The role of the globular heads of the C1q receptor in TcdA-induced human colonic epithelial cell apoptosis via a mitochondria-dependent pathway

Jinhua Liang
Hongqi Hospital affiliated to Mudanjiang Medical University
Yongzhong Ning
Chuiyangliu Hospital Beijing
Li Dong
Hongqi Hospital
Qi Li
Hongqi Hospital
Ling Chen
Hongqi Hospital
Yanmin Xu (xuyanmin20190909@163.com)
Hongqi Hospital

Research article

Keywords: Clostridium difficile, TcdA, gC1qR, Mitochondrial function

Posted Date: March 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-16745/v1

Version of Record: A version of this preprint was published on September 2nd, 2020. See the published version at https://doi.org/10.1186/s12866-020-01958-6.

Abstract

Background: Clostridioides (formerly Clostridium) difficile infection is the leading cause of antibiotic-associated colitis. Studies have demonstrated that C. difficile toxin A (TcdA) can cause apoptosis in many human cell types. The purpose of this study was to investigate the relationships among exposure to TcdA, the role of globular heads receptor of C1q (gC1qR) gene and the underlying intracellular apoptotic mechanism of human colonic epithelial cells (NCM 460).

Methods: In this study, gC1qR expression was examined using real-time polymerase chain reaction (PCR), western blot and immunohistochemical staining analysis. Cells viability was assessed by the water-soluble tetrazolium salt (WST-1) assay, apoptosis of cells was assessed by flow cytometry and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) Assay. Mitochondrial function was assessed via reactive oxygen species (ROS) generation, changes in the mitochondrial membrane potential (ΔΨm) and the content of ATP.

Results: Our study demonstrated that the concentration of TcdA increasing from 10 ng/ml to 20 ng/ml inhibited cell viability and induced cell apoptosis ($p < 0.01$). Moreover, TcdA induced gC1qR mRNA and protein expression. Overexpression of gC1qR could cause the mitochondrial dysfunction (including production of ROS, decrease of ΔΨm and the content of ATP) and cell apoptosis. However, silencing of gC1qR gene could reverse the TcdA-induced cell apoptosis and mitochondrial dysfunction.

Conclusion: Therefore, these data support a mechanism wherein gC1qR plays a crucial role in TcdA-induced human colonic epithelial cells apoptosis involving a mitochondrial dependent manner.

Background

Clostridium difficile is now also referred to as Clostridioides difficile (C. difficile). C. difficile infection (CDI) is the leading cause of antibiotic-associated colitis, a disease with high morbidity and mortality, and a major economic burden on hospitalized patients. In recent years, the incidence of CDI has been increasing significantly, and becoming the main cause of hospital infection in developed countries [1–3]. C. difficile produces intestinal damage and causes diarrhea mainly because it produces two key virulence determinants, toxin A and toxin B into the intestinal lumen [4–5]. TcdA was shown to produce an intense inflammatory response, including mucosal disruption, mast cell degranulation, fluid accumulation, epithelial cell death, edema, and severe neutrophil infiltration [6–9]. In vitro studies have demonstrated that TcdA can cause a wide apoptosis in many human cell types, including monocytes [10], HeLa cells [11], endothelial cells [12], and intestinal epithelial cells [13–15]. The mechanisms of apoptosis induced by TcdA remain to be fully characterized. Zhang et al. demonstrated that toxin A treatment resulted in significant viability loss and apoptosis in a neuronal cell line, this effect was found to depend on increased reactive oxygen species (ROS) production and the upregulation of p38 MAPK activity and p21 $^{Cip1/Waf1}$expression [16]. Carneiro et al. have demonstrated that TcdA induces cleavage of caspase 6, 8, 9, 3 and Bid leading to human intestinal epithelial cells death [17]. But, the role of mitochondrial dysfunction to TcdA-induced human intestinal epithelial cells death has not been thoroughly investigated.

During the process of cytotoxic injury, the activation of complement is an important biological characteristic. In the process of complement activation, C1q itself can act as a powerful extracellular signal to a wide range of cells, leading to ligand-specific biologic responses [18]. These responses are generally mediated by two expressed cell surface adaptive molecules, globular heads receptor of C1q (gC1qR) and collagen tail receptor of C1q (cC1qR). The receptor for the globular heads of C1q (gC1qR) is a ubiquitous cellular protein with high anionic properties, which was initially identified as a protein in mitochondrial matrix [19]. There is evidence that gC1qR mediates many biological responses, including infection, inflammation, and immune regulation. Examples of such reaction include growth disturbances, morphological abnormalities, and the initiation of apoptosis [20].

In this study, we explored the mechanism of TcdA-induced cell apoptosis using human colonic NCM 460 epithelial cell lines. We demonstrate that TcdA-induced cell death strongly dependent on the induction of the mitochondrial dysfunction pathway. Therefore, we provide evidence that gC1qR gene plays an important role in TcdA-induced epithelial cell apoptosis involving mitochondrial dependent manner.

Materials And Methods

Chemicals and reagents

Human colonic NCM 460 epithelial cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). For in vitro experiments, toxin A (TcdA) from *C. difficile* was obtained from Sigma-Aldrich (St. Louis, MO; C3977-2UG). Dulbecco's Modified Eagle's Medium (DMEM) powder, 1% L-glutamine, 100 g/ml streptomycin, 10 units/ml penicillin, and fetal bovine serum (FBS) were purchased from the Gibco (Grand Island, NY, USA); Lipofectin transfection reagent was purchased from Invitrogen (Burlington, ON, Canada). Dimethyl sulfoxide (DMSO) and Annexin V-FITC/Propidium Iodide (PI) Flow Cytometry Assay Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Small-interfering RNA (siRNA) were synthesised by Wuhan Genesil Biotechnology Co., Ltd (Wuhan, China). An unrelated gene siRNA was chosen as a negative control. All solvents and chemicals were analytical grade.

Cell culture

The human colonic NCM 460 epithelial cells were maintained in DMEM medium containing 10% FBS, 2 mM of glutamine, 1% nonessential amino acids, and antibiotics (100 units/ml of penicillin and streptomycin). The cells were cultured in a 5 % CO_2 incubator at 37°C. The human colonic NCM 460 epithelial cells were maintained to approximately 80 % confluence, then treated with a final concentration of 5 ng/ml, 10 ng/ml or 20 ng/ml TcdA for 24 h in a complete medium. Control group (Mock group) were exposed to media only. Toxin A concentrations were selected based on previous data [21].

gC1qR siRNA-expressing plasmid construction

The siRNA oligonucleotides were targeted to nucleotides 408 to 426. gC1qR siRNA sequenced as follows: 5'-AAC AAC AGC AUC CCA CCA ACA UU-3'. The pGenesil-1 vector using eGFP as the reporter gene was purchased from Wuhan Genesil Biotechnology Co., Ltd. The NCM 460 cells were transfected with a mixture containing Lipofectamine, optiMem, and siRNA oligonucleotides (50 μM) according to the manufacturer's instructions. The gC1qR siRNA-Lipofectamine complex was then added to NCM 460 cells in suspension and plated on 96-well plates (18,000 cells / well). After 48 h, the complete medium was added, along with TcdA (10 ng/ml). The cells were incubated for 24 h, and assays were performed to detect cell viability, cell apoptosis and cell mitochondrial function.

