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Bacterial secreted effectors and caspase-3 interactions

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Summary

Apoptosis is a critical process that intrinsically links organism survival to its ability to induce controlled death. Thus, functional apoptosis allows organisms to remove perceived threats to their survival by targeting those cells that it determines pose a direct risk. Central to this process are apoptotic caspases, enzymes that form a signalling cascade, converting danger signals via initiator caspases into activation of the executioner caspase, caspase-3. This enzyme begins disassembly of the cell by activating DNA degrading enzymes and degrading the cellular architecture. Interaction of pathogenic bacteria with caspases, and in particular, caspase-3, can therefore impact both host cell and bacterial survival. With roles outside cell death such as cell differentiation, control of signalling pathways and immunomodulation also being described for caspase-3, bacterial interactions with caspase-3 may be of far more significance in infection than previously recognized. In this review, we highlight the ways in which bacterial pathogens have evolved to subvert caspase-3 both through effector proteins that directly interact with the enzyme or by modulating pathways that influence its activation and activity.

Apoptosis – non-inflammatory cell death

Apoptosis was first discovered over forty years ago and has since been studied in intricate detail, generating a complex web of interactions that define this process (Kerr et al., 1972). Once initiated, the process of apoptosis proceeds rapidly with cell shrinkage, nuclear condensation, DNA fragmentation and the formation of apoptotic bodies, which eventually dissociate from the cell and become engulfed and destroyed by circulating phagocytes. Apoptosis has been linked to several disease states with increases in apoptosis leading to degenerative disease [i.e. Alzheimer’s, Parkinson’s, amyotrophic lateral sclerosis (Pasinelli and Brown, 2006; da Costa and Checler, 2010; Tischner et al., 2010; Crews et al., 2011; Song et al., 2011)] while decreases in, or mis-regulation of, apoptosis can lead to auto-immune disease [i.e. rheumatoid arthritis, systemic lupus erythematosus (Eguchi, 2001; Favaloro et al., 2012)] or tumour development (Favaloro et al., 2012). Apoptosis can be initiated from within or outside the host cell by stimuli including, microbial infection, oxidative stress, acquisition of tumorigenic potential, DNA damage or protein mis-folding, or simply when the cell has reached the end of its life cycle.

Once the process of apoptosis is induced, it engages a cascade of caspase enzyme activation, which is facilitated by upstream ‘initiator caspases’ that eventually results in controlled self-destruction of the cell caused by DNA fragmentation and cleavage of essential proteins by ‘executioner caspases’, namely caspases-3, -6 and -7 (Núñez et al., 1998). Executioner caspase activation occurs in cells as both intrinsic and extrinsic signals deemed detrimental to the cell are acted on, and one of two distinct signalling cascades becomes activated. Executioner caspases then trigger DNases, and cleave essential cellular proteins, effectively destroying the cell from within and choreographing the cellular events in apoptosis leading to the cell being removed or phagocytosed by circulating immune cells. Despite apoptosis being a well-defined process owing to its essential role in cellular survival, the function it plays during infection is still unclear. Contrary to the long accepted dogma of apoptosis serving a protective role for the host, recent investigation into bacterial infections reveals apoptosis and apoptotic caspases may actually promote infection by some bacterial pathogens (Molmeret et al., 2004; Srikanth et al., 2010).

Caspases – enzymatic mediators of apoptosis

Caspases, or cysteine aspartate proteases, are enzymes present in eukaryotic cells that play key roles in cellular...
differentiation, proliferation, inflammatory responses, and ultimately cell death (Connolly et al., 2014). The enzymes are divided into pro- and non-inflammatory forms based on their ability to initiate distinctive types of cell death, with those involved in pathways such as pyroptosis (caspase-1, -4, -5, -11, -12) classed as pro-inflammatory and those involved in apoptosis (caspase-2, -3, -6, -7, -8, -9, -10) classed as being non-inflammatory (Nuñez et al., 1998; Lamkanfi and Dixit, 2014). While the number and type of programmed cell death (PCD) pathways is constantly evolving (as we gain a better appreciation of the complex genetic and cellular regulation of PCD), in the context of bacterial infection, the activation of distinct inflammatory or non-inflammatory PCD can have a significant effect on the induction and severity of an immune response to bacterial pathogens. Crossover and feedback/activation between inflammatory and non-inflammatory pathways occurs during infection, while neighbouring cells may also undergo distinct types of cell death in close proximity and some pathogens are known to induce differing types of cell death depending on the host cell type infected (Fink and Cookson, 2005; Rosenzweig and Chopra, 2013). Moreover, apoptotic cells have also been shown to influence their environment inducing signalling changes and cell death in neighbouring bystander cells, and inducing the extracellular release of potent caspasases that can undermine epithelial integrity (Chin et al., 2006; Flynn and Buret, 2008; Grant et al., 2008).

