







# Proinflammatory Cytokines: Possible Accomplices for the Systemic Effects of *Clostridioides difficile* Toxin B

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**Abstract:** *Clostridioides difficile* infection (CDI) has a serious impact on the healthcare system, and most of its pathogenic effects are mainly due to the activity of toxins A and B (TcdA and TcdB, respectively). The molecular mechanisms of their cytotoxic activity are well known, especially in the colon, where the infection occurs and normally remains localized. However, the mechanisms causing toxic effects on various systemic organs (extraintestinal manifestations) with frequent lethal outcomes in some patients affected by CDI are still poorly understood. Few studies are available that demonstrate low serum levels of Tcds in both experimental animal models and patients with CDI. Until now, it has remained unclear how low levels of circulating Tcds could lead to serious toxic effects. On the basis of our previous in vitro studies, in which the proinflammatory cytokines TNF-alpha and IFN-gamma strongly potentiated the toxic activity of low doses of TcdB, we hypothesize that the presence of both TcdB in the circulation and a systemic proinflammatory cytokine storm may be responsible for the selective severe effects of TcdB in some patients. This may occur in patients with severe CDI and systemic Tcds, in whom proinflammatory cytokines such as TNF-alpha and IFN-gamma reach a significant concentration in the circulation. This hypothesis could identify therapeutic interventions based on the reduction or neutralization of the indirect toxic action of these cytokines.

**Keywords:** *Clostridioides difficile*, toxin B, proinflammatory cytokines, TNF-alpha, IFN-gamma, systemic effects

## Introduction

*Clostridioides*<sup>1</sup> *difficile* infection (CDI) represents an important burden on the healthcare systems of both Eastern and Western populations<sup>2,3</sup> as a major cause of iatrogenic diarrhoea, and it is increasingly present within the community.<sup>4</sup> The clinical picture of CDI is mutable and mainly involves the gastrointestinal tract, varying from asymptomatic carrier status to life-threatening diarrhoea.<sup>5</sup> However, the effects of CDI may sometimes become systemic with extraintestinal manifestations (liver, lung, kidney, cardiac, and neurological impairment) and multiorgan dysfunction syndrome with a clinical picture of toxemia.<sup>6-15</sup> Of interest, recurrent extraintestinal manifestations are also correlated with CDI relapse.<sup>16</sup>

It is worth noting that to date, the mechanisms responsible for the systemic effects of CDI are poorly known, and data on this topic are relatively scarce. Here, we hypothesize that these effects may be due to a “systemic proinflammatory cytokine storm” occurring during CDI, which enhances the toxicity of *C. difficile* toxins (Tcds) once they reach the systemic circulation.

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## Pathophysiological Aspects of CDI

The pathological effects of *C. difficile* are mainly due to the production of two large glucosylating toxins, toxin A (TcdA) and toxin B (TcdB).<sup>17–20</sup> TcdA and TcdB inactivate Rho-GTPases by monoglucosylation, with the following effects:

- (a) loss of the cytoskeletal structure, disassembly of focal adhesions and disruption of tight junctions<sup>17–21</sup> (in cultured cells, these effects cause cell rounding (cytopathic effect),<sup>17–20</sup>
- (b) arrest of the cell cycle, reduced expression of cyclins and activation of cyclin-dependent kinases involved in cell cycle phase progression,<sup>17–20,22</sup>
- (c) cell death by apoptosis or necrosis (cytotoxic effect).<sup>17–20,22</sup>

In vivo, TcdA and TcdB disrupt epithelial tight junctions and induce cell death, causing direct injury to the colonic epithelium. Furthermore, Tcds stimulate colonic epithelial cells to release proinflammatory cytokines and neutrophil chemoattractants, which in turn lead to an acute inflammatory response, a key characteristic of the clinical picture of CDI.<sup>5,17–21</sup> An impaired barrier within the context of active inflammation subsequently leads to enhanced intestinal and vascular permeability. Thus, the loss of a protective barrier favours the entry of Tcds and/or bacteria into the lamina propria, resulting in increased intestinal inflammation.<sup>17–21</sup> The deepening of the lesion at the level of the submucosa could therefore favour the passage into the systemic circulation of Tcds.

