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Cytotoxic synergism of *Clostridioides difficile* toxin B with proinflammatory cytokines in subjects with inflammatory bowel diseases

Bassotti G *et al*. Synergism toxin B/cytokines in IBD

Abstract

Clostridioides difficile (*C. difficile*) is progressively colonizing humans and animals living with humans. During this process, hypervirulent strains and mutated toxin A and B of *C. difficile* (TcdA and TcdB) are originating and developing. While in healthy subjects colonization by *C. difficile* becomes a risk after the use of antibiotics that alter the microbiome, other categories are more susceptible to infection and at risk of relapse such as those with inflammatory bowel disease (IBD). Recent *in vitro* studies suggest that this increased susceptibility could be due to the strong cytotoxic synergism between TcdB and pro-inflammatory cytokines, tumour necrosis factor-α (TNF-α) and interferon-g (IFN-g) (CKs). Therefore, in subjects with IBD the presence of an inflammatory state in the colon could be the driver that increases the susceptibility to *C. difficile* infection (CDI), its progression and relapses. TcdB is internalized in the cell *via* three receptors: Chondroitin sulphate proteoglycan 4 (CSPG4), poliovirus receptor-like 3 (PVRL3), and Wnt receptor frizzled family (FZD). CSPG4 and FZD are involved in cell death by apoptosis or necrosis depending on the concentration of TcdB and cell types while PVRL3 induces only necrosis. It is possible that cytokines could also induce a greater expression of receptors for TcdB more involved in necrosis that in apoptosis. Therefore, in subjects with IBD there are the conditions: (1) For greater susceptibility to CDI, such as the inflammatory state, and abnormalities of the microbiome and of the immune system; (2) for the enhancement of the cytotoxic activity of TcdB + CKs; and (3) for a greater expression of TcdB-receptors, stimulated by cytokines, that induce cell death by necrosis rather than apoptosis. The only therapeutic approach currently possible in IBD patient is monitoring of *C. difficile* colonization for interventions aimed

at reducing TNF-α and IFN-g levels when the infection begins. The future perspective is that to generate bacteriophages against *C. difficile* for a targeted therapy.

Key Words: Inflammatory bowel diseases; *Clostridioides difficile* infection; Cytokines; Tumour necrosis factor-alpha; Interferon-gamma; necrosis; Apoptosis; Cytotoxic synergism

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Core Tip: *Clostridioides difficile* (*C. difficile*) is an opportunistic pathogen which diffusion is progressively increasing worldwide. Among the categories at risk, particularly susceptible are patients with inflammatory bowel diseases, due to altered immunological status and the therapies adopted that favour intestinal dysbiosis and colonization by the germ. Recent *in vitro* studies also suggest that the infection might be favoured by the strong cytotoxic synergism between *C. difficile* toxin B and pro-inflammatory cytokines tumour necrosis factor-a and interferon-g. The therapeutic approaches are however still limited, and those presently available rely on antibiotic therapy and faecal transplantation.

INTRODUCTION

Clostridioides difficile (*C. difficile*) is a bacterium gram-positive, anaerobic, and spore-forming[1-6], responsible for the most widespread health care associated infection worldwide[7-11]. In the United States every year about 500000 individuals become infected, leading to approximately 29000 deaths, and in Europe there are 124000 cases of infected individuals per year with a mortality ranging from 3% to 30%[7-11]. *C. difficile* infection (CDI) accounts at present for more than 15%-25% of all opportunistic gastrointestinal infections[7-11]. The rate of *C. difficile* colonization is about 18%-90% of

healthy infants in relation to infant age[12], and 4%-15% of healthy individuals[13]. Its transmission occurs by the faecal-oral route[6,14], mainly by spores. Hospitals and community healthcare settings are an important source of infection due to the presence of *C. difficile*-infected patients[15,16]. The latter create a microenvironment highly contaminated by *C. difficile* spores extremely resistant to common strong disinfectants and radiation[17-19].

The clinical manifestations of CDI vary from asymptomatic carriage or mild self-limiting diarrhoea to pseudomembranous colitis (PMC), with complications as toxic megacolon, fulminant infection and also death[2-6,10]. The disease strictly depends by *C. difficile* germination and release of three toxins[2-6,10]. The Rho-glycosylating *C. difficile* toxins A (TcdA) and B (TcdB) are major toxins that are clearly responsible for diarrhoea and colitis[2-6,10]. In addition, 5%-30% of clinical *C. difficile* strains produce a binary autoprotease domain (ADP)-ribosylating toxin, defined *C. difficile* binary toxin (CDT), that modifies actin[20,21].

It has previously been highlighted how the continuous progressive spread of *C. difficile* in the anthropized environment and the ability to develop more virulent strains will allow it to colonize most of the human population in the near future[22,23]. Among the subjects who will be more progressively colonized/infected with the progressively expanding *C. difficile* are those with inflammatory bowel diseases (IBD)[24-29], a category which is increasing in number in both Western and Eastern countries[30,31].

The progressive *C. difficile* endemic spread and the growing number of IBD subjects[25,29,32,33] are already interacting, as evidenced by studies showing how the rate of CDI cases in patients with IBD has increased by approximately four times in recent years[25]. It is therefore important to understand the molecular and pathogenic events by which *C. difficile* colonizes and infects, and how these can impact on subjects with IBD characterized by a profound alteration of the microbiome, of the immune system and of the inflammatory response.