Cell Viability Assay

The NCM 460 cells viability was assessed by WST-1 assay. Cells were cultured in 96-well plates (1×10^5/well) and treated with 5 ng/ml, 10 ng/ml or 20 ng/ml TcdA. After 24 h of incubation, 10 μL of WST-1 solution (stock solution of 5 mg/mL in PBS) was added to 96-well plates, and the plates were maintained for an additional 4 h at 37 ◦C. The reducing activity of the cells was examined by treatment with dimethyl sulfoxide (DMSO, 150 μl) prior to reading at an optical density (OD) of 490 nm with an automatic microplate reader (Elx808; BioTek Instruments, Inc., Winooski, VT, USA).

Detection of apoptotic cells

The NCM 460 cells apoptosis were examined by Annexin V-FITC/ propidium iodide with flow cytometry analysis. The NCM 460 cells were washed and resuspended in binding buffer including 10 mM HEPES, 140 mM NaCl , 2.5 mM $CaCl_2$, pH 7.4 for 20 min at room temperature. The cell suspension (1×10^6/well) were mixed with 10 μL of Annexin V-FITC, and incubated for 30 min at room temperature. Then the mix stained with 10 μL of PI solution for additional 10 min on ice. The scatter parameters of the cells were calculated by Coulter EPICS XL flow cytometer (EasyCyte; Guava Technologies).

Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNNEL) Assay

The NCM 460 cells were exposed to the 5 ng/ml, 10 ng/ml or 20 ng/ml TcdA for 24 h in a complete medium, and then fixed with 4% paraformaldehyde for additional 20 min and embedded in paraffin at room temperature. The slices were treated with hydrogen peroxide block for 10 minutes and incubated with 50 microliters of TUNEL reaction mixtures for 60 minutes at 37 °C in the dark. After being washed twice with PBS, 50 μL of converter-peroxidase were added on the slices and incubated for 40 minutes at 37 °C, finally, the slices were added to 50 μL of diaminobenzidine substrates for 15 min at 25 °C. After being washed twice with PBS, cells apoptosis level was assessed under a light microscope. Cells with shrunken brown-stained nuclei were considered positive.

Real-time quantitative polymerase chain reaction (real-time qPCR)

Total RNA was extracted from cultured NCM 460 cells with a Total RNA Extraction Kit (Promega, Beijing, China), 10 μL of RNA was reverse-transcribed into complementary DNA (cDNA) according to protocol provided by the manufacturer (Invitrogen, Carlsbad, CA). The following sequences were used to detect gC1qR level: Primer-F (5'-AAT CAC ACG GTA GAC ACT GAA ATG CC-3') and Primer-R (5'-CAT CAT CCC ATC TAA AAT GTC CCC TG-3') and β-actin: Primer-F (5'-TCA CCC AVA CTG TGC CCA TCT ATG A-3') and Primer-R (5'-CAT CGG AAC CGC TCA TTG CCG ATA G-3'). Real-time qPCR was performed using a SYBR Green PCR Kit (Invitrogen) on an ABI PRISM 7500 real-time PCR system (Applied Biosystems). The gC1qR mRNA level was determined using the threshold cycle ($2^{-\Delta\Delta CT}$) method [22]. The relative amounts of target gene were normalized to the average of the endogenous control.

Western blot analysis

The NCM 460 cells were treated with various treatments, and total protein was harvested and lysed in buffer containing 1 mM of EDTA, 0.5% NP-40, 50 mM of Tris-HCl (pH 7.4), 50 mM of NaF, 1% Triton X-100, 1 mM of PMSF, 10% glycerol, 150 mM of NaCl, 1 mM of Na_3VO_4, and 1% protease inhibitor cocktail. The equal amount of concentrated proteins were separated using an 10% gradient SDS–polyacrylamide gel running at 100 V for 2 h and transferred onto a polyvinylidene fluoride (PVDF) membrane at 300 mA for 90 min. Non-specific membrane binding sites were blocked in 5% non-fat milk in PBST I for 1 h, and then incubated with primary antibodies specific to gC1qR (1: 1000 dilution, a recombinant rabbit monoclonal

antibody, Abcam: ab131284) and actin (1: 2000; ab8227, Abcam) in blocking solution at 37 °C for 2 h. After being washed twice with TBST, the membrane was incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (1: 4000; Santa Cruz). The protein band visualization was detected using enhanced chemiluminescence from Cell Signaling Technology (Beverly, MA, USA). The values used for the histogram were normalized to the endogenous control.

Immunohistochemistry

The cultured NCM 460 cells were trypsinized and pelleted at 1000 X for 20 min at 4 °C. Removing the supernatant, the cells mass were embedded in paraffin and cut into 5 μm and dried at 70 °C for 2 h. According to the standard procedures, the slice was treated with 3 % H_2O_2 for 25 min in the dark. After being washed twice with PBS, the section were underwent epitope retrieval (5 min 750 W, 15 min 350 W in a microwave) in 0.01 mol/L citrate buffer (pH 6.0). The section was incubated with primary antibodies specific to gC1qR (1: 100 dilution, Abcam: ab131284). After being washed twice with PBS, the section was incubated for 30 min with horseradish peroxidase (HRP)-conjugated secondary antibody (1: 4000; Santa Cruz). After being washed twice with PBS, each section was treated with streptavidin-peroxidase complex, and images were recorded under a confocal laser-scanning microscope (Leica, Germany). Since gC1qR localizes to the mitochondrial matrix, which is expressed in the cytoplasm, cells staining brown in their cytoplasm were considered positive.

Electron microscope

The cultured NCM 460 cells were digested by trypsin and pelleted at 1000 X for 20 min at 4 °C. After the supernatant was removed, cells were fixed with 2 % glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4), the cells mass were post-fixed in 1 % OsO4 for 1 h and stained with 1 % uranyl acetate for 2 h. Then the cell mass was dehydrated at an acetone series concentration of 50 % for 15 min, 70 % for 15 min, 80 % for 15 min, 90 % for 15 min and 100 % for 15 min respectively. The mass embedded in Durcupan and sectioned to 60-70 nm thickness. The ultrastructure of NCM 460 cells was examined at 3700 X magnification, and photographs were observed under a Zeiss 10^9 electron microscope (Carl Zeiss, Oberkochen, Germany).

Assay of intracellular ROS

Intracellular ROS production was quantified by a ROS assay kit (Beyotime, Shanghai, China). Briefly, at least 1×10^5 NCM 460 cells were incubated with 10 μM final concentration of H_2DCFDA, followed by incubation for 40 min at 37 °C. After being washed twice with PBS, cells were harvested. ROS level were analyzed with flow cytometry (BD FACSCalibur, San Jose, CA, USA) using 488 nm excitation and 530 nm emission wavelength.

Measurement of mitochondrial membrane potential (ΔΨm)

Loss of mitochondrial membrane potential (ΔΨm) was examined in NCM 460 cells using the membrane-permeable JC-1 dye (Beyotime). According to the manufacturer's instructions, NCM 460 cells were loaded with 10 μM JC-1 for 20 min at room temperature. Depolarization of ΔΨm was analyzed by monitoring the fluorescence intensities at the excitation wavelength 485 nm and the emission wavelength 530 nm using fluorescence microscopy.

