The Commonalities in Bacterial Effector Inhibition of Apoptosis

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Abstract

Anti-apoptotic pathways of the host cell play integral roles in bacterial pathogenesis, with inhibition of those pathways resulting in halted disease pathology. Certain pathogens have developed elegant mechanisms to modulate the fate of the host cell, many of which target novel pathways that are poorly understood in the context of the cell biology. Bacterial pathogenesis research not only promotes the understanding of the role of anti-apoptotic pathways in bacterial infection, but has a broader context in understanding the epitome of human disease, i.e. developing the understanding of tumorigenic or inflammatory pathways. Here we review host anti-apoptotic signalling pathways manipulated by translocated bacterial effectors that propagate the disease state, drawing common parallels and showing the novel differences.

Apoptosis and Bacterial Pathogenesis

Bacterial infection of human epithelial cells activates multiple host signalling pathways, which stimulate host immunity and impose cellular stresses. Prolonged activation of these pathways often results in programmed cell death, also termed apoptosis [1, 2]. Apoptosis is a highly regulated, innate immune response that occurs canonically by two different pathways: extrinsic and intrinsic. In the context of infection, apoptosis eliminates pathogens at the point of infection without emitting systemic alarms [3-9]. Apoptosis is controlled and progressed by the activation of cysteine-dependent aspartate-specific proteases (caspases), which cleave signal transduction, cytoskeletal and nuclear proteins to induce cell death. It should be noted a caspase-independent pathway exists, mediated by apoptosis inducing factors, but this is outside the scope of this review. Apoptotic signalling pathways are supported by other signalling pathways, such as the nuclear factor-kappa B cells (NF- κ B) pathway. NF- κ B is a collective term to describe a family of closely related transcription factors that are involved in inflammation, the immune and stress response, and can increase/decrease the transcription of pro- or anti-apoptotic proteins depending upon the type and duration of apoptotic stimuli.

For some pathogens, the timely induction of apoptosis enables dissemination, evasion of immune cells, and nutrient access. As part of an effective infection strategy bacterial

pathogens have evolved mechanisms to manipulate apoptosis by expressing or translocating effector proteins to control host signalling pathways. The mechanisms used to manipulate apoptotic pathways vary greatly among pathogens and host cell types. However, in recent years commonalities have emerged that show translocated bacterial effectors target specific points in apoptotic and apoptotic-related pathways. This review focuses on why translocated bacterial effectors share common targets and modes of action (Table 1) covering the extrinsic (Figure 1), intrinsic (Figure 1), generalist anti-apoptotic, and the NF- κ B-dependent pro-survival pathway (Figure 1) to promote cell survival.

It should be noted as this review focuses on translocated effectors examples of non-translocated effectors such as the *Chlamydia* cell surface tyrosine kinase receptor Ephrin2A, which activates the pro-survival phosphoinositide 3-kinase (PI3K)/Akt pathway [10], or chlamydial inclusion membrane protein G (IncG) homolog on the surface of inclusions, which promote mitochondrial membrane integrity [11-14] will be omitted. Nevertheless, non-translocated effectors are important and highlight that multiple components act in unison during pathogenesis to promote cell survival.

Extrinsic Apoptosis and its Regulation

Extrinsic apoptosis is activated by external signals [e.g. Fas ligand, pathogen-associated molecular patterns (PAMPs)] binding to host surface death receptors (Figure 1). Death receptors, such as those in the TNFR family, cross-link via their ligand binding domain, recruiting adaptor proteins inclusive of Fas-associated death domain protein (FADD) and TNFR1-associated death domain (TRADD). FADD/TRADD possesses a death domain (DD) to recruit other DD family members resulting in the formation of the death inducing signalling complex (DISC) at the activated receptor [15]. FADD also bears a death effector domain (DED), which directly promotes downstream apoptotic events, i.e. initiator caspase-8 and RIP1 activation. Interestingly, two alternative extrinsic pathways exist; the type 1 pathway recruits large levels of caspase-8 to the DISC, which cleaves procaspase-3 and induces apoptosis. The type 2 pathway recruits a small amount of caspase-8 to the activated receptor, which is targeted to the mitochondria where it cleaves BID, a BH-3 only member of the BCL-2 family [16, 17]. BID acts to promote oligomerization of pro-apoptotic BCL-2

members, Bax and Bak, to induce mitochondrial outer membrane permeabilisation (MOMP) and pro-apoptotic protein release. Unlike the type 1 pathway, the type 2 pathway relies largely on mitochondrial lysis to promote apoptotic cascades. Nevertheless, a commonality exists in that both pathways converge on mitochondrial lysis to promote or amplify the apoptotic cascade. Therefore, targeting DISC formation would halt extrinsic apoptosis at the point of initiation, irrespective of the cell being type I or type II..

Death Domain Targeting in Extrinsic Apoptosis

The extracellular pathogens enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC and EHEC, respectively) secrete NleB1 via their type III secretion system. NleB1 interacts with the DD on adaptor proteins at the activated receptor, specifically FADD, TRADD, TNFR1 and RIPK1 [18, 19]. Furthermore, NleB1 possesses N-acetyl-D-glucosamine (GlcNAc) transferase activity, which it utilises to modify the DD by adding a single GlcNAc moiety to the highly conserved arginine-117 of FADD or arginine-235 of TRADD. This modification blocks their recruitment to the activated receptor, thereby inhibiting formation of the DISC and stopping the extrinsic apoptotic pathway at initiation [20]. EPEC/EHEC also contain an NleB1 homologue; NleB2, which like NleB1 contains a Rossman fold, aspartate-any residue-aspartate (DXD) catalytic motif and can glycosylate TRADD, but with reduced efficacy than NleB1.

Similarly *Salmonella enterica* translocates three *Salmonella* pathogenicity island-2 (SPI-2) encoded effectors, SseK1/2/3, that bear 57-76% identity and 80-92% similarity to NleB1. SseK1 and SseK2 are found in most *Salmonella* genomes, with SseK3 having limited distribution as it is located on an active phage lysogen [21]. SseK1 was shown to function comparably to NleB1 and inhibits tumour necrosis factor- α (TNF- α) and NF- κ B signalling via GlcNAcylation of TRADD [20].

