MODULATION OF APOPTOSIS IN HUMAN URETHRAL EPITHELIAL CELLS BY WILD-TYPE NEISSERIA GONORRHOEAE AND ITS ISOGENIC MinD MUTANT STRAIN

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By

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ABSTRACT

The obligate human pathogen, *Neisseria gonorrhoeae*, has evolved mechanisms to manipulate the apoptotic machinery in human epithelial cells in favor of host niche adaptation. In the present research, I investigated the apoptotic effect of *N. gonorrhoeae* on transduced human urethral epithelial cells (THUEC) and the underlying mechanism of apoptosis modulation. Flow cytometric analysis showed that gonococcal infection conferred an anti-apoptotic effect in early infection, but induced apoptosis during prolonged infection periods in THUEC. Intracellular gonococci were required to provide the anti-apoptotic effect. Furthermore, immunoblotting analysis of the mitogen-activated protein kinase (MAPK) pathways mapped the apoptosis resistance to a signaling cascade involving epidermal growth factor receptor (EGFR), extracellular signal-regulated kinases (ERK), and Bim/Bad, in which the inhibition of ERK activation by *N. gonorrhoeae* contributed to the anti-apoptotic effect on THUEC.

A *N. gonorrhoeae minD* mutant strain harboring an insertionally inactivated *minD* gene that encodes an essential component within the cell division system, exhibits aberrant cell morphology and reduced adherence to and invasion of THUEC when compared to the parent strain. I investigated the impact of *minD* mutation on apoptosis and MAPK signaling in THUEC. Compared to the parent strain, infection with the *minD* mutant displayed reduced and delayed apoptosis during prolonged infection, and enhanced the inhibition of ERK activation from 6 h onwards. No alterations were observed on p38 and Jun N-terminal kinases (JNK) activation. Interestingly, the unexpected regulation of Bim and Bad coupled with enhanced inhibition of ERK activation in the *minD* mutant infected THUEC, followed by the stronger actin

rearrangement induced by the *minD* mutant relative to the parent strain as revealed by confocal microscopy, suggest a mechanism of bacterial cell shape-mediated modulation of host cell signaling through cytoskeleton rearrangement. Collectively, my data indicate the role for ERK pathway in *N. gonorrhoeae* mediated apoptosis resistance in THUEC, as well as a plausible impact of bacterial cell shape on host cell signaling.

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GuanQun

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LIST OF ABBREVIATIONS

A431	human epidermoid cells
ADP	adenosine diphosphate
AG1478	tyrphostin, EGFR inhibitor
Akt	protein kinase B
AMR	antimicrobial resistance
AP-1	activator protein 1
ASGP-R	asialoglycoprotein receptor
ASM	acid sphingomyelinase
ATP	adenosine triphosphate
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2-antagonist/killer-1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2
Bcl-xL	a BCL-2-like protein
Bfl-1	Bcl-2-related protein A1
BH3	Bcl-2 homology domain 3
BHI	brain heart infusion
Bik	Bcl-2-interacting killer
Bim	Bcl-2-like-11
Bmf	Bcl-2-modifying factor
BSA	bovine serum albumin
C3	complement component 3
C4BP	C4b-binding proteins
Caco-2	human epithelial colorectal adenocarcinoma cells
CD	cytochalasin D
Cdc42	cell division control protein 42 homolog
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CFU	colony-forming unit
Chang	human conjunctival cells
CHO	Chinese hamster ovary

c-IAP-2	baculoviral IAP repeat-containing protein 3
Cox-2	cyclooxygenase-2
CPAF	*chlamydia* protease-like activity factor
CR3	complement receptor 3
Ct	*Chlamydia trachomati*
Cyt *c*	cytrochrome c
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
EHEC	enterohemorrhagic *Escherichia coli*
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
Fgr	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
fH	factor H
FN	fibronectin
Gp96	heat shock protein 90kDa beta member 1
GTP	guanosine triphosphate
Hck	hemopoietic cell kinase
HEC-1-B	human endometrial cells
HeLa	human cervical epithelial cells
HEp2	human laryngeal epidermoid cells
HIV	human immunodeficiency virus
HPV	human papillomavirus
HSPGs	heparan-sulfate proteoglycans
IAP	inhibitors of apoptosis
ICAM-1	intercellular adhesion molecule 1, CD54
IgG	immunoglobulin G

IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin
IMM	inner mitochondrial membrane
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
JNK	c-Jun N-terminal kinase
JOSK-M	human myelomonocytic cells
LOS	lipooligosaccharide
MAPK	mitogen-activated protein kinases
Mcl-1	myeloid cell leukemia sequence-1
ME180	human cervical epithelial cells
MEK	mitogen-activated protein kinase kinase
min	minicell
MOI	multiplicity of infection
MOMP	mitochondrial outer membrane permeabilization
MreB	rod shape-determining protein
NF-κB	nuclear factor-κB
Ng	*Neisseria gonorrhoeae*
NHS	natural human serum
NI	non-infected
O/N	overnight
Opa	colony opacity-associated outer membrane proteins
p38	the 38-kDa stress-activated kinases
PBS	phosphate-buffered saline
PC-PLC	phosphatidylcholine-dependent phospholipase C
qPCR	quantitative real time polymerase chain reaction
PHUEC	primary urethral epithelial cells
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
P.IA/IB	PorB porin isoform 1A/1B

PilC	pilus assembly protein
PilT	twitching motility protein
PKC	protein kinase C
PMN	polymorphonuclear neutrophils
Puma	Bcl-2 binding component-3
Raf	MAP kinase kinase kinase
Ras	small GTPase
rhEGF	recombinant human epidermal growth factor
RLU	relative light units
RNA	ribonucleic acid
RT	room temperature
RTK	receptor tyrosine kinases
SB	SB202190, p38 inhibitor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHP	SH2-containing tyrosine phosphatases
SREC	scavenger receptor
STI	sexually transmitted infection
STS	staurosporine
Syk	spleen tyrosine kinase
T84	human colonic epidermoid cells
TCR	T cell receptor
Tfp	type IV pili
THUEC	transduced human urethral epithelial cells
TNF(R)	tumor necrosis factor (receptor)
TOM	translocase of the outer membrane
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
UO	U0126, MEK1/2 inhibitor
UPEC	uropathogenic *Escherichia coli*
Vav	guanine nucleotide exchange factor
VN	vitronectin

CHAPTER 1 INTRODUCTION

1.1. General Introduction of *Neisseria gonorrhoeae*

1.1.1. Gonococcal Infection in Men and Women

Gonorrhoea is the second most prevalent bacterial sexually transmitted infection (STI) worldwide with 106 million new cases each year (WHO, 2012). It is caused by the Gram-negative obligate human pathogen - *Neisseria gonorrhoeae* (Ng). Historical records of gonococcal infection can be traced back to biblical times (Leviticus 15:1-19) and the link of gonorrhoea to the causative agent Ng was initially described by Neisser in 1879 followed by the cultivation of the gonococcus by Leistikow and Löffler in 1882 (Sparling, 2007).

Ng infects mucosal surfaces including the urogenital tracts of men and women, the conjunctiva, the pharynx and rectum (Edwards and Apicella, 2004). In women, common symptoms of uncomplicated gonococcal infection include odorless vaginal discharge, vaginal bleeding, and dyspareunia. However, about 50% of women with cervical infections are asymptomatic carriers and 10 to 20 percent of unrecognized infections can develop ascending infection that causes acute salpingitis with endometritis (also known as pelvic inflammatory disease, PID), which can lead to ectopic pregnancy and infertility (CDC, 2010; Edwards and Butler, 2011; Miller, 2006). In men, urethral infections are usually symptomatic, characterized by acute urethritis concomitant with a purulent penile discharge containing polymorphonuclear neutrophils and urethral epithelial cells (Edwards and Apicella, 2004). Complications include epididymitis, which can manifest as unilateral testicular pain without discharge or dysuria (Miller, 2006). Moreover, gonococcal infection facilitates human immunodeficiency virus (HIV)

transmission by 3-5-fold, probably by the Ng-induced inflammatory response and the compromise of mucosal integrity (Jarvis and Chang, 2012; Wang et al., 2003).

The control of gonococcal infection relies on effective diagnosis and antibiotic treatment, preventive measures such as contact tracing and safe sex, and strengthened surveillance systems. However, effective antibiotic therapy is being eroded by the emergence of multidrug-resistant clinical isolates, leading gonococcal infection towards an untreatable status (Bolan et al., 2012; Dillon, 2011; WHO, 2012). This increased antimicrobial resistance (AMR) is hastening the search for novel antibiotic targets and particularly the development of vaccine candidates. Unfortunately, no effective vaccine is yet available despite decades of research. This is mainly because of the highly variable nature of Ng outer membrane proteins that undergo antigenic and phase variation, and the suppression and redirection of immune responses during naturally acquired gonococcal infection (Zhu et al., 2011).

1.1.2. Biology of *N. gonorrhoeae*

1.1.2.1. Gonococcal Infection Process

Gonococcal infection is comprised of at least four steps, including adherence, internalization, host niche adaptation, and dissemination (Merz and So, 2000). The intimate association between Ng and host cells (including epithelial cells, neutrophils, and lymphocytes) involves a number of interactions of bacterial outer membrane proteins (i.e. virulence factors) with host cell receptors (Edwards and Apicella, 2004). These virulence factors mainly include Tfp (Type IV pili), Opa proteins (colony opacity-associated outer membrane proteins), LOS (lipooligosaccharide), and PorB (Porin proteins) (Burch et al., 1997; Danielsson et al., 1986; Hagblom et al., 1985; Sadarangani

et al., 2011). These virulence factors, through their interaction with host receptors, enable Ng to invade host cells and to establish an intracellular niche by direct or indirect activation of signaling cascades in host cells during the course of infection. Furthermore, by using the mechanisms of antigenic and phase variation, they aid in Ng interacting with distinct cell types over time of infection, and successfully escaping the surveillance of the human immune system (Gray-Owen et al., 1997; Popp et al., 1999).

1.1.2.2. *N. gonorrhoeae* Major Virulence Factors

1.1.2.2.1. Type IV Pili

Tfp are retractile appendages that undergo antigenic variation through considerable and frequent intra- and inter-genomic recombination of pilin genes (Cahoon and Seifert, 2011). They confer initial attachment of gonococci to epithelial cells and mediate the formation of bacterial microcolonies, which are bacterial aggregates of 10-100 diplococci (Merz and So, 2000). Cortical plaques are then formed beneath the microcolonies, composed of extended microvilli, cortical actin and accumulated transmembrane receptors, such as CD44, EGFR, and ICAM-1 (Higashi et al., 2007; Higashi et al., 2009; Merz et al., 1999; Weyand et al., 2006). To date, two pilus-associated proteins of the Ng Tfp, designated PilC and PilT, have been identified as participating in the pathogenesis of epithelial cell infection. PilC mediates the binding of piliated gonococci to human cells in either a CD46 (Membrane Cofactor Protein)-dependent or independent manner, defined as a cell/tissue tropism (Kirchner and Meyer, 2005). PilC-CD46 interaction initiates host cell signaling including calcium fluxes and redistribution of host cell receptors (Ayala et al., 2001; Kallstrom et al., 1998). PilT is an ATPase that mediates pilus retraction. This retraction exerts mechanical forces on the host

cell membrane upon gonococcal adherence and initiates two mechanosensitive signaling cascades in host cells. The first one favors bacterial invasion and microcolony formation through the activation of phosphoinositide 3-kinase (PI3K)/Akt (PKB) pathways while the second signaling protects the infected cells from apoptosis, conferred by the activation of mitogen-activated protein kinases (MAPKs) pathways (Howie et al., 2005; Lee et al., 2005).

1.1.2.2.2. Colony Opacity-associated Outer Membrane Proteins

The Opa proteins mediate the intimate adherence of Ng to host cells. Ng contains 11 *opa* genes and their expression undergoes phase variation at the translational level due to the modulation of the number of 5'-CTCTT-3' repeats within the sequence encoding the N-terminal leader peptide by slipped-strand mispairing mediated frameshifting during DNA replication. The large reservoir of Opa proteins determines the cell tropism of gonococci through their interaction with two classes of host cell receptor, heparan-sulfate proteoglycans (HSPGs) and carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family, which are the targets of the Opa_{50} and the Opa_{CEA}, respectively (Sadarangani et al., 2011).

HSPGs are mainly expressed on the basolateral side of polarized epithelial cells, and their interaction with Opa_{50} mediates the phagocytic uptake of gonococci. This event involves the cytoskeletal rearrangements induced by two lipid second messengers, diacylglycerol (DAG) and ceramide, which are enzymatic products of membrane-localized phosphatidylcholine-dependent phospholipase C (PC-PLC) and acid sphingomyelinase (ASM) (Naumann et al., 1999). Alternatively, Opa_{50} can bind to host integrins expressed on the epithelial cells through two serum glycoproteins, vitronectin

(VN) and fibronectin (FN). However, this bridged interaction requires the prerequisite of Opa_{50} association with HSPGs and activation of protein kinase C (PKC) (Dehio et al., 1998).

Only four (i.e. CEACAM1, CEACAM3, CEACAM5 and CEACAM6) of the 12 members of the human CEACAM family have been shown to directly associate with Opa_{CEA} proteins. Among them, CEACAM1, CEACAM5 and CEACAM6 are expressed on the apical side of the polarized epithelial cells, while CEACAM 1, CEACAM 3 and CEACAM6 are expressed in neutrophils and CEACAM 1 is expressed in T and B lymphocytes (Sadarangani et al., 2011). In conjunction with the basolateral distribution of the Opa_{50} specific HSPGs, the apical expression of CEACAMs on polarized epithelial cells allows the transcellular transcytosis of gonococci. This provides a plausible mechanism of prolonged gonococcal transmission that gonococci exploit CEACAMs to efficiently invade subepithelial spaces and travel back to the mucosal surface from the infected tissues via HSPG-mediated endocytosis.

In neutrophils, internalization of Opa^{+} gonococci and the potently bactericidal cellular response are mediated by CEACAM3 (McCaw et al., 2003; Sarantis and Gray-Owen, 2007). CEACAM3 is a neutrophil-restricted receptor and harbors an immunoreceptor tyrosine-based activation motif (ITAM). Opa binding triggers the phosphorylation of tyrosine residues within ITAM by Src tyrosine kinases, such as Hck and Fgr. These phosphorylated tyrosine residues serve as the docking sites for different downstream effectors, such as Syk, Vav, and the PI3K regulatory subunit. While the guanine nucleotide exchange factor Vav activates small GTPase Rac and Cdc42 to promote cytoskeletal rearrangement and bacterial uptake, activation of Syk and PI3K

signaling has been shown to initiate bactericidal oxidative burst (Billker et al., 2002; Buntru et al., 2011; Sarantis and Gray-Owen, 2007).

Opa_{CEA} binding to CEACAM1 has been shown to arrest T cell activation and proliferation and to inhibit antibody production from B cells resulting from induction of cell death (Boulton and Gray-Owen, 2002; Pantelic et al., 2005). These effects in part explain the suppression of the immune response upon gonococcal infection. T cell activation is mediated by the activation of the T cell receptor (TCR) and subsequent stimulation of Src kinases, which leads to the tyrosine phosphorylation of the immunoreceptor tyrosine-based inhibition motif (ITIM) within CEACAM1 and the activation of downstream signaling cascades. Upon Opa_{CEA} binding to CEACAM1, tyrosine phosphatases SHP-1 and SHP-2 are recruited to ITIM and dephosphorylate CEACAM1 and TCR, which terminates T cell activation (Lee et al., 2008).

1.1.2.2.3. Lipooligosaccharide

The phase variable LOS is comprised of three major components: the oligosaccharide chain, the core region, and lipid A (Kerwood et al., 1992). All three structures have been shown to participate in gonococcal pathogenesis, including adherence, invasion, and inflammation. In primary male urethral epithelial cells, the lacto-*N*-neotetraose-terminal LOS is critical for efficient Ng invasion by binding to asialoglycoprotein receptor (ASGP-R), while a lipid A *msbB* mutant of Ng with a pentaacyl rather than the hexaacyl lipid A structure exhibited compromised ability in intracellular survival (Harvey et al., 2001; Post et al., 2002). Ng infection of epithelial cells is accompanied by production of cytokines, including interleukin (IL)-1β, IL-6, IL-8, and TNF-α, to recruit PMNs to the site of infection. This mutant also induced reduced

IL-6 and IL-8 production compared to the parent strain infection (Harvey et al., 2002). In addition, lipid A has been shown to serve as a site of deposition for complement fragment 3b (C3b), which is in turn converted to inactive complement fragment 3b (iC3b) and aids gonococcal invasion of female cervical epithelial cells by binding to complement receptor type 3 (CR3) (Edwards and Apicella, 2002). Sialylation of lacto-*N*-neotetraose-containing LOS on the variable oligosaccharide moiety renders those strains resistant to complement-mediated killing (i.e. unstable serum resistance) but may impair bacterial invasion of some cell types. This adaptive mechanism may prolong gonococcal infection by enabling Ng to switch between serum-resistant and invasive phenotypes (van Putten, 1993).

1.1.2.2.4. PorB Porin

Another gonococcal virulence factor, the PorB porins, are the most represented outer membrane proteins in the pathogenic *Neisseria* species and share the same trimeric structure and selective ion channel function with other members of Gram-negative porin superfamily (Massari et al., 2003). Ng PorB molecules confer stable serum resistance on gonococci by their abilities to bind plasma complement inhibitory proteins such as factor H (fH) and C4b-binding proteins (C4BP) (Ram et al., 2001). Ng PorB porins include two isoforms, PorBIA (P.IA) and PorBIB (P.IB). Whereas P.IA-expressing strains correlate with severe disseminating gonococcal infection and are often hyper-susceptible to antibiotics, P.IB strains are usually associated with local urogenital disease and reduced susceptibility to antibiotics (Brunham et al., 1985; Cannon et al., 1983; Liao et al., 2008). In addition, compared with gonococci expressing P.IB, gonococcal strains carrying P.IA are more resistant to killing by natural human serum (NHS) (van Putten et al., 1998).

Two recent studies have shown the role of P.IA in gonococcal adherence to and invasion of epithelial cells. The first study revealed a fH-mediated interaction of unsialylated P.IA gonococci with human complement receptor 3 (CR3) expressed on Chinese hamster ovary (CHO) cells (Agarwal et al., 2010a), while the second study identified the human heat shock glycoprotein Gp96 as a P.IA binding factor that facilitates gonococcal adherence and the scavenger receptor SREC as P.IA-specific receptors that mediate invasion of gonococci into human epithelial cells (Rechner et al., 2007). In contrast to P.IA, the host receptor for P.IB has not been identified.

Both PorB isoforms can be translocated into epithelial cell membranes, which results in the establishment of membrane-inserted anionic channels regulated by ATP/GTP (Muller et al., 1999). These channels rapidly elicit a transient calcium influx from the extracellular space, which in turn activates the Ca^{2+} dependent protease calpain and caspases and induces apoptosis (Muller et al., 1999). PorB molecules can be further integrated into the inner mitochondrial membrane (IMM) by the translocase of the outer mitochondrial membrane (TOM) complex, in which they induce dissipation of mitochondrial membrane potential and crista reorganization and initiate apoptosis (Muller et al., 2000; Muller et al., 2002). Interestingly, the effect of P.IA and P.IB on apoptosis has been shown to be opposite in different cell types. This will be discussed in the following sections.

1.2. Manipulation of Apoptosis in Human Epithelial Cells by *N. gonorrhoeae*

1.2.1. Apoptosis-related Cell Signaling Cascades in Human Epithelial Cells Modulated by *N. gonorrhoeae*

1.2.1.1. The MAP Kinase Signaling Cascades

Three major MAPK signaling cascades, including ERK, p38 MAPK and JNK, are targets of bacterial pathogens for modulating host cell responses. Previous studies have shown that ERK1/2 and p38 MAPK were activated in a Ng-infected T84 cell line (human colonic epidermoid cells) (Howie et al., 2005), while JNK was activated in Ng-infected T84, HeLa (human cervical epithelial cells), Chang (human conjunctival cells), and JOSK-M (human myelomonocytic cells) cell lines (Hauck et al., 1998; Howie et al., 2005; Kepp et al., 2009; Naumann et al., 1998). Mitogen-activated protein kinases (MAPKs) are serine/threonine-specific protein kinases and include the extracellular signal-regulated kinases (ERK1/2, also known as p44/42), the 38-kDa stress-activated kinases (p38 α, β, γ, δ), the c-Jun N-terminal kinases (JNK1/2/3), and ERK5 (Ramos, 2008). These kinases are activated through a sequential phosphorylation cascade in response to diverse extracellular stimuli and regulate various cellular activities, such as cell cycle progression, proliferation, differentiation, and cell survival/apoptosis (Cagnol and Chambard, 2010) (Fig 1.1). The ability of a number of bacteria to modulate host MAPK signaling is an effective virulence strategy. For example, a secreted type III effector protein from *Yersinia* spp., YopJ, acts as an acetyltransferase and counteracts p38 MAPK, JNK, and NF-κB signaling in infected cells by disrupting posttranslational modification (Mukherjee et al., 2006; Orth et al., 2000). A similar observation has shown that activation of ERK and p38 MAPK in macrophages upon virulent *Mycobacterium avium* invasion diminished quicker than that by the non-pathogenic strains, suggesting a mechanism that is beneficial to immune intervention and intracellular survival (Roach and Schorey, 2002).

In the context of gonococcal pathogenesis, ERK, p38 MAPK and JNK pathways

have all been reported to be activated in different cell types. JNK activation has been shown to induce Opa-dependent phagocytosis, activator protein 1 (AP-1)-dependent expression of proinflammatory cytokines, and Opa-mediated apoptosis (Hauck et al., 1998; Kepp et al., 2009; Naumann et al., 1998). Evidence for the independent involvement of p38 MAPK in gonococcal infection has not been fully resolved; however, short-term activation of JNK and p38 MAPK concomitant with prolonged ERK activation mediates apoptosis resistance in T84 human colonic epidermoid cells infected with piliated, Opa^- Ng (Howie et al., 2005).

Although signaling components involved in the ERK pathway upon gonococcal infection are not fully understood, the canonical ERK pathway (Fig 1.1) depicts the binding of extracellular growth factors such as epidermal growth factor (EGF) to its transmembrane receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor (EGFR). Downstream activation of the membrane-localized small GTPase Ras leads to the sequential recruitment and activation of Raf, MEK, and ERK (Kolch, 2000). Indeed, the recruitment of EGF receptors (EGFR) within the cortical plaque beneath gonococcal microcolonies has been observed (Merz et al., 1999), indicating the importance of microcolony formation in activating the ERK pathway in host cells. Moreover, a recent study has shown that Ng induces the transactivation of EGFR, which enhances gonococcal invasion into genital epithelial cells (Swanson et al., 2011).

For most bacteria, the orchestration of ERK, p38 MAPK and JNK signaling determines host responses, such as uptake of bacteria and apoptosis. For example, coordinate signaling of ERK and JNK mediates the uptake of pneumococci by host epithelial cells (Agarwal et al., 2010b). *Francisella tularensis*-induced macrophage

apoptosis requires the activation of the ERK pathway associated with reduced p38 MAPK activity (Hrstka et al., 2005). Effects of MAPK activation on apoptosis seem dependent on the magnitude and duration of signaling. In epithelial cells infected with piliated, Opa non-expressing Ng, a transient JNK activation associated with prolonged ERK activity displays an anti-apoptotic effect, whereas prolonged JNK activity without ERK activation induces apoptosis in P^- Opa^+ Ng-infected cells (Howie et al., 2005; Kepp et al., 2009).

