Future MICROBIOLOGY

RESEARCH ARTICLE

For reprint orders, please contact: reprints@futuremedicine.com

Involvement of cholesterol in *Campylobacter jejuni* cytolethal distending toxin-induced pathogenesis

Cheng-Kuo Lai[‡,1,2,3], Jing-Chi Su[1], Yu-Hsin Lin[3,4], Chia-Shuo Chang[1], Chun-Lung Feng[5], Hwai-Jeng Lin[‡,6], Chun-Jung Lin[‡,1,3], Guan-Chin Tseng[7], Hsin-Ho Liu[3,8], Jer-Tsong Hsieh[3], Hong-Lin Su[**,2,9] & Chih-Ho Lai[*,1,10,11]

ABSTRACT **Aim:** The aim of this study was to investigate whether cholesterol plays a pivotal role in cytolethal distending toxin (CDT) mediated pathogenic effects in hosts. **Materials & methods:** The molecular mechanisms underlying cholesterol sequestering conferred resistance to CDT-induced DNA double-strand breaks (DSBs) and cell cycle arrest were investigated. Histopathological analysis was conducted for evaluating CDT-induced intestinal inflammation in mouse. **Results:** CDT actions were attenuated by treatment of cells with methyl-β-cyclodextrin (MβCD). Severe intestinal inflammation induced by CDT treatment was observed in high-cholesterol diet-fed mice, but not in normal diet-fed mice, indicating that cholesterol is essential for CDT intoxication. **Conclusion:** Our findings demonstrate a molecular link between *Campylobacter jejuni* CDT and cholesterol, which is crucial to facilitate CDT-induced pathogenesis in hosts.

KEYWORDS
- *Campylobacter jejuni*
- cell cycle
- cholesterol
- cytolethal distending toxin
- DNA double-strand breaks

Cytolethal distending toxin (CDT) is produced by several Gram-negative bacteria including *Aggregatibacter actinomycetemcomitans* [1], *Campylobacter jejuni* [2], *Escherichia coli* [3], *Haemophilus ducreyi* [4], and *Shigella dysenteriae* [5]. The CDT holotoxin includes three subunits, CdtA, CdtB and CdtC, and functions as a genotoxin [6]. The CdtA and CdtC subunits are not only required for holotoxin binding to the cell surface, but necessary to associate with membrane cholesterol-rich microdomains [7,8]. After binding of CdtA/CdtC to the cell membrane, CdtB is internalized into the cytoplasm and then translocated into the nucleus [9]. The nuclear-translocated CdtB contains type I deoxyribonuclease (DNase) activity that leads to DNA double-strand breaks (DSBs) followed by cell cycle arrest at G2/M [10].

Lipid rafts are membrane microdomains that contain relatively high levels of cholesterol, glycosphingolipids and phospholipids [11,12]. The structure of lipid rafts is stabilized by cold nonionic detergents such as Triton X-100, but it can be disrupted by cholesterol-sequestering agents such

[1]School of Medicine & Graduate Institute of Clinical Medical Science, China Medical University, Taichung, Taiwan
[2]Department of Life Sciences, Agricultural Biotechnology Center, National Chung Hsing University, Taichung, Taiwan
[3]Department of Urology, University of Texas Southwestern Medical Center, Dallas, TX, USA
[4]Department of Biological Science & Technology, China Medical University, Taichung, Taiwan
[5]Department of Internal Medicine, China Medical University Hospital, Taichung, Taiwan
[6]Department of Internal Medicine, Taipei Medical University Hospital, Taipei, Taiwan
[7]Department of Pathology, China Medical University Hospital, Taichung, Taiwan
[8]Department of Urology, Taichung Tzu Chi Hospital, Taichung, Taiwan
[9]Department of Physical Therapy, China Medical University, Taichung, Taiwan
[10]Department of Nursing, Asia University, Taichung, Taiwan
[11]Department of Microbiology and Immunology, Chang Gung University, Taoyuan, Taiwan
*Author for correspondence: Tel.: +886 4 2205 2121 ext. 7729; Fax: +886 4 2233 3641; chl@mail.cmu.edu.tw
**Author for correspondence: Tel.: +886 4 2284 0416 ext. 417; Fax: +886 4 2285 4391; suhonglin@gmail.com
‡Authors contributed equally

Future Medicine part of fsg

as methyl-β-cyclodextrin (MβCD) and filipin [13]. Depletion of cholesterol by filipin has been shown to perturb lipid raft formation and reduces the ability of *C. jejuni* to enter epithelial cells [14,15]. Moreover, *C. jejuni* invasion of epithelial cells is mediated through unique plasma membrane invaginations termed caveolae, which are mainly localized in lipid rafts [16,17]. However, it remains unclear whether CDT is involved in the initial infection of the host cells in a cholesterol-dependent manner.

Accumulating studies examining CDT produced by *A. actinomycetemcomitans* [7], *C. jejuni* [18] and *H. ducreyi* [19,20] have demonstrated that both CdtA and CdtC not only bind to the cell surface, but also associate with membrane lipid rafts. Additionally, functional studies have revealed that the cholesterol recognition/interaction amino acid consensus (CRAC) motif in CdtC is necessary for toxin binding and the enhancement of intoxication [8,21] However, limited information is available on the role of cholesterol in *C. jejuni* CDT-induced pathogenesis in hosts.

Given the evidence that interactions between *C. jejuni* CDT and cholesterol may facilitate toxin activity, we examined potential pathogenic mechanisms that may form the basis for this association. In this study, we investigated whether cholesterol plays a pivotal role in CDT-mediated pathogenic effects on epithelial cells. Cholesterol sequestering in epithelial cells by MβCD conferred resistance to CDT-induced DSBs, apoptosis and cell cycle arrest. Moreover, CDT administration induced severe intestinal inflammation in high-cholesterol diet-fed mice, but not in the control group, revealing that high cholesterol diet may contribute to more severe CDT-mediated pathological derangement. Our data provide novel insights into the correlation between cholesterol and *C. jejuni* CDT, and demonstrate that cholesterol might be critical to facilitate CDT-induced pathogenesis.

Materials & methods

• Reagents & antibodies

Antibodies against cytochrome c, 53BP1, Bax, Bak, Bcl-2 and Bcl-xL were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies specific to p-ATM, p-CHK2, cleaved-PARP, NBS1 and p-p53 were obtained from Cell Signaling Technology (MA, USA). Antibodies against pro-caspase-3 and caspase-3 were obtained from Biolegend (CA, USA). An anti-β-actin mouse monoclonal antibody was purchased from Upstate Biotechnology (NY, USA). Antiserum against each CDT subunit was prepared as described previously [18]. MβCD and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (MO, USA).

• Purification of each CDT subunit

The recombinant CdtA, CdtB or CdtC were prepared and expressed by following our previous protocol [18]. Each purified subunit was analyzed by SDS-PAGE and western blot.

• SDS-PAGE & western blot analyses

AGS cells treated with CDT holotoxin were washed with PBS and the cell lysate was prepared. Each sample was subjected to SDS-PAGE (6–12%) and transferred onto polyvinylidene difluoride membranes (Millipore, CA, USA). The membranes were incubated with antiserum against each CDT subunit, or specific antibodies against p-ATM, p-CHK2, p-p53, NBS1, Bax, Bak, Bxl-xL, Bcl-2, cytochrome *c*, pro-caspase-3, caspase-3, cleaved-PARP, Bcl-2, Bax and β-actin for 2 h, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen, CA, USA). The target proteins were detected using the ECL Western Blotting Detection Reagents (GE Healthcare, NJ, USA). The intensity of each protein was quantified by using Image J software (NIH, MD, USA).

