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REVIEW ARTICLE

Biochemical regulation of the inflammasome

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Abstract

The extensively studied cytokine IL-1β is an important mediator of the inflammatory response. However, dysregulated release of IL-1β can be detrimental and is attributed to the progression and pathogenesis of multiple inflammatory diseases including, rhuematoid arthritis (RA), atherosclerosis, type 2 diabetes (T2D), Alzheimers disease and gout. IL-1β is encoded as a pro-protein. A multi-protein molecular scaffold termed the "Inflammasome" is responsible for the tightly controlled and coordinated processing of pro-IL-1β. The activation of several NLR (nucleotide-binding oligomerization domain (NOD)-like receptor) family members and PYHIN (pyrin and HIN domain) proteins can drive the formation of inflammasomes. However, the exact biochemical mechanisms governing their activation have been the subject of much research. Different inflammasomes have been demonstrated to respond to the same pathogen inducing a cooperative immune response accountable for the clearance of infection. Here, we review current knowledge surrounding the biochemical regulation of the NLRP1, NLRP3, NLRC4, AIM2 and IFI16 inflammasomes.

Keywords: IL-1β, inflammasome, AIM2, NLRP3

Introduction

The general strategy of innate immune detection relies on the recognition of microbial molecules with conserved molecular structures known as "pathogen associated molecular patterns" (PAMPs). This recognition initiates an inflammatory response vital to host defense and clearance of infection. In addition to sensing microbial products, the immune system has evolved to detect endogenous "danger" molecules or "danger associated molecular patterns" (DAMPs) which provoke inflammation. These DAMPs include products from damaged or dying cells such as adenosine triphosphate (ATP) and uric acid crystals or noxiuos exogenous factors including environmental insults asbestos and UV radiation (Chen & Nunez, 2010). DAMPs trigger a sterile inflammatory response paramount to tissue and wound repair.

Detection is executed by a limited number of specialized receptors commonly referred to as "pattern recognition receptors" (PRRs) (Hoebe *et al.*, 2004). Recognition by PRRs triggers the activation of intracellular pathways which results in the production of anti-microbial molecules and pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), interleukin-1β (IL-1β) and type-I interferons (IFN). The five main classes of PRRs comprise the membrane and endosomal Toll-like receptors (TLRs) (Takeda & Akira, 2005), the cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Ye & Ting, 2008), C-type lectin receptors (CLRs) (Osorio & Reis e Sousa, 2011), retinoic acid inducible gene-I (RIG-I)-like proteins (RLRs) (Yoneyama *et al.*, 2008) and certain proteins of the pyrin and HIN domain (PYHIN) family termed AIM2-like receptors (ALRs) (Keating *et al.*, 2011).

When an infectious or injurious insult causes extensive damage, PRRs trigger the acute inflammatory response. Normally, this leads to the clearance of the insult in question and repair of damaged tissues. However, in certain circumstances, when not dealt with effectively continued and uncontrolled inflammatory signaling can be deleterious for the host and hamper resolution.

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The extensively studied cytokine IL-1β was originally identified as an endogenous pyrogen causing fever and is an important mediator of the inflammatory response. However, dysregulated release of IL-1β can be detrimental and is attributed to the progression and pathogenesis of multiple inflammatory diseases including rheumatoid arthritis (RA), atherosclerosis, type 2 diabetes (T2D), Alzheimers disease, gout and a family of inherited autoinflammatory diseases which include familial mediteranean fever (FMF) and TNF-receptor-associated periodic syndrome or TRAPS (Dinarello, 1996). Several of these have been shown to be responsive to the blockade of IL-1β, as reviewed extensively by Dinarello (Dinarello, 2011).