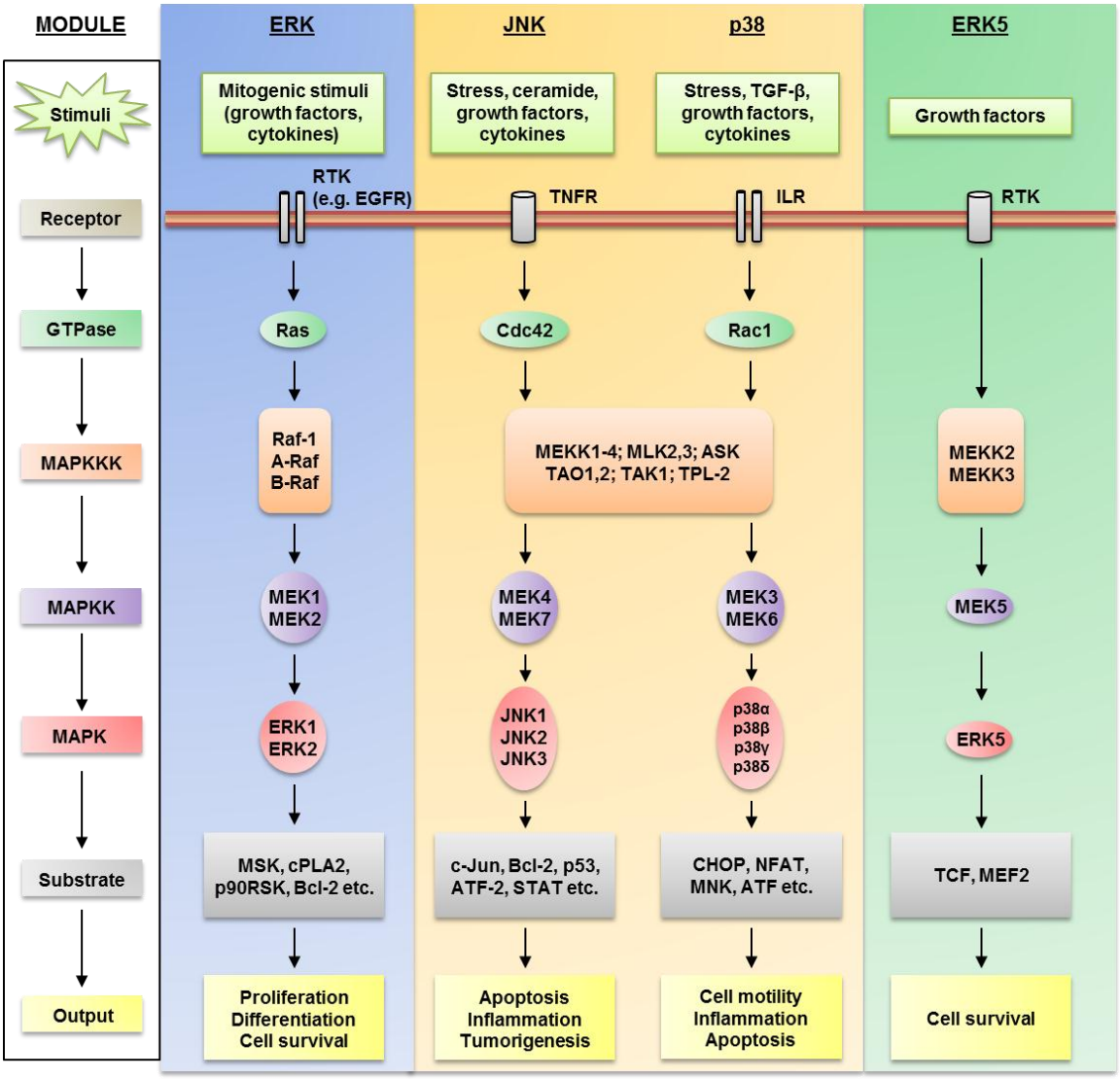

FIGURE 1.1. A simplified diagram showing the architecture of mammalian MAPK signaling pathways. Upon stimulation by a diverse array of stimuli, such as growth factors, cytokines, and stress, host cell receptors are recruited and the components involved in MAPK signaling pathways are activated in a sequential phosphorylation manner. The core architecture of MAPK signaling cascades (shown in the box) is the sequential activation of three-tiered kinase cascade from MAP3K to MAPK, which

includes ERK, p38, JNK, and ERK5. These MAPKs subsequently phosphorylate downstream substrates and regulate diverse cellular processes, such as cell proliferation, differentiation, and apoptosis. MAPKKK, MAP kinase kinase kinase; MAPKK, MAP kinase kinase; MAPK, MAP kinase; RTK, receptor tyrosine kinase; TNFR, tumor necrosis factor receptor; ILR, interleukin receptor. Modified from (Krishna and Narang, 2008; Qi and Elion, 2005).

1.2.1.2. The NF-κB Signaling Cascade

Gonococcal infections of human mucosal epithelial cells are often associated with a rapid local release of pro-inflammatory cytokines and chemokines, including IL-1β, IL-6, IL-8, and TNF-α (Harvey et al., 2002; Naumann et al., 1997). Some of these proteins are controlled by the classical nuclear factor-κB (NF-κB) signaling. The NF-κB complexes in mammalian cells are homo- or heterodimers comprised of the members of the Rel domain-containing protein family, including RelA (p65), RelB, c-Rel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52) (Rahman and McFadden, 2011). Normally, the ubiquitous p105/RelA heterodimers in the canonical NF-κB pathway are sequestered in the cytoplasm by binding to proteins of the inhibitor of NF-κB (IκB) family such as IκBα (Fig 1.2). Following phosphorylation of IκBα and p105 by the activated IκB kinase (IKK) complex (IKKα/IKKβ/NEMO), IκBα is degraded and p105 is cleaved into the p50 subunit. Eventually, the p50/RelA complexes are released and translocated into the nucleus, in which they drive the transcription of a broad spectrum of pro-inflammatory and pro-survival genes (Fig 1.2). In Ng-infected epithelial cells, NF-κB p50/RelA heterodimers have been shown to drive cytokine expression and to up-regulate CEACAM 1 expression via a Toll-like receptor-4-dependent pathway which enhances Opa-mediated Ng adherence (Muenzner et al., 2002; Muenzner et al., 2001; Naumann et al., 1997).

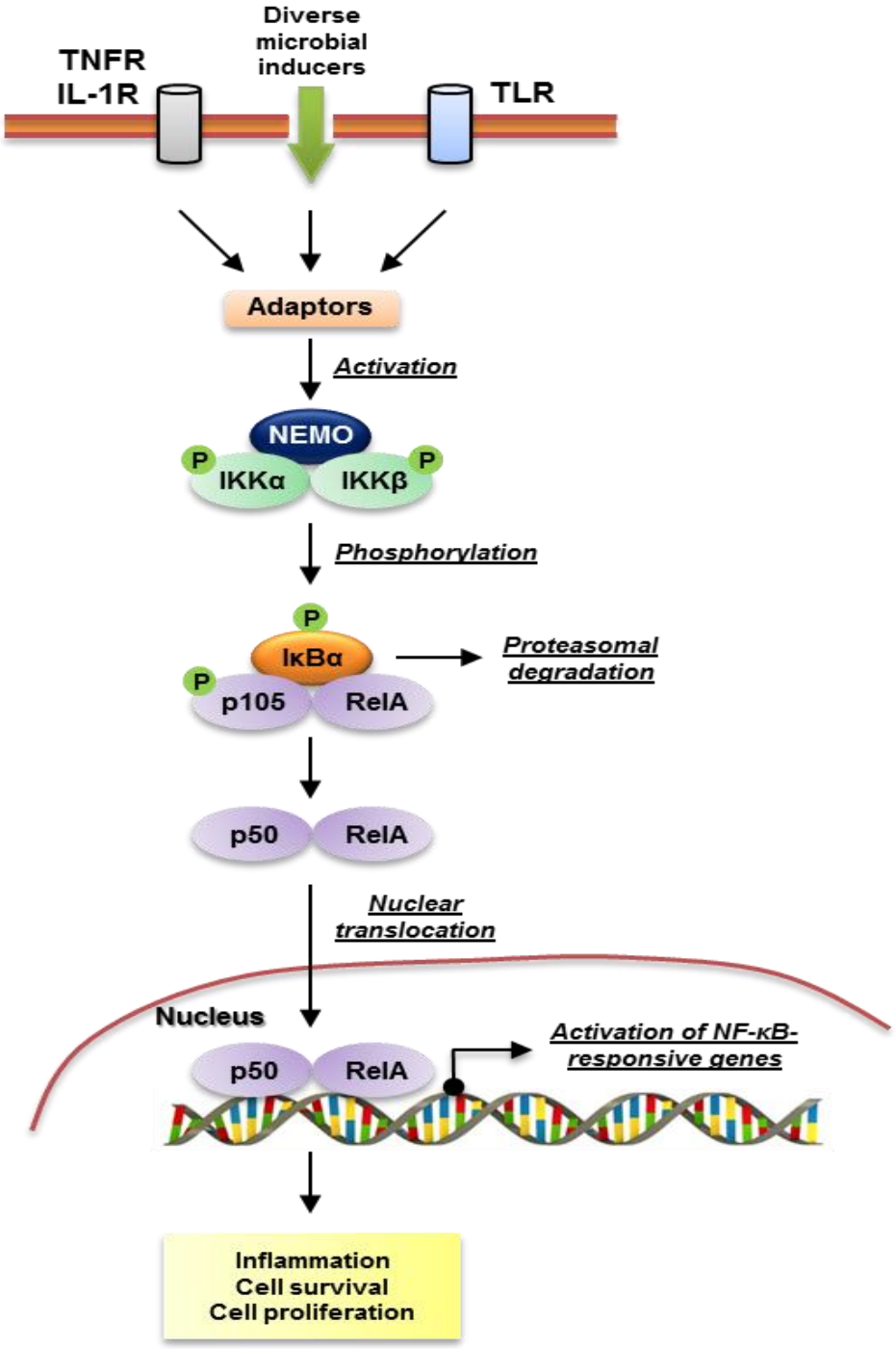

FIGURE 1.2. The canonical NF-κB pathway. The cognate ligand binding to receptors or the translocation of bacterial effector proteins leads to the activation of the IKK complex comprising IKKα and IKKβ catalytic subunits, and NEMO. Phosphorylation of

IκBα and p105 by the activated IKK complex leads to the degradation of IκBα by the proteasome and processing of p105 to p50 subunit. Released NF-κB heterodimers (i.e. p50/RelA) are then translocated into the nucleus and drive the transcription of NF-κB-responsive genes. These genes encode proteins that are involved in host responses including inflammation, cell survival, and cell proliferation. IκBα, NF-κB inhibitor α; IKK, IκB kinase; IL-1R, interleukin-1 receptor; NEMO, NF-κB essential modulator (also known as IKKγ); TLR, Toll-like receptor; TNFR, TNF receptor. Modified from (Rahman and McFadden, 2011)

Recent research has provided insight into the association of pilus retraction and microcolony growth of Ng with NF-κB activation. A Ng MS11Δ*pilT* mutant deficient in Tfp retraction forms disordered microcolonies and triggers markedly reduced NF-κB activation in epithelial cells as compared to the wild-type strain. In addition, the level of NF-κB activation correlates consistently with the initial number of gonococci within a microcolony, as well as the growth and fusion of microcolonies (Dietrich et al., 2011). The authors hypothesized a role for bacterial cell division, which controls the number of bacteria, in microcolony growth and NF-κB activation (Dietrich et al., 2011).

The effect of gonococcal PorB porin on apoptosis is also associated with the activation of NF-κB signaling. In contrast to the apoptosis-inducing effect of P.IA on epithelial cells, P.IB alone (in Δ*pil* or Δ*Opa* Ng mutant strains) has been demonstrated to activate the NF-κB pathway and the expression of its controlled anti-apoptotic genes in transduced human urethral epithelial cells (THUEC), including *bfl-1*, *cox-2*, and *cIAP-2* (Binnicker et al., 2004). The increased expression of anti-apoptotic genes provides evidence that P.IB may regulate the host apoptosis machinery; however, purified P.IB alone failed to protect THUEC from apoptosis, which was otherwise observed by infection with the wild-type gonococci (Binnicker et al., 2003, 2004). This underscores a synergistic effect of P.IB with other virulence factors on Ng-modulated apoptosis.

1.2.2. Extrinsic and Intrinsic Apoptotic Pathways and the Bcl-2 Family Proteins

Programmed cell death by apoptosis is an adaptive process by which organisms remove irreparably damaged and potentially dangerous cells to maintain homeostasis without eliciting inflammation (Lamkanfi and Dixit, 2010). Bacteria are equipped with apoptosis-modulating strategies for host niche adaptation (Rudel et al., 2010). Ng can

either induce or inhibit apoptosis in human epithelial cells (Binnicker et al., 2003, 2004; Follows et al., 2009; Howie et al., 2005; Howie et al., 2008; Kepp et al., 2009; Morales et al., 2006; Muller et al., 2000; Muller et al., 1999; Muller et al., 2002). In most cases, the core event in apoptosis is the activation of effector caspases (i.e. caspase-3, -6 and -7) by initiator caspases (i.e. caspase-8, -9 and -10), namely the caspase cascade to initiate morphological and biochemical changes of the cells (Pop and Salvesen, 2009). Two apoptotic pathways, extrinsic and intrinsic pathways, are characterized by the different involvement of initiator caspases (Fig 1.3). The extrinsic apoptotic pathway is triggered by the binding of extracellular death receptor ligands (i.e. FasL/TNF/TRAIL) to cell death receptors (i.e. Fas/TNFR/DR) which promotes the recruitment and activation of caspase-8 or -10, while the intrinsic apoptotic pathway is initiated by intracellular stimuli that activate the Bcl-2 family proteins (e.g. Bax and Bak) and regulate mitochondrial membrane integrity. Mitochondrial outer membrane permeabilization (MOMP) subsequently leads to the release of pro-apoptotic factors such as cytochrome *c* (Cyt *c*) from the mitochondria into the cytoplasm and the activation of caspase-9 (Elmore, 2007; Rudel et al., 2010).

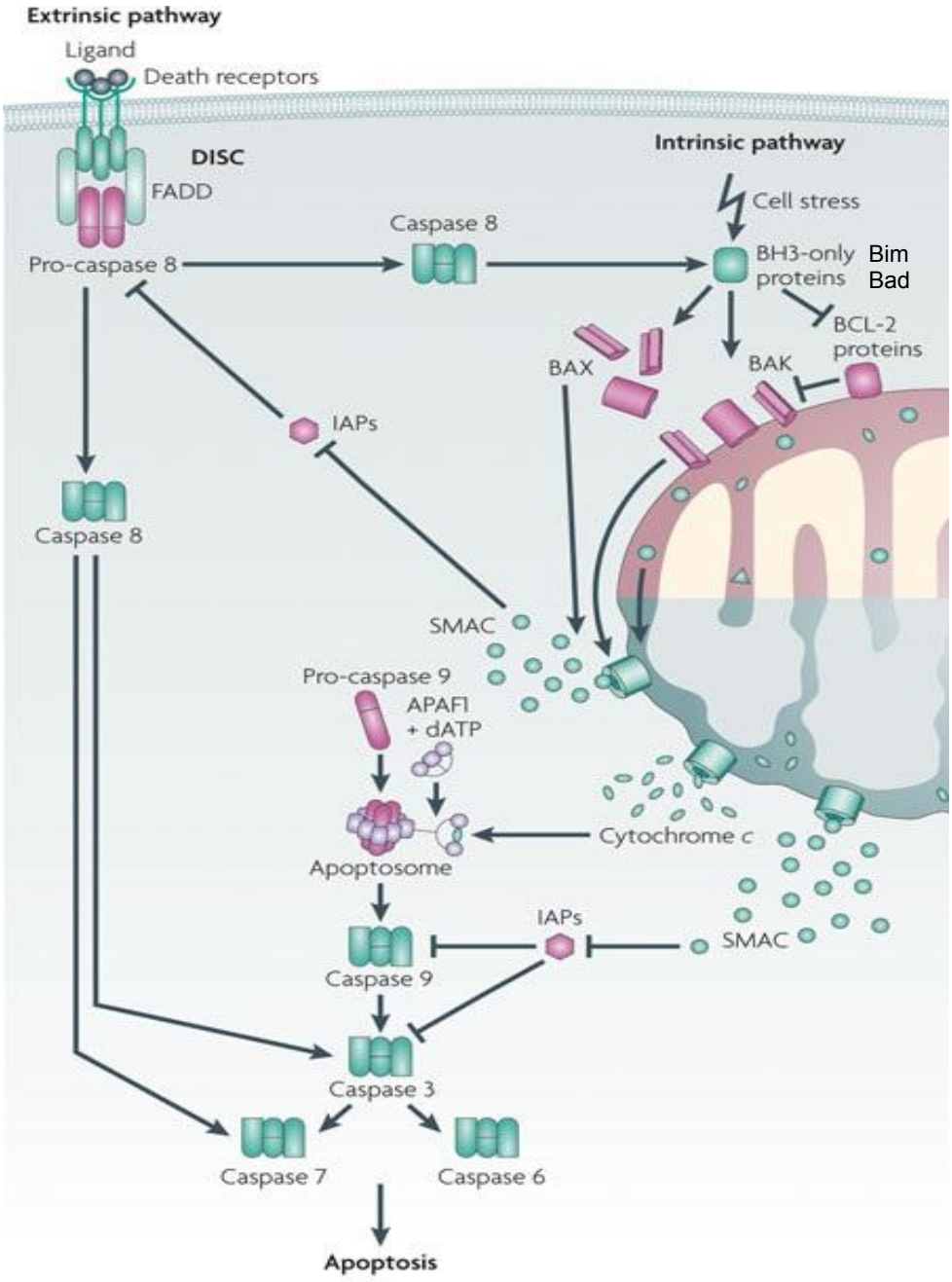

FIGURE 1.3. Extrinsic and intrinsic apoptotic pathways. Apoptosis is activated either by the death receptor-mediated extrinsic pathway or by the Bcl-2 family protein-mediated intrinsic pathways. The extrinsic apoptotic pathway is initiated by the cognate binding of extracellular death receptor ligands to their cognate cell death receptors followed by the

formation of the intracellular death-inducing signaling complex (DISC) composed of FAS-associated death domain (FADD) and pro-caspase 8. Auto-processing of pro-caspase 8 releases active caspase 8, which in turn proteolytically activates effector caspases (e.g. caspase 3, 6, and 7). The intrinsic pathway is triggered by various intracellular stresses, which activate the pro-apoptotic B cell lymphoma 2 (Bcl-2) homology 3 (BH3)-only proteins (e.g. Bim and Bad) that neutralize the anti-apoptotic Bcl-2 proteins (e.g. Bcl-2 and Bcl-xL). This effect liberates Bak and Bax from the inhibition by anti-apoptotic Bcl-2 proteins and the activation and oligomerization of Bak and Bax in the outer mitochondrial membrane lead to mitochondrial outer membrane permeabilization (MOMP). This results in the release of intermembrane space proteins, such as cytochrome *c*, second mitochondrion-derived activator of caspase (SMAC) from the mitochondria into the cytosol. Cytochrome *c* stimulates the assembly of the apoptosome by binding to the adaptor apoptotic protease-activating factor 1 (APAF1), which in turn activates caspase 9 and downstream effector caspases. SMAC relieves the inhibition of initiator caspases (e.g. caspase 8 and caspase 9) by inhibitor of apoptosis proteins (IAPs). (Rudel et al., 2010).

Regulation of MOMP is controlled by the balance of pro-apoptotic and anti-apoptotic Bcl-2 family protein members (Table 1.1). In unstimulated cells, pro-apoptotic proteins Bax and Bak are sequestered by anti-apoptotic proteins, such as Bcl-2, Bcl-xL and Mcl-1. This sequestration prevents the activation and oligomerization of Bax and Bak in the mitochondrial outer membrane. Activation of pro-apoptotic BH3-only proteins such as Bim, Bad and Bmf, releases Bax and Bak from Bcl-2 inhibition, thereby recovering the pore-forming activity of Bax and Bak in the mitochondrial outer membrane (Youle and Strasser, 2008).

Resistance to apoptosis induced by bacteria is beneficial to their intracellular survival. *Chlamydia* spp. and *Neisseria* are capable of manipulating the host's apoptotic machinery by modulating Bcl-2 family proteins. The pro-apoptotic BH3-only proteins including Bim, Puma, Bad, and Bik have been shown to be degraded in HeLa cells infected with *Chlamydia trachomatis* (Ct) by the secreted *chlamydia* protease-like activity factor (CPAF) (Dong et al., 2005; Fischer et al., 2004; Pirbhai et al., 2006). In addition, the PI3K/Akt-dependent phosphorylation of Bad in Ct-infected HeLa cells leads to the sequestration of phosphorylated Bad (p-Bad) via the host-cell adaptor protein 14-3-3β, thereby abolishing its access to the mitochondria (Verbeke et al., 2006).

A similar anti-apoptotic effect in Ng-infected T84 cells (human colonic epidermoid cells) is conferred by the *pilT*-enhanced extracellular-signal regulated kinases (ERK) activation, leading to the downregulation of BH3-only proteins Bim and Bad. While Bim undergoes degradation through the proteasome, Bad is inactivated by its phosphorylation at Serine-112 (Howie et al., 2008). In contrast, a Ng Opa-dependent activation of Bim and Bmf via c-Jun N-terminal kinase (JNK) pathway induces apoptosis

in HeLa, ME180, and HEp2 cells (Kepp et al., 2009).

The contradictory effects of Ng on apoptosis induction and inhibition indicate an adaptive mechanism exploited by Ng to regulate its cell cycle within host niches. These conflicting observations are also likely to result from the differences in bacterial strains, cell lines, cell culture conditions, and infection protocols used in the studies (Table 1.2) By comparing P.IA- or Opa-mediated apoptosis induction (Kepp et al., 2009; Muller et al., 2000; Muller et al., 1999; Muller et al., 2002) with P.IB- or Tfp-mediated inhibition of apoptosis (Binnicker et al., 2004; Howie et al., 2005; Howie et al., 2008), it is believed that no individual virulence factor solely accounts for the pro-/anti-apoptotic response observed with live Ng infection (Binnicker et al., 2003; Follows et al., 2009; Morales et al., 2006).

TABLE 1.1. Pro-apoptotic and anti-apoptotic Bcl-2 family members

Category	Members
Pro-apoptotic BH3-only proteins	Bad: BCL-2 antagonist of cell death Bid: BH3-interacting domain death agonist Bik: BCL-2-interacting killer Bim: BCL-2-like-11 Hrk: harakiri (also known as death protein-5) NOXA: Phorbol-12-myristate-13-acetate-induced protein 1 PUMA: BCL-2 binding component-3
Pro-apoptotic BAX/BAK family	Bak: BCL-2-antagonist/killer-1 Bax: BCL-2-associated X protein Bok: Bcl-2-related ovarian killer
Anti-apoptotic family	A1A: BCL-2-related protein A1A Bcl-2: B-cell lymphoma-2 Bcl-w: BCL-2-like-2 Bcl-xL: a BCL-2-like protein Mcl-1: myeloid cell leukemia sequence-1

The Bcl-2 family proteins can be simply classified into two subgroups according to their apoptotic effect: the pro-apoptotic and the anti-apoptotic families. The pro-apoptotic family is further divided into the Bax/Bak family and the BH3-only proteins, as the latter contains only one Bcl-2 homology (BH) domain. The balance between the pro-apoptotic and anti-apoptotic Bcl-2 proteins governs the integrity of the mitochondrial outer membrane and the initiation of MOMP (Youle and Strasser, 2008).

