Apoptosis of intestinal epithelial cells restricts *Clostridium difficile* infection in a model of pseudomembranous colitis

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*Clostridium difficile* is the leading cause of pseudomembranous colitis in hospitalized patients. *C. difficile* enterotoxins TcdA and TcdB promote this inflammatory condition via a cytotoxic response on intestinal epithelial cells (IECs), but the underlying mechanisms are incompletely understood. Additionally, TcdA and TcdB engage the Pyrin inflammasome in macrophages, but whether Pyrin modulates CDI pathophysiology is unknown. Here we show that the Pyrin inflammasome is not functional in IECs and that Pyrin signaling is dispensable for CDI-associated IEC death and for in vivo pathogenesis. Instead, our studies establish that *C. difficile* enterotoxins induce activation of executioner caspases 3/7 via the intrinsic apoptosis pathway, and demonstrate that caspase-3/7-mediated IEC apoptosis is critical for in vivo host defense during early stages of CDI. In conclusion, our findings dismiss a critical role for inflammasomes in CDI pathogenesis, and identify IEC apoptosis as a host defense mechanism that restricts *C. difficile* infection in vivo.
Clostridium difficile is a Gram-positive, toxin-producing and spore-forming obligatory anaerobic bacterial pathogen. It most commonly infects patients that are on antibiotics treatment, which licenses C. difficile outgrowth and intestinal colonization by disrupting the gut microbiota. However, we identified C. difficile infection (CDI) is thought to account for 15–30% of patients suffering from antibiotic-associated diarrhea, and 3–8% of patients with CDI progress to fulminant infection, a potentially life-threatening inflammatory condition that may involve severe ileus, toxic megacolon, colonic perforation with subsequent peritonitis, and septic shock. The pathophysiology of CDI is strictly associated with the production of TcdA and TcdB, two major pathogenic enterotoxins with cytotoxic properties that drive the hallmark symptoms of pseudomembranous colitis. However, these toxins primarily target IECs in the context of CDI, but whether the Pyrin inflammasome drives toxin-induced IEC death and plays a pathophysiological role during CDI is not known. To explore effector mechanisms that promote IEC cytotoxicity, cell death induction in primary IEC organoids was monitored by time-lapse imaging following intoxication with TcdA and TcdB. Wild-type intestinal organoids displayed a shrinking and disorganized cellular architecture that was associated with incorporation of the cell-impermeant DNA-intercalating agent propidium iodide (PI) already during initial hours of intoxication, and appeared dead by 16 h post-treatment. Similarly, intestinal organoids from Mefv+/− mice (lacking expression of the inflammasome sensor Pyrin) incorporated PI and displayed cell death features with kinetics resembling that of wild-type IEC organoids (Fig. 1a), indicating that Pyrin signaling is dispensable for TcdA-induced cytotoxicity of IECs. We next intoxicated intestinal organoids from Asc−/−, C1−/−, C11−/− and Gsdmd−/− mice to verify whether TcdA-induced IEC death was routed through inflammasomes other than the Pyrin pathway. Notably, IECs lacking the core inflammasome components ASC, caspase-1/11 or GSDMD all were equally sensitive to TcdA-induced cytotoxicity and died with kinetics resembling that of wild-type IEC organoids (Fig. 1b–d). Importantly, TcdB-induced IEC cytotoxicity was unaltered in intestinal organoids from Mefv+/− mice (Supplementary Fig. 1). IEC death was toxin-induced because mock-treated organoids of all analyzed genotypes failed to undergo cell death during the observed timeframe (Supplementary Fig. 2A–D). Together, these results demonstrate that unlike in monocytes and macrophages, Pyrin inflammasome activation and inflammasome-induced pyroptosis are dispensable for IEC cytotoxicity induced by C. difficile toxins TcdA and TcdB.