• Cell culture

AGS cells (human gastric adenocarcinoma cells; CRL 1739) were cultured in F12 medium (HyClone, UT, USA) supplemented with 10% complement-inactivated fetal bovine serum (HyClone) and penicillin/streptomycin (Invitrogen) at 37°C in a humid atmosphere containing 5% CO_2.

• Detection of cellular cholesterol & cell viability assay

To measure the cholesterol levels in total cell lysates, AGS cells (2×10^5) were treated with various concentrations of MβCD (0, 1, 2.5 and 5 mM), as described previously [22]. The cholesterol content was measured using an Amplex Red cholesterol assay kit (Molecular Probes, OR, USA). The amount (%) of cholesterol retained after pretreatment with MβCD was determined as described previously [23]. To test the effect of MβCD on cell viability, the cells were incubated

with various concentrations of MβCD (0, 1, 2.5, 5 and 10 mM) at 37°C for 30 min. Then, the cells were washed with PBS and incubated in fresh medium for 24 h. The cells viability was measured by the ability of the cells to reduce MTT (Sigma-Aldrich) to formazan, which was quantified using a spectrophotometer (BioRad, CA, USA) at a wavelength of 570 nm [24].

• Cell cycle analysis

The cell cycle distribution of each treatment was analyzed with an FACSCalibur flow cytometer (Becton Dickinson, CA, USA) as described previously [21]. Briefly, AGS cells (2×10^5) were pretreated with various concentrations of MβCD (0, 1, 2.5 and 5mM) for 30 min, followed by treatment with 100 nM CDT holotoxin for 24 h. The treated cells were fixed with ice-cold 70% ethanol for 2 h and stained with 20 μg/ml propidium iodide (Sigma-Aldrich) containing 1 mg/ml RNase (Sigma-Aldrich) and 0.1% Triton X-100 for additional 1 h. The stained cells were analyzed by flow cytometry by collecting 10,000 cells from each sample. Data were prepared using CellQuest (Becton Dickinson) and analyzed using WinMDI (Verity Software House, ME, USA). All samples were representative of three independent experiments.

• Immunofluorescence

The immunofluorescence staining of cells was described previously with slight modifications [18]. Briefly, cells (3×10^5/well) were plated in six-well plates and incubated for 24 h. The cells were pretreated with MβCD for 30 min and incubated with CDT holotoxin for 24 h. Then, the cells were fixed in 1% paraformaldehyde (Sigma-Aldrich) for 30 min followed by permeabilization with 0.1% Triton X-100 for 30 min. The cells were then incubated with the 53BP1 antibody and probed with Alexa Fluor® 594-conjugated anti-rabbit IgG (Invitrogen), and counterstained with DAPI (Sigma-Aldrich) for 10 min. The stained cells were visualized using a fluorescence microscope (Carl Zeiss, Göttingen, Germany) at 400× magnification. Fifty nuclei from each sample were counted for evaluation. Samples were examined in three independent experiments.

• Analysis of apoptotic cells

DNA fragmentation was detected by the terminal deoxynucleotidyl transferase-meditated dUTP-fluorescein nick end-labeling (TUNEL) assay using a fragmented DNA detection kit (Millipore) following the manufacturer's instructions. Cells (5×10^5) were seeded in 6-cm plates and pretreated with MβCD for 30 min. The cells were then treated with or without CDT holotoxin for 48 h. The cells were washed, fixed in 1% paraformaldehyde for 15 min and re-suspended in 70% ice-cold ethanol. The cells were then permeabilized with 0.1% Triton X-100 and stained with the TUNEL reaction mixture at 37°C for 30 min. The frequency of TUNEL-positive cells was determined by flow cytometry.

Alternatively, apoptotic cell death was detected using fluorescein isothiocyanate (FITC)-labeled Annexin V (Bio-Vision, CA, USA) and propidium iodide [25]. Cells (5×10^5) were pretreated with MβCD (5 mM) for 30 min, followed by incubation in the presence or absence of CDT holotoxin. After incubation for 48 h, the cells were washed with PBS and then re-suspended in Annexin V binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl and 2.5 Mm CaCl). The cells were then stained with FITC-labeled Annexin V (0.2 μg/ml) for 15 min followed by incubation with propidium iodide (2.5 μg/ml) for 5 min, and then analyzed using the FACSCalibur® flow cytometer (Becton Dickinson).

• Animals & treatments

Six-week-old male BALB/c mice were obtained from the National Laboratory Animal Center of Taiwan. The mice were cared for in accordance with the Animal Care and Use Guidelines of China Medical University under a protocol approved by the Institutional Animal Care Use Committee. Mice were randomly divided into two groups (ten mice each) and fed a high cholesterol diet (HCD; 60% kcal from fat, TestDiet 58Y1; Purina, IN, USA) or a normal diet (ND, 2% kcal) for 35 days starting from 11 weeks of age. Their body weight was recorded at the beginning of the study period. For experiments, HCD and ND mice were then randomly divided into two groups (five mice each) that received either an intragastric gavage with the vehicle alone (PBS) or CDT holotoxin (2.5 mg/kg) once every 3 days, for a total of five administrations (at days 42, 45, 48, 51 and 54). Blood samples were collected in microcentrifuge tubes and sera were separated by centrifugation at 1000 × *g* for 10 min at room temperature. The mice were then sacrificed after 14 h of fasting and the intestinal tissues were prepared for hematoxylin–eosin (H&E) or immunohistochemistry (IHC) staining.

• Assay of cholesterol concentration in serum

Blood was collected by cardiac puncture and centrifuged immediately to separate the serum. Total cholesterol was assayed using a Randox® total cholesterol kit (Randox Laboratories, Crumlin, UK).

• Immunohistochemistry analysis

Intestinal tissues from mice were formalin-fixed and then subjected to H&E or IHC staining. Briefly, tissue sections were de-paraffinized, rehydrated, blocked with 3% bovine serum albumin, and then stained with rabbit monoclonal antibodies against IL-1β (H-153) and TNF-α (CA, USA) for 24 h at 4°C. After washing, the samples were probed with a peroxidase-labeled goat anti-rabbit secondary antibody (Epitomics, CA, USA) and developed with an ABC kit (Vector Laboratories, CA, USA).

• Statistical analysis

The Student's *t*-test was used to determine the statistical significance of the difference between the two groups. A p-value of less than 0.05 was considered statistically significant.

Results

• Depletion of cholesterol reduces CDT intoxication

To investigate the biological activity of *C. jejuni* CDT in epithelial cells, each recombinant His-tagged CDT subunit was purified and analyzed by SDS-PAGE (**Supplementary Figure 1A**, see online at www.futuremedicine.com/doi/suppl/10.2217/fmb.14.119) and western blot **(Supplementary Figure 1B)**. MβCD was then employed to deplete membrane cholesterol and the cytotoxicity in AGS cells was evaluated. The cell viability was not affected by treatment with 0–5 mM MβCD, but slightly decreased by treatment with 10 mM MβCD **(Supplementary Figure 2A)**. After MβCD treatment, cholesterol levels in the cells decreased significantly in a dose-dependent manner **(Supplementary Figure 2B)**. These data indicate that MβCD at concentrations below 5 mM has the capacity to reduce the membrane cholesterol content while barely influencing cell viability.

To further determine whether membrane cholesterol is required for CDT intoxication, flow cytometry was used to assess the cell cycle distribution. Cells were treated with CDT holotoxin for 24 h, resulting in 81% of the cells arrested in G2/M **(Figure 1B)**. Cells were pretreated with various concentrations of MβCD (1, 2.5 and 5 mM) followed by exposure to CDT holotoxin for 24 h, resulting in a dose-dependent decrease of the proportion of cells arrested in G2/M **(Figure 1C–E)**. However, after restoring cholesterol (400 μg/ml), we observed a reversal of the MβCD inhibitory effect on the CDT-induced G2/M arrest **(Figure 1F)**. These results indicate that membrane cholesterol plays an important role in CDT-induced cellular intoxication.

