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MINIREVIEW

Cooperative Immune Suppression by *Escherichia coli* and *Shigella* Effector Proteins

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ABSTRACT The enteric attaching and effacing (A/E) pathogens enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) and the invasive pathogens enteroinvasive *E. coli* (EIEC) and *Shigella* encode type III secretion systems (T3SS) used to inject effector proteins into human host cells during infection. Among these are a group of effectors required for NF-κB-mediated host immune evasion. Recent studies have identified several effector proteins from A/E pathogens and EIEC/*Shigella* that are involved in suppression of NF-κB and have uncovered their cellular and molecular functions. A novel mechanism among these effectors from both groups of pathogens is to coordinate effector function during infection. This cooperativity among effector proteins explains how bacterial pathogens are able to effectively suppress innate immune defense mechanisms in response to diverse classes of immune receptor signaling complexes (RSCs) stimulated during infection.

KEYWORDS NF-κB, effector functions, immune suppression

The human pathogens enterohemorrhagic *Escherichia coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and *Shigella* are closely related to commensal *E. coli* but have evolved different strategies to survive in the host's gut (1–4). The attaching and effacing (A/E) pathogens EHEC and EPEC remain in an extracellular niche and encode effectors to attach to epithelial cells in the small intestines and colon through pedestal formation, whereas *Shigella* and EIEC actively invade epithelial cells in the colon and are able to survive in the cytosol of infected cells and spread from cell to cell. Compared with *Shigella*, human A/E pathogens elicit little inflammation and mucosal destruction (5–7). Despite this observed difference in inflammatory potential during infection, both *Shigella* and A/E pathogens encode multiple effectors that modulate innate immune pathways, including mitogen-activated protein kinase (MAPK)-, NF-κB-, and interferon (IFN)-regulated genes (6, 8–11). Inhibition of innate immune responses could allow both extracellular A/E pathogens and intracellular *Shigella*/EIEC to establish infection by reducing the infiltration of immune cells, attracted by the secretion cytokines and chemokines such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), and IL-8. Alternatively, suppression of immune responses may be beneficial for pathogens by reducing expression of cell intrinsic antibacterial factors or secretion of extracellular antimicrobial peptides. This aspect of infection and immunity is not yet fully understood and will likely be resolved with development of novel model systems that better mimic *in vivo* infection conditions.

In this review, we focus on the mechanisms of type III secretion systems (T3SS) effector proteins secreted by A/E pathogens and *Shigella*/EIEC that suppress host inflammation by inhibiting NF-κB signal transduction.

THE NF-κB PATHWAY

NF-κB is an essential transcription factor of the innate immune response to pathogens and is required for the production of proinflammatory cytokines and chemokines (reviewed in references 12 and 13). Activation of NF-κB occurs by cellular stimulation by

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cytokines, including tumor necrosis factor (TNF) and interleukin 1β (IL-1β), or by detection of bacterial and viral molecules (pathogen-associated molecular patterns [PAMPs]) or activities (patterns of pathogenesis) (reviewed in reference 14). More specifically, A/E pathogens activate NF-κB through host cell detection of the extracellular bacterial PAMPs lipopolysaccharide (LPS) and flagellin by the Toll-like receptor (TLR) family members TLR4 and TLR5, respectively (6, 15) (Fig. 1A). In addition, cellular detection of an active EPEC type III secretion system (T3SS) apparatus leads to NF-κB-mediated gene expression via a yet-unknown signaling pathway (16). On the other hand, invasive *Shigella* triggers the intracellular nucleotide-binding oligomerization domain-containing protein (NOD1) receptor, leading to NF-κB activation (17, 18). It was found that the *Shigella* effectors IpgB2 and OspB activate NOD1 in a guanine nucleotide exchange factor H1 (GEF-H1)-dependent manner upon *Shigella* invasion of epithelial cells (17). In addition to this mechanism, recent reports demonstrate that a ubiquitin coat on cytosolic *Shigella* stimulates further methionine 1 (M1)-linked ubiquitination. This host defense strategy leads to autophagy as well as NF-κB pathway activation (19) (Fig. 1B).