C. DIFFICILE

CDI and clinical manifestations

CDI causes nosocomial/antibiotic-associated and community healthcare diarrhoea with abdominal pain and cramps[6,10,32,33]. Colitis without pseudomembrane formation features watery diarrhoea, trace blood in stool, nausea, abdominal pain, malaise, anorexia, low-grade fever, dehydration, pyrexia, and leucocytosis[6,10,32,33]. Clinical manifestations of PMC consist of abdominal cramps, watery diarrhoea with dehydration, hypoalbuminemia, and increasing of serum proteins, mucus and inflammatory cells; sometimes plaques (pseudomembranes) are found in the colorectal mucosa[6,10,32,33]. Fulminant colitis, observed in about 3% of CDI patients, induces serious complications such as perforation, prolonged ileus, megacolon, and death[6,10,32,33]. CDI may not be limited to the colon, and extra-colonic *manifestations have been reported, including* small bowel disease with the formation of pseudomembranes on the ileal mucosa, bacteraemia, reactive arthritis, appendicitis, intra-abdominal abscesses, osteomyelitis, and empyema[34,35] In recent years, a significant rise in cases of fulminant colitis, which results in the development of symptoms, multiple organ failure, and increased mortality, has been associated with hypervirulent strains of *C. difficile*[6,10,32,33]. The disease strictly depends on *C. difficile* production and release of three toxins[2-6,10]. TcdA and TcdB are mainly responsible for clinical manifestations of disease[2-6,10]. However, 5%-30% of *C. difficile* clinical strains produce CDT, that contributes to disease by means of actin modification[20,21].

TcdA and TcdB: molecular structure and their receptors

TcdA and TcdB are single chain proteins, with a molecular weight of 308 kDa for TcdA and 270 kDa for TcdB[2-6,32]. Tcds show a 48% of sequence identity and 66% of sequence similarity, where the diversity of sequence is mainly limited to the C-terminal binding domain. TcdA and TcdB are constituted by four domains, each characterized by specific biological and functional properties: (1) A glucosyltransferase N-terminal domain (GTD); (2) an ADP; (3) a pore forming and translocation domain (TD); and (4) a C-terminal binding repetitive oligopeptides domain (CROPs)[2-6,32]. CROPs domain and

other amino acids outside this domain allow the binding of Tcds to the cells for subsequent internalization[2-5]. Although TcdA and TcdB CROPs display the solenoid fold, they present distinct spatial and sequential arrangements of their repeat units. This is in agreement with findings that suggest that both TcdA and TcdB bind to different receptors[2-6,32]; therefore, TcdA and TcdB do not follow the rule of one toxin-one receptor[2-6,32,36].

Two different cell surface receptors have been proposed for TcdA, rabbit sucrase isomaltase, and gp96[2-6,32,36]. Since many cells and tissues that are sensitive to TcdA do not express sucrase isomaltase and cells lacking gp96 are only partially resistant to TcdA intoxication, TcdA could bind to other receptor structures[2-6,32,36]. Regarding TcdB, three different receptors have been identified[2-6,32,36], chondroitin sulphate proteoglycan 4 (CSPG4), poliovirus receptor-like 3 (PVRL3), and Wnt receptor frizzled family (FZD; FZD1, 2 and 7)[2-6,32,36]. TcdB binding to CSPG4 receptor in HeLa and HT29 cells induces cell rounding and apoptosis at concentrations picomolar of TcdB, but induces necrosis at higher concentration of TcdB[32,36-38]. TcdB binding to PVRL3 receptor induces necrosis at high doses (the nanomolar range) of TcdB[32,36-39]. TcdB binding to FZD receptor induces cytopathic effects and apoptosis at picomolar concentrations of TcdB, indicating that FZD functions as an alternative receptor to CSPG4[32,36-40]. A further significant distinctiveness of TcdB is that it can bind to the membrane receptor with amino acid sequences that extend beyond the CROP sequences[32,36-40].

This description, in agreement with recent studies, indicates that TcdA and TcdB use more than one receptor for cell binding and uptake, and highlights how the heterogeneity of the TcdB-bound receptors may impact on the diversity of the effects in relation to the receptor binding, concentrations of TcdB, and cell types[20,32,36-43]. It is possible that TcdB may utilize multiple receptors to broaden the selection of mammalian cells it can target[36-41]. Further, TcdB variants are highly diverse for their receptor preference, with relevant implications on colonic pathology[20,32,36-43].

The role played by CROP domains is also demonstrated by the fact that antibodies directed toward the CROP domains of TcdA and TcdB prevent uptake[2-5,36], and excess of TcdA CROP domain compete with TcdA for cell binding[2-5,36]. However, TcdA and TcdB that lack CROPs domains are still capable to be internalised by the cells[2-5,36]. To understand some important aspects of the pathogenic strategy of *C. difficile* it is necessary to know what receptors for Tcds one type of cell can express. Receptors for Tcds do not have well-defined molecular structures[20,32,36-43]; they are likely formed by a complex configuration of the polysaccharide chains that are recognized by the TcdA or TcdB binding domains with a lectin-like structure[42]. Furthermore, TcdA and TcdB have some important features of intrinsically disordered proteins[44] which allow Tcds to modify their conformation to adapt and more efficiently bind to the target structure of Tcd receptors[20,32,36-43]. The complex and intrinsically disordered structural features of the Tcds allow them to bind very different cell types[2-6,32], such as the cells of surface epithelium of the human colon[43], colonocytes[45], hepatic cells[46], nervous cells[47], enteric glial cells (EGCs)[48], and cardiac cells[49].