To clarify why TcdA and TcdB failed to induce Pyrin-dependent pyroptosis in IECs, we analyzed the transcript levels of Mefv (that encode Pyrin) in a public gene expression profile dataset from BioGPS (Fig. 2a) and by tissue real-time qPCR analysis (Fig. 2b). Consistent with a recent report demonstrating that Mefv expression in the intestinal tract is confined to lamina propria cells and absent from the IEC fraction, we detected high Mefv transcript levels in bone marrow and myeloid cells (macrophages, monocytes, dendritic cells and neutrophils) whereas adrenal gland, bladder, heart, kidney, thymus, liver, lung and small and large intestines were virtually devoid of Mefv expression (Fig. 2a, b). Consistent with the low transcript levels in intestinal IECs (Fig. 2a), we could also not detect Pyrin expression at the protein level in cell lysates of intestinal organoids grown in vitro (Fig. 2c). As expected, robust Pyrin expression was detected in BMDM cell lysates (Fig. 2c). Together, these results suggest that Pyrin inflammasome signaling is restricted to cells of the myeloid lineage, contrary to the NLRC4 and NLRP3 inflammasomes that are functional in both myeloid cells and IECs. In agreement, TcdA, TcdB and FlaTox (a biochemical ligand of the NALP5/NLRC4 inflammasome) all triggered IEC death in primary organoids (Fig. 2d and Supplementary Article NATURE COMMUNICATIONS |DOI: 10.1038/s41467-018-07386-5
Fig. 1 Inflammasome activation and pyroptosis are dispensable for *C. difficile* TcdA-induced IEC cytotoxicity. a–d Primary intestinal organoids from wild-type (WT) and a *Mefv*<sup>−/−</sup>, b *Asc*<sup>−/−</sup>, c *Casp1/11*<sup>−/−</sup> or d *Gsdmd*<sup>−/−</sup> mice were stimulated with TcdA and PI incorporation analyzed by live-imaging for 16 h. Graphs correspond to PI quantification plotted by organoid area. Scale bars: 30 µm. The data are representative of at least 3 independent experiments.
Movie 1), but only FlaTox-induced cytotoxicity was associated with release of the inflammasome-dependent cytokine IL-18 (Fig. 2e). Moreover, the morphological changes of IECs undergoing TcdA/TcdB-induced cell death differed dramatically from FlaTox-stimulated IECs undergoing pyroptosis (Supplementary Movie 1), adding further credence to the notion that TcdA/TcdB-induced cytotoxicity differs from pyroptosis. In conclusion, these results demonstrate that Pyrin expression and Pyrin inflammasome signaling likely are restricted to the myeloid compartment, and that C. difficile toxins trigger a cell death mode in IECs that differs from inflammasome-induced pyroptosis.

**Caspases 3 and 7 drive C. difficile-induced IEC death.** Having ruled out pyroptosis induction as the mechanism of TcdA/B-induced IEC killing and based on our previous findings (Figs 1 and 2), we next asked whether C. difficile toxins elicited an apoptotic response in intoxicated IEC organoids. To this end, lysates of wild-type intestinal organoids that had been challenged with TcdA and TcdB, respectively, were examined by immunoblotting for activation of the apoptotic executioner caspases 3 and 7 (Fig. 3a). We observed robust cleavage of both caspases that was comparable to caspase-3/7 activation levels that were elicited by staurosporine, a broad-spectrum kinase inhibitor and a potent pro-apoptotic chemotherapeutic agent (Fig. 3a). Given these results, we next infected IEC organoids with either a toxicogenic wild-type strain of C. difficile or a mutant strain lacking expression of both TcdA and TcdB. Notably, wild-type C. difficile infection elicited robust activation of caspases 3 and 7, a response that was blunted in IEC organoids that had been infected with the
toxin-deficient mutant strain (Fig. 3b), in agreement with the established requirement for TcdA/TcdB in *C. difficile*-induced cytotoxicity. Moreover, PARP1 cleavage - a hallmark feature of apoptosis - was readily detected in intestinal organoids that had been intoxicated with TcdA or TcdB (Fig. 3a) or infected with toxigenic *C. difficile* (Fig. 3b), further demonstrating activation of executioner caspases 3 and 7 in these conditions.

