Distinct Roles of the Antiapoptotic Effectors NleB and NleF from Enteropathogenic *Escherichia coli*

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ABSTRACT During infection, enteropathogenic *Escherichia coli* (EPEC) translocates effector proteins directly into the cytosol of infected enterocytes using a type III secretion system (T3SS). Once inside the host cell, these effector proteins subvert various immune signaling pathways, including death receptor-induced apoptosis. One such effector protein is the non-locus of enterocyte effacement (LEE)-encoded effector NleB1, which inhibits extrinsic apoptotic signaling via the FAS death receptor. NleB1 transfers a single *N*-acetylglucosamine (GlcNAc) residue to Arg₁₁₇ in the death domain of Fas-associated protein with death domain (FADD) and inhibits FAS ligand (FasL)-stimulated caspase-8 cleavage. Another effector secreted by the T3SS is NleF. Previous studies have shown that NleF binds to and inhibits the activity of caspase-4, -8, and -9 *in vitro*. Here, we investigated a role for NleF in the inhibition of FAS signaling and apoptosis during EPEC infection. We show that NleF prevents the cleavage of caspase-8, caspase-3, and receptor-interacting serine/threonine protein kinase 1 (RIPK1) in response to FasL stimulation. When translocated into host cells by the T3SS or expressed ectopically, NleF also blocked FasL-induced cell death. Using the EPEC-like mouse pathogen *Citrobacter rodentium*, we found that NleB but not NleF contributed to colonization of mice in the intestine. Hence, despite their shared ability to block FasL/FAS signaling, NleB and NleF have distinct roles during infection.

KEYWORDS NleB1, NleF, EPEC, FasL signaling, caspase inhibitor, cell death

During infection, the extracellular enteric bacterial pathogens enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and *Citrobacter rodentium* utilize a type III secretion system (T3SS) to translocate virulence (effector) proteins directly into the cytosol of infected enterocytes (1). The genes encoding the T3SS, as well as several of these effectors, are encoded on the locus of enterocyte effacement (LEE) pathogenicity island and are required for the formation of intestinal attaching/effacing (A/E) lesions that are characteristic of these pathogens (2). A/E lesions are characterized by intimate attachment of the bacteria to the surface of the host cell and cytoskeletal rearrangements leading to pedestal formation (3, 4).

EPEC, EHEC, and *C. rodentium* also translocate many effector proteins encoded outside the LEE, termed non-LEE-encoded (Nle) effectors (5). Several of these effectors are involved in suppressing the host innate immune response by subverting various inflammatory pathways, including NF- κ B, mitogen-activated protein kinase (MAPK), and FAS signaling (6, 7). For example, the EPEC effector protein NleE is a cysteine methyltransferase that inhibits NF- κ B signaling by modifying host adapter proteins TAB2 and TAB3, preventing their recruitment to tumor necrosis factor (TNF)-stimulated signaling complexes (8–10). NleC is a zinc metalloprotease that also functions to inhibit inflammatory signaling by directly cleaving the NF- κ B subunit p65 (11–14). Together,

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these effectors synergistically reduce the production of inflammatory cytokines, such as interleukin-8 (IL-8), produced in response to EPEC infection.

The EPEC effector protein NleB1 subverts Fas ligand (FasL)-induced apoptosis (15). Upon engagement of FAS with FasL, receptor multimerization occurs, and the death domain (DD)-containing adapter protein Fas-associated protein with death domain (FADD) is recruited through heterotypic DD interactions. Procaspase-8 is then recruited to the complex via homotypic death effector domain (DED) interactions with FADD to form the death-inducing signaling complex (DISC). At the DISC, caspase-8 undergoes dimerization and autoproteolytic processing. Active caspase-8 acts on a range of cytosolic substrates, including receptor-interacting serine/threonine protein kinase 1 (RIPK1) and caspase-3, an executioner caspase of apoptosis (16).

NleB1 was recently identified as a novel glycosyltransferase that modifies a conserved arginine residue in death domain-containing host proteins, including FADD, tumor necrosis factor receptor type 1-associated death domain protein (TRADD), and RIPK1. Inhibition of FasL-induced apoptosis by NleB1 is mediated by the transfer of a single *N*-acetylglucosamine (GlcNAc) sugar moiety to Arg₁₁₇ in the death domain of FADD. This modification renders FADD unable to interact with FAS, thereby preventing assembly of the DISC and caspase-8 cleavage. The enzymatic activity of NleB1 depends on a DXD_{221–223} catalytic motif, as well as Tyr₂₁₉ and Glu₂₅₃ (15, 17–19). The importance of FAS signaling in the immune response to *C. rodentium* infection was demonstrated *in vivo*, where C57BL/6 mice deficient in either FasL or FAS were unable to efficiently clear infection (15).

