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PERSPECTIVES

Clostridioides difficile Infection in Patients with Inflammatory Bowel Disease May be Favoured by the Effects of Proinflammatory Cytokines on the Enteroglial Network

Gabrio Bassotti[1,2]
Alessandro Fruganti[3]
Giovanni Maconi[4]
Pierfrancesco Marconi[5]
Katia Fettucciari[5]

[1]Department of Medicine and Surgery, Gastroenterology, Hepatology & Digestive Endoscopy Section, University of Perugia, Perugia, Italy; [2]Gastroenterology & Hepatology Unit, Santa Maria della Misericordia Hospital, Perugia, Italy; [3]School of Biosciences and Veterinary Medicine, University of Camerino, Macerata, Italy; [4]Department of Biomedical and Clinical Sciences, Gastroenterology Unit, "L. Sacco" Hospital, University of Milano, Milano, Italy; [5]Department of Medicine and Surgery, Biosciences & Medical Embryology Section, University of Perugia, Perugia, Italy

Abstract: *Clostridioides difficile* infection is widespread throughout countries and represents an important cause of nosocomial diarrhoea, with relatively high morbidity. This infection often occurs in patients with inflammatory bowel diseases and may complicate their clinical picture. Here, we propose, on the basis of evidence from basic science studies, that in patients affected by inflammatory bowel diseases, this infection might be facilitated by a derangement of the enteric glial cell (EGC) network caused by the effects of proinflammatory cytokines, such as tumour necrosis factor alpha and interferon gamma, which enhance the cytotoxic effects of *C. difficile* toxin B on EGCs. This hypothesis, if confirmed, could open the door to alternative treatment approaches to fight *C. difficile* infection.

Keywords: *Clostridioides difficile* infection, cytokines, inflammatory bowel diseases, interferon gamma, tumour necrosis factor alpha

Introduction

One of the most widespread health-care-associated infections worldwide is that induced by *Clostridioides* (formerly *Clostridium*)[1] *difficile*.[2–4] In fact, in 2012, in the United States, 500,000 individuals were infected per year, with approximately 29,000 deaths, and in Europe, there were approximately 124,000 cases, with an overall mortality of 3–30%. *C. difficile* infection (CDI) represents 15–25% of all opportunistic gastrointestinal infections,[2–5] and the infection can be acquired after antibiotic exposure or hospitalization but also in the community in individuals who are younger, have a lower risk of antibiotic exposure and have not been hospitalized.[2–5] Recently, an increasing incidence of CDI has been demonstrated, especially of community-acquired CDI.[2–5] Many patients who get community-acquired CDI have underlying inflammatory bowel disease (IBD) and fit the demographic and risk factor profile for CDI.[2–5] The rate of CDI cases occurring in IBD patients has increased more than 4-fold in recent years.[2–5] A serious complication of CDI is a high risk of recurrence, with a recurrence rate of 20–25% after the first episode and up to 60% after the third episode.[2–5]

CDI is an important cause of nosocomial/antibiotic-associated diarrhoea, with clinical manifestations ranging from asymptomatic carriage or mild self-limiting diarrhoea to colitis without pseudomembrane formation to pseudomembranous

Correspondence: Gabrio Bassotti
Department of Medicine and Surgery, Gastroenterology, Hepatology & Digestive Endoscopy Section, University of Perugia, Perugia, Italy
Email gabassot@tin.it

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colitis and fulminant colitis,[2–5] and the severity of CDI is likely not affected by other coinfections.[6] Nosocomial/antibiotic-associated diarrhoea is defined as diarrhoea occurring between 2 hours and 2 months after the use of antibiotics and is frequently accompanied by abdominal pain and cramps.[2–5,7] The symptoms of colitis without pseudomembrane formation include watery diarrhoea, possible presence of trace blood in the stool, nausea, abdominal pain, malaise, anorexia, low-grade fever, dehydration, pyrexia, and leucocytosis.[2–5,7] Clinical manifestations of PMC include watery diarrhoea, abdominal cramps, dehydration, hypoalbuminemia, and rising inflammatory cells, serum proteins, and mucus. Furthermore, following sigmoidoscopic examination, plaques are observed in the colorectal mucosa and sometimes in the terminal ileum.[2–5,7] Fulminant colitis, which occurs in approximately 3% of CDI patients, accounts for most serious complications, including perforation, prolonged ileus, megacolon, and death.[2–5,7] CDI is not limited to the colon because extracolonic manifestations have been reported, and the clinical manifestations of disease include small bowel disease with the formation of pseudomembranes on the ileal mucosa, bacteraemia, reactive arthritis, visceral abscess, appendicitis, intraabdominal abscess, osteomyelitis, and empyema.[8,9] 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*.[2–5,7] CDI can be classified as endogenous or exogenous.[2–5,7] Endogenous infection originates via carrier strains, whereas exogenous infection occurs through infected individuals, contaminated health care workers, nosocomial sources, and contaminated environments.[2–5,7] *C. difficile* is spread via the oral-faecal route.[2–5,7] *C. difficile* is acquired by oral ingestion of spores that are resistant in the environment, are tolerant to the acidity of the stomach and have a process of spore germination that is both complex and unique.[2–5,7,10] Then, after the acquisition of *C. difficile* from an exogenous source, in the small intestine, the disruption of the normal gastrointestinal microbiome together with other factors, such as advanced age, the genetic and immune system of the host, the virulence of the *C. difficile* strain, the nature and extent of antimicrobial exposure, the use of proton-pump inhibitors (PPIs), the types of foods consumed, medication use, physical environment, obesity, renal disease, hypoalbuminemia, immune system impairment, autoimmune and allergic diseases, diabetes, and IBD, lead to diminished colonization resistance, which favours the germination of the ingested spores to the vegetative form, which then favours the colonization of *C. difficile* in the large intestine.[2–5,7,10–14] Subsequently, bacterial growth, multiplication, and toxin production damage enterocytes in the intestinal crypts.[2–5,7,10–14]

