Review

Sensing and reacting to dangers by caspases: Caspase activation *via* inflammasomes

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ABSTRACT: Caspases are well known as mediators of programmed cell death, or apoptosis, in which their functions are highly conserved throughout evolution. In addition to inducing apoptosis, caspases have important roles in immune reactions. As part of a cell's response to pathogens or alarm (cellular danger) signals from damaged cells, caspase-1 is activated by forming an inflammatory complex with apoptosis-associated speck like protein containing a caspase recruitment domain (ASC) and nucleotide-binding oligomerization domain like receptor (NLR) family proteins. The activated caspase-1 then cleaves and promotes the maturation of cytokines, such as IL-1β, IL-18, and IL-33. Although it has long been unclear how hosts recognize diverse stresses, including injury and pathogen infection, and react appropriately, recent analyses have revealed many details about the sensing mechanisms provided by NLRs. Members of the NLR family are activated and yield different outcomes depending on the stimulus. For example, the NLR member cryopyrin/NALP3 induces cytokine secretion and lipid synthesis in response to viral dsRNA and K^+ efflux, while another NLR, IPAF, induces IL-1β in response to the virulence protein, flagellin. Cryopyrin/NALP3-mediated caspase-1 activation is involved not only in the immune response to pathogens but also in the stress response to UV irradiation in human skin. In this review, we focus on the stress responses that particularly involve inflammatory caspases. Since host reactions to stresses have been studied in invertebrates as well as in mammals, we also review the caspase-mediated immune responses that have been identified in the fruit fly *Drosophila melanogaster*, and suggest that the contribution of caspases to general stress responses is evolutionally conserved.

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Introduction

Caspases are a family of cysteine proteases that are highly conserved in multicellular organisms. They are known as the central regulators of apoptosis. A cell death-executing caspase was first identified as Ced-3 in *Caenorhabditis elegans*, and since then, the regulatory functions of caspases following apoptotic stimulation have been studied in detail (*1,2*). On the other hand, recent studies have also indicated various non-apoptotic functions of caspases (*3*). In mammals, the first Ced-3 homolog reported was caspase-1 (interleukin-1β-converting enzyme, ICE), a cysteine protease responsible for the processing of prointerleukin 1β (proIL-1β) and the secretion of mature IL-1β (*4-6*). Thus, the first-described function of the mammalian Ced-3 homolog was in the immune response. Not only caspase-1, but also caspase-5 and caspase-11 have been reported as inflammatory caspases (*7,8*).

Caspase-1 is synthesized as an inactive procaspase-1. When activated, the enzyme is cleaved into subunits p20 and p10, which form a heterotetramer (*8,9*). In macrophages, caspase-1 activation promotes the secretion of IL-1β in response to pathogen associated molecular patterns (PAMPS), which include bacterial compounds and viral infections, or to endogenous molecules released from damaged cells (*10*). Caspase-1 also processes IL-18 and IL-33, and thus is responsible for both inflammatory and innate immune responses (*11*). However, many questions about the mechanism remain: How do cells sense various PAMPs and endogenous alarms, and distinguish them from the cell itself? Does each "danger signal" activate caspase-1 differently? And how does caspase-1 respond to each signal? Here, we review some of the pathways in various stress responses that occur through caspase-1.

Inflammasomes

The caspase-1-activating complex is called the "inflammasome" (*9,12*). It is composed of caspase-1 and apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD) (ASC/ PYCARD/ CARD5/ TMS1ASC) in addition to apoptotic protease activating factor-1 (Apaf-1)-like inflammasome components such as NALP1 (Defcap/ Nac/ CARD7), cryopyrin (NALP3/ Cias1/ Pypaf1), and ICE protease activating factor (IPAF/ CARD12), which can sense different bacteria, toxins, or endogenous danger signals released from damaged cells (*13-16*) (Figure 1).

In the case of microbial pathogens, host cells recognize them by germ-line encoded pattern recognition receptors (PRRs), which contain leucine-rich repeats (LRRs). The best known of these signaling systems are triggered by the LRR-containing Toll-like receptors (TLRs) on the surface of the cells. For instance, TLR4 senses lipopolysaccharide (LPS), TLR5 senses flagellin, TLR7 and 8 recognize toxins and antiviral imidazoquinoline R837 and R848, and TLR9 recognizes bacterial and viral nucleic-acid motifs (*17-21*). TLR activation induces the synthesis of proIL-1β which assembles into an inflammasome with caspase-1 and constitutively expresses ASC. TLR activation also leads to the induction of IL-16 and tumor necrosis factor (TNF) production (*22*).

