Inhibitors of apoptosis proteins (IAPs) govern cell death and inflammatory processes associated with microbial pathogenesis

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ABSTRACT

Inhibitors of apoptosis proteins (IAPs) are crucial mediators of cell death and immune signaling. While they were originally described for their role in apoptosis, recent studies have extended the function of several IAP members to cell death processes beyond apoptosis, such as necroptosis and pyroptosis. It is now generally recognized that IAPs influence inflammatory pathways, including those activated through the inflammasome and NF-κB transduction. While the connection between programmed cell death and inflammation has always been recognized, the characterization of IAPs has further bridged the two fields. These processes are critical for innate immune responses and are commonly employed by host cells to clear pathogenic microorganisms that are potentially encountered. As a consequence, a number of successful bacterial and viral pathogens have evolved mechanisms to manipulate the signaling cascades that regulate both host cell death and inflammation to favor their own survival and persistence. Here we discuss the roles of cIAP1, cIAP2 and XIAP in host cell survival and inflammation, and how this impacts microbial pathogenesis.

KEYWORDS: microbes, IAP, apoptosis, pyroptosis, necroptosis, innate immunity

INTRODUCTION

Inhibitors of apoptosis proteins (IAPs) are mostly known for their role in cell death via the apoptotic pathway; however their regulatory function extends beyond apoptosis to other forms of programmed cell death, such as necroptosis and pyroptosis, each with distinct inflammatory outcomes. In addition, IAPs also influence NF-κB transduction, which is ultimately responsible for induction of genes involved in both inflammation and cell survival processes that are central to immunity [1]. Recent studies have defined the complexity of signaling cascades associated with cIAP1, cIAP2 and XIAP, and revealed that they are uniquely situated at the junction of cell survival and inflammation, processes that are critical during microbial infection [2]. In this review we explore the role of cIAP1, cIAP2 and XIAP in the overlapping cell death and immune signal transduction pathways in the context of the recent studies on their role in microbial infections.

The first identified IAP was reported in a baculoviral system and was shown to inhibit host cell apoptosis [3]. Subsequently, a larger number of IAP family members have been reported, all with at least one shared baculoviral IAP repeat (BIR) (Figure 1). In humans, eight IAPs have been reported, namely BIRC1/NAIP, BIRC2/cIAP1, BIRC3/cIAP2, BIRC4/XIAP, BIRC5/Survivin, BIRC6/BRUCE, BIRC7/ML-IAP and BIRC8/ILP2 [4, 5]. In addition to the BIR domain, several IAPs also possess both an E3 ubiquitin ligase domain (RING) [6] and a ubiquitin-binding domain (UBA) [7] (Figure 1). These domains confer self-ubiquitylation and trans-ubiquitylation properties to IAPs and place them in a central position for ubiquitin-mediated immune signaling [8, 9].
Through ubiquitylation, IAPs propagate signaling downstream of numerous immune complexes and pathogen recognition receptors (PRRs) including Toll-like receptors (TLRs), NOD like receptors (NLRs), TNF receptor, the TNF superfamily, and inflammasomes [9-14]. IAPs utilize two different ubiquitylation patterns; the first consists of ubiquitin chains connected by a lysine in position 63 (K63), which creates scaffolds for protein-protein interaction. The second ubiquitin pattern, K48, targets proteins for proteasome degradation. Interestingly, IAPs can utilize both ubiquitylation patterns to positively regulate signaling, with variations among immune receptors.

Ubiquitin-mediated pathways drive both inflammation and cell survival, particularly via regulation of NF-κB, a central modulator of immune responses via activation of proliferative, pro-survival and pro-inflammatory genes. IAPs are key effectors of NF-κB signaling downstream of TNF receptor, TLR2, TLR4, NOD1/2, and retinoic acid-inducible gene (RIG1) [2, 8, 9, 15-20]. For example, following engagement of the TNF receptor (TNFR), IAPs utilize their E3 ligase domain to ligate K63-ubiquitin chains to the receptor-interacting protein kinase 1 (RIPK1). The ubiquitin chain then serves as a scaffold to recruit more kinases, which eventually leads to translocation of NF-κB to the nucleus. Although the proteins that are ubiquitylated by IAPs and the type of ubiquitylation vary by the engaged receptor, IAP ubiquitylation eventually leads to NF-κB activation. For example, following NOD activation, IAPs ligate K63 ubiquitin chains to RIPK2 to act as signaling scaffolds, but following TLR4 activation, IAPs ligate K48 ubiquitin chains to TRAF3 to target TRAF3 for proteasome degradation [18]. Both lead to NF-κB activation. In this way, IAPs can propagate NF-κB-dependent activation of cell survival genes (i.e. cyclin D1 [21], Bel-2 and IAP anti-apoptotic proteins [22], as well as inflammatory mediators (i.e. TNFα, IL-1, IL-6 and IL-8 [23]). However, if activation of these pathways occurs in the absence of IAPs, programmed cell death pathways such as necroptosis or apoptosis are initiated (Figure 2) [13, 24-28].