It has been hypothesized that when *C. difficile* spores germinate into their vegetative forms and replicate, their susceptibility to the cytotoxic activity of macrophages, polymorphonucleates and lymphocytes will increase[32,50-52]. These immune cells induce and increase the inflammatory response[2-6,32,50-53] characterized by secretion of several pro-inflammatory cytokines as interleukin (IL)-1, IL-6, IL-8, interferon-gamma (IFN-γ), and tumour necrosis factor-alpha (TNF-α)[32,54-58]. *C. difficile* struggles against these immune cells by means of Tcds, capable to bind to receptors with different forms of carbohydrates that give rise to distinct structures. This result is obtained by TcdB ability to recognize three types of receptors[32,36-40] and by its intrinsically disordered structure[44]. Therefore, due to accidental molecular homology, Tcds could also be cytotoxic to other cell types not present in the infection site, that express one or more of the Tcds receptors (*e.g.*, endothelial, hepatic, nervous, EGCs, and cardiac cells)[2-6,32,46-49]. Furthermore, the binding capability of Tcds toward colonocytes deepens the tissue damage within and beyond the submucosa. The subsequent damage to the muscle and

enteric nervous system cells creates conditions to expel *via* diarrhoea the *C. difficile* vegetative forms that rapidly become again spores, and to start again a new infection cycle. It is also possible that if the Tcd receptor domain mutates, the pathogenicity of *C. difficile* may become more severe.

Tcds uptake and internalization

Tcds, after binding to their cell membrane receptors, promote their uptake by endocytosis[2-6,32]. Tcds differ in uptake pathways: TcdB uptake is mediated by clathrin[2], while TcdA uptake is mediated by PACSIN2/Syndapin-II[2]. In the endocytic vacuole the progressive pH decrease promotes a Tcd conformational change that leads to translocation across the endosomal membrane of the catalytic domain for cleavage through vacuole pore formation[2-6,32]. Then, Tcds undergo autoprocessing by the cysteine protease domain (CPD), that follows the N-terminal GTD[2-6,32] in a host-factor dependent manner (*e.g.*, inositolphosphates, mainly inositol hexakisphosphate)[2-6,32], releasing the GTD into the host cell cytosol[2-6,32].

Tcds intracellular effects

The Tcds GTD, by the monoglycosylation of the catalytic site of Rho-GTPases, inhibits their activity[2-6,32]. The monoglycosylation of Rho-GTPases induces different effects *in vitro* and *in vivo*[2-6,32]. The effects mainly documented *in vitro* are: Actin condensation, rearrangement of the cytoskeleton, disruption of focal adhesions and tight junctions[2-6,32]; all these effects induce cell rounding in cultured cells (cytopathic effect)[2-6,32,48]; arrest of the cell-cycle, by reduction of both the expression of cyclins and the activation of kinases cyclin-dependent that together mediate progression in the different cell-cycle phase[2-6,32,48]; cell death by apoptosis or necrosis (cytotoxic effect)[2-6,32,48]. Cell death occurs in a glycosylation-dependent/glycosylation-independent way, mainly by apoptosis with caspase-dependent or caspase-independent mechanisms[2-6,32,48]. Furthermore, the cytotoxic effects are dependent on the dose of Tcds, receptors involved and cell types[2-6,32]. In fact, TcdB at high concentration by binding to CSPG4,

FZDs or PVRL3 induces cell death by necrosis[36-39,59], while at low concentration and by binding to CSPG4 or FZDs cell death occurs by apoptosis[2-5,36-39,48]. However, it must be taken into account that the effects of Tcds could depend also by cell types, likely by selective expression of Tcd receptors and by different expression levels of Tcd receptors[2-6,32].

Tcds effect in vivo

TcdA and TcdB, *in vivo*, disrupt epithelial tight junctions and induce cell death, provoking direct damage to the colonic epithelium[2-6,32]. In addition, Tcds induce an acute inflammatory response stimulating colonic epithelial cells to release pro-inflammatory cytokines and chemoattractants of neutrophils[2-6,32] that can induce tissue damage, modifying the barrier effect of the intestinal mucosa. A compromised intestinal barrier within the context of active inflammation subsequently leads to enhanced intestinal and vascular permeability[2-6,26,29,32,34]. Thus, following the loss of a protective barrier, there is the access of Tcds and/or bacteria into the *lamina propria*, which in turn increases intestinal inflammation[2-6,26,29,32,34]. TcdA primarily affects the intestinal epithelium, while TcdB has a broader cell tropism and is probably mainly responsible for the major clinical effects of *C. difficile* due to its toxicity, which is approximately 1000 times higher than that of TcdA. Thus, TcdB represents the main virulence factor of CDI[2-6,26,29,32,34]. TcdB causes death in many different cell types[2-6,26,29,32,34] other than epithelial cells and colonic myofibroblasts, such as hepatocytes, cardiomyocytes, lung fibroblasts, immunocytes, enteric neurons and EGCs[2-6,32,46-49].

