Entry of spores into intestinal epithelial cells contributes to recurrence of *Clostridioides difficile* infection

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*Clostridioides difficile* spores produced during infection are important for the recurrence of the disease. Here, we show that *C. difficile* spores gain entry into the intestinal mucosa via pathways dependent on host fibronectin-α5β1 and vitronectin-αvβ3. The exosporium protein BclA3, on the spore surface, is required for both entry pathways. Deletion of the *bclA3* gene in *C. difficile*, or pharmacological inhibition of endocytosis using nystatin, leads to reduced entry into the intestinal mucosa and reduced recurrence of the disease in a mouse model. Our findings indicate that *C. difficile* spore entry into the intestinal barrier can contribute to spore persistence and infection recurrence, and suggest potential avenues for new therapies.
Clostridioles difficile is a strict anaerobic Gram-positive pathogenic bacterium that forms highly resistant spores that easily persist in the environment and contribute to the transmission of *C. difficile* infections (CDI) through fecal–oral route\(^1\). Disruption of the gut microbiota by broad-spectrum antibiotics leads to an optimal environment for *C. difficile* colonization and proliferation in the colon and disease manifestation. CDI currently leads hospital-acquired diarrhea associated to antibiotics in the United States and worldwide\(^2\). In the US alone, ~500,000 patients per year become infected with CDI, and mortality rates reach ~8% of total patients\(^3\). The annual cost of CDI to the health care system is estimated at ~US 4.8 billion\(^4\). Treatment of CDI usually involves antibiotic therapy, typically vancomycin or metronidazole and, most recently, fidaxomicin\(^5\), which, although resolves the infection in ~95% of the cases, leads to recurrence of CDI (R-CDI) in 15–30% of the individuals\(^6\–^8\).

During infection, *C. difficile* produces two major virulence factors, toxins TcdA and TcdB, responsible for the clinical manifestation of the disease, induce pro-inflammatory cytokines, disruption of tight junctions, detachment of intestinal epithelial cells (IECs), and loss of transepithelial barriers. *C. difficile* also initiates a sporulation pathway that leads to the production of new metabolically dormant spores in the host’s intestine\(^9\). In vivo, spore formation is essential for the recurrence of the disease\(^9\). Moreover, spore-based therapies that remove *C. difficile* spores from the intestinal mucosa contribute to reducing the recurrence of the disease in animal models\(^10\).

Recent in vivo studies in the laboratory strain 630 suggest that the spore-surface mucus-binding protein, peroxiredoxin–chitinase CotE, and the exosporium collagen-like BclA1 proteins are required for the colonization and infectivity in a mouse model of CDI\(^11\). However, the surface layer of 630 spores does not resemble that of clinically relevant strains, which exhibit hair-like projections in their spore surface, structures that are absent in strain 630\(^12\). Notably, most clinically relevant sequenced *C. difficile* isolates, including isolates of the epidemiologically relevant 027 ribotype, have a truncated *bclA1* due to a premature stop codon in the N-terminal domain\(^13\), resulting in the translation of a small polypeptide, which localizes to the spore surface\(^14\), thus limiting the breadth and depth of these results. *C. difficile* spores exhibit high levels of adherence to IECs in vitro\(^14\–^16\), and that the hair-like projections of *C. difficile* spores come in close proximity with the microvilli of differentiated Caco-2 cells; furthermore, *C. difficile* spores interact in a dose-dependent manner with fibronectin (Fn) and vitronectin (Vn)\(^15\), two extracellular matrix proteins used by several enteric pathogens to infect the host\(^16\–^18\). However, the mechanisms that underlie how these interactions contribute to *C. difficile* spore persistence in vivo and contribute to the recurrence of the disease remain unclear.

Herein, we first demonstrate that *C. difficile* spores gain entry into the intestinal epithelial barrier of mice and that spore entry into IECs requires serum molecules, specifically Fn and Vn, that are luminally accessible in the colon mucosa. We also demonstrate that the spore entry pathway is Fn-α5β1 and Vn-αvβ3 integrin-dependent. Next, we show that the spore-surface collagen-like BclA3 protein is essential for spore entry into IECs through these pathways in vitro and for spore adherence to the intestinal mucosa. Our results also show that BclA3 contributes to the recurrence of the disease in mice. We also observed the therapeutic potential of blocking spore entry into the intestinal epithelial barrier, and how coadministration of nystatin with vancomycin reduces spore persistence and R-CDI in mice. Together, our results reveal a novel mechanism employed by *C. difficile* spores that contributes to R-CDI, which involves gaining intracellular access into the intestinal barrier via BclA3-Fn-α5β1 and BclA3-Vn-αvβ3 specific, and that blocking spore entry contributes to reduced recurrence of the disease.

### Results

**C. difficile** spores internalize into the intestinal barrier in vivo.

To study the interaction of *C. difficile* spores and the host’s intestinal barrier, we used a colonic/ileal loop assay infected with *C. difficile* spores for 5 h\(^18\), where *C. difficile* R20291 spores were labeled with anti-spore antibodies\(^15\). We observed similar levels of adherence of *C. difficile* spores to the colonic and ileum mucosa (Fig. 1a–c), with no preference for the site of spore adherence in both colonic and ileum mucosa (Fig. 1d, and Supplementary Figs. 1 and 2). Strikingly, we observed that *C. difficile* spores were able to cross the mucosal barrier in the colonic/ileal loop assay (Fig. 1a, b, e, f, Supplementary Movies 1 and 2, and Supplementary Figs. 3 and 4). We observed that 4.6 and 3.7 spores per 10\(^5\) µm\(^2\) were able to cross the mucosal barrier in colonic and ileal loops (Fig. 1e), corresponding to 0.92 ± 0.30% and 1.04 ± 0.48% of the total spores, respectively. In the colonic mucosa, internalized *C. difficile* spores were found to homogeneously localize 10–30 µm from the colonic surface and 5–50 µm from the closest crypt membrane, while in the ileum mucosa spores were homogeneously found at 15–70 µm from the villus tip and 10–50 µm from the villus membrane in ileal loops, (Supplementary Fig. 5), indicating multiple sites of entry in colon and ileum.

**C. difficile** spore entry into intestinal epithelial cells requires serum components in vitro. Our previous in vitro studies in IECs were conducted in the absence of fetal bovine serum (FBS) and did not evidence internalized spores\(^14\). \(^15\). \(^18\), \(^19\). Therefore, we assess if FBS contributed to spore entry by confocal fluorescence microscopy by analyzing monolayers of polarized T84 IECs (Fig. 2a and Supplementary Fig. 6a, b) and differentiated Caco-2 cells (Supplementary Fig. 6c, d), which were infected with *C. difficile* spores of the epidemiologically relevant R20291 and the commonly used strain 630 in the presence of FBS. In both cell lines, several intracellular spores of strain *C. difficile* 630 were found to be located between the apical and basal actin cytoskeleton (Fig. 2a and Supplementary Fig. 6). To obtain convincing evidence of entry of *C. difficile* spores into IECs, we analyzed polarized monolayers of T84 and Caco-2 cell lines infected with *C. difficile* 630 or R20291 spores using transmission electron microscopy (TEM). Electron micrographs evidence that some *C. difficile* spores were found extracellularly in the apical membrane, while others were found intracellularly (Fig. 2b–d). Intracellular *C. difficile* spores were surrounded by an endosomal-like membrane (Fig. 2c, d). Notably, the formation of membrane lamellipodia-like protrusions and circular ruffles surrounding *C. difficile* 630 spores were evidenced at the site of attachment of *C. difficile* spores to the apical membrane (Fig. 2e), suggesting macropinocytosis-like endocytosis of *C. difficile* spores. Intracellular spores of the strain, R20291, were also evidenced in differentiated Caco-2 cells (Fig. 2f–h).