To understand the exquisite specificity by which effector proteins suppress NF-κB, it is first necessary to review the upstream signaling pathways required for its activation. Receptor-mediated activation of NF-κB occurs in a concerted series of steps that involve the assembly of adapter proteins and recruitment of E3 ubiquitin ligases and kinases (Fig. 1). Perhaps the best-studied pathway for NF-κB stimulation is through the inflammatory cytokine TNF. TNF-induced multimerization of TNF receptor 1 (TNF-R1) results in intracellular recruitment of the cytoplasmic adapter proteins TRADD and RIPK1 (20, 21). These proteins function to recruit the E3-ubiquitin ligase complexes TRAF2/5 and cIAP1/2 to TNF-R1. Importantly, this event results in the ligation of K63-linked ubiquitin chains to RIPK1 and cIAP1/2 (22–25). Next, a second E3-ubiquitin ligase known as the linear ubiquitin chain assembly complex (LUBAC) is recruited to K63-linked ubiquitin chains and catalyzes linear M1-linked ubiquitin chains to RIPK1 and NEMO (26–31). Importantly, this concerted recruitment of E3 ligases establishes a signaling platform composed of both M1 and K63 ubiquitin chains that function to recruit the essential kinases required for NF-κB activation to TNF-R1 (32). For example, TAK1 kinase is recruited to K63-linked ubiquitin chains through its interaction with the ubiquitin-binding subunits TAB2 and TAB3 (33). IKKα and IKKβ are recruited to M1-linked ubiquitin chains through their association with IKKγ (NEMO) (34, 35). Once these kinases are brought together, TAK1 phosphorylates IKKβ (36) and IKKβ phosphorylates the IκBα-inhibitory subunit of NF-κB, leading to IκBα degradation by the 20S proteasome (13). The transcription factor NF-κB (composed of subunits p65 and p50) is released from IκBα, exposing a nuclear localization signal resulting in movement into the nucleus and activation of transcription of inflammatory genes (12).

While TNF stimulation of TNF receptor signaling complexes (TNF RSCs) is the prototypic example of NF-κB activation, other cytokines and PAMPs induce inflammation through similar signaling mechanisms (13). However, each pathway utilizes distinct components, including adapter molecules and K63-catalyzing E3 ubiquitin ligases. Here we discuss the current knowledge on effector molecules from EHEC, EPEC, EIEC, and *Shigella* that evolved to target NF-κB pathways at different steps in the signaling cascade (Fig. 1 and Table 1).

EFFECTORS THAT INTERFERE WITH ADAPTER ASSEMBLY

NleB is one of the first effector proteins from A/E pathogens that was found to prevent translocation of the p65 subunit of NF-κB to the nucleus (37, 38). Subsequent studies demonstrated that NleB possesses glycosyltransferase activity and transfers the sugar moiety GlcNac onto GAPDH (glyceraldehyde-3-phosphate dehydrogenase), thereby interfering with GAPDH-TRAF2 complex formation and TRAF2 polyubiquitination upon TNF RSC activation (39). Shortly after this report, another function of NleB in interfering with TNF RSC assembly was uncovered in two reports that showed that NleB transfers GlcNac on arginine residues in death domains in TRADD (Arg 235), FADD (Arg

