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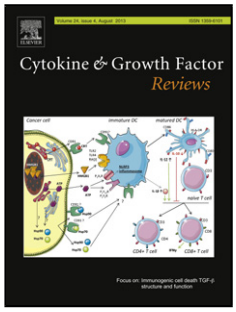

Title: Inhibition of death receptor signaling by bacterial gut pathogens

Author: <ce:author id="aut0005" biographyid="vt0015"> Cristina Giogha Tania Wong Fok Lung<ce:author id="aut0015" biographyid="vt0010"> Jaclyn S. Pearson<ce:author id="aut0020" biographyid="vt0005"> Elizabeth L. Hartland

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Elizabeth L Hartland obtained her Ph.D in 1996 from the University of Melbourne, Australia. She has held a Royal Society/NHMRC Howard Florey Fellowship in the Department of Biochemistry, Imperial College London, United Kingdom and Lecturer/Senior Lecturer positions in the Department of Microbiology, Monash University, Australia. She was an inaugural Australian Research Council Future Fellow at the University of Melbourne and is currently Professor and Head of the Department of Microbiology and Immunology at the University of Melbourne. She has a long-standing research interest in the pathogenesis of infections caused by *Escherichia coli* and *Legionella*, with a focus on mechanisms of bacterial colonization and immune evasion.

Jaclyn Pearson obtained her Ph.D in 2013 from the University of Melbourne, Australia and has an interest in the role of death receptor signaling in fighting bacterial gut infection.

Cristina Giogha and Tania Wong Fok Lung are graduate students in the Department of Microbiology and Immunology, University of Melbourne, Australia researching the biochemical function of T3SS effector proteins from bacterial enteric pathogens.

Inhibition of death receptor signaling by bacterial gut pathogens

Cristina Giogha[1], Tania Wong Fok Lung[1], Jaclyn S Pearson[1] and Elizabeth L Hartland[1,2*]

[1]Department of Microbiology and Immunology, University of Melbourne, Victoria 3010, Australia

[2]Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia

* Correspondence: hartland@unimelb.edu.au

Abstract

Gastrointestinal bacterial pathogens such as enteropathogenic *E. coli*, *Salmonella* and *Shigella* control inflammatory and apoptotic signaling in human intestinal cells to establish infection, replicate and disseminate to other hosts. These pathogens manipulate host cell signaling through the translocation of virulence effector proteins directly into the host cell cytoplasm, which then target various signaling pathways. Death receptors such as TNFR1, FAS and TRAIL-R induce signaling cascades that are crucial to the clearance of pathogens, and as such are major targets for inhibition by pathogens. This review focuses on what is known about how bacterial gut pathogens inhibit death receptor signaling to suppress inflammation and prevent apoptosis.

Keywords

Death receptor
Bacterial pathogens
T3SS effectors
Apoptosis
Inflammation
TNF

Contents

1. Introduction

Bacterial pathogens activate a number of signaling cascades within host cells during infection, many of which subsequently induce inflammation. Alternatively, and often in parallel, microbial detection can activate apoptotic signaling, which leads to the eradication of infected cells. The benefits of inhibiting or inducing cell death or inflammation for a pathogen differ depending on the specific pathogen and type of host cell targeted. For some pathogens, the induction of cell death in epithelial cells facilitates invasion to deeper tissues, while inducing cell death in immune cells can promote pathogen survival [1]. Bacterial gut pathogens have evolved highly specific mechanisms to modulate cell death and inflammatory signaling pathways in order to successfully establish infection, replicate and disseminate to other hosts. Ultimately, the inhibition of inflammation allows the pathogen to evade the innate immune response. However, inflammation can also be useful to pathogens, for example *Salmonella* induces inflammation to outcompete commensal bacteria in the gut [2].

Inflammation and cell death are induced by a variety of extrinsic and intrinsic factors targeting different cellular receptors. Key signaling pathways involved in the host anti-microbial defences include the nuclear factor-kappa B (NFκB) transcriptional regulator and mitogen-activated protein kinase (MAPK) pathways. This review will focus on how bacterial gut pathogens inhibit death receptor signaling to prevent inflammation and host cell death.

2. Death receptors and bacterial gut pathogens

Death receptor signaling is a significant component of the host response to bacterial gut pathogens. Death receptors including TNFR1, FAS (TNFSFR6) and the TRAIL (TNF-associated apoptosis-inducing ligand) receptors, DR4 and DR5, are defined by the presence of a cytoplasmic death domain (DD), which recruits DD-containing adapter proteins to an oligomeric signalosome via homo- and heterotypic DD interactions [3, 4]. The stimulation of death receptors occurs through an extracellular cysteine-rich domains (CRD) leading either to an inflammatory response or death of the cell.

In response to TNF, TNFR1 recruits adapter proteins to form different signaling complexes that have distinct and diverse outcomes [3]. Complex I requires binding of TRADD to TNFR1 via DD interactions, followed by recruitment of TRAF2, RIPK1 and cIAPs to the receptor complex. This signaling platform results in the activation of NF-κB and MAPK signaling, inducing an inflammatory response [5]. Complex IIa is formed upon dissociation of TRADD from TNFR1, recruitment of FADD and procaspase-8 to TRADD, followed by the activation of caspase-8 and

apoptosis of the cell. Complex IIb leading to necroptosis is formed upon de-ubiquitination and phosphorylation of RIPK1 and involves the components, RIPK3, FADD and procaspase-8 [6]. The formation of each of these signaling complexes is tightly regulated so that not all complexes can be activated at once and tissue homeostasis is maintained.

In the canonical extrinsic apoptosis pathway, recognition of FAS ligand (FasL) by FAS leads to the recruitment of FADD and procaspase-8 and formation of the death-inducing signaling complex (DISC), which initiates cell death though the activation of caspase-8 [7]. The signaling pathway in lymphoid cells differs slightly to that of non-lymphoid cells. For the latter, processing of the pro-apoptotic protein Bid is required to induce cell death [8, 9].