Bioinformatic searches of the primary sequence for NleB and SseK1 revealed no similarity to host conserved domains or host proteins. Nevertheless, as shown in Figure 2, these effectors share common targets and mode of action; blocking both complex I and II extrinsic pathways by inhibiting DISC formation, irrespective of the cell type or activated receptor. As such, it is easy to speculate that these effectors must share structural commonalities to

host DDs to enable interaction with DD containing proteins and permit effector mediated GlcNAcylation to block DISC and extrinsic apoptosis; however, this has yet to be confirmed.

Intrinsic Apoptosis and its Regulation

In addition to extrinsic apoptosis, infection can activate the intrinsic apoptotic pathway (Figure 1) via a number of mechanisms including the release of Ca^{2+} from the endoplasmic reticulum (ER), DNA damage, metabolic burden/stress or via a host intracellular detection system. Intrinsic pathways centralise around involvement of the B Cell Lymphoma-2 (BCL-2) family of proteins, which are categorised into BH-3 only (BIB, BIM, and PUMA), pro-apoptotic (BAX, BAK, Bcl-RAMBO and BOK) or anti-apoptotic (Bcl-2, Bcl-2xL, and MCL-1) members. Pro-apoptotic BCL-2 member activity is largely orchestrated by the activation of BH-3 only proteins, which promote their oligomerization at the mitochondria, inducing Mitochondrial Outer Membrane Permeabilisation (MOMP) [22]. MOMP releases additional pro-apoptotic proteins, inclusive of cytochrome c, which induce the formation of a death signalling complex (apoptosome) activating initiator caspase-9. Active caspase-9 cleaves effector caspase-3 inducing the caspase cascade and promoting apoptosis. Intrinsic apoptosis is moderated by anti-apoptotic BCL-2 members, with the ratio between anti- and pro-apoptotic BCL-2 members determining the susceptibility of cells to apoptosis [23]. This intricate control system provides a unique point for bacterial manipulation to promote cell survival.

Regulating BCL-2 Family Members

The following respectively highlights the commonalities in how different effectors modulate the expression, degradation or activity of BCL-2 members to benefit the pathogen.

Helicobacter pylori translocates CagA, which is tyrosine phosphorylated by host Src kinases. This phosphorylation is required to enable interaction between CagA and host GRB2, c-Met, ZO-1, and CRK. In turn this activates the MEK/ERK pathway and promotes Mcl-1 expression (Figure 2) [24]. Mcl-1, an anti-apoptotic BCL-2 member with a high turnover rate, acts by binding and inhibiting BH-3 only protein BIM [25]. BIM acts by binding BAK to assemble large homo-oligomeric pores in the mitochondrial outer membrane (MOM) inducing MOMP

and apoptosis [26]. In addition, CagA activates supporting pathways including MAPK, PI3K/Akt and NF- κ B to respectively promote post-translational modifications or expression of anti- and pro-apoptotic BCL-2 members, further regulating their activity [26]. Similarly *Mycobacterium tuberculosis* regulates anti- and pro-apoptotic BCL-2 member expression to inhibit apoptosis [27, 28]. The *Mycobacterium* effectors responsible have yet to be elucidated, nevertheless, this shows that two distinct bacteria adopt common approaches by targeting the expression of BCL-2 members to regulate apoptotic cascades. Further work is required to understand the intricacies of this approach and how host pathways are manipulated to reach common goals.

Alternatively, bacteria may degrade BH-3 only BCL-2 members, to promote cell survival. *Chlamydia spp.* translocate the chlamydial protease-like activity factor (CPAF), which has a broad range of activities. CPAF, a serine protease, degrades BH-3 only proteins BIM, PUMA and BAD, all of which bind to and antagonise anti-apoptotic BCL-2 members [29-32]. As such CPAF promotes anti-apoptotic BCL-2 member activity by removing their inhibitors. It should be noted that there is discussion regarding the role of CPAF during infection and its pertinence to inhibiting apoptosis. Due to the lack of clarity regarding this matter this is not something that will be addressed in this review. Nevertheless, we speculate that CPAF is one of a number of Chlamydial anti-apoptotic effectors that act in orchestration to promote cell survival during infection. Interestingly, CPAF is the sole example of this novel approach adopted by translocated effectors to promote cell survival. However, it has been observed that infection by a number of bacteria have resulted in the degradation of BCL-2 members to purport cell survival, indicating this strategy may be adopted by other bacteria. Nevertheless, other CPAF-like effectors remain to be identified.

Finally, effectors can also act as competitive inhibitors/substrate mimics or prevent pro-apoptotic members from reaching their target. SidF from *Legionella pneumophila* inhibits apoptosis by directly interacting with and neutralizing the effects of Bcl-RAMBO and BNIP3 [33]. Bcl-RAMBO promotes MOMP by inducing a permeability transition pore (PTP) in the MOM through the adenine nucleotide translocase, whereas BNIP3 induces apoptosis mediated by the PARP-1 polymerase to induce a PTP in the MOM and promotes the nuclear transport of apoptosis inducing factors [34]. A similar strategy of blocking pro-apoptotic BCL-2 member activity is taken by the emerging pathogen *Anaplasma phagocytophilum*,

which secretes Ats1 to block Bax. Ats1 utilises an N-terminal targeting sequence to target and insert itself into mitochondria, thereby enabling cleavage by the matrix-located mitochondrial processing peptidase. Cleaved Ats-1 translocates from the mitochondrial matrix and stacks into the MOM to block Bax insertion and pore formation, thus stopping MOMP, cytochrome c release and apoptosis. [35]. Again, this strategy of inhibiting BCL-2 family member activity is also adopted by *Coxiella burnetii* which translocates the effector CaeB via the Dot/Icm system. The specifics of CaeB inhibition is unknown, nevertheless, CaeB was shown to localize to the mitochondria and inhibit MOMP and caspase-9 activation [36]. It should be noted that CaeB did not affect the expression levels or localization of pro- or anti-apoptotic BCL-2 members [36]. CaeB possesses a coil-coil domain enabling protein interaction; as such we speculate that CaeB can act in one of two methods. First, by interacting with Bax to block mitochondrial pore formation or second by interacting with anti-apoptotic BCL-2 members to promote their activity and block Bax pore formation [36]. Like SidF and CaeB, *L. pneumophila* translocates the effector SdhA, via the Dot/Icm system, to block MOMP and cytochrome c release. The specifics of its activity remain unknown, however, its effects indicate it acts either at the point of MOMP or upstream of this [37].

