The Inflammatory Response during Enterohemorrhagic Escherichia coli Infection

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ABSTRACT The inflammatory response is an integral part of host defense against enterohemorrhagic Escherichia coli (EHEC) infection and also contributes to disease pathology. In this article we explore the factors leading to inflammation during EHEC infection and the mechanisms EHEC and other attaching and effacing (A/E) pathogens have evolved to suppress inflammatory signaling. EHEC stimulates an inflammatory response in the intestine through host recognition of bacterial components such as flagellin and lipopolysaccharide. In addition, the activity of Shiga toxin and some type III secretion system effectors leads to increased tissue inflammation. Various infection models of EHEC and other A/E pathogens have revealed many of the immune factors that mediate this response. In particular, the outcome of infection is greatly influenced by the ability of an infected epithelial cell to mount an effective host inflammatory response. The inflammatory response of infected enterocytes is counterbalanced by the activity of type III secretion system effectors such as NleE and NleC that modify and inhibit components of the signaling pathways that lead to proinflammatory cytokine production. Overall, A/E pathogens have taught us that innate mucosal immune responses in the gastrointestinal tract during infection with A/E pathogens are highly complex and ultimate clearance of the pathogen depends on multiple factors, including inflammatory mediators, bacterial burden, and the function and integrity of resident intestinal epithelial cells.

INTRODUCTION

The mammalian host is equipped with two major types of immune response, innate and adaptive, that are essential for effective control and elimination of infectious agents. The innate immune system is the first line of host defense against invading microbial pathogens and is promptly activated by the recognition of pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), flagellin, peptidoglycan, and CpG DNA (1). Pathogen-associated molecular patterns are recognized by specialized germline-encoded pattern recognition receptors (PRRs) expressed by immune cells. To date, a number of PRR families have been described, including the Toll-like receptors (TLRs), retinoic acid-inducible gene-I-like receptors, and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (2, 3). The first and most significant consequence of PRR-mediated pathogen recognition in the host is the rapid production of proinflammatory cytokines that stimulates the innate immune response (2, 3). In addition, the innate immune response directs the development of the more specific and long-term adaptive response to a particular pathogen, mediated by B and T cells.

The primary site of infection with the attaching and effacing (A/E) pathogens, enterohemorrhagic and enteropathogenic Escherichia coli (EHEC/EPEC), is the...
epithelium lining the mucosal surface of the gastrointestinal tract (4). Not only do these gastrointestinal epithelial cells play a pivotal role in ion transport, fluid uptake, and secretion that are critical to the homeostatic state of the digestive system, they also coordinate the expression and upregulation of specific antimicrobial products in response to infection, including cytokines with proinflammatory (tumor necrosis factor [TNF] and interleukin-1 [IL-1]) and chemoattractant (IL-8, macrophage inflammatory protein 1-alpha [MIP1-α], monocyte chemoattractant protein-1 [MCP-1]) functions (3). In vitro, the initial and most potent activation of inflammation by EHEC/EPEC occurs by TLR5 recognition of flagellin and, to a lesser extent, TLR4 recognition of LPS (6–8). Infection studies conducted in gnotobiotic piglets show EHEC and EPEC induce extensive inflammatory cell infiltration in the lamina propria as well as transmigration of inflammatory cells across the intestinal epithelium into the intestinal lumen (9–11). Depletion of neutrophils in mice infected with the mouse A/E pathogen Citrobacter rodentium results in elevated bacterial loads in the liver and spleen, suggesting an important role for neutrophils in controlling infection with A/E pathogens (12). Similarly, patients with EHEC infection consistently have significantly increased numbers of leukocytes in their feces, more so than with Salmonella or Shigella infections (13), whereas antibodies against the leukocyte adhesion molecule CD18 reduce clinical symptoms and pathology of EHEC infections in rabbits (14).

Neutrophils are a type of polymorphonuclear (PMN) leukocyte that play a key regulatory role in acute inflammation (15–18). They represent the first leukocytes recruited to the site of infection with a pathogen and can mediate killing by phagocytosis, degranulation (release of antibacterial proteins), or, in the case of extracellular pathogens, the release of neutrophil extracellular traps. Transmigration of neutrophils from the vasculature to the lamina propria and into epithelial tissues occurs in response to chemoattractive factors released by either resident sentinel leukocytes or the epithelium itself upon recognition of a pathogen (17–19). IL-8 is a potent chemoattractant for neutrophils, and a number of early studies showed that EHEC (20), EPEC (21), and other bacterial enteric pathogens stimulate epithelium-derived IL-8 expression during infection (22–24). Furthermore, increased IL-8 levels, along with neopterin and IL-10, are markers for increased risk of hemolytic-uremic syndrome (HUS) in EHEC-infected children. Despite this, it is not entirely clear to what extent epithelium-derived IL-8 contributes to mucosal defense and the inflammatory response generated against A/E pathogens.

