INTERACTION BETWEEN CYTOLETHAL DISTENDING TOXIN PRODUCED BY CAMPYLOBACTER JEJUNI AND HELICOBACTER HEPATICUS AND HOST INNATE IMMUNE DEFENSE

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Cytolethal distending toxin (CDT) of *C. jejuni*, a leading cause of intestinal illness worldwide, and *H. hepaticus*, a laboratory mice pathogen causes cell cycle arrest and induces apoptosis in eukaryotic cells. In the present studies, the interactions of CDT with host innate defense mechanisms were investigated within the context of intestinal infection. The acute stage of intestinal infection by *C. jejuni* is characterized by massive transepithelial migration of activated PMNs into the intestinal lumen. Recently, neutrophil extracellular traps (NETs) have emerged as an important innate host defense mechanism against bacterial pathogens. We hypothesize that CDT nuclease mediates resistance against extracellular killing in NETs. First, NETs were discovered to capture *C. jejuni* in non-human primates with acute campylobacteriosis. The lack of direct induction of NETs formation *in vitro* by *C. jejuni* indicates NETs formation in *C. jejuni* infection might be indirectly mediated by components present within the intestinal microenvironment. Although *C. jejuni* were efficiently captured within NETs, they themselves did not induce NET fromation. However, once captured, *C. jejuni* survived within NETs, and CDT did not play a role in escape of *C. jejuni* from NETs. Taken together, the data suggest that within the context of the intestinal tract, NETs likely provide innate defense by a mechanism of bacterial exclusion and disposal by intestinal peristalsis.

Given that CDT-induced intoxication of epithelial cells leads to a DDR, we hypothesized that CDT-intoxicated intestinal epithelial cells display a senescence-associated secretory phenotype (SASP). Following treatment of human intestinal epithelial cells with sub-lethal doses of CDT from either *C. jejuni* or *H. hepaticus* or *C. jejuni* whole cell lysates, we observed persistent DDR including cell growth arrest and upregulation of γ-H2AX, accumulation of intracytoplasmic beta-galactosidase, and expression of pro-inflammatory cytokines IL-6 and IL-24 and CXCL-8 chemokine characteristic of SASP. In sum, the data reveal that activation of senescence pathways is a novel and potentially important downstream mechanism of CDT-induced genotoxicity that might contribute to host innate defense during intestinal infection by bacterial pathogens.

BIOGRAPHICAL SKETCH

With a great interest in veterinary medicine, Changyou Lin became a veterinary student at College of Veterinary Medicine in China Agricultural University in 1997. After he got his BVM degree in 2001, he continued to pursuing his master degree in preventive veterinary medicine, and mainly focused on infectious diseases and immune related gene analysis. During this period, he contributed to several publications about immunology and infectious disease, and developed a great interest in comparative biomedical sciences.

In 2006, one year after he got accepted by Ph.D. program in China, he quit and came to the US for a family reunion. His dream to do research in veterinary medicine did not cease, and he entered graduate school in Cornell University and became a fresh Ph.D. student again in the field of immunology and infectious disease at College of Veterinary Medicine in 2007. He is currently studying in Dr. Gerald E. Duhamel's lab, and his research is mainly about the role of cytolethal distending toxin, a genotoxin produced by *Campylobacter jejuni* and *Helicobacter hepaticus* in host innate immune defense.

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INTRODUCTION

Cytolethal Distending Toxin

Cytolethal distending toxin (CDT) was first discovered about 27 years ago from the culture filtrates of *Escherichia coli* (1) and *Campylobacter jejuni* (2), and it was defined as a heat labile toxin factor that caused progressive mammalian cell distension. After the genes encoding *E. coli* CDT (EcolCDT) were cloned and sequenced (3), the molecular biology and pathogenetic mechanisms of disease were widely investigated. To date, CDT has been found in over two dozen of Gram-negative human and animal mucocutaneous bacterial pathogens primarily associated with major food- and water-borne diseases including *Helicobacter hepaticus* and several serotypes of *Salmonella enterica* (4).

With the exception of the intestinal pathogen *S. enterica* which encodes only the CdtB subunit (5-7), CDT from all other bacterial pathogens consists of *cdtA*, *cdtB*, and *cdtC* genes, respectively encoding the corresponding tripartite polypeptide subunits A, B, and C. The CDT holotoxin displays features of an AB type 2 component toxin, in which the "A" component, represented by CdtB, is the biologically active subunit, while the "B" component, represented by CdtA and CdtC, binds to host cell lipid raft membrane microdomains and assists in the delivery of CdtB into host cell cytoplasm (8-10). The mechanism of CDT uptake into target cells and its intracellular trafficking are incompletely understood. A receptor-mediated uptake process has been proposed for CDT; however, the exact receptor is controversial. *Actinobacillus actinomycetemcomitans* CDT (AactCDT) co-localizes with GM1-enriched membrane

fractions and their association is reduced by cholesterol depletion (10). Inactivation of SGMS1 and TMEM181 genes in mammalian cells results in reduced cell cycle arrest induction by EcolCDT; TMEM181 binds EcolCDT and might be part of the complex on the cell surface that interacts with CDT (11). After up take into host cells, *Haemophilus ducreyi* CDT (HducCDT) is transported from the endoplasmic reticulum (ER) to the Golgi complex through a retrograde pathway; blocking of CDT internalization abolishes its cytotoxicity (12). No evidence shows the translocation of CDT from ER to cytosol, and the active CdtB subunit may move directly from ER into nucleus (12, 13). Disruption of endosome acidification prevents HducCDT-mediated cell cycle arrest, but not EcolCDT-induced cell cycle arrest, suggesting at least two different endocytic pathways before reaching the nucleus. These divergent pathways correlate with low CdtA and CdtC sequences identity (22% and 19%) between these two bacterial species (14).

The B subunit displays conserved structural and functional homologies to members of the phosphodiesterase family including mammalian deoxyribonuclease I (DNase I) (8). Recombinant CdtB is capable of digesting plasmid DNA *in vitro* (8, 15). Endonuclease inhibitors prevent intoxication of B cells by recombinant CDT, suggesting cellular toxicity of CdtB is mediated by its nuclease activity (16). Furthermore, CdtB alone exhibits intracellular DNase I activity characterized by disruption and induction of G2/M cell cycle arrest, when expressed transiently, or microinjected into mammalian cells (17). Demonstration of DNA double-strand breaks (DSB) together with γ-H2AX expression following intoxication of HeLa cells

with HducCDT confirmes CDT-induced DNA damage results in a DNA damage response (DDR) (18, 19). Consistent with these observations is activation of tumor suppressor p53, cyclin dependent kinase inhibitor p21 and check point kinase CHK2 in HducCDT treated fibroblasts and keratinocytes and delayed DDR in HducCDT-intoxicated Ataxia Telangiectasia-mutated gene (ATM)-deficient lymphoblastoid cell lines (20). Fibroblasts exposed to HhepCDT exhibit MYC and ATM-dependent checkpoint activation similar to ionizing radiation-induced DDR (21). HducCDT induces DSB in HeLa cells similar to ionizing radiation, and activation of ATM-dependent RhoA GTPase leads to actin cytoskeleton rearrangement (18). Rad50 foci, which are involved in telomere maintenance and DNA damage (22), are also formed around the DSB in primary human fibroblasts intoxicated with CjejCDT (23).

It is generally accepted, in response to CDT induced DSB, activated ATM initiates DDR including activation of γ-H2AX, CHK2, p53 and p21, which in turn results in cell cycle arrest. The cell cycle arrest allows repair of the damaged DNA. CDT from many bacterial species also induces apoptosis in various cell lines including fibroblast, endothelial cell, epithelial and lymphoid (4). Few studies from a single investigator using human lymphoid cell lines have reported that CDT also displays phosphatidylinositol (PI)-3,4,5-triphosphate (PIP_3) phosphatase activity (24, 25), and might contribute to host immune response modulation. Cytotoxicity of CDT observed in studies differs according to target cells, toxin preparation including bacterial source, method of purification and resulting specific activity and concentration. CDT arrests epithelial and endothelial cell lines at G_2/M phase (9, 26-28), and fibroblast cell lines

at either G_1/S or G_2/M phase (20, 23, 29). Immune cells appear to be more sensitive to CDT, and usually undergo apoptosis after a short cell cycle arrest (20, 29, 30).

In our study, *C. jejuni* and *H. hepaticus* were used to investigate the role of CDT in host innate immune defense and pathogenesis of CDT within the context of the intestinal tract by using the human intestinal epithelial INT407 cell line.

Helicobacter hepaticus

The prototype for enterohepatic *Helicobacter* species, *H. hepaticus* is a spiral Gram negative microaerophilic bacteria, which was originally isolated from A/JCr, and later from several strains of inbred laboratory mice with chronic active hepatitis (31, 32). *H. hepaticus* colonizes the lower intestine and hepatobiliary tracts of susceptible inbred strains of mice causing persistent typhlocolitis and hepatitis leading to hepatocellular carcinoma (6, 31-37). *H. hepaticus* DNA has also been detected by PCR of specimens obtained from patients with biliary disease (38), hepatocellular carcinoma (39), cholangiocarcinoma, and inflammatory bowel disease (39, 40). Experimental infection of mice with *H. hepaticus* has been used to investigate inflammatory bowel disease (41) and bacteria-induced neoplastic diseases of the intestine, liver and mammary gland (40, 42, 43).

The complete genome of *H. hepaticus* reveals that genome content varies in different strains with many orthologs of *C. jejuni*. Most importantly, CDT is the only known

virulence factor found in *H. hepaticus* (44), its role in the pathogenesis of *H. hepaticus* infection and disease are incompletely understood (45-47). We reported previously that *H. hepaticus* CDT causes apoptotic cell death of intestinal epithelial cells through the mitochondrial pathway (48). HhepCDT also cause cell cycle arrest in human epithelial cell line *in vitro* (49). These observations are consistent with induction of apoptotic cell death in hepatitis and hepatocellular carcinoma of laboratory mice infected with *H. hepaticus* (6, 36, 39, 50, 51). CDT-deficient *H. hepaticus* fail to colonize the large intestine persistently in IL-10 knockout mice and cause less severe typhlocolitis (36, 37).

Campylobacter jejuni

Campylobacter jejuni is a Gram-negative bacterial pathogen which is the leading cause of food-borne diarrhea in humans worldwide. Prevalence of *C. jejuni* infection in the US is second closely behind *Salmonella*, and is about 14 laboratory-confirmed cases per 100,000 population in 2011 excluding many more undiagnosed and unreported cases (52); thus from the current total population of 316.5 million, 4.4 million people develop campylobacteriosis each year. According to a CDC estimate (52); *Campylobacter* infection affects approximately 0.8% or 25.3 million of the US population each year. Most of the *Campylobacter* infection is caused by *C. jejuni*, which are microaerobic, motile, spiral to curve-shaped, flagellated Gram negative bacteria that colonize the intestinal tract (53). The main sources of human infections are consumption and handling of contaminated poultry and the ingestion of

contaminated water (54, 55). Clinical signs range from mild, watery diarrhea to severe inflammatory bloody diarrhea, which last between 24-48 h, but generally, resolve within 10 days (54, 56). Although the infection is self-limiting in healthy persons, severe disease is seen in children, elderly and immunocompromised individuals. In some cases, *C. jejuni* infection can result in severe sequelae including Guillain–Barre syndrome (GBS) (57), relapse of inflammatory bowel diseases (58), reactive arthritis (59), and post-infectious irritable bowel syndrome (60).

Because there are no well-established laboratory animal models to study *C. jejuni* infection, the pathogenesis of campylobacteriosis and the contribution of virulence factors are incompletely understood. Murine models of *C. jejuni* are considered "colonization" models and lack clinical signs or intestinal lesions unless major alterations in inflammatory or immune functions are genetically engineered (61-71). IL-$10^{-/-}$ and SCID mice have been utilized to investigate *C. jejuni* colonization, inflammation, and infection, but bacterial colonization and disease varies widely with different *C. jejuni* strains (66, 72, 73). In contrast, animal models in which *C. jejuni* produces clinical disease and intestinal lesions such as dogs (74), ferrets (75-77), hamsters (78), one-day-old chicks (79-82), colostrum-deprived neonatal piglets (81, 83, 84), and non-human primates (85) are limited by the need for extensive surgical, chemical or genetic manipulations, immaturity or lack of research reagents.

The most studied factors of *C. jejuni* involved in bacterial colonization and virulence are flagella-mediated motility, fibronectin-binding outer membrane protein (CadF), *Campylobacter* invasive antigens (Cia), surface-exposed lipoprotein JlpA,

lipooligosaccharide (LOS) and cytolethal distending toxin (CDT) (86, 87). On the basis of *C. jejuni* complete genome sequencing analysis, CDT is the only toxin present (88). A PCR-based virulence factor prevalence investigation shows that *cdt* genes are present in 97.5% of *C. jejuni* strains isolated from diarrheal patients in Bangladesh (89). In *C. jejuni*, CDT does not appear to be secreted, but associated with the bacterial cell membrane (90). *In vitro* experiments show that outer membrane vesicles (OMVs) are the potential mechanism of CDT delivery to host cells (91). *In vitro,* CjejCDT induces cell distension and cell cycle arrest in several mammalian cell lines (92, 93). Nearly all studies about the role of CDT in disease have focused on *in vitro* models of eukaryotic cell genotoxicity characterized by cell cycle arrest and apoptotic cell death (94). CdtB mutant *C. jejuni* has impaired ability to invade SCID mice compared with isogenic wild type strain (68). *C. jejuni* with disrupted CdtB gene is less efficient in adherence and invasion of Hela cells *in vitro* than isogenic wild type strain (95). When inoculated into conventional and IL-10–/– deficient mice, *H. hepaticus* CdtB-negative mutants fail to establish a persistent infection when compared with wild-type parent strain (36, 96). These few studies in laboratory mice, mostly with *H. hepaticus* (36, 37, 96), but also *C. jejuni* (64), and others (97, 98) suggest a role for CDT in virulence, but the role of CDT in *C. jejuni* infection remains poorly understood.

Neutrophil extracellular traps (NETs)

Polymorphonuclear neutrophils (PMNs) play a central role as the first line of host innate immune defense against microbial infections (99). A novel mechanism of

microbial killing that involves de-condensation of PMN's nuclear chromatin leading to extracellular release of DNA and formation of web-like structures was first described by Brinkmann and coworkers in 2004 (100). Because extracellular DNA fibers and histones decorated with antimicrobial proteins efficiently trap and kill microbes, this process was called Neutrophil Extracellular Traps (NETs) (100). Soon after that, this process was found in other cells involved in innate defense including eosinophils, mast cells and macrophages (101-103). Because this process involves cell death by a mechanism different from apoptosis and necrosis, it has been referred to as ETosis and NETosis when referring to neutrophils (104). PMNs from diverse vertebrate species, including human, mouse, cat, cow, chicken and zebra fish have now been shown to form NETs (105-109). Except for neutrophil phagocytosis and release of ROS, NETosis is now recognized as a major defense mechanism for capture and/or killing of not only bacteria, but also fungi and protozoan parasites (110-114). A deficiency in NET formation has now been shown to account for increased susceptibility of human infants to microbial infections (115). Under certain circumstances NETs can also be detrimental. Excessive or persistent release of DNA and granule proteins from NETs has been implicated in development of certain autoimmune disorders such as systemic lupus erythematosus (116) and small-vessel vasculitis (117), but also contributes to disease progression in bacterial sepsis (118).

Multiple stimuli induce NET formation, including chemokines, cytokines, bacteria and their components, viruses and protozoa and many other microorganisms (119, 120). Microorganisms induce NETs formation and most of them are trapped and/or killed by

NETs. For example, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Escherichia coli* (100, 106, 121), *Toxoplasma gondii* and *Candida albicans* (111, 122) can trigger NET formation and are also killed by NETs. Group A *Streptococcus* resist killing by NETs through secretion of a DNase-Sda1 that destroys NETs' backbone DNA and M1 protein to inhibit cathelicidin activity (123, 124). Secretion of nuclease by *Staphylococcus aureus* degradea NETs and their survivability in NETs is much higher than nuclease-deficient mutant (125). In addition, phorbol myristate acetate (PMA), IL-8, LPS, hydrogen peroxide (H_2O_2) and interferon induce NETs formation (126), but among them, PMA appears to be the most potent inducer of NET formation by human peripheral blood neutrophils, and is therefore widely used in NETosis study. So far, the signaling mechanisms regulating NET formation are incompletely understood. PMNs from patients with inherited chronic granulomatous disease (CGD), which involves a mutation in a gene encoding NADPH are incapable of generating reactive oxygen species (ROS). PMNs from CGD patients lack the ability to form NETs, suggesting ROS is required for NETosis (127). Rac2, a small GTPase involved in regulation of ROS generation is also essential for NET formation (128). Moreover, peptidylarginine deiminase 4 (PAD4) was shown to be associated with the initiation of nuclear chromatin de-condensation and appears to be important in NETosis (129). PMNs from individuals deficient in myeloperoxidase (MPO) fail to form NETs (130), and genetically engineered neutrophil elastase (NE) knockout mice do not form NETs in a pulmonary model of *Klebsiella pneumoniae* infection (131). This data indicate that MPO and NE are also required for NET formation. In the PMA-induced NETosis, autophagy or superoxide is involved in intracellular chromatin de-condensation, and

inhibition of either of them prevents NETosis (132). Recently, c-Raf kinase and MEK inhibitors were found to inhibit PMA-induced NET formation, providing indirect evidence that Raf-MEK-ERK pathway contributes to NETosis (133, 134). The most recent novel discovery shows that NETosing PMNs, which have intact cell membrane, but carry de-condensed ruptured nuclei, are still able to chase bacteria and execute their phagocytic function (135). In general, NETosis appears to be a dynamic process, and sequential dissection of this process will reveal fundamental mechanisms of NET induction, formation and function.

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CHAPTER 2

CAMPYLOBACTER JEJUNI ARE CAPTURED, BUT NOT KILLED BY NEUTROPHIL EXTRACELLULAR TRAPS

Abstract

The acute stage of intestinal infection by *C. jejuni* is characterized by IL-8-mediated transepithelial migration of activated PMNs into the intestinal lumen. To date, studies examining the role of PMNs in defense against *C. jejuni* have focused on phagocytic uptake and intracellular killing within phagolysosomes; however, phagocytosis-independent extracellular bacterial killing by release of nuclear DNA and antimicrobial effector molecules, so-called neutrophil extracellular trap (NET) has emerged as an important innate host defense mechanism against bacterial pathogens. Given that PMNs are critical to host innate defense in *C. jejuni* infection, we hypothesize that nuclease activity of CDT mediates resistance to extracellular killing in NETs. First, the clinical relevance of NETs in host defense against *C. jejuni* infection was confirmed in two non-human primates that died of acute campylobacteriosis. The mouse and human PMNs together with wild-type *C. jejuni* and isogenic CDT nuclease mutant and complemented strains were used to evaluate iteration between NETs and *C. jejuni* for: (i) induction of NETs formation, (ii) capture within NETs, (iii) survival within NETs.

The lack of direct induction of NETs formation by *C. jejuni* incubated either under aerobic or microaerobic conditions suggested that NETs formation in *C. jejuni* infection might be indirectly mediated by components present within the intestinal microenvironment. Although *C. jejuni* were efficiently captured, they were not killed within a two hour incubation period. Moreover, the lack of significance differences in

NETs' capture and killing between wild-type and isogenic CDT nuclease mutants suggested that CDT does not play a role in free *C. jejuni* from NETs. Given that approximately 50% *C. jejuni* were captured in NETs, we speculate that NETs might prevent *C. jejuni* from disseminating and invading mucosa. Therefore, we propose that within the context of the intestinal tract, NETs provide a novel innate defense mechanism for bacterial exclusion through intestinal peristalsis.