• Membrane cholesterol is important for CDT-induced DSBs

We next determined whether DSB-related proteins are involved in *C. jejuni* CDT-induced intoxication of cells. The expression levels of phosphorylated ATM, p53 and CHK2 in cells treated with CDT holotoxin were higher than that in cells pretreated with MβCD followed by treated with CDT holotoxin **(Figure 2A)**. However, Nijmegen Breakage Syndrome 1 (NBS1) expression was increased compared with that in cells treated with CDT holotoxin. Immunofluorescence analysis showed that the expression of p53-binding protein (53BP1) was highly elevated upon CDT treatment, and this was maintained for up to 24 h **(Figure 2B)**. Additionally, the formation of 53BP1 foci was attenuated by the depletion of cholesterol and was restored by the replenishment of cholesterol **(Figure 2C)**. These findings reveal that the induction of DSB by *C. jejuni* CDT is mediated through the ATM-pathway and is associated with the cellular cholesterol level.

• Involvement of membrane cholesterol in the early stage of CDT-induced apoptosis

The percentages of apoptotic cells were then analyzed by Annexin V/propidium iodide staining and flow cytometry. Compared with the mock treatment **(Figure 3A)** or MβCD alone **(Figure 3B)**, the number of apoptotic cells was significantly elevated by treatment with CDT holotoxin **(Figure 3C)**. In contrast, depletion of cholesterol by MβCD resulted in a decrease in the CDT-induced apoptosis **(Figure 3D)**, and this effect was reversed by the replenishment of cholesterol **(Figure 3E)**. These results suggest that a reduction in the cellular cholesterol levels inhibits *C. jejuni* CDT-induced early apoptosis.

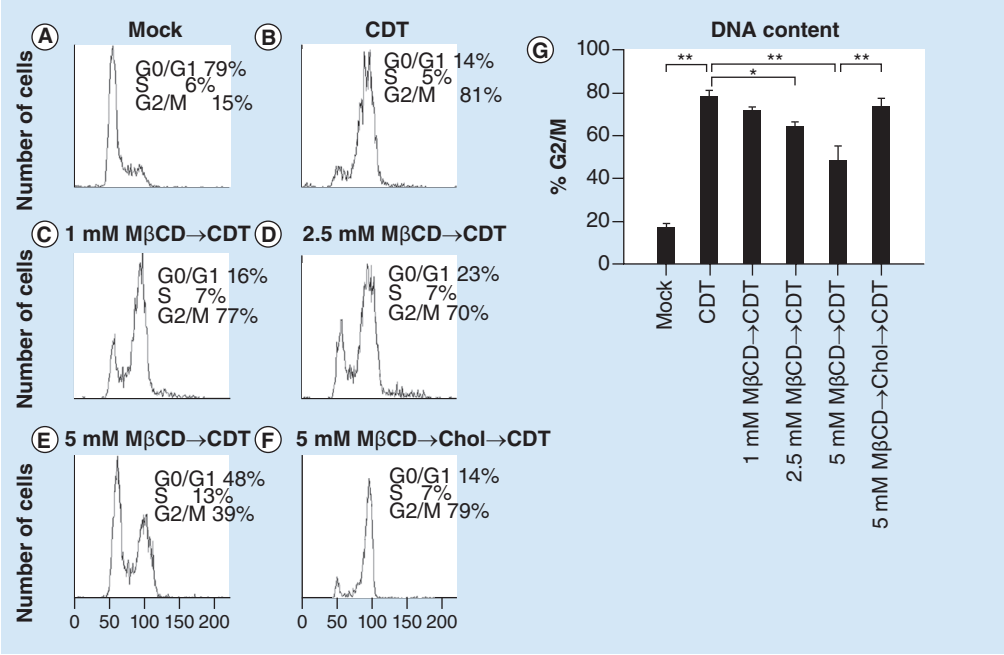

Figure 1. Cholesterol is required for *Campylobacter jejuni* cytolethal distending toxin-induced cell cycle arrest. AGS cells were incubated with **(A)** medium alone, **(B)** 100 nM CDT or **(C)** pretreated with 1 mM MβCD, **(D)** 2.5 mM MβCD, **(E)** 5 mM MβCD or **(F)** 5 mM MβCD, and then replenished with cholesterol (400 μg/ml) for 30 min at 37°C followed by treatment with 100 nM CDT for 24 h. The cells were stained with propidium iodide, and the cell cycle distribution was analyzed by flow cytometry. The proportions of cells in the G0/G1, S and G2/M phases of the cell cycle are shown at the right of each histogram. **(G)** The percentage of cells in G2/M were calculated and plotted as intensity histograms. The results represent the mean and standard deviation from three independent experiments.
*$p < 0.05$.
**$p < 0.01$.
CDT: Cytolethal distending toxin; MβCD: Methyl-β-cyclodextrin.

• CDT-induced apoptosis occurs via activation of the mitochondrial-dependent pathway

We further investigated whether *C. jejuni* CDT induced late apoptotic pathway. CDT holotoxin increased the proportion of cells in the sub-G1 phase **(Figure 4C)** compared with that of cells pretreated with MβCD **(Figure 4D)**. Similar results were also obtained in the TUNEL assay **(Supplementary Figure 3)**. These data suggest that membrane cholesterol is permissive for the apoptotic effect of CDT.

Next, we investigated whether *C. jejuni* CDT-induced apoptosis is mediated through the mitochondrial pathway. Compared with CDT treatment alone, the expressions of several apoptotic proteins including cytochrome *c*, cleaved caspase-3 and cleaved PARP were decreased in MβCD-pretreated cells followed by exposure to CDT **(Figure 5A)**. We further examined the protein expression levels of the Bcl-2 family by western blot. Compared with CDT treatment alone, after pretreatment with MβCD and subsequent exposure to CDT, there was a decrease of the protein levels of proapoptotic proteins Bax and Bak **(Figure 5B)**. In contrast, a significant increase in the levels of anti-apoptotic proteins Bcl-xL and Bcl-2 was observed in cells treated with MβCD and CDT. In parallel, depletion of cholesterol attenuated the CDT-elevated Bax/Bcl-2 and Bak/Bcl-2 ratios **(Figure 5C & D)**. Taken together, these results reveal that cholesterol depletion is able to attenuate the progression of *C. jejuni* CDT-induced cell apoptosis that occurs through DSBs and mitochondria-dependent pathways.

• CDT-induces intestinal inflammation in mice fed with high cholesterol diet

To elucidate whether cholesterol is a key factor involved in facilitating CDT intoxication *in vivo*,

