MOLECULAR MECHANISMS OF CYCLE ARREST AND APOPTOSIS IN LYMPHOID CELLS ELICITED BY CYTOLETHAL DISTENDING TOXIN, A GENOTOXIN PRODUCED BY CAMPYLOBACTER JEJUNI AND HELICOBACTER HEPATICUS

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Molecular mechanisms of cycle arrest and apoptosis in lymphoid cells elicited by cytolethal distending toxin, a genotoxin produced by *Campylobacter jejuni* and *Helicobacter hepaticus*

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It is well-established that the genotoxin, cytolethal distending toxin (CDT) which is produced by nearly two dozens of clinically-important bacterial pathogens causes DNA damage in a wide range of eukaryotic cells *in vitro.* More recently, it has become clear that irrespective of the origin of CDT, genotoxic damage results in a robust DNA damage response (DDR) in which the serine/threonine protein kinase ataxia telangiectasia mutated (ATM) plays a central role. The CDT-induced DDR closely resembles the response to DNA double strand breaks (DSBs) elicited by ionizing radiation (IR) which can lead to irreversible cell cycle arrest and apoptosis of mammalian cells. It is currently believed that CDT-induces ATM-dependent arrest at the G1/S and G2/M transitions of the cell cycle by activation of p53/Chk2 and p21(for G1/S) and Chk2 and CDC25C (for G2/M) respectively. By contrast, the mechanism of CDT-induced apoptosis remains incompletely understood; however, there is some evidence that it results from activation of the intrinsic apoptotic pathway through up-regulation of *Bax* together with down-regulation of *Bcl-2* and the release of mitochondrial cytochrome *c* causing down-stream activation of caspase-9. Moreover, on the basis of a higher sensitivity of hematopoietic cells to the cytotoxic effects of CDT, it has been suggested that CDT modulates the host immune response *in vivo.*

To provide a well-characterized *in vitro* model system that complements *in vivo* studies in a mouse model of defective DNA damage, we first generated several cells lines from spontaneous thymic lymphomas of $Atm^{-/-}$ and $p53^{-/-}$ mice. Then by comparing the responses of primary thymic lymphocytes with that of thymic lymphoma cell lines originated from $Atm^{-/-}$ and $p53^{-/-}$ mice, we clearly showed that treatment with *H. hepaticus* CDT causes a transient reduction in DNA synthesis which

is characteristic of activation of the ATM-dependent intra-S checkpoint. By demonstrating an ATM-dependent reduction in phosphorylated histone H3, we further confirmed CDT-mediated G2/M cell cycle arrest. These are the first conclusive evidences that CDT is capable of inducing cell cycle arrest in all phases of lymphoid cell cycle. Finally, we used our *Atm*$^{-/-}$ and *p53*$^{-/-}$ mouse thymic lymphoma cells lines to show that CDT-induced apoptosis is also p53-dependent.

Next, we examined the *in vitro* DDR of several human leukemia and lymphoma cell lines to genotoxic damage induced by CDT obtained from the human intestinal pathogen *Campylobacter jejuni.* While all human leukemia and lymphoma cell lines irrespective of their p53 status display comparable levels of activation of the ATM effector γ-H2AX in response to CDT treatment, two leukemic cell lines, MOLT-3 and MOLT-4 with wild-type p53 showed time- and dose-dependent increased susceptibility to *C. jejuni* CDT-induced apoptosis when compared to Jurkat, CEM and CA46 (also *Bax* deficient) cell lines with mutated p53. Demonstration of CDT-induced Chk1 activation in human leukemic cell lines suggests for the first time the involvement of ATR in the DDR to CDT-induced genotoxic damage. Taken together, we again conclude that p53 mutational status is a major determinant of the variable susceptibility of human lymphoma and leukemia cell lines to CDT. Activation of intra-S checkpoint by CDT may contribute to bacterial virulence by modulating rapidly dividing host lymphocytes, and thus, host adaptive immune response following bacterial infection.

To address this question directly, we assessed the adaptive humoral immune response following *H. hepaticus* infection of laboratory mice with a defective DDR due to a mutation in *Atm.* While infected wild type and heterozygous mice had high levels of IgG1 antibodies to *H. hepaticus* whole cell lysate (WCL), *Atm* null mice had approximately 60% reduction in serum antibody responses to both WCL and CdtB. The data suggest that DDR mechanisms are essential for bacterial and CDT-specific host adaptive immune responses.

BIOGRAPHICAL SKETCH

Rasika Jinadasa was born in Sri Lanka on June 06, 1977. He studied biology at Royal College Colombo and entered the Faculty of Veterinary Medicine and Animal Science at the University of Peradeniya, Sri Lanka in 1999. He earned his BVSc from the University of Peradeniya in 2004. Immediately following graduation he joined the Department of Basic Veterinary Sciences as a temporary demonstrator and later worked as a temporary lecturer attached to the Department of Farm Animal Production and Health for a year. Then he joined the laboratory of Dr. Gerald Duhamel at University of Nebraska-Lincoln, USA in 2005 and completed a Master of Science degree in Veterinary and Biomedical Sciences in 2007. He is currently reading for a PhD in the field of Comparative Biomedical Sciences under the mentorship of Dr. Gerald E. Duhamel, who moved to Cornell University in 2007. He is studying the molecular mechanisms of cell cycle arrest and apoptosis elicited by cytolethal distending toxin, a genotoxin produced by *Campylobacter jejuni* and *Helicobacter hepaticus*.

Dedicated to the memory of my mother and grandmother, Mrs. C. M. Gurusinghe, my high school science teacher and Professor Malcolm Alwis and Dr. S. Mahalingam, the gentlemen who taught me Bacteriology and Virology at Vet School

This would have been impossible without the support from my family, particularly from my wife and daughter

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CHAPTER 1

Cytolethal Distending Toxin: A Conserved Bacterial Genotoxin that Blocks Cell Cycle Progression Leading to Apoptosis of a Broad Range of Mammalian Cell Lineages[1]

Abstract

Cytolethal distending toxin (CDT) is a heterotrimeric AB type genotoxin produced by several clinically important Gram negative mucocutaneous bacterial pathogens. Irrespective of the bacterial species of origin, CDT causes characteristic and irreversible cell cycle arrest and apoptosis in a broad range of cultured mammalian cell lineages. The active subunit CdtB has structural homology with phosphodiesterase family of enzymes including mammalian deoxyribonuclease I, and alone is necessary and sufficient to account for cellular toxicity. Indeed, mammalian cells treated with CDT initiate a DNA damage response similar to that elicited by ionizing radiation-induced DNA double strand breaks resulting in cell cycle arrest and apoptosis. The mechanism of CDT-induced apoptosis remains incompletely understood, but appears to involve both p53-dependent and -independent pathways. While epithelial, endothelial and fibroblast cell lines respond to CDT by undergoing arrest of cell cycle progression resulting in nuclear and cytoplasmic distension that precedes apoptotic cell death, cells of hematopoietic origin display rapid apoptosis following a brief period of cell cycle arrest. In this review, the ecology and molecular biology of bacterial CDT and the molecular mechanisms of CDT-induced cytoxicity are critically appraised. Understanding the contribution of a broadly-conserved bacterial genotoxin that blocks progression of the cell cycle and causes cell death should assist with elucidating disease mechanisms for these important pathogens.

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Introduction

Johnson and Lior's seminal observations over two decades ago demonstrating a novel heat-labile toxin in culture filtrates obtained from certain *Escherichia coli*, *Shigella dysenteriae*, and *Campylobacter jejuni* which caused distinctive and progressive cytoplasmic and nuclear enlargement of cultured mammalian cells, so called cytolethal distending toxin (CDT) uncovered a novel paradigm amongst bacterial toxins and virulence mechanisms (Johnson & Lior, 1987; Johnson & Lior, 1988a; Johnson & Lior, 1988b). It was not until several years later that Scott and Kaper (1994) identified the genes encoding CDT in *E. coli*, which set the stage for fundamental investigations into the ecology, biochemistry, and molecular mechanisms of cellular toxicity associated with this novel bacterial toxin **(Table 1.1)**. Few years later, Peres and coworkers (1997) noted for the first time that the cellular enlargement associated with CDT intoxication was attributable to cell cycle arrest (Peres *et al.*, 1997). Soon after that, Gelfanova and coworkers (1999) demonstrated that the underlying mechanism of CDT-induced cell death involved apoptosis (Gelfanova *et al.*, 1999).

Table 1.1. Major milestones in CDT discovery time line.

Discovery	Bacteria	Reference
Biological activity	*Shigella dysenteriae*	Johnson & Lior, 1987
	Escherichia coli	Johnson & Lior, 1988a
	Campylobacter jejuni	Johnson & Lior, 1988b
Gene sequence	*Escherichia coli*	Scott & Kaper, 1994
Cell cycle arrest	*Escherichia coli*	Peres *et al.*, 1997
Apoptosis	*Haemophilus ducreyi*	Gelfanova *et al.*, 1999
Nuclease activity	*Campylobacter jejuni*	Lara-Tejero & Galan, 2000
	Escherichia coli	Elwell & Dreyfus, 2000
Crystal structure	*Haemophilus ducreyi*	Nesic *et al.*, 2004
Cell surface receptor	*Escherichia coli*	Carette *et al.*, 2009

The next milestone in CDT research discoveries came with the independent demonstration of structural conservation of amino acid residues and functional homology of CDTs from *E. coli* and *C. jejuni* to mammalian deoxyribonuclease-I (DNase-I) enzyme and correlation with DNA double strand breaks (DSBs) as the molecular basis of CDT-mediated mammalian cell cycle arrest (Elwell & Dreyfus, 2000; Lara-Tejero & Galan, 2000). With the understanding that CDT holotoxin consists of a heterotrimeric complex of three subunits and reconstitution of the CDT complex with individually expressed recombinant subunits, Lara-Tejero and Galan (2001) later proposed an AB toxin molecular model for CDT-induced cellular toxicity [as described below;(Lara-Tejero & Galan, 2001)]. This model has since been validated on the basis of high resolution crystal structure analysis of CDT from *Haemophilus ducreyi* and *Aggregatibacter (Actinobacillus) actinomycetemcomitans* (Nesic *et al.*, 2004; Yamada *et al.*, 2006). The identification of a protein receptor required for CDT binding to a myeloid leukemia cell line recently linked cell surface binding of the toxin to DNA damage, and further confirmed the AB toxin mechanistic model (Carette *et al.*, 2009).

Currently, CDT is the only member of the bacterial AB toxins that exhibits deoxyribonuclease activity, and irrespective of the bacterial species of origin, exerts genotoxic damage by causing DSB leading to irreversible cell cycle arrest and apoptosis in a broad range of mammalian cell lineages (Alouf, 2006; Dassanayake *et al.*, 2005a; Frisan *et al.*, 2003; Gelfanova *et al.*, 1999; Hickey *et al.*, 2005; Hontz *et al.*, 2006a; Hontz *et al.*, 2006b; Nesic *et al.*, 2004; Scott & Kaper, 1994; Whitehouse *et al.*, 1998; Yamada *et al.*, 2006; Young *et al.*, 2000b). The genes encoding CDT have now been found in more than two dozen bacteria belonging to the Gamma and Epsilon class of Proteobacteria. Many of these Gram negative bacteria are

clinically-important mucocutaneous pathogens of humans and animals that are responsible for major food-borne and water-borne bacterial illnesses worldwide **(Table 1.2)**.

The biological activity of CDT is highly dependent on cellular targets; however, hematopoietic cells display several orders of magnitude higher susceptibility than all other cell types suggesting a potential immunomodulatory role of CDT in the pathogenesis of diseases caused by CDT-producing bacterial pathogens. In this review, we highlight the ecology and molecular biology of bacterial CDT, summarize current structural features and biological characteristics of CDT, and critically address the molecular mechanisms of cellular toxicity. The respective roles of ATM and p53 in CDT-induced DNA damage response (DDR) leading to arrest of the cell cycle and ultimately cell death by apoptosis are reviewed in detail.

Table 1.2. Gram negative mucocutaneous bacteria harboring cytolethal distending toxin (CDT), their respective colonization niches, and associated clinical diseases in human and animal hosts.

Bacteria	CDT[a]	Host	Niche	Associated Disease	Reference
Class Gammaproteobacteria					
Family Pasteurellaceae					
***Haemophilus* species**					
H. ducreyi	HducCDT	Human	External genitalia	Chancroid	Cortes-Bratti *et al.*, 1999
H. parasuis	HparCDT	Pig	Upper respiratory mucosa	Septicemia	Yue *et al.*, 2009
***Aggregatibacter* (formerly *Actinobacillus*) species**					
A. actinomycetemcomitans	AactCDT	Human	Oral mucosa	Periodontitis	Ohguchi *et al.*, 1998
Family Enterobacteriaceae					
***Escherichia coli*[b]**					
EPEC[c]/ExPEC[d]	EcolCdtB-I	Human	Intestinal mucosa	Enterocolitis/septicemia	Asakura *et al.*, 2007a; Pickett & Whitehouse, 1999; Scott & Kaper, 1994; Toth *et al.*, 2003
			Urogenital mucosa	UTI[f]	Toth *et al.*, 2003
APEC[e]		Chicken	Intestinal mucosa	Septicemia	Johnson *et al.*, 2007
EPEC	EcolCdtB-II	Human	Intestinal mucosa	Enterocolitis	Pickett *et al.*, 1994; Pickett & Whitehouse, 1999
EPEC/ExPEC	EcolCdtB-III	Human	Intestinal mucosa	Enterocolitis	Bielaszewska *et al.*, 2004; Peres *et al.*, 1997; Toth *et al.*, 2003
NTEC[g]	EcolCdtB-III	Cattle	Intestinal mucosa	Enterocolitis/septicemia	Johnson *et al.*, 2009; Peres *et al.*, 1997; Pickett & Whitehouse, 1999

EPEC/ExPEC	EcolCdtB-IV	Human	Intestinal mucosa	Enterocolitis/septicemia	Toth *et al.*, 2003; Toth *et al.*, 2009
NTEC	EcolCdtB-IV	Human	Urogenital mucosa	UTI	Toth *et al.*, 2003
		Pig	Intestinal mucosa	Enterocolitis/septicemia	Toth *et al.*, 2003; Toth *et al.*, 2009
ExPEC		Chicken	Unknown	Septicemia	Toth *et al.*, 2003; Toth *et al.*, 2009
EHEC[h]/STEC[i]	EcolCdtB-V	Human	Intestinal mucosa	Enterocolitis/HUS[j]	Bielaszewska *et al.*, 2004
***Shigella* species**					
***S. boydii* serotype 13 (*E. albertii*)**	SboyCDT	Human	Intestinal mucosa	Dysentery	Hyma *et al.*, 2005; Johnson & Lior, 1987
S. dysenteriae	SdysCDT	Human	Intestinal mucosa	Dysentery	Johnson & Lior, 1987; Okuda *et al.*, 1997
***Salmonella* species**					
***S.enterica* serotype Typhi**	StypCdtB	Human	Intestinal mucosa	Typhoid fever	Haghjoo & Galan, 2004
Class Epsilonproteobacteria					
Family Campylobacteraceae					
***Campylobacter* species**					
C. jejuni	CjejCDT	Human	Intestinal mucosa	Enterocolitis	Fouts *et al.*, 2005; Johnson & Lior, 1988b; Young *et al.*, 2007
		NHP[k]	Intestinal mucosa	Enterocolitis	Fouts *et al.*, 2005; Johnson & Lior, 1988b; Young *et al.*, 2007
		Cattle, sheep, pig	Intestinal mucosa	Enterocolitis	Bang *et al.*, 2003; Inglis *et al.*, 2005
		Sheep	Intestinal mucosa	Reproductive loss	Sahin *et al*, 2008
		Dog, cat, ferret	Intestinal mucosa	Enterocolitis	Fox *et al.*, 1987; Young & Mansfield, 2005

		Chicken	Intestinal mucosa	Commensal	Young *et al.*, 2007
C. coli	CcolCDT	Human	Intestinal mucosa	Enterocolitis	Fouts *et al.*, 2005; Pickett *et al.*, 1996
		NHP	Intestinal mucosa	Enterocolitis	Dassanayake *et al.*, 2005b
		Cattle, pig	Intestinal mucosa	Commensal	Bang *et al.*, 2003
		Sheep, chicken	Intestinal mucosa	Commensal	Garrity, 2005
C. upsaliensis	CupsCDT	Human	Intestinal mucosa	Enterocolitis/bacteremia	Fouts *et al.*, 2005; Mooney *et al.*, 2001; Pickett *et al.*, 1996
		Pig,dog,cat,chicken	Intestinal mucosa	Commensal	Garrity, 2005
C. hyointestinalis	ChyoCDT	Human	Intestinal mucosa	Enterocolitis	Edmonds *et al.*, 1987
		Cattle	Intestinal mucosa	Commensal	Inglis *et al.*, 2005
		Pig	Intestinal mucosa	Enterocolitis	Gebhart *et al.*, 1983; Pickett *et al.*, 1996
C. lari	ClarCDT	Human	Intestinal mucosa	Enterocolitis	Fouts *et al.*, 2005; Pickett *et al.*, 1996; Shigematsu *et al.*, 2006
C. fetus* subsp. *fetus	CfetCDT	Human	Intestinal mucosa	Enterocolitis/bacteremia	Garrity, 2005; Johnson & Lior, 1988b; Pickett *et al.*, 1996
		Cattle	Intestinal mucosa	Enterocolitis	Ohya *et al.*, 1993
		Cattle, sheep	Urogenital mucosa	Reproductive loss	Garrity, 2005
C. fetus* subsp. *venerealis	CvenCDT	Human	Intestinal mucosa	Enterocolitis	Asakura *et al.*, 2008; Moolhuijzen *et al.*, 2009
		Cattle	Urogenital mucosa	Reproductive loss	Garrity, 2005; Moolhuijzen *et al.*, 2009

Family Helicobacteraceae					
Enterohepatic *Helicobacter* species					
H. hepaticus	HhepCDT	Laboratory mice	Intestinal mucosa	Enterocolitis/hepatitis	Young *et al.*, 2000b
H. bilis	HbilCDT	Laboratory mice	Intestinal/biliary mucosa	Typhlocolitis/hepatitis	Fox *et al.*, 2004b; Fox, 2007
		Dog	Intestinal mucosa	Commensal	Hanninen *et al.*, 2005; Kostia *et al.*, 2003
H. mastomyrinus	HmasCDT	Laboratory mice	Intestinal mucosa	Proctitis	Shen *et al.*, 2005
		Mastomys	Liver	Hepatitis	Shen *et al.*, 2005
H. cinaedi	HcinCDT	Human	Intestinal mucosa	Septicemia	Taylor *et al.*, 2003
		NHP	Intestinal mucosa/liver	Colitis/hepatitis	Fernandez *et al.*, 2002; Fox *et al.*, 2001
		Laboratory mice	Intestinal mucosa	Typhlocolitis	Shen *et al.*, 2009
H. canis	HcanCDT	Human	Intestinal mucosa	Bacteremia	Leemann *et al.*, 2006
		Dog	Intestinal mucosa/liver	Enterocolitis/hepatitis	Fox *et al.*, 1996
H. pullorum	HpulCDT	Human	Intestinal mucosa	Enteritis	Ceelen *et al.*, 2006; Young *et al.*, 2000a
		Chicken	Intestinal mucosa	Enteritis/hepatitis	Ceelen *et al.*, 2006; Young *et al.*, 2000a
		Laboratory mice	Intestinal mucosa	Commensal	Boutin *et al.*, 2010
H. winghamensis	HwinCDT	Human	Intestinal mucosa	Enteritis	Melito *et al.*, 2001

[a]CDT designation according to a modification of the nomenclature proposed by Cortes-Bratti, 2001 (Cortes-Bratti *et al.*, 2001a); only the genes encoding CDT have been described in *H. parasuis* and *C. fetus* subsp. *Venerealis*; the biological activity of CDT has not been determined in these bacteria.

[b]Variants of CdtB identified amongst pathotypes of *E. coli* are designated as EcolCdtB-I to -V

[c]EPEC, enteropathogenic *E. coli*; [d]ExPEC, extraintestinal pathogenic *E. coli*; [e]APEC, avian pathogenic *E. coli*; [f]UTI, urinary tract infection;

[g]NTEC, necrotoxigenic *E. coli*; [h]EHEC, enterohemorrhagic *E. coli*; [i]STEC, Shiga toxin-producing *E. coli*; [j]HUS, hemolytic uremic syndrome; [k]NHP, non-human primates

CDT genes and encoded proteins

Distribution of CDT genes amongst bacteria

Currently, bacteria that harbor CDT gene and display biological activity are restricted to the Gamma and Epsilon classes in the Proteobacteria phylum **(Table 1.2).** Within the Gammaproteobacteria one subset belongs to the family Pasteurellaceae and includes *H. ducreyi,* the cause of a sexually transmitted disease known as chancroid (Abeck *et al.*, 1997), *H. parasuis,* a commensal organism of the upper respiratory tract associated with septicemia in pigs (Yue *et al.*, 2009), and *A. actinomycetemcomitans,* a common contributing agent to aggressive periodontitis (Henderson *et al.*, 2002). A second subset of Gammaproteobacteria belongs to the Enterobacteriaceae family, and collectively, these organisms are responsible for intestinal and urinary tract infections that can lead to systemic spread. All members of the Epsilonproteobacteria belong to the order Campylobacterales and include several species of *Campylobacter* and enterohepatic *Helicobacter* species (EHS) which are primarily associated with enterocolitis; however, some species cause bacteremia/septicemia, hepatitis, and reproductive losses in humans and animals (Dassanayake *et al.*, 2005a; Dassanayake *et al.*, 2005b; Ge *et al.*, 2008; Johnson & Lior, 1987; Liyanage *et al.*, 2010). It is noteworthy that in addition to mammalian bacterial pathogens, a monophyletic group of bacteriophage-encoded CdtB orthologs has been found amongst facultative endosymbionts of sap-feeding insects (Degnan & Moran, 2008). Thus, the ecology of CDT-harboring bacteria is restricted to certain Gram negative bacteria that primarily occupy mucocutaneous niches where persistent colonization can occur as either a commensal or result in localized or disseminated infections and diseases in a broad range of mammalian hosts.

Nomenclature of CDT proteins

To simplify the designation of CDT produced by various bacteria, Cortes-Bratti and co-workers proposed a nomenclature system which has been widely adopted by the scientific community (Cortes-Bratti *et al.*, 2001a). Since then, the list of bacteria that harbor CDT has expanded significantly **(Table 1.2)**. Consequently, the original system consisting of the capitalized first letter of the genus followed by a single lower case species letter initial is no longer adequate, and some bacterial species now have overlapping designations; for example, Hp for *Haemophilus parasuis* and *Helicobacter pullorum* or Hc for *Helicobacter cinaedi* and *Helicobacter canis*. Therefore, we propose a modification of the previous nomenclature system in which the first three letters of the bacterial species in lowercases are placed after the capitalized first letter of the bacterial genus followed by CDT **(Table 1.2)**.

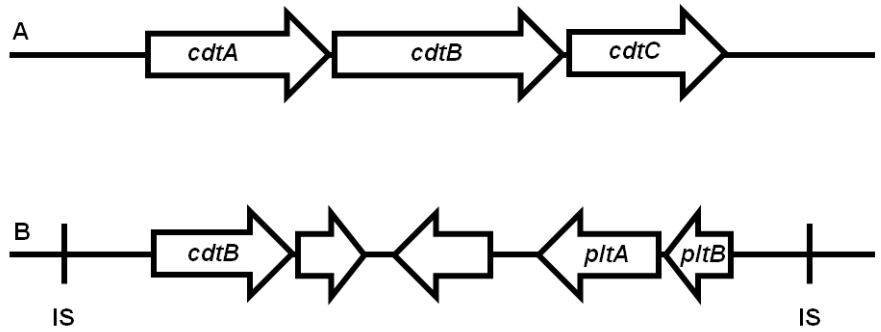
CDT operon

The CDT holotoxin consists of a heterotrimeric complex of three subunits designated CdtA, CdtB and CdtC with corresponding approximate molecular masses of 23 to 30, 28 to 29 and 19 to 21 kDa according to bacterial host species (Haghjoo & Galan, 2004; Hu & Stebbins, 2006). In nearly all bacteria, CdtA, CdtB and CdtC subunits are encoded by adjacent or slightly overlapping *cdtA, cdtB* and *cdtC* genes which together form a constitutively expressed operon on the chromosome (**Fig.1.1**). *Escherichia coli* is unique amongst CDT-producing bacteria in that at least five divergent variants of CdtB are found within this single species which also differentially segregate according to *E. coli* pathotypes [**Table 1.2**; (Janka *et al.*, 2003)]. Since these variants refer to the CdtB subunit, and to be consistent with our proposed nomenclature, they should be designated EcolCdtB-I through -V. Similar to other CDT-producing bacteria, EcolCdtB-I, -II, -IV and -V are encoded by chromosomal gene loci (Asakura *et al.*, 2007a; Johnson *et al.*, 2007; Oswald *et al.*, 1994;

Peres *et al.*, 1997; Toth *et al.*, 2009). However, analyses of sequences upstream and downstream of operons encoding CdtB-I and -IV variants revealed lambdoid prophage genes (Asakura *et al.*, 2007a; Toth *et al.*, 2009), whereas homologues of bacteriophages P2 and lambda are found in strain O157:H^- 493/89 harboring CdtB-V (Janka *et al.*, 2003). These gene arrangements are reminiscent of bacteriophage-encoded CdtB orthologs found amongst facultative endosymbionts of sap-feeding insects in which acquisition of *cdtB* has been attributed to horizontal gene transfer [HGT;(Degnan & Moran, 2008)]. Indeed, carriage of the CDT operon by certain enteropathogenic *E. coli* with EcolCdtB-I on a lysogenic phage background mediates HGT among related species, and confers enhanced bacterial toxicity during phage induction (Asakura *et al.*, 2007a; Johnson *et al.*, 2007; Oswald *et al.*, 1994; Peres *et al.*, 1997; Toth *et al.*, 2009). Also unique to *E. coli*, is the location of the operon encoding EcolCdtB-III which is found on a large conjugative plasmid called pVir (Johnson *et al.*, 2009). Finally, a notable exception to the heterotrimeric CDT model is *Salmonella enterica* serotype Typhi in which the genes encoding CdtA and CdtC are missing (Haghjoo & Galan, 2004). In keeping with our proposed CDT nomenclature, and since only the CdtB subunit is present in *S.* Typhi, the toxin should be designated StypCdtB. The *cdtB* gene of *S.* Typhi is located in a region of the chromosome with features consistent with a pathogenicity islet acquired by HGT and delineated by insertion sequences and a transposase gene (Haghjoo & Galan, 2004). Although the significance of these various CDT gene arrangements are incompletely understood, it is likely that they represent mechanisms of HGT which together might play a role in expanding the range of pathogenic bacteria that can establish persistent colonization and potentially cause infection and disease.

Figure 1.1. Organization of the CDT gene locus. In nearly all CDT-producing bacteria, the CDT gene cluster consists of three adjacent or slightly overlapping *cdtA*, *cdtB* and *cdtC* genes encoding the corresponding CdtA, CdtB and CdtC protein subunits is located on the chromosome (A), except for EcolCdtB-III in which the CDT gene cluster is found on a large conjugative plasmid called pVir. In *Salmonella enterica* serotype Typhi, the genes encoding CdtA and CdtC are missing (B), instead the StypCdtB protein subunit is encoded by *cdtB* gene located upstream of *pltA* and *pltB* genes which respectively encode *p*ertussis-*l*ike *t*oxin A (PltA) and PltB, homologs of the pertussis toxin ADP-ribosylating "A" subunit and one of the 5 components of its heteropentameric "B" subunit, respectively, which together are located on a pathogenicity islet delineated by insertion sequences (IS).

Figure 1.1

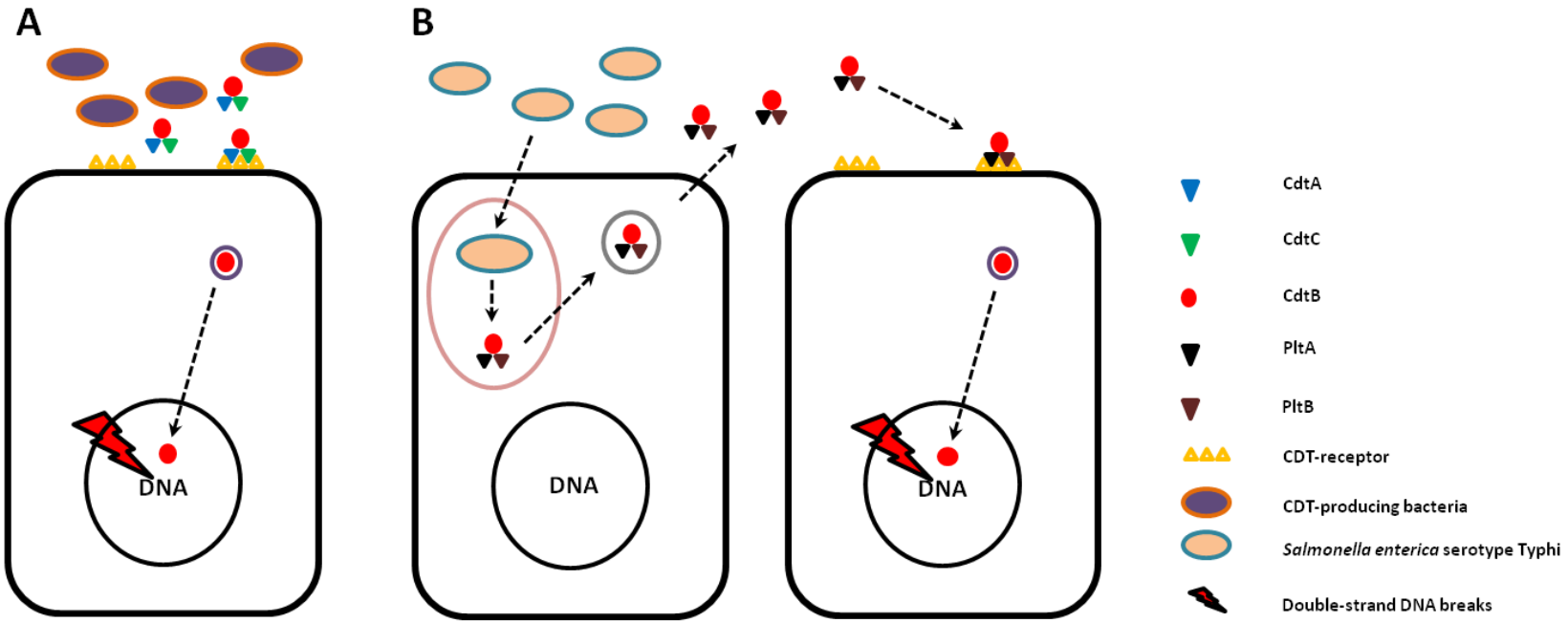


CDT protein structure and function

On the basis of structural and functional characteristics of reconstituted recombinant heterotrimeric CDT subunits, Lara-Tejero and Galan (2001) proposed an AB toxin molecular model for CDT-induced cellular toxicity (Lara-Tejero & Galan, 2001). Accordingly, the catalytically active "A" subunit is attributable to the CdtB subunit, whereas the CdtA and CdtC subunits, which together display a high degree of sequence diversity, are assembled as a heterodimeric binding "B" subunit mediating target host cell membrane specificity and cellular uptake of CdtB effector protein (Hu *et al.*, 2006; Hu & Stebbins, 2006; Nesic *et al.*, 2004). In *S.* Typhi, the *cdtB* gene which encodes the "A" subunit StypCdtB is located on a pathogenicity islet upstream of *pltA* and *pltB* genes, respectively encoding *p*ertussis-*l*ike *t*oxin A (PltA) and PltB which are homologs of the pertussis toxin ADP-ribosylating "A" subunit and one of the 5 components of its heteropentameric "B" subunit, respectively [**Fig. 1.2B**; (Spano *et al.*, 2008)]. The PltA and PltB form a heterotrimeric complex with StypCdtB, and together mediate the binding specificity or "B" subunit function required for cellular toxicity (Spano *et al.*, 2008).

Figure 1.2. Interaction of CDT with mammalian cells. In nearly all CDT-producing bacteria, binding of CdtA and CdtC protein subunits of functional holotoxin complex or "B" subunit to host cell membrane receptor is followed by internalization of CdtB protein subunit or active "A" subunit by clathrin coated pit-mediated endocytosis, nuclear translocation and host cell DNA damage (A). In *Salmonella enterica* serotype Typhi, intracellular uptake of bacteria into a *Salmonella*-containing vacuole is followed by concurrent expression of StypCdtB, PltA and PltB protein subunits, secretion and assembly into functional holotoxin complexes that are packaged into transport vesicles destined for extracellular secretion (B). Binding of PltA and PltB protein subunits or "B" subunit of functional holotoxin complex to the infected host cell (autocrine) and to uninfected neighboring (paracrine) host cell membrane receptors is followed by internalization of StypCdtB protein subunit or active "A" subunit, nuclear translocation and DNA damage.

Figure 1.2.