Caspase-3 – the executioner caspase
Apoptotic caspases are present in inactive pro-forms, and each is cleaved to induce its activation. Following activation of either the intrinsic or extrinsic pathways of the apoptotic cascade, initiator caspases cleave and activate the executioner caspases-3, -6 or -7. Pro-caspase-3 becomes an active enzyme when two cleaved monomers come together to form an active dimer (Nuñez et al., 1998); this has potent activity, including an ability to autocatalytically activate that contributes to the observed cascades effect of increasing caspase-3 activity as apoptosis progresses. Active caspase-3 recognizes a specific short peptide cleavage motif (DXXD) and cleaves cellular proteins where this motif is present and accessible (Fischer et al., 2003; Ju et al., 2007). The alternative executioner caspase, caspase-7, also recognizes an identical motif and has extensive functional redundancy with caspase-3 although caspase-3 is regarded as a more significant player in apoptosis and cell death because of its substrate promiscuity. (Walsh et al., 2008; Lamkanfi and Kanneganti, 2010). Nevertheless, caspase-7 can also play an important role in the outcome of bacterial infection by providing an adaptive mechanism whereby the host membrane is protected from damage from pore forming toxins (Cassidy et al., 2012).

The potential of caspase-3 to cause apoptosis once activated means that its activity must be tightly controlled. This control is achieved through constant turnover of the enzyme, which ensures that a threshold level of enzyme activation is not reached without an apoptotic stimulus (Tan et al., 2006; Jiang et al., 2009; Choi et al., 2009; Lai et al., 2011). Additionally, eukaryotic cells have been documented to have low levels of caspase-3 activity in non-apoptotic states implying that sub-apoptotic levels of this enzyme are expressed independent of apoptosis (Boland et al., 2013; Connolly et al., 2014). Of note for microbial infection, this caspase-3 activity plays roles in fundamental processes aside from apoptosis, most significantly in host cell proliferation and differentiation, but also in immunomodulation, signal transduction and cell migration. Therefore, perturbation of caspase-3 by bacterial pathogens may have consequences beyond simply deciding the fate of infected cells.

Apoptosis and bacterial infection
Apoptosis has been established as a critical point in viral infection, acting as either a facilitator or inhibitor of viral replication (Best, 2008; Richard and Tulasne, 2012). Similarly, apoptosis was thought to be a deliberate host response to a bacterial infection that ultimately results in the removal of compromised cells. More recently, however, studies have challenged this dogma, such that apoptosis has been described as a fundamental pathway in bacterial–host interactions, but its role in inhibiting or facilitating infection is yet to be clearly defined. While apoptosis can remove infected and compromised cells to benefit the host, induction of apoptosis may carry this out in a non-inflammatory fashion while also disrupting, for example, epithelial barriers to infection or removing circulating immune cells (Grant et al., 2008; Nogueira et al., 2009; Peters et al., 2013). This apparent paradox puts into question who benefits the most from apoptosis during infection – the bacteria or the host? While this question will remain a subject of debate, emerging evidence suggests that in some cases direct targeting of caspases is being employed by bacterial pathogens through effector proteins (Table 1). Indeed, such subversion occurs at nearly all points of the apoptotic cascade with different bacterial pathogens having evolved distinct modes to induce or inhibit specific apoptotic pathways in an attempt to manipulate the lifespan of infected cells and/or influence their behaviour in a manner that supports infection. Therefore, the critical role caspases play in determining cellular fate makes these serine proteases a high-risk target for bacterial pathogens, but when successfully manipulated,
Table 1. Summary of secreted bacterial effector proteins that activate or inhibit caspase-3.