Introduction

C. jejuni colonizes the mucus on the surface and crypts of the intestine and following epithelial invasion elicits host innate and adaptive immune responses (1-5). Vigorous *in vitro* studies show that *C. jejuni* CDT stimulates IL-8 secretion in polarized human colonic epithelial T84 cells, INT407 cells and many other mammalian cell lines (6, 7). Culture of *C. jejuni* with human intestinal cell line INT407 or monocytic cell line THP-1 increases IL-8 secretion (8, 9). It implicates the recruitment of PMNs is probably initiated by the pro-inflammatory cytokines such as IL-8 during *C. jejuni* infection. Three stages of host response have been identified as the basis of histological examination of colonic biopsies taken from individuals with spontaneous *C. jejuni* infections (10). Stage I (<5 days.) consists of mucosal inflammation dominated by polymorphonuclear neutrophils (PMNs) together with crypt abscesses, which corresponds with the onset of bloody diarrhea. Stages II and III (7 to >14 days.) show gradual transition to mononuclear cell inflammation and recovery. In the *C. jejuni* infected IL-$10^{-/-}$ mice, PMNs infiltration is found in colon even 12-day post infection, and contribute to the pathogenesis of *C jejuni* infection (11). Thus, the hallmark of acute *C. jejuni* enterocolitis is intestinal recruitment of PMNs which transmigrate through the epithelium into the gut lumen, presumably to eliminate *C. jejuni*.

Studies on the role of PMNs in defense against *C. jejuni* are limited to phagocytic uptake and killing by toxic reactive oxygen species, proteolytic enzymes, and antimicrobial proteins within phagolysosomes (12). In this study, we investigated the interaction of PMNs and PMNs in terms of NETosis, and the role that CDT might play as a nuclease in this process by using PMNs isolated from mice bone marrow and human peripheral blood. Our finding demonstrates that NETs are formed and capture *C. jejuni* in the intestine during *C. jejuni* infection *in vitro*; *C. jejuni* did not actively induce NETs by mouse or human PMNs *in vitro*; *C. jejuni* were not killed by NETs in 2 hour co-incubation with NETs, but a significant amount of *C. jejuni* was captured by NETs; CDT did not seem to free bacteria from NETs trap. NETs might be still an important defensive mechanism to control *C. jejuni* infection by entrapping bacteria.

Materials and methods

C. jejuni growth condition

Three different serum type *C. jejuni* isolated and serum typed by Dr. Gerald Duhamel lab were used in the study including ET46, EB53 and ET26. *C. jejuni* were grown and maintained on Mueller-Hinton blood agar plates (5% sheep blood, Colorado Serum Company, Denver, Colorado) at 37°C in the microaerobic condition generated by Pack-MicroAero gas pack (Mitsubishi Gas Chemical America, INC., New York, NY). After 3 days, bacteria were harvested by cold PBS, washed and re-suspended with RPMI 1640 (Invitrogen, Invitrogen, Grand Island, NY) for further use.

Construction of CDT isogenic mutant strains

The cdtB genes of *C. jejuni* strains ET46-36-20, isolated from macaques with campylobacteriosis (13) were disrupted to produce ΔcdtB mutants, and strain ET46-36-20 was complemented with a full-length cdtABC operon. Briefly, for directional cloning, the full length cdtABC operon of *C. jejuni* was amplified by PCR with cdtAF and cdtCR oligonucleotides to make pBSIIKS+-cdtABC (Strategen, La Jolla, CA). The cdtB gene was disrupted by insertional mutagenesis of a kanamycin resistance cassette which can function in both *C. jejuni* and E. coli, (shuttle vector pRY107, kindly provided by Dr. P. Guerry, Enteric Diseases Department, Naval Medical Research Center, MD), into a unique EcoRI site of cdtB at position 419 and selection with 50 μg/ml of kanamycin. The ET46-36-20 strain with a disrupted cdtB gene was complemented with a 2.9-kb fragment encompassing the cdtABC operon amplified by

PCR, ligated into pRY111, and selection with 20µg/ml of chloramphenicol. Sequencing of the recombinant shuttle vector cdtABC-01 revealed the cdtABC transcribed in the same orientation as the lacZ promoter. Complementation of the ET46-36-20 strain with a disrupted cdtB gene was accomplished by conjugation with E. coli harboring either empty pRY111 or cdtABC-01and selection with 200 g/ml of kanamycin and 15 g/ml of chloramphenicol. The constructs have been sequenced and immunoblotted to confirm the lost or gain of CDT in bacteria.

Mice

Female CD-1 mice were purchased from the Charles River (Wilmington, MA) and used between 6 and 12 weeks old. Mice were maintained in the Transgenic Mouse Core Facility at the Cornell University College of Veterinary Medicine. All animal experiments were approved by the Cornell University Institutional Animal Care and Use Committee.

Mouse PMN purification

In all experiments, mice were injected intraperitoneally (IP) with 1 mL of sterile 5% (v/v) thioglycolate (BD Biosciences, San Jose, CA), and PMNs were harvested 18 h later for assessment of the kinetics of NET induction and ROS production following activation. In each experiment, the activation of bone marrow PMNs were compared with that of PMNs obtained from the peritoneal cavity of the same mouse. Briefly, bone marrow from both femurs and tibias of a mouse were harvested by lavage with PBS, and PMNs were purified by discontinuous Percoll density gradient

centrifugation (14). PMNs from the peritoneal cavity of the same mouse were harvested by lavage and purification by continuous Percoll density gradient centrifugation as previously described (15), and erythrocytes were lysed hypertonically. PMNs were re-suspended in RPMI 1640 medium (Invitrogen, Grand Island, NY).

Human PMN purification

PMNs were isolated as described previously (16-18). Briefly, human peripheral blood was obtained from healthy blood donors via venipuncture and collected using sterile sodium heparin-containing tubes (BD Biosciences, San Jose, CA) after informed consent. PMNs were isolated from blood by centrifugation at 480 X g for 50 min at 23°C in a Marathon 8K centrifuge (Fisher Scientific, Pittsburgh, PA) using 1-StepTM Polymorphs (Accurate Chemical & Scientific Corporation, Westbury, NY). This centrifugation method creates a density gradient to separate blood into visible layers of plasma, mononuclear cells, PMNs, and erythrocytes and platelets. PMNs were extracted and washed in Mg^{2+} and Ca^{2+} free HBSS, and all remaining red blood cells in the suspension were lysed hypotonically. Neutrophils were suspended at a concentration of 1.0 x 10^6 cell/mL in HBSS containing 0.5% HSA, 2mM Ca^{2+}, and 10mM HEPES (Invitrogen), buffered to pH 7.4.

NET killing and catching assay

500 ul total number of 5 x 10^5 mouse or human PMNs were plated into each well of 24-well plates (Corning, Tewksbury, MA), and then activated with 4uM PMA (Sigma,

St. Louis, MO) for 4 h at 37^{o}C in air. Medium was replaced with 300 ul *C. jejuni* WT, KO, Comp or blank medium (control) at MOI about 1:10 in RPMI 1640with 10mg/ml cytochlasin D (Sigma, St. Louis, MO). After 700 x g 5 min centrifugation, plates were incubated 2 h at 37^{o}C in air or in microaerobic condition. For NET killing assay, after 2 h incubation 10 U/ml bovine DNase I (ThermoFisher Scientific, Waltham, MA) was used to destroy NETs and release bacterial at the end of incubation. Total bacteria in the well were diluted serially and plated onto Mueller-Hinton blood agar plates to determine CFU. For NET catching assay, at the end of 2 h incubation, free bacteria in the supernatant were removed by washing twice, and then trapped bacteria were freed by bovine DNase I for CFU determination.

Immunoblottig

Mouse or human PMNs plated into 24-well plates were incubated at 37^{o}C in 5% CO_2 and air in control medium or medium containing of appropriate concentrations of PMA (Sigma, St. Louis, MO) or *C. jejuni* isogenic mutant strains. At 5, 15, 30, 60 and 120 min PMNs were lyzed by RIPA buffer, and protein concentration was determined by Bradford Protein Assay kit (Bio-Rad, Hercules, CA). The same amount of proteins from different treatments were loaded and separated by SDS-PAGE and transferred onto PVDF membrane (Bio-Rad, Hercules, CA) to assess phosphorylated ERK1&2 and β-actin by using anti p-ERK1&2 (Cell Signaling, Danvers, MA) anti β-actin (Sigma, St. Louis, MO) antibodies. After incubation with HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (Santa Cruz, Santa Cruz, CA), the bands were visualized by using Westernbright ECL western blotting detection kit (Advansta,

Menlo Park, CA). Images were taken by The V3 Western Workflow (Bio-Rad, Hercules, CA.).

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections of Colons from NENPRC Cotton Top Tamarins were stained for detection of *C. jejuni* and NETs. Briefly, tissue sections were de-paraffinized by xylene (Sigma, St. Louis, MO) and rehydrated with 100%, 95% and 80 % ethanol (Sigma, St. Louis, MO) consecutively, and then were autoclaved in fast cycle for 7 min to retrieve the antigen. After blocking with 5% BSA (Sigma, St. Louis, MO) for 30 min, antibody to myeloperoxidase (Abcam, Cambridge, MA) and *C. jejuni* (Abcam, Cambridge, MA) were added onto the tissue section, and slides were kept in 4^{o}C overnight. Following three washes, secondary goat anti-mouse or rabbit antibodies labeled with Alexa 488 or 550 (Invitrogen, Carlsbad, CA) were applied to detect MPO and *C. jejuni*. Images were taken by using Zeiss 510 LSM confocal microscope.

Fluoresence and immunofluorescence microscopy

Mouse or human PMNs plated onto round coverslip sitting in 24-well plates were incubated for up to 4 h at 37^{o}C in 5% CO_2 and in control medium or medium containing appropriate concentrations of either PMA (Sigma, St. Louis, MO) or *C. jejuni* isogenic mutant strains. For live cell real time fluorescence staining, at 0, 2, and 4 h, 500 nM SYTOX green (Invitrogen, Carlsbad, CA) and 10ug/ml Hoechst 33342 (Invitrogen, Carlsbad, CA) were added into the well, and then coverslips were

transferred onto slides with ProLong Gold Antifade mounting fluid (Invitrogen, Carlsbad, CA). Fluorescence images were taken immediately after 5 min incubation at room temperature by using Zeiss 510 LSM confocal microscope. For immunofluorescence staining, PMNs were fixed with 4% (V/V) paraformaldehyde (PFA; Sigma, St. Louis, MO) in PBS, and incubated with antibodies to *C. jejuni* (Accurate Chem. & Scientific Corp., Westbury, NY) or myeloperoxidase (Abcam, Cambridge, MA) at 4^{o}C overnight. After application of secondary antibodies to mouse or rabbit labeled with Alexa Fluo 488 or 550 (Invitrogen, Carlsbad, CA), coverslips were transferred onto slides with ProLong Gold Antifade mounting fluid containing DAPI (Invitrogen, Carlsbad, CA). Fluorescence images were taken by using Zeiss 510 LSM confocal microscope.

NET induction and picogreen based extracellular DNA release determination.

Measurement of extracellular DNA release was adopted and revised from previous publication (19). Mouse or human PMNs plated into 96-well plates were incubated for up to 4 h at 37^{o}C in 5% CO_2 and in control medium or medium containing appropriate concentrations of either PMA (Sigma, St. Louis, MO) or *C. jejuni* isogenic mutant strains. 1% Human serum was added to investigate the role of opsonization in during NET formation. And then, plates were centrifuged at 700 x g for 10 min to make a close contact of PMNs and bacteria. At the indicated time points, 50ul 500 mU/ml micrococcal nuclease (Thermo Scientific, Waltham, MA) was added into each well of the plates. After 10 min digestion at 37^{o}C, enzymatic reaction was stopped by adding 5 mM EDTA (pH 8.0). Plates were spun at 1500 x g for 5 min, and 100 ul supernatant

was transferred into a flat-bottom 96-well plate for later extracellular DNA evaluation. PMNs and bacteria were lysed with 2% triton x-100 to release the entire nuclear DNA for determination of the total amount of PMN and bacteria DNA. After all of the supernatant at all of time points were collected, 100 ul diluted Picogreen (Invitrogen, Carlsbad, CA) was added into samples, and plates were incubated in dark for 5 min at room temperature. Fluorescence intensity was measured with a spectrofluorometer at 480-nm excitation and 520-nm emission.

Results

C. jejuni are co-localized with NETs present in the Colonic Lumen of a Monkey with Campylobacteriosis.

Spontaneous *C. jejuni* infection in New World primates (NWP) is characterized by acute watery diarrhea that may contain blood or mucus and enterocolitis with PMNs and formation of crypt abscesses similar to Stage I human infection (20). Considering the PMN response is the hallmark of the acute *C. jejuni* infection, we checked the existence of NETs in the intestine of monkey infected with *C. jejuni*, and the possible interaction between *C. jejuni* and NETs. DNA and *C. jejuni* staining of in Macaque monkey colonic tissue section (Figure 1.1A&B) showed that a large amount of *C. jejuni* colonized intestine, and many of them were co-localized with DNA, implicating that NETs might be formed to catch *C. jejuni* in the reality. One of the characteristics of NETs is the de-congested DNA decorated PMN granule derived proteins such as MPO, elastase, etc. (21). In order to confirm the NET formation, MPO, a PMN marker during acute inflammation, was stained to identify NETs in *C. jejuni* infection. Immunofluorescence staining of MPO and *C. jejuni* in colonic section of monkey with acute *C. jejuni* infection showed MPO staining was almost all over intestinal content in the intestinal lumen of *C. jejuni* infected monkey (Figure 1.1C). It indicates a large volume of PMNs had infiltrated the intestinal lumen during the acute *C. jejuni* infection. Interestingly, NET like DNA and MPO staining structures were visualized in Figure 1.1 C and D, and close-up view of the NETs (pointed by arrow in Figure 1.1 D) exhibited the co-localized staining of a group of *C. jejuni*, presumably captured by

NETs. However, in the intestine without MPO staining, no NET like structure of either DNA was observed (Figure 1.1E). The existence NET structure together with its co-localization with *C. jejuni* provided direct evidence that NETs were formed in the intestinal lumen during *C. jejuni* infection, and was able to capture *C. jejuni*.

***C. jejuni* do not induce mouse or human NET formation.**

Since several bacteria are able to induce NET fromation in *vitro* (22), but this has not been investigated with *C. jejuni*. We asked whether or not *C. jejuni* induce PMNs to form NETs, and whether or not *C. jejuni* CDT interferes with NET formation. PMNs used in the experiments were purified from human peripheral blood and mouse bone marrow, and the purity was greater than 90% (Figure 1.2). After PMNs were incubated in the presence of with wild-type (WT) and isogenic *C. jejuni* mutant strains with inactivated CDT (KO) or KO strain complemented with CDT gene (Comp), extracellular DNA release was evaluated by picogreen assay. PMN viability and NETs formation was visualized by double fluorescence staining with CYTOX green (a cell membrane non-permeable DNA dye; extracellular DNA staining) and Hoechst 33342 (cell membrane permeable dye; total DNA staining). Although PMA-activated mouse PMNs released extracellular DNA, incubation with *C. jejuni* WT, KO or Comp strains did not show significant release of nuclear DNA when compared with untreated control PMNs (Figure 1.3A). Double-fluorescence staining with Hoechst33342 and SYTOX green showed that a majority of PMNs remained alive after 4 hour co-incubated with *C. jejuni* (Figure 1.3B). There were some (<5%) spontaneous NETs formed by PMNs either in control or in *C. jejuni* infected (Figure 1.3B). Activation of

ERK1&2 pathway is required for bacterial and PMA-induced NET formation (23). To further confirm the lack of NETs induction by *C. jejuni*, ERK1&2 activation was assessed by immunoblotting of the whole cell lysate from PMNs activated by PMA and PMNs co-incubated with *C. jejuni* WT and KO strains over time. Phosphorylated ERK1&2 were detected within 5 min in the PMNs stimulated with PMA, but not detected in the PMNs co-incubation with *C. jejuni* WT or KO up to 120 min, which was similar to the control non-activated PMNs (Figure 1.3C). Taken together, these data suggest that unlike other bacteria, *C. jejuni* do not induce NET formation *in vitro*, and CDT does not seem to induce any PMN death.

To rule out the possibility that the lack of DNA release is unique to mouse PMNs, we examined human NET induction. There was no significant NET release and ERK activation (Figure 1.4A&B) detected in human PMNs infected with *C. jejuni*. The lack of NETs induction by *C. jejuni* with human PMNs and mouse PMNs confirmed that *C. jejuni* do not induce NETs *in vitro*. Given that *C. jejuni* is a strict microaerophile (156), environmental stress factors intend to affect *C. jejuni* survival and virulence (24). We evaluated the human PMNs NET formation under microaerobic condition, which is the actual condition that *C. jejuni* face during infection. No significant extracellular DNA release was detected among PMNs stimulated with different *C. jejuni* strains (Figure 1.4C). Fluorescence staining showed, even though there were few spontaneously NETs formed, almost all PMNs in control and *C. jejuni* infected groups remained alive and kept intact cell membrane (Figure 1.4G). However, nearly all PMNs treated with PMA lost their membrane integrity; their nuclear chromatin

DNA de-condensed and extruded outside. (Figure 1.4G). The lack of inducing NET formation brings out one direct question is whether or not *C. jejuni* inhibit NET formation. Under microaerobic condition, human PMNs were pre-incubated with *C. jejuni* for 2 h for a full settlement between *C. jejuni* and PMNs, and then stimulated with PMA for another 4 h. After 4 h PMA activation, human PMNs in presence of *C. jejuni* formed the same amount of NETs as non-treated control PMNs (Figure 1.4D). Fluorescence staining confirmed that regardless of the presence of *C. jejuni*, similar amount of NETs was formed after PMA activation (Figure 1.4H); there were no differences in NETs formation, observed among the groups of human PMNs co-incubated with PMA and different *C. jejuni* CDT isogenic strains (Figure 1.4H). All of the three different serum type *C. jejuni* ET46, EB53 and ET26 did not induce significant human NET formation with or without 1% human serum (Figure 1.4E&F), implicating all the *C. jejuni* strains might not be potent NET inducer, and serum opsonization does not promote NET formation. It suggests *C. jejuni* do not inhibit PMA-induced human NET formation *in vitro*. These results demonstrate that *C. jejuni* neither induce human NET formation nor inhibit human NET formation *in vitro*; CDT does not appear to interfere with NET formation.

C. jejuni are captured by NETs, and CDT does not free bacterial from NETs capture.

Since *C. jejuni* were captured by NETs during *C. jejuni* infection *in vivo*, we wondered if *C. jejuni* could be captured *in vitro*. Mouse PMNs were incubated with PMA for 4 h to obtain mouse NETs, and then *C. jejuni* WT, KO and Comp were added onto NETs.

After bacteria free in the supernatant were washed away, immunofluorescence was performed to detect NETs and *C. jejuni*. As shown in Figure 1.5, *C. jejuni* WT, KO and Comp were entrapped in the NETs, and bacteria staining co-localized with the track of NETs. When NETs were dismantled by bovine DNase I, only nucleus from intact PMNs were left, and no bacteria were seen (Figure 1.5). Generally, NETs are capable of capturing *C. jejuni*.

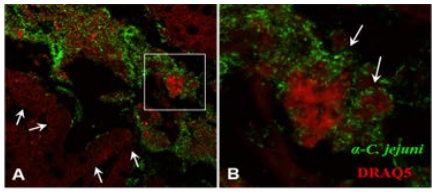
We next checked the efficacy of NET capture and whether or not CDT could free bacteria from NETs capture by digesting NETs in terms of its nuclease activity. Under microaerobic condition, approximately 50% of *C. jejuni* WT, KO and Comp inoculum was captured by human NETs (Figure 1.6A). Nearly all of the *C. jejuni* were released after NETs were dismantled by bovine DNase I (Figure 1.6A). Immunofluorescence images in 63x magnification clearly showed that *C. jejuni* were entrapped by NETs (Figure 1.6B). However, the percentages of NET captured bacteria among *C. jejuni* WT, KO and Comp isogenic strains were not significant different (Figure 1.6A), demonstrating that CDT might not contribute to fighting against NET entrapment. In sum, approximately half of the *C. jejuni* were trapped by NETs; however *C. jejuni* CDT did not free bacteria from NET capture I our *in vitro* experiments.

NETs do not exert efficient extracellular killing of *C. jejun in vitro*.

C. jejuni are captured by NETs, and we assessed the ability of NETs to exert killing of *C. jejuni* and whether or not CDT contributes to bacterial survival. *C. jejuni* WT, KO and Comp were added onto NETs made from PMA induced mouse or human PMNs.