Figure 1. Human inhibitor of apoptosis protein (IAP) family.
Members of the human IAP family (8 total, only 3 shown here) are all defined by the presence of at least one baculoviral IAP repeat (BIR), that mediate interaction with factors such as TRAFs, NODs and caspases. IAPs also contain a “really interesting new gene” (RING) E3 ubiquitin ligase domain for substrate ubiquitylation, a ubiquitin-associated domain (UBA) that binds to ubiquitin and polyubiquitylated chains, and a caspase recruitment domain (CARD) that suppresses the E3 ligase activity under steady-state conditions.

The role of IAPs in programmed cell death
Programmed cell death is a critical process in immune cell homeostasis and pathogen clearance, and its induction during microbial infection is a common host cell defense strategy. Several modalities of programmed cell death have been identified, each with distinct morphological and inflammatory outcomes [29] (Figure 3). The best-studied form of
Figure 2. NF-κB signaling by IAPs.
A. When IAPs are active, TNFR ligation is followed by coupling of IAPs with TNF receptor associated factors (TRAFs) and recruitment of RIPK1. In this complex, IAPs utilize their E3 ligase domain to ligate K63-ubiquitin chains to RIPK1 (black chains), proceeding to activation of NF-κB, which promotes transcription of proliferative, pro-survival and pro-inflammatory genes. B. In the absence of active IAPs, RIPK1 is not ubiquitylated and TNFR signaling initiates cell death pathways in which RIPK1 is found in a complex with Fas-associated death domain (FADD) and caspase 8, causing apoptosis, or with FADD and RIPK3, causing necroptosis.

Figure 3. Morphology distinction and inflammatory outcomes of cell death mechanisms.
A. Apoptosis is immunologically silent: the cell maintains membrane integrity while organelles are degraded and extruded in sealed compartments (apoptotic bodies) via cell blebbing. B. Necroptosis and pyroptosis are inherently inflammatory: the cellular membrane ruptures, leaking cytoplasmic content containing endogenous, danger-associated molecular patterns (DAMPs) that lead to production of inflammatory cytokines. Both apoptosis and pyroptosis cause nuclear condensation and DNA fragmentation, whereas necroptosis causes nuclear swelling, rupture and release of DNA (another DAMP). Lastly, apoptosis is characterized by mitochondrial depolarization while necroptosis is thought to lead to release of mitochondrial reactive oxygen species (ROS).
cell death is apoptosis, a tightly regulated process for removal of damaged cells with minimal inflammation and collateral damage to surrounding cells. This process eliminates abnormal or infected host cells through activation of signaling cascades defined as intrinsic or extrinsic pathways. The intrinsic pathway is initiated by intracellular stress signals such as genotoxic or nutrient stress, while the extrinsic pathway is initiated by ligation to cell death receptors such as TNF and CD95/Fas [30]. Both pathways are dependent on members of an intracellular protein family, the cysteine-dependent aspartate-specific proteases (caspases). Caspase 8 is the initiator caspase in the extrinsic pathway while caspase 9 represents the initiator caspase of the intrinsic pathway. In both cases, the effect of initiator caspases converges on the mitochondria, causing mitochondrial membrane depolarization and activation of the executioner caspases, caspase 3 and caspase 7. The executioner caspases represent the final step in the apoptotic signaling transduction that leads to DNA degradation, cell shrinkage and membrane blebbing, which are hallmarks of apoptosis [30, 31].