TcdA primarily affects the intestinal epithelium, while TcdB has a broader cell tropism and represents the main virulence factor of *C. difficile*.<sup>19</sup> There is evidence suggesting that Tcds can reach the circulation during CDI, causing systemic effects.<sup>6–15</sup> In experimental animal models (mouse and guinea pig), the presence of circulating and quantifiable amounts of Tcds has been related to the systemic effects of CDI and found to be associated with fatal diseases.<sup>10–13,23</sup> The presence of circulating Tcds has also been demonstrated in patients with CDI.<sup>6,8,24</sup>

Of the two Tcds produced by *C. difficile*, TcdB is probably mainly responsible for the systemic effects<sup>12,13,23</sup> due to its toxicity, which is approximately 1000 times higher than that of TcdA.<sup>17–21</sup> The mechanism by which Tcds reach the systemic circulation in some individuals with CDI is likely due to the important tissue damage that profoundly alters the

barrier effect of the intestinal mucosa.<sup>10–13,25,26</sup> Thus, when the two Tcds enter the circulation, cells of various organs may be damaged. In fact, in vitro cytotoxic studies have demonstrated that apart from causing the death of epithelial cells and colonic myofibroblasts,<sup>17–22,27–29</sup> these Tcds target hepatocytes,<sup>30</sup> cardiomyocytes,<sup>31</sup> lung fibroblasts,<sup>32</sup> immunocytes,<sup>17–22,33</sup> enteric neurons,<sup>17–22,34–36</sup> and enteric glial cells.<sup>22,37–39</sup>

In cell culture models, death induced by TcdA and TcdB occurs in a glucosylation-dependent or glucosylation-independent manner, mainly by apoptosis with caspase-dependent or caspase-independent mechanisms.<sup>17–20,22,39</sup> Apoptosis is induced by activation of the executioner caspases-3 and -7, which can occur via a death receptor-dependent extrinsic or by a mitochondria-dependent intrinsic pathway.<sup>17–20,40,41</sup> Apoptosis can also be activated in a caspase-independent manner by the cleavage and activation of pro-apoptotic Bcl-2 family proteins and non-caspase proteases such as cathepsins and calpains.<sup>17–20,42,43</sup>

## Cytokines: Possible Accomplices of Systemic Effects During CDI

The inflammatory response to CDI is characterized by increased local and systemic levels of cytokines,<sup>17–21,44–48</sup> some of which are associated with disease severity and prognosis.<sup>46–48</sup> However, it is important to understand the relationship between circulating Tcds and proinflammatory cytokines in CDI.

Human studies have shown that increased serum concentrations of tumour necrosis factor-alpha (TNF- $\alpha$ ) are associated with poor prognosis in patients with CDI,<sup>48</sup> and in experimental animal models, substances able to decrease proinflammatory cytokine levels exert a protective effect against CDI.<sup>49</sup> However, the production of proinflammatory cytokines during severe CDI cannot be the sole cause of the relevant systemic effects found in only some patients, since their production is high in all subjects with severe CDI.<sup>45</sup>

Susceptibility to the toxic action of Tcds varies between different cell types, and the systemic concentrations of Tcds can be so low as to cause only marginal toxic effects,<sup>3,10–13,22,50</sup> thus, we hypothesized that proinflammatory cytokines may strengthen the toxic effects of low doses of Tcds. This hypothesis stems from our recent in vitro studies in which TNF- $\alpha$  and interferon-gamma (IFN- $\gamma$ ) given before, concomitantly, or after low doses of TcdB (0.1 ng/mL or 1 ng/mL) strongly enhanced the apoptosis

induced by TcdB in enteric glial cells.<sup>22,50</sup> Apoptosis was characterized by an increase in caspase-3, caspase-7, caspase-9 and PARP activation without any change in the expression of Bcl-2 family members (Figure 1).<sup>22</sup>

## Discussion

According to our hypothesis, two conditions must exist to have toxic systemic effects during CDI:

