The binary toxin CDT enhances *Clostridium difficile* virulence by suppressing protective colonic eosinophilia

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*Clostridium difficile* is the most common hospital acquired pathogen in the USA, and infection is, in many cases, fatal. Toxins A and B are its major virulence factors, but expression of a third toxin, known as C. difficile transferase (CDT), is increasingly common. An adenosine diphosphate (ADP)-ribosyltransferase that causes actin cytoskeletal disruption, CDT is typically produced by the major, hypervirulent strains and has been associated with more severe disease. Here, we show that CDT enhances the virulence of two PCR-ribotype 027 strains in mice. The toxin induces pathogenic host inflammation via a Toll-like receptor 2 (TLR2)-dependent pathway, resulting in the suppression of a protective host eosinophilic response. Finally, we show that restoration of TLR2-deficient eosinophils is sufficient for protection from a strain producing CDT. These findings offer an explanation for the enhanced virulence of CDT-expressing *C. difficile* and demonstrate a mechanism by which this binary toxin subverts the host immune response.

The Gram-positive anaerobe *Clostridium difficile* causes mild to severe antibiotic-associated diarrhoea, pseudomembranous colitis, toxic megacolon and death. The Rho-glucosylating toxins A and B (TcdA and TcdB) induce host cell death and profound inflammation and are required for symptomatic infection. Production of the binary toxin *C. difficile* transferase (CDT) in addition to toxins A and B by *C. difficile* is associated with higher mortality, increased peripheral white blood cell count and elevated risk of recurrence in clinical studies. CDT-expressing strains have also become increasingly common over the last ten years, paralleling the overall increase in incidence and severity of *Clostridium difficile* infection (CDI), and now account for up to 20% of isolates in the hospital setting.

CDT consists of two components, which act cooperatively to intoxicate cells. CDTb, the binding component of CDT, is produced as a precursor protein and requires proteolytic cleavage before intoxication. Following cleavage, CDTb forms a heptamer and associates with the lipopolysaccharide stimulated lipoprotein receptor (LSR). This receptor is highly expressed within the liver, small intestine, colon and various other tissues, and is thought to be involved in the uptake and removal of lipopolysaccharides and in the formation of tricellular tight junctions. Following formation of the CDTb heptamer and LSR binding, CDTa, the enzymatic component of CDT, binds the CDTb heptamer. This complex is endocytosed, and endosomal acidification triggers insertion of the CDTb heptamer into the endosomal membrane, through which CDTa is released into the cytoplasm. CDTa then transfers an ADP–ribose moiety to globular actin, which subsequently acts as a capping protein to prevent actin filament elongation. This results in collapse of the actin cytoskeleton, allowing the formation of microtubule protrusions on the surface of host cells that are thought to increase *C. difficile* adherence.

Although CDT production is associated with more severe disease, the role of CDT during infection is not well understood. In a hamster model of CDI, CDT was shown to enhance virulence in the presence of toxin A, but not toxin B. In humans, the intensity of the host inflammatory response is critical in determining disease outcome and in murine models, innate IL-23 production is detrimental during infection. Toxins A and B shift the immune response towards this pathogenic inflammatory state by inducing IL-1β secretion via activation of the inflammasome. Therefore, we hypothesized that CDT may play an additional role during infection by influencing host inflammatory signalling.

To investigate the role of CDT during disease, here we use isogenic CDT mutants of two distinct PCR–ribotype 027 strains in a mouse model of *C. difficile* colitis (R20291 and M7404). Both strains are human isolates that express toxins A and B as well as CDT, but R20291 was originally isolated from an outbreak in the UK and M7404 originated in Canada. In this system, we show that CDT is a true virulence factor capable of enhancing disease severity in conjunction with toxins A and B. We report that CDT increases pathogenic host inflammation via a novel Toll-like receptor-2-dependent pathway, which is required for suppression of a protective host eosinophilic response during infection.