In addition to TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs/ CATERPILLER proteins) also sense pathogens. NLRs are cytosolic proteins that recognize microbial pathogens within the compromised cells, which means they function as intracellular PRRs (*23,24*). Both extracellular and intracellular PRRs trigger intracellular immune responses, such as assembly of the inflammasome, activation of caspase-1, and secretion of cytokines including IL-1β, IL-18, and IL-33 (*11*) (Figure 2).

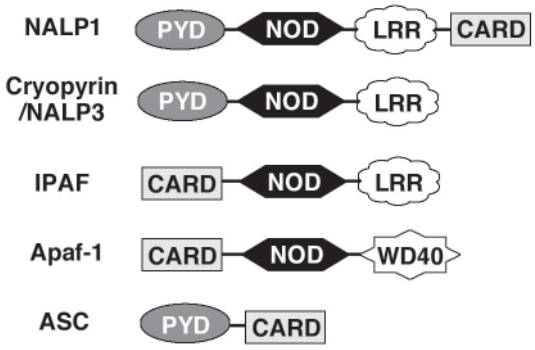

Figure 1. NLR family proteins and ASC. Members of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family contain common domains such as the Pyrin domain (PYD), caspase recruitment domain (CARD), NOD, leucine-rich repeat (LRR), or WD40, which contribute to the NLR's ability to sense and react to various stresses. The apoptosis-associated speck like protein containing a CARD (ASC) structure is also illustrated.

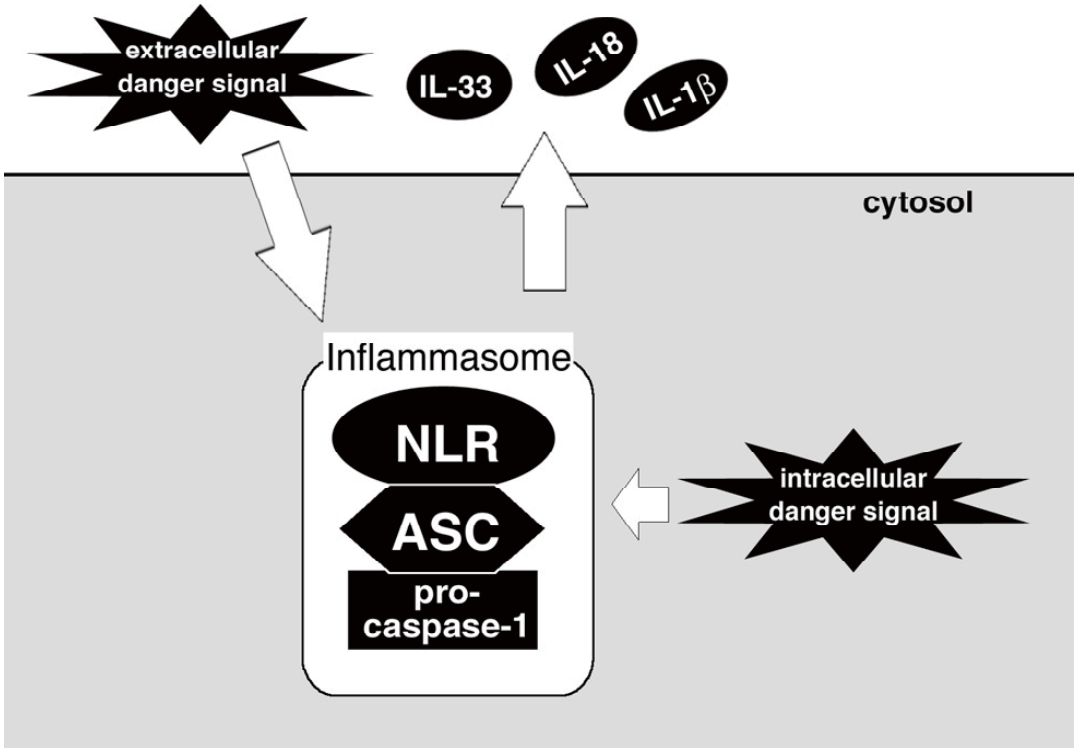

Figure 2. Mechanism for the intracellular and extracellular stress response. Both intracellular and extracellular danger signals induce the assembly of the inflammasome, which is generally composed of NLR, ASC, and procaspase-1. Activated caspase-1 then functions to process and promote the secretion of cytokines such as IL-1β, IL-18, and IL-33.