After 2 h co-incubation with NETs in either aerobic or microaerobic condition, total number of *C. jejuni* was determined by CFU assay. Interestingly, in aerobic condition no CFU was seen in neither of the three *C. jejuni* isogenic strains that were left in the blank medium without NETs (Data not shown), but not the *C. jejuni* incubated with NETs; however, *C. jejuni* in the blank medium under microaerobic condition retained similar amount of CFU as inoculum. It indicates microaerobic condition is critical for *C. jejuni*, and *C. jejuni* may take advantage of NETs to survive in aerobic condition (Figure 1.7 D-I). No significant numbers of CFU were found between inoculum and bacteria treated with NETs among all three *C. jejuni* CDT isogenic strains (Figure 1.7A-I). These results suggest *C. jejuni* were not killed by NETs *in vitro*, and *C. jejuni* CDT does not seem to play a role in increasing bacterial survival in NET capture.

Figure 1.1. *C. jejuni* trapped in NETs within the colonic lumen of a Macaque with campylobacterioisis. Double-staining and merged confocal laser scanning microscopy images of colon from a macaque monkey with naturally-occurring acute colitis associated with *C. jejuni* infection shows large numbers of *C. jejuni* (green) along the colonic surface epithelium (A, arrows). Close up view of the boxed area in A with *C. jejuni* (green) trapped within large aggregates of luminal extracellular DNA (red) (B). Colonic tissue section of a Tamarin monkey with naturally-occurring acute colitis associated with *C. jejuni* infection was stained for detection of *C. jejuni* (red) MPO (green) and DNA (blue) (C). Close-up view of the boxed area in (C) with *C. jejuni* (red) trapped within large aggregates of luminal extracellular DNA (blue) and MPO (green) (D). (40X original magnification). Colonic tissue section of a Tamarin monkey with *C. jejuni* infection (without PMN infiltration) was stained for detection of *C. jejuni* (red) MPO (green) and DNA (blue) (E). (40X original magnification).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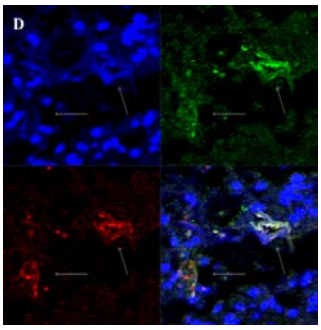
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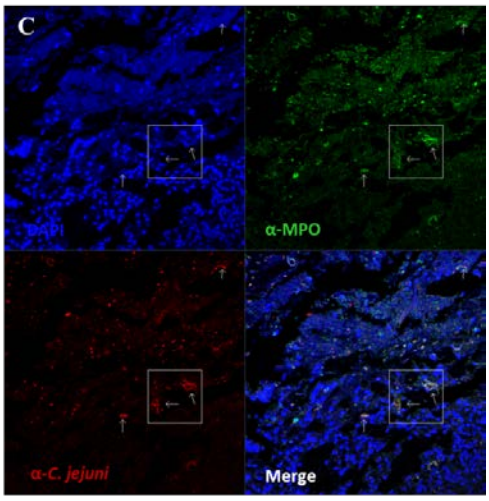
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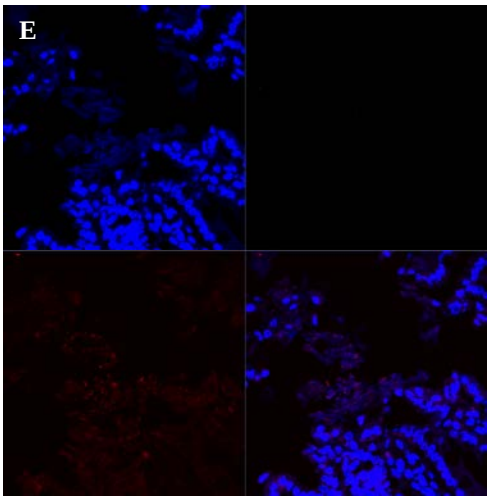
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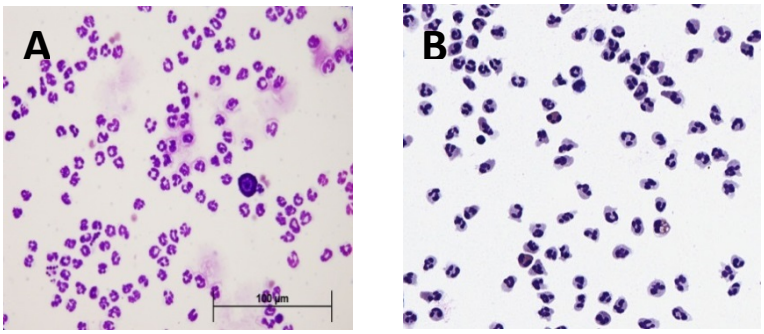
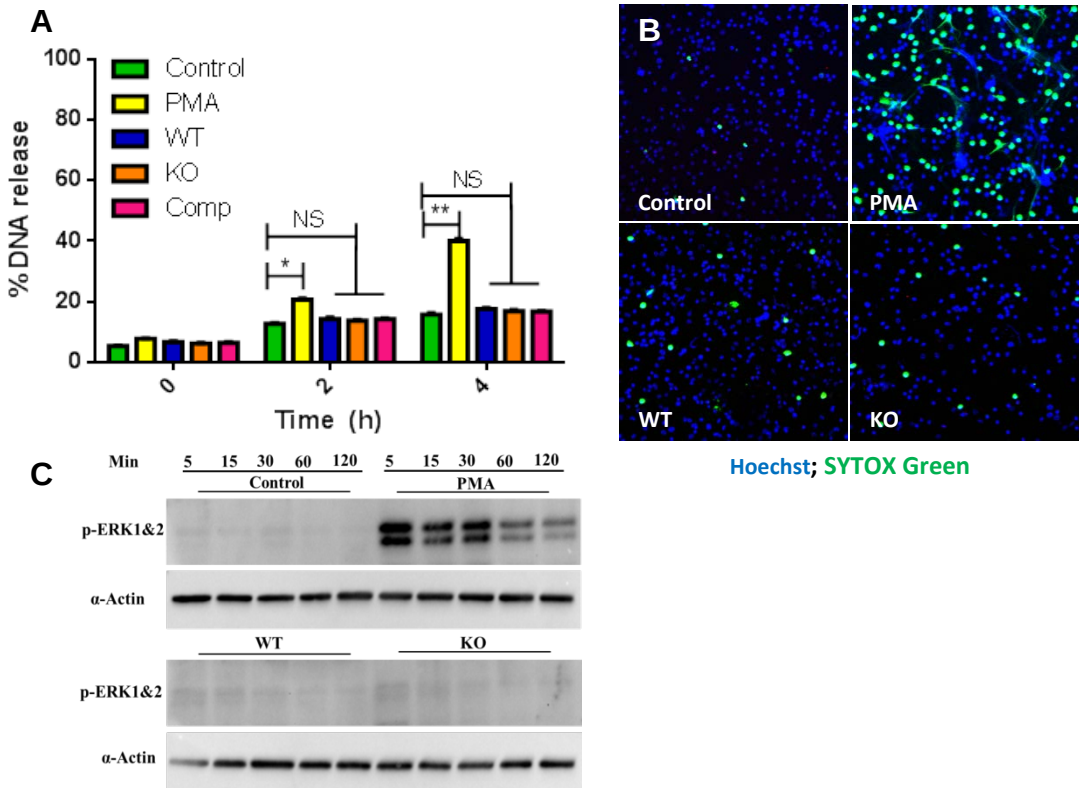


Figure 1.2 Purified thioglycolate-elicited mouse bone marrow and human peripheral blood derived PMNs. Photomicrographs of thioglycolate-elicited mouse bone marrow derived PMNs (A) harvested 18 h after intraperitoneal injection with 5% (v/v) thioglycolate medium to elicit sterile inflammation. (B) Human PMNs were purified from peripheral blood. Greater than 90% PMNs are obtained from mouse and human (Diff-Quick stain; 60 x original magnification).

Figure 1.3 *C. jejuni* do not induce mouse NETs *in vitro*. The percent extracellular DNA release by non-activated BMDM-PMNs (negative control) and PMA-activated PMNs (1µM PMA; positive control) was compared with that of PMNs incubated with *C. jejuni* WT, KO or Comp strains for up to 4 h at 37^{o}C in air (A). At the end of the incubation period, extracellular DNA was digested with monococcal nuclease (500 mU/mL final concentration) for 10 min at 37^{o}C, and the percent DNA release was quantified by using a Picogreen Assay. Data are expressed as percent total DNA release from TritonX100 lyzed PMNs. (B) Double immunofluorescence LCFM image of extracellular DNA release from non-activated BMDM-PMNs (negative control) and PMA-activated PMNs (1µM PMA; positive control) compared with PMNs incubated in the presence of *C. jejuni* WT and KO strains for 4 h at 37^{o}C in air and stained with Hoechst33342 (Hoechst) and SYTOX Green. (C) BMDM-PMNs obtained from control and samples incubated in the presence of PMA or of *C. jejuni* WT and KO strains for 5, 15, 30, 60 and 120 min were lyzed and phosphorylated ERK1&2 and β-actin were assessed in immunoblotting assay by using anti p-ERK1&2 antibody. Each experiment was done in triplicate, and representative data from two independent experiments is shown (Error bars represent Mean±SD; *P<0.05; **P<0.01, NS, not significant).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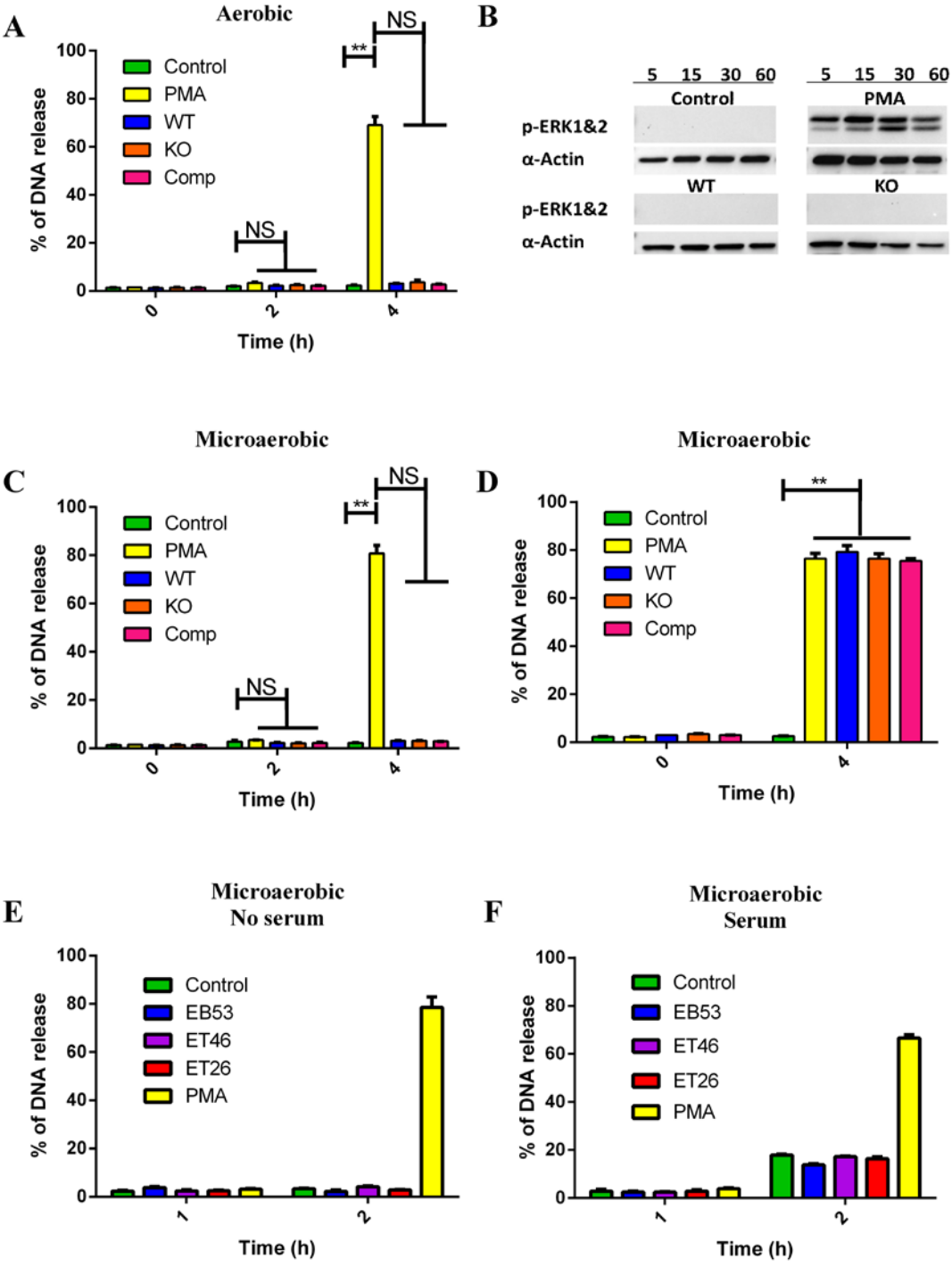
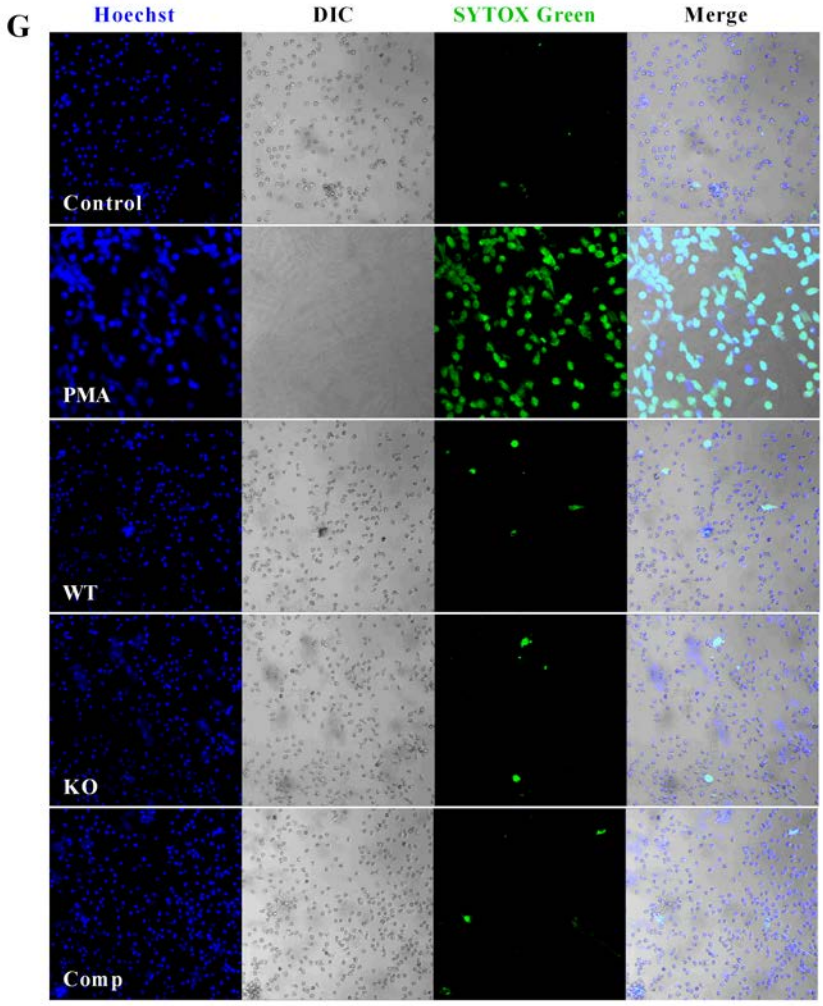
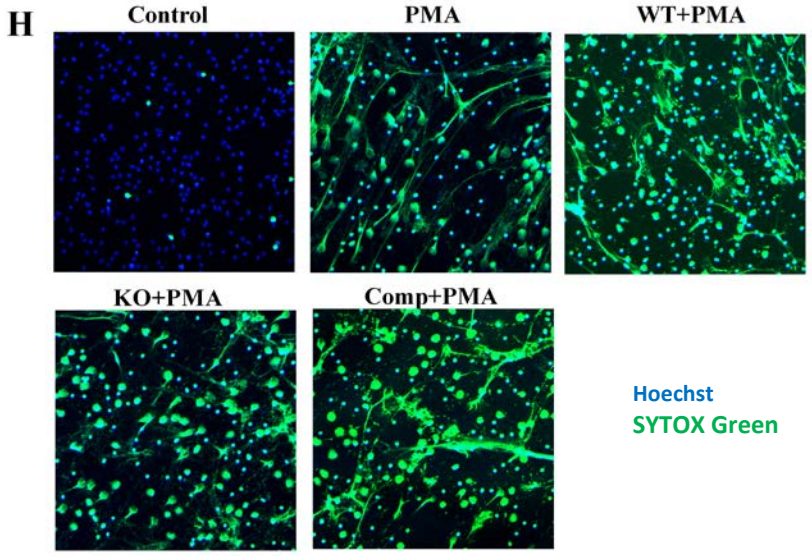
Control
PMA
p-ERK1&2
α-Actin
WT
KO
p-ERK1&2
α-Actin

Figure 1.4 *C. jejuni* do not induce human NETs *in vitro* either in air or microaerobic condition, and do not inhibit PMA-induced NET formation. The percent extracellular DNA release by non-activated human PMNs (negative control) and PMA-activated PMNs (200nM PMA; positive control) was compared with that of PMNs incubated with *C. jejuni* WT, KO or Comp strains at MOI of 100:1 for up to 4 h at 37^{o}C in air. (A) At the end of the incubation period, extracellular DNA was digested with monococcal nuclease (500 mU/mL final concentration) for 10 min at 37^{o}C and the percent DNA release was quantified by using a Picogreen Assay. (B) Human PMNs obtained from control and samples co-incubated with PMA or *C. jejuni* WT and KO strains for 5, 15, 30, 60 min were lysed and phosphorylated ERK1&2 and β-actin were assessed in immunoblotting assay by using anti p-ERK1&2 antibody. (C & G) The same treatment was set up as described in (A), but activation was performed at 37^{o}C in microaerobic condition. Double immunofluorescence LCFM image of extracellular DNA release stained with Hoechst33342 (Hoechst), and SYTOX Green was taken at 20 x magnification by Zeiss 510 laser scanning confocal microscope. (D) Human PMNs were pre-infected by *C. jejuni* WT, KO or Comp strains at MOI of 100:1 for 2h at 37^{o}C in microaerobic condition, and then 200nM PMA was added to the non-*C. jejuni* infected PMNs and control human PMNs. After 4 h incubation at 37^{o}C microaerobically, the percent extracellular DNA release was quantified by Picogreen assay. (E&F) Human PMNs were co-incubated with other two different serum type of *C. jejuni* EB53 & ET26 together with ET46 in the presence of 1% human serum or without human serum. Extracellular DNA release was evaluated as described above. (H) After human PMNs were plated onto coverslips sitting in 24-

well plates, they were pre-treated with *C. jejuni*, and then activated with PMA consequently. PMNs were stained with Hoechst 33342 and SYTOX Green 4 h post treatment. Immunofluorescence images were taken at 20 x magnification by Zeiss 510 laser scanning confocal microscope. Each experiment was done in triplicate, and representative data from two independent experiments is shown (Error bars represent Mean ±SEM; *P<0.05; **P<0.01, NS, not significant).