**CDT production enhances *C. difficile* virulence**

To test the role of CDT during disease, we infected mice with the PCR–ribotype 027 strain R20291 or one of the isogenic mutants R20291 CdtB+ (lacking the binding component of CDT) or

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Figure 1 | CDT expression enhances the virulence of PCR-ribotype 027 C. difficile in a murine model of infection. a,b, Eight-week-old C57BL/6J mice underwent an antibiotic regimen before infection with 1 × 10^7 c.f.u. of vegetative C. difficile strain R20291 or the isogenic mutants R20291 CdtA− or R20291 CdtB− (data are combined from two independent experiments, n = 16) and were monitored for survival (a) and weight loss (b). c,d, Mice were treated with the same antibiotic regimen before infection with 2 × 10^5 c.f.u. of M7404, M7404 CdtA− or M7404 CdtAComp (data are combined from two independent experiments, n = 15) and were monitored for survival (c) and clinical score (d). e, Mice were killed on day 2 of infection and caecal sections were isolated and fixed in Bouin’s solution for 18 h before undergoing paraffin embedding, sectioning and H&E staining. f, Samples were scored blindly by three independent observers. Data are representative (e) or combined from two independent experiments (n = 13) (f). *P < 0.05, **P < 0.01, ***P < 0.001 by Kaplan–Meier curve (a), two-tailed t-test (b,c) and Mann–Whitney test (df). Error bars represent standard deviation (s.d.) (b,c) or standard error of the mean (s.e.m.) (df).

R20291 CdtA− (lacking the enzymatic component of CDT)\(^{28,30}\). Infection with R20291 led to significantly greater mortality and weight loss than infection with an equivalent dose of R20291 CdtA− or CdtB− (60% survival versus 100% survival, n = 14, P = 0.001, Fig. 1a,b and Supplementary Fig. 1a–c). By day 3 of infection, mice infected with the CDT mutant strains began to recover weight and showed decreased clinical signs. At the same time point, groups infected with R20291 displayed increased weight loss and a high mortality rate. Thus, we concluded that the mutants lacking CDT were significantly attenuated at this dose.

We confirmed this phenotype in a second PCR-ribotype 027 strain, M7404, using an isogenic mutant lacking CdtA (M7404 CdtA−) as well as a third, CdtA complemented strain (M7404 CdtAComp)\(^{29}\). Although we initially used a wide range of infectious doses (1 × 10^5 to 1 × 10^7 c.f.u. per mouse) in an attempt to determine an LD50 inoculum, infection with M7404 did not result in significant mortality in our model. However, we did observe differences in weight loss and clinical scores between the groups. We found that M7404 CdtA− caused significantly less weight loss and lower disease severity scores on days 2 and 3 of infection than either the wild type or CDT complemented strains (Fig. 1c,d). These data suggest that although M7404 and R20291 may inherently differ in virulence in this model, CDT was able to enhance disease severity in both strains.

To further characterize the differences in disease manifestation in the presence or absence of CDT, we focused on strain R20291 as a model of severe C. difficile infection due to its significant mortality. The R20291 CdtB− mutant was chosen as a comparison to eliminate any potential effects of the known pore-forming ability of CDTb alone\(^{30}\). Haematoxylin and eosin (H&E) stained histopathological sections of the caeca from both groups were examined (Fig. 1ef). Sections taken from mice infected with R20291 displayed significantly more overall tissue damage in the form of epithelial barrier disruption (characterized by disorganization and sloughing of epithelial cells), submucosal oedema and lumenal exudate than those infected with R20291 CdtB− (Supplementary Fig. 1d). We next measured the production of toxins A and B in these mice on day 3 of infection, as expression of these major virulence factors...
were killed on day 3 of infection, and caecal cytokines were assessed by lysing whole caecal sections and quantifying protein via ELISA (data are combined from two independent experiments, and are shown normalized to total protein concentration, n = 16). c. Serum IL-6 was measured via ELISA at the same time point (n = 14). d. BMDCs were treated with 200 ng ml⁻¹ purified CDTa and CDTb (CDT) or 2 ng ml⁻¹ toxin A and 2 ng ml⁻¹ toxin B (TcdA/B) for 24 h. Secreted IL-1β was measured by ELISA: e. NFκB activation was detected in Raw Blue NFκB reporter cells by measuring secreted embryonic alkaline phosphatase (SEAP) in the culture media. f. BMDCs were treated with 200 ng ml⁻¹ CDTa and 200 ng ml⁻¹ CDTb or with 100 ng ml⁻¹ lipopolysaccharide (LPS) as a positive control for 8 h. Pro-IL-1β gene expression was assessed by qRT-PCR and is shown normalized to the S14 housekeeping gene. g. BMDCs were treated with 200 ng ml⁻¹ CDTa and 200 ng ml⁻¹ CDTb plus 2 ng ml⁻¹ toxin A and 2 ng ml⁻¹ toxin B in combination with decreasing amounts of anti-CDTa nanobody or anti-CDTb nanobody as indicated. **200 ng ml⁻¹, *20 ng ml⁻¹, +2 ng ml⁻¹. Secreted IL-1β was measured by ELISA. Data are combined from three independent experiments with three replicates each (d–g). *P < 0.05, **P < 0.01, ***P < 0.001 by Welch’s unequal variance t-test (a–f) or Mann–Whitney test (g). NS, not significant. Error bars represent s.e.m.