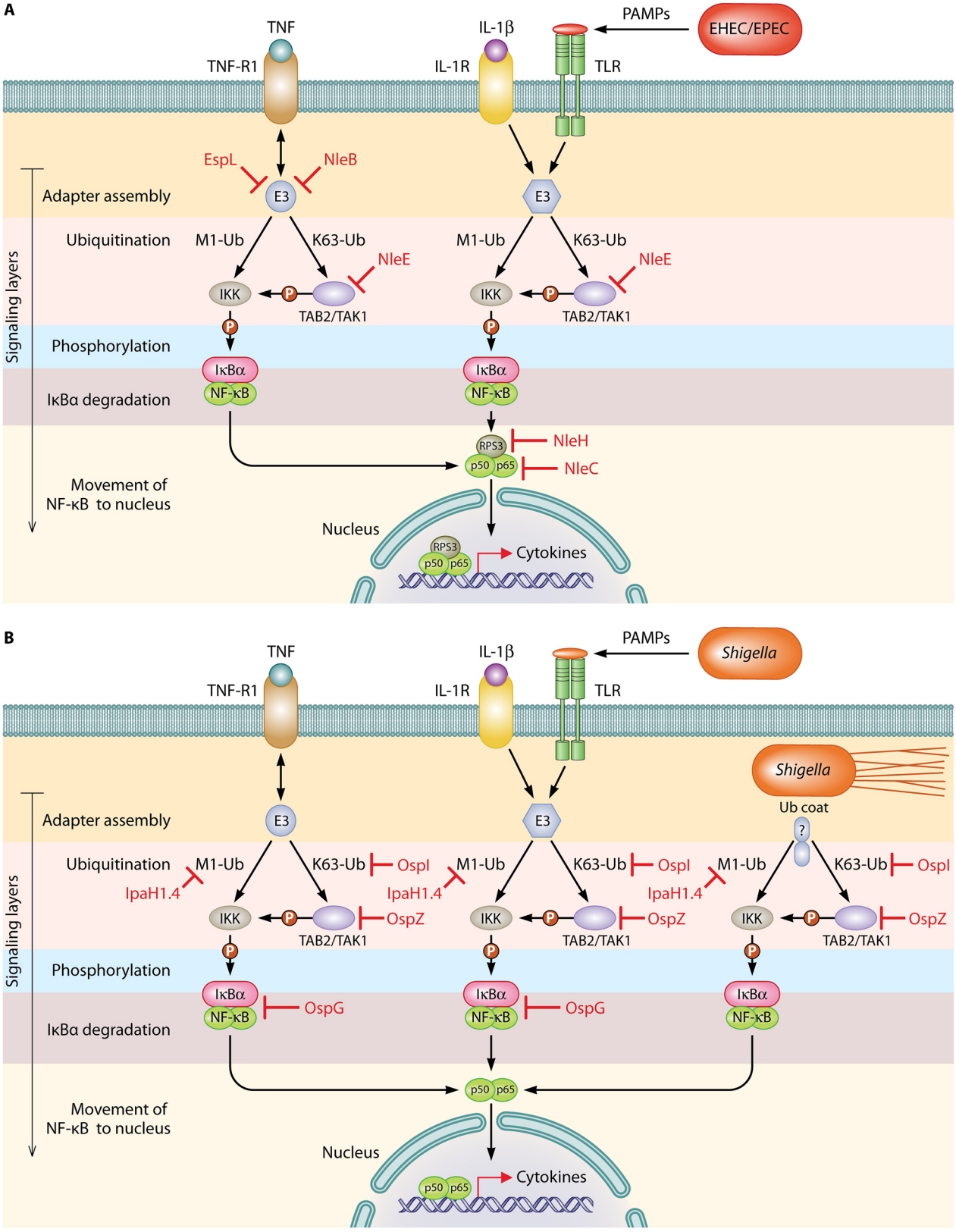

FIG 1 Schematic of NF-κB signaling pathways activated during EHEC/EPEC (A) and *Shigella* (B) infections. Signaling events are shown as layers, starting with the assembly and recruitment of adapter proteins and E3 ligases triggered by cytokine-receptor interaction and propagation of signaling by M1- and K63-linked ubiquitin chains, followed by signaling by TAK- and IKK-mediated phosphorylation, degradation of IκBα, and movement of the transcription factor NF-κB into the nucleus. EHEC/EPEC effector proteins NleB, EspL, NleE, NleH1/2, and NleC and *Shigella*/EIEC effector proteins OspI, OspG, OspZ, and IpaH1.4 and their inhibitory positions within these signaling cascades are shown.

TABLE 1 EHEC, EPEC, and *Shigella* effectors that modulate the NF-κB pathway

Effector(s)	Encoding organisms	Host target(s)	Activity	Reference(s)
NleB	A/E pathogens	TRADD, FADD, RIPK1	Glycosyltransferase	37–43
EspL	A/E pathogens	RIPK1, RIPK3	Cysteine protease	45
OspI	*Shigella* spp.	Ubc13	Deamidase	46–48
IpaH1.4	*Shigella* spp.	HOIP	E3 ubiquitin ligase	19, 52
NleE, OspZ	A/E pathogens, *Shigella* spp.	TAB2, TAB3	Methyltransferase	37–38, 59–62
OspG	*Shigella* spp.	Unknown	Kinase	65–68
NleH1/2	A/E pathogens	CRKL	Kinase	69–71, 73–74
NleC	A/E pathogens	NF-κB (p65)	Zinc protease	11, 75–78, 80, 82–84