A recently described genetically-programmed cell death mechanism is programmed necrosis, or necroptosis. Unlike apoptosis, necroptosis results in cellular swelling and cell membrane rupture, making it inherently inflammatory (Figure 3) [32]. Necrosis was classically thought of as an “accidental” cell death, in contrast to apoptosis or “programmed” cell death. However, recent evidence and the use of chemical inhibitors of necrosis have demonstrated that necrosis is also a programmed mechanism [33, 34]. Necroptosis is morphologically distinct from apoptosis and is characterized by swelling organelles, absence of DNA fragmentation, release of mitochondrial reactive oxygen species, and eventual cell membrane rupture and lysis [32, 35]. Instead of caspases, necroptosis is dependent on the activation of RIPK1 and RIPK3 [36, 37].

As mentioned above, IAPs are required for NF-κB signal transduction downstream of PRRs and TNFR. However, in the absence of IAPs, cell death occurs either in the form of apoptosis or necroptosis (Figure 2) [9, 38, 39]. For example, following TNFR ligation, IAPs conjugate polyubiquitin chains to RIPK1, leading to the recruitment of kinases and activation of canonical NF-κB signaling. In the absence of IAPs, RIPK1 ubiquitylation is not achieved, triggering the formation of a Fas-associated death domain (FADD), caspase 8 and RIPK1 complex, which causes apoptosis via the extrinsic pathway [40]. When caspase 8 is not available (due to inhibition or inactivation), TNFR ligation proceeds to cause RIPK1- and RIPK3-mediated necroptosis [41]. Cellular FLICE-like inhibitory proteins (cFLIPs) control caspase 8 activity, and thus the choice between apoptosis or necroptosis. It has also been reported that IAPs may influence the outcome of cell death through regulation of RIPK3 expression and, potentially, caspase 8 activation [42-44]. Ultimately, variations of IAP expression can influence NF-κB signaling and lead to these two different cell death pathways. It is important to note that apoptosis or necroptosis in the absence of IAPs have also been reported in a TNFR-independent fashion [44, 45], although the mechanisms of this process are not well-defined. Finally, while cIAP1 and cIAP2 have mostly been described in the TNFR-dependent cell death mechanism, XIAP also plays a role in both intrinsic and extrinsic apoptosis through a direct inhibition of caspases [46, 47].

IAP expression during microbial infection

Since IAPs modulate immune signaling and cell death pathways that are important in microbial clearance, it is not surprising that their expression is also influenced in response to microbial infections. Expression of cIAP1, cIAP2 and XIAP does not appear to be regulated by a similar mechanism as shown by several studies in which each IAP was upregulated or downregulated in different experimental conditions (i.e. microbial infection or cell stimulation). For example, several studies have demonstrated that ligands of microbial origin as well as whole live bacteria induce cIAP2 expression but not cIAP1 or XIAP [24, 48-50]. One study demonstrated that intestinal epithelial cells stimulated with Bacteroides fragilis enterotoxin induced cIAP2 expression via the COX-2 controlled prostaglandin E2, thereby connecting cIAP2 regulation to MAPK signaling [49]. It should also be noted that IAP expression may be controlled not only by induction (shown for cIAP2), but also by a stabilization mechanism (shown for XIAP). Stimulation of Kupffer cells with Pseudomonas aeruginosa and stimulation
The use of IAP knockout mice and small pharmacological inhibitors of IAPs (SMAC mimetics (SM) (Box 1)) have provided evidence that IAPs are critical for both pathogen clearance and survival of host animal. For example, treatment of mice with SM during lymphocytic choriomeningitis virus (LCMV) infection dampens T-cell expansion and survival, due to inhibition of IAPs and sensitization of the T-cells to TNFα-induced apoptosis, thus contributing to increased viral titers and dissemination to surrounding organs [60]. Similarly, infection of XIAP KO mice with C. pneumoniae results in higher bacterial burden and higher lung TNFα levels as compared to wild type mice. While this phenotype was attributed to increased susceptibility of macrophages to apoptosis, this did not completely account for the observed increased bacterial burden, since ex vivo macrophages from XIAP KO mice also exhibited reduced inducible nitric oxide synthase (iNOS) activity, a critical antibacterial host response, and increased TNFα production. These results suggested that XIAP controls both innate immune pathways and apoptosis during microbial infection [61].