TRAIL-R is another death receptor which upon binding of the ligand TRAIL, recruits FADD and procaspase-8 to form the DISC [10]. TRAIL has been studied extensively in the context of tumor cell apoptosis, but a role for TRAIL during infection with bacterial pathogens is not well established. While the involvement of TRAIL-R and FAS in apoptotic signaling is well accepted, their potential influence on anti-apoptotic, inflammatory and pro-survival signaling are controversial. Non-apoptotic signaling via these receptors seems to involve NF-κB and MAPK pathways, but the physiological relevance remains unclear [10, 11].

The evolution of bacterial pathogens to inhibit death receptor signaling can be attributed to the acquisition of virulence genes on mobile genetic elements such as prophages and integrative elements, which can be horizontally transferred between bacteria. Enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) are extracellular pathogens that infect epithelial cells of the human gut. EPEC and EHEC utilize a type III secretion system (T3SS) to inject virulence effector proteins directly into host cells, which manipulate host cell function [12]. One such effector, termed the translocated intimin receptor (Tir) mediates the formation of attaching and effacing (A/E) lesions which are characterized by intimate attachment of the bacteria to host cells and the effacement of brush-border microvilli around the adherent bacteria. The T3SS and several effectors, including Tir, are encoded by the locus of enterocyte effacement (LEE) pathogenicity island. There are also non-LEE encoded (Nle) effector proteins, many of which inhibit inflammation and cell death by blocking death receptor signaling. The effects of these proteins during infection *in vivo* have been studied using *Citrobacter rodentium*, an A/E pathogen of mice that is highly related to EPEC and EHEC.

Unlike EPEC, *Salmonella enterica* serovar Typhimurium is an invasive gastrointestinal pathogen

that possesses two T3SSs encoded by *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). While the SPI-1 T3SS is required to facilitate entry into host cells, the SPI-2 T3SS is required to establish intracellular replication. However, similar to EPEC, *S.* Typhimurium has evolved to evade host immune defenses through the injection of T3SS effectors that subvert innate immune and apoptotic signaling pathways.

Shigella is the causative agent of bacillary dysentery, or shigellosis, an invasive infection of the human colon. While highly genetically related to *E. coli*, *Shigella* spp. are different to A/E pathogens as they are invasive gastrointestinal pathogens. Once inside the cell, the bacteria lyse the endocytic vacuole, replicate in the cytoplasm and spread to adjacent cells via the polymerisation of F-actin at one pole. *Shigella* also uses a T3SS to translocate effector proteins directly in the host cell cytosol that are essential for invasion, vacuolar escape, and cell-to-cell spread [13]. As with A/E pathogens and *Salmonella*, a number of additional T3SS effectors target inflammation and cytoskeletal dynamics to promote the survival and dissemination of the pathogen [13, 14]. Many of these effectors share significant sequence homology with T3SS effectors of EPEC, EHEC and *Salmonella* and are likely to have similar functions within the cell.

3. Inhibition of death receptor induced inflammation

Gut bacterial pathogens trigger innate immune signaling via recognition of their pathogen associated molecular patterns (PAMPs) including flagellin and LPS [15]. Inflammatory cytokines such as TNF can then induce death receptor signaling and further inflammation via the activation of NF-κB or MAPK pathways [5].

Early studies showed that while EPEC PAMPS induced inflammation, the pathogen possessed the ability to inhibit the production of inflammatory cytokines [16, 17]. Prior EPEC infection led to the inhibition of IL-8 production in infected cells even when stimulated with TNF, IL-1β or bacterial flagellin. The inhibition was T3SS dependent, and subsequently several effectors of EPEC and EHEC were shown to inhibit NF-κB signaling by targeting different host cell components using diverse mechanisms of action.

TNF produced during *Shigella* and *Salmonella* infection also triggers MAPK and NF-κB activation. Indeed, patients infected with *S. dysenteriae* or *S. flexneri* have consistently higher levels of cytokines including TNF in their serum, intestinal tissue and stools during both the acute and convalescent phase of infection [18, 19]. Likewise, increased levels of inflammatory cytokines such as TNF are observed in sera from patients suffering from gastrointestinal *Salmonella* infections

[20]. Similar to EPEC, T3SS effectors from *Shigella* and *Salmonella* have been described that inhibit inflammatory signaling pathways.

3.1. Inhibition of NF-κB signaling by bacterial T3SS effectors

3.1.1. Targeting of TAB2 and TAB3

NleE is a T3SS effector of A/E pathogens that blocks NF-κB signaling in response to TNF and IL-1β. Initial studies showed that cells infected with A/E pathogens or expressing NleE ectopically were unable to respond to stimulation with TNF or IL-1β and that NleE prevented IκB degradation and p65 nuclear translocation [21, 22]. Recently, NleE was shown to target the adapter proteins TAB2 and TAB3 upstream of IκB in the NF-κB signaling pathway [23] (Fig. 1). NleE is a novel cysteine methyltransferase that modifies TAB2 and TAB3 by transferring a methyl group onto a zinc coordinating cysteine residue within the Npl4 zinc finger domain. This prevents recognition of the ubiquitin chains on TRAF2 and TRAF6, the ubiquitin ligases involved in the TNFR1 and IL-1 receptor complexes respectively [23]. The activity of NleE depends on a conserved six amino acid motif, ^{209}IDSYMK^{214}, within the C-terminal region that is essential for the effector to block NF-κB activation and modify TAB2/3 [22, 23]. Although several EPEC effectors inhibit NF-κB signaling, NleE appears to contribute significantly to the prevention of IL-8 secretion during infection of epithelial cells [22]. However, despite the potency of its activity *in vitro*, the importance of NleE during infection *in vivo* has been hard to define. During *C. rodentium* infection of mice, *nleE* null mutants show only a marginal defect in virulence in comparison to wild-type *C. rodentium* infection [24, 25], perhaps due to redundancy in activity with other T3SS effectors.

OspZ is a homologue of NleE, found in all *Shigella* species that also inhibits NF-κB activation and p65 nuclear translocation [22] (Fig. 2). Given the high amino acid sequence similarity with NleE in all species except *S. flexneri* serotype 2a [22], OspZ presumably also exhibits methyltransferase activity and targets TAB2/3 during *Shigella* infection. Curiously, OspZ from *S. flexneri* 2a is truncated by 36 amino acids at the C-terminus, lacks the IDSYMK motif and is non-functional [22, 26]. The non-functional form of OspZ is highly conserved among strains of *S. flexneri* 2a and it is unclear why the truncated gene is maintained in the bacterial genome.

