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***Clostridium difficile* colitis: pathogenesis and host defence**

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Abstract

Clostridium difficile is a major cause of intestinal infection and diarrhoea in individuals following antibiotic treatment. Recent studies have begun to elucidate the mechanisms that induce spore formation and germination and have determined the roles of *C. difficile* toxins in disease pathogenesis. Exciting progress has also been made in defining the role of the microbiome, specific commensal bacterial species and host immunity in defence against infection with *C. difficile*. This Review will summarize the recent discoveries and developments in our understanding of *C. difficile* infection and pathogenesis.

Clostridium difficile-induced colitis is the most common and costly healthcare-associated infection with an estimate of nearly half a million cases and approximately 29,000 deaths occurring annually in the United States¹. Disease associated with *C. difficile* infection ranges from mild diarrhoea to pseudomembranous colitis, which was first shown to be caused by *C. difficile* 40 years ago². A pristine intestinal microbiota provides resistance against *C. difficile* infection and disruption of the microbiota (for example, through antibiotic treatment), allows the bacterium to proliferate in the gut. *C. difficile* is a Gram-positive, spore-forming, obligate anaerobic bacterium. The formation of spores enables *C. difficile* to survive in oxic conditions, which contributes to transmission in healthcare settings and maybe also in the community (BOX 1). Once inside the gastrointestinal tract, pathogenesis is tightly linked to spore germination and the production of toxins. In this Review, we highlight factors that regulate the spore-forming life cycle of *C. difficile*, virulence and mechanisms that are mediated by the host and the microbiota that contribute to protection from disease. A more comprehensive understanding of *C. difficile* pathogenesis is emerging that may lead to new and innovative therapeutic and diagnostic options, which are urgently required to treat infection with this bacterium (BOX 2).

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***C. difficile* sporulation**

The lumen of the human colon is anoxic, which enables obligate anaerobic bacteria such as *C. difficile* to survive and, if the conditions are suitable, to proliferate, produce toxins and damage the intestinal epithelium. *C. difficile* forms spores that are resistant to heat, oxygen and common disinfectants, such as ethanol-based hand sanitizers, which facilitates spread³⁻⁵. Efforts to determine the mechanism of *C. difficile* sporulation benefit from more than 50 years of work on sporulation in *Bacillus subtilis* and, mechanistically, much is conserved between these two members of the Firmicutes phylum⁶.

Formation of the forespore

In culture, sporulation occurs at stationary phase when nutrients become limiting. At one pole of a *C. difficile* (or a *B. subtilis*) cell a septum is constructed that results in asymmetric division and the creation of two unequally sized compartments. The smaller compartment — the forespore — will develop into the spore, whereas the larger compartment — the mother cell — will prepare the forespore for dormancy. The forespore matures into a desiccated, stress-resistant chromosome-storage vessel, which is released into the environment through lysis of the mother cell. Spores can germinate and produce new vegetative cells when conditions become favourable again (FIG. 1). The transcription factors that are responsible for sporulation seem to be well conserved, which enables sporulation in *B. subtilis* to act as a template for understanding the regulation of sporulation in *C. difficile*⁶. The development of increasingly sophisticated tools for reverse genetics^{7,8} and forward genetics⁹, combined with transcriptional profiling, has enabled the identification of hundreds of genes that are involved in sporulation in *C. difficile*¹⁰.

Transcriptional regulation of sporulation

Although the exact environmental signals that trigger sporulation in *C. difficile* have remained elusive, the molecular circuitry that results in the formation of spores is now well described. In *C. difficile*, sporulation is initiated by signalling through sensor histidine kinases that ultimately results in the phosphorylation and activation of the transcription factor stage 0 sporulation protein A (Spo0A)¹¹. Five such kinases have been identified in *C. difficile* strain CD630 (REF. 12) and one kinase (CD1579) directly phosphorylates Spo0A *in vitro*¹³. Phosphorylated Spo0A drives the sporulation regulatory network and directly regulates dozens of genes^{14,15}. Spo0A is essential for *C. difficile* sporulation and mutants that lack this gene only exist as vegetative cells. The inability of Spo0A mutants to form spores profoundly decreases the spread of the organism from infected mice to susceptible, uninfected mice¹⁶. Spo0A induces the expression of the first sporulation-specific sigma factor, σ^H , which forms a positive feed-forward loop with Spo0A¹⁷.

Expression levels of Spo0A are also controlled by two transcription factors, CodY and CcpA, which may integrate nutritional state into the decision to form spores. CodY is mainly a transcriptional repressor that binds to DNA in the presence of GTP and branched-chain amino acids and represses the transcription of dozens of operons in *C. difficile*¹⁸, including the toxin locus¹⁹ and two regulators of sporulation²⁰. CcpA, a global regulator of carbon catabolite repression, directly represses *spo0A*²¹ and leads to decreased toxin

production when *C. difficile* is cultured in the presence of glucose²². Finally, deletion of the oligopeptide permeases *opp* and *app* increases the expression of Spo0A, spore production and virulence in a hamster model²³. Deletion of *opp* and *app* may decrease the ability of *C. difficile* to harvest nutrients from peptides in the local environment, which leads to the starvation of cells and accelerates the transition to stationary phase. Taken together, these data suggest that *C. difficile* represses sporulation in the presence of nutrients.

Downstream of Spo0A is a transcriptional programme that is driven by the sequential activation of four compartment-specific alternative sigma factors — σ F, σ E, σ G and σ K. In *B. subtilis*, σ F is activated in the forespore following asymmetric division²⁴. Inter-compartmental signalling across the division septum then activates σ E in the mother cell, followed by σ G in the forespore and finally σ K in the mother cell²⁴. The activation of these four sporulation sigma factors in *C. difficile* differs from that described for *B. subtilis*^{10,25} (FIG. 1). Specifically, the checkpoints that ensure the sequential, inter-compartmental progression of sigma factor activation in *B. subtilis* seem to be missing from late-stage sporulation in *C. difficile*. In both systems, σ F is activated only in the forespore, which results in the production of the SpoIIR signalling protein and the subsequent activation of σ E in the mother cell^{10,26}. Individual deletion of sigma factors, complemented by morphological characterization of *C. difficile* sporangia, demonstrated that σ F induces the activation of σ G in the forespore, whereas σ E promotes the activity of σ K in the mother cell. However, in contrast to *B. subtilis*, the activity of σ G does not depend on σ E and σ K is independent of σ G, which suggests that inter-compartmental signalling for the activation of sigma factors is not conserved (FIG. 1). In addition, the SpoIIIA–SpoIIQ ‘feeding tube’ channel, which is necessary for the activation of σ G in *B. subtilis*, is dispensable for the activation of σ G in *C. difficile*^{27,28}. A recent study reported a transposon mutant library in *C. difficile* that identified 404 candidate genes that are involved in sporulation, including known sporulation-associated genes, such as *spo0A*, but also genes such as *splA* the contribution of which to sporulation is unknown⁹.

The spore envelope

The spore core is encased in three protective layers: the peptidoglycan cortex, a coat that predominantly consists of proteins, and a third and outermost layer, the exosporium, which predominantly consists of glycoproteins²⁹. Only 25% of the more than 70 proteins that comprise the spore coat are conserved between *B. subtilis* and *C. difficile*³⁰, which suggests that the spore surface may be an important source of evolutionary adaptation among members of the Firmicutes⁶. Deletion of the major spore coat morphogenetic protein SpoIVA in *C. difficile* caused the coat to fail to localize to the spore surface³¹. Screening for envelope proteins from extracts of uncoated spores has uncovered a high degree of enzymatic activity among *C. difficile* spore coat proteins including a catalase with superoxide dismutase activity³², and CdeC, a cysteine-rich protein that localizes to the exosporium and enhances resistance to heat, lysozyme and ethanol³³. The *C. difficile* exosporium contains three collagen-like glycoproteins, BclA1, BclA2 and BclA3, which are conserved with the exosporium of *Bacillus anthracis*. Deletion of *bclA1* results in a slight decrease in virulence, but an increase in both spore germination and adherence, which suggests that changes to the structure of the spore envelope may affect disease^{34,35}.