As such it is not a surprise that the biological activity of IL-1β is tightly controlled and that several endogenous inhibitors of IL-1β exist, most notably an endogenous IL-1 receptor antagonist (IL-1Ra). IL-1β lacks a signal sequence and is produced as a cytosolic precursor which requires further processing (van de Veerdonk *et al.*, 2011). Specifically, two steps, synthesis and processing describe how IL-1β is produced. Synthesis of pro-IL-1β is initiated by the ligation of a PRR with its respective ligand, the most characterized being TLR4 which is activated by the gram-negative microbial product lipopolysaccharide (LPS) (Poltorak *et al.*, 1998). Nuclear factor–κB (NF-κB) and mitogen activated protein kinase (MAPK) signaling have been implicated in the induction of pro-IL-1β (Arnush *et al.*, 1998; Sharma *et al.*, 2003) yet the exact mechanisms regulating the transcription of IL-1β remains an area of great interest. Secondly, pro-IL-1β requires processing by caspase-1 into mature IL-1β.

The activation of certain NLR family members and PHYIN proteins can drive the formation of a multi-protein molecular scaffold termed the "Inflammasome" that is responsible for caspase-1 activation. To date, six PRRs are known to form an inflammasome complex capable of caspase-1 activation. These are the NLRs NLRP1, NLRP3, NLRP6 and NLRC4, and the PYHIN family proteins AIM2 and IFI16. In most cases, the recruitment and activation of caspase-1 is facilitated by the adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC) which forms part of the inflammasome. ASC is a 22-kDa protein and contains an N-terminal pyrin domain (PYD) and a C-terminal caspase activating recruitment domain (CARD) (Masumoto *et al.*, 1999; Conway *et al.*, 2000). Activation of caspase-1 via the inflammasome leads to the processing of IL-1β and also IL-18 (Rock *et al.*, 2010).

Structural domains of the NLRs includes an N-terminal effector domain, a central nucleotide-binding domain (NBD or NACHT) and a C-terminal domain composed of a series of leucine-rich repeats (LRRs) (Ye & Ting, 2008). The N-terminal effector domain is used to subclassify the NLR proteins. In addition to inflammasome activation, NLRs trigger a specific type of cell death termed "pyroptosis" and so are critically placed at the interface of innate immunity and cell-death signaling. The exact mechanisms governing the activation of inflammasomes are not fully understood. In certain cases, the different inflammasomes have been shown to respond to the same bacteria, but are likely to sense divergent bacterial products and induce a cooperative immune response accountable for the clearance of infection. Furthermore, inflammasomes are known to have different roles in different cell types. Here, we review current knowledge surrounding the biochemical regulation of NLRP1, NLRP3, NLRC4, AIM2 and IFI16 (Figure 1). These proteins are proving very important for both host defense and inflammation and their dysregulation is providing insight into the molecular basis of inflammatory diseases.

NLRP3

The prominent NLR, NLRP3 (also known as cryopyrin, NALP3 or PYPAF1) contains typical domains for an NLR; an N-terminal pyrin domain (PYD) followed by a central nucleotide-binding domain (NBD) and C-terminal leucine-rich repeats (LRR) (O'Connor *et al.*, 2003). Expressed in myeloid cells it is mainly cytoplasmic and highly upregulated upon stimulation of macrophage