Measurement of Intracellular ATP Levels

The ATP content in NCM 460 cells lysates was detected using an ATP Bioluminescent Cell Assay Kit (S0026, Beyotime, Shanghai, China) according to the manufacturer's instruction, NCM 460 cells were washed twice in cold PBS buffer, 1 mL of 2 % trichloroacetic acid was added into 0.1 mol/L Tris and 2 mmol/L EDTA to stop the luciferase reaction. Cells were removed with a scraper and collected in 1.8-mL Eppendorf centrifuge tubes for 10 min at 15,000 rpm at 4°C. Supernatants were diluted at 0.1 mol/L Tris–2 mmol/L EDTA (Tris-EDTA) (1: 50), and then ATP was determined and protein was analyzed by pellets. The absorbance of samples was examined using a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). A standard curve of ATP concentrations ranging from 0 to 200 nmol/mL was used in this experiment.

Statistical analysis

All data are displayed as the mean ± standard deviation (SD). Student's t test was used to compare the means of two groups. *p*-values less than 0.05 were considered significant (*$p < 0.05$; **$p < 0.01$; $^{\#}p > 0.05$). Statistical analysis of the data was performed using SPSS18.0. All experiments were performed in triplicate.

Results

C. difficile TcdA induces cell apoptosis in human colonic epithelial cells.

In this study, we used the human colonic NCM 460 epithelial cell lines as model to investigate the mechanisms of TcdA-induced cell apoptosis. NCM 460 cells were treated with different concentrations of TcdA (5 ng/ml, 10 ng/ml, 20 ng/ml). The results demonstrated that TcdA at 5 ng/ml treatment slightly decreased cell viability and increased cell apoptosis, but did not reach a statistically significant level ($p > 0.05$). In contrast, the concentration of TcdA increasing from 10 ng/ml to 20 ng/ml significantly decreased cell viability and apparently increased cell apoptosis when compared with the mock group ($p < 0.01$, $p < 0.001$,). Quantification of the data indicated that cell viability decreased (Fig. 1a) and cell apoptosis (Fig. 1b-c) increased in a dose-dependent manner following TcdA treatment.

Effects of TcdA on the expression of gC1qR in human colonic NCM 460 epithelial cells

Many features point to gC1qR localizes to the mitochondrial matrix, which mediates morphological abnormalities, growth perturbations and the initiation of apoptosis. To clarify whether the gC1qR gene is required for TcdA-induced cell apoptosis, we measured the gC1qR expression in the presence of TcdA at 10 ng/ml, the mRNA and protein expression of gC1qR were analyzed by qRT-PCR and western blot, respectively. The data revealed that gC1qR expression in the treatment TcdA group were significantly increased in NCM 460 cells compared with the mock group (Fig. 2a and 2b). Meanwhile, immunohistochemical staining analysis showed that gC1qR expression was apparently enhanced in the cytoplasm of human colonic NCM 460 epithelial cells in the treatment TcdA group (Fig. 2c).

Overexpression of gC1qR induced mitochondrial dysfunction in human colonic NCM 460 epithelial cell lines

The effect of the gC1qR gene on mitochondrial function in human colonic NCM 460 epithelial cells was explored in this study. To do this, we enhanced the gC1qR gene expression (gC1qR vector can effectively induce the expression of gC1qR protein, see Supplementary Fig. 1), the ROS generation data showed that ROS levels in the gC1qR vector group were increased by approximately 2.67-fold compared with the mock group, but there was no difference between the mock group and empty vector group (Fig. 3a). Meanwhile, the effect of gC1qR on mitochondrial membrane potential was monitored by the uptake of JC-1 following transfection 48 h. The value of mitochondrial membrane potential in the gC1qR vector group decreased approximately 61.2% compared with the mock group ($p < 0.01$), indicating the mitochondrial membrane depolarization. There was no difference between the mock group and empty vector group (Fig. 3b). As shown in Fig. 3c, the accumulation of the gC1qR gene significantly decreased the content of ATP of NCM 460 cells when compared with mock group ($p < 0.01$), however, empty vector group and mock group, the mitochondrial ATP content showed no obvious changes.

Effect of silencing of gC1qR gene on TcdA-induced cell apoptosis in human colonic epithelial cells

To more completely understand the role of gC1qR in TcdA-induced cell apoptosis in human colonic NCM 460 epithelial cells, the cell viability and cells apoptosis were detected, respectively. TcdA significantly inhibited NCM 460 cells viability compared with the mock group ($p < 0.01$). Pretransfection with gC1qR siRNA (gC1qR siRNA can effectively silence the expression of gC1qR gene, see Supplementary Fig. 2) reversed TcdA-inhibited cell viability, as shown in Fig. 4a. The cell viability in the TcdA (+), gC1qR siRNA (+)-treated group significantly increased in comparison with those of the TcdA treatment alone group ($p < 0.01$), moreover, cell viability in the TcdA (+), negative siRNA (+) group significantly decreased compared with the TcdA (+), gC1qR siRNA (+) group ($p < 0.01$, Fig. 4a). Meanwhile, silence of gC1qR could alleviate the TcdA-induced cell apoptosis, the cell apoptosis in the TcdA (+), gC1qR siRNA (+)-treated group significantly decreased in comparison with those of the TcdA treatment alone group ($p < 0.01$), cell apoptosis in the TcdA (+), negative siRNA (+) group significantly increased compared with the TcdA (+), gC1qR siRNA (+) group ($p < 0.01$, Fig. 4b). Next, the expression of apoptosis-related proteins, such as activated caspase-3 in NCM 460 cells were analyzed by Western blotting, the data revealed that the caspase-3 protein expression in the TcdA (+), gC1qR siRNA (+)-treated group significantly decreased in comparison with those of the TcdA treatment alone group ($p < 0.01$), moreover, compared with the TcdA (+), gC1qR siRNA (+) group, TcdA (+) and negative siRNA (+) group enhanced the expression of caspase-3 protein ($p < 0.01$, Fig. 4c). The apoptotic morphology image of electron microscope was indicated in the TcdA (+) group and the TcdA (+), negative siRNA (+) group. These apoptotic morphology changes included nuclei condensed and fragmentation, chromatin condensation and marginalization, cell shrinkage, and apoptosis bodies consisting of the cytoplasm with tightly packed organelles (Fig. 4d, red arrows).