TABLE 1.2. Effect of gonococcal infection on apoptosis in different cell types

Cell type	Strain	Apoptotic effect	Signaling pathway	Reference
PMN	Ng strain FA1090	Inhibits	--	(Chen and Seifert, 2011)
	Ng strain 1291			(Simons et al., 2006)
HeLa	Ng strain VP1 and MS11	Induces	--	(Kozjak-Pavlovic et al., 2009)
			Rac-1/JNK-1/Bim and Bmf	(Kepp et al., 2009)
			--	(Muller et al., 2000)
			Ca^{2+} signaling	(Muller et al., 1999)
T84	Ng strain N400 and N400 *pilT*	Inhibits	ERK/Bim and Bad	(Howie et al., 2008)
			ERK, p38, and JNK	(Howie et al., 2005)
A431	Ng strain MS11 and MS11 *pilT*		--	(Higashi et al., 2007)
Endocervical	Ng strain FA1090B and F62	Inhibits	NF-κB	(Follows et al., 2009)
Fallopian Tubes	Ng strain P9	Inhibits (MOI=100); Induces (MOI=1)	--	(Morales et al., 2006)
UEC	Ng strain 1291 and FA1090	Inhibits	NF-κB	(Binnicker et al., 2004)
UEC	Ng strain 1291		--	(Binnicker et al., 2003)

1.3. Contribution of Bacterial Morphology to Virulence

1.3.1. Association of Bacterial Shape with Virulence

Accumulating evidence has provided insights into the correlation of bacterial cell morphology with pathogenicity. In response to distinct physiological stresses such as oxidative killing and immune insults in ecological niches, pathogenic bacteria exploit diverse adaptive strategies by morphological change to enhance intracellular survival (Justice et al., 2008; Young, 2006). Filamentation derived from inhibition of cell division in uropathogenic *Escherichia coli* (UPEC), *Legionella pneumophila*, and *Haemophilus influenza* provides resistance to phagocytosis, thereby promoting biofilm development and sustained infection (Justice et al., 2004; Leroy et al., 2007; Piao et al., 2006). *Mycobacterium tuberculosis*, the etiological agent of tuberculosis, exhibits a filamentous phenotype resulting from compromised midcell FtsZ assembly during intraphagosomal survival (Chauhan et al., 2006). Growth as coccoid forms of *E. coli*, *Acinetobacter*, and *Vibrio parahaemolyticus* upon entry into stationary phase renders the bacterial population resistant to limited nutrient status (James et al., 1995; Jiang and Chai, 1996; Lange and Hengge-Aronis, 1991). Dilution back into high-nutrient medium rescues the rod shape of *Acinetobacter* (James et al., 1995), emphasizing the importance of morphological plasticity as a bacterial survival strategy.

With regard to the indispensable role of bacterial cell division in maintenance of cell shape, mutations in the cell division genes are likely to affect bacterial virulence. Interestingly, transposon mutagenesis-derived genome-wide screening identified a *Francisella tularensis* mutant with a disruption in the 3' region of *minD* (encodes a cell division inhibitor) which showed significant compromise of *in vivo* survival (Su et al.,

2007). Similar defective survival in macrophages and increased susceptibility to serum killing has been observed in a *F. tularensis minD* mutant constructed by insertional mutagenesis (Anthony et al., 1994).

1.3.2. The *min* Mutants of *N. gonorrhoeae*

1.3.2.1. The *min* Genes and Cell Division in *N. gonorrhoeae*

The midcell placement of division septum in Ng relies on the MinCDE system, which is encoded by part of a 17-kb gene cluster, termed the *min* operon (Ramirez-Arcos et al., 2001a). In rod-shaped bacteria, such as *Escherichia coli*, the Min system is classified as one of the four well-established bacterial cytoskeletal systems, which also includes the MreB, FtsZ, and Crescentin systems (Cabeen and Jacobs-Wagner, 2010; Shih and Rothfield, 2006). Within the MinCDE system, MinC and MinD form a nonspecific septum-inhibitory complex to antagonize FtsZ assembly while MinE acts as a topological specificity factor (de Boer et al., 1989). These three proteins operate in concert to determine the midcell placement of the FtsZ ring, the essential cell division scaffold.

The MinCDE structure oscillates from pole to pole to build up a higher concentration of inhibitor at both poles but relieves the midcell to ensure the selection of the midcell at the proper division site. The alternative growth of polar helical MinCDE zones towards the midcell from two cell poles is blocked by the MinE ring (E-ring) assembling at the leading edge (Lutkenhaus, 2007; Rothfield et al., 2005). While the E-ring blocks further extension of the polar zone across the midcell, it activates the latent MinD ATPase activity, thereby disassembling the MinCDE structure. This disassembly

results in the release of MinD: ADP from one pole and the formation of a polar zone at the opposite end of the cell (Lutkenhaus, 2007; Rothfield et al., 2005).

Ng divides in two alternating perpendicular planes over consecutive division cycles (Szeto, 2004; Westling-Haggstrom et al., 1977; Zapun et al., 2008). Pioneering research in the Dillon laboratory has established the conservative function of the Ng Min system in the midcell septum placement and has shown that Min proteins interact in concert via specific domains/regions (Eng et al., 2006; Greco-Stewart et al., 2007; Ramirez-Arcos et al., 2004; Ramirez-Arcos et al., 2001a; Ramirez-Arcos et al., 2001b; Szeto et al., 2004; Szeto et al., 2005; Szeto et al., 2001). However, determination of alternating perpendicular planes in spherical-shaped cell division probably requires the reorientation of Min protein helical arrays to direct their oscillations parallel to the nascent septum and in the direction of the new longitudinal axis (Szeto, 2004).

1.3.2.2. Impact of Mutations in the *min* Genes on Gonococcal Pathogenicity

Insertional inactivation of Ng *minC* and *minD* genes has been shown to disrupt normal cytokinesis patterns and gives rise to aberrant cell morphology (Ramirez-Arcos et al., 2001b; Szeto et al., 2001) (Fig 1.4). Research in our laboratory therefore sought to understand the role of *min* genes in gonococcal pathogenesis given the growing literature on the contribution of bacterial morphology to virulence. It has been shown that Ng *minC* and *minD* mutant strains (pil^+ opa^+) display abnormal microcolony formation, impaired invasion of ME-180, HeLa, and THUEC cell lines, and compromised adherence to THUEC (Parti et al., 2011a) and unpublished data. However, expression levels of major virulence factors in these mutant strains, i.e. PilE (the major pilin subunit in Tfp), Opa, and LOS, are similar to that of the isogenic parent strain (Parti et al., 2011a). These

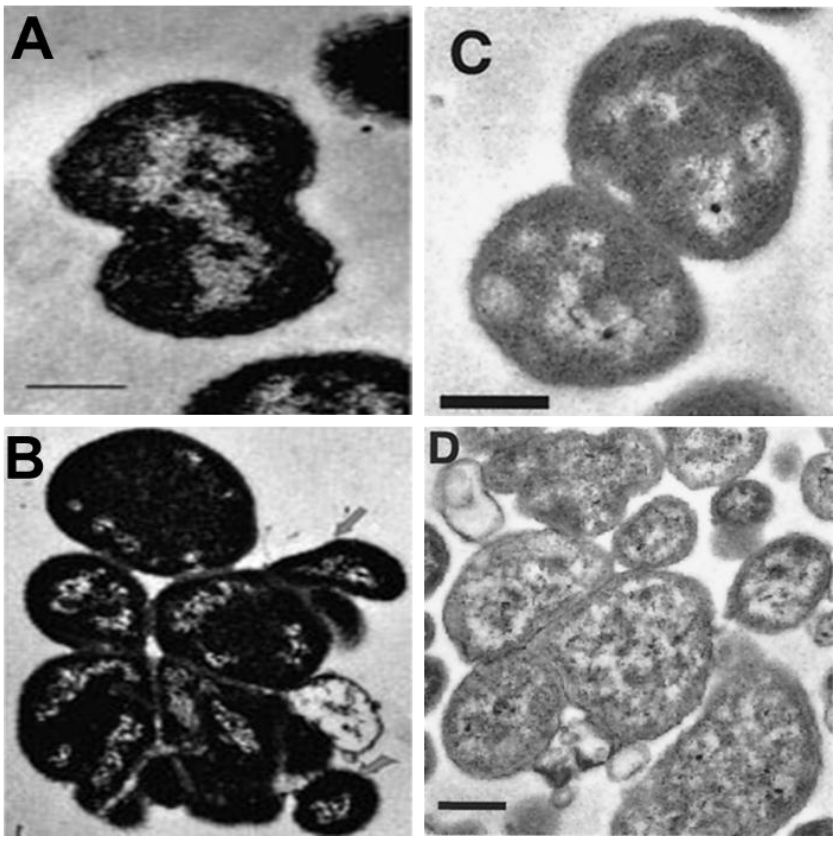

FIGURE 1.4. Aberrant cytokinesis in Ng *min* mutant strains. Electron microscopy shows wild-type Ng CH811Str^R strain as typical diplococcal morphology (A, C), whereas Ng *minC* mutant strain CSRC1 (B), *minD* mutant strain CJSD1 (D) exhibit aberrant cell morphology, resulting from abnormal cell division (Ramirez-Arcos et al., 2001b; Szeto et al., 2001).

observations demonstrate that the impaired virulence of these "shape mutants" cannot plausibly be attributable to the alteration of outer membrane proteins and indicate a virulence mechanism possibly dependent on cell morphology. Similarly, reduced adherence has also been observed in Caco-2 cells infected with an enterohemorrhagic *Escherichia coli* (EHEC) *minD* mutant strain (Parti et al., 2011b), highlighting that MinD /cell shape is likely to be an ubiquitous factor that contributes to bacterial pathogenicity. Interestingly, a recent microarray study has shown a 1.66-fold downregulation of *minD* expression upon adherence to human epithelial cell line A431 (i.e. 3 h post-infection) (Du et al., 2005). This observation directly connected *minD* regulation with gonococcal adherence.

The way in which MinD impacts pathogenicity is also suggested by some studies regarding the MinD-membrane interaction. Within the Min system, the ATP-dependent MinD helical structure is the essential framework of interaction of the Min protein complex with the cytoplasmic membrane. The C-terminal domain of MinD is an amphipathic α-helix and displays preferential association with anionic phospholipids in the membrane (Mileykovskaya et al., 2003; Szeto et al., 2003). This suggests a prerequisite of membrane lipid composition in bacterial division-site selection. An *in vitro* model, retroactively, has hypothesized that the MinD-membrane interaction may induce changes in membrane dynamics and structural properties, including fluidity gradient, membrane potential, and proteolipid domain formation (Mazor et al., 2008a, b; Mileykovskaya and Dowhan, 2005; Strahl and Hamoen, 2010). This hints that the distribution and/or the activities of surface macromolecules such as virulence factors may be affected when the *minD* gene is mutated, thereby altering the host cell interaction of

Ng. As Ng:epithelial cell interaction determines the host cell response through activation of corresponding signaling cascades, it is likely that the altered pattern of interaction by the *minD* mutant impacts host responses (e.g. apoptosis) by differentially regulating host cell signaling pathways.

1.4. Hypothesis and Objectives

Ng has been reported to confer an anti-apoptotic effect on human urethral epithelial cells. However, the detailed underlying mechanism is largely unknown. P.IB-mediated activation of NF-κB signaling pathway has been shown to contribute to this apoptosis resistance. The activation of MAPK signaling pathways regulates diverse cellular responses and has been observed in the context of gonococcal infection of epithelial cells, I was therefore interested in investigating the involvement of these pathways in the anti-apoptotic response in urethral epithelial cells.

Previous studies attributed the apoptotic effect of Ng to the activation of either pro-apoptotic or anti-apoptotic signaling pathways by the major virulence factors such as Tfp, Opa, and PorB. However, no individual virulence factor solely determines the apoptotic effect of Ng. Are there any other factors or how the whole bacterial cell contributing to the apoptotic effect are therefore of great interest. By studying Ng cell division mutants in parallel, I investigated the apoptotic effect of whole bacterial cell on human urethral epithelial cells and the impact of cell division on host cell signaling pathways.

<u>My work was motivated by the following hypotheses:</u>

Manipulation of MAPK and NF-κB pathways by Ng CH811Str^{R} contributes to the modulation of apoptosis in THUEC; Ng CJSD1 performs differently from the parent

strain in apoptosis modulation by differentially regulating these pathways.

To test these hypotheses, I established the following objectives:

Objective 1: To investigate the activation of MAP kinase signaling cascades and their role in apoptosis modulation in THUEC infected with Ng CH811Str^R and Ng CJSD1 by studying:

1) The time course of activation of MAP kinases (i.e. ERK, p38, and JNK);
2) The levels of expression/activation of Bim and Bad;
3) The involvement of EGFR in ERK activation;
4) The requirement of bacterial cell viability in ERK activation;
5) The effect of inhibition of ERK activation on apoptosis by flow cytometry.

Objective 2: To assess apoptosis in THUEC infected with Ng CH811Str^R and Ng CJSD1 by:

1) Evaluating the effects of intracellular or both extracellular and intracellular Ng on apoptosis modulation by flow cytometry;
2) Investigating the role of bacterial invasion in apoptosis modulation;
3) Detecting caspase 3/7 and 8 activities;
4) Profiling the expression of apoptotic genes in infected cells by real-time PCR (Appendix A1).

Objective 3: To examine the interaction of Ng CH811Str^R and Ng CJSD1 with the actin cytoskeleton in THUEC by confocal microscopy.

CHAPTER 2 MATERIALS AND METHODS

2.1. Bacterial Strains and Cell Cultures

2.1.1. Bacterial Strains and Growth Conditions

Neisseria gonorrhoeae wild-type strain CH811Str^R and its isogenic *minD* mutant strain CJSD1 were used in this research (Picard and Dillon, 1989; Szeto et al., 2001)(Table 2.1). Both strains were grown on gonococcal medium base agar (Difco, Sparks, MD, USA) plus 1% Kellogg's defined supplement (GCMBK) for 16 to 18 h at 37°C in a 7% CO_2 humid environment (Kellogg et al., 1963; Pagotto et al., 2000). When necessary, antibiotics were added to the medium in the following concentrations: 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) for Ng CH811Str^R and 100 μg/ml streptomycin plus 25 μg/ml chloramphenicol (Sigma) for Ng CJSD1. When indicated, gonococci were killed by incubation with 100 μg/ml gentamicin sulfate (Gibco, Grand Island, NY, USA) at 37°C for 2h, followed by overnight incubation at 4°C (Swanson et al., 2011). Piliation and Opa phenotypes were monitored by dissection microscopy of colony morphology. Gonococci expressing pili (P^+) and Opa (Opa^+) form smaller colonies which appear yellow and refract the light to give a granular appearance (Dillard, 2011; Kellogg et al., 1963). Both strains were stored in cryovials at -80 °C in 1 ml of BHI broth (Difco) supplemented with 20% glycerol.

2.1.2. Transduced Human Urethral Epithelial Cells and Culture Conditions

Transduced human urethral epithelial cells (THUEC, a gift from Dr. Michael A. Apicella, Department of Microbiology, University of Iowa, USA) were derived from the primary urethral epithelial cells (PHUEC) by transduction with a retroviral vector expressing the human papillomavirus (HPV) E6E7 oncogenes and exhibit

TABLE 2.1. Bacterial strains used in this research

Strain	Relevant genotype or phenotype	Source
N. gonorrhoeae CH811	Auxotype (A)/serotype (S)/plasmid content (P) class: nonrequiring/IB-2/ plasmid-free, Str^r	(Picard and Dillon, 1989)
N.gonorrhoeae CJSD1	CH811 Str^r *minD*::Cm	(Szeto et al., 2001)

comparable phenotypes to PHUECs (Harvey et al., 2002). THUEC were maintained in 75 cm^2 flasks at 37°C and 5% (v/v) CO_2 in FBS-free prostate epithelial growth medium (PrEGM, Clonetics, San Diego, CA, USA) and the medium was changed on the day after subculture and then every other day until 90% - 95% confluency by visual estimation. Cells were passaged every two to three days as previously described with some modifications (Binnicker et al., 2004). Cells in a 75 cm^2 flask were incubated with 3 ml 0.25% trypsin / 0.1% EDTA (Gibco) for 2 min at room temperature (RT). The trypsin was removed, and the cells were then incubated for another 5 min at 37°C. Lifted cells were suspended in 10 ml of PrEGM, centrifuged for 5 min at 1,500 rpm, and resuspended in fresh FBS-free PrEGM. Cell density was determined using a hemocytometer (Reichert, Buffalo, NY, USA) and ~ 2×10^6 cells were seeded to a new 75 cm^2 flask. To avoid significant divergence from the parent line, the passage number of THUEC never exceeded 10 passages before an older batch with a low passage number was revived from liquid nitrogen. For freezing THUEC, cells were suspended at a concentration of ~ 10^6 cells/ml in filtered PrEGM containing 10% dimethyl sulfoxide (DMSO, Calbiochem, La Jolla, CA, USA), and one-milliliter aliquots in cryovials were then kept at -20°C for 2 h and - 80°C overnight (O/N), before transfer to liquid nitrogen tank. For reviving THUEC, cell stocks were taken out from liquid nitrogen and immediately thawed in a 37°C water bath. One-ml cell suspensions were diluted in 10 ml FBS-free PrEGM and centrifuged to remove DMSO. The cell pellets were then suspended in fresh FBS-free PrEGM and seeded in 75 cm^2 flasks for growth (Harvey et al., 2002).

2.2. Challenge of THUEC with *Neisseria gonorrhoeae*

2.2.1. Determination of Colony-forming Unit

Viable counts were performed by using the droplet method with slight modifications (Sharpe and Kilsby, 1971). Following lysis of cell monolayers grown in 24-well culture plates with 1 ml of 2% saponin in PrEGM, 100 µl of this lysate was serially added to 900 µl of GC broth and 25 µl of each dilution was plated onto GCMBK agar (Difco) containing 100 µg/ml streptomycin (Sigma). Specifically, for adherence and invasion assays, 25 µl of the 10^{-2} to 10^{-4} dilutions and the 10^{0} to 10^{-2} dilutions were plated in triplicate onto antibiotic-containing plates, respectively. For enumerating original inoculum, seven 10-fold dilutions were made and 25 µl of the 10^{-4} to 10^{-7} dilutions were plated in triplicate. These dilutions normally generate < 80 CFUs/drop, which are discernible numbers for counting.

2.2.2. Bacterial Adherence and Invasion Assays

For infection, THUEC were seeded in 24-well tissue culture plates (Corning, NY, USA) at a density of ~ 10^5 cells per well and cultured 20-22 h to reach a sub-confluent monolayer. Overnight cultured gonococci on GCMBK agar were suspended in gentamycin-free PrEGM and the cell density was adjusted to an OD_{600} = 0.3 - 0.35 (~ 10^8 CFU/ml) (the growth curves of Ng CH811Str^R and Ng CJSD1 are shown in appendix A5). Of this bacterial suspension, 100 µl was added to each well (~10^5 cells) to reach a multiplicity of infection (MOI) of 100. Serial dilutions of each bacterial inoculum were plated on streptomycin-containing GCMBK agar plates to determine the actual number of colony-forming units (CFU) added. Infected cells were incubated at 37°C with 5% CO_2 for 4 h. Following infection, the samples were divided into three sets. For the first set (total CFU), the supernatant was transferred to an Eppendorf tube and the cell monolayer with adherent bacteria was incubated with 2% saponin (Sigma) in PrEGM for 10 min at

37°C followed by scraping and vigorous pipetting to lyse the THUEC cells thoroughly. This cell lysate was combined with the supernatant and serial dilutions were plated onto GCMBK agar. It is worth noting that this total number of bacterial CFU in the well is a necessary control in consideration of the multiplication of gonococci throughout the experiment. For the second set (cell-associated CFU), infected cell monolayers were washed with phosphate-buffered saline (PBS) five times to remove unattached bacteria. Serial dilutions of the cell lysate were plated to determine the cell-associated CFU. For the third set (intracellular CFU), infected cells were washed with PBS five times followed by incubation with fresh PrEGM containing 100 μg/ml gentamicin sulfate for 1 h at 37°C to kill extracellular bacteria. Cells were then washed with PBS five times to remove gentamicin, lysed, and plated as described above to determine the intracellular CFU. The adhesion frequency was expressed as the ratio of cell-associated CFU to total CFU while the invasion frequency was expressed as the ratio of intracellular CFU (Gm^R) to cell-associated CFU.

2.3. Immunoblotting

2.3.1. Antibodies and Inhibitors

Primary Rabbit antibodies against phospho-p44/42 MAPK (Erk1/2) (T202/Y204) (catalogue no. 9101), phospho-p38 MAPK (T180/Y182) (catalogue no. 9211), phospho-JNK (T183/Y185) (81E11), IκBα (catalogue no. 9242), phospho-Bad (S112) (40A9, catalogue no. 5284), Bad (D24A9, catalogue no. 9239), Bim (C34C5, catalogue no. 2933), Bax (D2E11, catalogue no. 5023), and Bak (D2D3, catalogue no. 6947) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody for the internal control β-tubulin (sc-9014) was purchased from Santa Cruz Biotechnology, Inc

(Santa Cruz, CA, USA). An IRDye 800CW conjugated goat anti-rabbit or mouse IgG secondary antibody (LI-COR, Lincoln, NE, USA) was used as described below.

MEK1/2 inhibitor U0126 (CST), p38 inhibitor SB202190 (Sigma), EGFR inhibitor Tyrphostin AG1478 (Sigma), actin polymerization inhibitor Cytochalasin D (Sigma), and apoptosis inducer Staurosporine (CST) were all dissolved in dimethyl sulfoxide (DMSO) for the stock concentration and applied to the cells at a final concentration as following: U0126 (10 μM); SB202190 (10 μM); AG1478 (5 μM); Cytochalasin D (4 μM); STS (1 μM). All chemical inhibitors were diluted in antibiotic-free PrEGM before adding to the cells. U0126, SB202190, and Cytochalasin D were preincubated with cells for 30 min while AG1478 was preincubated for 1 h before addition of bacterial suspension. All chemical inhibitors were maintained in the infection medium throughout the course of infection.

2.3.2. Protein Sample Preparation and Determination of Protein Concentration

At distinct time points postinfection, cell monolayers grown in 24-well plates were washed three times in ice-cold phosphate-buffered saline (PBS) and lysed with 40 μl of Mammalian protein extraction reagent (Thermo, Rockford, IL, USA) supplemented with a protease and phosphatase inhibitor cocktail (Thermo). The whole-cell lysates were scraped by tips into Eppendorf tubes followed by a 10 min centrifugation at 14,000 × *g* at 4°C. The supernatants were collected and protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA). Briefly, eight dilutions of BSA protein standard (Bio-Rad) were made and the OD_{590} values were measured spectroscopically along with the sample proteins. A standard curve for protein concentration was generated

from the eight BSA standards and the concentration of sample proteins was calculated (Fig 2.1).

2.3.3. Western Blotting

Equal amounts of protein samples (25 or 30 μg) were mixed with 5 × Laemmli loading buffer [0.3 M Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 0.5% bromophenol blue] (Laemmli, 1970) supplemented with 0.1 M dithiothreitol (DTT), boiled for 10 min and centrifuged at 14,000 × *g* for 1 min. Samples were separated on 15% or 8 - 16% linear gradient SDS-PAGE gels (Bio-Rad) at 100 V for 2-3 h until the loading dye ran off the bottom of the gel. The separated proteins in the SDS-PAGE gel were subsequently transferred to 0.2 μm nitrocellulose membranes (Bio-Rad). The membranes were blocked in TBS buffer [50 mM Tris-HCl (pH 7.4) and 150 mM NaCl] containing 0.1% Tween-20 (TBST) and 5% BSA and probed with the indicated primary antibodies overnight with shaking at 4°C. The next day, membranes were washed with TBST three times and then incubated with secondary antibodies in TBST with 5% dry milk for 1 h with shaking at RT. Following incubation, the membranes were washed for three more times with TBST and rinsed with PBS once. Visualization of membranes was performed with an Odyssey infrared imaging system (LI-COR) and photographed pictures in TIFF format were saved. Each membrane was photographed at three different exposure levels.