***C. difficile* germination and vegetative growth**

Germination of *C. difficile* spores and the growth of vegetative forms occurs only in the lower gastrointestinal tract, in part, because oxygen concentration at this site is negligible³⁶. Substances that are present in the intestine, most notably bile acids, induce germination of the spore into an actively replicating vegetative cell, a process that is controlled by the *cspBAC* gene locus.

The role of bile acids in spore germination

An early study demonstrated that the addition of taurocholate, a conjugated primary bile acid that is present in the small intestine, to culture media greatly increased the growth of colonies from clostridial spores that were isolated from human, calf and rat faeces³⁷. Subsequent studies demonstrated that the addition of taurocholate to cycloserine cefoxitin fructose agar (CCFA) media, which is used to culture *C. difficile*, increased the recovery of colonies from spores³⁸. Importantly, bile acids differ in their ability to promote germination and to influence vegetative growth³⁹. Thus, whereas taurocholate together with the amino acid glycine act as co-germinants of spores without affecting the vegetative growth of *C. difficile*, the secondary bile acid deoxycholate promotes germination but markedly suppresses vegetative growth^{39,40}. Interestingly, chenodeoxycholate, another primary bile acid, inhibits spore germination in *C. difficile* and acts both as a competitive inhibitor of taurocholate and a suppressor of vegetative growth in liquid culture^{41,42}. The potential role of the intestinal bile acid pool in determining *C. difficile* colonization following exposure to spores was supported by *ex vivo* studies of intestinal extracts from antibiotic-treated and untreated mice, which revealed a correlation between the capacity to support the growth of *C. difficile* and decreased levels of secondary bile acids^{43,44}. These observations led to the hypothesis that the production of secondary bile acids by the commensal microbiota, and their ablation by antibiotic treatment, modulates susceptibility to *C. difficile* colitis (BOX 3).

Degradation of the spore cortex

Bile acid-induced germination leads to cortex degradation, the release of calcium and dipicolinic acid and rehydration of the spore, which are important early steps in the germination process^{45,46} (FIG. 1). The receptor for bile acids on *C. difficile* spores, CspC, was recently identified by screening ethyl methanesulfonate mutagenized bacteria⁴⁷. CspC is encoded by the *cspBAC* locus and is similar in sequence to subtilisin-family proteases in *Clostridium perfringens* that have been associated with the activation of SleC, a lytic enzyme that is essential for spore germination⁴⁷, but lacks the catalytic triad that is required for proteolytic activity⁴⁷. Deletion of CspC renders *C. difficile* spores unresponsive to taurocholate. Notably, a single amino acid substitution of glycine to arginine at residue 457 of CspC alters the germination-inhibitory effect of chenodeoxycholate to a germination-stimulatory effect, markedly supporting the notion that CspC directly associates with primary bile acids⁴⁷. Also encoded by the *cspBAC* locus is a hybrid protein, CspB-CspA, which consists of the subtilisin-family proteins CspB and CspA. Biochemical studies have demonstrated that the CspA pseudoprotease domain regulates the level of the CspC receptor in mature spores⁴⁸. The YabG protease functions to cleave both the CspBA fusion protein and full-length SleC during sporulation, releasing CspB and pro-SleC, which become

components of the spore coat^{48,49}. Only CspB contains an enzymatically active catalytic triad; however, structural analysis of CspB from *C. perfringens* revealed that its prodomain, even after autocleavage, remains tightly bound, thereby occluding the active site^{47,49}. CspB is required to complete the proteolytic activation of pro-SleC into mature and active SleC, which degrades the dense proteoglycan cortex during spore germination^{48,49,50} (FIG. 1). It remains unclear how the association of taurocholate with CspC activates CspB and subsequently SleC, and it is possible that additional factors are required for the activation of germination. Indeed, recent studies have demonstrated that the lipoprotein GerS is essential for the full enzymatic activity of SleC following CspB-mediated cleavage of pro-SleC²⁷. Once initiated, germination of *C. difficile* spores is a complex process that, as determined by microarray analyses, involves the upregulation or downregulation of more than 500 genes⁵¹. The efficiency of germination varies between clinical strains of *C. difficile*, and whether this correlates with virulence remains an active area of investigation^{52,53}.

Virulence factors

The ability of *C. difficile* to cause colitis depends on a range of virulence factors, including toxins, which are encoded in the pathogenicity locus⁵⁴, and adherence and motility factors. In response to limited nutrient availability, *C. difficile* produces toxins that primarily target intestinal epithelial cells. Following toxin endocytosis and activation in the cytosol, epithelial cells undergo necrosis, which leads to loss of intestinal membrane integrity, host exposure to intestinal microorganisms and activation of the host inflammatory response.

TcdA and TcdB toxins

The pathogenicity locus generally encodes five proteins and, in most strains, is localized at a specific site in the *C. difficile* chromosome⁵⁵, although a recent study characterized unusual strains in which the pathogenicity locus was localized in atypical regions⁵⁶. The major toxins that are encoded by the pathogenicity locus are TcdA (also known as ToxA) and TcdB (also known as ToxB), which are two large secreted proteins that contain four structurally homologous domains⁵⁷. TcdA and TcdB contain RHO and RAC glucosyl transferase domains (GTDs) at the amino terminus and mediate toxicity by glycosylating and thereby inactivating host RHO and RAC GTPases in the cytosol of targeted cells (FIG. 2a). This disrupts the cytoskeleton and leads to the disassociation of tight junctions between colonic epithelial cells and the loss of epithelial integrity⁵⁵.

Adjacent to the GTD, TcdA and TcdB contain a cysteine protease domain (CPD) that autocatalytically cleaves the glucosyl transferase in the cytosol of eukaryotic cells on association with inositol hexakisphosphate (myo-inositol)^{58,59}. More efficient autoprocessing by the cysteine protease has been associated with increased virulence in *C. difficile* strains⁶⁰. A potent small-molecule inhibitor of the toxin cysteine protease was recently described that blocked the release of the glucosyl transferase and abrogated toxicity⁶¹. Administration of this drug reduced intestinal epithelial damage in mice that were infected with *C. difficile* and offers a potential therapeutic avenue to limit toxin-mediated damage.

The other two domains of TcdA and TcdB consist of a hydrophobic protein sequence that is involved in host membrane insertion and the combined repetitive oligopeptide repeat (CROP) domains that are hypothesized to bind to cell surface receptors before TcdA and TcdB endocytosis and internalization. The CROP domain of TcdA contains up to 38 repeats⁶² with a structure that enables the binding of carbohydrates⁶³ and associates with gp96 on the apical surface of colonocytes⁶⁴. Conversely, the CROP domain of TcdB has fewer repeats and associates with the N-terminal, extracellular domain of chondroitin sulfate proteoglycan 4, although the expression of this receptor in intestinal epithelial cells has not yet been reported⁶⁵. TcdB has also been shown to bind to poliovirus receptor-like 3 (PVRL3), which is expressed on the surface of colonic epithelial cells, although the association of TcdB with PVRL3 does not involve the CROP domain⁶⁶.