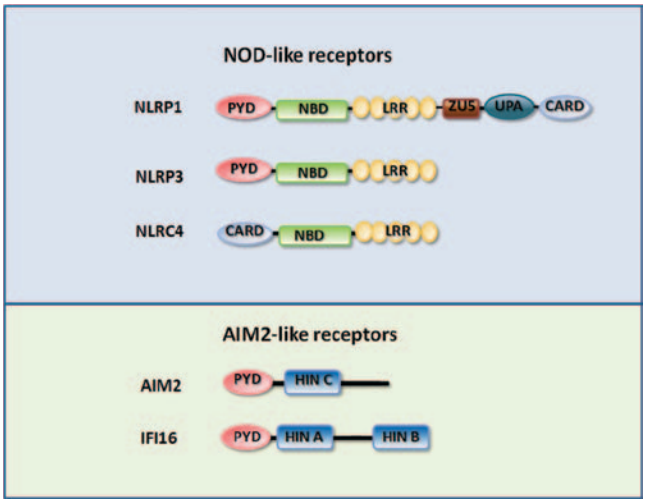

Figure 1. Domain structures of the human NOD-like receptors (NLRs) and AIM2-like receptors (ALRs) known to form inflammasomes. To date five PRRs are known to form an inflammasome complex capable of caspase-1 activation. These are the NLRs, NLRP1, NLRP3 and NLRC4, and the ALRs AIM2 and IFI16. The structural domains of the NLRs includes an N-terminal effector (i.e. PYD or CARD domain), a central nucleotide-binding domain (NBD or NACHT) and a C-terminal domain composed of a series of leucine-rich repeats (LRRs). NLRP3 contains a PYD domain which is used to physically interact with the PYD domain of ASC, facilitating the subsequent recruitment and activation of pro-caspase-1. NLRP1 and NLRC4 contain a CARD domain that can interact directly with caspase-1. The recruitment of ASC to the PYD domain of the NLRP1 been shown to enhance the activity of this inflammasome. The PYHIN proteins consist of an N-terminal PYD and one or more HIN-200 domains, which can be one of 3 subtypes (HIN A, HIN B, or HIN C) based on their sequences (Schattgen & Fitzgerald, 2011). AIM2 and IFI16 recruit ASC via a PYD to activate caspase-1.

with PAMPs (O'Connor *et al.*, 2003). Depending on the stimulus and cellular conditions activation culminates in the processing and secretion of the pro-inflammatory cytokines IL-1β, IL-18 and/or the induction of pyroptosis.

As mentioned, signal 1 drives production of pro-IL-1β, while signal 2 activates NLRP3, this is shown in Figure 2. However, it has also been shown that signal 1 is required for the upregulation of NLRP3 mRNA (Bauernfeind *et al.*, 2009). Typically activation triggers oligomerization of NLRP3 and the formation of an inflammasome responsible for IL-1β processing. Oligomerization is an ATP-dependent process mediated by the binding of ATP to the NACHT domain of NLRP3. The NACHT domain has ATPase activity which may possibly regulate disassembly (Petrilli *et al.*, 2007a). Only one NLRP3 inflammasome is formed per cell (Fernandes-Alnemri *et al.*, 2007). As NLRP3 lacks a CARD domain, the recruitment and activation of pro-caspase-1 is facilitated by the adaptor molecule ASC. Homotypic interactions are formed between the N-terminal PYD domains of NLRP3 and ASC. Subsequently, the CARD domain of associated ASC molecules recruits pro-caspase-1, resulting in its autocleavage and activation (Srinivasula *et al.*, 2002; Agostini *et al.*, 2004). Indeed *in vitro* ectopic expression of NLRP3 with ASC is sufficient to activate caspase-1 (Agostini *et al.*, 2004). The activation of caspase-1 subsequently results in the cleavage of pro-IL-1β and pro-IL-18 to their mature and biologically active forms (Figure 2).

Activating mutations in NLRP3 are associated with "cryopyrinopathies" or cryopyrin-associated periodic syndromes (CAPS) characterized by excessive production of IL-1β. They include familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal onset multisystem inflammatory disease (NOMIID) (also referred to as chronic infantile neurological, cutaneous and articular (CINCA) syndrome) (Hoffman & Wanderer, 2010). With such pathogenic effects, the need for and natural occurrence of inhibitors to IL-1β is clear.

Certainly, IL-1Ra is essential in controlling IL-1β induced inflammation as deletion of the IL-1Ra gene in mice leads to the spontaneous development of rheumatoid-like inflammatory joint disease (Horai *et al.*, 2000) and lethal arthritis (Nicklin *et al.*, 2000). In humans, mutations that affect the ability of IL-1Ra to block IL-1 or deletion of IL-ra results in severe and lethal systemic inflammation at birth (Reddy *et al.*, 2009). In the context of NLRP3, splice variants of ASC have been shown to compete with full length ASC for association and hamper processing of IL-1β (Bryan *et al.*, 2010). More recently in attempts to evade immune responses, the measles virus protein V has been shown to inhibit NLRP3-mediated IL-1β secretion (Komune *et al.*, 2011).