Several studies have reported the importance of CDI in IBD.[7,15–17] *C. difficile* has also been reported to be involved in the exacerbation of ulcerative colitis (UC).[7,15–17] This made it necessary to routinely evaluate CDI status in patients with severe IBD, especially before initiating further immunosuppressive therapy.[7,15–17] Recurrent CDI is one of the most challenging aspects of CDI that occurs either due to relapse or reinfection.[7,15–17] The main cause of recurrent CDI seems to be the disturbance of the normal bowel microflora and a defective immune response against *C. difficile* and/or its toxins (Tcds).[7,15–17]

CDI develops following intestinal colonization and the production of two large glucosylating toxins, *C. difficile* toxin A (TcdA) and toxin B (TcdB),[3,9,18–21] with subsequent disruption/modulation of the host microbiome.[22–24] TcdA and TcdB are the major virulence factors of *C. difficile* and contribute to its pathogenicity by inducing mucosal inflammation and diarrhoea.[3,4,18–21] However, *C. difficile* may produce several other presumed virulence factors, including *C. difficile* transferase toxin, fibronectin binding protein, fimbriae, and adhesins, which could contribute to the persistence of *C. difficile* by promoting adhesion and the penetration of *C. difficile*, antagonizing the natural immune response and contributing to an inflammatory state with cytokine induction.[3,4,7,18–21] In vivo, after the adhesion of the *C. difficile* spore to the surface of the intestinal epithelial cells and in the presence of conditions that favour germination,[2–5,7,10–14] *C. difficile* begins to synthesize and secrete TcdA and TcdB, which, after translocation to the cytosol of target cells, inactivate Rho-GTPases, including Rho, Rac, and Cdc42.[3,18–21] In vivo, TcdA and TcdB alter the cell cytoskeletal structure, disassemble focal adhesions, disrupt epithelial tight junctions and cause cell death.[3,4,18–21] These events provoke direct injury to intestinal epithelial cells, thus favouring the penetration of *C. difficile* in the deeper layers of the intestinal mucosa and the further penetration of Tcds, which progressively can reach myofibroblasts, submucosal cells, enteroglial cells (EGCs), resident immune cells (mast cells) or recruited immune cells (macrophages and polymorphonuclear cells) following the inflammatory response.[3,4,18–21]

Then, Tcds stimulate colonic epithelial cells and resident or recruited immune cells to release proinflammatory cytokines, chemokines and neutrophil chemoattractants, leading to an amplification of the acute inflammatory response, which is a main characteristic of the clinical picture of CDI.[3,4,18–21] The altered barrier and active inflammation increase intestinal and vascular permeability, which favours the entry of Tcds and/or bacteria into the lamina propria, resulting in an amplification of intestinal inflammation,[3,4,18–21] and finally, Tcds can reach neuronal cells and EGCs. In vitro, TcdA and TcdB inactivate Rho-GTPase leading to a loss of cytoskeletal structure, cell rounding, cell cycle arrest (cytopathic effects) and cell death for early necrosis or apoptosis (cytotoxic effects).[3,4,18–21] Furthermore, TcdA and TcdB can stimulate several cell types (intestinal epithelial cells, immune cells, neurons) to secrete cytokines and chemokines.[3,4,18–21]

