The Therapeutic Potential of Modifying Inflammasomes and NOD-Like Receptors

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Abstract—Inflammasomes are the central processing units (CPUs) responsible for decoding and integrating signals of foreignness, damage, danger, and distress released by pathogens, cells, and tissues. It was initially thought that the inflammasomes participated only in pathogen recognition and in the pathogenesis of a few, rare, hereditary inflammatory disorders. On the contrary, it is now clear that they have a central role in the pathogenesis of basically all types of chronic inflammation, in metabolic diseases and cancer. So far, six or possibly eight inflammasome subtypes have been identified. Their main, but by no means exclusive, function is to catalyze conversion of pro-IL-1β and pro-IL-18 into their respective mature forms. However, the different inflammasome subtypes may also participate in additional responses, e.g., proliferation, regulation of glycolytic metabolism, or cell activation, albeit it is not clear whether these effects are still mediated through IL-1β release or via modulation of other caspase-1-dependent or -independent pathways. Central to inflammasome organization and activity are proteins belonging to the nucleotide binding domain, leucine-rich repeat, or NOD-like receptor family. One relevant exception is the AIM2 inflammasome. NOD-like receptors belong to the superfamily of pattern recognition receptors, a group of highly conserved molecules specialized in the recognition of invariant molecular patterns diffused across species. Given their potent proinflammatory activity, it is anticipated that inflammasome activation is tightly controlled. In this review, I will summarize essential features of the known NOD-like receptors, the basic molecular structure of inflammasomes, their participation in pathophysiological responses, and their possible exploitation for therapy.

I. Introduction

Inflammation is the key pathophysiological response triggered by noxious agents in multicellular organisms. Virtually no perturbation in body homeostasis can occur without generating a local or systemic inflammatory response. Inflammation is intimately intertwined with innate and adaptive immunity and therefore deeply involved in the pathogenesis of immune-mediated diseases. Although often referred to as a “monotonous” process, inflammation can evolve in quite different fashions, thus producing substantially different clinical pictures (Medzhitov, 2008, 2010). To decipher the mechanisms underlying the different forms of inflammation (not only typical acute and chronic inflammation, but also the different presentations that both acute and chronic inflammation may have) and understand the different facets of the immune response (e.g., protective immunity, allergy and hypersensitivity, tolerance, suppression), we need to identify and dissect the events occurring during the initial few minutes following an injury or the encounter with a pathogen (Medzhitov and Janeway, 2002; Nathan, 2002). This rather short interval is crucial for all the following events and for the final outcome. Key factors determining the progression and the presentation of inflammation are PAMPs/MAMPs (pathogen-associated molecular patterns/microbe-associated molecular patterns), DAMPs (damage-associated molecular patterns), PRRs (pattern recognition receptors), cells of innate and adaptive immunity, and the inflammasomes (Janeway and Medzhitov, 2002; Schroder and Tschopp, 2010; Sims et al., 2010).

ABBREVIATIONS: Aβ, amyloid β; AIM2, absent in melanoma 2; Ag, antigen; AMD, age-related macular degeneration; ASC, apoptosis-associated speck-like protein containing a CARD; BIR, baculovirus inhibition of apoptosis protein repeat; CAPS, cyspryn-associated periodic syndromes; CARD, caspase-recruiting domain; CC, coiled coil; casp-1, caspase-1; CD, Crohn’s disease; CINCA, chronic infantile neurologic cutaneous and articular syndrome; CNL, CC-NB-LRR containing; CPPD, calcium pyrophosphate dihydrate; CPU, central processing unit; CIITA, class II transactivator; DAMP, damage-associated molecular pattern; DC, dendritic cell; ds, double strand; FCAS, familial cold-induced autoinflammatory syndrome; FIIND, function to find domain; FMF, familial Mediterranean fever; G-CSF, granulocyte-colony-stimulating factor; GM-CSF, granulocyte-macrophage-colony-stimulating factor; HET-E, heterokarion incompatibility protein; HIN, hematopoietic interferon-inducible nuclear protein; HMGBl, high mobility group box 1; IAPP, islet A polypeptide; ICE, interleukin-1-converting enzyme; IFN, interferon; IFN I, type I interferon; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; IPAF, inflammatory protease activating factor; LPS, lipopolysaccharide; LRR, leucine-rich repeat; LT, lethal toxin; MAMP, microorganism associated molecular pattern; MAPK, mitogen-activated protein kinase; MAVS, mitochondrial antiviral signaling; MDP, muramyl dipeptide; MHC I, major histocompatibility complex I; miRNA, microRNA; MSU, monosodium urate; NACHT, NAIP, CIITA, HET-E, and TP-1 containing; NAIP, NLR apoptosis inhibitory domain; NOD-LRR, nucleotide binding LRR containing; NK, natural killer; NLR, NOD-like receptor or nucleotide binding domain leucine-rich repeat containing; NO, nitric oxide; NOD, nucleotide binding and oligomerization domain; NOMID, neonatal onset multisystem inflammatory disorder; NF-κB, nuclear factor κB; PAMP, pathogen associated molecular pattern; panx, panxennin; PKR, protein kinase R; PGN, peptidoglycan; POP, pyrin only protein; PRR, pattern recognition receptor; PTX7, PTX7 receptor; RIG, retinoic acid inducible gene; RIP2, receptor-interacting protein 2; RLR, RIG-1-like receptor; ROS, reactive oxygen species; ss, single strand; STAND, signal transduction ATPases with numerous domains; T2DM, type 2 diabetes mellitus; TANK, TRAF family member-associated NF-κB activator; TBK1, TANK-binding kinase 1; Th, T helper; TXN, thioredoxin; TXNIP, thioredoxin inhibitory protein; TIR, Toll interleukin-1 receptor motif; TLRs, Toll-like receptors; TNF, tumor necrosis factor; TNL, TIR-NB-LRR-containing; TP-1, telomerase-associated protein; TTSS, type three secretion system; VEGF, vascular endothelial growth factor; VX-765, (S)-1-((S)-2-(4-amino-3-chlorobenzamido)-3,3-dimethylbutanoyl)-N-((2R,3S)-2-ethoxy-5-oxo-tetrahydrofuran-3-yl)pyrrolidine-2-carboxamide.
It is well known that inflammation can be started by a multiplicity of causes: microorganisms and viruses, trauma, cancer, foreign particles, ischemia, exposure to radiations, metabolic disorders, and many more. The common feature of all causes of inflammation is cell or tissue damage. This is obvious in the case of sterile inflammation and less obvious but nevertheless absolutely clear, for septic inflammation. The need for pathogens to cause cell damage to start inflammation has only recently been recognized, prompted by the discovery and the molecular characterization of DAMPs (Bianchi, 2007; Zeiser et al., 2011). DAMPs are molecules that are normally absent from the extracellular environment under healthy conditions but that are released when a foreign microorganism enters and multiplies within our body (infection) or as a consequence of tissue injury (sterile inflammation). In the case of infection, DAMPs are released by host cells injured by the pathogens. DAMPs may also originate extracellularly from damaged or abnormally processed extracellular matrix proteins, as in the case of biglycan, collagen fragments, fibronectin, or tenascin-C (Zeiser et al., 2011). In the ignition phase of inflammation a coordinate action between PAMPs/MAMPs and DAMPs is needed, or in other words, inflammation is started by recognition of both a foreign molecule and the associated cell damage. Foreignness is not by itself sufficient, unless a level of danger is also recognized (Heath and Carbone, 2003), or as stated by Carl Nathan “each cell commits to recruit and activate others based on multiple inputs, generally requiring evidence of both injury and infection” (Nathan, 2002).

From a teleological perspective, inappropriate as it might be for the interpretation of events occurring in an unintentional context such as the biologic world, it is absolutely logical that several checks and controls have to be passed before inflammation is started, because this is a powerful and potentially disruptive process that should be initiated only when absolutely necessary and when all due controls have been carried out. Risks associated with inflammation are well known to physicians and possibly even more to general practitioners, who spend a great deal of their time prescribing anti-inflammatory, rather than proinflammatory drugs, although we now know that stimulating inflammation may indeed be helpful in a few selected conditions (Lammers and Witjes, 2010; Sylvester, 2011). When a pathogen enters the body or tissues are injured, tissue resident cells of innate immunity (e.g., macrophages and dendritic cells, DCs), the first line of body defense, are confronted with a very important decision, because at this time a wealth of signals are generated that need to be properly decoded and integrated before a response is started. Substantial evidence suggest that the inflammasome (or rather the inflammasomes) is/are the fundamental intracellular structure responsible for this integration (Martinon et al., 2002; Schroder and Tschopp, 2010; Gross et al., 2011; Mankan et al., 2012; Strowig et al., 2012; von Moltke et al., 2012a) (Fig. 1).

In this light, inflammasomes might be considered the cellular central processing units (CPUs) responsible for the integration of information conveyed by PAMPs/MAMPs and DAMPs and for their translation into signals and responses. Such signals and responses may involve the release of diffusible cytokines [interleukin-1β (IL-1β) and interleukin-18 (IL-18)], activation of immune cells, or even cell death (pyroptosis). These events are necessary for the induction of further systemic responses and to spreading of inflammation (Dinarello, 2009).

Interleukin-1β is a proinflammatory cytokine that participates in almost all events involved in the activation and regulation of inflammation (Dinarello, 2011). Interleukin-1β stimulates the release of other cytokines such as IL-6, tumor necrosis factor (TNF)-α, and IL-1α, as well as IL-1β itself and other crucial factors responsible for growth and differentiation of immune cells [e.g., granulocyte-colony-stimulating factor (G-CSF) and granulocyte-monocyte-colony-stimulating factor (GM-CSF)]. In addition, IL-1β promotes release of polymorphonuclear cells into the blood (neutrophilia), lymphocyte activation, T helper (Th17 lymphocyte differentiation, DC activation, vascular endothelial
growth factor (VEGF) secretion, generation of reactive nitrogen and reactive oxygen (ROS) species, and importantly for the progression of inflammation, expression of adhesion molecules on vascular endothelial cells. Interleukin-1β is fundamental for innate as well as adaptive immunity, where it participates in T, B, and natural killer (NK) cell activation and differentiation and in antigen (Ag) presentation (Sims and Smith, 2010). Kinetics of IL-1β release support this key role because IL-1β is one of the earliest cytokines to be secreted during the initial phases of inflammation. Thus, it is not at all surprising that the immune system has evolved a complex and tightly modulated system to control its release. The other cytokine belonging to the IL-1 family that is converted into the mature form by inflammasomes is IL-18, previously known as interferon γ (IFNγ)-inducing factor. Interleukin-18 is known be involved in polarization of type 1 helper and cytotoxic T lymphocytes, in Th17 differentiation, in the production of Th2 cytokines by T cells, NK cells, basophils, and mast cells, and therefore it has a key role in autoimmune diseases in several chronic inflammatory pathologies and metabolic dysfunctions (Dinarello, 2007; Smith, 2011).

In addition to IL-1β and IL-18, the inflammasomes might also participate in secretion of additional leaderless cytokines and growth factors, such as IL-1α and fibroblast growth factor 2 (FGF2), or the danger signal high-mobility group box 1 (HMGB1) protein (Lamkanfi and Dixit, 2012).

The other process triggered by the inflammasomes (i.e., pyroptosis) is also conducive for inflammation. Pyroptosis is a type of cell death that shares features of apoptosis and necrosis, and as such it is a process that is tightly controlled (a distinctive feature of apoptosis) but at the same time capable of inducing an explosive release of proinflammatory DAMPs (a distinctive feature of necrosis) (Miao et al., 2011). Needless to say, the overall logic behind inflammasome activation is highly reminiscent of that governing activation of the apoptosome (Shi, 2006; Mace and Riedl, 2010): the final aim of both processes is caspase activation. This aim is reached via assembly of a heteroligomeric complex based on a scaffold component [i.e., apoptosis protease-activating factor (APAF), NOD-like receptor-pyrin-containing (NLRP) proteins or absent in melanoma 2 (AIM2) protein] and the recruitment of adaptor and effector partners, such as apoptosis-associated speck-like protein containing a CARD (ASC), and/or procaspases. Apoptosome and inflammasome complexes differ for the triggering stimulus, which is cytochrome c release from mitochondria in the former and PAMP/MAMP or DAMP detection in the latter case.

From a teleological perspective, the final aim of inflammation is to allow prompt wound repair and tissue regeneration. From this perspective, close control of inflammasome activity is required to allow activation of those processes that dampen inflammation and avoid excessive and long-lasting tissue destruction, a typical complication of chronic inflammation. This implies that ability to downmodulate inflammasome function should be intrinsic to inflammation. Furthermore, given that inflammasome activity is associated with production of highly specialized cell functions and to cell death, one cannot avoid hypothesizing that inflammasomes should be shut off during proliferation.

Inflammation is based on the efficient cooperation between signals, sensors, and effector systems. The inflammasome resides within the cytoplasm, therefore we can reasonably assume that it is perfectly equipped to sense intracellular pathogens with cytoplasmic location, but it is also clear that it needs a system of communication (extracellular sensor, intracellular messengers) that convey alarm signals delivered by extracellular PAMPs/MAMPs or DAMPs. Thus, an associated aspect of inflammasome biology is the necessity of a tight interaction on one hand with accessory molecules that allow scanning the pericellular environment for detection of PAMPs/MAMP or DAMPs, and on the other with the intracellular second messengers generated.

II. NLRs: What Are They?

Multicellular organisms have evolved highly sophisticated mechanisms to detect the presence of pathogens (or injurious agents) and activate the proper defense processes. Not all foreign microorganisms that multicellular organisms come in contact with are necessarily harmful; thus a complex mechanism of recognition of microbes as well as of their “dangerousness” had to be developed during evolution. This sophisticated system aimed at monitoring the extracellular and intracellular environment for the direct (the microorganism itself) or indirect (injury) detection of a pathogen is based on at least four different families of receptors: C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), Toll-like receptors (TLRs), and nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs), otherwise known as nucleotide-binding domain and leucine-reach repeat containing receptors (Kanneganti et al., 2007a; Chen et al., 2009; Davis et al., 2011; Sancho and Reis e Sousa, 2012). It is likely that all these sensors act in a concerted fashion to sense foreign microorganisms or danger and signal the host to mount (or abort) the proper response. CLRs, RLRs, TLRs, and NLRs make up the larger family of cellular receptors specialized in sensing microorganism patterns known as the PRR family (Hansen et al., 2011). TLRs are specialized to sense pathogens/microorganisms in the pericellular space or in the endosome lumen (which can be equated to the extracellular space) (Kawai and Akira, 2011). However, microorganisms can gain access to and even multiply within eukaryotic cell cytoplasm, thus it is crucial to be able to
sense their presence within this compartment. This is the likely evolutionary push that led to the development of cytoplasmic pathogen sensors.

The prototypic pathogen/danger-sensing receptors are NLRs (Davis et al., 2011; Kersse et al., 2011; Robertson et al., 2012). NLRs are characterized by a carboxy terminal, hypothetical pathogen-sensing (or autoinhibitory) domain made by the repetition of a classic leucine-rich motif [leucine-rich repeat (LRR)], a central sequence named NACHT (NAIP, CIITA, HET-E, and TPI-1) or NOD, and an N terminus that may consist in a pyrin domain (PYD), a caspase-recruitment domain (CARD), a baculovirus inhibition of apoptosis protein repeat (BIR), or other less defined motifs. Although NLRs are central to pathogen recognition, at least one member of this family named class II transactivator (CIITA) seems not to have a role in this process but rather to act as a transcriptional activator for MHC class II genes (Steimle et al., 1993). NLRs are conserved throughout the animal kingdom, from plants to mammals (Jones and Dangl, 2006).

