Modulation of Host Cell Processes by T3SS Effectors

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Abstract

The T3SS is a key virulence factor for many pathogenic bacteria including two of the enteric E. coli pathotypes; enteropathogenic and enterohaemorrhagic E. coli. Using this secretion system between 25 and 50 bacterial proteins are translocated directly into the host cell cytosol where they manipulate a variety of host cell processes to establish a successful infection. In this chapter we discuss these translocated proteins in the context of the host proteins and processes that they target - the actin cytoskeleton, small guanosine triphosphatases and innate immune signalling pathways. Many of these translocated proteins have been extensively characterized, which has helped us understand the cellular pathways they target in more detail. A challenge remains in understanding how the specific effector repertoire of each strain cooperates over the course of an infection.

Introduction

Most *E. coli* strains share a common genetic backbone of approximately 4.1 Mbp. However, significant divergence has occurred over the past 4.5 million years as a consequence of Horizontal Gene Transfer (HGT). Lineage specific acquisition of large groups of virulence genes (termed Pathogenicity Islands, PAIs), plasmids and prophages has given rise to a range of *E. coli* pathotypes with genomes up to 1 Mb larger than those of commensal *E. coli* strains. These strains, known as the pathogenic *E. coli*, have the ability to cause a broad range of diseases in different hosts (1, 2).

Six distinct enteric (also known as diarrheagenic) *E. coli* pathotypes are currently recognised. Of these, enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC), the murine pathogen *Citrobacter rodentium* (3), the rabbit diarrheagenic *E. coli* (RDEC) (4) and the emerging pathogen *Escherichia albertii* (5, 6) are characterised by the formation of an ultrastructural lesion on the apical surface of the intestinal epithelium known as an "Attaching and Effacing (A/E)" lesion (7, 8). Therefore, these pathogens are collectively referred to as the A/E pathogens. A/E lesions result from the effacement of the brush border microvilli and significant rearrangement of the actin cytoskeleton underneath adherent bacteria. This ability is conferred on the A/E pathogens by a 35 kb PAI known as the Locus of Enterocyte Effacement (LEE) (9) which encodes a Type 3 secretion system (T3SS) (10, 11) and a suite of effector proteins (12).

The T3SS is critically important for the virulence of the A/E pathogens, and the effectors delivered by the T3SS target many fundamental processes within the infected host cell. Different pathotypes and indeed different isolates within those pathotypes encode a unique T3SS effector repertoire and while we now have an excellent understanding of how many of these effectors function individually, we know less about the cooperation or antagonism of the entire effector repertoire for each strain.

This chapter describes many of the host cell processes targeted by T3SS effectors found in the A/E pathogens. Often this work has been performed with a single EPEC or EHEC strain (or in

some cases with *C. rodentium*). Throughout this chapter when we refer to the pathotype it is with the assumption that the effectors have the same function across the A/E pathogens, unless specifically stated otherwise.

Pathotype definitions: EPEC and EHEC

EPEC and EHEC are the most extensively studied of the *E.* coli pathotypes, and the original pathogenic *E. coli* strain described by Bray and colleagues in 1945 was an EPEC strain (13). The EPEC pathotype can be sub-divided into typical and atypical strains. Typical EPEC carry the EPEC adherence factor (EAF) plasmid that encodes the type IV bundle forming pilus (BFP) responsible for the classic localised adherence phenotype on epithelial cells (14). In contrast atypical EPEC are a diverse group of isolates that are often genetically more similar to other *E. coli* pathotypes than they are to typical EPEC (15). However, due to the presence of the LEE and the absence of shiga toxin these strains are classified as EPEC. The current genetic definition for EPEC and EHEC is therefore based on molecular detection of the EAF plasmid (*bfp*+), the LEE PAI (*eae*+) and shiga toxin genes (*stx*+) with typical EPEC defined as bfp+, eae+, stx-, atypical EPEC as bfp-, eae+, stx- and EHEC as bfp-, eae+, stx+. Phylogenetic analysis further categorises EHEC strains into EHEC1 and EHEC2 and typical EPEC into EPEC1-4 lineages (16, 17).
The diversity within the pathotypes includes diversity within the T3SS effectors that are encoded by each strain. The lineage of the LEE at least partly dictates the effector repertoire for a particular strain, despite many effectors being non-LEE encoded (NLE) (15) and whilst the prototypical EPEC strain E2348/69 encodes at least 25 T3SS effectors (18), some EPEC and EHEC strains can encode up to 50 effectors (19). Table 1 contains a summary of the activity of all currently recognised T3SS effectors from EPEC and EHEC and Figure 1 shows the genetic organisation of phage-encoded effectors from the EHEC strain Sakai. Beyond the effectors already described, it is likely that additional T3SS effectors remain to be discovered from recently sequenced aEPEC strains (15).

Table x.1: Summary of T3SS effector activities.

Effector*	Biochemical Activity	Host target or partner	Reported Function	References
Cif	Glutamine deamidase	Nedd8	Inhibits cell cycle progression	(20)
Efa1/LifA	-	-	A/E lesion formation	(21)
EspH	-	DH-PH domain of RhoGEFs	Actin manipulation, inhibition of phagocytosis	(22, 23)
EspF	-	SNX9, N-WASP, ABCF2, and various actin binding proteins	Membrane remodelling, actin nucleation and induction of apoptosis	(24-26)
EspG	TBC-like Rab GAP	Various Rab GTPases, Arf 1/5/6 and PAKs	Inhibits protein secretion and recycling	(27-29)
EspJ	Amidation and ADP ribosylation	Non-receptor tyrosine kinases	Inhibits phagocytosis	(30-32)
EspK	-	-	Predicted NK-κB inhibition (based on homology to GogB^{Stm})	(33, 34)
EspL	Cysteine protease	RIPK1, RIPK3,	Inhibits necroptosis	(35)

family		TRIF, ZBP1/DAI	and inflammation	
EspM family	WxxxE GEF	RhoA	Stress fibre formation and cell-cell junction disruption	(36-38)
EspN	-	-	-	(18)
EspO family	-	ILK	Predicted cell adhesion (based on homology to OspE^{Sf})	(39)
EspR family	-	-	-	(19)
EspS	-	-	-	(19)
EspT	WxxxE GEF	Rac1, Cdc42	Lamellipodia formation, host cell invasion	(36, 40)
EspV	-	-	Actin remodeling	(41)
EspW	-	Kif15	Actin remodeling	(42)
EspX family	-	-	-	(19)
EspY family	-	-	-	(19)
EspZ	-	CD98	Inhibits excessive cytotoxicity	(43, 44)
Map	WxxxE GEF	Cdc42, EBP50	Filopodia dynamics and cell barrier function	(36, 45)
NleA	-	Sec24, NLRP3	Inhibits protein secretion Inhibits inflammasome activation	(46, 47)
NleB family	Glycosyltransferase	FADD, TRADD, RIPK1	Inhibits extrinsic (death receptor–induced) apoptosis	(48, 49)
NleC	Zinc metalloprotease	P65, p50, p300	NF-kB inhibition	(50-52)
NleD	Zinc metalloprotease	P38, JNK	MAPK inhibition	(50)
NleE	S-adenosyl-l-methionine (SAM)-dependent cysteine methyltransferase	TAB2/3, ZRANB3	NF-kB inhibition and DNA repair	(53, 54)
NleF	-	Caspase 4, 8 and 9	Inhibits intrinsic and extrinsic apoptosis	(55)
NleG family	E3 ubiquitin ligase	-	-	(56)
NleH family	Ser/Thr kinase	Binds BI-1 and RPS3	Inhibition of NF-KB and intrinsic cell death signaling	(57, 58)
NleL (EspX7)	E3 ubiquitin ligase	JNK	A/E lesion formation	(59-61)
NleJ	-	-	-	(18)
TccP	-	IRSp53, IRTKS and various actin-binding proteins	Actin rearrangement and intimate attachment	(62-65)
Tir	-	SHIP2, Nck, IRTKS, IRSp53,	Actin rearrangement and intimate	(62-64, 66-71)

		PI3K,CK18, Talin, Vinculin, α-actinin, cortactin, 14-3-3tau	attachment	

*Family is used to indicate multiple homologs of the effector exist. In some cases binding partners and functions have only been demonstrated for one family member.

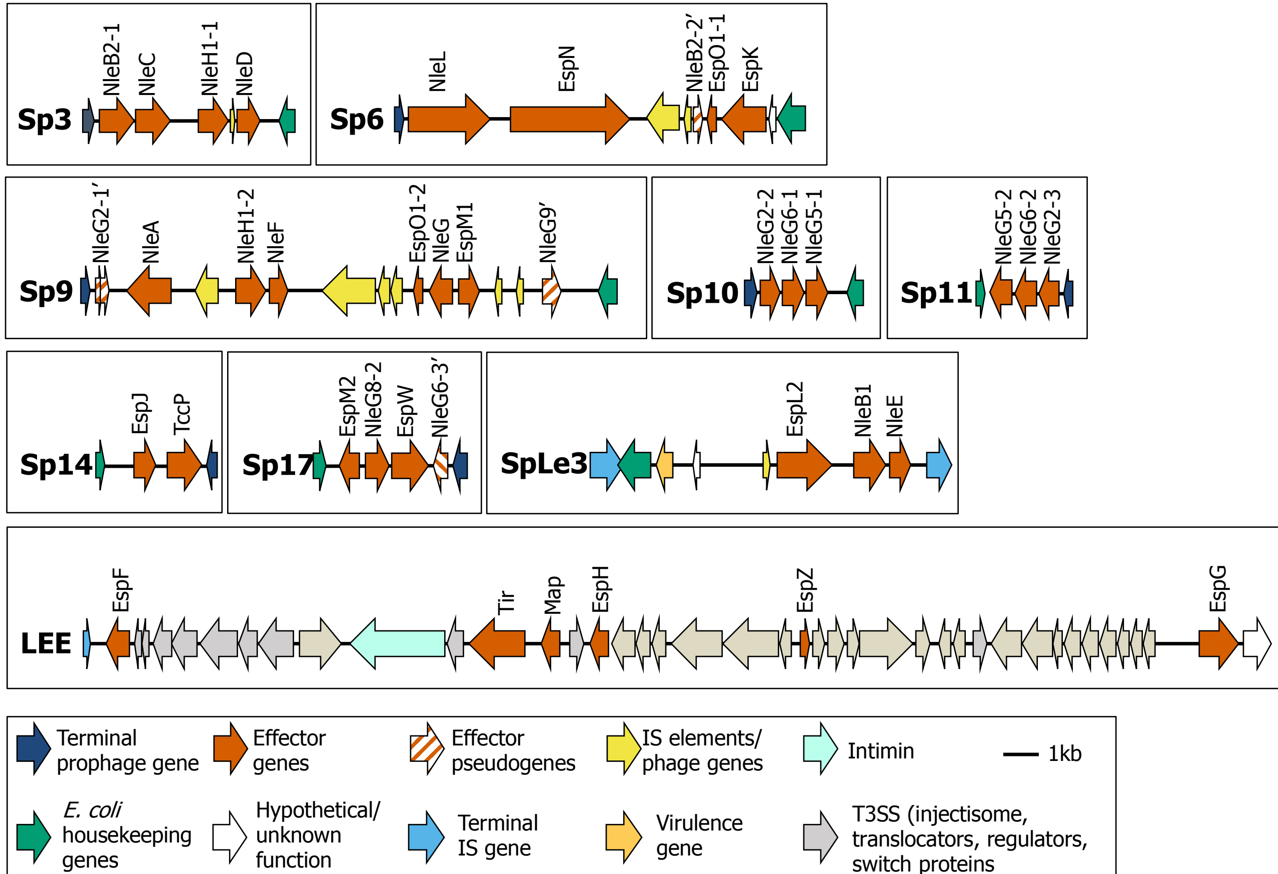

Fig. 1: Exchangeable Effector Loci (EEL) of EHEC O157. 7 EELs within prophages (Sp3, 6, 9, 10, 11, 14 and 17) and the 2 Pathogenicity Islands (SpLe3 and LEE) of EHEC O157 strain Sakai are depicted. Prophage EELs that are predicted to encode only effector pseudogenes have not been included (Sp4 and 12). All prophage EELs are flanked at one end by prophage genes, with only the terminal prophage gene indicated here. Similarly the SpLE3 and LEE PI are flanked by IS elements/remnants, only the terminal ones are shown. Effectors located on non-phage EELs are not shown. Gene and EEL nomenclature follows that of Tobe et al(19). (For further details on the T3SS genes encoded within the LEE PI refer to Figure 1)

In this Chapter we discuss effectors grouped based on their ability to manipulate the actin cytoskeleton, the small Guanosine Triphospatases (GTPases) and innate immune signalling pathways.