A
Aerobic
% of DNA release
Control
PMA
WT
KO
Comp
NS
**
NS
Time (h)
B
5 15 30 60
Control
5 15 30 60
PMA
p-ERK1&2
α-Actin
WT
KO
p-ERK1&2
α-Actin
C
Microaerobic
% of DNA release
Control
PMA
WT
KO
Comp
NS
**
NS
Time (h)
D
Microaerobic
% of DNA release
Control
PMA
WT
KO
Comp
**
Time (h)
E
Microaerobic
No serum
% of DNA release
Control
EB53
ET46
ET26
PMA
Time (h)
F
Microaerobic
Serum
% of DNA release
Control
EB53
ET46
ET26
PMA
Time (h)


G
Hoechst
DIC
SYTOX Green
Merge
Control
PMA
WT
KO
Comp


H
Control
PMA
WT+PMA
KO+PMA
Comp+PMA
Hoechst
SYTOX Green

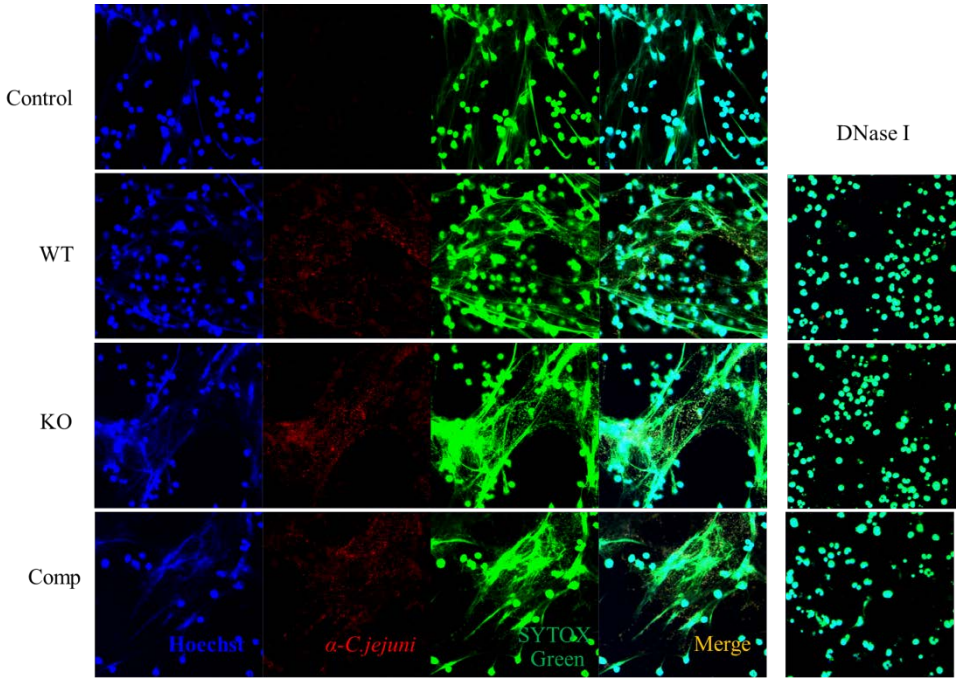
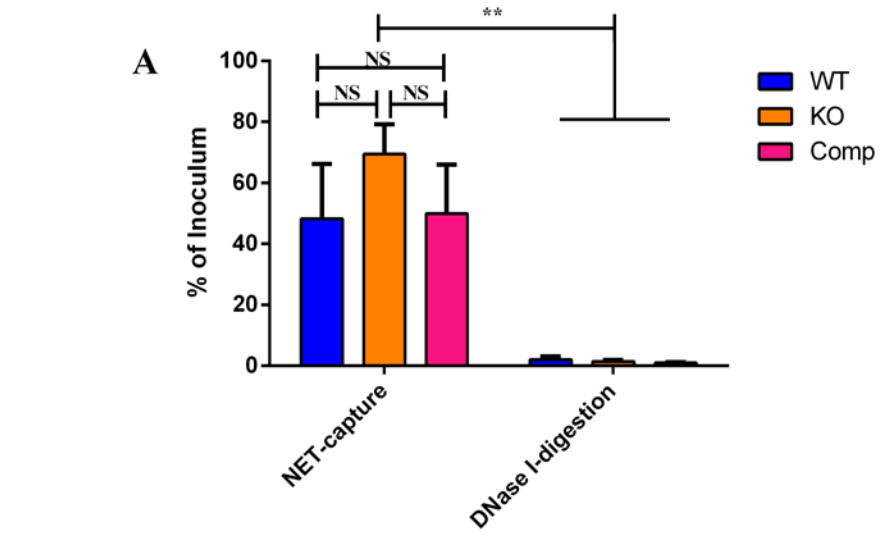


Figure 1.5 *C. jejuni* WT, KO and Comp were captured by mouse NETs. NETs from PMA-activated BMDM-PMNs were incubated with medium or medium containing either *C.jejuni* (WT), KO or Comp for 1 h (MOI 100:1). At the end of the incubation period, control NETs were digested for 15 min by adding bovine DNase I (10 U/ml final concentration) before removing free bacteria in the culture supernatant by washing, fixation with 4% paraformaldehyde and double-immunofluorescence staining LCFM. Extracellular DNA was stained with SYTOX Green, nuclear DNA was stained with Hoechst 33342 and *C. jejuni* with affinity purified rabbit polyclonal IgG to *C. jejuni* followed by Alexa Fluor (555; red) goat anti-rabbit IgG. 20X original magnification images captured with an inverted confocal laser scanning microscope (Zeiss LSM 510 Meta) at the Cornell Microscopy and Imaging Core Facility.

Figure 1.6 *C. jejuni* are captured by human NETs and CDT does not mediate bacterial escape from human NETs. (A) Following PMA activation of human PMNs and treatment with cytochalasin D to inhibit phagocytic uptake, NETs were removed by using bovine DNase I digestion after incubation with *C. jejuni* WT, KO, or Comp strains (MOI 1:1). After 1 h co-incubation at 37^{o}C in microaerobic condition, free bacteria in the culture supernatant were removed by washing, and the numbers of live bacteria associated with NETs were expressed as a percentage of the inoculum. (B) Following 4 h PMA activation of human PMNs plated onto coverslips in 24-well plates, PMNs were treated with cytochalasin D to inhibit phagocytic uptake. *C. jejuni* WT, KO, or Comp strains (MOI 100:1) were added onto human NETs. After 1 h incubation at 37^{o}C in microaerobic condition, free bacteria in the supernatant were removed by 3 washes. *C. jejuni* alone were fixed onto coverslip without PMNs for determination of the MPO antibody specificity. Immunofluorescence was performed to detect *C. jejuni* and MPO. DNA was stained with DAPI. (40x magnification). Each experiment was done in triplicate, and representative data from three independent experiments are shown (Error bars represent Mean±SEM; **P<0.01).


A
**
NS
NS
NS
100
80
60
40
20
0
% of Inoculum
WT
KO
Comp
NET-capture
DNase I-digestion

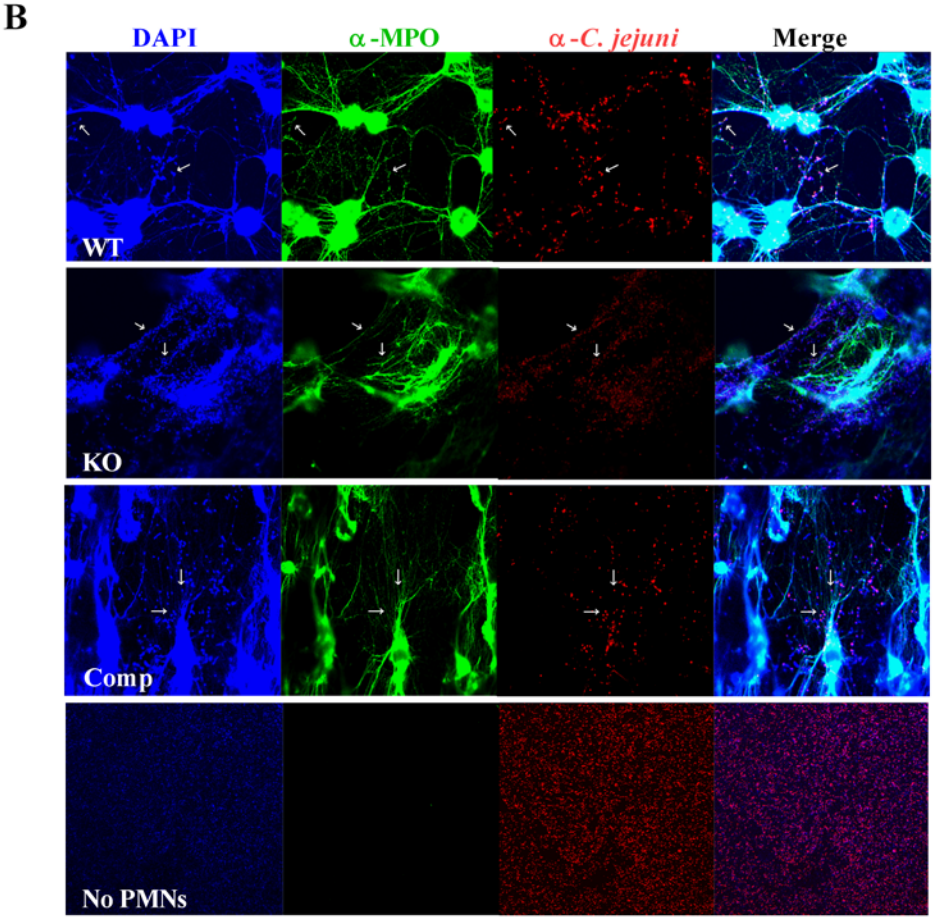
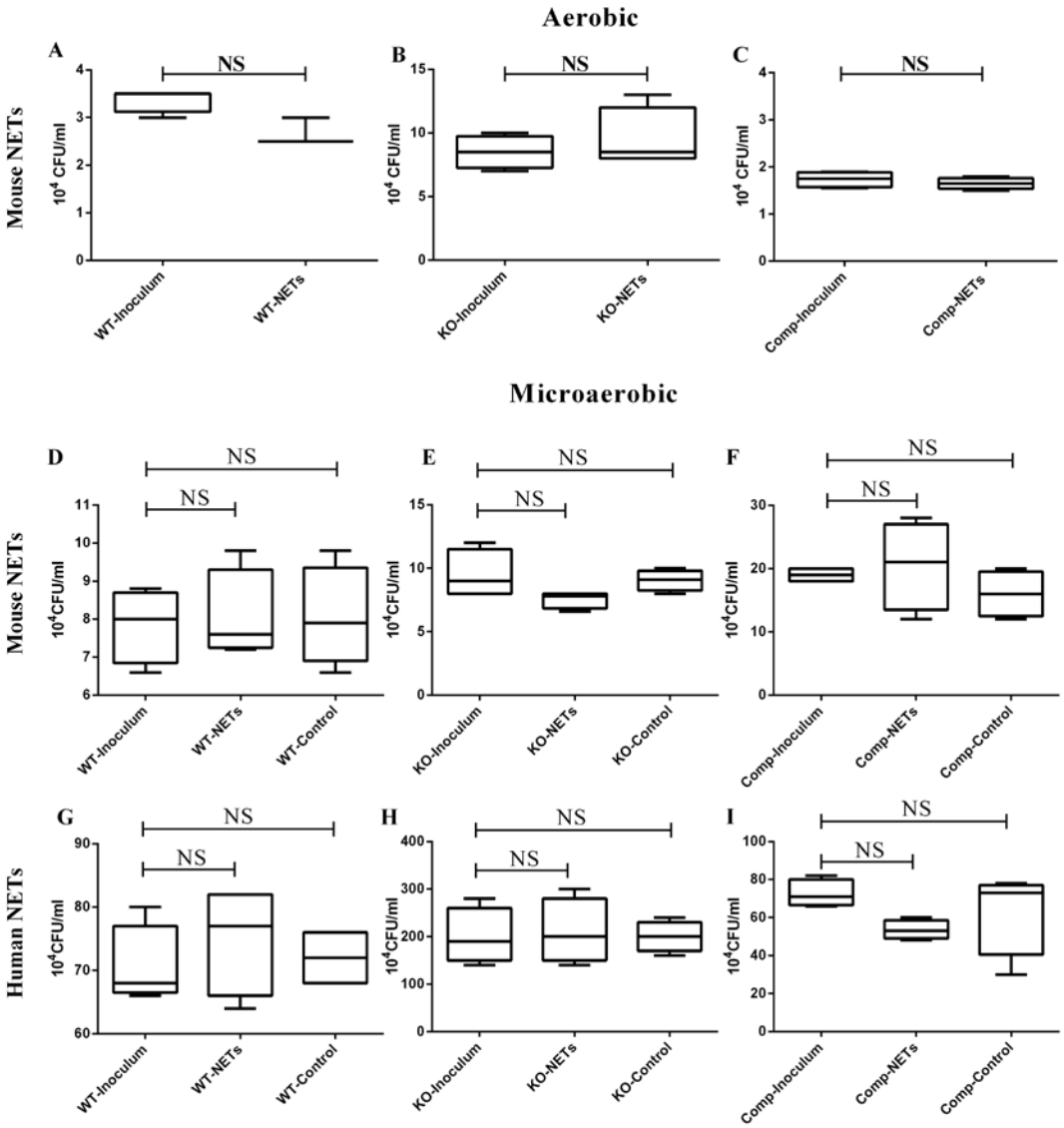

B
DAPI
α-MPO
α-C. jejuni
Merge
WT
KO
Comp
No PMNs

Figure 1.7 Mouse and human NETs do not kill *C. jejuni* WT, KO, and Comp strains. NETs obtained from PMA-activated BMDM-PMNs and human PMNs for 4 h were pre-treated with fresh medium containing cytochalasin D (10 µg/mL) to inhibit phagocytosis, and then incubated with *C. jejuni* CDT isogenic strains (MOI 1:1). After 2 h incubation at 37^{o}C in aerobic condition (A, B and C) or in microaerobic condition (D, E, F, G, H, I), *C. jejuni* were plated onto sheep blood agar plates to determine bacteria CFUs. No CFUs were detected in the control *C. jejuni* that were incubated in the aerobic condition. Each experiment was done in triplicate, and graphs are representative of three independent experiments. (Error bars represent Mean±SEM; NS, not significant if $P<0.05$).


Aerobic
Microaerobic
Mouse NETs
Human NETs
A
B
C
D
E
F
G
H
I
NS
10^4 CFU/ml
10^4CFU/ml
WT-Inoculum
WT-NETs
WT-Control
KO-Inoculum
KO-NETs
KO-Control
Comp-Inoculum
Comp-NETs
Comp-Control

Discussion

In this study, we discovered NETs in the intestinal lumen of monkey with *C. jejuni* infection, and *C. jejuni* were captured in NETs. We found that PMNs, recruited to the *C. jejuni* infected intesine, make NETs to control *C. jejuni* infection. *In vitro*, *C. jejuni* do not appear to induce NET formation; *C. jejuni* are largely captured by NETs, but manage to survive in NET capture; CDT does not seem to play a role in NET induction and releasing bacteria from capture. NETs might be an import mechanism to control *C. jejuni* infection by entrapping *C. jejuni*, and *C. jejuni* are able to avoid NETs killing by unknown mechanism.

Several bacteria have been reported to induce NET formation such as *E. coli* and *Shigella flexneri* (22, 25). However, *C. jejuni* did not induce mouse NET formation *in vitro.* Considering *C. jejuni* are strict microaerophile, and experiments were also performed under micoaerobic condition to favor *C. jejuni* living. No matter in aerobic or in microaerobic condition, *C. jejuni* did not induce human NET formation. ERK1/2 activation appears to be essential in NET formation. Inhibition of ERK1/2 by chemical inhibitors blocks NETs formation mediated by PMA (23, 26) and *Toxoplasma gondii* (15). Non-activation of ERK1/2 observed in the PMNs treated with *C. jejuni* confirmed that *C. jejuni* do not induce NET formation in our *in vitro* experiment. The fact that two other different type *C. jejuni* strains EB53 and ET26 do not induce NETs implicates that it might be common that *C. jejuni* do not induce NET formation in a wide range of this specie. The lack of PMN response to *C. jejuni* may result from the

maturity of the purified PMNs. Mouse bone marrow PMNs are in a resting state and have the capacity to become primed, while peritoneal exudate PMNs are already mature upon isolation. Peritoneal exudate PMNs generate more reactive oxygen species upon stimulation that bone marrow PMNs (27). Maturation of PMNs looks to be associated with transcellular migration, progressive starvation or sequential shifts in quantity and quality of tissue factors, chemokines and pro-inflammatory cytokines along their migration to the infection site. During *C. jejuni* infection *in vivo*, PMNs recruited into the intestinal lumen may be more mature than PMNs in bone marrow or peripheral blood derived PMNs *in vitro*. Even though *C. jejuni* do not induce NET formation, PMNs in the intestine might be more sensitive and intend to make NETs under the pressure from gut flora and contents, which are known as NET inducers.

One major function of NETs is to capture and kill microorganism (28, 29). Our Data show that more than 50% of *C. jejuni* was trapped in NETs. Group A *Streptococcus* resist NETs killing by secreting DNase-Sda1 to destroy NETs' backbone DNA and M1 protein to inhibit cathelicidin activity (30, 31). *Staphylococcus aureus* secret a nuclease to degrade NETs, and their survivability in NETs is much higher than nuclease-deficient mutant (32). The CdtB subunit of CDT has nuclease activity, and can digest plasmid DNA *in vitro* (33). However, no significant difference observed among CjWT, CjKO and CjComp strains in NET catching assay suggests that CDT is not able free bacteria from capture by digesting NETs. Several of the CDT properties may explain this observation. First, *Staphylococcus aureus* nuclease can digest DNA completely in a short time (32). However, CDT nuclease activity is much lower than

bovine DNase I. *In vitro* 6 h digestion with CdtB only cut a small portion of plasmid DNA open, and do not generate DNA smear (33). CDT may not be powerful enough to digest NET DNA, or it may require a fairly long period of time to digest NET DNA. Elongation of incubation time with NETs may help CjejCDT fully execute its nuclease function. However, due to the limitation of *in vitro* assay, our NET catching remained in 2 h incubation. Second, that CjejCDT is not detectable in the culture supernatant by immunoblotting, but present in the enriched bacterial cell membrane (7) infers *C. jejuni* CDT not only appears to be cell membrane associated, but also its abundance on the bacteria membrane is extremely low. Last, the association of PMN granule proteins with chromatin DNA may hamper CDT from reaching DNA. Generally, it suggests CDT does not seem to have the capacity to free bacteria from NET capture. That *C. jejuni* were captured and fully contacted with NETs in confocal microscopy means *C. jejuni* were under attack from the all bactericidal proteins on the NETs. However, *C. jejuni* were not killed in 2 h co-incubation with NETs in either aerobic or microaerobic condition. Genome wide analysis indicates that *C. jejuni* has no obvious homologues to the known virulence factors from other bacterial pathogens (34). Understanding of *C. jejuni* pathogenicity is still an ongoing effort, and the strategy that *C. jejuni* utilize to escape from NET killing needs more investigation.

In sum, NETs were visualized to trap *C. jejuni* in the infected intestine. *C. jejuni* were captured within NETs; however, bacterial killing was inefficient and *C. jejuni* CDT did not contribute to escape of bacteria from NETs *in vitro*. NETs might play an essential role in preventing *C. jejuni* from disseminating by entrapping bacteria;

eventually, the trapped bacteria may be evacuated by the host intestinal peristalsis and diarrhea. NETs might provide an efficient mechanism for the clearance of *C. jejuni* during infection.

Acknowledgments

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CHAPTER 3

THE CYTOLETHAL DISTENDING TOXINS OF HELICOBACTER HEPATICUS AND CAMPYLOBACTER JEJUNI INDUCE PREMATURE SENESCENCE OF HUMAN INTESTINAL EPITHELIAL CELLS *IN VITRO*

Abstract

Premature cellular senescence refers to the non-aging related cellular senescence. The hallmark of premature senescence is permanent arrest of cell proliferation, while remaining metabolically active. Cellular senescence is thought to be an anti-cancer mechanism, and has been studied mostly within the context of aging, DNA damage and oncogene-induced senescence. However, little is known about the contribution of premature senescence in infectious diseases. Cytolethal distending toxin, a genotoxin involved in bacterial persistent infection, is an AB type toxin composed of three subunits (CdtA, CdtB and CdtC). *Helicobacter hepaticus* (*H. hepaticus*) and *Campylobacter jejuni* (*C. jejuni*) cytolethal distending toxins cause DNA damage, induce cell cycle arrest, mediate apoptosis, and stimulate pro-inflammatory cytokine IL-6 and IL-8 secretion *in vitro*. Given that persistent DNA damage promotes cellular senescence, we hypothesized that a sub-lethal concentration of *H. hepaticus* CDT (HhepCDT) or *C. jejuni* CDT (CjejCDT) or *C. jejuni* whole cell lysate (WCL) induce premature cellular senescence and pro-inflammatory cytokine production by intestinal epithelial cells.

