NLRs at the intersection of cell death and immunity

Jenny P.-Y. Ting[*‡§], *Stephen B. Willingham*[*§] *and Daniel T. Bergstralh*[*||]

Abstract | Inflammation is a crucial element of the host response to cellular insult. Pathogen-induced inflammation includes a molecular pathway which proceeds through activation of the protease caspase-1 to the release of the inflammatory cytokines interleukin-1 (IL-1) and IL-18. Importantly, pathogens may also induce forms of cell death that have inherently pro-inflammatory features. Here, we review recent evidence demonstrating that NLR (nucleotide-binding domain, leucine-rich repeat containing) family proteins serve as a common component of both caspase-1-activated apoptotic pathways and caspase-independent necrotic pathways. Parallels are drawn between NLR protein function and the activity of structurally similar proteins involved in cell death: the apoptotic mediator APAF1 (apoptotic-protease-activating factor 1) and the plant disease resistance NBS-LRR (nucleotide-binding site leucine-rich repeats) proteins.

*Lineberger Comprehensive Cancer Center, ‡Department of Microbiology and Immunology, §Curriculum in Genetics, ||Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295, USA.
Correspondence to J.P.-Y.T.
e-mail:
jenny_ting@med.unc.edu
doi:10.1038/nri2296
Published online
25 March 2008

The NLR (nucleotide-binding domain, leucine-rich repeat containing) family of proteins (previously known as CATERPILLERs, NODs or NACHT–LRRs; see the HUGO Gene Nomenclature webpage on the NLR family) is rapidly emerging as being crucial to the regulation of immunity. Members of this family are distinguished by their domain architecture (FIG. 1), which consists of a variable N-terminal effector domain, a central nucleotide-binding domain (NBD) and C-terminal leucine-rich repeats (LRRs). More than twenty such proteins have been identified in humans, and marine organisms such as the sea urchin *Strongylocentrotus purpuratus* have an even greater complement, with more than 200 members predicted from recent genomic analysis[1]. To date, work on NLR proteins in animals has focused largely on their ability to mediate the initial immune response to pathogenic insult, particularly with regard to inflammation. However, a number of recently published papers show that NLR proteins also link innate immunity and cell-death signalling. Intriguingly, this signalling is not confined to apoptosis, but instead extends to two newly recognized cell-death programmes: pyroptosis and pyronecrosis.

Clues to the cell-death-related functions of the NLR proteins can be drawn from their structural relationship with molecules that are known effectors of cell death, such as the apoptotic mediator APAF1 (apoptotic-protease activating factor 1) and the NBS-LRR (nucleotide-binding site, leucine-rich repeats; also known as NB-LRR) plant disease resistance proteins[2]. APAF1 has an important role as a trigger protein for mitochondrial-dependent apoptosis. Similar to the NLRs, APAF1 has an N-terminal effector domain and a central NBD. APAF1 also contains C-terminal repeats, although these differ from those of the NLRs (FIG. 1). Members of the NLR family have an even closer structural resemblance to the NBS-LRR subset of plant resistance proteins, which are characterized by a variable N-terminal domain, a central NBD and C-terminal LRRs (FIG. 1). NBS-LRR proteins function in part by helping to induce the hypersensitive response, a form of programmed cell death with necrotic features[3]. In this article, we discuss the emerging theme that mammalian NLRs, similar to APAF1 and the NBS–LRR proteins, also act in the regulation of cell death.

Several routes to cell death

Although cell death is known to have an important role in the immune system, the majority of studies have focused on the role of apoptosis in cell death. Emerging evidence suggests that additional cell-death pathways are crucial for the triggering of inflammation and immunity. To begin to understand the contribution of cell death to immunity, it is useful to highlight a number of key differences between these types of cell death (TABLE 1). Cell-death nomenclature is outlined in BOX 1.

Apoptosis is a programmed form of cell death, in that it is a deliberate activity on the part of the cell and requires specific molecular mediators, most importantly the apoptotic caspases. Two caspase-dependent pathways,

the intrinsic and extrinsic pathways, regulate the final stages of apoptosis. The intrinsic pathway relies on the release of cytochrome *c* from mitochondria to induce formation of the apoptosome — a large protein complex comprised of cytochrome *c*, pro-caspase-9, APAF1 and deoxyribonucleic ATP[4]. Several models have been proposed concerning the mechanisms by which apoptosome formation results in caspase-9 activation[5]. The currently favoured model suggests that proximity-induced homodimerization of pro-caspase-9 within the apoptosome holoenzyme creates an active site that allows caspase-9 to become an initiator caspase that in turn cleaves and activates downstream effector caspases (caspase-3, caspase-6 and caspase-7)[6]. The extrinsic pathway of apoptosis begins on the cell surface, where cell-death receptors — proteins that contain an intracellular death domain (DD) — are activated by ligand binding. Receptor-triggered intracellular events result in the proteolytic activation of initiator caspase-8 and caspase-10, leading to cleavage of effector caspases (caspase-3, caspase-6 and caspase-7)[6]. Substrates of the effector caspases include poly-ADP-ribose polymerase (PARP), DNA-PK and other regulatory and structural proteins that maintain cellular and genomic integrity[7]. Cumulatively, the cleavage of these substrates leads to the death and breakdown of the cell.

In contrast to apoptosis, necrosis has been considered by some to be a passive, and therefore unprogrammed, form of cell death. In general, apoptosis relies on the protease activity of caspases, whereas necrosis is caspase independent. Apoptosis is an energy expensive process, whereas necrosis has been described as being the result of a bioenergetic failure, meaning that the cell lacks sufficient energy resources to maintain its metabolism. This condition may be triggered by the loss of ion-pump activity or overconsumption of ATP[8]. At the nuclear level, necrosis is distinguished from apoptosis by the persistence of the DNA content, which remains uncondensed. Perhaps the most striking difference between these forms of cell death is at the plasma membrane. Apoptosis is a slow process marked by membrane blebbing and the packaging of cellular material for recycling, but necrosis is characterized by rapid loss of plasma membrane integrity with the resultant release of cellular contents into the extracellular medium[9].

This last feature is central to the importance of necrosis in an immune and inflammatory context. Predictably, the release of cellular components has a drastic effect on the local environment. Some of these components, including uric acid, adenine phosphate, purine metabolites and heat-shock proteins, become pro-inflammatory effectors[8]. Necrotic macrophages can release pro-inflammatory cytokines such as tumour-necrosis factor (TNF) and interleukin-1 (IL-1)[10,11]. In addition, significant attention has been paid to another protein released from necrotic cells, the nuclear DNA-binding protein HMGB1 (high-mobility group box 1 protein). Once released, HMGB1 becomes an agonist for RAGE (receptor for advanced glycation end-products) and for the Toll-like receptors (TLRs) TLR2 and TLR4, which are expressed by monocytes and some other cell types[12,13].