3.1.2 Control of cellular ubiquitination by type III effectors

Ubiquitination is a key mechanism regulating many eukaryotic cellular processes, including cell cycle progression, gene transcription and death receptor signaling [27]. The *Shigella* effector OspG, is an atypical Ser/Thr protein kinase that inhibits NF-κB activation by preventing ubiquitination and subsequent proteasomal degradation of phospho-IκBα [28, 29]. OspG directly interacts with

ubiquitin conjugates and K63 or K48-linked poly-ubiquitin chains in host cells, blocking the progression of p65 nuclear translocation and transcriptional activation [29] (Fig. 2).

The EPEC homologues of OspG, NleH1 and NleH2 also inhibit degradation of IκB in response to TNF stimulation [30]. Ectopic expression of NleH1/2 inhibits IκB ubiquitination through an unknown mechanism dependent on conserved lysine residues, K159 and K169 in NleH1 and NleH2 respectively, that are implicated in kinase activity [30]. NleH1 has also been shown to inhibit NF-κB signaling independent of its kinase activity and its role in inhibition of IκB degradation. NleH1 targets ribosomal protein S3 (RPS3), a KH domain protein that binds to p65 and increases its affinity for a subset of NF-κB dependent genes [31, 32]. NleH1/2 both bind RPS3 however only NleH1 prevents nuclear translocation of RPS3, due to inhibition of IKKβ mediated phosphorylation of RPS3 [31]. NleH also prevents intrinsic apoptosis, possibly through binding Bax inhibitor 1 [33]. Animal experiments using NleH mutants have yielded conflicting results, and the function of NleH *in vivo* has still not been established (Fig. 1).

The *Salmonella* effector protein GogB was recently identified as an anti-inflammatory effector that manipulates the host ubiquitination system [34]. GogB targets the host Skp, Cullin, F-box (SCF) containing complex by binding to 2 of its components: S-phase kinase-associated protein 1 (Skp1) and F-box only protein 22 (FBOX22) [34]. The SCF complex is a multi-protein E3 ubiquitin ligase that catalyzes the addition of ubiquitin moieties to proteins fated for proteasomal degradation, one of which is IκB [34]. By targeting the SCF complex, GogB interferes with IκB degradation and inhibits NF-κB activation (Fig. 3).

The *Salmonella* effector SseL was also initially proposed as having anti-inflammatory activity. Le Negrate *et al*. suggested SseL dampens innate immune defences *in vivo* by deubiquitinating IκB, preventing its proteasomal degradation and interfering with NF-κB signaling [35] (Fig. 3). However, a recent study reassessed the involvement of SseL in the inhibition of the NF-κB pathway and found no evidence that SseL targets the NF-κB pathway [36]. Instead, SseL was found to contribute to macrophage cell death [36].

While some effectors can either inhibit inflammation or apoptosis, the *Salmonella* T3SS effector, AvrA, can dampen both the inflammatory and apoptotic pathways of a eukaryotic cell by inhibition of a number of signaling pathways. Initial studies suggested that AvrA blocks the NF-κB pathway downstream of IKK activation. Ectopically expressed AvrA inhibits p65 nuclear translocation in response to TNF as well as TNF-induced activation of an NF-κB-dependent IL-8 reporter in HeLa

cells [37]. Later work suggested that AvrA inhibits NF-κB signaling and apoptosis both *in vitro* and *in vivo* and proposed that AvrA acts as a deubiquitinase with suggested targets of IκBα and β-catenin [38]. Deubiquitination of IκBα rendered it more stable, thereby preventing p65 nuclear translocation [38] (Fig. 3).

While some effectors inhibit ubiquitination of IκBα to inhibit NF-κB signaling, other effectors promote ubiquitination to induce degradation of upstream signaling mediators. IpaH9.8 is an E3 ubiquitin ligase that targets the NF-κB signaling component NEMO/IKKγ during *Shigella* infection [39, 40]. The interaction of IpaH9.8 with NEMO and the ubiquitin-binding adaptor protein ABIN-1 promotes the polyubiquitination and subsequent proteasomal degradation of NEMO, resulting in a reduced NF-κB response during infection [39]. Studies have shown that IpaH9.8-mediated inhibition of NF-κB is more pronounced during TLR4 or NOD1 signaling compared to TNF-induced signaling [39], however given that NEMO/IKKγ is located downstream of TNFR1, it is plausible that IpaH9.8 would interfere with signaling induced as a result of TNF production in the gut during *Shigella* infection (Fig. 2).

The homologue of IpaH9.8 in *Salmonella*, SspH1, contributes to the down-regulation of IL-8 production after invasion of intestinal epithelial cells. SspH1 binds a mammalian Ser/Thr protein kinase called PKN1 through a leucine-rich repeat domain [41] which could explain the nuclear localization of SspH1 as well as its role in the inhibition of NF-κB-dependent gene expression including *IL8* [42]. SspH1 was later shown to function as an E3 ubiquitin ligase for PKN1 [43], which may be involved in the TRAF-NF-κB signaling pathway [44, 45] (Fig. 3). However, a recent study showed that SspH1-mediated ubiquitination and subsequent degradation of PKN1 did not inhibit NF-κB signaling and suggested that there may be other cellular targets of SspH1, which mediate this effect [46].

3.1.2. Inhibition of inflammation by direct cleavage of NF-κB

While some effector proteins promote the degradation of signaling components by regulating cellular ubiquitination, other effectors degrade NF-κB proteins directly. NleC is a T3SS effector of A/E pathogens that directly cleaves p65 and p50. NleC functions as a zinc metalloprotease and contains the catalytic consensus motif HEXXH [47-50]. Direct cleavage of p65 by NleC was shown using recombinant proteins [47, 50] and ectopic expression of NleC results in rapid degradation of p65 [49]. Cleavage of p65 occurs at the N-terminus within the Rel homology domain (RHD), however there is some disagreement on the precise cleavage site, with two studies identifying different cleavage points [47, 50]. NleC also cleaves other NF-κB proteins, p50 and c-Rel and

potentially other related signaling proteins, IκBα and the histone acetyltransferase p300 [48, 49, 51]. While deletion of *nleC* in *C. rodentium* does not result in colonization defects or lower pathogen load during infection in mice, increased colitis was observed in comparison to infection with wild-type *C. rodentium*, thereby supporting a role for NleC in inhibiting inflammation [52] (Fig. 1).