117), and RIPK1 (Arg 603, based on homology) (40, 41). The GlcNac moiety interferes with death domain oligomerization, preventing the assembly of the TNF death receptor complex as well as NF-κB activation by TNF (40–42). This unique posttranslational modification prevents activation of necroptosis and NF-κB-mediated transcription of downstream cytokines and chemokines (43). The structure of a FADD-FAS complex (44), which is involved in TNF-induced cell death signaling, reveals that Arg 117 in FADD is located at the binding interface of the two molecules (Fig. 2A), indicating that the GlcNAcylation of this residue prevents interaction between FADD and FAS. Deletion of *nleB* from *Citrobacter rodentium*, a model A/E pathogen, resulted in severe attenuation of colonization of infected mice, and this defect was restored in mice lacking functional FAS (40, 41). These results confirmed the importance of NleB in targeting TNF RSC assembly during infection with an A/E pathogen.

A second effector from A/E pathogens that interferes with TNF RSC assembly is EspL, a cysteine protease with predicted similarity to papain-like proteases. EspL was found to cleave the RIP homotypic interaction motif (RHIM)-containing regions of RIPK1 and RIPK3 during EPEC infection. EspL cleaves RIPK1 between Tyr 544 and Asn 545 and RIPK3 between Asn 463 and Asn 464, leading to complete loss of RIPK1 and RIPK3 and resulting in inhibition of TNF-induced necroptosis and NF-κB activation (45). *Shigella* encodes EspL