To investigate CDT genotoxicity we used *C. jejuni* wild-type strain (CjejWT) along with isogenic *C. jejuni* strains with disrupted CDT gene (CjejKO) and *C. jejuni* KO complemented with CDT gene (CjejComp) as well as recombinant HhepCDT and CjejCDT. After 7-day intoxication, a significant number of HhepCDT, CjejCDT, CjejWT and CjejComp WCL treated epithelial INT407 cells were arrested at G_2/M

phase of the cell cycle and showed a persistent DNA damage response (DDR). Consistent with this observation was positive staining for the presence of senescence-associated beta-galactosidase activity, and elevated mRNA and expression of IL-6 and IL-8 proteins. These features are consistent with CDT-induced premature cellular senescence. In addition, the anti-tumor and anti-inflammatory cytokine IL-24 was also up-regulated in intoxicated INT407 cells. Consistent with a very potent genotoxin, only 400 ng/mL *C. jejuni* WCL was required to induce cellular senescence, while 20 ug/mL was required for recombinant CDT. In conclusion, the data suggest that premature cellular senescence might be an important host defense mechanism against bacterial infection, and contributes to the inflammatory response seen in vivo.

Introduction

The phenomenon of cellular senescence was first described in the observation of infinite human diploid cell proliferation, and these cells eventually exit cell cycle irreversibly, but still remain alive metabolically (1). Cellular senescence is a preventive response to cease the proliferation of damaged cells. Substantial evidence shows that it is an anti-cancer mechanism (2-4).Cellular senescence is commonly seen in aging cells, and can also be induced by other cellular stresses such as DNA damage and oncogenic activation triggered by mutation (5-8). DNA damage response (DDR) is a very common element to induce cellular senescence (7). In order to differentiate it from the cellular senescence caused by the shortening of telomere in aging, senescence induced by other factors is termed as premature cellular senescence (9).

Senescent cells display several features (10, 11) including: (i) irreversible cell growth arrest while remaining metabolically active, (ii) up regulation of senescence associated β-galactosidase expression, which can be detected by histochemical staining at pH 6.0; (iii) changes in gene expression pattern, including upregulation of cell-cycle inhibitors p16 and p21; (iv) secretion of IL-6 and IL-8 so called senescence-associated secretory phenotype (SASP); (v) presence of senescence-associated DNA-damage foci. All the characteristics of cellular senescence may not be strictly relevant to every senescent phenotype, but three or more markers together with the morphological change can be a bona fide way to detect senescent cells (12).

Cellular senescence has been intensively studied in aging, DNA damage and oncogene induced senescence (10). However, less is known about the contribution of premature senescence in infectious diseases. A potent genotoxin cytolethal distending toxin (CDT) produced by about two dozen of bacterial species such as *Escherichia coli, Helicobacter hepeticu (H. hepaticus), Salmonella enterica and Campylobacter jejuni* (*C. jejuni*) is known to cause DDR and induce cell cycle arrest (reviewed in 37). Here we utilize *H. hepaticus* and *C. jejuni* CDT (CjejCDT) to investigate the potential role of CDT in inducing cellular senescence *in vitro*. CDT is found to be the only toxin present in *C. jejuni* when the complete genome sequencing analysis is accomplished (13). A PCR-based virulence factor prevalence investigation shows that the presence of *cdt* genes is 97.5% in *C. jejuni* species identified from diarrheal patients in Bangladesh (14).

C. jejuni CDT does not appear to be detected in the culture supernatant by immunoblotting, but it can be detected in the enriched bacterial cell membrane (15). *In vitro,* experiment shows that outer membrane vesicles (OMVs) are the potential vector that delivers CDT into the host cells and the surrounding environment (16). *C. jejuni* recombinant CDT induces typical cell distension and cell cycle arrest in different mammalian cell lines *in vitro* (17, 18). CdtB mutant *C. jejuni* has an impaired ability to invade SCID mice compared with the wild type strain (19). CdtB mutant *C. jejuni* is able to colonize NF-kB deficient mice, but does not induce gastroenteritis (20). *C. jejuni* with disrupted CdtB gene is less efficient in adherence and invasion to Hela cells *in vitro* than *C. jejuni* wild type strain (21). This implicates CjejCDT contributes

to bacteria invasion and colonization. The formation of γ-H2AX, a marker of DDR response, seen in Hela cells intoxicated with *Haemophilus ducreyi* CDT (22), INT407 cells exposed to *H. hepaticus* CDT (23) and fibroblasts treated with CjejCDT (24) suggests that CDT causes DNA damage and triggers DNA repair machinery. Direct consequences of DDR are cell distension and cell cycle arrest. CjejCDT causes G_2/M cell cycle arrest in fibroblasts by promoting DDR (18, 24). Except for initiating DDR and cell cycle arrest, CjejCDT is reported to stimulate IL-8 secretion in polarized human colonic epithelial T84 cells and INT407 cells (15, 25). IL-8 is a pro-inflammatory cytokine that recruits neutrophils to the infection sites, and neutrophil infiltration is a hall mark of acute *C. jejuni* enterocolitis (26, 27), and it implies that CDT may be involved in the host immune response against *C. jejuni* infection.

Recently, it is reported that fibroblasts or colon epithelial cells exposed to continuous sub-lethal recombinant *Helicobacter ducreyi* CDT stimulation over several weeks showed greater frequency of genomic instability (28). Interestingly, CDT from Shiga toxin-producing *E. coli* O157 induces G_2/M cell cycle arrest of human endothelial cells for up to 5 days, a feature indicative of cellular senescence (29, 30). CjejCDT causes epithelial cell distension and cycle arrest, and stimulates IL-8 expression, which is one of the SASP (15). Given that CDT cytotoxicities described above are also typical characteristics seen in senescent cells, we hypothesized that HhepCDT and CjejCDT will cause intestinal epithelial cells senescence if intoxicated long term.

Our study shows that human intestinal epithelial INT407 cells treated with sub-lethal doses of HhepCDT, CjejCDT, CjejWT WCL and CjejComp WCL display characteristic DDR, including arrest of the cell cycle and up regulation of γ-H2AX formation, intracytoplasmic accumulation of beta-galactosidase and secretion of SASP. However, senescence markers are not observed in HI-HhepCDT, HI-CjejCDT and CjejKO WCL treated cells, which look the same as normal cells. Based on evidence of these three cellular senescence markers, we can conclude *H. hepaticus* and *C. jejuni* CDT is a very potent genotoxin that induces cellular senescence in epithelial cells *in vitro*.

Materials and methods

C. jejuni growth condition and whole cell lysates preparation

C. jejuni strain and construction of CdtB isogenic mutants were described in supplemental material and methods. *C. jejuni* ET46-36-20 (CjejWT), *C. jejuni* ET46-36-20 CdtB knockout (CjejKO), and *C. jejuni* ET46-36-20 CdtB knockout complemented strain ABC01 (CjejComp) were grown on Mueller-Hinton blood agar plates (5% sheep blood, Colorado Serum Company, Denver, Colorado) at 37°C in the microaerobic condition generated by Pack-MicroAero gas pack (Mitsubishi Gas Chemical America, INC., New York, NY). After 3 days, bacteria were harvested by cold PBS, and sonicated to make whole cell lysate (WCL). WCL was centrifuged at 4000g for 10 min, and supernatant was collected and filtered through 0.22 um filter (Corning, Tewksbury, MA). WCL concentration was determined by using BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL).

Cell culture conditions

Human intestinal epithelial INT407 cell line (ATCC CCL-6) was maintained in Dulbecco's modification of Eagle's minimum essential medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and gentamicin (50 g/ml, Sigma, St. Louis, MO) at 37°C in a humidified atmosphere of 5% carbon dioxide in air as previously described (31). Cells were harvested by washing with phosphate-buffered saline (PBS; pH 7.4) followed by treatment with trypsin (Gibco, Grand Island, NY) at 37°C. For cell proliferation

assays, γ-H2AX immunoblot, and cytokine/chemokine determinations, INT407 cells were seeded in 24-well flat bottom culture plates (Corning Inc., Corning, NY) containing 1 mL of medium for up to 7 days. For determination of β-galactosidase expression, INT407 cells were grown on sterile 12 mm diameter round poly-L-lysine coated glass coverslips (Bellco Glass, Inc., Vineland, NJ) placed in 24-well flat bottom culture plates (Corning Inc.). For flow cytometry analysis, INT407 cells were seeded in 6-well flat bottom culture plates (Corning Inc.) containing 2 mL of medium for up to 7 days. To avoid cell overgrowth, all wells except those treated with HhepCDT were seeded with ten times fewer initial numbers of cells (approximately 1 x 10^4/mL in 24-well and 4 x 10^4/mL in 6-well culture plates).

Cell viability

Sequential determinations of INT407 cell proliferation was done by using trypan blue dye exclusion and a hemocytometer counting chamber (Hausser Scientific, Horsham, PA). After washing with PBS and treatment with trypsin, the cells were resuspended in DMEM, mixed 1:1 with 0.4% trypan blue solution (Gibco, Grand Island, NY) and the total numbers of viable cells were counted immediately.

Reconstituted recombinant *H. hepaticus* CDT (HhepCDT) and *C. jejuni* CDT (CjejCDT).

Reconstituted recombinant HhepCDT from *H. hepaticus* mouse strain 3B1/Hh-1 (ATCC 51449^T, Manassas, VA) (32) was prepared by mixing equal amounts of CdtA (HhepCdtA), CdtB (HhepCdtB), and CdtC (HhepCdtC) proteins expressed in

Escherichia coli, as previously described (23). Individual or combinations of recombinant fusion protein subunits were reconstituted as described previously (33, 34). Briefly, 20 µg of each recombinant fusion protein subunit was mixed in 100 µl of PBS (pH 7.4), and after incubation at 30°C for 1 h, reconstituted HhepCDT complexes were added to corresponding wells of 6-well or 24-well plates, followed by co-incubation for the indicated time.

Individual His-tagged *C. jejuni* CDT subunits were expressed in *E. coli*, and CDT holotoxin was reconstituted according to previously described protocols for *Helicobacter hepaticus* CDT with modifications (Dassanayake *et al.*, 2005; Liyanage *et al.*, 2010). Briefly, the gene sequence encoding each CjejCDT sub-unit was amplified by using specific primer pairs (Table 4.2) and high fidelity *Pfx* DNA polymerase (AccuPrime, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The amplified products were cloned in the pET-28a(+) expression vector (Novagen, Darmstadt, Germany) and transformed into *E. coli* BL21 (New England BioLabs, Ipswich, MA). Expression was induced when cultures grown at 37°C in Luria-Bertani medium containing kanamycin (Sigma, St. Louis, MO) reached an optical density at 600 nm of 0.6-0.8 by adding 1 mM isopropyl-β-D-thiogalactopyranoside (Invitrogen, Grand Island, NY) and grown an additional 5 h at 25°C. CjejCdtA and CjejCdtB were purified under denaturing conditions while CdtC was purified under native conditions. The purity of each recombinant protein preparation was determined by SDS-PAGE followed by Coomassie blue staining and Western blot as previously described (Dassanayake *et al.*, 2005). The CDT holotoxin

was reconstituted as previously described (Liyanage *et al.*, 2010) and stored at -80°C in 1:1 sterile glycerol. For negative controls, reconstituted recombinant CjejCDT was heat inactivated (HI) for 10 min at 70°C and stored at -80°C in 1:1 sterile glycerol. (**The clones were constructed and tested by Dr. Rasika Jinadasa when he was in Dr. Duhamel's laboratory. Since the data are not published yet, the method described here was directly adopted from Dr. Rasika Jinadasa Ph.D thesis entitled "Molecular mechanisms of cycle arrest and apoptosis in lymphoid cells elicited by cytolethal distending toxin, a genotoxin produced by *Campylobacter jejuni* and *Helicobacter hepaticus*"**)

Dtermination of sub-lethal dose of HhepCDT, CjejCDT, CjejWT WCL and CjejComp WCL to IN407 cell

Approximately, 1 X 10^5 INT407 cells were plated into 24-well plates, and treated with blank medium (control), HhepCDT, CjejCDT, CjejWT WCL and CjejComp WCL in a serial 5 x dilution manner. Each dilution of different protein was plotted in triplicate. Cells were checked every day (up to 7 days) for the cell distending toxicity. The dilution that did not kill the cells, and also made 100% cells enlarged was determined to be the sub-lethal dose to INT407 cells.

INT407 cell intoxication with recombinant proteins and *C. jejuni* WCL

Approximately, 1 X 10^5 or 3 x 10^5 INT407 cells were seeded into 6-well or 24-well plates (Costar, Cambridge, MA, USA) containing 2 ml or 1 ml medium with 20 μg/ml HhepCDT, 20 μg/ml CjejCDT, 20 μg/ml heat inactivated CjejCDT (HI-CjejCDT,

70°C for 30 min), 20 μg/ml heat inactivated HhepCDT (HI-HhepCDT, 70°C for 30 min), 400ng/ml CjejWT WCL, 400ng/ml CjejKO WCL, 80ng/ml CjejComp WCL, or blank medium (control). Treated INT407 cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for up to 7 days. To prepare the recombinant CDT holotoxin, individual or combinations of recombinant fusion protein subunits were reconstituted as described previously (33, 34). Briefly, 20μg of each recombinant fusion protein subunit were mixed in 100μl of PBS (pH 7.4), and after incubation at 30°C for 1 h, the reconstituted CDT complexes (HhepCDT or CjejCDT) were added into corresponding wells of 6-well or 24-well plates, followed by co-incubation for the indicated time.

Senescence-associated β-galactosidase staining

On day 1, 3, 5 and 7 of incubation in the presence of control medium or medium containing a sub-lethal concentration of reconstituted recombinant HhepCDT, CjejCDT, HI-HehCDT, HI-CjejCDT, CjejWT WCL, CjejKO WCL or CjejComp WCL, INT407 cells seeded on coverslips in 24-well plates were fixed with freshly made 4% (w/v) paraformaldehyde (PFA) and processed for histochemical detection of cytoplasmic β-galactosidase expression at pH 6 according to the manufacturer's instructions (Senescence β-galactosidase Staining Kit; Cell Signaling Technology, Danvers, MA). After counterstaining with nuclear fast red stain (Vector Laboratories, Burlingame, CA), photomicrograph images were recorded by using a camera mounted light microscope.

Immunoblotting

INT407 cells incubated in the presence of control medium or medium containing an either a sub-lethal concentration of reconstituted recombinant HhepCDT CjejCDT or the corresponding heat-inactivated HhepCDT (HI-HhepCDT), heat-inactivated CjejCDT (HI-CjejCDT), CjejWT WCL, CjejKO WCL, CjejComp WCL or individual recombinant HhepCdtA, HhepCdtB, HhepCdtC subunits were harvested day 0, 3, 5 and 7 incubation and lysed by using RIPA buffer according to the manufacturer instructions (Cell Signaling Technology, Beverly, MA). After separation by electrophoresis on 12% SDS-polyacrylamide gels, cell proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA), and Western blotting was done by using mouse monoclonal anti-human histone H2A.X phosphorylated at Ser 139 immunoglobulin (Ig) G1 antibody (Clone JBW301; EMD Millipore, Billerica, MA). To confirm equal loading of each cell preparation, a mouse monoclonal anti-human β-actin IgG1 antibody (Clone AC-15; Sigma, St. Louis, MO) was used. Specific protein bands were identified by incubation with horseradish peroxidase (HRP)-conjugated polyclonal goat anti-mouse IgG antibody (Santa Cruz, Santa Cruz, CA) followed by chemiluminescence detection (WesternBright ECL Western Blotting HRP Substrate; Advansta, Menlo Park, CA) and an image capture workstation (V3 Western Workflow; Bio-Rad, Hercules, CA).

Immunofluorescence

INT407 cells treated with CjejWT WCL, CjejKO WCL, CjejComp or blank medium were plated in 24-well plates with coverslips (ThermoFisher Scientific, Waltham,

MA) sitting on the bottoms of each well. After fixation with 4% PFA at day 1, 3, 5 and 7, cells were stained for detection of γ-H2AX or p21 by using monoclonal mouse anti-γ-H2AX antibody (1:1000 dilution, EMD Millipore, Billerica, MA), or monoclonal rabbit anti-human p21 antibody (1:1000 dilution, Cell Signaling Technology, Danvers, MA). Alexa-fluo labeled goat anti-mouse or goat anti-rabbit secondary antibody (Invotrogen, Grand Island, NY) was used to detect primary antibody. Alexa Fluo labeled Phalloidin (Invotrogen, Grand Island, NY) was added the same time with secondary antibody to stain actin. DAPI (Invotrogen, Grand Island, NY) was applied for DNA counter staining. After staining process, coverslips were taken out and mounted with Permount (ThermoFisher Scientific, Waltham, MA), and then images were taken at 20 x magnification by Zeiss laser confocal microscopy.

Flow cytometry

INT407 cells incubated in the presence of control medium or medium containing a sub-lethal concentration of reconstituted recombinant HhepCDT, CjejCDT, CjejWT WCL, CjejKO WCL, CjejComp WCL or corresponding HI-HhepCDT or HI-CjejCDT were harvested on day 0, 1, 3, 5 and 7 and the percent of cells in G0/G1 (green), S (blue) and G2/M (red) phases were determined by staining with propidium iodide and fluorescent flow cytometry as described previously (31). Briefly, harvested cells were transferred to 70% ethanol, held overnight at -20^0C, followed by rehydration with 1 mL PBS at room temperature for 15 min, and after centrifugation, were stained with propidium iodide (PI). For DNA content determinations, approximately 1.0 X 10^5 cells were examined by FACS analysis, with the excitation set at 488 nm and the

emission set at 630 nm (BD™ LSR II Flow Cytometer, Becton Dickinson, San Jose, CA, USA). The data was analyzed using BD FACSDiva software version 6.1.1 (Becton Dickinson), and the percentages of cells at each phase of the cell cycle were presented as donut charts by using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA).

Realtime PCR

Total mRNA of INT407 cells treated with HhepCDT, HI-HhepCDT, HhepCdtA, HhepCdtB, HhepCdtC, CjejCDT, HI-CjejCDT, CjejWT WCL, CjejKO WCL, CjejComp WCL or blank medium in 24-well plates was extracted by using mRNA purification kit (Qiagen, Valencia, CA). After first strand cDNA synthesis by using a reverse transcription kit (ABI, Grand Island, NY), RT-PCR amplification of IL-6, IL-8, IL-24 and 18S mRNA was carried out by using specific Taqman probes (ABI, Grand Island, NY) according to the manufacture's instruction and determination of relative mRNA expression level of each cytokine. Data were normalized to 18S mRNA and expressed as fold increase over corresponding levels of mRNA from INT407 cells incubated in the presence of control medium on day 0.

Cytokine protein expression assay

Supernatants from INT407 cells treated with HhepCDT, HI-HhepCDT, CjejCDT, HI-CjejCDT or blank medium were harvested on day 0, 3, 5, and 7, centrifuged at 10,000 x *g* for 1 minute, and the supernatant was placed at -80°C until needed. The concentrations of IL-6 and IL-8 were determined by using a commercially-available

fluorescent bead multiplex cytokine assay as described by the manufacturer (R&D Systems, Minneapolis, MN) and analyzed by using a Bio-plex 200 machine (Bio-Rad, Hercules, CA). The relative concentration of cytokine was normalized on the basis of total number of viable INT407 cells per well.

Data analysis

Statistical analysis was done by using Student t-test with $p<0.05$ considered significant and displayed by using GraphPad Prism 6 software (GraphPad Software). For cell growth determinations, the number of viable cells at one time point was compared with the previous time point within treatment.

Results

CDT causes cell growth arrest

C. jejuni recombinant CDT is known to cause cell cycle arrest, its activity requires the formation of tripartite holotoxin composed of three subunits CdtA, CdtB and CdtC, and none of the subunits alone exhibits any toxicity to intestinal epithelial cells (35). To investigate the possibility of cellular senescence induced by CjejCDT, we used recombinant HhepCDT and CjejCDT holotoxin to stimulate human intestinal epithelial cell INT407 cells in a long time of period (up to 7 days) in a concentration that did not promote cell towards apoptosis or kill them, but still made cell show CDT intoxication effect such as cell distension. HhepCDT and CjejCDT intoxicated INT407 cells did not proliferate, and the number of cell did not decrease significantly either in the 7-day observation period (Figure 2.1A&B.). In order to prevent the over-confluent growth in the 7-day long experiment, the starting number of non-intoxicated cells in Figure 2.1A was ten-fold fewer than that of the HhepCDT intoxicated cell cultures; the starting number of non-intoxicated cells for time point at day 7 in Figure1. B was ten-fold fewer than that of CjejCDT intoxicated. The medium control, HI-CjejCDT and HI-HhepCDT treated cells continued proliferating during the 7-day observation period. It suggests that CDT is able to cause cell growth arrest up to 7 days, but heat inactivated CDT is not. Any of HhepCdtA, HhepCdtB and HhepCdtC alone does not cause cell growth arrest, suggesting the single CDT subunit does not cause the same cytotoxicity, and a CDT holotoxin is required to arrest cell growth as previously described.

