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Caspase-8 functions as a key mediator of inflammation and pro-IL-1β processing via both canonical and non-canonical pathways.

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Running Title: Caspase-8 and the inflammasome

Summary: Caspase-8 is an apical component of cell death pathways. Activated caspase-8 can drive classical caspase-dependent apoptosis and actively inhibits cell death mediated by RIPK3-driven necroptosis. Genetic deletion of *Casp8* results in embryonic lethality as a result of uncontrolled necroptosis. This lethality can be rescued by simultaneous deletion of *Ripk3*. Recently, caspase-8 has been additionally connected to inflammatory pathways within the cell. In particular caspase-8 has been shown to be crucially involved in the induction of pro-IL-1β synthesis and processing via both non-canonical and canonical pathways. In this review, we bring together current knowledge regarding the role of caspase-8 in cellular inflammation with a particular emphasis on the interplay between caspase-8 and the classical and non-canonical inflammasomes.

Keywords: *interleukin-1β, caspase, inflammasome, apoptosis, death receptor, signal transduction*

Introduction

Caspases have been historically broadly classified into two types based upon their impact on cell fate: the inflammatory caspases ([caspases 1, 11 (4 and 5 in humans) and 12], and the apoptotic, or executioner, caspases (caspases 2, 3, 6, 7, 8, 9 and 10) (1). Over the last few years, our understanding of the different roles and functionality of some of these caspases has developed. As such, it has become apparent that this initial delineation is an oversimplification of caspase activity. In this review, we address the recent advances in our knowledge regarding the role of caspase-8 in the inflammatory response, particularly in relation to its function in inflammasome-mediated signalling, and in the synthesis and processing of pro-interleukin-1β (IL-1β).

Caspase-8

Caspase-8 is located on chromosome 1 in mice and chromosome 2 in humans. Genetic ablation of *Casp8* in mice results in embryonic lethality at the e10.5 stage (2). This phenotype can be rescued by the simultaneous removal of *Ripk3*(receptor-interacting serine/threonine-protein kinase 3), consistent with a role for caspase-8 in controlling RIPK3-dependent cell death processes (3, 4). Although these double knockout mice are viable, they ultimately develop lymphoproliferative disease. Like other caspases, caspase-8 is translated as a monomeric zymogen consisting of a prodomain followed by both a large and small catalytic subunit (*Fig. 1*). In caspase-8, the prodomain consists of two death effector domains (DEDs) that facilitate interaction with other cellular proteins and mediate homotypic interactions to induce caspase-8 dimerization and autoactivation by an induced proximity mechanism (*Fig. 1*). Activation of caspase-8 requires cleavage between the large and small catalytic subunits, specifically at the

aspartic acid residues D374 and D384. These cleavage events serve to stabilize the caspase-8 protein and can then be followed by further cleavage at D216 between the prodomain and large catalytic subunit (5). Interestingly, there is increasing evidence that the functionality of caspase-8 is related to the extent of molecular cleavage, with pro-apoptotic functions requiring a higher degree of processing. This may of course represent a cellular self-preservation strategy and ensure that apoptosis mediated by activation of death receptors is so finely tuned that cell death can only occur following a sufficiently robust level of receptor activation.

The traditional role of caspase-8 in apoptotic and necroptotic cell death

Classically caspase-8 has been associated with both apoptotic and necroptotic forms of cell death. Indeed when activated, caspase-8 serves both a pro-apopototic role and in addition functions as an inhibitor of necroptosis. Full consideration of these functions of caspase-8 is beyond the scope of this article. However, we refer the reader to a number of recent reviews that provide a detailed overview of the role of caspase-8 in cell death processing (1, 6–10). Briefly, activation of pattern recognition receptors such as TLR3 (Toll-like receptor 3), TLR4, DAI (DNA-dependent activator of interferons), and potentially RIG-I (retinoic acid inducible gene I) and MDA5 (melanoma differentiation-associated antigen 5) can stimulate necroptosis; however, it is ligation of death receptors in the plasma membrane that has been most extensively studied in relation to caspase-8 and cell death. Activation of CD95, TRAIL-R1/2 [tumor necrosis factor (TNF) apoptosis-inducing ligand receptor ½] or TNFR1 (TNF receptor 1) all result in the formation of multiprotein complexes involving caspase-8 (*Fig. 2*). The recruitment of FADD (Fas cell surface death receptor-associated death domain protein) to either CD95 or TRAIL-R1/2

induces formation of the membrane associated death-inducing signalling complex (DISC), which recruits and then activates caspase-8.

Caspase-8 activation occurs via a two-step mechanism which begins with dimerization (11, 12). Dimerization of caspase-8 leads to a proximity-induced mechanism of autoproteolytic activation. Dimeric caspase-8, however, shows only low levels of proteolytic activity. This results in cleavage being limited to other proximal caspase-8 molecules and a restricted set of substrates within the DISC. Subsequent processing of the linker peptide between the catalytic subunits leads to an enhancement in the proteolytic activity of caspase-8 and a related increase in the range of available substrates. Caspase-8 now becomes able to process classical apoptotic caspases, such as caspase-3 and caspase-9, and as a result leads to the initiation of apoptotic cell death (*Fig. 2*). The activation of caspase-8 can be regulated and controlled by two isoforms of a non-catalytic caspase-8 paralogue $cFLIP_L$ (cellular FADD-like IL-1β-converting enzyme-like inhibitory protein long form) and $cFLIP_s$ (short form), which heterodimerize with caspase-8 and inhibit its activity (13). Additional caspase-8 containing complexes are released into the cytoplasm from where they are believed to augment caspase processing and phagocytosis of apoptotic cells by macrophages (14, 15). Interestingly, application of mathematical modeling coupled to experimental observations has recently been employed to investigate the mechanism of caspase-8 activation at both the level of the single cell and also cell populations (16). The authors of this study have put forward a model of DISC-mediated caspase-8 activation in which cleavage between the prodomain and catalytic domains is mediated in *cis* within the individual DISC. However, cleavage between the catalytic domains is proposed to occur in a *trans*-based manner and therefore requires the association of multiple and distinct DISCs. It is suggested that this method of activation allows the cell to balance the degree of caspase-8 activation to reflect

either low-level or high-level stimulation. This would in turn provide a molecular timing switch for caspase-8-mediated cell death (16).