| Bacterium       | Effect on caspase-3? | Other caspases? | Mechanism of caspase-3 activation/inhibition | Bacterial effector responsible | Consequence                           | Reference               |
|-----------------|----------------------|----------------|---------------------------------------------|--------------------------------|---------------------------------------|-------------------------|
| *H. pylori*     | Activates            | Cas-8          | Caspase-3 possibly activated directly       | ND                             | Host cell killing                     | Ashktorab et al., 2002 |
| *L. pneumophila*| Activates            | No             | Caspase-3 activated directly                | Dot/Icm effector               | Prevents phagolysosome fusion         | Molmeret et al., 2004; Zhu et al., 2013 |
| *S. Dublin*     | Activates            | ND             | ADP ribosylating activity dependent         | SpvB/SpI-2 TTSS                | Important pathogenic mechanism        | Browne et al., 2008     |
| *S. Typhimurium*| Activates            | No             | ADP ribosylating activity dependent         | SpvB/SpI-2 TTSS                | Survival during systemic infection    | Valle and Guiney, 2005  |
| EPEC            | Activates            | No             | Direct caspase-3 activation                 | SpvB/SpI-2 TTSS                | Effector processing                   | Srikanth et al., 2010  |
| *E. coli* O157:H7| Activates            | Cas-9          | Mitochondrial disruption                    | N-terminal of EspF              | Leads to attaching/effacing lesions   | Zhoa et al., 2013      |
| Y. pestis       | Activates            | Cas-8          | Death inducing signalling complex assembly  | YopK/YopJ interplay            | Alters inflammatory signalling        | Peters et al., 2013     |
| V. vulnificus   | Activates            | No             | Direct cleavage of caspase-3               | Metalloprotease vEP            | Unknown, non-specific caspase-3       | Kim et al., 2007       |
| *A. salmonicida*| Activates            | Cas-9          | Multiple mechanisms                        | AexT/AexU, Act2 and Hcp        | Control of host inflammatory response | Rosenzweig and Chopra, 2013 |
| *A. hydrophila* | Activates            | Cas-8          | ADP-ribosylation, and possibly by caspase-3 binding | ExoS                           | Further dissemination                  | Kaufman et al., 2000   |
| *P. aeruginosa* | Activates            | Cas-1          | Cytochrome-c release                       | Putative effectors IgIC and IgII SopB | Further dissemination                  | Santic et al., 2010    |
| *F. tularensis* | Activates            | ND             | Sustains AK activation                     | Secreted protein possibly ExoA | Survival of intracellular replication niche | Knodler et al., 2005 |
| *P. aeruginosa* | Inhibits             | ND             | Stabilizes X-IAP                            | Secreted protein possibly ExoA | Inhibiting apoptosis aids bacterial survival | Ashare et al., 2007    |
| *F. tularensis* | Inhibits             | ND             | NF-kB induction prevents apoptosis          | T6SS effector, possibly IgIC   | Survival of infected cells/replication niche | Santic et al., 2010    |
| *L. pneumophila*| Inhibits             | No             | Dot/Icm dependent                          | T6SS effector                  | Inhibition allows time for replication | Abu-Zant et al., 2007  |
| *S. flexneri*   | Inhibits             | ND             | Anti-apoptosis expression/direct caspase binding | T6SS effector                  | Protects intracellular replication niche | Clark and Maurelli, 2007; Faherty and Maurelli, 2009; Faherty et al., 2010 |

Activation of caspase-3 by individual effectors is indicated as well as whether this activation is potentially direct or through upstream initiator or other caspases. ND indicates that the mechanism of activation of caspase-3 or the involvement of other caspases was not defined in the study.
disruption of caspase function and caspase-3 in particu-
lar, can significantly undermine the host response to
infection and can promote dissemination and further
bacterial infection.