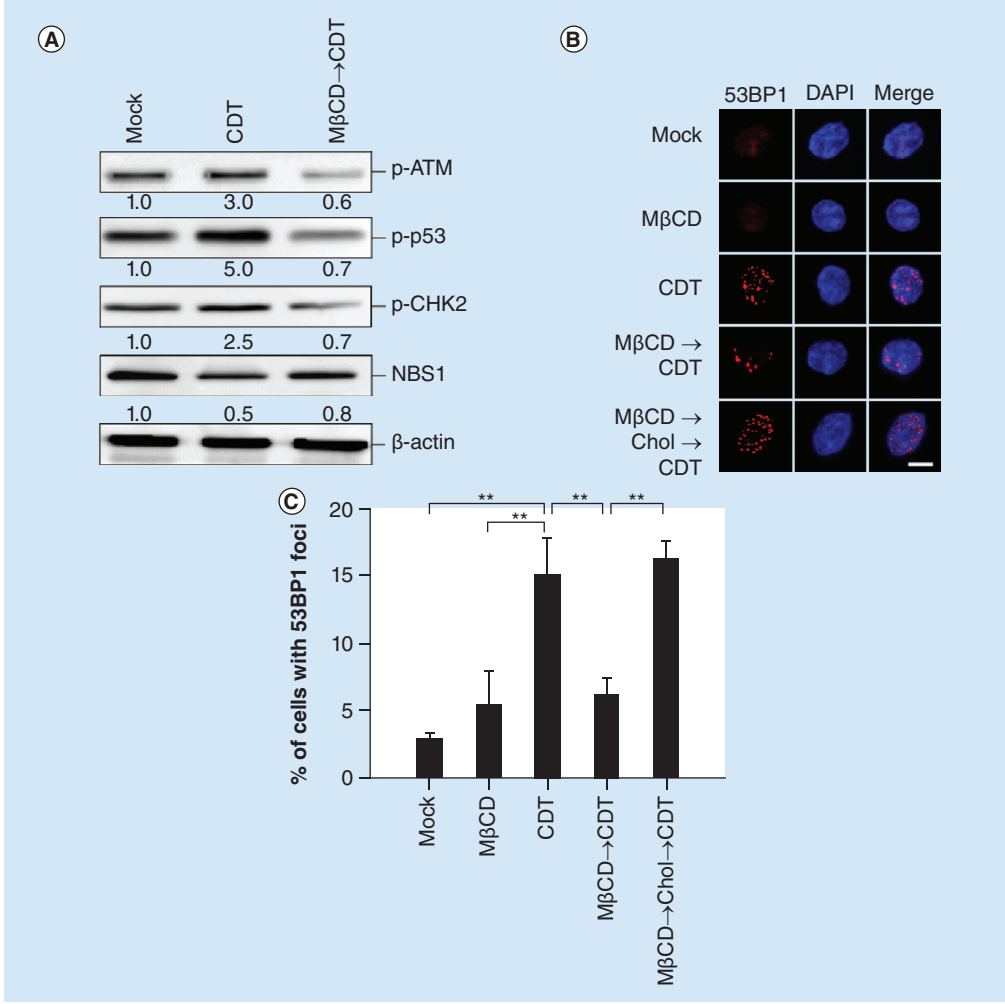

Figure 2. Depletion of cholesterol attenuates the cytolethal distending toxin-induced double-strand breaks pathway. AGS cells were incubated with medium alone, 100 nM CDT, or pretreated with 5 mM MβCD for 1 h and then exposed to CDT for 24 h. **(A)** The expression levels of the double-strand breaks-related proteins p-ATM, p-CHK2, p-p53 and NBS1 were examined by western blotting. The relative level of each protein expression was quantified by signal intensity and was indicated at the bottom of each lane. **(B)** AGS cells were treated with medium alone, 5 mM MβCD alone, 100 nM CDT, or pretreated with 5 mM MβCD and replenished with cholesterol followed by treatment with CDT for 24 h and then analyzed by fluorescence microscopy. Scale bars: 50 μm. **(C)** Histogram showing the percentages of cells with 53BP1 foci.
**p < 0.01, Student's *t*-test.
CDT: Cytolethal distending toxin.

mice were fed with normal diet (ND) or high cholesterol diet (HCD) (**Figure 6A**). After completing the course of the diets, the mice were euthanized and the intestinal tissues were subjected to the histological analyses. As shown in **Supplementary Figure 4**, the level of total cholesterol in the serum of HCD-fed mice was significantly higher than that in the ND-fed mice. Intestinal tissue specimens were stained with H&E for histological examination. As shown in **Figure 6B** (H&E), the intestinal tissue sections from the control group (ND-fed mice without CDT treatment) showed a clearly defined epithelium without inflammation. In contrast, the inflammatory score in ND-fed mice treated with CDT was more severe than that in the CDT untreated group. Further, CDT-induced heavy infiltration of inflammatory cells into the intestinal epithelium and tissue damage dramatically increased in HCD-fed mice compared with ND-fed mice.

We then examined the role of cholesterol in the CDT-induced production of pro-inflammatory cytokines by IHC analysis. As shown in **Figure 6B**, expression of IL-1β and TNF-α was not observed in the intestinal tissues from mice without CDT treatment. Strong expression of proinflammatory cytokines was identified in intestinal tissues from ND-fed mice treated with CDT. However, CDT-induced production of IL-1β and TNF-α was significantly higher in the intestinal epithelium of HCD-fed mice than in the intestinal epithelium of ND-fed mice. Although there was mild pathological derangement seen in the HCD-fed mice in the absence of CDT, no production of IL-1β and TNF-α was observed, suggesting that cholesterol alone could not recapitulate the effects of CDT. These results support that cholesterol plays an important role in facilitating *C. jejuni* CDT-induced pathogenesis in the intestinal epithelium.

Discussion

Little is known about the molecular mechanism of cholesterol-rich microdomain-mediated CDT pathogenicity during *C. jejuni* infection in hosts. Here, we employed an *in vitro* cell culture system to assess the role of membrane cholesterol in the *C. jejuni* CDT-induced cytopathic effects. Subsequent CDT effects, including DSBs and cell cycle arrest, were altered by cholesterol sequestering. Moreover, CDT-induced intestinal inflammation was significantly more pronounced in HCD-fed mice compared with ND-fed mice. The results support the notion

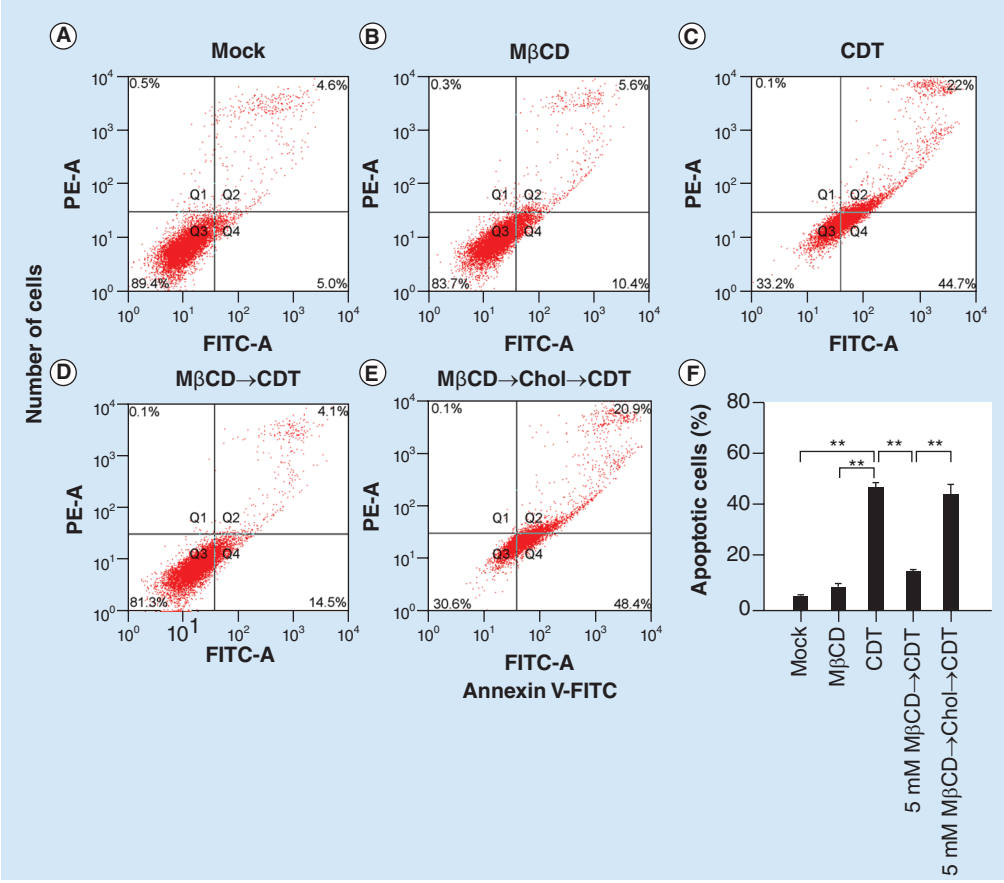

Figure 3. Involvement of cholesterol in cytolethal distending toxin-induced cell apoptosis. AGS cells were exposed to medium alone **(A)**, 5 mM MβCD **(B & D)**, or pretreated with 5 mM MβCD and replenished with cholesterol for 30 min **(E)**, followed by treatment with 100 nM CDT **(C–F)** for 24 h. The cells were then stained with Annexin V/propidium iodide and analyzed by flow cytometry. **(F)** The percentages of Annexin V-labeled cells were calculated and plotted as intensity histograms. The results represent the mean and standard deviation from three independent experiments.
**p < 0.01.
CDT: Cytolethal distending toxin; FITC: Fluorescein isothiocyanate; MβCD: Methyl-β-cyclodextrin.