Could explain the differences observed in tissue pathology. However, we did not observe significant differences (Supplementary Fig. 1e), suggesting that the mortality rates were driven by other factors. We also assessed C. difficile burden in caecal contents on day 3 of infection, but did not observe a difference at this time point (Supplementary Fig. 1f). We then assessed translocation of commensals from the gut to other organs as a possible consequence of epithelial damage. This process has been suggested to drive mortality in some models of CDT25. However, we did not observe differences in the translocation of commensals to the liver or spleen (Supplementary Fig. 1g). We conclude that tissue pathology due to CDT may influence disease outcome independently of toxin A and B production, C. difficile burden or liver commensal burden.

CDT causes increased inflammation

Based on clinical data demonstrating a correlation between the host inflammatory response and disease severity, we hypothesized that a systemic inflammatory response could result from increased tissue pathology and contribute to mortality during infection with R20291. To investigate this possibility, we quantified the inflammatory cytokines IL-1β and IL-6 within the caecal tissue of C57BL6 mice infected with R20291 or R20291 CdtB⁻ on day 3 post infection. Both IL-1β and IL-6 are known to be highly upregulated during CDT and to influence the type of immune response that develops. We found that R20291 induced significantly more caecal IL-1β and IL-6 than R20291 CdtB⁻ (Fig. 2a,b). Similarly, we observed significantly elevated serum IL-6 in R20291-infected mice (Fig. 2c). These results suggest that CDT production by R20291 results in a stronger local and systemic inflammatory response within the murine host.

We next sought to determine how CDT signalling directly influences inflammatory cytokine production. To investigate this, we exposed bone marrow derived dendritic cells (BMDCs) generated from C57BL6 mice to purified CDT in the presence or absence of 2 ng ml⁻¹ toxins A and B, and measured the amount of IL-1β secreted into the culture medium as a readout of inflammatory cytokine production (Fig. 2d). As we and other groups have shown previously, toxins A and B are sufficient to activate the inflammasome at this concentration, but a prior ‘priming’ signal is required for robust IL-1β secretion27. We found that CDT alone did not induce IL-1β secretion at this relatively long time point, even at a much higher concentration than toxins A and B (200 ng ml⁻¹). However, CDT did enhance IL-1β release in the presence of toxins A and B. These data suggest that CDT acts as a priming signal for inflammasome activation by toxins A and B. To investigate this further, we examined the ability of CDT to activate NFκB in a RAW macrophage reporter cell line. CDT alone was able to significantly activate NFκB over mock treated cells (Fig. 2e). Additionally, CDT induced significant pro-IL-1β gene expression in C57BL6 BMDCs as measured by quantitative reverse transcription polymerase chain reaction (Fig. 2f). To determine whether this phenotype was specific to CDT activity, we used anti-CDT targeted nanobodies, which have previously been demonstrated to block CDT intoxication28. At concentrations of 200 ng ml⁻¹ (12 nM) and 20 ng ml⁻¹ (1.2 nM), the anti-CDTa and anti-CDTb nanobodies both significantly decreased the amount of IL-1β secreted by BMDCs (Fig. 2g). Thus, we conclude that exposure to CDT is sufficient to activate NFκB and enhance inflammatory cytokine production.
CDT suppresses protective colonic eosinophilia

Because cytokine production shapes the immune response by influencing cell development, recruitment, proliferation and survival, we next used flow cytometry to examine the composition of effector cells in the colon during murine infection with R20291 and R20291 CdtB−. As expected, neutrophils (CD45+ CD11b+ Ly6G+ Ly6C+) dominated the innate compartment of both infected groups, representing roughly 20% of live cells in the colon (Fig. 3a–c). Monocytes (CD45+ CD11b+ Ly6Chi Ly6G−) were also significantly elevated in the colon of both infected groups compared to uninfected, antibiotic-treated controls. However, no difference was observed between neutrophils and monocytes in R20291 versus R20291 CdtB− infected groups, either as a percentage of live cells or by total cell number, suggesting that differences in neutrophil and monocyte recruitment were not responsible for infection outcome. In contrast, eosinophils were significantly elevated in R20291 CdtB− infected mice compared to R20291 infected animals (Fig. 3a–c). As with neutrophils and monocytes, eosinophils were significantly increased in both groups compared to uninfected, antibiotic-treated controls.