NLRs belong to a defined subgroup of ATP-binding and hydrolyzing proteins named STAND (signal transduction ATPases with numerous domains), which in turn is a member of the superfamily of ATPases associated with diverse cellular activities (Bonardi et al., 2012). STAND proteins, which are present from plants to mammals, are regulated via nucleotide binding, the ADP-bound form being the resting and the ATP-bound form being the activated state. It is thought that LRR binding of PAMPs/MAMPs or DAMPs, or of yet-to-be-identified intracellular messengers, triggers a conformational change that exchanges ADP for ATP within the NOD motif. ATP hydrolysis then reverts STAND proteins to a resting state. Although there is evidence that plant NLRs homodimerize, activation of effector responses by recruitment of additional proteins (homologous or heterologimerization) is better characterized in mammalian NLRs, which assemble to form specialized multimeric protein platforms (Bonardi et al., 2012). Oligomerization occurs via homotypic association of N-terminal domains (CARD or PYD in mammalian NLRs). This last mechanism appears to have a crucial role, especially in the control of activity of NLRs participating in the inflammasomes. As it is typical of many intracellular protein switches, it is likely that NLRs slowly cycle between active and inactive states, thus making the whole system rapidly responsive to stimuli.

A. Plant NLRs

Pathogens in plants are detected by surface receptors named receptor-like proteins and receptor-like kinases, which both trigger the so-called pattern-triggered immunity (Zipfel, 2008). A second type of immunity in plants is activated by recognition of pathogen products by specialized matching resistance (R) proteins prevalently found in the cytoplasm. This second type of plant immune response based on recognition of pathogen products is referred to as effector-triggered immunity (Boller and Felix, 2009). R proteins are highly polymorphic and belong to a family of intracellular receptors bearing a nucleotide-binding (NB) site and a LRR (and therefore named NB-LRR) structurally similar to mammalian NLRs. Plant NB-LRR can be further subdivided into two main families based on the presence at the N terminus of a Toll IL-1 receptor (TIR) or coiled-coil (CC) motif. These two families are accordingly named TNL (TIR-NB-LRR) or CNL (CC-NB-LRR) (Benko et al., 2008; Robertson et al., 2012). In plants, as well as in mammals, the core of NB-LRR activation seems to reside in ligand-driven conformational change and oligomerization, which then leads to activation of defense responses. The logic behind effector triggered immunity is that the host recognizes a target protein that is modified by effector molecules released by the pathogen, rather than the pathogen effector molecule itself (Jones and Dangl, 2006; Benko et al., 2008; Robertson et al., 2012). For example, the effector protein AvrPto produced by Pseudomonas syringae binds to and inhibits the host serine-threonine kinase Pto (Sessa and Martin, 2000). The AvrPto-bound/inhibited Pto is then sensed by the NB-LRR protein Prf, which triggers the defense response. “Sensing” the modified NB-LRR protein might consist in either physically binding the effector-NB-LRR complex or detecting a messenger released by the effector-activated NB-LRR protein (Jones and Dangl, 2006). Defense responses triggered by TNL or CNL proteins are mediated by at least two different pathways encoded by two systems: (1) the enhanced disease susceptibility 1 gene or (2) the non-race-specific disease resistance 1 gene (Aarts et al., 1998; Wiermer et al., 2005). However, both TNL and CNL share a final common pathway at the level of the synthesis of the defense hormone salicylic acid or of other defense factors such as jasmonic acid, ethylene, or nitric oxide (NO) (Elmore et al., 2011).

B. Invertebrate NLRs

The two most widely studied invertebrate model organisms, Drosophila melanogaster and Caenorhabditis elegans, lack NLR-encoding genes (Robertson et al., 2012). However, screening of other invertebrates has unveiled a large number of NLR-encoding genes. Invertebrate NLRs possess a carboxy-terminal LRR domain and a central NOD domain, but at the N terminus often show a death domain (Hibino et al., 2006). It is not clear whether invertebrate NLRs participate in host defense and which effector pathways are triggered, although it appears that in Hydra a NLR protein interacts with a death domain- or CARD-expressing protein to form a multimolecular complex akin to the mammalian inflammasome (Lange et al., 2011). Compared with plant and mammals, invertebrate NLRs have been the
subject of few studies and their structure and function are as yet very superficially known.

C. Mammalian NLRs

NLR structure and function has been more thoroughly studied and understood in mammals, i.e., in mice and humans. There are 22 and 34 NLR family members in humans and mice, respectively (Ting et al., 2008; Lamkanfi and Dixit, 2012; Robertson et al., 2012), subdivided into five subfamilies: NLRA, NLRB, NLRX, NLRP, and NLRX (Table 1). Human genes are written in upper case (e.g., NLRP3) and mouse genes are written with the uppercase for the first letter only (e.g., Nlrp3). Products of human genes are indicated in this review with uppercase (e.g., NLRP3), whereas those of mouse genes are indicated with upper case for the first letter only (e.g., Nlrp3). For the generic indication of NLR proteins, the notation for human products will be used.

1. NLRA. The NLRA (NLR family acidic domain containing) subfamily contains a CARD and transactivation domain in the amino terminus. The only member is CIITA, a class II transactivator that regulates expression of MHCII genes as well as of other genes involved in inflammation such as collagen type I, IL-4, IL-10, E-cathepsin, and Fas ligand (Wu et al., 2009). A possible role of CIITA in pathogen or danger sensing has not been as yet identified.

2. NLRB. The NLRB (NLR family BIR domain containing) subfamily, characterized by the presence of a BIR at the amino terminus, is comprised of one member in humans and seven members in mice (Ting et al., 2008). Members of the NLRB subfamily are named NAIP (NLR family-apoptosis inhibitory protein). In humans only one member is known (NAIP), whereas in mice this family numbers seven members (Naip1-7). NAIP proteins appear to be specialized in recognition of bacterial flagellin and type three secretion system (TTSS) (Molofsky et al., 2006). NAIP proteins do not seem to be able to assemble in an individual inflammasome complex, but rather to confer ligand specificity to the Nlrc4 inflammasome (see below). In mice it has been shown that different Naip paralogs determine the PAMP/MAMP specificity of the Nlrp4 inflammasome (Kofoed and Vance, 2011). Naip2 associates with Nlrc4 and allows the Nlrc4 inflammasome to drive IL-1β synthesis in response to the bacterial TTSS protein PrgJ. Naip5 on the other hand makes the Nlrc4 inflammasome responsive to bacterial flagellin. This suggests that Naip proteins are the direct binding sites for bacterial ligands, whereas Nlrc4 might be consider as an adaptor functional for caspase-1 activation and IL-1β processing (Kofoed and Vance, 2011). The function of human NAIP in host defense is not well understood, although there is evidence that human NAIP makes the Nlrc4 inflammasome responsive to the TTSS protein Cprl purified from Chromobacterium violaceum (Zhao et al., 2011) and that its overexpression makes A549 lung epithelial cells refractory to growth of Legionella pneumophila (Vinzing et al., 2008). In summary, it appears that both in mice and humans binding of NAIP by the corresponding bacterial ligands triggers association with Nlrc4, inflammasome assembly, and activation of innate immunity.

3. NLRX. The NLRX (NLR family with low homology to the N terminus of any other NLR subfamily

<table>
<thead>
<tr>
<th>NLR Family</th>
<th>HGNC-Approved Symbol</th>
<th>Domain Organization</th>
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<tbody>
<tr>
<td>NLRA</td>
<td>CIITA</td>
<td>CARD-NACTH-LRR</td>
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<tr>
<td>NAIP</td>
<td>Naip1</td>
<td>BIR-BIR-NACTH-LRR</td>
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<td>NOD1</td>
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<td>CARD-NACTH-LRR</td>
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<td>CARD-NACTH-LRR</td>
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<td>NLRB</td>
<td>NAIP</td>
<td>BIR-BIR-NACTH-LRR</td>
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<tr>
<td>NLRP</td>
<td>NLRP1</td>
<td>PYD-NACTH-FIIND-CARD</td>
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<td>NLRP14</td>
<td>Nlrp14</td>
<td>NACHT-LRR</td>
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<td>NLRX</td>
<td>NLRX1</td>
<td>X-NACTH-LRR</td>
</tr>
</tbody>
</table>

TA, transactivation domain; X, undefined domain.
infection with T cell activation and increased bacterial load upon 2012). NLRC5-deficient animals showed impaired CD8+ and fibroblasts (Neerincx et al., 2012; Staehli et al., 2012). These reports suggest that NLRC3 might be part of a feedback loop to attenuate TLR signaling and excessive inflammation. As described above (see NLRB), NLRC4 participates in the assembly of an inflammasome subtype activated by bacterial flagella (Amer et al., 2006; Franchi et al., 2006). NLRC5 has been described as an inhibitor of NF-κB and IFN I pathways (Cui et al., 2010), but more recently it has been shown to localize to the nucleus, where it modulates MHC I gene expression in lymphoid cells and fibroblasts (Neerincx et al., 2012; Staehli et al., 2012). NLRC5-deficient animals showed impaired CD8+ T cell activation and increased bacterial load upon infection with Listeria monocytogenes (Biswas et al., 2012).

NOD1 and NOD2, the most important noninflamma-
some NLRs, are both located in the cytoplasm and are well characterized for their ability to activate the transcription factor NF-κB. NOD1 and NOD2 are perhaps the NLR proteins for which a role in maintaining microbe-host homeostasis is better characterized. Specific ligands for NOD1 and NOD2 are structural components of bacterial peptidoglycan (PGN) such as diaminopimelic acid-containing muramyl tripeptide (muramyl-Tridap) and muramyl-dipeptide (MDP), respectively (Chen et al., 2009). Upon activation, NOD1 and NOD2 self-oligomerize to form a complex that interacts with the adaptor protein receptor-interacting protein 2 (RIP2) that in turn drives NF-κB and mitogen-activated protein kinase (MAPK) activation (Park et al., 2007). As will be further discussed below, NOD1 and NOD2 are central to control host-pathogen interactions and antibacterial immunity, especially in those tissues exposed to a very large bacterial burden such as the lower digestive tract. Nod1 deficiency was shown to impair host defense against Helicobacter pylori (Viala et al., 2004) and the intracellular parasite Trypanosoma cruzi (Silva et al., 2010). Furthermore neutrophils from Nod1−/− mice were shown to be unable to kill bacteria (Clarke et al., 2010). This latter study highlighted the priming role for maturation of an efficient antibacterial immunity of Nod1 ligands released in the gut by the microbiota and entering the systemic circulation. NOD2 reacts to a wide range of pathogens, including Streptococcus pneumonia, L. monocytogenes, Mycobacterium tuberculosis, Salmonella, Staphyloccocus aureus, and is intimately associated with the pathogenesis of Crohn’s disease (CD). About 50% of CD patients bear polymorphisms or frameshift mutations in the NOD2 LRR domain. A polymorphism in the NOD2 nucleotide-binding domain is associated with Blau syndrome, a granulomatous monogenic disease phenotypically characterized by arthritis and uveitis (Kanegangi et al., 2007a).

The mechanism by which NOD2 dysfunctions predispose to chronic inflammation in the digestive tract is unknown. A specific deficit observed in Nod2-deficient mice appears to consist of a reduced production of α-defensins in intestinal Paneth cells (Kobayashi et al., 2005). Since defensins are crucial in shaping the microbiota, it has been postulated that lack of defensin production in Nod2-deficient individuals might be a cause for a deranged composition of the intestinal microbiota and therefore important in the pathogenesis of CD. In support of this hypothesis, substantial changes in the microbiota were found in Nod2-deficient mice (Petnicki-Ocwieja et al., 2009; Rehman et al., 2011; Mondot et al., 2012). Alternatively, it has been proposed that Nod2 downmodulates TLR-mediated responses, thus preventing excessive reactivity to the resident intestinal bacterial flora (Watanabe et al., 2004, 2006). It is proposed that NOD2 mutations cause intestinal cells to overreact to otherwise harmless stimuli, thus producing an unnecessary chronic inflammatory response. A third hypothesis maintains that CD-associated NOD2 variants cause increased inflammation by inducing enhanced IL-1β release and sustained NF-κB activation.
Nod1 interacts with the oligomerizes and associates with RIP2. Upon infection ROS generation (Lipinski et al., 2009). Nod1 also self-complex, thus hinting at a role for NOD2 signaling in and Duox (Eitel et al., 2008). Some of these proteins membrane-associated proteins such as Erbin, Rac1, and Duox (Eitel et al., 2008). Some of these proteins participate in the constitution of the NADPH oxidase complex, thus hinting at a role for NOD2 signaling in ROS generation (Lipinski et al., 2009). Nod1 also self-oligomerizes and associates with RIP2. Upon infection with Shigella flexneri, a common intestinal pathogen, Nod1 interacts with the guanine nucleotide exchange factor H1 to promote RIP2-dependent NF-κB activation. (Fukazawa et al., 2008). To further stress their role in antibacterial defense, Nod1 and Nod2 were recently shown to take part in autophagy, a cellular process by which defective organelles, denatured proteins, or foreign material (bacteria included) are sequestered into double-membrane vesicles and targeted to the lysosomes for destruction. Both NOD1 and NOD2 were shown to interact with and recruit the ubiquitin-like autophagy-related protein ATG16L1 at sites of bacterial entry at the plasma membrane (Cooney et al., 2010; Homer et al., 2010; Travassos et al., 2010). This allows the full assembly of the autophagic machinery and bacterial killing. Interestingly, the ATG16L1 gene has also been associated with CD (Hampe et al., 2007; Rioux et al., 2007).

5. NLRP. The largest NLR subfamily is the NLRP (NLR family pyrin domain containing) subfamily, which consists of 14 human (NLRP1–14) and 20 mouse members, respectively (Mankan et al., 2012). NLRP subfamily members are the best characterized components of the inflammasomes. NLRP1 is the only NLR protein containing a CARD. NLRP1 responds to Bacillus anthracis lethal toxin (LT), the most important pathogenetic factor of anthrax (Lamkanfi and Dixit, 2009). Polymorphisms in the rat Nlrp1 gene determine susceptibility to LT (Newman et al., 2010). NLRP1 assembles with ASC and procaspase-1 to form an inflammasome that responds to LT and MDP to produce IL-1β. NLRP2 is thought to be a negative regulator of the NF-κB pathway by associating with the inhibitor of KB kinase complex, thus inhibiting IkB degradation (Bruyé et al., 2004).

NLRP3 is the scaffold component of the best characterized inflammasome complex, the NLRP3 inflammasome. Typical of NLRP3 is the ability to respond to a variety of unrelated factors of bacterial, viral, or host origin. This wide agonist specificity strongly suggests that the mechanism of activation must be indirect. The NLRP3 inflammasome is a powerful generator of mature IL-1β and point mutations are associated with rare genetic diseases (inflammasomopathies) that have had a strong impact in our understanding of the pathogenesis of chronic inflammatory diseases (Brydges et al., 2009; Kastner et al., 2010; Jounai et al., 2011).

NLRP4 was originally described as an inhibitor of autophagy via its binding to Beclin. However, very recent data show that NLRP4 negatively regulates IFN I signaling by targeting the TANK-binding kinase 1 (TBK1) kinase to proteasome-mediated degradation and thus preventing activation of the transcription factor IRF3 (Cui et al., 2012). NLRP6 participates in the formation of an inflammasome complex that seems to have a major role in regulating intestinal bacterial homeostasis (Chen et al., 2011; Elinav et al., 2011).