TcdA elicits effects primarily within the intestinal epithelium, while TcdB has a broader cell tropism.[3,18–21] Tcds induce cytopathic and cytotoxic effects on enteric cells (enterocytes, colonocytes, enteric neurons), immune cells, hepatic cells, nervous system cells, cardiac cells, and colon/pancreas tumour cell lines.[3,4,18–21] The effects of Tcds on mucosal and submucosal cell types in vivo and in vitro have been widely investigated for many years,[4,18–21] and our studies have contributed to the knowledge of the effects of TcdB (approximately 1000 times more potent than TcdA) on EGCs in vitro.[25] We recently demonstrated that TcdB induces cytopathic and cytotoxic effects in a dose- and time-dependent manner in EGCs.[25] In fact, TcdB induces the following in EGCs:[25] a) early Rac1 glucosylation; b) early cell rounding; c) cell cycle arrest, mediated by an upregulation of p27 and an inactivation of the cyclin B1/Cdc2 complex; and d) late apoptosis, mediated by a caspase-dependent but mitochondria-independent pathway, as demonstrated by early caspase-3 and PARP activation with later caspase-7 activation and ROCK1 overexpression but without alterations in pro- and antiapoptotic Bcl-2 family protein expression.[25] We have also demonstrated that TcdB in EGCs does not significantly affect ATP levels and mitochondrial functionality but activates caspase-3, confirming that TcdB-induced EGC apoptosis is mitochondria-independent and caspase-3-dependent.[26] Moreover, we also demonstrated that apoptosis is mediated through the ROS/JNK/caspase-3 axis; indeed, caspase-3 activation and apoptosis were reduced, inhibiting ROS production with ML171 or N-acetylcysteine or JNK activation with SP600125 (a JNK inhibitor).[26]

Furthermore, we also demonstrated that ECGs surviving the apoptosis caused by TcdB at 10 ng/mL acquire a state of senescence characterized by: a) irreversible cell cycle arrest, induced by an overexpression of p27, hypophosphorylation of phospho-RB, and downregulation of c-Myc, cyclin B1, cdc2 and phosphorylated-cdc2; b) flat morphology; c) positivity for senescence-associated-β-galactosidase; d) early and persistent DNA damage, indicated by ATM and H2AX phosphorylation; and e) an overexpression of Sirtuin-2 and Sirtuin-3.[27] TcdB-induced EGC senescence is JNK and AKT activation dependent but ROS, p16 and p53/p21 independent.[27] The senescence state of EGCs, in addition to altering functionality, can represent a potential preneoplastic stimulus danger to the surrounding cells.[25,27] This risk of senescence may be of some relevance in individuals with IBD, in which one or more recurrent CDIs, if accompanied by the formation of senescent EGCs, could contribute to the higher incidence of tumours reported.

Furthermore, our studies have highlighted an important phenomenon of an enhancement of the toxic effects of TcdB on EGCs through the proinflammatory cytokines tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ).[25] The proinflammatory cytokines TNF-α and IFN-γ, which alone are not cytotoxic against EGCs but only slightly cytostatic, given before (−18 h), concomitantly or after (+2 h) exposure to TcdB at low doses (0.1 ng/mL and 1 ng/mL), enhance the apoptosis of EGCs induced by TcdB, which is characterized by an increase in caspase-3, caspase-7, caspase-9 and PARP activation without any involvement of the Bcl-2 family.[25] This synergistic cytotoxic activity of TcdB with proinflammatory cytokines led us to hypothesize that even at low doses of TcdB, these cytokines might play a pathogenetic role in CDI and in its relapses.[8,28,29]

This synergism, which can be important in CDI, especially in the early stages of infection when the level of TcdB produced is still low, can become a relevant factor in patients with IBD with a persistent low level of inflammation, characterized by the presence of proinflammatory cytokines, including TNF-α and IFN-γ. Therefore, in individuals with IBD with persistent inflammation and frequent CDI relapses, the cytotoxic synergism between TcdB and the proinflammatory cytokines TNF-α and IFN-γ could contribute to the progressive disruption of

the EGC network and reduce the functionality of these cells.

It is now well recognized that IBD patients are at a high risk of developing CDI even in the absence of other traditional risk factors.[7,15–17] Therefore, persistent inflammation could contribute to the greater susceptibility to CDI in individuals with IBD.

As also reported above, among the risk factors for the occurrence of CDI are comorbidities, such as the known types of IBD, UC and Crohn's disease (CD).[7,15–17,30] IBD patients are often immunocompromised by the treatments used to control the disease (steroids, thiopurines, biological agents, etc.),[31] and CDI may complicate their clinical picture,[32] prolong hospitalization periods and increase surgical rates.[33] Of interest, experimental studies have shown that mice with IBD are more susceptible to CDI and that this infection provokes more severe disease and death.[34]

Several cytokines are consistently upregulated in IBD patients;[18,35–39] in particular, it is well known that the intestinal inflammatory state of both UC and CD patients is associated with increased levels of the proinflammatory cytokines TNF-α,[40] which correlates with disease activity and severity,[41,42] and IFN-γ.[43,44] This inflammatory state associated with IBD is present along with the strong inflammatory response provoked by CDI.