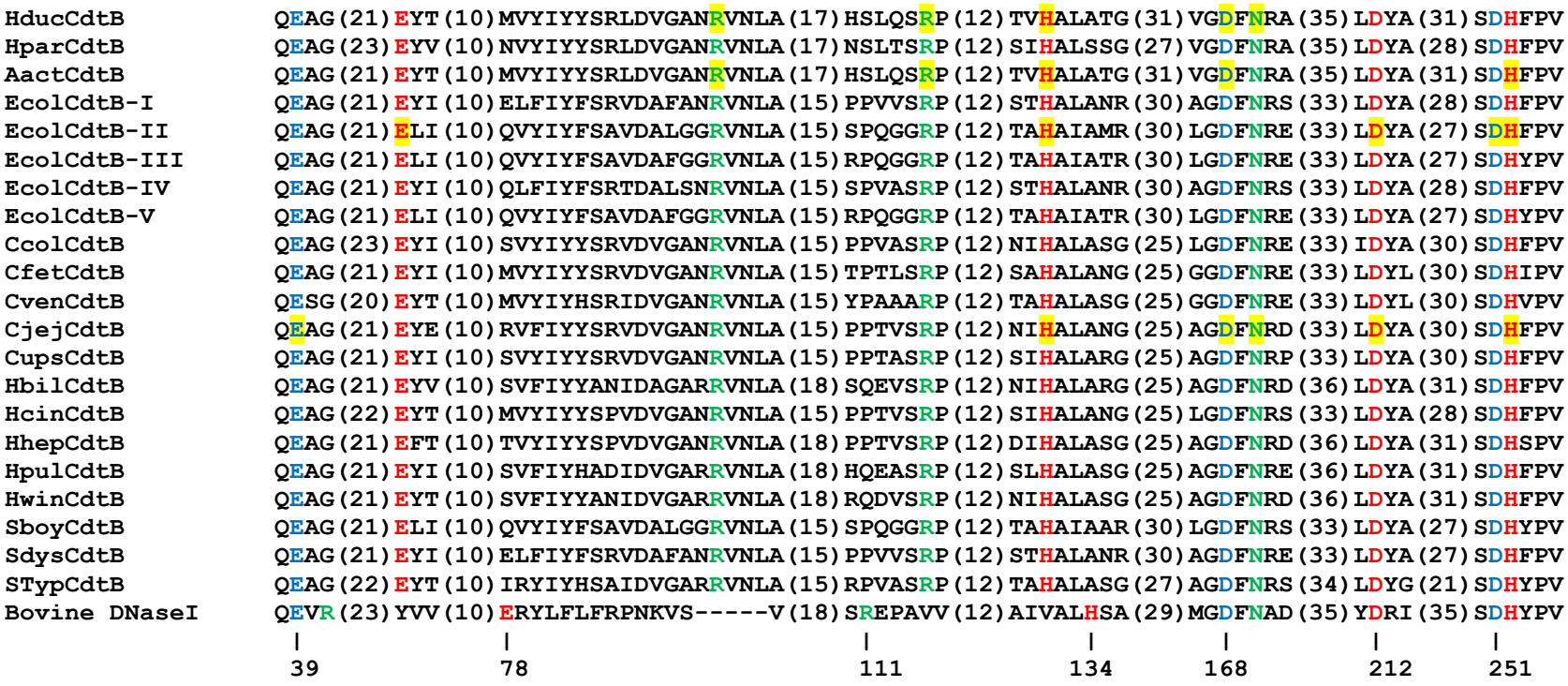

A
B
DNA
DNA
DNA
CdtA
CdtC
CdtB
PltA
PltB
CDT-receptor
CDT-producing bacteria
Salmonella enterica serotype Typhi
Double-strand DNA breaks

Consistent with the notion that CdtB is the active subunit, comparative *in silico* structural analysis of predicted *cdtB*-encoded amino acid sequences of human and animal bacterial pathogens revealed conserved endonuclease/exonuclease/phosphatase super family domain with approximately 25-40% sequence identity with phosphodiesterase enzymes including mammalian DNase-I (Elwell & Dreyfus, 2000; Lara-Tejero & Galan, 2000). Despite the limited overall sequence homology with DNase-I, *cdtB*-encoded catalytic, DNA- and divalent cation-binding residues are highly conserved among the various CDT-producing bacterial pathogens suggesting a critical role for this nuclease in host-pathogen interaction [**Fig. 1.3**; (Elwell & Dreyfus, 2000; Hu & Stebbins, 2006; Lara-Tejero & Galan, 2000; Nesic *et al.*, 2004)].

The catalytic residues corresponding to mammalian DNase-I-H134 (HducCdtB-H160), DNase-I-H252 (HducCdtB-H274) and DNase-I-D212 (HducCdtB-D238) are conserved among all bacterial CdtB (Nesic *et al.*, 2004). Moreover, mutagenesis analysis of EcolCdtB-II-E86 residue confirms the high degree of conservation of the corresponding DNase-I-E78 catalytic residue amongst all CdtB (Elwell & Dreyfus, 2000). Conversely, those residues corresponding to divalent cation binding DNase-I-E39 (CjejCdtB-E60), DNase-I-D168 (CjejCdtB-D185) and DNase-I-D251 (CjejCdtB-D260) as well as those corresponding to critical DNA binding DNase-I-R111 (HducCdtB-R144) and DNase-I-N170 (HducCdtB-N201) are highly conserved among all bacterial CdtB (Nesic *et al.*, 2004; Pickett & Lee, 2005). One additional DNA binding DNase-I-R117 (HducCdtB-R41) residue which is present in all CdtB also has been confirmed by mutational analysis in both AactCdtB and HducCdtB (Nesic *et al.*, 2004; Shenker *et al.*, 2007). These differences in DNA binding residue conservation between bacterial CdtB and mammalian DNase-I likely account for reduced *in vitro* nuclease activities found with AactCdtB, EcolCdtB-II, and HhepCdtB (Dassanayake *et al.*, 2005a; Elwell & Dreyfus, 2000; Mao & DiRienzo, 2002).

Figure 1.3. Comparative amino acid sequence of bacterial CdtB and mammalian DNase-I. Sequences alignment was done using ClustalW2. Numbers at the bottom correspond to amino acid positions of bovine or human DNAse-I. Residues required for enzymatic activity [catalytic (red), divalent cation binding (blue) and DNA binding (green)] in human DNase-I are indicated in bold. The residues predicted to be required for CdtB nuclease activity and which have been confirmed by site directed mutagenesis are highlighted in yellow. The numbers in parenthesis represent the number of intervening amino acid residues that are omitted (excluding gaps).

Figure 1.3.


HducCdtB QEAG(21)EYT(10)MVYIYYSRLDVGANRVNLA(17)HSLQSRP(12)TVHALATG(31)VGDFNRA(35)LDYA(31)SDHFPV
HparCdtB QEAG(23)EYV(10)NVYIYYSRLDVGANRVNLA(17)NSLTSRP(12)SIHALSSG(27)VGDFNRA(35)LDYA(28)SDHFPV
AactCdtB QEAG(21)EYT(10)MVYIYYSRLDVGANRVNLA(17)HSLQSRP(12)TVHALATG(31)VGDFNRA(35)LDYA(31)SDHFPV
EcolCdtB-I QEAG(21)EYI(10)ELFIYFSRVDAFANRVNLA(15)PPVVSRP(12)STHALANR(30)AGDFNRS(33)LDYA(28)SDHFPV
EcolCdtB-II QEAG(21)ELI(10)QVYIYFSAVDALGGRVNLA(15)SPQGGRP(12)TAHAIAMR(30)LGDFNRE(33)LDYA(27)SDHFPV
EcolCdtB-III QEAG(21)ELI(10)QVYIYFSAVDAFGGRVNLA(15)RPQGGRP(12)TAHAIATR(30)LGDFNRE(33)LDYA(27)SDHYPV
EcolCdtB-IV QEAG(21)EYI(10)QLFIYFSRTDALSNRVNLA(15)SPVASRP(12)STHALANR(30)AGDFNRS(33)LDYA(28)SDHFPV
EcolCdtB-V QEAG(21)ELI(10)QVYIYFSAVDAFGGRVNLA(15)RPQGGRP(12)TAHAIATR(30)LGDFNRE(33)LDYA(27)SDHYPV
CcolCdtB QEAG(23)EYI(10)SVYIYYSRVDVGANRVNLA(15)PPVASRP(12)NIHALASG(25)LGDFNRE(33)IDYA(30)SDHFPV
CfetCdtB QEAG(21)EYI(10)MVYIYYSRVDVGANRVNLA(15)TPTLSRP(12)SAHALANG(25)GGDFNRE(33)LDYL(30)SDHIPV
CvenCdtB QESG(20)EYT(10)MVYIYHSRIDVGANRVNLA(15)YPAAARP(12)TAHALASG(25)GGDFNRE(33)LDYL(30)SDHVPV
CjejCdtB QEAG(21)EYE(10)RVFIYYSRVDVGANRVNLA(15)PPTVSRP(12)NIHALANG(25)AGDFNRD(33)LDYA(30)SDHFPV
CupsCdtB QEAG(21)EYI(10)SVYIYYSRVDVGANRVNLA(15)PPTASRP(12)SIHALARG(25)AGDFNRP(33)LDYA(30)SDHFPV
HbilCdtB QEAG(21)EYV(10)SVFIYYANIDAGARRVNLA(18)SQEVSRP(12)NIHALARG(25)AGDFNRD(36)LDYA(31)SDHFPV
HcinCdtB QEAG(22)EYT(10)MVYIYYSPVDVGANRVNLA(15)PPTVSRP(12)SIHALANG(25)LGDFNRS(33)LDYA(28)SDHFPV
HhepCdtB QEAG(21)EFT(10)TVYIYYSPVDVGANRVNLA(18)PPTVSRP(12)DIHALASG(25)AGDFNRD(36)LDYA(31)SDHSPV
HpulCdtB QEAG(21)EYI(10)SVFIYHADIDVGARRVNLA(18)HQEASRP(12)SLHALASG(25)AGDFNRE(36)LDYA(31)SDHFPV
HwinCdtB QEAG(21)EYT(10)SVFIYYANIDVGARRVNLA(18)RQDVSRP(12)NIHALASG(25)AGDFNRD(36)LDYA(31)SDHFPV
SboyCdtB QEAG(21)ELI(10)QVYIYFSAVDALGGRVNLA(15)SPQGGRP(12)TAHAIAAR(30)LGDFNRS(33)LDYA(27)SDHYPV
SdysCdtB QEAG(21)EYI(10)ELFIYFSRVDAFANRVNLA(15)PPVVSRP(12)STHALANR(30)AGDFNRE(33)LDYA(27)SDHFPV
STypCdtB QEAG(22)EYT(10)IRYIYHSAIDVGARRVNLA(15)RPVASRP(12)TAHALASG(27)AGDFNRS(34)LDYG(21)SDHYPV
Bovine DNaseI QEVR(23)YVV(10)ERYLFLFRPNKVS-----V(18)SREPAVV(12)AIVALHSA(29)MGDFNAD(35)YDRI(35)SDHYPV
39
78
111
134
168
212
251

More recently, high resolution crystallographic analysis of reconstituted heterotrimeric HducCDT holotoxin from individually expressed recombinant subunits [2.0Å resolution; (Nesic *et al.*, 2004)] or complete AactCDT operon expressed as a holotoxin in *E. coli* [2.4 Å resolution; (Yamada *et al.*, 2006)] and a EcolCdtB-II subunit [(1.73Å resolution; (Hontz *et al.*, 2006b)] have provided further support to earlier observations and confirmed that CdtB closely resembles mammalian DNAse-I. Comparative protein modeling confirmed the high degree of key structural features conservation, namely canonical four-layered fold of mammalian DNAse-I family, deeply grooved aromatic patch and active site of CdtB from diverse bacterial species including CjejCDT, HhepCDT, EcolCDT-I, EcolCDT-II, EcolCDT-III, AactCDT and HducCDT (Hu *et al.*, 2006). While the heterotrimeric holotoxin forms a ternary complex between CdtB subunit and ricin-like lectin domains within CdtA and CdtB (Nesic *et al.*, 2004), the lectin domains form a deeply grooved, highly aromatic surface (aromatic patch) which is critical for cellular toxicity, presumably through binding to target cell surface biomolecules (Nesic *et al.*, 2004). Interaction between the N-terminal 13 amino acids of CdtC with the active nuclease site of CdtB suggests an auto-inhibitory function. Accordingly, the CdtB nuclease would be active only after release from CdtC and target cell entry.

Although the residues required for nuclease activity of CdtB are highly conserved, the overall amino acid sequence of CdtB varies among bacteria. While StypCdtB together with HducCdtB and AactCdtB form a distinct cluster, the CdtB produced by EHS and *Campylobacter* species are nearly similar forming a separate cluster which is distinct from CdtB produced by members of the *E.coli/Shigella* group (Degnan & Moran, 2008). Interestingly, a similar clustering pattern is seen when the amino acid sequences of CdtA and CdtC are compared between different bacterial pathogens (Eshraghi *et al.*, 2010). The clustering pattern of CDT subunit amino acid sequences

suggests differential infection niches that may correlate with bacterial adaptation to persistent colonization in specific hosts. Alternatively, the possibility that these structural differences indicate variable bacterial target cell binding specificities and intracellular nuclease subunit signaling pathways remain to be determined.

Production of CDT

Wide variations in biological activity of CDT are found among various CDT-producing bacteria. Moreover, biological activity is variously found either in culture supernatant (and thus presumably actively secreted), associated with bacterial cells or both **(Table 1.3)**. Moreover, the activity of CDT recovered from these different fractions can range from relatively high, intermediate, low to absent, and this may be relevant to disease pathogenesis for individual bacterial pathogen. Similar to other virulence factors, alterations in CDT production might be attributable to high numbers of passages on artificial media and selection of strains that are less virulent.

Table 1.3. Production of CDT by bacterial pathogens.

CDT	Bacterial location†		Reference
	Secreted	**Cell-associated**	
HducCDT	Present	Present	Ahmed *et al.*, 2001; Cope *et al.*, 1997; Gelfanova *et al.*, 1999
AactCDT	Present	Present	Ahmed *et al.*, 2001; Fabris *et al.*, 2002; Ohguchi *et al.*, 1998; Sugai *et al.*, 1998; Yamano *et al.*, 2003
EcolCdtB-I	High	High	Toth *et al.*, 2003
EcolCdtB-II	Present	Present	Pickett *et al.*, 1994
EcolCdtB-III	Present	Present	Toth *et al.*, 2003
EcolCdtB-IV	Absent	Present	Toth *et al.*, 2003
EcolCdtB-V	Present	ND	Bielaszewska *et al.*, 2005; Janka *et al.*, 2003

SboyCDT	Present	Present	Hyma *et al.*, 2005; Johnson & Lior, 1987
SdysCDT	Present	ND	Johnson & Lior, 1987
CjejCDT	High	High	Dassanayake *et al.*, 2005b; Pickett *et al.*, 1996
CcolCDT	Absent	Absent	Asakura *et al.*, 2007b; Dassanayake *et al.*, 2005b; Pickett *et al.*, 1996
CupsCDT	Present	High	Mooney *et al.*, 2001; Pickett *et al.*, 1996
ChyoCDT	ND	High	Pickett *et al.*, 1996
ClarCDT	ND	Low	Pickett *et al.*, 1996
CfetCDT	Present	High	Asakura *et al.*, 2007b; Ohya *et al.*, 1993; Pickett *et al.*, 1996
EHS	ND	Present	Chien *et al.*, 2000; Hanninen *et al.*, 2005; Kostia *et al.*, 2003; Shen *et al.*, 2005; Taylor *et al.*, 2003

†Secreted, CDT activity in unconcentrated culture supernatant; cell-associated, CDT activity in bacterial lysate or enriched outer membrane preparation; low, high, relative to secreted or cell-associated; ND, not determined; Strains of *H. ducreyi, A. actinomycetemcomitans, E. coli, Shigella* species, and enterohepatic *Helicobacter* species (EHS) that lack a full complement of CDT genes and/or activity have been described (Abuoun *et al.*, 2005; Ahmed *et al.*, 2001; Asakura *et al.*, 2007b; Dassanayake *et al.*, 2005b; Fabris *et al.*, 2002; Hyma *et al.*, 2005; Janka *et al.*, 2003; Johnson & Lior, 1987; Solnick & Schauer, 2001; Yamano *et al.*, 2003).

The early studies of Johnson and Lior (1988) assessed CjejCDT in culture supernatant; however, assessment of cell-associated CDT among clinical *C. jejuni* isolates revealed relatively high biological activities in most strains with few strains exhibiting comparatively lower levels of activity (Pickett *et al.*, 1996). Strains of *C. jejuni* that harbor CDT genes but lack biological activity have also been found (Abuoun *et al.*, 2005; Dassanayake *et al.*, 2005b). Two types of mutations within the CDT operon account for a lack of CDT activity in these strains; a deletion across *cdtA* and *cdtB*, and a nonsynonymous mutation in *cdtB* gene sequence (Abuoun *et al.*, 2005). In the closely related species *C. coli*, the genes encoding each of the three subunits are present, and although this species lacks biological activity, the underlying mechanism has not been identified (Asakura *et al.*, 2007b; Dassanayake *et al.*, 2005b; Pickett *et al.*, 1996). Clinical isolates which lack CDT genes and/or activity have also been found amongst *H. ducreyi, A. actinomycetemcomitans, E. coli, Shigella* species, and EHS (Abuoun *et al.*, 2005; Ahmed *et al.*, 2001; Asakura *et al.*, 2007b; Dassanayake *et al.*, 2005b; Fabris *et al.*, 2002; Hyma *et al.*, 2005; Janka *et al.*, 2003; Johnson & Lior, 1987; Solnick & Schauer, 2001; Yamano *et al.*, 2003).

Information concerning the molecular mechanism of bacterial synthesis and secretion of CDT is limited (Deng *et al.*, 2001; Ueno *et al.*, 2006). A difference of approximate 2 kDa between the observed and calculated molecular weights of EcolCdtB-II subunit attributable to cleavage of a putative N-terminal signal peptide sequence was suggested to facilitate the secretion across the inner membrane by a general export pathway (Dreyfus, 2003). Consistent with this observation, Ueno and coworkers demonstrated the presence of a lipid binding consensus motif (lipobox) and lipoprotein cleavage site in AactCdtA signal peptide (Ueno *et al.*, 2006). In addition to AactCdtA, putative lipobox motifs are also present in HducCdtA, CjejCdtA, HhepCdtA, EcolCdtA- I, -II and -III, EcoliCdtC-I, -II and -III (Ueno *et al.*, 2006);

unpublished observations). While the periplasmic CDT holotoxin consists of the un-cleaved AactCdtA, AactCdtB, and AactCdtC subunits, the secreted holotoxin complex in culture supernatant have cleaved signal sequences (Ueno *et al.*, 2006).

Cell-associated CjejCDT is found primarily in the periplasmic space of *C. jejuni*, whereas culture supernatant contains primarily CjejCDT tightly associated with outer membrane vesicles (Lindmark *et al.*, 2009). In the context of intestinal infection, packaging and release of CDT into outer membrane vesicles may serve a protective function against enzymatic digestion, thus allowing uptake of intact protein toxin by host absorptive enterocytes. Further studies on the mechanism of CDT production and release by bacterial pathogens should provide important insights for the design of therapeutic modalities aimed at inhibiting CDT production or neutralizing CDT activity during infection.

Molecular mechanisms of cellular toxicity

Cellular entry of CdtB

A causal relationship between the requirement for heterotrimeric CdtABC holotoxin and cellular toxicity is well established for AactCDT, CjejCDT, EcolCdtB-I and –II and HducCDT (Akifusa *et al.*, 2001; Lara-Tejero & Galan, 2001; Pickett *et al.*, 1994; Pickett *et al.*, 1996; Purven *et al.*, 1997; Scott & Kaper, 1994; Shenker *et al.*, 2005). In these pathogens, CdtA and CdtC subunits bind host cell membrane lipid raft microdomains before internalization of CdtB (Boesze-Battaglia *et al.*, 2006; Boesze-Battaglia *et al.*, 2009; Carette *et al.*, 2009; Cortes-Bratti *et al.*, 2000; Lara-Tejero & Galan, 2001; Nesic *et al.*, 2004; Shenker *et al.*, 2005). The integrity of lipid raft microdomains is critical for binding of AactCDT onto Jurkat cell membrane, presumably involving binding of host cell membrane cholesterol by a putative AactCdtC subunit cholesterol recognition/interaction amino acid consensus (CRAC) domain (Boesze-Battaglia *et al.*, 2009). As predicted, pretreatment of cells with CdtA-CdtC complex protects against intoxication by CdtABC holotoxin, but not by CdtB alone, again demonstrating that the internalization of CdtB is critical for toxicity (Deng & Hansen, 2003).

By using target cell lines with various surface membrane biomolecules, a cell surface polypeptide component rather than glycoconjugates identified in previous studies, appears as the most likely receptor responsible for CDT-host cell interaction (Eshraghi *et al.*, 2010). Recently, a putative G protein-coupled transmembrane protein, designated TMEM181, which localizes to membrane lipid rafts has been proposed as the cell membrane receptor for EcolCdtB-I by using a novel loss-of-function haploid genetic screen in a highly sensitive myeloid leukemia cell line (Carette *et al.*, 2009). However, the possibilities that TMEM181 is part of a complex that

constitutes a functional receptor, or that it plays a role in trafficking of a receptor-CdtB complex, or represents a ligand unique to EcoliCdtB-I cannot be ruled out completely. The demonstration that the expression level of TMEM181 is rate limiting for intoxication of cell lines with various degrees of sensitivity to CDT provides a molecular basis that explains the differential susceptibility of cells from different lineages to intoxication.

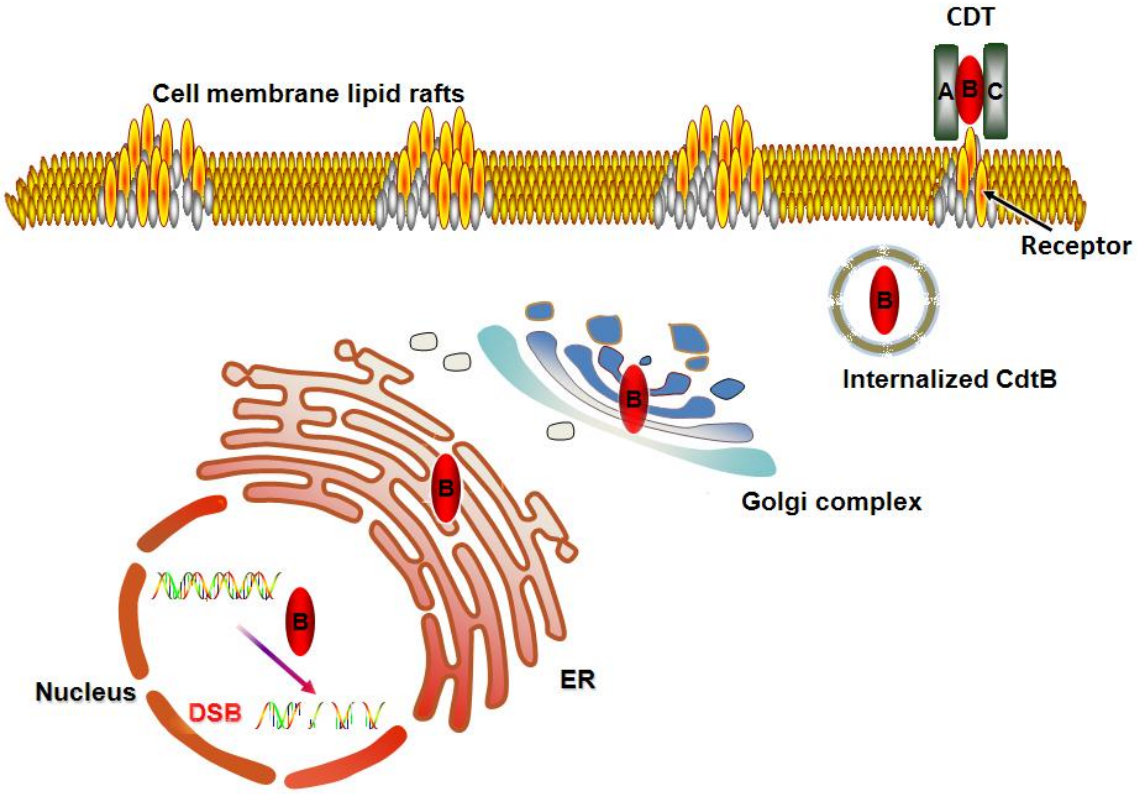
Following receptor-mediated internalization in clathrin-coated pits, CdtB undergoes retrograde transport to the endoplasmic reticulum (ER) via the Golgi complex [**Fig. 1.4**; (Cortes-Bratti *et al.*, 2000; Guerra *et al.*, 2005)]. The lack of protease inactivation or serum neutralization of HducCdtB and HducCdtC within minutes of cell surface binding suggests conformational changes of cell surface-bound toxin are followed by rapid internalization. Consistent with this observation, irreversible inhibition of Chinese hamster ovary (CHO) cell proliferation occurs within 2 minutes of exposure to EcoliCdtB-II (Aragon *et al.*, 1997). Pharmacological, chemical or genetic disruption of clathrin-mediated endocytic pathways, as well as- Golgi complex transport abolish CDT cytotoxicity (Cortes-Bratti *et al.*, 2000). Once taken up by mammalian cells, the catalytic subunit CdtB localizes to the nucleus presumably via alternate nuclear localization signals [NLS; (Lara-Tejero & Galan, 2000; McSweeney & Dreyfus, 2004; Nishikubo *et al.*, 2003)]. An N-terminal NLS has been proposed for AactCdtB, while two C-terminal NLS have been found in EcolCdtB-II (McSweeney & Dreyfus, 2004; Nishikubo *et al.*, 2003). On the basis of highly conserved N-terminal amino acid sequences corresponding to the putative NLS of AactCdtB among all known bacterial CdtB orthologs, a modular structure consisting of an N-terminal domain responsible for nuclear transport and a C-terminal DNase-like domain capable of exerting DSBs has been proposed (McSweeney & Dreyfus, 2004; Nishikubo *et al.*, 2003). Unlike the “A” subunit of other AB toxins which

generally translocates from the endoplasmic reticulum (ER) directly into the cell cytosol by a process of ER-associated degradation (ERAD), translocation of HducCdtB is ERAD-independent with the toxin subunit moving directly from the ER lumen to the nucleoplasm without unfolding (Guerra *et al.*, 2009).

In *S.* Typhi, bacterial internalization into *Salmonella*-containing vacuole of host cells leads to concurrent expression of StypCdtB, PltA and PltB which assemble into functional holotoxin complexes that are packaged into transport vesicles destined for extracellular secretion and intoxication of infected host cells (autocrine) and uninfected neighboring (paracrine) host cells (Spano *et al.*, 2008). The absence of CDT receptor on infected host cells might provide a protective mechanism against cytotoxicity which would allow intracellular survival of *S.* Typhi, and thus persistence in infected host tissues (Spano *et al.*, 2008).

Figure 1.4. CDT uptake and intracellular transport in mammalian cell. Binding of the "B" subunit consisting of CdtA and CdtC protein subunits of CDT heterotrimeric holotoxin to a cell membrane receptor located within lipid rafts is followed by rapid endocytosis of the "A" active CdtB protein subunit in a clathrin-coated pit and retrograde transport via the Golgi complex and endoplasmic reticulum (ER) before nuclear localization signal-mediated active transport to the nucleus and DNA double strand breaks (DSB).

Figure 1.4


CDT
A B C
Cell membrane lipid rafts
Receptor
B
Internalized CdtB
B
Golgi complex
B
B
Nucleus
DSB
ER

Nuclease and phosphatase activities of CdtB

The structural features of CdtB that predict a nuclease function are in agreement with DSBs observed in mammalian cells intoxicated with either CjejCDT, EcolCdtB-II or HducCDT (Dassanayake *et al.*, 2005a; Elwell & Dreyfus, 2000; Frisan *et al.*, 2003; Lara-Tejero & Galan, 2000). Transient expression, cellular transfection, or nuclear microinjection of CdtB cause fragmentation of mammalian cell nuclear DNA (Frisan *et al.*, 2003; Lara-Tejero & Galan, 2000). Similarly, rescue of a mouse B cell line from AactCDT-induced cytotoxic death with the DNA endonuclease inhibitor aurintricarboxylic acid further confirms the endonuclease-mediated DNA damage requirement for CdtB-induced cellular toxicity (Ohguchi *et al.*, 1998).

While it is well-established that cellular toxicity is mediated by the nuclease function of CdtB, other studies suggest phosphatidylinositol (PI)-3,4,5-triphosphate (PIP_3) phosphatase activity also might play a role in cellular toxicity (Shenker *et al.*, 2007). This is based in part on *in silico* comparative analysis demonstrating that several phosphatases involved in cell cycle regulation and signal transduction including inositol phosphatases contain a protein fold similar to $Mg2^{+}$-dependent endonucleases including mammalian DNase-I and bacterial CDT (Dlakic, 2000). Demonstration of PIP_3 phosphatase activity is more clearly seen when using human leukemia T-cell lines with constitutively elevated PIP_3 levels because of mutations in SHIP1 and/or PTEN (Shenker *et al.*, 2007). Therefore, an alternate mechanism of cellular toxicity might result from depletion of cell membrane PIP_3 and suppression of protein kinase B (PKB)/Akt signaling pathway (Shenker *et al.*, 2007). However, when compared with site specific phosphatase defective CdtB mutants or specific PIP_3 phosphatase inhibitors, the nuclease activity of AactCdtB alone is sufficient for induction of cell cycle arrest and apoptosis of proliferating U937 macrophage cell line (Rabin *et al.*, 2009). Similarly, cell cycle arrest and death of haploid

S. cerevisiae yeast strains requires CdtB DNase-I-like catalytic residues and nuclear localization (Matangkasombut *et al.*, 2009). Since yeasts lack PIP_3, CdtB-induced DSBs alone are sufficient to account for toxicity in this model (Matangkasombut *et al.*, 2009). Taken together the data suggest that the predominant mechanism of cellular toxicity varies according to target cell lineage with nuclease activity and DSBs as the most prevalent mechanism, and PIP_3 phosphatase activity playing an accessory role in certain situations.

CDT-induced DNA repair response and cell cycle arrest

Mammalian cell cycle regulation is responsible for the proper maintenance of several physiological processes, including the maintenance of intact mucosal epithelial barriers and clonal expansion of lymphocyte subsets during adaptive immune responses, both of which constitute important defense mechanisms against colonization and infection of mucocutaneous interfaces by bacterial pathogens (Oswald *et al.*, 2005). Therefore, CDT-producing bacteria might have evolved a specialized mechanism to disrupt key mammalian cell functions in order to establish persistent colonization and cause disease in certain niches (Nougayrede *et al.*, 2005; Oswald *et al.*, 2005). On the basis of their ability to modulate the cell cycle and cause cell death, CDT has been classified as an inhibitory cyclomodulin (Eshraghi *et al.*, 2010; Liyanage *et al.*, 2010; Nougayrede *et al.*, 2005; Oswald *et al.*, 2005). In fact, CDT was the first bacterial toxin shown to cause cell cycle arrest in mammalian cells (Toth *et al.*, 2009).

Cell cycle fidelity is maintained by redundant DNA damage checkpoint mechanisms, which are evolutionarily conserved signaling pathways that validate the integrity and accuracy of DNA replication at each phase of cell division (Bartek & Lukas, 2007; Jackson & Bartek, 2009). Activation of DNA damage checkpoints results in cell cycle arrest so that DNA can be repaired, or if damage is severe, progress to programmed cell death by apoptosis (Bartek & Lukas, 2007; Hoeijmakers, 2001). The DNA damage checkpoint network comprises upstream DNA damage sensors, signal transducers and downstream effectors (Bartek & Lukas, 2007). Specific pathways preferentially sense and respond to distinct types of DNA damage and initiate dedicated protective outputs (Hoeijmakers, 2001). Because DSBs are among the most severe DNA lesions, repair mechanisms involving error free homologous recombination (HR) and error prone non-homologous end joining (NHEJ) mechanisms will act synergistically to repair DSBs (Ciccia &

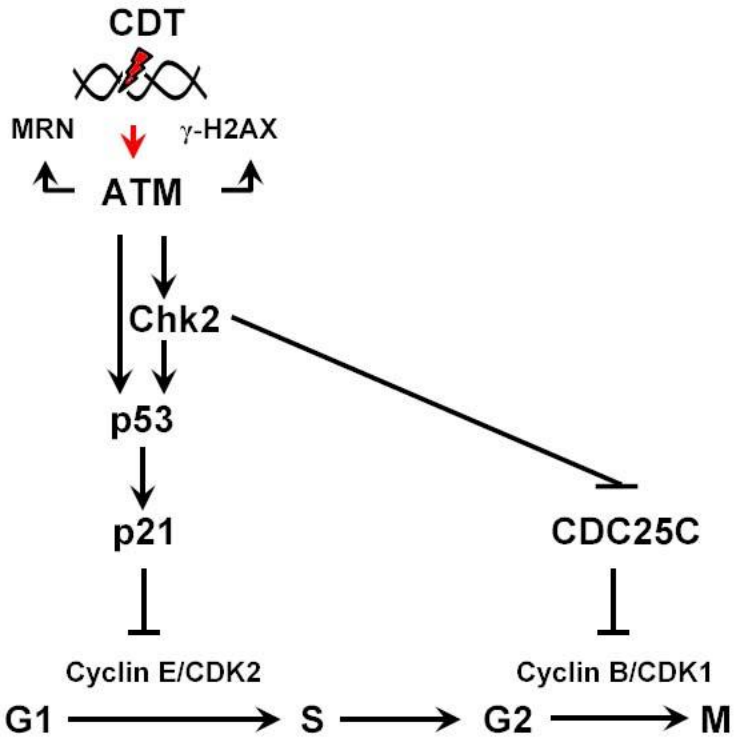
Elledge, 2010). It is currently believed that CDT-induced DSBs trigger a DDR similar to that caused by ionizing radiation (IR)-induced DSBs and mediated by the phosphoinositide 3-kinase related kinase ataxia telangiectasia mutated (ATM), encoded by the gene mutated in the rare autosomal recessive genetic disorder ataxia telangiectasia [AT; (Bartek & Lukas, 2007; Cortes-Bratti *et al.*, 2001b; Derheimer & Kastan, 2010; Jackson & Bartek, 2009)].

The ATM-dependent IR-induced DDR involves both induction of cell cycle arrest and initiation of DNA repair (Derheimer & Kastan, 2010). Major components of the ATM-dependent DNA damage signaling pathway include (*i*) the multifunctional MRN protein complex consisting of Mre11, Rad50 and Nbs1, (*ii*) histone H2AX, (*iii*) the cell cycle checkpoint regulator protein kinase Chk2, and (*iv*) the transcription factor p53 [**Fig. 1.5;** (Bartek & Lukas, 2007; Derheimer & Kastan, 2010; Jackson & Bartek, 2009)]. Initial DSBs are recognized by the MRN complex, which recruits ATM to the damage site (Bakkenist & Kastan, 2003; Bartek & Lukas, 2007; Hoeijmakers, 2001; Lavin & Kozlov, 2007; Lavin, 2008). The serine-threonine protein kinase ATM which normally exists as inactive dimers, dissociates and is activated by autophosphorylation in the presence of DSBs. Activated ATM subsequently phosphorylates a large array of substrates including, histone H2AX, Chk2, and p53 (Matsuoka *et al.*, 2007). Although the precise events surrounding ATM activation are incompletely understood, recent evidence suggests that ATM and MRN complex act in harmony to sense DSBs (Derheimer & Kastan, 2010). Activated MRN complex and phosphorylated H2AX (γH2AX) initiate and amplify the DNA repair process by stabilizing the DNA lesion and providing a platform for the binding of other DNA repair proteins (Derheimer & Kastan, 2010). Activated Chk2 reduces cyclin-dependent kinase (CDK) activity by several mechanisms including activation of the transcription factor p53 and subsequent induction of cyclin inhibitor p21 (Jackson & Bartek,

2009). CDKs are key regulators of cell cycle and the inhibition of CDKs can result in arrest of the cell-cycle progression at the G1-S, intra-S and G2-M checkpoints, allowing time for DNA repair before proceeding with replication or mitosis (Jackson & Bartek, 2009). Other ATM substrates additionally have key roles in affecting cell cycle checkpoint function, such as Smc1 for intra-S phase, as reviewed elsewhere (Derheimer & Kastan, 2010).