Underpinning the aforementioned strategies is the targeting of BCL-2 family members (Figure 2). Although the bacterial effectors modes of action vary each effector is unified in that their activity is centralised around BCL-2 member activity. Interestingly, certain BCL-2 members are core targets, specifically the BH-3 only proteins BIM, PUMA and BAD, the pro-apoptotic protein BAX and the anti-apoptotic proteins Mcl-1, Bcl-2 and Bcl-xL. Although other BCL-2 members exist this could indicate the essential nature of these members in apoptosis.

ER-Manipulated Apoptosis

The ER is commonly stressed by bacterial infection as it controls protein synthesis, initial post-translational modifications, lipid biosynthesis, calcium homeostasis, protein folding and trafficking [38]. Inhibition of these processes induces a stressed state on the cell, activating the unfolded protein response (UPR) to adapt to the environmental stresses and promoting cell survival [39]. The UPR is mediated via three signalling pathways which are kept inactive

via the ER luminal protein Grp78/Bip and regulated by Bax Inhibitor-1 (BI-1) [40, 41]. A prolonged UPR promotes apoptosis through the release of Ca^{2+} , which mitochondria can efficiently accumulate due to their negative (inside) membrane potential. Ca^{2+} increases mitochondrial metabolic activity, with excessive amounts resulting in the formation of the mitochondrial PTP, lysis and release of pro-apoptotic proteins [5-7, 42].

A major player in ER stress, BI-1, has a broad anti-apoptotic spectrum extensively reviewed in [43], making it a prime target for manipulation. EPEC/EHEC secrete the NleH family of effectors which have a broad range of anti-apoptotic activity and, like Bcl-2, interact with the N-terminus of BI-1 to promote its anti-apoptotic activity and block caspase-3 activation [44]. Infection with EPEC $\Delta nleH1\Delta nleH2$ mutants showed elevated cytosolic Ca^{2+} implicating BI-1 in regulating Ca^{2+} during EPEC infection and suggesting NleH effectors promote BI-1 activity. Furthermore, NleH effectors inhibit apoptosis induced by *Clostridium difficile* toxin B (TcdB), which induces apoptosis in three ways. Firstly, TcdB inhibits RhoGTPases blocking the assembly of the actin cytoskeleton resulting in loss of anchorage, secondly TcdB inhibits transcription factor activation/inactivation, and thirdly it disrupts mitochondrial membrane potential [45]. This implies NleH effectors have a global anti-apoptotic activity outside of the scope of some of BI-1s functions, suggesting NleH may have other interaction partners.

Similarly, *Salmonella bongori* translocates SboH which localises to the mitochondria and inhibits apoptosis induced by ER stress inducers [46]. SboH, a chimeric effector consisting of SopA and NleH, exerts its function via its NleH component (Figure 2). The binding partners and mode of action have yet to be elucidated, however, we speculate SboH exerts its anti-apoptotic effects via BI-1. As such SboH's localization to the mitochondria would bring it in close proximity to much of the ER enabling it to promote its anti-apoptotic activity via BI-1, assuming SboH also shares similar functional anti-apoptotic properties to NleH.

Underpinning both effectors is the commonality in the NleH component to exert anti-apoptotic function. The ER, a critically important organelle, is pivotal in regulating cellular stress and mitochondrial permeability. The shared commonality between these effectors indicates Bax Inhibitor 1, and potentially other ER resident proteins, may play a larger role in regulating global anti-apoptotic pathways than the majority of the focus of apoptotic literature to date. NleH has a multi-faceted approach to regulating cell death, however, the

importance of temporal orchestration of its abilities to inhibit apoptosis remains be identified.

Mimicking XIAPs

X-Linked inhibitors of apoptosis (XIAPs) are direct, endogenous caspase-3, -7 and -9 inhibitors, that interact via their baculovirus IAP repeats (BIR) to deliver a potent inhibition of the caspase cascade [47]. XIAP BIR domains use a site binding mechanism, termed the IAP-binding motif (IBM), which interacts with cleaved caspases, which structurally form an IBM. This enables tight interaction and blocks the catalytic binding cleft of those caspases [48]. Recently XIAPs were also shown to permeabilise and enter the mitochondria during times of stress to degrade the second mitochondria-derived activator of caspases (SMAC, also named DIABLO) which binds to the IAPs and promotes caspase activity, by using an E3 ligase activity to recruit endolysosomes into the mitochondria [49].

The EPEC/EHEC effector NleF is a functional XIAP mimic, however there is some difference in binding partners. NleF interacts with mitochondria, caspases-4, -8 and -9, and triggers nuclear translocation of NF- κ B p65 leading to the upregulation of proinflammatory IL-8 expression in a manner independent of caspase interaction [50] (Figure 2). As shown via caspase-9 inactivation, NleF acts by binding the S1-S4 pockets of two caspase monomers, making them proteolytically inactive [51]. Presently NleF remains unique in its mimicry of XIAPs, sharing no commonality with other bacterial effectors. Nevertheless, despite both NleF and XIAPs having a broad spectrum of anti-apoptotic activity, their mimicry does not block the downstream effects from stresses on the cell such as Ca^{2+} release from the ER. Such stresses, like Ca^{2+} release, has a significant number of undesirable effects during infection, therefore, other effectors help support NleF activity to create a homeostatic environment by modulating pathways such as the PI3K/Akt pathway.