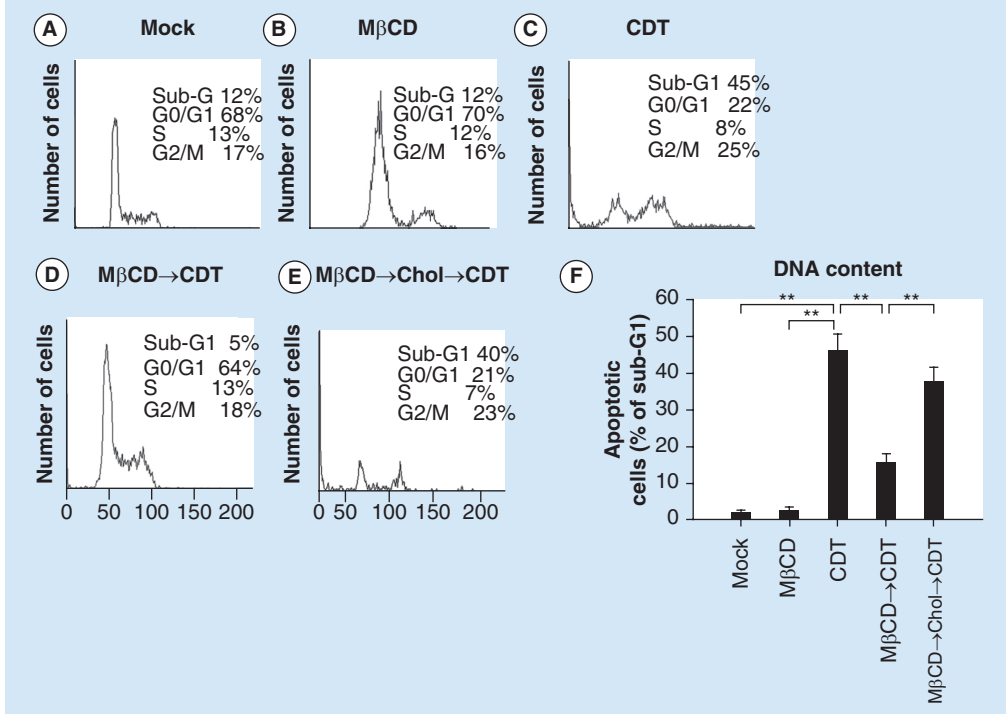

Figure 4. Cholesterol depletion attenuates cytolethal distending toxin induced cell death. AGS cells were incubated at 37°C for 48 h, followed by exposure to medium alone **(A)**, 5 mM MβCD **(B & D)**, or 5 mM MβCD and replenished with cholesterol **(E)**. The cells were then incubated for 24 h in the presence of **(A)** medium or **(C–E)** 100 nM CDT. The cell cycle distribution was determined based on DNA content that was analyzed by flow cytometry. The percentages of cells in sub-G1 phase of the cell cycle are indicated at the right of each histogram. **(F)** The percentages of cells in sub-G1 phase were calculated and plotted as histograms. The results represent the mean and standard deviation from three independent experiments.

**$p < 0.01$.

CDT: Cytolethal distending toxin; MβCD: Methyl-β-cyclodextrin.

that membrane cholesterol facilitates the ability of CDT to exert its cytotoxic effects in hosts.

Our recent findings demonstrated that the binding of CdtA and CdtC to cholesterol-rich microdomains is affected by treatment with MβCD, indicating that membrane cholesterol might act as a unique platform in response to CDT recognition, binding and internalization [18]. Consistently, in this study, we found that a high level of cholesterol is required to form raft-microdomains on the plasma membrane, which act as compartments to facilitate CDT intoxication in mice. It is plausible that some signaling molecules (e.g., tyrosine kinases) localized in membrane-rafts may facilitate the downstream signaling required for toxin action [26,27]. Indeed, this mechanism may provide a prominent advantage for *C. jejuni*, resulting in efficient CDT-induced pathogenesis of host cells.

In our previous study, we first tested whether CDT has the ability to intoxicate different cell types [18]. We employed three cell lines (AGS, Caco-2 and COLO205 cells) to determine the CDT intoxication activity. CDT-induced G2/M cell cycle arrest increased in these three cell lines with increase in CDT concentration. The saturation level for CDT intoxication by AGS cells was 200 nM; however, the two intestine-derived cell lines were not intoxicated by CDT at the saturation concentration, even at the highest concentration of 500 nM. Because the AGS cell line showed greater susceptibility to CDT-induced cell cycle arrest than did the other two cell lines, we selected AGS cells for analysis in this study.

In this study, the expression of several molecules was altered during *C. jejuni* CDT intoxication, including an upregulation of ATM, as well as activation of CHK2 and 53BP1. Eliciting ATM-dependent checkpoint responses by CDT requires

functional Myc [28], a proto-oncogene expressed abundantly in cancer cells. This event triggers substantial DNA damage that may facilitate genomic instability and promote tumor progression [29]. Additionally, accumulation of cholesterol may potentiate oncogenic signaling in some cancers [30–32]. Given that cholesterol is accumulated in cancer cells and is critical for CDT function, it is conceivable that alteration of cholesterol levels may be a novel strategy to prevent bacterial infections and their related cancers.

Increasing evidence indicates a link between *C. jejuni* infection and cardiovascular disease development [33–35]. However, the pathogenic mechanisms in *C. jejuni*-infected patients that increase the risk of cardiovascular disease are still unclear. Our present data show that *C. jejuni* CDT elicited production of IL-1β in the intestinal tissues of mice with high cholesterol levels. This finding is supported by previous studies showing that bacterial cell-free soluble components have the potential to induce pro-inflammatory responses [36], which may be important in atherogenesis and considered as biomarkers of cardiovascular disease risk [37]. Although it is tempting to speculate that CDT