Cryopyrin/NALP3 inflammasome

In the case of viral infection, viral DNA, single stranded RNA (ssRNA), and double stranded RNA (dsRNA) are produced during viral replication. After viruses are endocytosed into mammalian cells, DNA, ssRNA, and dsRNA are detected by TLRs in the endosomes, and the TLRs then activate NF-κB and mitogen-activated protein kinase (MAPK) pathways, which lead to IL-6 and TNFα secretion (*22*). IL-1β and IL-18 are not secreted *via* TLRs, but *via* the cryopyrin/NALP3-dependent pathway. Cryopyrin/NALP3 is a product of the cold-induced autoinflammatory syndrome 1 (CIAS1) gene and a member of the NLR family. It has some functional structures, such as CARD domains and carboxyl-terminal LRRs, that detect specific pathogens by the presence of monosodium urate (MSU), calcium pyrophosphate dehydrate crystals (CPPD), or PAMPs, including bacterial RNA and synthetic anti-viral purine analogs (*14*,*23*,*25*,*26*). Cryopyrin/NALP3 forms an inflammasome with ASC and caspase-1, but its specificity for sensing pathogens was unclear until recently.

It was recently reported that the ligand-recognition function of cryopyrin/NALP3 is highly specific for dsRNA (*27*) (Figure 3). When macrophages are infected with a virus, neither ssRNA nor dsDNA, but dsRNA is essential for the cryopyrin/NALP3-dependent induction of caspase-1 activation. Cryopyrin/NALP3 is necessary to induce IL-1β and IL-18 secretion, but is dispensable for interferon α (IFNα), TNFα, or IL-6 production. This sensing ability of cryopyrin/NALP3 that distinguishes dsRNA from ssRNA may have an important role in discriminating viral RNA from endogenous host RNA to avoid harmful activation of the inflammasome (*27*).

On the other hand, another report shows that cryopyrin/NALP3 activates caspase-1 in response to specific factors that induce an intracellular K^+ efflux (*15*) (Figure 3). When macrophages are infected by Gram-positive bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes*, ATP stimulates the $P2X_7$ receptor, which opens a channel for cytosolic K^+ efflux. This decrease in cytosolic K^+ level is a signal for formation of the inflammasome, and it triggers the caspase-1-dependent cleavage and secretion of IL-1β (*28*,*29*). In this pathway, TLR signaling still seems to be required for the expression of proteins other than cryopyrin/NALP3, including proIL-1β. In addition to these components, live bacteria may be necessary to trigger this pathway, since heat-killed *L. monocytogens* decreases the IL-1β induction (*15*).

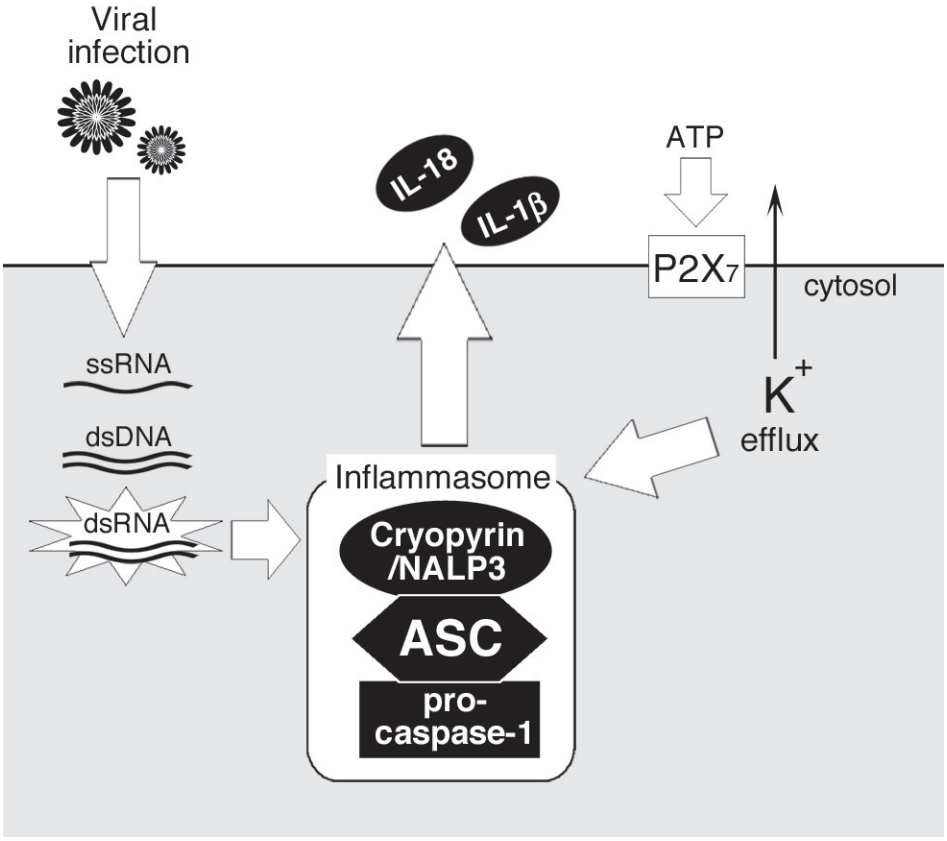

Figure 3. Cryopyrin/NALP3 inflammasome. Cryopyrin/NALP3, ASC, and procaspase-1 form the inflammasome in response to K^+ efflux and viral dsRNA produced during viral replication, to activate caspase-1, leading to cytokine secretion.

