Microreview

Manipulation of the host cell death pathway by Shigella

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Summary

Host cells deploy multiple defences against microbial infection. One prominent host defence mechanism, the death of infected cells, plays a pivotal role in clearing damaged cells, eliminating pathogens, removing replicative niches, exposing intracellular bacterial pathogens to extracellular immune surveillance and presenting bacteria-derived antigens to the adaptive immune system. Although cell death can occur under either physiological or pathophysiological conditions, it acts as an innate defence mechanism against bacterial pathogens by limiting their persistent colonization. However, many bacterial pathogens, including Shigella, have evolved mechanisms that manipulate host cell death for their own benefit.

Introduction

Host cells deploy multiple cell surface and cytosolic pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and Nod-like receptors (NLRs), which enable them to sense microbial intrusion as pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), bacterial secretion systems and bacterial cell wall components, or danger-associated molecular patterns (DAMPs), such as ATP and uric acids. Upon detecting these patterns, PRRs transmit alarm signals to the innate immune system, as well as activating and executing a multitude of defence mechanisms (Takeuchi and Akira, 2010; Franchi et al., 2012). One major host defence mechanism, the death of infected cells, plays a pivotal role in clearing damaged cells, eliminating pathogens, limiting microbial replication, locally confining tissue damage and inflammation, and emitting alarm signals. For example, when gut epithelial cells die as a result of infection, extrinsic or intrinsic stresses, or immune disorders, for example, they rapidly detach from the epithelial lining, and the gap is soon filled by neighbouring cells, thereby maintaining epithelial integrity (Ashida et al., 2012).

Cell death induced by bacterial infection can be categorized into three types: apoptosis, necrosis and pyroptosis (Fig. 1). Apoptotic cell death, which is non-inflammatory, is characterized by chromatin condensation and nuclear fragmentation, overall cell shrinkage, blebbing of the intact plasma membrane and eventual rounding of the cells (Kerr et al., 1972) (Fig. 1). There are two distinct apoptotic pathways: the intrinsic (mitochondria-mediated) pathway and the extrinsic (receptor-mediated) pathway. Both the intrinsic and extrinsic pathways require activation of caspase family proteases. Apoptotic stimuli trigger activation of initiator caspases (caspase-2, -8 and -9), which cleave and activate the executioner caspases (caspase-3, -6 and -7), which in turn cleave numerous substrates, ultimately resulting in apoptosis (Mace and Riedl, 2010; Rudel et al., 2010). Necrotic cell death, which occasionally occurs after non-physiological damage or during microbial infections, manifests as swollen cytoplasm, disorganized organelle structure, membrane rupture, intracellular content leakage and caspase-independent inflammation (Fig. 1). Necrotic cells expose DAMPs and emit alarms, such as pro-inflammatory mediators, thereby augmenting the inflammatory response and stimulating the innate immune system (Zitvogel et al., 2010; Sridharan and Upton, 2014; Vanden Berghe et al., 2014). Although necrotic cell death was long considered a form of accidental cell death, recent studies have shown that it sometimes occurs as a form of programmed cell death. In that context, death requires the activity of serine/threonine kinase receptor-interacting
protein 1 (RIP1) and RIP3, and this regulated process has occasionally been referred to as ‘necroptosis’ (Degterev et al., 2005; Cho et al., 2009; He et al., 2009; Zhang et al., 2009).