To functionally validate the role of caspases 3 and 7 in TcdA/B-induced IEC killing, we produced intestinal organoids from mice with a combined IEC-specific deletion of these executioner caspases (*Casp3/7IEC-KO*) by breeding mice with conditionally targeted *Casp3* and *Casp7* alleles (*Casp3/7F/F*) to animals expressing Cre recombinase under control of the IEC-specific Villin promoter (Villin-Cre). Western blot analysis for caspases 3 and 7 showed expression of both executioner caspases in wild-type IECs and confirmed their efficient deletion in *Casp3/7IEC-KO* organoids (Fig. 3c). Notably, TcdA and TcdB-induced IEC cytotoxicity was abolished in *Casp3/7IEC-KO* organoids, which was further supported by a failure to incorporate PI during the studied timeframe (Fig. 3d). As expected, mock-treated IEC organoids failed to undergo cell death during the observed timeframe (Supplementary Fig. 3), confirming specificity of these findings. Collectively, these results establish that apoptotic executioner caspases 3 and 7 are critical for *C. difficile*-induced IEC cytotoxicity.

**Bax/Bak pores contribute to TcdA/TcdB-induced IEC killing.**

Given that our studies in *Casp3/7IEC-KO* organoids established a role for apoptotic executioner caspases in *C. difficile* toxin-
induced IEC death, we next sought to analyze the upstream mechanisms promoting activation of caspases 3 and 7. Initiation of classical apoptosis is mediated by two distinct pathways; an intrinsic (mitochondrial) and extrinsic (death receptor) pathway. RIPK1 is a kinase that modulates a regulated necrotic cell death mode termed necroptosis as well as apoptosis induction under certain conditions. However, intestinal organoids from mutant mice that lack RIPK1 kinase activity (R1 KD mice) were killed by TcdA stimulation with similar efficiency as wild-type IECs (Fig. 4a). Moreover, absence of RIPK3—a related kinase that is essential for induction of necroptosis—failed to protect intestinal organoids against TcdA and TcdB-induced intoxication, regardless whether it was deleted alone or in combination with the apical initiator caspase-8 that drives death receptor-induced apoptosis (Fig. 4b and Supplementary Fig. 4A). Notably, transgenic expression of anti-apoptotic Bcl-2 under control of the LPK promoter for expression in IECs and hepatocytes, supported a slight but reproducible protection against TcdA/TcdB-induced cell death (Fig. 4c, Supplementary Fig. 5A and Supplementary Fig. 6). Intestinal organoid cell death was toxin-induced as

Fig. 4 RIP kinases and caspase-8 are dispensable, whereas Bax/Bak pores contribute to TcdA-induced IEC killing. a-c Primary intestinal organoids from wild-type (WT) and a Ripk-D138N (RIPK1 kinase dead), b Ripk3−/− and Ripk3−/− Casp8−/− or c Bcl-2LPK-Tg mice were stimulated with TcdA and PI incorporation analyzed by live-imaging for 16 h. Graphs correspond to PI quantification plotted by organoid area. Scale bars: 30 µm. d, e Cell lysates from primary intestinal organoids of d Ripk3−/− and Ripk3−/− Casp8−/− or e Bcl-2LPK-Tg stimulated with TcdA were prepared and immunoblotted for caspase-3, caspase-7 and tubulin or β-actin. Data are representative of 3 independent experiments. Data are shown as mean ± SD and were analyzed with 2-way ANOVA. *P < 0.05, **P < 0.01, and ***P < 0.001.
**Fig. 5** IEC apoptosis protects mice during in vivo *C. difficile* infection. **a** Scheme of *C. difficile* infection protocol. **b–d** Mevf+/+ (n = 9) and Mevf−/− (n = 11) littermates mice were infected with *C. difficile* and monitored for body weight (**b**), stool score (**c**) and bacterial burden in the stool (**d**). **e–g** Casp3/7+/+ (n = 7) and Casp3/7IEC-KO (n = 11) littersmates mice were infected with *C. difficile* and monitored for body weight (**e**), stool score (**f**) and bacterial burden in the stool (**g**). Nonparametric Mann-Whitney U test was used to analyze the data. Error bars represent SD. *P* < 0.05, **P** < 0.01, and ***P*** < 0.001