Another effector secreted by the T3SS of A/E pathogens is NleF. The amino acid sequence of NleF is 100% conserved between EPEC O127:H6 (strain E2348/69) and EHEC O157:H7 (strains EDL933 and Sakai), and this sequence is 85% identical to that of NleF from *C. rodentium* (20, 21). Caspase-9 was initially identified as an interacting partner of NleF in a yeast-2-hybrid (Y2H) screen (22). Analysis of the crystal structure of NleF bound to caspase-9 revealed that the four C-terminal residues of NleF (Leu₁₈₆, Gln₁₈₇, Cys₁₈₈, and Gly₁₈₉; referred to as the LQCG motif) occupied the active caspase-9 catalytic pocket. Further analysis of NleF and various caspases identified caspase-4 and caspase-8 as additional NleF interacting partners. The ability of these caspases to cleave synthetic substrates was inhibited in the presence of purified NleF (22). A further study confirmed the interaction of NleF with caspase-4 and the murine orthologue caspase-11 and subsequently demonstrated that this interaction led to the inhibition of IL-18 maturation and secretion from infected intestinal epithelial cells (IECs) (23).

Given the importance of FAS signaling in gut immune defense and the ability of NleF to inhibit caspase-8 activity, we investigated the importance of direct caspase-8 inhibition by NleF to EPEC pathogenesis. Here, we show that the effector protein NleF can inhibit FasL-induced caspase-8 cleavage and subsequent cell death. We demonstrate that during EPEC infection, NleB1 and NleF have complementary activities and can act synergistically to promote cell survival. However, *in vivo* experiments using *C. rodentium* suggested that the roles of NleB and NleF were distinct rather than synergistic.

RESULTS

Ectopic expression of NleB1 or NleF inhibits Fas signaling. Previously, NleF was shown to inhibit caspase-8 activity (22). In order to compare the roles of the EPEC effector proteins NleB1 and NleF in the inhibition of FasL-induced cell death, lentiviral transduction was employed to create HeLa cell lines that stably express Flag-NleB1, an NleB1 catalytic mutant (Flag-NleB1_{DXD}), Flag-NleF, or an NleF truncation mutant (Flag-NleF_{ΔLQCG}) under the control of a doxycycline-dependent promoter. Expression was assessed by immunoblotting over a 24-h period, and robust doxycycline induction of the Flag-tagged fusion proteins was observed (Fig. 1A). A high-molecular-mass product was detected upon the expression of Flag-NleF, as observed previously (Fig. 1A) (23).

FIG 1 Caspase activation in the presence of NleF. (A) Time course of Flag-NleB, Flag-NleB_{DXD}, Flag-NleF, or Flag-NleF_{ΔLQCG} induction in HeLa cell lines. Cells were treated with doxycycline for the indicated times, and the Flag epitope tag was detected by immunoblotting using anti-Flag antibodies. β-Actin was used as a loading control. A representative immunoblot from at least three independent experiments is shown. (B) Representative immunoblot of recombinant HeLa cell lines left untreated or treated with doxycycline to induce Flag-NleB, Flag-NleB_{DXD}, Flag-NleF, or Flag-NleF_{ΔLQCG} expression and FasL to induce caspase-8 cleavage. Epitope (Flag)-tagged-effector expression was detected using anti-Flag antibodies. Anti-caspase-8 antibodies were used to detect procaspase-8 and cleaved caspase-8 fragments (p43/41 and p18). Cleaved caspase-3 fragments (p17/19) were detected using anti-cleaved caspase-3 antibodies. RIPK1 and cleaved RIPK1 products were detected using anti-RIPK1 antibodies. Anti-β-actin Ab was used as a loading control. An immunoblot representative of at least three independent experiments is shown.

To test for inhibition of FAS signaling, the expression of Flag-NleB1, Flag-NleB1_{DXD}, Flag-NleF, and Flag-NleF_{ΔLQCG} was induced in the cell lines for 24 h before stimulation with FasL. As determined by immunoblot analysis, the expression of Flag-NleB1 but not the catalytically inactive form (Flag-NleB1_{DXD}) inhibited FasL-induced caspase-8,

FIG 2 FasL-induced cell death in the presence of NleF. (A) Confocal laser scanning microscopy showing recombinant HeLa cell lines treated with doxycycline to induce Flag-NleB, Flag-NleB_{DXD}, Flag-NleF, or Flag-NleF_{ΔLQCG} expression and FasL to induce cell death. Dead cells were stained with propidium iodide (PI) (red), and nuclei were stained with Hoechst (blue). Scale bar is 100 μm. Representative images from at least 3 independent experiments are shown. (B) Quantification by fluorescence microscopy of PI-positive recombinant HeLa cell lines used in the experiment whose results are shown in panel A, left untreated or treated with doxycycline and FasL. Results are the mean values ± standard errors of the means (SEM) for percentages of PI-positive cells out of total cells from three independent experiments in which ~60 cells in three distinct fields were counted. P values are from unpaired t test. ns, not significant.

caspase-3, and RIPK1 cleavage (Fig. 1B). NleF also inhibited the cleavage of caspase-8, caspase-3, and RIPK1, but the expression of NleF_{ΔLQCG} had no effect on FasL-induced cleavage of any of these host proteins (Fig. 1B). NleB1 and NleF, but not their inactive mutants, also inhibited cell death, as measured by propidium iodide (PI) uptake compared to the PI uptake in HeLa cells treated with FasL alone (Fig. 2A and B). Untreated HeLa cells displayed a low level of PI staining, which was unaffected by the addition of doxycycline (Fig. 2A and B).