Pyroptotic cell death involves caspase-1 or caspase-11 activation, resulting in production of IL-1β and IL-18 (Fig. 1) (Bergsbaken et al., 2009; von Moltke et al., 2013; Lamkanfi and Dixit, 2014). Pyroptosis shares several characteristics with apoptosis: both are accompanied by DNA damage and caspase-dependent cellular processes, although distinct executioner caspase species are involved. Pyroptosis also resembles necrosis in the sense that it is characterized by plasma membrane rupture, maintenance of mitochondrial membrane integrity and stimulation of the inflammatory response (Bergsbaken et al., 2009; Lamkanfi and Dixit, 2014). The most distinctive characteristic of pyroptosis is the involvement of PRRs, such as the NLRs (e.g. NLRP1, NLRP3 and NLRC4) and AIM2 (Mariathasan et al., 2004; 2006; Franchi et al., 2006; Miao et al., 2006; 2008; Fernandes-Alnemri et al., 2010; Rathinam et al., 2010). Upon recognition of PAMPs or DAMPs, PRRs form signal-transducing platforms, the canonical and non-canonical inflammasomes, which respectively send alarms that activate caspase-1 and caspase-11; these factors in turn induce pyroptotic cell death (Bergsbaken et al., 2009; Lamkanfi and Dixit, 2014). Both caspase-1 and caspase-11 induce pyroptosis as part of an essential host defence system against bacterial infection (Miao et al., 2010b; Kayagaki et al., 2011; 2013; Broz et al., 2012; Aachoui et al., 2013). Recent reports have shown that caspase-4 and -11 directly bind to cytoplasmic LPS of Gram-negative bacterial pathogens and underwent oligomerization, resulting in activation of caspase-11.
mediated inflammasomes and pyroptosis (Fig. 1) (Hagar et al., 2013; Kayagaki et al., 2013; Shi et al., 2014).

Thus, the cell death that occurs under both physiological and pathophysiological conditions limits microbial colonization, and by so doing acts as an innate immune defence (Zitvogel et al., 2010; Lamkanfi and Dixit, 2014; Sridharan and Upton, 2014). However, bacterial pathogens have also evolved mechanisms to manipulate the balance between host cell death and survival pathways; these strategies promote bacterial survival, maintain replicative niches and facilitate dissemination into other cells (as described in other excellent reviews, Lamkanfi and Dixit, 2010; Taxman et al., 2010; Ashida et al., 2011a; Higa et al., 2013; Cunha and Zamboni, 2013). In this review, we present selected examples of interactions between Shigella and host cell death pathways, with particular emphasis on strategies that bacteria use to manipulate host cell death and survival pathways and the outcome of the host cell response.

**Macrophages cell death**

*Shigella*, the causative agent of bacillary dysentery, have highly specialized invasive systems that enable bacteria to enter and colonize host cells, ultimately leading to severe inflammatory colitis. *Shigella* deliver a subset of effector proteins into host cells via the type III secretion system (T3SS), which subverts host cell functions and promote infection. *Shigella* target the M cells that overlay the solitary lymphoid nodules in the colon and rectum; bacteria endocytosed by M cells are transcytosed towards the M-cell pocket, where the resident macrophages and dendritic cells come in contact with the bacteria. *Shigella* then invade these macrophages, disrupt the phagosome vacuole, disseminate into the cytosol and multiply therein, eliciting rapid cell death (Fig. 2) (Ogawa et al., 2008; Ashida et al., 2011b,c). *Shigella* released from dying macrophages subsequently enter the intestinal epithelium via the basolateral surface of polarized enterocytes by delivering several effectors (via the T3SS) that subvert cell signalling pathways, including the Rac1–WAVE–Arp2/3 pathway, which then direct bacterial internalization by the epithelial cells. Soon after the invasion, *Shigella* are surrounded by a vacuolar membrane, but they disrupt this vacuole and disseminate into the cytosol. Subsequently, the bacteria multiply and move by directing actin polymerization at one pole of each bacterium, and these motile bacteria spread to neighbouring cells (Fig. 2) (Phalipon and Sansonetti, 2007; Ogawa et al., 2008; Carayol and Tran Van Nhipieu, 2013).