Manipulation of actin by Tir

Found in a wide range of eukaryotic cells, where it is often the most abundant protein in a cell, actin is involved in an array of different cellular processes. Actin is extremely dynamic and is found in both monomeric (G-actin) and various filamentous (F-actin) forms. Actin filaments, together with microtubules and intermediate filaments are key components of the cell

cytoskeleton, controlling cell shape and the organisation of cellular components. Considering its central role in the cell it is unsurprising that numerous pathogens target actin and/or actin binding proteins.

Tir, the translocated Initimin receptor, is involved in the subversion of host cell actin and allows the A/E pathogens to intimately adhere to the surface of infected cells and form the A/E lesion (12). Due to this central role, Tir has been studied extensively, and is arguably the most studied of the LEE effectors. Translocated Tir inserts into the host plasma membrane (PM) in a hairpin-loop topology and acts as the receptor for the bacterial outer membrane adhesin Intimin (72, 73). Binding to Tir by Intimin induces Tir clustering, leading to downstream signalling events that result in the formation of actin-rich 'pedestal'-like structures underneath adherent bacteria (65). However, despite its central role, there are differences in the mechanism by which Tir from different *E. coli* pathotypes acts. Typically, EPEC Tir relies on the phosphorylation of a tyrosine residue, Y_{474}, to recruit the host adaptor protein Nck and subsequently neural Wiskott-Aldrich syndrome protein (N-WASP) and the actin-related protein 2/3 (Arp2/3) complex, triggering actin polymerisation beneath attached bacteria (12). On the other hand, EHEC Tir lacks a Y_{474} equivalent and instead promotes Nck-independent actin polymerisation via a conserved Asn-Pro-Tyr motif (NPY_{458}, which is also found in EPEC Tir as NPY_{454}) to recruit N-WASP and Arp2/3 (74). Tir only produces weak actin polymerisation *in vitro* through the NPY_{458} pathway (75) and in order to overcome this and induce robust actin polymerisation EHEC Tir cooperates with the non-LEE encoded effector TccP/$EspF_U$. TccP interacts with Tir indirectly via the adapter proteins IRSp53 and IRTKS (insulin receptor tyrosine kinase substrate) (63, 64). Together, EHEC Tir and TccP/$EspF_U$ strongly activate Arp2/3, resulting in similar actin polymerisation to that seen during EPEC infection (76). Unusually, EPEC-2 strains can use both the Nck and TccP2 pathways to promote actin recruitment at the bacterial attachment site (77).

Intriguingly, whilst Tir is indispensable for A/E lesion formation *in vivo,* none of the pathways discussed above are necessary for the recruitment of N-WASP or A/E lesion formation at mucosal surfaces (78). Therefore other, as yet unknown, host factors must play a role in Tir-mediated actin polymerisation and A/E lesion formation *in vivo*. A growing body of work implicates Tir-mediated signalling in regulation of the lipid content of the host PM at the bacterial attachment site which may play a role in A/E lesion formation *in vivo* (62, 71). Recently an EPEC strain with all T3SS effectors deleted, excepting Tir, was found to form pedestals on cultured cells but not A/E lesions on mucosal tissue (21). When all NLE effectors were absent only marginal A/E lesions could be observed indicating that at least one NLE effector contribute to Tir-mediated A/E lesion formation. One example of this is the ubiquitination of JNK by NleL, which has been demonstrated to contribute to bacterial attachment and A/E lesion formation by EHEC, but is not present in EPEC (59). Therefore while Tir is central to actin polymerisation and A/E lesion formation it does not act alone in these processes.

Modulation of host small GTPases

After the translocation of Tir, the T3SS translocates additional effectors, detailed in Table 1. T3SS effectors function to repurpose host cell processes, creating a favourable environment in the gut for the replication and onward transmission of the infecting pathogen. Whilst EPEC/EHEC effectors manipulate a wide variety of host cell processes, subversion of host small GTPases is a recurring theme in effector function, and an important paradigm in host-pathogen interaction more broadly. Due to the involvement of small GTPases in almost all

essential cellular processes, a variety of pathogenic organisms have evolved to target small GTPases as a means to manipulate host cell function, with EPEC/EHEC being no exception.

Small GTPases are evolutionarily conserved hydrolase enzymes that function as molecular switches to control, amongst other things, protein recycling and actin dynamics at the plasma membrane (79, 80). Six major subfamilies make up the small GTPases; Ras, Rho, Rab, Ran, ARF and MIRO. These subfamilies are grouped together based on amino acid sequence, structure and cellular roles (81, 82), but common to all small GTPases is the ability to bind guanosine triphosphate (GTP) and hydrolyse GTP to guanosine diphosphate (GDP), via the universal 20 kDa "G-domain" (83).

In order to efficiently cycle between the GTP-bound active state and the GDP-bound inactive form at specific times and in specific locations within the cell, small GTPases interact with a number of accessory proteins, known as Guanine-nucleotide Exchange Factors (GEFs), GTPase Activating Proteins (GAPs) and in the case of the Ras, Rho and Rab subfamilies (all of which are prenylated at their C-termini) Guanine Dissociation Inhibitors (GDIs) (84). Together, these accessory proteins allow small GTPases to exert fine spatiotemporal control over a range of different cellular events.

GEFs activate small GTPases, and whilst many structurally distinct eukaryotic GEF families exist the mechanistic basis for their activation of small GTPases is conserved. In the cytosol, GEFs form a transient complex with the GDP-bound form of their cognate GTPase, promoting the dissociation of GDP and the recruitment of GTP to the nucleotide-free GEF/GTPase complex. GTP binding displaces the GEF, creating the GTP-bound, active form of the GTPase (85).All small GTPases undergo conformational changes when GTP-bound to allow the recruitment of effector proteins and the stimulation of downstream signalling cascades. For the Ras, Rho, Rab, ARF and MIRO GTPases activation results in both effector recruitment and insertion into cellular membranes, consistent with the roles of these GTPases in directing membrane traffic, cytoskeletal rearrangements and organelle movement (84, 86).

Acting in opposition to GEFs, GAPs promote the inactivation of small GTPases by catalysing GTP to GDP hydrolysis. Classically, nucleotide hydrolysis occurs through the provision of an arginine by the GAP (the conserved "arginine finger" motif) to the GTPase. This results in a conformational change leading to GTP-hydrolysis (85). In the case of the prenylated GTPases (Ras, Rho, Rab) GAPs act in concert with GDI's, a third family of regulatory proteins that aid in the extraction of these GTPases from membranes. Once a prenylated GTPase has been extracted from a membrane, the GDI remains associated with the GDP-bound GTPase, maintaining it in a soluble state (84).

Subversion of small GTPases by EPEC/EHEC effectors is frequently achieved through mimicry of these accessory proteins, often, but not exclusively, to modulate Rho family GTPases.

Rho GTPases

The Rho GTPases were the second family of small GTPases to be described in humans, following their initial description in *Aplysia* spp. (sea slugs) (87). The 20 Rho GTPases are subdivided into 'classically activated' and 'atypical' proteins. Classically activated Rho GTPases are regulated by GEFs and GAPs, as described above, and are subdivided into 4 families based on amino acid sequence, the Rho subfamily (RhoA, RhoB, RhoC); the Rac

subfamily (Rac1, Rac2, Rac3, RhoG); the Cdc42 subfamily (Cdc42, RhoQ and RhoJ) and the RhoF/RhoD subfamily.

The Rho subfamily (RhoA, RhoB and RhoC) are involved in the regulation of the actomyosin cytoskeleton and contractile stress-fibre formation. Activation of RhoA and/or RhoC (which share 92% amino acid identity (80)) in response to extracellular stimuli recruits the Rho-associated coiled-coil containing kinase (ROCK), a serine/threonine kinase involved in the formation of focal adhesions and actin stress fibres, acting in opposition to lamellipodia formation and cell migration (88). RhoB, which shares 84% amino acid identity with RhoA (80), is involved in the regulation of endocytic trafficking, and may play a role in the regulation of epithelial cell-cell contacts (89).

The Rac proteins act in opposition to the Rho subfamily and serve, in conjunction with other small GTPase families, to promote cell migration, membrane ruffling and lamellipodia formation by promoting cortical actin polymerisation (88, 90). This occurs primarily through the activation of N-WASP and subsequent recruitment of the WASP family veroprolin-homolog (WAVE) regulatory complex (WRC), leading to Arp2/3-mediated actin polymerisation.

The Cdc42 family coordinates the actin cytoskeleton and apical-basolateral polarity in many eukaryotes, through regulation of filopodia and recruitment of the Par complex. Cdc42 promotes actin polymerisation, the bundling of F-actin into filopodia and the membrane curvature necessary for cell protrusions to form (80) whilst also recruiting the Par complex (91) to the apical-lateral border of epithelial cells (80). Localisation of the Par complex at the apical domain allows the polarised trafficking of proteins and the formation of polarised cell-cell barriers, such as tight junctions.

Map, EspT and EspM mimic host Rho GEFs

Rho GTPase function is manipulated by both intracellular and extracellular pathogens during infection, including *Shigella* spp., *Salmonella enterica* sv. Typhimurium (STm), *Pseudomonas aeruginosa, Yersinia* spp., EPEC and EHEC to facilitate bacterial invasion, and, in the case of extracellular pathogens, avoid unwanted internalisation into host cells. The WxxxE family of proteins are a major group of effectors found across Gram-negative pathogens. These effectors acts as Rho GEFs, mimicking host proteins to activate endogenous Rho GTPases (36) (92, 93). The EPEC/EHEC effectors Map (92), EspT (94) and EspM (19, 37) are members of this family.