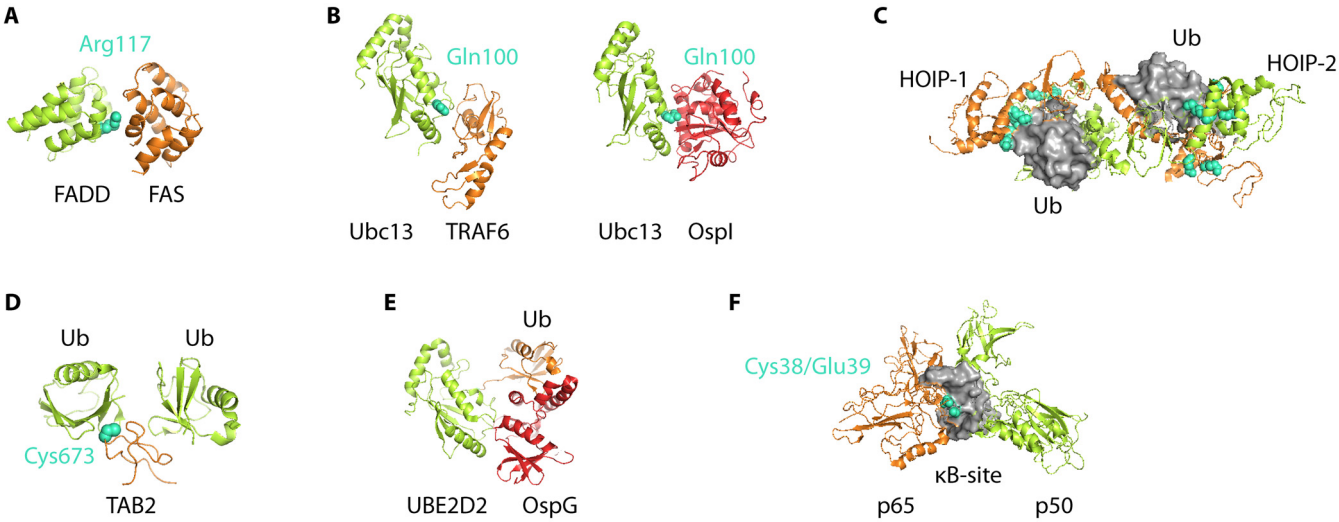

FIG 2 Structural insights into NF-κB suppression by T3SS effector proteins from EHEC, EPEC, EIEC, and *Shigella*. (A) FADD-FAS complex (PDB no. 3OQ9), with FADD residue arginine 117, targeted for GlcNAcylation by EHEC/EPEC NleB, shown in blue. (B) A Ubc13-TRAF2 complex (PDB no. 3HCU) and a Ubc13-OspI complex (PDB no. 3W31), with Ubc13 residue glutamine 100, targeted by *Shigella* OspI for deamidation, shown in blue. (C) Two HOIP molecules in complex with UbcH5b∼ubiquitin conjugate (PDB no. 5EDV). For clarity, the E2 molecules are not shown (ubiquitin is shown in gray). Six HOIP lysine residues, at positions 735, 737, 783, 784, 873, and 875 are targeted by *Shigella* IpaH1.4/2.5 and are shown in blue. (D) TAB2-K63 linked diubiquitin complex (PDB no. 3A9J), with TAB2 residue cysteine 673, targeted for methylation by EHEC/EPEC NleE and *Shigella* OspZ, shown in blue. (E) OspG from *Shigella* in complex with UbcH5b∼ubiquitin conjugate (PDB no. 4Q5E). (F) NF-κB dimer p65-p50 in complex with DNA (gray, IFN-β κB site). The residues cysteine 38 and glutamic acid 39, targeted by EHEC/EPEC NleC, in p65 are shown in blue (PDB no. 1LE5).

homologs OspD2 and OspD3, suggesting that intracellular pathogens could also target RIPK1 and RIPK3 during infection. It is possible that similar to the case for A/E pathogens, *Shigella* targets TNF-R1 assembly to prevent TNF-induced epithelial cell death. In summary, it has become clear that A/E pathogens have evolved several effectors that target the assembly of the TNF-R1 in infected cells with the dual purpose of preventing activation of NF-κB as well as activation of TNF-induced cell death.

EFFECTORS THAT INTERFERE WITH UBIQUITIN-MEDIATED SIGNAL PROPAGATION

As discussed above, K63-linked and M1-linked ubiquitin chains are two important platforms involved in cellular inflammatory signaling pathways. Both A/E pathogens and *Shigella* species have evolved several effector proteins that directly target synthesis of ubiquitin chains or target signal propagation by inhibiting kinase recruitment to the ubiquitin-mediated signaling platform.

OspI, encoded by *Shigella* and EIEC, inhibits the formation of K63-linked ubiquitin chains by deamidating the glutamine residue 100 in the E2-conjugating enzyme Ubc13 to a glutamic acid residue, resulting in inhibition of transfer of ubiquitin from Ubc13 to TRAF6 in active TLR and IL-1β RSCs (46–48). Although this has not been directly demonstrated, the targeting of Ubc13 by OspI could disrupt E2-E3 complex formation, as Gln 100 is present in the Ubc13-TRAF6 binding surface (49) (Fig. 2B).

IpaH1.4 and IpaH2.5, which are among a total of 12 IpaH proteins encoded on the *Shigella* and EIEC virulence plasmid and chromosome (50, 51), were recently found to inhibit activation of NF-κB mediated by M1-linked ubiquitin chains in TNF, IL-1β, and PAMP RSCs (52). Proteins of the IpaH family were first identified as E3 ligases with the discovery of IpaH9.8 from *Shigella* targeting the yeast mitogen-activated protein kinase kinase (MAPKK) Ste7 for degradation by the proteasome (53). In addition, an *in vitro* ubiquitination assay showed IpaH9.8-dependent ligation of K48-linked polyubiquitin chains to Ste7. Although IpaH proteins form an ubiquitin thioester intermediate similar to HECT E3 ligases, structures of IpaH1.4 and IpaH3 showed that IpaH proteins are E3 ligases with a novel fold (54, 55). Studies by our group demonstrated that IpaH1.4 and IpaH2.5 interact with both HOIP and HOIL-1, two subunits of the host LUBAC E3 ligase, and modify 6 lysine residues in the HOIP RING-between-RING catalytic domain with K48-linked ubiquitin chains, leading to HOIP degradation by the proteasome (52, 56, 57) (Fig. 2C). In cellular models of infection, IpaH1.4, but not its close homolog IpaH2.5, is secreted by *Shigella* and suppresses TNF-, IL-1β- and PAMP-mediated activation of NF-κB specifically through inactivation of M1-linked ubiquitin chain ligation by LUBAC (52). Recently, LUBAC was shown to be recruited to the ubiquitin coat of cytosolic bacteria and to generate M1-linked ubiquitin chains on the surfaces of these bacteria, leading to bacterial growth restriction though activation of autophagy and the NF-κB pathway (19, 58). *Shigella* IpaH1.4 was shown to inhibit the formation of this M1-linked ubiquitin signaling platform on the surfaces of cytosolic bacteria (19) (Fig. 1).