In order to further rule out that cell growth arrest does not result from CdtA and CdtC subunits, or any contamination from the preparation of recombinant CDT protein, *C. jejuni* whole cell lysate (WCL) made from CjejWT, CjejKO and CjejComp was used to intoxicate INT407 cells. As shown in Figure1.B, CjejWT WCL and CjejComp WCL lead to cell growth arrest, whereas CjejKO WCL did not. It strongly supports that CDT causes cell growth arrest, and CdtB is the active subunit responsible for cytotoxicity.

CDT is known to trigger DDR, which leads to cell cycle arrest in fibroblasts, epithelial and endothelial cell (22-24, 29). In less than 24 hours, *C. jejuni* CDT intoxicated HeLa cells show DDR such as the formation of Rad50 foci and γ-H2AX (24). Since recombinant HhepCDT, CjejCDT and *C. jejuni* WCL intoxicated INT407 cells exhibit significant growth arrest (Figure 2.1), DDR may be elevated consistently over time. We checked γ-H2AX formation over time in intoxicated INT407 cells. γ-H2AX in HhepCDT, CjejCDT, CjejWT WCL and CjejComp WCL intoxicated INT407 cells was formed at day 3 post-intoxication and maintained consistently at a significantly greater level over the 7-day observation period (Figure 2.2A&B), however only basal levels of γ-H2AX formation were observed in the non-intoxicated groups of cells (medium control, HI-HhepCDT, HI-CjejCDT and CjejKO WCL) (Figure 2.2A&B). Immunofluorescence staining of γ-H2AX (Figure 2.2C) showed that CjejWT WCL and CjejComp WCL intoxicated cells had γ-H2AX staining in their enlarged nucleus,

but CjejKO WCL treated cells looked the same as the medium control, and had very few spots of γ-H2AX staining. Cell cycle flow cytometry analysis confirmed that, at day 3, day 5 and day 7 post treatment, the percentage of G_2/M phase cells in HhepCDT intoxicated cells are 67.5%, 54.6% and 45.5%; in CjejCDT intoxicated cells are 73.2%, 64.2% and 60.5%; in CjejWT WCL intoxicated cells are70.9.0%, 69.9% and 62.7%; in CjejComp WCL intoxicated cells are 83.5%, 73.9% and 77.7%. HI-HhepCDT, HI-CjejCDT, and CjejKO WCL treated cells multiplied in the same pattern as medium control cells, and did not have cell cycle arrest (Figure 2.3). Overall, it clearly indicates that recombinant CDT or CjejWT WCL (which had active CdtB subunits) intoxicated cells stop proliferation, but are still viable throughout the 7-day period of intoxication; CDT induces cell cycle arrest by triggering DDR in a manner dependent on CdtB activity.

CDT induces senescence-associated β-galactosidase expression and senescence associated secretion phenotype

CDT intoxicated INT407 cells exit cell cycle permanently, but are still alive. Presumably, this occurs as a result of cellular senescence. In order to tell whether CDT intoxicated cells actually undergo cellular senescence or not, two more senescence markers were checked, As shown in Figure 2.4, at day 3, HhepCDT, CjejCDT, CjejWT WCL and CjejComp WCL intoxicated INT407 cells were distended, and some of the cells also showed large blue senescence-associated β-galactosidase positive staining; at day 5 and day 7, most of the cells became

senescence-associated β-galactosidase positive (Figure 2.4). However, medium control, HI-HepCDT, HI-CjejCDT and CjejKO WCL treated INT407 cells appeared normal in size and only few cells showed very small spots of blue senescence-associated β-galactosidase staining (Figure 2.4).

IL-6 and IL-8 are two cytokines up-regulated in senescence associated secretion phenotype, so realtime PCR was performed to evaluate the mRNA expression levels of IL-6 and IL-8. Compared with medium control INT40-7 cells, HhepCDT intoxicated INT407 cells had significantly increased IL-6 mRNA expression, from approximately 4-fold at day 3 to 20- fold at day 7, and IL-8 mRNA from 7-fold at day 5 to 14- fold at day 7 (Figure 2.5A); CjejCDT intoxicated had a gradual significantly increased IL-6 mRNA expression from approximately 24 fold at day 3 to 90 fold at day 7, and IL-8 mRNA expression from 4 fold at day 3 to 10 fold at day 7 (Figure 2.5B). In CjejWT WCL intoxicated INT407 cells, both of IL-6 and IL-8 mRNA expression increased approximately 2 fold for both at day 5 and 7. In CjejComp WCL intoxicated INT407 cells, IL-6 mRNA expression was up-regulated about 2, 2.5 and 6 fold at day 3, 5 and 7 separately, and IL-8 mRNA expression was also raised from about 2.5 and 10 fold at day 5 and 7. However, compared with medium control cells, HI-HhepCDT, HI-CjejCDT and CjejKO WCL treated cells did not have a significant up-regulation of IL-6 and IL-8 mRNA expression. Corresponding to mRNA expression, IL-6 protein concentration in the supernatant of HhepCDT and CjejCDT intoxicated cells started to increase significantly from day 3 or day5, and IL-8 protein concentration begun to escalate significantly from day 5 or day 7 (Figure 2.5D&E).

Except for the increased expression of IL-6 and IL-8, we occasionally also observed a striking increase of IL-24 mRNA expression in HhepCDT (Figure 2.5A) (12.6 fold at day 5, 59 fold at day 7), CjejCDT (Figure 2.5B) (2.8 fold at day 3; 21 fold at day 5; 29 fold at day 7), CjejWT WCL (Figure 2.5C) (2.9 fold at day 5; 7 fold at day 7) and CjejComp WCL (Figure 2.5C) (4 fold at day 5; 13.8 fold at day 7) intoxicated INT407 cells at day 5 and . IL-24, belonging to Il-10 family of cytokines, is a tumor growth suppressor (36), but its role in cellular senescence has not been well described. The significant increase of IL-24 mRNA expression in CDT intoxicated cells cellular senescence suggests it might get involved in CDT-induced cellular senescence. Generally, HhepCDT and CjejCDT intoxicated INT407 cells appear to show senescence-associated β-galactosidase expression and have senescence associated secretion phenotype – up-regulation of IL-6 and IL-8.

In conclusion, we have shown that recombinant HhepCDT and CjejCDT proteins and *C. jejuni* WCL cause cell growth arrest and DNA damage, induce senescence-associated β-galactosidase expression and stimulate SASP in human epithelial INT407 cell. Based with evidence of these three striking cellular senescence markers, we can conclude *in vitro C. jejuni* CDT is the very potent toxin that induces cellular senescence in epithelial cells; CdtB is critical for the CDT induced cytotoxicity, but the holotoxin formed by all of the CdtA, CdtB and CdtC are required to perform its cytotoxicity.

Figure 2.1 CDT intoxicated H407 cells do not preliterate.

H407 cells were plated into 24 well-plates, and treated with recombinant protein (HhepCDT, HI-HhepCDT, HhepCdtA, HhepCdtB, HhepCdtC and CjejCDT), *C. jejuni* WCL (CjejWT, CjejKO and CjejComp) or blank medium (control). The number of viable cells was determined by trypan blue exclusion at indicated time points. In Control, 10 x less of the initial number of cells were used for a confluent cell growth at day 7. In each treatment, the number of viable cells from day 1 was compared with the previous time point inside the same treatment for statistical significance analysis. (A), 10 x less of the initial number of cells were used for all non HhepCDT intoxicated cells; (B), 10 x less of the initial number of cells were used for a confluent cell growth at day 7 in medium control and CjejKO WCL treatments. In each treatment, the number of viable cells at each time point (except day 0) was compared with the previous time point inside the same treatment for statistical significance analysis of cell growth. The number of viable cells increases from day 3 to day 7 in the medium control, and HI-HhepCDT, HhepCdtA, HhepCdtB, HhepCdtC and CjejKO WCL treatments. There was not significant cell growth in the cells intoxicated with HhepCDT, CjejCDT, CjejWT WCL and CjejComp WCL. Each treatment at every time point was done in triplicate. Data shown are from one of three independent experiments. Error bars represent Mean±SEM. * $p<0.05$. **$p<0.01$.

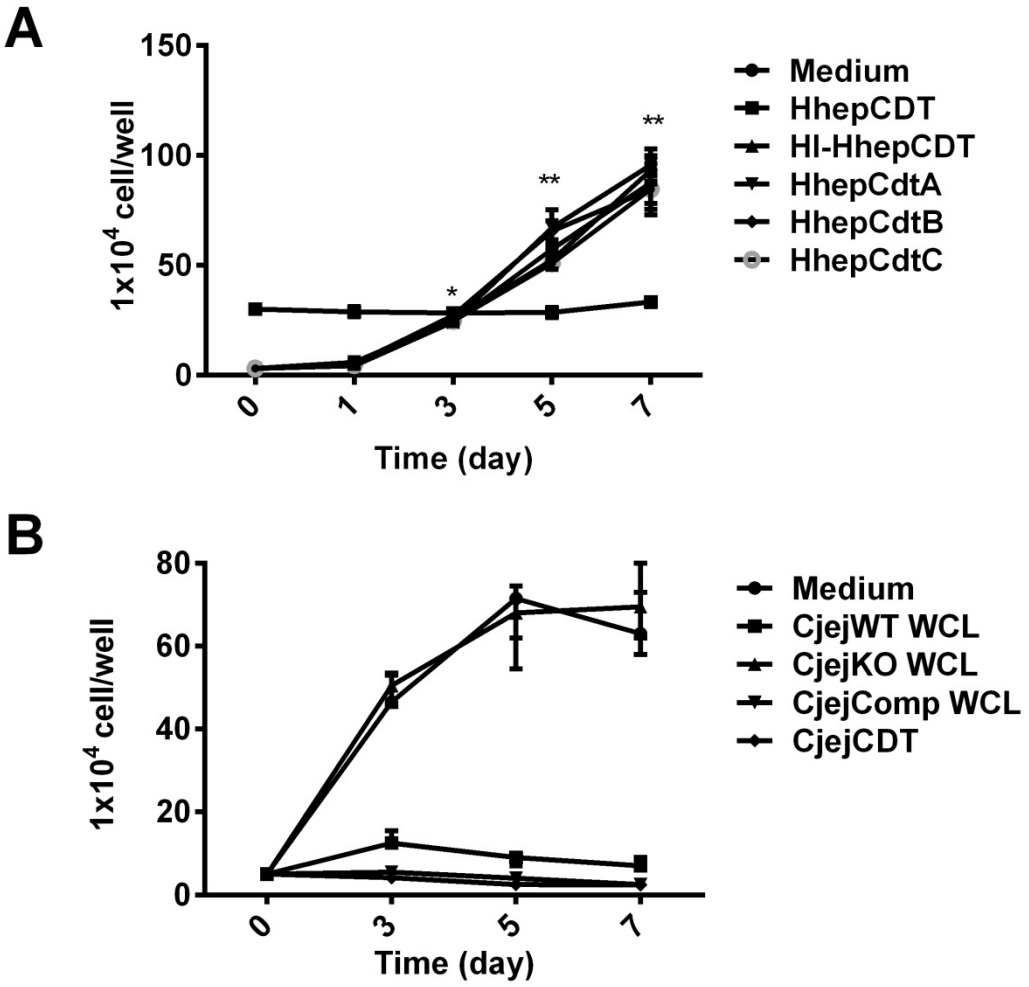

A
150
100
50
0
1x10^4 cell/well
0
1
3
5
7
Time (day)
*
**
**
Medium
HhepCDT
HI-HhepCDT
HhepCdtA
HhepCdtB
HhepCdtC
B
80
60
40
20
0
1x10^4 cell/well
0
3
5
7
Time (day)
Medium
CjejWT WCL
CjejKO WCL
CjejComp WCL
CjejCDT

Figure 2.2 Formation of γ-H2AX is up regulated in CDT intoxicated H407 cells.

Lysates of H407 with different treatments whole cell lysates were prepared at indicated time points. (A&B), Immunoblotting was performed by using anti-γ-H2AX antibody or anti-β-actin antibody. (C), Immunofluorescence was conducted by using primary anti-γ-H2AX antibody and Alexa-fluo labeled secondary antibody, and DNA was stained with DAPI. Basel level of γ-H2AX formation is observed in the control medium, HI-HhepCDT, HhCdtA, HhCdtB, HhCdtC HI-CjejCDT and CjejKO WCL treated H407 cells, but a significant amount γ-H2AX is formed in the HhepCDT, CjejCDT, CjejWT WCL and CjejComp WCL intoxicated H407 cells from day 3 and throughout the 7-day observation period. Data shown are from one of three independent experiments. Immunofluorescence shows that the nucleus of H407 cells intoxicated with CjejWT WCL and CjejComp WCL are stained strongly with γ-H2AX and are enlarged, while nucleus of cells treated with control medium and CjejKO WCL are not.

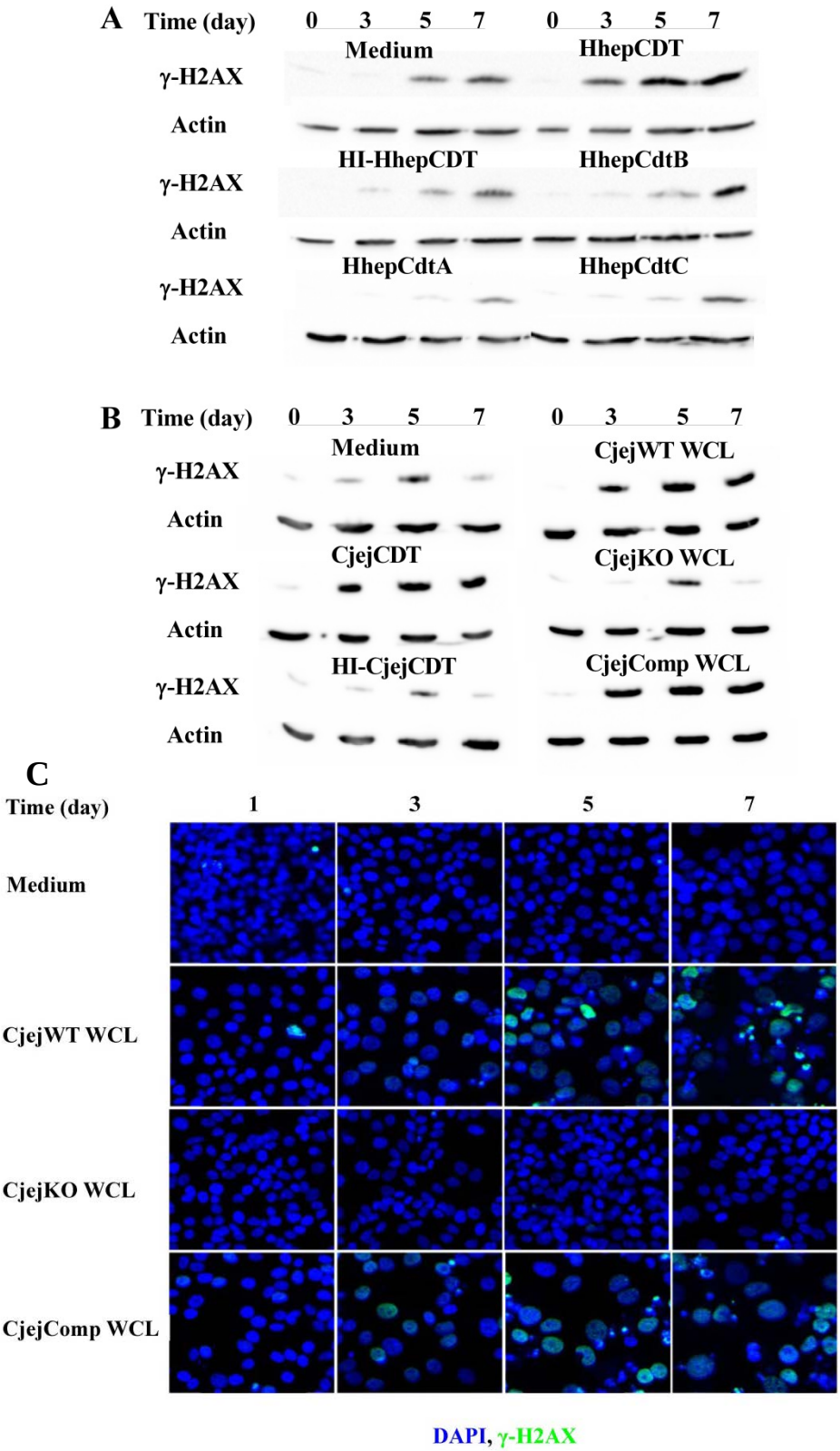
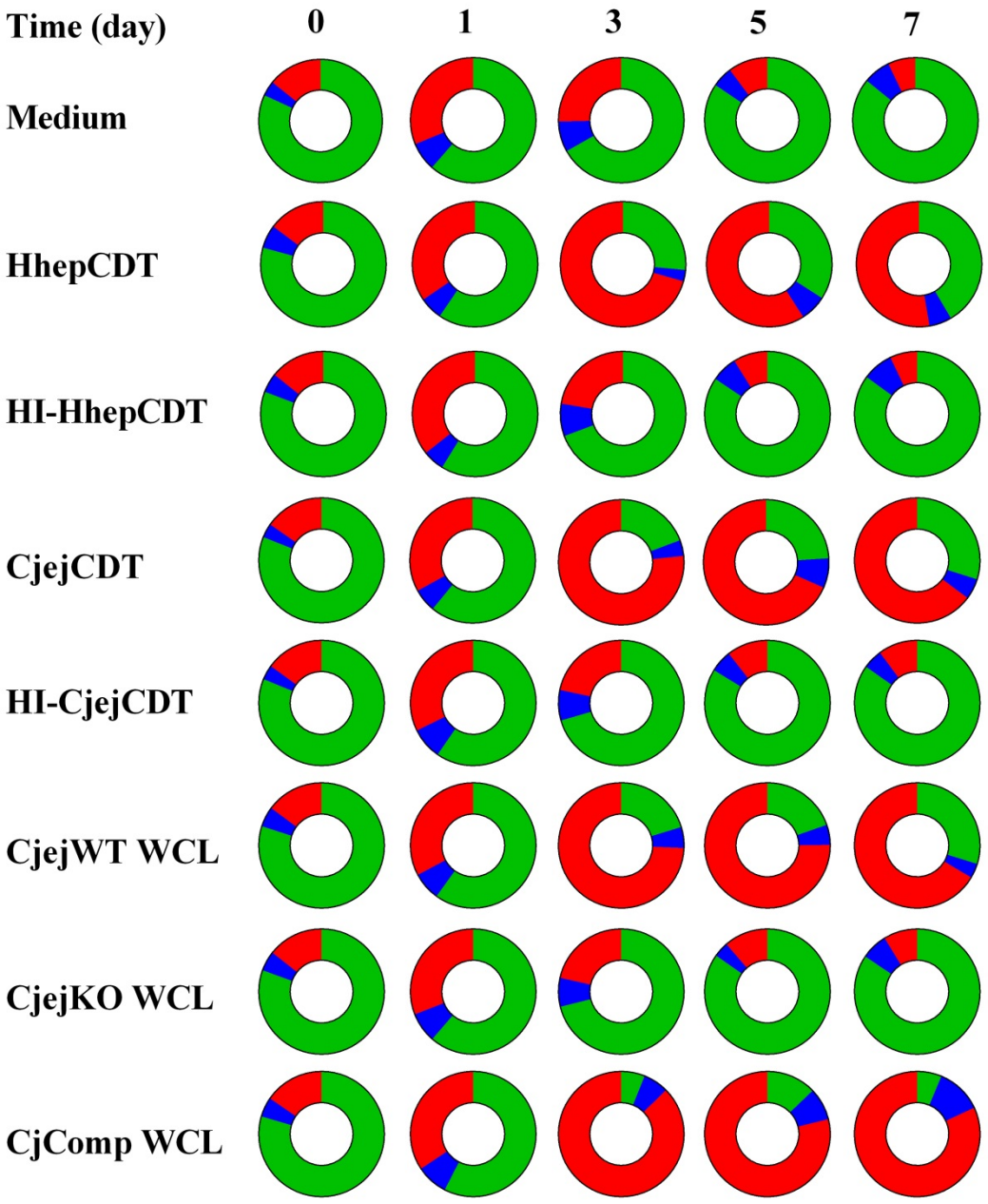

A
Time (day)
0 3 5 7
0 3 5 7
Medium
HhepCDT
γ-H2AX
Actin
HI-HhepCDT
HhepCdtB
γ-H2AX
Actin
HhepCdtA
HhepCdtC
γ-H2AX
Actin
B
Time (day)
0 3 5 7
0 3 5 7
Medium
CjejWT WCL
γ-H2AX
Actin
CjejCDT
CjejKO WCL
γ-H2AX
Actin
HI-CjejCDT
CjejComp WCL
γ-H2AX
Actin
C
Time (day)
1
3
5
7
Medium
CjejWT WCL
CjejKO WCL
CjejComp WCL
DAPI, γ-H2AX

Figure 2.3 CDT intoxicated H407 cells are arrested in G2/M cell phase.