Figure 1.5. Pathways of CDT-induced cell cycle arrest in mammalian cells. Following CDT-induced DNA double strand breaks (DSB), the ATM-mediated DNA damage response is initiated with subsequent activation of the multifunctional protein complex consisting of Mre11, Rad50 and Nbs1 (MRN), histone H2AX, the cell cycle regulator checkpoint kinase 2 (Chk2), and the transcription factor p53. Downstream p53-induced activation of p21 and Chk2-mediated inactivation of cell division cycle 25 (CDC25) C phosphatase block cell cycle progression by inhibition of cyclin dependent kinases (CDK)-Cyclin complexes, and thus, preventing entry into mitosis while promoting repair of DNA.

Figure 1.5



The main evidence in support of CDT-induced DDR is based on demonstration of MRN and γ-H2AX activation shortly following CDT exposure of susceptible cells. A large percentage of HeLa cells display γ-H2AX foci 2 h post-HducCDT exposure, and nearly all cells are positive for γ-H2AX foci within 6 to 8 h post-exposure (Li *et al.*, 2002). Both HducCDT and IR induce similar levels of Mre11 foci in HeLa cells and primary human dendritic cells (Li *et al.*, 2002). Nuclear microinjection of purified recombinant HducCdtB into HeLa cells induces Mre11 foci to the same extent as HducCDT holotoxin treated cells within 1 h, suggesting that the active subunit CdtB is sufficient and necessary to elicit DDR (Li *et al.*, 2002). Increased Rad50 foci and γ-H2AX are also seen in primary human fibroblasts treated with CjejCDT (Hassane *et al.*, 2003), in primary and established human endothelial cells treated with EcolCdtB-V (Bielaszewska *et*

al., 2005; Liyanage *et al.*, 2010), and cultured human intestinal epithelial H407 cells exposed to HhepCDT (Bielaszewska *et al.*, 2005; Liyanage *et al.*, 2010). As expected, γ-H2AX is readily detected in immortalized wild type human B lymphocyte lines post-HducCDT exposure, but it is absent in *Atm*-defective leukemic B cell lines obtained from AT patients (Li *et al.*, 2002). Analysis of diploid *S. cerevisiae* yeast strains provides additional evidence in support of CDT-induced DDR. Strains of *S. cerevisiae* that lack Rad51, the repair protein of budding yeast equivalent to the MRN complex of mammalian cells, are exquisitely sensitive to CjejCdtB, suggesting a critical role for HR in repairing CDT-induced DNA lesions (Kitagawa *et al.*, 2007).

Other evidence in support of a CDT-induced DDR is based on demonstration of cell cycle checkpoint activation, which further confirms data obtained by monitoring DNA damage signaling. Human HL fibroblast and larynx carcinoma HEp-2 cell lines display similar cell cycle checkpoint response kinetics consisting of p53, Chk2 and cdc2 phosphorylation and upregulation of p21 and p27, following exposure to IR or HducCDT (Cortes-Bratti *et al.*, 2001b). In common with IR, rat fibroblast cell lines exposed to HhepCDT exhibit c-Myc and ATM-dependent activation of DNA damage checkpoint responses (Guerra *et al.*, 2010). Even though not a genotoxin-specific phenomenon; formation of actin stress fibers through the ATM-dependent activation of small GTPase RhoA is seen in HeLa cells following HducCDT-induced DSBs (Frisan *et al.*, 2003). Formation of actin stress fibers is also seen in Hep-2 cells exposed to HpulCDT and CHO cells exposed to EcolCDT-II (Aragon *et al.*, 1997; Ceelen *et al.*, 2006). More recently, the formation of actin stress fibers in HeLa cells exposed to HducCDT was shown to result from the activation of nuclear RhoA-specific guanine nucleotide exchange factor (GEF) Net1 (Guerra *et al.*, 2008).

Currently CDT is known to cause ATM-dependent cell cycle arrest at the G2/M and G1/S transitions, although definitive molecular analysis could further strengthen these observations (**Fig.1.5**). The CDT-mediated G2/M arrest is a result, at least in part, of the activation of Chk2 by activated ATM upon sensing DSBs. Activated Chk2 phosphorylates and inactivates cell division cycle 25 (CDC25) C phosphatase. The resulting accumulation of phosphorylated cyclin B-cyclin dependent kinase (CDK) 1 complex prevents mitotic entry (Ge *et al.*, 2008; Smith & Bayles, 2006). The mechanism for CDT-mediated G1/S arrest is thought to be p53-dependent. Activated ATM phosphorylates p53, and the resulting upregulation of p21 inhibits cyclin E-CDK2, which blocks S-phase entry (Ge *et al.*, 2008; Smith & Bayles, 2006). However, p53 independent upregulation of p21 following CDT treatment has been reported (Smith & Bayles, 2006). Although there is limited evidence supporting a CDT-mediated intra S-phase checkpoint activation in yeast, the potential contribution of this checkpoint has yet to be investigated in mammalian systems (Matangkasombut *et al.*, 2009).

Limitations in our understanding of the molecular mechanisms of CDT-induced cell cycle arrest lie in the techniques that are commonly employed to address this question. With the exception of a few instances indicating accumulation of phosphorylated Cdk1 (cdc2) prior to cell cycle arrest, CDT-induced arrest of the cell cycle relies primarily on demonstration of altered patterns of DNA staining with propidium iodide and analysis by florescence-activated cell sorting (Bielaszewska *et al.*, 2005; Comayras *et al.*, 1997; Cortes-Bratti *et al.*, 2001b; Peres *et al.*, 1997). At least two G2/M checkpoints exist in mammalian cells, an Atm-dependent transient checkpoint which activates in G2 phase cells immediately after DNA damage, and prolonged Atm-independent checkpoint which is activated several hours after damage and reflects the G2 phase accumulation of cells that were initially damaged during S-phase (Weiss *et al.*, 2003; Xu

et al., 2002). Because CDT might induce arrest at multiple stages of the cell cycle, measurement of DNA content alone cannot distinguish between these two checkpoints highlighting the need of additional study of CDT-induced cell cycle arrest.

CDT-mediated apoptosis

Apoptosis is a physiological mechanism of cell death present in multicellular organisms for the controlled elimination of unwanted cells. Similar to creating a block in the cell cycle, bacterial toxins can induce apoptosis of host cells which can facilitate colonization, infection and disease (Nougayrede *et al.*, 2005; Oswald *et al.*, 2005). Diverse factors and stimuli can initiate signaling pathways leading to apoptotic cell death by two major signaling pathways; the extrinsic pathway involving death receptor activation, and the intrinsic (or mitochondrial) pathway that can be activated by several stimuli (environmental toxicants, drugs, toxins) that provoke cell stress or damage (Taylor *et al.*, 2008). The extrinsic pathway is activated by the binding of extracellular death ligands (for example FasL and TNFα) to the transmembrane death receptors (for example FAS), while the intrinsic pathway is activated by stimuli that alter the mitochondrial outer membrane permeability (Ow *et al.*, 2008). In some cases, the extrinsic pathway can also be activated by certain DNA-damaging drugs (Roos & Kaina, 2006). The activation of the intrinsic pathway depends on the activation of one or more members of the BH3 protein family including Bid, PUMA and NOXA (Taylor *et al.*, 2008). Accumulation of activated BH3 proteins beyond a critical level overrides the inhibitory effect of anti-apoptotic B-cell lymphoma-2 (BCL-2) family proteins (such as Bcl-2 itself) and promotes the formation of pro-apoptotic BCL-2 family protein oligomers Bax-Bak on mitochondrial outer membrane (Ow *et al.*, 2008; Taylor *et al.*, 2008). Accumulation of Bax-Bak complex results in the release of

mitochondrial intermembrane proteins, including cytochrome c into the cytosol (Ow *et al.*, 2008; Taylor *et al.*, 2008). A feature in common to both of these pathways is the activation of caspases that are involved in early signaling events as well as in final proteolysis phase of cell death (Creagh *et al.*, 2003). Stimulation of the extrinsic pathway leads to activation of caspase-8 that can process and activate downstream caspase-3 and caspase-7 (Taylor *et al.*, 2008). Stimulation of the intrinsic pathway involves multiple sequential alterations including signaling that results in release of cytochrome c from mitochondria and formation of the apoptosome (Ow *et al.*, 2008). This structure facilitates cytochrome c-APAF-1 dependent activation of caspase-9 which in turn activates caspase-3 leading to the execution phase of cell death by apoptosis (Creagh *et al.*, 2003; Taylor *et al.*, 2008).

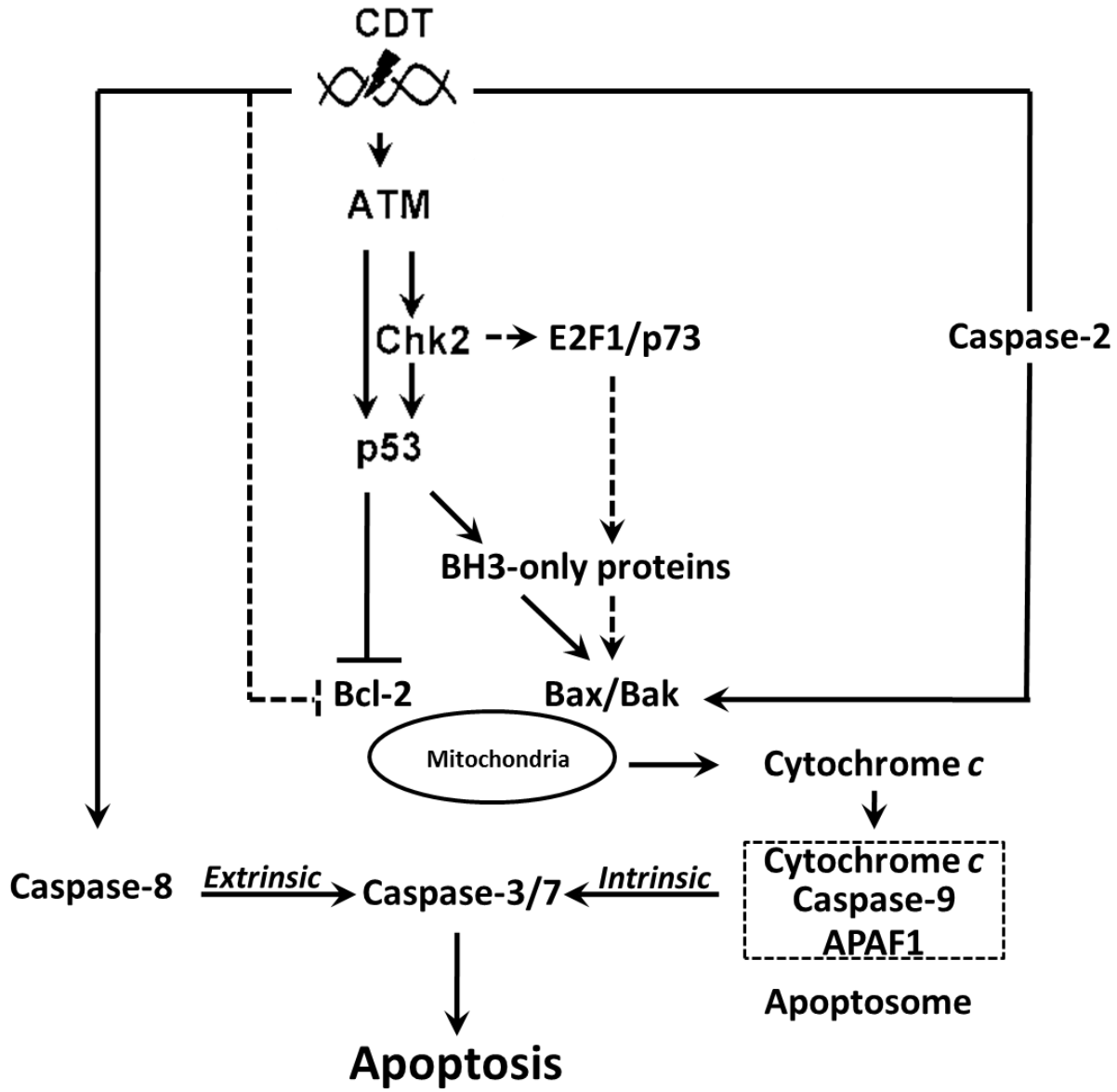
The status of p53 is a key factor that determines the sensitivity of eukaryotic cells to apoptosis, and this is particularly critical for genotoxin-induced apoptosis where cells with wild-type p53 genotype display greater sensitivity than cells with a p53-null genotype (Gudkov & Komarova, 2003; Roos & Kaina, 2006). In p53 wild-type cells, accumulation of phosphorylated p53 following DNA damage leads to the transcriptional activation of pro-apoptotic p53 targets including Bax, PUMA and NOXA, while in p53-null cells apoptosis sensitivity can be mediated through several mechanisms including the degradation of anti-apoptotic Bcl-2 and Chk1/Chk2-E2F1-p73 mediated upregulation of pro-apoptotic p53 targets (Gudkov & Komarova, 2003; Roos & Kaina, 2006). The p53-independent mechanism is somewhat less influential for facilitating apoptosis than direct activation of Bax by p53, and thus p53-null cells are generally more resistant to genotoxin-induced apoptosis (Roos & Kaina, 2006). Additionally, sensitivity to toxicant or drug-induced apoptosis has been shown to vary considerably in different cell lines, and this is attributed to differences in expression of Bcl-2 which has a protective function for

mitochondrial integrity (Zamzami *et al.*, 1996). High Bcl-2 expression in lymphoid tumor cell lines is associated with resistance to chemotherapeutic drug- and bacterial toxin-induced apoptosis (Bloom *et al.*, 2006; O'Brien *et al.*, 2001). Thus, the level of Bcl-2 expression can play an important role in target cell sensitivity to bacterial toxin-induced apoptosis. This may be particularly important in the case of toxins that induce DNA damage and an intrinsic or mitochondrial-based apoptotic pathway regulated by Bcl-2 proteins.

The mechanisms involved in CDT-induced apoptosis are incompletely understood and currently thought to be dependent on the particular type of target cell (Belibasakis *et al.*, 2004; Dreyfus, 2003). Based on the observations of early DNA fragmentation and activation of DDR in several p53 wild-type and p53-defective cell lines, it is now clear that apoptosis is a downstream event of CdtB-induced DDR which can be mediated through both p53-dependent and p53-independent mechanisms [**Figure 1.6**, Cortes-Bratti *et al.*, 2001b; Frisan *et al.*, 2003; Liyanage *et al.*, 2010)]. Similar to other genotoxins, cells with wild-type p53 are more sensitive to CDT-induced apoptosis than p53-deficient cells. For example, in human leukemia T-cell lines, caspases are activated earlier in p53 wild-type MOLT-4 cells (highly CDT sensitive) as compared to p53 mutated Jurkat cells (Cheng & Haas, 1990; Ohara *et al.*, 2004).

Figure 1.6. Pathways of CDT-mediated apoptosis in mammalian cells. Following CDT-induced DNA double strand breaks (DSB), the ATM-mediated DNA damage response is activated which directly or through Chk2 phosphorylates and activates p53 leading to activation of the intrinsic apoptotic pathway. Accumulation of fully phosphorylated active p53 overrides anti-apoptotic Bcl-2, while promoting the formation of pro-apoptotic Bcl-2-associated X (Bax) and Bcl-2 homologous antagonist killer (Bak) protein oligomers on the mitochondrial outer membrane by transcriptional upregulation of pro-apoptotic BH3-only proteins. These interactions result in the release of cytochrome *c* from mitochondria and activation of caspase-9 within the apoptosome. Activated caspase-9 in turn activates the executioner caspases-3 and -7 leading to apoptotic cell death. Activation of the extrinsic apoptotic pathway following DSB through caspase-8 has been suggested in human B lymphoblastoid JY and monocytic 28SC cell lines (Hickey *et al.*, 2005; Shenker *et al.*, 2001). In a p53-mutated background, the intrinsic apoptotic pathway may be activated by either caspase-2-mediated Bax/Bak translocation, Chk2 causing E2F1/p73-mediated transcriptional upregulation of pro-apoptotic BH3-only proteins causing Bax/Bak mitochondrial translocation or degradation of Bcl-2 (putative interactions are drawn as dotted lines).

Figure 1.6


CDT
ATM
Chk2
E2F1/p73
Caspase-2
p53
BH3-only proteins
Bcl-2
Bax/Bak
Mitochondria
Cytochrome c
Caspase-8
Extrinsic
Caspase-3/7
Intrinsic
Cytochrome c
Caspase-9
APAF1
Apoptosome
Apoptosis

The CDT produced by several bacteria can induce apoptosis in a broad range of cell types including established cell lines, proliferating and non-proliferating primary cells of epithelial, endothelial, fibroblastic and hematopoietic lineages (Bielaszewska *et al.*, 2005; Gelfanova *et al.*, 1999; Hickey *et al.*, 2005; Liyanage *et al.*, 2010; Mooney *et al.*, 2001; Ohara *et al.*, 2004; Shenker *et al.*, 1999; Shenker *et al.*, 2001; Yamamoto *et al.*, 2004). Activation of caspases following CDT exposure has been documented in cultured epithelial, endothelial and hematopoietic cell lines and caspase inhibitors protect cells from CDT-induced apoptosis (Alaoui-El-Azher *et al.*, 2010; Bielaszewska *et al.*, 2005; Gelfanova *et al.*, 1999; Hickey *et al.*, 2005; Ohara *et al.*, 2004; Shenker *et al.*, 2001; Wising *et al.*, 2005; Yamamoto *et al.*, 2004). Recently, we reported that activation of the intrinsic (mitochondrial) apoptotic pathway follows DDR activation by HhepCDT treatment of human intestinal epithelial H407 cells (Liyanage *et al.*, 2010); sequential up-regulation of Bax and down-regulation Bcl2 led to cytochrome c release and the subsequent activation of caspase-9 within 5 h following exposure to HhepCDT (Liyanage *et al.*, 2010). While the activation of caspase 9 peaked at 12h, caspase 3 was not activated until 24 h following HhepCDT exposure, and the activity of caspase 8 did not change (Liyanage *et al.*, 2010). Based on caspase activation patterns in this and other cell lines, and together with other experimental evidence, the major mechanism for CDT-induced apoptosis appears to be through the ATM-dependent intrinsic pathway (Alaoui-El-Azher *et al.*, 2010; Bielaszewska *et al.*, 2005; Hickey *et al.*, 2005; Ohara *et al.*, 2004; Shenker *et al.*, 2001; Yamamoto *et al.*, 2004). Inhibition of caspase-8 could block Fas-mediated apoptosis in Jurkat cells, but did not inhibit CDT-mediated apoptosis (Ohara *et al.*, 2004). Overexpression of Bcl-2 results in a significant reduction of CDT-mediated apoptosis in human B-cell lines, supporting the suggestion of an intrinsic pathway-dependent mechanism of apoptosis activation by CDT

(Ohguchi *et al.*, 1998; Shenker *et al.*, 2001). However, possible simultaneous activation of both intrinsic and extrinsic pathways has also been suggested for human B lymphoblastoid cell line, JY and the monocytic cell line, 28SC (Hickey *et al.*, 2005; Shenker *et al.*, 2001).

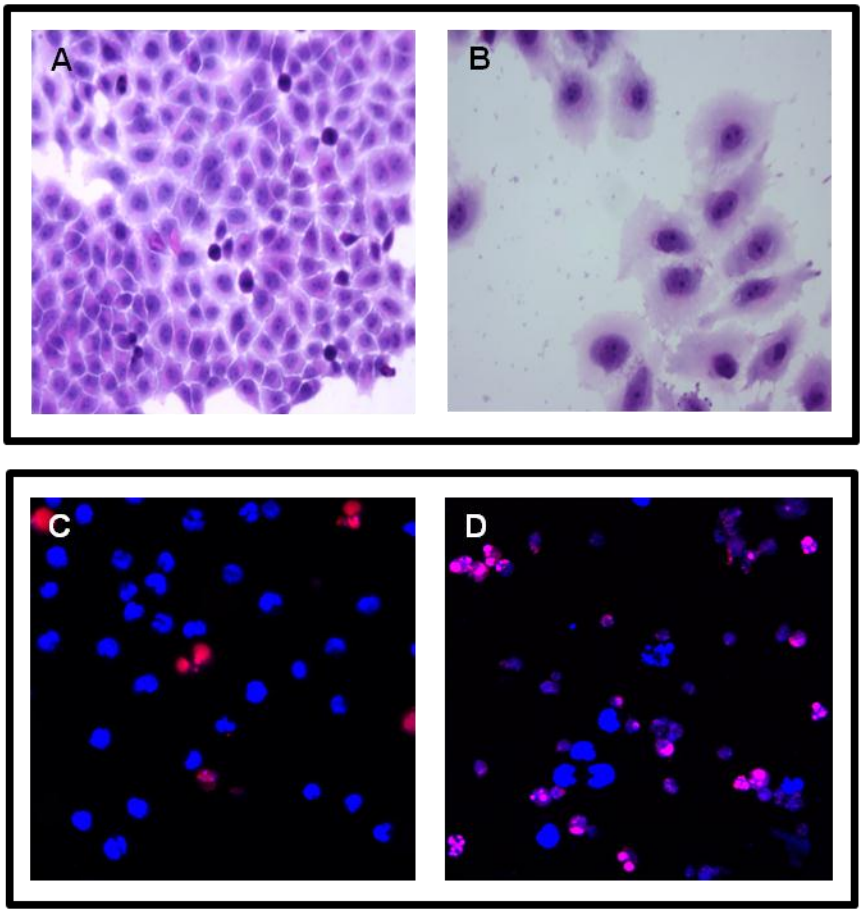
The role of ATM in CDT-mediated apoptosis is incompletely characterized. There is limited evidence suggesting ATM wild-type SN-B1 and JAC-B2 cells are more susceptible to HducCDT-induced apoptosis as compared to Atm defective cell lines (Cortes-Bratti *et al.*, 2001b). Based on caspase activation patterns, pharmacological inhibition of ATM or siRNA knock down of Chk2, it was recently shown that apoptosis induced by AactCDT in human immortalized gingival keratinocytes is mediated through the ATM-dependent DDR pathway (Alaoui-El-Azher *et al.*, 2010).

CDT cellular specificity and susceptibility

It has been proposed that the effects of CDT are cell type dependent (Belibasakis *et al.*, 2004; Dreyfus, 2003). Irrespective of the bacterial source of CDT and before characteristic nuclear and cytoplasmic distension are seen, cultured cell lines of epithelial and endothelial origins primarily arrest in the G2/M phase (Elwell *et al.*, 2001; Lara-Tejero & Galan, 2001; Smith & Bayles, 2006; Whitehouse *et al.*, 1998; Wising *et al.*, 2005; Young *et al.*, 2000b), whereas cells of fibroblastic origin arrest both in the G1/S and G2/M phases of the cell cycle (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001b; Hassane *et al.*, 2003; Smith & Bayles, 2006; Wising *et al.*, 2005). By contrast, cell lines of hematopoietic lineage including lymphocytes, monocytes, macrophages and dendritic cells, not only are several orders of magnitude more susceptible to CDT (pg versus μg, **Fig. 1.7**), but also display rapid apoptosis after a very transient arrest of the cell cycle (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001b; Hassane *et al.*, 2003; Smith & Bayles, 2006).

Figure 1.7. Comparison of CDT-mediated cytotoxicity in a human epithelial (cervical carcinoma HeLa; ATCC CCL-2) and a lymphoid (acute lymphoblastic T cell leukemia MOLT-4; ATCC CRL-1582) cell lines. *Campylobacter jejuni* CDT holotoxin (CjejCDT) reconstituted from purified recombinant His-tagged CdtA, CdtB and CdtC subunit fusion proteins was incubated with HeLa cells before fixation and staining. Light photomicrographs (60X original magnification) of formalin-fixed and hematoxylin and eosin stained HeLa cells incubated with control medium (D-MEM; A) or D-MEM containing 25 μg ml^{-1} of CjejCDT (B) for 72 hours. Note the marked nuclear and cytoplasmic enlargement of CjejCDT-treated cells compared to normal control cells. Confocal laser scanning photomicrographs (40X original magnification) of un-fixed Hoechst 33342 and propidium iodide double stained MOLT-4 cells incubated with control medium (RPMI;C) or RPMI containing 100 ng ml^{-1} of CjejCDT (D) for 24 hours. Greater than 85% of the cells treated with CjejCDT (D) display early apoptotic changes characterized by condensed and fragmented chromatin (blue) or late apoptotic changes consisting of similar nuclear changes and cytoplasmic uptake of propidium iodide (pink) compared with less than 5% dead (red) cells in the control.

Figure 1.6



The lowest effective dose of recombinant CDT that can intoxicate cell lines of hematopoietic lineage ranges between 10 and 50 pg ml^{-1} compared to 1 and 5 µg ml^{-1} for other cell types (Shenker *et al.*, 2007). More than 90% of either human Jurkat T cells or THP-1 monocytic cells undergo apoptosis within 24 to 48 hours after treatment with 100 ng ml^{-1} HducCDT, while only 30% of HeLa or HaCaT epithelial cells or primary human fibroblasts are affected under the same conditions (Wising *et al.*, 2005). Based on their extreme susceptibility, lymphocytes have been proposed as the *in vivo* target of CDT, suggesting immunomodulation allowing persistence of bacterial colonization (Ge *et al.*, 2005; Pratt *et al.*, 2006; Shenker *et al.*, 2007). Although CDT is a broad range genotoxin (Ge *et al.*, 2005; Pratt *et al.*, 2006; Shenker *et al.*, 2007), it remains to be demonstrated whether this apparent cell type specificity is in part attributable to inherent differences in cell surface receptor binding of CdtA and CdtC to host cell membrane depending on host cell surface biomolecule density, intrinsic differences in CdtB uptake and nuclear translocation, or variable target cell lineage DDR capability (Carette *et al.*, 2009; Eshraghi *et al.*, 2010). However, other factors that can determine the outcome of CDT interactions with susceptible cells including receptor chemical compositions and intrinsic affinities, but also amino acid sequence divergence of CdtA and CdtC binding subunits encoded by different bacteria need further detailed analysis (Carette *et al.*, 2009; Eshraghi *et al.*, 2010).

The mutational status of individual cell lines is another critical factor that can determine activation of specific checkpoint and apoptotic pathways, and thus the stage of cell cycle arrest and kinetic of progression to apoptosis in response to genotoxic injury. As described earlier, two of the most important mediators of CDT-induced DDR are ATM and p53, both of which play critical roles in determining the stage of cell cycle arrest and pathways to apoptotic execution. ATM is required for the initiation of G1/S, intra-S and G2/M checkpoint arrest (Derheimer &

Kastan, 2010; Xu *et al.*, 2002). However, there are at least two distinct G2/M checkpoints, and only the rapid-transient checkpoint is ATM-dependent (Xu *et al.*, 2002). Most established cell lines are tumor derived and frequently contain mutations in tumor suppressors, including *p53* (Cheng & Haas, 1990). Similar to ATM, p53 is required for the initiation and maintenance of G1/S checkpoint and can play a role in arrest at the G2/M stage of the cell cycle (Giono & Manfredi, 2006). As described earlier, *p53* wild-type cells are more susceptible to apoptosis compared to *p53* defective cells (Roos & Kaina, 2006).

Since the cellular responses to CDT-induced damage are very similar to IR-induced DDR, the inherent differential tissue susceptibility to IR-induced DNA damage may also be involved in the pathogenesis of diseases caused by CDT-producing bacterial pathogens (Gudkov & Komarova, 2003; Smith & Bayles, 2006). Rapidly proliferating cells including haematopoietic and intestinal epithelial cells are the most radiosensitive cell types, while non-proliferating cells of the nervous, respiratory, urinary, endocrine, musculoskeletal and mesenchymal tissues are relatively radioresistant (Gudkov & Komarova, 2003). The rate of cell division does not always correlate with cellular radiosensitivity, as extremely radiosensitive adult thymus, spleen, and bone marrow stem cells consist mostly of quiescent cells (Gudkov & Komarova, 2003). However, the expression levels of p53 and several p53-responsive pro-apoptotic genes including *Bax* and *Fas/Apo1* correlate with tissue radiosensitivity, where highly radiosensitive tissues have higher expression levels of *p53* and *p53*-responsive pro-apoptotic genes (Gudkov & Komarova, 2003). As a general rule, hematopoietic cells undergo rapid apoptosis following IR exposure, whereas fibroblasts undergo permanent growth arrest and epithelial cells with the exception of the intestinal epithelium, which undergo rapid p53-mediated apoptosis, exhibit reversible arrest,

while p53-deficient cells from all lineages tend to display a brief arrest (Gudkov & Komarova, 2003).

In this context it is interesting to note that the CDT-induced G1/S cell cycle arrest in fibroblasts have been observed exclusively in primary cell lines, which are likely to have wild type p53, and thus retain an intact G1/S checkpoint (Hassane *et al.*, 2003). In contrast G2/M cell cycle arrest does not depend on p53 for initiation, and therefore it is possible for established p53-deficient cell lines to retain a functional G2/M checkpoint (Giono & Manfredi, 2006). Indeed, the G2/M cell cycle arrest is the predominant type of cell cycle arrest associated with CDT toxicity (Smith & Bayles, 2006). At least in T-cell leukemia cell lines, susceptibility to CDT-induced apoptosis is dependent on p53 status of the cell line, as p53 wild-type cells are more susceptible to apoptosis compared to p53 deficient cells (Ohara *et al.*, 2004).

The differential tissue susceptibility to IR mirrors the cell type susceptibility to CDT (hematopoietic lineage hyper-susceptibility, growth arrest in epithelial, endothelial or fibroblast lineages and CDT-resistance in p53 defective cells from all lineages). To our knowledge, epithelial cell lines primarily show G2/M phase cell cycle arrest and a slow progression to apoptosis which can take several hours to days after CDT treatment. Investigating the mechanism of CDT intoxication in primary intestinal epithelial cells would provide additional insights.

It is conceivable that the CDT-induced DDR might be distinct from that induced by IR, yet some of the pathways may be overlapping. The ability of a cell line to generate DDR is also an important consideration which determines genotoxin susceptibility, particularly for agents that cause DSBs (Roos & Kaina, 2006). While DSBs can be repaired by either relatively error free

HR or error prone NHEJ; the later may lead to chromosomal rearrangements particularly in cells in which this mechanism is preferentially utilized such as cells in G1 phase of the cell cycle (i.e. resting fibroblasts, hepatocytes) or in lymphocytes undergoing V(D)J recombination-associated DSB repair during development (Callen *et al.*, 2007; Roos & Kaina, 2006). In HR-defective cells or cells that preferentially repair DSBs by NHEJ, the resulting chromosomal rearrangements can lead to apoptosis (Roos & Kaina, 2006). At least in *S. cerevisiae* yeast, two types of mutations display hyper-sensitivity to AactCdtB; strains defective in sensing DNA strand breaks and strains with defective HR repair (Matangkasombut *et al.*, 2009). Since yeast use HR as primary means of DSB repair, the data suggest that cells defective in DSB repair are more sensitive to CdtB.

Role of CDT in disease

Naturally-occurring infections with *H. ducreyi* and *A. actinomycetemcomitans* can elicit serum IgG antibodies to individual CdtABC protein subunits indicating development of a host adaptive immune response to toxin expressed during infection (Ando *et al.*, 2010; Mbwana *et al.*, 2003; Xynogala *et al.*, 2009). Whether serum IgG antibodies play a role in recovery from infection and protect against subsequent re-infection has not been determined. However, the presence of antibodies that can neutralize the biological activity of CDT has been demonstrated in sera obtained from patients with chancroid [anti-HducCDT; (Mbwana *et al.*, 2003)]; patients who are recovered from campylobacteriosis [anti-CjejCDT; (Abuoun *et al.*, 2005)]; and people with periodontitis [anti-AactCDT; (Ando *et al.*, 2010; Xynogala *et al.*, 2009)]. It has been suggested that the absence of AactCdtC-specific IgG in sera of patients with localized aggressive periodontitis compared with those with generalized aggressive periodontitis might be attributable to either differences in CdtC expression by different strains or differences in the extent or duration of these infections in individual patient (Ando *et al.*, 2010). Development of high levels of CDT-neutralizing IgG in serum and genital tissues of mice immunized with HducCDT toxoid compared to native toxin suggests a potential vaccine application for CDT toxoid in protection against chancroid (Lundqvist *et al.*, 2010). Aside from demonstrating adaptive humoral immune response to CDT following natural infection or immunization, nearly all studies on the role of CDT in disease have focused on *in vitro* models of eukaryotic cell genotoxicity and direct experimental evidence demonstrating the role of CDT in disease of human and animal hosts is lacking (Ge *et al.*, 2005; Smith & Bayles, 2006; Stevens *et al.*, 1999).

Failure to demonstrate differences in skin colonization and lesion development amongst human volunteers and rabbits inoculated with wild-type *H. ducreyi* strain compared with a

isogenic strain with inactivated *cdtC* gene suggests that CDT is not required for cutaneous infection with this pathogen (Stevens *et al.*, 1999; Young *et al.*, 2001). While *cdtA*-, *cdtB*- and *cdtC*-negative mutant strains are no longer cytotoxic for cultured HeLa cells *in vitro*, a difference in the onset and degree of cutaneous changes elicited by mutant strains compared to the corresponding wild-type *H. ducreyi* parent strain in a rabbit model of chancroid could not be demonstrated, further confirming that CDT is unlikely to contribute to the early stage of skin infection (Lewis *et al.*, 2001). Although a dose-dependent inflammatory response is seen following intradermal inoculation of rabbits with reconstituted recombinant HducCDT holotoxin, but not with individual subunits, incomplete characterization of the cellular infiltrate and mechanism of inflammation hinder proper interpretation of these observations (Wising *et al.*, 2002). Considering that HducCDT is highly toxic to a variety of mammalian cells *in vitro*, it is conceivable that local cellular damage might have been responsible for the dermal response seen with purified toxin in the rabbit model.

Since CDT is produced by several intestinal bacterial pathogens, early studies examined the role of purified CDT and mutant strains in the pathogenesis of diarrheal disease. Development of profuse watery diarrhea accompanied with intestinal fluid accumulation and colonic epithelial cell damage within 12 h after intragastric inoculation of conventional suckling mice with purified SdysCDT expressed in *E. coli* suggests a role for CDT in diarrheal disease seen with *Shigella dysenteriae* (Okuda *et al.*, 1997). These observations are further supported by demonstration of impaired translocation of *C. jejuni* CDT mutant compared with isogenic wild-type parent strain across the intestinal epithelial barrier of adult SCID mice at 2 h, but not at 6 h or 24 h after intragastrically inoculation with 10^9 colony forming units of each bacterial strain (Purdy *et al.*, 2000). Although the data suggest a potential role of CDT in direct intestinal epithelial cell

damage and disease, intragastric administration of massive doses of toxin or bacteria, respectively in immature and immunocompromized hosts is not representative of the natural disease. Therefore, it is still unclear whether CDT contributes to intestinal epithelial barrier dysfunction and systemic spread of CDT-producing bacteria.