NLRP5 and NLRP7 are involved in embryogenesis. NLRP5 has a role in preimplantation development, probably by controlling mitochondrial metabolism and cellular localization in ovulated oocytes (Fernandes et al., 2012). NLRP7 was initially identified as a factor involved in the pathogenesis of molar pregnancies, a rare genetic condition associated with appearance of hydatidiform moles (Murdoch et al., 2006; Wang et al., 2009). More recently NLRP7 was identified as the constituent of an inflammasome complex specifically assembled in response to microbial acylated lipopeptides, thus causing IL-1β and IL-18 secretion and restricting bacterial replication (Khare et al., 2012). This would bring NLRP7 into the restricted group of NLR proteins capable of forming a functional inflammasome.

NLRP9 was recently implicated in the pathogenesis of systemic-onset juvenile idiopathic arthritis (Tadaki et al., 2011). NLRP10 was described as an inhibitor of caspase-1-dependent IL-1β secretion and ASC-mediated NF-κB activation and thus as a negative regulator of the inflammasome (Wang et al., 2004b), but more recent data from Flavell’s laboratory indicate that this protein does not interact with the inflammasome or with inflammasome components; rather it affects the emigration of DCs from inflammatory sites and thus adaptive immunity (Eisenbarth et al., 2012).

Another NLR responsible for NF-κB inhibition is NLRP12 via direct association with the NF-κB-inducing kinase, thus causing proteasome-dependent degradation of NF-κB-inducing kinase. Deletion of the Nlrp12 gene in mouse is associated with enhanced susceptibility to intestinal inflammation and tumorigenesis, suggesting that Nlrp12 might participate in preserving intestinal homeostasis and preventing carcinogenesis (Zaki et al., 2011; Allen et al., 2012). Recently Nlrp12 has been shown to participate in the assembly of an inflammasome complex (Yersinia pestis inflammasome) specialized in the recognition of Yersinia pestis (Vladimer et al., 2012). This novel inflammasome appears to have an important role in controlling IL-1β and IL-18
secretion by *Yersinia*-infected cells, and thus indirectly promoting IFN-γ release by activated T lymphocytes. In addition, a specific inability to mount Th cell-driven immunity in response to a variety of stimulants such as lipopolysaccharide (LPS), aluminium hydroxide, and complete Freund’s adjuvant was found in dendritic cells from *Nlrp12*<sup>−/−</sup> mice. In two models of contact hypersensitivity *Nlrp12*<sup>−/−</sup> mice showed attenuated inflammation due to reduced migration of DCs to local lymph nodes (Arthur et al., 2010).

NLRP14 is exclusively expressed in the testis, where it seems to be involved in spermatogenesis. Mutations in this gene are responsible for spermatogenic failure in a patient subpopulation (Westerveld et al., 2006). NLRP8, NLRP11, and NLRP13 have no established function.

III. Molecular Organization of the Inflammasome

The inflammasome is an oligomeric protein complex with a tripartite structure: (1) a PAMP/MAMP or DAMP sensor, (2) an effector (IL-1β or IL-18-processing system), i.e., caspase-1, and (3) a coupling protein. This fundamental organization can change slightly in the different inflammasome subtypes. The logic behind such a molecular organization is to generate a molecular complex leading to an efficient and controlled activation of procaspase-1; this is because the end result of all the different inflammasomes so far characterized is cleavage of procaspase-1 and generation of activated caspase-1 (Fig. 2).

Two basic scaffold families of inflammasome constituents have been identified: (1) the NOD-like receptor (NLR) family and (2) the PYHIN-200 family of proteins (Schroder and Tschopp, 2010; Gross et al., 2011; Schattgen and Fitzgerald, 2011). In the NLR family, four inflammasome scaffold proteins have been most thoroughly characterized: NLRP1, NLRP3, NLRP6, and NLRC4 (IPAF). But recently, NLRP7 and NLRP12 have also been proposed to be able to assemble into inflammasomes with IL-1β- and IL-18-processing activity (Khare et al., 2012; Vladimer et al., 2012). In the PYHIN family, characterized by the presence of a pyrin (PY) domain and a HIN domain, so named after the conserved 200-amino acid motif “hematopoietic interferon-inducible nuclear protein,” only one member is known, the IFN-γ-inducible protein absent in melanoma 2 (AIM2). AIM2 was the first non-NLR scaffold constituent of the inflammasome identified. It was recently suggested that a member of the RIG-I-like helicases (RIG-I), a family of cytosolic proteins specialized in detection of viral RNA, might also assemble with ASC to drive IL-1β processing and release (Poock et al., 2010; Yu and Levine, 2011), thus giving another example of a non-NLR scaffold inflammasome. However, it is as yet uncertain whether RIG-I indeed participates in the composition of an additional inflammasome subtype specialized in viral RNA sensing, as another study showed that viral RNA activates the Nlrp3 inflammasome in the absence of RIG-I (Rajan et al., 2011). In addition, it has been proposed that pyrin may activate caspase-1 via ASC independently of NLRs, thus potentially forming a “pyrin inflammasome” (Mankan et al., 2012). Thus, possibly eight inflammasome subtypes have been identified, all sharing the common features of containing ASC and being able to drive procaspase-1 processing and activation.

The inflammasomes aggregate to generate supramolecular structures akin to those made by the apoptosome, i.e., wheel-shaped pentamer/heptamer complexes of MW in the 500- to 700-kDa range (Martinon et al., 2004; Hsu et al., 2008). Electron microscopy studies showed that the NLRP1 inflammasome (Faustin et al., 2007) assembles to form ring-like, donut-shaped structures with an outer diameter of 13 nm and an inner hollow core of 4 nm. For at least two types, Nlrp3 and Nlrc4, inflammasome oligomers were shown to assemble into single cytoplasmic foci (large speck-like structures) of diameter close to 1 μm (Broz et al., 2010). It needs to be stressed that large cytoplasmic quasicrystal aggregates (size 1 μm) containing Asc and low amount of caspase-1, but no Nlrps, have been detected in macrophages, where it is thought that they trigger inflammatory cell death (Fernandes-Alnemri et al., 2007). Upon pathogen stimulation of human macrophages, ASC translocates from the nucleus into the cytosol to form NALP3 and caspase-1-containing ring-shaped perinuclear aggregates from 4 to 6 μm of size (Bryan et al., 2009). Other studies have shown that ASC relocalizes to the cytoplasm after infection, where it assembles with caspase-1 to form dense bodies of 1–2 μm in diameter within which caspase-1 is activated (Broz et al., 2010). Interestingly, only one body per cell is formed.

A. NLRP1 Inflammasome

The first to be identified and the best characterized inflammasome is that formed by the NLR family member NLRP1 (previously known as NALP1), the NLRP1 inflammasome (Martinon et al., 2002). The NLRP1 inflammasome is made of the NLRP1 protein, containing, from the C- to the N-terminal end, a CARD, a FIIND (function to find domain), a LRR, a NACHT, and PYD residue. It is expressed in immune and non-immune cells (Kummer et al., 2007) and associates with caspase-1 and caspase-5 to drive IL-1β and IL-18 processing. Since NLRP1 possesses a CARD, it can interact with caspsases directly in the absence of ASC; however, inclusion of ASC into the reaction medium greatly accelerates NLRP1 inflammasome activity (Faustin et al., 2007). The NLRP1 protein contains a central NACHT domain with a predicted nucleoside triphosphatase activity found in bacterial, fungal, and animal proteins. The NACHT domain allows ATP-dependent oligomerization,
and therefore activation, of the inflammasome. The LRR domain, that NLRPs share with TLRs, is thought to be responsible for PAMP/MAMP/DAMP sensing and autoinhibition; however, at variance with TLRs, it is still debated whether PAMPs, MAMPs, or DAMPs bind to the LRR motif of NLR inflammasomes. On the other hand, there is consensus on the autoinhibitory role played by the LRR motif in the absence of inflammasome activators. It is thought that LRRs fold on the NACHT domain, thus preventing oligomerization and subsequent activation.

NLRP1 has been associated with the activation of pyroptosis and to activation of caspase-2 and -9 in the apoptosome (Hlaing et al., 2001). The main NLRP1 function is in immunity against *B. anthracis* as anthrax LT is a specific activator of the NLRP1 inflammasome. However, NLRP1 can also be activated by MDP. Interaction with LT causes IL-1β/IL-18 maturation and secretion as well as cell death. In mice, susceptibility to LT is linked to *Nlrp1b* polymorphisms. The pathophysiological significance of NLRP1 inflammasome-triggered cell death is poorly understood.

Upon interaction with LT, the NLRP1 protein undergoes cleavage and conformational change that relieves LRR-mediated inhibition, thus unmasking the NACHT domain, which promotes recruitment of further NLRP1 subunits to form penta and heptameric structures (Levinsohn et al., 2012). How NLRP1 senses LT or MDP is not known. No direct binding of these factors to NLRP1 has ever been shown; therefore, it is proposed that the mechanism of activation should be indirect, possibly involving K⁺ efflux or cathepsin B release into the cytoplasm. In this regard, a mechanism for LT-dependent Nlrp1 activation mediated by p38 MAPK and AKT inhibition, opening of connexin channels, ATP release, and P2X7 receptor (P2X7R) stimulation has been proposed. Karin and co-workers showed that LT triggers ATP release, which in turn stimulates the P2X7R to trigger IL-1β secretion (Ali et al., 2011). Inhibition of K⁺ efflux or P2X7R blockade with selective antagonists abrogated LT-stimulated inflammasome activation. It has also been proposed that NLRP1 activity can be triggered by autoproteolytic processing within FIIND (Finger et al., 2012). This

Fig. 2. Inflammasome subtypes. Inflammasome subtypes are classified according to their scaffold components. Exemplative activating factors are shown in boxes. For the NLRC4 inflammasome phosphorylation (P within red star symbol) is also indicated as a possible activating factor. Domain coding is shown at the bottom of the figure.
finding is of interest in view of the observation that LT-induced Nlrp1 activation is susceptible to inhibition by protease inhibitors (Levinsohn et al., 2012). The ability of NLRP1 inflammasome to trigger pyroptosis might have dire consequences to the host, especially in the case of systemic inflammasome activation. Masters and co-workers (2012) showed that in vivo activation of the Nlrp1a inflammasome caused extensive pyroptotic death of hematopoietic progenitor cells, with cytopenia, leukopenia, bone marrow hypoplasia, and immunosuppression.

B. NLRP3 Inflammasome

Due to the discovery in the NLRP3 gene of mutations responsible for the pathogenesis of three rare auto-inflammatory syndromes (Agostini et al., 2004), the NLRP3 inflammasome has quickly become a focus of considerable interest. The NLRP3 protein contains a central NACHT, a series of C-terminal LRRs, and a N-terminal PYD. As for NLRP1, the NACHT domain allows ATP-dependent oligomerization, and the LRR domain is thought to be responsible for PAMP/MAMP/DAMP sensing and autoinhibition. PYD mediates homotypic protein-protein interaction. It is thought that LRRs fold on the NACHT domain, thus preventing oligomerization and subsequent activation. Keeping Nlrp3 in an inactive state may be helped by interaction with the chaperone proteins SGT1 and HSP90 (Gross et al., 2011).

The NLRP3 inflammasome is probably the most versatile inflammasome subtype poised to react to a multiplicity of potentially harmful biologic and chemical agents and so far is the best characterized molecular trigger of IL-1β and IL-18 maturation and release. The scaffold protein NLRP3 lacks a CARD sequence; therefore, it has to rely on ASC to recruit caspase-1. Many different PAMPs/MAMPs and DAMPs activate the NLRP3 inflammasome: LPS, MDP, PGN, bacterial and viral RNA, viral DNA, the dinoflagellate activate the NLRP3 inflammasome: LPS, MDP, PGN, caspase-1. Many different PAMPs/MAMPs and DAMPs release. The scaffold protein NLRP3 lacks a CARD

masome activation by several bacteria or bacterial toxins that can cause ATP release. However ATP is by no means an obligatory intermediate for inflamma-
some activation by any bacteria, especially when the pathogens are capable of causing K+ efflux directly or via release of membrane-perturbing toxins. It has also been postulated that bacterial products activate the NLRP3 inflammasome by entering the cytoplasm via the P2X7R pore or via plasma membrane pores produced by bacterial toxins, but although it is undisputed that bacterial components can gain access to the cytoplasm by this second route, consistent failure to detect direct interaction of PAMPs/MAMPs with NLRP3 casts doubts on the physiologic relevance of this mechanism (Gross et al., 2011).

C. NLRP6 Inflammasome

The NLRP6 inflammasome has been implicated in preserving host-microbiota homeostasis in the digestive tract (Kempster et al., 2011). Nlrb6-deficient mice were reported to secrete less IL-18 and be more susceptible to chemically induced colitis and to colitis-induced cancer compared with wild-type mice (Chen et al., 2011; Elinav et al., 2011). Nlrp6-mediated modulation of the intestinal flora might also be relevant for the pathogenesis of dysmetabolisms and liver dysfunctions as mice deficient in Nlrp6 develop exacerbated hepatic steatosis and obesity (Henao-Mejia et al., 2012). On the other hand, Nlrp6-deficient mice are more resistant to systemic infection with several bacterial pathogens (e.g., L. monocytogenes, Salmonella typhimurium, E. coli) (Anand et al., 2012). Thus, it appears that Nlrp6, also thanks to its prevalent localization within intestinal
epithelia, has a protective role in preserving a healthy composition of the gut microbiota on the one hand and, on the other, aggravates systemic bacterial infections. The NLRP6 inhibitory activity on IL-1β and IL-18 secretion might be beneficial in the digestiv tract by preventing overactivation of the immune system in the presence of the symbiotic flora. On the other hand, the inhibitory activity of NLRP6 will be detrimental when coping with systemic infections, when inflammation would be undoubtedly protective for the host. Thus, the spatiotemporal context for NLRP6 activation is crucial (Anand et al., 2012).

**D. NLRP7 Inflammasome**

Microbial lipopeptides cause IL-1β maturation and release and are a cause of septic shock in mice. A study by Khare et al. (2012) identifies a NLRP7-containing inflammasome as the apparatus that transduces lipopeptide stimulation into IL-1β and IL-18 maturation and release in human macrophages. In this study, lipopeptide-stimulated IL-1β release did not require addition of exogenous ATP, but required ASC and was inhibited by NLRP7 but not by NLRP3, NLRP2, and NLRP9 silencing. Other cytokines, such as IL-6 and TNFα, were not inhibited by NLRP7 silencing. Finally, NLRP7, ASC, and caspase-1 assembled into high molecular weight complexes. Interestingly, NLRP7 activation was not inhibited by ROS scavengers and only marginally impaired by cathepsin B blockade or high K⁺.

**E. NLRP12 Inflammasome**

The NLRP12 inflammasome was identified in a recent study by Vladimer et al. (2012). It specifically participates in the recognition of *Y. pestis* and promotes IL-1β and IL-18 release and secretion IFN-γ by activated lymphocytes. The NLRP protein had been previously associated with negative regulation of inflammation via NF-κB inhibition (Zaki et al., 2011; Allen et al., 2012), and these new data now suggest that it might be also key in the activation of the immune response against selected pathogens. NLRP12 was shown to interact with ASC (Wang et al., 2002), and mutations in this molecule are associated with hereditary febrile inflammatory diseases (Savic et al., 2012). Findings by Vladimer et al. show that production of IL-18 in response to *Yersinia* requires both Nlpr12 and ASC, thus supporting the inclusion of the NLRP12 inflammasome in the inflammasome family.