NleB1 and NleF have complementary activities during EPEC infection. In order to observe a functional role for NleF during infection, HeLa cells were infected with various EPEC derivatives prior to stimulation with FasL. As with previous experiments, caspase activity was measured by immunoblotting and cell death was measured by PI staining. Consistent with previous findings (15), cells infected with wild-type EPEC were protected from caspase-8 and caspase-3 processing in response to FasL (Fig. 3). Neither the T3SS mutant (Δ escN) nor the *nleB1* mutant (Δ nleB1) was able to block caspase cleavage in response to FasL (Fig. 3), and as expected, this phenotype was restored upon complementation of the Δ nleB1 mutant with a plasmid encoding *nleB1* (pNleB1) but not with a plasmid encoding catalytically inactive *nleB1* (pNleB1_{DXD}). Cells infected

FIG 3 Caspase activation during EPEC infection *in vitro*. Representative immunoblot showing procaspase-8, cleaved caspase-8 fragments (p43/41 and p18), and cleaved caspase-3 fragments (p19/17) in HeLa cells infected with the indicated EPEC derivatives and stimulated with FasL for 1.5 h after infection. β -Actin was used as a loading control. An immunoblot representative of at least three independent experiments is shown.

with the NleF deletion mutant ($\Delta nleF$) showed a modest increase in caspase processing compared to the level in wild-type EPEC-infected cells (Fig. 3), and this was restored to wild-type levels with the reintroduction of full-length NleF but not the truncated NleF $_{\Delta LQCG}$ (Fig. 3).

To compare the relative contributions of NleB1 and NleF to inhibition of FasL-induced cell death, we constructed an *nleB1 nleF* double mutant ($\Delta nleB1 \Delta nleF$). HeLa cells infected with the $\Delta nleB1 \Delta nleF$ mutant displayed significant increases in caspase-8 and -3 cleavage upon FasL stimulation (Fig. 3), and this phenotype was complemented by the reintroduction of either *nleB1* [$\Delta nleB1 \Delta nleF$ (pNleB1)] or *nleF* [$\Delta nleB1 \Delta nleF$ (pNleF)] (Fig. 3) but not their inactive mutants [$\Delta nleB1 \Delta nleF$ (pNleB1 $_{DXD}$) and $\Delta nleB1 \Delta nleF$ (pNleF $_{\Delta LQCG}$)]. In addition, we examined cell death as determined by the PI uptake of cells infected with derivatives of EPEC E2348/69. As expected, cells infected with wild-type EPEC were protected from FasL-induced cell death compared to the FasL-induced cell death in uninfected cells or cells infected with the $\Delta escN$ mutant (Fig. 4A and B). In contrast, a substantial increase in PI uptake was observed in cells infected with the $\Delta nleB1 \Delta nleF$ mutant and treated with FasL (Fig. 4A and B). Reintroduction of either *nleB1* or *nleF* to the $\Delta nleB1 \Delta nleF$ mutant restored protection from FasL-induced cell death (Fig. 4A and B), which was consistent with the inhibition of caspase cleavage observed by immunoblotting (Fig. 3). Complementation of the $\Delta nleB1 \Delta nleF$ mutant with either *nleB1* $_{DXD}$ or *nleF* $_{\Delta LQCG}$ was insufficient to restore inhibition of FasL-induced cell death (Fig. 4A and B).

Distinct roles of NleB and NleF during *C. rodentium* infection. Previous work has shown that *nleB* is required for full colonization of mice by *C. rodentium* (24). Given the overlapping functions of NleB and NleF that we observed during EPEC infection *in vitro*, we hypothesized that a *C. rodentium* double *nleB nleF* mutant would be more attenuated for colonization than a single *nleB* mutant. To test this, C57BL/6 mice were orally infected with *C. rodentium* ICC169 and its $\Delta nleB$ and $\Delta nleB \Delta nleF$ derivatives. Fecal samples were collected over 18 days, and viable bacteria were measured by counting CFU per gram of feces. As expected, the *nleB* mutant of *C. rodentium* ($\Delta nleB$ mutant) was attenuated for colonization compared to wild-type *C. rodentium* ICC169 on days 8, 10, 12, 14, and 16 postinfection (Fig. 5). However, the additional deletion of *nleF* ($\Delta nleB \Delta nleF$ double mutant) did not increase this colonization defect (Fig. 5). Indeed, some-

FIG 4 FasL-induced cell death during EPEC infection. (A) Confocal laser scanning microscopy showing HeLa cells infected with EPEC derivatives and then treated with FasL for 1.5 h after infection. Cells positive for propidium iodide (PI) appear red, and nuclei stained with Hoechst appear blue. Scale bar is 100 μ m. (B) Quantification by fluorescence microscopy of PI-positive cells, comparing samples left untreated and treated with FasL. Results are the mean values $\pm$ SEM for percentages of PI-positive cells out of total cells from three independent experiments in which $\sim$ 60 cells in three distinct fields per sample were counted. *P* values are from unpaired *t* test comparing the results for all FasL-treated samples to the results for cells infected with EPEC E2348/69 and treated with FasL. ***, *P* < 0.001; ****, *P* < 0.0001; ns, not significant.

what surprisingly, the Δ nleB Δ nleF mutant was less attenuated than the Δ nleB mutant for up to 14 days after infection, whereas the single Δ nleF mutant was not attenuated for colonization at any time point during infection (Fig. 5). To test for possible redundancy in the functions of NleB and NleF *in vivo*, either *nleB* or *nleF* was reintroduced into the Δ nleB Δ nleF mutant by integration into the *attTn7* site in the *C. rodentium* genome. Gene expression was under the control of the *s12* promoter. The reintroduction of *nleB* but not *nleF* restored the bacterial load to wild-type levels (Fig. 5), suggesting that the effector proteins have distinct rather than overlapping functions *in vivo*.