Bacterial effector proteins and caspase-3 activation

Bacterial secretion systems and the proteins that tra-
verse them are essential components of the virulence
arsenal of many pathogens. Many effectors are secreted
through the type three secretion system, a sophisticated
organelle specific to gram-negative pathogens and com-
poased of a motor and needle complex through which
secreted effectors are injected into host cells (Dean,
2011; Raymond et al., 2013). The secreted effectors
promote disease by co-opting host cell signal trans-
duction pathways that facilitate cell attachment and
entry, suppress the host immune/defense response, and
modulate host cell biology. Consequently, these effectors
play a prominent role in bacterial pathogenesis and
host association. It is well appreciated that effectors consti-
tuate a large and diverse group of virulence proteins that
mimic eukaryotic proteins in structure and function. In
fact, up to 100 different type three secreted effector
(T3SEs) proteins may be delivered into individual host
cells by a single bacterium (Dean and Kenny, 2009).
Moreover, T3SEs are often multifunctional proteins with
many overlapping properties that orchestrate specific
host cell responses, which ultimately subvert fundamen-
tal pathways linked to cell survival, inflammation and
microbe destruction (Dean, 2011). Therefore, apoptosis
and caspase-3 targeting by bacterial effectors is not sur-
prising since coordinating the ability of a cell to survive
or die in controlled circumstances offers obvious benefits
to an invading microbe.

It appears that caspase-3 activation during bacterial
infection is a common by-product of bacterial invasion,
perhaps precipitated by the ensuing stress on the host cell
associated with intracellular replication. An example are
large molecular weight bacterial toxins that can target the
cell cycle or cell integrity with the resulting off-target effect
being cellular stress with subsequent cell death incited
through apoptotic caspases (Heine et al., 2008; Ionin
et al., 2008; Lee et al., 2008; Cheung et al., 2009). The
relationship between bacterial effectors and the activation
of caspase-3 is an area of increasing interest since in
addition to non-specific or indirect activation of caspase-3,
effectors are also able to promote caspase-3 activation
through subtle changes within cellular pathways or even
through direct interaction with the enzyme (Table 1). The
outcome for the pathogen responsible is often an increase
in infectivity rather than a clearing of the infection as
expected by the conventional understanding of the pro-
tective role of apoptosis.

**Salmonella effectors – divide and conquer**

*Salmonella* Typhimurium interactions with caspase-3 are
beginning to be understood, as effectors responsible have
been identified and the role that the enzyme plays in
infection has been studied in detail (Takaya et al., 2005;
Valle and Guiney, 2005; Browne et al., 2008; Srikanth
et al., 2010). S. Typhimurium uses an array of effectors to
exploit host cell function in both epithelial and immune
cells (McGhie et al., 2009). As mentioned prior, a promi-
nent feature shared by many effectors is their modular
architecture, which is often comprised of well-defined
regions that confer a subversive function. These distinct
modules within an effector often mediate very different,
unrelated functions, strongly suggesting that they evolved
independently of each other and subsequently combined
to form a chimeric protein (Kaniga et al., 1996; Dean,
2011; Fookes et al., 2011). This forms the basis of ‘termi-
nal reassortment’, a hypothesis proposed to explain the
diversity of bacterial effectors (Stavrinides et al., 2006).
The terminal reassortment tenet is strengthened by the
finding that 32% of all type three effector families con-
tain chimeric effectors and evidence that terminal
reassortment is important for the evolution of these viru-
ulence proteins (Stavrinides et al., 2006; Agbor and
McCormick, 2011; Fookes et al., 2011).

In keeping with this premise, we discovered that many
T3SEs from *S. Typhimurium* harbour a functional
caspase-3 cleavage site uniquely positioned at the junc-
tion separating their distinct functional domains, thereby
producing two independently functional proteins (Srikanth
et al., 2010). *Salmonella* invasion protein A (SipA), is a
bifunctional molecule with an actin-binding function of
SipA is localized to a C-terminal fragment (Liic et al.,
2003) while the N-terminal fragment triggers signal
transduction cascades that promote polymorphonuclear
leukocyte migration (Lee et al., 2000; Wall et al., 2007).
SipA also harbours a functionally active caspase-3 motif
that is precisely located at the junction separating the two
functional domains of this protein (Srikanth et al., 2010).
The outcome offers a compelling explanation as to how
diverse effectors with a modular architecture are able to
perform multiple unrelated functions in a manner pivotal
to the pathogenicity of the organism. Remarkably, SipA,
itsself, is necessary and sufficient for early caspase-3 acti-
vation, but in a process independent from the apoptotic
cascade (Srikanth et al., 2010). SipA therefore drives its
own cleavage upon cell entry, a novel mechanism for
activating a T3SE. Other caspase-3 cleavage sites iden-
tified in *S. Typhimurium* are also restricted to effector pro-
teins, with no sites in type three structural proteins or
chaperones, indicating this may be a general strategy
employed by *S. Typhimurium* for processing of its
secreted effectors.
Caspase-3 mediated proteolytic cleavage of viral effectors or capsid proteins has also been implicated in disease progression and virus spread (Zhimov et al., 1999; Wurzer et al., 2003; Best, 2008; Syrtzev, 2009; Richard and Tulasne, 2012). Identified caspase-3 cleavage sites, considered to play a role in processing of viral proteins, are highly relevant in pathogenesis of influenza virus and their disruption attenuates viral virulence (Wurzer et al., 2003; Zhimov and Klenk, 2009). Similarly, in the case of S Typhimurium infection, a single amino acid substitution in the caspase-3 motif of SipA to a sequence not recognized by caspase-3 profoundly attenuates the virulence of this pathogen both in vitro and in vivo (Srikanth et al., 2010). It is tempting to speculate that mutation of caspase-3 motifs in central virulence factors of both viral and bacterial pathogens may lead to novel vaccine approaches.