H407 cells treated with recombinant protein (HhepCDT, HI-HhepCDT, HhepCdtA, HhepCdtB, HhepCdtC and CjejCDT) and *C. jejuni* WCL (CjejWT, CjejKO and CjejComp) were disassociated from 6-well plates by trypsinization and stained with PI. Flow cytometric analysis was performed to estimate the proportion of cells in G_0/G_1 (green), S (blue) and G_2/M (red) phases. Cell cycle was represented as percent total number of cells in the donut chart. At day 1, there is no cell cycle difference observed among the H407 cells with different treatments. At day 3, 5, and 7, in contrast to control medium, HI-HhepCDT, HI-CjCDT and CjKO WCL treated H407 cells, significant amounts of cells are arrested in G_2/M phase in HhepCDT, CjCDT, CjWT WCL and CjComp WCL intoxicated H407 cells. Data shown are from one of three independent experiments.


Time (day)
0
1
3
5
7
Medium
HhepCDT
HI-HhepCDT
CjejCDT
HI-CjejCDT
CjejWT WCL
CjejKO WCL
CjComp WCL

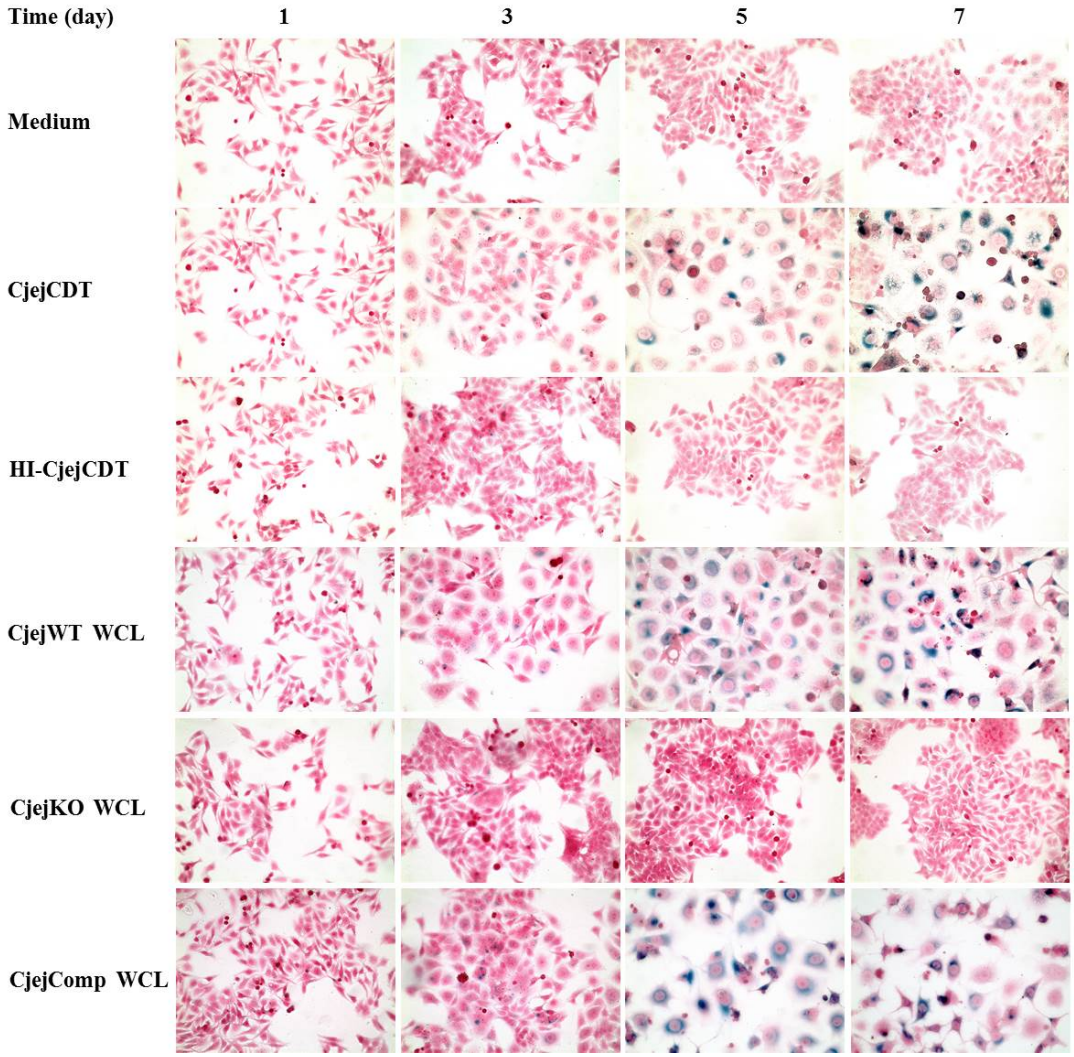
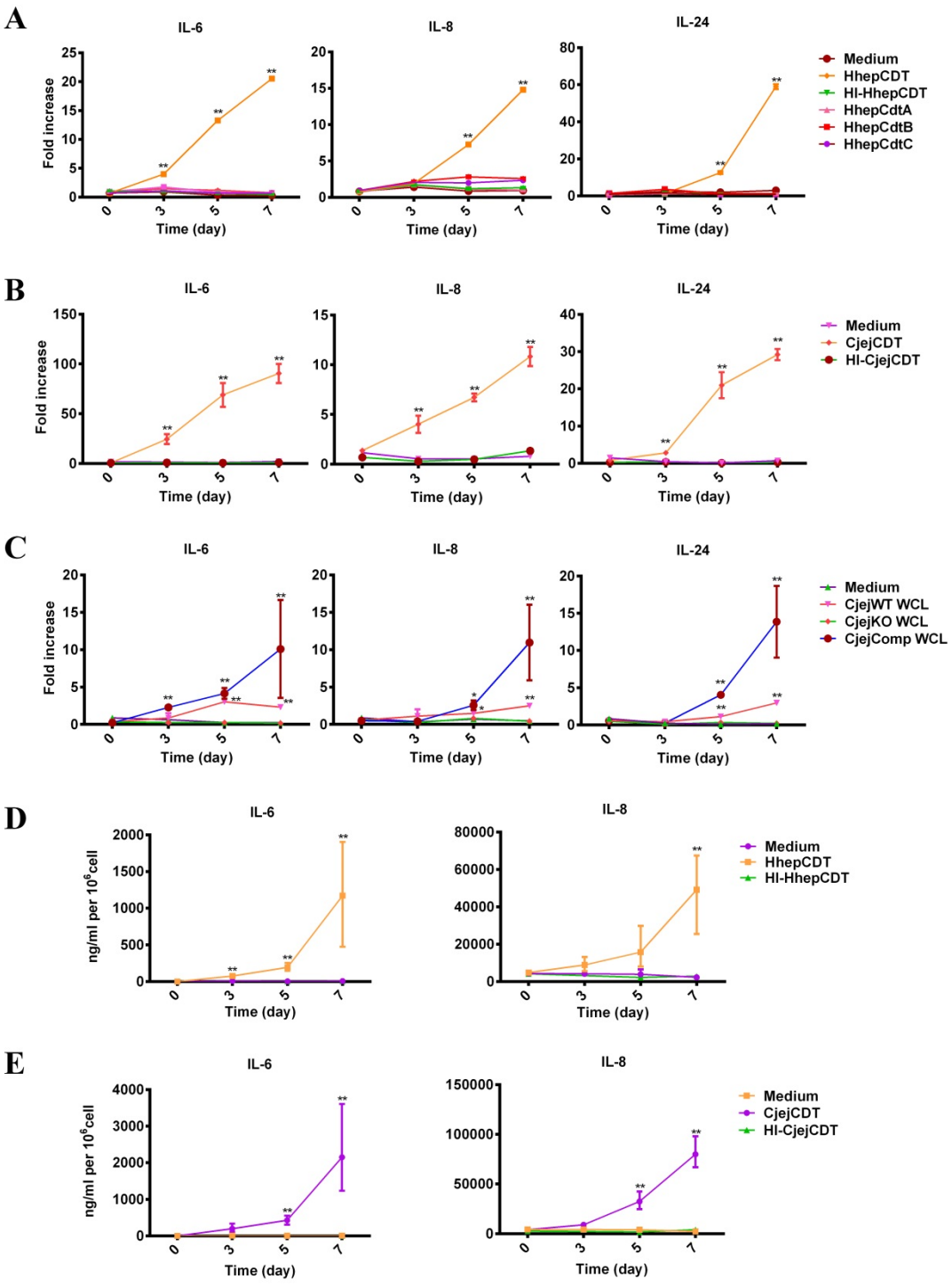


Figure 2.4 CDT intoxicated H407 cells show senescence associated β-galactosidase staining. After different treatments at indicated time points, H407 cells were processed for *in situ* cytochemical detection of β-galactosidase at pH 6.0. CjCDT, CjWT WCL and CjComp WCL intoxicated cells are enlarged, and a significant number of cells is stained blue (β-galactosidase positive) in contrast to control (Medium only), HI-CjCDT and CjKO WCL treated cells at day 3, 5 and 7. Images were taken at 20x magnification.

Figure 2.5 mRNA and protein expression levels of SASP were increased in CDT intoxicated H407 cells.

(A&B) After treatment with CjCDT, HI-CjCDT, CjWT WCL, CjKO WCL, CjComp WCL or medium only (control) at indicated time points, H407 cells total mRNA was purified and reverse-transcribed. IL-6, IL-8 and IL-24 cDNA levels were quantified by TaqMan real-time PCR, and normalized to 18S ribosomal RNA cDNA levels. Data were represented as fold of increase compared with the cDNA level at day 0. (C) Supernatant of H407 cells co-incubated with CjCDT, HI-CjCDT or blank medium (Control) was harvested, and the number of viable cells was also counted by trypan blue exclusion. Protein concentration of IL-6 and IL-8 in the supernatant was determined by using Fluorokine MAP human high sensitivity cytokine kit, and normalized to the production of cytokines by 1 x 10^6 cells. Error bars represent Mean±SEM. * $p<0.05$. ** $p<0.01$.


A
IL-6
IL-8
IL-24
Fold increase
Time (day)
Medium
HhepCDT
HI-HhepCDT
HhepCdtA
HhepCdtB
HhepCdtC
B
IL-6
IL-8
IL-24
Fold increase
Time (day)
Medium
CjejCDT
HI-CjejCDT
C
IL-6
IL-8
IL-24
Fold increase
Time (day)
Medium
CjejWT WCL
CjejKO WCL
CjejComp WCL
D
IL-6
IL-8
ng/ml per 10^6cell
Time (day)
Medium
HhepCDT
HI-HhepCDT
E
IL-6
IL-8
ng/ml per 10^6cell
Time (day)
Medium
CjejCDT
HI-CjejCDT

Discussion

Since the discovery of CDT in 1988, which causes progressive cell distension and death (37), it has caught attention to investigate how CDT causes those effects and its potential role in diseases. High dose of recombinant of *H. hepaticus* CDT is reported to kill epithelial cells by inducing apoptosis via mitochondrial apoptotic pathway, and increased level of γ-H2AX formation was observed in the cells indicating of DNA damage (23). However, low dose and consistent *H. hepaticus* lysates were reported to promote genome instability and trigger the cell survival signal (18, 24, 28). It appears that *in vitro* CDT cytotixity varies by the amount of toxin applied to the cells. So far, almost all of the studies focus on the cell death and cell cycle arrest induced by CDT *in vitro*. In this study, we characterized the changes of intestinal epithelial cells in 7-day long intoxication with sub-lethal doses of recombinant HhepCDT, CjejCDT or *C. jejuni* WCL, and observed that intoxicated cells eventually underwent premature cellular senescence. Our study illustrates that *C. jejuni* CDT induces cellular senescence, which is usually seen in aging and stresses such as drug induced DNA damage and oncogene malfunction (1). In addition, *H. hepaticus* and *C. jejuni* CDT elevates proinflammatory cytokine expression significantly during the process of cellular senescence.

CDT from different bacteria has been shown to have the capacity to induce cell death or growth arrest in many types of cells such as epithelial cells, keratinocytes, fibroblasts, and endothelial cell lines (18, 23, 24, 38-42), but most of the experiments

are mainly executed in a short period of time no more than 5 days. Here we went further to intoxicate epithelial cells with CDT or bacterial WCL for 7 days. CDT intoxicated cells did not die, but exhibited persistent DNA damage. The intoxicated cells were enlarged, arrested in G_2/M phase, and remained metabolically alive during the 7 day period intoxication. Similar phenomenon has been observed in a 5-day long intoxication of different endothelial cell lines with Escherichia coli CDT-V, and it appears to induce cell distension and irreversible G2/M arrest in a dose-dependent manner (29). We extended this report and demonstrated that *C. jejuni* CDT has the potential to cause long-term cell growth arrest in epithelial cells for up to 7 days, and intoxicated cells have persistent DDR. Considering that constitutive DDR contributes to the establishment and maintenance of cellular senescence (7, 43), another two senescent markers were checked to detect senescent cells in *H. hepaticus* and *C. jejuni* CDT intoxicated epithelial cells. The presence of senescence-associated β-galactosidase activity and SASP confirmed that *in vitro* the sub lethal dose of *C. jejuni* CDT intoxication induced cellular senescence in intestinal e pithelial cells, which is a very different phenomenon from apoptosis induced by *H. hepaticus* CDT in the *in vitro* experiments we have previously reported (23). It is well known that CdtA and CdtC are the subunits that are responsible to transport active CdtB subunit into the cell (42), and it is no surprise to see none of the three subunits of *H. hepaticus* CDT alone causes any cell damage, suggesting that the complete holotoxin of CDT is required for its cytotoxicity *in vitro*. *C. jejuni* CDT KO and Comp isogenic strains allowed us to exclude the possibility that contaminant from preparing recombinant CDT protein might be involved in inducing senescence, and validate that CDT intoxication results

in cellular senescence. Surprisingly, a very low concentration of CjejWT and CjejComp WCL (400ng/ml and 80ng/ml respectively) is enough to induce cellular senescence. Given that without cell membrane enrichment, CDT in the bacteria is too limited to be detected by immunoblotting (15), presumably, the actually amount of CDT in CjejWT and CjejComp WCL might be far less than 1 ng/ml. In contrast, much higher concentration of recombinant CDT protein (20ug/ml) is needed to induce cellular senescence. This discrepancy is probably due to the low efficacy in recombinant protein refolding and CDT complex resembling, when the recombinant CDT holotoxin was prepared.

Epithelial cells treated with high concentration of HhepCDT undergo apoptosis (23). Under intermediate concentration CDT, our data show, DNA damage is not repairable, but not enough to trigger the apoptosis either, and epithelial cells commit to senescence. Presumably less deleterious amount of CDT intoxication induces cell cycle arrest temporarily which allows the repair mechanisms to repair the damage. Persistent genotoxic stress during chronic infection by CDT-producing bacteria might contribute to neoplastic transformation by induction of genomic instability (28, 42). *H. hepaticus* has been correlated with the development of hepatocellular carcinoma in A/JCr mice and colorectal cancer in immunodeficient mice (44). In this scenario, cellular senescence would be beneficial in the defense to prevent or delay neoplastic transformation initiated by CDT-induced DNA damage. However cellular senescence could also be detrimental, as cellular senescence is connected with the disruption of tight junctions and increase of monolayer permeability *in vitro* (45). That *C. jejuni*

infection alters the tight junction formed by epithelial monolayer cells, and increases its permeability *in vitro* (46-48), implies CDT-induced cellular senescence could be a mechanism used by bacteria to break the integrity of tight junction and facilitate its invasion.

C. jejuni CDT induced senescent INT407 cells have a significant increased expression of IL-6, IL-8 and IL-24, of which IL-6 and IL-8 are among the cytokines found in SASP. Interestingly, persistent infection accompanied with chronic inflammation is a common feature of many CDT-producing bacteria (42), suggesting that induction of SASP might be more prevalent than previously recognized. Consistent with this suggestion is the demonstration of pro-inflammatory cytokine responses of various CDT intoxicated cells in short term *in vitro* experiments, including INT407 cells (49, 50), HeLa (51) and T84 (25, 51-53). Even though there are also CDT-independent mechanisms of IL-8 stimulation in *C. jejuni* infection, which depends on *C. jejuni* invasion (15), our data shows that CDT intoxication might still greatly contribute to the IL-8 production. In the immune response, continuous IL-8 expression results in consistent neutrophil infiltration, which is a definite observation in *C. jejuni* infected IL-$10^{-/-}$ mice (54). IL-6 is a proinflammatory cytokine, and critical in directing innate to adaptive immune response (55). After 10 month of infection with *H. hepeticus* or its isogenic CDT mutant strain, activation of NF-κB, and up-regulation of IL-6 mRNA and other proinflammatory cytokines were detected in *H. hepeticus* infected mouse liver, but not in CDT mutant strain infected mouse liver (56). NF-κB is reported to be the major inducer of SASP (57), and the IL-6 and IL-8 increase in the HhepCDT

induced senescence is likely through NF- κB pathway. LPS of *C. jejuni* and live *C. jejuni* infection stimulate IL-6 expression in intestinal epithelial cells. However, little is known about IL-6 function during *C. jejuni* infection *in vivo*. Proinflammatory cytokines IL-6 and IL-8 are commonly considered as enhancers of tumorigenesis (58) , and on the other hand cellular senescence is taken as anti-tumorigenesis mechanisms. IL-6 and IL8 may facilitate maintaining the phenotype of senescent cells, but they may also favor the tumorigenesis in the cells nearby non-autonomously. Further investigation is needed to illustrate the role of IL-6 and IL-8 in HhedpCDT and CjejCDT induced cellular senescence.

Significant increase of IL-24 was observed in CDT induced senescent cells, and its relationship with cellular senescence has not been described. IL-24 classified as IL-10 gene family has the property of inducing cancer cell apoptosis, and its expression is associated with immune system such as thymus, spleen and peripheral blood leukocytes (59). The expression of IL-24 can be detected from human colonic subepethelial myofibroblasts in the inflamed mucosa of IBD patients, and IL-24 suppresses inflammatory responses in the intestine mucosa (60). The greater level of IL-24 mRNA expression in CDT induced senescent epithelial cells implicates IL-24 may be essential in maintaining the intestinal homeostasis by eliminating cancer cells, and balancing the inflammatory response resulting from IL-6 and IL-8. The role IL-24 in cellular senescence is very unclear.

In conclusion, we demonstrated that bacteria CDT can damage DNA in INT407 epithelial cells, cause cell cycle arrest, and induce cellular senescence. Persistent CDT intoxication leads to significant increased expression of proinflammatory cytokines. It suggests CDT is a potent inducer of cellular senescence *in vitro*, which might not only be a defensive mechanism fighting against tumorigenesis during infection caused by *H. hepaticus* or *C. jejuni*, but also contributes to bacteria invasion and pathogenesis. Taken together, our findings elucidate a basic mechanism of disease and suggest potential pathway to neoplastic transformation associated with CDT-producing bacterial pathogens of humans and animals.