DISCUSSION

Apoptotic cell death is an important defense against many invading bacterial pathogens. EPEC-infected cells are protected from apoptotic cell death by several T3SS

FIG 5 Relative contributions of *nleB* and *nleF* to colonization of mice by *C. rodentium*. Bacterial loads in the feces of C57BL/6 mice infected with the indicated derivatives of *C. rodentium* are shown. Each data point represents the log₁₀ CFU per gram of feces per individual animal on day 4, 8, 10, 12, 14, 16, or 18 after infection. Mean values $\pm$ SEM are indicated; dotted line represents limit of detection. WT, wild-type *C. rodentium*. *P* values were determined by Mann-Whitney *U* test. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001.

effectors, including NleB1 and NleF. Previously, NleB1 from EPEC was shown to inhibit extrinsic apoptosis by modifying the adapter protein FADD, which is essential for FasL-induced formation of the DISC (15). Here, we investigated the role of NleF in the inhibition of FasL-induced cell death and demonstrated that, in addition to NleB1, NleF can inhibit caspase activity in FasL-treated apoptotic cells. When delivered into cells by the T3SS or when expressed ectopically, NleF reduced the amounts of cleaved caspase-8 and caspase-3 in epithelial cells treated with FasL. As a result, these cells were protected from cell death. We demonstrated that the C-terminal truncation of NleF (NleF_{ΔLQCG}) resulted in a loss of antiapoptotic activity, which is consistent with previous findings describing the C-terminal motif of NleF as essential for blocking caspase substrate binding (22).

We found that NleF inhibited the cleavage of caspase-8, as well as the caspase-8 substrates caspase-3 and RIPK1, implying that NleF contributes to the inhibition of extrinsic apoptosis, similar to NleB1. The proapoptotic BH3-only protein, Bid, is also cleaved by caspase-8, and this leads to mitochondrial damage, subsequent cytochrome *c* release, and the activation of caspase-9, leading to further activation of downstream caspases caspase-3 and -7 (16). Given that NleF was previously shown to inhibit caspase-9 activity (22), it is possible NleF acts on both caspase-8 and caspase-9 during FAS signaling to inhibit apoptosis.

NleF was initially reported to be important to *C. rodentium* colonization in C57BL/6 mice, as a *C. rodentium* Δ *nleF* mutant showed reduced colonization fitness compared to that of the wild-type strain in mixed infections (20). However, in contrast to these initial reports, we and others have shown that *nleF* mutants of *C. rodentium* are not defective for colonization compared to wild-type bacteria (23).

In terms of bacterial load, the double Δ *nleB* Δ *nleF* mutant appeared to have some increased fitness compared to the single Δ *nleB* mutant, which may result from dysregulated NF- κ B signaling. Recently, increased secretion of the proinflammatory chemokine CXCL1 was observed in the colons of mice infected with an *nleF* mutant of *C. rodentium* complemented with NleF (23). In addition, ectopic expression of NleF led to increased transcription of an NF- κ B-dependent reporter, as well as an increase in IL-8 mRNA expression. While this activity was dependent on the LQCG motif of NleF, it occurred independently of caspase involvement (25). Although both NleF and NleB1 can block FasL/FAS signaling, their distinct roles *in vivo* may be due to their ability to target other innate immune signaling pathways either directly or indirectly. For example, NleB1 modifies the death domain (DD)-containing proteins TRADD and RIPK1 to

TABLE 1 Bacterial strains used in this study

Strain	Characteristics	Reference or source
<i>Escherichia coli</i>		
EPEC E2348/69	Wild-type EPEC O127:H6	31
Δ escN mutant	E2348/69 Δ escN::Kan (Kan ^r)	32
Δ nleB1 mutant	E2348/69 Δ nleB1::Cm (Cm ^r)	15
Δ nleF mutant	E2348/69 Δ nleF::Kan (Kan ^r)	25
Δ nleB1 Δ nleF mutant	E2348/69 Δ nleB1::Cm Δ nleF::Kan (Cm ^r Kan ^r)	This study
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[F'proAB lacIqZΔM15 Tn10 (TetR)]</i>	Stratagene
<i>Citrobacter rodentium</i>		
ICC169	Spontaneous Nal ^r derivative of wild-type <i>C. rodentium</i> biotype 4280	33
Δ nleB mutant	ICC169 Δ nleB::Kan (Nal ^r Kan ^r)	15
Δ nleF mutant	ICC169 Δ nleF::Kan (Nal ^r Kan ^r)	23
Δ nleB Δ nleF mutant	ICC169 Δ nleB::Kan Δ nleF::Cm (Nal ^r Kan ^r Cm ^r)	This study
Δ nleB/nleF (<i>nleB</i>) mutant	ICC169 Δ nleB::Kan Δ nleF::Cm (Nal ^r Kan ^r Cm ^r) with <i>s12-nleB</i> transgene insertion at <i>attTn7</i>	This study
Δ nleB/nleF (<i>nleF</i>) mutant	ICC169 Δ nleB::Kan Δ nleF::Cm (Nal ^r Kan ^r Cm ^r) with <i>s12-nleF</i> transgene insertion at <i>attTn7</i>	This study

subvert TNF-induced NF- κ B activity and necroptotic cell death (18), and NleF inhibits other caspases (22). In addition, different cellular levels of effector proteins will influence their efficacy. It is possible that introducing higher levels of NleF expression in *C. rodentium* could compensate for the lack of NleB.