The LEE-encoded effector Map acts as a Dbl-family GEF mimic, activating Cdc42 (92) to induce the formation of transient filopodia around infecting bacteria (95) before re-localising to the mitochondria via an N-terminal mitochondrial targeting sequence (95, 96). Whilst the contribution of filopodia formation to the infectious process of A/E pathogens remains unclear, a Δ*map* mutant of *C. rodentium* is attenuated *in vivo* and shows a significant colonisation defect (97). Intriguingly, the induction of filopodia by Map has been shown to be dependent on Map's binding to ERM-binding phosphoprotein 50 (EBP50) at the plasma membrane (36, 98). However, the molecular details of Map's dependence on this interaction also remain unclear. In addition, Tir regulates Map-induced filopodia formation (45, 95) and together Tir and Map have been implicated in both the effacement of microvilli during infection and the rapid loss of function of the host sodium-glucose transporter SGLT-1 (99). This may have relevance for understanding the rapid-onset watery diarrhoea induced by

EPEC/EHEC, and provides a possible explanation as to why this watery-diarrhoea is refractive to oral rehydration therapy in severe cases of infection (99). In addition, the interaction between Map and EBP50 has been shown to contribute to the development of diarrhoea during infection (98), possibly though modulation of intestinal barrier function (100). The multiple phenotypes attributed to Map reflect the diverse roles of Cdc42 as a modulator of actin dynamics, cell cycle progression, cell polarity and membrane trafficking (101).

EspT is a WxxxE effector found in a small subset of EPEC strains (102). EspT-carrying strains are capable of invading non-phagocytic cells and forming intracellular actin pedestals (94). Like the other WxxxE effectors, EspT is a GEF mimic, activating the host Rho GTPases Rac1 and Cdc42 (40), resulting in the formation of lamellipodia and membrane ruffles on the surface of infected cells and subsequently the intracellular phenotype described above. The intracellular pathogens *Shigella flexneri* and STm also make use of this method of cell invasion (103). Interestingly, despite the small number of EPEC strains that carry EspT, the causative strain of an unusual outbreak of EPEC in Finland in the winter of 1987, in which adults as well as children were affected was found to carry EspT (104). It is tempting to speculate that the presence of EspT may have been responsible for the expanded host range of this particular strain.

The homologous WxxxE effectors EspM1 and EspM2 were initially identified in EHEC O157:H7 Sakai using a bioinformatics screen for homologues of known T3SS effectors (19), and share significant sequence identify with the EPEC B171 effector TrcA, another WxxxE effector (37). EspM effectors are RhoA GEFs (105) and EPEC/EHEC strains that carry EspM effectors are associated with severe human infections (102).

Whilst all EspM effectors induce the formation of actin stress fibres within infected cells (37, 38), the phenotypes displayed by the EspM effectors are subtly different. For example, whilst both EspM1 from EHEC O157:H7 Sakai and TrcA from EPEC B171 induce the formation of parallel stress fibres that are confined to the bacterial infection site, EspM2 from EHEC O157:H7 Sakai induces parallel stress fibre formation throughout infected cells, which are linked to the plasma membrane through focal adhesions (37). Stress fibre formation is a consequence of RhoA activation and downstream signalling mediated by the recruitment of the RhoA effector ROCK (37, 105).

In addition to promoting stress fibre formation, EspM1 and EspM2 disrupt the architecture of a polarised cell monolayer when translocated into epithelial cells during EHEC infection. This phenotype is dependent on RhoA (38) and is likely a consequence of the mislocalisation of both tight junction proteins and the basolateral protein β1-integrin that is induced by EspM, although it is important to note that EspM does not seem to decrease the barrier function of tight junctions (38). In conjunction with their effect on stress fibre formation, the ability of the EspM effectors to disrupt the integrity of the cell monolayer is indicative of the pleotropic consequences of aberrant RhoA activation. This is particularly noteworthy, as RhoA GTP-GDP exchange does not disrupt the RhoA/EspM complex, suggesting that RhoA is activated irreversibly upon binding of EspM (105). On this point, it is interesting to note that the EHEC T3SS effectors EspO1 and EspO2 have been shown to interact directly with EspM2 to counteract stress fibre formation and prevent cell detachment caused by excessive RhoA activation (106). Finally, both EspM1 and EspM2 have been reported to modulate pedestal formation, supressing this process early during EHEC infection. However, the relevance of this accessory role in pedestal formation during infection is unclear (38). Taken together, the diverse phenotypes attributed to EspM demonstrate the multifaceted nature of RhoA's regulation of the actin cytoskeleton and cell-cell junctions.

EspH inactivation of host GEFs

Operating in conjunction with the EPEC/EHEC WxxxE effectors (Map, EspT and EspM) the LEE-encoded effector EspH inactivates multiple host Rho GEFs through binding to their Dbl-homology and pleckstrin-homology (DH-PH) domain, preventing Rho GTPase activation (22). In this way EspH represses filopodia formation and enhances pedestal formation beneath adherent bacteria (23), acting in concert with Tir to recruit N-WASP to the bacterial attachment site (107). In addition, EspH in isolation promotes cell detachment and caspase-3 activation through the disassembly of focal adhesions (108) and is able to prevent phagocytosis of EPEC by macrophages (22). Ingeniously, EspH does not affect the WxxxE effectors, allowing EPEC/EHEC to replace the endogenous Rho GEFs with bacterial mimics to exclusively control Rho GTPase activation in infected cells (108). In fact, EspT and EspM (but not Map) counteract the focal adhesion disassembly induced by EspH, suggesting that EspH acts in collaboration with NLE WxxxE effectors that are also present in the infecting strain to negate its negative consequences and completely control host Rho GTPase function in infected cells (108).

EspW targets microtubules and control of cell shape

First identified in the same bioinformatics screen which identified EspM1 and EspM2 (19), EspW has subsequently been found throughout the sequenced EHEC O157:H7 strains as well as in a number of non-O157:H7 EHEC strains, EPEC O111:H9 and a range of EPEC clinical isolates (42). A truncated version of EspW, $EspW_{1-206}$ has also been observed in the EHEC O157:H7 progenitor strain EPEC O55:H7 (42, 109), although the function of this truncated protein remains unknown.

Unlike Map, EspT and EspM, EspW does not possess the WxxxE motif. Instead, full length EspW promotes Rac-1-dependent actin remodelling during infection via interaction with the C-terminus of the host microtubule motor Kinesin-12 (Kif15) (42), a homotetrameric protein (110) capable of forming parallel bundles of microtubules *in vitro* (111). Ectopically expressed EspW promotes the formation of large flower-shaped actin ruffles on the surface of cells, and during EHEC infection Kif15 is recruited to the actin pedestal where it localises with EspW. However, EspW is not required for the recruitment of Kif15 to the site of EHEC attachment, and instead Kif15 may serve to restrict EspW to the pedestal (42). Instead, the absence of *espW* results in significant cell shrinkage and rounding during EHEC infection, a phenotype that can be rescued by chemical activation of Rac1 (42). Therefore, it appears that EspW plays a role in maintaining cell shape during infection, via Rac1 mediated actin rearrangement, although the putative link between EspW's interacting partner, Kif15, and Rac1 remains to be elucidated.

EspG manipulates cell-cell contacts and the host cell surface

Like Map and EspH, EspG is a LEE-encoded effector and thus is highly conserved across EPEC and EHEC strains. In fact, EspG is one of the most conserved effectors across the A/E pathogens (16). However, unlike the effectors discussed above, EspG does not target host Rho GTPases, but rather ARF and Rab GTPases (Figure 2).

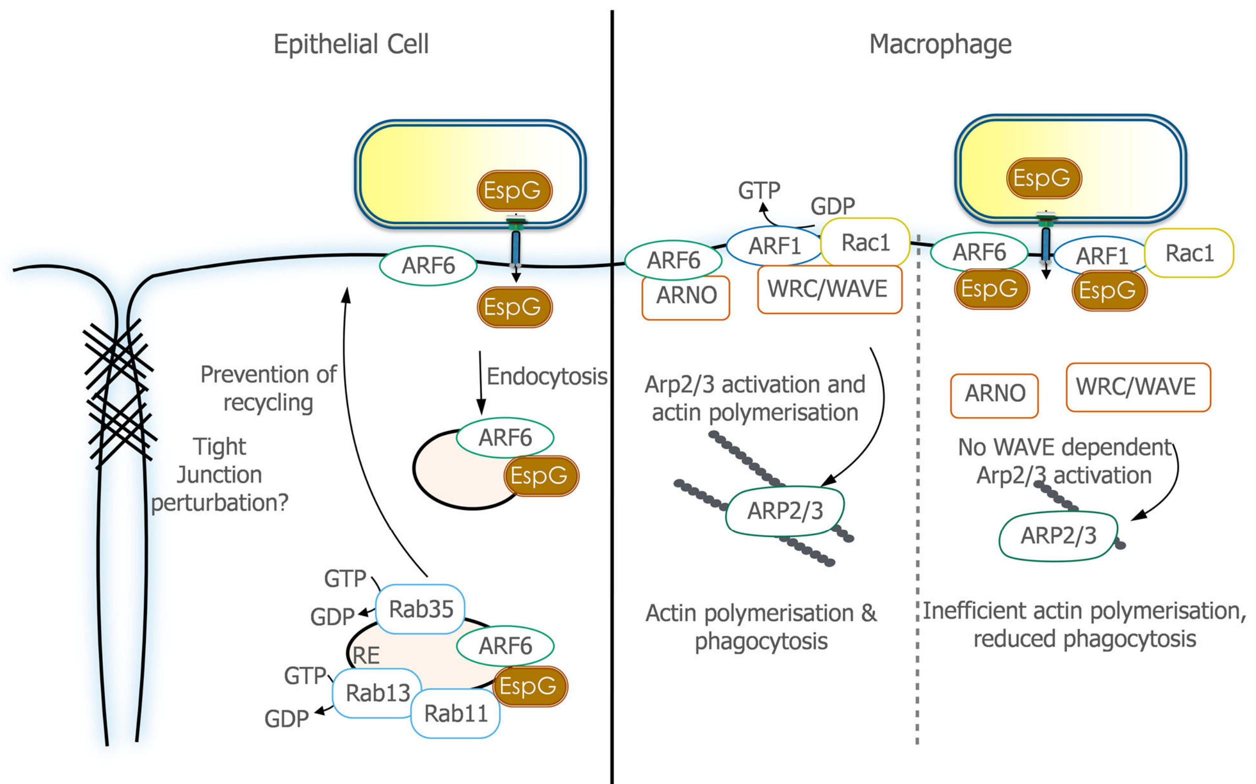

Fig. 2: Host cell targets of the LEE-encoded effector EspG. During infection of host epithelial cells, EspG binds to ARF6 whilst also interacting with Rab35. In this way EspG leads to the formation of enlarged "stalled recycling structures" (29) and prevents the recycling of endocytosed host proteins back to the PM (112). EspG also alters paracellular permeability via modulation of tight junctions, although the mechanism of this is unclear at present (113, 114). In macrophages, EspG prevents phagocytosis of attached bacteria by binding to ARF6 and ARF1, ultimately preventing the WAVE-dependent Arp2/3-mediated actin polymerisation necessary for efficient uptake of the bacteria into the macrophage (115).