The NLRP3 inflammasome can be activated by endogenous and exogenous stimuli recognised as "self" or "non-self" danger signals, respectively. A list

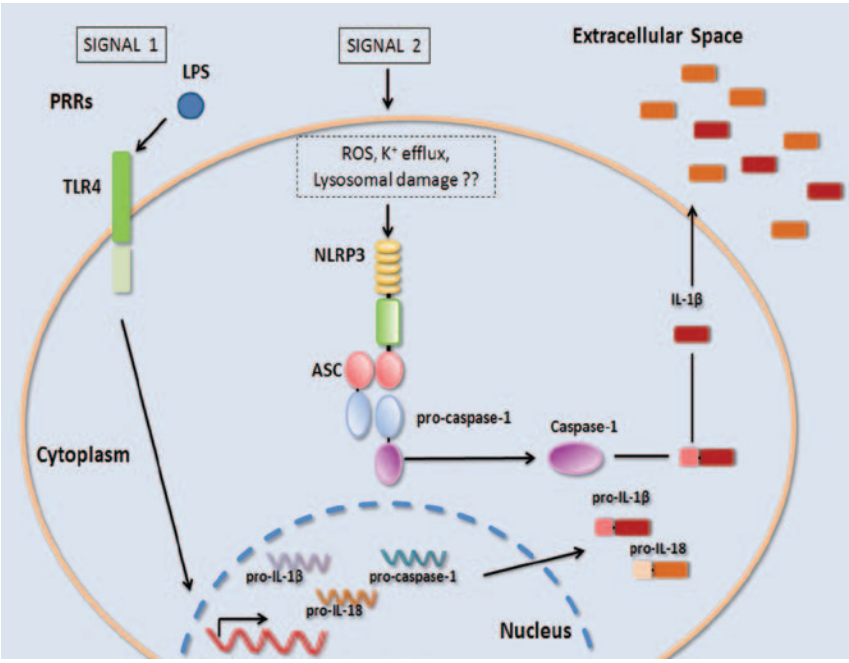

Figure 2. A model of NLRP3 inflammasome activation. The two signals required for the production of IL-1β and IL-18 are illustrated. Signal 1: Synthesis of both pro-IL-1β and pro-IL-18 is initiated by the ligation of a pathogen recognition receptor (PRR) with its respective ligand (i.e. Toll-like receptor 4 (TLR4) and lipopolysaccharide (LPS)). Signal 2: The activation of NLRP3 drives the formation of a multi-protein complex termed the inflammasome. Activation leads to the recruitment and activation of pro-caspase-1 and subsequent processing of pro-IL-1β and pro-IL-18 to their mature forms. In most cases the recruitment of pro-caspase-1 is facilitated by the adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC). The exact mechanisms governing the activation of inflammasomes are not fully understood. Several lines of evidence involving reactive oxygen species (ROS), lysosomal damage and potassium (K^+) efflux have been reported.

Table 1. Endogenous and exogenous activators of the NLRP3 inflammasome.