The recurrence of CDI has thus far been attributed to the persistence of the spores after antibiotic therapy or to reinfection after therapy in individuals who have a profoundly altered microbiome, the primary cause of CDI, which remains altered even after temporary cessation of antibiotic therapy.[2–5,7,10–12] An important new feature of *C. difficile* has recently been demonstrated, which further underlines the complex strategy by which it interacts with the host: the ability of *C. difficile* spores to adhere to gut epithelial cells and to penetrate them via a process of macropinocytosis-like endocytosis.[11,12] This entry pathway is Fr-95B_1 and Vn-$\alpha_v\beta_1$ integrin dependent, and the spore-surface collagen-like BclA3 exosporium protein plays a key role in the pathway.[11,12] In an in vivo model in mice, it was thus shown that the entry of spores into intestinal epithelial cells in a dormant but reactivable state contributes to the recurrence of CDI.[11,12] Taken together, this evidence in individuals with IBD highlights how several factors can contribute to the recurrence of relapses: a) persistent dysmicrobism, b) a persistent proinflammatory cytokine response, c) the reactivation of spores within epithelial cells, and d) cytotoxic synergism between TcdB and the proinflammatory cytokines TNF-α and IFN-γ. The recurrence of this phenomenon, even in a clinically more attenuated form, can lead to the progressive loss of EGCs and their reduced functionality. It must be kept in mind that, unlike other types of intestinal cells, which can be regenerated after cell death, this does not happen with EGCs; therefore, progressive dysfunction within the EGC functional network will be inevitable.

The Key Role of Proinflammatory Cytokines During CDI on the Enteric Glial Cell (EGC) Network: A Working Hypothesis

We recently assessed the in vitro effects of TcdB on EGCs, a cell population that plays a pivotal role in several gut functions, such as mucosal permeability, secretion, perception, motility, and neurotransmission,[45,46] and is likely to play an important pathophysiological role in IBD.[47–52] TcdB caused time- and dose-dependent cytopathic and cytotoxic effects on ECGs.[25] In fact, as reported above, TcdB causes cell rounding, Rac1 glucosylation, cell cycle arrest and cytotoxic effects by apoptosis in a time- and dose-dependent manner.[25] Apoptosis is mediated by a caspase-dependent but mitochondria-independent pathway.[25,26] In fact, we demonstrated that TcdB induces caspase-3, caspase-7 and PARP activation without alterations in pro- or antiapoptotic Bcl-2 family protein expression,[25] and Macchioni et al[26] confirmed that TcdB-induced EGC apoptosis is mitochondria-independent and mediated through the ROS/JNK/caspase-3 axis.[26]

Furthermore, we demonstrated that EGCs surviving the cytotoxic effects of TcdB undergo cellular senescence phenomena (irreversible cell cycle arrest, flat morphology, positivity for senescence-associated β-galactosidase, early and persistent DNA damage, an overexpression of Sirtuin-2/Sirtuin-3, JNK and AKT activation), with increasing GDNF production but persistent impairment of cell functions (persistent Rac1 glucosylation, cell cycle arrest, and resistance to apoptosis).[25,27] It is worth noting that the stimulation of EGCs with TNF-α and IFN-γ before (−18 h), concomitantly or after (+2 h) exposure to TcdB (0.1 ng/mL; 1 ng/mL), at doses of cytokines not affecting control cells, provoked a strong increase in TcdB-induced apoptosis.[25] In fact, we found that when EGCs were exposed to TNF-α plus IFN-γ 18 h before TcdB treatment, the percentage of apoptotic cells increased by approximately 3.3-fold with 0.1 ng/mL TcdB and 1.8-fold with 1 ng/mL TcdB, and when EGCs were exposed to TNF-α plus

IFN-γ 2 h after, 2 h before and concomitantly with TcdB treatment, the percentage of apoptotic cells increased by approximately 2.6-fold in EGCs treated with 0.1 ng/mL TcdB and by approximately 1.4-fold in EGCs treated with 1 ng/mL TcdB compared with TcdB-treated EGCs not stimulated with cytokines.[25] We also demonstrated that this synergistic increase in apoptosis was due to increased activation of caspase-3, caspase-7, caspase-9 and PARP without any involvement of Bcl-2 family members; indeed, Bax and Bcl-X_L expression was not significantly changed. Finally, we demonstrated that Rac1 glucosylation by TcdB persisted and was not affected by cytokines.[25] This synergistic cytotoxic activity of TcdB plus TNF-α and IFN-γ led us to hypothesize that even at low doses of TcdB, these cytokines might play a pathogenetic role in CDI and relapse.[8,28,29] Conditions that can be relevant in pathological gut conditions which are characterized by a persistent inflammatory picture, such as that featured in IBD. Therefore, the increased susceptibility of these patients to CDI might also be related to the synergistic cytotoxic effect between TcdB and proinflammatory cytokines on EGCs, in addition to the effect on other targets. However, since IBD patients often present important abnormalities of the gut microbiome,[22–24] although to date, the pivotal role of IBD in increased sensitivity to CDI has been evaluated,[24,53] we now, instead, must also consider the important role of the inflammatory environment that characterizes individuals with IBD in susceptibility to CDI.

