# 73068_Auto_Edited.docx

WORD COUNT 4614
TIME SUBMITTED 24-MAY-2022 04:21PM
PAPER ID 86437569

3
**Name of Journal:** *World Journal of Gastroenterology*

**Manuscript NO:** 73068

**Manuscript Type:** ORIGINAL ARTICLE

*Basic Study*

# P2X7 Receptor Blockage Decreases Inflammation, Apoptosis and Enteric Neuron Loss During Clostridioides difficile Toxin A-Induced Ileitis in Mice

Role of P2X7 receptor in enteric neuron loss induced by C. difficile toxins.

Ana A Q A Santos, Deiziane V S Costa, Danielle A Foschetti, Antoniella S G Duarte, Conceição S Martins, Pedro M G Soares, Patricia Castelucci, Gerly Anne de Castro Brito

**Abstract**

BACKGROUND

*Clostridioides difficile* (*C. difficile*) is the most common pathogen causing health care-associated infections. *C. difficile* toxins (TcdA and TcdB) have been shown to activate enteric neurons; however, the population of these cells that is more affected and the underlying mechanism remain unknown.

AIM

We aimed to characterize a specific population of TcdA-affected myenteric neurons and investigate the role of P2X7 in TcdA-induced ileum inflammation, cell death, and enteric nervous system (ENS) changes in mice.

METHODS

Swiss mice were subjected to TcdA-induced ileitis by using an ileal loop model and exposed to TcdA (50 µg/Loop) for 4 h. To investigate the role of P2X7 receptor, Brilliant Blue G (BBG, 50 mg/kg, i.p.), a nonspecific P2X7 receptor antagonist or A438079 (0.7 µg/mice, i.p.), a competitive P2X7 receptor antagonist, were injected one hour prior to a TcdA-challenge. Ileum samples were collected to analyze P2X7 expression (qPCR and immunohistochemistry), the population of myenteric enteric neurons (immunofluorescence), histological damage, intestinal inflammation (ELISA), cell death (TUNEL), neuronal loss and S100B synthesis (immunohistochemistry).

RESULTS

TcdA upregulated ($p<0.05$) P2X7 gene expression in ileum tissues, increasing this receptor in myenteric enteric neurons compared to that in the control mice. Compared to the control mice, TcdA also promoted ($p<0.05$) a loss of myenteric calretinin+ (Calr) and choline acetyltransferase+ (ChaT+) neurons and increased nitrergic+ (NOS+) and Calr+ neurons expressing P2X7. The blockage of the P2X7 receptor decreased the TcdA-

induced intestinal damage, cytokine release (IL-1β, IL-6, IL-8 and TNF-a), cell death, enteric neuron loss and S100B synthesis in the mouse ileum.

CONCLUSION

Our findings show that TcdA induces the upregulation of the P2X7 receptor, which promotes enteric neuron loss, S100B synthesis, tissue damage, inflammation and cell death in the ileum of mice. These findings contribute to future directions in understanding the mechanism involved in intestinal dysfunction reported in patients with pos-CDI.

**Key Words:** Clostridioides difficile; Clostridioides difficile toxin A; P2X7 receptor; enteric nervous system; enteric neuron; enteric glia

Santos AAQA, Costa DVS, Foschetti DA, Duarte ASG, Martins CS, Soares PMG, Castelucci P, Brito GAC. P2X7 Receptor Blockage Decreases Inflammation, Apoptosis and Enteric Neuron Loss During Clostridioides difficile Toxin A-Induced Ileitis in Mice. *World J Gastroenterol* 2022; In press

**Core Tip:** A knowledge gap remains regarding the population of enteric neurons affected by TcdA and the role of the P2X7 receptor, which is a low-sensitivity adenosine triphosphate (ATP)-gated cation channel, in TcdA-induced alterations in enteric neurons and enteric glial cell (EGC)-derived mediators, particularly S100B. Our findings showed the mechanism of P2X7-driven enteric neuronal loss induced by TcdA in the mouse ileum. TcdA promotes the upregulation of P2X7, which promotes cell death in enteric neurons and induces the release of proinflammatory mediators, which, in turn, promotes S100B synthesis in EGCs. However, the blockade of P2X7 abrogates ileum damage induced by TcdA.