Since immune cells that reach the replication area of the *C. difficile* vegetative form possess intrinsic motility, the molecular strategy to cause cytoskeleton alterations and cell cycle arrest is of crucial importance in order to decrease their functional ability to counteract CDI[2-6,32]. Tcds in these immune cells stimulate cytokine secretion, in particular of pro-inflammatory cytokines such as IFN-g and TNF-α[2-6,32,34], and also anti-inflammatory cytokines as IL-10[60]. Cytoskeletal disruption occurs in some cells after 30 min, representing the initial event that leads to cell rounding in most cell types

in vitro[32,61] with detachment of cells. Tcds *in vivo* induces both retraction of colonocytes and basal membrane cells, favouring the additional in-depth penetration of *C. difficile* and promoting an extremely inflammatory environment that causes the expulsion of *C. difficile* in the external environment by diarrhoea[32,62].

Tcds and cell death

Various Tcd-infected cells, after cell-cycle arrest, die by apoptosis[2-6,32]. This could represent an important aspect of the molecular strategy of this pathogen to reduce the inflammatory response. Apoptosis is a form of cell death that occurs without inflammation and is mainly mediated by activation of the effector caspases-3 and -7, which can be triggered by a death receptor-dependent extrinsic or a mitochondria-dependent intrinsic pathway[63-65]. However, apoptosis can also be activated in a caspase-independent manner by the cleavage/activation of pro-apoptotic Bcl-2 family members and non-caspase proteases such as calpains and cathepsins[66-69]. Caspase-dependent TcdA- and TcdB-induced apoptosis has been extensively investigated[2-6,32,48,70], while there is only one evidence that TcdA can also induce caspase-independent apoptosis following cathepsin[71] and calpain activation[71].

Recently, Fettucciari *et al*[72] demonstrated that the mechanism by which TcdB induces apoptosis is much more complex than previously thought[48,70]. TcdB induced apoptosis in EGCs, a cell population of paramount importance for colonic pathophysiology, by three signalling pathways activated by calpains, caspases and cathepsins, which are all involved in both induction and execution of apoptotic signalling[72]. Calpain activation by Ca^{2+} influx is the first pro-apoptotic event in TcdB-induced EGC apoptosis and causes caspase-3, caspase-7 and PARP activation. The latter is activated by caspases but also directly by calpains, which are responsible for the majority of apoptosis[72]. Caspase-3/caspase-7 and PARP activation is mediated also by activation by TcdB of initiator caspase-8, and it contributes to one third of apoptosis[72]. Finally, cathepsin B contributes to triggering the pro-apoptotic signal and to one third of apoptosis by a caspase-independent manner, and it seems mainly to control the levels of caspase-3 and

caspase-7 active fragments, highlighting the complex interaction between these cysteine protease families activated during TcdB-induced apoptosis[72]. Recently, we also demonstrated that pro-inflammatory cytokines TNF-α plus IFN-g (CKs) strongly increased apoptosis induced by TcdB, by an enhanced activation of the three pro-apoptotic pathways induced also by TcdB alone activated by calpains, caspases and cathepsins, all involved in both induction and execution of apoptotic signalling[72]. However, two important differences between TcdB- and TcdB + CK-induced apoptosis are: (1) Apoptotic signalling activation by TcdB + CKs is enriched by TNF-α-induced NF-kB signalling, inhibition of JNK activation and activation of AKT[72]; and (2) apoptosis induced by TcdB + CKs increased strongly in the time course, with more than 60% of the cells undergoing apoptosis at 72 h, while apoptosis induced by TcdB increased slowly, with only about 18% of cells undergoing apoptosis at 72 h[72].

This capability of TcdB to trigger three different cell death pathways represents an extremely important *C. difficile* strategy[72] to overcome the possible intrinsic resistance of a cell type to one or two of the three apoptotic pathways. In fact, as above reported, Tcds are able of cause cell death in different non immune[2-6,32,46-49] and immune cell types[2-6,32,73,74].

A further strategy adopted by *C. difficile* is its ability to induce different types of cell death that can lead to different consequences in the *C. difficile* pathogenesis[2-6,32]. In fact, TcdB causes cell death by both apoptosis and necrosis[2-6,32,75] that is dependent by the TcdB concentration and by the TcdB receptor expressed by the target cells[20,32,36-43]. TcdB, at lower concentrations and binding to CSPG4 or FZD receptor[20,32,36-43] induces apoptosis in a glycosylation-dependent manner[2-6,32], while at higher concentrations (100 pM or above) causes cell death by necrosis[20,36-43] which does not require either the autoprocessing or glucosyltransferase activities of the toxin[2-6,75]. Necrosis is an early process occurring after 2-4 h of intoxication and is found in both cell culture and colon explant models[2-6,75]. Necrosis is characterized by quick ATP depletion, early loss of plasma membrane permeability and cellular leakage, and chromatin condensation without caspase-3 and caspase-7 activation[2-6,32,75]. TcdB causes necrosis through