3.1.3. Inhibition of TNFR1 receptor complex formation

Unlike NleC and NleE, the T3SS NleB1 from A/E pathogens, was observed to inhibit NF-κB signaling in response to TNF, but not IL-1β [22]. Upon overexpression, NleB1 inhibited activation of NF-κB by preventing IκB degradation, however the specific cellular targets and mechanism of action of NleB were unknown until very recently. NleB1 was identified through sequence homology to be a glycosyl transferase, containing a Rossman fold and signature DXD catalytic motif [53]. The initial target of NleB from *C. rodentium* (NleB1 in EPEC) was suggested to be GAPDH, which was proposed to be a cofactor for TRAF2 that was O-GlcNAcylated by NleB to prevent TRAF2 polyubiquitination and downstream signaling [53]. However, the precise modification site within GAPDH was not identified and subsequent studies found NleB does not glycosylate GAPDH. Instead, NleB modifies the death domains of particular signaling mediators, including FADD, TRADD and RIPK1[54, 55]. Furthermore, NleB mediates a highly novel post-translational modification, which is N-linked glycosylation to arginine [54, 55], a modification that has only been described once for a self-glucosylating corn protein [56]. The arginine targeted by NleB is highly conserved within certain death domains, including arginine 235 within the death domain of TRADD. This modification prevents TRADD oligomerisation and recruitment to TNFR1 [54]. Infection of mouse embryonic fibroblasts (MEFs) with an *nleBE* double mutant of EPEC overexpressing NleB1 leads to inhibition of NF-κB signaling [54], yet despite the effect on NF-κB, NleB does not inhibit IL-8 secretion during EPEC infection [55]. Hence *in vivo*, NleB may not function to inhibit inflammation [11, 15]. NleB1 also modifies a conserved arginine in several other death domains (FADD, TNFR1 and RIPK1) some of which relate to its ability to inhibit apoptosis driven by death receptor signaling (see below) (Fig. 1).

EPEC and EHEC also contain a homologue of NleB1, NleB2 that also contains a Rossman fold and signature DXD catalytic motif. Recombinant NleB2 appears to glycosylate TRADD, however its activity is less than that of NleB1 [54]. Additionally, ectopic expression of NleB2 does not inhibit NF-κB activation in response to TNF to the same extent as NleB1 [54]. By co-immunoprecipitation, NleB2 binds only weakly to RIPK1 and not at all not to TRADD or FADD [55]. NleB2 also does not inhibit IL-8 secretion during infection of cultured cells [55]. Given its

inefficient binding to and modification of DD proteins, it is possible that the true cellular targets of NleB2 have not yet been found.

Strong homologues of NleB1 exist in *S. Typhimurium* where they are termed SseK1, SseK2 and SseK3. NleB1 and the SseK homologues share between 80-92% similarity and 57-76% identity. Importantly, all SseK effectors contain the signature DXD catalytic motif present in NleB1 and NleB2. SseK1 and SseK2 are encoded on distinct pathogenicity islets on the bacterial chromosome whereas SseK3 is encoded within the phage ST64B lysogen [57, 58]. While SseK1 and SseK2 are present in most available *Salmonella* genome sequences, SseK3 has a limited distribution in these genome sequences, consistent with it being encoded on an active phage lysogen [57]. All three SseK proteins are translocated by the SPI-2-encoded T3SS [57, 58]. In view of the strong homology amongst the NleB and SseK effectors, it is tempting to speculate that the SseK effectors also modify death domain-containing proteins through glycosyl transferase activity similar to NleB1 from EPEC. Additionally, Li *et al*. recently reported that SseK1 glycosylates the death domain of TRADD, and inhibits NF-κB signaling when expressed ectopically [54]. However, more work is needed to establish whether the SseK effectors function as glycosyltransferases and inhibit death receptor signaling pathways *in vivo* (Fig. 3).

Some studies have also shown that Tir has immunomodulatory functions unrelated to intimin binding and the formation of A/E lesions. Ectopically expressed Tir inhibits NF-κB activation in response to TNF stimulation of cultured epithelial cells, and this has been attributed to an interaction with TRAF2 [59]. Subsequent studies have revealed that Tir contains immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that interact with protein tyrosine phosphatases (SHP-1 and SHP-2) resulting in deubiquitination of TRAF6 and a block in signaling via the IL-1β receptor [60, 61]. However, since it is difficult to dissect the immunomodulatory function of Tir from its role in A/E lesion formation, the impact of this activity compared to NleE and NleC is hard to assess.

3.2. Inhibition of MAP kinase pathways to prevent inflammation

3.2.1 Specific inactivation of JNK and p38

NleD from EPEC and EHEC is another zinc metalloprotease effector that inhibits inflammatory signaling [47, 62]. NleD specifically cleaves JNK and p38 to inhibit MAPK signaling rather than NF-κB signaling [47]. Cleavage of JNK by NleD occurs within the activation loop of JNK2 and requires no additional host cofactors. While the role of NleD in inhibiting inflammation is not as pronounced as NleE and NleC, infection of cells with an EPEC mutant lacking *nleB, nleE* and *nleC* leads to less IL-8 secretion than infection with EPEC lacking *nleB, nleE, nleC* and *nleD* [47],

suggesting that NleD contributes to the combined suppression of inflammatory effectors. NleC has also been implicated in inhibition of p38 phosphorylation and activation, however NleC does not degrade p38, and its mechanism of action in this pathway remains to be elucidated [47, 52] (Fig. 1).

SpvC is another *Salmonella* effector with apparent anti-inflammatory properties [63]. SpvC inactivates p38 and JNK *in vitro* [64, 65] by removing phosphate from threonine in a conserved MAPK activation motif. SpvC inhibits the production of pro-inflammatory cytokines *in vivo* presumably through its function as a phosphothreonine lyase [64](Fig. 3).