## INTRODUCTION

*Clostridioides difficile* (*C. difficile*) continues to be the leading cause of nosocomial diarrhea worldwide [1]. *C. difficile* toxin A (TcdA), toxin B (TcdB) and binary toxin (CDT) are the main virulence factors of *C. difficile* infection (CDI)-related intestinal damage. These toxins have been shown to play an important role in secretory diarrhea and inflammation during infection [2, 3]. The clinical disease ranges from mild diarrhea to toxic megacolon, colonic perforation, and death.

Intestinal dysfunction has been identified in patients after the acute phase of CDI [4-7]. Growing evidence suggests that the enteric nervous system (ENS) plays an important role in regulating intestinal inflammation. Alterations in ENS components, including enteric neurons and glia, can contribute to amplifying the inflammatory immune response and intestinal dysfunction under inflammatory conditions.

The P2X7 receptor, which is a low-sensitivity adenosine triphosphate (ATP)-gated cation channel, can be expressed by several cell types, such as macrophages [8], EGCs [9], and enteric neurons [10]. Once activated, the P2X7 receptor increases the intracellular Ca2+ concentrations, which, in turn, promotes the release of proinflammatory cytokines and neuromodulators [11, 12]. Additionally, high levels of the P2X7 receptor have been reported in enteric neurons during colitis induced by dinitrobenzene sulfonic acid [13] and intestinal ischemia [14].

It has been shown that TcdA and TcdB excite enteric neurons, stimulating the release of substance P and vasoactive intestinal peptide (VIP) *via* the inhibition of noradrenergic transmission and the IL-1β pathway, respectively, resulting in neutrophil recruitment and secretory diarrhea [15-17]. However, a knowledge gap remains regarding the population of enteric neurons affected by TcdA and the role of the P2X7 receptor in TcdA-induced alterations in enteric neurons and enteric glial cell (EGC)-derived mediators, particularly S100B.

Here, we characterized the population of myenteric neurons affected by TcdA during ileitis in mice. In addition, we investigated the role of the P2X7 receptor in ileal damage, inflammation and enteric glial and neuronal changes in TcdA-induced ileitis in mice. Our hypothesis is that TcdA affects specific types of neurons and induces reactive

gliosis, and that activation of P2X7R, is involved not only in ileal damage and inflammation, but also, on enteric glia activation and neuronal loss induced by this toxin.

## MATERIALS AND METHODS

### *Animals*

Swiss mice (8 wk old) were provided by the central vivarium of the Federal University of Ceará. All mice were maintained under standard conditions at 24 °C with a 12-h light-dark cycle, and all groups were provided water and food *ad libitum*. All mouse procedures were conducted according to current regulations regarding animal experiments by the local Animals Care and Use Committee (protocol no. 7028200418).

### *Murine ileal loop model*

The TcdA-induced ileitis murine model was established as previously described [18, 19] with some modifications. Swiss mice ($n$ = 5 per group) were fasted for 4 h with free access to water before being deeply anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). After a midline laparotomy, one 4 cm ileal loop was ligated and injected with 50 µg of TcdA in 100 µL of phosphate-buffered saline (PBS). The control loops were injected with 100 µL of PBS alone. After 4 h, the mice were euthanized, and the ileal loops were removed for the subsequent analysis. Alternatively, some mice were injected with Brilliant Blue G (BBG, Sigma–Aldrich, 50 mg/kg, i.p.)[20], a nonspecific P2X7 receptor antagonist, or A438079 (Abcam, 10 µM/200 µL, i.p.), a competitive P2X7 receptor antagonist [21], one hour prior to the PBS or TcdA (50 µg) injection into the ileal loop. The experimental groups of this experiment were: control (loops were injected with 100 µL of PBS alone), TcdA (loops were injected with 50 µg of TcdA in 100 µL PBS), BBG (injected with Brilliant Blue G one hour prior to the injection of 100 µL of PBS in the loop), A438079 (injected with A438079 one hour prior to the injection of 100 µL of PBS in the loop), TcdA+BBG (injected with Brilliant Blue G one hour prior to the injection of 50 µg of TcdA in 100 µL