NLRP3 activator	Component (PAMPs)	Pathway	Reference
Microbial			
Aeromonas hydrophila	Aerolysin	Ion flux	(Gurcel *et al.*, 2006)
Clostridium difficile	Toxin A	ND	(Ng *et al.*, 2010)
Clostridium tetani	Tetanolysin O	Lysosomal damage	(Chu *et al.*, 2009)
Escherichia coli	ND	Pore formation and ion flux	(Kanneganti *et al.*, 2007)
Klebsiella pneumonia	ND	Lysosome damage	(Willingham *et al.*, 2009)
Listeria monocytogenes	Listerialysin O	ND	(Meixenberger *et al.*, 2010)
Neisseria gonorrhoeae	ND	Lysosome damage	(Duncan *et al.*, 2009)
Porphyromonas gingivalis	ND	Ion flux	(Yilmaz *et al.*, 2010)
Pseudomonas aeruginosa	Pilin	ND	(Arlehamn & Evans, 2011)
Saccharomyces cerevisiae	Mannan, Zymosan	ND	(Lamkanfi *et al.*, 2009)
Salmonella typhimurium	T3SS-independent signal	ND	(Broz *et al.*, 2010)
Streptococcus agalactiae	β-hemolysin	ND	(Costa *et al.*, 2012)
Shigella flexneri	ND	Lysosome damage	(Willingham *et al.*, 2007)
Staphylococcus aureus	Hemolysins	ROS	(Munoz-Planillo *et al.*, 2009; Craven *et al.*, 2009)
Fungal			
Aspergillus fumigatus	ND	ROS, SYK AND ion flux	(Said-Sadier *et al.*, 2010)
Candida albicans	Hyphae	ROS, SYK, hyphae	(Gross *et al.*, 2009; Joly *et al.*, 2009)
Chlamydia trachomatis/ muridarum	ND	SYK	(Abdul-Sater *et al.*, 2010)
Viral			
Sendai virus	dsRNA	ND	(Kanneganti *et al.*, 2006)
Influenza A	dsRNA, M2 protein	Pore formation and ion flux	(Thomas *et al.*, 2009; Ichinohe *et al.*, 2010)
Adenovirus	dsRNA	ROS	(Muruve *et al.*, 2008; Delaloye *et al.*, 2009)
Herpes simplex virus	dsDNA	ND	(Muruve *et al.*, 2008; Delaloye *et al.*, 2009)
Parasitic			
Plasmodium	Hemozoin	SYK	(Shio *et al.*, 2009)
Endogenous (DAMPs)			
	Extracellular ATP	Mislocalization	(Rock *et al.*, 2010)
	Hyaluronan	Mislocalization	(Rock *et al.*, 2010)
	Glucose	ROS	(Zhou *et al.*, 2010)
	Uric acid and MSU crystals	ROS	(Martinon *et al.*, 2006)
	Amyloid-β, IAPP*	Lysosome damage	(Rock *et al.*, 2010; Masters *et al.*, 2010)
	Cholesterol crystals	ROS, lysosome damage	(Rock *et al.*, 2010)
Environmental			
	Asbestos	ROS	(Hornung *et al.*, 2008)
	Silica	ROS	(Dostert *et al.*, 2008)
	Alum	ROS	(Li *et al.*, 2008)
	Metal alloys	ND	(Caicedo *et al.*, 2009)
	CPPD†	ROS, lysosome damage	(Rock *et al.*, 2010)

ND, Not defined.
*Islet amyloid particulate protein (IAPP).
†Calcium pyrophosphate dihydrate (CPPD).

of such activators is shown in Table 1. Endogenous activators include host derived molecules which are typically "crystalline" or polymeric in nature and associated with danger, damage or cell death (Masters & O'Neill, 2011) including cholesterol crystals (Rock *et al.*, 2010), islet amyloid polypeptide (IAPP) (Masters *et al.*, 2010), amyloid β, hyaluron, monosodium urate (MSU) (Martinon *et al.*, 2009), ATP and possibly glucose (Rock *et al.*, 2010). Amyloid β and IAPP are believed to contribute to the disease pathology of Alzheimer's and possibly type 2 diabetes (T2D), respectively via induction of the NLRP3 inflammasome. Free uric acid (UA) derived from necrotic or infected cells and tissue is thought to form crystalline structures upon exposure

to extracellular space. Chronically elevated levels of UA and the deposition of monosodium urate (MSU) within joints are characteristic of gout causing both inflammation, fibrosis and cartilage destruction (Cotran *et al.*, 1994). In addition, traumatic injury such as fibrosis and ischemia reperfusion release a cascade of NLRP3 agonists including extracellular ATP and hyaluron activating the inflammasome.

Non-self environmental contaminants and insults such as asbestos, silica and UV radiation have also been shown to activate NLRP3 (Martinon *et al.*, 2006). Indeed, chronic inhalation of asbestos can lead to the activation of alveolar macrophages and result in pulmonary interstitial fibrosis (Cotran *et al.*, 1994; Timblin *et al.*, 1998).