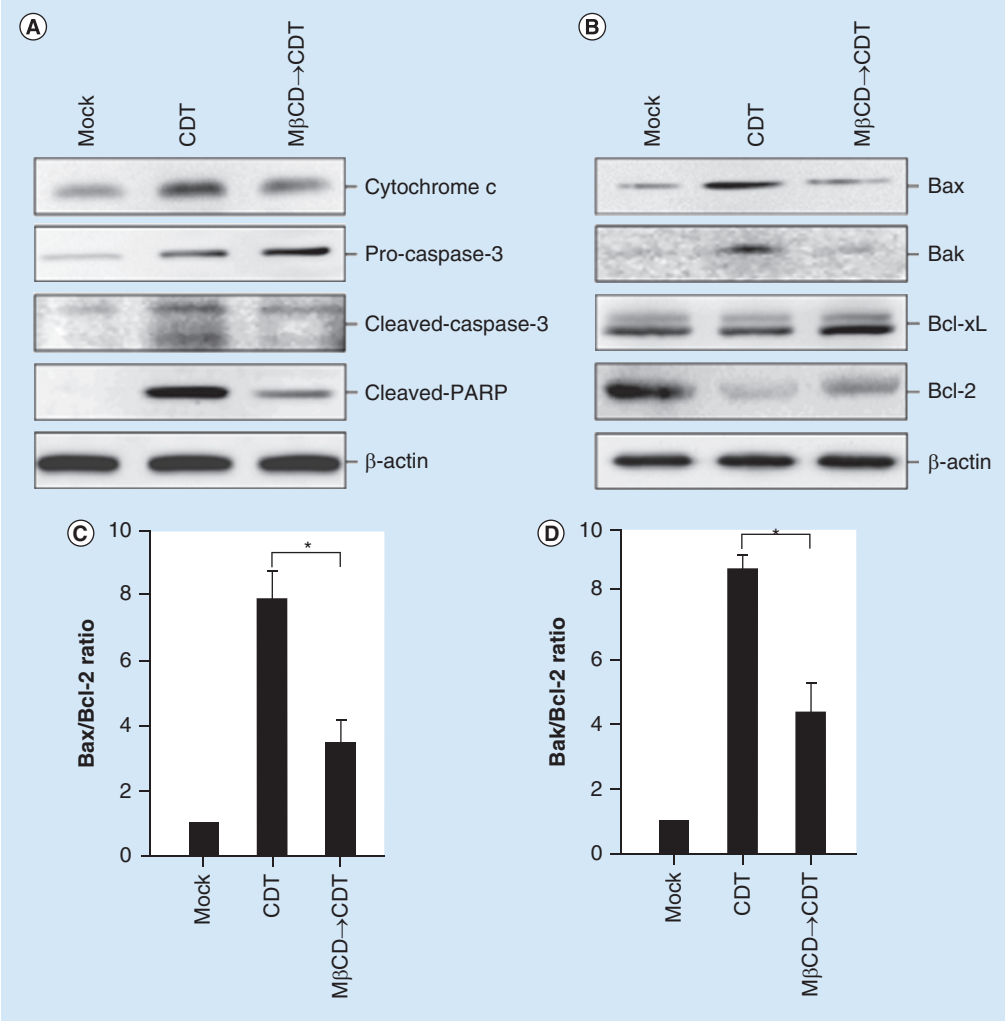

Figure 5. Involvement of cholesterol in cytolethal distending toxin induced apoptosis mediated through mitochondria-dependent and Bcl-2 family pathways. (A–B) AGS cells were treated with medium alone, 100 nM CDT or pretreated with 5 mM MβCD for 30 min and then exposed to CDT for 48 h. The protein expression levels were examined by western blot. **(C–D)** Histograms show the ratios of Bax/Bcl-2 and Bak/Bcl-2 expression as quantified by densitometry. The results represent the mean and standard deviation from three independent experiments.
*$p < 0.05$.
CDT: Cytolethal distending toxin; MβCD: Methyl-β-cyclodextrin.

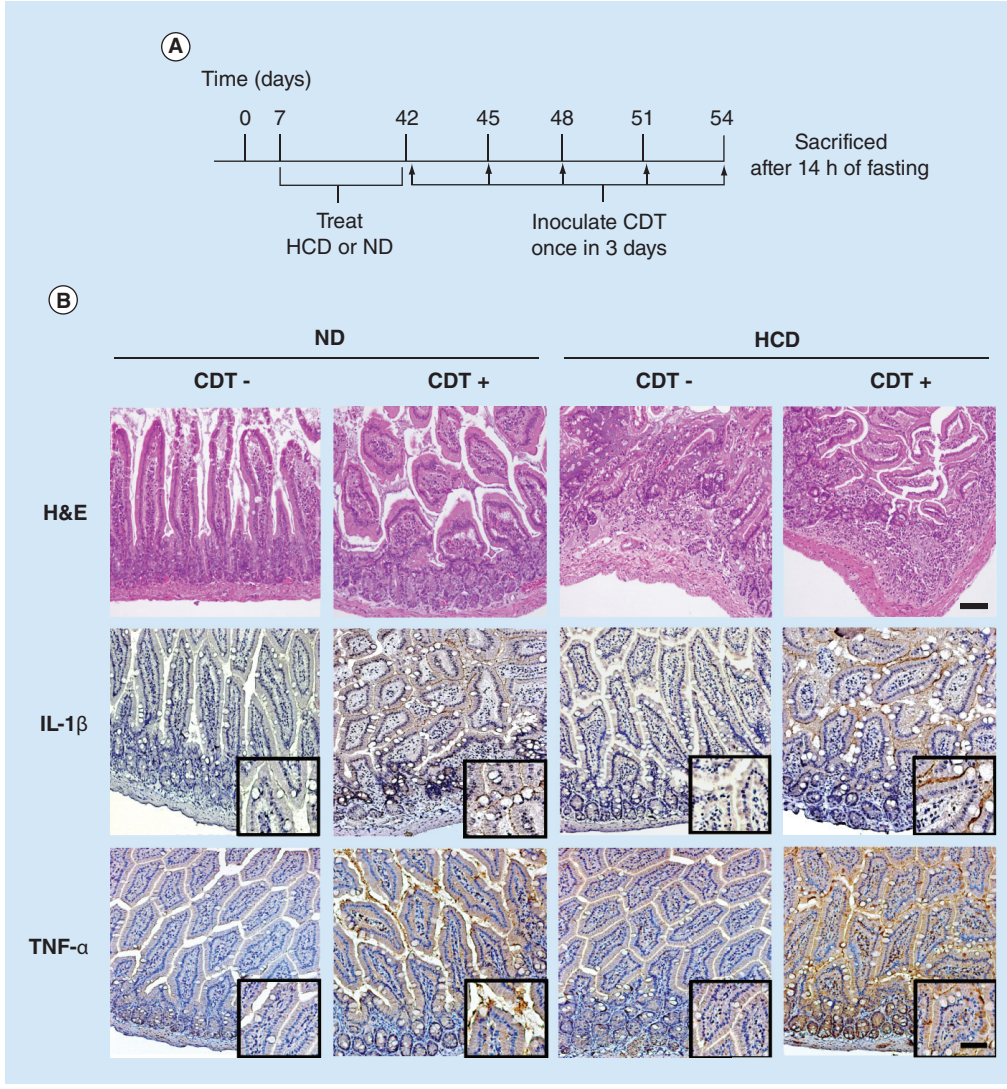

Figure 6. Cholesterol enhances cytolethal distending toxin-induced inflammation in mouse intestinal epithelium. (A) Mice were fed with ND or HCD for 35 days followed by intragastric gavage with or without CDT holotoxin (2.5 mg/kg) once every 3 days for five administrations. Arrows show the days of CDT administration. Mice were sacrificed and tissue specimens from the intestine were formalin-fixed and then subjected to **(B)** H&E or immunohistochemistry staining with specific antibodies against IL-1β and TNF-α (original magnification: 200×). The magnified images were shown in the lower right corner. Scale bars: 100 μm and 50 μm (insets).
CDT: Cytolethal distending toxin; H&E: Hematoxylin–eosin; HCD: High cholesterol diet; ND: Normal diet.

and increased cholesterol levels increase the risk of cardiovascular disease, further studies are required to investigate clinical associations between CDT-positive *C. jejuni* infection and cholesterol-related diseases.