Next, to quantitatively assess the internalization of *C. difficile* spores into non-phagocytic cells, we developed an exclusion assay in which, in nonpermeabilized cells, only extracellular spores are fluorescently labeled with anti-*C. difficile* spore antibody, while total spores can be quantified by phase-contrast microscopy; intracellular spores are not stained by anti-*C. difficile* spore antibody (absence of fluorescence) and are only detectable by phase-contrast microscopy (Supplementary Fig. 7a). With this assay, we probe that entry of 630, and R20291 spores into monolayers of Caco-2, T84, Vero, and HT29 cell lines significantly increased in the presence of FBS (Fig. 2i, j), as well as with serum from various mammalian...
The percentage of internalized spores of and R20291 strains was highest at 5 h post infection in Caco-2 and T84 cells (Supplementary Fig. 7b, c). Spores of various clinically relevant ribotypes were able to internalize into Caco-2 cells (Supplementary Fig. 7d). Overall, these results demonstrate that C. difficile spores are able to gain intracellular entry into non-phagocytic cells and that spore entry is serum-dependent in vitro.
C. difficile spore entry into intestinal epithelial cells requires Fn and Vn. Fn and Vn are extracellular matrix proteins, which are also present in mammal serum and are widely used by enteric pathogens to infect host cells.\(^1\)\(^6\),\(^17\). We have shown previously that both, Fn and Vn, bind in a concentration-dependent manner to C. difficile spores.\(^1\)\(^5\). To assess whether serum Fn and Vn contribute to C. difficile spore entry, we evaluated the internalization assay in the presence of RGD peptide to block the interaction of Fn and Vn with their cognate receptors through the RGD-binding domain.\(^2\)\(^0\),\(^2\)\(^1\). RGD significantly reduced the extent of spore entry into differentiated Caco-2 cells in the presence of human serum by ~45% (Fig. 3a and Supplementary Fig. 8a), indicating that serum Fn and Vn might be involved in C. difficile spore entry in an RGD-specific manner; by contrast, no decrease in adherence of C. difficile spores was evidenced in the presence of RGD (Fig. 3b and Supplementary Fig. 8b). Similar results were observed in undifferentiated Caco-2 cells (Supplementary Fig. 8c–f). We confirmed these results by showing that the

**Fig. 2 C. difficile spores are internalized by intestinal epithelial cells.** a Representative confocal micrograph of an internalized C. difficile 630 spores in T84 cells. C. difficile spores are shown in red, F-actin is shown in green (fluorophores colors were digitally reassigned for a better representation). The images were acquired from \(n = 8\) fields from two independent experiments. Yellow lines indicate an internalized spore. b–e TEM of differentiated monolayers of T84 cells infected with C. difficile 630 spores. Black and white arrows denote extracellular and intracellular C. difficile spores, respectively. c, d Magnifications of black squares of b, e An adhered C. difficile spore and an apical membrane extension of T84 cells surrounding C. difficile spores. f–h TEM of differentiated monolayers of Caco-2 cells infected with C. difficile R20291 spores. White arrows in f indicate internalized C. difficile spores. g, h Magnifications of black boxes in f. Micrographs are representative of \(n = 3\) independent experiments. Internalization of C. difficile spores t strain 630 and, j R20291 preincubated with FBS or culture media in undifferentiated (2 days) and differentiated (8 days) Caco-2, T84, Vero, and HT29. k Internalization of C. difficile spores R20291 strain preincubated with serum of different mammalian species in Caco-2 cells. i–k Representative of \(n = 3\) independent experiments. Error bars indicate the mean ± S.E.M. Statistical analysis was performed by two-tailed unpaired Student’s \(t\) test, \(p > 0.05\). Scale bars a 5 \(\mu\)m; c, d, 100 nm; e, 1 \(\mu\)m; f, 2 \(\mu\)m; g, 200 nm; h, 500 nm.
Fig. 3 *C. difficile* spore internalization requires Fn and Vn which are luminaly accessible in the intestinal barrier. **a** Internalization and adherence of *C. difficile* spores preincubated with NHS in differentiated monolayers of Caco-2 in the presence of RGD peptide. **c, d** Differentiated Caco-2 cells were infected with *C. difficile* spores preincubated with DMEM, NHS, Fn, or Vn. Data shown in each panel are normalized to the control. In bars, each dot represents one independent well from a to d three independent experiments. e–g Representative confocal micrographs of fixed whole-mount of the healthy colon of n = 2 mice for e accessible Fn (acc Fn); f accessible Vn (acc Vn); and g accessible Muc2 (acc Muc2) with accessible Ecad (acc Ecad). The main figure shows a 3D projection, below magnifications, and a z-stack of representative cells with different immunostaining. The figures h–n shows the cell repartition of cell immunodetected for h acc Ecad, i acc Fn, j total acc Fn cells that were immunodetected for acc Ecad, k cells immunodetected for acc Vn, l total acc Vn cells that were immunodetected for acc Ecad, m acc Muc2, n total acc Muc2 cells that were immunodetected for acc Ecad. h–n 1000–1200 cells were counted per field in two independent mice. **h** Four fields per mice, n = 2 mice; i, j two fields per mice, n = 2 mice; k, l one field per mice, n = 2 mice. Acc Fn, acc Vn, and Muc2 are shown in green, acc Ecad is shown in red, and F-actin in gray (fluorophores colors were digitally reassigned for a better representation). Scale bar, 20 μm. Error bars indicate mean ± S.E.M. Statistical analysis was performed by two-tailed unpaired Student’s t test, ns, p > 0.05; ****p < 0.0001. Scale bar, 20 μm.
infection with spores preincubated with Fn or Vn restored spore entry into differentiated (Fig. 3c) and undifferentiated Caco-2 cells (Supplementary Fig. 9a), but had no impact on spore adherence to differentiated (Fig. 3d) and undifferentiated Caco-2 cells (Supplementary Fig. 9b). Similar results were observed in differentiated Caco-2 cells pretreated with Fn or Vn before infection with C. difficile spores (Supplementary Fig. 9c, d), confirming that the presence of Fn and Vn mediates C. difficile spore entry.