**NF-κB AND MAPK SIGNALING**

The most crucial factor for initiation of IL-8 gene expression in the host is activation of the key transcriptional regulator of innate immune signaling, NF-κB (nuclear factor-κB) (25). NF-κB proteins are transcription factors that control gene expression during inflammation and are activated rapidly in response to various stimuli, including pathogens, stress signals, and proinflammatory cytokines such as TNF and IL-1β (26). There are five members of the NF-κB/Rel family of proteins: p65 (RelA), p50 (NF-κB1), p52 (NF-κB2), c-Rel, and RelB, which all share an N-terminal Rel homology domain that mediates DNA binding, dimerization, and nuclear translocation (27, 28). The p65, c-Rel, and RelB subunits harbor an additional C-terminal transactivation domain, which strongly activates transcription from NF-κB-binding sites in target genes. The p50 and p52 subunits lack the transactivation domain but still bind to NF-κB consensus sites and act as transcriptional repressors (29).

NF-κB activation is generally categorized as canonical, noncanonical, or alternative, depending on the stimuli. Canonical NF-κB signaling is representative of the general scheme of NF-κB signaling predominantly considered in this article, and is triggered by TLR ligands, proinflammatory cytokines, pathogens, and engagement of the T-cell receptor by antigen (30). Upon ligand recognition, the cognate receptor such as TNR1, IL-1R, or PRRs triggers signaling events that result in activation of the IkB kinase (IKK) complex, comprising IKKβ (IKK2), IKKα (IKK1), and IKKγ (NEMO). Activated IKK in turn phosphorylates the inhibitory protein of NF-κB (IkB), followed by subsequent ubiquitylation and degradation of IkB by the host cell proteasome (31). In a resting cell IkB is bound to p50/p65 dimers, which are released upon proteosomal degradation of IkB and transported into the nucleus through the nuclear pore complex where they can promote expression of multiple cytokine genes, including IL8 (26).

In addition to NF-κB, the IL-8 promoter region contains binding sites for several other transcription factors, including NF-IL-6, AP-1, and AP-3 (32). Several studies have demonstrated AP-1-dependent IL-8 production during EHEC and EPEC infection in cultured intestinal epithelial cells (20, 33, 34). AP-1 activation is regulated by mitogen-activated protein kinases (MAPK), which are highly conserved host proteins that play a central role in a number of cell responses, including regulation of cytokine expression, stress responses, and cytoskeletal reorganization (35, 36). The MAPKs comprise three subfamilies of serine/threonine kinases, including the
extracellular signal-related protein kinases ERK and two stress-activated protein kinases, p38 and JNK (c-Jun amino-terminal kinase). These kinases regulate cellular processes through phosphorylation of target protein substrates, including other protein kinases, phospholipases, transcription factors, and cytoskeletal proteins. Phosphorylation by MAPKs acts as an on/off switch for the activity of target substrates, a process that can be reversed by cellular protein phosphatases (37).

**PROINFLAMMATORY RESPONSES DURING EHEC INFECTION**

EHEC produces a number of factors that potentially up-regulate inflammatory cytokine production by intestinal epithelial cells. For example, early studies showed that purified Shiga toxin (Stx) could stimulate low-level production of IL-8 by cultured colon cancer cell lines (38–40). However, subsequent studies using primary human colonic epithelial cells showed that TLR5 recognition of H7 flagellin was the major factor inducing IL-8 production during EHEC infection, and not Stx signaling through the Gb3 receptor (7, 41). Indeed, in vitro the initial and most potent activation of inflammation by EHEC/EPEC occurs by TLR5 recognition of flagellin and, to a lesser extent, TLR4 recognition of LPS (6–8). Culture supernatants from the prototype EPEC strain E2348/69, but not from an EPEC flagellin (flIC) mutant, induced significant IL-8 production from cultured intestinal epithelial cells (42). In contrast, EHEC O157:H7 lacking Stx potently activates p38 and ERK1/2 MAPKs and NF-kB, and induced significant production of IL-8 by human intestinal epithelial cells (41). Flagellin/TLR5 signaling thus seems important to induce inflammatory signaling in vitro. In vivo studies using EHEC O157:H7-infected rabbits showed a marked increase in neutrophil infiltration in the colonic epithelium resulting in diarrhea and disruption of solute transport, none of which was dependent on Stx expression (43, 44). Although TLR5 is not generally located on the apical surface of enterocytes (45), A/E pathogens may gain access to basolateral receptors by compromising the epithelial barrier and cell polarity (46, 47). TLR5 is also expressed by lamina propria dendritic cells, which may sample luminal flagellin (48). In addition, some EHEC strains appear to have a predilection for the follicle-associated epithelium of the gastrointestinal tract that expresses TLR5 (49, 50). Hence it is likely that flagellin is sensed during EHEC infection.

Despite the fact that Stx alone induces relatively low levels of proinflammatory cytokine production, some studies have suggested that the loss of barrier function induced by neutrophil transmigration allows increased translocation of Stx from the lumen into the circulation (51), promoting a systemic cytokine response. Indeed, patients with HUS have increased levels of circulating proinflammatory cytokines, including IL-8, TNF, IL-6, IL-1β, and IL-10. Although no studies to date have been able to detect circulating Stx in patients with toxin-producing EHEC infections, others have demonstrated Stx bound to circulating neutrophils in patients with HUS (52). A recent study in nonhuman primates used purified Stx1 and Stx2 to evaluate their relative contribution to inflammatory cytokine production. Both toxins induced a significant increase in expression of IL-8 (CXCL8), MCP-1 (CCL2), and MIP1α (CCL3) in kidney tissue (53), whereas TNF and IL-12p35 levels were comparably low. Urine analysis indicated a substantial increase in the production of IL-6 and vascular endothelial growth factor in response to Stx1 compared to Stx2 48 h after challenge, which was consistent with an increase in leukocyte infiltration in the kidneys. Although both toxins were chemotactic in the kidneys, Stx1 induced a more rapid inflammatory response and time to euthanasia compared with Stx2 (53).