**F. NLRC4 Inflammasome**

The NLRC4 inflammasome is made of NLRC4 and caspase-1. Since NLRC4 possesses a CARD at the N terminus, interaction with ASC is not an absolute requirement for caspase recruitment, yet maximal production of IL-1β and IL-18 in response to different bacteria needs Asc, as shown by experiments in Asc⁻⁻ mice (Schröder and Tschopp, 2010). As for NLRP1, also in the case of NLRC4 deletion of LRR induces constitutive activation, further supporting an autoinhibitory role for this residue (Poyet et al., 2001). The best characterized activator of the NLRC4 inflammasome is bacterial flagellin, but endogenous cell cytoplasmic components have also been suggested to bind and activate NLRC4 (IPAF) (Mayor et al., 2007). Flagellin entry into the cell is very likely mediated by type III or IV secretion systems. However, bacteria lacking flagellin (i.e., *Shigella*) are also capable of activating the Nlr4 inflammasome (Davis et al., 2011). Additional data suggest that the NLRC4 is responsive to bacterial injection systems themselves, such as the rod protein component of TTSS (Miao et al., 2010). NLRC4 binds SGT1 and HSP90, probably keeping the inflammasome in an inactive but “competent” state, i.e., ready to shift into a fully active state upon binding with bacterial components (Mayor et al., 2007). The NLRC4 inflammasome plays a key role in protective responses against *Salmonella*, *Shigella*, *Pseudomonas*, and *Legionella* (Schröder and Tschopp, 2010).

The Nlr4 inflammasome is an interesting example of how mammalian NLRs might have exploited a recognition system based on a “modified self” similar to that present in plants to achieve a higher plasticity in the recognition of bacterial components. In fact, as anticipated above, coexpression of Naip5 is needed to make the Nlr4 inflammasome responsive to *Legionella* (Zamboni et al., 2006). The Naip5-Nlr4 recognition system seems to be involved in the pathogenesis of sepsis-like disease after gut injury in antibiotic-treated mice (Ayres et al., 2012). As with other inflammasome subtypes, the Nlr4 inflammasome is endowed with an ability to trigger IL-1β maturation and pyroptosis (Davis et al., 2011). The related protein Naip2 serves as a “Nlr4 inflammasome adaptor” for TTSS rod proteins like *Salmonella* PrgJ and *Burkholderia* BsaK (Zhao et al., 2011). On the other hand, human NAIP is the inflammasome receptor for the needle protein CPR1 from *C. violaceum*. Despite convincing evidence to support the role of NAIPs in conferring effector specificity to the NLRC4 inflammasome, details of this interaction are as yet obscure.

While ASC association to the NLRC4 inflammasome is needed for optimal IL-1β–IL-18 production, it is not needed for pyroptosis, which suggests that ASC might be a factor that redirects NLRC4 activity to specific functions. As for the other inflammasome subtypes, the mechanism of activation by PAMPs/MAMPs is a crucial and as yet unsolved problem. Furthermore, at variance from earlier evidence, it is now thought that K⁺ efflux is also a trigger for Nlr4 inflammasome activation (Arlehamn et al., 2010). A striking demonstration that the role of the Naip5-Nlr4 inflammasome in inflammation is much wider than so far anticipated is
provided by an elegant study by von Moltke et al. (2012b) showing that systemic Naip5-Nlr4 inflammasome activation by flagellin triggers a massive release of prostaglandins and leukotrienes (“eicosanoid storm”) and systemic inflammation independently of IL-1β and IL-18 release.

G. AIM2 Inflammasome

AIM2 is the best characterized non-NLRs scaffold component of the inflammasome (Fernandes-Alnemri et al., 2009; Hornung et al., 2009). The AIM2 protein bears an N-terminal pyrin domain and a C-terminal HIN200 domain. AIM2 recruits, possibly via ASC, and activates caspase-1. Stimulus for AIM2 activation is dsDNA binding to HIN200. It is understood that dsDNA binding drives AIM2 heteroduplex formation and ASC and caspase-1 recruitment. It is thought that AIM2 senses bacterial or viral DNA in the cytosol. Specifically, immunity against cytomegalovirus is faulty in Aim2−/− animals (Rathinam et al., 2010). AIM2 is also responsible for immunity against some cytoplasm-located intracellular pathogens such as Francisella tularensis. Aim2−/− mice develop a lower IL-1β–IL-18 titer, undergo a reduced extent of infected cell death, and readily succumb to the pathogen (Fernandes-Alnemri et al., 2010). Role of AIM2 against intracellular pathogens might be wider than previously thought. A study by Saiga et al. (2012) shows that clearance of M. tuberculosis by Aim2−/−-deficient macrophages is hampered, and infected animals develop a serious infection. Accordingly, IL-1 and IL-18 secretion is impaired, and mycobacterium DNA is found to localize to Aim2-containing cytoplasmic foci (Saiga et al., 2012). Recently, the AIM2 inflammasome has been implicated in a cross-talk between intracellular proinflammatory and proapoptotic pathways in response to infection with F. tularensis, as in caspase-1-deficient mice Aim2 triggers Asc-dependent activation of caspase-3, -8, and -9 and eventual apoptosis (Pierini et al., 2012). A role for AIM2-dependent cell death in restricting bacterial growth during infections has been postulated.

H. Pyrin Inflammasome

Pyrin is the product of the MEFV gene, responsible for the febrile disease named familial Mediterranean fever (FMF). It has been recently shown that transgenic (knock-in) mice for the pyrin gene harboring mutations responsible for FMF secrete large amounts of IL-1β in an Nlrp3-, Nlrp1-, or Aim2-independent fashion (Chae et al., 2011). This might be interpreted as a proof for the presence of an as yet to be identified inflammasome activated by pyrin or as a demonstration that pyrin itself can recruit ASC to form a caspase-1-activating complex (Mankan et al., 2012). The pyrin domain structure would allow this interaction as the pyrin molecule harbors an N-terminal PYD domain that permits ASC recruitment.

I. ASC

The adaptor protein ASC (previously known as CARD5, Pycard, TMS-1) has a central role in the activation of all inflammasome subtypes so far characterized, with the partial exception of NLRC4 (Schroder and Tschopp, 2010). The ASC gene, localized on chromosome 16p11.2-12, encodes a 22-kDa protein containing an aminoterminal PYD and a carboxyterminal CARD originally identified as the constituent of aggregates formed during retinoic acid-induced apoptosis (Masumoto et al., 1999). The key role of ASC in caspase-1 activation is clearly shown by failure of Asc−/− mice to secrete mature IL-1β and IL-18 and, by consequence, IFNγ (IL-18 is a potent inducer of IFNγ secretion from T lymphocytes). TNF and IL-6 release are unaffected. ASC is ideally suited to act as a “linker” between different intracellular sensors and the inflammasome-caspase-1 and thus bridge PAMP/MAMP/DAMP-sensing to initiation of inflammation. Given the broad sensitivity of the NLRP3 inflammasome (which is crucially dependent on ASC coupling for caspase-1 recruitment and activation) to a wide range of “danger” molecules, it can be suggested that ASC couples several (potentially all) distress conditions to inflammation.

It has been shown that in resting macrophages ASC is sequestered in the nucleus, only to be released into the cytoplasm upon macrophage interaction with pathogens (Bryan et al., 2009). In the cytosol ASC forms caspase-1-containing perinuclear aggregates. Co-localization of NLRP3 with ASC-containing aggregates has also been occasionally shown (Bryan et al., 2009). Finally, formation of ASC-containing aggregates is necessary for IL-1β maturation. Although the mechanism of ASC redistribution and the physiologic significance are not fully understood, it is likely that this nucleus-cytosol shift represents an additional checkpoint in the activation of inflammation. Additional roles for ASC, in addition to caspase-1 recruitment and activation, have been described, such as participation in NF-κB activation, in caspase-8-dependent apoptosis, and in antigen presentation and lymphocyte motility (Masumoto et al., 1999; Ippagunta et al., 2011). These latter inflammasome-independent ASC functions were shown to be mediated by the guanine nucleotide exchange factor protein Dock-2 and by the small GTPase Rac. Other inflammasome-independent effector functions of ASC are mediated via the phosphatase DUSP10/MKP5 and by MAPK (Taxman et al., 2011). These findings show that participation of ASC to immune cell responses is not restricted to IL-1β and IL-18 processing, but rather extend to antigen presentation, cell motility, and chemokine release (Hassan and Amer, 2011).
L. Caspase-1

Caspase-1 is a cysteine protease, i.e., an enzyme characterized by having a cysteine in the active site, belonging to the subfamily of inflammatory caspases, which is also comprised of human caspase-4, -5, and -12 and murine caspase-11 and -12. It is present in the cytoplasm as an inactive procaspase of about 45 kDa that has to be cleaved to generate active caspase-1. The active caspase is a heterodimer made by the assembly of a 10- and a 20-kDa cleavage fragments. Procaspase-1 is recruited within the inflammasome complex by interaction with the CARD domain of the scaffold proteins (e.g., NLRP1) or via the adaptor factor ASC and within the inflammasome undergoes auto-proteolysis. Caspase-1 was originally termed IL-1-converting enzyme (ICE) for its prominent activity in the conversion of pro-IL-1β into mature IL-1β and is considered the founding father of the caspase family (Broz et al., 2010). Caspase-1, like the other caspases, cleaves its substrates after an aspartic acid residue, specifically recognizing the Trp-Glu-His-Asp motif, a feature of great interest for the design of novel drugs targeting this enzyme.

M. Noncanonical Activation of Inflammatory Caspases

Caspase-11 is a murine inflammatory caspase recently implicated in pyroptosis triggered in response to E. coli, Vibrio cholera, and the B subunit of cholera toxin (Kayagaki et al., 2011; Broz et al., 2012). Interestingly, although caspase-11 can trigger pyroptosis, it is unable to trigger IL-1β release unless the NLRP3 inflammasome is also activated (Broz and Monack, 2013). The mechanism of caspase-11 activation is unclear as pyroptosis caused by activation of known inflammasomes subtypes does not seem to require caspase-11. Recent data by Broz et al. (2012) suggest that a coordinate activity of caspase-1 and caspase-11 is necessary to achieve efficient elimination of bacterial infections (e.g., Salmonella) and to limit inflammatory host damage. These authors have shown that caspase-1-deficient mice were strikingly susceptible to S. tiphimurium infection and were protected by concomitant deletion of the casp-11 gene (Broz et al., 2012). Relevance to human pathology is uncertain because humans do not have any direct ortholog of caspase-11.

IV. Is There a Logic in Inflammasome Organization?

Inflammation is the most important host response to any agent that threatens internal homeostasis. The weaponry that can be mobilized is ample and potentially very dangerous for the host itself, therefore the ignition phase of inflammation must be accurately controlled. Interleukin-1β is a key proinflammatory cytokine that affects basically all aspects of inflammation and immunity, from leukocyte diapedesis to ROS production, from antigen presentation to Th17 lymphocyte differentiation, from phagocytosis to fever (Dinarello, 2009, 2011) Therefore, tight control of its production is required at the transcriptional, translational, and release level. Interleukin-1β is a peculiar cytokine in that it lacks a typical signal sequence targeting the secretory system and it is therefore exported from the cells via an unusual, vesicle-mediated pathway. In response to endogenous or exogenous inflammatory agents, IL-1β is synthesized as a procytokine (pro-IL-1β) of molecular mass of ~34 kDa. Pro-IL-1β is then processed by caspase-1 to generate the 17-kDa biologically active mature cytokine. The mechanism of secretion is not fully understood, but the prevailing current opinion holds that mature IL-1β is loaded into vesicles, budding from either the plasma membrane or the lysosomal apparatus (Andrei et al., 1999; MacKenzie et al., 2001; Pizzirani et al., 2007), that are then shed into the pericellular space. Pro-IL-1β maturation and IL-1β-loaded vesicle release might be intimately linked because the P2X7R receptor (see below) is a very potent stimulus for both processes (Ferrari et al., 2006).

Other than IL-1β, caspase-1 is also responsible for processing IL-18, another proinflammatory cytokine that has a key role in T lymphocyte stimulation and IFN-γ release (Smith, 2011). Uncontrolled IL-1β release may have dire effects and can also precipitate shock. Therefore processes responsible for its maturation must be tightly controlled but at the same time must be readily available for activation when required.

Functions of caspase-1 are not restricted to IL-1β and IL-18 maturation but extend to control of an unconventional type of programmed cell death named pyroptosis (Miao et al., 2011). Pyroptosis is a form of cell death, often found during bacterial infections, that shares features of apoptosis (DNA fragmentation) and necrosis (brake-down of plasma membrane, release of intracellular content, and therefore stimulation of cytokine release and inflammation) (Miao et al., 2011). Pyroptosis might be considered a sort of “ultima ratio” of a cell that is unable to get rid of an intracellular pathogen and, therefore, suppresses itself but at the same time by igniting inflammation, alerts the body of the presence of the infectious agent. All the inflammasome subtypes so far known recruit caspase-1 either directly or indirectly via ASC (Mankan et al., 2012).

V. Mechanism of Activation of the Inflammasome

Although individual inflammasome subtypes are activated by specific triggers, because physical interaction with the different triggers has been demonstrated only for some inflammasome subtypes and individual inflammasomes may be activated by multiple agents, it is reasonable to think that all inflammasomes may share a common intracellular signal that causes assembly and activation (Fig. 3). A first level of control is
exerted by limiting availability of inflammasome components in resting cells. It is reported that while ASC and procaspase-1 are readily available, even in the absence of inflammatory stimuli, NLRP3 is normally expressed at very low levels, unless TLRs are engaged by a bacterial factor (e.g., LPS) or other PAMPs/MAMPs or DAMPs are sensed by the cell (Hornung and Latz, 2010). However, it should be kept in mind that a basal inflammasome activity occurs in quiescent immune cells, leading to ground level caspase-1 autocatalysis. Such a steady, basal activity enables a faster response to pathogens or endogenous DAMPs and a tighter regulation of inflammasome activity. Furthermore, the identification of endogenous inhibitors clearly suggests that cells have evolved a complex array of “throttles and brakes” to modulate the inflammasome. Given the function of the inflammasome as a sensor of foreign microorganisms and cellular stress, most models for its activation are based on its ability to recognize direct or indirect stress signals (Ogura et al., 2006; Strowig et al., 2012). A main difficulty in this respect is that direct interaction of PAMPs/MAMPs or DAMPs has been shown so far only for a few compounds, such as thioredoxin-interacting protein (TXNIP), dsDNA, or the cytoplasmic dsRNA-dependent protein kinase, also known as protein kinase R (PKR). However, the list of ligands for which there is convincing evidence of direct binding to NLRs is quickly growing (Monie, 2013).

It has been shown that the Nlrp3 inflammasome can be activated by ROS and that ROS scavenging is inhibitory (Meissner et al., 2008). However, the source and precise role of ROS in inflammasome activation are kind of dubious as absence of the main immune cell source of ROS, the NADPH oxidase, does not impair NLRP3 inflammasome activation (Meissner et al., 2010), and on the contrary knocking-down mitochondrial respiration inhibits Nlrp3 inflammasome function (Nakahira et al., 2011). Recently, it has been proposed that TXNIP might be the intermediary linking ROS generation to the NLRP3 inflammasome (Zhou et al., 2010; Xiang et al., 2011). Thioredoxin (TXN) belongs to a family of small redox-sensitive ubiquitous proteins found in many organisms, from bacteria to animal cells. TXN participates in the formation of a network of redox signaling pathways crucial to the regulation of cell metabolism, proliferation, differentiation, and apoptosis (Lee et al., 2013b). ROS generation causes dissociation of TXN from TXNIP, which is thus free to interact with NLRP3 and trigger inflammasome assembly. A key role of TXNIP in this process is shown by the finding that TXNIP−/− mice showed impaired activation of the Nlrp3 inflammasome (Zhou et al., 2010). Given the involvement of TXNIP in insulin resistance, these observations have highlighted the potential role of inflammasomes in metabolic diseases (Schröder et al., 2010). It is hypothesized that metabolic stress (hyperglycemia) releases TXNIP from TXN and triggers NLRP3-inflammasome-dependent IL-1β secretion, which is the final cause of pancreatic β-cell failure.