**Intestinal barrier sites with accessible Fn and Vn.** Both Fn and Vn are mainly located in the basal and basolateral membrane of epithelial cells, where tight and adherent junctions are formed\(^{16,17}\). However, several epithelial barrier suffers reorganization and/or disruption of tight and adherent junctions, such as cell extrusion sites, goblet cells (GCs), at cell–cell junctions with neighboring cells, and along villus epithelial folds\(^{22-24}\). Therefore, we hypothesized that sites undergoing adherent junction rearrangement also contained accessible Fn and Vn. We performed double staining, in which luminally accessible Fn and E-cadherin (Ecad), a marker for reorganization or disruption of adherent junctions\(^{22}\), were stained in nonpermeabilized tissue. We first determined the relative number of IECs in the colonic tissue that expresses luminally accessible Ecad and found that nearly 16% of the IECs have this feature (Fig. 3h). Accessible Fn and Vn were observed in 27 and 14% of the IECs cells (Fig. 3i, k). We observed that most of the cells that had luminally accessible Fn or Vn also had accessible Ecad (Fig. 3j, l) and are likely undergoing a major reorganization of the adherent junctions. However, a small fraction of epithelial cells with accessible Fn (33%) or Vn (12%) had no accessible Ecad (Fig. 3i, k). Luminally accessible Ecad has been previously found around mucus-expelling GCs in mice intestinal tissue\(^{22}\). Therefore, to quantify the relative abundance of GCs with accessible Ecad in our experimental conditions, we performed double immunostaining for accessible Ecad and the GC-specific marker Muc2\(^{22,25}\). We observed that 13% of the IECs were positive for Muc2 in colonic tissue (Fig. 3m), and all of Muc2-positive cells were positive for accessible Ecad (Fig. 3n). Luminally accessible Ecad has also been observed in mice ileal tissue\(^{22}\); we observed that nearly 17% of the IECs of mice ileal had luminal accessible Ecad (Supplementary Fig. 10a, b). Next, we quantified the relative abundance of GCs in the ileum mucosa and observed that nearly 9% of total IECs were positive for Muc2 (Supplementary Fig. 10c), of which 70% were positives for accessible Ecad (Supplementary Fig. 10d). This data supports the notion that Fn and Vn are also accessible in the intestinal epithelial barrier. Altogether, these results demonstrate the existence of sites in the intestinal barrier that undergo a reorganization of adherent junctions that exhibit accessible Fn and Vn through which C. difficile spores can gain entry into the intestinal epithelium.

**C. difficile spores internalize via Fn-α5β1 and Vn-α5β1 integrin in vitro.** The Fn RGD loop between domains FnIII9 and FnIII10 enhances binding between Fn and α5β1 integrin\(^{16}\); Vn also has a similar RGD loop that enhances binding to α5β1 integrin\(^{17}\). To address whether binding of Fn and Vn to their cognate integrin receptors is required for C. difficile spore entry into IECs, monolayers of Caco-2 cells were infected with C. difficile R20291 spores in the presence of the inhibitory RGD peptide, showing that in the presence of Fn or Vn, increasing concentrations of RGD progressively decreased spore entry into differentiated (Fig. 4a, b) and into undifferentiated Caco-2 cells (Supplementary Fig. 11a, c), but not spore adherence (Fig. 4c, d and Supplementary Fig. 10b, d) to Caco-2 cells. Next, through an antibody blocking assay, we assessed which integrin subunits are involved in Fn- and Vn-dependent entry of C. difficile spores into IECs. Results demonstrate that blocking the subunits of the collectin-binding, α5β1 integrin\(^{16,22}\) and β1 integrin subunit did not affect internalization nor adherence of C. difficile spores to Caco-2 cells in the presence of Fn (Fig. 4e, f) or Vn (Fig. 4g, h). However, a significant decrease in spore entry, but not spore adherence, to differentiated and undifferentiated Caco-2 cells was observed upon blocking each subunit of α5β1 integrin in the presence of Fn (Fig. 4e, f and Supplementary Fig. 11e, f), as well as blocking each subunit of α5β1 integrin in the presence of Vn (Fig. 4g, h and Supplementary Fig. 11g, h). These results were confirmed upon expressing each integrin subunit in Chinese hamster ovary (CHO) cells (Fig. 4i–k), a naive cell line that otherwise does not express integrins. CHO cells expressing individual α5 or β1 integrin subunits exhibited significant spore entry, but not adherence in the presence of Fn (Fig. 4l, m); equally, CHO cells expressing individual α5 or β1 integrin subunit exhibited significant spore entry, but not spore adherence in the presence of Vn (Fig. 4n, o). No increase in entry or adherence was detected in the absence of Fn and Vn (Supplementary Fig. 12a, b). Altogether, these observations demonstrate that the internalization of C. difficile spores into IECs occurs through Fn-α5β1 and Vn-α5β1 uptake pathways.

**Fn and Vn bind to the hair-like extensions of C. difficile spores, formed by the collagen-like BclA3 exosporium protein.** C. difficile spores of epidemiologically relevant strains exhibit hair-like projections that are likely to be formed by the collagen-like exosporium proteins\(^{1,13}\). Fn and Vn have a gelatin/collagen-binding domain\(^{16,17}\), suggesting that these molecules might interact with C. difficile spores through these hair-like projections. Indeed, through TEM coupled with immunogold labeling of Fn and Vn, we observed that more than ~50% of the spores were positive for Fn- or Vn-immunogold particles (Supplementary Fig. 13a, b); immunogold Fn- and Vn-specific particles were observed in proximity to the hair-like extensions of C. difficile R20291 spores (Fig. 5a, b), suggesting that these structures might be implicated in spore entry into IECs. Most epidemiologically relevant strains encode two collagen-like exosporium proteins, BclA2 and BclA3\(^{13}\). During the sporulation of R20291 strain, bclA3 expression levels are ~60-fold higher than those of bclA2\(^{28}\). Consequently, we first hypothesized whether BclA3 was responsible for the formation of the hair-like extensions. Therefore, we constructed a single bclA3 mutant strain, in an epidemic R20291 background, by removing the entire gene through a pyrE-based allelic exchange system\(^{29}\) (Supplementary Fig. 14). Electron micrographs demonstrate that, as expected, wild-type R20291 (Apr/E/pyrE\(^{+}\)) spores exhibited typical hair-like projections observed in previous reports\(^{4,11,12}\) (Fig. 5c). By contrast, the ΔbclA3 deletion mutant formed spores that lacked the hair-like projections (Fig. 5d) that were restored upon complementation of the ΔbclA3 mutant strain with a single wild-type copy of bclA3 in the pyrE locus (ΔbclA3/bclA3\(^{+}\); Supplementary Fig. 5e), indicating that BclA3 is required for the formation of these projections on the surface of C. difficile spores.