The key functions controlled by EGCs, such as mucosal permeability, secretion, perception, motility, and neurotransmission, justify the fact that abnormalities of this cell population are thought to play important pathophysiological roles, and particularly if alterations in EGCs are due to CDI, they can have important pathophysiological roles in the maintenance and/or progression of IBD.[47,50–52]

The progressive functional alteration of the EGC network following recurrent CDI could have relevant consequences when taking into account what functions perform this type of cell. EGCs are important cells of the enteric nervous system (ENS) that contribute to the modulation of inflammatory responses in the human gut.[45,46] EGCs possess several neurotrophic and neuroimmunomodulatory properties.[45,46] Under physiological conditions, EGCs, as reported above, regulate mucosal permeability, secretion, perception, motility, neurotransmission and host defence and play an important role in the maintenance of gut homeostasis and in the modulation of enteric neuronal activities.[45,46] Under pathological conditions, EGCs trigger and promote chronic inflammation in the intestinal mucosa since these cells release mediators that increase inflammation and are able to induce chronic inflammatory changes in the gut mucosa.[47–52,54] Such a detrimental loop is responsible for the substantial recruitment of other target cells, including immune cells. In fact, EGC-derived S100B is able to affect peripheral macrophages and intestinal mucosal immune cells. Furthermore, there is mounting evidence that EGCs might also be critically involved in IBD.[28,47–52,54] ENS alterations, characterized by apoptotic bodies of neurons and glia,[48] especially in submucosal plexi, are commonly observed in human IBD, and they have been postulated to play a fundamental role in the occurrence of disorders of intestinal motility and/or secretion.[47–52,54] In fact, in addition to the well-known involvement of macrophages and neutrophils, other cell types have been reported to substantially contribute to the onset and progression of IBD.[47–52,54] A better understanding of the molecular mechanisms underlying EGC dysfunction might constitute a new approach to increase the efficacy of new EGC-oriented drugs that may overcome the lack of long-term effectiveness of immunosuppressant agents used for IBD.

Thus, in IBD patients, the summation of several pathogenic *noxae*, including abnormalities of the immune system, intestinal dysmicrobism, and the "cytokine storm", may synergically act to induce or aggravate the disruption of the EGC network reported in these patients.[28] Of interest, the loss of EGCs might be increased by rapid enteric neurogenesis from glial progenitors, as shown in experimental animal models of colitis.[55]

Abnormalities of EGCs are likely to influence mucosal permeability, favouring both CDI and its relapses. Indeed, proinflammatory cytokines could play a pivotal role in these instances due to the extreme susceptibility of EGCs to TcdB when pretreated with very low doses of TNF-α and IFN-γ.[25] Therefore, it is possible that in IBD patients, even low bacterial loads of *C. difficile* might induce an infection and, due to the persistent inflammatory status often observed in these patients, also favour relapses. Moreover, the recurrence of CDI has thus far been attributed to the persistence of spores after antibiotic therapy or to reinfection after therapy in individuals who have a profoundly altered microbiome, the primary cause of CDI, which remains altered even after temporary cessation of antibiotic therapy.[2–5,7,10–12] However, the ability of *C. difficile* spores to adhere to gut epithelial cells and to

penetrate them via a process of macropinocytosis-like endocytosis and the ability of *C. difficile* spores to persist in a dormant but reactivable state could contribute to the recurrence of CDI.[11,12] Taken together, this evidence in individuals with IBD highlights how several factors can contribute to the recurrence of relapses, namely, persistent dysmicrobism, persistent proinflammatory cytokine response, a reactivation of the spores within the epithelial cells, cytotoxic synergism between TcdB and the proinflammatory cytokines TNF-α and IFN-γ. The recurrence of this phenomenon, even in a clinically more attenuated form, can lead to the progressive loss of EGCs and their reduced functionality.

Targeting Cytokines: A New Road to Fight CDI in IBD?

The above considerations suggest that therapeutically targeting proinflammatory cytokines might be a way to fight CDI in IBD patients. Indeed, immune profiling in these patients is being explored as a possible means for more focused interventions.[56,57]

An important implication of our in vitro results, namely that TNF-α and IFN-γ enhanced the cytotoxic effects of TcdB, should be that the reduction of TNF-α in vivo in patients with IBD is accompanied by a reduction in the incidence of CDI and recurrences.