Caspase inhibition by bacterial effector proteins is not unique to NleF. Effector proteins of both *Shigella* spp. and *Yersinia* spp. have similar reported activities. The effector protein OspC3 from *S. flexneri* interacts with active caspase-4 and inhibits caspase-4-dependent inflammatory cell death (26). The *Yersinia* type III effector YopM binds caspase-1, the activity of which is dependent on a motif on an exposed loop that is similar to the caspase-1 substrate YVAD. Despite possessing a motif similar to a caspase-1 cleavage motif, YopM itself is not cleaved by caspase-1, suggesting that it acts as a pseudosubstrate (27).

Direct binding of lipopolysaccharide (LPS) to human caspase-4 or murine caspase-11 activates the noncanonical inflammasome, resulting in the production of inflammatory cytokines IL-18 and IL-1 β , as well as pyroptotic cell death (28, 29). EPEC infection of human IECs activates this noncanonical inflammasome (30), and recently, the expression of NleF was shown to dampen this innate response by binding caspase-4, resulting in a decrease of IL-18 secretion from infected cells (23). Furthermore, NleF of *C. rodentium* binds murine caspase-11 to inhibit IL-18 secretion from colonic explants at an early time point during *C. rodentium* infection, resulting in decreased neutrophil recruitment to the colon (23). Taken together, we conclude that NleF functions as a caspase inhibitor to dampen apoptosis and inflammasome activation during infection with attaching and effacing pathogens and that NleB1 is the major effector blocking the FasL/FAS signaling pathway *in vivo*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth or Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) at 37°C, in the presence of ampicillin (100 μ g/ml), kanamycin (100 μ g/ml), chloramphenicol (25 μ g/ml), or nalidixic acid (50 μ g/ml) when required.

DNA cloning and purification. The plasmids and primers used in this study are listed in Table 2 and Table 3, respectively. DNA-modifying enzymes were used in accordance with the manufacturer's instructions (New England Biolabs). Plasmids were extracted using the AxyPrep plasmid miniprep kit. PCR products and restriction digests were purified using the Wizard SV gel and PCR clean-up system (Promega). To construct the complementation vectors, *nleF* was amplified from pEGFP-NleF (25) using primer pair NleF_{F1}/NleF_{R1} or NleF_{F1}/NleF_{R2}. PCR products were digested with EcoRI and BamHI and ligated into pTrc99A to create pTrc99A-NleF and pTrc99A-NleF _{Δ LQC}. To construct N-terminal Flag fusions to

TABLE 2 Plasmids used in this study

Plasmid	Characteristics ^a	Reference or source
pEGFP-NleF	<i>nleF</i> from EPEC E2348/69 in pEGFP-C2, Kan ^r	25
pTrc99A	Bacterial expression vector with inducible <i>lacI</i> promoter, Amp ^r	Pharmacia Biotech
pTrc99A-NleF	<i>nleF</i> from EPEC E2348/69 in pTrc99A, Amp ^r	This study
pTrc99A-NleF _{ΔLQCG}	<i>nleF</i> from EPEC E2348/69 in pTrc99A with LQCG _(186–189) motif deleted, Amp ^r	This study
pTrc99A-NleB1	<i>nleB1</i> from EPEC E2348/69 in pTrc99A, Amp ^r	9
pTrc99A-NleB1 _{DXD}	<i>nleB1</i> from EPEC E2348/69 in pTrc99A with DXD _(221–223) motif mutated to AAA, Amp ^r	15
p3XFlag-Myc-CMV-24	Dually tagged N-terminal Met-3XFlag and C-terminal c-myc expression vector, Kan ^r	Sigma-Aldrich
pFlag-NleF	<i>nleF</i> from EPEC E2348/69 in p3XFlag-Myc-CMV-24, Kan ^r	This study
pFlag-NleB1	<i>nleB</i> from EPEC E2348/69 in p3XFlag-Myc-CMV-24, Kan ^r	This study
pFlag-NleB1 _{DXD}	<i>nleB</i> from EPEC E2348/69 in p3XFlag-Myc-CMV-24 with DXD _(221–223) motif mutated to AAA, Kan ^r	This study
pF_TRE3G_PGK puro	Lentiviral transduction vector with Tet-responsive element, Amp ^r	34
pF_TRE-Flag-NleF	<i>nleF</i> from EPEC E2348/69 with N-terminal 3XFlag tag in pF_TRE3G_PGK puro, Amp ^r	This study
pF_TRE-Flag-NleF _{ΔLQCG}	<i>nleF</i> from EPEC E2348/69 with N-terminal 3XFlag tag and LQCG _(186–189) motif deleted in pF_TRE3G_PGK puro, Amp ^r	This study
pF_TRE-Flag-NleB1	<i>nleB1</i> from EPEC E2348/69 with N-terminal 3XFlag tag in pF_TRE3G_PGK puro, Amp ^r	This study
pF_TRE-Flag-NleB1 _{DXD}	<i>nleB1</i> from EPEC E2348/69 with N-terminal 3XFlag tag and DXD _(221–223) motif mutated to AAA in pF_TRE3G_PGK puro, Amp ^r	This study
pCMV-VSV-G	Mammalian expression vector encoding VSV-G glycoprotein, Amp ^r	John Silke
pCMVΔR8.2	Mammalian expression vector encoding HIV-1 Gag/Pol, Tat, and Rev, Amp ^r	John Silke
pKD46	Red recombinase helper plasmid, Amp ^r	35
pKD3	Red recombinase helper plasmid, Cm ^r Amp ^r	35
pKD4	Red recombinase helper plasmid, Kan ^r Amp ^r	35
pCR-Blunt II-TOPO	Cloning vector for blunt-end ligation, Kan ^r	Thermo Fisher Scientific
pCR-Blunt II-TOPO-NleB _{CR}	<i>nleB</i> from <i>C. rodentium</i> ICC169 in pCR-Blunt II-TOPO with ribosomal <i>s12</i> promoter and native NleB RBS, Kan ^r	This study
pCR-Blunt II-TOPO-NleF _{CR}	<i>nleF</i> from <i>C. rodentium</i> ICC169 in pCR-Blunt II-TOPO with <i>s12</i> promoter and RBS, Kan ^r	This study
pGRG36	Tn7 transgene insertion vector, Amp ^r	36
pGRG36-NleB _{CR}	<i>nleB</i> from <i>C. rodentium</i> ICC169 in pGRG36 with <i>s12</i> promoter and native NleB RBS, Amp ^r	This study
pGRG36-NleF _{CR}	<i>nleF</i> from <i>C. rodentium</i> ICC169 in pGRG36 with <i>s12</i> promoter and RBS, Amp ^r	This study