Because HhepCDT is the only known virulence factor found in *H. hepaticus,* a host-adapted pathogen of mice (Suerbaum *et al.,* 2003), laboratory mice have been used as a model to uncover pathogenetic mechanisms associated with CDT in infection and disease. Studies in laboratory mice, mostly with *H. hepaticus* (Ge *et al.,* 2005; Pratt *et al.*, 2006; Young *et al.*, 2004), but also with *H. cinaedi* (Shen *et al.,* 2009), and *C. jejuni* (Fox *et al.*, 2004a) and others [reviewed by Ge and co-workers; (Ge *et al.*, 2008)] suggest a potential contribution of CDT to bacterial virulence. On the basis of a more rapid intestinal clearance of *H. hepaticus* CdtB-negative mutants compared with wild-type parent strain in orally inoculated conventional and IL-10$^{-/-}$ deficient mice, a role for HhepCDT in resistance against host defense mechanisms has been suggested (Ge *et al.,* 2005; Pratt *et al.,* 2006). The lack of serum IgG1 and significantly lower IgG2c responses to *H. hepaticus* in IL-10$^{-/-}$ mice infected with *H. hepaticus* CdtB-negative mutant compared to mice infected with wild-type *H. hepaticus* up to 8 months post-inoculation suggest an immunomodulatory role for HhepCDT in this model (Pratt *et al.*, 2006). In these studies, IL-10$^{-/-}$ mice that recovered from infection with the CdtB-negative mutant strain were partially protected from subsequent challenge with either the mutant or wild-type *H. hepaticus* strains (Pratt *et al.*, 2006). However, because protection of mice recovered from infection with wild-type *H. hepaticus* was not evaluated, it is unknown whether partial protection is a function of a lack of exposure to CDT or a characteristic of *H. hepaticus* infection in IL-10$^{-/-}$ mice. In other studies using highly susceptible A/JCr male mice, the prevalence and level of cecal colonization by *H.*

hepaticus CdtB-negative mutant were reduced compared to wild-type parent strain at 4 and 10 months post-inoculation, whereas hepatic colonization levels were similar for both strains at 4 months, but reduced at 10 months post-inoculation in mice inoculated with the mutant strain (Ge *et al.*, 2007). Either strain produced similar degrees of hepatic inflammation early post-infection, but only mice infected with wild-type strain progressed to developed dysplastic changes by 10 months post-inoculation (Ge *et al.*, 2007). Taken together these studies demonstrate a role for HhepCDT in modulating the host adaptive immune response so that persistent intestinal colonization leading to systemic translocation of bacteria, and localization to the liver where chronic infection results in cancer development. Future studies with laboratory mice should uncover more precisely at what stage in the pathogenesis of disease and the specific cellular targets of CDT that are responsible for persistent infection and development of disease.

High concentrations of all three CjejCDT subunits are present in detergent extracts of purified outer membrane preparations of *C. jejuni* suggesting that it is primarily membrane-associated (Hickey *et al.*, 2000; Lindmark *et al.*, 2009). Incubation of *C. jejuni* in the presence of 25 mM or 0.1% bile acid sodium deoxycholate, a concentration physiologically relevant to the intestinal lumen, releases the membrane-associated CdtA, CdtB and CdtC subunits into the culture supernatant (Hickey *et al.*, 2005). Since CjejCDT holotoxin can elicit established human intestinal epithelial cell lines to produce CXCL8, a potent pro-inflammatory chemokine responsible for recruitment of polymorphonuclear neutrophils in the intestinal mucosa, a role for CDT in initiation of host innate defense has been suggested (Hickey *et al.*, 2000; Konkel *et al.*, 2001; Murphy *et al.*, 2010; Young *et al.*, 2007). However, since PMNs are expected to eliminate *C. jejuni*, the benefit of eliciting a pro-inflammatory response seems less desirable if bacterial infection is prevented. Given that massive translocation of PMNs across the intestinal

epithelium, as seen in the initial stages of campylobacteriosis, results in increased intestinal permeability and leakage of extracellular fluid into the gut lumen (Blikslager *et al.*, 2007; Dasti *et al.*, 2009), alterations in the gut microenvironment might indirectly promote local expansion of *C. jejuni*, thus allowing increased shedding of bacteria in feces, contamination of the environment and further spread of disease to susceptible hosts.

Development of a host adaptive immune response to individual CDT protein subunit is seen following spontaneous infection and disease caused by CDT-producing *A. actinomycetemcomitans*, *C. jejuni* and *H. ducreyi*. However, experimental infections in a laboratory mouse model of *H. hepaticus* clearly show that CDT can modulate both the level and isotype antibody response of the host and establish persistent infection. Future studies should define which bacterial clearance mechanism is specifically targeted by CDT and its relationship to host immune response modulation.

Conclusions and future directions

Currently, CDT is the only member of a novel class of AB-type bacterial toxins that exhibits nuclease activity *in vitro* and translocates to the nucleus of mammalian cells where it exerts genotoxic damage resulting in cell cycle arrest and apoptosis. Nearly a quarter of a century since the discovery of CDT, our understanding of the biology of this genotoxin and molecular mechanisms of cellular toxicity has greatly improved; however, the natural history and contributions of CDT to mucocutaneous colonization, chronic infection and pathogenesis of disease in various niches within their respective hosts are still incompletely understood. Of paramount importance is the production of CDT by all major food- and water-borne pathogens including several pathotypes of *E. coli* and certain species of *Campylobacter*, *Shigella*, and *Salmonella*. Clearly, further studies are needed in order to more specifically establish the *in vivo* relevance of nuclease and phosphatase activities, particularly in the context of host immune response modulation and contribution to persistent bacterial infection. In the context of intestinal diseases, a largely unexplored and provocative area of study is the potential role of CDT-producing *Campylobacter* and *Helicobacter* species in orchestrating host adaptive immune response leading to alterations in tolerance to gut microflora components and dietary antigens leading to development of inflammatory bowel disease. Determining the cellular targets of CDT produced by various bacterial pathogens within their respective hosts in relation to specific stages of the disease process is critical to understanding the role of this toxin in host-pathogen interactions. Given that CDT is the only known virulence factor produced by *H. hepaticus* and *H. bilis*, and that chronically infected mice develop inflammation-associated hepatic and colon cancer cancer (Ericsson *et al.*, 2010; Fox *et al.*, 2011), a provocative area of future research endeavors will be to determine whether or not CDT-producing bacteria represent co-factor that

promote cancer development in humans. We hope that this review will generate interest amongst our colleagues and stimulate the next generation of microbiologists to tackle this evolving field of CDT-mediated microbial pathogenesis.

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Rationale and hypothesis for the current study

It is currently known that CDT-induces a DNA damage response (DDR) similar to that elicited by ionizing radiation (IR)-induced DNA double strand breaks (DSBs) leading to ATM-dependent cell cycle arrest at the G2/M and G1/S transitions in mammalian cells. Moreover, on the basis of a higher sensitivity of hematopoietic cells to CDT, it has been suggested that CDT modulates the host immune response *in vivo*. However, the precise nature of the G2/M arrest in response to CDT has not been resolved and the possibility of CDT inducing the intra-S phase checkpoint has not been investigated. Additionally, role of ATM in pathogenesis of diseases caused by CDT-producing bacteria is not characterized. Furthermore, despite the critical importance of p53 in inducing apoptosis in response to genotoxic stress, the contribution of p53 to CDT-mediated genotoxicity is incompletely understood and CDT-induced DDR is not well characterized in lymphoid cells. Therefore the central hypothesis of the current study is that lymphocytes from ATM defective mice have increased susceptibility to CDT *in vitro* and mice with a defective ATM pathway have increased susceptibility to disease caused by *H. hepaticus*, a mouse pathogen with CDT as the only know virulence factor. Similarly it is also hypothesized that p53 defective lymphocytes have increased resistance to CDT-induced apoptosis.

In order to address these questions, several $Atm^{-/-}$ and $p53^{-/-}$ mouse thymic lymphoma cell lines were developed according to procedures described in chapter 1. Activation of the ATM-dependent intra-S and G2/M checkpoint by *Helicobacter hepaticus* CDT (HhepCDT) were characterized in chapter 3. Additionally the role of p53 in HhepCDT-induced apoptosis was also characterized in chapter 3. To complement these studies, the role of p53 in the differential

susceptibility of several human lymphoid cell lines to CDT was evaluated in chapter 4 using CDT from *Campylobacter jejuni*. Additionally, The CDT-induced DDR was further characterized in chapter 4. Finally the adaptive humoral immune response of Atm-defective mice was characterized chapter 5 following infection with *H. hepaticus*.

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CHAPTER 2

Derivation of thymic lymphoma T-cell lines from *Atm*$^{-/-}$ and *p53*$^{-/-}$ mice[1]

Abstract

Established cell lines are a critical research tool that can reduce the use of laboratory animals in research. Certain strains of genetically modified mice, such as *Atm*$^{-/-}$ and *p53*$^{-/-}$ consistently develop thymic lymphoma early in life (Barlow *et al.*, 1996; Elson *et al.*, 1996), and thus, can serve as a reliable source for derivation of murine T-cell lines. Here we present a detailed protocol for the development of established murine thymic lymphoma T-cell lines without the need to add interleukins as described in previous protocols (Barlow *et al.*, 1996; Kuang *et al.*, 2005). Tumors were harvested from mice aged three to six months, at the earliest indication of visible tumors based on the observation of hunched posture, labored breathing, poor grooming and wasting in a susceptible strain (Barlow *et al.*, 1996; Browne *et al.*, 2004). We have successfully established several T-cell lines using this protocol and inbred strains of *Atm*$^{-/-}$ [FVB/N-*Atm*^{tm1Led}/J] (Elson *et al.*, 1996) and *p53*$^{-/-}$ [129/S6-*Trp53*^{tm1Tyj}/J] (Jacks *et al.*, 1994) mice. We further demonstrate that more than 90% of the established T-cell population expresses CD3, CD4 and CD8. Consistent with stably established cell lines, the T-cells generated by using the present protocol have been passaged for over a year.

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Short Abstract

In this video we demonstrate a protocol to establish mouse thymic lymphoma cell lines. By following this protocol, we have successfully established several T-cell lines from *Atm*$^{-/-}$ and *p53*$^{-/-}$ mice with thymic lymphoma.

Materials

- Ice bucket
- 70% ethanol
- Dissection pad consisting of foam block covered with aluminum foil and pins
- Two sets of small sterile scissors and forceps
- Sterile 150 mm^2 Petri dish (Thermo Fisher Scientific, Waltham, MA; cat. #08-757-13)
- Sterile 18 gauge needles
- 50 ml conical tubes (Corning Inc., Corning NY; cat # 430829)
- 10% buffered formalin
- Tissue culture flasks
    T-25 (TPP, Trasadingen, Switzerland; cat # 90025)
    T-75 (TPP, Trasadingen, Switzerland; cat # 90075)
- 6-well plates (Corning Inc., Corning NY; cat # 3506)
- Sterile phosphate buffered saline pH 7.2 (PBS)
- Culture medium
    RPMI 1640 with 25 mM HEPES, 200 mM L-glutamine (Lonza, Walkersville, MD; cat#12-115F) supplemented with the following:
    10% heat inactivated (56°C, 30 min) fetal bovine serum (FBS; Hyclone, Logan, UT; cat# SH30088.03)

1% penicillin and streptomycin (Mediatech, Manassas,VA; cat# 30-002-CI)

1% nonessential amino acids (Mediatech; cat#25-025-CI)

55 mM 2β-mercaptoethanol (Sigma, St Louis, MO; cat#M752)

- Dimethyl sulfoxide (DMSO; Calbiochem, La Jolla, CA; cat # 317275)
- Antibodies (eBioScience, San Diego, CA)

anti-CD3-FITC (cat. #11-0031-85)

anti-CD4-APC (cat. #17-0041-83)

anti-CD8-PE (cat.#12-081-85)

- Bovine serum albumin (BSA; Life Technologies, Grand Island, NY; cat #. 11018-017)
- Concanavalin A (Sigma cat# C5275)

-1.8 ml freezing vials (Nunc, Roskilde, Denmark; cat# 368632)

Protocol

1. Dissection of tumor

1.1. For tumor dissection, a clean paper towel or disposable surgical drape is placed on the dissection pad and sprayed with 70% ethanol.

1.2. Mice for this protocol are routinely euthanized with CO_2. Place the euthanized mouse on the dissection pad and spray both sides of the animal with ethanol. The mouse is held in place onto the dissection pad by using four or more push pins inserted through the legs and sprayed thoroughly with 70% ethanol.

1.3. The skin over the midline is cut from the pubis to the base of the head by using sterile scissors, while avoiding the underlying muscles and penetrating into the body cavities.

1.4. Open the abdominal cavity with a new pair of sterile scissors and forceps being careful to not damage the internal organs.

1.5. Open the thoracic cavity by dissecting and deflecting the rib cage to reveal the tumor, without damaging blood vessels.

1.6. Separate the tumor from other organs, and transfer a piece approximately 40-50% of the total tumor (approximately up to 1.5 X 0.8 X 0.8 cm) into cold PBS kept on ice.

1.7. The remaining portion of the tumor can be saved for other assays and a complete necropsy can be performed at this time.

2. Culturing lymphoma cells

2.1. Spray the tube containing the dissected tumor with 70% ethanol and transfer to a biosafety cabinet.

2.2. Add 10 ml of culture medium to a sterile 150 mm^2 Petri dish.

2.3. Transfer the piece of tumor into the Petri dish by gentle suction with a pipette.

2.4. Bend two sterile 18 gauge needle facing the beveled side out.

2.5. Dissociate the tumor using the bent needles.

2.6. Swirl the dish, tap gently and place at an angle to collect the cell suspension to a side, while avoiding large pieces of tumor.

2.7. Aspirate the cell suspension and transfer into a 50 ml conical tube while avoiding large pieces of tumor.

2.8. Wash the plate with 10 ml of medium and transfer into the same tube while avoiding large pieces of tumor.

2.9. Mix the cell suspension composed of lymphocytes and stromal cells gently and transfer 5 ml into a T-25 tissue culture flask and 10 ml into a T-75 tissue culture flask. Gently swirl the flasks.

2.10. Use the remaining cell suspension to make 2, 4, 10,100 and 1000 fold dilutions in 2.5 ml final volume (culturing cells at different densities using several types of culture vessels maximizes the likelihood of establishing a T-cell line).

2.11. Plate 2.0 ml of each dilution into individual wells of a 6-well plate. Gently swirl the plate.

2.12. Incubate the cells for 48 h in humidified CO_2 atmosphere in air.

3. Feeding lymphoma cells

3.1. After 48 h of incubation, add 5 ml of medium to the T-25 flask and 10 ml of medium to the T-75 flask along the wall with minimal disturbance of the cells.

3.2. Add 2 ml of medium to each well of the 6-well plate along the wall with minimal disturbance of the cells.

3.3. Incubate the cells for an additional 72 h.

4. Passaging lymphoma cells

4.1. Initial passage

4.1.1. At the end of the fifth day of incubation, the cells are passaged at a 1:1 ratio, while retaining the original flasks and plates (maintaining the cell density at a concentration of approximately $3x10^6$/ml during the first passages promotes successful establishment of a continuous cell line).

a. For the T-25 flask, transfer 5 ml of cell suspension into a new flask containing 5 ml of medium. Add 5 ml of medium into the original flask.

b. For the T-75 flask, transfer 10 ml of cell suspension into a new flask containing 10ml of medium. Add 10 ml of medium into the original flask.

c. For the 6-well plate, transfer 2 ml of cell suspension from each well into corresponding wells of a new plate containing 2 ml of medium in each well. Add 2 ml of medium into each well of the original wells.

4.1.2. Incubate all flasks and plates for an additional 72 h. In our experience most cell lines are established from these passage one cultures (the presence of loosely adherent suspension cell aggregates over the attached stromal cells is an early indication of an established cell line).

4.1.3. For the cultures with fewer or no loosely adherent suspension cell aggregates, feed the flasks or plates by carefully replacing approximately 50% of the medium every 72 h. To avoid removing cells from the original culture, culture vessels are left standing and undisturbed for at least 5 min to let the cells sediment at the bottom by gravity before gentle aspiration of the supernatant medium.

4.2. Maintaining established cell lines

4.2.1. Established cell lines are passaged into new flasks and plates. For T-25 and T-75 flasks, add 5 ml of the original or passage one suspension into 5 ml of medium. For 6-well plates, add 3 ml of the original or passage one cell suspension into 7 ml of medium in a T-25 flask. Feed and retain the original or passage one culture by replacing the volume removed with fresh culture medium.

4.2.2. Passage established cell lines into new T-25 flasks. The cell lines have a doubling time of 18-24 h. The cultures are routinely maintained at a density of $1\text{-}3x10^6$/ml and passaged every 3 days. Add the required volume of previous passage culture to new medium to make up to 10 ml. Feed and retain the original culture.

4.2.3. After a few passages, the non-adherent cells will self-sustain without the adherent stromal cells and a smaller number of adherent cells are carried over with each passage. Beyond this point, the cultures can be maintained in non-tissue culture treated T-25 flasks.

5. Freezing and recovering lymphoma cell lines

5.1. The cell lines are frozen in freshly prepared RPMI 1640 medium containing 20% FBS and 10% DMSO.

5.2. Cells from a 48 h old culture in T-25 flask (approximately $1.5x10^6$ cells/ml) are centrifuged at 1500 rpm for 5 min at room temperature. After discarding the supernatant, the cells are re-suspended in 2 ml of cold freezing medium. Two 1.0 ml volumes of cell suspension from each flask are placed in freezing vials, immediately frozen at -80°C, and transferred to liquid nitrogen the following day.

5.3. Cells are recovered from liquid nitrogen storage by quick thawing, and resuspending the contents in 10 ml of fresh culture medium. Alternatively, freshly thawed cells are washed once in warm culture medium to remove the DMSO before resuspending in 10 ml of fresh culture medium. The cells are cultured for at least 72 h before subsequent passage.

Representative results

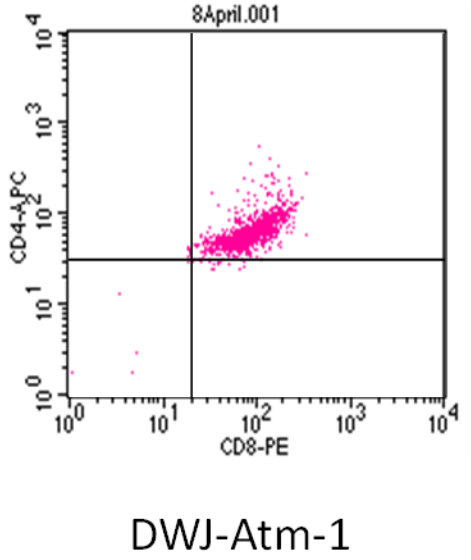
An *Atm*$^{-/-}$ T-cell line developed using this protocol and designated DWJ-Atm-1 was characterized by staining with anti-CD3-FITC, anti-CD4-APC and anti-CD8-PE and flow cytometry analysis (Figure 2.1). More than 90% of the T-cells were CD3, CD4 and CD8 triple-positive.

Figure 2.1: Characterization of a T-cell line developed using this protocol.

The cell surface markers of an established *Atm*$^{-/-}$ cell line designated DWJ-Atm-1 were characterized by staining with anti-CD3-FITC, anti-CD4-APC, anti-CD8-PE and flow cytometry analysis. Briefly, 1X10^6 fresh cells were washed once in cold PBS (1500 rpm/5 min), and incubated at 4°C for 10 min with 200 µl of antibody cocktail (each antibody at 1:200 dilution in PBS with 1% BSA. Cells can be examined immediately while fresh or fixed for later analysis. Fixed cells are prepared by adding neutral buffered formalin at a 4% (vol/vol) final concentration and incubated at 4°C for 30 min, harvested by centrifugation (1500 rpm/5 min) and resuspended in 200 µl of PBS with 1% BSA. A total of 1 X 10^4 cells were analyzed by using a BD FACS Calibur analyzer and CellQuest software (BD Biosciences, San Jose, CA).

Figure 2.1

(A)



DWJ-Atm-1

(B)

Cell Surface Marker	% Positive cells
$CD8^+$ $CD4^+$	96.2
$CD3^+$ $CD4^+$	85.7
$CD3^+$ $CD8^+$	91.9

Discussion

In this protocol, we provide detailed procedures to establish murine T-cell lines from two different mouse genotypes; *Atm*$^{-/-}$ [FVB/N-*Atm*^{tm1Led}/J] and *p53*$^{-/-}$ [129/S6-*Trp53*^{tm1Tyj}/J]. By using this protocol, a total of 6 (out of 7 attempts) *Atm*$^{-/-}$ and three (out of 5 attempts) *p53*$^{-/-}$ murine T-cell lines have been established in our laboratory. Representative data demonstrating that cell line DWJ-Atm-1, an *Atm*$^{-/-}$ cell line consists of a cell population in which greater than 90% of the cells express CD3, CD4 and CD8 surface markers confirm that clonal T-cell line can be established using our protocol.

Development of murine T-cell lines has been reported (Chervinsky *et al.*, 2001; Kuang *et al.*, 2005; Sharma *et al.*, 2006); however, detailed protocols for establishing these lines have not been described previously. Although only two mouse genotypes were used for derivation of murine T-cell lines in our laboratory, we believe that these procedures are applicable to a wide range of spontaneous T-cell lymphomas found in other strains of mice such as *Kras* defective, and potentially other animal species. Murine T-cell lines have potential applications in understanding basic questions in immunology and oncology. Currently, we are using these cell lines in studies aimed at characterizing host-pathogen interactions and genotoxin-induced cytotoxicity including mechanisms of cell cycle arrest and apoptosis. Given that murine T-cell lines share genomic alterations such as deletions of *PTEN* and *FBXW7* in common with acute human T-cell lymphoblastic leukemias and lymphomas, they are particularly well suited for research in cancer biology (Maser *et al.*, 2007), and may have further applications for rapid pre-clinical efficacy screening of immunological and cancer chemotherapeutic agents in vitro. Whether or not these cell lines can be propagated in vivo in either syngeneic or immunocompromised mice has not been determined.

In contrast to previous protocols in which successful establishment of a T-cell line took up to three months (Chervinsky *et al.*, 2001), cell lines developed using the current protocol were established within a month (between passage 2-8). We found that the presence of loosely adherent cell aggregates over attached stromal cells is a critical early indication of an established cell line. In order to allow murine T-cells to propagate indefinitely, we also found that it is important to leave the initial loosely adherent cell aggregates undisturbed during feedings. In addition, maintaining the cell density at a concentration of approximately $3x10^6$/ml during the first passages also promotes successful establishment of a continuous cell line. In our experience, culturing cells at different densities using several types of culture vessels maximizes the likelihood of establishing a T-cell line. Most T-cell lines were established from T-75 flasks or 6-well plates and 10 or 100 fold dilutions which correspond to approximately $1x10^7$ or $1x10^6$ cells/ml. A previous protocol recommended culturing cells at 5×10^6, 1×10^7, and 2×10^7 cells per well in 6-well plates, and feeding after 7 days (7). We observed that feeding initial cultures at 48 h and passage 72 h later, on day 5 is optimal. Waiting longer before the first feeding and passage will cause the cells to lose their viability and stop dividing.

The last parameter which we found is critical to successful propagation of murine T-cells in vitro is the presence of FBS in the culture medium that can support rapid cell growth. Different lots and suppliers of FBS should be screened prior to initiating the derivation of T-cell lines for identification of an appropriate reagent. If murine T-cell lines are not available for screening, primary cultures of thymic lymphocytes placed in wells of a 24-well plate at a concentration of $1x10^6$ per ml of test medium containing concanavalin A (1µg/ ml) to stimulate rapid cell growth is adequate. In the presence of an optimal source of FBS, the number of cells should double every 18 to 24 h.

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Disclosures

Authors do not declare any conflicts of interests.

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CHAPTER 3

Activation of ATM-dependent Intra-S-phase and G2/M checkpoints and p53-dependent apoptosis in mouse lymphocytes by *Helicobacter hepaticus* CDT[1, 2]

Abstract

Cytolethal distending toxin (CDT) is an AB type genotoxin produced by several important Gram negative bacteria. The active subunit CdtB has structural homology with mammalian deoxyribonuclease I. CDT-induces a DNA damage response (DDR) similar to that elicited by ionizing radiation (IR)-induced DNA double strand breaks (DSBs) leading to ATM-dependent cell cycle arrest at the G2/M and G1/S transitions in mammalian cells. The precise nature of the G2/M arrest in response to CDT has not been resolved and the possibility of CDT inducing the intra-S phase checkpoint has not been investigated. Here we demonstrate the activation of the ATM-dependent intra-S checkpoint by HhepCdtABC in primary mouse thymic lymphocytes and thymic lymphoma cell lines by observing a transient reduction in DNA synthesis. Further, we demonstrate rapid ATM-dependent reduction in phosphorylated histone H3 in HhepCdtABC treated cells thereby definitively demonstrating CDT-induced checkpoint mediated G2/M cell cycle arrest. Additionally we also demonstrate the CDT-induced apoptosis in thymic lymphoma cell lines is p53-dependent. Activation of intra-S checkpoint by CDT may contribute to bacterial virulence by modulating rapidly dividing lymphocytes and thus adaptive immune response following infection. Taken together, we provide first evidence to suggest that CDT induces a broad DDR including cell cycle arrest in all phases of the cell cycle.

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Introduction

Cytolethal distending toxin (CDT) is a heterotrimeric AB type toxin produced by several clinically important Gram negative bacteria (Ge *et al.*, 2008; Guerra *et al.*, 2011; Jinadasa *et al.*, 2011b). The active subunit CdtB has structural homology with phosphodiesterase family of enzymes including mammalian deoxyribonuclease I (Elwell & Dreyfus, 2000; Hu *et al.*, 2006; Hu & Stebbins, 2006; Lara-Tejero & Galan, 2000; Nesic *et al.*, 2004; Yamada *et al.*, 2006). Intoxication of mammalian cells with CDT causes DNA double strand breaks (DSBs) that initiates a DNA damage response (DDR) similar to that elicited by ionizing radiation (IR) and leading to irreversible cell-cycle arrest and apoptosis. Currently CDT is the only known AB type toxin with deoxyribonuclease activity (Alouf, 2006). Interestingly, CDT is the only known virulence factor in mouse pathogen *Helicobacter hepaticus*, which establishes long-term colonization of the lower intestinal and hepatobiliary tract resulting in chronic inflammation and ultimately hepatocellular and colonic carcinomas in susceptible strains (Fox *et al.*, 1994; Hailey *et al.*, 1998; Ihrig *et al.*, 1999; Suerbaum *et al.*, 2003; Ward *et al.*, 1994a; Ward *et al.*, 1994b).

Chronic colitis associated with *H. hepaticus* infection in mice mimics human inflammatory bowel disease (IBD), and therefore is widely used as a model for IBD (Fox *et al.*, 2011). Further, *H. hepaticus* is closely related to the major food-and-waterborne human pathogen *Campylobacter jejuni*. Since there are no effective mouse models to reproduce the pathogenesis of human *C. jejuni* infection, *H. hepaticus* infection in mice has been used as a surrogate model for human campylobateriosis (Suerbaum *et al.*, 2003; Young *et al.*, 2007). *C. jejuni* is the most frequently isolated food-and-water borne human pathogen worldwide, particularly in developed countries (Lindmark *et al.*, 2009; Young *et al.*, 2007). Even though the clinical illness may

manifest as mild non-inflammatory self-limiting enterocolitis, it can lead to severe inflammatory bloody diarrhea and fever (Lindmark *et al.*, 2009; Young *et al.*, 2007). Additionally, *C. jejuni* infection is also considered as a risk factor for subsequent development of Guillain-Barré syndrome; an acute inflammatory demyelization of the peripheral nervous system (Hughes & Cornblath, 2005; Lindmark *et al.*, 2009; Young *et al.*, 2007). The role of CDT in disease pathogenesis is incompletely understood; however, experimental infection of laboratory mice with *H. hepaticus* suggests that CDT plays an essential role in persistent infection and has adaptive immunomodulatory properties (Ge *et al.*, 2005; Ge *et al.*, 2008; Pratt *et al.*, 2006). On the basis of these *in vivo* observations and the comparatively lower dose-response of hematopoietic cells to CDT intoxication *in vitro,* lymphoid cells have been hypothesized as likely physiological targets of CDT (Ge *et al.*, 2005; Pratt *et al.*, 2006; Shenker *et al.*, 2007).

In response to DNA damage, DDR signaling cascades activate several checkpoints and prevent the cells from entering into S phase (G1/S checkpoint), slow the progression through S phase (intra-S or S-phase checkpoint), and block entry into mitosis [(G2/M checkpoint); (Weiss *et al.*, 2003)]. It is currently believed that CDT-induced DSB trigger a DDR mediated by the phosphoinositide 3-kinase related kinase ataxia telangiectasia mutated (ATM), encoded by the gene mutated in the rare autosomal recessive genetic disorder ataxia telangiectasia [AT;(Bartek & Lukas, 2007; Cortes-Bratti *et al.*, 2001; Derheimer & Kastan, 2010; Jackson & Bartek, 2009)]. It is also believed that the effects of CDT are cell type specific (Cortes-Bratti *et al.*, 2001; Hassane *et al.*, 2003; Li *et al.*, 2002; Liyanage *et al.*, 2010). On the basis of its ability to modulate the cell cycle and cause cell death, CDT has been classified as an inhibitory cyclomodulin (Nougayrede *et al.*, 2005; Oswald *et al.*, 2005). In fact, CDT was the first bacterial

toxin shown to cause cell cycle arrest in mammalian cells (Toth *et al.*, 2009). Cell lines of epithelial and endothelial origins has been reported to primarily arrest in the G2/M phase following CDT exposure (Elwell *et al.*, 2001; Jinadasa *et al.*, 2011b; Lara-Tejero & Galan, 2001; Smith & Bayles, 2006; Whitehouse *et al.*, 1998; Wising *et al.*, 2005; Young *et al.*, 2000), whereas cells of fibroblastic origin arrest both in the G1/S and G2/M phases of the cell cycle (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001; Hassane *et al.*, 2003; Smith & Bayles, 2006; Wising *et al.*, 2005). By contrast, hematopoietic lineage cell lines including lymphocytes, monocytes, macrophages and dendritic cells, are several orders of magnitude (10–50 pg/ml compared to 1–5 µg/ml for other cells) more susceptible to CDT, and undergo rapid apoptosis after a brief cell cycle arrest (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001; Hassane *et al.*, 2003; Smith & Bayles, 2006; Wising *et al.*, 2005). Human epithelial and fibroblastic cells that survive the acute phase of CDT intoxication demonstrate the markers of cells that undergo cellular senescence (Blazkova *et al.*, 2010).

Currently CDT is known to cause ATM-dependent cell cycle arrest at the G2/M and G1/S transitions of the cell cycle. The CDT-mediated G2/M arrest is mediated through the activation of Chk2 by ATM upon sensing DSBs. Activated Chk2 phosphorylates and inactivates CDC25 C and the resulting accumulation of phosphorylated cyclin B-CDK 1 complex prevents mitotic entry (Ge *et al.*, 2008; Smith & Bayles, 2006). The mechanism for CDT-mediated G1/S arrest is thought to be primarily p53-dependent. Activated ATM phosphorylates p53, and the resulting upregulation of p21 inhibits cyclin E-CDK2, which blocks S-phase entry (Ge *et al.*, 2008; Smith & Bayles, 2006). However, p53-independent upregulation of p21 following CDT treatment has also been reported (Smith & Bayles, 2006).

Although there is limited evidence supporting CDT-mediated intra S-phase checkpoint activation in yeast, it has yet to be investigated in mammalian systems (Matangkasombut *et al.*, 2009). DNA damage during S phase initiates a down-regulation of DNA synthesis, a phenomenon first evidenced by the failure of Atm-defective cells to inhibit DNA synthesis in response to IR [(radio-resistant DNA synthesis); (Weiss *et al.*, 2003)]. Additionally, the precise nature of the G2/M arrest in response to CDT has not been fully resolved, as at least two G2/M checkpoints exist in mammalian cells, an ATM-dependent transient checkpoint which activates in G2 phase cells immediately after DNA damage, and prolonged ATM-independent checkpoint which is activated several hours after damage and reflects the G2 phase accumulation of cells that were initially damaged during S-phase (Xu *et al.*, 2002).

In the current study we demonstrated the activation of the ATM-dependent intra-S checkpoint by HhepCdtABC in primary mouse thymic lymphocytes and thymic lymphoma cell lines by observing a transient reduction in DNA synthesis. Further, we demonstrate rapid ATM-dependent reduction in phosphorylated histone H3 in HhepCdtABC treated cells thereby definitively demonstrating CDT-induced checkpoint mediated G2/M cell cycle arrest. Additionally we also demonstrate the CDT-induced apoptosis in mouse thymic lymphoma cell lines is p53-dependent. Activation of intra-S checkpoint by CDT may contribute to bacterial virulence by modulating rapidly dividing host lymphocytes and thus adaptive immune response following infection. Taken together, we provide first evidence to suggest that broadly conserved genotoxin CDT is capable of inducing a broad DDR including cell cycle arrest in all phases of the cell cycle.

Materials and Methods

Cell lines and culture conditions. The cell lines used in this study were listed in Table 3.1. The derivation and maintenance of these cell lines was described earlier (Jinadasa *et al.*, 2011a). All cell lines were maintained in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with heat inactivated 10% fetal bovine serum (Hyclone, South Logan, UT) and 1% penicillin streptomycin (Mediatech, Manassas, VA) at 37°C in a humidified atmosphere of 5% carbon dioxide in air. All experiments were performed using cells cultured for 18 to 24 h after transfer to 24-well tissue culture plates unless otherwise indicated. Primary mouse thymic lymphocytes were cultured in same media with 1µg/ml concanavalin A (Sigma cat. # C2272) stimulation.

Table 3.1. Characterization of cell lines used in this study.

	Atm-1	Atm-2	Atm-3	p53-1	p53-2	p53-3
CD4+/CD8+	92.4 (3.8)	94.5(2.5)	91.5(1.2)	96.1(3.7)	91.0 (6.5)	88.1(5.5)
CD3+/CD4+	85.5(0.2)	91.8(0.6)	89.7 (0.7)	99.7(0.1)	88.3(0.4)	85.2(2.2)
CD3+/CD8+	87.5 (6.2)	93.9 (2.8)	90.4 (1.7)	99.5 (0.1)	93.8 (1.3)	88.7(3.4)

The cell surface markers of established $Atm^{-/-}$ and $p53^{-/-}$ were characterized by staining with anti-CD3-FITC, anti-CD4-APC and anti-CD8-PE by low cytometry as described earlier (1). Data is expressed as mean percentage and standard deviation of cells staining positive for each marker in two independent experiments (R. J).