The relative contribution of TcdA and TcdB to *in vivo* pathogenesis has been investigated by deletion mutagenesis and infection of antibiotic-treated hamsters^{67–69}. The combined deletion of TcdB and TcdA completely abolished *in vivo* virulence^{68,69}. Single deletion of TcdA did not alter *C. difficile* virulence in the hamster model, which indicates that TcdB alone is capable of mediating colitis. The potential role of TcdA is more controversial, with one report suggesting that *C. difficile* mutants that lack TcdB but express TcdA do not cause colitis⁶⁷, whereas two other reports showed TcdA-mediated disease^{68,69}. These inconsistent findings probably reflect differences in *C. difficile* strains, experimental animals (mice versus hamsters) and their microbiota composition and antibiotic sensitivity. Indeed, a carefully carried out, multi-laboratory follow-up study demonstrated that TcdB is the major virulence factor that mediates colonic epithelial damage, inflammation and mortality in the murine model, whereas TcdA is a relatively minor driver of inflammation in mice and is slightly more toxic in hamsters⁵⁴.

Remarkable sequence diversity in genes that encode TcdB exists among different *C. difficile* strains and it has been hypothesized that sequence differences in toxins may contribute to heterogeneity in virulence between strains^{12,70}. Studies with purified TcdB from the epidemic BI/NAP1/027 strain of *C. difficile* demonstrated 4-fold-greater toxicity in mice compared with TcdB from typical *C. difficile* strains⁷¹. It remains unresolved whether environmental factors promote toxin diversification and whether the host environment selects for increased or decreased virulence.

TcdR, TcdC and TcdE

The pathogenicity locus also encodes three other proteins, TcdR, TcdC and TcdE. TcdR is an alternative sigma factor that facilitates the binding of RNA polymerase to the promoters of the *tcdA* and *tcdB* genes and also, in a positive feedback loop, its own promoter⁷². On reaching stationary growth, the transcription of *tcdA* and *tcdB* is driven by TcdR in *C. difficile*⁷². During exponential growth, *C. difficile* expresses higher levels of TcdC, which has been hypothesized to act as an anti-sigma factor and thus suppresses the transcription of *tcdA* and *tcdB*⁷³. In contrast to typical anti-sigma factors, which associate with sigma factors to inhibit transcription, TcdC may directly associate with single-stranded DNA to inhibit *tcdA* and *tcdB* transcription⁷⁴. Hypervirulence has been associated with deletions in the *tcdC* sequence, which supports the potential role of TcdC in limiting toxin expression by *C.*

*difficile*⁷⁵. However, neither genetically engineered *tcdC* mutant strains nor restoration of *tcdC* expression in a hypervirulent 027 ribotype strain altered levels of toxin production when grown in culture media^{8,76}. These more recent studies cast doubt on the exact role of TcdC in negatively regulating the expression of *tcdA* and *tcdB*.

The fifth gene in the pathogenicity locus is *tcdE*, which encodes a holin-like protein that is believed to facilitate the secretion of TcdA and TcdB, which lack conventional secretion signal sequences. As with other proteins in *C. difficile*, controversy regarding the necessity of TcdE for toxin secretion exists, with one publication demonstrating its requirement for secretion⁷⁷ and another showing toxin secretion in its absence⁷⁸. More recent work suggests that TcdE is involved in the secretion of TcdA and TcdB in strains of *C. difficile* that secrete high amounts of toxin⁷⁹.

Binary toxin (CDT)

Some strains of *C. difficile*, in particular the hypervirulent BI/NAP1/027 strain, also express another toxin, referred to as binary toxin or *C. difficile* transferase (CDT), which may enhance virulence and is not encoded in the pathogenicity locus⁸⁰. The role of CDT in virulence remains unproven, although there is an association between its presence and higher mortality in patients⁸⁰. CDT is composed of two proteins, CdtA, an ADP-ribosyl transferase that ribosylates actin in eukaryotic cells, and CdtB, which forms pores in acidified endosomes and facilitates the transfer of CdtA to the cytosol. The cellular receptor for CDT is the lipolysis-stimulated lipoprotein receptor (LSR)⁸¹. Ribosylation interferes with polymerization of the actin meshwork that underlies the cell membrane, which results in cellular protrusions that are formed by microtubules and enhanced fibronectin delivery to the cell surface, thereby enhancing *C. difficile* adhesion to targeted cells⁸² (FIG. 2b).

Non-toxin virulence factors

The regulation of genes that control motility and adherence is an important factor that contributes to colonization efficiency and the virulence of *C. difficile*^{83,84}. Flagellar expression is highly variable among *C. difficile* strains⁸⁵ and lack of flagella has been linked to impaired adherence to the intestinal epithelium^{86,87}. Interestingly, mutant strains that lack components of the flagellar machinery exhibit dysregulated toxin expression and corresponding altered virulence *in vivo*, which suggests a link between flagellar expression and toxin regulation^{87,88}. In *C. difficile*, flagellum expression is regulated by the intracellular second messenger cyclic dimeric guanosine monophosphate (c-di-GMP)^{89,90}. c-di-GMP, which is synthesized from GTP by diguanylate cyclase, acts as a specific ligand and binds to a riboswitch upstream of the *flgB* operon, which is crucial in early flagellum formation, and terminates the transcription of *flgB*⁹⁰. High levels of intracellular c-di-GMP repress flagellum expression and thereby motility⁹¹, and also repress the synthesis of TcdA and TcdB⁹². Concurrently, c-di-GMP activates another riboswitch that induces the expression of type IV pili that interact with the intestinal epithelium and contribute to *C. difficile* aggregation and biofilm formation^{93,94}. In this manner, c-di-GMP acts as a key signal that can switch *C. difficile* between a highly motile, toxin-producing state and a strongly adherent biofilm-producing state. In addition, the adhesin fibronectin binding protein A, cell wall proteins such as Cwp66, S-layer protein A and its modifying protease

Cwp84, and even Spo0A (the master regulator for sporulation), all contribute to *C. difficile* adherence and have a role in biofilm formation^{95–97}. The extracellular matrix of the biofilm is composed of proteins, polysaccharides and free DNA from dead cells, which insulate vegetative cells from oxidative stress, antibodies and antibiotics, creating a protected niche for sporulation⁹⁸.

Microbiota-mediated resistance to *C. difficile*

Antibiotic treatment is the main risk factor for the development of *C. difficile* colitis through the disruption of colonization resistance. Restoration of the microbiota, for example, through faecal microbiota transplant, re-establishes resistance mechanisms that inhibit the growth of *C. difficile*.

The role of the microbiota in infection with *C. difficile*

Antibiotic treatment that precedes *C. difficile* infection substantially alters the intestinal metabolome, creating a more hospitable environment for the growth of *C. difficile*⁹⁹. In the colon, sialidase-producing commensal bacteria cleave sugars from glycosylated proteins that are bound to the epithelial cell membrane, which releases free sialic acid into the lumen¹⁰⁰. Primary fermenters break down complex carbohydrates into short-chain fatty acids¹⁰¹. Both of these metabolites are rapidly consumed as energy sources by commensal bacteria. However, antibiotic treatment can deplete competing commensal bacteria, which leads to an abundance of sialic acid and succinate, a short-chain fatty acid that is produced during fermentation. *C. difficile* has genes for both sialic acid catabolism and succinate transporters, which enables it to use the excess sialic acid and succinate for growth^{102,103} (FIG. 3). In addition to bacteria-derived metabolites, direct interactions between bacteria can limit the expansion of *C. difficile*. A limited number of bacteriocins have been identified that exhibit antimicrobial activity against Gram-positive pathogens such as *C. difficile*^{104,105}. These bacteriocins can target *C. difficile* while causing minimal disruption to intestinal microbial communities and could be used therapeutically as an alternative, or in addition, to standard antibiotic treatment.