Conclusion

In this study, we have demonstrated that membrane cholesterol plays a critical role in *C. jejuni* CDT intoxication of cells. The biological functions of *C. jejuni* CDT include not only DSBs, cell cycle arrest and apoptosis, but also inflammatory responses in the hosts. However, disruption of membrane rafts by a cholesterol-sequestering agent impairs CDT actions. Consistently, in the *in vivo* animal study, CDT-induced significant higher levels of proinflammatory cytokine production in the intestines of mice with high cholesterol levels

compared with that in the normal control group. Elucidating the molecular mechanism involved in the association of cholesterol with CDT may provide new insights into *C. jejuni*-induced pathogenesis and shed light on novel strategies to control the progression of infectious diseases.

Future perspective

Previous studies have revealed that lipid rafts, cholesterol-rich microdomains, might serve as entry portals for pathogens, including bacteria [14,23,38–44] and viruses [45–49]. Additionally, nonintracellular bacteria may hijack host membrane cholesterol for delivery of their toxins [50]. CDT produced by several Gram-negative bacteria including. *Actinobacillus actinomycetemcomitans*, *C. jejuni* and *Haemophilus ducreyi*, is found interacted with lipid rafts, followed by exerting its genotoxicity [7,18–19], indicating that the interaction of pathogens and toxins with cholesterol might magnify the signaling for their pathogenesis in the hosts. Therefore, controlling cholesterol may be a novel strategy to prevent hosts from CDT intoxication.

In recent years, the inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, known as statins, are widely prescribed for lowering serum cholesterol. Previous studies demonstrated that statins are used to reduce the risk of some bacterial infections, including *Chlamydia pneumonia* [51], *Clostridium difficile* [52], *H. pylori* [53], *Streptococcus pneumonia* [54] and *Staphylococcus aureus* [55]. These findings shed light on the novel approach that inhibition of cellular cholesterol may be useful to inhibit bacteria-induced pathogenesis. Therefore, it is worthy to develop new agents with extreme safety that reduce cholesterol and control the infection of these pathogens.

Acknowledgements

The authors thank the assistance of Shu-Chen Shen for providing the microscopic facility at the Scientific Instrument Center of Academia Sinica.

Financial & competing interests disclosure

This work was supported by Ministry of Science and Technology (103-2633-B-039-001 and 103-2811-B-039-018), China Medical University (CMU102-BC-2, CMU102-ASIA-21), Tomorrow Medicine Foundation and Taiwan Ministry of Health and Welfare Clinical Trial and Research Center of Excellence

EXECUTIVE SUMMARY

Structure & biological activity of cytolethal distending toxin

- Cytolethal distending toxin (CDT) is a genotoxin produced by *Campylobacter jejuni*, and is composed of three subunits, CdtA, CdtB and CdtC.
- The translocation of CdtB into the nucleus requires the association of CdtA/CdtC with lipid rafts, which are cholesterol-rich microdomains localized in the cell membrane.
- Nuclear-translocated CdtB contains type I deoxyribonuclease activity that induces DNA double-strand breaks followed by apoptosis.

CDT–cell interactions

- *Campylobacter jejuni* CDT not only induced DNA double-strand breaks and apoptosis, but also inflammatory responses in target cells.
- CDT effects are attenuated by depletion of cellular cholesterol.

CDT–host interactions

- Severe intestinal inflammation induced by CDT treatment is observed in high-cholesterol diet-fed mice, but not in normal diet-fed mice.
- Cholesterol is essential for CDT intoxication in hosts.

Conclusion & future perspective

- Our findings demonstrate a molecular link between *C. jejuni* CDT and cholesterol, which is crucial to facilitate CDT-mediated pathological derangement.
- Elucidating the molecular mechanism involved in the association of cholesterol with CDT may shed light on novel strategies to control the progression of infectious diseases.

(MOHW104-TDU-B-212-113002). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

Papers of special note have been highlighted as:
• of interest

1 Ohara M, Oswald E, Sugai M. Cytolethal distending toxin: a bacterial bullet targeted to nucleus. *J. Biochem.* 136(4), 409–413 (2004).

2 Whitehouse CA, Balbo PB, Pesci EC *et al.* *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. *Infect. Immun.* 66(5), 1934–1940 (1998).

3 Johnson WM, Lior H. A new heat-labile cytolethal distending toxin (CLDT) produced by *Campylobacter* spp. *Microb. Pathog.* 4(2), 115–126 (1988).

4 Cope LD, Lumbley S, Latimer JL *et al.* A diffusible cytotoxin of *Haemophilus ducreyi*. *Proc. Natl Acad. Sci. USA* 94(8), 4056–4061 (1997).

5 Johnson WM, Lior H. Production of Shiga toxin and a cytolethal distending toxin (Cldt) by serogroups of *Shigella* spp. *FEMS Microbiol. Lett.* 48(1–2), 235–238 (1987).

6 Lara-Tejero M, Galan JE. CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. *Infect. Immun.* 69(7), 4358–4365 (2001).

7 Boesze-Battaglia K, Besack D, McKay T *et al.* Cholesterol-rich membrane microdomains mediate cell cycle arrest induced by *Actinobacillus actinomycetemcomitans* cytolethal-distending toxin. *Cell. Microbiol.* 8(5), 823–836 (2006).

8 Boesze-Battaglia K, Brown A, Walker L *et al.* Cytolethal distending toxin-induced cell cycle arrest of lymphocytes is dependent upon recognition and binding to cholesterol. *J. Biol. Chem.* 284(16), 10650–10658 (2009).

9 McSweeney LA, Dreyfus LA. Nuclear localization of the *Escherichia coli* cytolethal distending toxin CdtB subunit. *Cell. Microbiol.* 6(5), 447–458 (2004).

10 Lara-Tejero M, Galan JE. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. *Science* 290(5490), 354–357 (2000).

• **Provided evidence that CdtB exhibits DNAase I activity, which is responsible for cytolethal distending toxin (CDT)-induced DNA damage and control cell cycle progression.**

11 Brown DA, London E. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell. Dev. Biol.* 14, 111–136 (1998).

12 Hooper NM. Detergent-insoluble glycosphingolipid/cholesterol-rich membrane domains, lipid rafts and caveolae (review). *Mol. Membr. Biol.* 16(2), 145–156 (1999).

13 Simons M, Kramer EM, Macchi P *et al.* Overexpression of the myelin proteolipid protein leads to accumulation of cholesterol and proteolipid protein in endosomes/lysosomes: implications for Pelizaeus–Merzbacher disease. *J. Cell. Biol.* 157(2), 327–336 (2002).

14 Wooldridge KG, Williams PH, Ketley JM. Host signal transduction and endocytosis of *Campylobacter jejuni*. *Microb. Pathog.* 21(4), 299–305 (1996).

15 Hu L, McDaniel JP, Kopecko DJ. Signal transduction events involved in human epithelial cell invasion by *Campylobacter jejuni*. 81–176. *Microb. Pathog.* 40(3), 91–100 (2006).

16 Zaas DW, Duncan M, Rae Wright J, Abraham SN. The role of lipid rafts in the pathogenesis of bacterial infections. *Biochim. Biophys. Acta* 1746(3), 305–313 (2005).

17 Watson RO, Galan JE. *Campylobacter jejuni* survives within epithelial cells by avoiding delivery to lysosomes. *PLoS Pathog.* 4(1), e14 (2008).

18 Lin CD, Lai CK, Lin YH *et al.* Cholesterol depletion reduces entry of *Campylobacter jejuni* cytolethal distending toxin and attenuates intoxication of host cells. *Infect. Immun.* 79(9), 3563–3575 (2011).

• **Demonstrated that cholesterol plays an important role in the binding of *C. jejuni* CDT to the cell membrane for induction of cytotoxicity.**

19 Guerra L, Teter K, Lilley BN *et al.* Cellular internalization of cytolethal distending toxin: a new end to a known pathway. *Cell. Microbiol.* 7(7), 921–934 (2005).