EspG is able to bind to ARF GTPases, likely promoting the recruitment of a specific subset of ARF-effectors (28, 116) whilst simultaneously acting as a TBC domain-like Rab GAP (27). Thus EspG has been referred to as a "catalytic scaffold". During infection of epithelial cells, EspG binds to the GTP-bound, active form of the ARF GTPase ARF6 (27-29) whilst simultaneously directing its Rab GAP activity towards the Rab GTPase Rab35 (29), disrupting this important signalling axis (79, 112). In epithelial cells, EspG has been implicated in the modulation of host tight junctions (113, 117, 118) as well as in the removal of a range of host cell surface proteins from the plasma membrane (112, 118-120). The ability of EspG to affect a range of cell surface and cell-cell junction proteins likely arises from its ability to prevent correct recycling of host cell surface proteins, likely via the formation of "stalled recycling structures" (29) a phenotype that is dependent on EspG's ability to function as a Rab GAP (112). However, the breadth of EspG's effect on the host cell surface is yet to be determined.

With regard to EspG's promotion of the GTP-bound, active form of ARF6, elegant *in vitro* work in which membrane-associated actin polymerisation was reconstituted using phospholipid-coated beads has revealed that EspG is able to block recruitment of the ARF6 effector ARNO, preventing formation of the WRC, an important mediator of macropinocytosis and phagocytosis. In this way EspG plays a role in resisting phagocytosis by macrophages (115).

Whether this role for EspG stabilised ARF6 is restricted to phagocytic cells, or plays a role during infection of epithelial cells, remains to be determined.

Conclusion

Modulation of host small GTPases is central to the infection strategy of EPEC and EHEC and represents a fundamental strategy employed by pathogenic organisms. Of the six LEE-encoded effectors, three (Map, EspH and EspG) are known to modulate one or more host small GTPases either directly (Map, EspG) or indirectly (EspH), whilst Tir also possesses a putative GAP domain (95). A number of NLE effectors also target small GTPases. The WxxxE effectors EspT and EspM act as Rho GEF mimics in order to modulate the host cytoskeleton, whilst EspW activates Rac1 through binding to the microtubule motor Kif15. The glutamine deamidase Cif reduces RhoA protein levels and increases stress fibres, which exemplifies yet another strategy of manipulating small GTPase function. Many effectors that target small GTPases do so in order to allow manipulation of the actin cytoskeleton, a hallmark of A/E pathogen infection. The mechanism by which these effectors cooperate with and/or antagonise each other and Tir, and how the spatial and temporal control necessary for these complex processes to occur is achieved remains to be determined.

In addition, whilst much progress has been made towards understanding the molecular details of GTPase subversion by individual EPEC/EHEC effectors, a number of unanswered questions remain, particularly regarding the effectors EspG and EspW. Simultaneously, bacterial effectors that manipulate host GTPase function are excellent tools for the study of fundamental eukaryotic cell biology.

NF-κB and Cell-death Signalling in Host Cells

Mammalian innate immunity relies on the detection of microbes by families of pattern-recognition receptors such as the Toll-like receptors (TLRs), NOD and leucine rich repeat domain containing proteins (NLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs), among others (121-123). Proteins from most of these families induce transcriptional upregulation of cytokines that are responsible for inflammation, which if left unchecked can lead to tissue damage. Among the most pro-inflammatory cytokines are Tumour necrosis factor (TNF) and Interleukin-1 (IL-1) family proteins, which also upregulate their own expression in a feed-forward mechanism (123). Thus, cytokine production amplifies inflammation and immunity, and as we discuss in the following sections, EPEC and EHEC have evolved several mechanisms to potently suppress or evade inflammatory and cell death signalling (summarised in Figures 3 and 4).

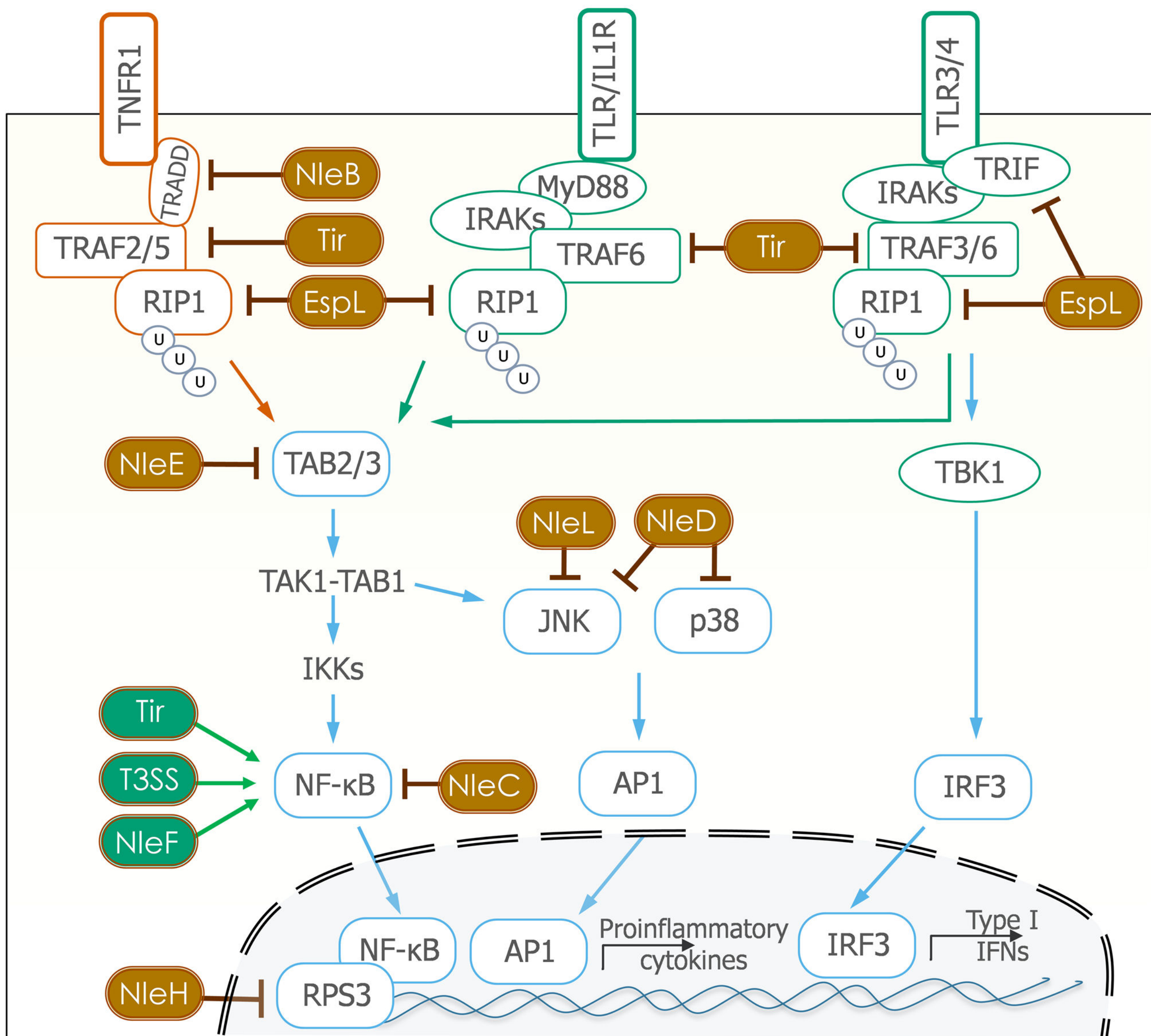

Fig. 3: Manipulation of TLR, TNFR1 and IL1R signalling by EPEC/EHEC. Schematics show signalling by TNFR1 via TRADD, TLR/IL-1-like receptors via MyD88 and TLR3/4 signalling via TRIF. Inhibitory effectors (EspL, NleB, NleC, NleD, NleE, NleL and Tir) are shown in brown and activating effectors (NleF, a functional T3SS and Tir) are shown in green. TRADD, TRAF6 and RIPK1 are prominent receptor-proximal proteins targets, whereas JNK, p38 and NF-κB exemplify downstream signalling targets in the host.

Detection of microbial molecules through the TLRs results in nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-dependent transcriptional upregulation of proinflammatory cytokines. The TLRs and IL-1 receptor-1 (IL1R1) share sequence similarity in their intracellular region called the Toll-IL-1 receptor domain (TIR) and signalling by these families of receptors is similar (123). Intestinal epithelial cells (IECs) express several pattern recognition receptors and cytokine receptors, including TLR4, TLR5, TNF-receptor, IL-1-receptor and IL-18-receptor (related to IL1R1), and are therefore capable of initiating as well as responding to inflammation.

TIR domains recruit the adaptor Myeloid differentiation primary response 88 (MyD88) to activate mitogen activate protein kinase (MAPK) and NF-κB pathways through the

recruitment of a number of protein kinases, ubiquitin E3 ligases and ubiquitin binding proteins, among others. Some TLRs, such as TLR4 and TLR3, also signal via the adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF) which also has a TIR. MyD88 recruits and activates IL1R-associated kinase (IRAK) family kinases that in turn recruit the TNF-R associated factor (TRAF)-family E3 ligases TRAF6 and TRAF3. Ubiquitylation of the receptor complex recruits the Transforming growth factor beta-activated kinase 1 (TAK1; also called MAP3K7) via the ubiquitin-binding proteins TAK1-binding proteins 2 and 3 (TAB2 and TAB3). TAB2/3 bind polyubiquitin chains and recruit a TAK1-TAB1 complex, resulting in TAK1 K63 ubiquitylation by TRAFs at the receptor. TAK1 is a central regulator of NF-κB and MAPK and is involved in a range of innate immune pathways. Activation of NF-κB via TAK1 is called canonical NF-κB signalling, which is common to TLRs, IL-1 receptors and TNFR1 signalling (123-125). In contrast, TLR3/4-signalling via TRIF induces type I interferons (IFNs) via the interferon stimulatory factor (IRF) family of transcription factors.

Active TAK1 phosphorylates and activates IκB kinases (IKKs) and MAPKs. The IKKs phosphorylate the Inhibitor of NF-κB inhibitory proteins (IκB), resulting in its proteasomal degradation, nuclear translocation of NF-κB and subsequently transcriptional activation of NF-κB regulated genes. The three MAPK branches, such as Jun N-terminal Kinase (JNK), Extracellular Signal-Regulated Kinase (ERK) and p38 MAPK phosphorylate and induce the nuclear translocation of the Activator protein 1 (AP1) transcription factor. The synergistic action of NF-κB and AP1 enhances gene transcription, inflammation and antimicrobial responses.

TNF signalling activates canonical NF-κB via TAK1 via markedly different receptor-proximal mechanisms (125, 126). TNF-family receptors have death domains (DD) in their intracellular regions that recruit DD-containing adaptors instead of TIR-containing adaptors. The TNFR1 adaptor TNFR1-associated DD-containing protein (TRADD) recruits receptor interacting serine/threonine kinase 1 (RIPK1) and a different set of TRAF E3 ligases, including TRAF2, TRAF5 and the cellular inhibitors of apoptosis (cIAP) proteins. Ubiquitylation of RIPK1, by both K63 and linear chains, recruits TAK1 via TAB2/TAB3. Downstream induction of NF-κB and MAPK-dependent genes sustains inflammation.