PI3K and Akt Activation

PI3K/Akt is a highly conserved pathway with a broad spectrum of activity including promoting cell survival, reviewed in [52] (Figure 3). Receptor activation directly stimulates class 1A PI3Ks resulting in the conversion of phosphoinositide-3,4,-bisphosphate (PIP2) lipids

to phosphoinositide-3,4,5-triphosphate (PIP3) lipids. At the plasma membrane, Akt binds PIP3, inducing conformational change enabling phosphoinositide-dependent kinase-1 (PDK1) to phosphorylate threonine-308, partially activating Akt. This induces further conformational change, exposing the Akt activation loop enabling mTOR1 or DNA-PK to phosphorylate Serine-473 for full Akt activity [52]. In the context of apoptosis, active Akt, a Ser/Thr Kinase goes on to inhibit activation of pro-apoptotic proteins (e.g., Bad, Bax), caspases (e.g. caspase-9), and transcription factors (e.g., p53), whilst activating anti-apoptotic transcription factors (e.g. FOXO), and caspase inhibitors (e.g., XIAP). Due to the broad spectrum of activity, Akt is a prime candidate for manipulation by bacteria to promote cell survival or modulate host activity.

S. enterica serovar Typhimurium secretes type III effector SopB/SigD to promote Akt- and Erk1/2-mediated protection [53, 54]. SopB does not activate PI3K, but localises to the plasma membrane and acts as an inositol phosphatase to activate Akt, seemingly in a manner similar to host inositol-4-phosphatase (INPP4B), [53-55]. Curiously, INPP4B has a tumour suppressor function by inhibiting PI3K signalling [56], however, SopB is both a 4 and 5 phosphatase with its 5 phosphatase component being essential for inducing ruffle formation [57]. SopB induced ruffles in the plasma membrane recruit PDK1 and Rictor, both important for initial activation of Akt [55]. Furthermore, SopB leads to an accumulation of PIP2 and PIP3 at the plasma membrane by recruiting Rab5 and Vps34 [58]. This accumulation further promotes initial Akt activation, which offers a degree of apoptotic protection until full Akt activation.

Similar to SopB, *Shigella flexneri* IpgD is a 4 -phosphatase but with different substrate specificities. IpgD removes the 4 phosphate on PI(4,5)P2 resulting in PI(5)P, which indirectly increases Akt phosphorylation by inhibiting PP2A phosphatase [59-61]. Unfortunately PI(5)P is a rare phosphoinositide and poorly understood, with further understanding of PI(5)P is required to fully appreciate the IpgD mode of action. Nevertheless, this underpins how different effectors share the same activity, act at the same point in a pathway and target phosphoinositides, albeit with differences in their substrate specificity, to modulate anti-apoptotic pathways.

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289 In a manner independent of phosphoinositides, the *E. coli* effector EspZ upregulates Akt and
290 focal adhesion kinase (FAK) activity [62]. The exact mechanism is unknown, however, EspZ
291 localises to the mitochondria, using a non-canonical mitochondrial targeting sequence, and
292 interacts with the inner mitochondrial membrane 17b (TMB17b) protein [63]. TMB17b is
293 part of a complex responsible for translocating proteins into the mitochondria. We
294 speculate that EspZ has a dual mode of action when inhibiting apoptosis, first by acting
295 directly/indirectly to activate Akt and FAK, and second by modulating mitochondrial
296 dependent pro-apoptotic pathways.

297

298 These effectors and the PI3K/Akt pathway they target highlight gaps in the current
299 understanding of phosphoinositide's involved in the PI3K/Akt pathway. This review shows
300 all effectors promote Akt activity, independent of PI3K, indicating the importance of
301 alternative activators of Akt on apoptotic cascades.

302

303 Modulating the NF- κ B and MAPK Pathway

304 The NF- κ B pathway can have a profound impact on the apoptotic response of a cell (Figure
305 3). NF- κ B collectively describes homo- and heterodimeric complexes formed by the Rel/NF-
306 κ B proteins, which include RelA (p65), RelB, c-Rel, NF- κ B1 (p50) and NF- κ B2 (p52). These
307 cytoplasmic proteins are held inactive by inhibitory I κ Bs proteins, with a number of
308 pathways activating I κ B kinases (IKKs), which phosphorylate I κ Bs resulting in their
309 ubiquitination and degradation thereby relinquishing inhibition of NF- κ B. In the context of
310 infection, the role of the NF- κ B pathway in the immune response has been the major focus,
311 however, depending upon the apoptotic stimuli, the NF- κ B pathways can play a critical role
312 in promoting cell survival.

313 The aforementioned EPEC and EHEC effector NleH, inhibits NF- κ B by binding to the
314 ribosomal protein S3 (Rps3); reducing its nuclear abundance. Rps3 interaction with the
315 transcription factor p65/RelA, is important for optimal binding and transactivation of a
316 subset of NF- κ B target genes. However, NleH did not inhibit p65/RelA nuclear transport

[64]. Unfortunately the NF- κ B modulation of apoptotic genes was not examined in the context of this work, leading to only speculative assessment of the data in the context of apoptosis. Similarly, CPAF cleaves the transcription factors RFX5 and USF1 which are required degradation of p65/RelA [65, 66]. These studies indicate that both NleH and CPAF both act to promote p65/RelA regulated gene expression. Due to the anti-apoptotic nature of both effectors, we hypothesise that p65/RelA promotes anti-apoptotic gene expression in the context of EPEC/EHEC and *Chlamydia* infection.