Membrane blebbing
Breakdown of the cytoskeleton during apoptosis results in 'blebbing' or bubbling of the plasma membrane. These blebs eventually separate from the cell to become apoptotic bodies, which are small membrane-enclosed packages of cytoplasm. Apoptotic bodies are ultimately engulfed by phagocytic cells and their contents are recycled.

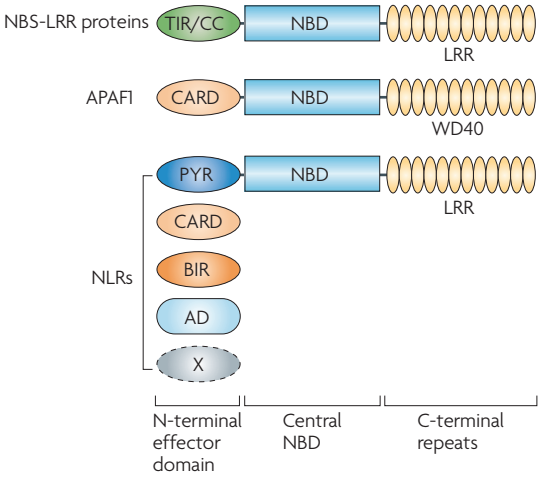

Figure 1 | **NLR proteins are structurally similar to the pro-apoptotic protein APAF1 and the plant cell-death mediating NBS-LRR proteins.** NLR (nucleotide binding, leucine-rich repeat containing) proteins are defined by three characteristics: an N-terminal effector domain, a central NBD (nucleotide-binding domain) and C-terminal repeats. When defined, NLR effector domains consist of either a pyrin domain (PYR), caspase recruit domain (CARD), baculovirus inhibitor of apoptosis repeat (BIR) domains or a transactivation domain (AD). One NLR protein has an undefined or uncharacterized effector domain (X). APAF1 (apoptotic-protease activating factor 1) also has an N-terminal effector CARD and a central NBD. However, its C-terminal repeats differ from those of the NLRs. NBS-LRR (nucleotide binding site, leucine-rich repeats) proteins are characterized by a Toll/interleukin-1-receptor (TIR) or coiled-coil (CC) N-terminal effector domains, a central NBD and C-terminal LRRs.

Activation of these receptors results in the exacerbation of inflammation in the microenvironment through the induction of additional pro-inflammatory cytokines[14]. Recent work has identified two more forms of cell death, pyroptosis and pyronecrosis, which appear to exploit the pro-inflammatory features of necrosis within the context of immunity. However, the extent to which each resembles apoptosis and necrosis is different.

Pyroptosis is a cell-death pathway activated by microbial pathogens, including *Salmonella* spp. and *Listeria* spp.[15,16]. Pyroptosis is similar to apoptosis in that DNA damage occurs and the process is caspase dependent[17]. However pyroptosis does not rely on the classical pro-apoptotic initiator and effector caspases (caspases-3, caspase-8 and caspase-9), but rather on caspase-1. In addition to its apoptotic qualities, pyroptosis exhibits some of the same features as necrosis. Similar to necrosis, pyroptosis is characterized by plasma-membrane breakdown. Moreover, mitochondrial membrane integrity is maintained during pyroptosis[16,17]. Ongoing studies are aimed at identifying additional molecular mediators of pyroptosis. Very recent work has described the pyroptosome, a large complex comprised of ASC (apoptosis-associated speck-like protein containing a caspase-recruitment domain (CARD); also known as PYCARD) dimers

that assembles as a feature of this process[18]. ASC is a common binding partner for the NLR family proteins. Although it has been demonstrated that a large ASC complex can assemble *in vitro* in the absence of NLR proteins, it has also been suggested that NLR proteins are required for pyroptosome formation *in vivo*[18]. From an inflammation standpoint, the two outstanding features of pyroptosis are the activation of caspase-1 and the breakdown of the plasma membrane.

Two very recent studies have identified another NLR-protein-dependent pathway of pro-inflammatory cell death, termed pyronecrosis, which has primarily necrotic features[19,20]. This form of cell death is found in genetic auto-inflammatory diseases involving mutations in the *NLRP3* (also known as *CIAS1, NALP3, PYPAF1* or cryopyrin) gene and is also associated with microbial pathogens such as *Shigella flexneri*[19,20]. Unlike pyroptosis, pyronecrosis is caspase independent; neither the activating cleavage of effector caspase-3 nor its substrate PARP occur during pyronecrosis, and cell death proceeds in the presence of caspase-1-specific inhibitors and pan-caspase inhibitors[19,20]. However, cell death is abrogated in the presence of an inhibitor of the lysosomal protease cathepsin B, implicating lysosome activity in the pathway[19,20]. Additional hallmarks of apoptosis are not observed. Pyronecrotic cells demonstrate neither DNA fragmentation nor the loss of mitochondrial membrane potential[20]. As determined by electron microscopy, the morphological changes characteristic of pyronecrosis are consistent with necrosis and include membrane degradation and uncondensed chromatin[19,20]. Similar to classical necrosis, pyronecrosis is accompanied by release of the pro-inflammatory cytokine HMGB1 (REF. 20). Recent work suggests an intriguing connection between cell-death pathways and the NLR proteins, which are early mediators of inflammation in response to cellular insult.

NLR proteins, apoptosis and pyroptosis

Owing to their structural similarities, the pathway involving the apoptotic mediator APAF1 has been used as a model to understand NLR-protein function. Whereas APAF1 activates a caspase-dependent programme of cell death, several NLR proteins act to promote a caspase-dependent programme of inflammation. Research on the latter has mainly focused on formation of protein complexes called inflammasomes[21,22]. Biochemical studies have identified a number of inflammasomes, which promote inflammation by activating caspase-1, resulting in the release of the pyrogenic cytokines IL-1β and IL-18 from cells stimulated with different factors[23,24]. Although differing slightly in their makeup, each inflammasome includes the IL-1β-converting enzyme pro-caspase-1, as well as one of four NLR proteins: NLRP3, NLRP2, NLRC4 (also known as IPAF or CLAN) or NLRP1 (also known as NALP1) (FIG. 2). Given that over 20 mammalian NLR genes have been recognized, it is likely that more NLR inflammasomes exist. A potential fifth inflammasome containing the related molecule pyrin has already been identified[25].