^aVSV, vesicular stomatitis virus; RBS, ribosome binding site.

NleB1 and NleF, the *nleB1* and *nleF* genes were amplified from EPEC E2348/69 genomic DNA (25) using primer pairs NleB1_{F1}/NleB1_{R1} and NleF_{F2}/NleF_{R1}, respectively. The PCR products were digested with EcoRI and BamHI and ligated into p3XFlag-Myc-CMV-24 to create pFlag-NleB1 or pFlag-NleF. The NleB1 catalytic mutant, pFlag-NleB1_{DXD}, was generated using the Stratagene QuikChange II site-directed mutagenesis kit. pFlag-NleB1 was used as the template DNA and amplified using the primer pair NleB1_{(DXD)F}/NleB1_{(DXD)R}. Plasmids were digested with DpnI at 37°C overnight before subsequent transformation into XL-1 Blue *E. coli*.

To construct the lentiviral transduction vectors, Flag fusions to NleB1 or NleB1_{DXD} were amplified from pFlag-NleB1 or pFlag-NleB1_{DXD} using the primer pair Flag_F/NleB1_{R2}. The Flag fusion to NleF was amplified from pFlag-NleF with primer pair Flag_F/NleF_{R3} or Flag_F/NleF_{R4}. PCR products were digested with BamHI and NheI and ligated into pF_TRE to create pF_TRE-Flag-NleB1, pF_TRE-Flag-NleB1_{DXD}, pF_TRE-Flag-NleF, and pF_TRE-Flag-NleF_{ΔLQCG}.

To construct transgene insertion vectors, *nleB_{CR}* and *nleF_{CR}* were amplified from *C. rodentium* ICC169 genomic DNA using primer pairs NleB_{F2}/NleB_{R3} and NleF_{F3}/NleF_{R5}, respectively. The *s12* promoter was amplified from *C. rodentium* ICC169 genomic DNA using primer pair S12_{F1}/S12_{R1} or S12_{F1}/S12_{R2}, before being annealed to a complementary region of the *nleB_{CR}* or *nleF_{CR}* amplification product. Annealed products were ligated into pCR-Blunt II-TOPO using the Zero Blunt TOPO kit (Thermo Fisher Scientific) to create pCR-Blunt II-TOPO-NleB_{CR} or pCR-Blunt II-TOPO-NleF_{CR}. *s12* promoter-gene fusions were then digested with XmaI and XhoI and ligated into pGRG36 to create pGRG36-NleB_{CR} and pGRG36-NleF_{CR}.

Construction of EPEC and *C. rodentium* mutants. The EPEC E2348/69 *ΔnleB1 ΔnleF* double mutant was created using the lambda Red mutagenesis system (35). Using genomic DNA from EPEC E2348/69, PCR was used to amplify regions from the 5' and 3' flanking sites of *nleF* using primer pairs NleF_{EP_1}/NleF_{EP_2} and NleF_{EP_3}/NleF_{EP_4}. The plasmid pKD4 was used as the template DNA to amplify the kanamycin resistance cassette using primer pair pKD_F/pKD_R. Overlapping PCR was used to assemble the linear fragment consisting of the kanamycin resistance cassette and the 5' and 3' *nleF* flanking regions using primer pair NleF_{EP_1}/NleF_{EP_4}. This fragment was then electroporated into a previously described *ΔnleB1* mutant of EPEC E2348/69 (15) containing the lambda Red helper plasmid pKD46, following the expression of lambda Red recombinase with 10 mM L-arabinose. The deletion of *nleF* was confirmed by PCR and DNA sequencing using the external primer pair NleF_{EP_1}/NleF_{EP_4}.