As it multiplies within macrophages, *Shigella* release T3SS effectors and components, which are recognized as PAMPs by NLRC4 and NLRP3, leading to the production of IL-1β and IL-18 and ultimately pyroptotic cell death. Recent reports have shown that members of the NAIP subfamily of NLRs act as direct pathogen sensors that determine the specificity of the NLRC4 inflammasome for various bacterial ligands. Indeed, NAIP5 and NAIP6...
specifically activate the NLRC4 inflammasome in response to flagellin, whereas NAIP2 is required for inflammasome activation by T3SS rod components (Lightfield et al., 2008; Kofoed and Vance, 2011; Zhao et al., 2011; Tenthorey et al., 2014). Notably, even though Shigella lack flagella, it is capable of stimulating macrophage cell death in a T3SS-dependent manner via NLRC4 and NLRP3 activation (Suzuki et al., 2007; 2014; Miao et al., 2010a; Davis et al., 2011). At low infection doses, for example, a multiplicity of infection (MOI) of 10 (i.e. 10 bacteria per cell), Shigella infection induces rapid (1–2 h) NLRC4/caspase-1-dependent pyroptosis via NAIP2 recognition of the T3SS rod component MxiI (Fig. 3A) (Suzuki et al., 2007; 2014; Miao et al., 2010a). Furthermore, human NAIP and mouse NAIP1 can detect Shigella T3SS needle protein MxiH, resulting in activation of NLRC4-dependent inflammasomes and pyroptosis (Fig. 3A) (Rayamajhi et al., 2013; Yang et al., 2013). At a higher infection dose (MOI 50) for a longer period (6 h), Shigella infection induces NLRP3-dependent, caspase-1-independent necrosis-like cell death accompanied by inflammation, implying a mixed form of cell death called pyronecrosis (Fig. 3A) (Willingham et al., 2007). A recent report showed that in addition to T3SS components, the T3SS effector IpaB also induces pyroptosis. IpaB oligomers form ion channels and permeabilize the host cell membrane, thereby promoting potassium influx, which in turn activates the NLRC4/ASC/caspase-1 inflammasome and induces pyroptosis (Fig. 3A) (Senerovic et al., 2012). Because pyroptosis results in release of inflammatory mediators (such as IL-1β and IL-18, which play important roles in restricting Shigella infection) and recruitment of neutrophils, it is unclear whether Shigella-induced pyroptotic cell death is optimal for the bacterium (Sansonetti et al., 2000; Miao et al., 2010b). Nevertheless, killing of macrophages is essential in order for Shigella to enter the surrounding epithelial cells and spread to neighbouring cells, because otherwise the macrophages would kill the Shigella themselves. Future studies of the relationship between Shigella and pyroptosis may reveal the role of macrophage cell death in Shigella infection.

Neutrophil cell death (NETosis)

As described above, once Shigella invade host cells, PRRs recognize the presence of PAMPs or DAMPs and stimulates host defence systems such as NF-κB and MAPK, triggering the secretion of pro-inflammatory cytokines and chemokines (such as IL-8), which in turn recruit neutrophils to the site of infection to limit and clear bacterial infection (Sansonetti et al., 1999). Neutrophils, which migrate from the circulating blood to infectious sites in response to inflammatory stimuli, are the major anti-

Fig. 3. Shigella induction of macrophage, neutrophil and B-cell death.
A. PAMPs or DAMPs generated by Shigella invasion and multiplication in macrophages trigger NLR inflammasome assembly and induce pyroptosis or pyronecrosis. The T3SS components (MxiI and MxiH) and potassium influx trigger NLRC4 inflammasome-mediated pyroptosis. An unknown Shigella factor triggers NLRP3-dependent caspase-1-independent pyronecrosis.
B. Neutrophils kill Shigella both intracellularly (phagocytosis) and extracellularly (NETosis), leading to release of NETs, which are composed of decondensed chromatin and antimicrobial proteins.
C. Shigella induce B-cell death in both invaded and non-invaded cells. In non-invaded cells, the T3SS needle tip protein IpaD interacts with TLR2 on B cells to induce apoptosis.
Manipulation of host cell death by Shigella

Crooked phagocytic cells in Shigella infection (Zhang et al., 2001). Neutrophils engulf Shigella into phagosomes that fuse with granules, which contain multiple bactericidal enzymes that contribute to resolving the infection (Fig. 3B) (Mandic-Mulec et al., 1997; Weinrauch et al., 2002). In addition, neutrophils can also kill Shigella extracellularly by releasing neutrophil extracellular traps (NETs). Shigella are trapped by NETs, which are composed of chromatin laced with antimicrobial peptides and granule proteins (e.g. elastase that degrades T3SS effector proteins and the outer membrane protein VirG), resulting in bacterial killing (Weinrauch et al., 2002; Brinkmann et al., 2004). Interestingly, recent studies have demonstrated that NET release is the result of a unique form of neutrophil cell death, called ‘NETosis’ (Fig. 3B). NETosis is now considered to be a specialized form of regulated neutrophil necrotic cell death, and is observed as an antibacterial immune defence system in infections by many types of bacteria, including Staphylococcus aureus and Streptococcus pyogenes (Fuchs et al., 2007; Branzk and Papayannopoulou, 2013). Although high-dose Shigella infection (MOI = 100) triggers necrotic cell death of neutrophils (François et al., 2000), it remains unclear whether Shigella actively induce NETosis to promote infection, or instead host cells trigger NETosis to resolve the infection.