The inflammasome complexes appear to differ from each other in respect to their activating stimuli. The NLRC4 inflammasome is activated in response to *Salmonella typhimurium* and *Legionella pneumophila*[26–28]. NLRP1 is required for caspase-1 activation in response

Table 1 | **A comparison of cell death pathways**

Cell-death pathway	Physiological consequence	Morphological features	Biochemical features	Activating factors	Pathogenic stimuli	Refs
Apoptosis	• Non-inflammatory cell death • Effects limited to dying cell	• Blebbing of plasma membrane • Recycling of cellular contents • Chromatin condensation	• Caspase dependent • PARP cleavage • Mitochondrial permeabilization • DNA laddering • HMGB1 maintained in nucleus	• Caspase-8 and caspase-10 (extrinsic) • APAF1 (intrinsic) • Cytochrome *c* • Caspase-9 (intrinsic) • Caspase-3, caspase-6 and caspase-7 (effectors)	ND	7,6,37
Necrosis	• Elicits substantial inflammation • Affects local environment	• Loss of plasma membrane integrity • Release of cellular contents • No chromatin condensation	• Caspase independent • No PARP cleavage • Mitochondrial swelling • HMGB1 release from nucleus	ND	ND	8,9
Pyroptosis	• Elicits substantial inflammation • Affects local environment	• Loss of plasma membrane integrity • Mitochondrial membrane maintained • No chromatin condensation	• Caspase-1 dependent • ASC dimerization into pyroptosomes	• NLRC4 • NAIP5 • NLRP1 • ASC • Caspase-1	• *Salmonella* spp. • *Shigella* spp. • *Listeria* spp. • Anthrax lethal toxin	15,16,18
Pyronecrosis	• Elicits substantial inflammation • Affects local environment	• Loss of plasma membrane integrity • Mitochondrial membrane maintained • No chromatin condensation	• Caspase independent • Requires ASC	• NLRP3 • ASC • Cathepsin B	• *Shigella* spp. • Nigericin (?) • Maitotoxin (?)	19,20, 32,34,35

APAF1, apoptotic-protease-activating factor 1; ASC, apoptosis-associated speck-like protein containing a CARD; HMGB1, high-mobility group box 1 protein; NAIP5, neuronal apoptosis inhibitory protein 5; ND, not determined; NLR, nucleotide-binding domain, leucine-rich-repeat-containing family; PARP, poly-ADP-ribose polymerase.

Box 1 | **Cell death terminology**

At one time, cell death terminology could essentially be distilled down to two terms: apoptosis and necrosis. Apoptosis was synonymous with programmed cell death, and necrosis was a catchall term for accidental death. However, recent studies have identified molecular mediators of morphologically necrotic cell death, including PARP (poly-ADP-ribose polymerase), and the kinase RIP (receptor interacting protein), demonstrating that adapted pathways may have been disguised by their necrotic appearance[9]. Earlier use of the term 'necrosis' for all non-apoptotic cell death has largely abated as a host of non-apoptotic programmes have emerged. NLR (nucleotide-binding domain, leucine-rich repeat containing) family proteins, which are involved in the regulation of innate immunity, have been implicated in several of these programmes: autophagy, pyroptosis and pyronecrosis.

Autophagy is a process whereby cellular components are collected and degraded in specific compartments called autophagosomes. Although most commonly associated with cell death, autophagy might also have other consequences. Recent work has demonstrated that the NLR protein NLRC4 can participate in autophagy in response to infection with *Shigella flexneri*, but this process does not result in cell death[43]. Instead, it appears to protect cells from *S. flexneri*-induced cell death[43].

'Pyroptosis' and 'pyronecrosis' are two more recent additions to the terminology of cell death. A defining feature of pyroptosis, a programme first shown to occur in macrophages infected with *Salmonella typhimurium*, is its dependence on caspase activity[15]. Importantly, pyroptosis does not rely on a traditional apoptotic effector (such as caspase-3), but rather on caspase-1, which has long been known to be associated with inflammation through cytokine maturation[15]. Given the importance of NLR inflammasomes to caspase-1 activation in response to pathogens, NLR proteins are likely to emerge as key effectors of pyroptosis. Intriguingly, one of these proteins, NLRP3, has been shown to mediate an alternative form of cell death in response to pathogens. Observed in macrophages infected with *S. flexneri*, pyronecrosis is a caspase-independent but cathepsin-dependent cell-death pathway with the morphological characteristics of necrosis[20].

The importance of NLR proteins to other cell-death pathways remains to be determined. Although the list of terms used to describe such pathways has expanded over the past decade, future work should continue to refine cell-death terminology and improve classification. Although pyroptosis and pyronecrosis are stimulated by similar factors and have similar outcomes, the reliance of pyroptosis but not pyronecrosis on caspase-1 activity represents no small difference between them. Current work is focused on further sharpening the distinction between these two forms of death. One more term, which may represent a difference between these pathways, merits discussion. 'Oncosis' has often been used interchangeably with necrosis, but is more specifically used to describe non-apoptotic cell death resulting from the swelling and bursting of the cell[65]. Caspase-1-dependent cellular swelling has been observed in pyroptotic cells, but has yet to be reported as a feature of pyronecrosis[66].

to anthrax lethal toxin[29]. To date, the NLRP3 inflammasome is associated with the widest range of stimuli, including lipopolysaccharide (LPS) in the presence of ATP, uric acid crystals, polyI:C (polyinosinic–polycytidylic acid), bacterial and viral RNA and both Gram-positive and Gram-negative bacteria[20,30–35]. Although the pathogen recognition steps that lead to inflammasome activation continue to be elucidated, recent work has shown that cytosolic bacterial molecules can induce NLRP3-mediated caspase-1 activity independently of TLR signalling[36].

In some respects the inflammasomes resemble the apoptosome, which includes APAF1 (FIG. 2). APAF1 is composed of an N-terminal CARD, a central NBD and C-terminal WD-40 repeats, and is thereby similar in domain structure to the NLR proteins. It is thought to be held inactive by intramolecular contact between its C-terminal WD40 repeats and N-terminal regions until cytochrome *c* and dATP relieve this inactive conformation and promote assembly of the apoptosome, leading to activation of pro-caspase-9 (REF. 37). The inflammasome NLR proteins appear to act in an analogous manner, in that intramolecular interaction mediated by the C-terminal LRRs is proposed to hold these proteins in an inactive formation until stimulation promotes inflammasome assembly and activating cleavage of pro-caspase-1.