EPEC also secretes NleB, which utilises its GlcNac transferase activity to inhibit NF- κ B by modifying glycolysis enzyme glyceraldehyde 3-phosphate (GADPH), a coactivator of TRAF2 [19]. TRAF2 is required for both NF- κ B activation and TNFR complex II formation, thereby offering further protection to extrinsic apoptosis and inhibition of NF- κ B gene expression. Interestingly, EPEC and EHEC secrete the translocated intimin receptor (Tir), which is used to promote intimate attachment between host and microbe, and Tir also blocks NF- κ B activation by interacting with TRAF2 [67]. Unfortunately the role of both Tir and NleB inhibition of TRAF2 on the NF- κ B pathway and its exact impact on apoptosis has yet to be elucidated. However, we speculate that these effectors target TRAF2 to halt the activation of the TAB2/3-TAK complex. This complex activates c-Jun N-terminal kinase (JNK), promoting BID cleavage and apoptosis. Furthermore, the TAB2/3-TAK pathway promotes NF- κ B pro-apoptotic and inflammatory gene expression. This hypothesis is supported by the EPEC/EHEC effector NleD, a zinc metalloprotease that directly cleaves JNK and p38-MAPK, indicating the potential importance of JNK in the context of infection [68]. These effectors seemingly share a common target, but act at different points in the pathway to block JNK activity and the apoptotic cascade.

Interestingly, other effectors target overlapping and different aspects of the NF- κ B pathway. In contrast to NleH and CPAF, the EPEC and EHEC effector NleE and the *S. flexneri* effector OspZ, which are more potent NF- κ B inhibitors, block p65/RelA and c-rel nuclear translocation, but not that of p50 or STAT1/2 [69, 70]. NleE is required for complete inhibition of NF- κ B translocation to the nucleus acting via the inhibition of IKK. IKK phosphorylates I κ BB (IKKB) and ultimately its degradation [69]. As such NleE must act downstream of death receptor activation but upstream of IKK activation, as NleE utilises its cysteine methyltransferase activity to inhibit transforming growth factor β -activated kinase

1 (TAK1) [70]. In support, NleE also targets the adaptor proteins TAB2/3 for methylation; thereby preventing recognition of the ubiquitin chain on TRAF2/6 affecting everything upstream of IKK and thus blocking inflammatory cytokine production. These actions block both JNK activation and transcription of pro-inflammatory and apoptotic genes. This again draws parallels to that of NleH, CPAF and NleB which target JNK to promote cell survival; however, this emphasises discrepancies in the modulation of transcription factors. EPEC and EHEC temporally translocate effectors dependent upon the stage of infection, with NleH being an early stage effector. As such we speculate that EPEC/EHEC effectors act in cohorts to turn on/off NF- κ B regulated genes to promote pathogenesis, modulate apoptosis and dissemination. This would then explain why a more potent NF- κ B inhibitor, NleE, would be released to override a weaker, but early stage effector, NleH, to progress pathogenesis.

S. Typhimurium translocates the effector AvrA, an acetyltransferase belonging to the same family as that of YopJ/P in *Yersinia pseudotuberculosis* and VopA in *Vibrio parahaemolyticus* [71]. YopJ/P modulates ERK, p38, and IKK, while VopA has the same effect except on IKK. In reference to AvrA, this is translocated during the initial stages of invasion and blocks JNK and MAPK activation. AvrA- infection results in an increased inflammatory response and dampens apoptosis, however, it may require phosphorylation to be activated [71, 72]. This further emphasises the importance of the JNK pathway during apoptosis and infection and highlights how a specific pathway is targeted by multiple bacterial effectors.

However, *L. pneumophila* translocates effectors LegK1 and LnaB, which activate NF- κ B, most likely by initiating the kinase cascade that ultimately causes phosphorylation and subsequent degradation of I κ B, the inhibitor of NF- κ B [73]. Activated NF- κ B translocates to the nucleus and can induce anti-apoptotic genes expression i.e. promote *xiap* and *pai-2* expression, whose product inhibits apoptosis by targeting caspases 3 and 7. LegK1 is a Ser/Thr kinase that activates the NF- κ B pathway independent of upstream components [74]. Specifically, LegK1 activates IKK β independently of IKK, seemingly by mimicry as p100 is also phosphorylated by LegK1 in the non-canonical pathway [74]. Conversely the mode of action for LnaB has yet to be fully elucidated. This is the inverse strategy to those effectors stated thus far, as LegK1 acts to promote rather than inhibit NF- κ B activation. Despite this difference *L. pneumophila* infection exerts a similar outcome to those bacteria that inhibit NF- κ B, which is the promotion of anti-apoptotic gene expression.

Interestingly *C. burnetii* utilises an alternative strategy to regulate gene expression. To this end *C. burnetii*, an intracellular pathogen, translocate AnkG, via a type IV secretion system to AnkG directly interacts, via an N-terminal arginine rich region, with the mitochondrial protein p32. Following a stressed response AnkG is then trafficked to the nucleus to inhibit apoptosis, whereupon it appears to interfere with nuclear function to prevent apoptosis, however, the exact mechanism is unknown [75, 76]. Although the exact mechanism has yet to be elucidated, this shows a commonality in how different effectors target expression of pro- or anti-apoptotic genes. Further work is required to fully understand the manner in which this is achieved across bacterial pathogens.

Interestingly, the same is true of the *H. pylori* secreted effector JHP0290, which exists in monomeric and dimeric forms and bind host cells to activate the NF- κ B and ERK pathways. This activation in turn inhibits apoptosis of *H. pylori* infected cells, the specifics of which remain unknown [77]. *H. pylori* infected cells up-regulate Mcl-1 and c-IAP expression, in an unknown manner [78]. As such there is a potential link between JHP0290 activation of NF- κ B or ERK and Mcl-1/c-IAP expression, however, this has yet to be shown. An entirely different approach is adopted by *Bartonella tribocorum* BepA, which is translocated via the VirB/VirD4 system, increasing cellular cyclic AMP levels [79]. BepA localises to the plasma membrane and directly interacts with the C2 catalytic subunit of the host adenylate cyclase (AC) to potentiate the α -subunit of the AC-stimulating G protein ($G_{\alpha s}$)-dependent cAMP production [80]. BepA could work in one of three ways; either by binding and activating $G_{\alpha s}$, by acting as a scaffold between AC and $G_{\alpha s}$ -complex forcing them into an active cAMP producing conformation or BepA interaction with AC could potentiate the $G_{\alpha s}$ -mediated activation of AC [80]. Irrespectively, BepA acts to promote the upregulation of cAMP response genes via the cAMP-response element binding protein (CREB), although the anti-apoptotic gene has yet to be elucidated. Elevated cAMP acts by numerous mechanisms to inhibit apoptosis, first by activating protein kinase A (PKA), the effector caspase of cAMP, to inhibit BAD by phosphorylation of Ser-114. Second, cAMP elevation has been shown to increase IAP expression, thereby inhibiting caspase activation [81]. Interestingly *Bartonella* also possess BepB and BepC, which are paralogous proteins of BepA, however, they exhibited no signs of anti-apoptotic function [79].