IPAF inflammasome

Intracellular Gram-negative bacteria *Salmonella typhimurium* translocate effector virulence proteins, including flagellin, to the cytosol of the host cell (Figure 4). Extracellular flagellin is sensed by TLR5, and stimulated TLR5 induces the activation of NF-κB and MAPK, leading to the secretion of IL-6 and a chemokine, monocyte chemoattractant protein-1 (MCP-1) (*30*). TLR5 also mediates the transcription and translocation of proIL-1β. However, the intracellular mechanism by which flagellin activates caspase-1 to lead to IL-1β secretion has remained poorly defined. Although the cytosolic invasion mechanism of the *S. typhimurium* flagellin is still unclear, it was recently shown that flagellin protein is essential for both caspase-1 activation and IL-1β secretion in *S. typhimurium*-infected macrophages. Macrophages in which *S. typhimurium* replicate, respond to the bacteria *via* IPAF, a NOD-LRR protein that was first identified as a human CED4/Apaf-1 family member (*31*,*32*). As IPAF-deficient macrophages cannot activate caspase-1 or secrete IL-1β, IPAF seems to be indispensable for the caspase-1 activation and IL-1β processing (*13*,*33*). Unlike cryopyrin/NALP3, ASC modulates but is not essential for the IPAF-dependent caspase-1 activation, since ASC-deficient cells show only a partial defect in their response to cytoplasmic flagellin (*34*). IPAF contains a CARD domain, and it may interact with the CARD of caspase-1 or ASC. Whether flagellin activates host cytosolic IPAF directly remains to be determined. This signaling pathway is independent of TLR5, given that extracellular flagellin stimulates neither caspase-1 nor IL-1β activation in macrophages. Moreover, the lack of caspase-1 activation in stimulated IPAF-deficient macrophages is not due to a decreased expression level of procaspase-1 (*34*,*35*). In addition, *S. typhimurium* induces apoptosis in cells *via* IPAF and caspase-1 activation (*13*,*36*). IPAF is necessary to control the caspase-1 activation in response to *S. typhimurium* infection, but it is dispensable for responses to heat-killed *S. typhimurium*, *F. tularensis*, LPS, and ATP (*35*). Thus, the function of IPAF in the innate immune response might be more restricted than those of cryopyrin/NALP3.