PBS), A438079 (injected with A438079 one hour prior to the injection of 50 µg of TcdA in 100 µL PBS).

TcdA was provided by Prof. Carlos Quesada from the University of Costa Rica. BBG was kindly provided by Dr. Patricia Castelucci from the University of São Paulo. A43807 was kindly provided by Dr. Henning Ulrich from the University of São Paulo.

***Analysis of ileum histologic damage***

The ileum samples were fixed in 10% formalin solution for 20 h and processed by the NEMPI-UFC Research Histology Core. The severity of the ileum damage was measured by a blinded histopathologic specialist based on a scoring system ranging from 0 to 3 as previously described with some modifications as follows: (0) absence of alterations; (1) mild loss of the integrity of villi, mild edema and neutrophil infiltrate; (2) partial loss of villi, moderate edema and neutrophil infiltrate; and 3) complete loss of villi, extensive edema and intense neutrophil infiltrate [22].

***Analysis of enteric neuron population***

Fresh ileal samples were flushed with PBS, dissected and opened along the mesenteric border. Then, the samples were fixed in 4% paraformaldehyde (in 0.2 M sodium phosphate buffer, pH 7.4) overnight at 4 °C. Then, the samples were washed three times with 100% dimethyl sulfoxide (DMSO) for 10 min, followed by three washes with PBS for 10 min each. All samples were stored at 4 °C in PBS containing 0.1% sodium azide. The fixed tissues were dissected to remove the mucosa, submucosa, and circular layers, yielding longitudinal muscle-myenteric plexus whole mounts as previously described [20].

Whole-mount preparations of ileum myenteric samples were preincubated in 10% horse serum in PBS containing 1.5% Triton X-100 for 45 min at room temperature to reduce nonspecific binding and permeabilize the tissue. The antibodies used in this

study are described in Table 1. Double labeling was achieved using combinations of primary antibodies (Table 1) overnight at 4 °C. Then, the samples were washed (with PBS three times for 10 min each) and incubated with secondary antibodies (Table 1). After washing with PBS, the samples were mounted in glycerol buffer (in 0.5 M sodium carbonate, pH 8.6). The immunostaining images were acquired using confocal microscopy on a Zeiss confocal scanning laser system installed on a Zeiss Axioplan 2 microscope. The images were acquired at 512 X 512 pixels, and the thickness of each optical section was 0.5 µm. Z-stacks of immunoreactive cells were captured as a series of optical sections with a center spacing of 0.2 µm. Confocal images were collected using Zeiss LSM 5 Image Processing Software and further processed using Corel Photo Paint and Corel Draw software programs [23].

***Quantitative analysis of myenteric neuron immunostaining***

The antigen colocalization was determined by examining the fluorescently labeled preparations. First, neurons were identified by immunofluorescence. Then, the filter was switched, and labeling of the second antigen was determined. In this manner, the proportion of neurons labeled with antigen pairs was determined. The cohort size was 100 neurons, and the data were collected from preparations obtained from five mice per experimental group. The percentage of double immunoreactive neurons was calculated and is expressed as the mean ± SEM. The density of neurons immunoreactive (neurons/$cm^2$) to the P2X7 receptor, nNOS, calretinin and ChAT and the neuronal morphological profiles were measured by examining the whole-mount preparations at x100 magnification. The immunoreactive neuronal cell bodies in the myenteric ganglia in each visual microscopic field (0.04909 $mm^2$) were counted. For the quantification of the two whole-mount preparations (1.0 $cm^2$ each), counts were performed in 40 microscopic fields chosen at random for each antigen in each animal. The cell perikaryal profile areas ($lm^2$) of 50 randomly selected neurons from each animal were obtained using a semiautomatic morphometry device and measured using the Image-Pro Plus software package.