Two recent papers describe biochemical stages in inflammasome activation[24,38]. Using purified components of the NLRP1 inflammasome, Faustin *et al.* demonstrated that assembly of this inflammasome complex required the microbial product muramyl dipeptide (MDP) as well as the presence of nucleotides[38]. Surprisingly, and in contrast to the apoptosome, the NLRP1 inflammasome exhibited little nucleotide specificity. However, this does not appear to be the case for the NLRP3 inflammasome. Duncan *et al.* showed that NLRP3 binds specifically to ATP or dATP and acts as an ATPase. NLRP3-catalysed nucleotide hydrolysis was shown to be vital for protein function. It is required for NLRP3 self-association, interaction with the inflammasome adaptor protein ASC, caspase-1 activation and IL-1 release[24].

Although a comparison between inflammasome and apoptosome activation may help to clarify steps in the induction of inflammation, any similarities found may also extend to function. Much attention has been paid to the role of the NLR protein NAIP5 (neuronal apoptosis inhibitory protein 5) in determining susceptibility to *L. pneumophila*[39]. In addition to inducing the release of IL-1β through the NLRC4 inflammasome, cytosolic *L. pneumophila* flagellin also activates a caspase-1-dependent form of cell death in macrophages that requires NAIP5 (REFS 40,41). One characteristic of this cell-death pathway is nuclear condensation, which is typical of apoptosis[40]. Moreover, membrane blebbing, another feature of apoptosis, was observed in NAIP5-expressing HEK293 cells following infection with *L. pneumophila*[42]. However, unlike classical apoptosis, *L. pneumophila* flagellin-induced macrophage cell death has been reported

Nuclear condensation
A hallmark of apoptosis is pyknosis, or the condensation of chromatin into compact spots along the nuclear membrane. During pyknosis, the nucleus itself may also shrink.