The type IV pilus, hemorrhagic coli pilus (HCP), of EHEC O157:H7 is another factor capable of inducing an inflammatory response in vitro. Production of IL-8 and TNF is significantly increased at the basolateral surface of polarized T84 and HT-29 and in nonpolarized HeLa cells during infection with HCP-expressing EHEC strains (54). Deletion of the HCP major pilin subunit hcpA significantly reduced IL-8 production in cultured intestinal epithelial cells during EHEC O157:H7 strain EDL933 infection, but not to the same extent as a flIC/hcpA double mutant, confirming the importance of EHEC flagellin in eliciting an inflammatory response. NF-kB and AP-1 activation were increased in the presence of purified HCP and after 3 h of infection with EHEC O157:H7 strain EDL933 in cultured intestinal epithelial cells (54).

Overall, the initial induction of proinflammatory cytokines in the intestinal mucosa during EHEC infection appears to result from activation of MAPK and NF-kB signaling by a variety of factors. This in turn drives the increased production of cytokines by intestinal epithelial cells and subsequent PMN transmigration. Intestinal tissue damage is sustained as a result of the large number of infiltrating PMNs releasing proteolytic enzymes, reactive oxygen intermediates, and phospholipid derivatives, contributing to diarrheal disease and the release of luminal contents (including Stx) into the circulation.
C. rodentium AND IMMUNE RESPONSES IN MICE

Studying the in vivo inflammatory response intensively in the human host is not a viable option for EHEC and EPEC infection. Therefore, animal models of infection have been invaluable in understanding the type of immune response elicited, including which cytokines and chemokines are released, and how they might contribute to clearance or pathogenesis. Rabbits, calves, and lambs are the primary animals used to test the characteristics of infection and pathology with EHEC strains, whereas the closely related mouse pathogen C. rodentium is also used as a model of EHEC and EPEC colonization. Besides the advantage of infecting a small laboratory animal, C. rodentium shares most key virulence factors of EHEC and EPEC, including a type III secretion system (T3SS) and effector proteins encoded by the locus of enterocyte effacement (LEE) pathogenicity island and the non-LEE effectors encoded on various genomic islands and integrative elements. The key virulence factors that C. rodentium lacks compared to EHEC are genes encoding Stx and flagellin. Despite these limitations, C. rodentium infection of mice has allowed two critically important research questions in the field to be partially addressed, namely, (i) does innate immunity provide protection against A/E pathogens or does it just induce tissue damage and (ii) what are the relative contributions of innate and adaptive immunity in controlling infection with A/E pathogens.

Infection with C. rodentium causes colitis characterized by inflammatory cell infiltration, hyperplasia of crypt cells, loss of goblet cells, and significant intestinal barrier disruption (55, 56). Both innate and adaptive responses contribute to the control of C. rodentium infection, dissemination, and elimination. For instance, mice lacking B cells, T cells, or both have greater pathogen loads in their colonic and peripheral tissues, and despite a severe disease phenotype in these mice, a significant percentage of mice survive, suggesting more than an adaptive response is involved (57, 58). For example, in the absence of neutrophils, mice infected with C. rodentium also have a high bacterial load, which highlights the importance of the inflammatory response in the early stages of infection (12). Activation of NF-κB has been demonstrated in vivo during C. rodentium infection (59); however, unlike EHEC and EPEC, C. rodentium is nonflagellated and does not activate TLR5 (60). Therefore, it is likely that activation is either T3SS-dependent and/or a result of TLR4 recognition of LPS.

Mice with an intact adaptive system but deficient in mast cells display increased colonic inflammation and increased production of proinflammatory cytokines (61). These mice also experience systemic infection, and most die within 4 to 7 days of C. rodentium infection (61), which suggests that mast cells play a role in control and clearance of C. rodentium rather than regulating inflammation.

A strong Th1/Th17 response, characterized by an increase in the expression of IL-1β, TNF, IL-12, gamma interferon (IFNγ), IL-17, and IL-22, is elicited in the colon of C. rodentium-infected mice and produces pathology similar to that seen in mouse models of inflammatory bowel disease (IBD) (62–64). Th17 cells are a subset of T-helper cells that have an innate function and secrete large amounts of the proinflammatory cytokines IL-17 and IL-22. C. rodentium infection induces a Th17 response within 2 weeks of infection, and this is needed for clearance of the pathogen (64). The induction of this response requires NOD1/NOD2 signaling and the proinflammatory cytokine IL-6 (63). IL-6-deficient mice develop severe mucosal ulcerations and increased crypt cell apoptosis, suggesting a protective role for the cytokine during gut infection (65). Although it is not clear if all these immune and inflammatory factors are required for mucosal defense during human infection with EHEC, some studies have highlighted the importance of IL-6 in clearance of EPEC in children (66, 67). In addition, a chronic Th17 response is strongly associated with the development of IBD (68).