Activation of another death receptor, TNFR1, leads to the formation of an alternative pro-survival multi-protein complex known as Complex I at the plasma membrane. Complex I consists of, amongst other proteins, TNFR-associated factors 2 and 5, TRADD (TNFR1-associated death domain), cellular inhibitor of apoptosis (cIAP) 1 and 2 and RIPK1, and is tightly regulated by ubiquitylation. Ultimately recruitment of the IKK [inhibitor of nuclear factor κB (NFκB) kinase] complex results in activation of NFκB signaling pathways. Internalization of ligand-bound TNFR1 leads to a compositional shift in the complex and the formation of a cytoplasmic Complex II. Complex II, also known as the 'TRADDosome' (17) normally contains TRADD, FADD, RIPK1, RIPK3, and caspase-8 and forms following the deubiquitylation of RIPK1 by either of the proteins A20 or cylindromatosis. This complex activates caspase-8 and promotes the apoptotic pathway of cell death and serves to actively inhibit necroptosis by direct cleavage of RIPK1 and RIPK3. If $cFLIP_L$ is incorporated into Complex II this inhibits the apoptotic pathway and helps maintain the induction of NFκB signaling pathways.

In the absence of caspase-8 following genetic ablation, pharmacological inhibition, or as a result of certain viral infections, RIPK1 and RIPK3 are not cleaved, and instead RIPK3 becomes phosphorylated by RIPK1 leading to the initiation of necroptotic cell death through formation of the necrosome (*Fig. 2*). Assembly of the necrosome can also occur as a result of either the loss or the inhibition of IAPs (inhibitor of apoptosis proteins), which are primarily known for inhibiting apoptotic caspase activity by binding via evolutionary conserved baculoviral IAP repeat motifs and acting as ubiquitin ligases through their RING (really interesting new gene) finger E3 ligase domain. IAPs also have a role in the negative regulation

of the inflammatory response by limiting RIPK3-dependent activation of the inflammasome receptor NLRP3 (NLR family, pyrin domain containing 3)(18). Following formation of the necrosome, a series of downstream effector molecules are activated. These include the serine/threonine protein phosphatase PGAM5, mitochondrial and the mixed lineage kinase domain-like protein (19, 20). It has been proposed that these proteins mediate the propagation of necroptosis through facilitation of pore formation in the cellular membrane (21). Necroptosis is an inherently inflammatory process in nature due to the release of cellular contents that act as either pro-inflammatory stimuli, such as ATP and high mobility group proteins, or are themselves pro-inflammatory cytokines, for example IL-33 and IL-1α (22).

Caspase-8 and the inflammasome

The cellular responses to infection, damage, and threat are wide and varied. Over the last decade or so, the importance of a cytoplasmic multi-protein complex known as the inflammasome has become apparent, not just in terms of its role combating infection but also more generically in relation to activation of a pro-inflammatory immune response (23, 24). Inflammasome activation requires two signals. 'Signal 1' to upregulate the cellular levels of pro-IL-1β as well as inflammasome components such as the NLR (nucleotide binding, leucine-rich repeat containing receptor) family member NLRP3 and 'Signal 2' to initiate inflammasome activation and cytokine processing and secretion (23, 25, 26). Signal 1 is often provided as a result of upregulated NFκB signaling following the activation of TLR or NOD1/2 (nucleotide oligomerization domain 1/2) pathways. Signal 2, on the other hand, takes the form of a particular activatory ligand specific for certain inflammasome receptors (27). Examples of activatory

ligands include ATP, uric acid crystals, nigericin, dsDNA, the bacterial protein flagellin, and the anthrax lethal toxin. The classical canonical inflammasome is a complex of an activated receptor, the adapter protein ASC (apoptosis-associated speck-like protein containing a CARD), and an effector caspase that triggers cell death and processes pro-IL-1β and pro-IL-18 into their active forms ready for release from the cell (23, 28). To date, there are four canonical inflammasomes, formation of which is mediated by activation of one or more of the NLR family members NLRP1, NLRP3, and NLRC4 (NLR family, CARD domain containing 4)/NAIP (neuronal apoptosis inhibitory protein) or the interferon-inducible protein AIM2 (absent in melanoma 2). Receptor activation serves to act as a nucleation signal for the self-association of ASC leading to the formation of a very large cellular complex, which has a rough diameter of around one micron (24). This multiprotein complex is often referred to as a 'speck'. The speck then serves as a focus for the recruitment of effector caspases, which under classical canonical conditions is caspase-1 (23, 28, 29). Caspase-1 is subsequently activated, also as with caspase-8 via a proximity-induced mechanism, and then processes pro-IL-1β and pro-IL-18 into their active forms prior to their release from the cell. Caspase-1 can also mediated pyroptosis, a caspase-1 dependent mechanism of cell death (30, 31). Non-canonical inflammasomes trigger similar cellular processes but the mechanisms by which this is achieved are different (24).

Caspase-8 was not originally thought to be involved in inflammasome activity. However, recent work suggests in fact that caspase-8 does play an unexpected and important effector role in both canonical and non-canonical inflammasomes, roles which we expand on below. As a point of clarification, much of the experimental data obtained in studying the role of caspase-8 in the inflammasome has been generated either using caspase-8 inhibitors, through the use of mice with lineage or cell-specific *Casp8* deletions (sometimes genetic, sometimes inducible), or in a

Casp8$^{-/-}$*RIPK3*$^{-/-}$ double knockout background. This approach is due to the embryonic lethality of caspase-8 ablation, which results in uncontrolled necroptotic death (2–4).

Integrating caspase-8 and ASC in cell death

ASC activation and speck formation has been associated with multiple types of cell death. These include apoptosis, necrosis, caspase-1 dependent pyroptosis, and pyronecrosis. First identified in human promyelocytic leukemia, HL-60 cells as an aggregating body formed during apoptosis (32). ASC was subsequently shown to interact directly with caspase-8, an interaction that could be competitively inhibited by the closely related Pyrin protein. The ASC and caspase-8 interaction occurs between the N-terminal Pyrin Domain of ASC and the DEDs of Caspase-8 (33). Currently a molecular understanding of this interaction is lacking. In fact it is not even known whether both caspase-8 DEDs are required for this interaction, let alone which specific residues in either ASC or caspase-8 are involved. Both Pyrin domains and DEDs are members of the Death Domain (DD) superfamily. DD-mediated interactions are common in inflammatory and cell death pathways underpinning the formation of multiprotein complexes such as: the Myddosome (34, 35), the Piddosome (36), the apoptosome (37, 38), the ripoptosome (39, 40), and the DISC (41, 42). However, usually DD interactions are homotypic in nature, meaning that they occur between protein folds of the same type. Hence the ASC:Caspase-8 interaction presents an intriguing target for future study and may provide a truly novel perspective on DD-family interactions.