Evidenced by the lack of cytotoxicity in mock-treated controls of all analyzed genotypes (Supplementary Fig. 7A–C). These results suggest that RIP kinases and death receptor-induced apoptosis are dispensable, whereas mitochondrial Bax/Bak pores partially contribute to TcdA/TcdB-induced caspase-3/7 activation and IEC apoptosis. In agreement, TcdA and TcdB intoxication of Ripk3−/− and Ripk3−/−Casp8−/− organoids failed to alter cleavage of caspases 3 and 7 (Fig. 4d and Supplementary Fig. 4B). Although activation of caspases 3 and 7 was not substantially altered in Bcl-2 transgenic IECs following TcdA intoxication (Supplementary Fig. 5B). In conclusion, these results exclude a role for death receptor-induced apoptosis and necroptosis signaling in *C. difficile* toxin-induced IEC cytotoxicity, and suggest that mechanisms redundant to the pro-apoptotic Bax/Bak pore that lead to mitochondrial outer membrane permeabilization (MOMP) may promote TcdA/B-induced caspase-3/7 activation and IEC apoptosis through the intrinsic apoptosis pathway.

**IEC apoptosis restricts *C. difficile* growth in vivo.** Having established that *C. difficile*-induced IEC death corresponds to caspase-3/7-mediated apoptosis via the intrinsic pathway, we next sought to investigate its pathophysiological role in an established mouse model of live CDI. In this in vivo infection model, littermate mice of the indicated genotypes are treated with a cocktail of antibiotics during 5 days prior to infection in order to create a niche in the intestinal tract that supports *C. difficile* growth (Fig. 5a). Following infection, mice are assessed daily for a number of clinical parameters during a period of 14 days and stool samples are taken at regular intervals to assess bacterial burden. Corroborating our findings that the Pyrin inflammasome is not functional in IECs (Fig. 1), Mevf+/+ and Mevf−/− littermate mice responded to *C. difficile* infection with a comparable decline in body weight over the course of infection (Fig. 5b). Moreover, stool consistency - which is a robust surrogate marker of CDI severity - was equally affected in infected Mevf+/+ and Mevf−/− littermate mice (Fig. 5c). Consistently, longitudinal...
quantitative assessment of C. difficile replication in stool samples of infected mice confirmed that systemic disruption of Pyrin inflammosome activation failed to impact C. difficile replication rates during infection as evidenced by the similar bacterial loads detected in the stool of Mefv+/− and Mefv−/− littermate mice (Fig. 5d).

In marked contrast to Pyrin-deficient mice, selective deletion of executioner caspases 3 and 7 in IECs caused Casp3/7IEC-KO mice to lose significantly more body weight during initial stages of infection relative to littermate Casp3/7+/− mice that do express these executioner caspases in IECs (Fig. 5e). Moreover, stool scores of Casp3/7−/−IEC-KO mice were significantly elevated relative to those of Casp3/7+/− control mice (Fig. 5f). We extended these results by quantifying C. difficile burdens in the stool. This analysis showed that Casp3/7−/−IEC-KO and littermate Casp3/7+/− mice had equal C. difficile counts on the first day of infection. However, Casp3/7−/−IEC-KO mice continued to present with high infection rates by day 3 post-infection, contrary to Casp3/7+/− mice that started to clear the infection (Fig. 5g). Casp3/7−/−IEC-KO mice eventually controlled C. difficile burdens and progressed towards self-limiting disease as evidenced by the reduced bacterial counts detected by day 7 post-infection (Fig. 5g). Collectively, these results demonstrate that the Pyrin inflammosome is dispensable, whereas caspase-3/7-mediated IEC apoptosis is a critical host defense mechanism that protects against C. difficile infection during early stages of CDI.

Discussion

CDI is a major public health concern. It is estimated that the number of people infected with C. difficile is higher than those infected with Salmonella species in Europe and the United States. The high incidence of CDI is associated with the emergence of hypervirulent C. difficile strains in Europe and North America in the new millennium, which was recently suggested to be associated with the widespread adoption of trehalose as an additive in human diets. Therefore, molecular analysis of C. difficile-host interactions and better understanding of how this bacterial pathogen instigates host pathology may improve disease prevention and inform novel therapeutic approaches.