Recombinant reconstituted *H. hepaticus* CDT (HhepCdtABC). The his-tagged proteins were expressed in *E. coli*. HhepCdtABC was produced as described earlier (Dassanayake *et al.*, 2005; Liyanage *et al.*, 2010). 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) and grown an additional 5 h at 25°C. HhepCdtA and HhepCdtB were purified under denaturing conditions while HhepCdtC 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. Fornegative controls, reconstituted recombinant HhepCdtABC was heat inactivated (HI) for 10 min at 70°C and stored at -80°C in 1:1 sterile glycerol. Briefly, the gene sequence encoding each CjejCDT sub-unit was amplified by using specific primer pairs (Table 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 DE (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) 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 CjejCdtABC was heat inactivated (HI) for 10 min at 70°C and stored at -80°C in 1:1 sterile glycerol.

Western blotting. Approximately $3x10^6$ cells (5 ml cultures in T25 flasks) were incubated with 100 ng/ml native or HI CjejCdtABC or γ-irradiated (5Gy; positive control). Western immunoblot was performed as described earlier with modifications (Liyanage *et al.*, 2010). Briefly, cells were lysed in RIPA buffer with protease and phosphatase inhibitors (aprotinin, leupeptin, phenylmethanesulfonyl fluoride and sodium orthovanadate (all from Sigma, 1 mM each) and incubated on ice for 10 min. The lysate was briefly sonicated (1 min at 0.5-2 power setting on a Misonix 3000 sonicator (Misonix Inc. Farmingdale, NY). Protein concentrations were determined by Bradford's method (Bradford, 1976). Proteins were separated on 12-13% (4% for ATM) SDS PAGE, transferred to PVDF membranes and immunoblotted with following antibodies; anti-phospho ATM (Rockland, cat. #200-301-400), anti- γ-H2AX (Millipore, cat.# 05-636), anti-beta actin (Sigma, cat.# A5441). Bands were detected with ECL (Advansta, cat. # K-12045) according to manufacturer's instructions using Versa Doc (Bio Rad) gel imaging system.

Detection of γ-H2AX fluorescence-activated cell sorter (FACS). Approximately $1x10^6$ cells were incubated with 100 ng/ml native or HI HhepCdtABC for 2 h to 8 hand phosphorylation of γ-H2AX was detected by flow cytometry as described earlier (Zhu & Weiss, 2007). A total of $2x10^4$ cells were analyzed on a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences).

Determination of apoptosis by fluorescence-activated cell sorter (FACS) analysis. Cultures grown to a density of 1x 10^6 cells/ml were incubated in the presence of native or HI CjejCdtABC (100 ng/ml) for 24 h and the number of apoptotic cells was determined by FACS analysis. Characteristic apoptotic conformational change in cell membrane associated with phosphatidylserine translocation was assessed by using annexin-V and propidium iodide (PI) staining according to previously described protocols with modifications (Martin *et al.*, 1995; Shounan *et al.*, 1998). Briefly, cells were washed once in cold PBS and resuspended in 1X binding buffer containing 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ and 0.1% bovine serum albumin (BSA), pH 7.4 at room temperature (RT). Cells were stained with 50 µg/ml annexin V-FITC (Santa Cruz Biotechnology, Santa Cruz, CA) and PI (Sigma) for 10 min at RT in the dark. A total of 2x10^4 cells were analyzed using a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences, San Jose, CA) and classified as early or late apoptotic, necrotic or viable as previously described (Shounan *et al.*, 1998; Vermes *et al.*, 1995).

Direct detection of apoptotic nuclear morphology using Hoechst 33342 and PI double staining. Cell cultures at a density of 1x10^6 cells/ml were incubated with 100 ng/ml CjejCdtABC or heat inactivated CDT. Apoptotic cell death was assessed by microscopic detection of characteristic apoptotic nuclear morphology using a double fluorescence method as described previously (Muscarella & Bloom, 1997; Muscarella & Bloom, 2003; O'Brien *et al.*, 2001). Briefly, 200 µl of cell suspension were stained with 20 µg/ml PI (red fluorescence, Sigma) and 100 µg/ml Hoechst 33342 (blue fluorescence; Sigma) for 10 min at 37°C in the dark. For each sample, a total of 200 cells were counted using a Leitz Aristoplan fluorescence microscope with long-pass filter cube A at 400X magnification. Apoptotic cells were identified based on the

presence of characteristic condensed, segregated chromatin bodies in intact but shrunken cells, which were discriminate from necrotic cells that were swollen, with irregular or damaged membranes and propidium iodide positive. Early apoptotic changes were characterized by condensed and fragmented chromatin (with blue fluorescence) and late apoptotic changes consisted similar nuclear changes and cytoplasmic uptake of propidium iodide (pink fluorescence) and classified as either apoptotic, necrotic, or normal/viable (O'Brien *et al.*, 2001).

Detection of phospho-Histone H3 by (FACS). Approximately $1x10^6$ cells were incubated with 100 ng/ml native or HI HhepCdtABC for 6 h with or without nocodazole. Phosphorylation of H3 was detected by flow cytometry as described earlier (Xu *et al.*, 2002). A total of $2x10^4$ cells were analyzed on a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences).

Radioresistant DNA synthesis (intra-S checkpoint activation) assay. The assay was performed as previously described with modifications (Weiss *et al.*, 2003). Briefly, triplicate cultures of $1x10^6$ cells in 200 µl volumes in 96 well plates were incubated with 100 ng/ml native or HI HhepCdtABC for 30 mins and the cells. The cells were pulsed for 1 h with 0.5 µCi/ml 3H-thymidine (Amersham Biosciences, Buckinghamshire, UK) and harvested onto glass-fiber filters (Brandel, Gaithersberg, MD) using a Brandel M-12 cell harvester (Brandel, Gaithersberg, MD). Radioactivity was measured by scintillation counting using a Beckman LS6500 scintillation counter (Beckman, Fullerton, CA).

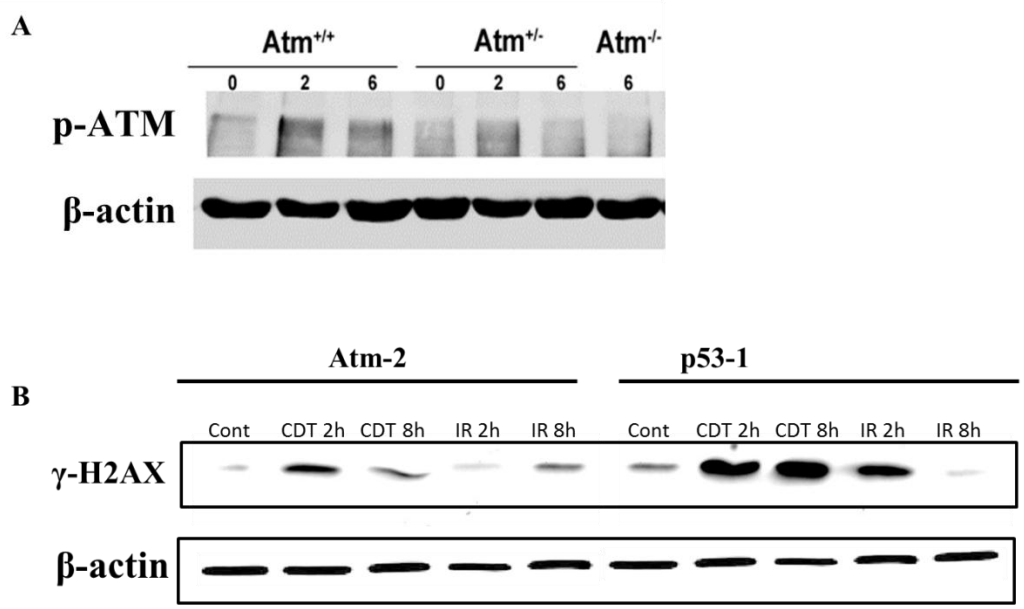
Statistical analysis. Statistical analysis was performed using JMP Pro version 9.02 (SAS Institute Inc, Cary, NC). Data are presented as mean ± SD. A p value < 0.05 was considered as significant.

Results

HhepCdtABC activates an ATM-dependent DNA damage responses (DDR). The CDT-induced DDR, particularly the phosphorylation of ATM in response to CDT-induced DNA damage is not well characterized in lymphoid cell lines. Therefore, HhepCdtABC-induced DDR was initially characterized using $Atm^{+/+}$, $Atm^{+/-}$, and $Atm^{-/-}$ primary mouse thymic lymphocytes or thymic lymphoma cell lines. Phosphorylation of ATM in primary mouse thymic lymphocytes as early as 2 h and 6 h after RecHhCdtABC treatment (Figure 3.1A). It was interesting to observe that the lymphocytes from $Atm^{+/-}$ mice have a lower level of CDT-induced ATM phosphorylation compared to those from wild type mice. This is the first demonstration (along with our parallel demonstration of ATM phosphorylation by CjejCdtABC in human lymphoid cell lines; chapter 4) of the phosphorylation of ATM by CDT from any bacteria providing direct evidence for initiating ATM dependent DDR by CDT-mediated DNA damage. Similarly, increase in γH2AX was observed by western blot 2 h post exposure to HhCdtABC in $p53^{-/-}$ ($Atm^{+/+}$ control) cell line compared to $Atm^{-/-}$ cell line. However, slight increase in γH2AX was evident at 2 h post exposure in $Atm^{-/-}$ cell line (Figure 3.1B). Significantly higher (**, $p< 0.05$) increase in γH2AX positive cells were observed in $p53^{-/-}$ ($Atm^{+/+}$ control) cell line compared to $Atm^{-/-}$ cell line 6 h after incubation with HhCdtABC (Figure 3.1C).

Figure 3.1: HhepCdtABC activates an ATM-dependent DNA damage responses (DDR). Representative Western blots from two independent experiments showing the phosphorylation of ATM in primary mouse thymocytes (A) and γ-H2AX in TLC (B) following HhepCdtABC exposure. Proteins were separated on 13% (4% for p-ATM) SDS PAGE and blotted with mouse mAb anti- p-ATM and anti- γ-H2AX. Membranes were blotted with mouse mAb anti- β-actin as loading control. Phosphorylation of γ-H2AX in TLC analyzed by flow cytometry (C). TLC incubated with native or HI RecHhCDT (100ng/ml) for 6 h, fixed in 70% ethanol and stained with mAb anti-γH2AX and FITC labeled secondary antibody. Data represents means of two independent experiments. Significance of differences between treated and control cultures were tested by using the two way ANOVA test [(***, $p < 0.001$; NS, not significant), (G. B and R. N. J)].

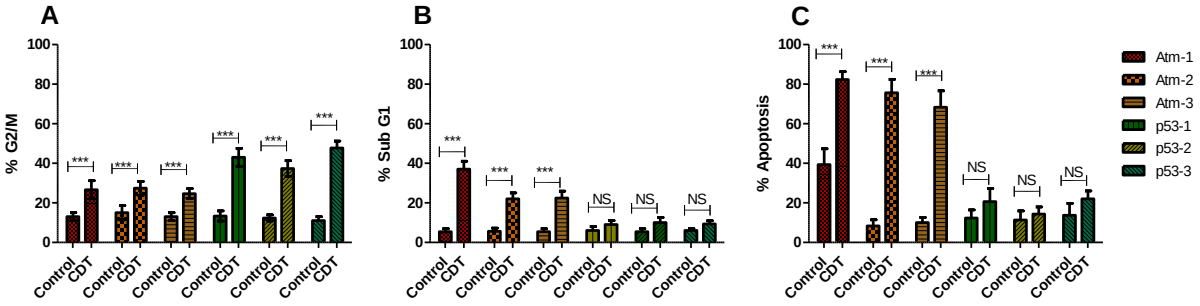
Fig.3.1.


A
Atm+/+
Atm+/-
Atm-/-
0
2
6
0
2
6
6
p-ATM
β-actin
B
Atm-2
p53-1
Cont
CDT 2h
CDT 8h
IR 2h
IR 8h
Cont
CDT 2h
CDT 8h
IR 2h
IR 8h
γ-H2AX
β-actin
C
% γ-H2AX positive cells
40
30
20
10
0
NS
NS
***
***
HI CDT
Native CDT
Atm-1
Atm-2
p53-1
p53-2

HhepCdtABC activates G2/M cell cycle arrest leading to p53-dependent apoptosis. The activation of G2/M checkpoint and apoptosis was initially characterized by flow cytometry using thymic lymphoma cell lines. The initial cell cycle experiments revealed the accumulation of significantly higher levels of cells with 4N DNA content in both *Atm*$^{-/-}$ and *p53*$^{-/-}$ thymic lymphoma cell lines treated with HhepCdtABC for 24 h (Figure 3.2A). However, as expected, the apoptosis (Sub G0 DNA content) was only significant in *Atm*$^{-/-}$ cell lines (Figure 3.2B). CDT-induced apoptosis was further characterized by double staining the cells with Annexin V and PI. Only the *Atm*$^{-/-}$ cell lines had a significant increase in apoptosis (figure 3.2C), which confirmed the previous observations with cell cycle experiments. These findings suggest that the HhepCdtABC activates G2/M checkpoint leading to apoptosis in thymic lymphoma cell lines.

Figure 3.2: HhepCdtABC activates G2/M cell cycle arrest leading to p53-dependent apoptosis. A total of $1x10^6$ TLC were incubated in the presence of native or heat inactivated HhepCdtABC (100 ng/ml) for 24 h and percentages of cells arresting at G2/M as detected by the 4N DNA content (A), and apoptosis as detected by Sub G1 DNA content (B) by analyzing $2x10^4$ cells fixed in 70% ethanol and stained with propidum iodide (PI). The percent apoptotic cell death was assessed by flow cytometric analysis of $2x10^4$ cells stained with annexin V-FITC and PI (C). Each bar represents the mean percent apoptosis ± SD by adding the percent cells positive for annexin V-FITC only (early apoptosis) with the percent cells positive for both annexin V-FITC and PI (late apoptosis) in three separate experiments. For each cell line, the mean percent apoptosis of treated and control cultures were analyzed by using two way ANOVA test [(***, $p < 0.001$; NS, not significant), (R. N. J.)].

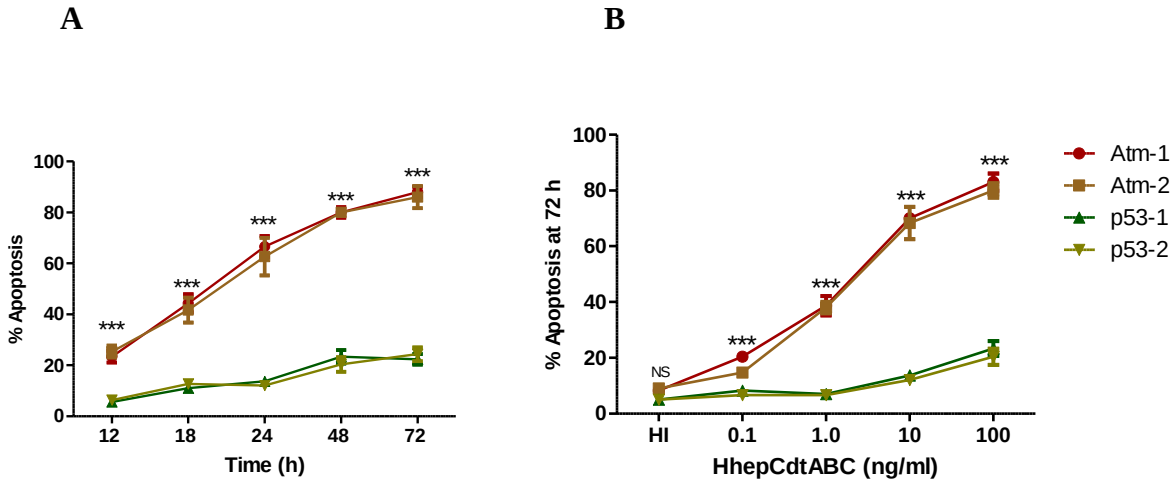
Fig.3.2.


A
% G2/M
B
% Sub G1
C
% Apoptosis
Control
CDT
***
NS
Atm-1
Atm-2
Atm-3
p53-1
p53-2
p53-3

The p53-dependent HhepCdtABC-induced apoptosis is time- and dose-dependent. In order to confirm the p53-dependent apoptosis observed in Figure 3.2, the kinetics of CDT-induced apoptosis was further investigated by simultaneous microscopic detection of characteristic apoptotic nuclear morphology and plasma membrane integrity by dye exclusion to detect necrosis according to well validated previously described methods (Bloom *et al.*, 2006; Jinadasa *et al.*, 2011b; Muscarella & Bloom, 1997; Muscarella *et al.*, 2003; O'Brien *et al.*, 2001). In agreement with our flow cytometry data, the Atm null cell lines with wild-type p53 displayed increased time- and dose-dependent susceptibility to CDT-induced apoptosis as compared to p53 null cell lines (Figure 3.3). When incubated with 100ng/ ml CDT, $p53^{-/-}$ cell lines had significantly less apoptosis compared to $Atm^{-/-}$ (p53 wild type) cell lines up to 72 h (Figure 3.3 A). The dose response to CDT was also highly correlated to the p53 status of the cell lines (Figure 3.3 B). The $Atm^{-/-}$ (p53 wild type) cell lines were significantly more susceptible to CDT within the entire 0.1 -100 ng/ml range of toxin concentrations (Figure 3.3 B).

Figure 3.3: The p53-dependent HhepCdtABC-induced apoptosis is time- and dose-dependent. A total of $1x10^6$ TLC were incubated in the presence of native or heat inactivated HhepCdtABC (100 ng/ml) and apoptotic cell death was assessed by simultaneous detection of plasma membrane integrity by dye exclusion and examination of characteristic apoptotic nuclear morphology. Apoptotic cells were identified based on the presence of characteristic condensed, segregated chromatin bodies in intact but shrunken cells, which were discriminate from necrotic cells that were swollen, with irregular or damaged membranes. Early apoptotic changes were characterized by condensed and fragmented chromatin (with blue fluorescence) and late apoptotic changes consisted similar nuclear changes and cytoplasmic uptake of propidium iodide (pink fluorescence). A total of 200 cells were scored for each sample and classified as either apoptotic, necrotic, or normal/viable. Values are means ±SD (n=3). For each time point or CDT concentration, the significance of differences between treated and control cultures were tested by using the two way ANOVA test with Bonferroni correction (***, $p < 0.001$; NS, not significant).Cells incubated with100 ng/ml HhepCdtABC up to 72 h (A).Cells incubated with varying concentrations of HhepCdtABC for 72 h [(B), (R. N. J)]

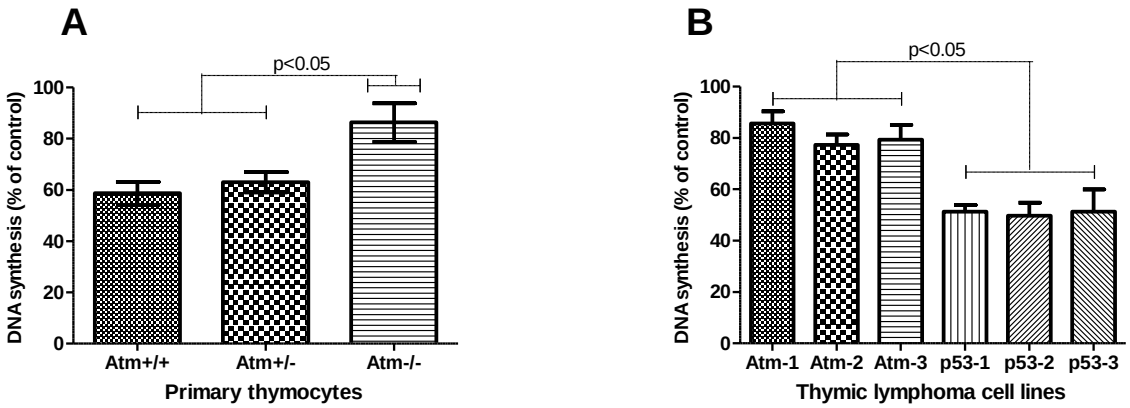
Fig.3.3.


A
B
% Apoptosis
Time (h)
12
18
24
48
72
***
% Apoptosis at 72 h
HhepCdtABC (ng/ml)
HI
0.1
1.0
10
100
NS
Atm-1
Atm-2
p53-1
p53-2

HhepCdtABC activates of intra-S checkpoint in an ATM-dependent manner. In order to explore the ability of CDT to activate intra-S checkpoint, we then characterized the transient reduction in DNA synthesis (classical marker for intra S-phase checkpoint) in HhepCdtABC treated cell lines. We observed a significant reduction in DNA synthesis in HhepCdtABC treated wild type and heterozygous primary mouse thymic lymphocytes and *p53*$^{-/-}$ thymic lymphoma cell lines compared to Atm null cells and cell lines (Figure 3.4). This is the first definitive demonstration of the activation of intra S-checkpoint by the broadly conserved bacterial genotoxin CDT, and we conclude that CDT is capable of inducing arrest at all phases of cell cycle.

Figure 3.4: HhepCdtABC activates of intra-S checkpoint in an ATM-dependent manner. A total of $1x10^6$ primary mouse thymic lymphocytes obtained from 6-8-week-old mice stimulated for 24 h with Concanavalin A (1µg/ml) (A), or thymic lymphoma cell lines (B) were incubated in the presence of native or heat inactivated HhepCdtABC (100 ng/ml) for 30 min. Transient reduction of DNA synthesis was measured by pulsing 1 h with ^{3}H-thymidine. Data is expressed as the mean and standard deviation of three independent experiments. Significance of differences between cell lines were tested by using the two way ANOVA test with Bonferroni correction (R. N. J).

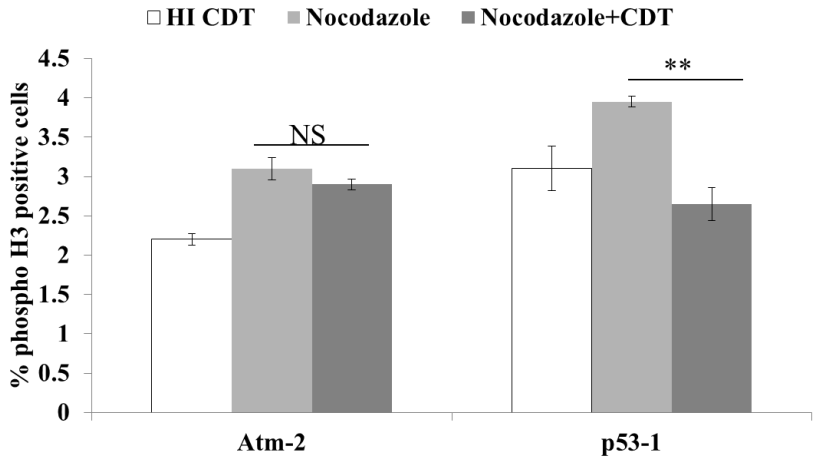
Fig. 3.4.


A
p<0.05
DNA synthesis (% of control)
100
80
60
40
20
0
Atm+/+
Atm+/-
Atm-/-
Primary thymocytes
B
p<0.05
DNA synthesis (% of control)
100
80
60
40
20
0
Atm-1
Atm-2
Atm-3
p53-1
p53-2
p53-3
Thymic lymphoma cell lines

HhepCdtABC activates ATM-dependent G2/M checkpoint. In order to further characterize the ATM-dependent G2/M checkpoint, we analyzed the HhepCdtABC treated cells by flow cytometry for the phophorylation of histone H3 (phospho-H3), which is a specific marker for mitotic cells. Nocodazole was used as a control to block the cells in mitosis (M phase). As expected, we observed a reduction in phospho-H3 positive cells in $p53^{-/-}$ cells as compared to $Atm^{-/-}$ cells. This is the first definitive demonstration of CDT-mediated G2/M cell cycle arrest (Figure 3.5).

Figure 3.5. HhepCdtABC activates ATM-dependent G2/M checkpoint. A total of $1x10^6$ primary mouse thymic lymphocytes obtained from 6-8-week-old mice stimulated for 24 h with Concanavalin A (1µg/ml) (A), or thymic lymphoma cell lines (B) were incubated with native or heat inactivated HhepCdtABC (100 ng/ml) with or without nocodazole for 6 h. Cells were fixed and stained with anti-Histone H3 antibody, FITC labeled secondary antibody and counter stained with PI for DNA content. Data represents means of two experiments [(**, $p < 0.01$; NS, not significant), (R. N. J.)].

Fig. 3.5.


HI CDT
Nocodazole
Nocodazole+CDT
% phospho H3 positive cells
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
NS
**
Atm-2
p53-1

Discussion

Mammalian cell cycle regulation is responsible for the maintenance of intact mucosal epithelial barriers and clonal expansion of lymphocyte subsets during adaptive immune response, both of which are important defense mechanisms against colonization and infection of mucocutaneous interfaces by bacterial pathogens (Oswald *et al.*, 2005). Therefore, CDT-producing bacteria might have evolved a specialized mechanisms to disrupt key mammalian cell cycle functions in order to establish persistent colonization and cause disease in certain niches (Nougayrede *et al.*, 2005; Oswald *et al.*, 2005). Cell cycle fidelity is maintained by redundant DNA damage checkpoint mechanisms (Bartek & Lukas, 2007; Jackson & Bartek, 2009). Activation of DNA damage checkpoints results in cell cycle arrest so that DNA can be repaired, or if damage is severe, progress to programmed cell death by apoptosis(Bartek & Lukas, 2007). The DNA damage checkpoint network comprises upstream DNA damage sensors, signal transducers and downstream effectors (Bartek & Lukas, 2007).

The ATM-dependent IR-induced DDR involves both induction of cell cycle arrest and initiation of DNA repair (Derheimer & Kastan, 2010). Major components of the ATM-dependent DNA damage signaling pathway include the multifunctional MRN protein complex consisting of Mre11, Rad50 and Nbs1, histone H2AX, the cell cycle checkpoint regulator protein kinase Chk2, and the transcription factor p53(Bartek & Lukas, 2007; Derheimer & Kastan, 2010; Jackson & Bartek, 2009). Initial DSBs are recognized by the MRN complex, which recruits ATM to the damage site (Bakkenist & Kastan, 2003; Bartek & Lukas, 2007; Lavin & Kozlov, 2007; Lavin, 2008). The serine-threonine protein kinase ATM which normally exists as inactive dimers, dissociates and is activated by autophosphorylation in the presence of DSBs. Activated ATM

subsequently phosphorylates a large array of substrates including, histone H2AX, Chk2, and p53(Matsuoka *et al.*, 2007). Although the precise events surrounding ATM activation are incompletely understood, recent evidence suggests that ATM and MRN complex act in harmony to sense DSBs (Derheimer & Kastan, 2010). Activated MRN complex and phosphorylated H2AX (γ-H2AX) initiate and amplify the DNA repair process by stabilizing the DNA lesion and providing a platform for the binding of other DNA repair proteins (Derheimer & Kastan, 2010). Activated Chk2 reduces cyclin-dependent kinase (CDK) activity by several mechanisms including activation of the transcription factor p53 and subsequent induction of cyclin inhibitor p21 (Jackson & Bartek, 2009). CDKs are key regulators of cell cycle and the inhibition of CDKs can result in arrest of the cell-cycle progression at the G1-S, intra-S and G2-M checkpoints, allowing time for DNA repair before proceeding with replication or mitosis (Jackson & Bartek, 2009). Other ATM substrates additionally have key roles in affecting cell cycle checkpoint function, such as Smc1 for intra-S phase, as reviewed elsewhere (Derheimer & Kastan, 2010).

The role of *Atm* in CDT induced cell cycle arrest and apoptosis was previously demonstrated only in several human leukemic cell lines. *Atm* wild-type SN-B1 and JAC-B2 cells were more susceptible to CDT-induced apoptosis compared to four cell lines *Atm* defective cell lines. γ-H2AX was readily detectable in $Atm^{+/+}$ cells but not in $Atm^{-/-}$ cells exposed to HducCDT. Taken together, current study is the first demonstration of the activation of ATM-dependent DNA repair response by CDT using well defined *Atm* defective primary cells or cell lines. Our observations on HhepCdtABC-induced γ--H2AX activation agrees with previous demonstrations of CDT-induced DDR. A large percentage of HeLa cells display γ-H2AX foci 2 h post-HducCDT exposure, and nearly all cells are positive for γ-H2AX foci within 6 to 8 h post-exposure (Li *et*

al., 2002). Increased Rad50 foci and γ-H2AX are also seen in primary human fibroblasts treated with CjejCDT (Hassane *et al.*, 2003), in primary and established human endothelial cells treated with EcolCdtB-V (Bielaszewska *et al.*, 2005), and cultured human intestinal epithelial H407 cells exposed to HhepCDT (Liyanage *et al.*, 2010). Higher levels of γ-H2AX was detected in immortalized wild type human B lymphocyte lines post-HducCDT exposure, but it is absent in *Atm*-defective leukemic B cell lines obtained from AT patients (Li *et al.*, 2002).

Currently accepted consensus for CDT-mediated G2/M arrest is the phosphorylation of ATM followed by Chk2 activation, which phosphorylates and inactivates CDC25C. Resulting accumulation of phosphorylated cyclin B-CDK1 complex prevents mitotic entry. Limitations in our understanding of the molecular mechanisms of CDT-induced cell cycle arrest are partially due to the techniques that are commonly employed to address this question. With the exception of a few instances indicating accumulation of phosphorylated Cdk1 (cdc2) prior to cell cycle arrest, CDT-induced arrest of the cell cycle relies primarily on demonstration of altered patterns of DNA staining with propidium iodide and analysis by florescence-activated cell sorting (Bielaszewska *et al.*, 2005; Comayras *et al.*, 1997; Cortes-Bratti *et al.*, 2001; Peres *et al.*, 1997). At least two G2/M checkpoints exist in mammalian cells, an ATM-dependent transient checkpoint which activates in G2 phase cells immediately after DNA damage, and prolonged ATM-independent checkpoint which is activated several hours after damage and reflects the G2 phase accumulation of cells that were initially damaged during S-phase (Weiss *et al.*, 2003; Xu *et al.*, 2002). Because CDT might induce arrest at multiple stages of the cell cycle, measurement of DNA content alone cannot distinguish between these two checkpoints highlighting the need to delineate the mechanism of CDT-induced cell cycle arrest in greater details. Therefore, we

analyzed the CDT treated cells by flow cytometry for the phophorylation of histone H3 (phospho-H3), and this is the first definitive demonstration of CDT-mediated G2/M cell cycle arrest.

We characterized the kinetics of CDT-induced apoptosis using simultaneous microscopic detection of characteristic apoptotic nuclear morphology and plasma membrane integrity by dye exclusion to detect necrosis according to well validated previously described methods and flow cytometry. Although there is limited evidence supporting the CDT-mediated intra S-phase checkpoint activation, the underlying mechanism is not completely investigated. We demonstrated the activation of the ATM-dependent intra-S checkpoint in primary mouse thymic lymphocytes and thymic lymphoma cell lines. This is the first definitive demonstration of the activation of intra S-checkpoint by the broadly conserved bacterial genotoxin CDT. Activation of intra-S checkpoint by CDT may contribute to bacterial virulence by modulating rapidly dividing host lymphocytes and thus adaptive immune response following infection. Taken together, we provide first evidence to suggest that broadly conserved genotoxin CDT is capable of inducing cell cycle arrest in all phases of the cell cycle.

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CHAPTER 4

Contribution of human lymphoid cell line p53 status to genotoxicity induced by *Campylobacter jejuni* cytolethal distending toxin[1, 2]

Abstract

Cytolethal distending toxin (CDT) is an AB type genotoxin produced by several clinically important human and animal pathogens including *Campylobacter jejuni*, a major cause of infectious diarrheal illness worldwide. Intoxication of mammalian cells with CDT causes DNA double strand breaks (DSBs) elicits a DNA damage response (DDR) characterized by irreversible cell cycle arrest and apoptosis. Although the DNA damage responsive tumor suppressor protein p53 is critical to the response of mammalian cells to genotoxins, the contribution of p53 to CDT-mediated genotoxicity is incompletely understood. Therefore the purpose of the current study was to further characterize the role of p53 in the differential susceptibility of several human lymphoid cell lines with different p53 status to the DDR induced by CDT *in vitro*. The human leukemic T-cell lines MOLT-3 and MOLT-4 with functionally wild-type p53 displayed increased time- and dose-dependent susceptibility to CDT-induced apoptosis compared to p53 mutated Jurkat and CEM cell lines. But in contrast, the *p53* mutated and proapoptotic Bcl-2 protein Bax deficient CA 46 Burkitt lymphoma cell line was resistant to CDT-induced apoptosis. The comparable γ-H2AX activation observed in all cell lines after CDT treatment confirmed similar levels of CDT-induced DSBs irrespective of p53 status. Taken together, the data suggest that p53 mutational status correlates with the differential CDT-susceptibility of human lymphoma and leukemia cell lines.

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[2]Author contributions: R. P. D constructed *E. coli* BL21 with p28a(+)CjejCdtB while R. N. J and G. B performed all other experiments (individual contributions are indicated in figure legends). R. S. W. and G. E. D. designed the experiments. R. N. J., S. E. B., R. S. W. and G. E. D. wrote the manuscript.

Introduction

Cytolethal distending toxin (CDT) is a heterotrimeric AB type genotoxin produced by several species of Gram negative Gamma and Epsilon classes of Proteobacteria (Alouf, 2006; Ge *et al.*, 2008; Guerra *et al.*, 2011; Jinadasa *et al.*, 2011; Lara-Tejero & Galan, 2001). Included in this group are members of *Campylobacter, Shigella, Salmonella* and certain *E. coli* pathotypes which together represent all major food and water borne bacterial pathogens of clinical importance worldwide (den Bakker *et al.*, 2011; Haghjoo & Galan, 2004; Jinadasa *et al.*, 2011; Okuda *et al.*, 1995; Pickett *et al.*, 1994; Pickett *et al.*, 1996). In this context, *Campylobacter jejuni* is the leading cause of infectious diarrheal illness worldwide (Lindmark *et al.*, 2009; Young *et al.*, 2007). The clinical presentation with campylobacteriosis can range from a mild self-limiting enterocolitis to severe inflammatory bloody diarrhea and fever (Lindmark *et al.*, 2009; Young *et al.*, 2007). Infection with *C. jejuni* is also a major risk factor for subsequent development of Guillain-Barré syndrome; an acute inflammatory demyelinating disease of the peripheral nervous system (Hughes & Cornblath, 2005; Janssen *et al.*, 2008; Lindmark *et al.*, 2009; Young *et al.*, 2007). The role of CDT in the pathogenesis of disease caused by *C. jejuni* is incompletely understood; however, experimental infection of laboratory mice by the related intestinal pathogen *Helicobacter hepaticus* suggests that CDT plays an essential role in persistent infection and has adaptive immunomodulatory properties (Ge *et al.*, 2005; Ge *et al.*, 2008; Pratt *et al.*, 2006). On the basis of these *in vivo* observations and the comparatively lower dose-response of hematopoietic cells to CDT intoxication *in vitro*, lymphoid cells have been hypothesized as likely physiological targets of CDT (Ge *et al.*, 2005; Pratt *et al.*, 2006; Shenker *et al.*, 2007).