It is possible that ROS act at multiple levels in NLRP3 inflammasome activation, i.e., at the level of NLRP3 gene expression and as final triggers of inflammasome activation (Bauerfeind et al., 2011). It has been shown that lysosomal destabilization causes Nlrp3 inflammasome activation, and cathepsin B has been identified as the triggering agent (Halle et al., 2008; Hornung et al., 2008). It is proposed that lysosomal destabilization and/or neutralization of intralysosomal pH, even without overt rupture of the lysosomal membrane, are sufficient to cause cathepsin B release (Hoegen et al., 2011). The hypothetical mechanism of action of cathepsin B is unclear, although it is proposed that this enzyme might cause proteolytic inactivation of some NLRP3 inhibitory factor, or stimulation of a NLRP3 activator (Strowig et al., 2012). However, it has also been reported that cathepsin B-deficient mice show no inhibition of inflammasome activation in response to C. albicans or malarial hemozoin (Dostert et al., 2009; Gross et al., 2009). While direct interaction of PAMPs/MAMPs or DAMPs with NLRP3 is still unclear, there is convincing evidence that three other scaffold proteins, NLRP1, NAIP, and AIM2 can directly bind their cognate ligands, MDP, flagellin, and dsDNA, respectively (Strowig et al., 2012).

In the case of activation of the NLRP1 inflammasome, a proteolytic mechanism of activation has been suggested. The only identified activator of Nlrp1 is the metalloprotease LT of B. anthracis, but its mechanism has remained elusive. Levinsohn and collaborators (2012) have shown that LT cleaves Nlrp1 in the N-terminal domain, and this results in inflammasome stimulation. However, how proteolysis triggers Nlrp1 activity has not been determined.

One of the most heavily investigated DAMPs is the HMGB1 protein, which is released by dsRNA or the mimetic agent poly(I:C) via an inflammasome-requiring pathway (Lamkanfi et al., 2010; Lamkanfi and Dixit, 2011). A recent study has shown that PKR, an intracellular kinase activated by HMGB1, is also activated by other prototypical DAMPs such as ATP, MSU, adjuvants, and E. coli; undergoes autophosphorylation; activates the inflammasome; and triggers IL-1β and IL-18 secretion (Lu et al., 2012). PKR was found to physically interact with Nlrp1, Nlrp3, Nlrc4, and Aim2. Interestingly, inflammasome activity could be reconstituted in a cell-free system with recombinant Nlrp3, Asc, and procaspase 1.

Phosphorylation reactions might have a broader significance in inflammasome activation given the recent demonstration by Dixit and co-workers that phosphorylation on a serine residue located between the NACHT and LRR domains of Nlrc4 by protein kinase Cδ is critical for NLRC4 inflammasome activity. Mutants defective in Nlrc4 phosphorylation were unable to recruit
procaspase-1 and form specks in response to *S. tiphimurium* infection (Qu et al., 2012).

Despite circumstantial evidence for direct activation of inflammasomes by a handful of ligands, the most common mechanism of activation shared by almost all inflammasome subtypes is the change in the ionic composition of the inflammasome microenvironment (Davis et al., 2011). A decrease in the peri-inflammasome K⁺ concentration is a powerful and fast stimulus for inflammasome assembly and procaspase-1 cleavage, as well as apoptosome aggregation and caspase-3 activation (Hughes et al., 1997; Cheneval et al., 1998; Yu et al., 1999; El Kebir et al., 2006). In vitro experiments show that the K⁺ threshold for inflammasome inhibition is in the 70 mM range (Petrilli et al., 2007). This seems to be a common feature of inflammatory and apoptotic caspases (Karki et al., 2007; Arlehamn et al., 2010). The trigger role of K⁺ depletion in caspase-1 activation was known long before the inflammasome was discovered thanks to the pioneering experiments of Gabel and co-workers who showed that the K⁺ selective ionophore nigericin, but not the Na⁺ selective ionophore monensin,
induced nonlytic release of 17-kDa IL-1β from mouse macrophages and human peripheral monocytes (Perregaux et al., 1992). Two years after, again Gabel and his coworkers showed that extracellular ATP was an extremely potent stimulus for IL-1β processing and release and that K⁺ efflux was the trigger (Perregaux and Gabel, 1994). With the identification and cloning of P2X7R, the plasma membrane pathway responsible for the large ATP-stimulated outward K⁺ fluxes became obvious (Di Virgilio et al., 1996; Surprenant et al., 1996). The P2X7R was soon identified as the sole P2 receptor coupled to IL-1β and IL-18 processing and release (Ferrari et al., 1997; Perregaux et al., 2000; Sanz and Di Virgilio, 2000; Mehta et al., 2001) and in fact as one of the most potent inducers of the maturation and release of these cytokines. The ability to sense the ionic composition of the intracellular environment provides a straightforward system for inflammasome activation, as virtually all pathogens or injurious chemical compounds (such as environmental pollutants and endogenous crystals) upset intracellular ion homeostasis causing an unbalance in transmembrane ion distribution. Early plasma membrane damage may participate in inflammasome activation by at least two main mechanisms: (1) by causing a drop in the cytoplasmic K⁺ concentration and (2) by inducing ATP release and the activation of an autocrine loop of cell stimulation mediated via the P2X7R. Several groups have reported that K⁺ efflux is necessary for Nlrp3 inflammasome activation by diverse stimuli such as MSU, PGN, R837 (an imidazoquinoline compound mimicking ssDNA or ssRNA), E. coli, LT of B. anthracis, L. monocytogenes, and S. aureus toxin (Mariathanasan et al., 2006; Petrilli et al., 2007; Gross et al., 2011). S. tiphimurium and S. flexneri, targeting the Nlrc4 inflammasome, seem not to be sensitive to the K⁺ concentration (Petrilli et al., 2007), although more recently activation of the Nlrc4 inflammasome by S. tiphimurium was also shown to be K⁺ sensitive (Arlehamn et al., 2010). Analogously, activation of the Nlrp3 inflammasome due to particulate material such as asbestos or MSU is blocked by increasing the extracellular K⁺ concentration, a maneuver that prevents intracellular K⁺ decrease (Dostert et al., 2008). It might be hypothesized that a localized drop in intracellular K⁺ is perceived by eukaryotic cells as a worrisome indicator of plasma membrane injury and potentially irreversible cell damage requiring a prompt defensive response (inflammasome activation), which may or may not be followed by outright cell suppression (pyroptosis). This K⁺ efflux-based, very sensitive danger detection system is tightly coupled to the P2 receptors (P2Rs), an additional very efficient, signaling system based on the ability to sense and decode the ATP concentration in the tissue microenvironment.

It is well known that the extracellular ATP concentration is negligible in healthy tissues (in the low nanomolar range), but it is equally well known that the extracellular ATP levels may rise to the high micromolar level following tissue damage at sites of inflammation or within the tumor microenvironment (Pellegratti et al., 2008; Wilhelm et al., 2010; Michaud et al., 2011). ATP itself is a very efficient extracellular distress signal because, with the extracellular level normally very low, even minute leaks can increase the pericellular concentration several fold. Furthermore, being highly hydrophilic, ATPdiffuses rapidly through the aqueous extracellular milieu where its presence is quickly detected by an array of selective receptors with affinities covering the full range of ATP concentrations found in physiologic and pathologic conditions, i.e., from the low nano to the high micromolar level. Last but not least, the ubiquitous presence of ecto-ATPases (CD39 and CD73) allows quick termination of the ATP signal.

In the P2R family, a special role in danger sensing and in the activation of cell defense (or cell death) is played by the P2X7R subtype. This receptor has a special place in inflammasome biology because its stimulation is still used as one of the most potent and reliable means to activate the NLRP3 inflammasome. On the contrary, P2X7R seems to have no role in the activation of other inflammasome subtypes such as Nlrp1, Nlrc4, and Aim2 (Franchi et al., 2007) despite their dependence on the K⁺ concentration (Gross et al., 2011). The P2X7R is an homotrimeric ATP-gated plasma membrane cation-selective channel made by the assembly of three P2X7 subunits endowed with the peculiar property to undergo a transition to a non-selective pore when exposed to elevated extracellular ATP concentrations. Opening of the nonselective P2X7R pore causes a large outwardly directed K⁺ flux as well as transmembrane fluxes of aqueous solutes of molecular weight up to 900 Da. This will not only cause uptake of small hydrophilic, normally membrane impermeant molecules but also release of small cytoplasmic solutes, among which is ATP. Nine human and one mouse P2X7 subunit splice variants have so far been identified, the canonical, full-length P2X7 being identified as isoform A (P2X7A). Since ATP itself is the P2X7R agonist, P2X7R opening is likely to initiate a process of auto-amplifying cell stimulation that, if not aborted by removing extracellular ATP or by shutting off P2X7R, may lead to extensive cell death in inflamed tissues (Di Virgilio et al., 1998; Wang et al., 2004a). This autocrine/paracrine process of cell stimulation is likely to have an important role as one of the early events that spreads and amplifies inflammation. Dampening of inflammation by supplementation of apyrase, a potato ortholog of mammalian CD39, by P2X7R blockade, or p2x7 deletion, is a strong proof of the role of ATP as a proinflammatory mediator and P2X7R as a proinflammatory receptor (Idzko et al., 2007; Wilhelm et al., 2010).

The P2X7R is ideally suited to respond to unusual, pathologic ATP tissue levels because of its very high
EC\textsubscript{50} for ATP (between 100 and 300 µM) and lack of desensitization. These features allow P2X7R to open most frequently when extracellular ATP is elevated above physiologic levels. The pore function is closely associated with P2X7R and to its pathophysiological functions. It is widely held that all relevant P2X7R-associated responses, i.e., NLRP3 inflammasome activation and cytotoxicity, depend on and require the pore function. Generally speaking, this is correct. However a word of caution is necessary because on the one hand we know that human immune cells express at least one truncated P2X7 splice variant, i.e., a P2X7 subunit lacking the 230 C-terminal amino acids named P2X7B, which is coupled to K\textsuperscript{+} fluxes and that might also participate in inflammasome activation, and on the other hand there is clear evidence that pore formation might not by itself be sufficient to trigger cell death (Murgia et al., 1992; Raffaghello et al., 2006). To further stress the complex role of P2X7R in cell physiology, there is now clear evidence that while stimulation of this receptor by pathologically elevated ATP levels triggers inflammation, tonic, basal level activation by endogenously released ATP on the contrary produces a strong trophic effect on cell metabolism and growth (Adinolfi et al., 2005, 2009, 2012). This multifaceted activity makes P2X7R an attractive target for the development of novel anti-inflammatory and anticancer drugs.

In consideration of the pivotal role of K\textsuperscript{+} efflux in the activation of at least four inflammasome subtypes (NLRP1, NLRP3, NLRC4, and AIM2) (Gross et al., 2011), it not surprising that close attention has been paid to the molecular identification of the pathways mediating K\textsuperscript{+} fluxes. A few years ago the plasma membrane channel pannexin-1 (panx-1) was tentatively identified as the P2X7R nonselective pore (Pelegrin and Surprenant, 2006). Accordingly, several bacterial products such as LPS, lipid A, Pam3, PGN, MDP, lipoteichoic acid, E. coli RNA and DNA, and zymosan were shown to require panx-1 for NLRP3 inflammasome stimulation (Kanneganti et al., 2007b; Lamkanfi et al., 2009a). However later experiments showed that panx-1 is dispensable for inflammasome activation by ATP, nigericin, alum, silica, flagellin, and cytoplasmic DNA (Qu et al., 2011). In addition, although panx-1 inhibition can under certain conditions impair P2X7R-dependent transmembrane ion fluxes, deletion of the panx-1 gene does not impair P2X7 responses (Qu et al., 2011; D. Cavagna and F. Di Virgilio, unpublished results). On the other hand, panx-1 inhibition was shown to decrease ATP-stimulated IL-1\textbeta release (Murphy et al., 2012), and panx-1 levels were shown to change in parallel with those of Nlrp3 and IL-1\textbeta in mice with steatohepatitis (Csak et al., 2011). Furthermore, an in vivo study by Riteau and co-workers (2010) has shown that both P2X7R and panx-1 are necessary for full blown inflammation and fibrosis in mice treated with bleomycin. Therefore, it is reasonable to conclude that panx-1 certainly has an important function in the early phases of inflammation but is not an obligatory component of the chain of events leading to inflammasome activation.

Extracellular ATP and the P2X7R are also important in inflammasome activation as triggers of ROS generation (Cruz et al., 2007; Pfeiffer et al., 2007). Many agents reported to drive NLRP3 activation via ROS generation are also potent triggers of ATP release (Aβ, hyperglycemia, skin irritants, infections, ultraviolet light) (Mizumoto et al., 2002; Solini et al., 2004; Inoue et al., 2007; Sanz et al., 2009; Weber et al., 2010). Thus, the increase in the extracellular ATP concentration following cell and tissue damage is likely to be a major contributing factor to ROS generation in response to a host of injurious stimuli. It might be worth mentioning that numerous inflammasome activators (uric acid, silica, alum) are also potent inducers of ATP and other nucleotide release (Riteau et al., 2012).

Recently, cell volume regulation has been shown to modulate the NLRP3 inflammasome. Pelegrin and co-workers reported that cell swelling caused by a decrease in extracellular osmolarity triggered NLRP3 inflammasome stimulation, caspase-1 activation, and IL-1\textbeta release via a mechanism involving K\textsuperscript{+} depletion and the regulatory volume decrease response (Compan et al., 2012). These observations were confirmed in vivo in a rat brain-injury model where kainic acid was perfused into the hippocampus to cause cell swelling. Kainic acid perfusion triggered a large IL-1\textbeta release that was reduced by perfusing a hypertonic sorbitol solution after kainic acid.

The role of cations in NLRP3 inflammasome activation might be more widespread than currently thought as in a recent study Lee and co-workers (2012) showed that extracellular Ca\textsuperscript{2+} triggers NLRP3-inflammasome-mediated IL-1\textbeta release via stimulation of plasma membrane Ca\textsuperscript{2+}-sensing receptor. Ca\textsuperscript{2+}-sensing receptor was shown to trigger an inositol 1,4,5-trisphosphate-mediated increase in cytoplasmic Ca\textsuperscript{2+} and inhibition of adenylate cyclase. Thus, inflammasome stimulation was the result of the concomitant Ca\textsuperscript{2+} increase and cyclic AMP decrease.

Ubiquitination/deubiquitination reactions might also have an important role in the control of inflammasome activation. Small molecular weight inhibitors of deubiquitinate enzymes were shown to inhibit NLRP3 (and to a smaller extent AIM2) inflammasome association, caspase-1 activation, and IL-1\textbeta release (Lopez-Castejon et al., 2013).