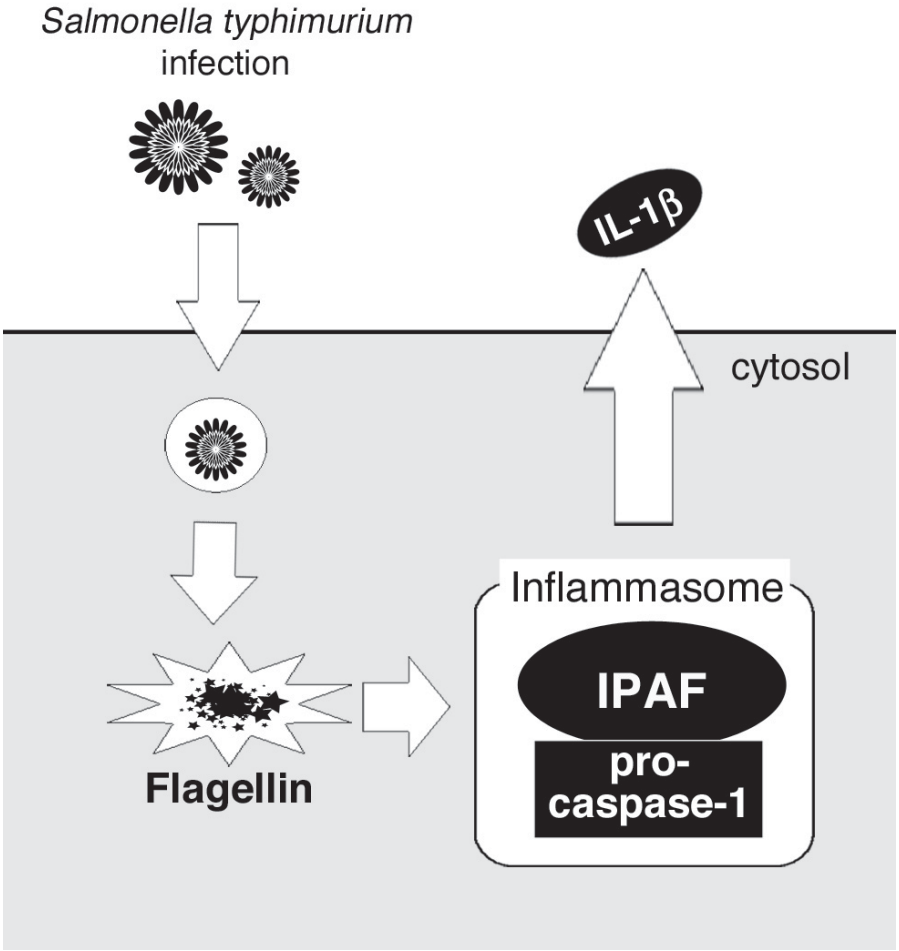

Figure 4. IPAF inflammasome. IPAF and procaspase-1 form an inflammasome without ASC in response to flagellin, a virulence protein produced by certain bacteria such as *Salmonella typhimurium*.

Caspase-1 mediates the SREBP pathway

There are many microorganisms and bacteria that produce pore-forming toxins to invade target cells. The infected cells respond to the toxin in various ways, such as by undergoing apoptosis or necrosis or by surviving, depending on the toxin, the target cells, and the size of the pores (*37*,*38*). These cellular mechanisms are still unclear, but one survival mechanism of such infected cells was recently reported (*39*). One of the pore-forming toxins, aerolysin, which is secreted by *Aeromonas hydrophila*, binds to glycosylphosphatid ylinositol (GPI)-anchored proteins at the surface of target cells. Aerolysin then exposes its hydrophobic domain to the cell membrane like a circular ring. These pores are not permeable for proteins but for ions, and lead to K^+ efflux (*40*). Some unique cellular reactions to *A. hydrophila* infection are reported, such as the release of calcium from the endoplasmic reticulum (ER), vacuolation of the ER, and the production of proinflammatory molecules including TNFα, IL-1β, IL-6, and prostaglandin E2 (*41*,*42*). Among these various responses, the K^+ efflux acts to trigger the assembly of the inflammasome to activate caspase-1. Activated caspase-1 stimulates the sterol regulatory element binding protein (SREBP) pathway in addition to IL-1β, -18, and -33 secretion (*39*) (Figure 5).

SREBPs promote lipid metabolism, which functions predominantly in cholesterol and fatty acid biosynthesis. Although SREBPs initially reside in the ER, their activation requires proteolitic cleavage in the Golgi, which means that SREBPs require transport from the ER to the Golgi (*43*), and cleaved SREBPs translocate to the nucleus to activate genes involved in lipid metabolism. SREBPs are regulated principally by cellular cholesterol levels, but they are also activated by phagocytosis, the depletion of ER Ca^{2+} stores, or the exposure of cells to hypotonic media (*44*,*45*). In the case of *A. hydrophila* infection, K^+ efflux-induced caspase-1 activation is required for the transportation and activation of SREBPs, and this signaling is independent of Ca^{2+} entry (*39*). Furthermore, this ionic perturbation is sensed not only by the cryopyrin/NALP3, but also by the IPAF inflammasome. Whether IPAF and cryopyrin/NALP3 sense the toxin-induced K^+ decrease directly *via* their LRR domains is not yet known. The caspase-1-dependent SREBP activation pathway promotes cell survival in response to pore