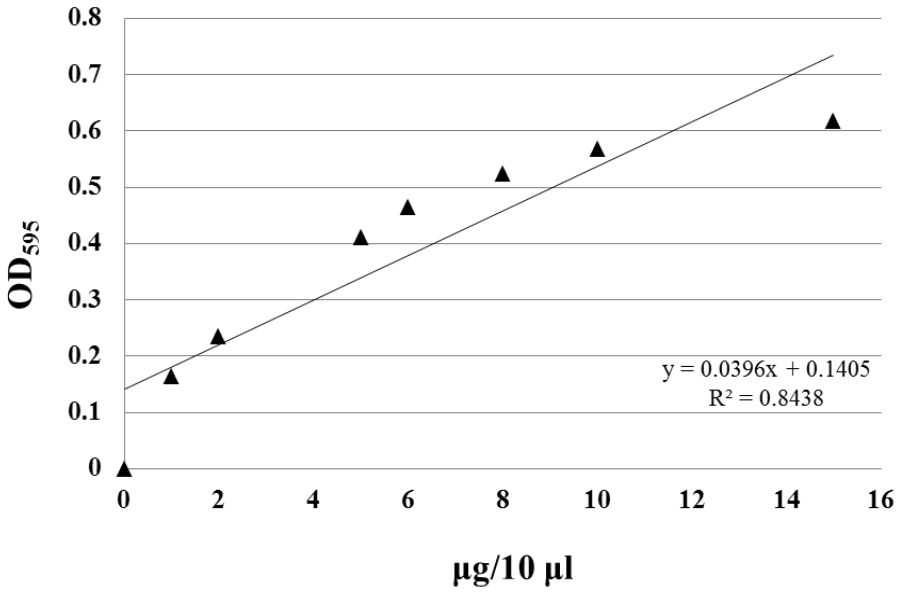

FIGURE 2.1. Representative standard curve for protein concentration. Eight dilutions (i.e. 0, 1, 2, 5, 6, 8, 10, 15 µg/10 µl) of the stock BSA protein standard (2 mg/ml) were made and the OD_{590} values were measured spectroscopically along with the sample proteins. A standard curve for BSA protein concentration was generated from the eight BSA dilutions and the concentration of sample proteins was calculated according to the linear fitting equation. (detailed method is shown in Appendix Table A3)

2.3.4. Densitometric Analysis

Densitometric analysis of the protein bands was performed using ImageJ software from NIH (v1.46, http://rsbweb.nih.gov/ij/). Briefly, the picture background was extracted and each lane was plotted using the "Rectangular selection" followed by "Gel analysis". The density value of each target protein band was determined with "Wand tool". To express relative protein levels, target proteins were normalized to the corresponding levels of the internal loading control (e.g. β-tubulin), and the value for non-infected control (NI) was set to 1.

2.4. Flow Cytometry

THUEC were grown to 90% confluence in six-well culture plates and infected as described in section 2.2. At 4 h post-infection, the supernatants were removed and the cell monolayers were washed twice with fresh antibiotic-free PrEGM. Two different conditions were used to differentiate the effects of intracellular Ng and extracellular Ng on apoptosis (Binnicker et al., 2003; Follows et al., 2009). For the first setting (intracellular Ng), the washed cells were further incubated with fresh medium containing 100 μg/ml gentamicin for 1 h, after which the cells were washed three times with PBS and incubated with antibiotic-free medium for the remainder of 12 h, 24 h, and 48 h. For the second setting (extracellular and intracellular Ng), the washed cells were directly incubated for the remainder of 12 h, 24 h, and 48 h in fresh medium without antibiotics. Apoptosis at distinct time points post-infection was measured by the classic Annexin V/Propidium iodide (PI) staining method (Vermes et al., 1995) using an Alexa Fluor 488 annexin V/Dead cell Apoptosis Kit with Alexa Fluor 488 annexin V and PI for Flow Cytometry (Invitrogen, Eugene, OR, USA) according to the manufacturer's instruction

with slight modifications. Briefly, a total of 1×10^6 infected cells were washed, trypsinized, and collected in 15 ml conical tubes. Cells were washed in ice-cold PBS (calcium/magnesium free) and resuspended in Annexin-binding buffer. 200µl of cell suspension was transferred to a 5 ml round bottom test tube and stained with 5 µl Alexa Fluor 488 annexin V and 1 µl 100 µg/ml PI for 15 min at room temperature (RT). After incubation, cells were immediately analyzed by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed in BD CellQuest Pro software by gating on the cells based on forward and side scatter and cell-associated fluorescence of 10,000 cells per sample was measured in fluorescence channel 1 (FL1-H) and channel 3 (FL3-H) detecting Alexa Fluor 488 and PI fluorescence, respectively.

2.5. Luminescent Caspase Activity Assay

The effects of Ng CH811Str^R and Ng CJSD1 on apoptosis induction/inhibition with respect to caspase activation was monitored by the Caspase-Glo 3/7 and 8 cell-based assays (Promega, Madison, WI, USA) according to the manufacturer's instruction. THUEC (~ 1.5×10^4 cells) were seeded in each well of a white walled 96-well plate (Nunc, Rochester, NY, USA). Following infection with Ng for 6 h, cells were washed twice with fresh antibiotic-free PrEGM and incubated with 100 µl of fresh PrEGM for another 4 h. Caspase reagent was then added to each well to reach a final volume of 200 µl. This caspase reagent contains cell lysis buffer, luciferase, and luminogenic caspase substrates, which contain the tetrapeptide sequence DEVD and LETD that can be recognized by caspase 3/7 and caspase 8, respectively. The released activated caspases from lysed eukaryotic cells are capable of cleaving these substrates to expose the

substrate for luciferase, resulting in the luciferase reaction and production of light. The contents of the wells were mixed gently using a plate shaker at 300 rpm for 2 min and then incubated at RT for 1 h in the dark. The luminescence of each sample was measured in a VICTOR *X* Multilabel Plate Reader (PerkinElmer, Turku, Finland) within 1 h after incubation and the relative light units (RLU) were calculated after blank subtraction (i.e. luminescence value in wells with caspase reagent but without THUEC).

2.6. Confocal Microscopy

2.6.1. Labeling of *N. gonorrhoeae* Cells

Overnight cultures of Ng CH811Str^R and Ng CJSD1 grown on GCMBK agar were suspended in PBS to reach an OD_{600} = ~ 0.3 - 0.35 (~ 10^8 CFU/ml), and labeling of bacterial cells was performed as described elsewhere with some modifications (Agerer et al., 2004). Briefly, 1 ml of bacterial suspension was centrifuged at 5,000 × *g* for 5 min and the pellet was resuspended in staining solution containing Alexa Fluor 488 carboxylic acid succinimidyl ester in PBS (Molecular Probes, Eugene, OR, USA). Labeling was performed at RT with shaking for 30 min followed by washing twice with PBS. Labeled Ng was resuspended in antibiotic-free PrEGM before adding to cells.

2.6.2. Staining Actin Filaments

THUEC were grown on MatTek (Ashland, MA, USA) 35 mm glass bottom culture dishes (~ 0.375 × 10^5 cells) and infected with Alexa Fluor 488-labeled gonococci as described above. Following a four-hour infection, cell monolayers were washed twice with PBS and actin staining was performed as described elsewhere with slight modifications (Giardina et al., 1998; Parti et al., 2011b). Cells were fixed in 3.2% paraformaldehyde in PBS for 15 min and incubated with 0.1% glycine in PBS for another

15 min. Cells were then permeabilized with 0.1% Triton X-100 in PBS for 15 min and blocked with 1% BSA in PBS for 20 min with gentle shaking. Actin filaments were stained with Alexa Fluor 546-phalloidin (Molecular Probes) at RT for 20 min. Samples were washed three times with PBS before mounting and visualizing with a Leica TCS SP5 Spectral Confocal Microscope (Buffalo Grove, IL, USA), equipped with an Argon (458, 477, 488, 514, 488 nm) and two Helium/Neon (543, 633 nm) lasers and 10×/20×/40×/63× PL APO objectives. All three channels (bacteria, actin, and eukaryotic cells) were recorded and the corresponding images were digitally processed with Photoshop CS4 (Adobe Systems, San Jose, CA, USA).

2.7. Statistical Analyses

The statistical significance of differences between groups was calculated using Graphpad Prism 5.0 with one-way or two-way ANOVA followed by the Bonferroni post-test to obtain the *P*-value. Data are shown as mean ± SD of at least two independent experiments. Significant differences between groups are denoted by an asterisk ($P < 0.05$), two asterisks ($P < 0.01$), or three asterisks ($P < 0.001$).

CHAPTER 3 RESULTS

3.1. Apoptosis in THUEC Infected with *Neisseria. gonorrhoeae* Strain CH811Str^R or CJSD1

3.1.1. Apoptotic Effect of Gonococcal Infection on THUEC

Ng can either induce or inhibit apoptosis in human epithelial cells (Binnicker et al., 2003, 2004; Follows et al., 2009; Howie et al., 2005; Howie et al., 2008; Kepp et al., 2009; Morales et al., 2006; Muller et al., 2000; Muller et al., 1999; Muller et al., 2002). In THUEC, infection with Ng strain 1291 protected cells from staurosporine (STS)-induced apoptosis (Binnicker et al., 2003). This anti-apoptotic effect was partly conferred by the P.IB-mediated activation of the NF-κB signaling pathway, which up-regulates the expression of anti-apoptotic genes, including *bfl-1*, *cox-2*, and *c-IAP-2* (Binnicker et al., 2004). To examine the apoptotic effect of our strains on THUEC, flow cytometric analysis was performed to quantitatively determine the percentage of apoptotic cells in the total cell population infected with Ng CH811Str^R or Ng CJSD1.

Following the four-hour infection, cells were washed to remove unattached bacteria and incubated in fresh antibiotic-free medium for the remainder of 12, 24, and 48 h. This washing step was applied to reduce the cytotoxic effect of Ng proliferation during prolonged infection periods. At 12 h post-infection, THUEC infected with Ng CH811Str^R or Ng CJSD1 displayed a reduced percentage of early apoptotic cells compared to the non-infected control (Fig 3.1A, black bar 1 and grey bar 1). In contrast, prolonged incubation periods of Ng CH811Str^R (24 h and 48 h post-infection) and Ng CJSD1 (48 h post-infection) with THUEC induced a noticeable increase in the percentage of early apoptotic cells compared to the non-infected control (Fig 3.1A, black bars 2 and 3; grey

bar 3). In addition, prolonged infection with Ng CH811Str^R (24 h and 48 h post-infection) and Ng CJSD1 (48 h post-infection) significantly increased the percentage of late apoptotic/necrotic cells in the total cell population (Fig 3.1B, black bars 2 and 3; grey bar 3).

Interestingly, infection with Ng CJSD1 alleviated the augmented apoptosis induction observed in prolonged Ng CH811Str^R infection. At 24 h post-infection, in contrast to the increase of the percentage of early apoptotic cells in the cell population infected with Ng CH811Str^R (Fig 3.1A, black bar 2), infection with Ng CJSD1 resulted in significant reduction of the percentage of early apoptotic cells (Fig 3.1A, grey bar 2) and retained the anti-apoptotic effect when compared to the non-infected control (Fig 3.1A, white bar 2). In addition, compared to the increase of the percentage of the late/necrotic apoptotic cells by Ng CH811Str^R (Fig 3.1B, black bar 2), Ng CJSD1-infected THUEC showed similar percentage of the late/necrotic apoptotic cells to the non-infected control (Fig 3.1B, grey bar 2 and white bar 2). Moreover, although both strains significantly induced late-stage apoptosis/necrosis at 48 h post-infection, the number of late apoptotic/necrotic cells in Ng CJSD1-infected THUEC was significantly less than that of infection with Ng CH811Str^R (Fig 3.1B, black bar 3 and grey bar 3).

To further illustrate the pro-apoptotic effect of Ng CH811Str^R and Ng CJSD1 on THUEC, representative histograms showing the annexin V and PI staining profiles of the total cell population infected for 48 h were generated. Compared to the cells infected with Ng CH811Str^R, more Ng CJSD1-infected cells displayed relatively lower fluorescence intensity of PI (Fig 3.1C, lower panel), indicating more healthy cells. Further evidence of the reduced and delayed apoptosis by Ng CJSD1 was provided by the presence of more

annexin V^- cells in the total infected cell population (Fig 3.1C, upper panel).

Taken together, these results indicate that *N. gonorrhoeae* confers an anti-apoptotic effect on THUEC in early infection, but induces apoptosis during prolonged infection. Ng CJSD1 partly loses the ability to induce apoptosis when compared to that of Ng CH811Str^R.

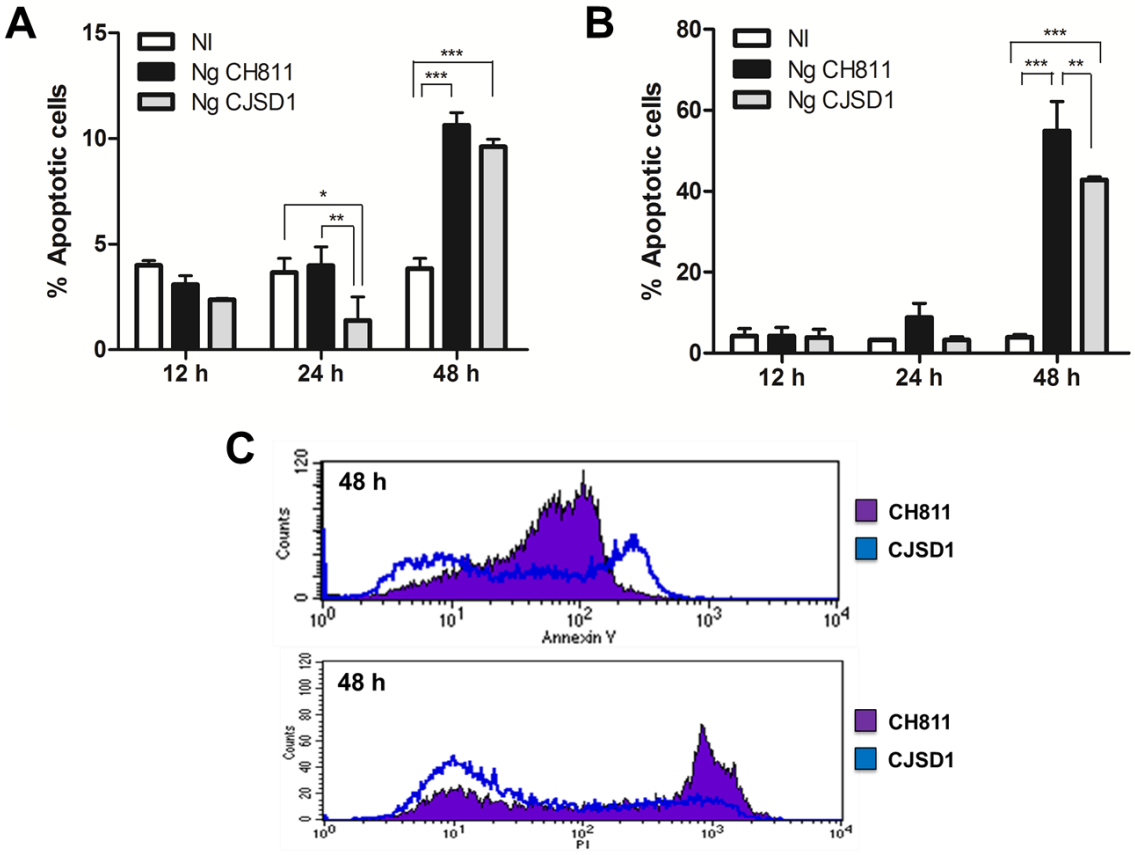
A
% Apoptotic cells
15
10
5
0
NI
Ng CH811
Ng CJSD1
*
**
***
***
12 h
24 h
48 h
B
% Apoptotic cells
80
60
40
20
0
NI
Ng CH811
Ng CJSD1
***
***
**
12 h
24 h
48 h
C
48 h
Counts
120
0
10^0
10^1
10^2
10^3
10^4
Annexin V
CH811
CJSD1
48 h
Counts
120
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
PI
CH811
CJSD1

FIGURE 3.1. Apoptotic effect of gonococcal infection on THUEC. THUEC were left non-infected, infected with Ng CH811Str^{R} or Ng CJSD1 for 4 h (MOI=100), followed by vigorous washing to remove unattached bacteria. Cell monolayers were further incubated with antibiotic-free medium for the remainder of 12, 24, and 48 h. Cells were harvested, stained with Alexa 488-annexin V and PI, and submitted to flow cytometric analysis.

(A, B) Percentage of early (Annexin V^{+}/PI^{-}) (A) or late (Annexin V^{+}/PI^{+}) (B) apoptotic cells in the total cell population at 12, 24, and 48 h post-infection. Data are the mean ± SD of two independent experiments performed in triplicate. Two-way ANOVA followed by Bonferroni post-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

(C) Annexin V (above) and PI (below) staining profiles of the total cell population infected with Ng CH811Str^{R} (purple) or Ng CJSD1 (blue) for 48 h.

3.1.2. Apoptotic Effect of Intracellular *N. gonorrhoeae* on THUEC

To specifically examine the apoptotic effect of intracellular gonococci on THUEC, extracellular gonococci were killed with gentamicin at 4 h post-infection, after which the cells were incubated in fresh medium for the remainder of infection periods. At 12 h post-infection, 2.25% of cells infected with Ng CH811Str^{R} were in early-stage apoptosis (annexin V^{+}/PI^{-}) while 4.31% early apoptotic cells were observed in non-infected control (Fig 3.2A, left and middle panels). This inhibition of early apoptosis by Ng CH811Str^{R} lasted at least until 48 h post-infection (Fig 3.2B, black bars). Similarly, infection with Ng CJSD1 for 12 h resulted in 2.01% early apoptotic cells in the total cell population (Fig 3.2A right panel) and the inhibition of early apoptosis conferred by Ng CJSD1 was comparable to that of Ng CH811Str^{R} at 12, 24, and 48 h post-infection (Fig 3.2B, grey bars). In addition, similar percentage of late apoptotic/necrotic (annexin V^{+}/PI^{+}) and dead cells (annexin V^{-}/PI^{+}) were observed in THUEC infected with either Ng strain to the non-infected control (Fig 3.2A). Taken together, these data demonstrate that intracellular gonococci of both Ng CH811Str^{R} and Ng CJSD1 confer an anti-apoptotic effect on THUEC.

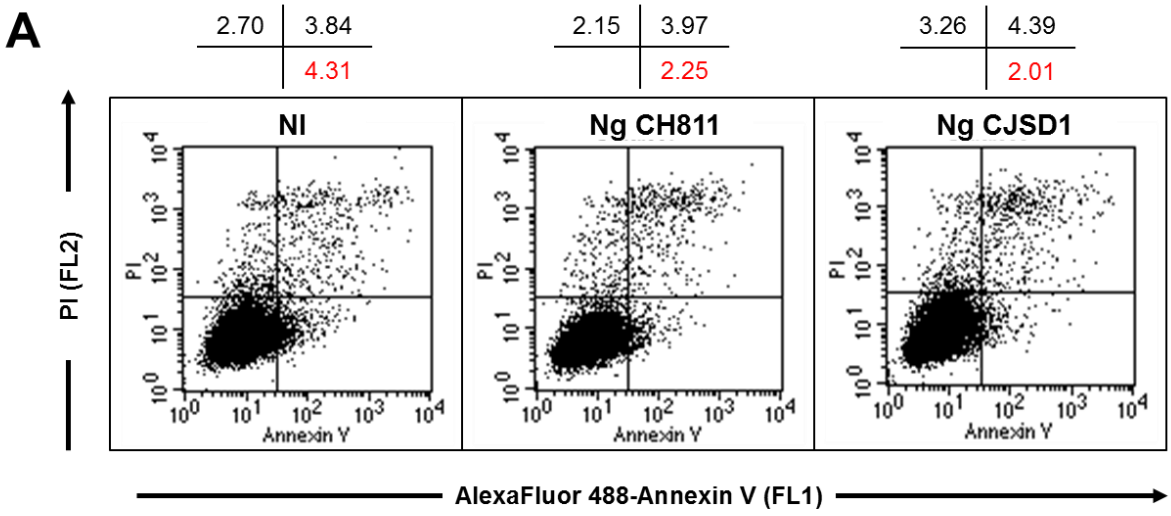
A
2.70
3.84
4.31
2.15
3.97
2.25
3.26
4.39
2.01
NI
Ng CH811
Ng CJSD1
PI (FL2)
PI
Annexin V
AlexaFluor 488-Annexin V (FL1)
B
% Apoptotic cells
NI
Ng CH811
Ng CJSD1
12 h
24 h
48 h
*
***

FIGURE 3.2. Apoptotic effect of intracellular *Neisseria gonorrhoeae* strain CH811Str^{R} and Ng CJSD1 on THUEC. THUEC were left non-infected (NI), infected with Ng CH811Str^{R} or Ng CJSD1 for 4 h (MOI=100), followed by a one-hour incubation with 100 μg/ml gentamicin to kill extracellular bacteria. Cells were then incubated in antibiotic-free medium for the remainder of 12, 24, and 48 h. At distinct time points post-infection, cells were harvested, stained with Alexa Fluor 488-annexin V and PI, and analyzed by flow cytometry.

(A) Plots show percentages of early apoptotic cells (Annexin V^{+}/PI^{-}, lower right), late apoptotic/necrotic cells (Annexin V^{+}/PI^{+}, upper right), and dead cells (Annexin V^{-}/PI^{+}, upper left) in the total cell population infected with Ng for 12 h. Outset numbers in the top of each plot correspond to the quadrant. Data are representative of at least three independent experiments with similar results.

(B) Percentage of early apoptotic cells in the total cell population at 12, 24, and 48 h post-infection. Data are the mean ± SD of three independent experiments performed in triplicate. Statistical significance is shown as comparing to NI control. Two-way ANOVA followed by Bonferroni post-test (*$P < 0.05$, ***$P < 0.001$).

3.1.3. Effect of Cytochalasin D on Apoptosis in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^R

Since both intracellular Ng CH811Str^R and Ng CJSD1 conferred an anti-apoptotic effect on THUEC, I next examined whether the inhibition of apoptosis is solely dependent on intracellular gonococci. Therefore, cytochalasin D, an inhibitor of actin polymerization, was used to impede the internalization of gonococci into THUEC as the uptake of gonococci by primary urethral epithelial cells involves actin rearrangement (Giardina et al., 1998). In contrast to the significant reduction of the percentage of early apoptotic cells in the total cell population by Ng CH811Str^R (Fig 3.3, bar 2), infection in the presence of cytochalasin D did not provide the anti-apoptotic effect (Fig 3.3, bar 3). Non-infected cells with or without cytochalasin D treatment exhibited comparable percentage of early apoptosis (data not shown), ruling out the effect of cytochalasin D on cell viability. Therefore, these results demonstrate that Ng-induced apoptosis resistance is mediated by the gonococci residing in THUEC (i.e. intracellular).

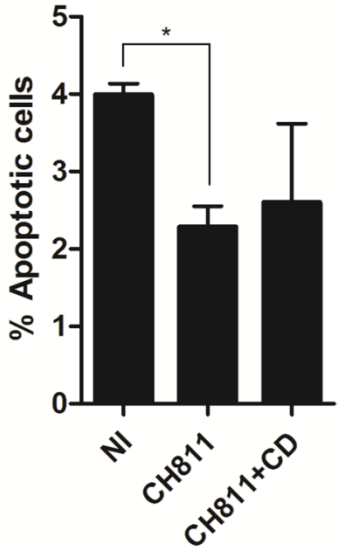

FIGURE 3.3. Effect of cytochalasin D on apoptosis in THUEC infected with *N. gonorrhoeae* strain CH811Str^R. THUEC were left non-infected (NI), infected with Ng CH811Str^R (MOI=100) for 4 h with or without 30 min pre-incubation with cytochalasin D (CD), followed by a one-hour incubation with 100 μg/ml gentamicin to kill extracellular bacteria. Cells were then incubated in antibiotic-free medium for the remainder of 12 h. At 12 h post-infection, cells were harvested, stained with Alexa Fluor 488-annexin V and PI, and analyzed by flow cytometry. Data show the percentage of early apoptotic cells in the total cell population and are the mean ± SD of three independent experiments performed in duplicate. One-way ANOVA followed by Bonferroni post-test (*$P < 0.05$).