A recent study characterized the composition of the microbiota of a set of antibiotic-treated mice that exhibited a range of susceptibilities to *C. difficile* colitis and used mathematical modelling to identify commensal bacterial species that were significantly associated with resistance to the development of infection¹⁰⁶. The same approach was also used to characterize intestinal members of the microbiota that were associated with resistance to the development of *C. difficile* colitis in patients who were undergoing haematopoietic stem cell transplantation^{106,107}. Combining the human and murine datasets identified the commensal species *Clostridium scindens* as most highly associated with resistance to *C. difficile* infection¹⁰⁶. *C. scindens* has a bile acid inducible (*bai*) operon that encodes dehydroxylating enzymes that are necessary to convert primary bile acids into secondary bile acids¹⁰⁸. The inhibitory effect of *C. scindens* on *C. difficile* was negated through the addition of the bile acid sequestrant cholestyramine to cultures, which indicated that secondary bile acids were probably mediators of *C. difficile* growth inhibition (FIG. 3). Bile acids are probably not the only metabolites present in the intestinal lumen that inhibit the expansion of *C. difficile*.

Through both direct and indirect mechanisms intestinal commensal microbial communities have a central role in determining whether *C. difficile* successfully colonizes the large intestine.

Faecal microbial transplants

Resolution of antibiotic-associated diarrhoea through the transfer of normal faecal microbiota to patients was demonstrated even before *C. difficile* was implicated as its cause¹⁰⁹. Anecdotal case reports and small uncontrolled trials that were conducted after the failure of antibiotic therapy demonstrated the effectiveness of faecal microbial transplants as a treatment for recurrent *C. difficile* colitis¹¹⁰. Scepticism among many clinicians limited the adoption of faecal microbial transplants as a routine treatment until a randomized clinical trial of faecal microbial transplant versus conventional antibiotic treatment clearly demonstrated the superiority of faecal microbial transplants to resolve recurrent episodes of disease associated with *C. difficile* infection¹¹¹.

Currently, faecal microbial transplant is only available for patients who experience recurrent *C. difficile* infection. Efficacy of treating primary *C. difficile* infection or patients who are actively receiving antibiotics with a faecal microbial transplant has not been carefully studied. Furthermore, an important concern about faecal microbial transplants is that the complete composition of the faeces cannot be determined. Thus, uncharacterized viruses and bacterial species may be transferred and the consequences are unpredictable to some extent, particularly in patients who have compromised immune systems. Admittedly, the short-term complications of faecal microbial transplants have been minimal, especially when balanced with its remarkable effectiveness. Nevertheless, several studies have demonstrated that consortia of a small number of commensal bacteria can resolve symptoms and infection in patients with *C. difficile* colitis. The first of these studies¹¹² demonstrated that administration of a mixture of 10 commensal bacterial species cured patients with recurrent *C. difficile* infection. For reasons that remain unclear, bacteriotherapy for *C. difficile* languished for several decades and only 25 years later did a follow-up paper, using mice, identify a smaller consortium of bacterial species that also reduced the severity of *C. difficile* infection¹¹³. Another study, using germ-free mice, demonstrated that administration of a single bacterial species in the Lachnospiraceae family could decrease the density of *C. difficile* growth *in vitro* and ameliorate disease severity¹¹⁴. The mechanism of clearance remained undefined although metabolomics studies suggested that clearance correlated with the re-establishment of normal secondary bile acid concentrations in the lower gastrointestinal tract¹¹⁵. Two separate phase II clinical trials of patients recovering from *C. difficile* infection following initial antibiotic treatment, reported that administration of a non-toxicogenic strain of *C. difficile*¹¹⁶ or a consortia of spore-forming commensal species¹¹⁷ significantly decreased the recurrence of disease. These clinical trials demonstrate the potential of a targeted approach to inhibit toxigenic *C. difficile*; however, the mechanism of action of these bacterial therapeutics remains undefined.

Host response to *C. difficile* infection

The immune system of the host rapidly responds to microbial molecules that traverse the epithelial barrier, a process that goes into overdrive in the setting of *C. difficile* toxin-mediated damage to the colonic epithelium. Loss of epithelial integrity results in increased intestinal permeability and the translocation of bacteria from the gut lumen into deeper tissues¹¹⁸. In response, resident immune cells and intoxicated epithelial cells release pro-inflammatory cytokines and chemokines that recruit circulating innate and adaptive immune cells and drive the expression of antimicrobial peptides, and the production of reactive nitrogen species (RNS) and reactive oxygen species (ROS)^{119,120} (FIG. 4). Although *C. difficile* has evolved resistance mechanisms against ROS and some antimicrobial peptides^{121–123}, these effector molecules limit the translocation of other intestinal bacteria. Furthermore, *S*-nitrosylation of the cysteine protease domain of TcdA and TcdB by RNS inhibits the release of the GTD into the cytosol, thereby attenuating toxin potency¹²⁴. Inflammatory responses are essential for host survival following *C. difficile* infection, but overly robust inflammation can be detrimental. For example, ROS that are produced in response to a toxin can exacerbate epithelial damage¹²⁵ and mice that lack the pro-inflammatory cytokine interleukin-23 (IL-23) show improved survival compared with wild-type mice¹²⁶. Furthermore, clinical studies demonstrate that the magnitude of the inflammatory response, as measured by faecal cytokine levels, correlates more closely with severity and duration of infection than with the *C. difficile* burden¹²⁷.

Inflammation and cytokine production

TcdA or TcdB in *C. difficile* can activate nuclear factor- κ B (NF- κ B) and activator protein 1 (AP-1) signalling pathways in intestinal epithelial cells through the phosphorylation of mitogen-activated protein kinases (MAPK), which leads to the transcription of pro-inflammatory chemokines, such as IL-8, chemokine C-X-C motif ligand 1 (CXCL1; also known as GRO α), CXCL2 and CC-chemokine ligand 2 (CCL2), and the recruitment of innate immune cells^{128–132}. Furthermore, TcdB glycosylation and the resulting inactivation of RHO GTPases in epithelial cells are detected by the intracellular pyrin receptor, which binds to apoptosis-associated speck-like protein containing a CARD (ASC), which leads to inflammasome formation and the secretion of IL-1 β ¹³³. The pattern-recognition receptors nucleotide-binding oligomerization domain-containing 1 (NOD1)¹³⁴, Toll-like receptor 4 (TLR4)¹³⁵ and downstream signalling proteins, such as myeloid differentiation primary response protein 88 (MYD88)¹³⁶ and ASC¹³⁷ support early host defence against *C. difficile* infection and mice that lack these proteins have increased mortality following infection. Notably however, *Asc*^{-/-} mice show decreased intestinal inflammation following direct instillation of TcdA or TcdB in the ileum¹³⁸, which exemplifies how differences in experimental protocols can lead to seemingly contrary results. The use of different antibiotic pre-treatment regimens to induce susceptibility to *C. difficile*^{139,140} or the composition of the infecting inoculum (vegetative bacilli versus spores)¹⁴¹ also affect *C. difficile* virulence in the murine infection model. These variables can provide a useful spectrum of disease severity to gain insights into when pro-inflammatory mediators have beneficial or deleterious effects on *C. difficile* pathogenesis.

Cellular responses

Resolution of diarrhoea and recovery of lost weight in mice infected with *C. difficile* are independent of adaptive immune defences as *Rag1*^{-/-} mice, which lack T cells and B cells, recover from acute infection similarly to wild-type mice^{141,142}. Intestinal innate lymphoid cells (ILCs) respond to IL-1 β , IL-12 and IL-23, which are pro-inflammatory cytokines that are upregulated during the acute phase of *C. difficile* infection^{133,143,144}, by producing effector cytokines, such as IL-22, IL-17a, interferon- γ (IFN γ) and tumour necrosis factor (TNF). The production of these effector cytokines by ILCs activates recruited neutrophils and macrophages and induces the expression of antimicrobial peptides, the production of RNS and ROS, and repair mechanisms in epithelial cells¹⁴⁵. In contrast to *Rag1*^{-/-} mice, *Rag1*^{-/-}*Il2rg*^{-/-} mice, which lack ILCs (in addition to T cells and B cells), fail to upregulate IFN γ , IL-22 or downstream effector molecules and exhibit high mortality following *C. difficile* infection¹⁴¹. Two ILC subsets, T-bet-expressing ILC1s, which produce IFN γ , and retinoic acid-related orphan receptor- γ t (Ror γ t)-expressing ILC3s, which produce IL-22, are activated during acute *C. difficile* infection and contribute to host defence¹⁴¹. IL-22 induces the production of antimicrobial peptides in the gut¹⁴⁶ immediately following *C. difficile* infection, and activates the complement pathway in the lung and liver to clear translocated bacteria¹⁴². IFN γ produced by ILC1s can increase phagocytic mechanisms and the expression of ROS-producing and RNS-producing enzymes in the colonic mucosa.