activation of a strong production of reactive oxygen species (ROS) by assembly of the NADPH oxidase complex on endosomes[2-6,75]. High levels of ROS trigger necrosis likely by DNA damage, lipid peroxidation, protein oxidation and/or mitochondrial dysfunction[2-6,32,75]. It has been suggested that pore formation in the endocytic vacuoles is important for the glycosylation-independent necrotic cell death caused by TcdB. Indeed, a TcdB mutant, defective in pore formation, does not induce necrosis even at high nanomolar concentrations[2-6,75]. Unlike TcdB, TcdA does not trigger ROS production through the NADPH oxidase complex and causes a glycosylation-dependent apoptosis at all concentrations tested[2-6]. The ability of TcdB, but not of TcdA, to cause necrosis may explain why a wild-type ($TcdA^+TcdB^+$) epidemic strain and an isogenic $TcdA^-TcdB^+$ mutant in animal infection models provoke considerably more damage to colon tissue than an isogenic $TcdA^+TcdB^-$ mutant strain[2-6]. The glycosylation-independent mechanism of TcdB might play a similar role in the context of human disease; TcdB-induced necrosis likely contributes to the extensive gut damage observed in patients with severe forms of CDI[2-6].

TcdB induces also an early cell death defined pyknotic cell death, characterized by chromatin condensation, cell cycle arrest and ballooning of the nuclear envelope that is both glucosyltransferase domain-dependent and -independent[2-6,75], and occurs at concentrations 5000 times greater than necessary for Rho protein glycosylation and ROS production[2-6,75].

It has also been reported that TcdA and TcdB trigger pyrin inflammasome activation in an ASC [apoptosis-associated speck-like protein with a caspase recruitment domain (CARD)]-dependent manner, causing release of IL-1β[2-6]. In particular, TcdB-induced inflammasome activation triggers a type of cell death defined "pyroptosis", characterized by cell swelling and lysis with gross release of cellular content and inflammatory cytokines like IL-1β through pore formation in target cells, caused by caspase-1-dependent activation of gasdermin D, that induces a strong inflammation[2-6].

Most importantly, EGCs intoxicated with low doses of Tcds might revert to their normal functions after a brief cell-cycle arrest[32,48,76], while EGCs that survive apoptotic

activity of TcdB become senescent as a TcdB-mediated survival response to stressful stimulus[32,48,76]. This ability of cells surviving the cytotoxic activity of Tcds to become senescent may affect functionality of EGCs, intestinal neurons and myocytes, contributing to alter bowel motility[32,77]. The acquisition of a senescence status by these cell types could have critical outcomes in the subsequent development of post-infectious irritable bowel syndrome (IBS) and stimulation of pre-neoplastic cells[32,77].

Thus, we can postulate that, when healing after an acute CDI, patients can later have some important consequences such as decrease of EGC number, impairment and alterations of EGC functionality[32]. After CDI, the structural and functional defects caused by the Tcds might be long-lasting in a significant percentage of patients and trigger low-grade inflammation and persistent dysmicrobism[25]. This implies that residual *C. difficile* bacteria that remain when healing after an acute CDI may benefit of this condition, and provoke relapses. The latter might be due to cytotoxic synergism with inflammatory cytokines, that can occur even after months, without any evident cause[78]. Therefore, it is possible that *C. difficile* changes the large bowel environment to remain for a long time and favour easier relapses. This means a continual expansion of *C. difficile* carriers in the large bowel environment, with induction of an IBS-like condition and with recurrences due to a latent or fluctuating inflammatory condition as in IBD subjects.

All this emphasises the complex molecular strategy of *C. difficile* based on the cytotoxic synergism with some components of the inflammatory response, as the IFN-g and TNF-α, which potentiate cytotoxicity of TcdB[48,72]. Therefore, it is conceivable that IFN-g and TNF-α behave as drivers of the infection from the beginning of the infection, amplifying apoptosis induced by low doses of Tcds, and opening the way to infection progression[26,32,34,48,72].

Therefore, it is likely that antibiotic treatment, other than provoking dysmicrobism, by means of bacteriolytic activity builds an inflamed environment in the large bowel by release of bacterial factors from killed bacteria. Additionally, whatever inflammatory

environment induced in the absence of antibiotic treatment could also help CDI in subjects with obesity, or various pathologies accompanied by an inflammatory state[28].

THE CONDITIONS THAT FAVOUR THE ENDEMIC SPREAD OF C. DIFFICILE IN PEOPLE WITH IBD

An endemic spread of *C. difficile* in IBD patients is favoured by: The extreme resistance of the spores to the external environment and the mechanism of spore germination[17-19,79].

C. difficile spores are extremely resistant to strong disinfectants and radiation[17-19]. The mechanism of germination is both complicated and distinctive compared to that of other microorganisms[79], due to the peculiar Tcds interaction with the host, and to cellular microbial factors which facilitate the colonization and successively infection and relapses[9,12,14-16,79-83]. Moreover, *C. difficile* spores can also adhere to gut epithelial cells and penetrate them *via* a process of macropinocytosis-like endocytosis[84,85]. This macropinocytosis-like endocytosis is dependent by Fr-95B_1 and Vn-a$_v\beta_1$ integrin and by the spore-surface collagen-like BclA3 exosporium protein[84,85]. In an *in vivo* model in mice, it has been shown that the entry of spores into intestinal epithelial cells in a dormant but reactivable state contributes to the recurrence of CDI[84,85].