3.2. Time-course Activation of MAP Kinases in THUEC Infected with *Neisseria gonorrhoeae* Strain CH811Str^R or CJSD1

I next investigated the involvement of cell signaling pathways implicated in the anti-apoptotic effect of Ng on THUEC. Three major MAPK signaling cascades, including ERK, p38 MAPK and JNK, are targets of bacterial pathogens for modulating host cell responses. Previous studies have shown that ERK1/2 and p38 MAPK were activated in a Ng-infected T84 cell line (human colonic epidermoid cells) (Howie et al., 2005), while JNK was activated in Ng-infected T84, HeLa (human *cervical epithelial* cells), Chang (human conjunctival cells), and JOSK-M (human myelomonocytic cells) cell lines (Hauck et al., 1998; Howie et al., 2005; Kepp et al., 2009; Naumann et al., 1998). To study whether these three signaling cascades are involved in THUEC upon gonococcal infection, immunoblotting was performed to monitor the time course of phosphorylation of ERK1/2 (two isoforms of ERK, i.e. p44/p42), p38, and JNK.

Incubation of THUEC with cell culture growth medium (non-infected) from 4 to 10 h induced high constitutive activation of ERK1/2 (Fig 3.4A, lanes 1, 4, 7, and 10). Within the same time frame, infection with Ng CH811Str^R decreased levels of phosphorylated ERK1/2 in THUEC over time (Fig 3.4A lanes 2, 5, 8, and 11). This effect was further enhanced by infection with Ng CJSD1 from 6 h post-infection onwards (Fig 3.4A lanes 3, 6, 9, and 12). These observations were further reinforced by the densitometric analysis of at least three separate experiments (Fig 3.4B). At 6 h post-infection, levels of phosphorylated ERK1/2 in Ng CJSD1-infected THUEC were significantly less than that in non-infected and Ng CH811Str^R-infected cells. The same pattern was observed at 10 h post-infection while Ng CH811Str^R infection displayed significantly reduced levels of

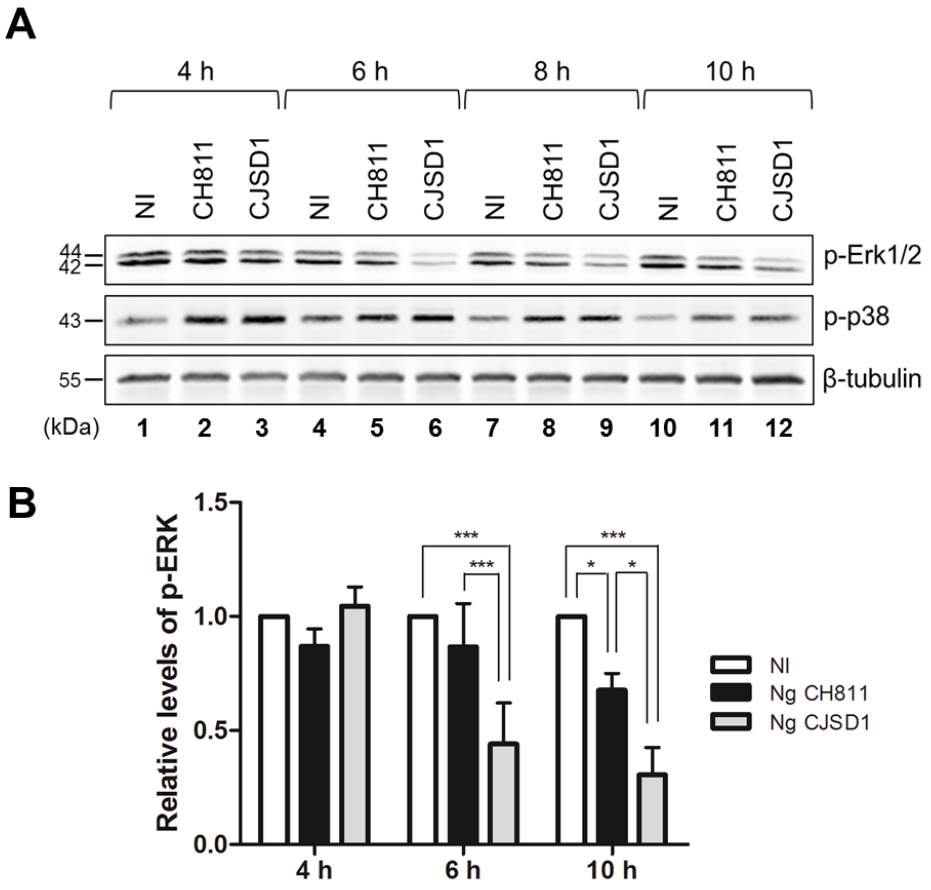

FIGURE 3.4. MAPK activation in THUEC infected with *N. gonorrhoeae* strain CH811Str^R or Ng CJSD1. THUEC were left non-infected (NI), infected with Ng CH811Str^R or Ng CJSD1 for 4, 6, 8, and 10 h (MOI=100).

(A) Representative immunoblots showing the levels of p-ERK1/2 and p-p38. β-tubulin served as the internal control.

(B) Densitometric analysis of p-ERK levels normalized to the corresponding β-tubulin levels. The value for non-infected control was set to 1. Data are mean ± SD of three independent experiments. Significant differences were determined by two-way ANOVA followed by Bonferroni post-test (*$P < 0.05$, ***$P < 0.001$).

phosphorylated ERK1/2 as compared to the non-infected control.

To rule out the possibility that the reduced levels of phosphorylated ERK by Ng CH811Str^R and Ng CJSD1 resulted from degradation of ERK proteins, total ERK proteins were probed as an internal loading control in addition to the β-tubulin at 6 h post-infection. Equal amounts of total ERK proteins were observed in both non-infected (Fig 3.5, lane 1) and Ng-infected samples (Fig 3.5, lanes 2 and 3), confirming that Ng CH811Str^R and Ng CJSD1 dampened ERK activation in THUEC without affecting total ERK protein levels.

Moreover, I investigated whether the considerable constitutive ERK activation was caused by the supplements (contain epidermal growth factor, an EGFR ligand that may activate ERK) added to the cell growth medium. Supplement starvation by using cell culture basal medium (without supplements) during infection did not diminish the background ERK activation (Fig 3.5, lane 1), suggesting that the constitutive ERK activation is THUEC specific.

p38 was activated in both non-infected (Fig 3.4, lanes 1, 4, 7, and 10) and Ng-infected THUEC throughout the course of infection. However, in contrast to ERK, infection with Ng CH811Str^R increased the levels of phosphorylated p38 throughout the course of infection and this augmentation appeared to decline with time (Fig 3.4, lanes 2, 5, 8, and 11). Ng CJSD1 infection exhibited similar pattern of p38 activation (Fig 3.4, lanes 3, 6, 9, and 12). Phosphorylated JNK was not detected within the same time frame (data not shown).

However, it has been reported that in T84 cells JNK activation dropped significantly to undetectable level by 3 h post-infection (Howie et al., 2005). So there is a possibility that JNK is activated in THUEC within the first four hours of infection.

Taken together, these results demonstrate that both Ng CH811Str^R and Ng CJSD1 induce prolonged p38 activation but decrease ERK phosphorylation, without activating JNK in THUEC from 4 to 10 h post-infection. Ng CJSD1 induces an enhanced ERK inhibition compared to the parent isogenic strain Ng CH811Str^R.

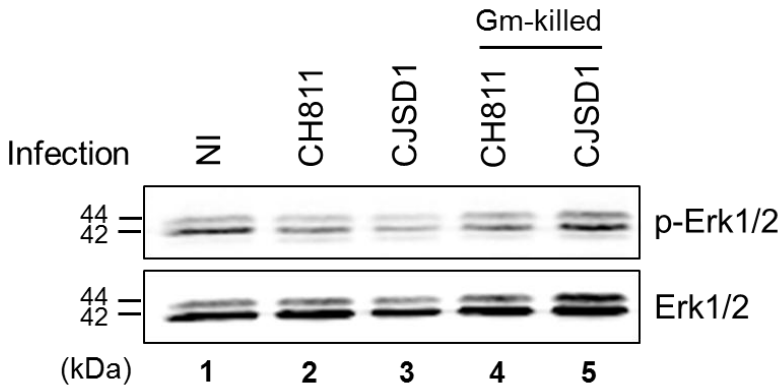

FIGURE 3.5. Effect of supplement starvation and gentamicin-killed *N. gonorrhoeae* strain CH811Str^{R} or Ng CJSD1 on ERK1/2 phosphorylation in THUEC. THUEC were left non-infected (NI), infected with live or gentamicin-killed gonococci for 6 h (MOI=100). Gonococci were killed by 2-hour incubation with 100 μg/ml gentamicin and the efficiency of gentamicin killing was confirmed by viable count. Gentamicin-killed bacteria were used at the same MOI as the live bacteria. Infection in this experiment was performed with PrEBM (basal medium without supplements). The total ERK 1/2 protein levels in each sample serve as the internal control.

3.3. ERK Phosphorylation in THUEC Infected with Gentamicin-killed *N. gonorrhoeae* Strain CH811Str^R or CJSD1

The requirement of live gonococci in mediating inhibition of ERK activation was determined by infecting THUEC with gentamicin-killed Ng CH811Str^R or Ng CJSD1 (Fig 3.5, lanes 4 and 5). Levels of phosphorylated ERK1/2 in gentamicin-killed Ng CH811Str^R-infected cells were similar to that in cells infected with live bacteria (Fig 3.5, compare lane 4 to lane 2), suggesting that Ng CH811Str^R-mediated inhibition of ERK activation is dependent on bacterial adherence and does not require bacterial internalization. In contrast, infection of THUEC with killed Ng CJSD1 totally reversed the reduced levels of phosphorylated ERK1/2, as observed in live bacterial infection, to the levels of ERK1/2 activation in the non-infected control (Fig 3.5, compare lane 5 to lanes 3 and 1). This indicates that the enhanced inhibition of ERK activation by Ng CJSD1 is mediated by bacterial internalization, and Ng CJSD1-mediated ERK inhibition requires bacterial cell viability.

3.4. Effect of ERK Inhibition on Apoptosis in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^R

To investigate the implication of Ng-mediated inhibition of ERK activation in apoptosis in THUEC, the MEK1/2 inhibitor U0126, which inhibits the activation of ERK1/2, was applied along with Ng CH811Str^R infection and the effect of ERK inhibition on apoptosis was examined by flow cytometric analysis. At 12 h post-infection, while intracellular Ng CH811Str^R decreased the percentage of early apoptotic cells in the total cell population compared to the non-infected control (Fig 3.6, bars 1 and 2), U0126 treatment further significantly lowered the percentage of early apoptotic cells relative to

the infection in the absence of inhibitor (Fig 3.6, bars 2 and 3). Non-infected THUEC with a four-hour U0126 treatment followed by removal of the inhibitor displayed a similar pattern of apoptosis to that without treatment (data not shown), indicating that the synergistic effect of U0126 and gonococcal infection on apoptosis inhibition is dominated by the presence of intracellular gonococci.

Therefore, ERK signaling is involved in the modulation of apoptosis in THUEC. Inhibition of ERK activation by the intracellular Ng CH811Str^{R} and Ng CJSD1 contributes to the anti-apoptotic effect of *N. gonorrhoeae* on THUEC.

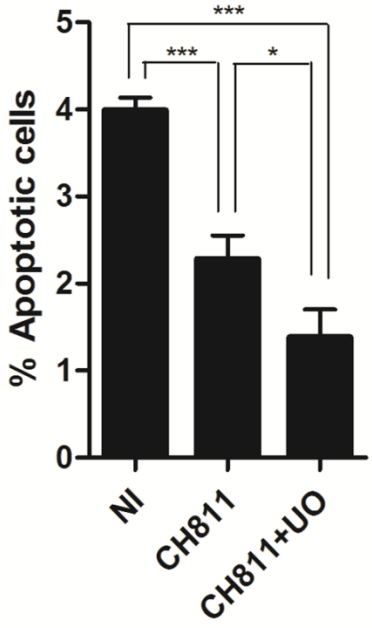

FIGURE 3.6. Effect of inhibition of ERK activation on apoptosis in THUEC challenged with *N. gonorrhoeae* strain CH811Str^{R}. THUEC were left non-infected (NI), infected with Ng CH811Str^{R} (MOI=100) for 4 h with or without 30 min pre-incubation with the MEK1/2 inhibitor U0126 (UO), followed by a 1 h incubation with 100 μg/ml gentamicin to kill extracellular bacteria. Cells were then incubated in antibiotic-free medium for the remainder of 12 h. At 12 h post-infection, cells were harvested, stained with Alexa Fluor 488-annexin V and PI, and analyzed by flow cytometry. Data show the percentage of early apoptotic cells in the total cell population and are the mean ± SD of three independent experiments performed in duplicate. One-way ANOVA followed by Bonferroni post-test (*$P < 0.05$, ***$P < 0.001$).

3.5. Effect of Inhibition of Epidermal Growth Factor Receptor on ERK Phosphorylation in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^R or CJSD1

I next sought to map the signaling molecules involved in the ERK signaling cascade that modulates apoptosis in THUEC. Ng adherence to A431 (human epidermoid cells), Chang, and HEC-1-B (human endometrial cells) cell lines has been shown to recruit EGFR to the attachment sites to facilitate bacterial internalization (Merz et al., 1999; Swanson et al., 2011). However, involvement of EGFR in ERK activation in Ng-infected human epithelial cells has not been identified.

By using the specific EGFR inhibitor tyrphostin AG1478, the effect of inhibition of EGFR activation on ERK phosphorylation in THUEC was examined by immunoblotting. Treatment with 5 μM AG1478 was sufficient to block ERK activation in non-infected cells (Fig 3.7, lane 4) compared to that without treatment (Fig 3.7, lane 1). A similar effect of inhibiting ERK phosphorylation was observed in cells infected with either Ng CH811Str^R or Ng CJSD1 in the presence of AG1478 (Fig 3.7 lanes 5 and 6) compared to the infection without treatment (Fig 3.7, lanes 2 and 3). Levels of total ERK1/2 were same in each sample. These results indicate that EGFR is an upstream signaling molecule (i.e. host cell receptor) that mediates ERK activation in THUEC.

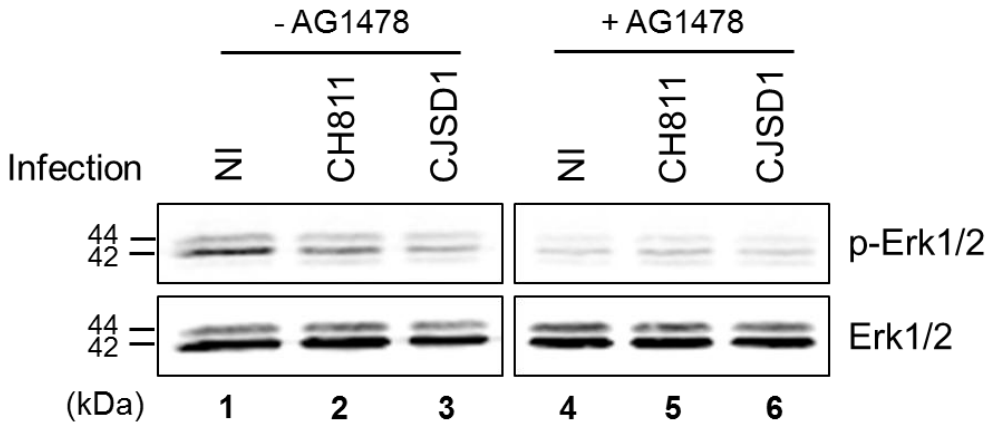

FIGURE 3.7. Effect of EGFR inhibitor AG1478 on ERK phosphorylation in non-infected THUEC and THUEC infected with *N. gonorrhoeae* strain CH811Str^{R} or Ng CJSD1. THUEC were pre-incubated with 5 μM EGFR inhibitor AG1478 for 1 h before the addition of bacteria and incubation for 6 h (MOI=100). Infection for this experiment was performed in PrEBM (basal medium) without supplements. The total ERK 1/2 protein levels in each sample serve as the internal control (Obtained from the same representative immunoblot in Fig. 3.5).

3.6. Effect of ERK inhibition and Cytochalasin D on Bim and Bad Expression in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^R

Rapid and sustained ERK activation in T84 cells infected with piliated, non-Opa-expressing Ng mediated an anti-apoptotic signaling through the downregulation of pro-apoptotic proteins Bim and Bad (Howie et al., 2005; Howie et al., 2008). Protein levels of Bim and Bad are negatively regulated by activated ERK through posttranslational modification (Sheridan et al., 2008). Phosphorylation of Bim and Bad renders them susceptible to degradation and sequestration, respectively (Datta et al., 1997; Meller et al., 2006). To further map the downstream signaling molecules involved in the ERK signaling cascade that modulates apoptosis, I examined whether Bim and Bad are regulated by activated ERK in THUEC. Compared to the non-infected control, infection with Ng CH811Str^R showed slightly increased levels of Bad, and similar levels of Bim$_{EL}$ and p-Bad (Ser112) (Fig 3.8, lanes 1 and 2). U0126 treatment along with Ng CH811Str^R infection resulted in significantly decreased levels of phosphorylated ERK1/2, in conjunction with elevated total proteins of Bim$_{EL}$ and Bad and slightly decreased levels of p-Bad (Fig 3.8, lane 3) as compared to the non-infected and infected cells without treatment (Fig 3.8, lanes 1 and 2). It is worth noting that three Bim isoforms, resulting from alternative RNA splicing, were probed and it has been reported that Bim$_{EL}$ and Bim$_L$ were mainly posttranslationally regulated by ERK and JNK, respectively, while no post-translational regulation occurred on Bim$_S$ (Ley et al., 2005). Therefore, similar to other cell types, Bim$_{EL}$ and Bad are negatively regulated by activated ERK in THUEC, and degradation of Bad is dependent on the phosphorylation of Bad at Ser112.

In addition, to examine the effect of bacterial internalization on the ERK signaling cascade in THUEC, cytochalasin D was applied to Ng CH811Str^{R}-infected cells to impede internalization of gonococci. Compared to the non-infected and infected cells without treatment (Fig 3.8, lanes 1 and 2), cytochalasin D treatment along with infection diminished the ERK1/2 activation, coupled with an augmentation of Bim_{EL} levels (Fig 3.8, lane 4). Interestingly, although both U0126 and cytochalasin D dampened ERK activation to a similar extent, levels of Bim_{EL} and Bad upon cytochalasin D treatment were noticeably lower than that of U0126 treatment, while p-Bad levels were the opposite (Fig 3.8, lanes 3 and 4). These observations are reminiscent of the side effect of cytochalasin D mediated disruption of cytoskeletal actin, as it has been reported that cytochalasin D abrogates ERK activation in some cell types (Bijian et al., 2005; Ingram et al., 2000; Numaguchi et al., 1999). Therefore, it is very likely that the inhibition of ERK activation and effect on Bim_{EL}, Bad, and p-Bad upon cytochalasin D treatment were the result of disruption of the actin cytoskeleton by cytochalasin D.

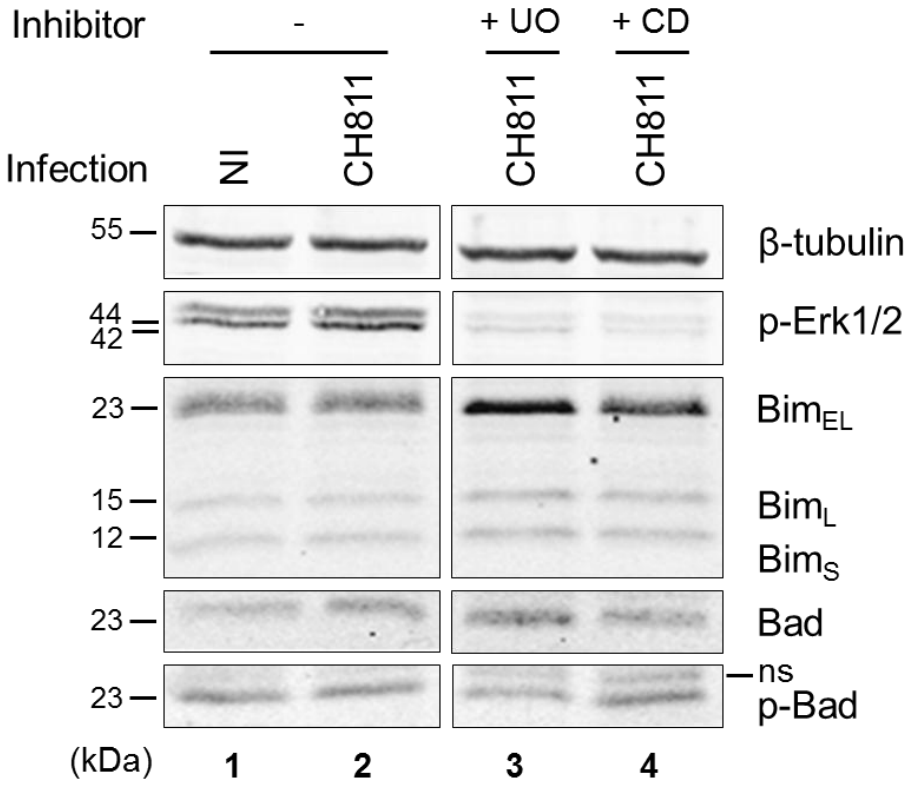

FIGURE 3.8. Effect of ERK inhibition and cytochalasin D on Bim and Bad expression in THUEC infected with *N. gonorrhoeae* strain CH811Str^R. THUEC were left untreated, or pre-incubated with U0126 (UO, MEK1/2 inhibitor) or cytochalasin D (CD, inhibitor of actin polymerization) for 30 min before the addition of bacteria and incubation for 6 h (MOI=100). Immunoblotting was performed with antibodies against p-ERK, Bim, Bad, and p-Bad (Ser112). Three different Bim isoforms are shown (Bim$_{EL}$-extra-long; Bim$_L$-long; Bim$_S$-short). β-tubulin serves as the internal control. Representative immunoblots are shown from at least two independent experiments.

3.7. Expression of Bim and Bad in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^R or CJSD1

To further investigate the implication of MinD in apoptosis in THUEC, levels of Bim, Bad, and p-Bad were monitored in Ng CJSD1-infected cells at 6 h post-infection. Although infection with Ng CJSD1 decreased the levels of phosphorylated ERK as that with U0126 treatment, the levels of Bim_{EL} and Bad in Ng CJSD1-infected cells were noticeably less than that of the Ng CH811Str^R-infected cells (Fig 3.9, lanes 2 and 3).

Since ERK-mediated Bad degradation occurs by phosphorylation of Bad at Ser112 (Howie et al., 2008), the Ser112 phosphorylated form of Bad (p-Bad) was also detected by immunoblotting. Ng CH811Str^R-infected cells had similar levels of p-Bad as the non-infected control (Fig 3.9, lanes 1 and 2). In contrast, Ng CJSD1-infected cells showed noticeably higher levels of p-Bad (Fig 3.9, lane 3). Taken together, the reduced levels of Bim_{EL} and Bad coupled with increased p-Bad levels in Ng CJSD1-infected cells partially explain the reduced and delayed apoptosis in THUEC infected with Ng CJSD1 as observed at later time points post-infection (Fig 3.1).

Interestingly, as Bim and Bad are negatively regulated by activated ERK in THUEC as shown in section 3.6, the enhanced inhibition of ERK activation by Ng CJSD1 (Fig 3.9, lane 3) should have translated into an enhancement of levels of Bim and Bad, coupled with decreased p-Bad levels. Therefore, the unexpected regulation of Bim and Bad in Ng CJSD1-infected cells suggests an unclear link between ERK and Bim/Bad which requires further investigation.