BclA3 is required for Fn-α5β1- and Vn-α5β1-mediated spore entry into IECs. To address whether BclA3 exosporium protein is implicated in C. difficile spore entry into IECs, we first assayed whether the absence of BclA3 protein affected the internalization of C. difficile spores into IECs in the presence of Fn or Vn. As a control, we ensured that the anti-C. difficile spore goat serum used to quantify extracellular C. difficile spores, recognized ΔbclA3 mutant spores (Supplementary Fig. 15a–c). Spores of the C. difficile ΔbclA3 mutant strain exhibited a significant decrease
in spore entry into Caco-2 cells, but not adherence to monolayers of Caco-2 cells was observed upon infection with *C. difficile* spores ΔbclA3 mutant in the presence of Fn (Fig. 5f, g) and Vn (Fig. 5h, i). Importantly, the defect in spore entry of the ΔbclA3 mutant strain in the presence of Fn or Vn was restored to wild-type levels of internalization ΔbclA3/bclA3+ strain (Fig. 5f–i), indicating that BclA3 is required for Fn- and Vn-mediated internalization into IECs. We further confirmed these results in monolayers of HeLa cells, evidencing essentially identical results (Supplementary Fig. 16a–d). Next, to address whether Fn-α5β1 and Vn-αvβ1-mediated spore entry is BclA3 specific, we carried out infection experiments with ΔbclA3 mutant spores in monolayers of CHO cells expressing individual integrin subunits. In the presence of Fn, a significant decrease in spore entry (Fig. 5j, k), but not in adherence (Supplementary Fig. 16e, f), was observed upon infection of CHO cells expressing the αv or β1 integrin receptors in the presence of Vn (Fig. 5l, m); however, the absence of BclA3 had no impact on spore adherence to CHO cells in the presence of Vn (Supplementary Fig. 16g, h). Fn- and Vn-mediated internalization of *C. difficile* spores into CHO cells expressing each integrin subunit was restored to wild-type
levels upon infection with spores ΔbclA3/bclA3+ (Fig. 5j–m). These results collectively demonstrate that BclA3-Fn-α5β1 and BclA3-Vn-αvβ1 are two pathways through which C. difficile spores can internalize into non-phagocytic cells.

Inactivation of the exosporium protein BclA3 decreases spore adherence, but not spore entry, of C. difficile spores to the intestinal mucosa. To assess whether the collagen-like BclA3 exosporium protein also contributed to the internalization of C. difficile spores into the intestinal mucosa in vivo, we used a colonic and ileal loop mouse model (Fig. 6a–c and Supplementary Fig. 17a–c). In contrast to our in vitro data, analysis of colonic mucosa sections show that inactivation of bclA3 leads to a significant decrease of ~60% in spore adherence per 10^5 µm² to the ileum mucosa (Fig. 6d); however, no differences were observed in spore internalization relative to the total adhered spores (Fig. 6e).
A similar trend was evidenced in ileal loops, where ΔbclA3 spores adhered in a ~50% lower than wild-type spores per 10^5 µm^2 (Fig. 6f); however, no differences were observed in spore internalization relative to total adhered spores (Fig. 6g). The defects in spore adherence to the colonic and ileum mucosa were restored to wild-type levels upon complementing the wild-type bclA3 allele in the ΔbclA3 mutant (Fig. 6d–g and Supplementary Fig. 17a–c). Strikingly, these data indicate that the collagen-like BclA3 exosporium protein is required for C. difficile spore adherence to the intestinal mucosa and that additional spore-surface proteins or uncharacterized host factors are contributing to spore entry pathways in vivo.
The *C. difficile* collagen-like BclA3 exosporium protein contributes to spore persistence and recurrence of the disease in mice. Since BclA3 is essential for spore entry into IECs in vitro and for adherence in the intestinal mucosa in vivo, we hypothesized that BclA3 might mediate spore persistence and contribute to R-CDI. Therefore, antibiotic-treated mice were infected with wild type spores (ΔpyrE/ΔpyrE+), ΔbclA3, and ΔbclA3/ΔbclA3+ strains (Fig. 6h). All three groups of mice exhibited similar weight loss during the initiation of CDI, and all manifested signs of diarrhea within 3 days post infection (Supplementary Fig. 17d, e). Similar levels of *C. difficile* spores shed in feces were observed during the initiation of CDI between mice infected with wild type spores (ΔpyrE/ΔpyrE+) and the mutants ΔbclA3 and ΔbclA3/ΔbclA3+ (Supplementary Fig. 17f). These results indicate that the absence of BclA3 does not affect the initiation of CDI. Next, the impact of BclA3 in the recurrence of the infection was assessed by treating *C. difficile*-infected mice with vancomycin for 5 days (Fig. 6h), R-CDI was monitored from day 8 post infection. No significant differences in weight loss were evidenced after vancomycin treatment (days 8–11; Fig. 6i). However, a significant delay in the onset of diarrhea during R-CDI was observed after vancomycin treatment in mice infected with ΔbclA3 mutant spores compared to wild-type-infected mice (Fig. 6i). R-CDI defect observed in ΔbclA3 mutant-infected mice was restored to wild-type levels with the agent, nystatin, a caveolin-related pathway inhibitor that disrupts membrane microdomains known to be implicated in integrin-mediated endocytosis and pathogen uptake30,32. Cells were preincubated with nystatin for 1 h at 37 °C and infected in the same medium containing the inhibitor and ΔbclA3 spores. *C. difficile* spore entry was inhibited in a dose-dependent manner into Caco-2 cells and T84 cells (Fig. 7a, b and Supplementary Fig. 18a, b). A total of 30 µM of nystatin inhibited the spore entry by ~80% in human cell lines Caco-2 and ~65% in T84 cells. We determined cell viability in the presence of nystatin by MTT at the highest concentration of the inhibitor. Cell viability was generally ~90% (Supplementary Fig. 18c). These results suggest that *C. difficile* spore entry is sensitive to cholesterol-sequestering compounds.

Vancomycin administration leads to increased fecal concentration of primary bile acids33 leading to enhanced *C. difficile* spore germination34,35, suggesting that luminal taurocholate would trigger germination of extracellular *C. difficile* spores that could subsequently become inactivated by vancomycin. Therefore, we hypothesize that intracellular spores should remain dormant in the presence of taurocholate. To test this hypothesis, monolayers of 1-h nystatin-treated or untreated Caco-2 cells were infected for 3 h with serum-treated *C. difficile* spores. Next, infected monolayers were washed and treated with taurocholate to trigger germination of extracellular spores, followed by ethanol treatment to inactivate germinated *C. difficile* spores. We observed that not all of the spores became ethanol-sensitive upon taurocholate treatment of infected Caco-2 monolayers (Fig. 7c), suggesting that internalized spores were protected from taurocholate-triggered germination. We confirmed this by evidencing a significant increase in ethanol-sensitive germinated spores in the presence of nystatin (Fig. 7c). These results indicate that blocking *C. difficile* spore entry contributes to taurocholate-triggered germination of *C. difficile* spores and subsequent spore inactivation.

**Inhibition of *C. difficile* spore entry into the intestinal barrier reduces recurrence of the disease in mice.** To address whether in vivo *C. difficile* spore entry into the intestinal barrier also required RGD-binding integrins36, colonic and ileal loop assays were assessed in the presence of RGD during *C. difficile* spore infection (Fig. 7d, e and Supplementary Fig 18d, e). Ileal and colonic loops were injected with RGD peptide and *C. difficile* spores for 5 h, then were processed and visualized in confocal microscopy. Consistent with our in vitro data, in the colonic loop sections, we observed that presence of RGD peptide reduced spore internalization by ~82% (Fig. 7g), while no difference in spore adherence was observed (Fig. 7h); similarly, in ileal loop sections, we observed that RGD peptide decreased spore internalization by ~90% (Fig. 7i) and did not affect the spore adherence to the ileum mucosa (Fig. 7i). These results demonstrate that *C. difficile* spore entry in vivo is RGD-binding integrin-dependent.