Neutrophils are crucial in the early host defence against *C. difficile*-mediated damage as depletion of these cells in mice results in acute mortality following infection¹³⁶. The pro-inflammatory cytokines IL-23 and granulocyte-macrophage-colony stimulating factor (GM-CSF) contribute to neutrophil migration to the site of infection by augmenting the expression of neutrophil chemotactic factors CXCL1 and CXCL2 (REFS 147,148). Once in the intestinal mucosa, neutrophils have several host protective functions, including the production of ROS in response to the activation of the *N*-formyl peptide receptor by TcdB¹⁴⁹ and the secretion of IFN γ ¹⁵⁰, potentially acting in concert with ILC1s to enhance phagocytosis and bacterial killing by macrophages.

Although T cells and B cells do not contribute to the resolution of the acute phase of *C. difficile* infection in mice, clinical data indicate that adaptive immune responses can have protective effects. Severity of disease is inversely correlated with the presence of toxin-specific immunoglobulin A (IgA) and IgG antibodies^{151,152}. Furthermore, a randomized, placebo-controlled trial demonstrated that patients who received humanized monoclonal antibodies that were specific for *C. difficile* TcdA and TcdB had reduced recurrences of disease¹⁵³. The role of T cells is less well understood. Major histocompatibility complex class II (MHC II)-deficient mice, which lack CD4⁺ T cells, exhibit unaltered acute infection¹⁵⁴, but toxin-specific antibody production and protection against secondary challenge with *C. difficile* is decreased compared with wild-type mice¹⁵⁴. Interestingly, even in fully immunocompetent mice, *C. difficile* colonization can persist following initial infection and recovery^{140,154,155}, which suggests that the main function of the immune system is to limit and repair *C. difficile*-mediated damage, not to sterilely clear *C. difficile* from the intestine.

Conclusions

Our understanding of the pathogenesis of *C. difficile* infection has increased markedly during the past decade. Deciphering the genes and proteins that are involved in sporulation, germination and toxin production has the potential to lead to new approaches to treatment (BOX 1). Dissection of the interactions between commensal bacterial species and *C. difficile* and the identification of commensal bacteria-derived inhibitory mechanisms may yield interventions that prevent or ameliorate *C. difficile* colitis. Greater understanding of host immune defence mechanisms may enable clinicians to reduce deleterious inflammatory responses while enhancing protective immune defences. As effective as infection control efforts have been to reduce transmission and protect patients from *C. difficile* infection, it is likely that new approaches, such as administration of protective probiotic bacterial strains, will ultimately be more effective at decreasing the incidence of infection. Because faecal microbial transplant is indisputably effective in curing *C. difficile* infections, *C. difficile* will be the first infection to be treated with bacteriotherapy.

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Eric Pamer received his M.D. from Case Western Reserve University, Cleveland, Ohio, USA. He is an infectious disease clinician scientist at the Memorial Sloan Kettering Cancer Center, New York, USA, and research in his laboratory focuses on defence against antibiotic-resistant pathogens.

Glossary

Germination

The transformation of a dormant spore to an actively replicating bacterial cell.

Reverse genetics

Targeted alterations of the genome. In *Clostridium difficile*, tools include ClosTron and allelic replacement.

Forward genetics

Untargeted alterations of the genome that are achieved by chemical mutagens or transposable elements.

Sensor histidine kinases

Signal-sensing proteins that pass phosphate to response regulator transcription factors that alter gene expression in response to extracellular stimuli.

Sigma factor

The DNA-binding subunit of RNA polymerase, each sigma factor binds to a distinct consensus sequence.

Catabolite repression

The regulation of gene expression such that preferred carbon sources are metabolized first.

Sporangia

A cell of a spore-forming bacterium that has completed asymmetric division.

Vegetative growth

Normal exponential growth of bacteria in rich media. *Clostridium difficile* switches between vegetative growth and sporulation.

Prodomain

A peptide sequence at the amino terminus of a protein that is cleaved for the protein to be active and fully functional.

Riboswitch

A secondary structure of mRNA, typically in the 5'-untranslated region, that binds to small molecules and regulates transcription and/or translation in *cis*.

Type IV pili

Polymer filaments on the surface of Gram-positive and Gram-negative bacteria that facilitate motility or adhesion.

Bacteriocins

Ribosomally synthesized antimicrobial peptides that are produced by bacteria that can selectively act against specific bacterial species or exhibit broad-spectrum activity.

Inflammasome

A cytosolic multiprotein complex that detects pathogen-associated molecular patterns. The detection of these 'danger signals' activates transcription of pro-inflammatory cytokine genes.

Innate lymphoid cells

(ILCs). Haematopoietic-derived innate immune cells that are capable of producing effector cytokines tailored to coordinate the early host response against distinct classes of pathogen.

Complement pathway

A series of interactions between plasma-derived proteins that lead to the opsonization of a pathogen and activation of the inflammatory immune response.v

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Box 1**Genomic insights into the taxonomy and transmission of *Clostridium difficile***

Clostridium difficile was first isolated from the intestines of infants and was shown to cause colitis in guinea pigs and rabbits¹⁵⁶. It was assigned to the genus *Clostridium* because of its morphology, ability to form spores and inability to undergo vegetative growth in the presence of oxygen. The *Clostridium* genus is complex and contains many distinct and dissimilar species that were grouped together based on traditional microbiological methods. Recent analyses, mostly based on sequences of 16S rRNA and ribosomal protein genes, place *C. difficile* in the Peptostreptococcaceae family and its genus name, therefore, has been changed to *Peptoclostridium*¹⁵⁷. The number of infections with *C. difficile* is increasing and the repeated emergence of new and evolved epidemic strains, such as the notorious BI/NAP1/027 strain, raises the possibility that new strains are spreading into human populations from environmental sources that are yet to be defined. Although healthcare and chronic care facilities have been typical places in which patients acquire *C. difficile* infections, in the past decade, more patients are presenting with community-acquired *C. difficile* infections¹⁵⁸. New evidence indicates that the transmission of *C. difficile* between patients who are hospitalized has been overestimated¹⁵⁹, which suggests that many patients who develop an infection with *C. difficile* did not acquire it during hospitalization and presumably harboured the organism asymptotically. Pigs, horses and a wide range of other mammals can be colonized with *C. difficile* and viable *C. difficile* spores have been detected in various locations¹⁶⁰. A deeper understanding of the environmental distribution of *C. difficile* strains will provide important insights into the epidemiology of human infections.

Box 2**Clinical dilemmas regarding *C. difficile* infection**

The diagnosis and treatment of *Clostridium difficile* infection in clinical settings are far from straightforward. Many clinical studies have been carried out to address these complex topics and our understanding has certainly improved. However, the development of more sensitive diagnostic tests, the application of whole-genome sequencing, the introduction of new antibiotics and greater acceptance of faecal microbial transplants have led to many new questions that require further investigation.