VI. The Inflammasome and Autophagy/
Mitophagy

Autophagy is an evolutionary-conserved cellular process involved in clearance of damaged intracellular
organelles, protein turnover, and disposal of intracellular parasites. Since elimination of degraded or misfolded intracellular material prevents extracellular release of potential DAMPs, autophagy is thought to have an important function as a negative regulator of inflammation (Deretic, 2012). Damaged mitochondria are also cleared by insertion into autophagic vacuoles, via a specialized form of autophagy often referred to as “mitophagy.” In view of the “anti-inflammatory” role of autophagy/mitophagy, it is not surprising that it is generally thought that this process is inhibitory for inflammasome activity. Autophagy removes “cytosolic DAMPs,” such as degraded intracellular organelles, damaged nucleic acids, or misfolded proteins, that might stimulate inflammasome activity. This “scavenging” activity is even more relevant in reference to the mitochondria. Injured mitochondria release high amounts of ROS as well as mitochondrial DNA, both agents being potent stimulators of the Nlrp3 inflammasome (Nakahira et al., 2011; Zhou et al., 2011). Rather contradictory with its hypothesized anti-inflammatory function, autophagy stimulation has also been directly implicated in the process of IL-1β release (Dupont et al., 2011; Deretic et al., 2012). It was previously reported that loss of the autophagy protein Atg16L1 induces an increase in IL-1β production (Saitoh et al., 2008), a finding that supports the anti-inflammatory function of autophagy. More recently, Deretic and collaborators have rather surprisingly shown that stimulation of autophagy accelerates release of mature IL-1β via a novel secretory pathway (Dupont et al., 2011; Deretic et al., 2012).

The process of extracellular IL-1β delivery is as yet poorly understood: this cytokine accumulates in the cytosol as pro-IL-1β, which is then cleaved into a IL-1β pro-piece and mature IL-1β following inflammasome-dependent caspase-1 activation. However, mature IL-1β lacks a secretory piece to allow its targeting to the cellular secretory apparatus and final extracellular delivery. Thus how it crosses the plasma membrane is obscure. A number of vesicular non-mutually exclusive pathways for IL-1β secretion have been proposed: (1) exocytosis of granules derived from lysosomes (Gardella et al., 2001; Andrei et al., 2004); (2) release of exosomes derived from multivesicular bodies (Qu et al., 2007); and (3) release of plasma membrane-derived microparticles (MacKenzie et al., 2001; Pizzirani et al., 2007; Turolla et al., 2012). Recently, Deretic and collaborators have suggested that an autophagy-based, inflammasome-associated unconventional secretion (named “autosecretion”), independent of the endoplasmic reticulum and Golgi apparatus, might be responsible for release of leaderless proteins (such as IL-1β) (Dupont et al., 2011; Deretic et al., 2012). These studies are of relevant interest because they might help clarify whether the inflammasome participates in IL-1β secretion other than being involved in processing. It is well known that pro-IL-1β is not normally secreted and that mature IL-1β is quickly released. However, whether the inflammasome components also take part in further steps involved in IL-1 secretion (e.g., IL-1β packaging into exosomes or microparticles) is unknown. Stimulation of inflammasome activity during autophagy might not only depend on the activity of an intrinsic autophagic pathway but also on the extrinsic stimulation by bystander cells undergoing autophagy. It has been shown that autophagy-competent cells have a higher ATP release rate than autophagy-deficient cells (Michaud et al., 2011) and, accordingly, that cells undergoing autophagy release large amounts of ATP that trigger P2X7R-dependent IL-1β release (Ayna et al., 2012). These findings show that autophagy may play both a negative and a positive role in the activation of the inflammasome and of the overall inflammatory response, depending on the specific conditions. Our understanding of factors determining these opposite choices is however at a very preliminary stage.

VII. Negative Regulation of the Inflammasome

Every tightly regulated homeostatic process includes feedback mechanisms used to prevent unnecessary activation or to dampen an exaggerated response. Inflammation is kept in check by a host of soluble factors or cellular elements that counterbalance the effect of exogenous or endogenous proinflammatory agents. Furthermore, ability to shut down inflammation is so crucial in host-pathogen interactions that many infectious agents have “learned” to synthesize and release anti-inflammatory factors. Immune cells themselves have an early anti-inflammatory, autoinhibitory, safeguard system based on the synthesis of alternatively spliced inflammasome components (Davis et al., 2011). NLRP10 was reported to inhibit caspase-1-mediated IL-1β secretion and ASC-mediated NF-κB activation (Wang et al., 2004b). Accordingly mice engineered to systemically overexpress Nlrp10 were resistant to endotoxic shock. Among other NLR proteins, Nlrp1 and Nod2 are alternatively spliced (Hlaing et al., 2001; Rosenstiel et al., 2006), producing proteins that might serve as negative regulators (dominant negatives?) of the activity of their full-length isoform. Naturally occurring isoforms of NLRP3 with NF-κB inhibitory activity have also been described, but their effect on inflammasome function has not been reported (O’Connor, et al., 2003). Three novel ASC isoforms, ASC-b, ASC-c, and ASC-d, were recently described in human THP-1 cells. Of these isoforms, ASC-c colocalized with and inhibited caspase-1 (Bryan et al., 2010). Colocalization or interaction with NLRP3 was not observed.

Among endogenous inhibitors of the inflammasome, we should not neglect alternatively spliced isoforms of the P2X7 subunit (Cheewatrakoolpong et al., 2005; Nicke et al., 2009). The mouse isoform, which is
truncated in the C-terminal tail, showed reduced trafficking to the plasma membrane and smaller agonist-evoked currents. When coexpressed with the full-length variant this isoform acted as a dominant negative (Masin et al., 2012). Functional responses of truncated P2X7R human variants were also investigated. While there is consensus on their inability to generate the large conductance P2X7R pore, one study reported that a truncated isoform, P2X7j, when coexpressed with the full-length P2X7A variant acts as a dominant negative (Feng et al., 2006); another study surprisingly showed that another truncated variant, P2X7B, enhances all P2X7R-dependent responses (Adinolfi et al., 2010). However, it is not known whether any of these variants affects inflammasome activity and IL-1β processing.

Among other proteins extrinsic to the inflammasome, an inhibitory function has been suggested for pyrin only proteins (POPs) and CARD-only proteins that might act as decoy receptors for ASC and caspase-1, respectively (Dorfleutner et al., 2007; Stehlik and Dorfleutner, 2007). Due to its implication in the pathogenesis of FMF, the best known protein with purported negative modulatory activity of the inflammasome is pyrin, the product of the \textit{MEFV} gene (Kastner, 2005; Kastner et al., 2010; Savic et al., 2012). However, the mechanism of action of pyrin is far from being understood, to the point that until recently there was equal support for a negative as well as for a positive role of pyrin in inflammasome modulation (Chae et al., 2006; Masters et al., 2006; Yu et al., 2007). More recently studies with genetically engineered mice have shed some light on this issue.

Kastner and co-workers have generated pyrin-deficient mice as well as “knock-in” mice bearing a mutated B30.2 pyrin domain implicated in FMF patients (Chae et al., 2011). Pyrin-deficient animals showed no clinical features of FMF, while those harboring the mutated copies showed signs consistent with the human disease, enhanced IL-1β release included. Surprisingly, the enhanced IL-1β release activity of the mice expressing mutated pyrin was shown to depend on Asc, but not Nlrp3 expression, a finding that lead the authors to postulate the existence of a previously unrecognized Asc-dependent, Nlrp3-independent inflammasome activated by pyrin. This finding clarifies the role of pyrin in the pathogenesis of FMF and in the regulation of inflammasome activity and provides definitive proof for the role of excessive IL-1β production in the pathogenesis of FMF.

Inhibitors of serine proteinases (serpins) have also been shown to act as endogenous inhibitors of caspase-1 (Young et al., 2000). Finally, the antiapoptotic proteins Bel-2 and Bel-X\textsubscript{L} interact with NLRP1, prevent its binding to ATP, and inhibit caspase-1 activation (Faustin et al., 2009).

Pathogens have evolved several inflammasome-targeted inhibitory factors (Taxman et al., 2010; von Moltke et al., 2012a). Mixoma virus and pox Shope fibroma virus produce proteins (vPOP) homologous to host POP (cPOP). In addition, poxviruses encode serpin-like proteins, such as cytokine response modifier A, that abrogate caspase-1 proteolytic activity and therefore prevent IL-1β release (Ray et al., 1992). A protein, NS1, encoded by an influenza A virus member of the \textit{Orthomyxoviridae} family and a protein expressed by baculoviruses, p35, are reported to prevent IL-1β and IL-18 secretion and are putatively thought to interact with the inflammasome. Several bacteria possess efficient systems to prevent inflammasome activation. \textit{Pseudomonas aeruginosa} and \textit{Yersinia} exploit TTSS to inject into the host cell inflammasome-inhibitory factors (ExoU, YopE, YopT, YopK). \textit{F. tularensis} produces two factors, RipA and MviN, that prevent caspase-1 activation (Huang et al., 2010; Ulland et al., 2010). Infection by \textit{M. tuberculosis} and \textit{Mycoplasma bovis} causes a modest release of IL-1β. This appears to be due, at least in the case of \textit{M. bovis}, to release of a factor, a metalloproteinase encoded by the \textit{zmp1} gene that inhibits IL-1β processing (Master et al., 2008). \textit{S. pneumoniae} produces a cholesterol-dependent pore-forming toxin, pneumolysin, that inhibits caspase-1 activation and IL-1β release (Littmann et al., 2009). Surprisingly, other pore-forming bacterial toxins on the contrary are potent inflammasome activators.

One of the most intriguing recently described inhibitors of the NLRP3 inflammasome is NO. Hernandez-Cuellar and co-workers (2012) showed that IFN-β and LPS pretreatment downmodulated Nlrp3 inflammasome activity by stimulating generation of endogenous NO. This inhibitory activity might have a crucial pathophysiological relevance, because during \textit{M. tuberculosis} infection endogenously generated IFN-γ inhibits inflammasome function and IL-1β release via NO-mediated thiol nitrosylation of NLRP3 (Mishra et al., 2013).

Recently, a myeloid-specific miRNA, miR-223, was shown to downmodulate the NLRP3 inflammasome (Bauernfeind et al., 2012; Haneklaus et al., 2012). Furthermore, it was also shown that an Epstein-Barr virus-specific miRNA, miR-BART15, can be transferred into noninfected cells by exosomes derived from infected cells to inhibit NLRP3 inflammasome activity (Haneklaus et al., 2012).

**VIII. Inflammasome and Diseases**

**A. Cryopyrinopathies**

Discovery of human diseases caused by inflammasome dysregulation has been instrumental for our understanding of inflammasome function. Three monogenic intrinsic inflammasomopathies due to autosomal dominant mutations in the NLRP3/CIA51 gene [cryopyrinopathies or cryopyrin-associated periodic syndromes (CAPS)] have been described so far: familial cold-induced autoinflammatory syndrome (FCAS),
Muckle-Wells syndrome (MWS), and neonatal onset multisystem inflammatory disorder (NOMID) otherwise known as chronic infantile neurologic cutaneous and articular (CINCA) syndrome (Kastner, 2005; Kastner et al., 2010; Savic et al., 2012). These three rare diseases of childhood onset share clinical manifestations and are likely to represent a continuum of increasing severity, where FCAS is the mildest and NOMID the most severe. Clinical manifestations are due to dysregulated IL-1β release and include periodic fever, rash, arthralgia, cold sensitivity, conjunctivitis, and aseptic meningitis. Mutations in the NLRP3/CIA51 gene are detected in 55–60% of CAPS patients. The mutated NLRP3 protein seems to have an increased tendency to spontaneous oligomerization, thus causing an enhanced NLRP3 inflammasome activation and IL-1β release (Savic et al., 2012). The human disease is replicated in two knock-in mouse models harboring two mutations associated with FCAS and Muckle-Wells syndrome (Brydges et al., 2009; Meng et al., 2009). Phenotype of the mice was characterized by formation of skin abscesses and erythema and increased IL-1β production. Myeloid cells from these mice did not spontaneously secrete IL-1β, but were exceedingly sensitive to stimulation with PAMPs/MAMPs. Intrinsic inflammasomopathies show that at least the NLRP3 inflammasome can undergo autoactivation, thus stressing the need for a tight regulation to prevent inappropriate release of IL-1β.

In addition to NLRP3, another NLR protein, NLRP12, has been implicated in the pathogenesis of periodic fever syndromes. NLRP12 is a negative regulator of NF-κB activity, which has only very recently been associated with the inflammasome (Vladimer et al., 2012). In 2008, a periodic fever syndrome was described due to mutations in the NLRP12 gene (Jeru et al., 2008). Patients affected by these mutations have attacks of urticarial rash, arthralgia, and myalgia triggered by exposure to cold. Due to its similarity to FCAS, this syndrome was named FCAS2 (Savic et al., 2012). As more patients with mutated NLRP12 are found, the mechanism by which mutations in this NLR affect the immune response becomes more complex. According to studies by Jeru et al. (2011) and Borghini et al. (2011), the caspase-1/IL-1β pathway might also be affected by a dysfunctional NLRP12, although response to IL-1β blockers is not as satisfactory as for classic CAPS.

B. Familial Mediterranean Fever

FMF is a periodic fever syndrome common among people of Armenian, Arab, Jewish, or Turkish ancestry and within other populations originating from countries lining the Mediterranean basin (The International FMF Consortium, 1997; French FMF Consortium, 1997). Its manifestations include recurrent fever episodes, sterile serositis, monoarthritis, and splenomegaly. Amyloidosis is a frequent complication in untreated patients. FMF is due to mutations in the MEFV gene that encodes pyrin, previously also known as marenostrin (French FMF Consortium, 1997). FMF is commonly classified as an autosomal recessive disease; however genetic analysis reveals that often only one allele is mutated in affected individuals. This may suggest that FMF could be due to MEFV haploinsufficiency or to a dominant-negative effect or to a dose-dependent gain of function (Park et al., 2012). Wild-type pyrin can accelerate procaspase-1 cleavage in an ASC-dependent fashion (Yu et al., 2006), thus functioning as a proinflammatory factor. However, it has also been reported that pyrin can inhibit the inflammasome (Chae et al., 2006; Seshadri et al., 2007). More recent findings obtained with genetically modified mice that express a human pyrin molecule bearing mutations associated with FMF show that indeed mutant pyrin enhances inflammasome via an ASC-dependent but Nlrp3-independent mechanism (Chae et al., 2011). The pathogenetic role of dysregulated caspase-1 in FMF is demonstrated by the clinical efficacy of IL-1β inhibition in patients refractory to colchicine, which remains the drug of choice (Stankovic Stojanovic et al., 2012; Ter et al., 2012). Interestingly, one of the as-yet-ill-characterized effects of colchicine in immune cells is to block P2X7R and P2X7R-associated IL-1β release (Marques-da-Silva et al., 2011), an effect that might be relevant to the anti-inflammatory activity of this drug.

C. Gout and Pseudogout

Colchicine is a very effective treatment of gout and pseudogout, diseases due to intra-articular deposition of MSU or CPPD crystals, respectively. Both MSU and CPPD are potent activators of the NLRP3 inflammasome, and accordingly mice lacking Nlrp3, Asc, and caspase-1 are unable to release IL-1β and IL-18 in response to MSU or CPPD stimulation and do not develop inflammation following intraperitoneal injection of these crystals (Martinon et al., 2006). It is suggested that MSU and CPPD crystals are phagocytosed by mononuclear phagocytes and are then released into the cytoplasm following endosome/phagosome damage, thus leading to inflammasome activation. The molecular mechanism of inflammasome activation by MSU and CPPD has not been clarified, and it is possible that inflammasome stimulation requires an intermediate and as yet unknown step (Kingsbury et al., 2011). In support of the pathogenetic role of the inflammasome in acute and chronic gout, administration of IL-1β blockers such as the recombinant IL-1Ra (IL-1 receptor antagonist) anakinra (McGonagle et al., 2007), the IL-1 Trap rilonacept (Terkeltaub et al., 2009), or the monoclonal anti-IL-1β antibody canakinumab (So et al., 2010) proved to be very effective. Recent reports suggest that activation of the inflammasome by
particulate agents might be a general paradigm for sterile inflammation. In addition to cholesterol crystals, MSU, CPPD, silica, asbestos, alum, and Aβ, activation of the inflammasome has been reported by polyester (Demento et al., 2009), titanium oxide (Yazdi et al., 2010; Winter et al., 2011), carbon (Reisseter et al., 2011), polystyrene (Lunov et al., 2011), gold (Nguyen et al., 2012), and silver (Yang et al., 2012) nanoparticles.

