPH oxidase (37), it appears that TcdB might induce aberrant activation of the Duox2 pathway within the intestinal epithelium during infection. Since ROS is known to cause organ damage and is also implicated in the pathogenesis of gastrointestinal conditions, such as small intestine ischemia and ulcerative colitis (39–41), it is possible that the uncontrolled Duox2-mediated burst of H2O2 and ROS resulting from C. difficile infection with TcdB-producing strains contributes to gut damage. Although further work is needed to understand the role that upregulation of Duox2 may play in C. difficile-induced gut damage, this pathway may represent a novel therapeutic target for reducing the level of gastrointestinal damage during CDI by reducing ROS production. Similar approaches have been used to develop a promising class of novel therapeutics that inhibit NADPH oxidases for the treatment of numerous inflammatory diseases and cancers (42, 43).

Overall, our results have important implications for the development and validation of treatment agents for CDI and for disease pathogenesis studies. Genetically, C. difficile is a heterogeneous species, with new disease-causing variants emerging regularly. Although many cases of CDI are acquired within hospitals, recent molecular epidemiological studies have shown that many other environmental sources may contribute to the infection reservoir. Regardless of the environmental source, TcdB appears to be the only toxin common to all current and emerging human and animal disease-causing isolates (44–46), reinforcing the outcomes presented here and the need for TcdB-targeted therapeutics. Our results also support the use of multiple animal infection models for the purpose of comprehensive evaluation of therapeutics. Finally, despite being an infection that is confined to the gut, C. difficile infection is clearly shown by this work to cause damage to organs distal to the infection site, and this work also provides new insights into the systemic host damage that can occur as a consequence of a localized gastrointestinal infection.

MATERIALS AND METHODS

Construction and characterization of toxin gene mutants. The tcdA, tcdB, and tcdAB C. difficile mutants were made using targetron technology and utilizing appropriately retargeted derivatives of plasmid pDLL1 and conjugative matings from a B. subtilis BS34A donor strain, as previously described (23). For isolation of the double toxin mutant, antibiotic selection could not be used, and so this strain was detected by PCR screening for both the tcdA− and tcdB− specific insertion of the targetron. The targetron element inserted after nucleotide 4068 of the sense strand for tcdA, nucleotide 1587 of the sense strand for tcdB, and nucleotide 421 of the sense strand for cdtA. The tcdAB double toxin mutant was constructed using tcdB mutant 1 as the parent strain. To verify that the targetron insertions had occurred as anticipated, PCR using the following oligonucleotide primers were used: for tcdA, EBS-universal (s)igma) and JRP3602 (TAAATGTACTCTACATAACACAGGG); for tcdB, EBS-universal and JRP1392 (GTTGCCCCCTGAAGCATATG); and for cdtA, EBS-universal and JRP1744 (GGAAGAAAAAGACGAGAAAG). Strain numbers were assigned as follows: for tcdA mutant 1, DLL3043; for tcdB mutant 2, DLL3045; for tcdB mutant 1, DLL3101; for tcdB mutant 2, DLL3102; and for the tcdAB mutant, DLL3121.

Each mutant was also confirmed by Southern hybridization analysis as described previously, using an ermA-specific PCR product (47), a tcdB− or tcdA-specific PCR product, or a cdt-specific PCR product amplified using primers JRP1746 (GGAAGACGAGATTGGCATACA) and JRP2505 (GGTTTACGTCAGCATAGGGA).

To confirm the correct toxin production profiles in each mutant and wild-type strain, TcdA-, TcdB-, and CdtA-specific Western blot analyses were performed as described previously (9, 13), except that toxins were precipitated from culture supernatants using chloroform-methanol (8), and Vero and HT29 cell cytotoxicity and neutralization assays were also performed as described previously (9).

Complementation of the tcdA and tcdB genes was attempted; however, despite multiple attempts, it did not prove possible to clone the intact tcdA or tcdB gene into an appropriate shuttle plasmid that would facilitate conjugative transfer into C. difficile.

Animal infection trials. Virulence trials using the Monash and Sanger mouse models and the Hines hamster model were performed as described previously (2) and are described in detail in the supplemental material. For the Monash mouse model, all monitoring was carried out in accor-
dance with Victorian State Government regulations and the Monash University Animal Ethics guidelines. All experiments were approved by the Monash University SOPS B Animal Ethics Committee. All Sanger mouse model experiments were performed in accordance with the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. All hamster experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Hines VA Hospital.

**Histopathological scoring and staining.** All histopathological analysis was performed by an independent, certified pathologist at the Australian Phenomics Network, University of Melbourne. Monash and Sanger colonic and cecal sections were assessed using a scoring system based on previously established parameters (26), described in detail in Table S3 in the supplemental material. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed on thymus sections using an *in situ* cell death detection kit with fluorescein (Roche), following the manufacturer’s instructions.

**Microarrays.** Total RNA was extracted from colonic tissues isolated from mice at Monash University and the terminal cecal tissues of mice from the Sanger Institute, using the Qiagen RNeasy kit according to the manufacturer’s instructions. RNA quality was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies), and microarray gene expression analyses performed on an Illumina GEX platform. Microarray data were analyzed using GeneSpring software (Agilent Technologies). Genes showing differential expression were selected with a Benjamin-Hochberg-corrected P value of <0.05 and fold change (compared to mock-infected controls) of >2 for Monash tissues or >1.5 for Sanger tissues. The transcriptomic responses of samples were arranged as a heat map (Fig. 4) by applying a Pearson hierarchical-clustering algorithm based on both entities and conditions. Analyses of enriched Gene Ontology terms and KEGG pathways associated with differentially expressed gene sets were subsequently performed using the DAVID (Database for Annotation, Visualization and Integrated Discovery) (27) functional classification tool and the InnateDB database (28) for innate immune response.

Note that additional methods are provided in the supplemental material.

**SUPPLEMENTAL MATERIAL**
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00551-15/-/DCSupplemental.

**Text S1,** DOC file, 0.1 MB.
**Figure S1,** JPG file, 0.3 MB.
**Figure S2,** JPG file, 0.3 MB.
**Figure S3,** JPG file, 0.6 MB.
**Figure S4,** JPG file, 0.8 MB.
**Figure S5,** JPG file, 1.5 MB.
**Figure S6,** JPG file, 0.8 MB.
**Table S1,** DOC file, 0.03 MB.
**Table S2,** XLSX file, 0.1 MB.
**Table S3,** DOC file, 0.04 MB.

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