Until now there are no studies that have directly addressed this issue, however a series of in vitro and in vivo results suggest the existence of this relationship.

Fidaxomicin (FDX) and its primary metabolite, OP-1118, inhibit TcdB-induced TNF-α production and histological damage in human colonic explants, thereby reducing TcdB cytotoxicity.[58] FDK and OP-1118 reduced TNF-α synthesis by blocking NF-kB activation.[58]

In a study on 629 patients with acute symptoms of CDI, FDX reduces recurrences more than vancomycin.[59]

Furthermore, in another study on 81 patients with IBD, all patients after a first episode of CDI treated with FDX had no recurrences.[60]

However, these results do not allow to identify which contribution has the greatest efficacy towards bacteria Gram+ anaerobic and the reduction of the inflammatory response by FDX.

Tcds are present in the serum in patients with IBD and the positive index for circulating TcdB is higher in those with active IBD than in those in remission and in controls. The treatment of patients with IBD with Infliximab (IFX) reduces TcdB levels in the sera as if reducing TNF-α decreases CDI.[61] This can be explained by the fact that IFX therapy can restore the gut epithelial barrier and thus block TNF-α induced inflammation.[61]

Based on our hypothesis, however, the neutralization of TNF-α would prevent the cytotoxic synergism between cytokines and the TcdB reducing or strongly attenuating its cytotoxic effects.

A further confirmation that IFX reduces the incidence of CDI occurs in a study on 521 patients with IBD where there is a clear-cut reduction in CDI compared to other treatments with corticosteroids or other immunosuppressive agents.[62]

In a study on 319 patients with IBD, in which 9% develop CDI, the genetic risk of developing IBD is associated with 6 genetic polymorphisms that are correlated with an increased risk of developing CDI.[63] What's most important, patients undergoing anti-TNF-α biologic therapy have a lower risk of developing CDI,[63] ie, anti-TNF-α therapy is protective against CDI. The proposed explanation is that anti-TNF-α therapy, reducing this cytokine, promoting mucosal healing, as assessed with the endoscopy, decreases the likelihood of disrupting the microbiome and thus lowering the risk of CDI.[63,64]

Although indirectly, the explanation of these results, according to our in vitro studies, is that the reduction/neutralization of TNF-α reduces inflammation and antagonizes the cytotoxic synergism between this proinflammatory cytokine and TcdB with the consequent reduction of both cytotoxicity at the level of the epithelium of the gut mucosa and inflammation, as well as of the risk of the amplification of a new CDI or recurrence.

The anti-TNF-α regimen is not effective in reducing CDI in patients with IBD, on the contrary it increases the CDI risk when combined with other immunosuppressive drugs, probably due to the summation of suppressive effects on the immune system.[65,66]

Furthermore, the combinations of IFX with antibiotics increase of 4-fold the incidence of CDI in patients with IBD, likely because of the decrease of TNF-α levels and therefore of antimicrobial resistance summed to further dysmicrobism induced by antibiotics.[16]

Certainly, a study of correlation in patients with IBD between the levels of TNF-α, their persistence over time and the incidence of CDI and relapses would be important, to help clarify these aspects.

In light of the fact that in patients with IBD, the possible persistence of *C. difficile* spores inside the epithelial cells of the colonic mucosa because they are not

eradicated by antibiotic therapy or because they are introduced from the outside into a patient with a persistent inflammatory state suggests that trying to reduce the levels of circulating TNF-α, a factor that predisposes and favours the onset of CDI, may be useful in these patients.

This preventive approach is relevant to prevent recurrences of CDI, even of low intensity, which progressively and irreversibly alter the functionality of the EGC network, which contributes to increasing the susceptibility to recurrent CDI.

Conclusion

C. difficile, an anaerobic Gram-positive spore-forming bacterium, can be acquired by exogenous (via the orofecal route) or endogenous sources. In susceptible hosts, the spores germinate into vegetative forms of *C. difficile*, through which the overgrowth and the production of TcdA and TcdB lead to inflammation. TcdA and TcdB by disruption of cytoskeletal structure leads to alterations of cell shape, cell adhesions and epithelial tight junctions and causes cell death and injury to the colonic mucosa, which favour the passage of Tcds into deeper layers of the mucosa that contain immune cells and EGCs (Figure 1A). In vitro Tcds inactivating Rho-GTPase lead to a loss of cytoskeletal structure, cell rounding, cell cycle arrest (cytopathic effects) and cell death for early necrosis or apoptosis (cytotoxic effects) in epithelial intestinal cells, as well as immune cells (Figure 1A). TcdB also induces cytopathic effects and cytotoxic effects (apoptosis) on EGCs, and the proinflammatory cytokines TNF-α and IFN-γ enhance the cytotoxic effects of TcdB on EGCs. Furthermore, EGCs that survive the cytotoxic effects of TcdB undergo senescence (Figure 1A). The clinical manifestations of CDI range from asymptomatic colonization and mild-moderate illness, to severe diarrhoea and