3.2.2 OspF targets MAPK signaling and additionally inhibits NF-κB

The homologue of SpvC, the *Shigella* effector OspF is also a phosphothreonine lyase that irreversibly inactivates MAPK [66, 67]. The activity of OspF prevents histone H3 phosphorylation in the host cell nucleus, thereby blocking access to NF-κB binding sites including in the *IL8* promoter [66, 68]. This results in decreased neutrophil recruitment at the site of infection [66] (Fig. 2).

While AvrA from *Salmonella* has been directly linked to NF-κB inhibition by deubiquitinating IκB, a different biochemical activity has also been suggested by Jones *et al.*, whereby AvrA inhibits inflammation and apoptosis both *in vivo* and *in vitro* by acetylating the mitogen-activated protein kinase kinases (MAPKK) MKK4 and MKK7, inhibiting their phosphorylation and thereby blocking JNK and NF-κB signaling pathways [69] (Fig. 3). Using the streptomycin pretreatment mouse model of enteric salmonellosis, AvrA was observed to prevent macrophage cell death and bacterial dissemination by blocking JNK phosphorylation [70]. The ability of AvrA to dampen both the inflammatory and the apoptotic pathways is consistent with the fact that *Salmonella* elicits a transient inflammation in intestinal epithelial cells without overtly destroying the epithelia, a pathology that is more characteristic of infections with *Shigella* or EHEC [71].

4. Inhibition of extrinsic apoptosis

As the subversion of inflammatory signaling can lead to apoptosis, bacterial gut pathogens have also evolved to inhibit apoptotic pathways induced by death receptor ligands. Apoptotic cell death is non-inflammatory due to the rapid engulfment of apoptotic bodies that do not release their contents, and is characterized by a lack of inflammatory cytokine production by macrophages during engulfment.

4.1. Inhibition of the death inducing signaling complex (DISC)

Although NleB1 was first described as having a role in the inhibition of NF-κB activation [21, 22], NleB1 can also antagonize death receptor signaling to prevent apoptosis [54, 55]. NleB1 binds to and modifies the death domain of FADD to inhibit TNF or FasL-induced DISC formation, thereby preventing caspase-8 activation and cell death [54, 55]. NleB1 modifies arginine 117 with GlcNAc in the DD of FADD, which is essential for formation of the FAS-FADD oligomeric complex and formation of the DISC [4, 72]. An EPEC *nleB1* mutant has reduced ability to inhibit caspase-8 activation and cell death *in vitro*, while a *C. rodentium nleB* mutant also shows diminished ability to inhibit caspase-8 activation *in vivo* [55] (Fig. 1).

The inhibition of FAS signaling by NleB suggests that the FAS pathway is important for controlling infection with A/E pathogens. Indeed the role of FAS signaling in controlling infection with *C. rodentium in vivo* is supported by the development of severe disease during infection of FAS and FasL deficient mice with *C. rodentium* [55]. Furthermore, similar phenotypes of severe disease are observed during infection of Bid deficient mice, suggesting that apoptosis of non-lymphoid cells helps control colitis. Interestingly, polymorphisms in the human *FASLG* gene encoding FasL have been implicated in the development of inflammatory bowel disease, suggesting a role for Fas signaling in controlling pathology in response to gut microbes [73]. For EPEC, NleB may prolong the survival of infected gut epithelial cells by preventing their removal to enhance bacterial colonization and increase bacterial shedding in feces. This would optimize subsequent dissemination to other hosts [74].

4.2. Prevention of apoptosis by direct inhibition of caspases

NleF has been implicated in inhibition of apoptosis induced by both intrinsic and extrinsic pathways. Although work has focused mainly on the role of NleF in inhibiting intrinsic apoptosis, NleF binds caspase-8 and inhibits TRAIL induced activation of caspase-8 and apoptosis [75]. It appears that NleF may act as a direct caspase inhibitor, as NleF was shown to directly bind caspase-9 similarly to previously reported caspase-9 inhibitors [75]. However, it appears that the role of NleF in inhibiting apoptosis may be secondary to other effectors such as NleB, as no differences in activation of effector caspases-3 and -7 were observed during infection of HeLa cells with EPEC Δ*nleF* in comparison to wild-type EPEC [75] (Fig. 1).

5. Lymphotoxin-α and alternative signaling via TNFR

Lymphotoxin-α (LTα) is a member of the TNF superfamily and has recently emerged as an important factor in controlling immune homeostasis and regulation of the intestinal microflora [76]. LTα is essential for the development of secondary lymphoid tissues and for the organization of

lymphoid tissues including the spleen and thymus [77, 78]. The predominant pathway for LT-induced lymphoid tissue development is via the lymphotoxin-β receptor (LTβR), which is found on a number of non-lymphoid cell types including, fetal stromal cells, cells of the myeloid lineage, endothelial cells, hepatocytes and intestinal epithelial cells [79, 80]. The LTβR is activated when engaged by a heterotrimer of LTα and LTβ [$LT\alpha_1\beta_2$), which can be expressed by B cells, T cells and innate lymphoid cells (ILCs) that express the RORγt receptor [76]. Mice deficient in LTα, LTβ or LTβR are unable to coordinate lymphoid organogenesis [76, 81]

Homotrimers of LTα can also bind and activate signaling via TNFR1 [82], although activation is not as potent as that induced by TNF [80, 83, 84]. As a natural pathogen of mice, *C. rodentium* infection provides a useful model to study the interaction between ILCs and intestinal epithelial cells *in vivo*. During *C. rodentium* infection LT is essential for IL-22 production by ILCs, and inhibition of LTβR signaling severely impairs ILC IL-22 production [85, 86]. These ILCs are predominantly located in lymphoid follicles in the colon and are closely associated with dendritic cells (DCs). LTβR-deficient mice are highly susceptible to infection with *C. rodentium* with mortality occurring as early as day 10 after infection [87] and clearance is dependent on expression of LTβR on both myeloid and intestinal epithelial cells [87]. TNFR-deficient mice are also susceptible to *C. rodentium* infection [88], which suggests that either TNF- and/or LT-induced TNFR signaling may play a role in clearance of the pathogen. Given that *C. rodentium* encodes all of the same LEE and non-LEE-encoded virulence factors that inhibit death receptor signaling [12, 89], it is likely that TNFR signaling would be blocked during *C. rodentium* infection.