Studies have suggested that TNF plays an important role in clearance of EPEC in children (67) and control of bacterial load in animal models of infection (69, 70). TNF is produced rapidly in response to local or systemic tissue damage and is a potent activator of macrophages and neutrophils. Failure to control TNF production can lead to severe tissue damage and organ-specific tissue pathology as a result of chronic activation of immune cells and inflammatory responses. In the intestine, increased TNF levels are highly associated with tissue pathology and disease, including Crohn’s disease and IBD (71). Mice that are TNF deficient (TNFRp55−/−) demonstrate increased tissue pathology during C. rodentium infection, including pronounced hyperplasia, increased T-cell infiltrate, and higher levels of mucosal IFNγ and IL-12 production (69). However, TNFRp55+/− mice are not compromised in their ability to clear infection compared to wild-type C57BL/6 mice, and despite the compensatory increase in cytokine production, they experience significantly higher bacterial loads than wild-type mice (69). Overall, it is plausible that the increased bacterial burden may be a major contributing factor to increased tissue pathology in the absence of...
TNF and that TNF plays a major role in limiting bacterial replication.

TLRs have been strongly implicated in mediating inflammation during *C. rodentium* infection. For instance, TLR4, although not protective, mediates chemokine induction and subsequent neutrophil and macrophage recruitment and is partially responsible for tissue pathology during *C. rodentium* infection (72). Conversely, TLR2 is not critical in mediating proinflammatory responses associated with *C. rodentium*-induced colitis but helps maintain mucosal integrity during *C. rodentium* infection (65). MyD88 is a key signaling adaptor protein that is shared among TLRs (except TLR3), IL-18R, and IL-1R and is essential in the control of mucosal inflammation by a number of pathogens (73–76). MyD88 activation initiates NF-κB activation and, in the intestine, plays an important role in maintaining tissue homeostasis (77). During *C. rodentium* infection MyD88 protects against bacteremia and severe pathology in C57BL/6 mice (12), and MyD88-deficient mice experience elevated bacterial load in the colon and peripheral tissues, which correlates with a decrease in neutrophil infiltration into the colonic tissue. MyD88-deficient mice also experience severe colonic ulceration and bleeding, resulting in high levels of morbidity and mortality. This severe disease phenotype is due to impaired epithelial barrier function and defective cellular proliferation, followed by an inability to repair mucosal damage.

MyD88 association with the IL-1R but not the IL-18R affords protection against increased mortality and pathology during infection with *C. rodentium* (78). IL-1R-deficient mice infected with *C. rodentium* have disease similar to that seen in MyD88-deficient mice, but they do not experience higher bacterial loads or an inability to recruit neutrophils and mediate tissue damage repair. The mice are unable to induce production of proinflammatory cytokines IL-6 and IFNγ during infection, suggesting that the bacteria are mediating severe tissue pathology rather than a dysregulated innate immune response and that IL-1R signaling regulates susceptibility to *C. rodentium* infection (78). The importance of IL-1R signaling is supported by work showing that mice deficient for the inflammasome components Nlrc4, Nlrp3, and caspase-1 that lead to IL-1β and IL-18 production were highly susceptible to *C. rodentium* and had exacerbated intestinal inflammation and increased bacterial load after day 10 of infection (79). Similar observations were made in IL-1β- and IL-18-deficient mice. Despite the fact that Nlrc4-deficient mice were highly susceptible to *C. rodentium* infection, only Nlrp3 and not Nlrc4 induced caspase-1 activation (79).

### INHIBITION OF INFLAMMATORY SIGNALING BY A/E PATHOGENS

Many of the early studies on EHEC/EPEC-mediated inflammation focused on the proinflammatory response characterized by marked infiltration and transmigration of PMNs in the intestinal epithelium. However, some 10 years ago, researchers made the remarkable observation that EHEC and EPEC strains could inhibit NF-κB and MAPK activation as well as IkB degradation and the production of proinflammatory cytokines, including IL-8 and IL-6, early in the course of infection (80, 81). Furthermore, this inhibitory mechanism was dependent on the presence of a functional T3SS, revealing for the first time that EHEC/EPEC suppressed host inflammatory responses in a type III-dependent manner (80). This suggested that EHEC/EPEC had evolved specialized mechanisms to dampen the early inflammatory response during infection, possibly to persist for longer periods before the overall immune response would inevitably clear the bacteria.

The early discovery of the LEE pathogenicity island and its association with A/E lesion formation led to the intensive study of LEE-encoded effectors and their involvement in cytoskeletal reorganization and disruption of tight junctions in the epithelium (82). However, the activity of these effectors could not explain how A/E pathogens suppressed the production of inflammatory cytokines. Characterization of several non-LEE-encoded effectors over the past 3 years has revealed that A/E pathogens inject multiple effector proteins into the host cell that specifically target innate immune factors. These effectors have highly specific and diverse functions that interfere with a range of innate signaling pathways, including NF-κB and MAPK activation (83–86) (Fig. 1).