Studies from our laboratory demonstrated a protective role for eosinophils after recombinant IL-25 administration. Because the
We did not observe a significant decrease in eosinophil numbers in the peripheral blood of infected mice, we noted a significantly decreased percentage of live eosinophils in R20291 infected mice (Fig. 4e). This mirrored the phenotype observed in the colon and indicated that the eosinophil defect was systemic, but did not originate in the bone marrow, suggesting that a recruitment defect was not responsible for the observed decrease in colonic eosinophils.

We also observed that a higher percentage of blood eosinophils in mice infected with R20291 stained positive for Annexin V, a marker of apoptosis, which could contribute to the observed defect in mature eosinophils (Fig. 4f). Taken together, these data suggest that eosinopoiesis functions similarly within both groups of infected mice, but that mature eosinophils that exit the bone marrow in R20291 infected mice undergo increased rates of apoptosis, resulting in the observed decrease in live eosinophils in both the blood and colon of these animals.

**CDT recognition by TLR2 mediates eosinophil suppression**

Because activation of Nfkb is classically associated with pattern recognition receptor (PRR) signalling, we next asked whether these signalling pathways were involved in the recognition of CDT to mediate eosinophil apoptosis. We analysed the ability of BMDCs generated from whole bone marrow of TLR2−/−, TLR4−/− and TLR5−/− mice to secrete IL-1β following exposure to CDT and Toxins A and B. We found that TLR2−/− BMDCs were essentially unable to respond to CDT, suggesting that this pathway may be involved in CDT recognition (Fig. 5a). To corroborate the role of TLR2, we treated wild-type C57BL6 BMDCs with CDT and toxins A and B following pre-treatment with an anti-TLR2 neutralizing antibody or an isotype control. The anti-TLR2 antibody significantly reduced the release of IL-1β (Fig. 5b). To determine whether this interaction occurs in vivo, we next infected TLR2−/− mice alongside C57BL/6 wild-type controls with R20291 as well as R20291 CdtB−. Neither TLR2−/− nor C57BL/6 mice experienced significant mortality after infection with R20291 CdtB− (Fig. 5c). However, TLR2−/− mice were significantly protected from mortality after infection with CDT-producing R20291, suggesting that TLR2 signalling enhances *C. difficile* virulence in the presence of CDT (Fig. 5d).

We next asked whether TLR2 may be involved in the marked decrease in colonic eosinophils observed during infection with R20291. We assessed eosinophilia in the colon lamina propria via flow cytometry and found that TLR2−/− mice displayed significantly higher numbers of colonic eosinophils than wild-type mice after infection with R20291 CdtB− (Fig. 5e). We found that TLR2−/− BM Eos and TLR4−/− BM Eos are unable to signal via TLR2. We next investigated the possibility that this could be due to direct killing of eosinophils by CDT. We incubated BM Eos with CDT in *vitro* and assessed cell death by Live/dead and Annexin V staining measured by flow cytometry.
Treatment with CDT did not significantly enhance the number of detected apoptotic (Fig. 5f) or dead eosinophils (Supplementary Fig. 4b). Similarly, CDT did not enhance apoptosis or death of eosinophils when added in conjunction with toxins A and B (Supplementary Fig. 4c,d). Therefore, we conclude that direct killing of eosinophils by CDT is unlikely, suggesting that TLR2 signalling on eosinophils when added in conjunction with toxins A and B. This required TLR2 signalling, and TLR2-deficient mice were protected against the CDT-producing strain R20291, correlating with increased colonic eosinophils compared to wild-type mice. Finally, adoptive transfer of TLR2-deficient eosinophils was sufficient to protect mice against CDT+ R20291, confirming an unexpected role for eosinophils in protection from CDI.