The immune response to C. difficile may be both protective and detrimental to the host, depending on its magnitude. Neutrophil recruitment into the intestinal tract consequent to NF-kB signaling via the TLR4 and Nod1 signaling axes is essential for protecting the host against C. difficile, as evidenced by decreased survival rates in mice lacking these PRRs. Notably, a polymorphism in the human IL8 gene that results in increased production of IL-8, a neutrophil chemoattractant, is associated with increased risk for C. difficile recurrence, highlighting the importance of a balanced immune response during infection. TLR5 engagement has also been linked to protection against CDI. In addition, innate lymphoid cells (ILCs), mainly ILC1, and to a lesser extent ILC3, also contribute to protection against acute CDI via the induction of IL-22 and IFN-γ.

Although inflammasomes are central conduits of innate immune responses, their role in CDI is incompletely understood. TcdA and TcdB were recently shown to engage the Pyrin inflammasome in mouse and human myeloid cells, leading to secretion of IL-1β and IL-18 and induction of pyroptosis. However, IECs are the primary target cells of C. difficile exotoxins in the context of CDI pathobiology. Previous studies suggested both protective and detrimental roles for the inflammasome adaptor ASC during CDI, but whether Pyrin inflammasome signaling impacts on C. difficile-induced IEC cytotoxicity and modulates the course of CDI pathobiology in vivo is unknown. Here, we showed in primary IEC organoid systems that C. difficile-induced IEC cytotoxicity is fully mediated by its exotoxins, and that the Pyrin inflammasome (and inflammasome activation in general) is dispensable for TcdA/TcdB-induced IEC killing, which we linked to absent Pyrin expression and functionality in IECs. This markedly contrasts to the situation in myeloid cells, where Pyrin is highly expressed and inflammasome activation elicits pyroptotic cell death in response to TcdA/TcdB-induced RhoA modification, thus highlighting the cell type specificity of host-pathogen interactions. Moreover, we provided genetic evidence that TcdA and TcdB-induced IEC death critically relied on caspase-3/7-mediated apoptosis through the intrinsic apoptotic pathway, whereas blockade of both caspase-8-mediated death receptor-induced apoptosis and necroptotic signaling failed to protect from TcdA/TcdB-induced IEC cytotoxicity. Notably, transgenic expression of the anti-apoptotic protein Bcl-2 in intestinal organoids did not fully recapitulate the protective phenotype of IECs lacking caspases 3 and 7, suggesting that mechanisms in addition to the Bax/Bak pore may promote MOMP during CDI. Indeed, Bcl2-transgenic IECs continued to engage caspases 3 and 7 in response to TcdA and TcdB. Thus, although Bax/Bak-independent mitochondrial membrane permeabilization has been reported to induce release of cytochrome c into the cytosol, future work should address the mechanisms by which C. difficile toxins cause MOMP and caspase-3/7 activation. Moreover, a recent report showed that TcdA and TcdB also induce cell death of human IEC organoids, and future studies should address whether the molecular cytotoxicity mechanisms we have uncovered in the murine system are conserved in humans.

Induction of cell death upon microbial infections is generally a host defense mechanism. Some pathogens have evolved virulence mechanisms that manipulate cell death pathways in order to promote their replication and dissemination in a hostile host environment. For instance, poxviruses and several bacterial pathogens encode effector proteins that interfere with activation of apoptotic caspases and/or inflammasomes, thereby preventing host apoptosis and pyroptosis, respectively. However, it is currently unknown whether IEC cytotoxicity in the context of CDI is detrimental or beneficial to the host. We found that mice lacking apoptotic executioner caspases 3 and 7 selectively in IECs were significantly more susceptible to CDI compared to littermate control mice. In agreement, we measured increased bacterial replication rates in the stool of Casp3/7IEC-KO mice. Notably, Casp3/7IEC-KO mice restored control of CDI infection with delayed kinetics, pointing to apoptosis-independent anti-microbial mechanisms that support C. difficile clearance. Although it is tempting to speculate that in rare cases of systemic C. difficile toxin dissemination, activation of the Pyrin inflammasome in myeloid cells might exert a detrimental role to the host by promoting excessive myeloid cell pyroptosis and inflammatory tissue damage, our results suggest that the Pyrin inflammasome is dispensable for host defense during self-limiting pseudomembranous colitis. Moreover, the lack of Pyrin expression in IECs prevents this cell type to undergo pyroptosis following C. difficile toxin exposure, and instead promotes IEC apoptosis as the dominant IEC death mode during CDI in vivo. These findings suggest that C. difficile-induced IEC apoptosis is an early host defense mechanism that contributes to bacterial restriction and toxin production, most likely by promoting clearance of infected cells in the intestinal epithelium by professional phagocytes and infiltrating neutrophils.