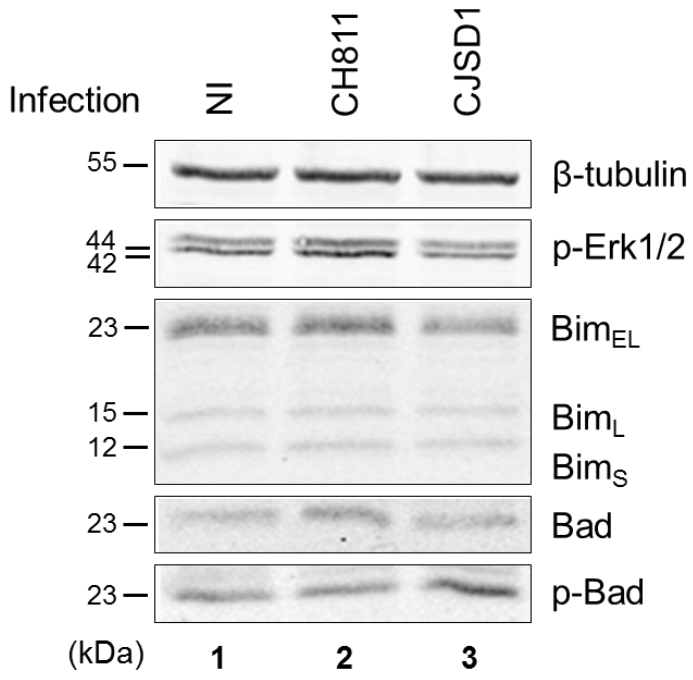

FIGURE 3.9. Levels of Bim, Bad, and p-Bad in THUEC infected with *N. gonorrhoeae* strain CH811Str^R or Ng CJSD1. THUEC were left non-infected (NI), infected with Ng CH811Str^R or Ng CJSD1 for 6 h (MOI=100). Immunoblotting was performed with antibodies against p-ERK, Bim, Bad, and p-Bad (Ser112). Three different Bim isoforms are shown (Bim$_{EL}$-extra-long; Bim$_L$-long; Bim$_S$-short). β-tubulin serves as the internal control. Representative immunoblots are shown from at least two independent experiments (Obtained from the same representative immunoblot in Fig. 3.8).

3.8. Caspase 8 and Caspase 3/7 Activities in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^R or CJSD1

The apoptotic machinery in eukaryotic cells functions in either a caspase-dependent or independent manner (Broker et al., 2005). To determine whether the Ng-modulated apoptosis in THUEC involves caspase activation, caspase 3/7 and 8 activities were measured in cells infected Ng CH811Str^R or Ng CJSD1. Compared to the non-infected cells, infection with either strain for 10 h induced similar caspase 3/7 activation in THUEC (Fig 3.10A, bars 1, 2, and 3). Similarly, caspase 8 activity was elevated in THUEC infected with Ng CH811Str^R and Ng CJSD1 (Fig 3.10B, bars 1, 2, and 3). These results suggest that Ng-modulated apoptosis in THUEC involves caspase activation.

In addition, six-hour ERK inhibition by U0126 followed by removal of the inhibitor upon Ng CH811Str^R infection did not affect caspase 3/7 and caspase 8 activities compared to infection without treatment (Fig 3.10A, bar 4; Fig 3.10B, bar 4), suggesting that U0126 does not affect the viability of THUEC. Given that the U0126 treatment along with intracellular Ng CH811Str^R infection enhanced the anti-apoptotic effect of intracellular gonococci, it is likely that the apoptosis resistance provided by *N. gonorrhoeae* during early infection was not reflected in reduced caspase activities.

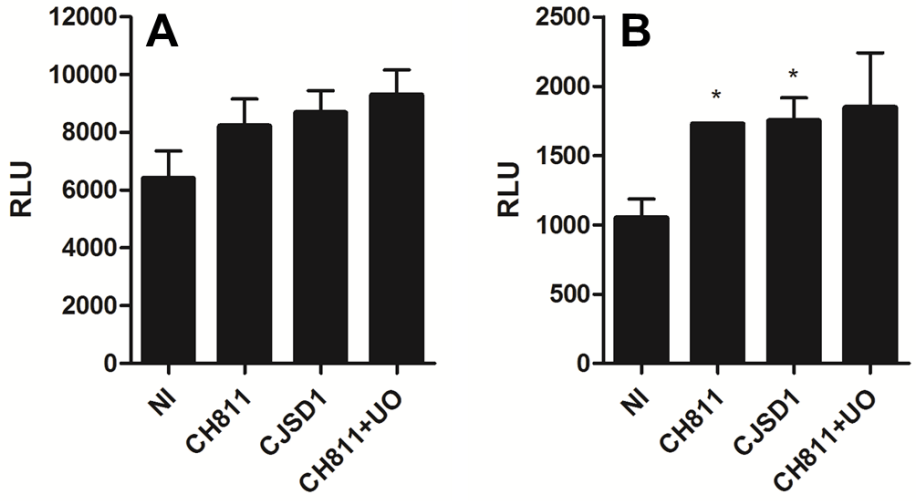

FIGURE 3.10. Cellular caspase 3/7 (A) and caspase 8 (B) activities in THUEC infected with *N. gonorrhoeae* strain CH811Str^R or Ng CJSD1. THUEC were left non-infected, infected with Ng CH811Str^R or Ng CJSD1 for 6 h followed by removal of unattached bacteria and further incubation for 4 h. U0126 (UO) treatment was preformed 30 min before infection. Luminescent caspase substrates were added into cell lysates and relative light units (RLU) were measured after 1 h room-temperature incubation in the dark. Data show mean ± SD of two independent experiments performed in duplicate. Statistical significance between non-infected control and Ng CH811Str^R / Ng CJSD1 infection is determined by unpaired two-tailed *t*-test (*$P < 0.05$).

3.9. Time-course Expression of IκBα in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^R and CJSD1

NF-κB signaling is involved in cytokine expression, bacterial internalization, and apoptosis in Ng-infected human epithelial cells (Binnicker et al., 2004; Muenzner et al., 2002; Muenzner et al., 2001; Naumann et al., 1997). To examine whether NF-κB signaling is activated in THUEC upon infection with Ng CH811Str^R and Ng CJSD1, IκBα levels were measured by immunoblotting. IκBα sequesters NF-κB complexes in the cytoplasm, and upon activation undergoes degradation, thereby releasing NF-κB complexes. NF-κB complexes are then translocated into the nucleus, in which they drive the transcription of a broad spectrum of genes.

While non-infected THUEC showed constant IκBα levels (Fig 3.11A, lanes 1, 4, 7, and 10) during 4 to 10 h incubation in culture medium, infection with Ng CH811Str^R induced significant IκBα degradation from 4 h onwards, and this effect strengthened over time (Fig 3.11A, lanes 2, 5, 8, and 11). Ng CJSD1 infection displayed a similar pattern of IκBα degradation from 6 h to 10 h post-infection (Fig 3.11A, lanes 3, 6, 9, and 12). However, IκBα degradation appears to be greater in Ng CJSD1-infected cells from 6 h onwards, indicating that Ng CJSD1 induced stronger activation of NF-κB signaling. These observations were further reinforced by the densitometric analysis of at least three separate experiments (Fig 3.11B).

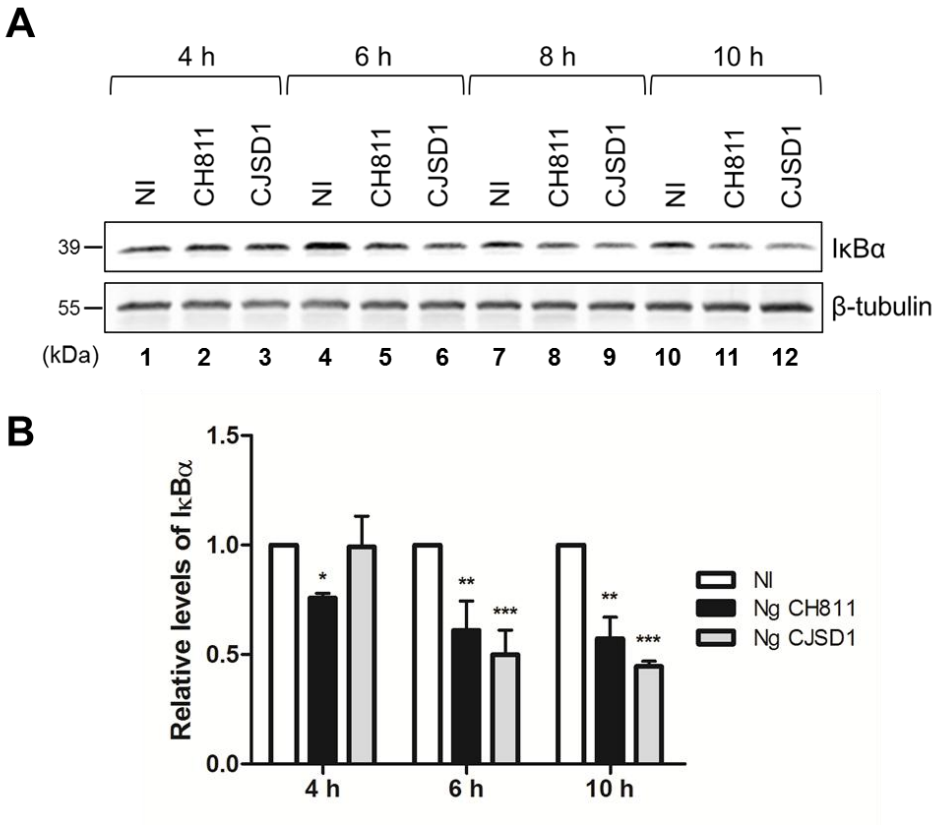

FIGURE 3.11. IκB*α* degradation in THUEC infected with *N. gonorrhoeae* strain CH811Str^{R} or Ng CJSD1. THUEC were left non-infected (NI), infected with Ng CH811Str^{R} or Ng CJSD1 for 4, 6, 8 and 10 h (MOI=100).

(A) Representative immunoblot showing the levels of IκBα. β-tubulin serves as the internal control.

(B) Densitometric analysis of IκBα levels normalized to the corresponding β-tubulin levels. The value for non-infected control was set to 1. Data are mean ± SD of three independent experiments. Significant differences were determined by two-way ANOVA followed by Bonferroni post-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3.10. Association of *N. gonorrhoeae* CH811Str^R and CJSD1 with Actin Cytoskeleton in THUEC

Previous work has shown that Ng CJSD1 formed diffused microcolonies compared to the well-organized, round microcolonies formed by Ng CH811Str^R upon adherence to THUEC (Parti et al., 2011a). Given the aberrant cell morphology of Ng CJSD1, I hypothesized that the pattern of interaction of either strain with epithelial cells would be different. Confocal microscopy was performed to visualize the interaction of Ng CH811Str^R and Ng CJSD1 with actin cytoskeleton, as gonococcal invasion of primary urethral epithelial cells induced actin rearrangement (Giardina et al., 1998). By overlaying the bacterial channel (Fig 3.12, A and B) and actin channel (Fig 3.12, C and D), the interaction of Ng CH811Str^R and Ng CJSD1 with actin filaments is shown in Fig 3.12 E and F. Ng CH811Str^R adhered to THUEC individually or by forming small aggregates and microcolonies (arrowhead). This association induced focal actin recruitment beneath the microcolonies. In contrast, under a similar field of view, more bacterial aggregates of Ng CJSD1 were observed. The size of these aggregates was visually larger than that formed by Ng CH811Str^R. This may be due to the relatively large size of individual Ng CJSD1 cells. However, we were unable to determine the number of cells within a bacterial aggregate. Association of Ng CJSD1 with THUEC seems to induce more mobilization of actin filaments (arrowhead), suggesting that actin rearrangement by Ng CJSD1 is stronger than that by Ng CH811Str^R.

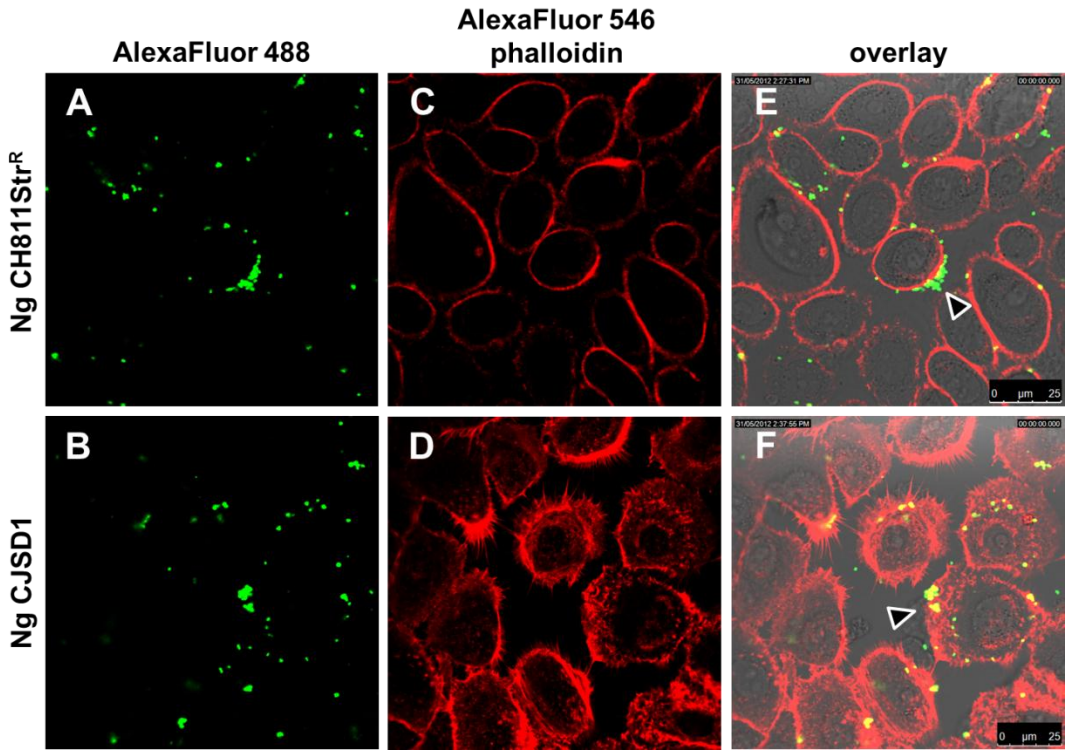

FIGURE 3.12. Confocal analysis of THUEC infected with *N. gonorrhoeae* strain CH811Str^R or Ng CJSD1. THUEC were infected with Alexa Fluor 488-labelled Ng CH811Str^R or Ng CJSD1 for 4 h, fixed, permeabilized, and then stained with Alexa Fluor 546 phalloidin for actin cytoskeleton. Arrowheads indicate interaction of gonococci (green) with actin filaments (red). Scale bars represent 5 μm. (A, B) Bacterial channel; (C, D) Actin channel; (E, F) Overlay.

CHAPTER 4 DISCUSSION

4.1. Inhibition of ERK Activation by *Neisseria gonorrhoeae* Contributes to Anti-apoptotic Effect on THUEC

The pathogen-mediated modulation of host cell death masters the teeter-totter of pathogen transmission and elimination. Ng has been shown to either induce or inhibit apoptosis in human epithelial cells. These conflicting observations are likely to result from the differences in bacterial strains, cell lines, cell culture conditions, and infection protocols used in the studies (Table 1.2). Gonococcal infection of urethral epithelial epithelium has been reported to protect cells from STS-induced apoptosis (Binnicker et al., 2003). Similar protection from apoptosis induced by exogenous apoptosis inducer has been observed in several cell types such as endocervical, fallopian tube epithelial cells, and PMNs (Morales et al., 2006; Follows et al., 2009; Chen and Seifert, 2011). In the present research, the apoptotic effect of gonococcal infection on THUEC was examined without using apoptosis inducer, which may provide evidences that are akin to natural infection. As revealed by the quantitative flow cytometric analyses, gonococcal infection of THUEC provides an anti-apoptotic effect during early infection (till at least 12 h post-infection) but induces apoptosis at prolonged time points. This observation appears to correspond to the clinical manifestation of gonococcal urethritis in men, which features an incubation period of gonococci before the onset of purulent discharge (Edwards and Apicella, 2004). During this incubation periods, urethral epithelial cells probably secure a niche for proliferation of intracellular gonococci, after which the bacteria disseminate to other host niches and infected epithelial cells are shed into the penile exudate. I further demonstrate that the anti-apoptotic effect conferred by Ng is primarily mediated by

intracellular gonococci, as impeding bacterial internalization abolishes the apoptosis resistance. This observation is consistent with two recent studies which demonstrate that the anti-apoptotic response of Ng-infected human endocervical and fallopian tube epithelial cells is dependent on their association with intracellular gonococci (Follows et al., 2009; Morales et al., 2006).

The impact of mind mutation, which leads to cell division deficiency, on Ng-modulated apoptosis in THUEC was investigated in parallel. Infection with Ng CJSD1 resulted in decreased and delayed apoptosis during prolonged infection periods relative to the parent strain. This suggests that bacterial load plays a role in mediating the progress of apoptotic response, as Ng CJSD1 showed significantly reduced viability compared to the parent strain after reaching stationary phase (6 h after inoculation) (Szeto et al., 2001). Interestingly, although a previous study in our laboratory showed that Ng CJSD1 invaded THUEC significantly less than the parent strain (Parti et al., 2011a), I observed that intracellular gonococci of Ng CJSD1 conferred a similar level of apoptosis inhibition as the parent strain. This observation suggests that bacterial load is not the only contributing factor to apoptosis.

Caspase activation plays a central role in both the extrinsic and the intrinsic apoptotic pathways (Elmore, 2007). Gonococcal infection can protect host cells from apoptosis by inhibiting caspase 3/7, 8, and 9 activities (Chen and Seifert, 2011; Follows et al., 2009; Howie et al., 2008; Simons et al., 2006). In contrast, Ng-induced apoptosis involves the activation of caspase 3 (Kepp et al., 2009; Muller et al., 2000; Muller et al., 1999; Muller et al., 2002). To investigate the implication of caspase activation in Ng-induced apoptosis in THUEC, the activities of caspase 8 and caspase 3/7 were examined

in cells infected with Ng CH811Str^R or Ng CJSD1 for 10 h. Both strains induced noticeable caspase activation. This result indicates that Ng-induced apoptosis in THUEC involves caspase activation. Ng CJSD1 induced similar caspase activity as Ng CH811Str^R at 10 h post-infection. However, it is very likely that the difference in caspase activities induced by Ng CH811Str^R and Ng CJSD1 will manifest at later time points, as the significant difference in the percentage of apoptotic cells between the two strains started from 24 h post-infection.

The ability of a number of bacteria to modulate host MAPK signaling cascades is an effective virulence strategy. While a number of bacteria, such as *Pseudomonas aeruginosa*, *Bartonella henselae,* and *Leishmania major* confer anti-apoptotic effects on host cells by activating ERK signaling (Sarkar et al., 2012; Schmid et al., 2006; Zhang et al., 2004), inhibition of ERK activation has been observed in virulent *Yersina enterocolitica* and *Mycobacterium avium*-infected macrophages to prevent cytokine production, thereby counteracting immune responses (Roach and Schorey, 2002; Ruckdeschel et al., 1997). I therefore investigated the involvement of MAPK pathways in apoptosis modulation by Ng in THUEC. Non-infected THUEC exhibited high constitutive ERK activation, which was not diminished by supplement starvation. Infection with Ng CH811Str^R dampened ERK activation from 6 h to 10 h post-infection. This result is in contrast to the observation in T84 cells infected with Ng N400 that gonococcal infection increased activation of ERK (Howie et al., 2005; Howie et al., 2008). Given that the activation of ERK in T84 cells by Ng initiates an anti-apoptotic signaling, I was interested in understanding the contribution of ERK inhibition by Ng to the apoptotic response in THUEC. Inhibition of ERK activation by U0126 and

gonococcal infection synergistically decreased apoptosis, indicating that ERK activation serves as a pro-apoptotic, rather than a pro-survival signaling in THUEC. To my knowledge, the present research is the first report linking inhibition of ERK activation to bacteria-mediated inhibition of apoptosis. Ng CH811Str^{R}-mediated attenuation of ERK activation therefore contributes to the anti-apoptotic effect of Ng, which may establish a favorable niche for intracellular Ng proliferation.

Similarly, infection with Ng CJSD1 also led to inhibition of ERK activation in THUEC. Intriguingly, Ng CJSD1 infection resulted in an enhanced ERK inhibition compared to the parent strain. This observation seems to partially explain the reduced and delayed apoptosis in Ng CJSD1-infected THUEC.

4.2. *N. gonorrhoeae*-modulated Apoptosis in THUEC Involves EGFR-ERK-Bim/Bad Signaling Cascades

I further mapped the signaling molecules involved in the ERK pathway that modulates apoptosis in Ng-infected THUEC. The canonical ERK pathway is initiated by activation of cell surface receptor tyrosine kinase (RTKs) followed by sequential recruitment and activation of Ras, Raf, MEK, and ERK (Krishna and Narang, 2008). Gonococcal attachment to human epithelial cells has been shown to recruit EGFR, one of the RTKs to the cortical plaque formed beneath bacterial microcolonies (Merz et al., 1999; Swanson et al., 2011). By using the EGFR-specific inhibitor AG1478, ERK activation was diminished in both non-infected and Ng-infected THUEC. This result demonstrates that EGFR is an upstream signaling molecule of ERK, which is consistent with observations in other cell types (Kim et al., 2009b; Kodama et al., 2002; Vacca et al., 2000).

Two pro-apoptotic Bcl-2 family proteins, Bim and Bad, have been shown to be regulated by activated ERK in Ng N400-infected T84 cells (Howie et al., 2008). Regulation of these two proteins occurs at the post-translational level rather than the mRNA level in Ng-infected epithelial cells (Binnicker et al., 2003; Howie et al., 2008). As with the previous observations in T84 cells (Howie et al., 2008), Bim_{EL} and Bad were both negatively regulated by activated ERK, as inhibition of ERK activation by U0126 in Ng CH811Str^{R}-infected THUEC significantly increased the levels of Bim_{EL} and Bad, accompanied by the decreased p-Bad (Ser112) levels. These results demonstrate that Bim and Bad are two downstream signaling molecules of ERK in THUEC.

Although infection with Ng CJSD1 decreased the levels of phosphorylated ERK, the levels of Bim_{EL} and Bad in Ng CJSD1-infected cells were unexpectedly less than that of Ng CH811Str^{R}-infected cells. I propose that other signaling cascades are specifically involved in the regulation of Bim_{EL} and Bad in THUEC in response to Ng CJSD1 infection. Bim has also been reported to be regulated by activated JNK (Lei and Davis, 2003; Ley et al., 2005). However, JNK activation was not observed in THUEC infected with Ng CH811Str^{R} and Ng CJSD1. Bad undergoes sequestration or degradation following phosphorylation at Ser112, Ser136, and Ser155 by ERK/p90RSK, PI3-K/Akt, and PKA, respectively (Bonni et al., 1999; Datta et al., 1997; Datta et al., 2000). In Ng N400-infected T84 cells, phosphorylation of Bad only occurred at Ser112 (Howie et al., 2008). I similarly observed ERK-dependent Bad phosphorylation at Ser112 and Bad degradation in Ng CH811Str^{R}-infected THUEC with U0126 treatment. However, the enhanced inhibition of ERK activation by Ng CJSD1 was unexpectedly coupled with increased phosphorylated Bad (Ser112). It is possible that the phosphorylation of Bad at

Ser112 by activated ERK was bypassed by other protein kinases activated in Ng CJSD1-infected THUEC, such as mitochondria-anchored PKA, which has been reported to phosphorylate Bad at Ser112 (Harada et al., 1999). Interestingly, although phosphorylation of Bad at Ser136 and Ser155 was not probed in the present research, I monitored the activation of PI3-K/Akt pathway and found no activated Akt in THUEC upon Ng infection (data not shown). This result indicates that PI3-K/Akt signaling is not involved in Bad phosphorylation and degradation in THUEC. Taken together, the mechanism by which Ng CJSD1 manipulates Bim and Bad in THUEC requires further investigation.

Therefore, I identified an EGFR-ERK-Bim/Bad signaling cascade that is involved in gonococcal infection in THUEC. This signaling cascade is modulated differently by Ng CJSD1 compared to the isogenic parent strain Ng CH811Str^{R}.