While SipA induces caspase-3 activation in intestinal epithelial cells, the SPI-2 T3SE SpvB induces caspase-3 activation in macrophages through its ADP-ribosylation of actin during infection (Table 1; Valle and Guiney, 2005; Browne et al., 2008). Although the exact mechanism of caspase-3 activation remains unclear, again, it was independent of the initiator caspases-8 and -9 (Valle and Guiney, 2005). SpvB also has two functional domains but a caspase-3 site has to date not been identified but other host or bacterial proteases may be responsible. Therefore, these two T3SEs induce caspase-3 activation in different cell types through very different means, with SipA inducing caspase-3 at the earliest time point in infection (i.e. epithelial cell entry) and SpvB later in infection upon SPI-2 expression in macrophages (Valle and Guiney, 2005; Browne et al., 2008; Srikanth et al., 2010). It appears that during S. Typhimurium infection, caspase-3 is under continuous targeting by effectors.

### Caspase-3 and Legionella intracellular survival

*Legionella pneumophila* is thought to use effectors to directly activate caspase-3 and bypass the classical intrinsic and extrinsic pathways of apoptosis activation. One of five Dot/Icm secreted effector(s) is thought to be responsible (VipD/Lpg2831, Lpg0716, Lpg0898, Lpg1625, LegS2/Lpg2176; Zhu et al., 2013). The consequences of caspase-3 activation during *L. pneumophila* infection also shed light on one of the more diverse roles for caspase-3 in promoting infection as its activation causes degradation of rabaptin-5, a phagosome/endosome marker that marks phagosomes for lysosome fusion and bacterial killing (Zhu et al., 2013). Degradation of rabaptin-5 is an essential step in mediating *L. pneumophila* intracellular replication and ensuring a successful infection. There is also speculation that multifunctional *L. pneumophila* proteins may also undergo some kind of processing post-delivery into host cells in a manner similar to S. Typhimurium effectors, though as yet no evidence has been presented to indicate caspase-3 may be involved (Zhu et al., 2013). While direct activation of caspase-3 by bacterial effectors such as those from *L. pneumophila* and *S. Typhimurium* is intriguing in the context of infection, a greater understanding of how these effectors mediate this activation, without inducing widespread apoptosis, would be of great significance in fighting these infections. The observed temporal delays in apoptosis post-caspase-3 activation leads to the hypothesis that bacterial pathogens may employ complementary strategies for both initial caspase-3 activation and later enzyme inhibition, most likely through modification or degradation of the enzyme by other effectors to prevent rapid apoptosis.