Effect of silencing of gC1qR gene on TcdA-induced mitochondrial dysfunction in human colonic epithelial cells

In previous experiments, our data indicated that the overexpression of the gC1qR gene could induce the mitochondrial dysfunction (including the production of ROS, decrease of mitochondrial membrane potential and the content of ATP). TcdA significantly enhanced ROS generation compared with the mock group ($p < 0.01$). Pretransfection with gC1qR siRNA prevented TcdA-induced ROS accumulation, the level of ROS in the TcdA (+), gC1qR siRNA (+)-treated group significantly decreased in comparison with those of the TcdA treatment alone group ($p < 0.01$), moreover, treatment with TcdA (+) and negative siRNA (+) caused a significantly increased in ROS accumulation when compared to the TcdA (+), gC1qR siRNA (+) group ($p < 0.01$, Fig. 5a). The silence of gC1qR gene attenuates damage induced by TcdA. Mitochondrial dysfunction was reflected by a decrease in mitochondrial membrane potential and the content of ATP. We examined the mitochondrial membrane potential and the content of ATP, which was lower in TcdA group than in the mock group, however, gC1qR siRNA maintained the mitochondrial membrane potential and the content of ATP at a normal level against TcdA exposure (Fig. 5b-c). In addition, the mitochondrial membrane potential and the content of ATP in the TcdA (+) and negative siRNA (+) group significantly decreased when compared with the TcdA (+) and gC1qR siRNA (+) group ($p < 0.01$).

Discussion

It has been well established that C.difficile TcdA induces cell apoptosis of human epithelial cells in vitro, with typical apoptotic changes including DNA fragmentation and caspase activation [23–25]. Human colonic epithelial cells apoptosis caused by TcdA may play an important role in the pathogenicity of C. difficile in humans. In this study, we investigated the mechanism of TcdA induces human colonic epithelial cells apoptosis. We found that TcdA can inhibit the viability of human colonic epithelial cells and induce apoptosis in a dose-dependent manner. These findings are consistent with the current view that TcdA induces apoptosis in other epithelial cells. Our findings also suggest that TcdA induced apoptosis of human colonic epithelial cells is closely related to TcdA induced expression of gC1qR protein. gC1qR is a multifunctional cellular protein expressed on a variety of tissues and cell types, including endothelial cells, dendritic cells, lymphocytes, and platelets [26]. In addition to being participated in regulating growth disturbances and initiating apoptosis [27]. In this study, we reported the cell apoptosis induced by TcdA mediated gC1qR gene, and also found that the pro-apoptotic protein caspase-3 was also involved in the process of cells apoptosis.

The biologic responses mediated by gC1qR are extensive, for example, gC1qR is involved in uptake, phagocytosis, apoptotic of macrophage cells [28]. When constitutively expressed in a normal mouse fibroblast lines, gC1qR induces cell apoptosis [29]. In previous reports, gC1qR has been widely studies, mainly as an induction factor of apoptosis. Chen reported that gC1qR induces apoptosis of cervical squamous cell carcinoma through the mitochondrial and p53-dependent pathways [30]. In this study, we found that when gC1qR overexpression in human colonic epithelial cells led to increasing rates of apoptosis. Recent cohort studies have shown that gC1qR is a conserved eukaryotic multifunctional protein, mainly located in mitochondrial matrix and cell surface. Human gC1qR is expressed as 282 amino acid proprotein, and its first 73 amino acids contain mitochondrial localization signal, which is necessary to locate the protein to mitochondria, and then it is lysed to generate mature gC1qR [31–32]. gC1qR in mature form is associated with apoptosis and autophagy by inducing mitochondrial dysfunction [33]. There is growing evidence that mitochondrial dysfunction is linked to apoptosis induced by cytotoxic factors, such as ROS, which are overproduced in defective mitochondria. These findings raise concerns about the role of the gC1qR-induced mitochondrial dysfunction. Our study indicated that gC1qR over-expression in NCM 460 cells led to the production of ROS, and the continuous accumulation of ROS was related to the loss of ATP, which in turn damage the integrity of the mitochondrial membrane potential, the resulting mitochondrial dysfunction, leads to colonic epithelial cells apoptosis. These data support the role of gC1qR-mediated mitochondrial dysfunction in apoptosis of NCM 460 cells.

Conclusion

In this report, we demonstrated that gC1qR expression is necessary in TcdA induced apoptosis of NCM460 cells. The silencing of gC1qR gene can protect mitochondrial function, including reducing ROS production, increasing ATP levels, and restoring mitochondrial membrane potential, thereby inhibiting NCM460 cell apoptosis. Our data showed that TcdA induced apoptosis of NCM460 cells through gC1qR-dependent and mitochondrial dependent pathways.

Abbreviation

Clostridioides difficile (*C. difficile*); *C. difficile* toxin A (TcdA); globular heads receptor of C1q (gC1qR); real-time polymerase chain reaction (PCR); water-soluble tetrazolium salt (WST-1); terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL); reactive oxygen species (ROS); mitochondrial membrane potential (ΔΨm);