### EFFECTORS THAT INHIBIT INFLAMMATORY SIGNALING

**The Cysteine Methyltransferase, NleE**

NleE was one of the first non-LEE-encoded effectors of EHEC implicated in the inhibition of NF-κB signaling (84, 85). This 27-kDa translocated effector is highly conserved across A/E pathogens and was first discovered as a type III secreted effector of *C. rodentium* (87, 88). In EHEC O157:H7 strain EDL933, NleE is located on the virulence-associated O-island (OI) 122, whereas in EPEC O127:H6 E2348/69, NleE is encoded on genomic pathogenicity island integrative element six (IE6) (89, 90). In both cases, NleE is encoded directly downstream of the type III effector NleB1. Homology at the amino
acid level between NleE from EHEC and EPEC is high (99% identity) and also between EHEC/EPEC and C. rodentium (~85% identity). Furthermore, all Shigella species carry a strong homolog of NleE known as OspZ, which share about 74% identity with NleE from EHEC/EPEC (91). Overall, the high level of similarity between the NleE/OspZ effectors suggests that their function is conserved across the species.

Initial in vivo studies revealed no significant colonization defect for a C. rodentium ΔnleE mutant compared to the wild type strain, although the ΔnleE mutant was outcompeted by the wild type in a competitive infection (88). A subsequent study, however, argued that NleE was essential for full colonization in mice and that NleE contributed to disease pathology during C. rodentium infection (90). In addition, NleE is consistently associated with the virulence profile of O157 and non-O157 EHEC and EPEC strains (92, 93).

More recent in vitro studies have shown that NleE from EHEC, EPEC, rabbit specific EPEC (REPEC), and
C. rodentium and full-length OspZ from Shigella species inhibit the activation of NF-κB in cultured epithelial cells (84, 85). Despite the translocation of numerous type III effectors into the host cell during infection, NleE is sufficient to block TNF-induced IkB degradation and nuclear translocation of activated NF-κB (p65) in epithelial cells when it is expressed ectopically (84, 85). Furthermore, NleE-dependent inhibition of NF-κB activation contributes significantly to the low levels of IL8 expression and IL-8 production in EPEC-infected epithelial cells (84, 85). A concurrent study also demonstrated the ability of NleE to inhibit nuclear translocation of activated NF-κB and proinflammatory cytokine production in human dendritic cells (DCs) (94). IL-8, TNF, and IL-6 expression and production were suppressed in the presence of EPEC-delivered NleE in human monocyte DCs and Peyer’s patch DCs. The follicle-associated epithelium is rich in DCs and forms the interface between the luminal contents of the intestinal tract and the gut-associated lymphoid tissue. Therefore it seems plausible that enteric pathogens such as EHEC, EPEC, and Shigella sp. would evolve a mechanism to evade immune detection at this junction.

The precise mechanism by which NleE inhibits NF-κB activation was described recently (95). NleE possesses a unique S-adenosyl-l-methionine-dependent methyltransferase activity that modifies a cysteine residue in the zinc finger domain of the signaling adaptor proteins TAB2 and TAB3 (TAK1-binding proteins 2 and 3) (95). TAB2/3 contain zinc finger domains, which bind K63-linked polyubiquitin chains on the target TNF receptor-associated proteins (TRAF) 2/6 following activation of TNF or TLR/IL-1R signaling. The binding of TAB2/3 to ubiquitinated TRAF allows TAK1 to form a complex with IKK and subsequently phosphorylate IKKβ, which leads to the degradation of IkB and activation of NF-κB. The modification by NleE abolishes ubiquitin-chain binding of the zinc finger domains of TAB2/3 and thereby disrupts NF-κB signaling in host cells. The activity of NleE depends on a conserved 6-amino-acid motif, IDSYMK (95), within the C-terminal region that is essential for its ability to block NF-κB activation and modify TAB2/3 (85, 95).

The Metalloprotease Effectors, NleC and NleD

The non-LEE effectors NleC and NleD are potent inhibitors of two key innate signaling networks through their proteolytic activities. While NleC targets NF-κB Rel proteins for degradation, NleD cleaves the MAPK enzymes JNK and p38 (96–99). NleC was first identified as an effector secreted by the LEE-encoded T3SS of C. rodentium (87). The 37-kDa effector is conserved across A/E pathogens, encoded on OI 36 in EHEC O157:H7 EDL933 and on prophage four (PP4) in EPEC O127:H6 E2348/69 (89, 100). EHEC and EPEC NleC share 100% amino acid sequence similarity and are 95% similar to NleC from C. rodentium. NleD is a 30-kDa effector that is also conserved across A/E pathogens and is also encoded on OI 36 in EHEC O157:H7 EDL933 and on PP4 in EPEC O127:H6 E2348/69. EPEC and EHEC NleD share 99% amino acid similarity and 77% with NleD from C. rodentium.

Initial studies on NleC and NleD revealed that both effectors were translocated into host cells by the EPEC T3SS. However, when tested in calves or lambs, ΔnleC and ΔnleD deletion mutants of EHEC O157:H7 exhibited wild-type virulence (101). Similarly, neither nleC nor nleD mutants of C. rodentium were attenuated in mice (88), although mice infected with an nleC deletion mutant showed increased pathology, suggesting that NleC assists in reducing the severity of colitis during C. rodentium infection (99).