Patients who are hospitalized and have diarrhoea are generally tested for *C. difficile* infection. The recent introduction of a fast and highly sensitive PCR test that detects *tcdB* has led to higher rates of detection but it also potentially identifies patients colonized with *C. difficile* whose diarrhoea is caused by other factors. The concern about false-positivity is particularly important in patient populations with diarrhoea that is associated with laxative administration or following irradiation for haematopoietic stem cell transplantation. Rates of *C. difficile* carriage in the overall population are variable, but in some cases can be quite high — for example, in infants in which carriage rates are very high but are not associated with colitis. The administration of antibiotics following a false-positive test in a patient who is asymptotically colonized with *C. difficile* has the potential to disrupt the protective gut microbiota and induce colitis.

The high recurrence rate of *C. difficile* infection in patients following conventional treatment with antibiotics is probably the result of collateral damage to the microbiota induced by metronidazole or oral vancomycin¹⁶¹. Indeed, these two antibiotics, which are used to treat *C. difficile* infection, are destructive to the commensal microbiota and render the host highly susceptible to reinfection. A recent addition to the treatment armamentarium is fidaxomicin, a non-absorbable antibiotic that is less toxic to obligate anaerobic commensal bacteria. Recurrence of *C. difficile* infection caused by some, but not all, strains is decreased following treatment with fidaxomicin, when compared with other antibiotics¹⁶².

The most rapidly evolving and certainly most effective treatment for recurrent infection is faecal microbial transplant. Donor selection, route of transplantation, timing of transplantation and screening for potentially transmissible pathogens remain important issues that, with continued studies, should be resolved. It is likely that faecal microbial transplant will be replaced with precision bacteriotherapy with defined consortia of commensal bacteria.

Box 3**Microbiota-mediated primary bile acid conversion**

Bile acids are produced in the liver and secreted into the small intestine. Most are reabsorbed in the terminal ileum; however, approximately 5% of bile acids flow into the large intestine in which a subset of anaerobic bacteria converts them into secondary bile acids¹⁶³. Primary bile acids, such as glycocholate and taurocholate, are deconjugated to yield cholate by bile salt hydrolases that are expressed on the surface of a wide range of bacterial species in the gut¹⁶⁴. A smaller subset of bacterial species that reside in the caecum and colon, such as *Clostridium scindens*¹⁶⁵, take up cholate and chenodeoxycholate and dehydroxylate the 7 α carbon of the bile acid backbone to produce deoxycholate or lithocholate, respectively¹⁶⁶. The benefit of this dehydroxylation step for the bacteria that carry it out remains unclear, but bile acids may simply represent an electron acceptor in the anaerobic environment of the lower intestinal tract. The association between the levels of secondary bile acids and colon cancer suggests that their production is deleterious to the host. Recent studies have determined that another common intestinal commensal bacterium, *Ruminococcus gnavus*, detoxifies secondary bile acids by converting them into iso-bile acids¹⁶⁷. Balancing the potential negative effects of secondary bile acids to the host is their ability to inhibit spore germination and suppress the vegetative growth of *C. difficile*. Indeed, loss of secondary bile acids in the caecum and colon following antibiotic treatment is strongly associated with susceptibility to infection with *C. difficile*, and recovery from recurrent *C. difficile* infection following microbiota reconstitution by faecal transplantation is highly correlated with the recovery of secondary bile acid levels¹¹⁵. For reasons that remain unclear, the effect of bile acids on spore germination and growth in *C. difficile* varies between strains, with some strains inhibited by chenodeoxycholate, whereas other strains are not¹⁶⁸. Bile acids vary somewhat between mammalian species. For example, muricholic acids that are produced by mice inhibit the germination of *C. difficile* spores¹⁶⁹, although it remains unclear whether this applies to spores of all strains. Ursodeoxycholate, which is present in human bile and is also used therapeutically to treat cholestatic liver disease, inhibits both germination and vegetative growth of *C. difficile*¹⁷⁰.

Key points

- Disease that is associated with infection by *Clostridium difficile* represents an urgent public health threat. The severity of *C. difficile* infection is determined by strain virulence, interactions with intestinal commensal microbial communities, and the host immune response to damage of the intestinal epithelium that is induced by *C. difficile*.
- The ability to sporulate and germinate is essential to *C. difficile* virulence. Hundreds of genes that are involved in sporulation and germination have been identified as well as a bile acid receptor that induces germination.
- *C. difficile* secretes toxin proteins that are internalized by host cells through receptor-mediated endocytosis and cause disruption to cytoskeletal architecture, which leads to cell death. Toxin-mediated cell death results in the loss of intestinal barrier integrity and the translocation of bacteria into underlying tissues.
- The intestinal microbiota provides colonization resistance against *C. difficile* infection. Commensal bacteria that are capable of converting primary bile acids to secondary bile acids inhibit the growth of *C. difficile* by depriving *C. difficile* spores of an important germinant and by increasing the concentration of secondary bile acids in the intestinal lumen, which are toxic to the vegetative form of *C. difficile*.
- Toxin-mediated damage to the epithelium activates the host inflammatory immune response. The role of the immune system is to limit epithelial damage and the dissemination of intestinal bacteria into the circulation. However, an overly robust inflammatory response can be damaging to the host and contribute to disease pathology.