Declarations

Acknowledgements

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China; Contract grant number: No. 81571437.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YMX made substantial contributions to the conception and design of the study. JHL drafted the manuscript and revised it critically for important intellectual content. LD and LC have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Larcombe S, Hutton ML, Lyras D. Involvement of Bacteria Other Than Clostridium difficile in Antibiotic-Associated Diarrhoea. Trends Microbiol. 2016; 24(6): 463-476.
2. White MB, Rajagopalan S, Yoshikawa TT. Infectious Diarrhea: Norovirus and Clostridium difficile in Older Adults. Clin Geriatr Med. 2016; 32(3): 509-522.
3. Kim HN, Kim H, Moon HW, Hur M, Yun YM. Toxin positivity and tcdB gene load in broad-spectrum Clostridium difficileinfection. Infection. 2018; 46(1): 113-117.
4. Kuehne SA, Cartman ST, Heap JT, Kelly ML, Cockayne A, Minton NP. The role of toxin A and toxin B in Clostridium difficile infection. Nature. 2010; 467(7316): 711-713.
5. Kroh HK, Chandrasekaran R, Zhang Z, Rosenthal K, Woods R, Jin X, Nyborg AC, Rainey GJ, Warrener P, Melnyk RA, et al. A neutralizing antibody that blocks delivery of the enzymatic cargo of Clostridium difficile toxin TcdB into host cells. J Biol Chem. 2018; 293(3): 941-952.
6. Carter GP, Chakravorty A, Pham Nguyen TA, Mileto S, Schreiber F, Li L, Howarth P, Clare S, Cunningham B, Sambol SP, et al. Defining the Roles of TcdA and TcdB in Localized Gastrointestinal Disease, Systemic Organ Damage, and the Host Response during Clostridium difficile Infections. mBio. 2015; 6: e00551.
7. D'Auria KM, Kolling GL, Donato GM, Warren CA, Gray MC, Hewlett EL, Papin JA. In vivo physiological and transcriptional profiling reveals host responses to Clostridium difficile toxin A and toxin B. Infect. Immun. 2013; 81: 3814-3824.
8. Lima AA, Lyerly DM, Wilkins TD, Innes D, Guerrant RL. Effects of Clostridium difficile toxins A and B in rabbit small and large intestine in vivo and on cultured cells in vitro. Infect Immun. 1988; 56: 582-588.
9. Lyerly DM, Krivan HC, Wilkins TD. Clostridium difficile: its disease and toxins. Clin Microbiol Rev. 1988; 1: 1-18.
10. Warny M, and Kelly CP. Monocytic cell necrosis is mediated by potassium depletion and caspase-like proteases. Am. J. Physiol. 1999; 276: C717-C724.
11. Qa'Dan M, Ramsey M, Daniel J, Spyres LM, Safiejko-Mroczka B, Ortiz-Leduc W, Ballard JD. Clostridium difficile toxin B activates dual caspase-dependent and caspase-independent apoptosis in intoxicated cells. Cell Microbiol. 2002; 4: 425-434.
12. Hippenstiel S, Schmeck B, NGuessan PD, Seybold J, Krull M, Preissner K, Von Eichel-Streiber C, Suttorp N. Rho protein inactivation induced apoptosis of cultured human endothelial cells. Am. J. Physiol. 2002; 238: L830-L838.
13. Frädrich C, Beer LA, Gerhard R. Reactive Oxygen Species as Additional Determinants for Cytotoxicity of Clostridium difficile Toxins A and B. Toxins (Basel). 2016; 8.
14. Carneiro BA, Fujii J, Brito GAC, Alcantara C, Oria RB, Lima AAM, Obrig T, Guerrant RL. Caspase and Bid involvement in Clostridium difficile toxin A-induced apoptosis and modulation of toxin A effects by glutamineandalanyl-glutamine in vivo and in vitro. Infect Immun. 2006; 74: 81-87.
15. Gerhard R, Nottrott S, Schoentaube J, Tatge H, Olling A, Just I. Glucosylation of Rho GTPases by Clostridium difficile toxin A triggers apoptosis in intestinal epithelial cells. J. Med. Microbiol. 2008; 57: 765-770.
16. Zhang P, Hong J, Yoon IN, Kang JK, Hwang JS, Kim H.
Clostridium difficile Toxin A Induces Reactive Oxygen Species Production and p38 MAPK Activation to Exert Cellular Toxicity in Neuronal Cells. J. Microbiol. Biotechnol. 2017; 27: 1163-1170.
17. Carneiro BA, Fujii J, Britto GA, Alcantara C, Oria RB, Lima AA, Obrig T, Guerrant RL. Caspase and bid involvement in Clostridium difficile toxin A-induced apoptosis and modulation of toxin A effects by glutamine and alanyl-glutamine in vivo and in vitro. Infect Immun. 2006; 74: 81-87.
18. Rubinstein DB, Stortchevoi A, Boosalis M. Receptor for the globular heads of C1q (gC1q-R; p33; Hyaluronan-binding protein is preferentially expressed by adenocarcinoma cells. Int Cancer Res. 2004; 110(5): 741-750.
19. Peerschke EI, Petrovan RJ, Ghebrehiwet B, Ruf W. Tissue factor pathway inhibitor-2 (TFPI-2) recognizes the complement and kininogen binding protein gC1qR/p33 (gC1qR): implications for vascular inflammation. Thromb Haemost. 2004; 92(4): 811-819.
20. Xu L, Xiao N, Liu F, Ren H, Gu J. Inhibition of RIG-I and MDA5dependent antiviral response by gC1qR at mitochondria. Proc Natl Acad Sci USA. 2009; 106(5): 1530-1535.

21. Johal SS, Solomon K, Dodson S, Borriello SP, Mahida YR. Differential effects of varying concentrations of Clostridium difficile toxin A on epithelial barrier function and expression of cytokines. J. Infect Dis. 2004; 189: 2110-2119.
22. Livak KJ, Schmittgen TD. Analyses of relative gene expression data using real-time quantitative PCR and the 2ΔΔCT method. Methods. 2001; 25: 402-408.
23. Fettucciari K, Ponsini P, Gioè D, Macchioni L, Palumbo C, Antonelli E, Coaccioli S, Villanacci V, Corazzi L, Marconi P, et al. Enteric glial cells are susceptible to Clostridium difficile toxin B. Cell Mol Life Sci. 2017; 74(8): 1527-1551.
24. Busch K, Suchodolski JS, Kühner KA, Minamoto Y, Steiner JM, Mueller RS, Hartmann K, Unterer S. Clostridium perfringens enterotoxin and Clostridium difficile toxin A/B do not play a role in acute haemorrhagic diarrhoea syndrome in dogs. Vet Rec. 2015; 176(10): 253.
25. Zhang BZ, Cai J, Yu B, Hua Y, Lau CC, Kao RY, Sze KH, Yuen KY, Huang JD. A DNA vaccine targeting TcdA and TcdB induces protective immunity against Clostridium difficile. BMC Infect Dis. 2016; 16(1): 596.
26. Fogal V, Richardson AD, Karmali PP, Scheffler IE, Smith JW, Ruoslahti E. Mitochondrial p32 protein is a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation. Mol Cell Biol. 2010; 30(6): 1303-1318.
27. PeerschkeEI, MintaJO, Zhou SZ. Expression of gC1q-R/p33 and its major ligands in human atherosclerotic lesions. Mol Immunol. 2004; 41(8): 759-766.
28. Chowdhury AR, Ghosh I, Datta K. Excessive reactive oxygen species induces apoptosis in fibroblasts: role of mitochondrially accumulated hyaluronic acid binding protein 1 (HABP1/p32/ gC1qR). Exp Cell Res. 2008; 314(3): 651-667.
29. Fogal V, Zhang L, Krajewski S, Ruoslahti E. Mitochondrial/ cell-surface protein p32/gC1qR as a molecular target in tumor cells and tumor stroma. Cancer Res. 2008; 68(17): 7210-7218.
30. Chen ZL, Gu PQ, Liu K, Su YJ, Gao LJ. The globular heads of the C1q receptor regulate apoptosis in human cervical squamous carcinoma cells via a p53-dependent pathway. J Transl Med. 2012; 10: 255.
31. Mallick J, Datta K. HABP1/p32/gC1qR induces aberrant growth and morphology in Schizosaccharomyces pombe through its N-terminal alpha helix. Exp Cell Res. 2005; 309(2): 250-263.
32. Peerschke EI, Murphy TK, Ghebrehiwet B. Activation-dependent surface expression of gC1qR/p33 on human blood platelets. Thromb Haemost. 2003; 89(2): 331-339.
33. Muta T, Kang D, Kitajima S, Fujiwara T, Hamasaki N. P32 protein, a splicing factor 2-associated protein, is localized in mitochondrial matrix and is functionally important in maintaining oxidative phosphorylation. J Biol Chem. 1997; 272(39): 24363-24370.

Supplemental Information Note

Supplementary Fig 1. gC1qR expression was detected by western blot analysis. The human colonic epithelial cells were transfected with gC1qR vector, empty vector or plain medium (Mock) for 48 h. The expression efficiency of gC1qR protein was analyzed by western blot assay. $^{**}p < 0.01$, $^{\#}p > 0.05$ versus Mock group.

Supplementary Fig 2. gC1qR expression was detected by western blot analysis. The human colonic epithelial cells were transfected with gC1qR siRNA, negative siRNA or plain medium (Mock) for 48 h. The silencing efficiency of gC1qR gene was analyzed by western blot assay. $^{**}p < 0.01$, $^{\#}p > 0.05$ versus Mock group.