B-lymphocytes cell death

In comparison with macrophages and epithelial cells, interactions between Shigella and T or B lymphocytes have been poorly investigated. However, previous studies observed massive T- and B-cell death in rectal biopsies of Shigella-infected humans (Raqib et al., 2002). Furthermore, a recent study by Phalipon’s group showed that Shigella target B cells and induce apoptosis in both Shigella-invaded and non-invaded cells (Notherfeller et al., 2014). Shigella invade B cells and replicate intracellularly, leading to B-cell death. On the other hand, the T3SS needle tip protein lpaD binds to TLR2 and triggers mitochondrial apoptosis in non-invaded B cells, which would help the bacterium avoid antibody-mediated immune responses (Fig. 3C) (Notherfeller et al., 2014). Therefore, this result implies that Shigella have ‘secret weapons’ that counteract host adaptive immunity.

Epithelial cell death

Some bacterial pathogens, including Chlamydia, enteropathogenic Escherichia coli, Shigella and Helicobacter pylori, which colonize within or upon epithelia, deploy various mechanisms to prevent host cell death, because cell survival is necessary for the bacteria to survive and maintain their replicative niches (Fischer et al., 2004; Pirbhai et al., 2006; Mimuro et al., 2007; Hemrajani et al., 2010; Li et al., 2013; Pearson et al., 2013).

Shigella invasion and multiplication within epithelial cells cause DNA damage, mitochondrial damage and oxidative stress, eventually inducing both necrosis-like and apoptosis-like cell death, efficiently preventing bacterial replication and spreading (Clark and Maurelli, 2007; Carneiro et al., 2009; Faherty and Maurelli, 2009; Bergounioux et al., 2012). For example, Shigella invasion of epithelial cells triggers the up-regulation of the pro-apoptotic protein Gadd45α, resulting in induction of mitochondria-dependent apoptosis (Lembo-Fazio et al., 2011). The phagocytic membrane remnants generated by Shigella are recognized as DAMPs, and subsequently recruit inflammasome components including NLRP3, NLRC4, ASC and caspase-1 (Dupont et al., 2009). Carneiro et al. (2009) indicated that Shigella infection of the epithelium stimulates oxidative stress, which triggers death signalling through the BH-3-only protein BNIP3 and CypD, resulting in mitochondrial permeability transition-dependent necrosis-like cell death in the later stages of infection (Fig. 4). As described above, host cells induce several types of cell death as part of a host defence system aimed at eliminating infected cells and clear bacteria. Nevertheless, Shigella are able to replicate within epithelial cells and spread to adjacent epithelial cells, strongly indicating that the bacterium must possess some activity that prevents host cell death (Mantis et al., 1996).

A recent report showed that the early stage of Shigella infection induces DNA damage, which is followed by activation of p53 and induction of apoptosis (Bergounioux et al., 2012). Interestingly, Shigella actively prevent this early stage of apoptosis induction via activation of calpain, mediated by the T3SS effector VirA, thereby maintaining the replicative niche. VirA binds to the calpain inhibitor calpastatin and promotes its degradation, resulting in the degradation of p53 by activated calpain protease, preventing pro-apoptotic signalling through the p53 pathway. Intriguingly, calpain activation by VirA also promotes bacterial uptake into the epithelial cells, and sustained calpain activation eventually induces necrosis and restricts bacterial proliferation at late stages of infection. Therefore, calpain activation is a strategy that prevents cells from undergoing the early stage of apoptosis until Shigella have succeeded in multiplying inside them (Fig. 4). Another study showed that Shigella have an activity that dampens mitochondrial dysfunction-mediated necrotic-like cell death by activating the Nod1/RIP2/NF-κB/Bcl-2 pro-survival pathway, indicating that epithelial cell survival is the pivotal strategy used by Shigella (Carneiro et al., 2009). Those authors performed kinetics experiments whose results suggested a scenario in which cytoprotective signalling
becomes dominant during bacterial multiplication within epithelial cells, but is ultimately overwhelmed by cell death signalling at a later stage, thereby enabling bacterial egress towards the intestinal lumen (Fig. 4).