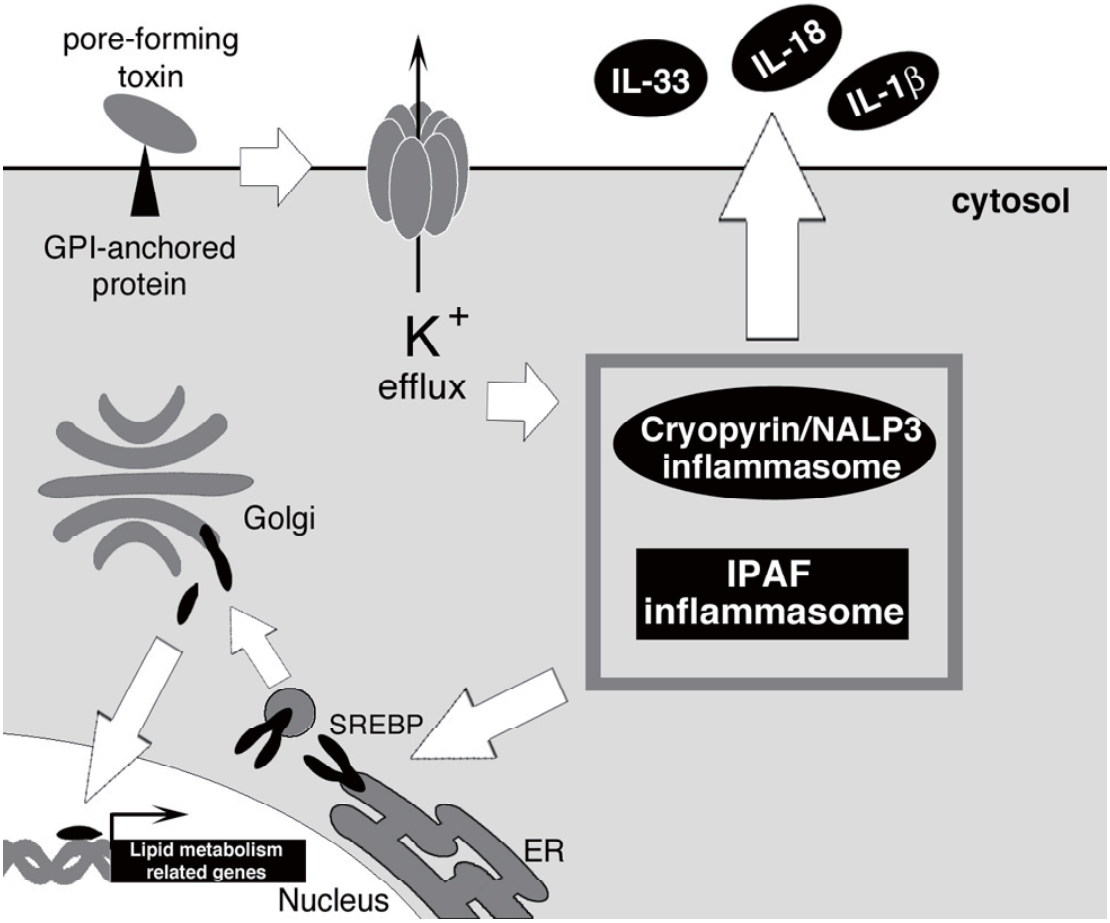

Figure 5. Host response to pore-forming toxins. Pore-forming toxin binds to glycosylphosphatidylinositol (GPI)-anchored protein and produces a pore in the host membrane. Both cryopyrin/NALP3 and the IPAF inflammasome recognize K^+ efflux through the pore and activate caspase-1, which induces cytokine secretion and lipid synthesis *via* the sterol regulatory element binding protein (SREBP) pathway.

formation, perhaps by facilitating membrane repair (*39*). On the other hand, caspase-2 is reported to be a transcriptional target of SREBPs and has been suggested to participate in lipid homeostasis under both physiological and pathogenic conditions (*46*).

Inflammasomes in nonprofessional immune cells

Inflammasomes are present even in the keratinocytes of human skin. Inflammasome proteins including NALP1, cryopyrin/NALP3, and IPAF are constitutively expressed in human skin. Likewise, although proIL-1α, -1β, and -18 exist constitutively, their secretion from keratinocytes is not detected under normal conditions. However, UVB irradiation induces caspase-1 activation and the secretion of IL-1β in human primary keratinocytes (*47*). The UVB-irradiated skin of caspase-1 knockout mice shows a defect in neutrophil infiltration, indicating that caspase-1 is indispensable for UVB-induced skin inflammation. Caspase-1 activation is also important in the response of human skin to irradiation, and similar to the case of pathogen infection, caspase-1 is activated by an intracellular response to UVB irradiation. An inflammasome containing cryopyrin/NALP3, ASC, and caspase-1 is necessary, but IPAF is dispensable for the IL-1β secretion by irradiated human keratinocytes. Since the binding of caspase-1 to ASC is detected only outside the cells, the activation of caspase-1 and the secretion may be coupled, or the secretion may occur just after activation (Figure 6). In Chinese hamster ovary (CHO) or HeLa cells, pore-forming toxin induced K^+ efflux triggers assembly of the inflammasome without requiring an increase in Ca^{2+}, and LPS stimulated macrophages require both Ca^{2+} increase and K^+ efflux (*39*,*48*). On the other hand, the triggering of inflammasome activation in irradiated keratinocytes is dependent only on the increase in cytoplasmic Ca^{2+} through its release from intracellular stores (*49*).