To construct the *C. rodentium* ICC169 *ΔnleB ΔnleF* double mutant, PCR was used to amplify regions from the 5' and 3' flanking sites of *nleF* from *C. rodentium* ICC169 using primer pairs NleF_{CR_1}/NleF_{CR_2} and NleF_{CR_3}/NleF_{CR_4}. The plasmid pKD3 was used as the template DNA to amplify the chloramphenicol

TABLE 3 Oligonucleotide primers used in this study

Primer	Sequence (5'–3')
NleF _{F1}	AAGAATTCATGTTACCAACAAGTGGTCT
NleF _{F2}	CCCGAATTCATGTTACCAACAAGTGGTTC
NleF _{F3}	ATGTTACCAGCTAGTAGC
NleF _{R1}	GCGGTGGATCCCTCATCCACATTGTAA AGATCC
NleF _{R2}	GGGGGATCCTCAAGATCCTTTGTTGTAAG
NleF _{R3}	GGGGCTAGCTCATCCACATTGTAAAGATCC
NleF _{R4}	GGGGCTAGCTCAAGATCCTTTGTTGTAAG
NleF _{R5}	ATCCTCGAGCTATCCAATTTGCAAAGATC
Flag _F	CAGGATCCATGGACTACAAAGACCATGACGGTG
NleB1 _{F1}	AAGCGAAT TCAATGTTATCTTCATTAAATGTCC
NleB1 _{F2}	GTGTTTACGAAGCAAAAGCTTAGTCTAAGGGGAATCTT
NleB1 _{R1}	GCGGTGGATCCCTTACCATGAACTGCTGGTATAC
NleB1 _{R2}	GGGGCTAGCTTACCATGAACTGCTGG
NleB1 _{R3}	ATCCTCGAGTTACCATGAACTGTTGGTAT
NleB1 _{(DXD)F}	GCGGATGTATATATCTTGCTGCCGCGCATGATTATTACCG
NleB1 _{(DXD)R}	CGGTAATAATCATGGCGGCAGCAAGATATATACATCCGC
NleF _{EP_1}	GGTTCGGGGAGTGCAGAGAAA
NleF _{EP_2}	GAAGCAGCTCCAGCCTACACAAAAACCCCTTAACAAATAAATCC
NleF _{EP_3}	CTAAGGAGGATATTCATATGATTAGAGGCCTGTGAGCCTGT
NleF _{EP_4}	ATGCATCGTGCATATGGTGC
NleF _{CR_1}	GTAAAGGTGGTAACGCCGTG
NleF _{CR_2}	GAAGCAGCTCCAGCCTACACATGCCTACTATAACCGGATAAAAA
NleF _{CR_3}	CTAAGGAGGATATTCATATGGGGCCTGGTTACCATCTGT
NleF _{CR_4}	CACCCCATCCTCTTAGCAG
S12 _{F1}	ATCCCGGGGCGCGATCGTTGTATATTTTC
S12 _{R1}	AGCTTTTGCTTCGTAAACAC
S12 _{R2}	GCTACTAGCTGGTAACATTAAATAGCTCCTGGTTTT
pKD _F	TGTGTAGGCTGGAGCTGCTTC
pKD _R	CATATGAATATCCTCCTTAG
CR _F	AGAGATCTCATACATTCACG
CR _R	TGGCTTATAATCTGATACGG

resistance cassette using primer pair pKD_F/pKD_R. Overlapping PCR was used to assemble the linear fragment using primer pair NleF_{CR_1}/NleF_{CR_4}. This fragment was then electroporated into a previously described *C. rodentium* Δ nleB mutant containing pKD46. The deletion of *nleF* was confirmed by PCR and DNA sequencing using the external primer pair NleF_{CR_1}/NleF_{CR_4}. The *C. rodentium* Δ nleB Δ nleF mutant was complemented with either *nleB*_{CR} or *nleF*_{CR} using a previously described method of transgene insertion (36). Briefly, pGRG36-NleB or pGRG36-NleF was electroporated into electrocompetent *C. rodentium* Δ nleB Δ nleF. The insertion of *nleB* or *nleF* at the attTn7 insertion site was confirmed by PCR and sequencing using primers CR_F/CR_R.

Construction of stable inducible cell lines expressing NleF and NleB1 in HeLa cells. HEK293T cells were grown on 10-cm plates to a confluence of 60% and transfected with 2.0 μ g pCMV Δ R8.2, 0.8 μ g pCMV-VSV-G, and 1.2 μ g pF_{TRE}-Flag-NleF, pF_{TRE}-Flag-NleF Δ LQCG, pF_{TRE}-Flag-NleB1, or pF_{TRE}-Flag-NleB1_{DXD}. The tissue culture medium was replaced 24 h after transfection. Supernatant containing packaged virus particles was harvested 48 h after transfection and filtered through a 0.45- μ m membrane filter. Hexadimethrine bromide (5 μ g/ml; Sigma) was added to virus-containing medium. HeLa cells were grown in 6-well plates to a confluence of 50%, and then cell culture medium was replaced with virus-containing medium added to DMEM with 10% fetal calf serum (FCS) in a 1:1 ratio. HeLa cells were centrifuged at 1,800 rpm for 45 min at room temperature. Forty-eight hours after infection, the tissue culture medium was replaced with DMEM with 10% FCS containing puromycin (5 μ g/ml). Cells were maintained in puromycin for at least three passages before use.