***Immunohistochemistry***

The immunostaining of S100B (an enteric glia-derived mediator), HuC/D (a neuronal marker) and the P2X7 receptor wasperformed in paraffin-embedded ileum sections (4 µm thick) using the streptavidin-biotin-peroxidase method in formalin-fixed sections mounted on poly(l)-lysine-coated microscope slides as previously described [24]. Briefly, the samples were deparaffinized and rehydrated by incubation with xylene and graded alcohols, respectively. Then, the samples were immersed in antigen retrieval solution (*EnVision ™ FLEX Target Retrieval Solution, pH=6.0; Dako Denmark A/S*) for 20 min at PT link (DAKO), incubated in 3% hydrogen peroxide (*EnVision™FLEX Peroxidase-Blocking Reagent; DAKO)* to block endogenous peroxidase for 15 min at room temperature and washed with PBS. Then, the samples were incubated with primary antibodies (rabbit P2X7-Invitrogen, mouse HuC/D-Invitrogen, or goat S100B-Santa Cruz Biotechnology, 1:100) in antibody diluent solution (*EnVisionTMFLEX antibody diluent; Dako*) overnight at 4 °C. Then, the samples were incubated with *EnVision™FLEX/HRP; Dako* as recommended by the manufacturer. P2X7, HuC/D and S100B were visualized with the chromogen 3,3′-diaminobenzidine (DAB, *EnVision™FLEX DAB+ Chromogen; Dako Denmark A/S*). The negative-control sections were processed simultaneously as described above, but the first antibody was replaced with antibody diluent solution (*EnVision™FLEXantibodydiluent; Dako*). The slides were counterstained with Mayer's hematoxylin. The images were acquired under a Leica DM100 microscope and analyzed using Adobe Photoshop 8.0 software. The percentages of P2X7-, S100B- and HuC/D-stained tissue sections were measured by using Adobe Photoshop as previously described [25].

***Total RNA extraction, reverse transcription, and real-time PCR***

The total RNA was isolated from the ileum using an *Aurum™ Total RNA Fatty and Fibrous Tissue* kit (Bio–Rad, CA, USA), and 1 µg of RNA was reverse transcribed

using iScript™ (Bio–Rad) according to the manufacturer's instructions. Real-time PCR (qPCR) was performed on a 7900HT fast real-time PCR system (Applied Biosystems) apparatus using the following specific primers (IDT, Coralville, IA): P2X7 receptor (*forward* GCACGAATTATGGCACCGTC and *reverse*CCCCACCCTCTGTGACATTCT) and GAPDH (*forward TGCACCACCAACTGCTTAG* and reverse *GGATGCAGGGATGATGTTC*) [21]. The reaction mix was prepared in a final volume of 20 µl as follows: 10 µl of master mix iQTM SYBR® Green (Applied Biosystems), 2 µL of each primer (200 nM), 1 µL of cDNA and 5 µL of nuclease-free water. The gene amplifications were obtained with the following steps: 10 min at 95 °C (initial denaturation), 15 s at 95 °C and 60 s at 60 °C for 40 cycles, and a melting curve. The relative gene expression was determined using the 2-ΔΔCt method with GAPDH as a housekeeping gene.

***TUNEL assay***

Ileum samples were processed for a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) analysis using an *ApopTagR S 7100* Kit (Merck Millipore, Germany) to quantify the apoptotic and necrotic cells. Briefly, paraffin-embedded sections were hydrated and incubated with proteinase K (Sigma, USA, 20 mg/mL) for 15 min at room temperature. Endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 5 min at room temperature. After a washing step, the sections were incubated with TdT buffer containing TdT enzyme and reaction buffer in a humidified chamber at 37 °C for 1 h. The specimens were incubated for 10 min at room temperature with stop/wash buffer and then incubated with anti-digoxigenin–peroxidase conjugate at room temperature in a humidified chamber for 30 min. After washing with PBS, the slides were covered with peroxidase substrate (DAB) to develop color and counterstained with methyl green.