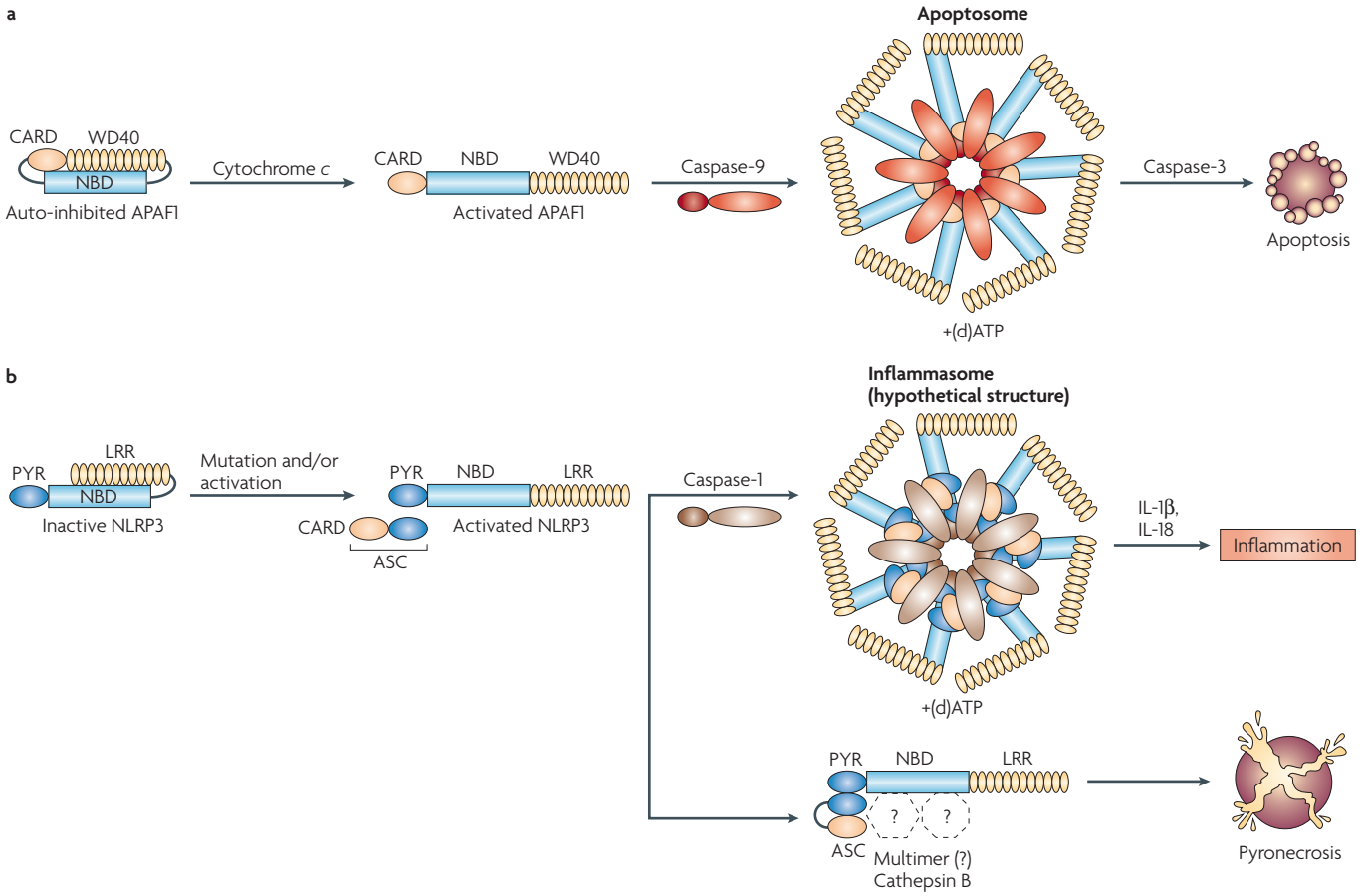

Figure 2 | **Comparison of APAF1 and NLRP3 cell-death pathways. a** | Release of cytochrome *c* from mitochondria initiates apoptosome-dependent apoptosis. Cytochrome *c* induces a conformational change in APAF1 to relieve the intramolecular interactions holding the molecule in an auto-inhibited state. Once activated, APAF1 (apoptotic-protease activating factor 1) and cytochrome *c* undergo deoxyribonucleic-ATP-dependent oligomerization into a heptameric wheel-like structure termed the apoptosome. Pro-caspase-9 molecules aggregate through homotypic interactions between their CARDs (caspase recruitment domains) and those of APAF1. Subsequent homodimerization of pro-caspase-9 generates active caspase-9 molecules which cleave and activate the effector caspases caspase-3 and caspase-7 to induce apoptotic cell death. **b** | Similarly, NLRP3 activation or disease-associated NLRP3 mutations might weaken putative inhibitory intramolecular interactions between the NLRP3 nucleotide binding domain (NBD) and its C-terminal leucine rich repeats (LRRs). Using the adaptors ASC (apoptosis-associated speck-like protein containing a CARD) and CARDINAL/TUCAN, activated NLRP3 aggregates caspase-1 molecules through the formation of a macromolecular inflammasome complex. Complex formation potentiates subsequent caspase-1 cleavage and activation. Based on studies of the NLRP1 inflammasome, the complex is probably comprised of 5–7 subunits, each with the ability to recruit pro-caspase-1 molecules[39]. Interleukin-1β (IL-1β) is activated by caspase-1-mediated cleavage of the inactive pro-IL-1β precursor molecule. However, formation of the inflammasome is not the only function of NLRP3. Activated NLRP3 also initiates pyronecrosis, a molecular pathway of necrotic cell death that is dependent on ASC and proceeds through cathepsin B. This cell-death pathway does not rely on caspase-1 or IL-1β, and therefore is independent of inflammasome function. The status of protein complex or oligomers required for this process has not been studied. The term '(d)ATP' is used to reflect that both dATP and ATP may be involved in regulating complex formation and activation.

to be independent of caspase-3 activity[40]. Therefore, the observed apoptotic features may instead be related to pyroptosis. Notably, caspase-1- and NLRC4-dependent cell death has been observed at time-points of less than 3 hours in *S. flexneri*-infected cells[43]. NLRC4 is also required for cell death in macrophages infected with *S. typhimurium* macrophages, which is mediated by bacterial flagellin[26,27]. Although features of the mechanism remain to be determined, these results demonstrate that an NLR protein is required to mediate an apoptosis-like cell death induced by a bacterial component.

The above mentioned work suggests a pro-apoptotic function for NLR proteins. Intriguingly, evidence supporting a functional relationship between NLRs and anti-apoptotic signalling factors has been provided in another system. In a cell-free system, the NLRP1 inflammasome is activated by the bacterial product MDP, resulting in the maturation of IL-1β. This process is regulated by two members of the anti-apoptotic BCL-2 (B-cell lymphoma 2) family of mitochondrial membrane proteins. Both BCL-2 and BCL-X_L bind to NLRP1 directly to suppress its activity[44]. These data illustrate a surprising cross-talk

between inflammatory and anti-apoptotic signalling, although the influence of this interaction on cell survival or death has not yet been determined. Although the regulation of inflammation is an unexpected role for BCL-2 family members, the interface between mitochondrial membrane factors and innate immunity is not unprecedented. Recent work has established the mitochondrial outer membrane as a critical staging area for antiviral signalling through MAVS (mitochondrial antiviral signalling protein; also known as IPS-1, VISA and CARDIF), the RIG-I (retinoic-acid-inducible gene I)-like RNA helicases and the NLR protein NLRX1 (REFS 45–47).

Additional work points to NLRP1 as a mediator for toxin-induced cell death. Boyden and Dietrich dissected mouse genetics to implicate NLRP1 as the primary mediator of mouse macrophage susceptibility to the anthrax lethal toxin[29]. In cells expressing functional NLRP1, anthrax lethal toxin elicited a form of cell death that is caspase-1 dependent (therefore, suggestive of pyroptosis). Without functional NLRP1, macrophages do not undergo this form of cell death and fail to activate caspase-1 in the presence of anthrax lethal toxin. These findings suggest that NLRP1 mediates macrophage cell death as a deliberate response to anthrax lethal toxin, and raise the interesting possibility that anti-apoptotic signalling factors may regulate NLRP1-induced death as well as NLRP1 inflammasome activity.

NLR proteins, necrosis and pyronecrosis

The induction of necrotic cell death as a crucial component of immunity is well established across phylogenic kingdoms. The plant NBS-LRR disease resistance proteins act in the defence against pathogens by helping to mediate the hypersensitive response, a form of rapid programmed cell death, and it has recently been shown that the NLR family protein NLRP3 mediates a similar pathway in monocytes[19,20]. *NLRP3* was first identified through its association with two dominantly inherited periodic fevers: FCAS (familial cold autoinflammatory syndrome) and Muckle-Wells Syndrome[48,49]. It has since been identified as the genetic locus for a third fever syndrome, CINCA/NOMID (chronic infantile neurological cutaneous articular syndrome/neonatal onset multisystem inflammatory disease)[50,51]. These three diseases are now considered to constitute a range of severity for one single condition, cryopyrin-associated periodic syndrome (CAPS), which is characterized by spontaneous inflammation[52]. This suggests that disease-associated variants of *NLRP3* may encode a hyperactive version of NLRP3 that promotes excessive production of IL-1β, a possibility that is consistent with the gain-of-function phenotype typically associated with dominant inheritance. Indeed, following stimulation, monocytes isolated from patients with *NLRP3* mutations demonstrate hyperactivation of IL-1β (REFS 23,53,54).

However, this is not the extent of the phenotypic changes associated with mutant *NLRP3*. Peripheral blood mononuclear cells isolated from patients with *NLRP3* mutations lose viability when exposed to LPS[20,55]. To identify more precisely the cellular consequences of mutant *NLRP3* expression, two groups developed constructs encoding known disease-associated variants of NLRP3 (REFS 19,20). The expression of these variants in the monocytic cell line THP-1 induced excessive IL-1β release, as expected, but also an inflammatory necrosis that we termed pyronecrosis[20]. Intriguingly, pyronecrosis is not dependent on IL-1β signalling or caspase-1, although it requires the presence of the inflammasome component ASC and intact cathepsin B[19,20]. The binding of ATP to NLRP3 is also necessary for this pathway to proceed[24]. Because NLRP3 and ASC appear to act together in a function that is independent of procaspase-1 activation, and hence independent of the inflammasome, we suggest that these two factors comprise an alternative complex to promote pyronecrosis (FIG. 2). Cumulatively, these observations offer insight into the consequence of NLRP3 hyperactivity. The inherent function of the protein and its relationship to pathogen resistance merit further consideration.