In addition, the estrogen-responsive B box protein (EBBP/TRIM16) pathway is reported to be involved in the IL-1β secretion in keratinocytes. Under UV irradiated condition, endogenous EBBP colocalizes with IL-1β at the cell membrane in the perinuclear region of keratinocytes and macrophages. EBBP does not interact with ASC but binds to procaspase-1, NALP1, and proIL-1β *via* its ret finger protein (RFP) domain. The coexpression of EBBP with proIL-1β, procaspase-1, and NALP1 enhances the IL-1β secretion (*50*), although the detailed mechanisms and functions of EBBP are still unknown. These findings together support the idea that the caspase-1 dependent inflammasome exists even in nonprofessional immune cells.

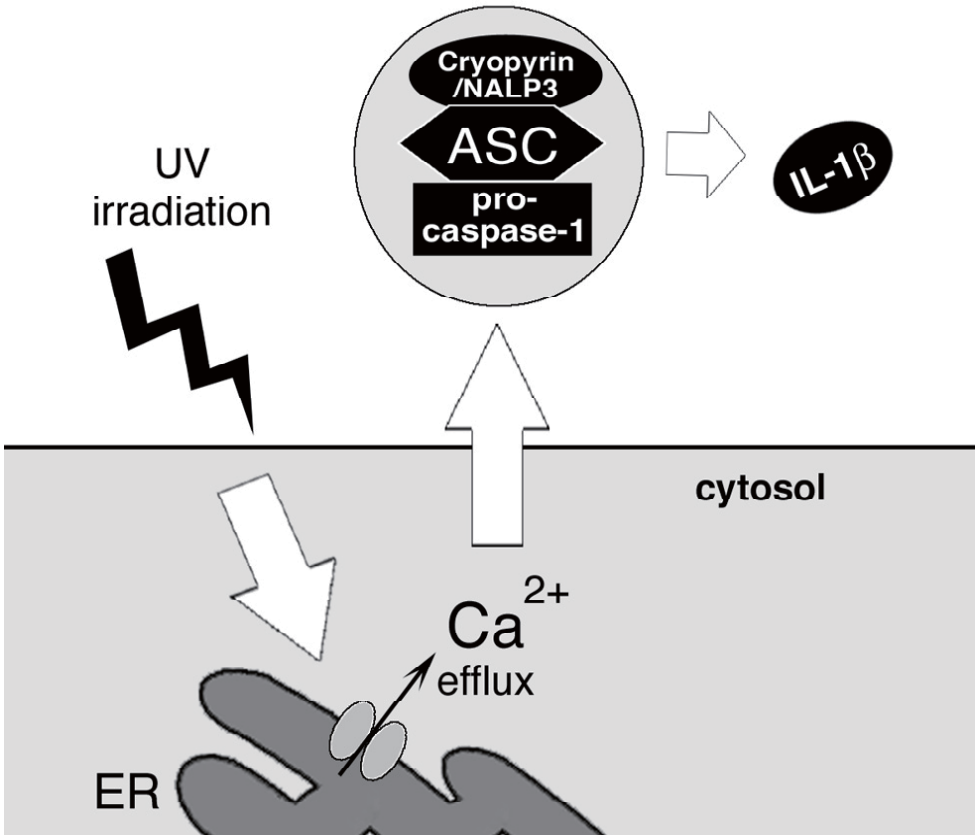

Figure 6. Inflammatory response in keratinocytes. The cryopyrin/NALP3-dependent inflammasome activates caspase-1 to induce cytokine secretion in response to Ca^{2+} efflux from the endoplasmic reticulum (ER), which is caused by UV irradiation.

Endogenous danger signals

Caspases are essential not only in the immune reaction to PAMPs, the invaders, but also, recent findings indicate that caspases mediate a greater range of immune responses, including those to endogenous danger signals. For example, in a course frequently leading to death, sepsis occurs after infection or injury and leads to the organs becoming dysfunctional. Caspases are thought to be involved in the mechanisms causing this disease, since apoptotic lymphocytes accumulate at the organs during sepsis, and the organ damage is reduced by blocking apoptosis with caspase inhibitors (*51-53*). Caspase-1 knockout mice are resistant and show less apoptosis in the splenocyte and macrophages during septic shock induced by bacteria than wild-type mice, although IL-1β knockout or double-knockout mice of IL-1β and IL-18 are not protected (*54*). On the other hand, high mobility group box 1 (HMGB1) is known to accumulate and mediate organ damage in sepsis (*55,56*). HMGB1 release occurs by the exposure of macrophages to necrotic cell debris, or to both apoptotic T cells and apoptotic macrophages. Although apoptotic cells have the ability to release HMGB1 from macrophages, and the inhibition of HMGB1 reduces sepsis, the inhibition of HMGB1 showed no significant decrease in the development of sepsis-induced apoptosis. These results indicate that at least part of the HMGB1 function is downstream of caspases (*57*).