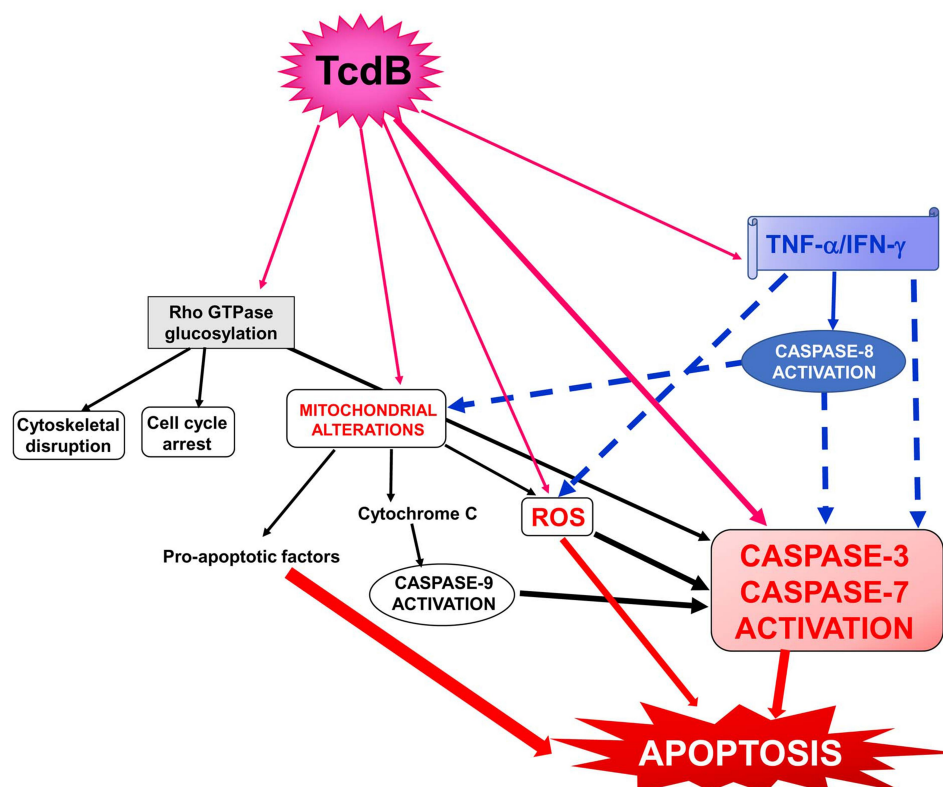
- (a) The production of high levels of proinflammatory cytokines is necessary.<sup>17–21,44–48</sup> In patients with severe CDI, the systemic inflammatory response (as documented in both patients and experimental animal models)<sup>6,10–13,48,51</sup> is due to not only local tissue damage but also the inflammatory action of the Tcds,<sup>17–21,44–48,52</sup> the structural and metabolic components released by *C. difficile* itself, and the modification of the intestinal microbial flora following *C. difficile*-specific therapy.<sup>5,23–26,53–56</sup>
- (b) Tcds have to reach the systemic circulation (a characteristic limited to only some patients with CDI).<sup>6–13</sup>

In these conditions, the interaction between TcdB, TNF- $\alpha$  and IFN- $\gamma$  can trigger an increase in the toxicity of TcdB and its systemic pathological effects (Figure 2). Of interest, this enhanced toxicity in vivo might occur at extremely low concentrations of Tcds,<sup>10</sup> ones comparable to those that synergize in vitro with inflammatory cytokines to induce strong cell death.<sup>22</sup> Since some evidence suggest that TcdA can also reach the circulation in patients with severe CDI and cause extraintestinal manifestations,<sup>6–16</sup> it is of great interest to know if proinflammatory cytokines can increase its toxicity as it happens for TcdB and if the two effects can further amplify the systemic effects of CDI.

In conclusion, one of the pivotal mechanisms underlying the systemic effects of CDI could be the pathological alliance between *C. difficile* and proinflammatory cytokines.<sup>50</sup> This relationship could promote investigations aimed at antagonizing this mechanism by the use of selective drugs targeting proinflammatory cytokines.