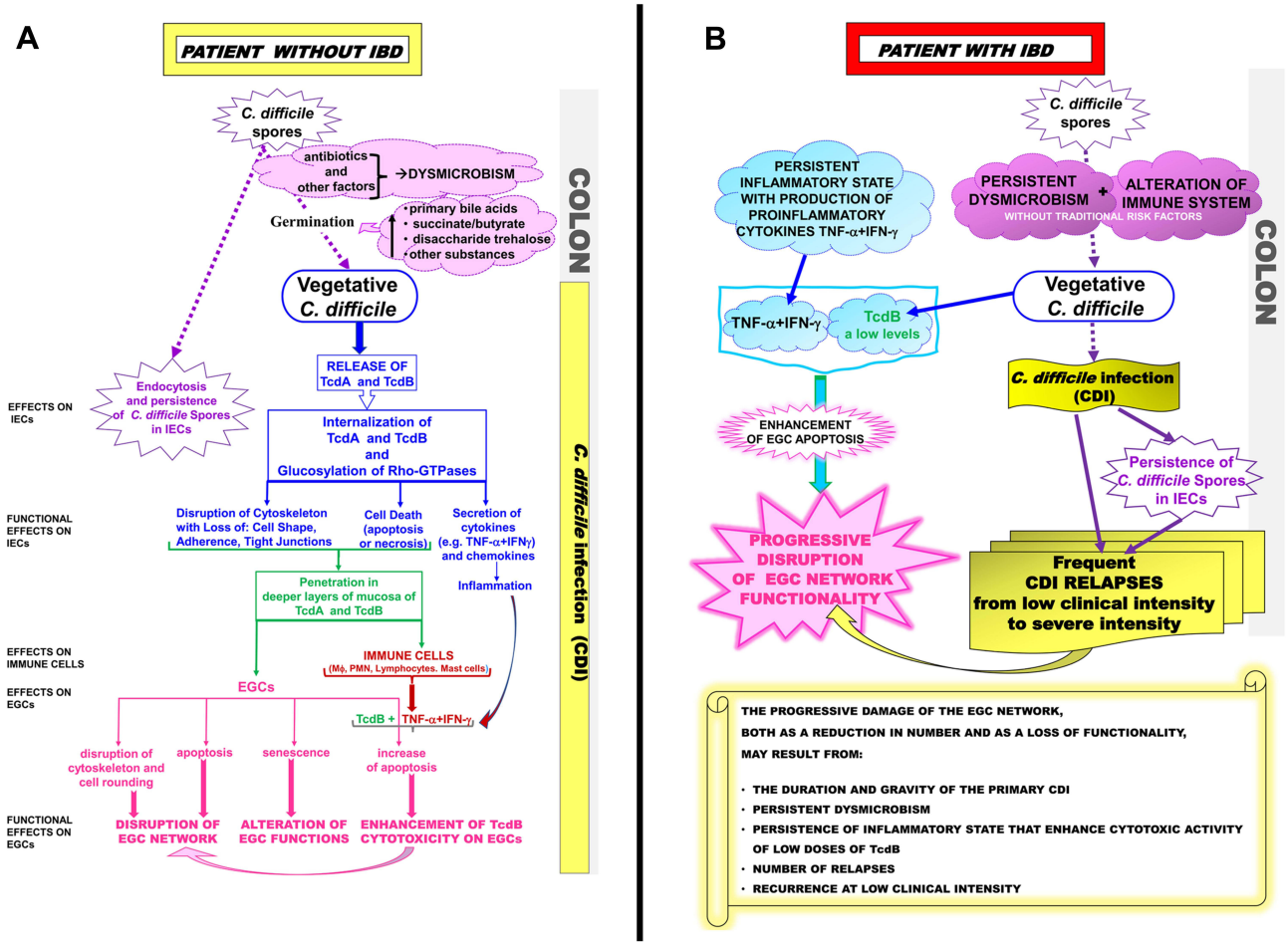

Figure 1 Schematic description of the events of CDI in patients without IBD and in patients with IBD. (**A**) Events of CDI in patient without IBD. (**B**) Events of CDI in patient with IBD.
Abbreviations: CDI, *C. difficile* infection; TcdA, *C. difficile* toxin A; TcdB, *C. difficile* toxin B; TNF-α, tumour necrosis factor-alpha; IFN-γ, interferon-gamma; EGCs, enteric glial cells; IECs, intestinal epithelial cells.

fulminant life-threatening infection. A serious complication of CDI is a high risk of recurrence.