### Extracellular release of Caspase-3 during Escherichia coli infection

The T3SEs Cif and EspF from *E. coli* activate caspase-3 indirectly through disruption of cellular pathways with both the cell cycle and the mitochondria being targeted (Samba-Louaka et al., 2009; Zhao et al., 2013). This results not only in caspase-3 activation but also its release extracellularly into the intestinal lumen during infection. Extracellular caspase-3 release has previously been described in the intestine and in the case of *E. coli* infection, it degrades tight junction proteins that are susceptible to cleavage through their caspase-3 motifs, resulting in reduced intestinal epithelial integrity (Hentze et al., 2001; Bojarski et al., 2004; Chin et al., 2006). These proteins are integral to the integrity and barrier function of the intestinal epithelium and such caspase-3 cleavage makes the intestinal barrier vulnerable to potential bacterial paracellular translocation. The mechanism of caspase-3 mobilization and release from the cell is unknown. However, ubiquitination, which is carried out so effectively by pathogens such as *E. coli*, *Pseudomonas aeruginosa* and *S. Typhimurium* through their ubiquitin ligase mimics (i.e. NleL, AvrPtoB, SopA, SspH2), can mobilize intracellular caspase-3 (Janjusevic et al., 2006; Zhang et al., 2006; Quezada et al., 2009; Lin et al., 2011a). A similar phenomenon occurs in *S. Typhimurium*-infected epithelial cells with activated caspase-3 moving from the cytosol to the cell membrane, but again, the trigger or pathway responsible for this migration is presently unknown (Srikanth et al., 2010).

### Yersinia spp. effectors and caspase-3

*Yersinia* spp. encodes a number of Yop proteins that manipulate pathways and caspases upstream of caspase-3 that dramatically alter its activation (Table 1).
Indeed, infection by *Yersinia* spp. amounts to a coordinated attack on PCD pathways with modulation of host cell death determining the outcome of infection through alteration of the innate inflammatory response (Bergsbaken and Cookson, 2009). Activation of caspase-3 is dependent on the interplay between Yop proteins in the case of *Y. pestis*, with YopK influencing the induction of caspase-3 activation by the deubiquitinase YopJ (Peters et al., 2013). While YopJ induces apoptosis through inhibiting the production of anti-apoptotic proteins, YopK further manipulates apoptosis by altering the role upstream caspases play in activating caspase-3. Perhaps most interestingly, while YopK controls YopJ induction of apoptosis depending on the cell type infected. Excess YopJ was expected to induce increased caspase-3 activation in infected cells but this occurs at varying levels in different macrophage cell lines leading to speculation that activation of caspase-3 by these Yop proteins may be related to the activation state of infected immune cells (Peters et al., 2013).

The outcome is that host cells infected by *Yersinia* spp. are driven towards non-inflammatory apoptosis through caspase-3 activation, reducing the influx of immune cells and increasing the likelihood of bacterial survival and dissemination. Again, like for many other pathogens discussed in this review, caspase-3 activation is most likely dependent on the cell type infected, and in the case of immune cells, their activation state (Bergsbaken and Cookson, 2009; Peters et al., 2013). Whether this increased complexity of interplay between caspase-3 and effectors in different cell types is due to differing host cell responses to infection or an adaptation by bacterial pathogens to their environment is still to be elucidated (Rosenzweig and Chopra, 2013).

**Vibrio metalloprotease Vibrio extracellular protease (vEP) cleaves caspase-3**

*Vibrio vulnificus* has perhaps one of the most intriguing mechanisms of caspase-3 activation, achieved through its secreted metalloprotease vEP. This small secreted enzyme not only directly activates caspase-3 but does so in a unique way, cleaving the enzyme at a site distinct from the normal cleavage motif targeted by initiator caspases to activate the enzyme (Kim et al., 2007). This novel mechanism of vEP cleavage of caspase-3 causes the enzymes activity to initially and profoundly increase before more cleavage of caspase-3 by vEP renders the enzyme inactive at later time points in infection. Such vEP activity is non-specific, and although vEP is seen intracellularly during infection, it has yet to be shown to be responsible for induction of apoptosis. However, such transient activation implies that caspase-3 activity would only present at early stages in infection, and is similar to that seen in other bacterial infections where an initial increase in caspase-3 activity is followed by a delay in triggering apoptosis thought to be as a result of the intervention of other anti-apoptotic effectors (Srikanth et al., 2010). This is yet another example in which diverse bacterial pathogens are using contrasting means to achieve similar goals, with *V. vulnificus* employing a single enzyme to control caspase-3 activity whereas other bacterial pathogens may use a number of effectors to achieve the same goal (Kim et al., 2007).