A specific role for caspase-8 in ASC-associated cell death was originally demonstrated through the inhibition of apoptosis following the overexpression of a catalytically inactive

caspase-8 construct as well as through the action of the viral proteins CrmA and baculovirus p35. In addition, the genetic removal of caspase-8 resulted in the inhibition of apoptotic death following ASC activation (33). Further work from the group of Suda and colleagues (43, 44) demonstrated that ASC activation and speck formation has the capacity to result in either apoptotic or necrotic death and that the preise death pathway initiated was cell-type specific. Using an overexpression system in COS-7 cells, they showed that ASC and caspase-8 co-localized to the speck structures and that either caspase-8 knockdown or caspase-8 inhibition led to a significant reduction in the degree of ASC-mediated apoptosis (44). Analysis of the apoptotic cascades activated by caspase-8 led to the proposition that caspase-8 promotes apoptosis through two distinct mechanisms. One route involves the direct cleavage of caspase-3, while the other is propagated through cleavage of the pro-apoptotic protein Bid. As with the decision to proceed via either apoptotic or necrotic death, the precise mechanism employed appears to be seemingly dependent on cell type (44). A more recent study has shown that the catalytic activity of caspase-1 is not needed for driving ASC-induced apoptosis or necrosis. However, in cells lacking or expressing low-levels of caspase-1 the process of caspase-8 dependent ASC-induced apoptosis is favored. In contrast, necrosis is preferred in cells expressing caspase-1 (45). This is indicative of a complex regulation strategy for the initiation of these cell death pathways.

Despite the well-documented connection between ASC and caspase-8, it required the results of a yeast-2-hybrid screen coupled with functional assays in A549 lung carcinoma cells before a direct link between caspase-8 and inflammasome activity was proposed (46). In this work, LRR-deleted NLRC4 was used as bait in a yeast-2-hybrid screen. Caspase-8 was not reported to interact directly with caspase-8, but instead ubiquitination of NLRC4 facilitated

interaction between NLRC4 and Sug1 (suppressor of gal 1), a component of the 26S proteasome. Using immunoprecipitation, NLRC4, Sug1, and caspase-8 were subsequently shown to co-localize. This association resulted in caspase-8 activation and cell death in a caspase-8 dependent manner (46). ASC is not essential for NLRC4 signaling but has been shown to enhance the activity of the NLRC4 inflammasome (47–49). Consequently, this study did not address whether ASC was also present in these assemblies and hence mediating the recruitment of and interaction with capase-8. This remains a likely possibility however.

Caspase-8 and the synthesis, processing, and release of pro-interleukin-1β

IL-1β is an important pro-inflammatory mediator that signals through the type 1 interleukin-1 receptor (50, 51). IL-1β is synthesized in an inactive precursor form, pro-IL-1β, which undergoes subsequent cleavage prior to secretion from the cell. The canonical activation pathway for IL-1β involves stimulation of pro-IL-1β synthesis as a consequence of the activation of NFκB signaling. This is followed by inflammasome-mediated caspase-1 activation and the sequential cleavage of pro-Il-1β to the active cytokine which is then released from the cell (23). A number of non-canonical pathways also contribute to or provide alternative routes for IL-1β release. These include pathways intrinsically linked to death receptor signaling and apoptosis (52, 53). Recent studies have assigned important roles for caspase-8 in these processes (54–57).

It has been known for a while that a selection of non-caspase proteases including elastase, collagenase and cathepsin G are themselves capable of processing pro-IL-1β (58). More recently it has been shown that caspase-8, in addition to caspase-1, is also capable of proteolytically

processing pro-IL-1β into its active form. This was first reported by Maelfait and colleagues who demonstrated that overexpression of TRIF (Toll interleukin-1 receptor-domain protein inducing interferon), an adaptor protein used by both TLR3 and TLR4, resulted in the processing of pro-IL-1β and that this could also be achieved by stimulation of the TLR3 and TLR4 signaling pathways with poly(I:C) and LPS (lipopolysaccharide), respectively (54). Neither the inhibition nor the absence of caspase-1 had a significant impact on TLR3, and TLR4 activated pro-IL-1β processing, whereas processing was significantly inhibited by the presence of either the viral protein CrmA or use of a pan-caspase inhibitor. Overexpression of caspase-8 resulted in an enhancement of pro-IL-1β processing, and the authors proceeded to demonstrate that recombinant pro-IL-1β was a substrate for direct cleavage by recombinant caspase-8 and that the cleavage site, D117, was the same as that utilized by caspase-1 (54).

Activation of Fas by FasL results in a significant upregulation in pro-IL-1β production. This is most likely as a direct result of NFκB signaling, which can be activated in an inflammasome-independent manner (55). The activation of IL-1β via the Fas pathway was dependent on caspase-8 as both the chemical inhibition of caspase-8 and the use of *Casp8/Ripk3* double knock out BMDMs resulted in a loss in the ability to cleave pro-IL-1β. In contrast, *Ripk3*$^{-/-}$ BMDMs (bone marrow derived macrophages) functioned normally in this respect. The processing of prol-IL-1β was dependent however on the adapter protein FADD, which plays a crucial role in recruiting caspase-8 to Fas. Together these experiments demonstrate an integral role for caspase-8 in Fas-mediated inflammasome-independent production of mature IL-1β (55).

Caspase-8 has been intrinsically connected more recently to the processing and release of IL-1β following the induction of apoptosis. IAPs function to limit the apoptotic response,

including caspase-8 dependent apoptotic pathways. Pharmacological antagonism of IAPs brought about by the application of SMAC (second mitochondrial-derived activator of caspases) mimetics resulted in a strong induction of IL-1β in macrophages and DCs primed with LPS. A significant proportion of the induced pro-IL-1β processing was shown to be inflammasome-independent but caspase-8 dependent (18). Moreover application of the proapoptotic chemotherapeutic agents doxorubicin and staurosporine to LPS-primed murine BMDMs resulted in the robust release of IL-1β (57). Somewhat surprisingly IL-1β release under these conditions was only minimally impaired in the absence of the inflammasome components ASC, NLRP3, NLRC4 or caspase-1. A detailed characterization of the signaling pathways involved showed that the release of IL-1β following doxorubicin or staurosporine treatment was in fact mediated by caspase-8. In this pathway caspase-8 was activated at ripoptosomes formed in a TRIF-dependent manner. This overall process was shown to be dependent on TLR4 (57). A more recent report has corroborated the importance of caspase-8 in mediating apoptosis related IL-1β release and also demonstrated that IL-1α release whilst independent of caspase-8 is regulated by necroptosis (56).