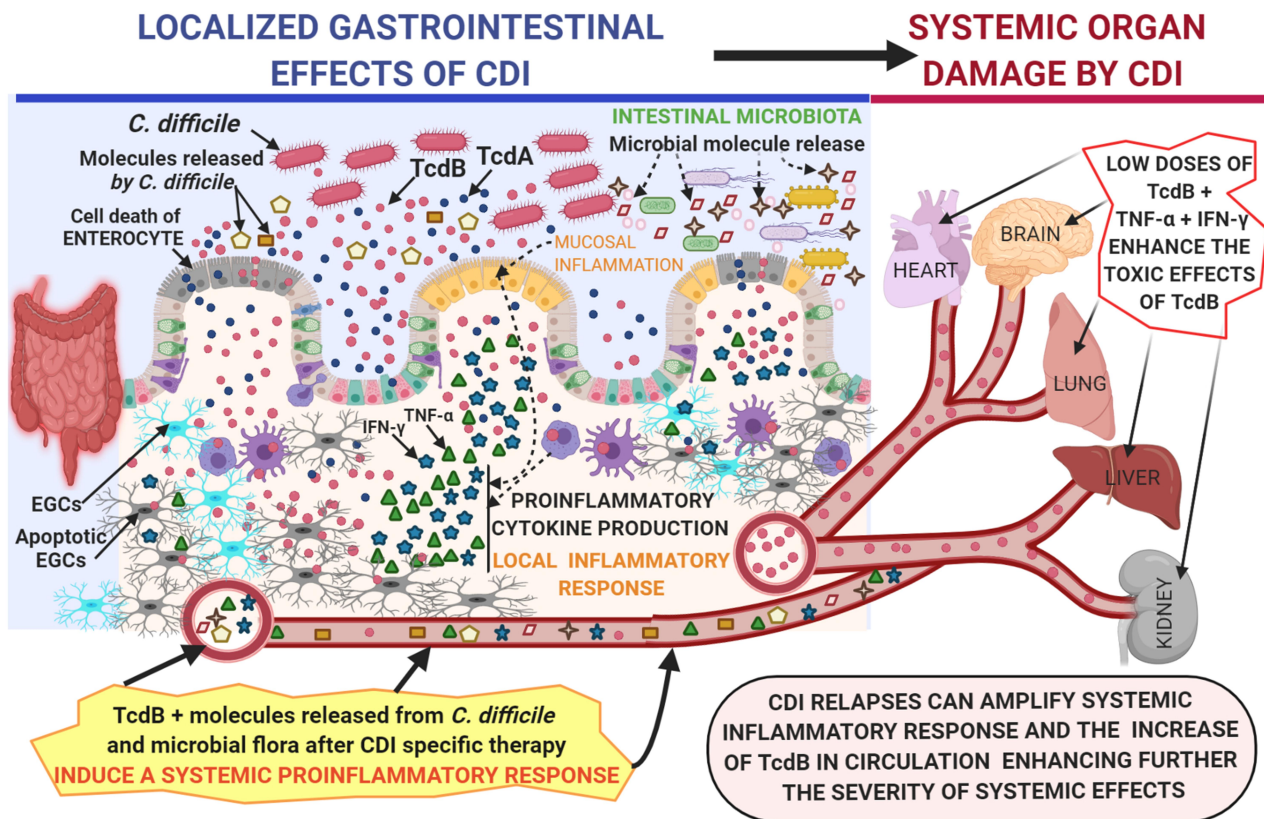
## Conclusions

In conclusion, here, we hypothesize that when TcdB enters the systemic circulation of patients affected by severe CDI,



**Figure 1** Schematic of the possible signalling pathways involved in the cytotoxic synergism between TcdB and the proinflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  in the induction of apoptosis. Full arrows indicate activation; dotted arrows indicate activation enhancement.

**Abbreviations:** TcdB, *C. difficile* toxin B; TNF- $\alpha$ , tumour necrosis factor-alpha; IFN- $\gamma$ , interferon-gamma; ROS, reactive oxygen species.



**Figure 2** Proposed mechanism by which *C. difficile* toxins, in particular TcdB, enter the circulation and cause toxic effects enhanced by the systemic proinflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$  on various systemic organs in severe CDI.

**Notes:** The figure was Created with BioRender.com and was Adapted from "Gut-Brain-Axis", by BioRender.com (2020). Retrieved from <https://app.biorender.com/biorender-templates>.

**Abbreviations:** TcdB, *C. difficile* toxin B; TcdA, *C. difficile* toxin A; TNF- $\alpha$ , tumour necrosis factor-alpha; IFN- $\gamma$ , interferon-gamma; EGCs, enteric glial cells.

there can be a strong enhancement of its toxic action on various organs due to the presence of a systemic cytokine storm, in which proinflammatory cytokines such as TNF- $\alpha$  and IFN- $\gamma$  reach a significant concentration in the circulation. This hypothesis could highlight new therapeutic interventions based on the reduction or neutralization of the indirect toxic action of these cytokines.

## Abbreviations

CDI, *Clostridioides difficile* infection; Tcds, *C. difficile* toxins; TcdA, *C. difficile* toxin A; TcdB, *C. difficile* toxin B; TNF- $\alpha$ , tumour necrosis factor-alpha; IFN- $\gamma$ , interferon-gamma.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or

critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors report no conflicts of interest for this work.

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