The study by Marchès et al. (2005) (101) first identified putative zinc metalloprotease motifs, HEXXH, within both NleC and NleD although no further research on the significance of the motif for protein function was conducted until recently. In fact, NleC degrades the NF-κB subunit, p65, and this function is dependent on the zinc metalloprotease motif, H183EIIH (96–99, 102). Degradation is direct, as recombinant His6-NleC cleaves both p65 and p50 in epithelial cell lysates (98). Furthermore, mutation of the histidines or the glutamate within the metalloprotease motif of NleC renders the protein inactive (96–99, 102), a phenomenon that had been previously observed for other zinc metalloproteases (103). Although two independent research groups concurrently identified specific cleavage sites of recombinant p65 by NleC, they were not consistent with each other (96, 102). Both groups identified the site within the N-terminal Rel homology domain of p65. However, whereas Baruch et al. (96) put the cleavage site between residue C38 and E39 by N-terminal sequencing, Yen et al. (102) proposed the cleavage site to be between residue P10 and A11. Either way, since the Rel homology domain of NF-κB proteins is required for binding to NF-κB consensus, dimerization, and nuclear localization (104), cleavage by NleC would render p65 unable to bind target genes and activate transcription of inflammatory cytokines.

To date, there is conflicting evidence on whether NleC degrades other NF-κB proteins in addition to p65. Whereas one study showed clear degradation of p50 with NleC expressed ectopically or delivered by the
T3SS (98), another group stated NleC could not cleave p50 directly (102). Similarly, while IxB degradation was observed upon ectopic expression of NleC (97), another study found that reduced levels of IxB in EPEC infected cells could not be attributed directly to NleC (102). Yet another study suggested that NleC cleaves the acetyltransferase p300, a transcriptional coactivator of many host cell genes, including p65 (105, 106). NleC delivered by the T3SS bound to endogenous p300 and reduced host cell nuclear levels of p300 in a metalloprotease-dependent manner (106). Furthermore, overexpression of p300 in host cells resulted in a significant increase in IL-8 production during wild-type EPEC infection, suggesting that NleC-dependent cleavage of p300 assists suppression of IL-8 during infection (106). In addition, NleC was observed to target MAPK signaling by inhibiting phosphorylation of p38 during EPEC infection, although this was independent of the zinc metalloprotease motif (99).

Given the diversity and complexity of innate immune signaling pathways, it is perhaps not surprising that A/E pathogens have evolved mechanisms to target specific pathways such as NF-kB signaling at multiple levels. Although there is apparent redundancy in the functions of NleE and NleC, secreted IL-8 levels are significantly higher upon infection with a double nleC/nleE mutant than single nleE or nleC mutants, suggesting that NleC and NleE act synergistically to inhibit IL-8 production (96, 98, 99, 102, 106).

NleD-mediated proteolytic degradation of the MAPKs p38 and JNK also depends on the [141]HELLH motif of NleD (96). JNK cleavage by wild-type EPEC is detected as early as 30 min post infection with the kinase completely degraded by 2.5 h post infection. NleD directly cleaves JNK and p38 within a conserved activation loop present in both signaling kinases. ERK, however, is unaffected by the metalloprotease, which is likely explained by the fact that it does not contain the activation loop present in p38 and JNK. The specific cleavage site of JNK as determined by N-terminal sequencing occurs after residue P184 within a TPY motif (96). Phosphorylation of T183 and Y185 in this motif is required for JNK activation; therefore, NleD selectively cleaves the signaling kinase at its site of activation. As with NleC, mutation of the glutamate within the metalloprotease motif of NleD renders NleD inactive, and furthermore, although not sufficient, NleD contributes to the relief of IL-8 suppression in wild-type EPEC infection of cultured epithelial cells (96).

Overall, NleC and NleD are zinc-containing metalloproteases that provide yet another mechanism by which EHEC and EPEC control the host inflammatory response during infection. Although the degradation of p65, JNK, and p38 may appear extraneous given the efficiency of NleE alone to inhibit IL-8 production, NleC and NleD clearly amplify this modulatory effect, demonstrating that EHEC and EPEC are highly evolved bacteria that specifically target signaling networks at multiple levels.

The Glycosyl Transferase, NleB

The non-LEE-encoded effector B (NleB) was first identified as a secreted effector of C. rodentium and is conserved across all A/E pathogens (87). EHEC and EPEC carry two copies of nleB (nleB1 and nleB2) whereas C. rodentium carries only one that is most similar to NleB1. NleB1 from EPEC and EHEC share a high level of amino acid identity (∼98%) with each other and about 89% amino acid identity and ∼96% similarity with NleB from C. rodentium. In EHEC O157:H7, the nleB1 gene is located on genomic OI 122, which is consistently present in Stx-producing EHEC strains that cause outbreaks and severe disease (107). NleB2 is located on a separate pathogenicity island in both EHEC and EPEC strains and has not yet been implicated in disease epidemiology of either pathotype. NleB2 is highly related to NleB1 from both EHEC and EPEC strains and shares 60 to 62% amino acid identity and ∼80% similarity.

Early work in vivo using a signature-tagged mutagenesis approach revealed that nleB is essential for full colonization of mice by C. rodentium, thereby establishing the effector as an important virulence determinant of A/E pathogens (88). Furthermore, a study on the transmissibility of C. rodentium showed that NleB contributes significantly to the fitness of the pathogen (90). Strong homologs of NleB (termed SseK1, SseK2, and SseK3) exist in a number of Salmonella spp., where their function and contribution to virulence are still under investigation (108).