6. Conclusions

There is much left to understand about the role of various effector proteins in inhibiting inflammation and/or apoptosis during infection. Several effectors appear to have redundant functions, so it remains to be seen how all the effectors act together and whether their activity is regulated by hierarchy of expression and/or translocation *in vivo*. What is certain is that bacterial effector proteins potently subvert the anti-microbial response of the host cell by inhibiting both death receptor induced inflammation and cell death.

In some cell types, the simultaneous inhibition of inflammatory and apoptotic pathways induces a form of cell death known as necroptosis, which can not only remove infected cells, but also induce inflammation [90]. The role of necroptosis in normal human physiology is unclear but the inflammation and cell death induced by necroptosis could potentially promote the clearance of infection where the primary innate responses are inhibited by the pathogen. However, considering

that bacterial effector proteins attack both inflammatory and apoptotic signaling at multiple points, it is reasonable to assume that some pathogens will also inhibit the last remaining innate anti-microbial response of necroptosis. Further study is required to investigate the potential role of necroptosis during infection and the possible inhibitory mechanisms exhibited by bacterial pathogens that may attack this pathway.

438 **Legend to the Figures**
439
440 **Figure 1. Inhibition of death receptor signaling by enteropathogenic *E. coli* (EPEC) and**
441 **enterohemorrhagic *E. coli* (EHEC).** The T3SS effector NleB1 is a glycosyltransferase that
442 modifies a conserved arginine in the DD of FADD, TRADD and RIPK1 with a single GlcNAc. The
443 modified DD proteins are not recruited to the death receptor complex and cell death is subsequently
444 blocked. Tir is a dual function effector that 1) mediates intimate attachment of EPEC/EHEC to the
445 host cell and 2) interacts with TRAF2 to inhibit NF-κB activation. NleE is a cysteine
446 methyltransferase that modifies the zinc finger domain of TAB2/3 thereby preventing recognition
447 of ubiquitinated TRAF2. NleD and NleC are zinc metalloproteases that specifically cleave JNK/p38
448 and Rel proteins, respectively. NleH binds the transcription factor RPS3 and prevents its nuclear
449 translocation, thereby dampening NF-κB activation. NleF binds caspases-4, -8 and -9 and prevents
450 apoptosis.
451
452 **Figure 2. Inhibition of death receptor signaling by *Shigella*.** OspF is a phosphothreonine lyase
453 that inactivates MAPK signaling. OspZ inhibits IκB degradation and although the mechanism has
454 not been tested, it is likely that OspZ is a cysteine methyltransferase given its high sequence
455 homology to NleE from EPEC/EHEC. OspG is a Ser/Thr protein kinase that inhibits ubiquitination
456 and proteasomal degradation of phospho-IκBα. IpaH9.8 is an E3 ubiquitin ligase that promotes
457 ployubiquitination and proteasomal degradation of NEMO.
458
459 **Figure 3. Inhibition of death receptor signaling by *Salmonella*.** SseK1 is highly homologous to
460 NleB1 from EPEC/EHEC and potentially GlcNAcylates the DD of TRADD, however no functional
461 studies have been published. AvrA and SseL inactivate NF-κB signaling by deubiquitinating IκB.
462 AvrA also inhibits phosphorylation of MAPK components MKK4 and MKK7, further inactivating
463 NF-κB and JNK signaling. SpvC is a phosphothreonine lyase that inactivates p38 and JNK. GogB
464 targets the host SCF E3 ligase complex to inhibit ubiquitination and subsequent degradation of
465 IκBα.
466
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468

469 **Table 1**. Type III effector proteins from bacterial gut pathogens and their effect on death receptor
470 signaling

Effector	Host targets	Enzymatic activity	Function	References
Attaching and effacing pathogens (EPEC, EHEC, *C. rodentium*)				
Tir	SHP-1, SHP-2, TRAF 2		Inhibits NF-κB signaling.	[59-61]
NleB1/NleB2	Death domain containing proteins (FADD, TRADD, RIPK1, TNFR1)	N-linked Glycosyl transferase	Inhibits DISC formation, inhibiting apoptosis. Inhibits NF-κB signaling.	[53-55]
NleC	p65(RelA), p50, c-rel, IκB, p300	Zinc metalloprotease	Cleaves NF-κB, inhibits inflammation.	[47-51]
NleD	JNK, p38	Zinc metalloprotease	Cleaves JNK and p38. Inhibits inflammation	[47]
NleE	TAB2, TAB3	Cysteine methyltransferase	Inhibits ubiquitin chain binding by TAB2 and TAB3, inhibiting NF-κB signaling.	[21-23]
NleF	Caspase-4, -8 and -9		Caspase inhibitor, inhibits apoptosis.	[75]
NleH1/NleH2	RPS3, Bax inhibitor 1	Ser/Thr kinase	Inhibits NF-κB and apoptosis	[30, 31, 33]
Salmonella				
AvrA	IκBα, MKK4, MKK7	Deubiquitinase, acetyltransferase	Inhibits NF-κB and MAPK signaling, anti-inflammatory and anti-apoptotic	[37, 38, 91]
GogB	Skp1, FBOX22		Targets SCF complex to inhibit IκBα degradation and NF-κB activation.	[34]
SpvC	ERK, p38, JNK	Phosphothreonine lyase	Inactivates MAPK, inhibits inflammation	[63-65]
SseL	IκBα	Deubiquitinase	Deubiquitinates IκBα to inhibit NF-κB signaling, contested by [36]	[35]
SseK1/2/3	DD of TRADD (for SseK1)	N-linked Glycosyl transferase	Inhibits NF--κB activation	[54]
SspH1	PKN1	E3 ubiquitin ligase	Inhibits NF-κB activation	[42, 92, 93]
Shigella				
IpaH9.8	NEMO, ABIN-1	E3 ubiquitin ligase	Inhibits NF-κB activation by promoting ubiquitinationa and proteasomal degradation of NEMO	[39]
OspG	K63 or K48-linked polyubiquitinated proteins	Ser/Thr protein kinase	Prevents ubiquitination of IκBα	[28, 29]
OspF	MAPK	Phosphothreonine lyase	Inactivates MAPK, prevents access of NF-κB to the *IL8* promoter.	[66-68]
OspZ	TAB2, TAB3 (by homology to NleE)	Cysteine methyltransferase	Inhibits NF-κB activation	[22)