**Aeromonas effectors and caspase-3**

*Aeromonas salmonicida* and *A. hydrophila* employ a number of effectors that activate caspase-3 (Table 1). Three effectors have been implicated; AexT/AexU, Act2 and Hcp. AexT from *A. salmonicida* is a bifunctional effector protein, homologous to ExoT/S from *Pseudomonas*, which is capable of caspase-3 activation through induction of caspase-9 activity (Rosenzweig and Chopra, 2013). When its homologue from *A. hydrophila*, AexU, is mutated, mutants are far more virulent resulting in increased cytokine production and mouse mortality during infection. AexU therefore is speculated to play an important role in inducing apoptosis as a means of controlling the host inflammatory response and prolonging *A. hydrophila* infection. The effector Act2 also induces caspase-3 activation and apoptosis but the mechanisms are incompletely understood while the effector Hcp, once translocated into the host cell, induces rapid caspase-3 activation (Rosenzweig and Chopra, 2013). Macrophages treated with Hcp also lose the ability to carry out phagocytosis indicating this may be a means of protection and escape from infected immune cells. Multiple functional copies of Hcp are present on *Aeromonas* genomes and these can be expressed simultaneously allowing rapid induction of apoptosis, emphasizing the important role that manipulation of host life span plays during infection by this *Aeromonas*.

**Francisella, Pseudomonas and caspase-3 activation**

Other effectors known to target caspase-3 indirectly include ExoS from *P. aeruginosa* and type six secretion system (T6SS) delivered effectors from *Francisella tularensis* (Lai and Sjöstedt, 2003; Alaaoui-El-Azher et al., 2006; Jansson et al., 2006; Santic et al., 2010; Zivna et al., 2010). *P. aeruginosa* ExoS, a bifunctional homologue of AexT/AexU from *Aeromonas*, inhibits phosphorylation of cellular proteins such as FOXO3a, inducing caspase-3 activation and the apoptotic cascade (Jansson et al., 2006). Interestingly, in the case of *P. aeruginosa*, caspase-3 activation by ExoS was noted...
earlier than that of the upstream initiator caspase, caspase-8, suggesting that ExoS may directly activate caspase-3, in addition to indirectly activating the enzyme through caspase-8 (Kaufman et al., 2000). *F. tularensis* induction of caspase-3 is dependent on a functional T6SS, leading to cytochrome-C release and nuclear-factor kappaB (NF-κB) translocation (Santic et al., 2010). This effect depends on IgIC and IgI, putative Francisella effectors that also play a structural role in the T6SS. For both *P. aeruginosa* and *F. tularensis*, caspase-3 activation and apoptosis appears to result in the death of host cells with subsequent bacterial dissemination. This is yet another example in which the role of apoptosis rather than combating infection contributes to infection by aiding bacterial spread.

**Mechanisms of caspase-3 activation**

Activation of caspase-3 by bacterial pathogens is increasingly being recognized as a bacterial infection strategy but yet little is known of the mechanisms by which bacteria interact with caspase-3 directly. Upstream initiator caspsases such as caspase-8 and -9 are routinely activated during infection resulting from the perturbation of numerous cellular pathways, often simultaneously, leading to indirect induction of caspase-3 activity and the cell being overwhelmed by the pathogen. While this activation can be tracked over time giving an indication of the pathways involved and how caspsases are activated, direct or alternative activation of caspase-3 as described during bacterial infection is more difficult to understand mechanistically (Kaufman et al., 2000; Kim et al., 2007; Srikanth et al., 2010; Zhu et al., 2013). Intrinsic and extrinsic apoptotic pathways are thought to be the means by which the majority of apoptosis occurs but activation of caspase-3 independently of these pathways suggests an alternative pathway(s) for caspase-3 activation is induced during bacterial infection. As such, a pathway has yet to be identified; it cannot be discounted that direct effector enzyme interaction may also be responsible for caspase-3 activation. Direct effector binding of caspsases, as shown with *E. coli* NleF (caspases-4, -8 and -9), *Shigella flexneri* OspC3 (cleaved caspase-4) and YopM of *Y. pestis* (caspase-1), may also occur with caspase-3, although to date, this remains largely speculation (LaRock and Cookson, 2012; Blasche et al., 2013; Kobayashi et al., 2013). The presence within effectors of short amino acid motifs that are known to stimulate caspase-3 activation, such as the RGD motif, may also contribute to activation, and indeed, an evolutionary conservation of a prokaryotic caspase-3 activity has also been described, which could play a role (Buckle et al., 1999; Bidle et al., 2010). However if, as the evidence suggests, some bacterial pathogens do indeed utilize unique means of target-