FasL treatment of inducible cell lines. HeLa cells and inducible HeLa cell line derivatives were seeded into 24-well tissue culture plates 24 h prior to being left untreated or treated with doxycycline (20 ng/ml) to induce effector protein expression. An additional 24 h later, the medium was replaced with DMEM containing FasL (20 ng/ml; gift from Andreas Strasser) for 1.5 h. Cell lysates were collected and analyzed by immunoblotting as described below.

For propidium iodide (PI) staining, HeLa cells and inducible HeLa derivatives were seeded on poly-L-lysine-coated coverslips in 24-well tissue culture plates 24 h prior to being treated with doxycycline and FasL as described above. PI (20 μ g/ml; Cayman Chemicals) was added to each well for the final 15 min of FasL treatment. Cells were prepared for confocal microscopy as described below.

FasL treatment of EPEC-infected cells. HeLa cells were seeded onto 24-well tissue culture trays for 24 h prior to infection. EPEC cultures grown with shaking overnight in LB were inoculated 1:100 into DMEM and subcultured for a further 3 h, including 30 min of induction with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) in 5% CO₂. HeLa cells were left uninfected or infected with EPEC cultures at an optical density at 600 nm (OD₆₀₀) of 0.03. After 2 h, the tissue culture medium was replaced with

DMEM containing gentamicin (100 μ g/ml) with or without FasL (20 ng/ml; gift from Andreas Strasser) for a further 1.5 h. For detection of caspase cleavage, cells were harvested for immunoblotting as described below. For quantification of PI-positive cells, PI was added to each well for the final 15 min of FasL treatment. Cells were prepared for confocal microscopy as described below.

Immunoblotting. For analysis of cell monolayers, cells were lysed in cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM Na_3VO_4 with 1 $\times$ EDTA-free Complete protease inhibitor cocktail [Roche]). Cell lysates were pelleted, and equal volumes of supernatant were collected and added to 4 $\times$ lithium dodecyl sulfate (LDS) sample buffer (Thermo Fisher) supplemented with dithiothreitol (DTT; 50 mM), heated to 70°C for 10 min, and resolved on 4 to 12% Bis-Tris gels (Thermo Fisher) by PAGE. Proteins were transferred to nitrocellulose membranes before being incubated for 1 h in 5% skimmed milk powder in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.5, 150 mM NaCl) with 0.1% Tween 20. Membranes were washed and probed with one of the following primary antibodies diluted 1:1,000 in 5% bovine serum albumin (BSA) in TBS with 0.1% Tween 20: horseradish peroxidase (HRP)-conjugated monoclonal mouse anti-FLAG-M2 antibody (Ab) (Sigma-Aldrich), monoclonal mouse anti-caspase-8 Ab (1C12) (Cell Signaling Technology), polyclonal rabbit anti-cleaved caspase-3 Ab (Asp175) (Cell Signaling Technology), monoclonal rabbit anti-RIP (D94C12) XP Ab (Cell Signaling Technology), or monoclonal mouse anti- β -actin Ab (Sigma-Aldrich) diluted 1:5,000. The secondary antibodies used were HRP-conjugated anti-mouse Ab (PerkinElmer) and HRP-conjugated anti-rabbit Ab (PerkinElmer) diluted 1:3,000 in TBS with 5% BSA and 0.1% Tween 20. Immunoblots were developed with Amersham ECL Western blotting detection reagents (GE Healthcare) or Western Lightning ultra (PerkinElmer), and chemiluminescence was detected using the DNR MF-ChemiBIS Bio-Imaging System.

Confocal microscopy. For visualization of PI-positive HeLa cells, treated cells were fixed with paraformaldehyde (4% in phosphate-buffered saline [PBS]; Sigma) for 15 min, followed by thorough washing with PBS. Cells were incubated for a further 15 min with Hoechst (2.4 μ g/ml; Thermo Fisher) to stain DNA.

Coverslips were mounted onto microscope slides with Prolong Gold antifade reagent (Invitrogen). Images were acquired using a Zeiss confocal laser scanning microscope with a 20 $\times$ objective. For quantification of PI-positive cells, three distinct fields per coverslip containing approximately 60 cells each were counted blind. Results were obtained from at least three independent experiments.

Infection of mice with *C. rodentium*. All animal experimentation was approved by the University of Melbourne Animal Ethics Committee. Strains of *C. rodentium* were cultured in LB broth supplemented with appropriate antibiotics overnight at 37°C with shaking. Bacteria were pelleted by centrifugation and resuspended in PBS. C57BL/6 mice (6 to 8 weeks old, male and female) were inoculated by oral gavage with 200 μ l containing approximately 1×10^9 CFU of *C. rodentium* derivatives. The viable count of the inoculum was determined by retrospective serial dilution and plating on Luria-Bertani agar (LA) containing appropriate antibiotic. Fecal samples were collected from each mouse on days 4, 8, 10, 12, 14, 16, and 18 postinfection and emulsified in PBS to a concentration of 100 mg/ml. The number of viable bacteria per gram of feces was determined by plating serial dilutions of fecal samples onto antibiotic-containing LA. The limit of detection was 400 CFU/g feces.

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