Since gonococcal infection of urethral epithelial cells induced up-regulation of two anti-apoptotic Bcl-2 family members, *bfl-1* and *mcl-1* (Binnicker et al., 2003), it would be worthwhile in future studies to monitor the levels of anti-apoptotic proteins in THUEC infected with Ng CH811Str^{R} and Ng CJSD1. The importance of studying anti-apoptotic proteins is also supported by the observation that inhibition of ERK activation in Ng CH811Str^{R}-infected THUEC by U0126, which increased the levels of pro-apoptotic proteins Bim and Bad, further lowered the percentage of apoptotic cells compared to Ng CH811Str^{R} infection without treatment. Given that apoptosis is controlled by the balance between pro-apoptotic and anti-apoptotic proteins, it is possible that the pro-apoptotic effect of Bim and Bad was outweighed by the activation of anti-apoptotic proteins through an unknown mechanism, thereby giving rise to decreased apoptosis.

4.3. Modulation of Host Response by *N. gonorrhoeae* in a Cell Shape-dependent Manner

Modulation of the host response, including apoptosis in Ng-infected cells has been attributed to bacterial cell surface virulence factors, such as Tfp, Opa, porin, and LOS. These virulence factors aid bacterial attachment, internalization, and niche adaptation through their interplay with corresponding host receptors, such as Tfp-CD46, Opa-CEACEM, and LOS-ASGP-R (Harvey et al., 2001; Kirchner and Meyer, 2005; Sadarangani et al., 2011). The *minD* mutant strain Ng CJSD1 evaluated in the present research has been shown to express comparable levels of pilin, Opa, and LOS as compared to its isogenic parent strain Ng CH811Str^{R}. However, its ability to form organized microcolonies, to adhere to and invade THUEC was significantly impaired (Parti et al., 2011a). In the present research, I further compared Ng CJSD1 to Ng CH811Str^{R} with regard to their impacts on cell signaling in THUEC. While infection with Ng CH811Str^{R} dampened ERK activation and increased the levels of Bim and Bad, Ng CJSD1 displayed enhanced inhibition of ERK activation and unexpected down-regulation of Bim and Bad.

How does the *minD* mutant strain Ng CJSD1 differentially regulate host cell signaling without apparently changing the expression of major virulence factors? In vitro *E. coli* models have hypothesized that the MinD-membrane interaction induces changes in membrane dynamics and structural properties, including fluidity gradient, membrane potential, and proteolipid domain formation (Mazor et al., 2008a, b; Mileykovskaya and Dowhan, 2005; Strahl and Hamoen, 2010). Therefore, it may be that the cell division deficiency resulting from the *minD* inactivation alters the distribution of virulence factors

on bacterial cell surface, thereby disrupting the normal pattern of interaction between gonococci and host cells.

Furthermore, Ng-induced ERK activation has been correlated to the mechanical force initiated by Tfp retraction, which requires the functional pilus subunit PilT (Howie et al., 2005; Howie et al., 2008). Although Ng CJSD1 expresses similar levels of pilin subunit (PilE) to the parent strain Ng CH811Str^{R}, whether the enhanced inhibition of ERK activation by Ng CJSD1 results from the alteration of pilus retraction is unclear. Interestingly, a recent study has correlated the pilus retraction with microcolony formation and NF-κB activation. A *pilT* mutant, which lacked pilus retraction, formed disordered microcolonies and triggered reduced NF-κB activation (Dietrich et al., 2011). In the present research, I also examined NF-κB activation by Ng CJSD1 given that it similarly formed abnormal microcolonies upon attachment to THUEC (Parti et al., 2011a). Compared to Ng CH811Str^{R}, infection with Ng CJSD1 delayed the onset of NF-κB activation. However, once activated, it appears to induce stronger NF-κB activity than Ng CH811Str^{R}. As NF-κB signaling mediated pro-survival effect in THUEC through inducing expression of anti-apoptotic genes, including *bfl-1*, *cox-2*, and *c-IAP-2* (Binnicker et al., 2004), elevated NF-κB activity by Ng CJSD1 partially explains the reduced and delayed apoptosis observed in Ng CJSD1 infection.

I observed a significant inhibition of ERK activation when treating THUEC with the inhibitor of actin polymerization, cytochalasin D. This inhibition of ERK activation by cytochalasin D-mediated actin disruption has been reported in several cell types (Bijian et al., 2005; Ingram et al., 2000; Numaguchi et al., 1999). I therefore hypothesized that the enhanced inhibition of ERK activation and unexpected modulation

of Bim and Bad by Ng CJSD1 may result from its aberrant association with the host cell cytoskeleton upon attachment to and internalization of THUEC. Confocal microscopic analysis was therefore performed to visualize the interaction of Ng CH811Str^R and Ng CJSD1 with actin cytoskeleton. Compared to Ng CH811Str^R, the *minD* mutant strain Ng CJSD1 formed larger bacterial cell aggregates, possibly due to the relatively large size of individual *minD* mutant cells resulting from the cell division deficiency. Relatively diffused distribution of actin filaments in THUEC infected with Ng CJSD1 strongly suggests that the actin rearrangement by Ng CJSD1 is stronger than that by Ng CH811Str^R.

Cytoskeleton rearrangement is also linked to the regulation of Bim and Bad. Bim is normally sequestered in the cytoskeleton via the association with motor complexes bound to dynein light chain LC8. The release of Bim from the cytoskeleton in response to apoptotic stimuli initiates the translocation of Bim to mitochondria, in which it activates Bak and Bax-mediated MOMP (Puthalakath et al., 1999). Cell survival signaling conferred by Bad phosphorylation is also dependent on sequestration of phosphorylated Bad in the cytoplasm by binding to 14-3-3 proteins that are associated with the cytoskeleton (Sluchanko and Gusev, 2010; Zha et al., 1996). As unexpectedly decreased levels of Bim and Bad coupled with ERK inhibition were observed in Ng CJSD1-infected cells, it is likely that both effects are strongly mediated by Ng CJSD1-induced cytoskeleton rearrangement in THUEC, and the positive regulation of Bim and Bad by inhibition of ERK activation is outweighed by the effect of cytoskeleton rearrangement. However, the detailed mechanism by which Ng CJSD1-mediated cytoskeleton rearrangement modulates Bim and Bad requires further investigation. It is worthwhile to

probe for Bim and Bad in different cellular compartments by fractionation (e.g. the cytoskeleton, mitochondria from infected cells).

Initiation of ERK signaling by pilus retraction occurs upon gonococcal attachment to epithelial cells (Howie et al., 2005). I examined which gonococcal infection step, i.e. attachment or internalization is responsible for the inhibition of ERK activation in Ng-infected THUEC. Infection with the live and gentamicin-killed Ng CH811Str^{R} led to the same pattern of inhibition of ERK activation, suggesting that bacterial attachment, rather than internalization is required for the inhibition of ERK activation in Ng CH811Str^{R}-infected THUEC. Intriguingly, infection with dead Ng CJSD1 totally abolished the enhanced inhibition of ERK activation observed with live Ng CJSD1 infection. This result strongly indicates that the enhanced inhibition of ERK activation by Ng CJSD1 is dependent on the internalization of live Ng CJSD1 bacteria.

I asked the question whether gonococcal virulence factors are contributors to Ng-mediated inhibition of ERK activation. Both strains used in this research express pili, Opa, and LOS. As primary male urethral epithelial cells do not express CEACAM (Edwards and Apicella, 2004), the involvement of Opa in gonococcal infection of THUEC is excluded. Activation of ERK signaling can be either pilus-dependent (Howie et al., 2005; Howie et al., 2008) or independent (Rodriguez-Tirado et al., 2012). It is likely that pili are involved in the inhibition of ERK activation in THUEC as bacterial attachment was sufficient for Ng CH811Str^{R}-mediated inhibition of ERK activation and pili are the major virulence factors involved in attachment of gonococci to epithelial cells. However, to fully understand whether pili are involved in the inhibition of ERK activation in THUEC, a pilus negative strain is needed for comparison in future research.

Uptake of < 5% gonococci by primary human urethral epithelial cells occurred through non-receptor mediated process, such as macropinocytosis (Zenni et al., 2000). This mechanism requires cytoskeleton rearrangement and does not involve interaction between virulence factor and host receptor (i.e. non-receptor mediated invasion). Therefore, impaired ability of Ng CJSD1 to invade THUEC can be explained by: 1) reduced receptor-mediated invasion (i.e. redistribution of bacterial cell surface virulence factors); 2) impaired non-receptor mediated invasion (i.e. large size of individual bacteria).

Given that the enhanced inhibition of ERK activation by Ng CJSD1 is bacterial internalization-dependent and Ng CJSD1 infection noticeably disrupted the cytoskeletal actin, I propose a model that the aberrant shape of Ng CJSD1 is the major contributor to its impact on cell signaling, including inhibition of ERK activation and regulation of Bim and Bad, which in turn modulates apoptosis in THUEC (Fig 4.1).

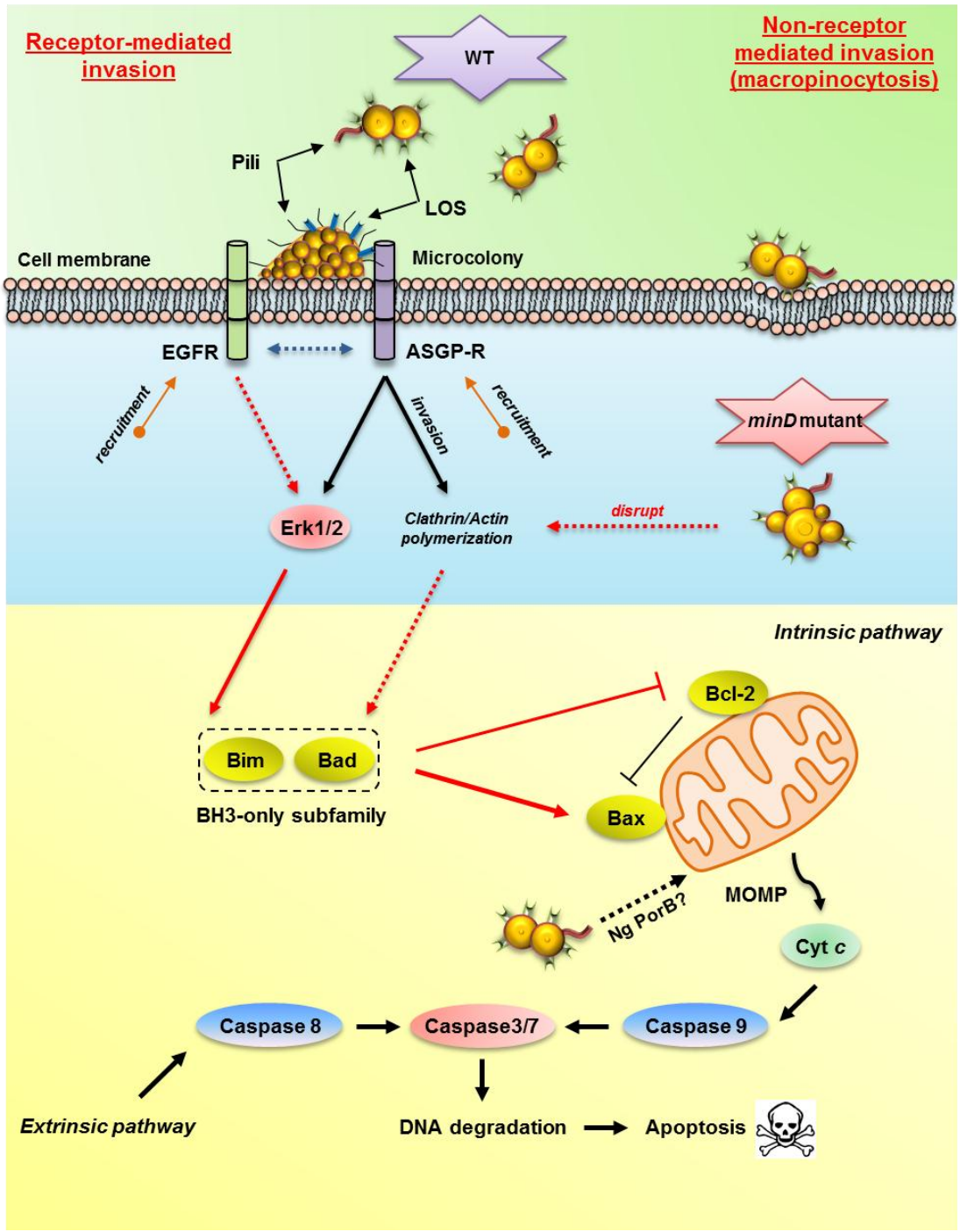
Receptor-mediated invasion
WT
Non-receptor mediated invasion (macropinocytosis)
Pili
LOS
Cell membrane
Microcolony
EGFR
ASGP-R
recruitment
invasion
recruitment
minD mutant
Erk1/2
Clathrin/Actin polymerization
disrupt
Intrinsic pathway
Bcl-2
Bim
Bad
BH3-only subfamily
Bax
MOMP
Ng PorB?
Cyt c
Caspase 8
Caspase3/7
Caspase 9
Extrinsic pathway
DNA degradation
Apoptosis

FIGURE 4.1. Proposed model for interaction of *Neisseria gonorrhoeae* with THUEC. Gonococcal invasion of human urethral epithelial cells can occur through either receptor-mediated or non-receptor mediated mechanisms (Harvey et al., 2001; Zenni et al., 2000). Both mechanisms require the rearrangement of cytoskeleton. The former further involves the binding of bacterial LOS to ASGP-R. Meanwhile, the direct or indirect interaction between gonococci and EGFR activates the EGFR-ERK-Bim/Bad signaling cascade. This signaling pathway partially confers apoptosis resistance through mitochondria-mediated intrinsic apoptotic pathway. Released cytokine (e.g. TNF-α) from infected THUEC may initiate the extrinsic apoptotic pathway (Rudel et al., 2010). Ng CJSD1 (the *minD* mutant strain) seems to regulate the EGFR-ERK-Bim/Bad signaling cascade by a mechanism involving aberrant cell shape-mediated cytoskeleton rearrangement.

CHAPTER 5 CONCLUSION AND IMPACT

In the present research, I investigated the modulation of apoptosis in THUEC by *Neisseria gonorrhoeae* strain CH811Str^{R} and its isogenic *minD* mutant CJSD1. Gonococcal infection confers an anti-apoptotic effect on THUEC in early infection, but induces apoptosis during prolonged infection periods. Intracellular gonococci are the primary contributor to the anti-apoptotic response. I further correlated the Ng-modulated apoptosis in THUEC with an EGFR-ERK-Bim/Bad signaling cascade. To my knowledge, this is the first report mapping this signaling pathway in male urethral epithelial cells. Both strains conferred anti-apoptotic effects by dampening ERK activation, suggesting that ERK pathway serves as a pro-apoptotic signaling in THUEC.

By studying the *minD* mutant Ng CJSD1 in parallel with the parent strain, I explored the contribution of cell division to gonococcal pathogenesis in THUEC. While Ng CJSD1 infection led to reduced and delayed apoptosis in THUEC during prolonged infection periods compared to the parent strain, the enhanced inhibition of ERK activation, which requires internalization of live bacteria, and unexpected regulation of Bim and Bad by Ng CJSD1 drove me to speculate a mechanism of bacterial cell shape dependent modulation of host cell signaling through the pattern of interaction with the cytoskeleton. This speculation was further supported by a preliminary confocal analysis showing that Ng CJSD1 appeared to induce stronger actin rearrangement than the parent strain. Therefore, both the enhanced ERK inhibition and unexpected regulation of Bim and Bad in Ng CJSD1-infected THUEC are likely due to the abnormal pattern of cytoskeleton rearrangement by Ng CJSD1, and the aberrant shape is a major contributor to this scenario.

Previous reports from our laboratory hypothesized that the reduced adherence to and invasion of THUEC by Ng CJSD1 is likely to be attributable to the aberrant cell shape. The present research provided further evidence of the impact of aberrant cell shape on cell signaling and host response during gonococcal infection. In accordance with the growing body of literature on the contribution of bacterial morphology to pathogenesis, Ng cell division mutants serve as a novel paradigm for studying this aspect in terms of gonococcal infection.

APPENDIX

A1. Apoptotic Gene Expression Profiling in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^R and CJSD1

An apoptosis pathway-focused RT^2 Profiler PCR Array System (SABiosciences, Maryland, USA) was used to examine the expression levels of apoptotic genes in Ng CH811Str^R and Ng CJSD1-infected THUEC according to the manufacturer's instruction. This system profiles the expression of 84 key genes (Appendix A2) involved in apoptosis, which include the TNF ligands and receptors, Bcl-2 family members, caspase, IAP, TRAF, CARD, death domain, death effector domain, and CIDE families; as well as genes involved in the p53 and ATM pathways.

THUEC were infected with Ng CH811Str^R or Ng CJSD1 for 6 h, after which the supernatants were removed and the cell monolayers were washed twice with antibiotic-free PrEGM. Apoptosis induction was performed by further incubating with 1 μM STS for 4 h. Total RNA from infected THUEC was isolated by using the RNeasy Mini Kit (QIAGEN, Mississauga, ON, CA) according to the manufacturer's instructions. Briefly, THUEC grown in 6-well culture plates (~ 0.75×10^6 cells) were lysed and homogenized by loading onto the QIAshredder homogenizers (QIAGEN) and spinning down. Homogenized cell lysates were then loaded onto the RNeasy spin column and genomic DNA was eliminated by on-column DNase digestion using the RNase-Free DNase Set (QIAGEN). Total RNA was eluted with RNase-free water (QIAGEN) and the final concentration was measured by using a NanoDrop ND-1000 Spectrophotometer (Wilmington, DE, USA). cDNA was then synthesized from 1 μg RNA using the RT^2 First Strand Kit (SABiosciences) and mixed with a ready-to-use qPCR Master mixes

containing SYBR Green and fluorescein reference dye. Aliquots of the mixture were dispensed to the primer sets of 84 apoptosis-related genes in each well of the same plate array. Relative gene expression levels were determined by the $\Delta\Delta C_t$ method following the real-time amplification (Schmittgen and Livak, 2008) carried out by the Bio-Rad iCycler detection system with iQ5 optical system software v2.1.

As a preliminary screening, this experiment did not include the non-infected control. Data are expressed as fold change of Ng CJSD1 relative to Ng CH811Str^R infection. Among the 84 apoptotic genes, four genes were downregulated greater than two-fold in Ng CJSD1-infected THUEC relative to Ng CH811Str^R (Table A1). Akt is a serine/threonine-specific protein kinase that is involved in the pro-survival PI3K/Akt pathway. Bcl-xL belongs to the Bcl-2 protein family and acts as an anti-apoptotic member. TNFRSF25 (tumor necrosis factor receptor superfamily member 25) and CD27 (TNFRSF7, tumor necrosis factor receptor superfamily member 7) belong to the TNF-receptor superfamily and have been shown to activate NF-κB signaling (Akiba et al., 1998; Chinnaiyan et al., 1996).

Symbol	Gene	Gene Description	Fold Change (CJSD1/CH811)
AKT1	AKT/PKB	V-akt murine thymoma viral oncogene homolog 1	-2.10
BCL2L1	BCL-XL/S	BCL2-like 1	-2.87
TNFRSF25	APO-3/DDR3	Tumor necrosis factor receptor superfamily, member 25	-2.70
CD27	S152/T14	CD27 molecule	-2.28*

TABLE A1. Apoptotic genes regulated greater than 2-fold in response to *N. gonorrhoeae* strain CJSD1 infection relative to the Ng CH811Str^R infection. THUEC were infected with Ng CH811Str^R or Ng CJSD1 for 6 h followed by 4 hours of apoptosis induction with staurosporine. Total RNA was extracted and synthesized cDNA was submitted to real-time PCR analysis. Negative values indicate fold down-regulation (*the average threshold cycle of this gene is relatively high (>30), meaning that its relative expression level is low in both samples). Data are from only one experiment.

TABLE A2. 84 key apoptotic genes profiled by the qPCR array

Position	UniGene	GenBank	Symbol	Description
A01	Hs.431048	NM_005157	ABL1	V-abl Abelson murine leukemia viral oncogene homolog 1
A02	Hs.525622	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1
A03	Hs.552567	NM_001160	APAF1	Apoptotic peptidase activating factor 1
A04	Hs.370254	NM_004322	BAD	BCL2-antagonist of cell death
A05	Hs.377484	NM_004323	BAG1	BCL2-associated athanogene
A06	Hs.523309	NM_004281	BAG3	BCL2-associated athanogene 3
A07	Hs.194726	NM_004874	BAG4	BCL2-associated athanogene 4
A08	Hs.485139	NM_001188	BAK1	BCL2-antagonist/killer 1
A09	Hs.159428	NM_004324	BAX	BCL2-associated X protein
A10	Hs.193516	NM_003921	BCL10	B-cell CLL/lymphoma 10
A11	Hs.150749	NM_000633	BCL2	B-cell CLL/lymphoma 2
A12	Hs.227817	NM_004049	BCL2A1	BCL2-related protein A1
B01	Hs.516966	NM_138578	BCL2L1	BCL2-like 1
B02	Hs.283672	NM_020396	BCL2L10	BCL2-like 10 (apoptosis facilitator)
B03	Hs.469658	NM_006538	BCL2L11	BCL2-like 11 (apoptosis facilitator)
B04	Hs.410026	NM_004050	BCL2L2	BCL2-like 2
B05	Hs.486542	NM_014739	BCLAF1	BCL2-associated transcription factor 1
B06	Hs.435556	NM_016561	BFAR	Bifunctional apoptosis regulator
B07	Hs.591054	NM_001196	BID	BH3 interacting domain death agonist
B08	Hs.475055	NM_001197	BIK	BCL2-interacting killer (apoptosis-inducing)
B09	Hs.558359	NM_004536	NAIP	NLR family, apoptosis inhibitory protein
B10	Hs.503704	NM_001166	BIRC2	Baculoviral IAP repeat-containing 2
B11	Hs.127799	NM_001165	BIRC3	Baculoviral IAP repeat-containing 3
B12	Hs.356076	NM_001167	BIRC4	Baculoviral IAP repeat-containing 4
C01	Hs.150107	NM_016252	BIRC6	Baculoviral IAP repeat-containing 6 (apollon)
C02	Hs.348263	NM_033341	BIRC8	Baculoviral IAP repeat-containing 8
C03	Hs.145726	NM_001205	BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1
C04	Hs.646490	NM_004330	BNIP2	BCL2/adenovirus E1B 19kDa interacting protein 2
C05	Hs.144873	NM_004052	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
C06	Hs.131226	NM_004331	BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like
C07	Hs.550061	NM_004333	BRAF	V-raf murine sarcoma viral oncogene homolog B1
C08	Hs.405153	NM_006092	NOD1	Nucleotide-binding oligomerization domain containing 1
C09	Hs.200242	NM_032587	CARD6	Caspase recruitment domain family, member 6
C10	Hs.446146	NM_014959	CARD8	Caspase recruitment domain family, member 8
C11	Hs.2490	NM_033292	CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
C12	Hs.5353	NM_001230	CASP10	Caspase 10, apoptosis-related cysteine peptidase
D01	Hs.466057	NM_012114	CASP14	Caspase 14, apoptosis-related cysteine peptidase
D02	Hs.368982	NM_032982	CASP2	Caspase 2, apoptosis-related cysteine peptidase (neural precursor cell expressed, developmentally down-regulated 2)
D03	Hs.141125	NM_004346	CASP3	Caspase 3, apoptosis-related cysteine peptidase
D04	Hs.138378	NM_001225	CASP4	Caspase 4, apoptosis-related cysteine peptidase