Methods

Mice. Mefv+/−, Asc−/−, Casp1−/−, Casp11−/−, Gsdmd−/−, Ripk1−/−, Ripk3−/−, Casp8−/− and Ripk1D138N−/− mice have been described. C57BL/6 ES cells (JNA3A.11) with conditionally targeted casp3 (clone HEPD0716_4_A08) and casp7 (clone EPD0398_5_C04) alleles were obtained from the European Conditional Mouse
Intestinal organoid culture and live imaging. Primary intestinal epithelial organoids were grown as described before. Briefly, the small intestine and colon were flushed and cut into small pieces that were dissociated in PBS containing 2 mM EDTA for 30 min at 4 °C. After extensive washing, the isolated crypts were pelleted and mixed with 25 μL of Matrigel (Corning) and put in a 24-well plate. After polymerization of the Matrigel, complete culture medium containing advanced DMEM/F12 (Gibco) supplemented with B27 supplement (0.02%, Invitrogen), N2 supplement (0.1%, Invitrogen), N-acetylcysteine (0.0025%, Sigma-Aldrich), mouse epidermal growth factor (mEGF; 0.001%, Invitrogen), and conditioned Rpondin and mNoggin medium was added to the wells. Organoids were seeded and imaged in an 8-well chamber (iBidi) for cell death analysis by real time lapse microscopy or in 24-well plates for Western blotting analysis. Cell death was induced with TcdA (1 μg/ml, Enzo Life Sciences), TcdB (1 μg/ml, List Laboratories) or TcdA (1 μg/ml, Enzo Life Sciences) and TcdB (1 μg/ml, List Laboratories). Cell death analysis was performed with TcdA or TcdB while data analysis and image reconstruction were performed with ImageJ (NIH) by measuring PI intensity and organoid area. PI intensity was divided by organoid area to obtain final values.

Clostridium difficile infection. Clostridium difficile strains VPI10463 (toxigenic; TcdA+TcdB+) and VPI11186 (nontoxigenic; TcdA–TcdB–) were purchased from ATCC. Glycerol stocks were cultured overnight at 37 °C in anaerobic conditions in BHIS enriched medium (37.5 g/l brain heart infusion—Gibco; 5 g/l yeast extract—Gibco; 0.03% l-cysteine—Sigma; 0.1% sodium taurocholate—Sigma). For C. difficile in vivo infection, mice received antibiotics cocktail (2 mg/ml ampicillin, 0.4 mg/ml kanamycin, 0.035 mg/ml gentamicin, 850 U/ml colistin, 0.215 mg/ml metronidazole and 0.045 mg/ml vancomycin) in drinking water for 5 days followed by 2 days in normal drinking water. One day prior infection, mice were infected with a single intraperitoneal injection of the broad-spectrum antibiotic clindamycin (10 mg/kg). On the day of infection, mice were infected with 10⁶ vegetative cells of Clostridium difficile. Fecal homogenates were incubated with proteinase K followed by ethanol pre-cation of Rho proteins. 

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Cytokine analysis. IL-18 levels in cell culture medium were determined by mouse head based multiplex assay using Luminex technology (Bio-Rad) according to the manufacturer’s instructions.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7.0 software. Data are shown as mean ± SD. The data were compared with the nonparametric Mann–Whitney U test for in vivo studies and two-way ANOVA for in vitro studies. P < 0.05 was considered statistically significant; *P < 0.05, **P < 0.01, and ***P < 0.001.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

Data availability. The data that support the findings of this study are available from the corresponding author upon request. A reporting summary for this article is available as a Supplementary Information file.
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Authors contribution:
P.H.Y.S. and M.L. conceptualized the project; P.H.Y.S. and L.H. performed the experiments; F.G., S.K., T.V.B., N.T. and P.V. provided essential reagents; P.H.Y.S. and M.L. analyzed the data and wrote the manuscript; M.L. oversaw the project.

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