Progressive colonization of human by the *C. difficile* spores, in which the latter wait for the appropriate conditions that favour transition to the vegetative form capable of induce infection and the clinical picture.

Colonization is due to *C. difficile* transmission *via* the faecal-oral route, with the spores that traverse the acidic pH of the stomach to colonize the large bowel, where remain inactive until appropriate conditions favour passage to the vegetative form[83]. The conditions that favour intestinal germination of *C. difficile* spores are an increase of primary bile acids, butyrate, disaccharide, trehalose, and other substances produced by some bacterial species taxonomically identified present in a perturbed microbiome and that favours *C. difficile* overgrowth *vs* other pathogens, and a reduction of secondary bile acids[79,80,86-91]. Of interest, these conditions have been described in patients with

IBD[92,93]. In fact, *C. difficile* colonizes and infects the colon following antibiotic-immunosuppressant-induced gut dysbiosis[9,90,94-101]. The dysbiosis also depends from other different factors such as age, type of drug used, administration of proton-pump inhibitors, types of foods, physical environment, the genetic and immune system of individuals, and concomitant pathologies (*e.g.*, diabetes, obesity, autoimmune and allergic diseases, IBD)[9,90,94-101]. These predisposing factors have progressively broadened the range of subjects susceptible to colonization/infection by *C. difficile*[9,90,94-101]. In turn, *C. difficile* colonization causes gut flora perturbations that increases dysmicrobism and inflammation, promoting CDI and CDI relapses[9,90,94-101]. Moreover, changes in normal gut microflora after a first CDI could predispose individuals to recurrent CDIs, and the protracted antimicrobic therapy for *C. difficile* can cause, in a gut microbiota already modified, further and persistent dysbiosis and inflammation.

Although following primary CDI episodes the bacterial taxa restore with time, in some subjects some taxa may not recover fully and maintain a decreased resistance to colonization. This in turn promotes the subsequent growth of pathogens (including *C. difficile*), altering the composition of the gut microbiome. The frequent use of antibiotics to treat *C. difficile* increases the pool of antibiotic-resistant genes in the gut microbiome, thereby favouring recurrent CDIs[9,90,96-103]. Although antibiotic exposure, hospitalization, advanced age and immunocompromised status increase the risk for disease, community-acquired infections in otherwise healthy young and adults not previously exposed to antibiotics are not infrequent[9,90,96-103].

The favourable conditions for *C. difficile* colonization are more widespread in "developed countries", due to the increase in antibiotic therapies[15] through all ages and changes in microbiota for various external factors[104]. Thus, a progressive increase of CDI and CDI-related deaths (at present in the range of 5%-30% with primary infection)[16] is foreseeable, with a more progressive rise of death rates following CDI relapses[105,106].

Persistent dysmicrobism, mainly due to the use of antibiotics and immunosuppressive drugs and, most importantly, a colonic environment characterized by waves of inflammatory response with a continuously active basal level.

Persistent dysmicrobism enables the overgrowth of several intestinal pathogens, including *C. difficile.* Some particularities of *C. difficile* favour its growth in an altered environment characterized by a low-grade inflammation[15,80-82,90,98-101,104], such as in subject with IBD[24-31]. For primary CDI, the changes in gut microbial flora that favour overgrowth of *C. difficile* are crucial, also compared to other various intestinal pathogens (*e.g., C. perfringens), even though* the role of gut flora in regulating *C. difficile* is more complex than previously hypothesized. Indeed, in preventing *C. difficile* colonization, disease, and recurrence, the maintenance of enough density of the species that create an environment hostile to *C. difficile* expansion by means of both changes of biomass and composition rather than the simple reduction of some taxonomic groups plays a key role[9,90,94-101]. Furthermore, *dysmicrobism* depends primary by the factors and pathologies reported above (*e.g.*, IBD)[9,90,94-101].

Continued emergence of new *C. difficile* strains that are more hypervirulent or multidrug-resistant (*e.g.*, ribotypes 015, 027, 078 or 176), many of which produce the binary toxin CDT[2-6,20,51,91,107,108].

Production of variant of Tcds by some *C. difficile* strains[51,80,91,99,102,107-111]. The expansion of CDI could be enhanced by *C. difficile* strains that produces Tcd variant[51,80,91,99,102,107-111]. In fact, many of these are hypervirulent and release the binary toxin CTD linked with enhanced morbidity and mortality[51,80,91,99,102,107-111]. CTD stimulates the formation of long cellular filaments, which become anchor points for other *C. difficile* to epithelial cells, potentiating the infection[2-6,20,51,91,107,108]. The Tcd variants are also greatly different for enzymatic activity, immunogenicity and in their receptor preference, with important implications on colonic pathology[2-6,32,51,91,107,108].