With the exception of *Salmonella enterica* serovars which only has the active subunit CdtB, all other bacterial pathogens produce CDT holotoxin consisting of a complex of three subunits designated CdtA, CdtB and CdtC (Jinadasa *et al.*, 2011). The CDT subunits are encoded by adjacent or slightly overlapping *cdtA, cdtB* and *cdtC* genes forming a constitutively expressed operon (Jinadasa *et al.*, 2011) which in *C. jejuni* have approximate molecular masses of 27-, 30- and 20-kDa, respectively (Pickett *et al.*, 1996). Because the active subunit of CDT, CdtB has structural and functional homology with members of the phosphodiesterase family of enzymes including mammalian deoxyribonuclease I, it is the only known AB type toxin that has DNase activity (Alouf, 2006; Elwell & Dreyfus, 2000; Hu *et al.*, 2006; Hu & Stebbins, 2006; Lara-Tejero & Galan, 2000; Nesic *et al.*, 2004; Yamada *et al.*, 2006).

Intoxication of mammalian cells with CDT causes DNA double strand breaks (DSBs) that initiates a DNA damage response (DDR) similar to that elicited by ionizing radiation (IR) and characterized by activation of γ-H2AX leading to irreversible cell-cycle arrest and apoptosis. The genotoxic effects of CDT *in vitro* are cell-type specific (Cortes-Bratti *et al.*, 2001; Eshraghi *et al.*, 2010; Hassane *et al.*, 2003; Li *et al.*, 2002; Smith & Bayles, 2006). Intoxicated epithelial and endothelial cells primarily arrest in the G2/M phase (Elwell *et al.*, 2001; Jinadasa *et al.*, 2011; Lara-Tejero & Galan, 2001; Smith & Bayles, 2006; Whitehouse *et al.*, 1998; Wising *et al.*, 2005; Young *et al.*, 2000), whereas mesenchymal cells like fibroblasts typically arrest both in the G1/S and G2/M phases of the cell-cycle (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001; Hassane *et al.*, 2003; Smith & Bayles, 2006; Wising *et al.*, 2005). By contrast, cells of hematopoietic lineage including lymphocytes, monocytes, macrophages and dendritic cells, are several orders of

magnitude more susceptible to CDT, and undergo rapid apoptosis (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001; Hassane *et al.*, 2003; Smith & Bayles, 2006; Wising *et al.*, 2005).

The molecular mechanism of CDT-induced apoptosis remains incompletely understood; however, some evidence suggests the involvement of both p53-dependent and -independent pathways (Jinadasa *et al.*, 2011; Liyanage *et al.*, 2010; Ohara *et al.*, 2004; Ohara *et al.*, 2008). Following CDT exposure, epithelial cells appear to undergo both p53-dependent and -independent apoptosis with comparable kinetics, while lymphocytes, a primarily p53-dependent mitochondrial pathway has been suggested (Jinadasa *et al.*, 2011; Ohara *et al.*, 2004). However, the contribution of p53 mutational status of cell lines to CDT-mediated genotoxicityin general and CDT-induced DDR of lymphoid cells in particular are incompletely characterized. While it is well-established that cellular toxicity of CDT is due to the nuclease activity of CdtB, a recent study suggests a potential role of phosphatidylinositol (PI)-3,4,5-triphosphate (PIP_3) phosphatase activity in CdtB in cellular toxicity through AKT pathway in human leukemia T-cell lines with constitutively elevated PIP_3 levels (Shenker *et al.*, 2007).

Although the DNA damage responsive tumor suppressor protein p53 is critical to the response of mammalian cells to genotoxic stress leading to cell-cycle arrest and apoptosis, the contribution of p53 to CDT-mediated genotoxicity in general, and the increased susceptibility of lymphoid cells in particular, is incompletely understood. In the current study we investigated the role of p53 in CDT-susceptibility of human lymphoma and leukemia cell lines. Consistent with a recently proposed modification of a widely accepted nomenclature of CDT proteins produced by various bacteria originally proposed by Cortes-Bratti and co-workers this revised nomenclature will be

used throughout this paper (Cortes-Bratti *et al.*, 1999; Jinadasa *et al.*, 2011). Cell lines with functionally wild-type p53 displayed increased time- and dose-dependent susceptibility to recombinant reconstituted *C. jejuni* CDT (CjejCdtABC) induced apoptosis as compared to p53 mutated cell lines. The comparable γ-H2AX activation observed in all cell lines after CjejCdtABC treatment confirmed similar levels of CjejCdtABC-induced DNA damage irrespective of p53 status. Additionally we did not observed any detectable change in phosphorylated Akt levels following CjejCdtABC treatment, suggesting that nuclease activity of CjejCdtABC is the primary contributor of genotoxicity. Taken together, these results suggest that p53 mutational status may be a major determinant of the differential CjejCdtABC-susceptibility of human lymphoma and leukemia cell lines.

Materials and Methods

Cell lines and culture conditions. The cell lines used in the present study and their respective p53 genomic status are presented in Table 4.1. All cell lines were maintained in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with heat inactivated 10% fetal bovine serum (Hyclone, South Logan, UT) and 1% penicillin streptomycin (Mediatech, Manassas, VA) at 37°C in a humidified atmosphere of 5% carbon dioxide in air. All experiments were performed using cells cultured for 18 to 24 h after transferring to 24-well culture plates unless otherwise indicated.

Table 1: Lymphoma and leukemia cell lines used in this study.

Table 4.1. Cell lines used in this study.

Cell line	ATCC #[a]	p53 genomic status	Reference
MOLT-3	CRL-1552	Wild type	(Cai *et al.*, 2001)
MOLT-4	CRL-1582	Heterozygous	(Cheng & Haas, 1990; Hasegawa *et al.*, 2009; Ohara *et al.*, 2004; Rodrigues *et al.*, 1990)
Jurkat	TIB-152	Mutant	(Cheng & Haas, 1990; Hasegawa *et al.*, 2009; Ohara *et al.*, 2004)
CCRF-CEM	CCL-119	Mutant	(Cheng & Haas, 1990)
CA 46	CRL-1648	Mutant [b]	(Cherney *et al.*, 1997; Gutierrez *et al.*, 1999; O'Brien *et al.*, 2001)

[a]ATCC, American Type Culture Collection, Manassas, Virginia

[b] also *Bax* deficient

Reconstituted recombinant *C. jejuni* CDT (CjejCdtABC). 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) 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 CjejCdtABC was heat inactivated (HI) for 10 min at 70°C and stored at -80°C in 1:1 sterile glycerol.

Table 4.2: Primers for expression of *Campylobacter jejuni* cytolethal distending toxin subunits.

Primer[a]	Sequence[b]
CjejCdtA F:	5'-CGGAATTCTTTGAAAATG TAAATC -3'
CjejCdtA R:	5'-CCGCTCGAGTCATCGTACCTCTC-3'
CjejCdtB F:	5'- CGGAATTCCAATTTAGAAAATTTTAATGTTGGC -3'
CjejCdtB R:	5'-CCGCTCGAGCTAAAATTTTCTAAAATTTACTG -3'
CjejCdtC F:	5'-CGGAATTCACTCCTACTGGAG -3'
CjejCdtC R:	5'- CCGCTCGAGTTATTCTAAAG -3'

[a] CjejCdtA, B, C: *C. jejuni* cytolethal distending toxin A, B and C; F, forward; R, reverse.

[b] 5'-EcoR I and 3'-Xho I restriction sites incorporated for directional cloning are underlined.

Nuclease assays. Digestion of plasmid DNA with recombinant CjejCdtB was performed as described previously with modifications (Dassanayake *et al.*, 2005). Briefly, a plasmid (pCaggs) with a single *Sal I* restriction site was linearized with FastDigest *Sal I* (Fermentas, Glen Burnie, MA) according to the manufacturer's instructions. Complete digestion of plasmid DNA was confirmed by gel electrophoresis before purification by phenol-chloroform extraction. Intact super coiled plasmid DNA (1µg) or linearized plasmid DNA (1µg) were incubated with either CjejCdtB (1µg) or bovine DNaseI (1 unit; Fermentas) at 37^{o}C for up to 6 h in two independent experiments. In each experiment, control plasmid DNA (1µg) was incubated at 37^{o}C for 6 h in digestion buffer alone. Approximately 100 ng of each sample was loaded onto a 0.8% agarose gel, and after electrophoresis, the DNA was visualized by staining with ethidium bromide.

Cell proliferation. Duplicate cultures of MOLT-4, Jurkat, CEM and CA 46 cell lines grown to a density of 1x10^{6} cells per mL in 2 mL volume in 6-well plates were incubated with native or HI CjejCdtABC (100 ng/mL) at 37°C in a humidified atmosphere of 5% carbon dioxide in air for 72 h. Every 24 h, a volume of 75 µL of cell suspension was mixed with 25 µL of 0.4% trypan blue stain (Invitrogen), and the total number of viable cells per mL was determined by counting cells that excluded the dye using a hemacytometer (Hausser Scientific, Horsham, PA) and a light microscope at 100X magnification. Data was expressed as mean total numbers of viable cells ± SD of three independent experiments. For each time point, the total numbers of viable cells in treated and control cultures were compared by using two way ANOVA test with Bonferroni correction.

Determination of apoptosis by fluorescence-activated cell sorter (FACS) analysis. Cultures grown to a density of 1x 10^6 cells/mL were incubated in the presence of native or HI CjejCdtABC (100 ng/mL) for 24 h and the number of apoptotic cells was determined by FACS analysis. Characteristic apoptotic conformational change in cell membrane associated with phosphatidylserine translocation was assessed by using annexin-V and propidium iodide (PI) staining according to previously described protocols with modifications (Martin *et al.*, 1995; Shounan *et al.*, 1998). Briefly, cells were washed once in cold PBS and resuspended in 1X binding buffer containing 10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ and 0.1% bovine serum albumin (BSA), pH 7.4 at room temperature (RT). Cells were stained with 50 µg/ml annexin V-FITC (Santa Cruz Biotechnology, Santa Cruz, CA) and PI (Sigma) for 10 min at RT in the dark. A total of 2x10^4 cells were analyzed using a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences, San Jose, CA) and classified as early or late apoptotic, necrotic or viable as previously described (Shounan *et al.*, 1998; Vermes *et al.*, 1995).

Direct detection of apoptotic nuclear morphology using Hoechst 33342 and PI double fluorescence staining. Cell cultures at a density of 1x10^6 cells/mL were incubated in the presence of CjejCdtABC (100 ng/mL) for up to 72 h and the percent apoptotic cell death was determined sequentially by fluorescent microscopy following double staining with PI and Hoechst 33342. In separate experiments, each cell line was incubated in control medium or medium containing increasing concentrations of CjejCdtABC from 0.1 up to 100 ng/mL and examined after 72 h. Briefly, 200 µl of cell suspension was stained by adding PI (red fluorescence; 20 µg/mL final concentration; Sigma) and Hoechst 33342 (blue fluorescence;100 µg/mL final concentration; Sigma) and incubation for 10 min at 37°C in the dark. For each

sample, a total of 200 cells were classified as either viable, apoptotic or necrotic on the basis of characteristic cellular morphology by direct examination using a Leitz Aristoplan fluorescence microscope with long-pass filter cube A at 400X magnification as previously described (Jinadasa *et al.*, 2011; Muscarella & Bloom, 1997; Muscarella & Bloom, 2003; O'Brien *et al.*, 2001). Viable cells were characterized by intact cytoplasmic and nuclear membranes with homogenous light blue chromatin pattern, while cells that had undergone early apoptotic changes had intact but shrunken cell margins with condensed, segregated or fragmented blue white chromatin pattern. Cells in late apoptosis displayed nuclear changes similar to those in early apoptosis, but also showed diffuse pink cytoplasmic uptake of PI. By contrast, necrotic cells appeared swollen with irregular cytoplasmic membranes and were positive for PI uptake (red).

Immunofluorescence staining. Cell cultures at a density of 1 $x10^6$ cells/mL were incubated in the presence of CjejCdtABC (100 ng/mL) for up to 72 h or γ-irradiated (5Gy; positive control) and incubated for 2 h. Cells were fixed in 4% paraformaldehyde (pH 7.4) for 1h at 4 °C, blocked with 3% BSA in PBS with 0.2% Triton X-100 for 1h at 37 °C, stained with mouse monoclonal (mAb) anti- γ-H2AX (Millipore, cat.# 05-636) antibody (1:500 dilution) in PBS with 3% BSA and 0.02% Triton X-100 for 2h at room temperature (RT). Cells were washed twice (2000 rpm/ 5 min) in PBS with 3% BSA and stained with anti-mouse IgG-Alexa Flour 488 (1:1000 dilution, Invitrogen, cat. # A11017) at RT in 3% BSA with PBS and 0.02% Triton X-100. Cells were washed twice (2000 rpm/ 5 min) in PBS with 3% BSA, nuclei were stained with DAPI and spun down (400 rpm/ 4 min) on to glass slides using a CytoSpin III cytocentrifuge (Shandon Inc., Pittsburgh, PA). The slides were imaged with Leica DMRE fluorescence microscope at 1000X magnification.

Western blotting. Approximately $3x10^6$ cells (5 ml cultures in T25 flasks) were incubated with 100 ng/ml native or HI CjejCdtABC or γ-irradiated (5Gy; positive control). Western immunoblot was performed as described earlier with modifications (Liyanage *et al.*, 2010). Briefly, cells were lysed in RIPA buffer with protease and phosphatase inhibitors (1 mM each aprotinin, leupeptin, phenylmethanesulfonyl fluoride and sodium orthovanadate; Sigma) while incubating on ice for 10 min. The lysate was sonicated for 1 min at 0.5-2 power setting (Misonix 3000 Sonicator; Misonix Inc. Farmingdale, NY), centrifuged for 15,000 rpm for 5 min . Protein concentrations were determined by Bradford's method (Bradford, 1976). Proteins were separated on 12-13% (4% for ATM) SDS PAGE, transferred to PVDF membranes and immunoblotted with following antibodies; anti-phospho ATM (Rockland, cat. #200-310-400), anti- γ-H2AX (Millipore, cat.# 05-636), anti-DNA PKcs (abcam, cat. # ab18356), anti-phospho Chk1 (Cell Signaling, cat. #2341), ani-Chk2 (Millipore, cat. # 05-649), anti-phospho Akt (Cell Signaling, cat.# C31E5E), anti-beta actin (Sigma, cat.# A5441). Bands were detected with ECL (Advansta, cat. # K-12045) according to manufacturer's instructions using Versa Doc (Bio Rad) gel imaging system.

Metaphase chromosomal spreads. Chromosomal spreads were prepared and scored as described earlier with following modifications (Toller *et al.*, 2011; Zhu & Weiss, 2007). Approximately $1x10^6$ MOLT-4 or Jurkat cells were incubated with 100 ng/ml native or HI CjejCdtABC for 7 h. Colcemid was added to the cells (0.1μg/ml final concentration, Gibco, cat.# 15210-040) and incubated for an additional 1 h. Cells were harvested by centrifugation (2000rpm/ 5 min) and incubated with 14 ml of pre-warmed hypotonic 75mM KCl at 37 °C for 15 min. Cells were fixed by adding 1 ml of freshly prepared methanol/acetic acid (3:1) and gently

inverting the tubes. Cells were recovered by centrifugation (2000rpm/ 5 min) and washed three times with freshly prepared methanol/acetic acid (3:1) at room temperature. At the final wash, cell pellet was resuspended in 400 µl of methanol/acetic acid (3:1) and spotted on to glass slides in humid chamber, by dropping 40 µl of cell suspension on to each slide. The slides were stained with Giemsa stain as described previously and examined under a light microscope at 1000X magnification (Zhu & Weiss, 2007).

Statistical analysis

Statistical analysis was performed using JMP Pro version 9.02 (SAS Institute Inc, Cary, NC). Data are presented as mean ± SD. A p value < 0.05 was considered significant.

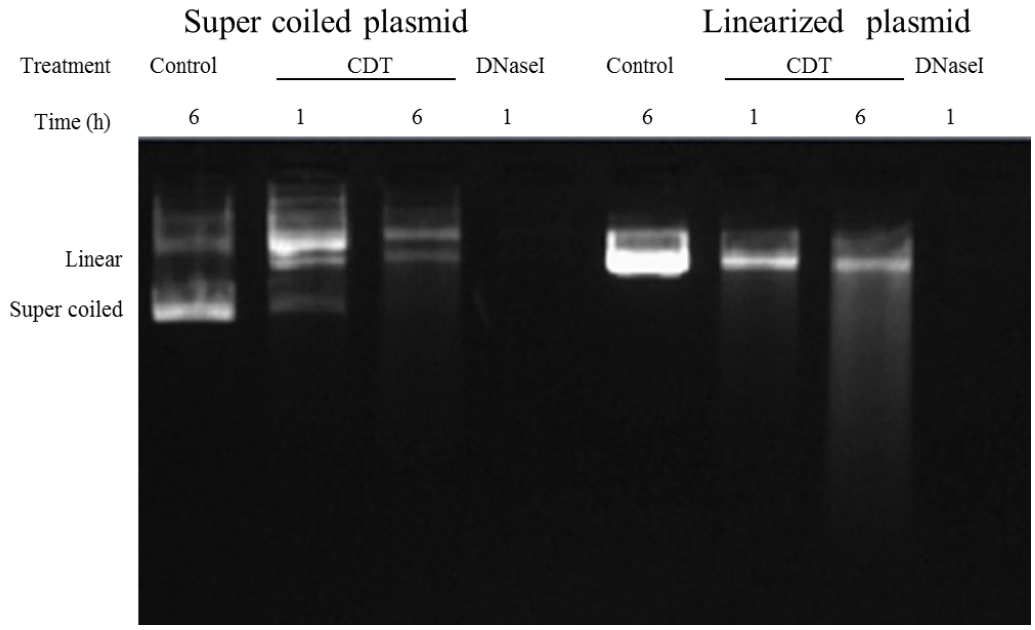
Results

There are some evidence indicating that CDT-induced apoptosis by HhepCDT and AactCDT primarily involves activation of p53-mediated mitochondrial pathway in epithelial cells and lymphoid cells, respectively (Jinadasa *et al.*, 2011; Liyanage *et al.*, 2010; Ohara *et al.*, 2004). However, this has not been clearly established for CjejCDT and the contribution of individual cell line p53 mutational status to CDT susceptibility remains to be clearly established. In this study, the contribution of p53 to CjejCDT-induced apoptosis of human leukemic T-cell lines with either functionally wild type p53 (MOLT-3 and MOLT-4) or mutated p53 (Jurkat, CEM) was investigated in details (Table 4.1). By evaluating CA 46 cell line, a Burkitt's lymphoma cell line which has an additional defective *Bax*, a key component of the mitochondrial apoptotic pathway that confers a highly resistant phenotype to a wide range of genotoxins and chemotherapeutic drugs, the activation of p53-mediated mitochondrial pathway of lymphoid cells by CjejCDT was further characterized.

Recombinant CjejCdtB subunit display nuclease activity *in vitro*. The nuclease activity of the CjejCdtB was confirmed by qualitative assessment of changes in band intensity after digestion of either supercoiled or linearized plasmid DNA (Figure 4.1). Nicking of super coiled DNA was clearly evident at 1 h, with the onset of DNA smearing in both supercoiled and linearized DNA. By 6 h, there were prominent DNA smearing in both super coiled and linearized DNA samples. By comparison, one unit of bovine DNaseI almost completely digested both super coiled and linearized DNA by 1 h incubation suggesting that CjejCdtB has nuclease activity in vitro.

Fig. 4.1. Nuclease activity of recombinant *Campylobacter jejuni* CdtB subunit (CjejCdtB) *in vitro*. Intact super coiled plasmid DNA (1µg) or linearized plasmid DNA (1µg) by prior digestion with *Sal I* were incubated in the presence of CjejCdtB (1µg) or bovine DNaseI (1 unit) for up to 6 h at 37^{o}C. Control plasmid DNA (1µg) was incubated for 6 h in digestion buffer alone. Each lane was loaded with approximately 100 ng of DNA. Representative gel from two independent experiments (R. N. J).

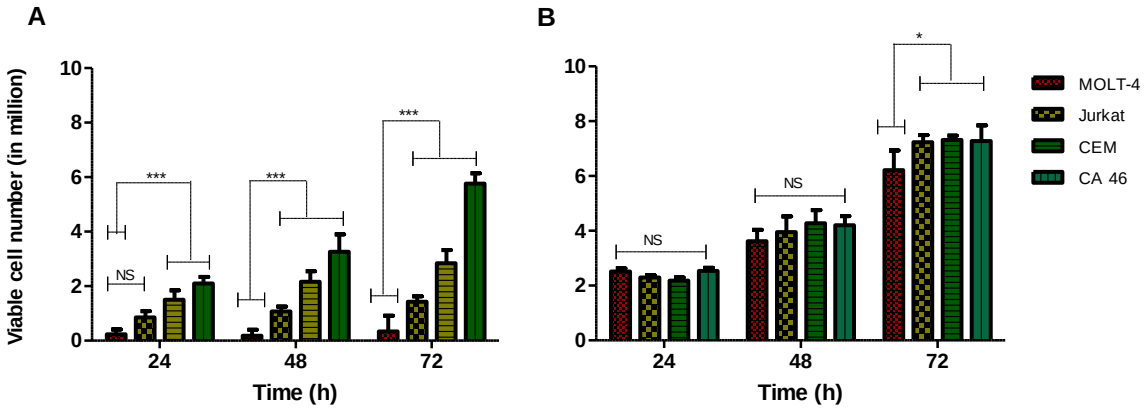
Fig. 4.1.


Super coiled plasmid
Linearized plasmid
Treatment
Control
CDT
DNaseI
Control
CDT
DNaseI
Time (h)
6
1
6
1
6
1
6
1
Linear
Super coiled

Inhibition of human lymphoma and leukemia cell line proliferation by CjejCdtABC is p53-dependent. The CDT susceptibility of lymphoid and leukemia cell lines was initially determined by the ability of CjejCdtABC to inhibit proliferation in MOLT-4, CEM, Jurkat and CA 46 cell lines (Figure 4.2). As expected, p53 mutated cell lines (CEM, Jurkat and CA 46) had significantly less growth inhibition (except for Jurkat cells at 24 h) compared to p53 wild type MOLT-4 cells. The MOLT-4 cell line was highly susceptible to CDT and failed to proliferate following 24 h post CDT exposure. The cell number did not regain the base line and CDT-treated MOLT-4 cells were completely non-viable by 72 h. In contrast the p53 mutated *Bax* deficient CA 46 cell line was almost completely resistant to CDT and was able to proliferate at a rate comparable to control cells at 24 and 48 h. The p53 mutated cell lines Jurkat and CEM displayed intermediate levels of CDT susceptibility, where Jukat cells were more susceptible than CEM. The Jurkat cells showed limited proliferation initially (at 24 h), but more substantial proliferation was observed at 48 and 72 h. The CEM cell line continued to proliferate at a lower rate, suggesting that CDT-susceptibility in human lymphoma and leukemia cell lines could be dependent on p53 mutational status of the cell line.

Fig. 4.2. Inhibition of human lymphoma and leukemia cell line proliferation by CjejCdtABC is p53-dependent. The number of viable MOLT-4, Jurkat, CEM and CA 46 in duplicate cultures incubated in the presence of native (A) or heat-inactivated (B) CjejCdtABC (100 ng/ml) at 37°C were determined by trypan blue dye exclusion every 24 h for up to 72 h. Data is expressed as mean total numbers of viable cells ± SD of three independent experiments. For each time point, the numbers of viable cells in treated and control cultures were compared by using two way ANOVA test with Bonferroni correction [(**, $p < 0.01$; ***, $p < 0.001$; NS, not significant), (R. J)].

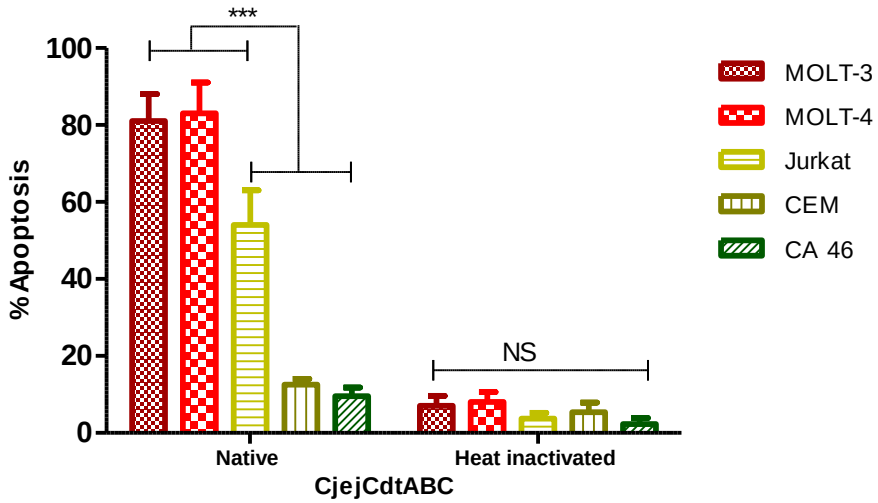
Fig. 4.2.


A
B
Viable cell number (in million)
10
8
6
4
2
0
NS
***
***
***
*
24
48
72
Time (h)
MOLT-4
Jurkat
CEM
CA 46

CjejCdtABC-induced apoptosis of human lymphoma and leukemia cell lines is p53-dependent. CjejCdtABC-induced apoptosis was then assessed in all cell lines by flow cytometry, 24 h following CDT exposure. Apoptotic cell death was assessed by analysis of conformational changes in membrane phospholipid phosphatidylserine (PS) following annexin-V and PI staining. As expected p53 wild type MOLT-3 and MOLT-4 cell lines displayed the highest susceptibility to CDT-induced apoptosis (approximately 80% apoptosis). The p53 mutated Jurkat cell line displayed approximately 50% apoptosis at 24 h post CDT treatment, while the CEM and CA 46 cell line were extremely resistant (less than 15% apoptosis) to CDT-induced apoptosis (Figure 4.3), suggesting that CDT-induced apoptosis in human lymphoma and leukemia cell lines is dependent on p53 mutational status of the cell line.

Fig. 4.3. CjejCdtABC-induced apoptosis of human lymphoma and leukemia cell lines is p53-dependent. A total of $1x10^6$ of each cell line were incubated in the presence of native or heat-inactivated CjejCdtABC (100 ng/ml) for 24 h and the percent apoptotic cell death was determined by flow cytometric analysis of $2x10^4$ cells stained with annexin V-FITC and propidium iodide (PI). Each bar represents the mean percent apoptosis ± SD of cells positive for annexin V-FITC only (early apoptosis) and both annexin V-FITC and PI (late apoptosis) in three separate experiments. The mean percent apoptosis of control and treated cells were compared by using two way ANOVA test [(***, $p < 0.001$; NS, not significant) (R. N. J)].

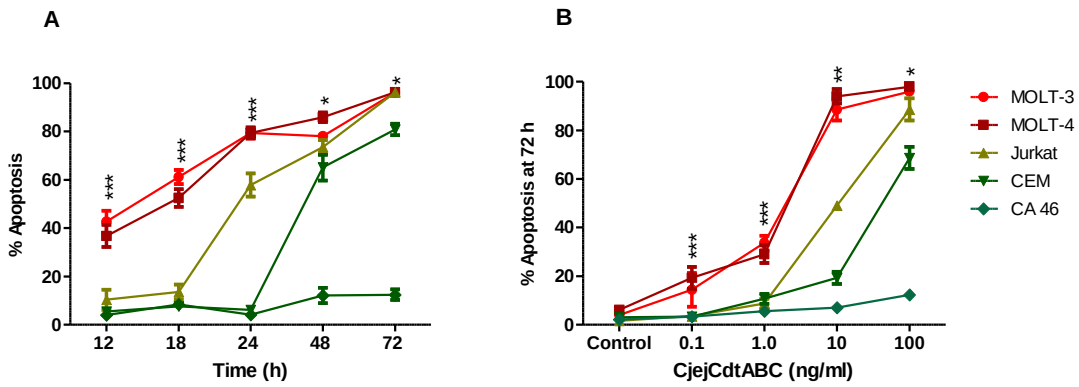
Fig. 4.3.


***
100
80
60
40
20
0
% Apoptosis
NS
Native
Heat inactivated
CjejCdtABC
MOLT-3
MOLT-4
Jurkat
CEM
CA 46

The p53-dependent CjejCdtABC-induced apoptosis of human lymphoma and leukemia cell lines is time- and dose-dependent. The kinetics of CDT-induced apoptosis was further investigated by simultaneous microscopic detection of characteristic apoptotic nuclear morphology and plasma membrane integrity by dye exclusion to detect necrosis according to well validated previously described methods (Bloom *et al.*, 2006; Jinadasa *et al.*, 2011; Muscarella & Bloom, 1997; Muscarella *et al.*, 2003; O'Brien *et al.*, 2001). In agreement with our flow cytometry data, the MOLT-3 and MOLT-4 cell lines with functionally wild-type p53 displayed increased time- and dose-dependent susceptibility to CDT-induced apoptosis as compared to p53 mutated Jurkat, CEM, CA46 cell lines (Figure 4.4 A and B). When incubated with 100ng/ ml CDT, all p53 mutated cell lines had significantly less apoptosis compared to p53 wild type cell lines up to 48 h (Figure 4.4 A). At 72h post CDT exposure, there were no significant differences between the apoptotic populations in Jurkat cells compared to MOLT-3 and MOLT-4 cells. However, even at 72 h post exposure there were significant differences between CEM and CA 46 cells compared to MOLT-3 and MOLT-4 cells (Figure 4.4 A). The dose response to CDT was also highly correlated to the p53 status of the cell lines (Figure 4.4 B). The p53 wild type MOLT-3 and MOLT-4 cells were significantly more susceptible to CDT within the entire 0.1 -100 ng/ml range of toxin concentrations (Figure 4.4 B). Furthermore, among the p53 mutated cell lines Jurkat and CEM displayed increasing time and dose dependent susceptibility to CDT while the *Bax* deficient CA 46 cell line showed significant resistance to CDT-induced apoptosis.

Fig. 4.4. The p53-dependent CjejCdtABC-induced apoptosis of human lymphoma and leukemia cell lines is time- and dose-dependent. A total of $1x10^6$ of each cell line were incubated in the presence of CjejCdtABC (100 ng/mL) for up to 72 h and the percent apoptotic cell death was determined sequentially by fluorescent microscopy following double fluorescence staining with PI and Hoechst 33342 (A). In separate experiments, each cell line was incubated in control medium or medium containing increasing concentrations of CjejCdtABC from 0.1 up to 100 ng/mL and examined after 72 h (B). For each time point (A) or CjejCdtABC concentration (B), a total of 200 cells were counted and the data was expressed as percent mean apoptosis ± SD of three independent experiments. The mean percent apoptosis of each cell line at different times after addition of CDT or different concentrations of CDT were compared by using the two way ANOVA test with Bonferroni correction [(*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$), (R. J)].

Fig. 4.4.


A
% Apoptosis
100
80
60
40
20
0
12
18
24
48
72
Time (h)
***
***
***
*
*
B
% Apoptosis at 72 h
100
80
60
40
20
0
Control
0.1
1.0
10
100
CjejCdtABC (ng/ml)
***
***
**
*
MOLT-3
MOLT-4
Jurkat
CEM
CA 46

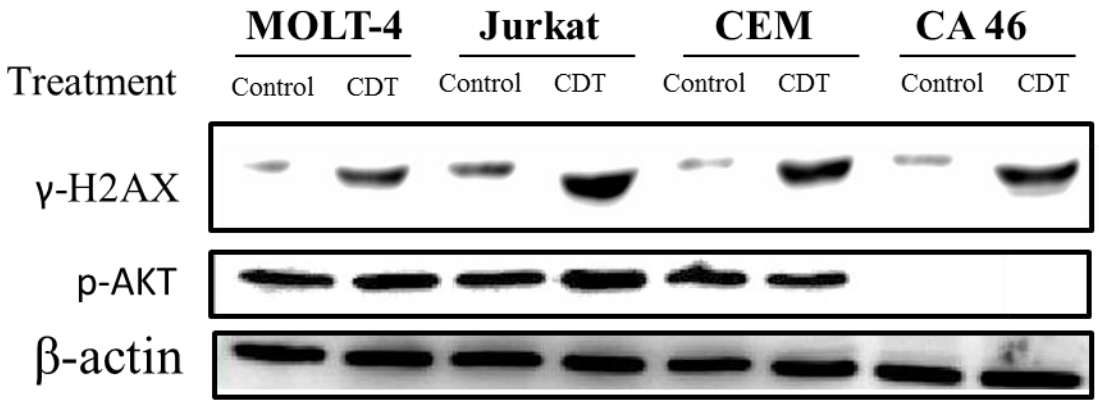
Induction of p53 phosphorylation by CjejCdtABC correlates with upregulation of p53 target PUMA in wild-type p53 but not p53 mutated human lymphoid cell lines.

We found that p53 was phosphorylated within 2 h of incubation in the presence of CjejCdtABC in MOLT-3 and MOLT-4 cells, whereas phosphorylated p53 was not detected in treated Jurkat or CEM cells (Fig. 5). By contrast, CA 46 cells had comparatively higher basal level of phosphorylated p53 and the level did not change in response to treatment with CjejCdtABC indicating that phosphorylated p53 is constitutively elevated in this cell line (Figure 4.5). In order to demonstrate the activation of p53 dependent apoptotic pathway, we then looked at the up regulation of p53 upregulated modulator of apoptosis (PUMA), a pro-apoptotic protein and a member of the Bcl-2 protein family that is upregulated by p53 in response to DNA damage. As expected, increased levels of PUMA were found in MOLT-3 and MOLT-4 cells 2 h after incubation in the presence of CjejCdtABC, while the level of PUMA expression did not change in Jurkat and CEM (Fig. 4.5). Consistent with our earlier interpretation that phosphorylated p53 is constitutively elevated in CA 46 cells, high level of PUMA expression was present in control CA 46 cells, and the level was not affected by treatment with CjejCdtABC for 2 h (Figure 4.5).

Fig. 4.5. CjejCdtABC induction of p53 phosphorylation correlates with upregulation of p53 target PUMA in wild-type p53 but not p53 mutated human lymphoid cell lines.