In addition, before bacterial internalization into the epithelial cells, *Shigella* deliver IpgD via the T3SS. IpgD acts as phosphoinositide phosphatase that specifically transforms PtdIns(4,5)P$_2$ to PtdIns(5)P at the bacterial entry site, promoting bacterial invasion. Pendaries *et al.* showed that at this step, PtdIns(5)P generated by IpgD contributes to activation of the PI3K/Akt pro-survival pathway, thereby indirectly augmenting pro-survival signalling (Fig. 4). Consistent with this, infection with the *Shigella* ΔipgD mutant resulted in an increased proportion of apoptotic cells (Pendaries *et al.*, 2006). Other studies have shown that *Shigella* have an activity that inhibits staurosporine-induced apoptosis in epithelial cells by delivering several T3SS-secreted effectors, including Spa15, during multiplication within epithelia (Fig. 4) (Clark and Maurelli, 2007; Faherty and Maurelli, 2009; Faherty *et al.*, 2010).

A recent study showed that invasive bacterial pathogens, including enteroinvasive *E. coli*, *Salmonella* and *Shigella*, inhibit apoptosis at the early stage of infection via the function of a conserved bacterial pilus component protein, FimA (Sukumaran *et al.*, 2010), in addition to the T3SS effector proteins. Secreted FimA localizes to mitochondria, where it inhibits Bax-mediated cytochrome c release. In general, apoptotic stimuli dissociate mitochondrial hexokinase (HK) from the outer mitochondrial membrane protein VDAC1, blocking the anti-apoptotic function of the VDAC–HK interaction. This triggers translocation of Bax into the outer mitochondria membrane and release of cytochrome c, which in turn

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Fig. 4. *Shigella* deploy multiple countermeasures against epithelial cell death. *Shigella* invasion of the epithelium stimulates genotoxic stress and oxidative stress. Genotoxic stress triggers apoptosis through p53 activation, whereas the T3SS effector VirA activates calpain, which degrades p53 and prevents apoptosis in order to promote bacterial replication for a while. However, sustained calpain activation eventually induces necrosis in the later stage of infection. Oxidative stress triggers death signalling through the BH-3-only protein BNIP3 and CypD, resulting in mitochondrial permeability transition (MPT)-dependent necrosis-like cell death. *Shigella*-induced necrosis-like cell death is counterbalanced by the peptidoglycan (PGN)/NOD1/RIP2/NF-κB/Bcl2 pro-survival pathway. The secreted pilus protein FimA binds to the mitochondrial outer membrane protein VDAC, strengthening the VDAC–HK interaction, which prevents cytochrome c release. Via the T3SS, *Shigella* deliver Spa15 and IpgD, which inhibit apoptosis by preventing caspase-3 activation and activating the PI3K/Akt pro-survival pathway respectively. Another T3SS effector, OspC3, blocks caspase-4-mediated acute inflammatory cell death by binding and inhibiting caspase-4 activation, thereby prolonging epithelial cell viability.
activates apoptotic signalling. By contrast, mitochondria-localized FimA binds to VDAC and strengthens the VDAC–HK interactions, preventing Bax integration into the mitochondrial membrane and suppressing host cell apoptosis (Fig. 4) (Sukumaran et al., 2010).