D. Adjuvants

Identification of the inflammasome as a target of alum is of particular interest. Alum, hydrated potassium aluminum sulfate, is the most commonly adjuvant used in human vaccines. The mechanism of action of adjuvants (the biologic basis of “adjuvanticity”) has long remained mysterious. In recent years it has been shown that release of DAMPs and stimulation of a local inflammatory response, is one of the main mechanisms by which adjuvants potentiate antigen immunogenicity (Kool et al., 2012). It was then proposed that the inflammasome is involved (Kool et al., 2008), although mechanistic details have not been resolved yet and discrepancy exists between in vitro and in vivo data (Kool et al., 2012).

E. Misfolded Proteins

Misfolded proteins are at the basis of several chronic human pathologies that share an important inflammatory basis (Buxbaum, 2004; Masters et al., 2009; Masters and O’Neill, 2011). Overproduction of IL-1β and IL-18 via the inflammasome (most commonly NLRP3) is common in these pathologies (Masters and O’Neill, 2011). Evidence suggests that a priming and a triggering step is required. Priming is necessary to drive synthesis of the procytokines, and in some instances of NLRP3, may be due to agents that engage plasma membrane TLRs (TLR2 or TLR4). Misfolded proteins are thought to act as triggers to cause inflammasome assembly and activation via phagolysosomal destabilization and cathepsin B release or ROS generation (Halle et al., 2008; Meissner et al., 2008). Participation of the inflammasome and of inflammasome accessory proteins to detection of misfolded proteins might be more complex than currently understood. Two groups reported that the P2X7R is an obligatory partner in the cascade of events leading to Aβ- or serum amyloid A-dependent IL-1β release (Sanz et al., 2009; Niemi et al., 2011). Sanz et al. (2009) showed that soluble Aβ activates mouse microglial cells in a P2X7R-dependent fashion as either P2X7R blockade or genetic p2x7 deletion completely abrogated response to Aβ. In vitro, Aβ acts as an incomplete stimulus for IL-1β release since a priming step (i.e., LPS) is also required. Very interestingly, priming is not required for Aβ-dependent IL-1β release in vivo (Sanz et al., 2009). It is unclear whether Aβ directly interacts with the P2X7R or whether receptor activation is the consequence of Aβ-dependent ATP release, but it is a fact that Aβ causes ATP release from glial cells (Kim et al., 2007, 2012; Sanz et al., 2009; Orellana et al., 2011). Niemi et al. (2011) reported that serum amyloid A causes accumulation of pro-IL-1β, at least in part, via TLR2 and TLR4 and triggers IL-1β maturation via P2X7R. Interleukin-1β secretion was markedly decreased by Nlrp3 silencing or in Asc−/− mice. Niemi et al. (2011) implicated cathepsin B in the P2X7R-dependent mechanism of IL-1β secretion, but they were unable to detect any lysosomal destabilization. The observation that P2X7R-mediated superoxide generation is upregulated in microglia from a transgenic mouse model of Alzheimer’s disease (Parvathenani et al., 2003) suggests a pathway for inflammasome activation by Aβ.

The inflammasome might be involved in the pathogenesis of Parkinson’s disease, because in an animal model, IL-1β oversecretion or antagonism aggravates or ameliorated symptoms, respectively (Pott Godoy et al., 2008). Unfortunately, no other studies have addressed the possible involvement of the inflammasome in Parkinson’s disease, except for a report describing the protective effect of P2X7R blockers in a rat model of this condition (Marcellino et al., 2010).

A wide-scale analysis of disease-linked single nucleotide polymorphisms of miRNAs and mRNAs, which included Huntington’s disease, showed that nearly all disease-associated miRNAs analyzed have the potential to target mRNA of the inflammasome pathways (NLRP1 and NLRP3 included) (Glinsky, 2008), suggesting a dysregulation of inflammasome also in Huntington’s disease. Interestingly, microarray analysis from patients treated with chloroquine showed a reversal of disease-linked NLRP1 and NLRP3 gene expression phenotypes, further stressing the participation of the inflammasome in this disease.

F. Atherosclerosis and Metabolic Diseases

In view of the central role of the inflammasome in inflammation, it is not surprising that its participation in atherosclerosis, a leading cause of cardiovascular morbidity, has been closely investigated. It has been reported that cholesterol crystals destabilize the phagosomes and cause inflammasome activation (Duewell et al., 2010). Reconstitution of mice deficient in the low density lipoprotein receptor (a condition that predisposes to early atherosclerosis) with bone marrow cells deficient of Nlrp3, Asc, or IL-1α/IL-1β was protective against induction of atherosclerosis. Investigation of the role of the inflammasome in a different animal model of atherosclerosis, the ApoE-deficient mouse, has produced conflicting results. One study found no difference in atherosclerosis progression in ApoE−/− Nlrp3−/−, ApoE−/− Asc−/−, ApoE−/− casp-1−/− double KO mice (Menu et al., 2011). Another study reported
that atherosclerotic plaque areas, infiltrating macrophages, and smooth muscle cell number were reduced in the aortic wall of ApoE<sup>−/−</sup> casp-1<sup>−/−</sup> mice (Usui et al., 2012). Although further studies are certainly necessary to settle this issue, additional “ex adjuvanti-bus” evidence seems to support a pathogenic function of the inflammasome in atherosclerosis, because it has been known for a while that IL-1β deficiency or antagonism ameliorates atherosclerosis in the ApoE<sup>−/−</sup> mouse model (Elhage et al., 1998; Kirii et al., 2003; Wen et al., 2012).

The inflammatory pathogenesis of metabolic diseases is increasingly recognized. Multisystemic dysfunctions occurring in the course of “metabolic syndrome” include inflammatory alterations in the cardiac, adipose, pancreatic, and hepatic tissue (Strowig et al., 2012). Interleukin-1β and IL-18 have a pre- eminent role as causative agents in all these dysfunctions (Netaea et al., 2006; Feve and Bastard, 2009). Although it is to be expected that a crucial proinflammatory pathway such as the inflammasome pathway is implicated in diseases that recognize a strong inflammatory pathogenesis, it is less obvious that some of the metabolic factors dysregulated in these diseases directly affect inflammasome activity. This is, for example, the case of hyperglycemia. Chronic hyperglycemia, such as that occurring in type 2 diabetes mellitus (T2DM), is toxic to pancreatic β cells ending with extensive β cell death and reduced insulin secretion (Dinarello et al., 2010). Loss of pancreatic tissue is largely due to stimulation of IL-1β secretion by infiltrating inflammatory macrophages and activated β cells. The mechanism of glucose toxicity has been unveiled and shown to be due to TXNIP-dependent NLRP3 inflammasome activation. In brief, hyperglycemia triggers ROS generation, probably at the mitochondrial level, followed by TXN dissociation from TXNIP and TXNIP binding to NLRP3 (Zhou et al., 2010). Another NLRP3 activating factor that accumulates into the diabetic pancreas is the A peptide (islet A polypeptide, IAPP). IAPP has been shown to activate NLRP3 in macrophages primed with LPS (Masters et al., 2010). Very interestingly, LPS could be replaced by minimally oxidized low density lipoprotein as priming agents. IAPP-stimulated IL-1β release required an active glucose metabolism and was independent of TXNIP. The mechanism by which glucose metabolism affects inflammasome activity was not investigated in this study. Inflammasome might also be the target of metabolic derangements outside the pancreas. NLRP3 and caspase-1 are overexpressed in the liver and adipose tissue of obese individuals (Strowig et al., 2012). Adipose tissue-infiltrating macrophages overexpress Nlrp3, Asc, and caspase-1 and are the target of saturated fatty acids with proinflammatory activity such as palmitate and ceramides (Vandanmagsar et al., 2011; Wen et al., 2011). Overactivation of the inflammasome and of caspase-1 may have several untoward effects on adipose tissue metabolism by amplifying the local inflammatory response and increasing insulin resistance (Henao-Mejia et al., 2012). A pathogenetic role of the NLRP3 inflammasome in T2DM is also supported by the finding that macrophages from T2DM patients overexpress NLRP3, ASC, and IL-1ß and that treatment with the antidiabetic drug metformin inhibits IL-1β maturation (Lee et al., 2013a).

Accruing evidence suggests that inflammasomes may have an important function outside the innate immune system. An example of this noninflammatory role of inflammasomes is found in adipose tissue. It is reported that caspase-1 is upregulated during adipocyte differentiation and skews the adipocytes toward an insulin-resistant phenotype (Stienstra et al., 2011, 2012). Conversely, caspase-1-deficient animals are more insulin-sensitive and their adipocytes show higher fat oxidation rates. In addition, mice deficient in Nlrp3, Asc, and casp-1 are resistant to high fat diet-induced obesity and are protected from obesity-induced insulin resistance (Stienstra et al., 2011, 2012). Infiltration of adipose tissue by inflammatory macrophages is also reduced in Nlrp3<sup>−/−</sup>, Asc<sup>−/−</sup>, and casp1<sup>−/−</sup> mice, likely due to reduced monocyte chemoattractant protein (MCP)-1 secretion. Several other obesity-related endocrine factors are also altered in these inflammasome-deficient mice. Collectively, these data show that inflammation, via the key inflammasome effector pathway, is a crucial linker between metabolic stress, obesity, and T2DM on the one hand and hyperglycemia and pancreatic β cell damage, on the other hand. It would be of the utmost therapeutic interest if targeting the inflammasome proved useful to prevent both obesity-induced insulin resistance and hyperglycemia-dependent insulin deficiency.

The intriguing connections between metabolism and inflammasomes are only now beginning to be investigated, but it is likely that these molecular pathways play a central role in the biochemical changes occurring during inflammation as well as during metabolic disorders and cancer, as recently pointed out by O’Neill and co-workers (Wen et al., 2012). Glucose stimulates IL-1β secretion from pancreatic β cells, and inhibition of glucose metabolism prevents IL-1β gene expression (Masters et al., 2010); reciprocally LPS promotes aerobic glycolysis, the so-called Warburg effect (Krawczyk et al., 2010). Thus, there seems to be a tight and as yet largely unexplored link between inflammation and glucose metabolism. A site of intersection between glycolysis and the inflammasomes might be the P2X7R. Very recently, we reported that cells expressing the P2X7R have a very active aerobic glycolysis and that agents known to stimulate aerobic glycolysis, such as mitochondrial uncouplers, require P2X7R expression (Amoroso et al., 2012). Interestingly, cells expressing the P2X7R overexpress key glycolytic enzymes such as pyruvate kinase M2 (PKM2) and hexokinase type 2 (HK2), whereas adipose tissue and macrophages of P2X7R−/− mice have reduced levels of these enzymes (Tosti et al., 2014). It will be important to determine whether the P2X7R activation of the inflammasome is a consequence of metabolic stress or a cause of metabolic stress induction. It is clear that understanding these connections is a major challenge for future research.
enzymes such as glyceraldehyde 3-dehydrogenase, pyruvate kinase M2, pyruvate dehydrogenase kinase, and phosphofructokinase, as well as the transcription factor hypoxia inducible factor (HIF)-1α. In addition, P2X7R causes overexpression of the glucose transporter Glut1 and increased accumulation of intracellular glycogen stores. These effects on glycolytic metabolism mimic several of the effects elicited by LPS, pointing to P2X7 as a possible crossroad in the glycolytic and inflammatory pathways.

G. Retinopathies

Given the relevance of the retina as a target tissue in diabetes, it is not surprising that TXNIP and the inflammasome have been found to contribute to the pathogenesis of diabetic retinopathy (Devi et al., 2012). Very interestingly, the inflammasome appears to be also involved in ischemia retinopathy (Kawaguchi et al., 2011). To further stress the importance of the inflammasome in eye pathophysiology, it was very recently shown that deficiency of the miRNA-processing enzyme DICER1 in retinal pigmented epithelia causes Alu RNA accumulation, which in turn activates the NLRP3 inflammasome, triggers IL-1β and IL-18 secretion and leads to retinal epithelial cell degeneration, which is responsible for age-related macular degeneration (AMD) (Tarallo et al., 2012). In another study addressing the pathogenesis of AMD, it was shown that NLRP3 inflammasome activation by abnormal proteins (known as drusen) that accumulate under the retina during AMD might be protective (Doyle et al., 2012). This latter study showed that inflammasome activation causes IL-18 secretion that in turn downregulates VEGF, thus reducing the excessive neoangiogenesis associated with AMD.

H. Cardiac Diseases

Further data support involvement of the inflammasome in crucial responses outside the immune system. For example, it is known that cardiac tissue is severely injured during ischemia/reperfusion. Now data from Kawaguchi et al. (2011) show that hypoxia/reoxygenation stimulate the inflammasome in cardiac fibroblasts (but not in cardiomyocytes). Inflammasome activation then causes IL-1β secretion and inflammatory cell infiltration. Infarct size caused by ischemia/reperfusion and associated fibrosis and dysfunction were markedly reduced in Asc/-/- animals. These observations were confirmed and extended by a following paper showing that activation of the Nlrp3 inflammasome in cardiomyocytes causes increased infarct size and promotes adverse cardiac remodeling (Mezzaroma et al., 2011). Infarct size and pathologic cardiac remodeling could be reduced by inhibition of Nlrp3 as well as P2X7R.

I. Cancer

The inflammatory microenvironment is a fundamental factor determining tumor progression. Interleukin-1β and IL-18 are commonly overexpressed in many tumors, and their concentration within biologic fluids may offer prognostic indications (Zitvogel et al., 2012). These observations indicate a dysregulation of inflammasome activity in cancer, but it has not been easy so far to pinpoint exactly in which way a dysregulated inflammasome may predispose to or sustain malignant degeneration. For example, Nlrp3- but not Nlrc4-deficient mice are more susceptible to dextran sulfate-induced colitis-associated colon cancer (Allen et al., 2010), but another study reported on the contrary that dextran-sulfate-induced colitis degenerates more often in cancer in Nlrc4- than in Nlrp3-deficient mice (Hu et al., 2011). A protective effect of Nlrp3 and casp1 deletion against dextran sulfate-induced colitis has been also shown by Siegmund et al. (2001) and Bauer et al. (2010). Deletion of other inflammasome components such as Nlrp6, Nlrp12, Asc, or casp1 enhanced susceptibility to colon inflammation and tumorigenesis, likely due to failure to downmodulate tumor-infiltrating inflammatory cell activity (Dupaul-Chicoine et al., 2010; Zaki et al., 2010, 2011; Chen et al., 2011; Normand et al., 2011). These reports show that the role played by the inflammasome in tumorigenesis is far from clear. One of the mechanisms by which the inflammasome may contribute to colonic carcinogenesis is by modulating the composition of the colonic microbiota and host/pathogen equilibrium. This is suggested by the observation that mice deleted of Nlrp6 are more susceptible to spontaneous inflammation and dextran-induced colitis (Elinav et al., 2011). However, in general it is likely that an active inflammasome may affect carcinogenesis by modulating the cytokine content of the tumor microenvironment (Zitvogel et al., 2012).