Figures

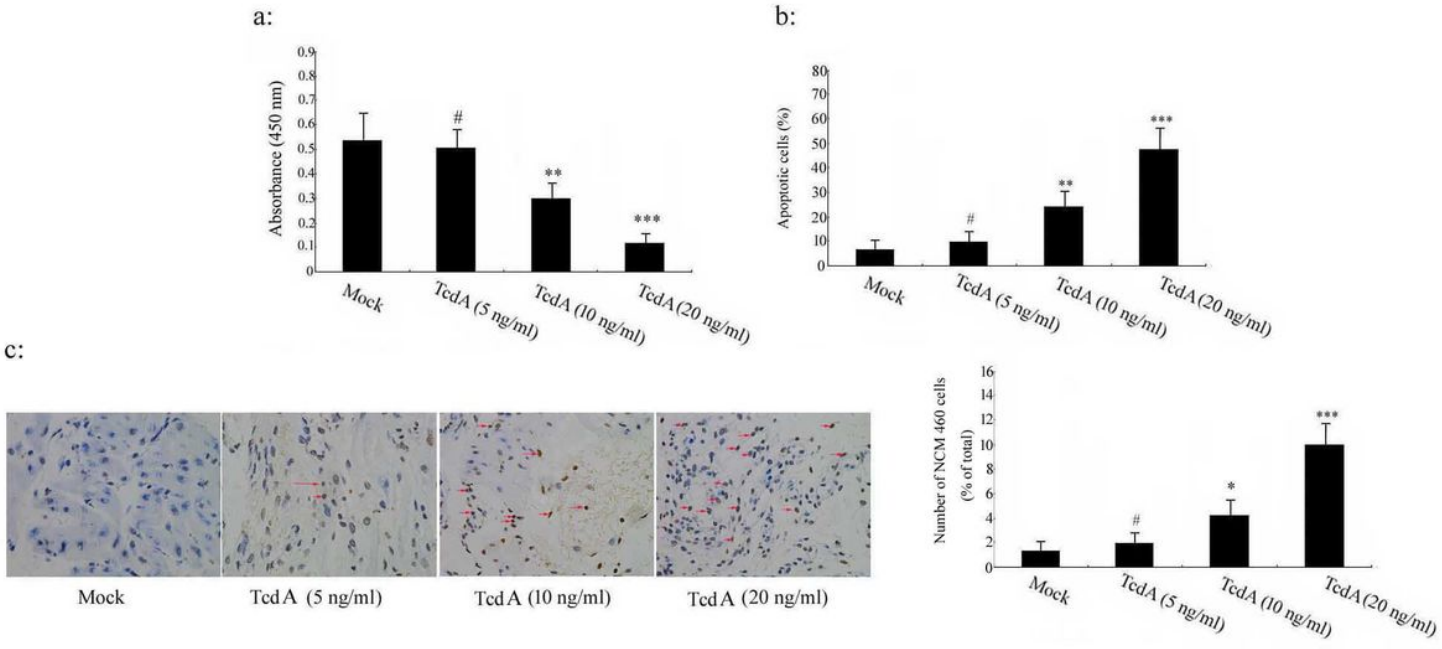

Figure 1

Sensitivity of human colonic epithelial cell to TcdA-induced cell apoptosis. The human colonic epithelial cells were exposed to 5 ng/ml, 10 ng/ml, 20 ng/ml TcdA for 24 h. (a) The viability of cells was detected by WST-1 assay (n = 3 individual experiments). (b) Apoptotic death of NCM 460 cells was assessed by flow cytometric analysis (n = 3). (c) TUNNEL staining of human colonic epithelial cells. Cells with shrunken brown stained nuclei were considered positive (Red arrows). ***p < 0.001, **p < 0.01, *p < 0.05, #p > 0.05 versus Mock group.

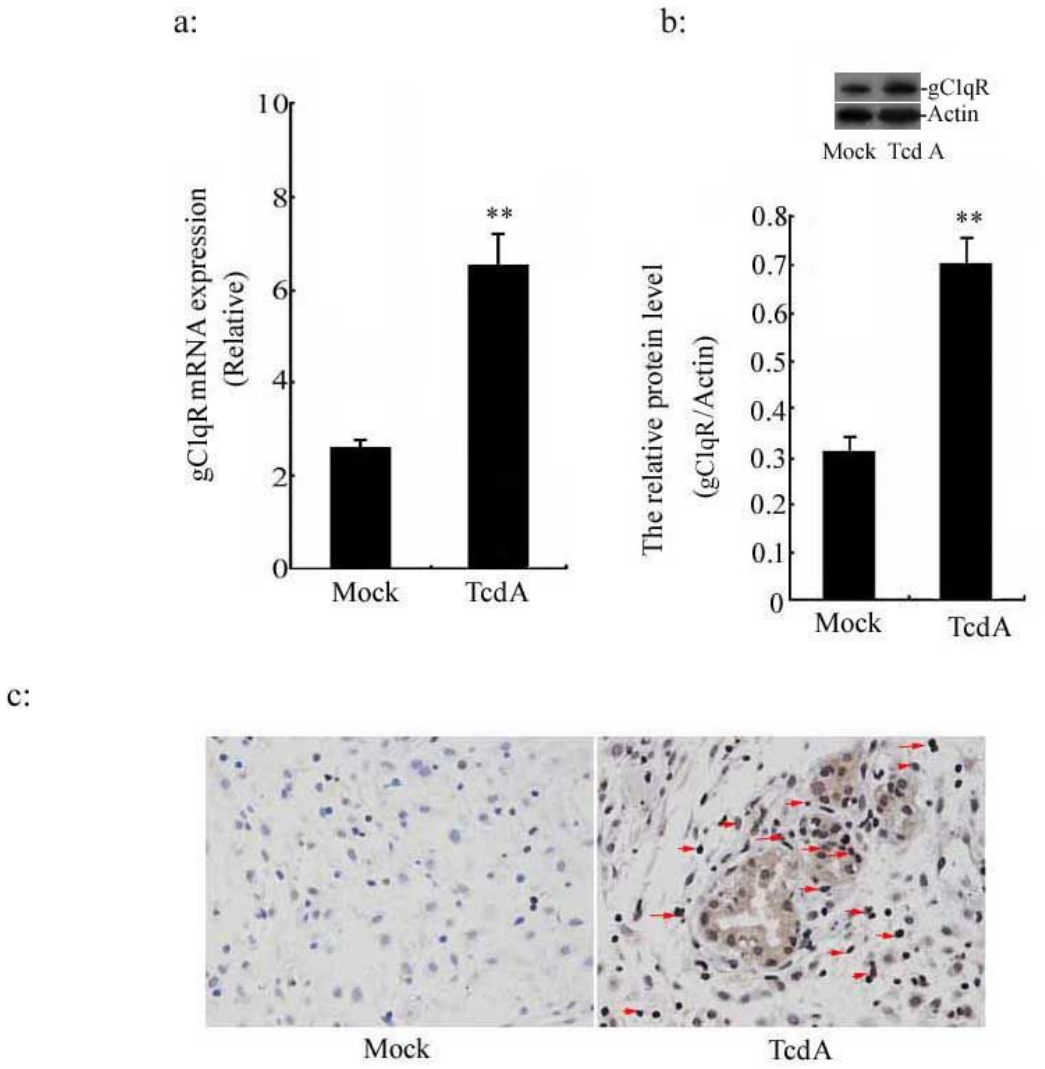

Figure 2

gC1qR expression in human colonic epithelial cell lines. The human colonic epithelial cells were treated with TcdA at 10 ng/ml for 24 h. (a) gC1qR mRNA level was detected using qRT-PCR; (b) The expression of gC1qR protein in lysates of NCM460 cells was measured by western blot assay. (c) Localization and expression of gC1qR protein was examined using immunohistochemical staining analysis. Cells with brown stained cytoplasm were considered positive (Red arrows). **$p < 0.01$ versus Mock group.