Necrosis has long been observed in monocytic cells infected with intracellular bacteria or exposed to toxins. Although in some cases pathogen-induced cell death is almost certainly passive, the active and programmed process of pyronecrosis might be a critical feature of macrophage function. Notable among the necrosis-inducing pathogens is the Gram-negative bacteria *S. flexneri*. At early timepoints and/or low MOI (<10 MOI), macrophage cell death induced by *S. flexneri* has long been recognized as being caspase-1 dependent[43]. It has recently been demonstrated that gene deletion of the NLR family member NLRC4, but not the inflammasome component ASC, also diminishes this process[46]. Cumulatively, these findings are suggestive of pyroptosis. At later timepoints and a higher MOI, *S. flexneri* induces caspase-1-independent cell death in human monocyte-derived macrophages[57-59]. This phenomenon is characterized by morphologically necrotic characteristics closely mirroring those of mutant NLRP3-induced cell death. Through the use of knockout and knockdown techniques, *S. flexneri*-induced necrotic cell death was shown to be dependent on NLRP3 in both mouse macrophages and in the human monocytic cell line THP-1. Similar to mutant NLRP3-induced cell death, this process also depends on ASC and requires the protease cathepsin B[20]. It thereby appears that NLRC4 mediates *S. flexneri*-induced pyroptosis, while NLRP3 mediates pyronecrosis in response to the same bacteria. These findings suggest the intriguing possibility that different NLR proteins mediate different forms of cell death with distinct biologic outcome. Not only does rapid cell death deny the pathogen an environment in which to replicate, the process of necrosis is inherently pro-inflammatory, leading to release of IL-1β and other factors from surrounding cells. Thereby, pyronecrosis is likely to contribute substantially to the disease state in patients with CAPS, who suffer from spontaneous inflammation characterized by IL-1β production.

In its more severe forms, CAPS is also characterized by joint deformities and arthralgias[52]. NLRP3 expression is not limited to monocytic cells but also extends to osteoblasts, and the joint-related symptoms of CAPS are probably due to excessive NLRP3 activity in these cells. As with *S. flexneri* in macrophages, wild-type NLRP3 appears to

Cryopyrin-associated periodic syndrome
(CAPS). An autosomal dominant condition arising from mutations in *NLRP3*. CAPS has only recently been recognized as a single condition, and represents a range of disease severity that was formerly thought to be three distinct diseases: FCAS (familial cold autoinflammatory syndrome), Muckle-Wells syndrome and CINCA/NOMID (chronic infantile neurological cutaneous articular syndrome / neonatal onset multisystem inflammatory disease). Patients suffering from CAPS develop spontaneous inflammation and excessive release of the cytokine IL-1β, and may also suffer from arthralgia, deafness and hives.

Knockdown
This term is used to describe the decrease in mRNA or protein expression that results from RNA interference.

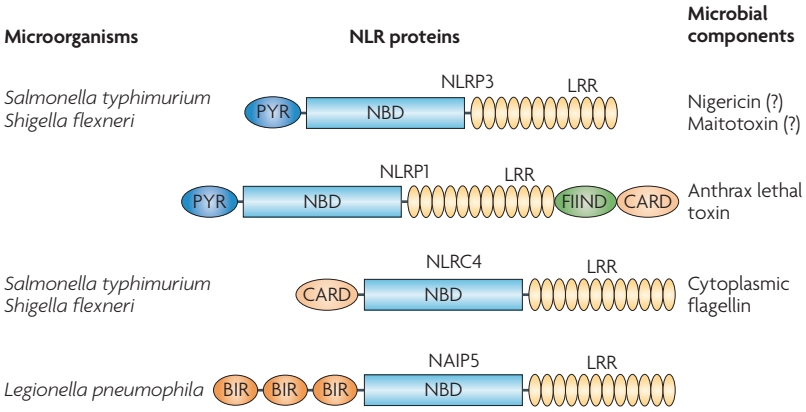

Figure 3 | **Specificity of the NLR proteins involved in the induction of cell death.** NLR (nucleotide-binding domain, leucine-rich repeat containing)-mediated cell death appears to be activated by specific microorganisms (left) or microbial products (right). In response to *Shigella flexneri* infection of macrophages, NLRP3 mediates a caspase-1 independent necrotic-like cell death called pyronecrosis. NLRP3 is also required for *Salmonella typhimurium*-induced cell death in osteoblasts, and may be involved in cell death induced by the toxins nigericin and maitotoxin. A caspase-1-dependent form of cell death called pyroptosis is thought to be mediated by NLRP1 in response to anthrax lethal toxin, NAIP5 (neuronal apoptosis inhibitory protein 5) in response to *Legionella pneumophila*, and NLRC4 in response to *S. typhimurium*. NLRC4 is also involved in *S. flexneri* at early time-points and low MOI. BIR, baculovirus inhibitor of apoptosis repeat; CARD, caspase recruit domain; FIIND, F interacting domain; LRR, leucine-rich repeat; NBD, nucleotide-binding domain; PYR, pyrin domain.

also regulate pathogen-induced cell death in osteoblasts. Though *S. typhimurium* activates the NLRC4 inflammasome in macrophages, mouse primary osteoblasts do not express NLRC4. In these cells, NLRP3 is partly required for maximal *S. typhimurium*-induced cell death[60]. This finding suggests that pyronecrosis might contribute to the joint-related symptoms of CAPS. Moreover, it demonstrates an additional level of complexity to NLR-mediated cell death. In the absence of NLRC4, NLRP3 assumes a role in the response to *S. typhimurium* that it would not otherwise have.

The induction of necrosis by *S. flexneri, S. typhimurium* and other microbial pathogens may be mediated through toxins. Some of these toxins have been examined directly with respect to caspase-1 activation and cell death. Nigericin is a toxin produced by *Streptomyces hygroscopicus*. This molecule functions as a potassium ionophore and is a potent inducer of both IL-1β release and necrosis in monocytes[61,62]. As with pyronecrosis, both functions are dependent on the activity of cathepsin B[61]. Another potent toxin, maitotoxin, is produced by the dinoflagellate *Gambierdiscus toxicus*. Maitotoxin has been demonstrated to induce necrosis in a manner dependent on the calcium-activated cysteine protease calpain and also promotes IL-1β release by mouse macrophages[63,64]. Similar to *S. flexneri*, both nigericin and maitotoxin activate the NLRP3 inflammasome[32,34]. Moreover, these toxins also alter the levels of intracellular potassium[32,34]. Although molecular mediators of nigericin and maitotoxin continue to be identified, the work outlined above indicates the participation of NLRP3 or another NLR family protein in macrophage response to these toxins.

Concluding remarks

Emerging evidence reveals that some NLR proteins contribute to the host-cell response to insult by not only facilitating the maturation of IL-1β, but also by mediating cell death (FIG. 3). Both processes have a strong impact on immunity. IL-1β release is a well-established signal for the onset of inflammation and initiation of the adaptive immune response. The consequences of pathogen-induced cell death in the context of immunity have not been studied as thoroughly, although a plethora of reports have now shed light on this topic. One obvious result is that invading bacteria are denied an environment in which to replicate. However, cell-death programmes which result in a loss of plasma-membrane integrity can also exacerbate inflammation through the discharge of such intracellular inflammatory cytokines and factors such as IL-1, TNF and HMGB1.