Another unique aspect of DD-containing receptors is their ability to activate apoptosis or necroptosis by forming distinct receptor complexes. These complexes differ depending on the context (e.g. differential ubiquitylation) and cell-type (e.g. myeloid versus epithelial cells). For example, TNF does not induce death in most cells but increases NF-κB-dependent proinflammatory cytokine production and inflammation. NF-κB-activation by TNF results in the upregulation of several NF-κB-dependent genes that prevent cell death, including cellular inhibitor of apoptosis (cIAPs), B-cell lymphoma 2 (BCL2) proteins, the caspase-8-inhibitor cellular FLICE-like inhibitory protein (cFLIP) and the ubiquitin-editing enzyme A20, among others. Altered signalling by TNF can, however, induce apoptosis via a process called receptor-induced apoptosis signalling, also known as the cell-extrinsic signalling pathway (126).

The outcome of TNF-signalling is finetuned by ubiquitylation and caspase-8 activity. Reduced ubiquitylation of the TNFR1-complex, for example by deubiquitylating enzymes or reduced activity of cIAPs E3 ligases, results in the formation of an apoptosis-inducing cytosolic complex that contains a related adaptor Fas-associated protein with death domain (FADD), RIPK1 and caspase-8 (126). Catalytic activation of caspase-8 into its p18/p10 processed form leads to cleavage of the BH3 interacting-domain death agonist (BID) protein that induces

mitochondrial damage, activation of caspases-9, -3, -6 and -7 and apoptosis. In some cases, such as LPS-induced sepsis or hepatic toxicity induced by TNF, BID-independent apoptosis proceeds via a mechanism that relies on JNK and reactive oxygen species (ROS) (127-129).

TNF-induced apoptosis is also prevented by cFLIP which prevents full caspase-8 activation but promotes cell survival by facilitating caspase-8-mediated cleavage and inactivation of RIPK1. An alternative scenario emerges when the proteolytic activity of caspase-8 is impaired or protein kinase activity of RIPK1 is increased, both of which can lead to necroptosis via the activation of RIPK3 and mixed lineage kinase domain like pseudokinase (MLKL) (126). Just as in the TNF-pathway, reduced caspase-8 activity during TLR4 or TLR3 signalling also induces necroptotic cell death.

In the case of the TNF-like molecules First Apoptosis Signal receptor Ligand (FASL) and TNF-related apoptosis-inducing ligand (TRAIL), which signal via TNFR-like receptors FAS and Death Receptor 5 (DR5) respectively, FADD directly recruits caspase-8 to the receptor complex to trigger apoptosis. FADD is thus critical for apoptosis and/or necroptosis by TNFR-family receptors. Although traditionally thought to be the initiator caspase in the extrinsic apoptosis pathway, the cell-survival role of caspase-8 is now better understood, including in IECs.

The nucleotide-binding oligomerization domain (NOD) and leucine-rich repeat domain (LRR) containing (NLR) proteins NOD1 and NOD2 can also activate NF-κB and MAPK pathways. These two cytosolic receptors of peptidoglycan components require RIPK2 to activate TAK1 (130).

EPEC/EHEC-induced NF-κB activation

As flagellated Gram-negative bacteria, EPEC/EHEC molecules such as LPS and flagellin, among others, are likely to serve as potent triggers of inflammation by activating TLR-pathways. Subsequent release of TLR/NLR-induced proinflammatory cytokines could further amplify inflammation during infection. A large body of work exists on the redundancy of effectors used by EPEC to block NF-κB activation. This has provided credence to the idea that EPEC actively subverts innate immune detection and stealthily limits intestinal inflammation to cause disease. The ablation of various LEE and non-LEE effectors in EPEC (for example, loss of *nleB, nleC, nleD* and *nleE;* see below for a description on their roles) or additional removal of flagellin (*fliC*) revealed that these strains strongly induce NF-κB activation and IL-8 secretion in epithelial cells (131). However, the host signalling pathway(s) responsible for NF-κB activation remained a mystery. By reconstituting the EPEC LEE locus encoding the T3SS and related effectors in *E. coli* K12, it was established that a functional T3SS system, but not effectors, was essential for NF-κB activation in infected epithelial cells. Further, while host cell contact-induced T3SS triggering induced NF-κB activation, ectopic expression of individual T3SS structural protein in host cells did not. Loss of *Myd88, Traf6* and *Ripk2* in the host did not affect NF-κB activation, ruling out TLR/IL1R-MyD88 or NOD1/NOD2-RIPK2 signalling as the underlying NF-κB-activating pathways. However, other possibilities remain to be tested. As *nleB, nleC* and *nleE* were essential to inhibit T3SS-dependent NF-κB activity, the as yet unknown pathway must still converge on the canonical NF-κB-pathway which are targeted by these effectors. For example, the involvement of TRIF-dependent NF-κB signalling, autocrine activation by cytokines such as TNF (via TNFR1-TRADD), oligosaccharide-sensing by C-type lectin receptors or cytosolic RNA-sensing by RIG-I-like helicases has not been ruled out (131).

Tir-mediated modulation of TLR and TNF signalling

EPEC Tir induces actin-pedestals via the phosphorylation of Tyr_{474} and Tyr_{454} residues. The *C. rodentium* model of *in vivo* infection allowed an investigation into the role of Tir phosphorylation and inflammation. Notably, translocation of Tir with both tyrosines intact led to higher CXCL1 and CXCL2 production by purified enterocytes from infected mice (132). Mutation of both tyrosine residues (Y451A/Y471A in *C. rodentium* Tir) reduced CXLC1 and CXCL2 production. Intriguingly, while actin-rich pedestals were absent on enterocytes during *in vivo* during infection with *C. rodentium* Y451A/Y471A Tir, colonisation and formation of A/E lesions was comparable to WT *C. rodentium* Tir. Importantly, loss of both tyrosine residues and actin polymerisation correlated with reduced colonic neutrophil influx at day 14 post-infection, which is the initial stage of pathogen clearance. While further investigation is required, these findings suggest a positive regulation of NF-κB signalling by Tir in a manner that requires both Tyr471 and Tyr451 (132).

Tir can also inhibit NF-κB activation (133-135). The tyrosine phosphorylation motifs around Tyr483 or Tyr511 residues in EPEC Tir are similar to immune tyrosine-based inhibitory motifs (ITIMs). In the host, tyrosine phosphatases recruited via ITIMs dephosphorylate various signalling proteins and dampen signal transduction. Recruitment of Src homology region 2 domain-containing phosphatase-1 (SHP1; also called PTPN6) to tyrosine phosphorylated EPEC Tir enhanced interaction between SHP1 and TRAF6 leading to reduced TRAF6-ubiquitylation. This impaired the production of proinflammatory TNF and IL-6 from EPEC-infected macrophages. Mutational-inactivation of the tyrosine residue in individual ITIM increased inflammatory cytokine production and mutation of both ITIMs increased it further, pointing to an additive role of the two motifs. Similar effects are reported via the recruitment of the related SHP2 phosphatase (134). Another study reported that EPEC-infection of epithelial cells inhibited their response to exogenous TNF in a Tir-dependent manner. They found Tir-dependent proteasomal degradation of TRAF2 was responsible for reduced TNF-induced IL-8 production in HeLa and polarised Caco2 cells. Interestingly, these effects could be recapitulated by delivery of Tir by an almost effectorless strain of *Yersinia,* which pointed to a non-essential role of Tir in suppressing NF-κB (133).

NleE and inhibition of TAB2 and TAB3

NleE was the first EPEC/EHEC effector demonstrated to have an NF-κB inhibitory activity (136, 137). Ectopic expression of NleE alone was sufficient to block NF-κB activation by exogenous TNF and IL-1β. This suggested that NleE acted at a common hub, the most upstream of which is TAK1. A subsequent study revealed that NleE methylates a critical zinc-coordinating cysteine residue in TAB2 (C673) and TAB3 (C692) and impairs their ability to bind polyubiquitin chains (138). Methylated TAB2/3 fail to recruit TAB1-TAK1 to active TRAFs, which results in potent inhibition of NF-κB via both cytokines. Unusually, NleE uses S-adenosine L-methionine as a cofactor for methylation, and a C-terminal IDSY(M/I)K motif is essential for catalytic activity. The broad involvement of TAK1 in canonical NF-κB and MAPK activation in many cell types underscores the importance of NleE in A/E and other enteric pathogens such as *Shigella flexneri* and *S. boydii* which express the related OspZ effector. The DBS 100 strain of *C. rodentium* lacking *nleE* is markedly impaired in colonization in mice and thus cause less intestinal pathology (139); in contrast, nleE-lacking ICC 169 *C. rodentium* have similar virulence as wildtype bacteria (140).

NleB subverts FADD-dependent signalling

NleB has N-acetyl glucosamine transferase (GlcNAc) activity and adds a single GlcNAc to its target proteins TRADD, FADD and GAPDH that are involved in signalling via the TNFR family of receptors (48, 49, 141-143). Thus, ectopic expression of NleB inhibits TNF-induced, but not

IL-1β-induced, NF-κB activity. In addition, NleB also blocks apoptosis induced by FAS-L, which directly recruits FADD and triggers apoptosis (137). While O-GlcNAc'ylation is known in host cells, and is reversible, N-GlcNAc'ylation is presumably irreversible, which helps explain the potency of NleB action. Mutational analyses of NleB indicated that catalytic activity requires the DXD motif, Tyr219 and Glu253. *C. rodentium* lacking *nleB* are highly defective for virulence and cannot colonise mice (49, 144, 145). Infection of FASL- or FAS-deficient mice with *C. rodentium* showed increased morbidity and delayed clearance of the pathogen. This suggested that inhibition of enterocyte apoptosis by A/E pathogens may promote colonization and disease pathogenesis. *C. rodentium* NleB was also shown to GlcNAc'cylate GAPDH, which binds TRAF3 to promote its ubiquitylation (141). Ectopic expression of NleB reduced TRAF3 ubiquitylation and impaired type I interferon (IFN) production in response to stimulation of TLR4 or TLR3 with LPS or poly(I:C) respectively (144).

NleD and proteolytic inactivation of MAPKs

As an *nleB/nleE* double-mutant strain still blocked JNK activation by TNF, further studies based on this initial finding led to the identification of NleC and NleD as suppressive factors with protease activities (50). NleD and NleC have related HExxH motifs for zinc coordination and metalloprotease activity. NleD cleaves the p38 and JNK kinases, but not ERK. Direct cleavage of JNK2 by NleD occurred within the protein kinase activation loop. UV-irradiation induces JNK-dependent apoptosis that can be blocked by NleD. More recent biochemical studies have identified the molecular specificity of NleD, for example Arg203 in NleD and NleD-like proteases is essential for cleavage of p38 but dispensable for JNK proteolysis (146). NleD has also been reported to block RNAseL expression and production of type I IFNs in human Caco2 IECs infected with EPEC. IFNs maintain the expression of tight junction proteins whose reduced expression accelerated barrier breakdown and increased trans-epithelial electrical resistance (147).