Although we have demonstrated that CDT is able to activate NFκB and enhance inflammatory cytokine production, it is unclear where this signalling occurs in vivo. CDT may intoxicate epithelial cells to promote recruitment of immune cells, which may in turn enhance the inflammatory response. Alternatively, CDT may act directly on immune cells to enhance NFκB activation. The role of the lipolysis-stimulated lipoprotein receptor in inflammatory response by suppressing protective eosinophils within the colon and blood via the indirect induction of eosinophil apoptosis. Additionally, purified CDT was able to activate NFκB and induce inflammatory IL-1β production in conjunction with toxins A and B. This required TLR2 signalling, and TLR2-deficient mice were protected against the CDT-producing strain R20291, correlating with increased colonic eosinophils compared to wild-type mice. Finally, adoptive transfer of TLR2-deficient eosinophils was sufficient to protect mice against CDT+ R20291, confirming an unexpected role for eosinophils in protection from CDI.

Discussion

Despite understanding the enzymatic function of CDT, the role of this toxin in enhancing C. difficile virulence remains incompletely characterized, and the influence of CDT on the host immune response has not been examined. We found that CDT was able to enhance the virulence of two PCR-ribotype 027 C. difficile strains, resulting in increased tissue pathology. CDT skewed the host inflammatory response by suppressing protective eosinophils within the colon and blood via the indirect induction of eosinophil apoptosis. Additionally, purified CDT was able to activate NFκB and induce inflammatory IL-1β production in conjunction with toxins A and B. This required TLR2 signalling, and TLR2-deficient mice were protected against the CDT-producing strain R20291, correlating with increased colonic eosinophils compared to wild-type mice. Finally, adoptive transfer of TLR2-deficient eosinophils was sufficient to protect mice against CDT+ R20291, confirming an unexpected role for eosinophils in protection from CDI.

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Figure 5 | TLR2 mediates CDT recognition and is required for eosinophil suppression. a, BMDCs were generated from TLR2−/−, TLR4−/− and TLR5−/− mice before treatment with 200 ng ml−1 CDTa and 200 ng ml−1 CDTb (CDT) and 2 ng ml−1 toxin A and 2 ng ml−1 toxin B (TcdA/B) for 24 h. IL-1β secretion was assessed by ELISA. b, BMDCs were treated with 200 ng ml−1 CDTa and 200 ng ml−1 CDTb (CDT) and 2 ng ml−1 toxin A and 2 ng ml−1 toxin B (TcdA/B) for 24 h in the presence of a TLR2 neutralizing antibody or an isotype control. IL-1β was assessed by ELISA. Data are combined from three independent experiments of three replicates each (a,b). c, Eight-week-old TLR2 knockout mice or C57BL/6J mice were infected with R20291 or R20291 CdtB− and monitored for survival (data combined from two independent experiments, n = 14). d, Mice were killed on day 3 of infection and colonic eosinophils (CD45+ CD11b+ SiglecF+ SSC−) were measured by flow cytometry following tissue processing and staining (data combined from two independent experiments, n = 8). e, C57BL/6 mice received 4 × 10⁵ TLR2−/− or B6 bone marrow-derived eosinophils (TLR2−/− or B6 Eo) via IP injection one day before and for three subsequent days following infection with R20291 or R20291 CdtB−. Mice were monitored daily for survival (data are combined from two independent experiments, n = 13). f, BM Eos were incubated for 8 h with 200 ng ml−1 CDTa and 200 ng ml−1 CDTb in the presence or absence of anti-TLR2 neutralizing antibody (aTLR2) or anti-CDT neutralizing nanobody (aCDT). Eosinophils were stained with Live dead or Annexin V and cell death was assessed by flow cytometry. Data are representative of three independent experiments assayed in duplicate. *P < 0.05, **P < 0.01, ***P < 0.001 by Mann–Whitney test (a), Welch’s unequal variance t-test (b,d,f) or Kaplan–Meier analysis (c,e). NS, not significant. Error bars represent s.e.m.

mediating an immune response is also unknown. It is conceivable that LSR and TLR2 mediate separate pathways in response to CDT. Alternatively, these receptors may cooperate to permit CDT recognition and intoxication.