Recent evidence has shown that the interstitium of solid tumors is highly conducive for inflammasome activity due to the high concentration of extracellular ATP and to high expression of P2X7R by tumor and infiltrating inflammatory cells (Adinolfi et al., 2012). Accordingly, several cancers are characterized by high release of IL-1β (Krelin et al., 2007; Reed et al., 2009; Dunn et al., 2012; Li et al., 2012). In some cancer histotypes (e.g., stomach cancer and mammary carcinomas) the role of IL-1β in cancer progression and metastasis has been ascribed to its ability to drive intratumor recruitment of myeloid-derived suppressor cells (Bunt et al., 2007; Tu et al., 2008), a cell type endowed with a potent immunosuppressing activity. These findings suggest that inflammasome-driven oversecretion of IL-1β in the tumor interstitium should
be detrimental for the host and that inflammasome inhibition or IL-1β blockade should be advisable. Indeed, Dinarello (2010) has proposed IL-1β blockade as a therapy for primary and metastatic tumors. Furthermore, overactivation of NLRP3 has been described in the tumor cells themselves, a finding that raises the issue of a cell autonomous function of the inflammasome in supporting cancer cell growth (Okamoto et al., 2010). Interleukin-1β targeting in cancer therapy is an attractive strategy that should, however, be carefully weighed in the light of the crucial role of this cytokine in the modulation of anticancer-specific immunity.

It is a recent acquisition that, in the treatment of several tumors, chemotherapeutics are more efficient in the presence of an active, tumor antigen-specific immune response, probably elicited by antigens and other immunostimulating factors released from dying tumor cells (Ghiringhelli et al., 2009; Zitvogel et al., 2011). This type of cell death (“immunogenic cell death”) provides a strong and tumor cell-specific activation of cytotoxic T cells that is thought to be crucial in controlling residual disease. In this process of chemotherapy-driven instruction of adaptive immunity the Nlrp3 inflammasome has a central role (Ghiringhelli et al., 2009; Aymeric et al., 2010). Tumor cells injured by chemotherapeutics release intracellular DAMPs, pivotal among which is ATP, which act on the P2X7R expressed by nearby DCs, leading to Nlrp3 inflammasome activation and IL-1β secretion. Interleukin-1β in turn stimulates IL-17 secretion by γδ T cells and activation of cytotoxic CD8+ T lymphocytes (Zitvogel et al., 2012). This mechanism for the immunostimulating activity of immunogenic cell death is supported by the direct in vivo demonstration that the tumor microenvironment is an ATP-rich milieu (Pellegratti et al., 2008; Michaud et al., 2011).

Additional hints to the role of the inflammasomes in cancer come from a recent study by a Taiwan research team that has shown that NLRP3, AIM2, and RIG-1 inflammasomes are overexpressed in Epstein-Barr virus-associated nasopharyngeal carcinoma, and expression levels correlate with patient survival (Chen et al., 2012). Inflammasome overexpression correlates with high IL-1β levels, which drive a rich intratumor neutrophil infiltrate. In this study, neutrophil infiltration is associated with better survival of cancer patients. In the light of this activity of IL-1β in the potentiation of antitumor-specific immunity, treatments aimed at antagonizing this cytokine should be carefully weighed.

Interleukin-18 has been implicated as a protective factor in colonic inflammation and tumorigenesis (Elinav et al., 2011), as well as in prostate tumors (Tse et al., 2011), although its role is controversial in other cancers (Dunn et al., 2012). In addition, interpretation of its activity is made complicated by its complex modulation of anticancer responses mediated by NK and T cells (Terme et al., 2012). A phase II clinical trial in patients with metastatic melanoma suggested a limited efficacy of IL-18 administration as a single immune therapeutic (Srivastava et al., 2010).

Light on the role of inflammasome/IL-18 axis in cancer might be shed by the finding that activation of the NLRP3 or NLRP6 inflammasomes by danger signals in the intestinal mucosa drives IL-18 secretion, which in turn represses synthesis of the IL-22 soluble receptor [also known as IL-22 binding protein (IL-22BP)], thereby enhancing the growth-promoting activity of IL-22 and therefore colon tumorigenesis (Huber et al., 2012).

Collectively, the evidence so far discussed suggests that the inflammasome has an important but as yet controversial role in primary and metastatic tumor formation. The most daunting task will be to dissect inflammasome effects intrinsic to the tumor cells (tumor cell autonomous effects) from those due to activation of the inflammasome within infiltrating inflammatory cells.

**IX. Inflammasome Blockers as Novel Therapeutics**

All data accumulated to date have confirmed that the inflammasome is the CPU that integrates signals responsible for (1) the initiation and amplification of inflammation, (2) the stimulation of innate and adaptive immunity, and (3) the modulation of cell growth and differentiation. Thus it is no surprise that increasing attention is paid to the development of drugs targeting this organelle (Lopez-Castejon and Pelegrin, 2012). Simply considering the two inflammasome-dependent best characterized responses (pyroptosis and cytokine release), blockade of inflammasome activity could be simplistically achieved at the level of final effector pathways, i.e., caspase-1 or IL-1β and IL-18 blockade. However, as stressed throughout this review, inflammasome functions are far more complex than the mere stimulation of inflammatory cell death or cytokine release; thus efficient inflammasome interference must target all the different steps involved in stimulation of inflammasome assembly, caspase-1 activation, and cytokine maturation and release. As summarized by Lopez-Castejon and Pelegrin (2012), in principle it is possible to antagonize the inflammasome by targeting pathways upstream or downstream of the inflammasome, as well as the specific inflammasome components. Schematically, the following targets can be identified:

1. Plasma membrane receptors involved in inflammasome activation (i.e., the P2X7R or panx-1);
2. Cytoplasmic mediators or second messengers (i.e., ROS, TXNIP, PKR, cathepsin B, K+ ions);
3. Inflammasome intrinsic components (i.e., NLRPs, AIM2, ASC, caspase-1);
4. Cytokines processed via the inflammasome (i.e., IL-1β and/or IL-18);
5. Cytokine targets (i.e., receptors for IL-1β and IL-18).

Targeting inflammasome-activating plasma membrane receptors is one of the most promising avenues for drug development, at least for those inflammasome subtypes that depend on P2X7R or panx-1 for their activation. This opinion is based on a twofold consideration: (1) P2X7R is increasingly shown to be crucial for a multiplicity of responses involving the inflammasome and (2) over the last 10 years there has been a large effort by the pharmaceutical industry toward the development of P2X7R blockers. As a result, over 20 clinical trials have been run as of 2011 to test safety and efficacy of P2X7R blockers as anti-inflammatory drugs (Arulkumaran et al., 2011). Therefore, a large panoply of compounds are available that might potentially interfere with the inflammasome, although so far none has been approved for clinical use. Phase I/Phase II clinical trials have been concluded or are still underway by Astra-Zeneca, Pfizer, Evotec, and GlaxoSmithKline to test safety and efficacy of drug-like P2X7R blockers in several chronic inflammatory diseases such as osteoarthritis, rheumatoid arthritis, chronic obstructive pulmonary disease, and CD (Arulkumaran et al., 2011). Main compound classes from which selective P2X7R blockers were developed are benzamide derivatives, bicycloheteroaryl compounds, acylhydrazine antagonists, aromatic amine antagonists, amine derivatives, amide derivatives, biaromatic antagonists, and heterocyclic ion channel blockers (Friedle et al., 2010). Recently, Affectis Pharmaceuticals (Martinsried, Germany) patented a central nervous system-penetrant oral P2X7R antagonist, AFC-5128, an N-indol-3-yl-acetamide and N-azaindol-3-yl-acetamide compound, for the treatment of neuropathic pain and multiple sclerosis. Of interest in view of the possible therapeutic applications of P2X7R antagonists is the demonstration in preclinical studies that P2X7R blockers have a potent antitumor effect (Adinolfi et al., 2012). The use of this class of antagonists as anticancer drugs is further supported by the in vivo observation that stimulation of P2X7R triggers, while blockade abrogates, VEGF release (Adinolfi et al., 2012).

Targeting panx-1 to antagonize the inflammasome is more problematic essentially for two reasons: (1) panx-1 is dispensable for P2X7R-induced inflammasome activation (Qu et al., 2011) and (2) no drug-like selective panx-1 blockers are available so far. However, it is reported that an inhibitor of organic anion transporter(s), probenecid, long used to lower uric acid levels in the body, is a potent inhibitor of panx-1 (Silverman et al., 2008; Ma et al., 2009).

Downstream of P2X7R and panx-1 it would be possible to target the ion perturbation (cytosol K+ depletion) caused by opening of the large-conductance, nonselective P2X7R/panx-1 pore. It has been reported that glyburide, a sulfonylurea compound targeting KATP channels used to treat T2DM, blocks IL-1β processing and release as well as NLRP3 inflammasome activation (Hamon et al., 1997; Lamkanfi et al., 2009b). However, none of the relevant molecules responsible for inflammasome activation, nor the inflammasome components themselves, seem to be targets of glyburide activity (Lamkanfi et al., 2009b); thus its mechanism of action is still mysterious. Inhibition of K+ efflux is certainly a powerful means to inhibit almost all inflammasome subtypes, but this necessitates targeting of the P2X7R or panx-1 channels (preferably the former!), which brings us back to P2X7R pharmacology. Alternatively, scattered evidence suggests that drugs inhibiting voltage-gated K+ channels, i.e., idebenone, may also prevent inflammasome activation (Newman et al., 2011).

ROS generation is another pathway for inflammasome activation. TXNIP has been identified as a key component linking ROS formation to NLRP3 inflammasome stimulation (Zhou et al., 2010); thus it is reasonable to assume that TXNIP inhibition might be useful to downmodulate inflammasome activity. In view of its involvement in lipid/glucose metabolism, various avenues to control TXNIP expression and function have been explored, but attempts to inhibit inflammasome activity by this route are still very preliminary (Watanabe et al., 2010). In alternative to conventional small drug therapeutics, increasing the synthesis of TXNIP-destabilizing miRNA (miRNA-17) might be pursued to downmodulate TXNIP activity (Lerner et al., 2012). As a more general therapeutic approach, antioxidants such as n-acetyl cysteine, might be used to inhibit the inflammasomes (Dostert et al., 2008), although use of antioxidants must be weighed against the evidence that inhibition of ROS generation might indeed stimulate inflammasome activity (van de Veerendonk et al., 2010).

A few compounds directly interacting with and inhibiting the inflammasome have been described. One of them is a diarylsulfanilurea compound named CRID, CP-456,773, which is thought to prevent formation of Asc complexes during the process of assembly of the Nlrp3 and AIM2 inflammasomes (Coll and O’Neill, 2011). Hundreds of caspase-1 inhibitors have been synthesized based on the optimal peptide recognition sequence of caspase-1, but unfortunately most of these tetrapeptide-aldehyde blockers (Ac-YVAD-CHO) are unsuitable for in vivo use (MacKenzie et al., 2010). Therefore peptidomimetic inhibitors of caspase-1 have been developed. Some have entered clinical trials (e.g., pralnacasan, VX-765, emricasan) for the treatment of rheumatoid arthritis, osteoarthritis, psoriasis, or transplant rejection, but none of these compounds has been developed further due to liver
toxicity or undisclosed reasons (MacKenzie et al., 2010). Allosteric inhibitors are also under intense scrutiny. This novel strategy targets an allosteric site at the dimer interface of caspases by using small molecules that have better drug-like properties (MacKenzie et al., 2010). More recently the organogold compound auranofin was shown to prevent anthrax LT-mediated activation of the Nlrp1 and Nlrp3 inflammasomes, probably by direct inhibition of caspase-1 (Newman et al., 2011). Two protease inhibitors, ritonavir and disulfiram, were shown to inhibit caspase-1 activation and suggested to be useful in abrogating IL-18 release (Nobel et al., 1997; Sloand et al., 2000; Kast, 2008). Very recently, we showed that the widely used Ca²⁺ channel blocker nimodipine and its congener nitrendipine abrogate Aβ-stimulated IL-1β release from mouse microglial cells, both in vitro and in vivo, but the mechanism of this inhibitory action is as yet unknown (Sanz et al., 2012). Finally, it is possible to target the products of inflammasome activity, IL-1β and IL-18. As summarized by Lopez-Castejon and Pelegrin (2012), the process of IL-1β interaction with the target cells is tightly controlled at the level of the target cell plasma membrane and in the extracellular space where IL-1β may be bound by soluble IL-1RI and IL-1RII receptors shed by activated immune cells. At the plasma membrane, IL-1β stimulation is modulated in two ways: (1) by mutually exclusive binding to the functional IL-1RI or to the decoy IL-1RII receptor and (2) by competition for IL-1RI binding with IL-1Ra. Thus, therapeutic IL-1β blockade has been so far pursued via three avenues: (1) by preventing binding of IL-1β to its receptor by using the recombinant IL-1Ra (anakinra); (2) by using an anti-IL-1β mAb (canakinumab); and (3) by using an IL-1β trap made by fusing the binding domains of IL-1RI and IL-1RAcP to the Fc portion of human IgG (rilonacept) (Dinarello et al., 2012).

X. Conclusion

The discovery of the inflammasome is one of the most important recent discoveries in immunology and medicine. Identification and characterization of this novel organelle has provided the molecular mechanism to bridge cell stimulation by pathogens or physical and chemical injury to activation of the earliest responses by immune cells. The inflammasome has clarified the long-standing conundrum concerning the mechanisms responsible for pro-caspase-1 activation and consequently of IL-1β and IL-18 processing and will likely help solve remaining uncertainties on the process of mature IL-1β release. Given the accruing evidence demonstrating the fundamental role played by this cytokine in chronic inflammatory diseases, metabolic disorders, and cancer, inflammasomes will no doubt become targets for the development of innovative therapeutics (Fig. 4). Already now several of the inflammasome accessory molecules are the focus of intense investigation for the development of potent and selective blockers able to inhibit inflammasome activity. It is as yet unclear how the inflammasomes are activated and whether they all share a common mechanism. However, recent experiments have provided convincing evidence that, contrary to the previously held opinion, some agonists (e.g., dsDNA or PKR) may indeed directly interact with the inflammasome.
while more general and indirect mechanisms of activation (e.g., ROS, K⁺ drop, cathepsin B) are not effective in all inflammasome subtypes so far characterized. It is possible that more than one mechanism is involved and that full inflammasome activation is the result of multiple events acting in synergy. It is anticipated that several tightly controlled mechanisms have been developed to turn on caspase-1, and therefore multiple inflammasome subtypes are likely to be present. Only two years ago, only four inflammasomes had been identified [NLRP1, NLRP3, NLRC4, and AIM2 (Gross et al., 2011; van de Veerdonk et al., 2011)]. At the time this review was written, this number had already increased to eight. Other inflammasome subtypes, maybe atypical such as the “pyrin inflamma-

...soma,” will certainly be identified in the next few years. Coupling of the inflammasomes to other intracellular organelles (e.g., the mitochondria or the endoplasmic reticulum) will be another important field of investigation in coming years, as well as illuminating the intriguing links between glycolytic metabolism (the Warburg effect), inflammasomes, and inflammation. The relevance of the inflammasome is not by any means restricted to inflammation, as sound evidence links this organelle to metabolic diseases, cell differen-
tiation, and cancer. These will be other exciting areas where understanding inflammasome function will provide new insights into the pathogenesis of a range of diseases and provide novel approaches to treatment.

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References

The Inflammasomes