NleC and proteolytic inactivation of NF-κB

NleC is a zinc metalloprotease that cleaves NF-κB family proteins, including p65, c-Rel, RelB and p50 (50-52, 148, 149). Like NleD, the protease activity relies on an HExxH motif that coordinates zinc ions. In addition, NleC also cleaves the acetyltransferase p300, which is a positive regulator of NF-κB-dependent IL-8 production (150). Cleavage occurs within the DNA-binding domains of NF-κB subunits, which results in their inactivation (151-154). The conserved ^{22}EIIE^{25} and ^{177}PVLS^{180} motifs in p65 are involved in binding to NleC. Different homo- or hetero-dimers of NF-κB subunits have subtly different gene targets in cells and by acting on multiple subunits, NleC has broadly suppressive effects on NF-κB-mediated transcription.

EspT and activation of NF-κB

EspT (discussed in 'Map, EspT and EspM mimic host Rho GEFs ') activates Rac1 and promotes invasion. However, EspT-mediated activation of Rac1 also induces NF-κB in a manner that is independent of bacterial invasion (40, 94, 102). The related STm effector SopE activates Rac1 and triggers NOD1-dependent NF-κB activation (155). Whether EspT functions similarly remains to be tested.

NleF in inhibiting apoptosis and activating NF-κB

NleF interacts with caspases-4, -8 and -9 (55, 156) and the COP1 vesicle protein Tmp21 (157). Recombinant NleF potently inhibits caspase-4 (IC_{50} ~14 nM), caspase-8 (IC_{50} ~40 nM) and

caspase-9 (IC_{50} ~80 nM) (55). Here we discuss the cellular effects of NleF-mediated inhibition of caspase-8/9; NleF-caspase-4 interactions are discussed in the section on Inflammasomes. The co-crystal structure of caspase-9-NleF revealed that insertion of four C-terminal residues of NleF into the caspase-9 active site inhibits its proteolytic activity in a manner similar to the inhibitor peptide zEAD-Dcmbk. NleF, which partially localises to mitochondria, can block the intrinsic caspase-9-dependent apoptosis induced by staurosporine. As a direct inhibitor of caspase-8, ectopic expression of NleF could potently block FASL- and TRAIL-induced receptor-mediated apoptosis. While NleF and NleB both block apoptosis, they act at different steps in the pathway. By inhibiting a receptor-proximal step, NleB causes severe dampening of the immune response compared to NleF which acts 'downstream' on caspases (55, 156, 158, 159). This is reflected by the relatively marked attenuation caused by *nleB* deletion as compared to *nleF* deletion in *C. rodentium* (145, 156).

A second role of NleF is activation of NF-κB. EPEC increases NF-κB activity early during infection (1.5 h post-infection), dependent on the presence of NleF (160). Consistent with this, EPEC infection-induced IL-8 production in IECs *in vitro* was NleF-dependent. The underlying mechanisms of NleF action on NF-κB were caspase-4, -8 and -9-independent.

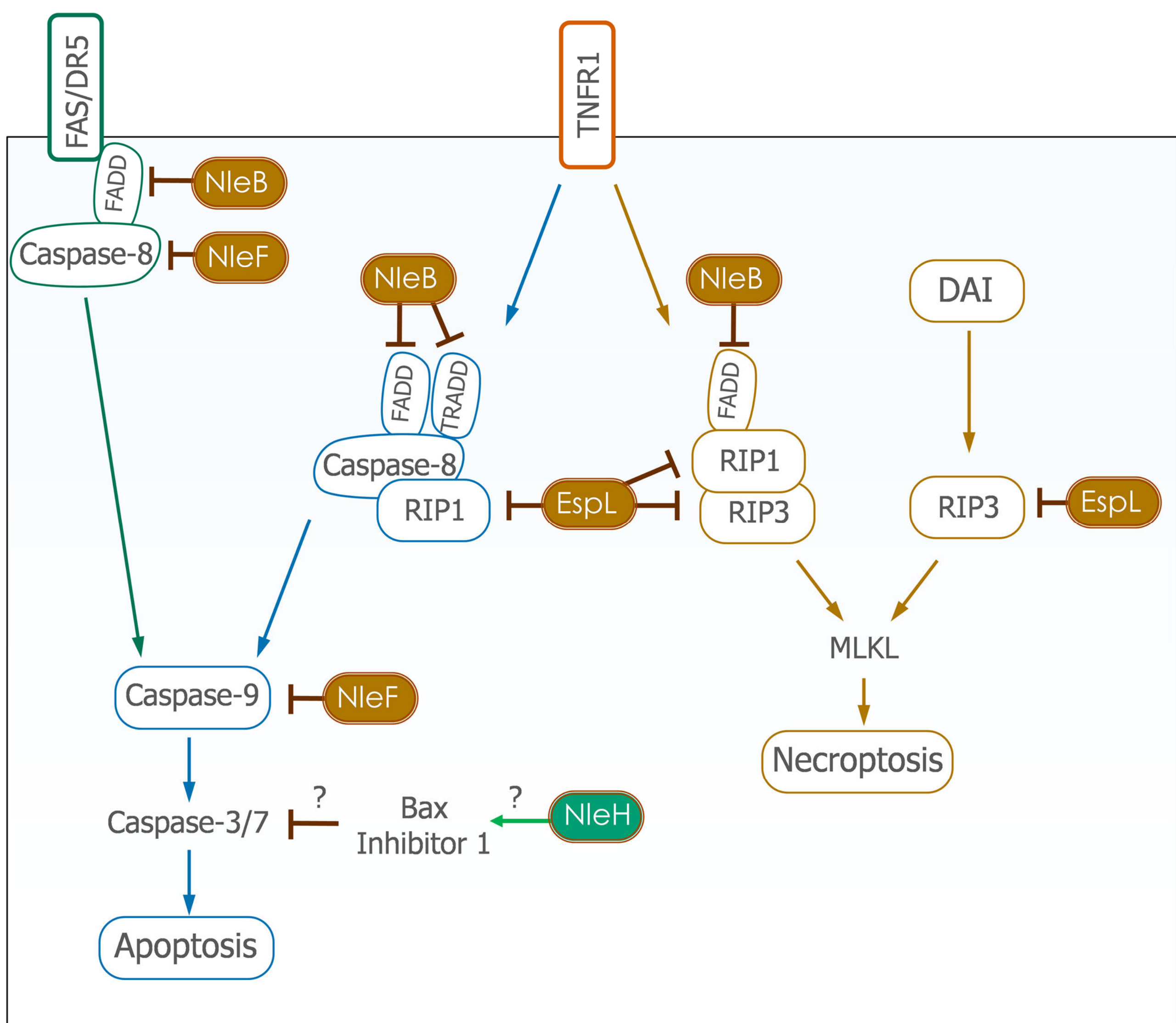

Fig. 4. Suppression of apoptosis and necroptosis by EPEC/EHEC. FAS and death receptor 5 (DR5) mediate FADD and caspase-8 dependent apoptosis. TNFR1 mediates apoptosis or necroptosis in a context-specific manner (see text). TNFR1-induced apoptosis via RIPK1 and caspase-8 typically requires inhibition of NF-κB activity and/or protein translation, and

necroptosis requires inhibition of caspase-8 or reduced RIPK1 ubiquitylation. The cytosolic protein DAI directly induces necroptosis via RIPK3. The mechanisms of NleH-mediated inhibition of apoptosis via Bax-inhibitor 1 are poorly understood (depicted by ? marks).

NleH-mediated inhibition of NF-κB and apoptosis

NleH1 and NleH2 reportedly inhibit NF-κB activity in cells via two different mechanisms. There is evidence that NleH1/2 binding to ribosomal protein S3 (RPS3), a subunit of NF-κB, prevents NF-κB nuclear translocation (57). Another study reported that transfection of EPEC NleH1/2 reduced IKK-β activity, IκB degradation and NF-kB activation in response to TNF-stimulation (161). However, an alternative mechanism was suggested based on the finding that NleH1 inhibited IKK-β-mediated RPS3 phosphorylation on the key Ser209 residue required for its nuclear translocation (162, 163). In contrast to NleH1, NleH2 inhibits NF-κB by blocking IκB degradation (141). Deletion of both NleH proteins in EPEC revealed a relatively weak inhibitory activity on TNF-induced NF-κB activation and KC-induction *in vivo*. *C. rodentium* encodes a single NleH and its deletion reduced NF-κB-reporter activity in the colonic mucosa *in vivo*. Complementation of *ΔnleH C. rodentium* suggested that NleH1 but not NleH2 has more anti-NF-κB activity (141). EHEC lacking NleH1 and NleH2 showed increased colonisation of calves however, the mechanisms for this are unclear (164). Thus, while several reports exist on NF-κB-suppressive roles for NleH proteins, the underlying mechanisms that have been proposed remain to be reconciled.

NleH proteins are related to Shigella OspG, and have an atypical protein kinase structure and a C-terminal PSD-95/Disc Large/ZO-1 (PDZ) motif (163, 165, 166). NleH1 is reported to undergo autophosphorylation but does not phosphorylate RPS3 or IKK-β. The v-Crk sarcoma virus CT10 oncogene-like protein (CRKL) protein was identified as an NleH1 substrate and implicated in inhibiting RPS3 phosphorylation (167). The crystal structure of NleH2 revealed that it might not require autophosphorylation for full activity due to the lack of the conserved Arg residue in the HRD motif typically found in other protein kinases (166). Furthermore, p38 and JNK inhibition independently of the kinase domain has also been reported for NleH proteins. The PDZ motif, also implicated in NF-κB inhibition, may also have additional subversive roles, for example via binding to PDZ-containing proteins such as Na(+) /H(+) exchanger regulatory factor 2 (NHERF2) (165).

In addition, EPEC NleH1 and NleH2 block pro-caspase-3 proteolysis and apoptosis induced by staurosporine, brefeldin A, tunicamycin or *Clostridium difficile* TcdB toxin (58, 168). The ER-resident BAX-inhibitor protein 1 (BI-1) has been identified as an NleH1-binding partner, and is required for NleH1-mediated inhibition of cellular apoptosis. BI-1 inhibits apoptosis, particularly when induced by ER-stress or Ca+2 elevation by blocking the action of BCL2-family protein BAX. Notably, the kinase activity of ectopically expressed NleH1 is dispensable for its anti-apoptotic roles. Consistent with this, *C. rodentium ΔnleH* induces reduced pro-caspase-3 cleavage *in vivo* (58).

EspL cleaves RHIM-domain proteins

The receptor-interacting protein (RIP) homotypic interaction motifs (RHIM) present in signalling proteins such as TRIF, RIPK1, RIPK3, DAI (DNA-dependent activator of IFN-regulatory factors, also called ZBP1), among others, are essential for signal transduction by these proteins. An unconventional Cys-His-Asp catalytic triad and protease activity of EspL against these RHIM-containing proteins inactivates their respective signalling pathways (35).