Caspase-8 and non-canonical inflammasomes

Evidence for a direct role for caspase-8 in non-canonical inflammasomes has recently emerged. Gringhuis and coworkers (59) have shown that activation of dectin-1, a member of the C-type lectin receptor family and important in the detection of fungal infection, also resulted in caspase-8 mediated processing of pro-IL-1β. Stimulation of dectin-1 by different strains of pathogenic *Candida albicans* and the specific ligand curdlan resulted in the recruitment of the proteins

CARD9, Bcl-10 (B-cell lymphoma/leukemia 10), and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation protein 1) into a scaffold complex to drive pro-IL-1β transcription and also led to the secretion of IL-1β from human dendritic cells. IL-1β release occurred in both caspase-1-dependent and -independent manners. The exact nature and extent of this dependency was influenced by the precise yeast strain being used as a stimulus. Pro-IL-1β processing was shown to be entirely dependent on ASC, but displayed only a small contribution from NLRP3 driven pathways. Through the use of chemical inhibition, gene silencing and immunoprecipitation studies the molecular pathways involved in the caspase-1-independent processing of pro-IL-1β following dectin-1 stimulation were pieced together. The process was shown to be additionally dependent upon various components of the dectin-1 signaling pathway including dectin-1, Syk (spleen tyrosine kinase), CARD9, MALT1, and Bcl-10, while caspase-8 was identified as the protease responsible for pro-IL-1β processing. Full-length uncleaved caspase-8 associated with MALT-1 prior to receptor activation. Following stimulation this complex subsequently associated with another MALT1 containing complex, MALT1-CARD9-Bcl10, resulting in caspase-8 undergoing partial proteolysis to a 43 kDa form. No evidence was found to support further processing to the pro-apoptotic p18 form of caspase-8. The multiprotein complex formed post dectin-1 stimulation then recruits ASC in a caspase-8 dependent manner resulting in the formation of a non-canonical caspase-1 independent inflammasome in which caspase-8 serves to process pro-IL-1β. Together these data suggest that fungal pathogens can stimulate both IL-1β production and maturation and that this is driven through a noncanonical dectin-1 dependent caspase-8-dependent inflammasome (59). Further work has subsequently reported an essential role for caspase-8 in the promotion of β-glucan-induced cell death and also, in contrast to the Gringhuis study (59), NLRP3 inflammasome-dependent IL-1β maturation (60).

These differences may result from variations in the experimental approaches or reflect species based differences resulting from the use of either human (59) or murine (60) derived DCs (dendritic cells). Without question, it is becoming clear that β-glucan recognition receptors and the inflammatory proteases caspase-8 and caspase-1 coordinate cytokine secretion and cell death in response to fungal infections (59, 60).

Caspase-8 and the canonical inflammasome: a controversial association?

Much of the evidence outlined above suggests an important role for caspase-8 in mediating IL-1β release due to the action of both non-canonical inflammasomes and cell death signaling pathways. However, when assimilating the different pieces of work performed studying the classical inflammasome, it rapidly becomes apparent that there is still a large degree of controversy within the field regarding the actual, or precise, role of caspase-8 in canonical inflammasome pathways. This is contextualized in the following sections.

Given that caspase-8 is able to process pro-IL-1β following TLR and C-type lectin receptor activation, we wondered whether caspase-8 was also associated with classical inflammasome formation via an NLR-mediated pathway. Infecting murine BMDMs with *Salmonella* resulted in the co-localisation of caspase-8 and ASC at speck-like inflammasome structures (61). In wildtype cells, roughly half these specks also contained caspase-1 indicating that both caspases could be efficiently recruited to the same inflammasome. Studies in knockout cells showed that whilst ASC is essential for speck formation, caspase-1 (or caspase-11) is not needed for the efficient recruitment of caspase-8. Similarly, caspase-1 can be efficiently recruited independently of caspase-1. Recruitment of caspase-8 to the inflammasome causes caspase-8

activation as around eighty percent of the caspase-8 positive specks were shown to harbor active caspase-8.

Infection of macrophages with *Salmonella* Typhimurium is known to activate both the NLRC4 and NLRP3 inflammasomes. Our study showed that NLRC4 activation by *Salmonella* Typhimurium, or through cytosolic delivery of the ligand flagellin, leads to direct recruitment of caspase-8 to the inflammasome via ASC and that caspase-8 has a direct role in the processing of pro-IL-1β. However, we saw no evidence that caspase-8 plays any role in mediating the rapid macrophage pyroptosis seen in response to *Salmonella* infection (61). In the same study, it was shown that stimulation of the NLRC4 pathway is critical for caspase-8 activation, as neither *Nlrc4*$^{-/-}$ nor *Asc*$^{-/-}$ cells were able to cleave and activate caspase-8, whereas *Nlrp3*$^{-/-}$ and *Caspase-1/11*$^{-/-}$ cells both still produced the activate p18 caspase-8 isoform. Analysis of the production of pro-IL-1β revealed that this was significantly impaired following infection of *Casp8*$^{-/-}$*RIPK3*$^{-/-}$ cells consistent with an important role for caspase-8 in the synthesis of pro-IL-1β. In contrast to the activation of the NLRC4 inflammasome, activation of the NLRP3 inflammasome by *Salmonella* did not induce any role for caspase-8 in the processing of pro-IL-1β (61).

Further examination of the protein interactions indicated a dynamic pattern of association between caspase-8 and ASC and NLRP3. In unprimed cells, caspase-8 could only be immunoprecipitated with ASC and not NLRP3, following LPS-mediated priming caspase-8 associated with NLRP3 but not ASC, and with both proteins post *Salmonella* infection. Relating this to the requirement for Signal 1 and Signal 2 in the activation of the NLRP3 inflammasome suggests that Signal 1 (priming) leads to an association with NLRP3 and that Signal 2 (*Salmonella* infection) results in the formation of a caspase-1 caspase-8 containing

inflammasome that drives pro-IL-1β processing. Together, these observations are indicative of a major functional role for caspase-8 in classical inflammasome signaling but demonstrate discrete and distinct physiological roles depending on the precise inflammasome activated.