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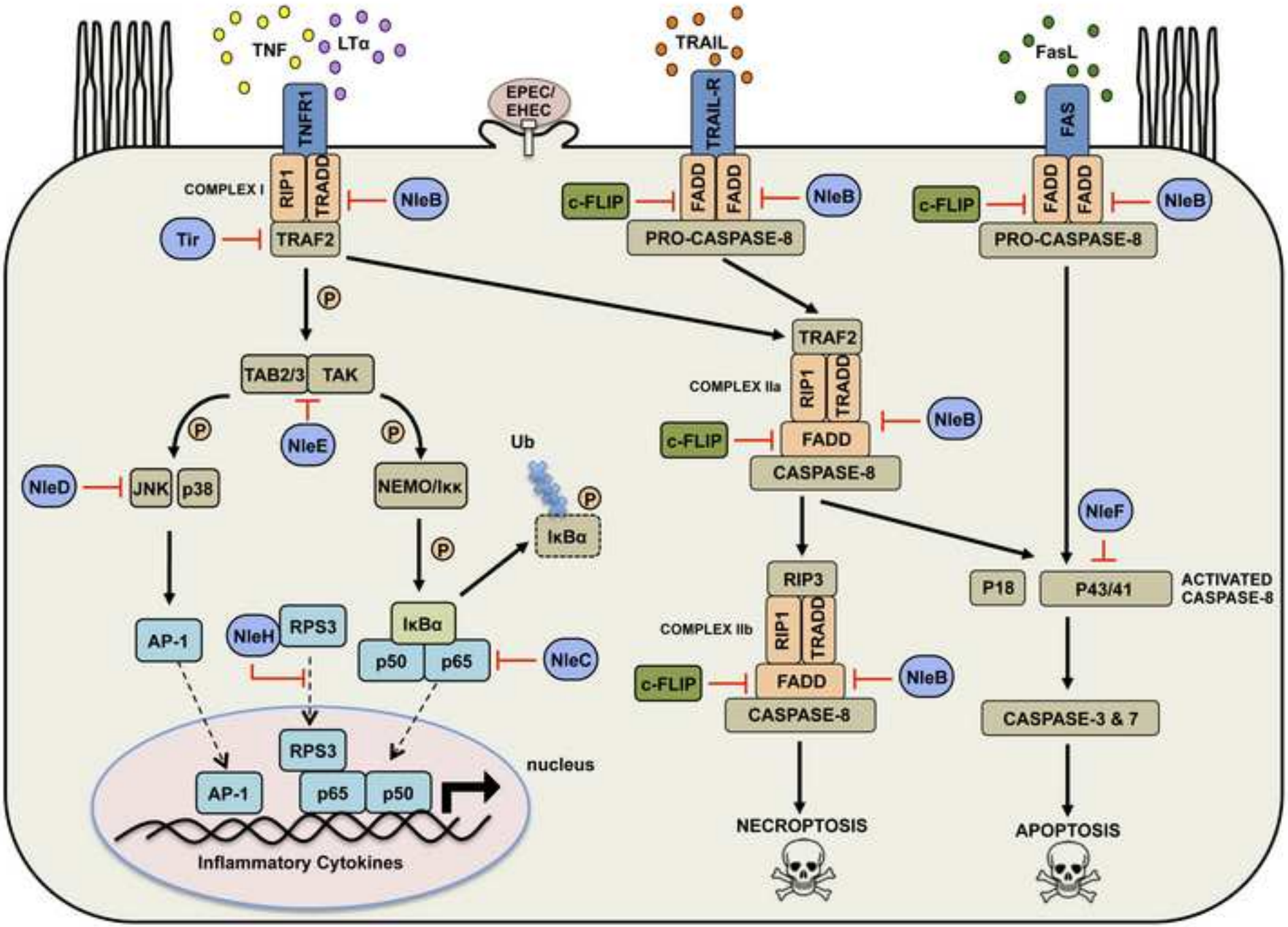
Figure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Tir
TRAF2
c-FLIP
FADD
PRO-CASPASE-8
TAB2/3
TAK
NleE
NleD
JNK
p38
NEMO/IκK
Ub
IκBα
AP-1
NleH
RPS3
p50
p65
NleC
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Inflammatory Cytokines
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CASPASE-8
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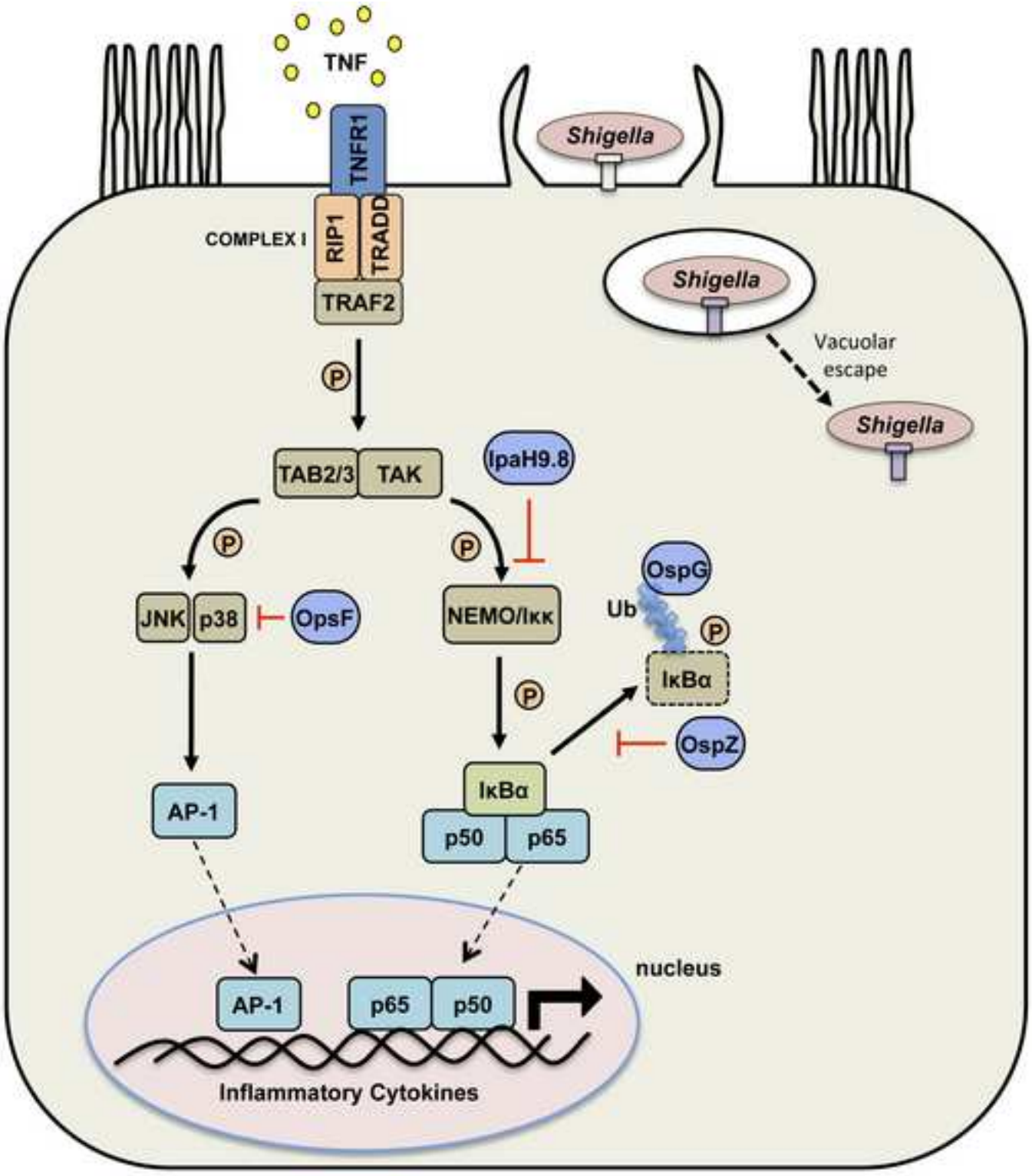