For example, inactivation of RIPK1 by EspL redundantly inhibits TNF-dependent NF-κB activation in EspL expressing cells. Similarly, cleavage of TRIF abrogates TLR3 and TLR4-induced type I IFN production in EspL expressing cells. Importantly, cleavage of RIPK1 and RIPK3 also abrogates necroptosis induced by TNRF1 and DAI. During EPEC infection, levels of RIPK1 drop early post-infection in an EspL activity-dependent manner, and RIPK3 levels drop later during infection, suggesting a preference for RIPK1. Furthermore, EspL is only active against RIPK1 before it assembles into oligomeric amyloid-like fibrils that trigger necroptosis. *C. rodentium ΔespL* is cleared much faster during infection as compared to wildtype *C. rodentium* which points to the importance of EspL *in vivo* (35).

NleL-mediated inhibition of JNK

NleL has ubiquitin E3-ligase activity that is biochemically similar to eukaryotic E6-AP Carboxyl Terminus (HECT) family proteins (60, 61); however, NleL is structurally unrelated to HECT-ligases. NleL can monoubiquitylate JNK1 at Lys68 and reduce its interaction and phosphorylation by the upstream kinase MKK7 (59). NleL can also target JNK2 and JNK3 and thus reduce AP1-activity in cells. JNK was also found to regulate EHEC Tir-mediated pedestal formation and bacterial attachment to cells, thus suggesting a role for NleL in this process.

EspJ and non-receptor tyrosine kinases

EspJ is uniquely able to couple amidation and ADP-ribosylation (30) and targets this biochemical activity to non-receptor tyrosine kinases (32). EspJ can ADP-ribosylate Src to inhibit complement receptor 3 (CR3) and FcγR-mediated phagocytosis (30). Proteomic analysis of IEC's isolated from mice infected with *C. rodentium* (WT, Δ*espJ* or Δ*espJ* complemented with a catalytically inactive EspJ) indicate a broad immunomodulatory effect of EspJ through regulation of multiple tyrosine kinases including Src, Abl, Csk, Tek and Syk families (32)

Inflammasome Signalling Pathways

Inflammasomes are multimolecular scaffolds that activate caspase-1 (122, 169). Oligomeric inflammasome complexes form a single 'speck' or 'focus' (~1-3 μm in size) per cell upon their activation by microbial and environmental cues. The inflammatory outcomes of caspase-1 activation include the release of proinflammatory cytokines such as IL-1β and IL-18, alarmins such as IL-1α and HMGB1, and lytic cell death via pyroptosis. The inactive pro-caspase-1 zymogen undergoes autoproteolytic activation within inflammasome foci. Pro-IL-1β and pro-IL-18 are biologically inert precursors that require proteolytic maturation. Gasdermin-D (GSDMD) is also inert until it is processed, which releases the N-terminus fragment that inserts within membranes and forms pores. GSDMD pores cause release of ions leading to swelling and eventual cell lysis, and may also facilitate the release of small proteins, including mature IL-1β and IL-18 from macrophages. IL-1α and HMGB1 release is also regulated by inflammasomes through mechanisms that are poorly understood; however, caspase-1 does not proteolytically process either protein (122, 169). Mature IL-1β and IL-1α have overlapping immune roles, for example in elevating body temperature (causing fever), inducing proinflammatory cytokines and acute phase proteins, and as neutrophil attractants. IL-18 is especially important for neutrophil recruitment, the induction of type II interferon (IFNγ) from lymphocytes and tissue-repair in the intestine. Inflammasomes thus orchestrate the early innate immune responses to infection and help launch effective adaptive immune responses. Differential expression of inflammasome sensors and substrates results in distinct outcomes following inflammasome activation in different cell types. Inflammasome signalling

is best understood in macrophages and dendritic cells, however, intestinal epithelial cells also express several inflammasome-signalling proteins and the pro-IL-18 substrate.

Inflammasomes sensors are modular proteins and share conserved domains. Examples include proteins from the NLR proteins with a PYD (pyrin domain; NLRPs) or NLRs with a CARD (caspase activation and recruitment domain; NLRCs), AIM2-like receptors (ALRs) and the non-NLR/ALR sensor called PYRIN. Caspase-1 has a CARD at its N-terminus which recruits it to inflammasome complexes, typically via the small adaptor protein PYCARD (protein with a PYD and CARD) also called ASC (apoptotic speck-associated protein containing a CARD). While CARD-containing NLRs (e.g. NLRC4) can directly recruit caspase-1 for pyroptosis, for reasons not completely clear, ASC is required for optimal IL-1β and IL-18 processing by NLRC4 inflammasomes. EPEC and EHEC T3SS components, RNA and LPS have previously been suggested to activate inflammasomes in various cell types.

EPEC infection activates NLRC4 and NLRP3 inflammasomes, both of which rely on upstream receptors or molecules for their activation. The NLRC4 inflammasome requires proteins of a NLR sub-family called NAIPs (NLRs with apoptosis inhibitor repeat proteins) which contain N-terminal BIRs (baculovirus inhibitor of apoptosis repeat domains). *Naip* genes have diversified in the mouse, four of which have been characterised extensively: NAIP1 binds the T3SS needle proteins, NAIP2 binds the T3SS rods, and NAIP5 and NAIP6 bind flagellins. In contrast, a single human *NAIP* produces at least two isoforms that are capable of binding T3SS needle, rod and flagellin. Ligand-bound NAIPs stimulate NLRC4 oligomerization, ASC recruitment and caspase-1 activation (170, 171).

The NLRP3 inflammasome can be activated in two ways; by loss of cellular K^+ through bacterial pore-forming toxins or host-proteins that can form pores ('canonical' signalling), or by cytosolic LPS ('non-canonical' signalling). Non-canonical NLRP3 activation requires upstream activation of caspase-4 (previously called caspase-11 in the mouse) or caspase-4 and caspase-5 in the human (122, 169). Caspase-4, -5 are cytosolic receptors for LPS and are directly activated by LPS-binding. Therefore, unlike caspase-1 which is oligomerised within inflammasome scaffolds, current evidence suggests LPS-binding is sufficient to induce oligomerisation and activation of caspase-4. Active caspase-4 can also proteolytically process GSDMD and cause pyroptosis in myeloid cells and IECs. The efflux of K^+ via GSDMD or pannexin-1 pores activates NLRP3-ASC-caspase-1 inflammasome which is essential for IL-1β and IL-18 processing in macrophages (172-174). Recombinant Shiga-like toxins Stx1 and Stx2 activate the NLRP3 inflammasome in THP1 macrophage-like cells in a manner that depended on their N-glycosidase activity (175). In contrast, Stx was dispensable during EHEC infection or mouse macrophages in which NLRP3 activation was reported via *E. coli* RNA:DNA hybrids that gain access to the host cytosol independently of the T3SS (176). As discussed further below, EPEC LPS can activate caspase-4 in different cell types. The mouse *C. rodentium* infection model has highlighted the importance of inflammasomes in host defence against A/E pathogens. Loss of inflammasome genes, such as *Nlrp3, Nlrc4, Casp1/4, Il1b* and *Il18* resulted in greater pathogen-burdens at late stage of infection and higher morbidity (177-181). More recent work has highlighted the critical role of the microbiome in *C. rodentium* colonisation (182). Additional studies are required to dissect the contribution of the host genotype and the microbiome to the outcomes of *C. rodentium* infection in *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice to identify the relative contribution of these inflammasomes to host-defence against A/E pathogens. *In vivo* studies have pointed to an important role for IEC-intrinsic inflammasome in innate immunity to *C. rodentium.* How EPEC proteins activate, suppress or evade detection by inflammasomes is discussed next and summarised in Figure 5.

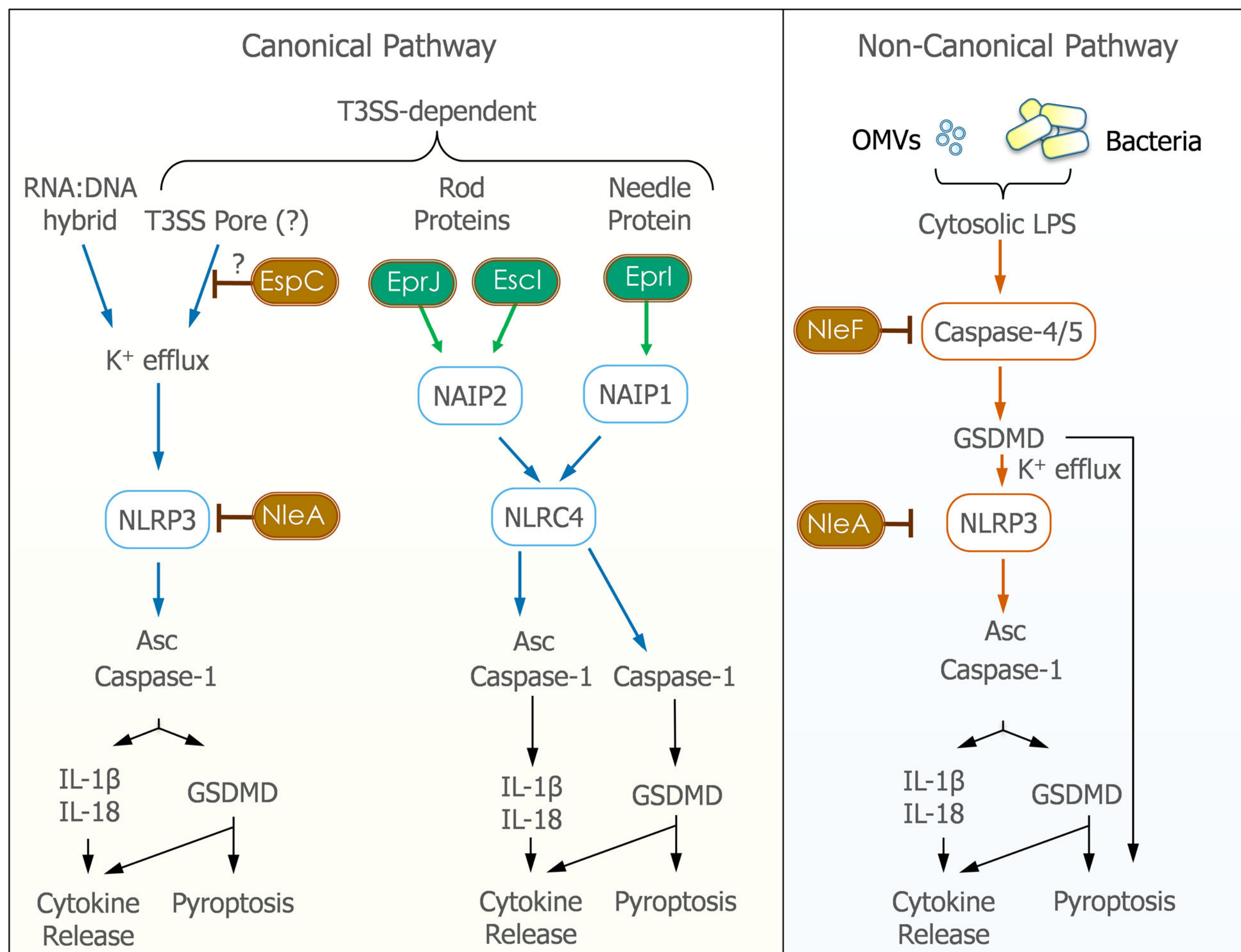

Figure x.5: Inflammasomes signalling during EPEC/EHEC infection. Schematics depict the canonical (left) and non-canonical (right) inflammasome activation pathways. T3SS structural proteins that activate inflammasomes are shown in green (EscI, EprJ and EprI), and effectors that suppress inflammasomes are shown in yellow (EspC, NleA and NleF). EHEC RNA:DNA hybrids gain access to the cytosol independently of the LEE T3SS. The EHEC ETT2 needle protein EprI is a ligand for human NAIP (not depicted) and mouse NAIP1. The LEE T3SS and ETT2 rod proteins are reported to activate murine NLRC4 via NAIP2. EspC blocks necrosis by regulating the T3SS pore, and whether this form of death is pyroptotic is not known (depicted by ?). EHEC bacteria and purified OMVs stimulate non-canonical signalling via caspase-4 in mouse macrophages. EPEC and EHEC NleF can block caspase-4 in IECs. The transcriptional upregulation of NLRP3 and pro-IL-1β require NF-κB activity which may be supressed by the actions of other effectors that are not depicted (see text and Figure 3).