Since the RGD dependency of spore entry into the intestinal barrier is likely attributed to integrin receptors, we address whether the cholesterol-sequestering drug, nystatin, could block internalization of *C. difficile* spores into the intestinal barrier in vivo in the colonic and ileum mouse mucosa. Mice were treated for 24 h with nystatin or saline as a control before surgery.
and during intestinal loop infection (Fig. 7d, f and Supplementary Fig 18d, f), then were infected with *C. difficile* spores for 5 h, then tissues were processed for confocal microscopy. In the colonic loop section, we observed that nystatin had no effect on spore internalization (Fig. 7g) and in spore adherence to the colonic mucosa (Fig. 7h); however, in the ileal loop sections, the presence of nystatin significantly decreased spore internalization by ~96% (Fig. 7i), and no effect in *C. difficile* spore adherence to the ileum mucosa was observed (Fig. 7j).

Since *C. difficile* spore entry prevents taurocholate germination, contributing to the persistence of *C. difficile* spores during the disease; we hypothesized that administration of the inhibitor of
spore entry, nystatin, during CDI treatment with vancomycin, could reduce the recurrence of the infection in a previously developed mouse model of R-CDI8 (Fig. 7k). To address this question, antibiotic-treated mice were infected with C. difficile R20291 spores. During the first episode of CDI, both groups of mice had similar weight loss, the timing of the onset of diarrhea, and shed similar amounts of C. difficile spores during the initiation of CDI (days 1–3; Supplementary Fig. 18g–i). On day 3 post-infection, animals were treated with vancomycin or a mixture of vancomycin and nystatin for 5 days (Fig. 7k). Vancomycin-treated mice exhibited a significant decrease in weight during R-CDI, which became highest at day 11 post-infection (fourth day after vancomycin treatment; Fig. 7k, m). By contrast, CDI animals treated with vancomycin and nystatin had no significant decrease in weight loss during the recurrence of the infection (Fig. 7k, n). These observations were confirmed upon monitoring the onset of diarrhea during R-CDI (Fig. 7o); we observed a significant delay in the onset of recurrent diarrhea in CDI mice treated with the mixture of vancomycin and nystatin compared to vancomycin alone (Fig. 7o). The animals shed similar amounts of C. difficile spores during R-CDI (Supplementary Fig. 18i). Collectively, these results demonstrate that the administration of a pharmacological inhibitor of internalization of C. difficile spores during vancomycin treatment delays the incidence of recurrence of the infection.

Discussion

During CDI, C. difficile spore formation is essential in the recurrence of the disease7, yet the underlying mechanisms that correlate C. difficile spore persistence and recurrence of the disease remain unclear. In this study, we unraveled a novel and unexpected mechanism employed by C. difficile spores to interact with the intestinal mucosa that contributes to the recurrence of disease. Our results have identified host molecules, cellular receptors, and a spore-surface ligand involved in spore entry into IECs. Notably, intracellular spores remain dormant in the presence of germinant. Using nystatin, a pharmacological inhibitor of spore entry in combination with antibiotic treatment leads to a reduction in the recurrence of the disease in mice. Together, these observations open a new angle for therapeutic interventions of CDI to prevent the recurrence of the disease.

Our results identified host molecules and cellular receptors involved in the entry of C. difficile spores into IECs. The presence of Fn or Vn allows C. difficile spores to gain intracellular access to IECs, in an RGD-specific manner and through specific integrin receptors (i.e., α5β1 and αvβ3). These observations were confirmed by the in vivo inhibition of C. difficile spore entry in the presence of the RGD peptide, which inhibits specifically interactions between Fn-α5β1 and Vn-αvβ3. Although Fn and Vn are mainly located in the basal and basolateral membrane of IECs, contributing to cell polarity16,17, antibody staining of healthy ileum and colonic tissue demonstrates that Fn and Vn are luminal accessible in a significant fraction of the IECs. Most of these cells were positive for luminally accessible Ecad and suggests that these cell types include cell extrusion cells, next to extrusion sites, and epithelial folds that typically undergo adherent junction reorganization22–24. However, a small fraction of cells positive for luminally accessible Fn and Vn were negative for luminally accessible Ecad, suggesting a novel phenotype within cells at the intestinal epithelial barrier. We also confirmed previous observations in mice that identified GCs have luminally accessible Ecad22, suggesting that C. difficile spores might also target these cell types to gain entry into the epithelial barrier. M cells are an additional cell type that might contribute to C. difficile spore entry into the intestinal epithelial barrier includes since they express β1-integrin at the apical surface in contrast to its normal basolateral location in enterocytes22,37,38. The fact that in vivo spore entry was RGD-binding integrin-specific suggests that Fn and Vn are accessible and employed by C. difficile spores to gain entry into IECs, which is consistent with the presence of accessible Fn and Vn in ileal and colonic loops. It is noteworthy that while RGD-specific entry was observed in both ileal and colonic loops, nystatin was only able to reduce spore entry into the ileum, but not colonic mucosa. This suggests that caveolae-independent endocytosis of C. difficile spores might prime in the colonic epithelia. During CDI, C. difficile toxins disrupt adherent junctions, leading to progressive exposure of deep regions of the colonic epithelium as infection advances. One consequence of this cellular disorganization may alter the distribution of cell receptors that may lead to increased adherence and internalization of C. difficile spores into the intestinal mucosa. Together, these observations prompt further studies to address how epithelium remodeling contributes to persistence of C. difficile spores and recurrence of the disease.

Another major contribution of this work is the role of the spore-surface collagen-like BclA3 exosporium protein in C. difficile spore entry into IECs in a Fn-α5β1- and Vn-αvβ3-dependent manner. Our previous work shows that Fn and Vn bind in a dose-dependent manner to C. difficile spores15. By immunogold-electron microscopy, our results demonstrate that Fn and Vn bind to the hair-like projections of C. difficile spores. We also demonstrate that they are formed by the collagen-like exosporium
glycoprotein BclA3. It is noteworthy that experiments with monolayers of Caco-2 cells and CHO cells expressing integrin subunits demonstrate that BclA3 is essential for spore entry in the presence of Fn and Vn in an integrin-dependent manner; results that contrast with BclA3 being essential for adherence to the intestinal mucosa, but not for spore entry into the intestinal barrier. Coupling these results with those of in vivo RGD-specific C. difficile spore entry into the intestinal barrier indicates that additional spore-surface proteins might play redundant roles during in vivo spore entry or some uncharacterized host factors may also contribute. Regardless of these incongruencies, we observed that BclA3 contributes to the recurrence of the disease in a mouse model, suggesting that BclA3-mediated spore adherence to the intestinal mucosa might contribute to spore persistence and recurrence of the disease. The differences in spore adherence to the colonic tissue after R-CDI observed in the medium colonic tissue of mice might relate to the absence of mucosal folds typically observed in the distal and proximal colon of mice. Here, we have shown that BclA3 uses Fn and Vn, and their specific integrins to gain entry into IECs and that BclA3 is essential for C. difficile spore adherence to the intestinal mucosa and contributes to the recurrence of the disease.