Another major question concerns the mechanism of eosinophil killing by CDT. Although our data suggest that CDT does not directly kill eosinophils, intoxication by CDT and the subsequent increase in inflammatory cytokine production by innate cells may shape the inflammatory environment to support eosinophil apoptosis. Indeed, innate inflammation has long been known to suppress eosinophilia by unknown mechanisms.22,23. TLR2-dependent eosinophil suppression is also not unprecedented, as multiple groups have reported in different murine models of allergic inflammation44–46. Administration of a TLR2 agonist has been shown to reduce eosinophilia by inducing T cell apoptosis in a model of allergic conjunctivitis, as well as by enhancing T regulatory cells and inducing Th1 cytokines in murine asthma models. It remains to be investigated whether these mechanisms underlie eosinophil suppression during CDI. The protective role of eosinophils is similarly unknown, and clarifying how TLR2 signalling impacts the function of these cells may provide clues to their activity during infection.

Understanding the mechanism by which CDT enhances C. difficile virulence is essential to understanding the virulence of PCR–ribotype 027 strains and other hypervirulent strains that express CDT. These isolates are increasingly common, and their spread is probably contributing to the overall increase in CDI incidence and severity.
Our data clarify the protective role eosiophils play during CDI and suggest that targeting CDT in the development of vaccines and therapeutic inhibitors is essential to successfully treating infection with these strains. Our knowledge of the C. difficile virulence factors that result in pathogenic immune responses during CDI continues to evolve, presenting new potential drug targets for treating this common and life-threatening infection.

Methods
Backyard strains and culture. C. difficile strains R20291 CdA+ and R20291 CdB− were generated using the Clostron system, and functional inactivation of the targeted genes was confirmed by western blot, as described previously29. Strains M7404 CdA+ and M7404 CdB+ were generated using the targetron system as previously described30. To prepare the infection inoculum, strains were inoculated onto brain heart infusion (BHI) agar supplemented with the appropriate selective antibiotic from frozen stocks and incubated at 37 °C overnight in an anaerobic work station (Shel Labs). Single colonies were inoculated into BHI medium supplemented with cycloserine and cefoxitin (C. difficile supplement, Sigma) and grown anaerobically overnight at 37 °C. The next day, cultures were spun for 1 min at 6,000g and washed twice in anaerobic PBS. The optical densities of the cultures were measured, and the culture density was adjusted to 1 × 10^7 CFU/ml (R20291 strains) or 2 × 10^8 CFU/ml (M7404 strains) in sterile, anaerobic PBS. Syringes were loaded with the inoculum and sealed in airtight bags before and during transport to the infection facility. Mice received 100 µl of inoculum each by oral gavage. To enumerate caecal samples, the caecal contents were resuspended in 50 µl of supernatant with 150 µl of bovine serum (FBS), 2 mM L-glutamine and 100 U/ml of antibiotics (penicillin and streptomycin) and plated on BHI agar supplemented with cycloserine and cefoxitin (Sigma) to quantify CDI30. Briefer, CDI was scored blinded, with a score from 0 to 3 assigned based on the presence of clinical symptoms.

CDT complementation. The recombinant plasmid used for complementation of the cdtA mutant was pBB3167 (described previously)43. C. difficile strains were grown in tryptic yeast (TY) broth (3.0% tryptone, 2.0% yeast extract and 0.1% sodium thioglycollate) at 37 °C in a Don Whitley A35 anaerobic work station in an atmosphere of 10% (vol/vol) H2, 10% (vol/vol) CO2 and 80% (vol/vol) N2. Toxins were partially purified and concentrated eight-fold from 72 h cultures of C. difficile culture supernatants with methanol–chloroform precipitation48. Protein concentrations were determined using the BCA protein assay kit (Pierce) according to the manufacturer's instructions, and blots were probed as previously described29. Briefly, the Western Lightning Chemiluminescence reagent kit (Perkin-Elmer) was used to detect the blots, following the manufacturer's instructions (also donated by Techlab). Toxins were detected within caecal contents using the C. difficile TOXA/B enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (also donated by Techlab).

Cytokine detection. IL-1β and IL-6 were detected in protein supernatants from BMDCs, serum and tissue lysates using the Mouse IL-1β and IL-6 Ready-Set-Go! ELISA kit (E Bioscience) according to the manufacturer's instructions. Eotaxin-1 and eotaxin-2 were detected in caecal lysates using R&D Systems DuoSet ELISA kits according to the manufacturer's instructions. BMDC pro-IL-1β production was assessed by quantitative reverse transcription PCR. cDNA was isolated using the RNeasy isolation kit (Qiagen). The resulting cDNA was quantified by Quantitect Primer assay (Qiagen) using Sensifast SYBR & Fluorescein Master Mix (BioWare). The results were normalized using the housekeeping genes HPRT1, G3PDH and 18S for data presentation.