***Cytokine quantification by ELISA***

To measure inflammation markers, ileum samples were processed to measure the levels of IL-1β, IL-6, KC (keratinocyte chemoattractant, a human IL-8 analog) and TNF-a by enzyme-linked immunosorbent assay (ELISA) using a mouse cytokine kit assay (R&D Systems) according to the manufacturer's instructions. The absorbance of the samples was detected at 450 nm using an ELISA reader (Biotech Epoch, US). The data are expressed as pg per mg of tissue.

***Statistical analysis***

The results are expressed as the means ± standard errors of the mean (SEM) as determined by GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA). The differences among more than two experimental groups were evaluated using a one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test. Student's t-test was performed to analyze the differences between two groups. The histopathologic score data was compared by using a Kruskal-Wallis nonparametric test followed by Dunn's. Statistical significance was set at $p < 0.05$.

## RESULTS

***TcdA upregulates P2X7 receptor transcripts in the ileum of mice and increases the population of myenteric enteric neurons expressing P2X7***

First, we investigated whether TcdA could alter *P2X7* gene expression in the ileum of mice by using qPCR. We found that TcdA upregulated P2X7 in the ileum of mice compared to that in the control mice ($p<0.05$, Figure 1A). The P2X7 protein measurement by the immunofluorescence analysis showed an increase in the percentage of P2X7 immunostaining positivity in the TcdA-challenged mouse ileum samples compared to that in the control samples ($p<0.05$, Figure 1B). An increase in P2X7 expression was observed in epithelial cells, the lamina propria and the myenteric plexus (Figure 1C).

Given that enteric neurons are an important component of the myenteric plexus, which is a part of the ENS, we investigated whether the P2X7 receptor was increased in

these cells from the myenteric plexus by using an immunofluorescence analysis. Compared to the control group, we found an increased density of enteric neurons expressing P2X7 in the ileum myenteric plexus in the mice challenged with TcdA ($P$ = 0.01, Figure 1D, E).

***TcdA decreases the density of Calr+ and ChaT+ enteric neurons in the ileum myenteric plexus of mice***

Subsequently, to better understand how TcdA affects the myenteric enteric neuron population, we immunostained the ileum myenteric plexus with nNOS, Calr and Chat, which represent the main population of enteric neurons. As shown in Figure 2A, the density of Calr+ ($p<0.03$) and ChaT+ neurons ($p<0.002$) in the ileum myenteric plexus of the mice challenged with TcdA was decreased compared to that in the control mice. In addition, these subtypes of neurons all expressed P2X7 (Figure 2B).

In the enteric neuron population expressing P2X7, compared to the control group, we found increased nNOS+P2X7+ and Calr+P2X7+ neurons, but not ChaT+P2X7+ neurons, in the ileum myenteric plexus of the mice ($p<0.05$, Figure 2C-E).

Taken together, these findings indicate that TcdA decreases the enteric neuron population, specifically Calr+ and ChaT+, but upregulates the P2X7 receptor in a specific population (nNOS and Calr) of neurons in the ileum myenteric plexus of mice.

***Blockage of P2X7 decreases ileal damage induced by TcdA in mice***

Then, we blocked the P2X7 receptor by pretreating the mice with a pharmacological approach, BBG and A438079, one hour prior to the challenge with TcdA to determine whether P2X7 receptor activity was required for the ileal damage induced by this toxin. H&E-stained slides of ileum samples were analyzed for evidence of epithelial damage, edema, and neutrophil infiltration, with a maximal severity score of 3 (Figure 3A and B). TcdA induced complete epithelial disruption, extensive edema and intense neutrophil infiltrate in the ileum of the mice, resulting in a high damage score (score=3) compared to that of the undamaged ileum in the control mice ($p<0.007$, Figure 3A and

B). However, both P2X7 antagonists (BBG and 438079) impressively decreased the ileal damage promoted by TcdA, resulting in a reduction in the damage score (score=1) compared to that in the nontreated TcdA-challenged mice ($p<0.04$, Figure 3A and B).