Meanwhile in *Yersinia* infection models, caspase-8 and RIPK1 have both been shown to be key regulators of macrophage cell death, NF-κB and inflammasome activation playing a key role in host resistance to infection (62, 63). These two pieces of work simultaneously but independently demonstrated that the cell death resulting from infection of BMDMs with *Yersinia* strains was independent of caspase-1, caspase-11, NLRP3, ASC, NLRC4 and unaffected by FasL or TNFα. Extending the studies to include different combinations of *RIPK1*, *RIPK3*, and *Casp8* knockouts demonstrated that death was dependent on RIPK1 and Caspase-8 and that this occurred via FADD-dependent mechanisms as well as TRIF-dependent and TRIF-independent routes (62, 63). The release of IL-1β was lessened when *Casp8* was knocked down or caspase-8 inhibited, and both groups subsequently demonstrated that caspase-8 controlled the activation of caspase-1 and hence pro-IL-1β processing. Caspase-8 activation however was not influenced by caspase-1 (62, 63).

Caspase-8 deficiency, in a *RIPK3*$^{-/-}$ background, has been shown to reduce both the transcriptional priming and the posttranslational activation of the canonical and non-canonical NLRP3 inflammasomes. The same effect is also seen with the absence of FADD (64). This study also reported that deletion of *Casp8* resulted in a reduction in the activation of caspase-1 and caspase-11 by the NLRP3 inflammasome. The authors demonstrated that capsase-1 is a substrate for caspase-8, thereby providing a potential mechanism for this defect. The NLRC4 inflammasome was however unaffected (64). Consist with these observations mice lacking either

caspase-8 or FADD showed an increased susceptibility to the effects of either LPS challenge or *Citrobacter rodentium* infection. Further evidence for the role of caspase-8 in the canonical inflammasome comes from the action of the endogenous caspase-8 inhibitor c-FLIP$_L$ (65). Deletion of c-FLIP impairs ATP- and monosodium uric acid-induced, NLRP3 dependent, IL-1β production in macrophages, as well as dsDNA-dependent activation of the AIM2 inflammasome. The precise manner in which c-FLIP$_L$ mediates NLRP3 or AIM2 inflammasome activity remains uncertain but may involve interaction with caspase-1, an impact on inflammasome assembly, or mitochondrial localization. Interestingly, while c-FLIP$_L$ is seemingly required, somewhat counter-intuitively given the evidence of caspase-8-mediated activation of caspase-1, for full NLRP3 and AIM2 inflammasome activation, this is not the case for Dectin-1-induced caspase-8 dependent IL-1β generation. In this instance, c-FLIP$_L$ provides an inhibitory function (65).

Caspase-8 has also recently been intrinsically connected to activation of the inflammasome formed by the cytosolic DNA sensor AIM2. Like NLRP1, NLRP3, and NLRC4, AIM2 can drive the formation of a canonical inflammasome. Infection with *Francisella tularensis*, the bacterial cause of tularaemia, or the delivery of DNA to the cytosol triggers AIM2/ASC-dependent caspase-3-mediated apoptosis in caspase-1-deficient macrophages (66, 67). These studies showed that AIM2 activation can result in the ASC-dependent, caspase-1-independent activation of both caspase-8 and caspase-9 leading to cell death by apoptosis. In the presence of casapse-8 inhibition or following caspase-8 knockdown, activation of AIM2 results in caspase-1-dependent death (i.e. pyroptosis) occurring. Through the utilization of a variety of co-localization, immunoprecipitation, and pro-IL-1β processing experiments using chimeric caspase constructs, it was demonstrated that caspase-8 interacts with the Pyrin domain of ASC via its own DEDs. This confirmed earlier observations regarding the domains involved in

mediating caspase-8 and ASC association (33). This work also showed that caspase-8 specifically colocalizes with the AIM2/ASC speck (66, 67). Interestingly, Sagulenko and colleagues (67) showed that there is a molecular switch in the AIM2-mediated response to cytosolic DNA. Specifically, they demonstrated that low concentrations of cytosolic DNA result in the initiation of a caspase-8 driven apoptotic death pathway but high concentrations of cytosolic DNA instead induce a rapid caspase-1 dependent pyroptotic death. Caspase-8 therefore functions as an apical caspase in the AIM2- dependent cell death pathway (67).

Caspase-8 as a negative regulator of the inflammasome

In conjunction with their work in identifying the role of caspase-8 in AIM2-dependent inflammasome function, Sagulenko and colleagues (67) also connected caspase-8 to the NLRP3 inflammasome. Specifically, they showed that nigericin stimulation of the NLRP3 inflammsome caused the efficient activation of caspase-8. As outlined above, a number of other studies have also connected a positive requirement for caspase-8 function with efficient activation of the NLRP3 inflammasome. However, the role of caspase-8 in the NLRP3 inflammasome is not clear cut. Using mice specifically lacking caspase-8 in dendritic cells rather than cells from caspase $8^{-/-}$ /$RIPK3^{-/-}$ mice, Wallach's group (68) has shown that caspase-8 deficiency facilitated an enhancement in the LPS-induced assembly and function of the NLRP3 inflammasome. Enhanced inflammasome assembly in the caspase-8-deficient cells lead to elevated processing of pro-IL-1β and pro-IL-18 and led to an increased mortality following challenge with LPS but had no effect on pyroptosis. An intriguing observation in this study was that *Casp8*-deficient DCs were capable of secreting both IL-1β and IL-18 following just LPS stimulation, i.e. after receiving

only Signal 1 and not Signal 2. This ability was lost following the deletion of *RIPK3* or the addition of the RIPK1 inhibitor Necrostatin-1. The authors (68) verified that their cells were not receiving an endogenous Signal 2 as a result of the accumulation of damage-associated molecular patterns in the DCs themselves. This led to the hypothesis that caspase-8 exerts a more direct effect on inflammasome function by directly regulating or inhibiting inflammasome activity, potentially through a simultaneous association with FADD and NLRP3 (68). It is difficult however to reconcile these observations with data showing that caspase 8 is an effector mechanism in both the canonical and non-canonical inflammasomes, so further work is required to explain these discrepancies.