A total of $5x10^6$ of each cell line were incubated in the presence of CjejCdtABC (100 ng/mL) for 2 h and after lysis, proteins were separated by gel electrophoresis, transferred to PVDF membranes and after blocking were immunoblotted with polyclonal rabbit IgG antibody to phosphorylated p53 or PUMA. Membranes were blotted with mouse monoclonal antibody to β-actin as loading control. Representative Western blots from two independent experiments (R. N. J).

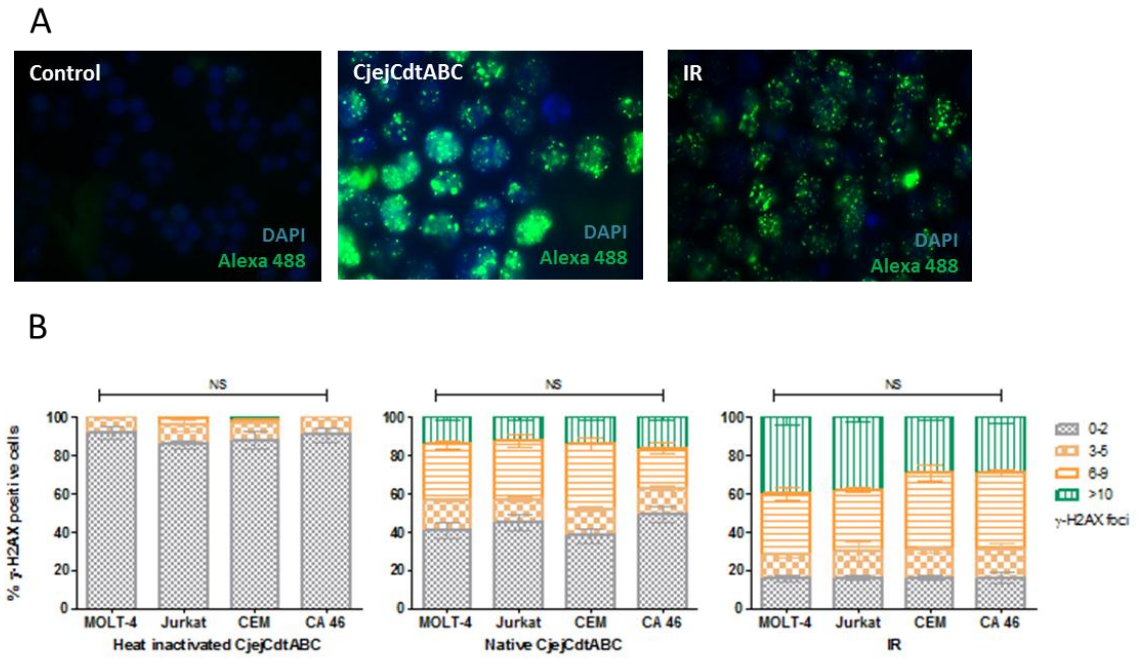
Fig. 4.5


MOLT-4
Jurkat
CEM
CA 46
Treatment
Control
CDT
Control
CDT
Control
CDT
Control
CDT
γ-H2AX
p-AKT
β-actin

The DNA damage response of human lymphoid cell lines to CjejCdtABC is similar to irradiation and independent of p53 cellular status. To confirm that observed differences in apoptosis susceptibility was due to the mutational status of cell lines and not to differences in the amount of DNA damage sustained by individual cell lines, we then investigated the activation of histone H2AX (γ-H2AX, a hallmark of DNA damage) in cells exposed to CDT (Figure 4.5). The background level of γ-H2AX levels slightly varied among cell lines, possible due to differences in spontaneous DNA damage levels in individual cell lines. Nevertheless, Comparable levels of γ-H2AX activation were observed in all cell lines by Western blot (Figure 4.5) and immunofluorescence (Figure 4.6) 2 h after CDT treatment suggesting comparable levels of CDT-induced DNA damage (and DDR activation) in various cell lines irrespective of p53 status.

Fig. 4.6. The DNA damage response of human lymphoid cell lines to CjejCdtABC is similar to irradiation and independent of p53 cellular status. A total of $1x10^6$ of each cell line were incubated in the presence of CjejCdtABC (100 ng/mL) or γ-irradiated (5Gy), and incubated for 2 h before fixation and staining with mouse monoclonal antibody anti-γ-H2AX followed by anti-mouse IgG-Alexaflour 488 and nuclear staining with DAPI. At least 100 cells were counted in control and treated cell lines and the data were expressed as percent γ-H2AX positive (green nuclear foci). Representative photomicrographs of control MOLT-4 cells incubated in the presence of HI (control), native CjejCdtABC or after irradiating (IR) for 2 h before double staining and fluorescent microscopy (A). Each bar represents mean percent γ-H2AX positive cells with 0-2, 3-5, 6-9 and >10 nuclear γ-H2AX foci per cell ± SD from two independent experiments [(B), (R. N. J)].

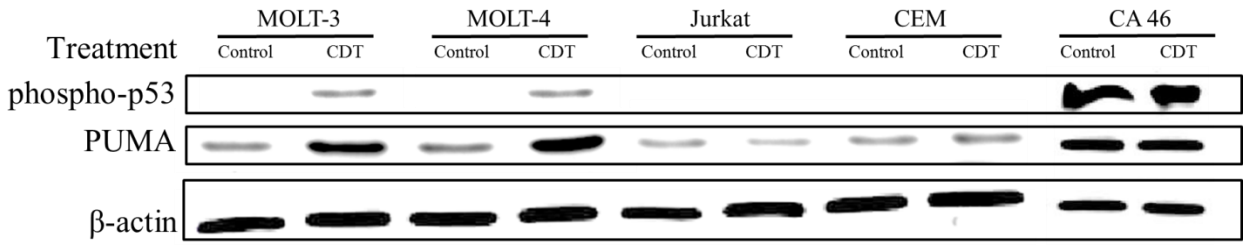
Fig.4.6.


A
Control
DAPI
Alexa 488
CjejCdtABC
DAPI
Alexa 488
IR
DAPI
Alexa 488
B
% γ-H2AX positive cells
NS
MOLT-4
Jurkat
CEM
CA 46
Heat inactivated CjejCdtABC
Native CjejCdtABC
IR
0-2
3-5
6-9
>10
γ-H2AX foci

The CjejCdtABC-induced DNA damage response of human lymphoid cell lines does not alter AKT phosphorylation level. There is some evidence that CDT might act as a PIP3 phosphatase, and thus, according to this hypothesis, CDT genotoxity would be mediated through depletion of PIP3 levels resulting in reduced pAKT (Shenker *et al.*, 2007). Consequently, the human leukemic T-cell lines MOLT-4, Jurkat and CEM with known mutations in SHIP/PTEN and constitutively elevated PIP3 levels were highly susceptible to CDT (Shenker *et al.*, 2007). As expected, MOLT-4, Jurkat and CEM cells lines had similarly elevated baseline levels of pAKT while CA 46 cells did not (Shenker *et al.*, 2007). However, despite comparable levels of γ-H2AX activation after 2 h incubation in the presence of CjejCdtABC, and thus a well-established DDR, the levels of pAkt did not change in any of the cell lines (Figure 4.7). These results suggest that changes in the level of AKT phosphorylation either do not play a significant role or play a minor role in CjejCdtABC-mediated apoptosis of both p53-functional MOLT-4 and p53-mutated Jurkat and CEM human leukemic T-cell lines.

Fig. 4.7. The CjejCdtABC-induced DNA damage response of human lymphoid cell lines does not alter AKT phosphorylation level. A total of $5x10^6$ of each cell line were incubated in the presence of CjejCdtABC (100 ng/mL) for 2 h and after lysis, proteins were separated by gel electrophoresis, transferred to PVDF membranes and after blocking were immunoblotted with mouse monoclonal antibody to γ-H2AX and rabbit polyclonal IgG antibody to phosphorylated AKT (p-AKT). Membranes were blotted with mouse monoclonal antibody to β-actin as loading control. Representative Western blots from two independent experiments (R. N. J).

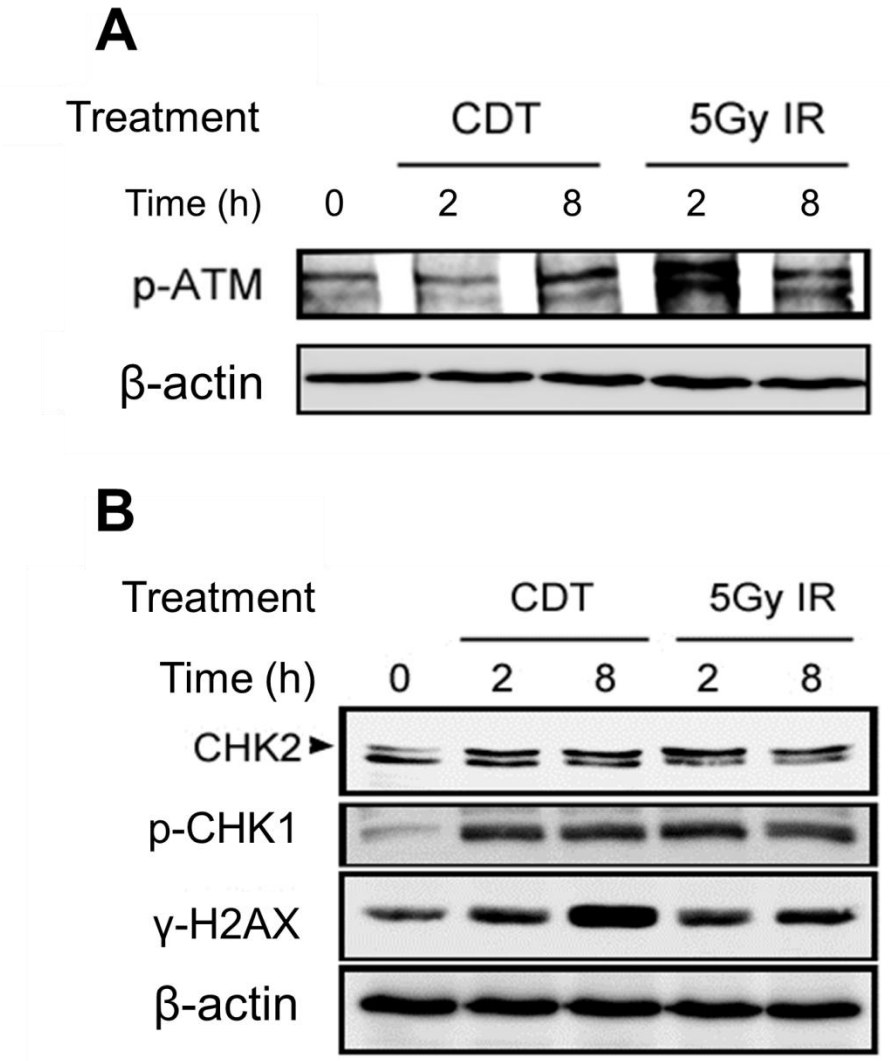
Fig. 4.7.


MOLT-3
MOLT-4
Jurkat
CEM
CA 46
Treatment
Control
CDT
Control
CDT
Control
CDT
Control
CDT
Control
CDT
phospho-p53
PUMA
β-actin

CjejCdtABC activates MOLT-4 cellular ATM- and ATR-dependent DDR without causing chromosomal breaks. Even though it has been previously suggested that CDT-induced DDR in lymphoid cells is ATM-dependent, phophorylation of ATM due to CDT-induced DNA damage has not been demonstrated. Therefore, in order to provide direct evidence to show that CDT-induced DDR is ATM-dependent, the DDR in lymphoid cells was further characterized by using MOLT-4 cells. For the first time, we demonstrate the phosphorylation of ATM (pATM) in lymphoid cells 2 h post CjejCdtABC exposure, suggesting the activation of ATM-dependent DDR (Figure 4.8). Increased pATM levels were observed in CDT-treated cells at 8 h post exposure, however irradiated cells demonstrated highest level of pATM at 2 h post exposure. Comparable with this, increased γ-H2AX was observed in 8 h post CjejCdtABC exposure compared to cell 2 h post exposure to CjejCdtABC (Figure 4.8).

Fig. 4.8. CjejCdtABC activates MOLT-4 cellular ATM and ATR responses. A total of $5x10^6$ of MOLT-4 cells were incubated in the presence of CjejCdtABC (100 ng/mL) or γ-irradiated (5Gy) for 2 or 8 h and after lysis, proteins were separated by gel electrophoresis, transferred to PVDF membranes and after blocking were immunoblotted with either mouse monoclonal antibody to either phosphorylated ATM (A), CHK2, or γ-H2AX and rabbit polyclonal IgG antibody to phosphorylated CHK1 (B). Membranes were blotted with mouse monoclonal antibody to β-actin as loading control. Representative Western blots from two independent experiments (G.B).

Fig. 4.8.


A
Treatment
CDT
5Gy IR
Time (h)
0
2
8
2
8
p-ATM
β-actin
B
Treatment
CDT
5Gy IR
Time (h)
0
2
8
2
8
CHK2
p-CHK1
γ-H2AX
β-actin

Since we and others have shown here and elsewhere that CdtB from various bacterial species can cause in vitro DSBs and activate H2AX and ATM in several cell lines, we examined the metaphase chromosomal spreads made from MOLT-4 (or Jurkat) cells incubated with CjejCdtABC for the presence of chromosomal breaks. Interestingly, we did not find obvious evidence for chromosomal breaks in either of the cell lines tested, up to 8 h post CjejCdtABC-exposure (data not shown), suggesting that CjejCdtABC induced DNA damage is more subtle than chromosomal breaks, probably involving single stranded DNA breaks. However, there was a clear reduction in the number of metaphases per slide in CjejCdtABC-treated cells compared to controls, indirectly indicating the cells undergoing G2/M arrest with CjejCdtABC treatment (data not shown). Encouraged by these results, and cell cycle checkpoints can be activated by related kinases ATM, ATR or DNA-PK, we then investigated the phosphorylation of DNA-PK, Chk1 and Chk2 in CjejCdtABC-treated MOLT-4 cells. The phosphorylation of Chk1 is a classical marker for the activation of ATR. We did not find a detectable increase in phosphorylation of DNA-PK (data not shown). As expected, significant increase in phosphorylation of Chk2 was observed 2 h post CDT (or IR) exposure, further confirming the activation of ATM-dependent DDR by CDT (Figure 4.8). Interestingly, we also observed the phosphorylation of Chk1 2 h post CDT-exposure. Phosphorylation of Chk1 is a primary indication of ATR activation, and here we suggest the activation of ATR by CjejCdtABC-induced DNA damage.

Discussion

The mutational status of p53 is a major determinant of the sensitivity of mammalian cells to apoptosis, particularly for genotoxin-induced apoptosis where p53 wild type cells display greater sensitivity than p53 null cells (Gudkov & Komarova, 2003; Roos & Kaina, 2006). Despite its potentially critical importance, the contribution of p53 mutational status of cell lines to CDT susceptibility has not been fully investigated. Additionally, CDT-induced DDR is not well characterized in lymphoid cell lines. In order to address these questions, we utilized two p53 wild type cell lines (MOLT-3 and MLOT-4) and three cell lines with mutated p53 (Jurkat, CEM and CA 46) to further investigate the role of p53 in CDT-induced apoptosis in human lymphoid cell lines.

The nuclease activity of the CjejCdtB was initially confirmed by in vitro digestion of plasmid DNA as described earlier (Dassanayake *et al.*, 2005). In vitro digestion of plasmid DNA by recombinant CdtB was previously observed with EcolCdtB-II, ActCDT, and HhepCdtB (Cao *et al.*, 2005; Dassanayake *et al.*, 2005; Elwell & Dreyfus, 2000; Nishikubo *et al.*, 2006). Here we extend these observations by demonstrating the weak in vitro nuclease activity of CjejCdtB. By using linearized plasmid DNA as a control, we confirm for the first time that CjejCdtB is capable of creating double stranded DNA breaks (DSBs) in vitro. Additionally appearance of DNA smears in both super coiled and plasmid DNA with 6 h digestion suggests that CdtB-induced DSBs are not sequence specific.

The CjejCdtABC susceptibility of lymphoid cell lines was initially assed using viable cell counts. As expected p53 wild type MOLT-4 cells were extremely susceptible to CjejCdtABC.

All p53 mutated cell lines (Jurkat, CEM and CA-46) had significantly higher growth rates after CjejCdtABC exposure (except for Jurkat cells at 24 h) compared to p53 wild type MOLT-4 cells indicating that p53 mutational status correlates with CjejCdtABC susceptibility in human lymphoma and leukemia cell lines. Similar to the initial findings, further characterizing of the kinetics of CjejCdtABC-induced apoptosis revealed that p53 wild type cell lines (MOLT-3 and MOLT-4) are highly susceptible to CjejCdtABC-induced apoptosis compared to p53 mutated cell lines (Jurkat, CEM and CA 46). These findings extend the early observations on p53 wild type MOLT-4 and p53 mutated Jurkat cells (Ohara *et al.*, 2004), where caspases were activated earlier in MOLT-4 cells. Additionally we further observed that p53 mutated Jurkat cell line displayed an intermediate susceptibility to CjejCdtABC. The p53 mutated CEM and CA 46 cell lines, particularly the *Bax*-deficient CA 46 cell were extremely resistant to apoptosis, suggesting that CjejCdtABC-induced apoptosis in human lymphoid cell lines is dependent on p53 mutational status of the cell line.

Interestingly, among the p53 mutated cell lines Jurkat and CEM displayed noticeable time and dose dependent increasing susceptibility to CjejCdtABC while the *Bax* deficient CA 46 cell line showed minimal time and dose dependent increasing susceptibility to CjejCdtABC, suggesting the potential late activation of p53-independent mechanisms. Additionally, the extreme CjejCdtABC resistance in CA 46 cell line having a *Bax* deficient genotype, indirectly suggest that CjejCdtABC-induced apoptosis in human lymphoid cell lines can be mediated through mitochondrial pathway. These observations are comparable with previous demonstrations by us and others that CDT-induced apoptosis is primarily mediated through mitochondrial pathway (Alaoui-El-Azher *et al.*, 2010; Bielaszewska *et al.*, 2005; Hickey *et al.*, 2005; Liyanage *et al.*,

2010; Ohara *et al.*, 2004; Shenker *et al.*, 2001; Wising *et al.*, 2005; Yamamoto *et al.*, 2004). We previously reported sequential up-regulation of Bax and down-regulation Bcl-2 leading to cytochrome *c* release and the subsequent activation of caspase-9 by HhepCDT in human intestinal epithelial H407 cells (Liyanage *et al.*, 2010). Activation of mitochondrial apoptotic pathway was also suggested by the significant reduction of CDT-induced apoptosis in human B-cell lines over-expressing Bcl-2 (Ohguchi *et al.*, 1998; Shenker *et al.*, 2001).

To confirm the differential apoptosis susceptibility in human lymphoma and leukemia cell lines is due to the mutational status of cell lines and not the amount of DNA damage sustained by individual cell lines, we then characterized the phosphorylation of histone H2AX. Comparable levels of γ-H2AX were observed in all cell lines by immunofluorescence and Western blot 2 h after CjejCdtABC treatment suggesting comparable levels of CjejCdtABC-induced DNA damage (and activation of DDR) in various cell lines irrespective of p53 status. CDT-induced γ-H2AX activation was previously demonstrated in HeLa cells 2 h post *Haemophilus ducreyi* CDT (HducCDT) exposure, and nearly all cells are positive for γ-H2AX foci within 6 to 8 h post-exposure (Li *et al.*, 2002). Increased γ-H2AX foci are seen in primary human fibroblasts treated with CjejCDT (Hassane *et al.*, 2003), in primary and established human endothelial cells treated with *Escherichia coli* CdtB-V (EcolCdtB-V), and human intestinal epithelial H407 cells exposed to HhepCDT (Bielaszewska *et al.*, 2005; Liyanage *et al.*, 2010).

In this study for the first time we provide direct evidence that CjejCdtABC-induced DDR in human lymphoma and leukemia cell lines involve ATM activation. Phosphorylation of ATM

was detectable at 2 h in CjejCdtABC treated MOLT-4 cells and became more prominent by 8 h post exposure to CjejCdtABC. In comparison IR induced similarly higher levels of phopho-ATM at 2 h post exposure, and had reduced phopho-ATM levels by 8 h. Similar kinetics were observed for the γ-H2AX levels in MOLT-4 cells, where CjejCdtABC induced γ-H2AX level increased from 2 h to 8h while it remained constant or appeared be lower by 8 h following IR exposure. The delayed onset of CjejCdtABC-induced phopho-ATM and γ-H2AX levels compared to IR exposure could be attributable to the fact that the extent and severity of CjejCdtABC-induced DNA lesions depends on the efficiency of cell surface binding, cellular internalization, delivery and enzymatic action of CjejCdtB, while IR is a rapid and almost instantaneous mechanical injury to DNA. In the same notion, the reduction of phopho-ATM and γ-H2AX levels in IR-exposed MOLT-4 cells by 8 h (compared to IR-exposed cells at 2 h) could be attributable to the activation of DNA repair mechanisms. Induction of ATM-dependent DDR by CDT in human lymphocytes has been indirectly demonstrated by characterizing the γ-H2AX levels in previous studies (Li *et al.*, 2002). Immortalized ATM-wild type human B lymphocyte lines had readily detectable γ-H2AX levels following HducCDT exposure, but it was absent in ATM- defective leukemic B cell lines obtained from AT patients (Li *et al.*, 2002).

Since nuclease activity of CdtB is the well-established molecular basis of cellular toxicity, and CDT is potentially causing DNA strand breaks, we then investigated the mediators besides ATM that can be involved in CDT-induced DDR. Three major phosphoinositide 3-kinase related kinases (PIKKs) involved in DDR are ATM, ATR and DNA-PK (Bartek & Lukas, 2007). We observed the phosphorylation of Chk 1in MOLT-4 cells 2h post exposure to CjejCdtABC, providing evidence to suggest that the CjejCdtABC-induced DDR can also be mediated through

ATR. We did not find any evidence to suggest the activation of DNA-PK by CjejCdtABC in MOLT-4 cells. Interestingly, even though we observe weak in vitro plasmid DNA digestion by CjejCdtB, we did not observe chromosomal breaks in CjejCdtABC-treated cells, and together with the observation of the activation of ATR, it is possible to suggest that CjejCdtABC-induces a more subtle type of DNA damage such as single strand DNA breaks in vivo, as suggested by previous studies (Cortes-Bratti *et al.*, 2001; Sert *et al.*, 1999). Sert and colleagues did not observe DNA strand breaks in EcolCDT treated HeLa cells analyzed by single cell gel electrophoresis (comet) assay (Sert *et al.*, 1999). Additionally, based on the evidence that nearly half of the CjejCDT sensitive mutants are not sensitive to HO endonuclease (which is known to cause DSBs) in a *Saccharomyces cerevisiae* systematic transformation array it has been suggested that at least in yeast CjeCdtB primarily induces single strand DNA breaks (SSB), which will be converted to DSBs during the replication of nicked strand (Kitagawa *et al.*, 2007). However, chromosomal DNA fragmentation by CDT has been observed in epithelial cells exposed to relatively high doses of CDT (Frisan *et al.*, 2003; Lara-Tejero & Galan, 2000). Similar, we also observed chromosomal breaks in 3T3 fibroblasts with relatively very high (25 ug/ml) levels of CjejCdtABC (data not shown).

In p53 wild type cells, DNA damage results in the accumulation of fully phosphorylated active p53, which leads to the transcriptional activation of the pro-apoptotic p53 targets including Bax, Bid, Puma and Noxa (Gudkov & Komarova, 2003; Roos & Kaina, 2006). In p53-null cells apoptosis sensitivity is mediated through several alternative mechanisms including the degradation of anti-apoptotic Bcl-2 (Gudkov & Komarova, 2003; Roos & Kaina, 2006). The p53-independent mechanism is relatively less efficient for facilitating apoptosis than direct

activation of Bax by p53, and thus p53-null cells are generally more resistant to genotoxin-induced apoptosis (Roos & Kaina, 2006). Additionally, sensitivity to toxin or drug-induced apoptosis can vary considerably in different cell lines depending on the differences in the expression levels of other mediators in apoptotic pathway, for example the anti-apoptotic protein Bcl-2 which has a protective function for mitochondrial integrity (Zamzami *et al.*, 1996).

The CDT-induced apoptosis in p53 mutated cell lines may be mediated through several pathways. One of the possible pathway is through Chk1/Chk2 and E2F1/p73(Jinadasa *et al.*, 2011). The Chk1/Chk2-E2F1/p73 pathway is one of the major pathways leading to apoptosis in p53 mutated cells in response to DNA damage (Roos & Kaina, 2006). Additionally, Chk1 also play a major role in replication stress induced apoptosis, which can be mediated independent of p53 and Chk2 (Myers *et al.*, 2009; Rodriguez & Meuth, 2006; Sidi *et al.*, 2008). Since we observed the activation of Chk1 in MOLT-4 cells, and we did not observe chromosomal breaks in either MIOLT-4 or Jurkat cells, suggesting a more subtle DNA damage induced by CDT, it is highly likely that apoptosis in p53 mutated cells will mediated through Chk1.

While it is well-established that the nuclease activity of CdtB is the molecular basis of CDT cellular toxicity, other than structural and biochemical evidence of PIP_3 phosphatase activity with purified recombinant AactCdtB, the evidence for this activity playing a role in cellular toxicity is limited (Dlakic, 2000; Shenker *et al.*, 2007). The PIP_3 phosphatase activity was demonstrated only in human leukemia cell lines with constitutively elevated PIP_3 levels due to mutations in SHIP1 and/or PTEN and the suppression of protein kinase B (PKB)/AKT pathway

(due to the depletion of PIP_3) was speculated as the mechanism of cellular toxicity (Shenker *et al.*, 2007). Our data suggest that changes in the level of AKT phosphorylation either do not play a significant role or play a minor role in CjejCdtABC-mediated apoptosis of both p53-functional MOLT-4 and p53-mutated Jurkat and CEM human leukemic T-cell lines. While we show that the pAKT levels of these leukemic cell lines is constitutively elevated (Figure 4.7), the hypothesis that this accounts for their greater susceptibility to CdtB phosphatase is not consistent with the observed resistance of CEM and to a lesser degree Jurkat cell lines (Figure 4.3), both with defective p53 pathway when treated with CjejCdtABC. Based on previous suggestions that the interaction of CjejCDT with cellular receptors is different from that of AactCDT (Eshraghi *et al.*, 2010), the possibility that these discrepancies are attributed to different molecular mechanisms of genotoxicity cannot be excluded completely. However, our data is consistent with previous studies with site specific phosphatase defective AactCdtB mutants or specific PIP_3 phosphatase inhibitors demonstrating that nuclease activity is sufficient for induction of cell cycle arrest and apoptosis in proliferating human monocytic cell line U937 (Rabin *et al.*, 2009). Further studies using haploid *S. cerevisiae* that lacks PIP_3, confirmed that AactCdtB-induced DSB is sufficient to cause cell cycle arrest and death (Matangkasombut *et al.*, 2009). Additionally, cellular toxicity in *S. cerevisiae* requires DNase-I-like catalytic residues of CdtB and its nuclear localization (Matangkasombut *et al.*, 2009). Taken together the data suggest that the predominant mechanism of cellular toxicity of CDT is the CdtB nuclease activity and DSB, and PIP_3 phosphatase plays an accessory role in certain situations.

Although we cannot rule out the contribution of mutations in genes other than p53, our data clearly indicate that p53 mutational status correlates with the differential CjejCdtABC-susceptibility of human lymphoma and leukemia cell lines. For the first time we show the

activation of ATM-dependent DDR in human lymphoma and leukemia cell lines and provide the first evidence of involvement of CHK1 in CDT-induced DDR. The absence of detectable change in phosphorylated Akt levels following CjejCdtABC treatment suggesting that nuclease activity of CjejCdtABC is the primary contributor for cytotoxicity.

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CHAPTER 5

ATM is essential for development of adaptive immune responses to bacterial whole cell antigens and cytolethal distending toxin in a laboratory mouse model of *Helicobacter hepaticus* infection[1, 2]

Abstract

It is well-established that the bacterial genotoxin, cytolethal distending toxin (CDT) causes DNA damage in a wide range of eukaryotic cells *in vitro* leading to a robust DNA damage response (DDR) in which ATM plays a central role. Moreover, on the basis of a higher sensitivity of hematopoietic cells to the cytotoxic effects of CDT, it has been suggested that CDT modulates the host immune response *in vivo.* Here we assess the adaptive humoral immune response of laboratory mice with a defective DDR following infection with *H. hepaticus*. Assessment of serum IgG1 responses to *H. hepaticus* whole cell lysate (WCL) and CdtB subunits revealed differential responses in Atm wild type ($Atm^{+/+}$) compared with Atm-defective ($Atm^{+/-}$) and null ($Atm^{-/-}$) genotypes. While infected $Atm^{+/+}$ and $Atm^{+/-}$ mice had high levels of IgG1 antibodies to *H. hepaticus* WCL, $Atm^{-/-}$ mice had a greater than a 50% reduction in their serum antibody responses to both WCL and CdtB. Histopathological examination of control uninfected and infected mice of any genotype euthanized four or 10 weeks post challenge revealed no significant intestinal inflammation. The data suggest that DDR mechanisms are essential for bacterial and CDT-specific host adaptive immune responses.

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Introduction

Enterohepatic *Helicobacter* species (EHS) are a major cause of enterocolitis, hepatitis and bacteremia several laboratory animal species particularly mice and non-human primates (Duhamel *et al.*, 2003; Fox *et al.*, 2001a; Fox *et al.*, 2001b; Fox *et al.*, 2011; Ge *et al.*, 2008a; Kemper *et al.*, 1993; Liyanage *et al.*, 2010; Solnick & Schauer, 2001); however, their pathogenicity in human beings is incompletely understood (3, 20, 53). The prototype for EHS is *Helicobacter hepaticus,* which establish long-term colonization of the lower intestinal and hepatobiliary tract resulting in chronic inflammation and ultimately hepatocellular and colonic carcinomas in susceptible strains of mice including A/JCr, SCID/NCr, C3H/HeNCr (Fox *et al.*, 1994; Hailey *et al.*, 1998; Ihrig *et al.*, 1999; Suerbaum *et al.*, 2003; Ward *et al.*, 1994a; Ward *et al.*, 1994b). The chronic colitis associated with *H. hepaticus* infection has features of inflammatory bowel disease (IBD), and thus, it has been widely used as a model to define aspects of IBD pathogenesis (Fox *et al.*, 2011). Further, *H. hepaticus* is closely related to the major food- and waterborne human pathogen *Campylobacter jejuni,* and because of a lack of laboratory animal models, *H. hepaticus* infection in mice has been used as a surrogate model for human campylobateriosis (Suerbaum *et al.*, 2003; Young *et al.*, 2007).

Although cytolethal distending toxin (CDT) is the only known virulence factor produced by *H. hepaticus,* the contribution of this genotoxin to disease pathogenesis has remained incompletely understood (Ge *et al.*, 2008a; Jinadasa *et al.*, 2011; Liyanage *et al.*, 2010; Suerbaum *et al.*, 2003). CDT is a heterotrimeric AB type toxin produced by over two dozens of clinically important Gram negative bacteria beside *H. hepaticus,* that inhabit a wide range of hosts' mucocutaneous surfaces (Alouf, 2006; Ge *et al.*, 2008a; Guerra *et al.*, 2011; Jinadasa *et al.*, 2011; Johnson &

Lior, 1987; Johnson & Lior, 1988a; Johnson & Lior, 1988b; Lara-Tejero & Galan, 2001; Purven & Lagergard, 1992; Smith & Bayles, 2006; Sugai *et al.,* 1998; Young *et al.,* 2000). The active subunit CdtB has structural homology with phosphodiesterase family of enzymes including mammalian deoxyribonuclease I (Elwell & Dreyfus, 2000; Hu *et al.*, 2006; Hu & Stebbins, 2006; Lara-Tejero & Galan, 2000; Nesic *et al.*, 2004; Yamada *et al.*, 2006). The CdtB subunit is necessary and sufficient to cause cellular toxicity (Elwell & Dreyfus, 2000; Lara-Tejero & Galan, 2000). Currently CDT is the only known AB type toxin with deoxyribonuclease activity (Alouf, 2006). Mammalian cells exposed to CDT initiate a DNA damage response (DDR) similar to that elicited by ionizing radiation (IR)-induced DNA double strand breaks (DSBs) leading to irreversible cell cycle arrest and apoptosis (Cortes-Bratti *et al.*, 2001; Hassane *et al.*, 2003; Li *et al.*, 2002; Liyanage *et al.*, 2010). Based on its ability to modulate mammalian cell cycle, CDT has been proposed as a cyclomodulin, a novel group of bacterial toxins which are capable of modulating the host cell cycle during the pathogenesis of disease (Nougayrede *et al.*, 2005). Cell lines of epithelial and endothelial origins primarily arrest in the G2/M phase following CDT exposure (Elwell *et al.*, 2001; Jinadasa *et al.*, 2011; Lara-Tejero & Galan, 2001; Smith & Bayles, 2006; Whitehouse *et al.*, 1998; Wising *et al.*, 2005; Young *et al.*, 2000), whereas cells of fibroblastic origin arrest both in the G1/S and G2/M phases of the cell cycle (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001; Hassane *et al.*, 2003; Smith & Bayles, 2006; Wising *et al.*, 2005). By contrast with all other cell types, cells of hematopoietic lineage including lymphocytes, monocytes, macrophages and dendritic cells are several orders of magnitude (10–50 pg/ml compared to 1–5 µg/ml for other cells) more susceptible to the action of CDT and undergo rapid apoptosis after a brief cell cycle arrest (Belibasakis *et al.*, 2004; Cortes-Bratti *et al.*, 2001; Hassane *et al.*, 2003; Smith & Bayles, 2006; Wising *et al.*, 2005).

The CDTs produced by several bacteria have been shown to inhibit the proliferation and cause functional impairment and apoptosis of immune cells. *Aggregatibacter actinomycetemcomitans* CdtB (AactCdtB) inhibits the proliferation of mitogen stimulated human B cells, impairs the production of IgG and IgM, and causes G2/M arrest and apoptosis in mitogen stimulated human T lymphocytes (Shenker *et al.*, 1991; Shenker *et al.*, 1999; Shenker *et al.*, 2001). Inhibition of proliferation and apoptotic cell death of were observed in *Haemophilus ducreyi* CDT(HducCDT) treated mitogen stimulated human peripheral blood mononuclear cells (PBMCs) and enriched T cells, and also in Jurkat cells (Gelfanova *et al.*, 1999). Inhibition of proliferation was also observed in viral-stimulated human B cells exposed to HducCDT, with a decrease in IgG and IgM production (Svensson *et al.*, 2001). Similarly, viral-stimulated human B cell lines are also undergoing apoptosis when treated with HducCDT (Cortes-Bratti *et al.*, 2001; Frisan *et al.*, 2003).