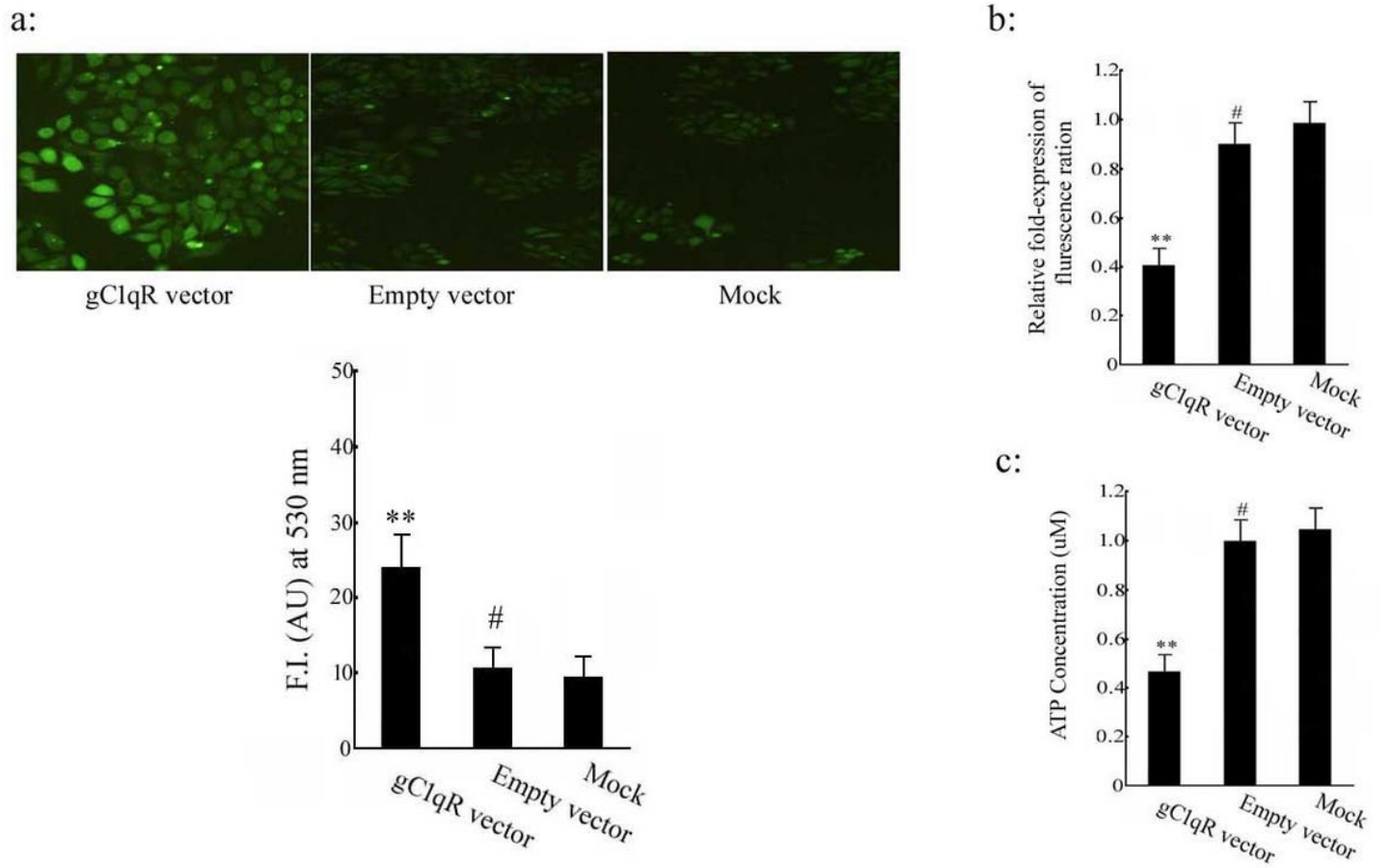

Figure 3

The effect of overexpression of gC1qR on mitochondrial function of human colonic NCM 460 epithelial cell lines. The human colonic epithelial cells were transfected with gC1qR vector or empty vector for 48 h. (a) Intracellular ROS generation was determined by fluorescence of H2DCFDA (Green). (b) The change of mitochondrial membrane potential was detected. The relative Δψm value was measured by monitoring the fluorescence of JC-1 (590: 527 nm fluorescence ratio). (c) ATP content was detected in NCM 460 epithelial cell. The data are means ± S.D. of three separate experiments performed in triplicate. **p < 0.01, #p > 0.05 versus Mock group.