The structural organization of caspase-8-containing inflammasomes

The current experimental evidence suggests the precise role of caspase 8 in the inflammasome is unclear and remains to be fully elucidated and may indeed serve different purposes in different cell types and under the influence of different stimuli. It is clear, however, that caspase 8 can be recruited directly into the inflammasome complex. The ability of the DEDs of caspase-8 to mediate interaction with the pyrin domain of ASC has been reported by a number of groups using techniques such as immunoprecipitation, confocal microscopy and through the construction of chimeric caspase constructs (33, 66, 67). Recent studies using immunofluoresence and super-resolution microscopy have enabled the direct visualization of caspase-8 within the endogenous ASC speck structure (61, 69). While not only confirming that caspase-8 can be recruited to the canonical inflammasome, the enhanced levels of resolution achieved using super-resolution microscopy have revealed a concentric ring-like structure to the

endogenous inflammasome, consisting of an outer ring of ASC, an internal NLR-containing ring, and a central caspase component (69). This study also confirmed that caspase-1 and caspase-8 were present in the same inflammasome speck consistent with the reported ability of caspase-8 to act as a direct activator of caspase-1 (64).

The precise molecular interactions involved in recruitment of caspase-8 remain elusive, and how this occurs in respect of the molecular interactions between ASC, caspase 1, and caspase 8 is only starting to be elucidated. It is clear that caspase-8 and caspase-1 can interact independently of one another with ASC via the pyrin and CARD of ASC respectively. Consistent with the independent nature of the caspase:ASC interaction the recruitment of caspase-8 to the inflammasome is dependent upon ASC and not caspase-1. Equally caspase-1 recruitment is independent of caspase-8. This leads to the possible formation of inflammasome specks containing just caspase-1, just caspase-8, neither caspase, or both caspases (61) (*Fig. 3*). However, the dynamics of these events are uncertain. For example, are the caspases and ASC pre-associated or is the caspase recruited to the preformed speck? How do other inflammasome-linked proteins influence caspase recruitment? Quite likely the inflammasome should be viewed as a dynamic protein platform in which the decision over which effector caspases to recruit is regulated by the nature of the stimulus received and the cell type. Indeed, we have no clue currently as to the stoichiometric relationship of any of the inflammasome components and caspase-8 is no different. However, a recent quantitative mass spectroscopy based study of the stoichiometry of the DISC revealed that in this macromolecular signaling complex caspase-8 is likely to be present at around a ninefold molar excess to FADD (70). More intriguingly, overexpression of the caspase-8 DED has shown them to be capable of forming filamentous structures in the cell (71). Although these filaments were not reported to form in the context of

the full-length pro-caspase-8 protein, it raises interesting parallels with the recent reports of filamentous ASC induced upon overexpression (72). It remains to be seen whether similar aggregation is behavior for caspase-8 is observed in relation to its role in inflammasome function.

Caspase-8 and inflammatory disease

Aside from or in some instances associated with its role in inflammasome functionality, caspase-8 has been directly implicated in a number of inflammatory conditions. Specific deletion of *Casp8* from murine intestinal epithelial cells, keratinocytes, and hepatocytes leads to the induction of ileitis and chronic skin and liver inflammation respectively (73–75). The absence of *Casp8* from intestinal epithelial cells resulted in the dysregulation of necroptosis and increased cell death (75). Interestingly, these mice did not appear to possess Paneth cells and contained a significantly reduced number of Goblet cells. As a result, intestinal homeostasis was severely disrupted, spontaneous inflammation occurred, especially in the ileum, and they showed an enhanced susceptibility to the development of colitis (75). Conditional deletion of *Casp8* from hepatocytes led to an increase in the susceptibility of the mice to infection with *Listeria monocytogenes* and an alteration in the pattern of recovery following partial hepatectomy (74). In the absence of caspase-8 initial cellular proliferation was impaired; however, the rate of new growth became more rapid than that of the control mice and lasted for longer. This led to an overall increase in liver size and ultimately induced a chronic inflammatory state. Interestingly the prolonged enhanced proliferation only occurred in mice of a mixed genetic background and not in a pure C57Bl/6 background (74). Removal of Casp8 from keratinocytes caused chronic

skin inflammation due to an increase in the IRF3 (interferon response factor 3)-dependent response to endogenous mediators of inflammation and also resulted in enhanced gene activation following DNA transfection (73).

The above examples all showcase inflammation that occurs as a result of *Casp8* deletion. This suggests that at least in these tissues caspase-8 plays an important role in the suppression of cellular inflammation. However, the clinical effects of caspase-8 are far from straightforward, and caspase-8 has also been directly implicated with a number of conditions: the progression of geographic atrophy, an age-related form of macular degeneration (76), the progression of acute glaucoma (77), and neurotoxicity resulting from microglia activation (78). More specifically, the retinal pigmented epithelium of individuals with geographic atrophy showed an increase in caspase-8 expression and a role for Fas-ligand dependent activation of caspase-8 in the reduced levels of endoribonuclease DICER1, the upregulation of IL-18, and the accumulation of Alu RNA characteristic of disease progression (76). Similarly in rodent models of acute glaucoma, caspase-8 was shown to be crucial for the TLR4-dependent upregulation of pro-IL-1β required for retinal ganglion cell death in a caspase-1-dependent NLRP3-dependent manner. Inhibition of caspase-8 protected against cell death (77). Meanwhile the addition of LPS to microglial cultures resulted in TLR4-driven activation of caspase-8 and the subsequent caspase-8-dependent activation of caspase-3 and caspase-7. Other caspase-8 substrates, including Bid and RIPK1, were not cleaved in this system. Active caspase-8 was also identified in the microglial of patients with either Parkinson's disease or Alzheimer's disease, suggesting an important role for caspase-8 in these neurodegenerative disorders (78).

Virus-mediated inhibition of caspase-8

The role of caspase-8 is not just limited to a direct part in the inflammatory and death pathways or even as a contributor to the control of bacterial infection. Caspase-8 is also an important component of the antiviral response, a role that has long been acknowledged since the first reports of viral targeted inhibition of caspase-8 function (79). The importance of caspase-8 for this purpose is exemplified by the wide repertoire of viral proteins that function directly either as caspase-8 inhibitors, or target other points in the caspase-8, FADD, and RIPK1-RIPK3 signaling pathways (80). For example, poxviral proteins from the serpin family have been shown to bind directly to caspase-8 and as a result disrupt its functionality. In addition the activation of caspase-8 can be specifically inhibited by viral proteins such as the adenoviral E3 protein, p35 from baculovirus and the vICA (viral inhibitor of caspase-8 induced apoptosis) protein found in strains of cytomegalovirus (81–85). The net result of viral induced caspase-8 inhibition is normally to limit or reduce the inhibition of necroptotic signaling as a result of reducing the extent of RIPK1 and RIPK3 cleavage. Disruption of capase-8-mediated RIPK1-RIPK3 necrosome formation is also mediated by the poxviral proteins CrmA and B13R. Indeed, CrmA is capable of inhibiting caspase-1, -4, -5, and -8 activity (86), attributes that have made CrmA a useful tool for the non-pharmacological inhibition of caspase functionality over the years. A whole subset of viral proteins homologous to cFLIP-long and cFLIP-short, and known as vFLIPs, function to inhibit the apoptotic role of caspase-8. In some cases, these vFLIPs also target the necroptotic RIPK1-RIPK3 pathway. For example, the rodent cytomegalovirus protein vIRA interferes with the interaction between RIPK1 and RIPK3. This has the effect of stopping the progression of necroptosis, which would otherwise be initiated following the inhibition of caspase-8 by the virally encoded protein vICA (87). While this is an example of both the caspase-8-associated