Two such modes of cell death, pyroptosis and pyronecrosis, have been recently identified. Alhough there are significant differences between the two, such as their differential dependence on caspase-1, they are evidently both pathways which respond to pathogens by promoting the inherently pro-inflammatory release of cellular contents. NLR proteins have emerged as important regulators of both these pathways. Future studies should aim to find additional mediators of pyroptosis and pyronecrosis. Recent work has described inhibitory interaction of mitochondrial anti-apoptotic proteins with the NLR family protein NLRP1. Accordingly, pro-pyroptotic and pro-pyronecrotic factors may be found within the pool of recognized pro-apoptotic cell-death proteins. Given that the activity of the NLR proteins extends beyond caspase-1 activation to cell death, it will be interesting to determine whether known cell-death regulatory factors contribute to this new role.

1. Rast, J. P., Smith, L. C., Loza-Coll, M., Hibino, T. & Litman, G. W. Genomic insights into the immune system of the sea urchin. *Science* **314**, 952–956 (2006).
2. Belkhadir, Y., Subramaniam, R. & Dangl, J. L. Plant disease resistance protein signaling: NBS-LRR proteins and their partners. *Curr. Opin. Plant. Biol.* **7**, 391–399 (2004).
3. Lam, E., Kato, N. & Lawton, M. Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* **411**, 848–853 (2001).
4. Zou, H., Henzel, W. J., Liu, X., Lutschg, A. & Wang, X. Apaf-1, a human protein homologous to *C. elegans* CED-4, participates in cytochrome *c*-dependent activation of caspase-3. *Cell* **90**, 405–413 (1997).
5. Riedl, S. J. & Salvesen, G. S. The apoptosome: signalling platform of cell death. *Nature Rev. Mol. Cell Biol.* **8**, 405–413 (2007).
6. Zimmermann, K. C., Bonzon, C. & Green, D. R. The machinery of programmed cell death. *Pharmacol. Ther.* **92**, 57–70 (2001).
7. Nicholson, D. W. Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.* **6**, 1028–1042 (1999).
8. Zong, W. X. & Thompson, C. B. Necrotic death as a cell fate. *Genes Dev.* **20**, 1–15 (2006).
9. Edinger, A. L. & Thompson, C. B. Death by design: apoptosis, necrosis and autophagy. *Curr. Opin. Cell Biol.* **16**, 663–669 (2004).
10. Chen, C. J. *et al.* Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nature Med.* **13**, 851–856 (2007).
11. Dinarello, C. A. Biologic basis for interleukin-1 in disease. *Blood* **87**, 2095–2147 (1996).
12. Lotze, M. T. & Tracey, K. J. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nature Rev. Immunol.* **5**, 331–342 (2005).
13. Park, J. S. *et al.* Involvement of Toll-like receptors 2 and 4 in cellular activation by high mobility group box1 protein. *J. Biol. Chem.* **279**, 7370–7377 (2004).
14. Sunden-Cullberg, J., Norrby-Teglund, A. & Treutiger, C. J. The role of high mobility group box-1 protein in severe sepsis. *Curr. Opin. Infect. Dis.* **19**, 231–236 (2006).