In the case of gout and pseudogout, which are common joint diseases, aberrant activation of the cryopyrin/NALP3 inflammasome induces IL-1β constitutively. The known symptom of gout is the deposition of MSU or CPPD crystals at the joints and periarticular tissues (*58*). These unique pathogenic agents, MSU and CPPD, activate caspase-1 to secrete IL-1β in an ASC- and cryopyrin/NALP3-dependent manner. This notion is supported by the observation that caspase-1- or ASC-deficient mice show reduced neutrophile influx when MSU or CPPD is injected (*14*). Moreover, macrophages from Muckle-Wells syndrome patients spontaneously secrete active IL-1β in a caspase-1-dependent manner, even without stimulation (*59*). Because many of these patients have a mutation in the cryopyrin/NALP3 gene, Muckle-Wells syndrome is thought to be caused by the increased processing and secretion of IL-1β *via* caspase-1 and the mutated cryopyrin/NALP3 (*60*). Therefore, caspases also play important roles in the reaction to various endogenous danger signals.

Invertebrate immune system

To date, *Drosophila melanogaster* has contributed to the study of numerous apoptotic and non-apoptotic functions of caspases. Is the immune system in invertebrates completely different from that in mammals? To overcome PAMPs, flies have several immune mechanisms such as the humoral immune response, melanization, and the cellular immune response. Among these, the most well-characterized is probably the humoral immune response, which is composed of two major pathways, immune deficiency (IMD) and Toll. These pathways independently regulate distinct classes of NF-κB proteins. The IMD pathway is activated by Gram-negative bacteria and induces Relish activation. The Toll pathway is triggered by fungi or Gram-positive bacteria and leads to Dorsal and Dif activation (*61*). Genetic screening experiments designed to dissect the IMD pathway identified *dredd*, a *Drosophila* ortholog of caspase-8. A loss-of-function mutant of *dredd* is viable and fertile, but highly susceptible to Gram-negative bacteria, and it is defective in the production of antibacterial peptides, such as diptericin and attacin. In addition, *dredd* has been shown physically interact with the NF-κB homolog, Relish, in *Drosophila* mbn-2 (hemocyte-like) cells. *Dredd* mutant flies fail to process Relish, which contains Rel-homology domains at the N-terminus and lκB-like domains at the C-terminus. These observations suggest that *dredd* is involved in NF-κB activation (*3,62*).

Another report indicates that *eiger*, the only fly homolog of TNF (*63,64*), is involved in the fly's immune machinery. Microarray analysis showed that *eiger* is up-regulated after LPS exposure to mbn-2 cells (*65*). *Eiger* acts to protect the cells against extracellular pathogens independently from Toll and IMD signals. *Eiger* mutant flies show decreased phagocytosis, which functions to exclude pathogens, indicating that *eiger* has a role in limiting the growth of pathogens *via* phagocytosis. These mutants are also unable to suppress pathogens that have phagocyte-defeating activity (*67*). However, induced *eiger* can be harmful for flies if there is no proper target for it (*66,67*). Mammals as well as flies die as a result of too much TNF secretion (*68*). Thus, the immune responses of the fly have similarities to those of mammals in terms of cytokine secretion mechanisms and autoinflammatory diseases.

In the nematode *C. elegans*, caspase is involved in the immune responses, and the *ced-3* mutant is sensitive to some pathogens, including *S. typhimurium* infection (*69*). *Drosophila* has just one NOD-like protein, *dark/dapaf-1/HAC-1*, which forms the apoptosome. In addition to the domains required to trigger caspase activation, *dark* also contains a WD40 region, which may be functionally equivalent to the LRR that recognizes some pathogens or danger signals because cytochrome c released from mitochondria can be considered as an intracellular alert signal in stressed cells. Therefore, we expect *dark* to sense dangers and trigger caspase activation in response to stresses even in *Drosophila*, perhaps by forming an inflammasome-like

complex.

Although each organism seems to have diverse machinery for eliciting stress responses, the major mediators might be represented by caspase. Inflammatory caspases function to get rid of exogenous and endogenous dangers, and apoptotic caspases function to remove the organism's own cells that are dangerous. Since these systems for eliminating harmful cells are indispensable for all species to develop normally and survive in this stressful world, the substantial roles of caspase in stress responses, not only in the apoptosome but also in the inflammasome, may have been conserved evolutionally in both vertebrates and invertebrates.

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