Only a few studies have examined IAPs, apoptosis and infection, and therefore broad generalizations cannot be made of the role of cIAP1, cIAP2 and XIAP in this context. In vitro studies have demonstrated that the increased IAP expression achieved following microbial stimulation inhibits apoptosis, but it is unclear whether this is beneficial for the host or pathogen. In vivo studies, however, suggest that XIAP controls both innate immune pathways and apoptosis in response to microbial infection [61].

IAPs and apoptotic outcomes

Induction of host cell death is a fundamental immune defense process during microbial infection that favors clearance of infected cells. However, pathogens can often manipulate host cell death pathways to favor their own replication and persistence [54-56]. Several recent in vitro and in vivo studies have begun to establish a role for IAPs in cell death during microbial infection (Table 1). In oral keratinocytes, expression of the human papilloma virus (HPV) oncoproteins E6 and E7 can upregulate cIAP2 gene expression, resulting in enhanced cell resistance to exogenous induction of apoptosis [57]. In gastric cancer cells, Helicobacter pylori infection also induces cIAP2 expression, with anti-apoptotic consequences during infection [50]. Chlamydia trachomatis infection of human epithelial cells suppresses host cell apoptosis via increased cIAP2 protein expression [48, 58, 59]. Inhibition of cIAP2, cIAP1 and XIAP in C. trachomatis-infected epithelial cells resulted in sensitization to TNF-induced apoptosis, suggesting that multiple IAPs are involved in prevention of cell death. It has been hypothesized that cIAP1, cIAP2 and XIAP form a heteromeric complex, and that changes in expression of one IAP could alter the expression of the others. However, the mechanism of IAP-IAP complex formation and of regulation of cell death and immune signaling pathways are not yet fully defined.

IAPs and necroptotic functional outcomes

Unlike apoptosis, necroptosis is characterized by cell membrane rupture and inflammation. Similar to apoptosis, necroptosis is also modulated by several microbial pathogens [63] and recent studies have demonstrated that IAPs inhibit necroptosis during microbial infection [42, 64, 65]. In macrophages, depletion of cIAP1 and cIAP2 by the use of SM or by siRNA ex vivo leads to spontaneous activation of RIPKs, resulting in programmed necrosis. This was shown in a Listeria monocytogenes mouse infection model, in which IAP depletion by SM resulted in necrosis of HET cells with Sendai virus both result in enhanced stabilization of XIAP via induction of the phosphatidylinositol 3-kinase (PI3K) signaling network [51, 52]. Other pathogens have also been shown to decrease IAP expression; for example, stimulation of HEK cells with mammalian reovirus leads to degradation of XIAP and subsequent downregulation of cIAP1, resulting in apoptosis [53]. Additional work is needed to define the mechanisms by which each IAP is regulated and whether this is an IAP-specific process. However, it is clear that pathogens can influence IAP expression with consequences on both inflammation and cell death. While inflammation and cell death are intertwined, in this review we will separately review IAPs in apoptosis, necroptosis, and immune signaling during microbial infection.
of peritoneal macrophages and increased bacterial burden [42]. In a recent study, Rodrigue-Gervais et al. demonstrated that mice infected with a sub-lethal dose of a mouse-adapted H1N1 virus were protected when cIAP2 was expressed [64]. While there were no differences in viral titers, inflammatory cytokines, or immune cell recruitment to the lungs in wild type and cIAP2 KO mice, lung tissue