There are other effectors where a phenotype has been observed but their binding partner or mode of action has yet to be elucidated. *C. burnetii* CaeA interferes with UV induced apoptosis and localises to the nucleus. It is hypothesised that it modulates the transcription of anti-apoptotic proteins, however that has yet to be evidenced [82]. It should be noted other *Coxiella* effectors, CBU1314 and CBU1976, also localise to the nucleus, however, their role during infection has yet to be shown.

The implication of the NF- κ B pathway or other transcription related pathways on anti-apoptotic effects of those effectors is often unclear. Underpinning most effectors is the modulation of the JNK pathway, which regulates BH-3 only BCL-2 members. Where JNK is not regulated other effectors use elaborate mechanisms to affect BCL-2 family member activity. Again, these examples show that all effectors target BCL-2 members by regulating NF- κ B or MAPK pathways. However, these pathways often have both pro- and anti-apoptotic abilities, therefore without further assessing downstream gene expression one can only speculate as to the contribution of these pathways on the apoptotic inhibition in pathogenesis.

Concluding Remarks

Here we reviewed multiple effectors translocated by different bacteria, highlighting commonalities in the mechanisms used to disrupt key signalling pathways to promote cell survival. The focus of the majority of bacterial effectors is on the manipulation of BCL-2 members by modulating their expression or activity. Bacteria regulate BCL-2 members directly or indirectly through pathways such as PI3K/Akt, NF- κ B and MAPK. Interestingly when regulating anti-apoptotic BCL-2 member proteins many bacteria target MCL-1. It is evident that bacteria share common strategies to counteract host defence mechanisms. The similarities could be due to bacteria sharing genetic material via vertical and horizontal gene transfer, by replication or sharing mobile genetic elements such as prophages and integrative elements.

Although the intricate details of the mechanisms bacterial pathogens use to manipulate host cell death pathways may vary, it is evident that the three specific pathways; extrinsic,

439 intrinsic and transcription-mediated pathways are widely used as targets for bacterial
440 effectors to enhance pathogenesis. This review has highlighted, using select examples, that
441 common strategies, targets and modes of action are employed by bacterial pathogens to
442 manipulate host cell pathways during infection. It is highly likely that there are a large
443 number of effectors that have yet to be discovered to help regulate apoptosis during
444 infection (see Outstanding Questions). Furthermore, this review highlights an information
445 gap on those effectors reviewed, to wholly appreciate their impacts on cell survival. Some
446 of this is due to gaps in our knowledge of apoptosis whilst others likely to be a work in
447 progress, for example, NleH global anti-apoptotic activity is likely to be due to a range of
448 binding partners. Irrespective of our understanding to date, the inhibition of apoptosis will
449 continue to remain central to pathogenesis, enabling bacteria to replicate and disseminate.

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Figure Legends

Figure 1. Schematic of the Extrinsic and Intrinsic Apoptotic Pathways. Extrinsic apoptotic stimuli, such as Fas ligand (FasL), Tumour necrosis factor alpha (TNF α), TNF-related apoptosis-inducing ligand (TRAIL) interact with cell surface receptors and promote trimerization/oligomerization, enabling cytosolic recruitment of adaptor molecules such as FAS-associated death domain protein (FADD), TNFR1-associated death domain protein (TRADD) and additional proteins such as receptor interaction protein (RIP) kinase, which help to detect cellular stresses. Adaptor molecules recruit initiator caspase-8, which then oligomerises and activated via autocatalysis. Activating caspase-8 induces apoptosis either by directly cleaving executioner caspase-3 or by cross-talking to the intrinsic apoptotic pathways by cleaving BH-3 only protein BID to truncated BID (tBID). tBID translocates to mitochondria whereupon they promote oligomerization of BCL-2-associated X protein (BAX), inducing mitochondrial outer membrane permeabilization (MOMP). MOMP enables the release of mitochondrial pro-apoptotic proteins, such as cytochrome c to promote executioner caspase activation and apoptosis. Cytochrome c binds to apoptotic protease activating factor 1 (APAF-1), inducing oligomerization and formation of the apoptosome, which recruits and activates initiator caspase-9 via cleavage. Cleaved Caspase-9 cleaves executioner caspases-3 and -7, leading to apoptosis. Anti-apoptotic BCL-2 members act to inhibit apoptotic progression directly binding to pro-apoptotic effectors (BAX, BAK) or BH-3 only proteins (BID, BIM, PUMA). In support of this X-linked inhibitor of apoptosis proteins (XIAPs) and FLICE inhibitory proteins (FLIPs) inhibit caspase activation. In addition, receptor activation by extrinsic apoptotic stimuli can activate the NF- κ B pathway via NIK/IKK, which depending upon the stimuli and cell type, induces the expression of pro-survival genes like Bcl-2 and FLIPs. Alternatively, receptors may activate JNK via ASK1/MKK7, which promotes pro-apoptotic gene expression by activation other proteins and JNK also directly activates BAX and inhibits Bcl-2.