**Inhibition of caspase-3 by bacterial pathogens**

Intracellular survival of bacterial pathogens determines the success or failure of an infection and bacterial pathogens have evolved to protect their intracellular niche to increase their chances of success. While activation of apoptosis-related proteins, as described above, appears an unusual strategy and detrimental to intracellular survival, many bacterial pathogens actively engage in complementary strategies to inhibit apoptosis (Faherty and Maurelli, 2008). *F. tularensis, L. pneumophila, P. aeruginosa* and *S. Typhimurium* all employ effectors to manipulate caspase-3 activation, but in this case, in order to inhibit its activity (Knodler et al., 2005; Abu-Zant et al., 2007; Ashare et al., 2007; Santic et al., 2010). Each, however, affects caspase-3 indirectly, primarily through activation of pathways or proteins that prevent caspase-3 activity such as NF-κB, inhibitor of apoptosis protein (IAP) or Akt. Inhibition by both *L. pneumophila* and *F. tularensis* is dependent on their Dot/Icm and T6SS (Abu-Zant et al., 2007; Santic et al., 2010). Although the effectors responsible for manipulating caspase-3 activity are not definitively known, IgIC is suggested as being responsible in the case of *F. tularensis*, The outcome is similar for both pathogens with NF-κB translocated to the nucleus where it increases expression of anti-apoptotic proteins. The net result for these pathogens is increased replication time in their intracellular niche and protection from circulating immune cells. *P. aeruginosa* and *S. Typhimurium* both target/ phosphorylate and stabilize Akt reducing caspase-3 activation while *P. aeruginosa* also stabilizes X-linked IAP, preventing activation of apoptotic caspsases (Knodler
et al., 2005; Ashare et al., 2007). The strategy of stabilizing anti-apoptotic proteins is a common approach for inhibiting caspase-3 utilized by bacterial pathogens. S. flexneri uses a combination of these approaches, including up-regulating the IAP family (Faherty et al., 2010). In addition, S. flexneri inhibits caspase-3 through a membrane expression of invasion plasmid antigen E (MxiE)-dependent mechanism that may involve direct binding of caspase-3 or caspase-9 (Clark and Maurelli, 2007). The effector responsible is not yet known but Spa15 is known to have an anti-apoptotic effect (Faherty and Maurelli, 2009). Enteropathogenic E. coli (EPEC) NleH, a homologue of the Shigella effector OspG, also inhibits caspase-3 activation through interaction with Bax inhibitor 1 (Hemrajani et al., 2010). In the case of Shigella and E. coli infections, this inhibition of caspase-3 activation and subsequent apoptosis may slow exfoliation of the intestinal epithelium and promote infection by these pathogens.

Mechanisms of inhibition
Recent studies have shown that effector binding of caspases other than caspase-3 can have an inhibitory effect on enzyme activity (Blasche et al., 2013). While direct binding can inhibit caspase enzyme activity, subtle caspase-3 modifications can also have a significant effect on enzyme activity, intracellular location and its lifespan (Choi et al., 2009; Jiang et al., 2009; Dunne et al., 2013). Bacteria not only possess the effectors to inhibit caspases in this way but pathways such as ubiquitination and S-nitrosylation are up-regulated in infected host cells (Janjusevic et al., 2006; Zhang et al., 2006; Quezada et al., 2009; Lin et al., 2011b; Dunne et al., 2013). Ubiquitination of caspase-3 reduces activity of caspase-3 by altering the active site while also targeting the enzyme for proteasomal degradation. This mechanism of proteasomal recycling is employed by host cells to maintain caspase-3 at basal non-apoptotic levels at times when no danger is perceived (Tan et al., 2006). The presence of large numbers of ubiquitin ligase mimics in the bacterial effector repertoire means ubiquitination may be a means for bacterial pathogens to secure their intracellular niche for prolonged periods, and also could explain the mobilization of caspase-3 to the extremities of the cell seen during infection with S. Typhimurium and E. coli (Flynn and Buret, 2008; Srikanth et al., 2010).

An alternative means of caspase-3 inhibition recently identified in long-term E. coli infection of immune cells, although not yet attributed to a specific effector or pathway, was S-nitrosylation (Dunne et al., 2013). This modification occurs in all host cell types but was seen to be up-regulated in infected dendritic cells and macrophages leading to both increased proteasomal deg-
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