***Blockage of P2X7 decreases ileal inflammation and cell death induced by TcdA in mice***

Subsequently, we evaluated whether P2X7 receptor activity was involved in ileal inflammation and cell death induced by TcdA in mice. We found that both P2X7 blockers (BBG and A438079) reverted the TcdA-induced increase in IL-1β ($P = 0.008$ and $P = 0.03$, Figure 4A), TNF-a ($P = 0.0002$ and $P = 0.0001$, Figure 4B) and IL-6 ($p=0.03$ and $p<0.0001$, Figure 4C) in the ileum samples of the mice. However, compared to the nonpretreated TcdA-challenged mice, the blockade of the P2X7 receptor by A438079, but not BBG, decreased the levels of KC ($p=0.01$, Figure 4D) and the number of TUNEL+ cells ($p=0.01$, Figure 4E) in the ileum of the mice challenged with TcdA.

Taken together, these data indicate that P2X7 is involved in intestinal damage, inflammation and cell death induced by TcdA in mice.

***Blockage of P2X7 decreases enteric neuron loss and S100B synthesis induced by TcdA in mice***

Since we observed that TcdA promoted a decrease in ileum enteric neurons in mice, we assessed whether the P2X7 receptor could account for this alteration. We found that compared to the nonpretreated TcdA-challenged mice, a P2X7 blocker (A438079) increased the percentage of HuC/D, a panmarker of enteric neuron, positive immunostaining in the ileum in the mice challenged with TcdA (Figure 5A and B).

Furthermore, we evaluated whether P2X7 activation is required to induce S100B expression, which is released by EGCs at high levels under inflammatory conditions, in the ileum of TcdA-challenged mice. As shown in Figure 5A and 5B, the P2X7 antagonist A438079 accentually decreased the percentage of S100B-positive immunostaining in the ileum of the mice challenged with TcdA in comparison to that in the ileum of the nonpretreated TcdA-challenged mice ($P = 0.009$).

Taken together, these data indicate that P2X7 is involved in enteric neuronal loss and S100B synthesis induced by TcdA in mice.

## DISCUSSION

Our data show that TcdA upregulated the P2X7 receptor in the ileum of mice. As shown here, an increased expression of P2X7R has been reported in colonic biopsies from Crohn's disease patients [26] and preclinical models of intestinal inflammation, such as colitis induced by trinitrobenzene sulfonic (TNBS) acid [27] and sepsis [28]. Thus, P2X7 receptor upregulation is a common finding under inflammatory conditions [29].

In this study, we also demonstrated that the P2X7 receptor was increased in the epithelial layer, lamina propria and myenteric plexus. In the myenteric plexus, we found an increased density of neurons expressing P2X7, including the nNOS+ and Calr+ subtypes. In addition to enteric neurons, as shown here, other cell types can express P2X7 receptors, such as mast cells, T cells and dendritic cells [26]. However, we focused on enteric neurons in the myenteric plexus because this component of the ENS is a major functional unit of this system that moves luminal contents along the intestine by coordinating muscle contraction and relaxation [30]. In addition, CDI is characterized by intense diarrhea in the acute phase of disease, and the mechanism is poorly understood.