20 Eshraghi A, Maldonado-Arocho FJ, Gargi A *et al.* Cytolethal distending toxin family members are differentially affected by alterations in host glycans and membrane cholesterol. *J. Biol. Chem.* 285(24), 18199–18207 (2010).

21 Lai CH, Lai CK, Lin YJ *et al.* Characterization of putative cholesterol recognition/interaction amino acid consensus-like motif of *Campylobacter jejuni* cytolethal distending toxin C. *PLoS ONE* 8(6), e66202 (2013).

22 Lu DY, Chen HC, Yang MS *et al.* Ceramide and Toll-like receptor 4 are mobilized into membrane rafts in response to *Helicobacter pylori* infection in gastric epithelial cells. *Infect. Immun.* 80(5), 1823–1833 (2012).

23 Lai CH, Chang YC, Du SY *et al.* Cholesterol depletion reduces *Helicobacter pylori* CagA translocation and CagA-induced responses in AGS cells. *Infect. Immun.* 76(7), 3293–3303 (2008).

24 Lu DY, Tang CH, Chang CH *et al.* *Helicobacter pylori* attenuates lipopolysaccharide-induced nitric oxide production by murine macrophages. *Innate Immun.* 18(3), 406–417 (2012).

25 Lin CD, Kao MC, Tsai MH *et al.* Transient ischemia/hypoxia enhances gentamicin ototoxicity via caspase-dependent cell death pathway. *Lab. Invest.* 91(7), 1092–1106 (2011).

26 Oda M, Shiihara R, Ohmae Y *et al.* *Clostridium perfringens* alpha-toxin induces the release of IL-8 through a dual pathway via TrkA in A549 cells. *Biochim. Biophys. Acta* 1822(10), 1581–1589 (2012).

27 Simons K, Toomre D. Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell. Biol.* 1(1), 31–39 (2000).

28 Guerra L, Albihn A, Tronnersjo S *et al.* Myc is required for activation of the ATM-dependent checkpoints in response to DNA damage. *PLoS ONE* 5(1), e8924 (2010).

29 Guerra L, Guidi R, Frisan T. Do bacterial genotoxins contribute to chronic inflammation, genomic instability and tumor progression? *FEBS J.* 278(23), 4577–4588 (2011).

30 Zhuang L, Kim J, Adam RM, Solomon KR, Freeman MR. Cholesterol targeting alters

lipid raft composition and cell survival in prostate cancer cells and xenografts. *J. Clin. Invest.* 115(4), 959–968 (2005).

31 Freeman MR, Solomon KR. Cholesterol and prostate cancer. *J. Cell. Biochem.* 91(1), 54–69 (2004).

32 Kolanjiappan K, Ramachandran CR, Manoharan S. Biochemical changes in tumor tissues of oral cancer patients. *Clin. Biochem.* 36(1), 61–65 (2003).

33 Cunningham C, Lee CH. Myocarditis related to *Campylobacter jejuni* infection: a case report. *BMC Infect. Dis.* 3, 16 (2003).

34 Alzand BS, Ilhan M, Heesen WF, Meeder JG. *Campylobacter jejuni*: enterocolitis and myopericarditis. *Int. J. Cardiol.* 144(1), e14–16 (2010).

35 De Cock D, Hiltrop N, Timmermans P, Dymarkowski S, Van Cleemput J. Myocarditis associated with campylobacter enteritis: report of three cases. *Circ. Heart Fail.* 5(2), e19–e21 (2012).

36 Oscarsson J, Karched M, Thay B, Chen C, Asikainen S. Proinflammatory effect in whole blood by free soluble bacterial components released from planktonic and biofilm cells. *BMC Microbiol.* 8, 206 (2008).

37 Packard RR, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clin. Chem.* 54(1), 24–38 (2008).

38 Lafont F, Tran Van Nhieu G, Hanada K, Sansonetti P, van der Goot FG. Initial steps of *Shigella* infection depend on the cholesterol/sphingolipid raft-mediated CD44-IpaB interaction. *EMBO J.* 21(17), 4449–4457 (2002).

39 Duncan MJ, Li G, Shin JS, Carson JL, Abraham SN. Bacterial penetration of bladder epithelium through lipid rafts. *J. Biol. Chem.* 279(18), 18944–18951 (2004).

40 Grassme H, Jendrossek V, Riehle A *et al.* Host defense against *Pseudomonas aeruginosa* requires ceramide-rich membrane rafts. *Nat. Med.* 9(3), 322–330 (2003).

41 Lin M, Rikihisa Y. Obligatory intracellular parasitism by *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* involves caveolae and glycosylphosphatidylinositol-anchored proteins. *Cell. Microbiol.* 5(11), 809–820 (2003).

42 Kowalski MP, Pier GB. Localization of cystic fibrosis transmembrane conductance regulator to lipid rafts of epithelial cells is required for *Pseudomonas aeruginosa*-induced cellular activation. *J. Immunol.* 172(1), 418–425 (2004).

43 Wang M, Hajishengallis G. Lipid raft-dependent uptake, signalling and intracellular fate of *Porphyromonas gingivalis* in mouse macrophages. *Cell. Microbiol.* 10(10), 2029–2042 (2008).

44 Garner MJ, Hayward RD, Koronakis V. The *Salmonella* pathogenicity island 1 secretion system directs cellular cholesterol redistribution during mammalian cell entry and intracellular trafficking. *Cell. Microbiol.* 4(3), 153–165 (2002).

45 Panchal RG, Ruthel G, Kenny TA *et al. In vivo* oligomerization and raft localization of Ebola virus protein VP40 during vesicular budding. *Proc. Natl Acad. Sci. USA* 100(26), 15936–15941 (2003).

46 Favoreel HW, Mettenleiter TC, Nauwynck HJ. Copatching and lipid raft association of different viral glycoproteins expressed on the surfaces of pseudorabies virus-infected cells. *J. Virol.* 78(10), 5279–5287 (2004).

47 Chung CS, Huang CY, Chang W. Vaccinia virus penetration requires cholesterol and results in specific viral envelope proteins associated with lipid rafts. *J. Virol.* 79(3), 1623–1634 (2005).

48 Bremer CM, Bung C, Kott N, Hardt M, Glebe D. Hepatitis B virus infection is dependent on cholesterol in the viral envelope. *Cell. Microbiol.* 11(2), 249–260 (2009).

49 Pelkmans L, Puntener D, Helenius A. Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae. *Science* 296(5567), 535–539 (2002).

50 Manes S, del Real G, Martinez AC. Pathogens: raft hijackers. *Nat. Rev. Immunol.* 3(7), 557–568 (2003).

- **Review article that clearly discussed the critical role of cholesterol for the infectious diseases.**

51 Erkkila L, Jauhiainen M, Laitinen K *et al.* Effect of simvastatin, an established lipid-lowering drug, on pulmonary *Chlamydia pneumoniae* infection in mice. *Antimicrob. Agents Chemother.* 49(9), 3959–3962 (2005).

52 Motzkus-Feagans CA, Pakyz A, Polk R, Gambassi G, Lapane KL. Statin use and the risk of *Clostridium difficile* in academic medical centres. *Gut* 61(11), 1538–1542 (2012).

53 Nseir W, Khateeb J, Tatour I *et al.* Long-term statin therapy affects the severity of chronic gastritis. *Helicobacter* 15(6), 510–515 (2010).

54 Boyd AR, Hinojosa CA, Rodriguez PJ, Orihuela CJ. Impact of oral simvastatin therapy on acute lung injury in mice during pneumococcal pneumonia. *BMC Microbiol.* 12, 73 (2012).

55 Chow OA, von Kockritz-Blickwede M, Bright AT *et al.* Statins enhance formation of phagocyte extracellular traps. *Cell Host Microbe* 8(5), 445–454 (2010).

- **Report that demonstrated that reduced cholesterol enhanced bacterial clearance by phagocytes.**