In addition to the extreme in vitro CDT susceptibility of hematopoietic cells, CDT has also been associated with immunomodulation *in vivo* (Pratt *et al.*, 2006; Young *et al.*, 2004). Infection of interleukin 10 deficient (IL-$10^{-/-}$) C57BL/6 mice with *H. hepaticus* lacking CdtB resulted in the clearance of infection in four months, while the wild type bacteria persisted for over eight months (Young *et al.*, 2004). The mice infected with wild type *H. hepaticus* developed severe typhlocolitis and produced significantly higher levels of IgG1 and IgG2 compared to the animals infected with CDT-deficient mutant *H. hepaticus* suggesting that CDT is having an immunomodulatory effect (Pratt *et al.*, 2006). It has also been shown that CDT is essential for the colonization of *H. hepaticus* in outbred Swiss Webster mice (Ge *et al.*, 2005). The infection was cleared in eight weeks post infection (w.p.i) in females and 16 w.p.i. in males infected with

isogenic CDT-deficient *H. hepaticus*, whereas the wild type bacteria persisted in both males and females for over 16 w.p.i. (Ge *et al.*, 2005). Based on the extreme in vitro CDT susceptibility of hematopoietic lineage cells and association of CDT with immunomodulation in vivo, it has been proposed that hematopoietic lineage cells could be the in vivo target of CDT (Pratt *et al.*, 2006; Shenker *et al.*, 2007).

Even though the precise nature of secretion of CDT during bacterial infection is not completely understood, increasing evidence suggests that CDT is secreted in bacterial outer membrane vesicles (Lindmark *et al.*, 2009; Rompikuntal *et al.*, 2012). Additionally, physiological levels of bile acid sodium deoxycholate enhance the outer membrane vesicle mediated secretion of CDT in vitro (Hickey *et al.*, 2005). Natural infections with *H. ducreyi* and *A. actinomycetemcomitans* can elicit serum IgG antibodies to individual CdtABC subunits indicating development of an adaptive immune response to toxin during infection (Ando *et al.*, 2010; Mbwana *et al.*, 2003; Xynogala *et al.*, 2009). Serum antibodies that can neutralize the biological activity of CDT have been demonstrated in patients with chancroid (Mbwana *et al.*, 2003); those with periodontitis (Ando *et al.*, 2010; Xynogala *et al.*, 2009), and patients recovered from campylobacteriosis (Abuoun *et al.*, 2005). Moreover, development of high levels of CDT-neutralizing IgG in sera and genital tissues of mice immunized with HducCDT toxoid compared to native toxin suggests a potential vaccine application for CDT toxoid in protection against chancroid (Lundqvist *et al.*, 2010).

Development of a host adaptive immune response to individual CDT protein subunits is seen following spontaneous infection and disease caused by CDT-producing *A.*

actinomycetemcomitans, C. jejuni and *H. ducreyi.* However, experimental infections in a laboratory mouse model of *H. hepaticus* clearly show that CDT can modulate both the level and isotype antibody response of the host and establish persistent infection (Ge *et al.*, 2005; Pratt *et al.*, 2006). The purpose of the current study is to determine the effect of ATM on the immune response to *H. hepaticus* infection. The $Atm^{-/-}$ mice used in this study were initially designed and characterized by Elson et al., 1996 and has defective immune systems characterized by smaller thymi compared to wild type mice, reduced number of thymocyets, reduced cortex and a large medulla (Elson *et al.*, 1996). Additionally, $Atm^{-/-}$ mice develop spontaneous thymic lymphomas between 3-4 months of age (Elson *et al.*, 1996). Further, spleen and peripheral blood from $Atm^{-/-}$ mice had significant reduction in T cells; however, no significant alterations were observed in the relative proportion of B cells (Elson *et al.*, 1996). An independent $Atm^{-/-}$ mice model developed by Barlow et al., 1996 had similar observations regarding lymphocyte development and T cell maturation while no significant defects were observed in B cell development (Barlow *et al.*, 1996). However, independent studies have demonstrated similar developmental defects in B cells (Xu *et al.*, 1996).

We characterized the immune responses against *H. hepaticus* bacterium and CdtB. Further this study also explores the effect of a high cholate diet in modulating the host immune response. Assessment of serum IgG1 responses to *H. hepaticus* WCL and CdtB subunits revealed differential responses in $Atm^{+/+}$ compared with $Atm^{+/-}$ and null $Atm^{-/-}$ genotypes. While infected $Atm^{+/+}$ and $Atm^{+/-}$ mice had high levels of IgG1 antibodies to H. hepaticus WCL, $Atm^{-/-}$ mice had a greater than a 50% reduction in their serum antibody responses to both WCL and CdtB. Histopathological examination of control uninfected and infected mice of any genotype

euthanized four or 10 weeks post challenge revealed no significant intestinal inflammation. The data suggest that DDR mechanisms are essential for bacterial and CDT-specific host adaptive immune responses. Additionally, a diet that alters the bile acid composition resulted in an overall reduced immune response against Hhep WCL and CdtB.

Materials and Methods

Mice and *H. hepaticus* infection

The *Atm*$^{-/-}$ mice used in this study were originally designed and characterized by Elson et al., 1996 (Elson *et al.*, 1996) and a kind gift to R. S. W from Dr. Phillip Leder and maintained on FVB background. The colony was re-derived at the Cornell University transgenic mouse facility (TMCF) to generate *Helicobacter*-free mice colony by embryo transfer and maintained in the high security Cornell University East Campus Research Facility (ECRF) for breeding and transferred to a barrier facility for infection. The mice were fed either normal Harlan rodent chow or diet containing 1.0% cholesterol, 0.5% cholic acid, and 15% dairy (Harlan diet TD.88051) as described earlier (Maurer *et al.*, 2006). Mice in each experimental group are listed in tables 5.1 and 5.2.

Table 5.1. Mice in the conventional diet group.

Genotype	Weeks p.i					
	4		8		10	
	Infected	Control	Infected	Control	Infected	Control
$Atm^{+/+}$	8	4	7	4	7	4
$Atm^{+/-}$	8	8	6	6	6	6
$Atm^{-/-}$	10	6	7	4	3	4

Table 5.2. Mice in the high cholate diet group.

Genotype	Weeks p.i					
	4		8		10	
	Infected	Control	Infected	Control	Infected	Control
$Atm^{+/+}$	3	3	2	2	2	2
$Atm^{+/-}$	8	5	4	4	4	4
$Atm^{-/-}$	4	2	1	1	1	1

The *H. hepaticus* strain (TMCF 5664) used in this study had been isolated from Cornell university transgenic mouse core facility (TMCF) according to previously described protocols (Fox *et al.*, 1994). The presence of *cdtB* and HH252, HH260 and HH275within *H. hepaticus* pathogenicity island 1 (HHGI1) was confirmed by PCR assays according to previously described protocols (Dassanayake *et al.*, 2005a; Ge *et al.*, 2008b). The *in vitro* CDT biological activity also was confirmed by treating HeLa cells with whole cell lysate (HhepWCL) and demonstrating cell cycle arrest and apoptosis as previously earlier (Dassanayake *et al.*, 2005b). Six to eight week old weaned mice received 3 doses (0.5ml of approximately $2x10^8$ bacteria/ml suspension in brucella broth) of *H. hepaticus* by oral gavage every other day according to protocols described earlier (Ge *et al.*, 2008b). The infection was verified by PCR for HhepCdtB gene two weeks post infection (p.i.) using individual fecal pellets. Serum was collected by submandibular bleeding 4 and 8 weeks post infection (p.i) and at 10 weeks p.i. during necropsy by allowing the blood to clot at room temperate and centrifuging at 10000g for 10 min. The serum samples were immediately frozen until further use. Bacterial load in cecal tissue was determined by quantitative real time PCR as described earlier (Ge *et al.*, 2001). All animal procedures were approved by Cornell University institutional animal care and use committee (IACUC).

Recombinant *H.hepaticus* CdtB (HhepCdtB) and HhepWCL antigens

His-tagged HhepCdtB was expressed in *E. coli* and purified under denaturing conditions according to previously described protocols (Dassanayake *et al.*, 2005a; Liyanage *et al.*, 2010). The purity of proteins was characterized by SDS-PAGE and Coomassie blue staining and/or Western blot as described (Dassanayake *et al.*, 2005a). HhepWCL was prepared according to

previously described protocols (Dassanayake *et al.*, 2005b). Concentrations of the antigens were adjusted to 200 µg/ml in PBS with 10% glycerol and stored at -80 °C.

ELISA

Immulone flat bottom 96 well plates (Fisher, cat. #3455) were coated with 100 µl of antigen (diluted 1:100 in $NaCO_3$ pH 9.6) and incubated at 4°C overnight. After washing the plate 4 times with wash buffer (8.5% NaCl with 0.1% Tween 20), 100 µl of dilute serum [1:100 dilution in PBST (PBS with 1% BSA and 0.1% Tween 20)] was added to triplicate wells and incubated at 37°C for 2h. Plates were washed 4 times with wash buffer and incubated with 100 µl of dilute (1:2000 in PBST) secondary antibody (goat anti-mouse IgG1-HRP, Santa Cruz Biotechnology cat. # sc2969) at 37°C for 1h. Plates were washed 4 times with wash buffer and 100 µl of ABTS peroxidase substrate (KPL cat. # 50-66-18) was added and incubated at room temperature for color development (5-20 mins). The reaction was stopped by adding 5% SDS (100 µl per well) when the negative control wells were just beginning to show color. Absorbance at 405 nm was recorded using a Synergy 2 multi-mode microplate reader (BioTek instruments).

Histopathology

Histopathological analysis was performed by a pathologist blind to the identity of the samples and a score was given to each sample based on the intensity and severity of the lesions.

Statistical analysis

Statistical analysis for FACS data was performed using JMP Pro version 9.02 (SAS Institute Inc, Cary, NC). Data are presented as mean ± SD. A p value < 0.05 was considered as significant.

Results

Isolation and characterization of *H. hepaticus* strain TMCF 5664.

A field strain of *H. hepaticus* was isolated from the liver of a mouse from a conventional mouse colony at TMCF. The strain was PCR positive for CdtB and selected components of the HHGI 1 genes HH252, HH260 and HH275 (Figure 5.1). The in vitro CDT activity of the infecting strain was verified by the ability to induce cell cycle arrest in HeLa cells. The HhepWCL from the strain TMCF 5664 induced significantly higher G2/M cell cycle arrest in HeLa cells confirming the CDT activity (Figure 5. 2).

Figure 5.1. Detection of HHGI 1 genes HH252, HH260 and HH275 by PCR.

Each lane of the 0.8% agarose gel was loaded with 12 µl of PCR product and visualized by ethidium bromide staining. Lane 1, molecular weight marker; lane 2, HH252; lane 3, HH260; lane 4, HH275 (R. N. J.).

Fig. 5.1.

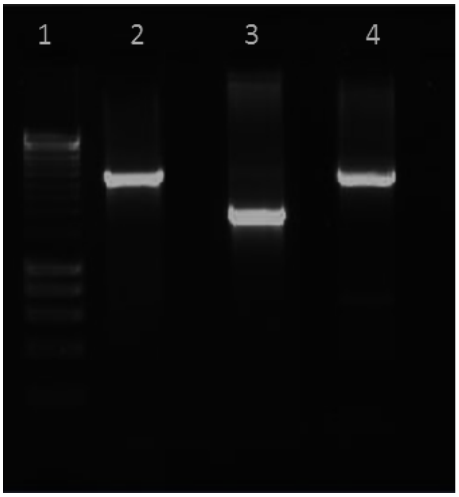
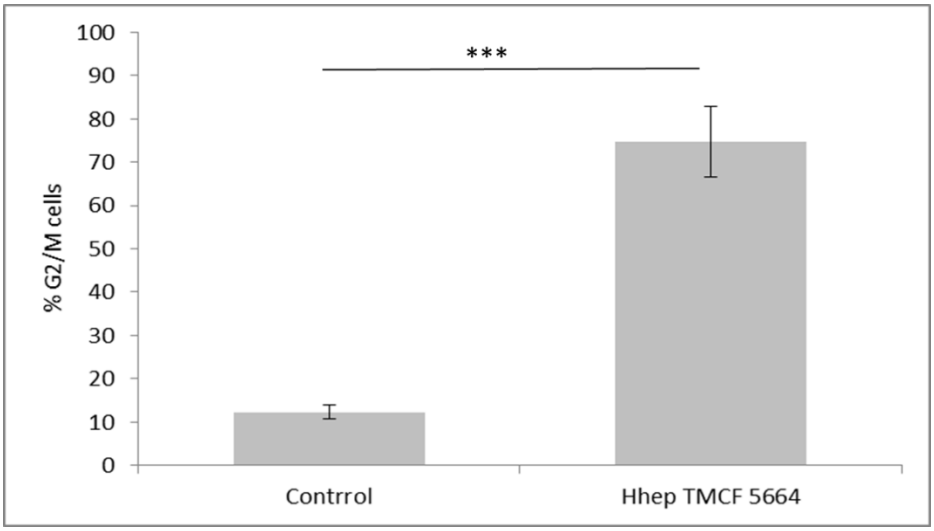


Figure 5.2. Hhep TMCF 5664 displays CDT activity in vitro.

HeLa cells were exposed to 20ug/ml Hhep TMCF 5664 WCL for 72 h. Cell cycle arrest was determined by FACS. Data shows the mean percentage of cells arresting at G/M phase as determined by the presence of 4N DNA content. Data represent the means of two independent experiments. The mean values were compared by using Student's t test [(***, $p<0.001$), S. Y. C.].

Fig. 5.2.


100
90
80
70
60
50
40
30
20
10
0
% G2/M cells
***
Contrrol
Hhep TMCF 5664

***H. hepaticus* infection elicits an Atm-dependent immune response.**

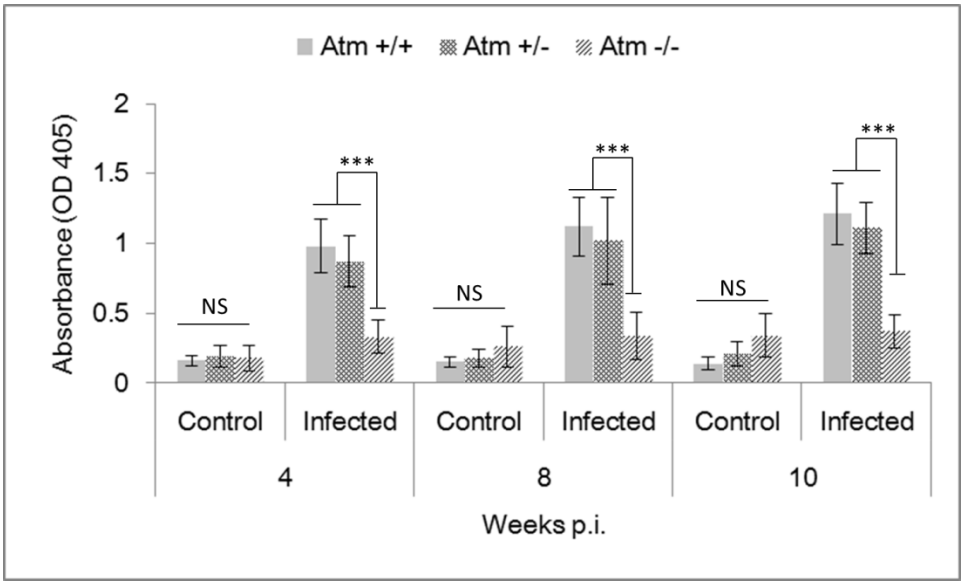
Even though it is well established that Atm null mice have defective immune responses characterized by lower serum IgG levels, role of Atm in developing specific adaptive immune response against a bacterial infection has not been characterized. Particularly, it is of particular interest to characterize the response of Atm heterozygous mice, as 1-2 % of human population is heterozygous for Atm, they might be in an increased risk for infection with CDT producing bacteria (Smilenov *et al.*, 2001). Control mice had minimal levels of serum IgG against HhepWCL or HhepCdtB. The responses against HhepWCL always higher as compared to responses against HhepCdtB (Figure 5.3). As expected wild type and heterozygous mice had a significantly higher IgG1 response compared to Atm-null mice. Interestingly, the heterozygous mice had a marginally lower IgG1 response as compared to wild type and Atm-null mice; however, this difference was not statistically significant. The specific IgG responses against HhepWCL and HhepCdtB had a slight increase over time; however, the increase was not statistically significant.

Figure 5.3. *H. hepaticus* infection elicits an Atm-dependent immune response

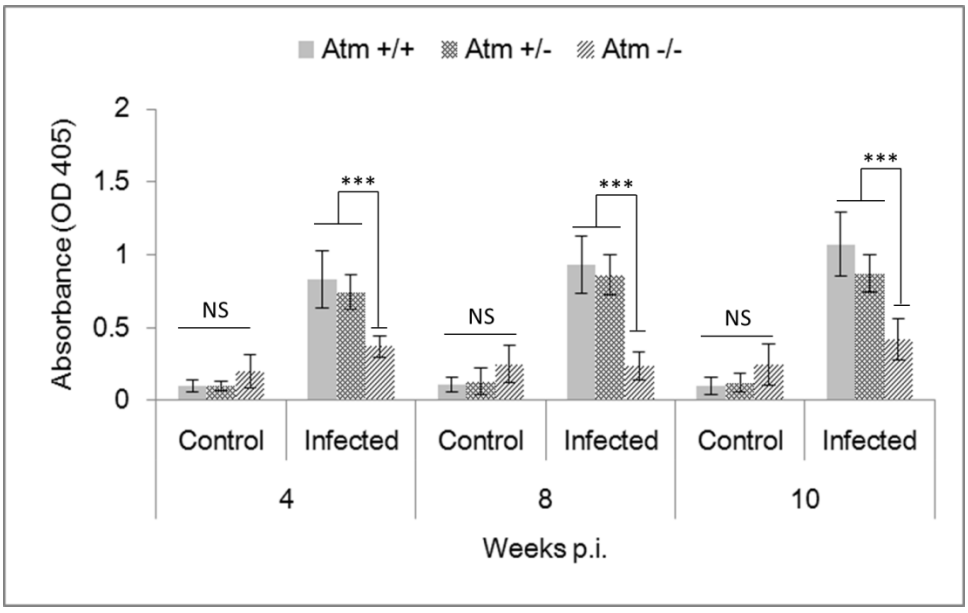
Serum IgG1 responses (absorbance at 405 nm) were measured by ELISA against HhepWCL (A) and HhepCdtB (B) in mice fed with conventional diet over 10 weeks p.i. The plates were coated with 0.02 µg of antigen and incubated with 1:100 diluted sera for 2 h at 37 °C. Following wash, the plates were incubated with 1:2000 dilutes anti-mouse IgG1-HRP. Following wash, peroxidase substrate was added to the plates and color development up to 20 mins was detected by spectrophotometer after adding 5% SDS stop reagent. The data represent mean values of three technical replicates for each data point. Number of mice used in each group is listed in table 5.1. For each time point, the mean values were compared by using two way ANOVA test with Bonferroni correction [(***, $p < 0.001$; NS, not significant), (R. N. J. and G. E. D).

Fig. 5.3.

(A)


Atm +/+
Atm +/-
Atm -/-
Absorbance (OD 405)
2
1.5
1
0.5
0
NS
***
NS
***
NS
***
Control
Infected
Control
Infected
Control
Infected
4
8
10
Weeks p.i.

(B)


Atm +/+
Atm +/-
Atm -/-
Absorbance (OD 405)
2
1.5
1
0.5
0
NS
***
NS
***
NS
***
Control
Infected
Control
Infected
Control
Infected
4
8
10
Weeks p.i.

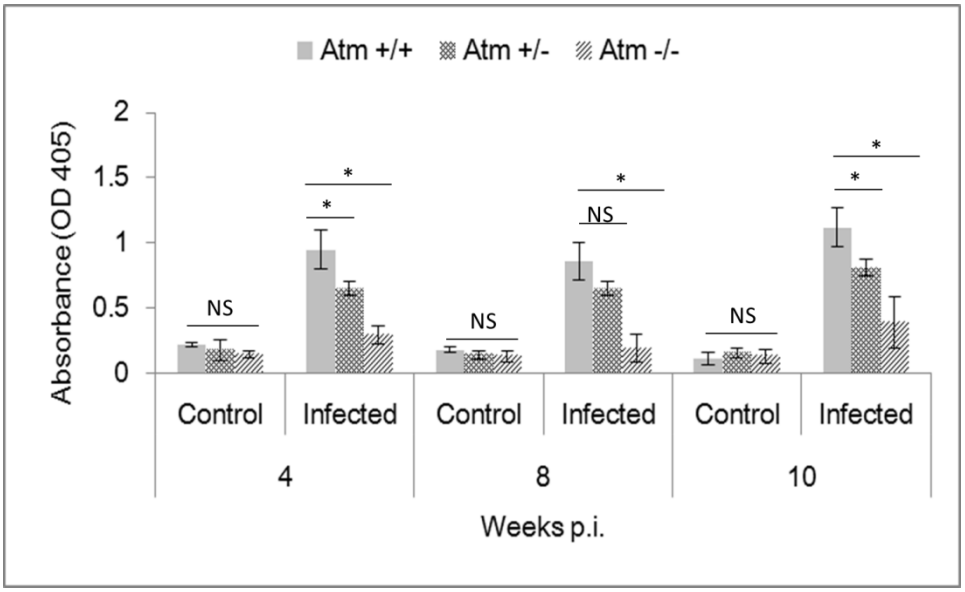
***H. hepaticus* elicited Atm-dependent immune response is modulated by host diet.**

Even though the precise nature of secretion of CDT during bacterial infection is not completely understood, increasing evidence suggests that CDT is secreted in bacterial outer membrane vesicles (Lindmark *et al.*, 2009; Rompikuntal *et al.*, 2012). Additionally, physiological levels of bile acid sodium deoxycholate enhance the outer membrane vesicle mediated secretion of CDT in vitro (Hickey *et al.*, 2005). Similar to mice fed with conventional diet, control mice had a basal level IgG response. The high cholate diet had an overall effect of reducing IgG1 responses against HhepWCL and HhepCdtB compared to conventional diet. Further, the response did not increase over time as compared to mice fed with conventional diet. Similar to mice fed with conventional diet, wild type mice had a significantly higher IgG1 response compared to Atm-null mice and the heterozygous mice had an intermediate IgG1 response as compared to wild type and Atm-null mice. The difference between IgG1 responses of wild type and heterozygous mice were more pronounce in high cholate diet fed mice compared to mice fed with conventional diet and reached statistical significance at 4 weeks p.i. and 10 weeks p. i. time points (Figure 5.4).

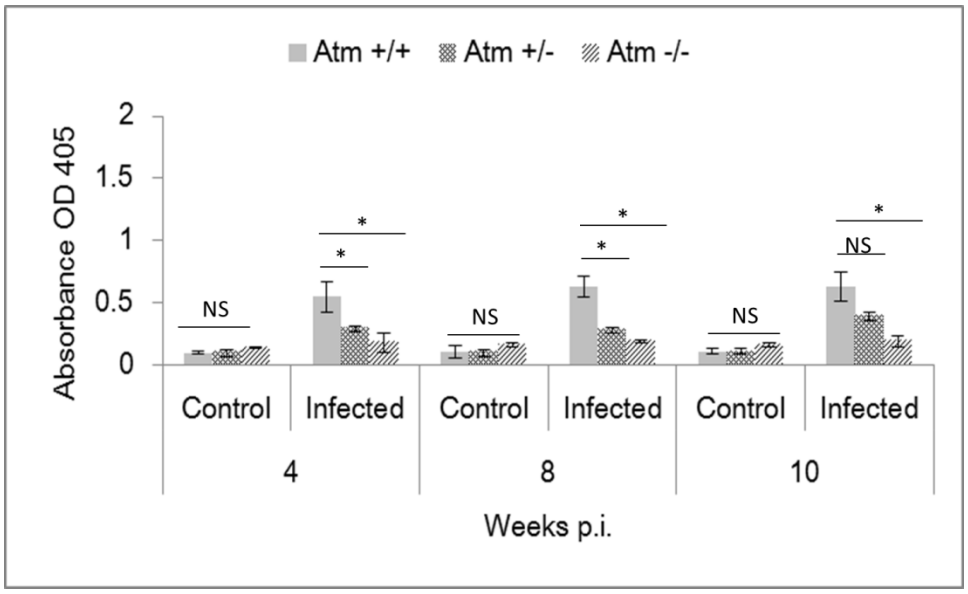
Figure 5.4 *H. hepaticus* elicited Atm-dependent immune response is modulated by host diet. Specific IgG1 responses (absorbance at 405 nm) were measured by ELISA against HhepWCL (A) and HhepCdtB (B) in mice fed with high cholate diet over 10 weeks p.i. The plates were coated with 0.02 µg of antigen and incubated with 1:100 diluted sera for 2 h at 37 °C. Following wash, the plates were incubated with 1:2000 dilutes anti-mouse IgG1-HRP. Following wash, peroxidase substrate was added to the plates and color development up to 20 mins was detected by spectrophotometer after adding 5% SDS stop reagent. The data represent mean values of three technical replicates. Number of mice used in each group is listed in Table 5.2. For each time point, the mean values were compared by using two way ANOVA test with Bonferroni correction [(*, $p < 0.05$; NS, not significant), (R. N. J. and G. E. D)].

Fig. 5. 4.

(A) WCL



(B) CdtB



Quantitative assessment of bacterial load in cecal tissue at10 weeks p.i.

In a representative preliminary experiment for quantitative assessment of bacterial load in cecal tissue at10 weeks p.i. in mice fed with conventional diet revealed a marginally high level of

colonization in Atm null mice. However, the mice fed with high cholate diet had a lower bacterial load compared to mice fed with conventional diet (Table 5.3). Even in the mice fed with high cholate diet, high level of colonization was detected in Atm null mice.

Table 5.3. Quantitative assessment of bacterial load in cecal tissue 10 weeks p.i.

Genotype	Diet			
	Conventional		High cholate	
	C_T Mean	SD	C_T Mean	SD
$Atm^{+/+}$	25.53 (n=2)	4.27	31.10(n=3)	0.53
$Atm^{+/-}$	25.73(n=3)	2.38	29.49(n=3)	0.53
$Atm^{-/-}$	19.84(n=2)	0.44	21.01(n=1)	0.08

Preliminary data from a representative experiment depicting the mean threshold (C_T Mean) values and standard deviations for detecting *H. hepaticus* genomic DNA in total DNA extracts of ceca from mice infected with *H. hepaticus* 10 weeks p.i. Each sample was assayed in triplicate (R. N. J.).

Discussion

As most of the previous studies on *H. hepaticus* infection in mice have been performed using laboratory strains of *H. hepaticus*, we used a field strain of *H. hepaticus* isolated from the liver of a mouse from the conventional mouse colony at TMCF in order to get a better understanding of the immune response during *H. hepaticus* infection. In mice fed with either type of diet, a significant IgG1 response was observed against both HhepWCL and HhepCdtB in infected group compared to controls. This indicates the development of adaptive immune responses against *H. hepaticus* and HhepCdtB, suggesting the production of CDT during pathogenesis. Adoptive immune responses for HhepWCL has been reported before, however this is the first report characterizing the adoptive immune response against HhepCdtB (Ge *et al.*, 2005; Pratt *et al.*, 2006; Young *et al.*, 2004). As expected wild type and heterozygous mice had a significantly higher IgG1 response compared to Atm-null mice and this is the first demonstration of the inability of Atm null mice to mount an adaptive immune response against a bacterial pathogen. Interestingly, the heterozygous mice had a marginally lower IgG1 response as compared to wild type and Atm-null mice. This is an interesting preliminary finding given the fact that 1-2 % of human population is heterozygous for Atm, they might be in an increased risk for infection with CDT producing bacteria (Lu *et al.*, 2006; Smilenov *et al.*, 2001).

The *Atm*$^{-/-}$ mice used in this study were initially designed and characterized by Elson et al., 1996 and has defective immune systems characterized by smaller thymi compared to wild type mice, reduced number of thymocyets, reduced cortex and a large medulla (Elson *et al.*, 1996). Additionally, *Atm*$^{-/-}$ mice develop spontaneous thymic lymphomas between 3-4 months of age (Elson *et al.*, 1996). Further, spleen and peripheral blood from *Atm*$^{-/-}$ mice had significant

reduction in T cells; however, no significant alterations were observed in the relative proportion of B cells (Elson *et al.*, 1996). An independent *Atm*$^{-/-}$ mice model developed by Barlow et al., 1996 had similar observations regarding lymphocyte development and T cell maturation while no significant defects were observed in B cell development (Barlow *et al.*, 1996). However, independent studies have demonstrated similar developmental defects in B cells (Xu *et al.*, 1996). However, more recent evidence suggest that ATM deficiency also impairs IgG class-switch recombination (Lumsden *et al.*, 2004) and result in low antibody titer (Reina-San-Martin *et al.*, 2004; Xu *et al.*, 1996), even though ATM is not essential for somatic hyper mutation (Reina-San-Martin *et al.*, 2004).

Based on the observations that high concentrations of all three CjejCDT subunits are present in detergent extracts of purified outer membrane preparations of *C. jejuni* suggests that it is primarily membrane-associated (Hickey *et al.*, 2000; Lindmark *et al.*, 2009). Incubation of *C. jejuni* in the presence of 25 mM or 0.1% bile acid sodium deoxycholate, a concentration physiologically relevant to the intestinal lumen, releases the membrane-associated CdtA, CdtB and CdtC subunits into the culture supernatant (Hickey *et al.*, 2005). We hypothesized that the increased release of CDT in the high deoxycholate diet environment could have a negative impact on the adoptive immune response because increasing concentrations of CDT might inhibit the proliferation or cause increased apoptosis in rapidly proliferating lymphocytes. The high cholate diet had an overall effect of reducing IgG1 responses against HhepWCL and HhepCdtB. Further, the response did not increase over time as compared to mice fed with conventional diet. Similar to mice fed with conventional diet, wild type mice had a significantly higher IgG1 response compared to Atm-null mice and the heterozygous mice had an

intermediate IgG1 response as compared to wild type and Atm-null mice. The difference between IgG1 responses of wild type and heterozygous mice were more pronounce in high cholate diet fed mice compared to mice fed with conventional diet. Even though our mouse model does not fully reproduce human AT phenotype, which is caused by several point and truncating mutations, there is some evidence to support that Atm heterozygous mice are more susceptible to cancer and IR (Barlow *et al.*, 1999; Spring *et al.*, 2002; Worgul *et al.*, 2002)

However, preliminary quantitative assessment of bacterial load in cecal tissue at10 weeks p.i. in mice fed with conventional diet reveals a marginally high level of colonization in Atm null mice. However, the mice fed with high cholate diet had lower bacterial load compared to mice fed with conventional diet. Even in the mice fed with high cholate diet, high level of colonization was detected in Atm null mice. In this context, even though we can assume Atm defective mice are more susceptible to *H. hepaticus* infection, we cannot rule out any additional effects by the high cholate diet that might cause reduction in colonization levels of *H. hepaticus*.

Additionally, we did not observe any significant intestinal inflammation during histopathological examination of uninfected or infected mice of any genotype euthanized at 4 or 10 weeks post challenge. This could be in part due to the shorter duration of the experiment or strain differences. Almost all previous studies *H. hepaticus* infection mice were performed with ATTCC reference strains investigation chronic infection and usually lasted for at lasted at least 16 weeks post infection (Ge *et al.*, 2005; Pratt *et al.*, 2006; Young *et al.*, 2004). Overall here we report for the first time that Atm null mice have reduced adaptive immune response against the infection with mouse pathogen *H. hepaticus* suggesting that DDR mechanisms are essential for

bacterial and CDT-specific host adaptive immune responses. Additionally, we provide interesting preliminary data for the future characterization of immune responses in Atm heterozygous mice and the effect of host diet in modulation of immune responses against CDT-producing bacteria.

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CHAPTER 6

Summary and Future Directions

It is well-established that the bacterial genotoxin, cytolethal distending toxin causes DNA damage in a wide range of eukaryotic cells *in vitro* and this result in a robust DNA damage response (DDR) in which ataxia telangiectasia mutated (ATM) plays a central role. Moreover, on the basis of a higher sensitivity of hematopoietic cells to CDT, it has been suggested that CDT modulates the host immune response *in vivo.* In chapter one, we proposed the models for CDT-induced cell cycle arrest and apoptosis based on most current literature and highlight the importance of investigating the CDT-induced G2/M and intra S-phase checkpoint. In chapter two we described the methods for the generation of mouse thymic lymphoma cell lines. We were able to generate several cell lines using this protocol and had been successfully used for our investigations. In chapter three, for the first time we demonstrated the activation of the ATM-dependent intra-S checkpoint by *H. hepaticus* CDT in primary mouse thymic lymphocytes and thymic lymphoma cell lines by observing a transient reduction in DNA synthesis (classical marker for intra S-phase checkpoint). Further in chapter three, we demonstrated an ATM-dependent reduction in phosphorylated histone H3 (classical marker for mitotic cells) and thereby definitively demonstrating CDT-mediated G2/M cell cycle arrest. Taken together, we provide first evidence to suggest that broadly conserved genotoxin CDT is capable of inducing cell cycle arrest in all phases of the cell cycle.

In chapter four, using CDT from *C. jejuni*, we conclude that p53 mutational status highly correlates with the variable CDT-susceptibility of human lymphoma and leukemia cell lines. We also conclude that variable CDT-susceptibility of human lymphoma and leukemia cell lines does

not significantly depend on the degree of activation of Akt pathway, which suggest that the major molecular mechanism of CDT-induced apoptosis is the nuclease activity of CdtB. We also report for the first time, the activation of Chk1 in human leukemia cell lines by CDT, suggesting the involvement of ATR in the DDR process and together with the absence of chromosomal breaks; this provides a new perspective for CDT-induced DDR. Finally in chapter five we assessed the adaptive humoral immune response induced by *H. hepaticus* infection in laboratory mice with a defective DDR due to mutation in *Atm* and demonstrate that ATM is are essential for bacterial and CDT-specific host adaptive immune responses.

Therefore further investigation of molecular mechanisms involved in a potential synergistic activity between ATM and ATR, including the immune responses in ATR defective mouse model is suggested. Additionally investigating the pathways involved in p53-independent apoptosis, particularly Chk1/Chk2-E2F1/p73 axis will elucidate the molecular mechanisms of p53-indepent late apoptosis observed in certain cell lines. Finally, based on our clear *in vitro* evidence showing that p53 status highly correlates with differential CDT susceptibility of cell lines, investigating a *H. hepaticus* infection model on a p53 defective background will further enhance the understanding of CDT-induced DDR.