We showed that TcdA promoted neuron loss, specifically by reducing the density of Calr+ and ChaT+ neuronal populations. Acetylcholine, which is synthesized by a reaction between choline and acetyl-CoA catalyzed by ChaT, is the primary transmitter in excitatory motor neurons, intrinsic afferent neurons and interneurons, and calretinin is the primary transmitter in excitatory cholinergic neurons [31]. Excitatory motor neurons are involved in coordinated muscle contraction [30], thus the reduction on the density of Calr+ and ChaT+ neuronal population induced by TcdA, may be involved in functional disorders after CDI Accordingly,a study performed in the US military found functional gastrointestinal disorders (including gastroesophageal reflux disease, dyspepsia, irritable bowel syndrome or constipation) post-CDI recovery [6].

Here, the alterations in the myenteric enteric neuron population induced by TcdA may be related to these post-CDI-related intestinal dysfunctions. However, more studies are needed to better understand how these alterations specifically contribute to this intestinal dysfunction induced by CDI.

In this study, we also show that the activation of the P2X7 receptor is involved in the TcdA-induced enteric neuron loss as its inhibition by known P2X7R antagonists (BBG and A438079) accentually decreased the loss of these ENS cells during ileitis induced by TcdA. Consistent with our data, the activation of P2X7R has been shown to promote cell death in mucosal regulatory T cells in colitis induced by TNBS [32]. The P2X7 receptor regulates cell death pathways, such as apoptosis, pyroptosis, necrosis and autophagy [33].

ATP released from dead cells can increase the activation of the P2X7 receptor and promote the secretion of proinflammatory cytokines, such as IL-1β, which, in turn, can induce the secretion of other cytokines [34, 35]. Here, the blockade of P2X7 markedly decreased IL-1β, IL-6, KC and TNF-α synthesis in the TcdA-challenged mouse ileum, suggesting that this receptor plays an important role in inflammation induced by this *C. difficile* toxin. Similarly, in a model of TNBS-induced colitis, P2X7 receptor blockade reduced the severity of inflammation by decreasing the infiltration of macrophages in the lamina propria [36]. In contrast, deletion of P2X7R increased the susceptibility to toxoplasmic ileitis [37], suggesting that the activation of this receptor plays a role against intracellular pathogens. Differently, *C. difficile* releases toxins, which in turn entry into the cells to inhibit the Rho GTPases and, here, P2X7R antagonists have positive effects.

In addition, we demonstrated that the blockade of the P2X7 receptor decreased S100B synthesis in the ileum of the mice challenged with TcdA. S100B functions as a proinflammatory mediator when released at higher levels by activating nuclear activation factor-κB (NFκB) [25] and has been shown to be an important mediator during CDI [38]. In the myenteric plexus, enteric glial cells can express S100B [39] and are involved in controlling motility and the epithelial barrier [40]. In a rat glioblastoma cell line, IL-6 promoted S100B synthesis [41]. In the present study, the P2X7 blockade-related reduction

in proinflammatory cytokines may contribute to the decrease in S100B synthesis induced by TcdA, which in turn, reduces the intestinal inflammation and neuronal death.

More studies are needed, for example, using a *C. difficile* infection model for exploring how the P2X7R blockage can affect the CDI outcome and to better explore its physiological benefits for improving intestinal permeability and dysmotility during infection. However, it is important to emphasize that exploring the role of this receptor in the damage induced by one of the main virulence factors released by *C. difficile*, helps to understanding its pathogenesis and developing alternatives co-treatments to control the deleterious exacerbated host response to the *C. difficile* toxins.

## CONCLUSION

In conclusion, our results highlight the mechanism of P2X7-driven enteric neuronal loss induced by TcdA in the mouse ileum. TcdA promotes the upregulation of P2X7, which promotes cell death in enteric neurons and induces the release of proinflammatory mediators (IL-1β, IL-6, KC and TNF-α) in epithelial/immune cells, which, in turn, promotes S100B synthesis in EGCs. However, the blockade of P2X7 abrogates ileum damage induced by TcdA (Figure 6). Altogether, our findings open avenues to better understand how *C. difficile* toxins promote changes in ENS components that can be related to intestinal dysfunction post-CDI.