The complex equipment of surface antigens of *C. difficile* as flagella, fimbriae, pili, cell wall proteins and biofilm, which act as colonization factors or mediate innate immune responses which can play a key role on the persistence of *C. difficile*[2-6,10,32] and on the

inflammatory state with induction of pro-inflammatory cytokines such as TNF-α and IFN-g[2-6].

IMPACT THAT THE LATEST KNOWLEDGE ON THE MOLECULAR PATHOGENESIS OF C. DIFFICILE TOXINS COULD HAVE IN COLONIZATION/INFECTION IN INDIVIDUALS WITH IBD

The receptors for TcdA and TcdB

TcdB is considered the most involved in the CDI, due to its presence in most toxic strains and to its degree of pathogenicity 1000 times greater than that of TcdA[2-6]. To date, as reported above, three receptors have been identified for TcdB: CSPG4, PVRL3, and FZD[32,36-39]. TcdB binding to CSPG4 receptor in HeLa and HT29 cells induces cell rounding and apoptosis, at concentrations picomolar of TcdB, but induces necrosis at higher concentration of TcdB[32,36-39]. TcdB binding to PVRL3 receptor induces necrosis at high doses (nanomolar range) of TcdB[32,36-39]. TcdB binding to FZD receptor induces cytopathic effects and apoptosis at picomolar concentrations of TcdB, indicating that FZD function as an alternative receptor to CSPG4[32,36-39]. TcdB can bind other membrane receptors as consequences of amino acids that extend beyond the sequence CROP, considered the molecular area of TcdB most involved in the receptor binding[32,36-40] and for its properties of intrinsically disorder protein[44]. Therefore, there is a relevant heterogeneity of the receptors bound by TcdB, and of the diversity of the effects in relation to the types of receptor bound and to the concentration of TcdB[32,36-41]. However, and more importantly, the variants of TcdB are further diversified for their receptor preference with relevant implications in the pathology of CDI[2-6,32,51,91,107,108], first of all the ability to bind extremely different cell types[2-6,32], as epithelium cells the human colon[43] and nerve cells[47], EGCs[48], neurons, liver cells, and heart cells[49].

What are the elements that can therefore impact on subjects with IBD as a consequence of the heterogeneity of Tcds and of their receptors on the various cell types? The intestinal mucosa of IBD subjects is altered by the inflammation resulting

from the immune response and the dysmicrobism. Although direct data on how this altered colonic environment may modify the expression of the various receptors for TcdB are not available, it is likely that this could occur, as suggested by some *in vitro* data. Therefore, it is possible that conditions whereby a different expression of receptors for TcdB are expressed on colonic epithelial cells favouring the more necrotic effects of the TcdB could arise, characterizing the trend of infection. Moreover, even the inflammatory immune cells that try to fight infections could be induced to express receptors that lead to their cell death by necrosis. Thus, in subjects with IBD an initial CDI could quickly become more serious due to a strong inflammatory response that favours the expression of receptors for the TcdB that lead to death mainly by necrosis. All this would profoundly change the environment already altered by necrosis to favour further relapses.

It is therefore possible that the progress and severity of CDI in subjects with IBD depends on an inflammatory environment inducing the prevalent expression of receptors that favour cell necrosis. Indeed, it has been demonstrated that expression of CSPG4 it is increased by inflammatory conditions such as that induced by TNF-α and LPS[112,113].

The inflammatory response in synergy with the TcdB enhances the toxicity of TcdB

CKs release potentiates the apoptosis of EGCs treated with low doses of TcdB[48,72]; this phenomenon is relevant with profound implications *in vivo*[26,29,32,34,48,72], especially in subjects with IBD[29].

First, the enhancement of apoptosis induced by the synergism between TcdB and CKs also occurs when CKs are given to EGCs 18 h before TcdB[48]. Therefore, in an already inflamed environment, as soon as *C. difficile* begins to produce Tcds, cytotoxicity is immediately increased by the presence of pre-existing cytokines, paving the way for the progression of CDI.

Second, the cytotoxic synergism between the TcdB and CKs is triggered even three days after infection with TcdB[48,72], implying that even if at the beginning of the

infections there is no significant inflammatory response, the enhancement of cytotoxicity by cytokines can occur later.

Third, the cytotoxic synergism between TcdB and CKs at 24 h is mainly characterized by death by apoptosis[48,72], while cells surviving in the following days progressively die for apoptosis/necrosis[72]. This in contrast to the cells treated only with TcdB, that die by apoptosis at 24 h and in the following days there is only a slight increase in cell death by this mechanism[72].

Fourth, cell death induced by TcdB + CKs is characterized by the activation of three apoptotic pathways[72], with a primary role played by calpains and subsequently cathepsin B activation, which either directly or converging on the effector caspases (caspases-3 and -7) lead the cell to death, bypassing any anti-apoptotic barrier in the first 24 h[72]. Thereafter, a process of amplification of the cell death process begins in the cells who have resisted the apoptotic thrust, with consequent death by apoptosis and necrosis[72]. It is therefore clear how the cytotoxic synergism between TcdB + CKs finds in subjects with IBD a particularly favourable environment that will favour CDI, characterized by a strong cell death response by necrosis, with an increase of the mortality and major incidence of CDI relapses. These are due to the fact that, although partially restored with antibiotic therapy, the intestinal environment remains very susceptible to further CDI relapses for the characteristics of necrotic cell damage and the persistence of the inflammatory response.