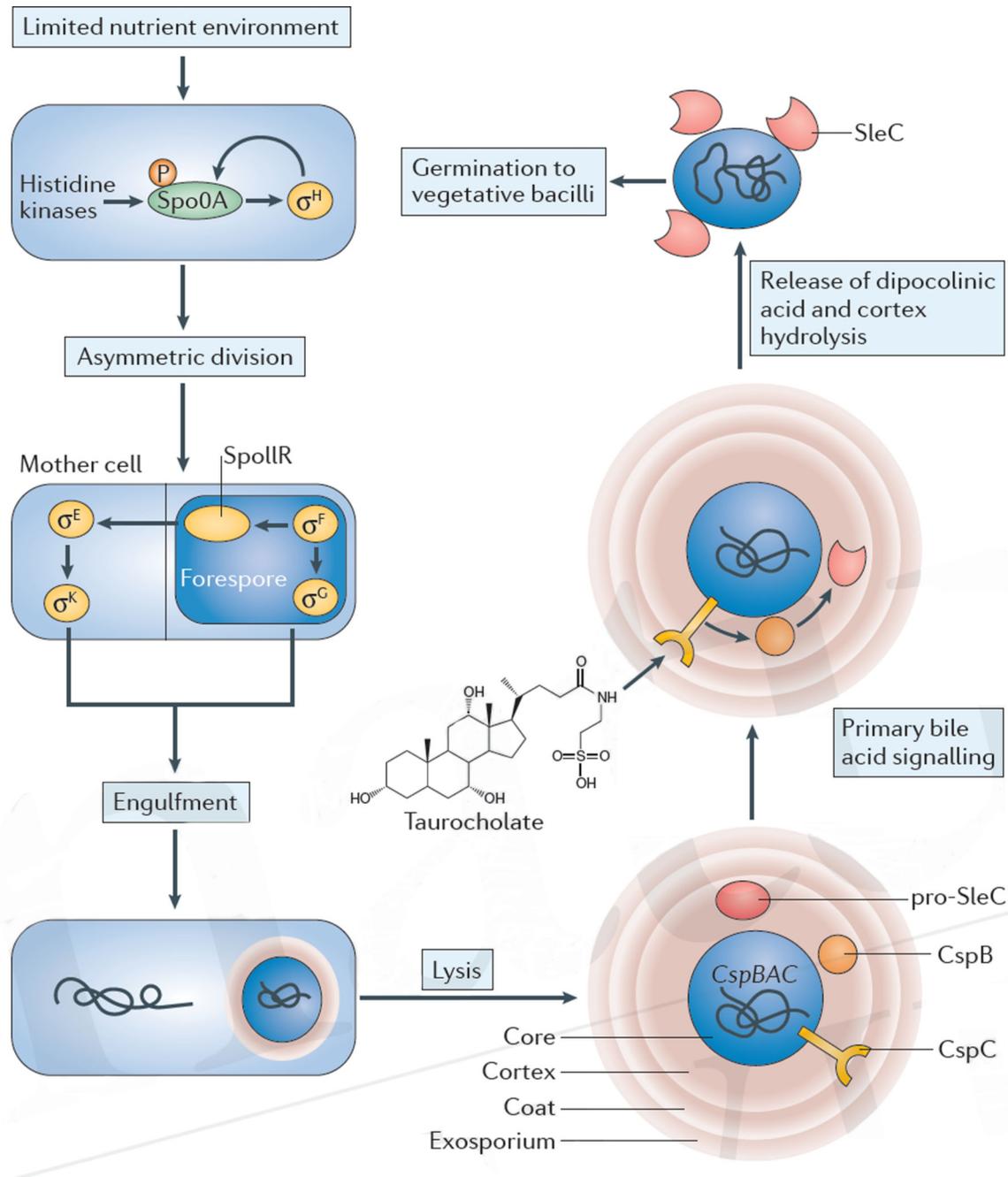


Figure 1. Sporulation and germination of *C. difficile*

A limited nutrient environment induces sporulation. The transcription factor stage 0 sporulation protein A (Spo0A) is phosphorylated by histidine kinases, activating a cascade of signalling and morphological events that create a forespore within the mother cell of the bacterium. After lysis, the spore is released into the environment. The core of the spore, which contains the condensed chromosome, is encapsulated by three protective layers: the cortex, coat and exosporium. Germination of the spore can be initiated by bile acids, such as taurocholate, which signal through the CspC receptor. Activation of the SleC enzyme by

CspB leads to the degradation of the cortex of the spore and eventually leads to outgrowth of a new vegetative cell. P, phosphate.

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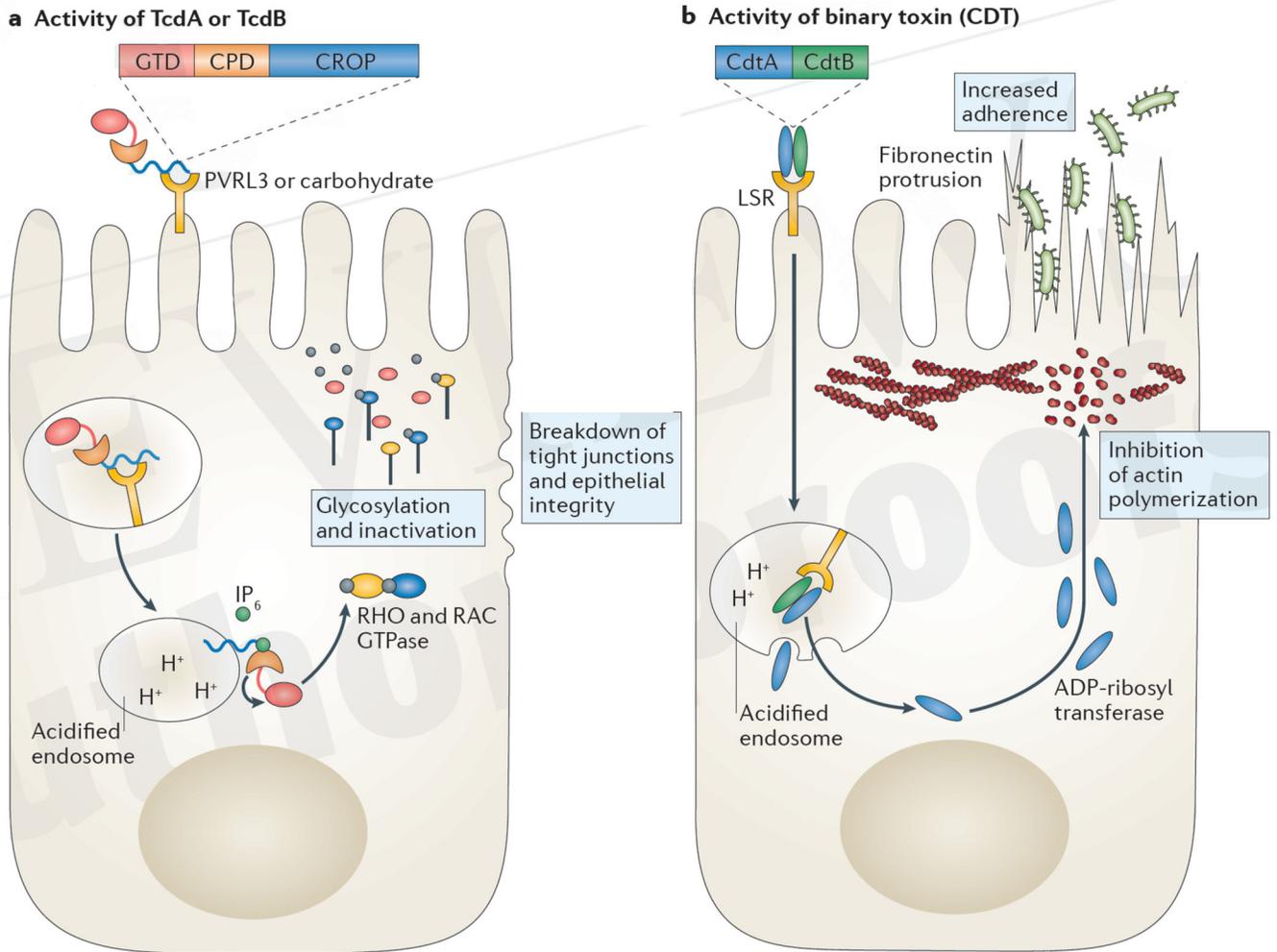


Figure 2. Mechanism of action of *C. difficile* toxin in epithelial cells

a | The combined repetitive oligopeptide repeat (CROP) domain of TcdA binds to carbohydrates on the apical surface of epithelial cells, whereas TcdB binds to poliovirus receptor-like 3 (PVRL3) expressed on colonic epithelial cells. Toxin is internalized and acidification of the endosome enables the CROP domain to embed into the endosomal membrane and the subsequent transport of the cysteine protease domain (CPD) and the glucosyl transferase domain (GTD) into the cytosol. Inositol hexakisphosphate (IP₆) activates the cysteine protease to cleave and release the toxin glycotransferase. Glycosylation and thereby inactivation of RHO or RAC GTPases ultimately results in the breakdown of tight junctions and epithelial integrity. **b** | Binary toxin (also known as *Clostridium difficile* transferase (CDT)) binds to the lipolysis-stimulated lipoprotein receptor (LSR) and is internalized. The CdtB subunit creates pores in the acidified endosome that enable the release of the CdtA subunit into the cytosol. The ADP-ribosyl transferase activity of CdtA inhibits actin polymerization near the cell membrane, enabling fibronectin microtubules to elongate and protrude through microvilli, which increases *C. difficile* adherence to the epithelium through type IV pili.