The work presented here also shows that C. difficile spore entry into IECs contributes to spore dormancy in the presence of primary bile salts (i.e., taurocholate) and that blocking in vivo spore entry during antibiotic treatment (vancomycin) leads to reduced dormancy in the presence of primary bile salts (i.e., taurocholate) and that blocking in vivo spore entry during antibiotic treatment (vancomycin) leads to reduced dormancy in the presence of pri-

Table 1) were routinely grown at 37 °C under anaerobic conditions in a Bactron III-2 (Shellab, USA). Then plates were removed from the chamber, and colonies were scraped out with ice-cold sterile Milli-Q water. Then the sporulated culture was loaded in 45% weight vol

Bacterial strains and growth conditions. C. difficile strains (see Supplementary Table 1) were routinely grown at 37 °C under anaerobic conditions in a Bactron III-2 anaerobic chamber (Shellab, USA) in BHIS medium: 3.7% weight vol −1 brain heart infusion broth (BD, USA) supplemented with 0.5% weight vol −1 yeast extract (BD, USA) and 0.1% weight vol −1 l-cysteine (Merck, USA) or on BHIS agar plates. Escherichia coli strains were routinely grown aerobically at 37 °C under aerobic conditions with shaking at 1 x g in Luria-Bertani medium (BD, USA), supplemented with 25 µg mL −1 chloramphenicol (Merck, USA), where appropriate.

For minimal medium (C. difficile minimal medium with muramyl dipeptide) media was prepared and uracil-free medium when performing genetic selections. For CDMM broth preparation, 5× amino acids (50 mg mL −1 casamino acids, 2.5 mg mL −1 L-tryptophan, and 2.5 mg mL −1 l-cysteine), 10× salts (50 mg mL −1 Na2HPO4, 50 mg mL −1 NaHCO3, 9 mg mL −1 KH2PO4, and 9 mg mL −1 NaCl), 2× glucose (200 mg mL −1 glucose), 10× traces salts (2.0 mg mL −1 (NH4)2SO4, 1.3 mg mL −1 CaCl2·2H2O, 1.0 mg mL −1 MgCl2·6H2O, 0.5 mg mL −1 MnCl2·4H2O, and 0.05 mg mL −1 CoCl2·6H2O), 100× iron (0.4 mg mL −1 FeSO4·7H2O), and 100× vitamins (0.1 mg mL −1 d-biotin, 0.1 mg mL −1 calcium-pantothenate, and 0.01 mg mL −1 pyridoxine). Cells were plated on DHIS plates supplemented with 25 µg mL −1 (NH4)2SO4, 0.02 mg mL −1 CaCl2·2H2O, 0.01 mg mL −1 MgCl2·6H2O, and 0.001 mg mL −1 CoCl2·6H2O. 0.04 mg mL −1 FeSO4·7H2O, 0.001 mg mL −1 d-biotin, 0.001 mg mL −1 calcium-pantothenate, and 0.001 mg mL −1 pyridoxine for 18,400 × g for 5 min each. The sporulated culture was loaded in 45% weight vol −1 autoclaved Nycodenz (Axell, USA) solution and centrifuged at 18,400 × g for 40 min to separate spores. Spore pellet was washed twice at 18,400 × g for 5 min with ice-cold sterile Milli-Q water. Then the sporulated culture was washed five times with washed Milli-Q water for 40 min each. The resulting pellet was resuspended in 1× PBS and taken up in 24 well plates for 2 days post confluence (undifferentiated) or 8 days post confluence (differentiated), changing the culture medium every other day.

Spor preparation. A total of 100 µL of 1:1000 dilution of an overnight culture of BHIS was plated in 70:30 agar plates that were prepared as follows: 63% weight vol −1 bacto peptone (BD, USA), 0.35% weight vol −1 protease peptone (BD, USA), 0.07% ammonium sulfate (NH4)2SO4 (Merck, USA), 0.10% weight vol −1 Tris base (ONMIPUR, Germany), 1.11% weight vol −1 brain heart infusion extract (BD, USA), and 0.15% weight vol −1 yeast extract (BD, USA), 1.5% weight vol −1 Bacto agar (BD, USA). Plates were incubated for 7 days at 37 °C under anaerobic conditions in anaerobic chamber Bactron III-2 (Shellab, USA). Then plates were removed from the chamber, and colonies were scraped out with ice-cold sterile Milli-Q water. Then the sporulated culture was washed five times with washed Milli-Q water for 40 min each. The sporulated culture was loaded in 45% weight vol −1 autoclaved Nycodenz (Axell, USA) solution and centrifuged at 18,400 × g for 40 min to separate spores. Spore pellet was washed and separated twice at 18,400 × g for 5 min with ice-cold sterile Milli-Q water to remove Nycodenz. Spores were counted in Neubauer chamber, and volume adjusts at 5 × 109 spores mL −1 and stored at −80 °C.

C. difficile mutant construction by allelic exchange. Primer design and amplification of C. difficile R20291 strain were based on the available C. difficile genomes from the EMBL/GenBank databases with accession number FN545816. The oligonucleotides and the plasmids/plasmids used in this study are listed in Supplementary Tables 1 and 2, respectively. In-frame deletions in C. difficile R20291 were made by allelic exchange using pyrE alleles.

To remove the bclA3 gene, a 1086 bp allelic exchange cassette was obtained by overlap extension PCR of the LHA and RHA originated by amplification with primer pairs P332 (FP-LHA-bclA3-pyrE)P334 (RP-LHA-bclA3-pyrE) and P335 (FP-RHA-bclA3-pyrE)P336 (RP-RHA-bclA3-pyrE), each of 544 and 542 bp in size. The resulting cassette yielded complete removal of the entire bclA3 cassette. The above cassette was cloned into SfiI/Axl sites in pPM138, giving plasmid pDP376. To verify the correct construction of the plasmids, all constructs were sequenced.

The plasmids obtained were transformed into E. coli CA434 (RP4) and mated with C. difficile R20291 ΔpyrE. C. difficile transconjugants were selected by subculturing on BHIS agar containing 15 µg/mL thiopropamine (Sigma-Aldrich, USA) and 25 µg/mL cefoxitin (Sigma-Aldrich, USA) and re-streaked five times. The single-crossover mutants identified were streaked onto CDMM with 1.5% weight vol −1 agar supplemented with 2 mg mL −1 5-FOA (USBiological, USA) and 5 µg mL −1 uracil (Sigma-Aldrich, USA) to select for plasmid excision. Confirmation of plasmid excision was made by negative selection in BHIS-thiopropamine plates. The isolated FOA-resistant and thiopropamine-resistant colonies were screened using the primer pair P664 (FP-bclA3-detect)-P665

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differentiated Caco-2, T84, Vero, and HT29 cells were infected at 37 °C with an MOI of 10 with *C. difficile* spores preincubated 1 h at 37 °C with PBS or DMEM as control, as was described above. Were washed gently in PBS before immunostaining, as described above.