Recently, Kobayashi et al. (2013) showed that the *Shigella* T3SS effector OspC3 antagonizes inflammatory (pyroptosis-like) cell death dependent on caspase-4, the human homolog of murine caspase-11, and thereby promotes epithelial infection. Intriguingly, caspase-4-dependent inflammatory epithelial cell death is also observed in infections by other enteric pathogens, such as *Salmonella* and EPEC, indicating that type of cell death is a major innate immune defence against bacterial infection. OspC3 interacts with the p19 catalytic subunit of caspase-4, and inhibits its activation by preventing heterodimerization between p19 and the p10 catalytic subunit, thereby reducing caspase-4 catalytic activity and decreasing the level of cell death (Fig. 4). Interaction and inhibition of caspase-4 activity requires the X-Y-X-D-X motif of the OspC3 ankyrin-repeat (ANK) region, which is conserved in other bacterial and viral proteins, including *Shigella* other OspC (OspC1 and OspC2), *Legionella pneumophila* and *Rickettsia rickettsii*, and might interact with catalytic pocket of caspase-4 p19. A similar mechanism has been observed in inhibition of caspase-1 by the *Yersinia* T3SS effector YopM. An aspartic acid residue in the exposed loop of YopM is essential for the YopM–caspase-1 interaction and inhibition of caspase-1 activation (LaRock and Cookson, 2012). Of note, infection by a *Shigella* ΔospC3 mutant induces early cell death and increased cytokine production in human epithelial cell lines. Furthermore, guinea pigs infected with a *Shigella* ΔospC3 mutant exhibited severe colitis with mucosal cell death, inflammatory cell infiltration and a reduction in the number of colonizing bacteria relative to that of WT *Shigella* (Kobayashi et al., 2013). Therefore, although *Shigella* infection triggers caspase-4-dependent inflammatory epithelial cell death, *Shigella* OspC3 antagonizes and delays epithelial cell death and promotes infection.

As described above, *Shigella* manipulate the host cell survival pathway by dampening the cell death signal via a subset of effector proteins or virulence factors, until the bacteria have fully multiplied within epithelial cells and spread among them. Therefore, it is tempting to speculate that after a successful colonization within the intestinal mucosa, *Shigella* activate cell death in order to break down the epithelium by delivering as-yet-uncharacterized T3SS-secreted effectors, thereby facilitating bacterial egress from epithelium. *Shigella*’s ability to maintain a delicate balance between cell survival and cell death during infection, for its own benefit, prolongs the survival of infected epithelial cells, which is important for intercellular spread of the bacteria.

**Conclusion**

Bacterial infection elicits diverse host cell responses that include activation of cell death and survival signalling, inflammatory responses and the innate immune system. One of these responses, host cell death, is critical to the fate of bacterial infection, the host innate defence barrier and disease outcome. Bacterial-triggered cell death is manifested in multiple modes. Importantly, the outcome of each cell death modality varies greatly depending on the stage of infection (early versus late), intensity of infection (low versus high MOI), host cell type (myeloid versus non-myeloid cells), targeted signalling pathway and the physiological state of the host cells (naïve versus activated myeloid cells) (Bergsbaken and Cookson, 2007; Lamkanfi and Dixit, 2010; Rudel et al., 2010). Nevertheless, bacterial pathogens deploy multiple highly specialized factors that manipulate host cell death and cell survival pathways, modulate the host’s inflammatory response and circumvent host innate barriers, thereby promoting infection. As a survival strategy, some bacteria actively induce cell death, whereas others inhibit cell death in infected cells. Indeed, the outcome of bacterial modifications of host cells is dependent on cell type (myeloid versus non-myeloid cells), because the bacterial stratagem and host responses in each cell type are quite different. In fact, *Shigella* trigger pyroptotic cell death in macrophages, but inhibit cell death in epithelial cells because *Shigella* prefer these cells as habitats for replication, spreading to neighbouring cells, and evasion of immune cells. Bacterial pathogens utilize three ways to inhibit cell death: (i) prevention of mitochondria damage; (ii) prevention of caspase activation; and (iii) activation of cell survival signalling. Because mitochondria and caspsases play key roles in triggering host cell death (apoptosis, necrosis and pyroptosis) during bacterial infection, many bacteria target these factors in order to modulate host cell death (Lamkanfi and Dixit, 2010; Rudel et al., 2010; Ashida et al., 2011a). Currently, our understanding of the dynamic interplay between pathogens and host innate defence responses remains incomplete. Studying the type of cell death induced by *Shigella* infection *in vivo* would provide the clue to understand what is beneficial and what is disadvantageous for bacteria to promote gut colonization. Furthermore, by investigating the mechanisms by which bacteria manipulate host cell death and innate immune pathways, we will gain greater insight into the control of cell death, including the inflammatory response, during infection. The resultant insights will prove useful in the identification of new drug targets and vaccine candidates.
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Conflict of interest

The authors declare no competing financial interests.

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