D05	Hs.213327	NM_004347	CASP5	Caspase 5, apoptosis-related cysteine peptidase
D06	Hs.389452	NM_032992	CASP6	Caspase 6, apoptosis-related cysteine peptidase
D07	Hs.9216	NM_001227	CASP7	Caspase 7, apoptosis-related cysteine peptidase
D08	Hs.591630	NM_001228	CASP8	Caspase 8, apoptosis-related cysteine peptidase
D09	Hs.329502	NM_001229	CASP9	Caspase 9, apoptosis-related cysteine peptidase
D10	Hs.472860	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5
D11	Hs.592244	NM_000074	CD40LG	CD40 ligand (TNF superfamily, member 5, hyper-IgM syndrome)
D12	Hs.390736	NM_003879	CFLAR	CASP8 and FADD-like apoptosis regulator
E01	Hs.249129	NM_001279	CIDEA	Cell death-inducing DFFA-like effector a
E02	Hs.642693	NM_014430	CIDEB	Cell death-inducing DFFA-like effector b
E03	Hs.38533	NM_003805	CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
E04	Hs.380277	NM_004938	DAPK1	Death-associated protein kinase 1
E05	Hs.484782	NM_004401	DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide
E06	Hs.86131	NM_003824	FADD	Fas (TNFRSF6)-associated via death domain
E07	Hs.244139	NM_000043	FAS	Fas (TNF receptor superfamily, member 6)
E08	Hs.2007	NM_000639	FASLG	Fas ligand (TNF superfamily, member 6)
E09	Hs.80409	NM_001924	GADD45A	Growth arrest and DNA-damage-inducible, alpha
E10	Hs.87247	NM_003806	HRK	Harakiri, BCL2 interacting protein (contains only BH3 domain)
E11	Hs.643120	NM_000875	IGF1R	Insulin-like growth factor 1 receptor
E12	Hs.36	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)
F01	Hs.1116	NM_002342	LTBR	Lymphotoxin beta receptor (TNFR superfamily, member 3)
F02	Hs.632486	NM_021960	MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)
F03	Hs.513667	NM_003946	NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)
F04	Hs.499094	NM_013258	PYCARD	PYD and CARD domain containing
F05	Hs.103755	NM_003821	RIPK2	Receptor-interacting serine-threonine kinase 2
F06	Hs.241570	NM_000594	TNF	Tumor necrosis factor (TNF superfamily, member 2)
F07	Hs.591834	NM_003844	TNFRSF10A	Tumor necrosis factor receptor superfamily, member 10a
F08	Hs.521456	NM_003842	TNFRSF10B	Tumor necrosis factor receptor superfamily, member 10b
F09	Hs.81791	NM_002546	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
F10	Hs.279594	NM_001065	TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
F11	Hs.443577	NM_014452	TNFRSF21	Tumor necrosis factor receptor superfamily, member 21
F12	Hs.462529	NM_003790	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25
G01	Hs.355307	NM_001242	CD27	CD27 molecule
G02	Hs.193418	NM_001561	TNFRSF9	Tumor necrosis factor receptor superfamily, member 9
G03	Hs.478275	NM_003810	TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10

G04	Hs.501497	NM_001252	CD70	CD70 molecule
G05	Hs.494901	NM_001244	TNFSF8	Tumor necrosis factor (ligand) superfamily, member 8
G06	Hs.408312	NM_000546	TP53	Tumor protein p53 (Li-Fraumeni syndrome)
G07	Hs.523968	NM_005426	TP53BP2	Tumor protein p53 binding protein, 2
G08	Hs.192132	NM_005427	TP73	Tumor protein p73
G09	Hs.460996	NM_003789	TRADD	TNFRSF1A-associated via death domain
G10	Hs.522506	NM_021138	TRAF2	TNF receptor-associated factor 2
G11	Hs.510528	NM_003300	TRAF3	TNF receptor-associated factor 3
G12	Hs.8375	NM_004295	TRAF4	TNF receptor-associated factor 4
H01	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H02	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)
H03	Hs.546356	NM_012423	RPL13A	Ribosomal protein L13a
H04	Hs.544577	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H05	Hs.520640	NM_001101	ACTB	Actin, beta
H06	N/A	N/A	HGDC	Human Genomic DNA Contamination
H07	N/A	N/A	RTC	Reverse Transcription Control
H08	N/A	N/A	RTC	Reverse Transcription Control
H09	N/A	N/A	RTC	Reverse Transcription Control
H10	N/A	N/A	PPC	Positive PCR Control
H11	N/A	N/A	PPC	Positive PCR Control
H12	N/A	N/A	PPC	Positive PCR Control

A2. Expression of MMP-9 in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^{R}

In HEC-1B and ME180 cells, Ng microcolonies recruited and activated EGFR to aid bacterial invasion (Swanson et al., 2011). The activation of EGFR was dependent on the shedding of Ng-induced EGFR ligands by matrix metalloproteinase (MMP). MMP-9 expression has been shown to be regulated by the EGFR-ERK pathway in cancer cells (Kim et al., 2009a; Ueno et al., 2011). Infection with Ng CH811Str^{R} increased the expression of MMP-9 (Fig A1, lane 2) compared to the non-infected control (Fig A1, lane 1). Treatment with U0126, AG1748, and cytochalasin D along with infection abolished the induction of MMP-9 expression (Fig A1, lanes 4, 6, and 8), suggesting that MMP-9 expression is dependent on the activation of EGFR/ERK and Ng invasion.

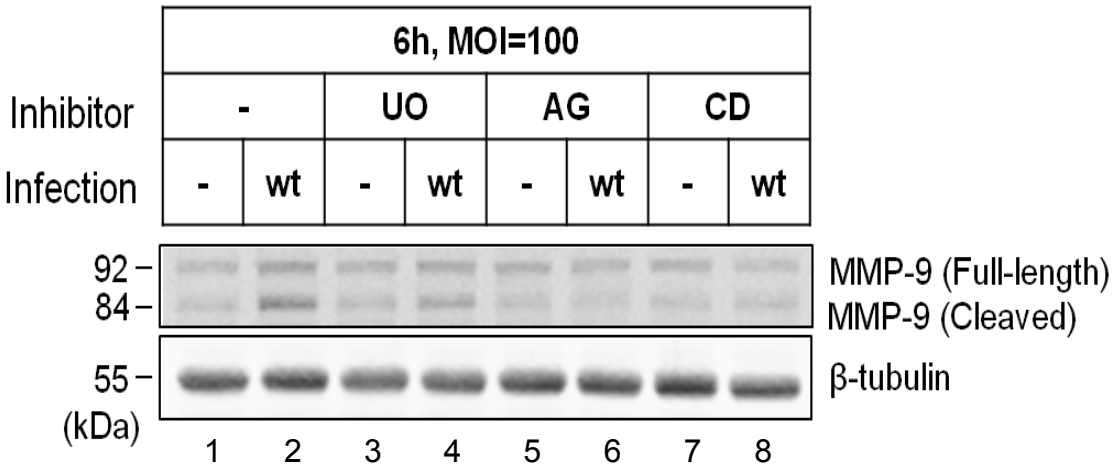

FIGURE A1. Ng CH811Str^{R}-induced expression and activation of matrix metallopeptidase 9 (MMP-9) in THUEC. THUEC were left non-infected or infected with Ng CH811Str^{R} for 6 h with or without inhibitors. Representative immunoblots showing the levels of full-length (proenzyme, 92kDa) and cleaved (active enzyme, 84kDa) MMP-9. β-tubulin served as the internal control. UO-U0126, MEK1/2 inhibitor; AG-AG1478, EGFR inhibitor; CD-Cytochalasin D, inhibitor of actin polymerization.

A3. Effect of Apoptosis Induction on Caspase 3/7 and Caspase 8 Activities in THUEC Infected with *N. gonorrhoeae* Strain CH811Str^R and CJSD1

Ng infection has been shown to protect cells from STS-induced apoptosis (Binnicker et al., 2003; Follows et al., 2009; Howie et al., 2008). To study the cytoprotective effects of Ng CH811Str^R and Ng CJSD1 on THUEC, staurosporine (STS) was used to induce apoptosis. STS induces apoptosis by inhibiting the activity of protein kinases. I examined the cytoprotection conferred by two strains from the perspective of caspase activation. While both strains induced increased activity of caspase 3/7, STS treatment after infection further significantly increased the activation of caspase 3/7, indicating that STS-induced apoptosis in THUEC involves caspase activation. Compared to non-infected cells with STS treatment, STS-treated THUEC infected with CH811Str^R and CJSD1 exhibited significant increase in caspase 3/7 activities. Similarly, infection in the presence of STS further increased the caspase 8 activity, suggesting that the extrinsic apoptotic pathway is involved in the STS-induced apoptosis in THUEC. However, upon STS induction, no difference in caspase activities was observed between Ng CH811Str^R and Ng CJSD1.

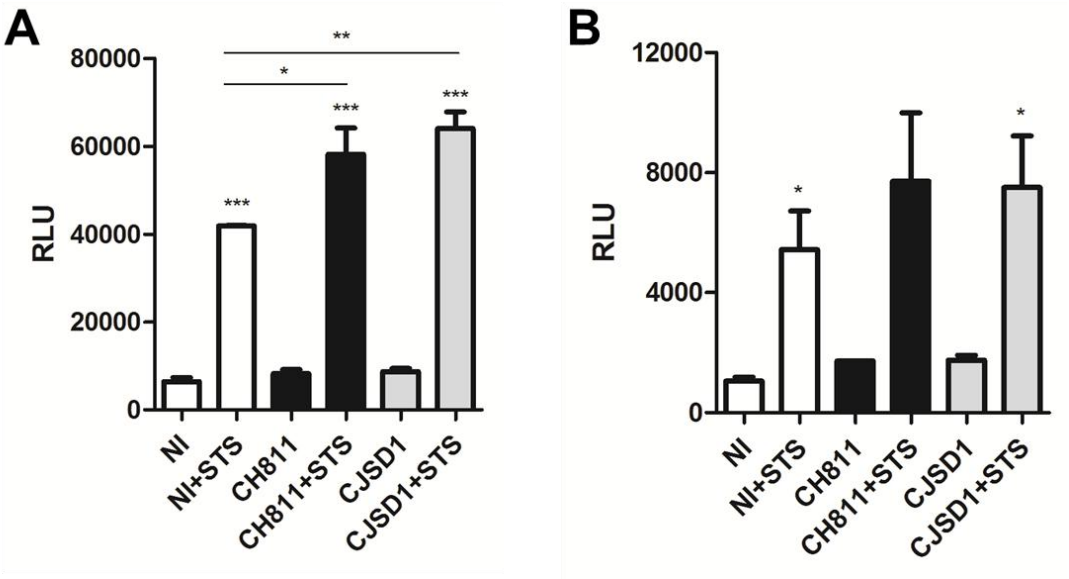

FIGURE A2. Effect of apoptosis induction on caspase 3/7 (A) and caspase 8 (B) activities in THUEC infected with Ng CH811Str^{R} or Ng CJSD1. THUEC were left non-infected, infected with Ng CH811Str^{R} or Ng CJSD1 for 6 h followed by removal of unattached bacteria and further incubation for 4 h with 1 μM staurosporine (STS). Luminescent caspase substrates were added into cell lysates and relative light units (RLU) were measured after 1 h room-temperature incubation in dark. Data show mean ± SD of two independent experiments performed in duplicate. Statistical significance between non-STS treated and STS-treated samples is determined by One-way ANOVA followed by Bonferroni post-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

A4. PCR Confirmation of *N. gonorrhoeae* Strain CJSD1

To verify the insertional inactivation of the *minD* gene in Ng CJSD1 used in this research, PCR was performed to amplify the *minD* fragment containing the chloramphenicol resistance cassette (Cm^R) in Ng CJSD1. Either purified genomic DNA or single colonies were served as templates in the PCR reaction system. As shown in Figure A3, compared to the *minD* fragment amplified from the parent strain Ng CH811Str^R (~ 600 bp), the fragment size amplified from Ng CJSD1 was ~1500 bp, containing the *minD* fragment and the Cm^R cassette (~900 bp). The amplified PCR fragments were further sequenced to confirm the insertion of the Cm^R cassette (data not shown).

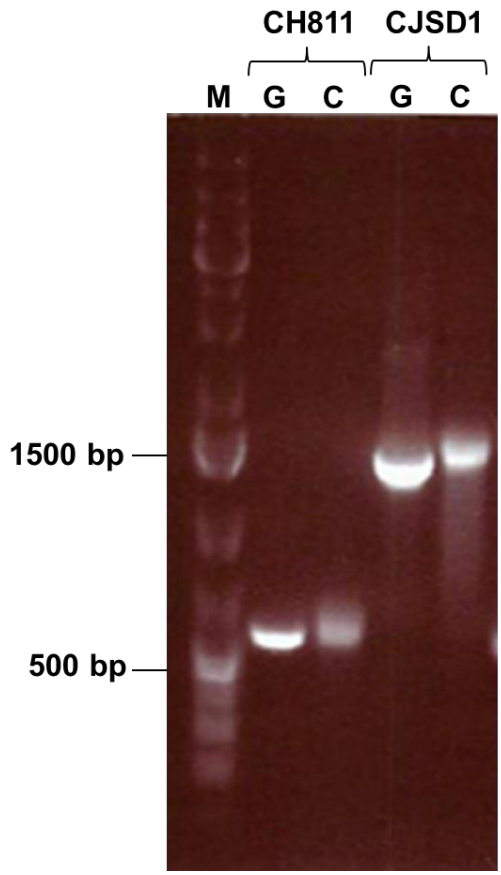

FIGURE A3. PCR confirmation of the *minD* mutant strain Ng CJSD1. The fragment of the *minD* gene in Ng CH811Str^R and Ng CJSD1 was amplified from the purified genomic DNA (G) or colony PCR (C). Overnight cultured gonococci were harvested from the GCMBK plates and the genomic DNA of two strains was extracted using a QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Purified genomic DNA or single colonies were added to the PCR reaction system and the fragment of the *minD* gene was amplified with primers MinD4 and MinD7. This primer set amplified the fragment spanning the chloramphenicol resistance cassette (Cm^R) in Ng CJSD1. M, DNA Marker.

A5. Growth Curves for *N. gonorrhoeae* Strain CH811Str^R and CJSD1

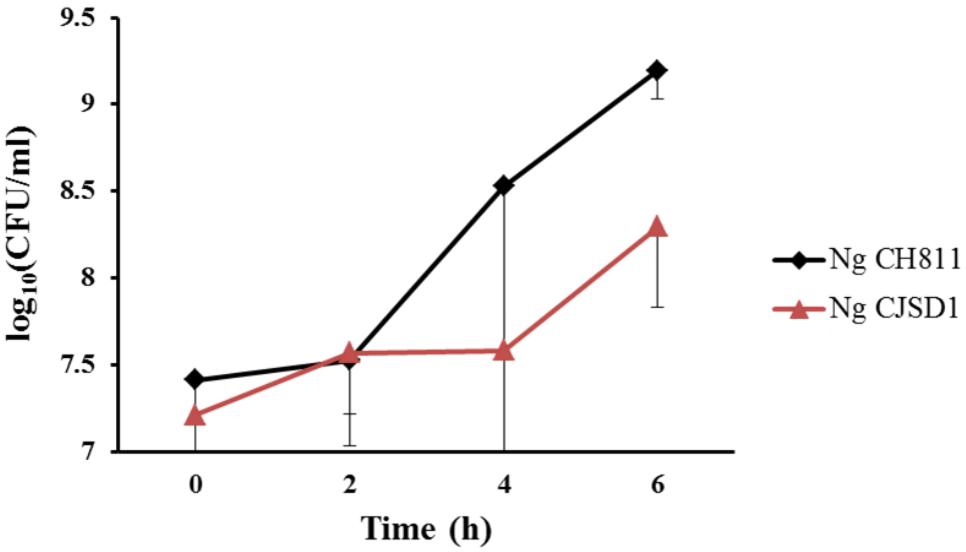

FIGURE A4. *N. gonorrhoeae* growth curve colony-forming units. Overnight grown Ng CH811Str^R and Ng CJSD1 were harvested from the GCMBK plates with a sterile loop and suspended in PrEGM at a concentration of 10^7 CFU/ml. Bacterial suspensions were incubated for 2, 4, 6 h at 37 °C and 5% CO_2 and serial dilutions were plated to enumerate CFU at different time points. Data are expressed as the mean ± SEM of three independent experiments. At each time point, there is no statistically significant difference ($p < 0.05$) between the CFU of each strain (Kruskal-Wallis non-parametric analysis of variance).

TABLE A3. Layout of the 96-well plate used to determine protein concentration

		1 µg	2 µg	5 µg	6 µg	8 µg	10 µg	15 µg				µg/10 µl
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	10/0	9.5/0.5	9/1	7.5/2.5	7/3	6/4	5/5	2.5/7.5	-	-	-	dH_2O/BSA
B	10/0	9.5/0.5	9/1	7.5/2.5	7/3	6/4	5/5	2.5/7.5	-	-	-	dH_2O/BSA
C	8/2	8/2	8/2	8/2	8/2	8/2	8/2	8/2	-	-	-	dH_2O/Sample
D	8/2	8/2	8/2	8/2	8/2	8/2	8/2	8/2	-	-	-	dH_2O/Sample
E	-	-	-	-	-	-	-	-	-	-	-	-
F	-	-	-	-	-	-	-	-	-	-	-	-
G	-	-	-	-	-	-	-	-	-	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

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EDUCATION

- **M.Sc.**, Department of Biology, College of Arts and Science, University of Saskatchewan (U of S), Canada (Sept. 2010 - Nov. 2012). Research topic: Differential regulation of signal transduction pathways in human epithelial cells by *Neisseria gonorrhoeae min* operon mutants (Supervisor: Dr. Jo-Anne R. Dillon).

- **B.Sc.**, **Biological Technology**: School of Life Science, Beijing Institute of Technology (BIT), China (Sept. 2006 - Jul. 2010). Undergraduate research topic: Molecular mechanisms of trinucleotide repeat instability in neurological and neuromuscular disorders (Supervisor: Dr. Xuefeng Pan).

RESEARCH EXPERIENCE

- **Sept. 2010 - Nov. 2012**: M.Sc., Dept. of Biology, U of S, Canada. (Supervisor: Dr. Jo-Anne R. Dillon)
    - M.Sc. thesis: Modulation of apoptosis in human urethral epithelial cells by *Neisseria gonorrhoeae* and a minD mutant strain
        - Studied the pathogenesis of *N. gonorrhoeae* infection in terms of bacteria-host interaction (adherence & invasion), cell signaling (MAP kinases & apoptotic pathways), and host responses (apoptosis & inflammation).
        - Examined the pathogenicity of a *N. gonorrhoeae* cell division gene (*minD*) mutant with an *in vitro* cell culture system, and revealed how this mutant modulates host apoptosis by differentially regulating cell signaling cascades.
    - Practical experience: Tissue culture; immunoblotting; protein/DNA electrophoresis; quantitative PCR; flow cytometry; confocal microscopy; ELISA
    - Relevant courses: Advanced nucleic acids; Advanced topics in cell and molecular biology; molecular basis of microbial pathogenesis

- **Jul. 2009 - Jul. 2010**: Research Assistant, Laboratory of Molecular Biology and Genetics, BIT, China. (Supervisor: Dr. Xuefeng Pan)
    - Undergraduate thesis (equivalent to honors project): Molecular mechanisms of trinucleotide repeat expansion in Fragile X syndrome and gene therapy approach to

Friedreich's ataxia

- Studied the coordination of DNA replication, repair and recombination in the maintenance of genome stability by using *E. coli* as an *in vivo* model, implicated by the expansion or contraction of genome/plasmid-carried CGG·CCG (FXS) and GAA·TTC (FRDA) triplet repeats in *E. coli* mutants defective in the major DNA repair/recombination systems.
- Investigated the application of ODN for gene therapy of FRDA by specific shortening of GAA·TTC repeat size via ODN-mediated homologous recombination.

- Practical experience: Site-specific mutagenesis; P1 transduction; molecular cloning; PCR; DNA electrophoresis; DNA sequencing; bioinformatics

- **Feb. 2009 - Jun. 2009**: Research Assistant, Laboratory of Proteomics, BIT, China. (Supervisor: Dr. Shengyuan Xiao); Intern, Open Laboratory of Chemical Analysis, Agilent Technologies, China. (Supervisor: Dr. Hong Zhang)
  - Research projects: Development of a micro system for volatile amine trapping and determination from tissue and smoke; Detection of chlorpyrifos in market fruits/vegetables
    - Performed quantitative assessment of methylamine in mouse tissues and smoke by FMOC (9-fluorenylmethyl chloroformate-Cl) derivatization followed by HPLC analysis.
    - Developed a GC method for quantitative determination of chlorpyrifos in market fruits and vegetables.
    - Obtained national vocational qualification in chemical investigation, issued by the Ministry of Labor and Social Security, China.
  - Practical experience: Animal experimentation; intensive training in principles and operations of GC, HPLC, and MS for proteomics and food safety applications

TEACHING EXPERIENCE

- **Sept. 2011 - Apr. 2012**: Teaching Assistant, BIOL 120.3 (The Nature of Life), U of S
  - On-site experimental demonstration, lab instruction, and marking

CONFERENCE PRESENTATIONS

1. **G. Liu**, R.P. Parti, J.R. Dillon. 2011. *Neisseria gonorrhoeae* cell division mutants display attenuated virulence during human epithelial cell infection. Poster presentation. Prairie University Biology Symposium (PUBS), Feb 24 - 26, 2011. Saskatoon, Canada.
2. **G. Liu**, R.P. Parti, J.R. Dillon. 2011. *Neisseria gonorrhoeae min* operon gene mutants differentially regulate MAPK and PI3K/Akt pathways during epithelial cell infection. Poster presentation (#6). Biology Graduate Student Symposium - University of Saskatchewan, Apr 1, 2011. Saskatoon, Canada. *Honorable mention
3. **G. Liu**, R.P. Parti, J.R. Dillon. 2011. Differential regulation of MAPK and PI3K/Akt pathways in *Neisseria gonorrhoeae min* operon gene mutants-induced epithelial cell

infection. Poster presentation (#67). Prairie Infectious Immunology Network Meeting (PIIN), Jun 15 - 17, 2011. Russell, Canada.

4. R.P. Parti, D. Biswas, **G. Liu**, A. Cox, J.R. Dillon. 2011. Mutations in *min* operon genes of *Neisseria gonorrhoeae* result in altered virulence. Poster presentation (#70). Prairie Infectious Immunology Network Meeting (PIIN), Jun 15 - 17, 2011. Russell, Canada.
5. **G. Liu**, R.P. Parti, J.R. Dillon. 2012. Infection by a MinD mutant of *Neisseria gonorrhoeae* causes reduced activation of extracellular signal-regulated kinases and differentially regulates host apoptosis in human urethral epithelial cells. Oral presentation. Biology Graduate Student Symposium - University of Saskatchewan, Apr 10, 2012. Saskatoon, Canada.

PUBLICATIONS

1. J. Long, P. Chang, **G. Liu**, X. Pan. 2012. Orientation-dependent propagation of AGG repeats in *Escherichia coli* relies on the RecBCD pathway of homologous recombination. Gene. Under revision
2. **G. Liu**, R.P. Parti, J.R. Dillon. 2012. Inhibition of ERK activation by *Neisseria gonorrhoeae* contributes to the anti-apoptotic effect on human urethral epithelial cells. To be submitted to Infect. Immun.

AWARDS, HONORS & QUALIFICATIONS

- Honorable Mention (Poster Presentation), Biology Graduate Student Symposium, U of S (2011)
- CGSR Scholarship, U of S (2010 - 2011)
- National Vocational Qualification (Certificate #: 0970003006306716), Chemical Investigation, Ministry of Labor and Social Security, PRC (2009)
- Chemical Analysis Engineer - Food Safety (Certificate #: AC11000010), Agilent University (2009)
- Distinct Undergraduate Award , BIT (5 times, 2006 - 2010)

VOLUNTEER WORK

- Receptionist, 2009 Cross-Straits Energy Economics Conference (Oct. 2009)
- Receptionist, 13th International Amine Oxidase and Related Diseases Workshop (Oct. 2008)
- Spectator services, Wukesong Basketball Gymnasium, Beijing Olympics (Aug. 2008)
- Instructional guide, Orangutan House, Beijing Zoo (Apr. 2007 - Apr. 2008)