NLRP3 detects exogenous PAMPs from bacteria, viruses and fungi. Pore-forming toxins from multiple bacteria such as hemolysin (*Staphylococcus aureus*) (Munoz-Planillo *et al.*, 2009), listerialysin O (*Listeria monocytogenes*) (Meixenberger *et al.*, 2010), toxin A (*Clostridium difficile*) (Ng *et al.*, 2010), tetanolysin O (*Clostridium tetani*) (Chu *et al.*, 2009) and aerolysin (*Aeromonas hydrophila*) (Gurcel *et al.*, 2006) have all been shown to activate the NLRP3 inflammasome. While a distinct role for AIM2 as a major receptor for pathogenic DNA and activation of the inflammasome has emerged (Rathinam *et al.*, 2010), it has been demonstrated that NLRP3 plays a minor but definite role in response to certain viruses. NLRP3 is necessary for caspase-1 activation in response to adenoviral DNA, herpes simplex virus and modified vaccinia virus (Muruve *et al.*, 2008; Delaloye *et al.*, 2009). Double stranded-RNA from influenza and sendai virus can also stimulate the NLRP3 inflammasome with a prominent requirement for NLRP3 particularly at high doses of virus i.e. influenza A (Ichinohe *et al.*, 2009).

NLRP3 is also necessary for host survival and responses to fungal pathogens including *Candida albicans* (Gross *et al.*, 2009), *Saccharomyces cerevisiae* (Lamkanfi *et al.*, 2009) and *Aspergillus fumigatus* (Said-Sadier *et al.*, 2010). With such a broad range of microbial, endogenous and environmental activators, attributing the activation of NLRP3 to a single PAMP or DAMP seems next to impossible.

Biochemical regulation of NLRP3

The precise biochemical mechanism of NLRP3 activation remains unknown. There is no evidence of direct ligand binding and the list of molecules described to activate it is extensive. The hypothesis that NLRP3 is a "sensor" to changes in the cellular environment rather than a "receptor" has gained favor. Several lines of evidence involving reactive oxygen species (ROS), lysosomal damage and potassium efflux exist to support this and will be discussed herein (Figure 3).

Lysosomal damage

The first line of evidence to support a common molecular signal for NLRP3 activation was work performed by Latz and colleagues (Hornung *et al.*, 2008). They observed that inefficient clearance of particulates including silica crystals following phagocytosis caused lysosomal damage and rupture. This process has been referred to as "frustrated phagocytosis". They demonstrated that rupture led to a release of the lysosomal protease cathepsin B which they believe triggered NLRP3 activation in the cytosol. Certainly, phagocytic uptake of a majority of particles (including MSU) has been shown to lead to swelling and rupture of phagocytic lysosomes and the release of cathepsin B. It is believed cathepsin B may act on NLRP3 itself or indeed cleave the ever elusive "endogenous" ligand for NLRP3 triggering its activation (Hornung *et al.*, 2008).

However, it has to be noted that macrophages from cathepsin B deficient mice display minimal reduction in inflammasome activation (Duewell *et al.*, 2010). Lysosomal rupture is not necessary for the non-particulate activators of NLRP3 such as ATP or nigericin (Hornung *et al.*, 2008). In support of a role for lysosomal damage and by default cathepsin B as a key activator of the NLRP3 inflammasome, studies continue to identify the involvement of both in the process (Niemi *et al.*, 2011; Hoegen *et al.*, 2011; Jin *et al.*, 2011). Hoegen *et al.*, demonstrate that the pneumococcal pore-forming toxin pneumolysin, a causative agent of *pneumococcal pneumonia and meningitis*, is a key inducer of IL-1β. Induction of IL-1β in this case is initiated via activation of the NLRP3 inflammasome and depends not only on the release of ATP but lysosomal destabilization and cathepsin B activation (Hoegen *et al.*, 2011). Similarly, serum amyloid A has been shown to activate the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway (Niemi *et al.*, 2011).

Reactive oxygen species

All known activators of NLRP3 including particulates, cause the generation of reactive oxygen species (ROS). Furthermore, chemical scavengers of ROS have been used to block the inflammasome in response to a range of NLRP3 activators (Dostert *et al.*, 2008; Newman *et al.*, 2009). Similar to the apoptosome, increased levels of ROS are necessary for NLRP3 activation (Cruz *et al.*, 2007). The source of such ROS was first believed to be the phagosome-associated NADPH oxidases which are activated upon phagocytosis of particulate matter that in turn activates the inflammasome (Dostert *et al.*, 2008). However, macrophages from cells deficient in four out of the seven NADPH oxidase complexes (NOX1, NOX2, NOX3 and NOX4) responded normally to activators of NLRP3, some displaying even slightly elevated responses (Latz, 2010).