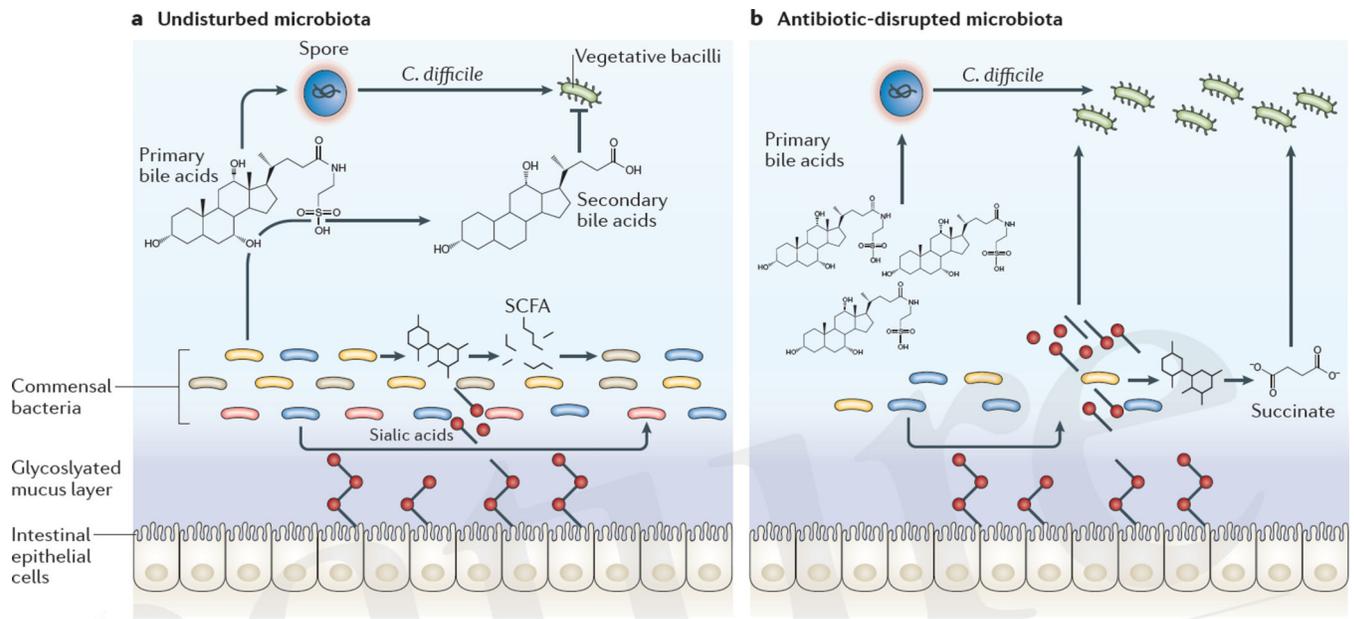


Figure 3. Microbiota-mediated defences against *C. difficile*

a | The intact microbiota converts primary bile acids into secondary bile acids, several derivatives of which inhibit the growth of *Clostridium difficile* through detergent-induced toxicity to vegetative bacilli. Commensal bacteria that express sialidases cleave sugars that are attached to epithelial cells and release sialic acid into the intestinal lumen. Fermenting commensal bacterial species convert carbohydrates into short-chain fatty acids (SCFAs), such as succinate. Bystander commensal bacterial populations can consume these metabolites as energy sources. **b** | Antibiotic-mediated disruption of the microbiota depletes primary bile acid converters, which enables *C. difficile* sporulation and growth. Antibiotics can also deplete competing sialic acid and succinate consumers, liberating an energy source for *C. difficile*.

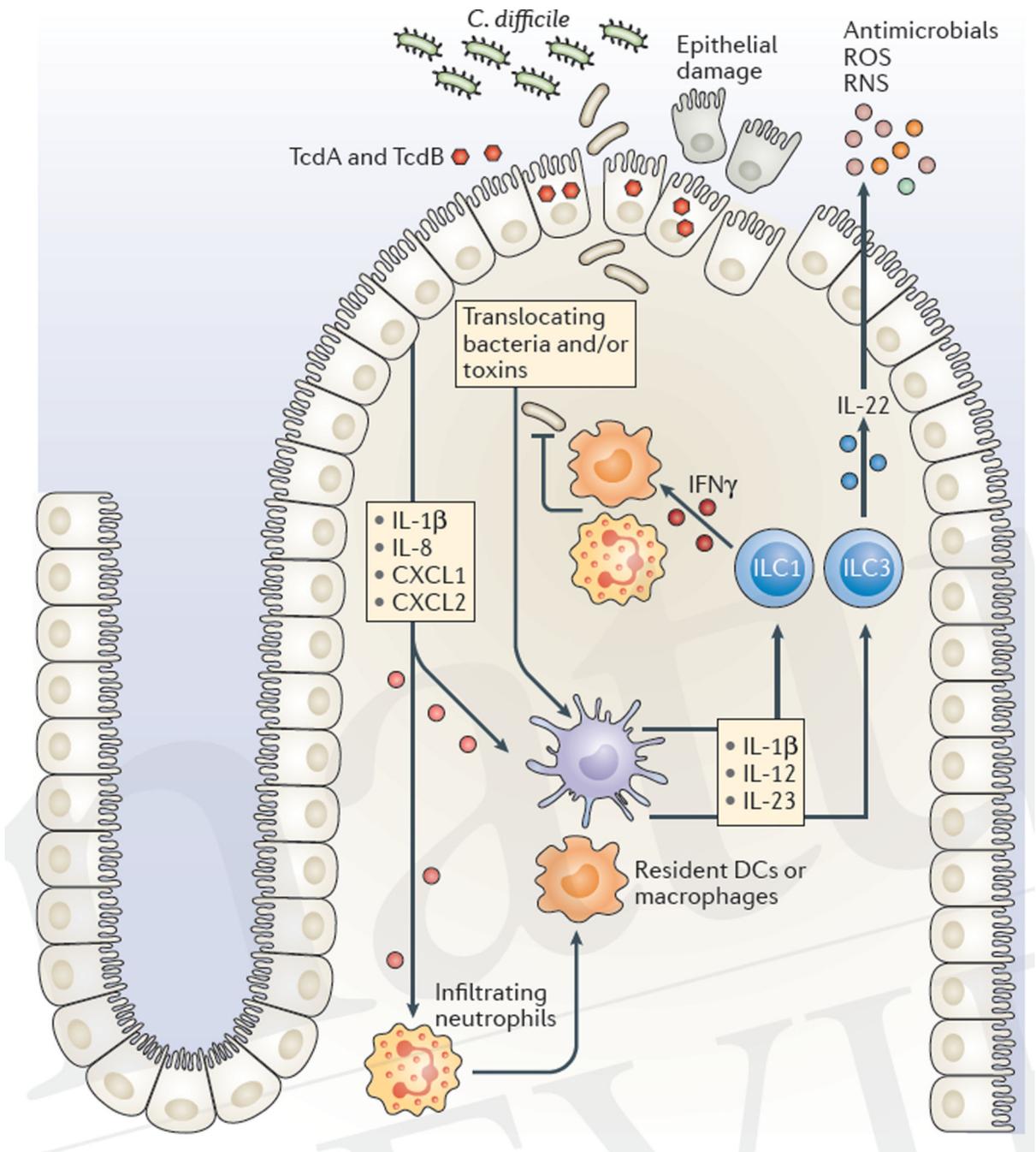


Figure 4. Innate immune-mediated defences against *C. difficile*

The acute host response to *Clostridium difficile* is initiated by toxin-mediated damage, loss of epithelial integrity and the detection of translocating bacteria. Intestinal epithelial cells and resident innate immune cells secrete pro-inflammatory chemokines (such as chemokine C-X-C motif ligand 1 (CXCL1), CXCL2, and interleukin-8 (IL-8)) and pro-inflammatory cytokines (such as IL-1 β , IL-12 and IL-23), which leads to the recruitment of neutrophils and the activation of innate lymphoid cells (ILCs). IL-12 signalling drives the expression of interferon- γ (IFN γ), whereas IL-1 β and IL-23 signalling induces the production of IL-22.

The effector cytokines IFN γ and IL-22 induce defence mechanisms such as increased phagocytic activity of macrophages and neutrophils and the production of antimicrobial peptides and enzymes that synthesize reactive oxygen species (ROS) and reactive nitrogen species (RNS). These defence mechanisms limit bacterial dissemination, attenuate toxin activity and repair epithelial damage. DCs, dendritic cells.

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