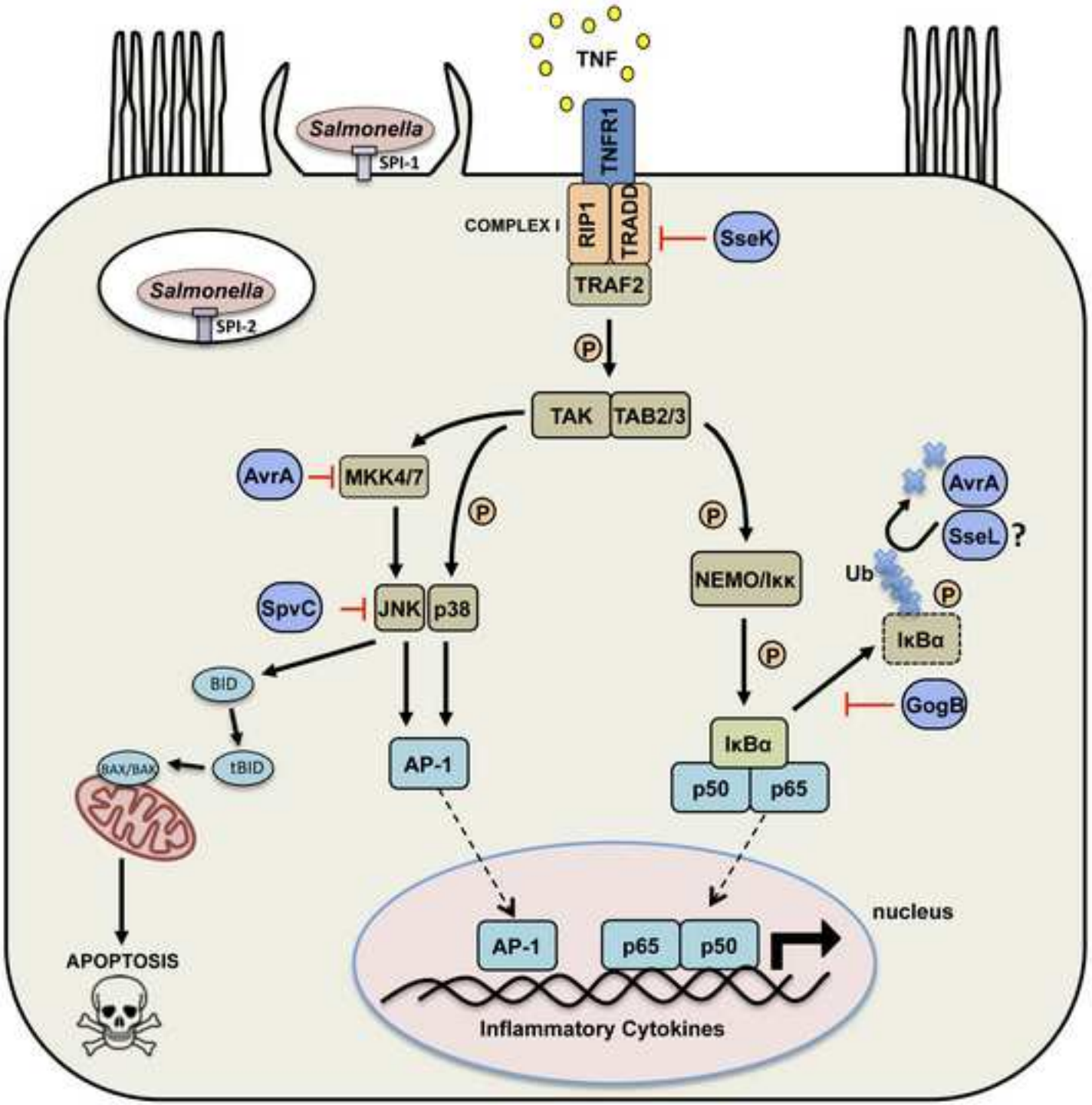

Author/s:
Giogha, C; Lung, TWF; Pearson, JS; Hartland, EL

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