apoptotic and necroptotic death pathways being disrupted by viral infection, in many cases necroptotic death does occur. This observation suggests that necroptotic death may be a more favorable outcome for the virus than other more classical cell defense or death routes. In addition to its role in antiviral apoptotic and necroptotic death, alternative functions for caspase-8 in the cellular response to viral infection have also been reported. These include the activation of capase-8 in response to cell stimulation with dsRNA, a role in the regulation of IRF3 signaling following RIG-I activation, and formation of a TRADDosome-like complex associated with MAVS (mitochondrial antiviral associated protein) (88–90). Clearly caspase-8 is a pivotal molecule in the antiviral response and it is likely that our understanding of the functional repertoire and protective involvement of caspase-8 against viral pathogens will continue to expand over the next few years.

Burning questions and future directions

The role of caspase-8 in inflammation, particularly in relation to inflammasome-mediated signaling, will remain an important area of study in the coming years. Caspase-8 provides a pivotal connection between the death and inflammatory pathways—pathways intrinsically linked in cell fate and disease pathogenesis. Understanding more fully how precisely caspase-8 contributes to inflammation will help our understanding of the delicate balance between inflammatory and death cell phenotypes.

Caspase-8 already functions as a molecular switch: in the cellular decision whether to proceed down NFκB-mediated pro-survival pathways or initiate apoptotic death; in the switch between apoptotic or necroptotic death; and in the cases of some viruses, whether to inhibit

apoptotic death, necroptotic death, or both. Do bacterial infections target caspase-8 at all? Are some of the inflammasome inhibitors with an unknown mechanism of action actually caspase-8 inhibitors? DISC-mediated activation of caspase-8 could also be viewed as a molecular switch dependent on the extent of external stimuli and hence the number of DISCs formed. Could caspase-8 also act as a molecular switch for inflammatory signaling? Determining whether or not caspase-8 acts as a negative regulator of inflammasome signaling, how precisely it assists in the activation of caspase-1 and the processing of pro-IL-1β, and identifying how the role of caspase-8 differs in the face of different inflammatory stimuli and in different cell types are all important questions to address. Finally, will caspase-8 present itself as a suitable target for the modulation of the inflammatory response and the treatment of either acute of chronic inflammatory conditions?

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Figure Legends

Fig. 1. **The organization of caspase-8.** (A) Syntenic comparison of the immediate environment surrounding *Casp8,* the gene encoding caspase-8, between humans and mice. Genes in blue are on the forward strand, genes in green are on the reverse strand. (B) Graphical representation of the domain organization of the caspase-8 protein. The key aspartic acid residues acting as cleavage sites during protein activation are marked with arrows and labelled. DED, death effector domain; p18 and p10, catalytic sub-domains.

Fig. 2. **Simple schematic representation of the involvement of caspase-8 in death-receptor signaling.** (A) Engagement of CD95 or TRAIL1/2 results in the recruitment of FADD and pro-caspase-8 to the cytosolic death domain of the receptor, which in turn results in the formation of a DISC (death-inducing signaling complex). Sites of caspase-8 cleavage are marked by a yellow star. (B) Following TNFR1 activation caspase-8 can ultimately form a complex, the 'TRADDosome' with TRADD, FADD, RIPK1, and RIPK3. Caspase-8-mediated cleavage of RIPK1 and RIPK3 inhibits necroptosis and directs cell death down the apoptotic pathway. (C) When caspase-8 is inhibited, degraded, or deleted, RIPK1 and RIPK3 do not get cleaved. RIPK1 phosphorylates RIPK3 which activates neroptotic death. DD, death domain; DED, death effector domain.

Fig. 3. **Schematic of the possible interactions between ASC and caspase-1 and caspase-8.** Prior to inflammasome stimulation, it is possible that ASC may bind to both, either, or neither of pro-caspase-8 and pro-caspase-1 (left hand side). Upon receipt of 'Signal 2', the inflammasome is activated leading to formation of an ASC-driven speck. Pro-caspase-8 and pro-caspase-1 can undergo autoproteolytic activation through a proximity induced mechanism. However, in the

case when both caspases are present in the same inflammasome, this can facilitate caspase-8-mediated processing of pro-caspase-1 and has the potential to lead to enhanced pro-IL-1β processing. The molecular structures of the ASC pyrin (deep pink) and CARD (light blue) were generated in PYMOL from the protein data bank file 2KN6 (91).

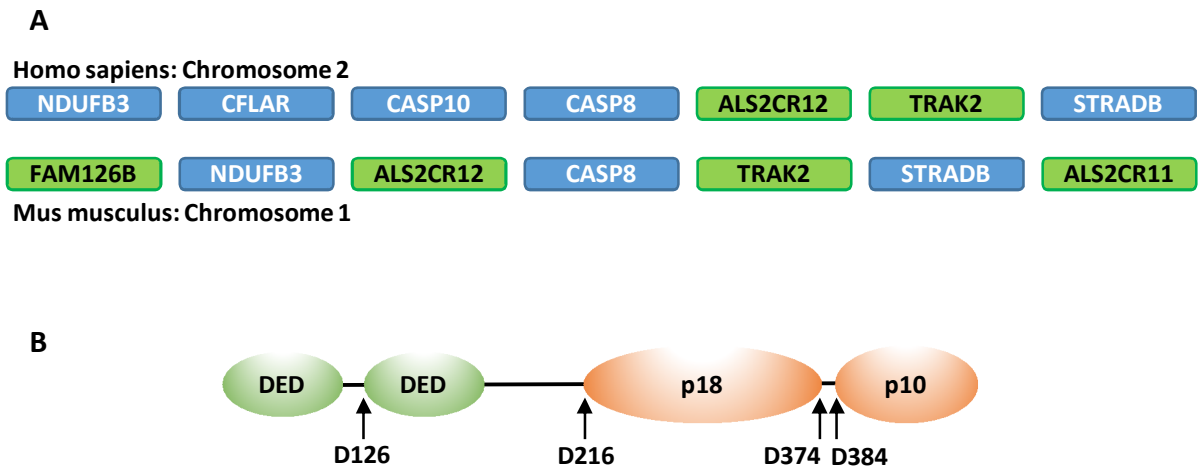
A
Homo sapiens: Chromosome 2
NDUFB3
CFLAR
CASP10
CASP8
ALS2CR12
TRAK2
STRADB
FAM126B
NDUFB3
ALS2CR12
CASP8
TRAK2
STRADB
ALS2CR11
Mus musculus: Chromosome 1
B
DED
DED
p18
p10
D126
D216
D374
D384