NleE from A/E pathogens and its *Shigella* homolog OspZ were initially found to inhibit NF-κB activation by blocking p65 translocation into the nucleus (37, 38, 59). However, a subsequent study clarified this mechanism by establishing NleE as an *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase that methylates Cys 673 in TAB2 and TAB3 of the essential TAB-TAK kinase complex (60, 61) (Fig. 2D). A conserved motif present in both NleE and OspZ (amino acids 49-GITR-52) is required for binding to TAB2/3 (61). Methylation of Cys 673 in the Npl4 zinc finger (NZF) domain of TAB2/3 by NleE resulted in loss of the zinc ion and disruption of TAB2/3 binding to K63-linked ubiquitin chains (60, 62). A structure of TAB2 in complex with K63-linked diubiquitin revealed that Cys 673 in TAB2 is involved in forming a hydrophobic surface that interacts with ubiquitin (63, 64). The addition of a methyl group by NleE on this critical cysteine likely interrupts the TAB2-ubiquitin binding interface directly and also indirectly through loss of the zinc ion from the NZF domain (60). Thus, NleE and OspZ provide A/E pathogens and *Shigella* with another effector to suppress NF-κB activation by disrupting TAB-TAK complex recruitment to K63-linked ubiquitin chains.

EFFECTORS THAT DIRECTLY TARGET NF-κB OR PREVENT ITS TRANSLOCATION TO THE NUCLEUS

The first effector found to be involved in immune modulation by *Shigella* was OspG (65). OspG is encoded on the virulence plasmid of EIEC and all four *Shigella* serogroups (4, 51) and shares sequence similarity with eukaryotic protein kinases of the RIO family (65). Interestingly, OspG interacts with host E2∼ubiquitin (UbcH7∼ubiquitin and UbcH5b∼ubiquitin) conjugates that stabilize the N-terminal and C-terminal lobes and the OspG active site (Fig. 2E). OspG has been shown to reduce degradation of IκBα, thereby inhibiting NF-κB translocation to the nucleus and suppressing inflammation in cellular and animal models of *Shigella* infection (65–68). Surprisingly, no direct OspG substrate has been identified, and it will be interesting for future studies to understand the role of the OspG kinase domain in NF-κB activation. It is possible that the main function of OspG in this pathway is to sequester an E2∼ubiquitin conjugate that is required for function of an essential RSC E3 ligase.

Both NleH1 and NleH2, from A/E pathogens, are homologous to *Shigella* OspG kinase and also suppress activation of NF-κB (69–71). However, unlike OspG, ubiquitin does not promote catalytic site activation in NleH1 and NleH2 (68). NleH1 and NleH2 bind but do not phosphorylate 40S ribosomal protein S3 (RPS3). RPS3 has been shown to be part of NF-κB complexes in the nucleus and enhances binding activity of p65 to a subset of NF-κB-dependent promoters (72). In *in vitro* kinase assays NleH1 was found to phosphorylate CRKL, resulting in recruitment of NleH1 to the IKKβ complex (73), and this recruitment was proposed to reduce nuclear translocation of RPS3 as well as of p65, leading to suppressed activation of RPS3-dependent NF-κB promoters (69, 72, 74).

An effector protein present in A/E pathogens, but not in *Shigella* or EIEC, is NleC, a zinc protease that directly cleaves the p65 subunit of NF-κB (11, 75–78). Cleavage occurs between Cys 38 and Glu 39 of p65, in a region of p65 that is important for NF-κB contact with the κB site in DNA (79–84). In particular, Cys 38 is an important residue that directly interacts with phosphate in the DNA backbone (79, 81) (Fig. 2F). The N-terminal 20 amino acids of p65, which are not conserved in other NF-κB Rel family members, are required for cleavage by NleC, suggesting that NleC specifically targets the p65 subunit of NF-κB (80). As the cytoplasmic pool of p65 is too large for NleC to cleave, it has been suggested that the 38-amino-acid N-terminal p65 fragment binds to RPS3 and inhibits interaction with functional NF-κB, thereby preventing the transcriptional activation of a subset of RPS3-dependent NF-κB genes (72, 80). The finding that A/E pathogens encode multiple effector proteins that target the transcription of RPS3-dependent NF-κB genes suggests that this pathway is important in controlling infection with these extracellular pathogens.