## ARTICLE HIGHLIGHTS

### *Research background*

The P2X7 receptor, a low-sensitivity adenosine triphosphate (ATP)-gated cation channel, can be expressed by several cell types, including enteric neurons. Once activated, the P2X7 receptor (P2X7R) promotes the release of proinflammatory cytokines and neuromodulators. High levels of P2X7R have been reported in enteric neurons during experimental colitis.

*Research motivation*

A knowledge gap remains regarding the population of enteric neurons affected by TcdA and the role of the P2X7 receptor in TcdA-induced alterations in enteric neurons and enteric glial cell (EGC)-derived mediators, particularly S100B.

*Research objectives*

We characterized the population of myenteric neurons affected by TcdA during ileitis in mice. In addition, we investigated the role of the P2X7 receptor in ileal damage, inflammation and enteric glial and neuronal changes in TcdA-induced ileitis in mice.

*Research methods*

Swiss mice were subjected to TcdA-induced ileitis by using an ileal loop model and exposed to TcdA (50 µg/Loop) for 4 h. To investigate the role of P2X7 receptor, Brilliant Blue G (BBG, 50 mg/kg, i.p.), a nonspecific P2X7 receptor antagonist or A438079 (0.7 µg/mice, i.p.), a competitive P2X7 receptor antagonist, were injected one hour prior to a TcdA-challenge. Ileum samples were collected to analyze P2X7 expression (qPCR and immunohistochemistry), the population of myenteric enteric neurons (immunofluorescence), histological damage, intestinal inflammation (ELISA), cell death (TUNEL), neuronal loss and S100B synthesis (immunohistochemistry).

*Research results*

TcdA upregulated ($p<0.05$) P2X7 gene expression in ileum tissues, increasing this receptor in myenteric enteric neurons compared to that in the control mice. Compared to the control mice, TcdA also promoted ($p<0.05$) a loss of myenteric calretinin+ (Calr) and choline acetyltransferase+ (ChaT+) neurons and increased nitrergic+ (NOS+) and Calr+ neurons expressing P2X7. The blockage of the P2X7 receptor decreased the TcdA-induced intestinal damage, cytokine release (IL-1β, IL-6, IL-8 and TNF-a), cell death, enteric neuron loss and S100B synthesis in the mouse ileum.

*Research conclusions*

Our findings show that TcdA induces the upregulation of the P2X7 receptor, which promotes enteric neuron loss, S100B synthesis, tissue damage, inflammation and cell death in the ileum of mice.

*Research perspectives*

These findings contribute to future directions in understanding the mechanism involved in intestinal dysfunction reported in patients with pos-CDI.

**ACKNOWLEDGEMENTS**

The authors would like to thank Darlyane V.S. Costa for kindly drawing Figure 6 and Socorro França and Flávia A. Silva for their technical assistance.

# 73068_Auto_Edited.docx

ORIGINALITY REPORT

3%
SIMILARITY INDEX

PRIMARY SOURCES

| | | |
|---|---|---|
| 1 | mts.intechopen.com<br>Internet | 48 words — 1% |
| 2 | Conceição S. Martins, Deiziane V. S. Costa, Bruno B. Lima, Renata F. C. Leitäo et al. "Clostridioides difficile Toxin A-Induced Wnt/β-Catenin Pathway Inhibition Is Mediated by Rac1 Glucosylation", Frontiers in Microbiology, 2020<br>Crossref | 31 words — 1% |
| 3 | f6publishing.blob.core.windows.net<br>Internet | 16 words — < 1% |
| 4 | www.science.gov<br>Internet | 15 words — < 1% |
| 5 | creativecommons.org<br>Internet | 13 words — < 1% |
| 6 | www.nature.com<br>Internet | 13 words — < 1% |

| | | | |
|---|---|---|---|
| EXCLUDE QUOTES | ON | EXCLUDE SOURCES | < 12 WORDS |
| EXCLUDE BIBLIOGRAPHY | ON | EXCLUDE MATCHES | < 12 WORDS |