Figure 1

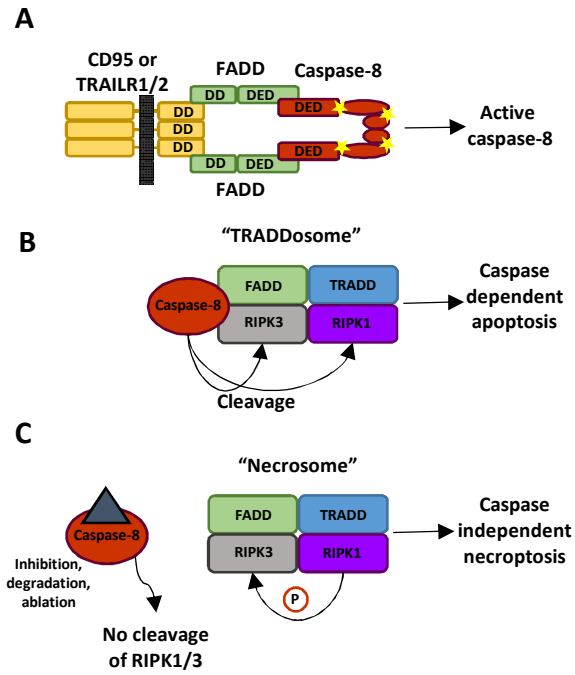
A
CD95 or TRAILR1/2
FADD
Caspase-8
DD
DED
Active caspase-8
FADD
B
"TRADDosome"
Caspase-8
FADD
TRADD
RIPK3
RIPK1
Caspase dependent apoptosis
Cleavage
C
"Necrosome"
Caspase-8
Inhibition, degradation, ablation
No cleavage of RIPK1/3
FADD
TRADD
RIPK3
RIPK1
P
Caspase independent necroptosis

Figure 2

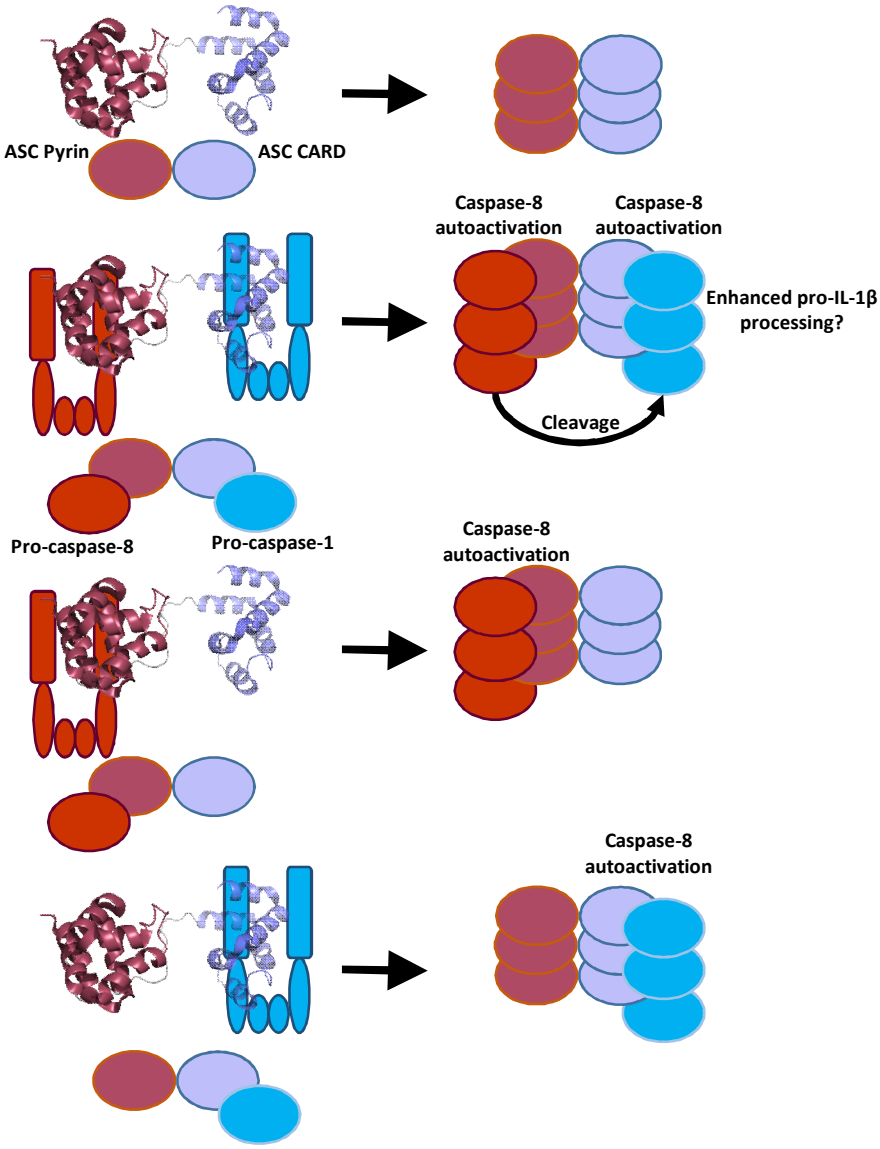
ASC Pyrin
ASC CARD
Caspase-8 autoactivation
Caspase-8 autoactivation
Enhanced pro-IL-1β processing?
Cleavage
Pro-caspase-8
Pro-caspase-1
Caspase-8 autoactivation
Caspase-8 autoactivation
Pre stimulation
'Signal 1'
Post-stimulation
'Signal 2'

Figure 3