EPEC FliC, EscI, EscF and the NLRC4 inflammasome

Due to its presence in diverse pathogenic bacteria, the T3SS is a major target for detection by the host immune system. The T3SS is evolutionarily related to the bacterial flagellar basal body, and the first studies on NLRC4 identified a role for it in detecting STm flagellin (183). The role of NLRC4 in detecting EPEC is evident from the lack of inflammasome response by *Nlrc4*$^{-/-}$ mouse macrophages (184). During *C. rodentium* infection, *Nlrc4*$^{-/-}$ show marked weight loss between days 6-12 post-infection and increased pathogen burden (181). This was accompanied by increased intestinal inflammation and pathology. However, the production of antibodies, IFNγ and IL17A were higher in *Nlrc4*$^{-/-}$ animals, pointing to reduced early innate immune responses but a relatively competent adaptive immune response later during infection. Bone-marrow chimera experiments indicated that NLRC4 signalling in the non-

haemopoietic compartment was required for host-defence. Consistent with this, NLRC4 expression was detected in intestinal crypts, which may respond by producing IL-18. However, as discussed next, EPEC has evolved mechanism that largely allow it to escape detection by the NAIP-NLRC4 system, especially in human cells.

A novel cytosolic protein-delivery approach using flagellin or T3SS proteins fused to the non-catalytic N-terminal regions of the anthrax lethal factor (LnF) plus protective antigen (PA) helped dissect NLRC4 signalling induced by individual bacterial molecules (184). Flagellin proteins from STm (FliC) and *Legionella* (FlaA) interact with NAIP5 when delivered via LnF-PA. However, EPEC and EHEC flagella do not interact with NAIP5 or activate NLRC4 inflammasomes (184). In agreement with this, a *ΔfliC* EPEC E2348/69 strain has unaltered inflammasome activation in mouse macrophages, which suggests that EPEC/EHEC flagellins evade detection by inflammasomes.

As a T3SS-deficient EPEC (*ΔescN*) fails to activate inflammasomes in macrophages, NLRC4 likely detects a T3SS component. Almost all strains of EHEC and EPEC also encode remnants of a second T3SS called ETT2 (185). Frame shift inactivation and deletion of several genes within ETT2 point to a non-functional injectisome. EPEC strains have a deletion in ETT2 resulting in the loss of rod and needle genes; in contrast, EHEC strain Sakai encodes the complete ORFs for rod (EprJ) and needle (EprI). In a study that used transient transfection of the EPEC LEE T3SS rod protein EscI or the EHEC ETT2 rod EprJ in mouse macrophages, both proteins induced NLRC4-dependent pyroptosis (186). A subsequent study confirmed that EPEC EscI is a ligand for mouse NAIP2 and that a *ΔesfFΔfliC* EPEC strain fails to activate inflammasomes during infection of mouse macrophage (184). Various T3SS rod proteins, including the *Salmonella* pathogenicity island 1 (SPI-1) rod protein PrgJ, activate the Naip2-NLRC4 system. However, as the extensively used human macrophage cell lines (THP1 and U937) do not respond to flagellin or T3SS rods, the search for activators of human NLRC4 continued, leading to the identification of the T3SS needle CprI from *Chromobacterium violaceum* as a ligand for human NAIP and mouse NAIP1 (184, 187). Interestingly, the LEE T3SS needle protein EscF from EPEC and EHEC fails to bind to human or mouse NAIP, and exemplifies another evasion mechanism (184, 187). In contrast, the EHEC ETT2 needle protein EprI, which is related in sequence to SPI-1 needle PrgI, readily activates human and mouse NAIP-NLRC4 (186). Whether EprI or EprJ are expressed and secreted by EHEC remains to be tested during infection.

EPEC EspC and regulation of the T3SS pore

The T3SS pore has the potential to activate NLRP3 by promoting K+ efflux (188, 189). While this has been suggested in the case of effectorless strains of *Yersinia,* whether this happens during EPEC infection has not been tested. However, in epithelial cells, the EPEC serine protease autotransporter of enterobacteriaceae (SPATE) family effector EspC targets EspA-EspD subunits of the T3SS translocon upon host cell-contact. This effectively down-regulates pore formation and reduces EPEC-induced cytotoxicity. Therefore, a *ΔespC* strain is more cytotoxic to epithelial cells. However, the mechanisms of cytotoxicity and indeed whether this is inflammasome-dependent pyroptosis remains to be tested.

NleA and suppression of NLRP3 inflammasomes

The EPEC effector protein NleA (also called EspI) can inhibit NLRP3 inflammasomes in human THP1 cells (47). A screen of deletion mutants of non-LEE islands identified NleA in inhibiting NLRP3 activation by preventing its deubiquitylation. In naïve cells, ubiquitylation restrains NLRP3 activity, which is relieved by its deubiquitylation by BRCC3. NleA interaction with

NLRP3 impaired its deubiquitylation, thus reducing oligomeric foci formation. How NleA affects NLRP3 ubiquitylation remains to be elucidated mechanistically. NleA was previously reported to block COPII vesicle trafficking and protein secretion by directly binding to host Sec24 (46). However, NleA-dependent reduced IL-1β secretion can be attributed to caspase-1 inhibition and not protein secretion inhibition as IL-1β (and IL-18) do not have signal peptides for ER-Golgi-mediated trafficking and secretion, and are released via an unconventional secretion mechanism. Loss of *nleA* in *C. rodentium* severely reduces intestinal colonisation and inflammation, indicating its importance *in vivo* (97).

NleE and suppression of inflammasome-priming

Inflammasome sensors such as NLRP3 and mouse caspase-4 require priming for post-translational licensing and increased expression (47). Importantly, pro-IL-1β expression is upregulated by NF-κB signalling in myeloid cells. As discussed previously, NF-κB inhibition by several EPEC effectors could potentially reduce mature IL-1β production in macrophages. A role for NleE in reducing pro-IL-1β expression was evident during infection of THP1 macrophages. The other NF-κB inhibitory effectors such as NleC and NleB are predicted to have similar suppressive roles on pro-IL-1β production.

EHEC OMVs/LPS and the non-canonical activation of inflammasomes

EHEC, EPEC and *C. rodentium* were among the first Gram-negative pathogens shown to activate caspase-4 in macrophages in a TLR4- and TRIF-dependent manner (177, 190, 191). However, these studies used EPEC/EHEC/Cr that were grown in condition that do not induce T3SS, LEE or non-LEE effectors, presumably to avoid NLRC4 activation. How LPS from these bacteria gains access to cytosolic caspase-4 has remained elusive. Studies using outer membrane vesicles (OMVs) from non-LEE expressing EHEC or *E. coli* K12 showed that OMVs are endocytosed via processes that require Rab7 (192) and TLR4-TRIF (193, 194). OMVs gain access to the cytosol via mechanisms that are not entirely clear but require the guanylate-binding proteins (GBPs), including GBP2 and GBP5 (193-195). Mouse caspase-4 and GBPs are IFN-inducible genes and their expression needs to be upregulated for optimal inflammasome activation (191). Thus, in naïve mouse macrophages, caspase-4 activation is delayed and can take up to 10 h. In contrast, both human caspase-4 and caspase-5 are constitutively expressed, further pointing to likely differences in human macrophages.

NleF and suppression of caspase-4 activity

Human caspase-4 was reported to be inhibited (IC_{50} ~ 5 nM) by purified NleF in a manner that required its four C-terminal residues, which were also critical for NleF-caspase-4 interaction (156). In addition, mouse caspase-4 was potently inhibited by *C. rodentium* NleF. Moreover, EPEC induced caspase-4-dependent pro-IL-18 processing in human Caco2 IECs. The direct processing of pro-IL-18 by caspase-4 in IECs, independently of NLRP3, was also reported during STm infection. EPEC *ΔnleF* affected markedly increased mature IL-18 production by IECs, pointing to a physiologically relevant subversive role for NleF during infection. In the *C. rodentium* infection model, higher IL-18 release was observed in colonic explants of mice infected with *ΔnleF C. rodentium* than the wildtype pathogen. Colonic explants from *C. rodentium* or *ΔnleF* infected *Casp4*$^{-/-}$ mice did not secrete detectable IL-18, which indicated that early IL-18 production was caspase-4-dependent *in vivo*. Importantly, reduced IL-18 levels correlated with lower colonic influx of neutrophils. Thus, NleF-mediated caspase-4-inhibtion blocked early neutrophil responses to infection (156). Similarly, EHEC NleF is reported to block caspase-4-dependent pyroptosis and IL-1β conversion in HT29 IECs (PMCID: PMC5448047). Subversion of IEC caspase-4 by A/E pathogens points to

inflammasome-dependent antimicrobial host defence. An important outstanding question is how LPS from extracellular A/E pathogens is able to localize to the cytosol, and whether any effectors may be involved in the process.

Conclusion

Innate immune signalling leading to the transcriptional upregulation of proinflammatory cytokines and type I IFNs as well as removal of infected cells via programmed cell death are essential for effective antimicrobial immunity. A/E pathogens encode effectors with distinct biochemical activities to block multiple steps of signal transduction by a broad range of pattern recognition receptors and cytokine receptors. Transcriptional responses as well as rapid post-translational pathways, exemplified by TLRs and inflammasomes respectively, are targeted by A/E pathogens for robust inhibition of host responses. Future studies should focus on the temporal nature of stimulatory and inhibitory actions of various effectors during infection.

Summary and perspectives

A/E pathogens predominantly cause diarrheagenic disease in children but distinct pathotypes are increasingly associated with disease in adults. A number of LEE and NLE effectors are together responsible for damaging the intestinal lining, suppressing host responses and causing disease. Studies on T3SS delivered effectors have led to the identification of exciting biochemical activities such as GEF-mimics, arginine-GlcNac'lase, deamidase, combined deamidase and ADP-ribosylase, unconventional proteases, caspase-inhibitors and ubiquitin ligases. Future studies should focus on the collective spatiotemporal nature of effector function and their contribution to intestinal disease.

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