<table>
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<td>XIAP KO mouse</td>
<td>High bacterial burden, low pro-inflammatory cytokines</td>
<td>Not examined [20]</td>
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<td>XIAP</td>
<td><em>C. pneumonia</em></td>
<td>XIAP KO mouse</td>
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<td>cIAP1 KO mouse</td>
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<td>cIAP1, cIAP2, XIAP</td>
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<td>Gastric cancer cells, cIAP2 siRNA</td>
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<td>Apoptosis [51]</td>
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<tr>
<td>cIAP2</td>
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<td>Human oral keratinocyte siRNA</td>
<td>Not examined</td>
<td>Apoptosis [58]</td>
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<tr>
<td>cIAP2</td>
<td><em>B. fragilis</em> enterotoxin</td>
<td>Intestinal epithelial cell line, cIAP2 siRNA</td>
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<td>cIAP2</td>
<td><em>N. gonorrhoeae</em></td>
<td>Human cervical epithelial cells</td>
<td>High IL-1β</td>
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IAPs are predominantly studied in cancer, where a poor patient prognosis has been correlated to over-expression of IAPs at the site of the tumor [62]. To prevent these high levels of IAP and promote tumor cell apoptosis, small molecular inhibitors of IAPs have been designed that mimic the effect of the known intrinsic inhibitor of IAP, SMAC. In physiologic conditions, SMAC is released from mitochondria, binds to the BIR domain of IAPs and blocks their ability to inhibit caspases. SMAC mimetics (SM) were originally designed to prevent XIAP-mediated inhibition of caspase 3 and caspase 7 activation, but it has later been shown that SM preferentially bind to cIAPs [10]. Once bound, SM trigger auto- and trans-ubiquitylation of cIAPs, rapidly leading to proteasomal degradation. While IAP degradation may cause TNFα-induced apoptosis, SM are presently being used to elucidate regulation of IAP-dependent pathways.

Box 1. SMAC mimetics (SM).

IAPs and potential roles in pyroptosis

Another form of programmed cell death involved in the cellular response to pathogen infection is pyroptosis. Pyroptosis is an inflammatory form of cell death mediated by caspase 1 in the context of the inflammasome complexes. The inflammasome is a multiprotein intracellular complex that recognizes pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Upon ligand recognition, NOD like receptors (NLRs) assemble and recruit apoptosis-associated speck-like protein containing a CARD (ASC) and caspase 1; this complex then cleaves cytosolic IL-1β and IL-18 precursors into their active form and initiates pyroptosis. Similar to the forms of cell death discussed above, pyroptosis is also modulated by pathogens. The induction or inhibition of pyroptosis by pathogens may depend on whether the pathogen seeks to escape and spread, or instead to dampen immune activation by inhibiting the release of inflammatory mediators [72].

Although IAPs have never been directly linked to pyroptosis, recently a role for cIAP1 and cIAP2 in regulation of the inflammasome has been proposed [73]. Work from Vince et al. demonstrated that complete inhibition of cIAP1, cIAP2 and XIAP by compound A (a SM) in macrophages enhanced inflammasome activity, measured by increased activation of caspase 1 and generation of IL-1β [74]. Production of active IL-1β was independent of caspase 1, but dependent on caspase 8 and RIPK3, mediators of a TNFR-independent death complex known as the ripoptosome. It was also found that depletion of IAPs resulted in activation of the inflammasome by the ripoptosome [74]. In contrast, Labbé et al. found that depletion of cIAP1 and cIAP2 in macrophages by BV6, another SM, reduced activation of the inflammasome and IL-1β activity [75]. These investigators also demonstrated that cIAP1 and cIAP2 ubiquitylate caspase 1, which in turn enhances its association with the inflammasome.

analysis demonstrated epithelial cell apoptosis in wild type mice and caspase-independent cell death in the cIAP2 KO mice. Administration of a RIPK1 inhibitor demonstrated that necroptosis caused lung epithelial cell death in the cIAP2 KO mice, as the RIPK1 inhibitor maintained lung integrity and enhanced animal survival. Using bone marrow chimeras, it was established that cIAP2 KO epithelial cells undergo necroptosis due to release of FasL from hematopoietic cells. This study demonstrated a protective role of cIAP2 in lung epithelium during influenza infection [64]. Whether other IAPs have specific cell death functions in specific tissues or during certain infections has not been elucidated [66].

The studies from McComb et al. and Rodrigue-Gervais et al. have demonstrated that cIAPs protect the host from microbial induced necroptosis, suggesting that pathogens may utilize necrosis as a means for dissemination [67]. However, multiple studies have suggested that necroptosis can also represent a host defense mechanism against pathogens such as vaccinia virus or pathogenic E. coli [63, 68-71]. More studies are needed to demonstrate a definitive role for IAPs in programmed necrosis during infection.

IAPs and microbial pathogenesis

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While it is important to note that both studies were carried out using purified ligands, these observations suggest that similar changes in IAPs due to microbial infection may impact the inflammasome and potentially, pyroptosis outcomes (Figure 4).