Patients with IBD, including those with CD and UC, have increased CDI rates, recurrences, and disproportionately higher morbidity and mortality than CDI patients without IBD. Traditional risk factors for CDI (eg, antibiotic use, hospitalization, advanced age, PPI use, severe underlying illness, anti-neoplastic chemotherapy, and immunosuppressants) may not be found in many IBD patients. Furthermore, recurrent CDI is more common in IBD patients than in patients without IBD.

The most recent findings on the molecular mechanisms of *C. difficile* pathogenicity and spore biology may have profound new implications for CDI in patients with IBD and suggest new methodologies to reduce their incidence and pathological consequences.

The newly acquired knowledge and the possible links with CDI pathology in IBD patients can be summarized as follows (Figure 1B):

First, EGCs are susceptible to the cytotoxic effects of TcdB in vitro. It is therefore foreseeable that even in vivo, during the phase in which infection spreads to the submucosal tissue, EGCs are affected by the toxic effects of TcdB.

Second, the proinflammatory cytokines TNF-α and IFN-γ potentiate the cytotoxic effects of TcdB on EGCs, and this effect is more evident at relatively lower doses of TcdB.

Third, EGCs that survive the cytotoxic effects of TcdB acquire a state of senescence that causes altered functionality and thus the potential to favour the onset of IBS, IBD and tumours.

Fourth, *C. difficile* spores can survive inside the epithelial cells of the colon.

Therefore, in vivo, the most relevant effects of TcdB on EGCs could be the decrease in EGC number due to cytotoxicity and the transformation of many of the surviving EGCs with reduced or a loss of function into senescent cells. Either of these two events has profound consequences on the functionality of the EGC network (Figure 1B).

These new insights into the effects of TcdB on EGCs (Figure 1A) may have important implications for individuals with IBD who are more susceptible to CDI and recurrence (Figure 1B).

In fact, it is important to note the following:

A) IBD patients are characterized by profound alterations of the microbiota and the immune system that contribute significantly to the increased susceptibility to CDI and recurrences. These patients are also characterized by a persistent inflammatory state, including the production of proinflammatory cytokines such as TNF-α and IFN-γ, which represents the first important link with the results that demonstrate how these cytokines increase the cytotoxic effects of TcdB. Thus, in patients with IBD, a mild CDI may be exacerbated due to the cytotoxic synergism between TcdB and proinflammatory cytokines.

B) Spores remaining protected within the epithelial cells of the colon favour the onset of new CDI, even in the absence of reinfections. Therefore, the possibility of a new CDI depends not only on the causes thus far well known, such as non-eradication after antibiotic therapy or reinfection, but also on the endocellular persistence of *C. difficile*.

C) The persistent state of inflammation is added to the known conditions that favour CDI in patients with IBD, namely, the alteration of the microbiota and the reduced functionality of the immune system.

D) The increased numbers of CDI episodes and relapses cumulatively increase the damage to EGCs, which are progressively reduced in number due to the cytotoxic effects of TcdB, and most of the EGCs that survive to the cytotoxic effects of TcdB reduce their functionality due to the transition to a senescent state.

E) All this implies that in patients with IBD, CDI can progressively reduce the functionality of the EGC network, with serious consequences for the intestinal physiology regulated by this network, thus contributing to a greater susceptibility to reinfections or relapses.

If the persistent inflammatory state in patients with IBD, even if modulated over time, favours CDI, then it is possible to reduce CDIs and recurrences by reducing TNF-α levels in the circulation whenever there are significant increases compared to what may be considered baseline levels in patients with IBD.

In support of this, there are in vitro results that clearly demonstrate that the cytotoxic synergism between TcdB and TNF-α plus IFN-γ is dependent on the dose of cytokines but not dependent on the time of the addition of cytokines.

Therefore, the monitoring of TNF-α levels in patients with IBD and their reduction if certain levels are exceeded also have relevant clinical importance in mitigating the more general toxic effects of TNF-α.

Abbreviations

CDI, *Clostridioides difficile* infection; IBD, inflammatory bowel disease; UC, ulcerative colitis; Tcds, *Clostridioides difficile* toxins; TcdA, *Clostridioides difficile* toxin A; TcdB, *Clostridioides difficile* toxin B; EGCs, enteric glial cells; TNF-α, tumour necrosis factor-alpha; IFN-γ, interferon-gamma; CD, Crohn's disease; FDX, fidaxomicin; IFX, infliximab.

Author Contributions

All authors made a significant contribution to the work reported, whether in the study conception, design, and execution, data acquisition, analysis and interpretation or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval to the version to be published; agreed on the journal to which the article has been submitted; and agreed to be accountable for all aspects of the work.

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