Mice and infection. Experiments were carried out using sex-matched 8- to 12-week-old C57BL/6, BALB/c and C57BL/6 mice from the Jackson Laboratory. Experiments contained fewer than four mice, with a goal of eight mice, per experimental group. Based on an expected protection from 50% to 0% mortality, six mice per group will have >90% power to detect a statistically significant difference in mortality. All animals were housed under specific pathogen-free conditions at the University of Virginia's animal facility, and procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia. No animals were excluded from the study, and sample harvesting was randomized between groups. Blinded scoring of animals was not possible because of the separate equipment used to prevent contamination between C. difficile strains. Before infection, bedding was exchanged every two days between BMDC2+ and C57BL6 mice for a minimum of two weeks to equilibrate microbiota between strains. Mice were infected using a modified version of a previously published model for C. difficile. Briefly, mice were given an antibiotic cocktail consisting of 45 mg 1.1 vancomycin (Mylan), 35 mg 1.1 colistin (Sigma), 35 mg 1.1 gentamicin (Sigma), 15 mg metronidazole (Hospira), and 10 mg cefoxitin (Sigma) before incubation at 37 °C. The next day, mice were then switched back to regular drinking water for two days (days 3 onward), followed by an intraperitoneal (IP) injection (0.032 mg 1.1 clindamycin (Hospira) on day 1. On day 0, mice were gavaged with C. difficile. Mice were monitored twice daily throughout the course of the infection and immediately scored (if severe illness developed) according to clinical scoring parameters. Scores were based on weight loss, coat condition, activity level, diarrhoea, posture and eye condition for a cumulative clinical score between 1 and 20.

Tissue protein and histology. Mice were humanely euthanized and tissue was immediately removed for analysis. Total caecal lysate was generated by removing the caecum and rinsing gently with PBS. Tissue samples were homogenized with a solution (Sigma) for 18 h. Tissue samples were then switched back to regular drinking water for two days (days 3 onward), followed by an intraperitoneal (IP) injection (0.032 mg 1.1 clindamycin (Hospira) on day −1. On day 0, mice were gavaged with C. difficile. Mice were monitored twice daily throughout the course of the infection and immediately scored (if severe illness developed) according to clinical scoring parameters.

Eosinophil depletion. Eosinophils were depleted using an anti-mouse SiglecF monoclonal antibody (R&D Systems) as previously shown36. Control groups received IgG2A isotype control antibody (R&D Systems). Both groups were given 40 µg of antibody per mouse on day −1 and day +1 of infection (80 µg total per mouse)
via intraperitoneal injection. Eosinophil deactivation was evaluated by flow cytometry, which showed an 82% average reduction in colonic eosinophils.

Eosinophil adoptive transfer. Bone marrow derived eosinophils were generated as previously described and examined on day 10 for eosinophil markers and viability via flow cytometry6,7. Cultures routinely consisted of >75% live CD45+ CD11b+ Siglec-F+ eosinophils. For adoptive transfer, cultures were synthesized such that each injection occurred on day 10 of culture. Cultures were collecting using a cell scraper and washed twice in sterile PBS. A total of 4 x 10^6 eosinophils were given intraperitoneally per mouse. Mice received four total injections of eosinophils, starting the day before infection and for three subsequent days.

Statistical analysis. Statistical analysis was calculated and significance determined (P < 0.05) using the Mann–Whitney test or other suitable analysis, including Welch’s t-test for unequal variance. All statistical analysis was performed using GraphPad Prism software.

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**Author contributions**
C.A.C. conceived and designed the experiments, performed the experiments, analysed the data and wrote the paper. E.L.B. performed the experiments, provided valuable advice and contributed materials. M.M.S., M.G.W. and S.L.B. performed the experiments. S.A.K., C.S., A.M.E., F.K.-N., D.L., K.A. and N.P.M. contributed materials and valuable advice on experimental design. W.A.P. assisted with the experimental design and edited the paper.

**Additional information**
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**Competing interests**
The authors declare no competing financial interests.