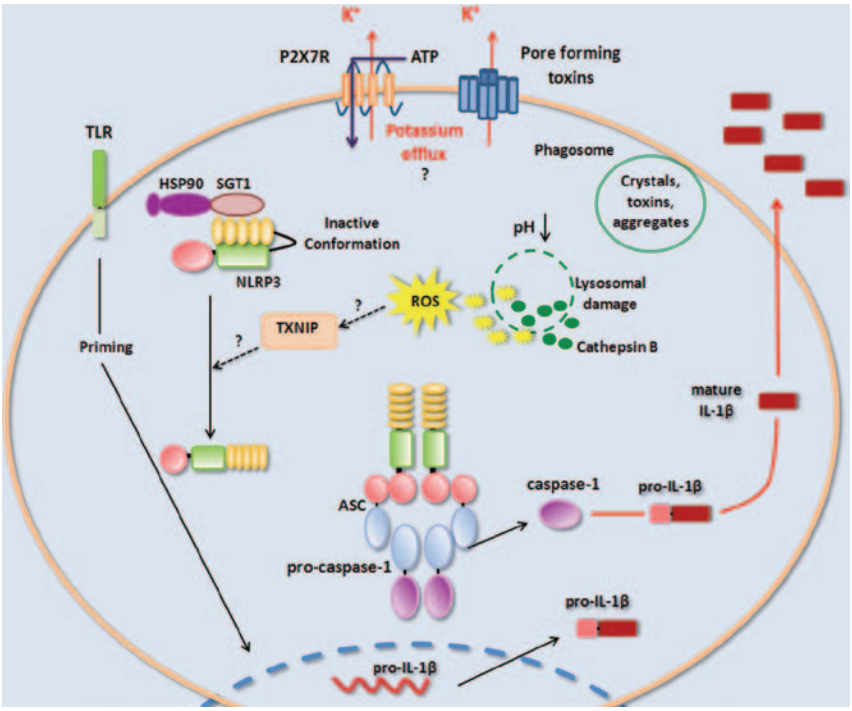

Figure 3. Biochemical regulation of the NLRP3 inflammasome. Activation of the NLRP3 inflammasome culminates in the processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18. The precise biochemical mechanisms underlying this process remain unknown. However, processing by NLRP3 is known to require 1) a priming step and 2) an activation step. 1) Priming: stimulation of PRRs leads to the upregulation of pro-1β, pro-IL-18 and most importantly the expression of NLRP3 (a rate limiting factor in the activation of the NLRP3 inflammasome). 2) Activation: NLRP3 can be activated by endogenous and exogenous stimuli (See Table 1). Activators include host derived molecules including cholesterol crystals, amyloid β, hyaluron, monosodium urate (MSU) and ATP. There is no evidence of direct ligand binding. Several lines of evidence involving reactive oxygen species (ROS), lysosomal damage and potassium efflux have been reported. Pore-forming toxins initiate potassium efflux via the P2X7 receptor and pannexin-1 structure which leads to NLRP3 inflammasome assembly by an unknown mechanism. Lysosomal damage which leads to the leakage of lysosomal enzymes including cathepsin B can trigger NLRP3 activation. The exact role of cathepsin B in NLRP3 activation is unclear. It is thought that the production of ROS in response to infection and or hazardous stimuli leads to the indirect activation of NLRP3 through the release of thioredoxin-interacting protein (TXNIP) from thioredoxin. TXNIP can bind to NLRP3 possibly by competing with HSP90 and SGT1 which retain NLRP3 in an inactive state. Ultimately upon activation homotypic interactions are formed between the N-terminal PYD domains of NLRP3 and ASC. Subsequently, the CARD domain of ASC molecules recruits pro-caspase-1 resulting in its autocleavage and activation which leads to the processing of IL-1β.

Currently, mitochondria are believed to be the main source of ROS for NLRP3 activation (Zhou *et al.*, 2011; Nakahira *et al.*, 2011). Inflammasome activation is extremely impaired in macrophages in which the mitochondrial outer membrane protein voltage-dependent anion channel (VDAC) has been inactivated or mitochondrial activity dampened via the removal of mitochondrial DNA (Zhou *et al.*, 2011; Nakahira *et al.*, 2011). Both mitochondrial DNA and VDAC are essential for the metabolic activity of mitochondria and by default ROS production via the electron transport chain.