Therefore, in subjects with IBD a circuit of progressive cell damage can be activated, which feeds on itself based on the elements that characterize the first event of colonization/infection by *C. difficile*, i.e., the presence of an inflammatory state that enhances the cytotoxic synergism of TcdB + CKs, with induction of apoptosis and necrosis that in turn could lead to increased expression of receptors for TcdB. The latter, based on TcdB receptors involved and TcdB concentration, will promote cell death by apoptosis and/or necrosis. Cell death causes a deeper tissue damage with a progressive increase in this loop that can only be stopped with antibiotic therapy, which adds an

additional level of inflammatory response and once the infection is resolved leaves an environment even more susceptible to relapses.

It is clear that if CDI occurs in a subject with IBD the clinical picture can progress to a more acute form for the following reasons: (1) The expression of high levels of receptors for TcdB that induce cell death by necrosis; and (2) higher levels of cytokines that enhance the development of receptors for TcdB, inducing mainly necrosis. The final result is enhancement of cell death by apoptosis/necrosis.

The above considerations could explain the difference in colonic environment during CDI in individuals with IBD and therefore the susceptibility to one or more relapses[29] (Figure 1).

Perspectives for future approaches to CDI treatment

Based on some peculiarities of the pathogenesis mechanisms of *C. difficile* in subjects with IBD, we can try to adopt new strategies to counteract CDI in these subjects, bearing in mind that to date efficient contrast strategies had strong limitations for the following reasons: It is not possible to block the endemic spread of *C. difficile* and to eradicate it in hospitals or nursing homes, which represent one of the most contaminated environments and the greatest cause of spread.

Antibiotics or treatments preventing the development of conditions that favours relapses are not available. Faecal transplantation, although effective, it is still a limited therapeutic option due to several limitations to its wider use.

Immunotherapy with monoclonal antibodies to TcdA and TcdB has yielded very limited results. Vaccination against TcdA and TcdB did not yield significant clinical and eradication results for *C. difficile*.

Therefore, assuming that the spread of *C. difficile* cannot be stopped and that subjects with IBD are one of the categories more at risk of contracting CDI, based on the most recent knowledge on the molecular pathogenesis of *C. difficile*, which methodology of interventions can we try to develop? Here we propose some approaches.

Monitor subjects with IBD for *C. difficile* with greater frequency to identify the onset of the active phase of the disease as early as possible. In subjects with active IBD, the presence of colonization by *C. difficile* because represents a serious risk will requires a strong reduction in inflammatory response before the infection begins or spreads.

In subjects already colonized with *C. difficile* and quiescent IBD, continue the microbiological monitoring by evaluating the extent of colonization over time. In case of IBD exacerbation intervene to reduce TNF-α and IFN-g levels using appropriate targeting drugs.

Due to the limited availability of specific antibiotics for CDI and the continuous emergence of antibiotic resistance, the research for alternative methods is under way. For instance, pangenomic analysis of this bacterium has revealed specific drug targets toward the core genome of *C. difficile*[114]. This, in the next future, will likely pave the road for more targeted therapeutic approaches.

In high-risk subjects it would be important to develop a selective prophylaxis and therapy based on highly specific bacteriophages for *C. difficile*. Indeed, this is becoming a hot topic, due the impending problem of multidrug-resistant bacteria. Since it is presently possible to analyse intestinal phages, this could represent a tool of paramount importance in the future development of phage therapy[115,116]. Of interest, there is the recent demonstration of the feasibility of combination phage therapy to treat infections associated with IBD[117].

CONCLUSION

The increasing worldwide diffusion of CDI represents a serious health problem, enforced by resistance of many bacterial strains to antibiotic therapy. This is particularly worrisome for some patient categories, such as aged institutionalized subjects, immunodepressed ones, IBD[118]. The latter are particularly at risk subjects, due to the basal impaired immunological status and the frequent use of antibiotics and immunosuppressant agents used for their treatment. It is therefore of paramount importance to understand the mechanisms favouring CDI in these patients, in order to

develop more targeted and effective therapeutic strategies to limit/abolish relapses, morbidity and mortality due to this infection. Today we begin to understand that *C. difficile* evolution, for the progressive colonization of humans, is using some key elements of the inflammatory response such as pro-inflammatory cytokines TNF-α and IFN-g to favour a necrotic trend of cell death which increases the inflammation and the expression of TcdB receptors that promote necrotic cell death. It is absolutely mandatory to find prophylactic and therapeutic methodologies to antagonize this negative alliance between *C. difficile* and inflammatory response. This is important not only to protect patients with IBD, but also to prevent that in other risk categories this process of pathological self-supply between TcdB and CKs, which could be at the basis of relapses and mortality, gets active.

Figure 1 Causes of increased susceptibility and increased cytotoxic effect of *Clostridioides difficile* in subjects with inflammatory bowel disease. CDI: *Clostridioides difficile* infection; CK: Cytokines; EGC: Enteric glial cells; IBD: Inflammatory bowel disease; IFN-γ: Interferon-gamma; TcdB: Toxin B of *Clostridioides difficile*; TNF-α: Tumour necrosis factor-alpha.

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