Also, undifferentiated Caco-2 cells were infected at an MOI of 10 with *C. difficile* spores preincubated 1 h at 37 °C with PBS, mouse serum (Pacific Immunology, USA), rabbit serum (Pacific Immunology, USA), and NHS (Complement Technology, USA), as was described above. Then were washed gently in PBS before immunostaining, as described above.

To evaluate if *C. difficile* internalization occurs in different strains, undifferentiated Caco-2 cells were infected at an MOI of 10 with *C. difficile* spores R20291, M120, and spores of *C. difficile* clinical isolates PUC25, PUC30, PUC25+, PUC31, PUC26, and PUC 131[18], which were preincubated in CFU mL−1 with PBS, as was described above. Then were washed gently in PBS before immunostaining, as described above.

To assess that the internalization of *C. difficile* spores into IECs is through the specific interaction between Fn or Vn and their cognate integrin receptors, infection experiments were done in the presence of the RGD peptide[39,40]. Brefly, differentiated and undifferentiated Caco-2 cells were incubated with 0, 1, 3, and 5 µg mL−1 of RGD peptide (Abcam, USA) for 1 h, 37 °C then were infected for 3 h at 37 °C with spores preincubated 1 h at 37 °C with NHS, as was described above. Then, samples were washed gently in PBS before immunostaining, as described above.

To evaluate whether the internalization of the spores is mediated by Fn and Vn, differentiated and undifferentiated Caco-2 cells were treated for 1 h 37 °C with 10 µg mL−1 of purified human Fn or human Vn in DMEM, and then were infected with an MOI 10 of untreated *C. difficile* spores R20291. The infection was also performed using untreated Caco-2 cells that were infected for 3 h at 37 °C with an MOI 10 of *C. difficile* spores preincubated for 1 h at 37 °C with 10 µg mL−1 of human Fn or human Vn in DMEM. Then samples were washed in PBS before the immunostaining, as described above.

To confirm that the internalization of *C. difficile* spores is dependent on Fn and Vn, we perform an infection assay in differentiated and undifferentiated Caco-2 cells that were preincubated with 0, 1, 3, and 5 µg mL−1 of RGD peptide (Abcam, USA) for 1 h, 37 °C then were infected with an MOI of 10 with *C. difficile* spores preincubated for 1 h 37 °C with 10 µg mL−1 of purified human Fn or human Vn in DMEM. Then samples were washed in PBS before the immunostaining, as described above.

To identify the integrin subunits implicated in spore entry, an antibody blocking assay was performed using mouse monoclonal antibodies against individual integrin subunits: anti-human integrin αι and αι, (ab78614, ab68621; Abcam, USA), αι, and βι (MAB19502, MAB1999Z, and MAB20232Z; Millipore, USA); and control nonimmune IgG antibody (15066, Sigma-Aldrich, USA). Caco-2 cells were incubated with 200 µL of DMEM with the appropriate antibodies at 5 µg mL−1 for 1 h at 37 °C. The cells were infected for 3 h at 37 °C with spores preincubated for 1 h at 37 °C with 10 µg mL−1 of purified human Fn or human Vn in DMEM. Then samples were washed in PBS prior to the immunostaining, as described above.

In order to demonstrate that *C. difficile* spore entry requires the integrins αι, and αι, then CHO cells with ectopic expression of αι, αι, and βι integrins were infected 3 h at 37 °C with an MOI of 10 of *C. difficile* spores that were preincubated with 10 µg mL−1 of purified human Fn or human Vn in DMEM. Then samples were washed in PBS before the immunostaining, as described above.

To evaluate whether the collagen-like exosporium protein BdaA3 is required for *C. difficile* spore entry into IECs is dependent of differentiated Caco-2 cells and monolayers of HeLa cells, were infected for 3 h at 37 °C with an MOI of 10 with wild type (Aprr/pyre+), BdaA3, and BdaA3/bdaA3* *C. difficile* R20291 spores that were preincubated for 1 h at 37 °C with 10 µg mL−1 of purified human Fn or human Vn in DMEM. Then samples were washed in PBS before the immunostaining, as described above.

In order to demonstrate that *C. difficile* spore entry into the monolayers of IECs is dependent of differentiated Caco-2 cells, spores were preincubated with 0, 1, 3, and 5 µg mL−1 of RGD peptide (Abcam, USA) for 1 h, 37 °C then were infected for 3 h at 37 °C with spores at an MOI 10 preincubated with FBS, as was described above. At the used concentration of nystatin, the cell viability of treated Caco-2 cells and T84 for 4 h was ~90%, as was observed by trypan blue (Invitrogen, USA) and MTT assay (Life Technologies, USA) according to manufacturer protocols.

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**Immunofluorescence of adhered *C. difficile* spores in infected monolayers and epifluorescence analysis.** The aforementioned infected monolayers of cells were subsequently fixed with PBS−4% paraformaldehyde for 10 min. Then they were washed three times and blocked with PBS−1% BSA overnight at 4 °C; in non-processed monolayers, the monolayers were washed with 1:50 anti- *C. difficile* spore goat serum (recognize specifically to *C. difficile* spores in infections of IECs infection assays; PAC 5573 Pacific Immunology, USA) in PBS−1% BSA 1 h room temperature (RT) and 1:400 anti-goat conjugated with CFL 488 secondary antibody (green; SC362255, Santa Cruz Biotechsologies, USA) in PBS−1% BSA for 1 h before being washed three times with PBS and covered with Dako Fluorescence Mounting Medium (Dako, Denmark) and
sealed with nail polish. Samples were observed on an Olympus BX53 fluorescence microscope with UPLFLN 100x oil objective (numerical aperture 1.30). Images were captured with the microscope camera for fluorescence imaging (Imaging Bte Retiga and pictures were analyzed with ImageJ (NIH, USA). Extracellular spores or adhered were considered as spores in phase contrast that were marked in fluorescence. Internalized spores were considered as spores visible in phase contrast, but that does not have fluorescence. A total of ~300 spores were analyzed per experimental condition.

Mice used. A 6–8 weeks C57BL/6 (male or female) were obtained from the breeding colony at the Biological Science Department of Universidad Andrés Bello derived from Jackson Laboratories. Mice were housed with ad libitum access to food and water. Bedding and cages were autoclaved, and mice had a 12-h cycle of light and darkness. Mice were housed at 20–24°C with 40–60% of humidity. All procedures complied with all relevant ethical regulations for animal testing and research and were approved by the Institutional Animal Care and Use Committee of the Universidad Andrés Bello.