Recent studies in cell signaling have shown that transient expression of NleB1 in cultured epithelial cells inhibits IxB degradation and NF-kB activation in response to TNF but not IL-1β stimulation (85, 96). This suggested that the point at which NleB1 interrupted NF-kB signaling was downstream of TNF-R1 but upstream of IKK and TAK1, where the TNF and IL-1 pathways converge. Despite compelling evidence for NleB-mediated inhibition of NF-kB activation from TNF-R1, subsequent work showed that the effector had no significant role in blocking production of the NF-kB-dependent cytokine, IL-8, during EPEC infection (J. S. Pearson, unpublished data).
A recent study revealed that NleB of *C. rodentium* is a member of the GT-8 family of glycosyltransferase proteins and exhibits GlcNAc transferase activity (109). NleB1 and NleB2 contain a catalytic motif (DxD) that is conserved across all A/E pathogens and the SseK proteins from *Salmonella* spp. The host protein GAPDH was proposed as the target for NleB binding and O-GlcNAc modification. Coincidently, the study proposed a role for GAPDH binding and promoting TRAF2 ubiquitylation and subsequent NF-κB activation, and thus concluded that GlcNAcylation of GAPDH by NleB hindered TRAF2-mediated NF-κB activation (109). In this instance, modification of GAPDH may explain previous observations that NleB could block TNF-induced IkB degradation and p65 nuclear translocation (84, 85); however, this study did not examine the cytokine production that would implicate NleB in dampening the inflammatory response during infection. This work did, however, show that a *C. rodentium nleB* mutant complemented with a catalytic mutant of NleB (NleBAAA) was attenuated to similar levels as the nleB mutant of *A/E* pathogens (109).

NleH and the Inhibition of NF-κB Signaling

NleH1 and NleH2 are non-LEE-encoded effectors of A/E pathogens that also contribute to suppression of NF-κB activation. The effectors share 84% amino acid identity and are located on separate genomic islands in both EHEC and EPEC strains. Studies to date have been varied in defining the mechanism by which the NleH effectors interfere with NF-κB signaling. Initial work showed that EHEC nleH1 and nleH2 bind to a recently discovered non-Rel NF-κB subunit, ribosomal protein S3 (RPS3), a K homology domain protein that regulates NF-κB-dependent transcription (83, 110). NleH has significant sequence similarity to the *Shigella* effector OspG, a Ser/Thr protein kinase that prevents ubiquitylation and subsequent degradation of phospho-IκBα and downstream activation of NF-κB (111). Alanine replacement of the conserved lysine residue at position 53 abolishes the kinase activity of OspG (83). NleH1 and NleH2 are also autophosphorylated Ser/Thr protein kinases, but their kinase activity is independent of their interaction with RPS3 (110). NleH1 but not NleH2 reduces the nuclear abundance of RPS3 with no effect on other NF-κB signaling factors, and in fact, the N-terminal region of NleH1 is sufficient to perform this function (83). The mechanism by which NleH1 executes this through inhibition of IκKB-mediated phosphorylation of RPS3 at serine residue 209 was demonstrated during EHEC O157:H7 infection in vitro and in vivo (112). NleH2 does not inhibit RPS3 nuclear translocation, but it does increase expression from an AP-1-dependent luciferase reporter; hence it may affect a different signaling pathway (83). Another study suggested that NleH1 and NleH2 suppress TNF-induced IkBα degradation in cultured epithelial cells by interfering with phospho-IκBα ubiquitylation, which is dependent on conserved lysine residues, K159 and K169, in NleH1 and NleH2, respectively, that are implicated in kinase activity (86).

A number of studies conducted with animal models have tried to dissect the contribution of NleH to pathogenesis and inflammatory responses in the host; however, they have been somewhat inconsistent. *C. rodentium* carries only one copy of NleH and is functionally most similar to NleH1 of EHEC/EPEC (83). Initial work showed that an nleH mutant was attenuated early in infection (6 days post infection) in C57BL/6 mice, but not at later time points (10 days post infection) (113), plus the mutant strain was cleared more rapidly than the wild-type strain (114), suggesting the effector plays a role in persistence of the pathogen. As for inflammation, one study showed that wild-type *C. rodentium* infection in C57BL/6 mice induces higher transcription of TNF mRNA in mouse tissues than an nleH mutant at 14 days post infection (114), whereas a more recent study showed the opposite at 7 days post infection (115), suggesting an anti-inflammatory role for NleH in vivo earlier in infection. The former study also showed that NleH induced an increase in NF-κB activity in the colonic mucosa of C57BL/6 mice at later time points; however, there was no overall significant tissue damage or increase in T-cell infiltrate in the colons of mice infected with either wild type or an nleH mutant of *C. rodentium* over 14 days of infection (114). Another study suggested that KC (a functional homolog of human IL-8) secretion in streptomycin-treated C57BL/6 mice increases upon infection with an EPEC nleH1nleH2 double mutant, suggesting that NleH contributes to the inhibition of inflammation in EPEC-infected mice (86). However, it is difficult to reconcile this result with the fact that EPEC colonization of mice does not lead to a productive infection (116).