Acknowledgments

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Supplemental material and methods

Construction of *C. jejuni* CDT isogenic mutants

The cdtB genes of *C. jejuni* strains ET46-36-20, isolated from macaques with campylobacteriosis (29) were disrupted to produce ΔcdtB mutants, and strain ET46-36-20 was complemented with a full-length cdtABC operon. The constructs have been sequenced and immunoblotted (Figure 2.6). Briefly, for directional cloning, the full length cdtABC operon of *C. jejuni* was amplified by PCR with cdtAF and cdtCR oligonucleotides (Figure 2.6A), to make pBSIIKS+-cdtABC (Strategen, La Jolla, CA). The cdtB gene was disrupted by insertional mutagenesis of a kanamycin resistance cassette which can function in both *C. jejuni* and E. coli, (shuttle vector pRY107, kindly provided by Dr. P. Guerry, Enteric Diseases Department, Naval Medical Research Center, MD; 139), into a unique EcoRI site of cdtB at position 419 and selection with 50 μg/ml of kanamycin. The ET46-36-20 strain with a disrupted cdtB gene was complemented with a 2.9-kb fragment encompassing the cdtABC operon amplified by PCR, ligated into pRY111, and selection with 20μg/ml of chloramphenicol. Sequencing of the recombinant shuttle vector cdtABC-01 revealed the cdtABC transcribed in the same orientation as the lacZ promoter, and cdtABC-02, with the cdtABC in the opposite orientation. Complementation of the ET46-36-20 strain with a disrupted cdtB gene was accomplished by conjugation with E. coli harboring empty pRY111, cdtABC-01, or cdtABC-02 and selection with 200 μg/ml of kanamycin and 15 μg/ml of chloramphenicol. Enriched membrane preparations of

each strain were examined by immunoblot with hyperimmune rabbit polyclonal antibodies prepared against *C. jejuni* His6-CdtB fusion protein. As expected, the *C. jejuni* ET46-36-20 WT and isogenic strains with inactivated cdtB gene complemented with the full length cdtABC operon (ΔcdtBComp), and recombinant *C. jejuni* CdtB fusion protein displayed a band of approximately 29-kDa molecular mass (Figure 2.6B). Conversely, the *C. jejuni* ET46-36-20 ΔcdtB mutant and *C. jejuni* ET46-36-20 transformed with the empty vector pRY111 showed no band, confirming that these strains no longer produced CdtB.

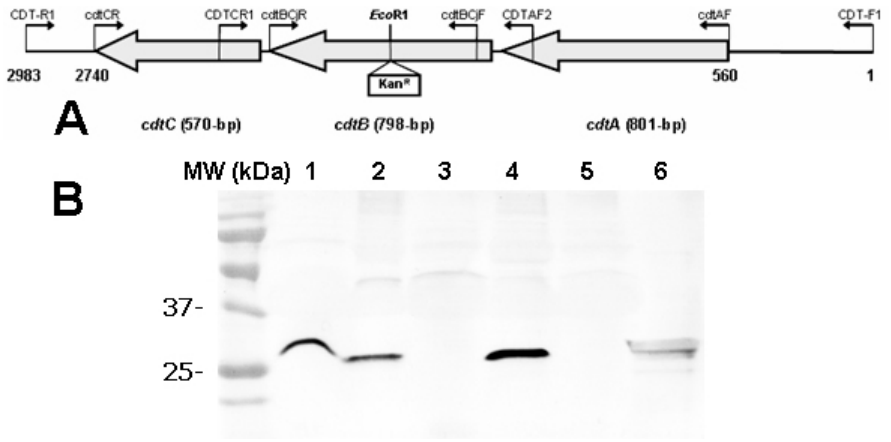

Figure 2.6. Construction and expression of recombinant *C. jejuni* CdtA, CdtB and CdtC.

A. *C. jejuni* cdtABC operon with location of oligonucleotide primers for overexpression of CdtB fusion protein (cdtBCjF and cdtBCjR), directional inactivation of cdtB by insertion of a kanamycin (KanR) antibiotic resistance cassette (CDTAF2 and CDTCR1; cdtAF and cdtCR), and complementation with full length cdtABC operon (CDT-F1 and CDT-R1). B. Immunoblot analysis of CdtB obtained from enriched membrane fractions of *C. jejuni* ET46-36-20. Lanes: (1) wild-type (WT), (2) cdtB isogenic mutant strain complemented with full length cdtABC operon in a 3' to 5' orientation (ΔcdtB + pcdtCBA-01); (3) ΔcdtB; (4) ΔcdtB complemented with full length cdtABC operon in a 5' to 3' orientation (ΔcdtB + pcdtABC-02); (5) ΔcdtB complemented with empty vector (ΔcdtB + pRY111); and purified recombinant His6-tagged *C. jejuni* CdtB fusion protein as a positive control. The membrane was incubated with rabbit anti-*C. jejuni* His6-CdtB fusion protein followed by horseradish peroxidase-conjugated goat anti-rabbit antibody and 4-chloro-1-naphtol substrate.

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CHAPTER4

DISCUSSION

Summary of findings

CDT is a novel AB type genotoxins that has been found in a growing list of clinically-important Gram negative bacterial pathogens (1). There are substantial *in vitro* evidences to support CDT contribution to DNA damage resulting in cell cycle arrest and apoptosis (1). *In vivo,* CDT has been linked to persistent bacterial colonization and pathogenesis of *H. hepaticus* and *C. jejuni* infection (2-4), and modulation of host immune response in *H. hepaticus* infection (5). Our studies focused on understanding the contribution of CDT produced by *C. jejuni* and *H. hepaticus* in induction of host innate immune response by intestinal epithelial cells and interaction with NETs.

Because large numbers of PMNs are recruited at the site of intestinal infection during acute *C. jejuni* infection, Chapter 2 examined the contribution of CDT nuclease in NETs. This was justified on the basis of demonstrating NET formation within the intestinal lumen of rhesus macaque with spontaneous campylobacteriosis and co-localization of *C. jejuni* within NETs. To investigate the interaction of *C. jejuni* with NETs we used PMNs derived from bone marrow of mice and peripheral blood of human beings. Although *C. jejuni* did not induce mouse or human NET formation *in vitro,* efficient trapping within NETs that was rescued by adding exogenous mammalian DNaseI was found. The lack of NET induction was not serotype-specific since three different strains of *C. jejuni* showed similar phenotypes, suggesting *C. jejuni* is not a potent NET inducer. The lack of significant differences in NETs induction, capture and killing between *C. jejuni* wild-type (CjejWT) compared to

isogenic ΔCDT (CjejKO) and ΔCDT complemented with CDT (CjejComp) mutant strains indicates that CDT might not contribute to bacterial escape from NETs. Nevertheless, NETs likely represent an important mechanism to control *C. jejuni* infection during acute infection of the gut (Figure 3.1).

Because *H. hepaticus* and *C. jejuni* can persist in the intestine and interact with host intestinal epithelium, Capter 3 examined the kinetic of CDT interaction with human intestinal epithelial cells over 7 days. For these studies we used reconstituted recombinant *C. jejuni* and *H. hepaticus* CDT (CjejCDT and HhepCDT) and whole-cell lysates (WCL) of CjejWT and CjejKO and CjejComp isogenic strains. Our data showed that recombinant CDT, WCL from CjejWT and CjejComp induced premature cellular senescence characterized by arrest of cell proliferation that correlated with persistent DDR together with senescence-associated β-galactosidase expression and senescence-associate secretory phenotype (SASP). While greater concentration of recombinant CDT (20 ug/ml) was required for induction of cellular senescence, several fold less WCL (8 ng/ml) induced similar level of senescence-associated phenotype. Taken together our data demonstrate that in addition to cell cycle arrest and apoptosis, CDT can induce premature senescence of intestinal epithelial cells which might contribute to the pathogenesis of disease.

Interaction of *Campylobacter jejuni* with NETs

The lack of suitable laboratory animal models that mimic acute human intestinal infection (6) has greatly limited our understanding of the contribution of PMNs in

control of *C. jejuni* infection. Nevertheless, it is well-established that *C. jejuni* can elicit a robust IL-8 response *in vitro* (7-9) which is thought to play key role in host innate immune recruitment of PMNs at the site of intestinal infection. There are only few reports describing phagocytic killing of *C. jejuni* by PMNs (10); however, the recent discovery of NETs urged us to investigate the potential contribution of NETs to control of acute *C. jejuni* infection. Our demonstration of *C. jejuni* association with NETs in the intestinal tract of non-human primates with spontaneous acute campylobacteriosis suggested that NETs might be an important mechanism of host innate defense against *C. jejuni* infection. Moreover, on the basis of previous reports on the role of bacterial nucleases mediating escape of bacterial pathogens from NETs, we investigated the contribution of CjejCDT nuclease in interaction of *C. jejuni* with NETs. The *in vitro* findings that although *C. jejuni* did not induce mouse or human NET formation, but were efficiently captured and survived NET killing was unexpected. However, considering that PMNs interact with *C. jejuni* in the lumen of the intestine, an environment rich in bacteria and their products which are potent NET inducers (11) suggested that trapping of *C. jejuni* within pre-formed NETs might assist control of infection by eliminating bacteria through peristalsis, and thus, reduce bacteria load. Given that the stereotypical host response to *C. jejuni* infection is diarrhea, the release of large numbers of live bacteria in feces might promote transmission of the disease between susceptible hosts. We speculate that the close proximity of large numbers of *C. jejuni* trapped within NETs might offer the

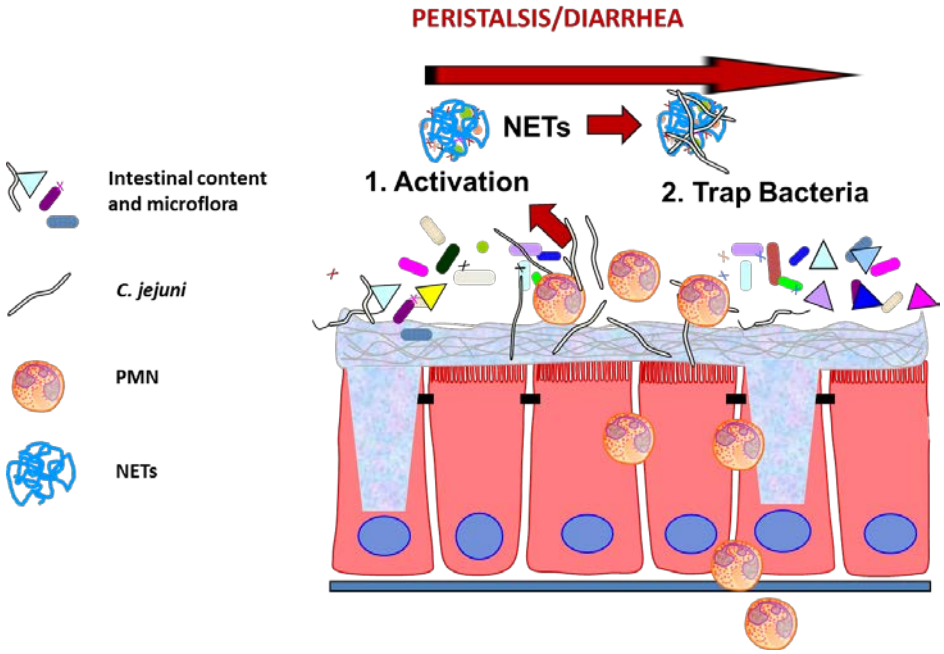

Figure 3.1 NETs in innate immune defense against *Campylobacter jejuni* intestinal infection. *C. jejuni* colonization of the intestine elicits recruitment of host PMNs to the site of infection through bacterial induced intestinal epithelial cell expression of IL-8 chemokine. Non-specific NET induction by intestinal flora bacteria and their products can entrap *C. jejuni* preventing further colonization and epithelial cell invasion. Eventually, intestinal peristalsis and secretory diarrhea expedite the elimination of *C. jejuni* entrapped within NETs and further dissemination of the pathogens in the environment, and thus, transmission of disease.

advantage of providing an efficient mechanism of horizontal gene transfer including antibiotic resistance.

The unexpected findings that CDT nuclease did not inhibit NET formation or mediate bacterial escape from NETs suggested that it is unlikely to contribute to bacterial resistance against this host innate defense mechansim. Because *C. jejuni* CDT is membrane-associated and has a weak DNase activity when compared with other bacterial nucleases that have been shown to mediate NET escape, the lack of contribution of CDT to NET interaction suggested a different pathogenetic role for this toxin. However, because of the limited viability of *C. jejuni in vitro* (maximum 4 hour observation period in our studies), the interaction of *C. jejuni* with NETs *in vivo* was not fully assessed. Therefore possibility that *C. jejuni* are killed over a longer period of time cannot be excluded completely. Moreover, additional factors within the intestinal lumen that might affect the biology of *C. jejuni* cannot be duplicated in our *in vitro* experiments including the role of bile acids increasing CDT expression and release (12).

Induction of premature cellular senescence by CDT

Cellular senescence is a well-established process associated with aging and various cellular stresses including chemical- and radiation-induced DNA damage and mitogenic oncogene (13-16); however, less is known about induction of premature cellular senescence in infectious diseases. Recently, the CDT of *Haemophilus ducreyi* (HducCDT) was shown to induce premature cellular senescence of fibroblast (17) and

promoted genome instability over long-term intoxication (18). Because *H. hepaticus* and *C. jejuni* are intestinal pathogens, we used human intestinal epithelial INT407 cell line to investigate the outcome of intoxication with sub-lethal concentrations of recombinant CDT and CDT holotoxin from whole cell lysate (WCL) within the context of a relevant *in vivo* infection microenvironment for these pathogens. Recombinant CjejCDT and HhepCDT proteins induced premature cellular senescence. The essential role of CdtB in induction of cellular senescence was further confirmed with *C. jejuni* CDT isogenic mutants and individual recombinant CDT subunits. The observation of CDT mediated cellular senescence and its senescence associate secretory phenotype (SASP) provides an exciting new perspective to our interpretation of the mechanism of *C. jejuni* pathogenesis within the context of host intestinal inflammatory bowel disease.

Although persistent *C. jejuni* infection has only been documented in AIDS patients (19), chronic infection of immunocompetent marmosets with *C. jejuni* is characterized by large number of bacteria within the lumen of the colon extending into crypt lumena (Figure 3.2 A and B). on the basis of these observations we hypothesized that long-term CDT-induced intoxication of intestinal epithelial cells might play a critical role in intestinal homeostasis and chronic inflammation.

Within the intestine, stem cells located at the base of colonic crypts give rise to progenitor cells that differentiate into absorptive epithelial cells (20). On the basis of

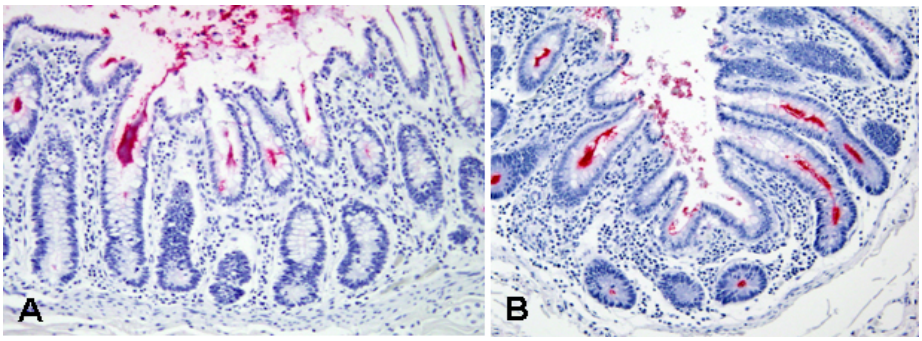

Figure 3.2 Captive cotton top tamarins (CCT) with *C. jejuni*-associated colitis. Immunohistochemical staining of formalin-fixed and paraffin-embedded sections of colons obtained from CTT with naturally-acquired colitis with a *C. jejuni*-specific mouse monoclonal IgG2b antibody (MED-CLA201; Accurate Chem. & Scientific Corp., Westbury, NY). Note the large numbers of bacteria (red) in the lumen and inside crypt lumena. Avidin-biotin-alkaline phosphatase complex method and hematoxylin counterstain (20X original magnification).

our observations, we hypothesize that persistent colonization of intestinal crypts by large numbers of *C. jejuni* and the release of CDT induces premature senescence and induction of a SASP that contributes to chronic host inflammatory response. Because senescence is known to alter tight junctions and increase epithelial barrier permeability (21), this in turn might provide a suitable crypt microenvironment that promotes bacterial persistence within intestinal crypt lumena. Concurrently, the CDT-induced SASP might also contribute to alterations of the crypt microenvironment, but also to the chronic intestinal inflammatory response (22). Whether chronic intestinal inflammation promotes survival of *C. jejuni* within crypt lumena and dissemination into the environment, thus promoting disease transmission remains to be determined.

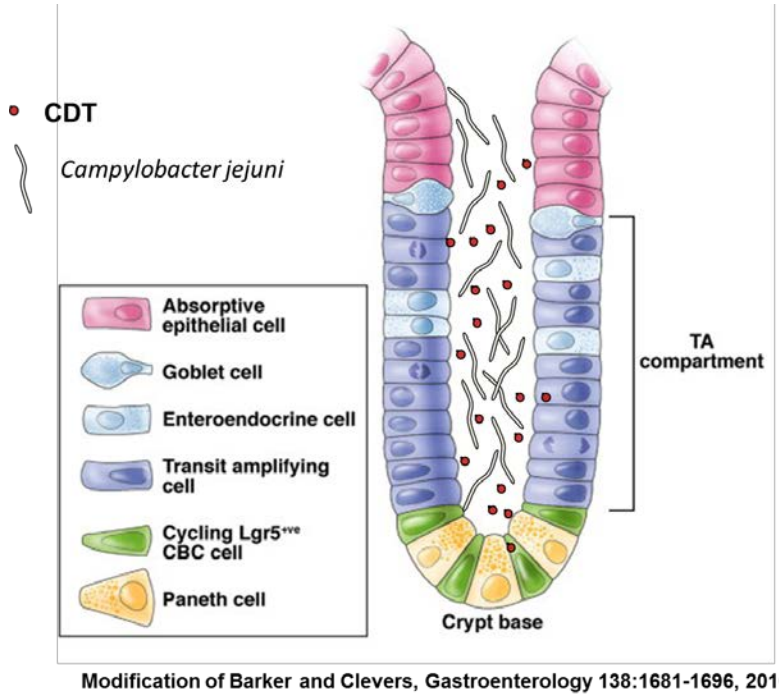

Figure 3.3 CDT interaction with colonic intestinal epithelial cells. The current proposed model of intestinal epithelial cell structure and kinetics consists of $Lgr5^{+ve}$ crypt base columnar (CBC) stem cells giving rise to transit amplifying (TA) progenitor cells which generate differentiated absorptive epithelial cells. Absorptive epithelial cells and TA cells are being replaced regularly; however, the stem cells are limited and have an extended life span. We propose that persistent colonization of intestinal crypts by *C. jejuni* can lead to CjejCDT-induced premature cellular senescence in stem cells leading to SASP and chronic inflammation. Both of these mechanisms adversely increase the intestinal epithelial barrier permeability favoring bacteria survival, multiplication and environmental dissemination.

Future directions

The combined medical burden of campylobacteriosis, inflammatory bowel disease and colon cancer to human health is beyond that of any other diseases of mankind. The pathogenesis of *C. jejuni* and *H. hepaticus* infection in general and the contribution of CDT to disease in particular are incompletely understood and represent a major hindrance to development of disease control strategies. Because of the lack of suitable laboratory animal model to study *C. jejuni* infection and disease, most of the current data including our own is based on *in vitro* model systems. Because CDT induces persistent premature senescence of cultured intestinal epithelial cells *in vitro*, future studies will correlate sequential intestinal epithelial cell kinetics with expression of DDR in animal models experimentally infected with CDT-producing bacterial pathogens.

Because *C. jejuni* causes persistent intestinal colonization without significant intestinal inflammation in conventional laboratory mice, it is unlikely that premature cellular senescence is involved in this model. By contrast, experimental infection of immunodeficient mice with *C. jejuni* might provide an *in vivo* model to further delineate the role of NET in host innate defense. Alternatively, a fetal rabbit intestine xenograft model can be extended to examine factors involved in short term host response to acute *C. jejuni* infection, particularly the role of NET. By adding different bacterial components, the contribution of the intestinal flora could be further investigated. Infection of xenografts with *C. jejuni* CDT isogenic mutant strains could

provide direct evidences about the role of CDT in *C. jejuni* interaction with NETs.

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