Colonic and ileal loop assay. C57BL/6 mice were anesthetized in an isoflurane chamber (RWD, USA) with 4% vol–vol isoflurane (Baxter, USA) and were maintained with 2% vol–vol during the surgery administered by air. A midline laparotomy was performed, making 1-cm incision in the abdomen, 1.5 cm ileal, and proximal colon (at 1.0–1.5 cm from the cecum as a reference) were ligated with silk surgical suture8. No postoperative treatment was used. To evaluate the kinetics of nystatin uptake and absorption of NFON in C. difficile spore internalization in vivo, 24 h prior to surgery, mice (n = 4) were treated with nystatin (17,000 IU kg–1) in 100 µL of DPBS by oral gavage; control mice (n = 8; 4 for control and 4 for RGD treatment) where treated with 100 µL of DPBS by oral gavage. On the day of surgery, ileal and colonic ligated loops of control mice (n = 4) were treated with DPBS containing RGD (20 µM) and colonic and ileal ligated loops of nystatin-treated mice (n = 4) were injected with 100 µL of DPBS containing 3 × 108 C. difficile R20291 spores and 340 µL (17,000 IU kg–1) nystatin; ileal and colonic ligated loops of RGD-treated mice (n = 4) were injected with 100 µL of DPBS containing 3 × 108 C. difficile R20291 spores and 86.6 µg (250 nM) of RGD peptide. Peptide was up to resuspend nystatin and RGD peptide by rendered higher solubility than saline solution (0.9% weight vol–1 NaCl). To evaluate the role of BclA3 protein in C. difficile spore internalization, the ligated loops were injected with 100 µL of 0.9% weight vol–1 NaCl containing 5 × 108 wild-type spore (AtypEp/rpe+) (at 4°C overnight. After mucus fixation with cross-linking agents, such as paraformaldehyde, cause mucus layer of colon to collapse and rendering the intestinal barrier or to evaluate spore adherence and internalization in mice treated with RGD and nystatin, confocal images were acquired in Leica SP8 was used with HPL APO CS2 40× oil, numerical aperture 1.3. For signals detection, three (PMT) spectral detectors were used; PMT1 (410–483) DAPI, PMT2 (510–550) Alexa-Fluor 568, and PMT3 (587–635) Alexa-Fluor 555. Emitted fluorescence was split with four dichroic mirrors (QD 405, 488, 561, and 635 nm). Images (1024 × 1024 pixels). To observe the sites with accessible Fn and Vn in the intestinal barrier, and to evaluate the adherence and internalization of the ΔbclA3 spore mutant to the intestinal barrier or to evaluate spore adherence and internalization in mice treated with RGD and nystatin, confocal images were acquired in Leica SP8 was used with HPL APO CS2 40× oil, numerical aperture 1.3. For signals detection, three (3D) reconstructions of intestinal epithelium were performed using ImageJ software (NIH, USA). Villi and crypts were visualized by Hoechst and phalloidin signals. 3D reconstruction movies were performed with software Leica Application Suite X (Leica, Germany).

To quantify cells of the colonic and ileal mucosa with accessible proteins undetected, confocal images with a 1-µm Z step size were filtered with Gaussian Blur 3D (sigma: 0.6; r: 0.6; z: 0.6) and quantify with cell counting plug-in of ImageJ (1000–1200 cells were counted in an area 84,628 µm2 per mice. To quantify spore adherence and internalization, confocal images with a 0.7-µm Z step size were analyzed. Adhered spores were considered within the intestinal epithelium or to the closest crypt membrane. For ileum mucosa, we measure the perpendicular distance from the center of the spore to the villus membrane.

Visualization of spore internalization in intestinal epithelial cells in vitro by confocal microscopy. Differentiated Caco-2 cells and T84 cells cultured onto Transwell (Corning, USA) until 1000–2000 µL. Cells were infected for 5 h with an MOI of 10 with C. difficile spores previously stained with Alexa-Fluor 488 Protein Labeling Kit (Molecular Probes, USA), according to the manufacturer’s instruction. Cells were washed twice with PBS and were permeabilized with PBS–0.06% Triton X-100 (Merck, USA) for 10 min at RT, were washed and incubated with 1:150 phalloidin Alexa-Fluor 568 (12 µg/mL) for 30 min at RT. Finally, tissues were incubated with 1:100 phallolidin Alexa Fluor 647 (A22287, Invitrogen, USA) for 90 min at RT. The aforementioned immune-stained tissues were subsequently mounted with the luminal side-up. Thus, the colonic crypts and the intestinal villi were oriented under light microscopy with x10 or x40 magnification and were oriented side-up toward the coverslip. The tissue segment was placed over 5 µL of Dako fluorescent mounting medium (Dako, Denmark) applied onto a glass slide. The tissue covered with 15 µL Dako fluorescent mounting medium and closed with a coverslip. Coverslips were affixed to the glass slide with vinyl tape to hold the tissue sections in place and were allowed to cure for at least 24 h before imaging.
R20291 spores preincubated 1 h at 37 °C with 100 µL of NHS (Complement, Technology USA) for each well, and then was suspended in the infection volume of 1 mL. 10% final vol vol−1 PBS. Untreated spores rinsed off, and cells were washed, mixed with 500 µL of absolute ethanol (Merck, USA) for 60 min at RT. Then, serial dilutions of the sample were plated onto selective medium supplemented with 0.1% weight vol−1 taurocholate, 16 mg µL−1 cefoxitin, 250 µg mL−1 cycloheximide, and 1.5% weight vol−1 agar (BD, USA) (TCCFA plates). The plates were incubated anaerobically at 37 °C for 48 h. The colony count was expressed as the log_{10} CFU g−1 of the tissue.

Cecum content cytotoxicity assay in Vero cells infected mice during R-CDI. A 96-well flat-bottom microtiter plates were seeded with Vero cells at a density of 10^5 cells well−1. Mice cecum contents were suspended in PBS at a ratio of 1:10 (100 mg mL−1 of cecum content), vortexed, and centrifuged at 18,400 × g for 5 min, the supernatant was sterilized with a 0.22 µm filter and serially diluted in DMEM supplemented with 10% vol vol−1 FBS and 100 U mL−1 penicillin, and 100 µg mL−1 streptomycin; then 100 µL of each dilution was added to wells containing Vero cells. Plates were screened for cell rounding 16 h after incubation at 37 °C. The cytotoxic titer was defined as the reciprocal of the highest dilution that produced rounding in at least 80% of Vero cells per gram of luminal samples under ×20 magnification.

Statistical analysis. Prism 7 (GraphPad Software, Inc.) was used for statistical analysis. Student’s t test and the nonparametric test was used for pairwise comparison. Significance between groups was done by Mann–Whitney unpaired t test. Comparative study between groups for in vitro experiments was analyzed by analysis of variance with post hoc Student’s t tests with Bonferroni corrections for multiple comparisons, as appropriate. A P value of ≤0.05 was accepted as the level of statistical significance. Differences in the percentages of mice with normal stools, as well as percentages of mice with CDI, were determined by Gehan–Breslow–Wilcoxon test.

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Author contributions
Study concept and design: P.C.-C., M.P.-G., and D.P.-S. Acquisition of data: P.C.-C., P.M.-U., G.C.-A., and D.P.-S. Drafting of the manuscript: D.P.-S. Critical revision of the manuscript for important intellectual content: P.C.-C. and D.P.-S. Statistical analysis: P.C.-C., M.P.-G., and D.P.-S. Obtained funding: D.P.-S. Technical or material support: D.P.-S., N.M., and S.K. Study supervision: D.P.-S.

Competing interests
P.C.-C. and D.P.-S. are inventors on the PCT WO2020035720 (A1) patent relating to a method and pharmacological composition for the prevention of recurrent infections caused by *Clostridoides difficile*, submitted by Universidad Andrés Bello. The other authors declare no competing interests.
Additional information
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