COOPERATIVITY BETWEEN EFFECTOR PROTEINS

The presence of multiple immune-suppressive effectors in both A/E pathogens, *Shigella*, and EIEC suggests that targeting one RSC component by one effector is not sufficient to deactivate NF-κB activation during infection. This could be explained by several mechanisms at play in the host cell. A target protein could be too abundant for an effector to quickly and efficiently deactivate signal propagation, thus necessitating inactivation of a second site. Alternatively, signaling pathways may encode compensatory mechanisms to overcome loss of a single pathway component. In this case, two or more effectors would be needed to overcome the functional redundancies encoded by these systems. Lastly, multiple signaling pathways could be induced simultaneously during infection (e.g., TLR, IL-1R, and TNFR), leading to NF-κB activation. To target a broad range of receptors, which depend on different signaling components, a larger effector repertoire would be required. Thus, although bacterial effector proteins likely target the weakest link in a pathway or a common signaling node, one effector is likely not sufficient to function as a master NF-κB repressor, explaining the growing number of NF-κB-suppressing effector proteins identified in A/E pathogens and *Shigella* species. In fact, by studying NF-κB activation during infection with strains lacking multiple

immune-suppressive effectors, it was revealed that, in both A/E pathogens and *Shigella*, effectors cooperate to fully repress NF-κB (11, 52).

Recent studies indicate that a combination of NleB, NleC, and NleE is likely required for EPEC to fully suppress NF-κB. It was argued that because not enough effector molecules are translocated during infection, only partial inhibition of TNF-induced NF-κB activation was achieved by each individual effector (11, 37, 38). In addition, the finding that both NleB and EspL target RIPK1 during infection (45) further supports the idea that multiple effectors are required to target a critical signaling hub. NleC possibly also cooperates with NleH1 and NleH2 to suppress activation of RPS3-NF-κB-dependent genes (69, 74, 80). Future studies will be needed to better understand the coordination of EHEC and EPEC effectors during infection.

During infection, the *S. flexneri* effector IpaH1.4 cooperates with OspI by targeting the redundant host signaling platforms M1- and K63-linked ubiquitin chains in IL-1R and pattern recognition receptor (PRR) pathways. Surprisingly, in TNF-R1 signaling, inhibition of signaling through either M1- or K63-linked ubiquitin chains by IpaH1.4 or OspI, respectively, was sufficient to fully suppress NF-κB activation. This shows that the relative requirement of M1- and K63-linked ubiquitin chains varies between receptors. In addition to IL-1R, *Shigella* required the cooperative action of both IpaH1.4 and OspI to deactivate one or more pathways that detect *Shigella* PAMPs during infection (52). One of these pathways is likely the recently described pathway that depends on M1-linked ubiquitin chains for detection of ubiquitin coating on cytosolic bacteria. Formation of these ubiquitin chains was found to be inhibited by IpaH1.4 (19), and it will be interesting to determine the role of OspI in arresting NF-κB activation by this intracellular pathway. In addition to OspI, some *Shigella* strains express functional OspZ that could add to suppression of signaling through K63-linked ubiquitin chains (37). Furthermore, OspG and yet-unidentified effectors may, similar to the case for effector proteins in EHEC and EPEC, target components within the same pathway or in other pathways to effectively inhibit NF-κB activation.

CONCLUSIONS

By comparing bacterial strategies to subvert human NF-κB-mediated immune responses, two general mechanisms of T3SS effector function emerged. (i) Although effector proteins from A/E pathogens and *Shigella*/EIEC display a wide variety of enzymatic activities, a common mode of action appears to be to hinder host protein-protein or protein-DNA interactions to prevent the formation of critical signaling hubs that activate genes involved in innate immunity. (ii) T3SS effectors from enteric pathogens cooperate during infection to effectively suppress host immune receptor signaling pathways. This is likely required due to the complexity and redundancy within host signaling pathways that lead to NF-κB activation during infection. Thus, the study of pathogenic immune-modulatory effectors not only elucidates the molecular mechanisms of these effectors but also reveals the wiring of host inflammatory pathways in more detail.

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