Despite intense interest in the mechanism of action and function of NleH, neither NleH1 nor NleH2 inhibits NF-κB activity to the same extent as NleE or NleC in vitro (85). Given the possible additional role of NleH in cell death signaling (117), the mechanism of action of NleH and its contribution to EPEC/EHEC pathogenesis warrant further investigation.
Another Role for the Translocated Intimin Receptor (Tir)?

Two studies have identified a role for Tir in inhibiting host innate signaling mechanisms. Ruchaud-Sparagano et al. (2012) (118) showed that transient expression of Tir from EPEC in HeLa cells could inhibit TNF-induced NF-κB activation by binding and degrading the cytoplasmic TRAF2 in a proteasome-independent manner. Consequently, a slight yet significant increase in IL-8 production in HeLa cells infected with an EPEC tir deletion mutant was observed (118). A subsequent study showed that Tir shares sequence similarity with host cellular immunoreceptor tyrosine-based inhibition motifs (ITIMs) and inhibits TLR signaling (119) these motifs are critical negative regulators of eukaryotic immunoreceptor signaling pathways (120, 121). Tir was required for the inhibition of proinflammatory cytokine (TNF and IL-6) expression during EPEC and C. rodentium infection in vitro and in vivo, respectively (119). However, the essential role of Tir in intimate adherence and hence effector translocation makes it difficult to attribute this property directly to Tir, particularly as the background strain used was JPN15, which has been cured of the plasmid that encodes bundle-forming pili and already adheres less efficiently to cells than wild-type EPEC. Likewise, while deletion of tir enhanced NF-κB, Erk, JNK, and p38 MAPK activation during EPEC infection of cultured epithelial cells (119), this was not distinguished from the role of Tir in adherence and the translocation of other effectors. Nevertheless, this study provided evidence that Tir binds directly to SHP-1 (119), a phosphatase that suppresses cellular immune responses by dephosphorylation of NF-κB and MAPKs (122). Downregulation or mutagenesis of SHP-1 increased TNF and IL-6 production in EPEC-infected cells, and recruitment of SHP-1 by Tir was essential for its inhibitory function. Finally, Tir also enhanced SHP-1 association with TRAF6 by an unknown mechanism, which in turn prevented TRAF6 ubiquitylation and subsequent signaling cascades.

Proinflammatory T3SS Effectors

Evidence for the proinflammatory activity of some T3SS effector proteins comes primarily from the study of genomic island deletion mutants. An EPEC mutant lacking genomic islands PP4 and IE6 (encoding seven effectors including NleE, NleC, and NleD) induced p65 nuclear translocation and pronounced IL-8 secretion independently of TNF stimulation (85, 28). Nuclear translocation of p65 induced by the PP4/IE6 double mutant was greater than for a T3SS mutant (escN), suggesting that other translocated effectors stimulated NF-κB activity (85). Recent work on the WxxxE effector, EspT, supports the idea that some effectors directly activate NF-κB signaling (123). EspT induced IL-8 and IL-1β secretion, which required the small GTPase Rac1 (123). A similar phenomenon was evident during Salmonella sp. infection where activation of Rac1 and Cdc42 by the WxxxE effector, SopE, also induced NF-κB activation. NF-κB activation by Rac1 occurred via NOD1 and RIPK2 signaling and was also applicable to the sensing of peptidoglycan by NOD1 (124). This study concluded that NOD1 senses microbial infection by monitoring the activation state of small GTPases, such as Rac1 and Cdc42. Because the WxxxE effectors have known roles in cytoskeletal rearrangement and bacterial invasion, this illustrates the point that the activity of an effector protein on a host cell protein may inadvertently stimulate an inflammatory response.

The pO157-Encoded SteC Protease from EHEC Impairs Neutrophil Function

In addition to the type III effectors, a number of other EHEC-related virulence factors are believed to counteract host inflammatory processes. StcE (secreted protease of Cl-esterase inhibitor) is a type II secreted protease encoded by the pO157 virulence plasmid of EHEC strains that cleaves the protein backbone of mucin-type glycoproteins (125). Mucins are found on almost all hematopoietic cells, including neutrophils, and play critical roles in cellular interactions within the immune system. CD43 and CD45 are neutrophil-specific mucins (126, 127) specifically targeted by StcE for proteolysis, which leads to increased neutrophil adhesion, impaired migratory capacity, and an enhanced oxidative burst (128). The impaired neutrophil function may contribute to increased tissue damage and inflammation or, conversely, may act to dampen the inflammatory response due to a lack of neutrophil mobility. Indeed, neutrophils isolated from children with HUS are more adherent to the vascular endothelium (129).

CONCLUSIONS

Given that the gastrointestinal tract is the largest inflammatory organ in the mammalian body, it is not surprising that EHEC/EPEC and other enteric bacterial pathogens including Salmonella sp. and Shigella sp. have evolved numerous mechanisms for diverting or arresting inflammatory processes in order to persist and cause disease. Although there is a certain level of redundancy between the effectors and their functions, each one plays
a contributing role in counteracting the inflammatory responses of the host. This diversity of effector protein function exemplifies how A/E pathogens have evolved to evade numerous host defense mechanisms, especially innate immunity. Current research efforts to determine effector function are revealing more novel enzymatic functions and targets for effectors and at the same time refining our understanding of host-pathogen interactions.

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