Figure 2. Schematic of bacterial effector manipulation of extrinsic and intrinsic apoptotic pathways. Upon infection intrinsic and extrinsic pathways are initiated as part of host innate immunity. In response, bacteria have developed a number of mechanisms to manipulate these pathways to proliferate host cell survival for bacterial propagation and

disease progression. Bacteria translocate effector proteins, by a secretion system, to block all aspects of both pathways sharing commonalities between bacterial effectors in both targets and the manner in where they exert their effects. *Escherichia coli* and *Salmonella enterica* secretes NleB and SseK1 to block extrinsic apoptosis at the point of initiation through interaction with death domain containing proteins. NleB NleF binds to and inhibits caspases-4, -8 and -9, inhibiting both intrinsic and extrinsic pathways. CagA from *H. pylori* activates the ERK pathway to promote XIAP activity and the expression of MCL-1, with *M. tuberculosis* also promoting MCL-1 expression, but in an unknown manner. Multiple *Chlamydia* sp. secrete CPAF which actively degrades BH-3 only proteins BIM, BAD and PUMA, thereby preventing them from inactivating anti-apoptotic BCL-2 members. *Chlamydia* also secrete IncG which interacts with and promotes 14-3-3b activity; binding to BH-3 only protein BAD to block its inhibitory activity. EPEC/EHEC NleH family blocks the intrinsic apoptotic pathway by binding to Bax Inhibitor-1 (BI-1), stopping elevated cytosolic Ca^{2+} and the ER stress response. SidF from *L. pneumophila* blocks Bcl-RAMBO induced MOMP, with Ats-1 from *Anaplasma* forming a stack inside the mitochondria to block BAX anchorage and mitochondrial permeabilization. SdhA, CaeB, SboH, BepA, from multiple bacteria, all localise to the mitochondria and block cytochrome c release in different manners, none of which have been elucidated in full. CaeA, CBU1314 and CBU1976 all from *C. brunetti* are thought to be anti-apoptotic by regulating gene expression, this has yet to be elucidated.

Figure 3. Schematic of bacterial effector manipulation of PI3K/Akt, associated survival pathways and transcription-related pathways. Receptor stimulation triggers activation of the PI3K/Akt pathway to promote cell survival or targets the NF- κ B, MAPK and transcription of pro- and anti-apoptotic proteins. Bacteria have developed mechanisms to manipulate these pathways to promote host cell survival for bacterial propagation and disease progression. Bacteria translocate effector proteins, by a secretion system, to block all aspects of both pathways sharing commonalities between bacterial effectors in both targets and the manner in where they exert their effects. The PI3K/Akt Pathway and associated survival pathways are modulated by *H. pylori* effector JHP0290, which promotes ERK1/2 activation and *B. tribocorum* effector BepA, which promotes Protein kinase A (PKA) activity

703 both of which result in pro-apoptotic BCL-2 member inhibition. With regards to PI3K/Akt *S.*
704 *enterica* and *S. flexneri* adopt similar strategies by acting as inositol phosphatases to
705 promote Akt activation. *E. coli* EspZ also activates Akt in a manner that is unknown.
706 Transcription can also be modulated via the NF- κ B and MPAK pathways. The *E. coli* effector
707 Tir has been shown to block host receptor signalling by binding TRAF and having
708 downstream impact on TAB2/3 and JNK signalling. JNK signalling is also inhibited by *E. coli*
709 NleD and *S. enterica* AvrA by more direct mechanisms. Finally other effectors target NF-
710 κ B signalling to promote the transcription of pro-survival genes, including *E. coli* NleH, *L.*
711 *pneumophila* LegK1 and LnaB, *Y. pseudotuberculosis* YopJ, *V. parahemolyticus* VopA and *C.*
712 *burnetii* AnkG all by targeting transcription factors or non-Rel subunits

Outstanding Questions

- Does targeting a pathway specific to apoptosis offer an advantage to a particular bacterium, as opposed to targeting a more general pathway such as the PI3K/Akt pathway?
- Is there an appropriate balance between targeting specific and generalist anti-apoptotic pathways?
- With NF- κ B having a dual role in both pro- and anti-apoptosis does the nature of infection, for example extracellular versus intracellular, affect whether a pro- or anti-apoptotic NF- κ B pathway is activated?
- What roles do post-translational modifications play in the regulation of mitochondria-dependent apoptosis by bacterial effectors?
- Why does the primary anti-apoptotic BCL-2 family target appear to be MCL-1 as opposed to other anti-apoptotic BCL-2 members?
- Does bacterial targeting of certain host proteins give an indication of their importance in the cell or are they merely more easily targeted?
- Would the use of selected anti-apoptotic inhibitors during infection provide a human benefit in pathogen clearance?