15. Brennan, M. A. & Cookson, B. T. *Salmonella* induces macrophage death by caspase-1-dependent necrosis. *Mol. Microbiol.* **38**, 31–40 (2000).
This is the first report to describe pyroptosis.
16. Cervantes, J., Nagata, T., Uchijima, M., Shibata, K. & Koide, Y. Intracytosolic *Listeria monocytogenes* induces cell death through caspase-1 activation in murine macrophages. *Cell. Microbiol.* **10**, 41–52 (2008).
17. Fink, S. L. & Cookson, B. T. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. *Infect. Immun.* **73**, 1907–1916 (2005).
18. Fernandes-Alnemri, T. *et al.* The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell Death Differ.* **14**, 1590–1604 (2007).
The authors of this study demonstrate the assembly of a large ASC-containing complex, termed the pyroptosome, during pyroptosis.
19. Fujisawa, A. *et al.* Disease-associated mutations in CIAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. *Blood* **109**, 2903–2911 (2007).
This article shows that the expression of disease-associated mutants of NLRP3 results in a caspase-1-independent, cathepsin B-dependent form of cell death.
20. Willingham, S. B. *et al.* Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/Cryopyrin/NLRP3 and ASC. *Cell Host Microbe* **2**, 147–159 (2007).
The work presented in this article confirms the findings of Fujisawa *et al.* and demonstrates that the previously observed form of cell death, which is herein named pyronecrosis, occurs in monocytic cells infected with the pathogen *S. flexneri*.
21. Srinivasula, S. M. *et al.* The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. *J. Biol. Chem.* **277**, 21119–21122 (2002).
22. Martinon, F., Burns, K. & Tschopp, J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-β. *Mol. Cell* **10**, 417–426 (2002).
23. Agostini, L. *et al.* NALP3 forms an IL-1β-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* **20**, 319–325 (2004).
24. Duncan, J. A. *et al.* Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling. *Proc. Natl. Acad. Sci. USA* **104**, 8041–8046 (2007).
25. Yu, J. W. *et al.* Cryopyrin and pyrin activate caspase-1, but not NF-κB, via ASC oligomerization. *Cell Death Differ.* **13**, 236–249 (2006).
26. Franchi, L. *et al.* Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1β in salmonella-infected macrophages. *Nature Immunol.* **7**, 576–582 (2006).
27. Mariathasan, S. *et al.* Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* **430**, 213–218 (2004).
28. Miao, E. A. *et al.* Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nature Immunol.* **7**, 569–575 (2006).
29. Boyden, E. D. & Dietrich, W. F. Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nature Genet.* **38**, 240–244 (2006).
The authors of this study show that functional NLRP1 is required for cell death in mouse macrophages exposed to anthrax lethal toxin.
30. Kanneganti, T. D. *et al.* Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J. Biol. Chem.* **281**, 36560–36568 (2006).
31. Kanneganti, T. D. *et al.* Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* **440**, 233–236 (2006).
32. Mariathasan, S. *et al.* Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* **440**, 228–232 (2006).
33. Martinon, F., Petrilli, V., Mayor, A., Tardivel, A. & Tschopp, J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**, 237–241 (2006).
34. Sutterwala, F. S. *et al.* Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1. *Immunity* **24**, 317–327 (2006).
35. Mariathasan, S. & Monack, D. M. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. *Nature Rev. Immunol.* **7**, 31–40 (2007).
This article discusses current research into the specificity and function of the inflammasomes.
36. Kanneganti, T. D. *et al.* Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling. *Immunity* **26**, 433–443 (2007).
37. Schafer, Z. T. & Kornbluth, S. The apoptosome: physiological, developmental, and pathological modes of regulation. *Dev. Cell* **10**, 549–561 (2006).
38. Faustin, B. *et al.* Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation. *Mol. Cell* **25**, 713–724 (2007).
39. Wright, E. K. *et al.* Naip5 affects host susceptibility to the intracellular pathogen *Legionella pneumophila*. *Curr. Biol.* **13**, 27–36 (2003).
40. Molofsky, A. B. *et al.* Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J. Exp. Med.* **203**, 1093–1104 (2006).
41. Ren, T., Zamboni, D. S., Roy, C. R., Dietrich, W. F. & Vance, R. E. Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS Pathog.* **2**, e18 (2006).
42. Zamboni, D. S. *et al.* The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of *Legionella pneumophila* infection. *Nature Immunol.* **7**, 318–325 (2006).
43. Suzuki, T. *et al.* Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. *PLoS Pathog.* **3**, e111 (2007).
44. Bruey, J. M. *et al.* Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1. *Cell* **129**, 45–56 (2007).
This paper demonstrates the surprising finding that NLRP1 inflammasome activity is regulated by the anti-apoptotic factors BCL-2 and BCL-X_L.
45. Seth, R. B., Sun, L., Ea, C. K. & Chen, Z. J. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF 3. *Cell* **122**, 669–682 (2005).
46. Yoneyama, M. & Fujita, T. RIG-I family RNA helicases: Cytoplasmic sensor for antiviral innate immunity. *Cytokine Growth Factor Rev.* **18**, 545–551 (2007).
47. Moore, C. B. *et al.* NLRX1 is a regulator of mitochondrial antiviral immunity. Nature **451**, 573–577 (2008).
48. Hoffman, H. M., Mueller, J. L., Broide, D. H., Wanderer, A. A. & Kolodner, R. D. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nature Genet.* **29**, 301–305 (2001).
49. Aganna, E. *et al.* Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis. *Arthritis Rheum.* **46**, 2445–2452 (2002).
50. Aksentijevich, I. *et al. De novo* CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new member of the expanding family of pyrin-associated autoinflammatory diseases. *Arthritis Rheum.* **46**, 3340–3348 (2002).
51. Feldmann, J. *et al.* Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. *Am. J. Hum. Genet.* **71**, 198–203 (2002).
52. Ting, J. P., Kastner, D. L. & Hoffman, H. M. CATERPILLERs, pyrin and hereditary immunological disorders. *Nature Rev. Immunol.* **6**, 183–195 (2006).
53. Janssen, R., Verhard, E., Lankester, A., Ten Cate, R. & van Dissel, J. T. Enhanced interleukin-1beta and interleukin-18 release in a patient with chronic infantile neurologic, cutaneous, articular syndrome. *Arthritis Rheum.* **50**, 3329–3333 (2004).
54. Stack, J. H. *et al.* IL-converting enzyme/caspase-1 inhibitor VX-765 blocks the hypersensitive response to an inflammatory stimulus in monocytes from familial cold autoinflammatory syndrome patients. *J. Immunol.* **175**, 2630–2634 (2005).
55. Saito, M. *et al.* Disease-associated CIAS1 mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients. *Blood* **111**, 2132–2141 (2008).
56. Navarre, W. W. & Zychlinsky, A. Pathogen-induced apoptosis of macrophages: a common end for different pathogenic strategies. *Cell. Microbiol.* **2**, 265–273 (2000).
57. Fernandez-Prada, C. M., Hoover, D. L., Tall, B. D. & Venkatesan, M. M. Human monocyte-derived macrophages infected with virulent *Shigella flexneri in vitro* undergo a rapid cytolytic event similar to oncosis but not apoptosis. *Infect. Immun.* **65**, 1486–1496 (1997).
58. Koterski, J. F., Nahvi, M., Venkatesan, M. M. & Haimovich, B. Virulent *Shigella flexneri* causes damage to mitochondria and triggers necrosis in infected human monocyte-derived macrophages. *Infect. Immun.* **73**, 504–513 (2005).
59. Suzuki, T. *et al.* A novel caspase-1/toll-like receptor 4-independent pathway of cell death induced by cytosolic Shigella in infected macrophages. *J. Biol. Chem.* **280**, 14042–14050 (2005).
60. McCall, S. H. *et al.* Osteoblasts express NLRP3, a nucleotide-binding domain and leucine-rich repeat region containing receptor implicated in bacterially induced cell death. *J, Bone Miner. Res.* **23**, 30–40 (2008).
61. Hentze, H., Lin, X. Y., Choi, M. S. & Porter, A. G. Critical role for cathepsin B in mediating caspase-1-dependent interleukin-18 maturation and caspase-1-independent necrosis triggered by the microbial toxin nigericin. *Cell Death Differ.* **10**, 956–968 (2003).
62. Perregaux, D. *et al.* IL-1β maturation: evidence that mature cytokine formation can be induced specifically by nigericin. *J. Immunol.* **149**, 1294–1303 (1992).
63. Verhoef, P. A., Kertesy, S. B., Estacion, M., Schilling, W. P. & Dubyak, G. R. Maitotoxin induces biphasic interleukin-1β secretion and membrane blebbing in murine macrophages. *Mol. Pharmacol.* **66**, 909–920 (2004).
64. Zhao, X. *et al.* Maitotoxin induces calpain but not caspase-3 activation and necrotic cell death in primary septo-hippocampal cultures. *Neurochem. Res.* **24**, 371–382 (1999).
65. Majno, G. & Joris, I. Apoptosis, oncosis, and necrosis. An overview of cell death. *Am. J. Pathol.* **146**, 3–15 (1995).
66. Fink, S. L. & Cookson, B. T. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cell. Microbiol.* **8**, 1812–1825 (2006).

Acknowledgements
J.P.-Y.T. is supported by NIH grants and the SPAR award. D.T.B. is supported by the Lineberger postdoctoral award.

DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
APAF1 | ASC | BCL-2 | HMGB1 | IL-1 | NLRC4 | NLRP1 | NLRP3 | TNF

FURTHER INFORMATION
HUGO gene nomenclature, NLR family: http://www.genenames.org/genefamily/nlr.php

ALL LINKS ARE ACTIVE IN THE ONLINE PDF