**IAPs in immune signaling and inflammation**

IAPs can regulate NF-κB and MAP kinases downstream of activation of PRRs, pathways that influence both cell survival and inflammation in response to stress signals. Some studies have suggested that the main function of IAPs is regulation of innate immune signaling for production of inflammatory and antimicrobial mediators [9, 28]. Evidence for this hypothesis includes an observed reduction of cytokine and iNOS production in vitro in cells lacking expression of IAPs [61, 75-77].

NLRs are intracellular pathogen recognition receptors that recognize bacterial cell wall components and induce NF-κB and MAPK signaling through RIP2 [78]. Utilizing NLR agonists, Bertrand et al. found that the ubiquitin ligases of cIAP1 and cIAP2 were required for RIP2 signaling, a pathway not directly involved in apoptosis [15]. Macrophages from cIAP1 and cIAP2 KO mice ex vivo exhibited attenuated cytokine profiles in response to NOD ligands, and in vivo cIAP KO mice were protected from experimental colitis as compared to wild type.

**Figure 4. Role of IAPs in apoptosis, necroptosis and pyroptosis.**

A. Induction of apoptosis by extracellular death receptors (extrinsic pathway) or by intracellular stress (intrinsic pathway) converges on activation of caspase 3 and caspase 7, the final reversible steps in this cascade that are susceptible to inhibition by XIAP. Up-stream of these caspases in the extrinsic pathway, cIAPs can block formation of the caspase 8 death complex. B. Necroptosis is also initiated by death receptors or intracellular stress and is dependent on RIPK1 and RIPK3 activation. cIAPs and XIAP block formation of the necroptosis death complex, which leads to activation of mixed lineage kinase domain-like (MLKL) and production of mitochondrial ROS. ROS and RIPK3 then lead to activation of the inflammasome and release of IL-1β. C. Pyroptosis is induced by detection of intracellular PAMPs or DAMPs by the inflammasome and is caspase 1 dependent. Inflammasome activation is enhanced through ubiquitylation of caspase 1 by cIAPs (black), leading to IL-1β release. All factors required for execution of each specific cell death pathway are shown in grey.
mice. In these studies, no differences in cell death were reported between wild type, cIAP1 KO or cIAP2 KO bone marrow-derived macrophages (BMDMs) or in peritoneal macrophages in vivo during induction of experimental colitis [15]. These findings suggest a role for cIAPs downstream of NLRs independent of cell death. In XIAP KO macrophages, infection with L. monocytogenes (a potent inducer of NLRs) ex vivo reduced NF-κB activation and cytokine production as compared to macrophages isolated from wild type mice [19]. In vivo, XIAP KO mice infected with L. monocytogenes presented increased bacterial burden and decreased animal survival as compared to wild type mice. No differences were observed in macrophage apoptosis in vivo between the mice strains, and the in vivo phenotype was attributed to the ability of XIAP to promote signaling downstream of both TLR2 and NLRs [19]. It has also been reported that cIAP1 KO mice exhibit an increased sensitivity to C. pneumoniae lung infection [76], impairment of macrophage recruitment, increased interferon gamma and reduced TNFα. In ex vivo experiments, macrophages from cIAP1 KO mice mirrored the dysregulated cytokine profile and also had blunted nitric oxide production, a critical antibacterial host response. However, cIAP1 KO macrophages exhibited increased sensitivity to caspase-independent cell death, a phenotype that could contribute to the small number of lung macrophages and resulting susceptibility to C. pneumoniae infection [76].

CONCLUSION
A connection between cellular processes leading to cell death and inflammation is well accepted, and IAPs have been identified as molecular mediators shared by these pathways [79]. IAPs participate in multiple programmed cell death pathways, including those with inflammatory outcomes, in response to endogenous causes and microbial infection. A more in depth understanding of both recruitment and activation of IAPs during microbial infections will clarify the mechanisms by which regulation of cell death pathways and inflammation by both pathogens and host cells may influence immune responses. In the specific case of cIAP1, cIAP2 and XIAP, distinct and yet redundant functions have been attributed to these intracellular proteins in the context of different cell death pathways and in different cell types. Additional studies will be needed to further clarify the interplay between host cell death and inflammatory pathways mediated by microbial pathogens and to potentially develop IAP-targeted therapies.

CONFLICT OF INTEREST STATEMENT
None of the authors has a conflict of interest.

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