Table 1. Overview of the anti-apoptotic bacterial effectors discussed

Bacterium	Effector Name	Target	Targeted Pathway	Enzymatic Activity	Identified Mode of Action on Apoptosis	Similar Homologue	Ref
Extrinsic Apoptosis							
<i>Escherichia coli</i>	NleB1	DD Containing Proteins: FADD/TRADD/TNFR1/ RIPK1	Extrinsic / Transcription	N-linked glycosyl transferase	1. Blocks DISC formation to inhibit caspase-8 activation 2. Modulates GADPH to activity to block NF- κ B activation and prevent pro-apoptotic protein transcription	SseK	[18-19]
<i>Escherichia coli</i>	NleB2	DD Containing Proteins: TRADD	Extrinsic	N-linked glycosyl transferase	Blocks DISC formation to inhibit caspase-8 activation	NleB1	[18]
<i>Salmonella enterica</i> (multiple serovars)	SseK 1 /2 /3	TRADD (Identified for SseK1)	Extrinsic	N-linked glycosyl transferase	Inhibits NF- κ B signalling	NleB1	[20-21]
Intrinsic Apoptosis							
<i>Helicobacter pylori</i>	CagA	GRB2/c-MET/ZO-1/CRK	Intrinsic	None Identified	1. Up-regulates anti-apoptotic protein Mcl-1 2. Activates PI3K, MAPK and NF- κ B to induces post-translational modification of c-IAPs, Bcl-xL and BAD		[24-26]
<i>Chlamydia trachomatis</i>	CPAF	BH-3 only proteins	Intrinsic / Transcription	Serine protease	1. Degradation of pro-apoptotic BCL-2 family members BIM, PUMA and BAD 2. Degrades PARP-1 to regulate apoptotic response 3. Cleaves transcription factors RFX5, USF1 and p65/RelA to modulate apoptotic gene expression		[29-32] [65-66]
<i>Legionella pneumophila</i>	SidF	BNIP3, Bcl-Rambo	Intrinsic	None Identified	Inhibits formation of the permeability transition pore in mitochondria		[33-34]
<i>Anaplasma phagocitoiphilum</i>	Ats-1	BAX	Intrinsic	None identified	Up taken into the mitochondria and processed by the host for insertion into the mitochondrial membrane thereby blocking BAX insertion and MOMP		[35]
<i>Coxiella burnetii</i>	CaeB	Unknown	Intrinsic	None Identified	Blocks MOMP via unknown mechanism		[36]
<i>Legionella pneumophila</i>	SdhA	Unknown	Intrinsic	None Identified	Inhibits mitochondrial disruption and caspase activation	SidH	[37]
<i>Escherichia coli</i>	NleH	RPS3 BI-1	Intrinsic / Transcription	Ser/Thr Kinase	1. Binds BI-1 to inhibit ER apoptosis 2. Modulates NF- κ B pathway via Rps3 binding, permitting p65/RelA mediated transcription	OspG	[44-45]
<i>Salmonella bongori</i>	SboH	Unknown	Intrinsic	None Identified	Blocks ER stress induced apoptosis and cell detachment following infection	SopA, NleH	[46]
<i>Escherichia coli</i>	NleF	p43/41 component of caspases-4, 8 and 9	Intrinsic / Extrinsic	None Identified	Caspase-4, -8 and -9 inhibitor		[50-51]
PI3K/Akt Pathway							

<i>Salmonella</i>	SopB/SigD	PI3K/Akt	PI3K/Akt	Inositol phosphatase	Induces sustained activation of Akt and ERK1/2 mediated protection		[53-55, 57]
<i>Shigella flexneri</i>	IpgD	PI3K/Akt	PI3K/Akt	Inositol phosphatase	Induces sustained activation of Akt		[59-61]
<i>Escherichia coli</i>	EspZ	CD98 / Akt	PI3K/Akt	Unknown	Promote Akt and FAK anti-apoptotic activity, as well as regulating mitochondrial protein translocation		[62-63]
Transcription Mediated Pathway							
<i>Mycobacterium tuberculosis</i>	Unknown	Unknown	Transcription	Unknown	Promotes transcription of anti-apoptotic BCL-2 members, A1 and Mcl-1 whilst inhibiting pro-apoptotic BAX expression		[27-28]
<i>Escherichia coli</i>	Tir	SHP-1, SHP-2, TRAF-2	Transcription	None Identified	Interacts with TRAF family to inhibit NF- κ B, MAPK and JNK signalling		[67]
<i>Escherichia coli</i>	NleD	JNK	Transcription	Zinc metalloprotease	Cleaves JNK and p38-MAPK, thereby blocking JNK signalling and BAD activation		[68]
<i>Escherichia coli</i>	NleE	TAB2/3, TAK1	Transcription	Cysteine methyltransferase	Modifies TAB2/3 stopping recognition of TRAF2 ubiquitin chain. Blocks IKK activation and NF- κ B inhibitor	OspZ	[69-70]
<i>Shigella flexneri</i>	OspZ	TAB2/3, I κ B	Transcription	Cysteine methyltransferase	Inhibits NF- κ B activation	NleE	[70]
<i>Vibrio parahemolyticus</i>	VopA	ERK, p38, JNK	Transcription	Acetyltransferase activity	Inhibition of MAPKs and NF- κ B signalling	AvrA, YopJ	[71]
<i>Yersinia pseudotuberculosis</i>	YopJ	ERK, p38, JNK	Transcription	Acetyltransferase activity	Inhibition of MAPKs and NF- κ B signalling	AvrA, VopA	[71]
<i>Salmonella enterica</i> serovar typhimurium	AvrA	I κ B, MKK4, MKK7, JNK	Transcription	Deubiquitinase, acetyl transferase	Inhibits JNK and MAPK signalling, having anti-inflammatory and anti-apoptotic effects	YopJ, VopA	[71-72]
<i>Legionella pneumophila</i>	LnaB	Unknown	Transcription	None Identified	Promotes NF- κ B pathway		[73]
<i>Legionella pneumophila</i>	LegK1	I κ B, p100	Transcription	Ser/Thr Kinase	Promotes NF- κ B pathway		[73-74]
<i>Coxiella burnetii</i>	AnkG	p32	Transcription	None Identified	The mode of action is unknown, however, nuclear transport following stress detection by p32 is required for AnkG induced cell survival		[75-76]
<i>Helicobacter pylori</i>	JHP0290	ERK	PI3K/Akt / Transcription	None Identified	Activates ERK pathway and promotes MCL-1 expression, in addition to PI3K/Akt and NF- κ B activation		[77-78]
<i>Bartonella tribocorum</i>	BepA	Adenylate cyclase	Transcription	None identified	The mode of action is unknown, irrespectively, BepA increases cytosolic cAMP, inhibiting apoptosis by activating PKA and inducing BAD phosphorylation	BepB, BepC	[79-80]
<i>Coxiella burnetii</i>	CaeA	Unknown	Transcription	None Identified	Transcriptional modulation promoting cell survival		[82]

FasL / Death Stimuli

Pro-Survival Signals

PI3K/Akt Pathway

NF-κB and MAPK

TNF, FasL, TRAIL

Key:

- Transcription factor
- Pro-apoptotic
- Kinase
- Anti-apoptotic
- Caspase
- Bacterial effector