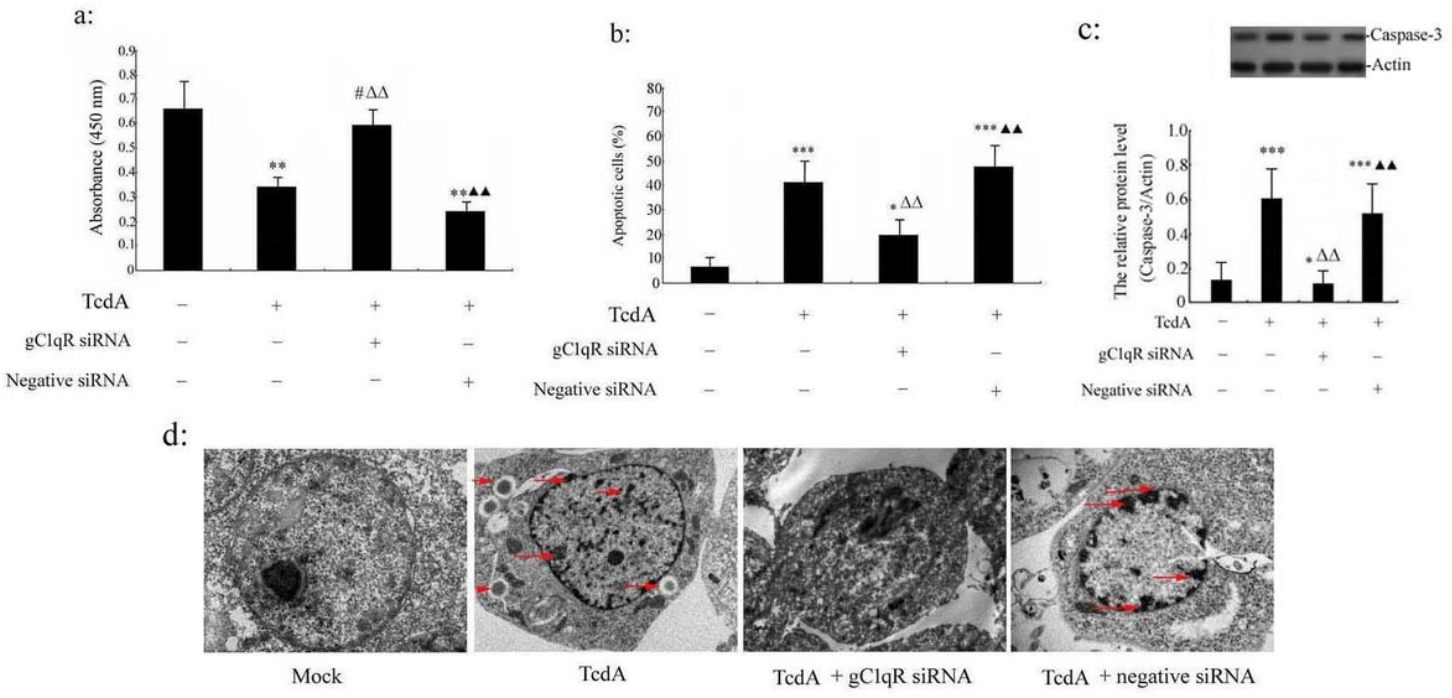

Figure 4

Effect of silencing of gC1qR gene on TcdA-induced cell apoptosis in human colonic epithelial cells. NCM 460 cells were transfected with gC1qR siRNA or negative siRNA for 48 h respectively, and then TcdA (10 ng/ml) was added for 24 h. (a) Cell viability was determined by WST-1 assay as described previously; (b) Apoptotic death of NCM 460 cells was examined by flow cytometric analysis. The data are means ± S.D. of three separate experiments performed in triplicate. (c) The expression of caspase-3 protein in lysates of NCM460 cells was measured by western blot assay. ***p < 0.001, **p < 0.01, *p < 0.05, #p > 0.05 versus TcdA (-), gC1qR siRNA (-) and negative siRNA (-) group (Mock group); △△p < 0.01 versus TcdA (+), gC1qR siRNA (-) and negative siRNA (-) group; ▲▲p < 0.01 versus TcdA (+), gC1qR siRNA (+) and negative siRNA (-) group. (d) The change of morphology of the NCM 460 cells was observed with an electron microscope. Red arrows point to the nuclei condensed and fractured, and chromatin increased and marginalized in the nucleus (3700 X).

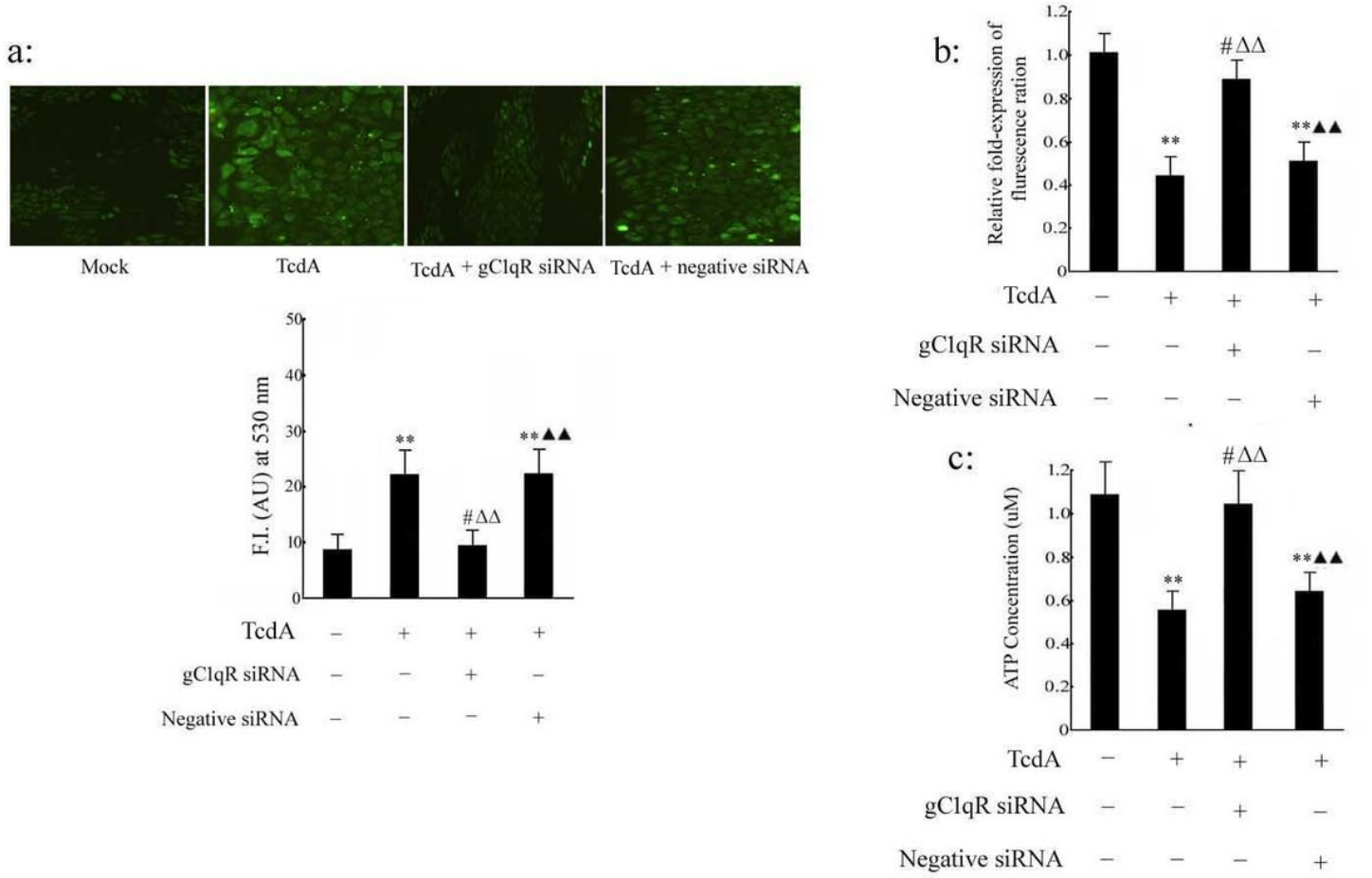

Figure 5

Effect of silencing of gC1qR gene on TcdA-induced mitochondrial dysfunction in human colonic epithelial cells. NCM 460 cells were transfected with gC1qR siRNA or negative siRNA for 48 h respectively, and then TcdA (10 ng/ml) was added for 24 h. (a) Intracellular ROS generation was measured by fluorescence of H2DCFDA (Green). (b) NCM 460 cells were stained with JC-1 and were subjected to flow cytometry. The relative Δψm value was examined by monitoring the fluorescence of JC-1 (590: 527 nm fluorescence ratio). (c) The ATP levels in NCM 460 epithelial cell were assessed. The data are presented as mean ± S.D. (n = 3). **p < 0.01, #p > 0.05 versus TcdA (-), gC1qR siRNA (-) and negative siRNA (-) group; △△p < 0.01versus TcdA (+), gC1qR siRNA (-) and negative siRNA (-) group; ▲▲p < 0.01 versus TcdA (+), gC1qR siRNA (+) and negative siRNA (-) group.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigure1.jpg
- SupplementaryFigure2.jpg