Cells with diminished VDAC expression display impaired caspase-1 activation in response to NLRP3 activators. Furthermore, Bcl-2 family members are known to regulate VDAC activity. Bcl-2-transgenic mice display partial VDAC closure and a decrease in both mitochondrial Ca^{+} levels and ROS production. As such, macrophages from these mice display diminished levels of IL-1β. Interestingly, VDAC and by default mitochondria are not essential for the activation of the NLRC4 or AIM2 inflammasomes, highlighting the specificity in which inflammasomes are tightly regulated (Zhou *et al.*, 2011).

A plausible link between NLRP3 and mitochondria is strengthened by the fact that in resting cells, both endogenous and overexpressed NLRP3 have been shown to localize with the ER (Hayashi *et al.*, 2009). Following stimulation, NLRP3 relocates to perinuclear areas that stain positively for ER and mitochondria. This ER/mitochondrial staining is indicative of mitochondrial associated membranes (MAMs) and suggests that upon stimulation NLRP3 is critically located to receive signals from mitochondria. The adaptor molecule ASC has also been shown to relocate to these areas following stimulation. Furthermore, blockade of the electron transport chain by targeting Complex I and II via rotenone and antimycin A, respectively, leads to ROS production and has been shown to induce NLRP3 activation (Zhou *et al.*, 2010).

In the case of cellular stress or damage, ROS generating mitochondria are constantly removed from the cell by autophagy. Inhibiting autophagy enhances levels of ROS by damaged mitochondria and subsequently NLRP3 activation (Zhou *et al.*, 2011; Nakahira *et al.*, 2011). Taken together, this provides good evidence that there is a

significant role for ROS from mitochondria in NLRP3 activation. A recent study by Bauernfeind and colleagues indicated that the role of ROS in the NLRP3 inflammasome might be in the induction of NLRP3. As such transcriptional upregulation of NLRP3 can be blocked by the ROS inhibitors diphenylene iodonium (DPI) or N-acetylcysteine (NAC) (Bauernfeind *et al.*, 2011). Interestingly DPI is an inhibitor of both NOX-dependent ROS production and mitochondrial ROS while NAC will scavange ROS regardless of source. Using IL-18 as a read out to circumvent blockade of IL-1β transcription, the release of IL-1β in response to NLRP3 stimuli and not AIM2 or NLRC4 was blocked by DPI. The specificity on the NLRP3 inflammasome can be explained by the fact that NLRP3 is expressed at limiting levels and requires priming. In contrast, NLRC4 and AIM2 are constitutively expressed and independent of de novo translation. This work does not rule out a general role of ROS in triggering the NLRP3 inflammasome but places ROS upstream of NLRP3 induction. Finally, elsewhere it has been reported that thioredoxin-interacting protein (TXNIP) might be a direct ligand for NLRP3. TXNIP is activated by ROS, since upon oxidation it dissociates from thioredoxin and has been shown to interact with NLRP3. Furthermore, the expression of TXNIP is induced by glucose in β cells (Zhou *et al.*, 2010), repressed by insulin and elevated in T2D (Parikh *et al.*, 2007) a disease in which an imbalance of IL-1β activity impacts on islet inflammation. As such T2D patients have been successfully treated with inhibitors of IL-1 (Dinarello, 2011). However, the exact role of TXNIP in activation of the NLRP3 inflammasome has recently been disputed (Masters *et al.*, 2010).

Potassium efflux

In addition to lysosomal destabilization and the production of ROS, a decrease in K^+ levels in the cytosol is required for NLRP3 inflammasome activation. In the absence of priming high cytoplasmic levels of K^+ (150 mM) block activation by ATP, which as a potent activator of the NLRP3 inflammasome acts via the P2X7 receptor to reduce intracellular K^+ levels by approx. 50% (Perregaux & Gabel, 1994). This K^+ efflux is believed to trigger the formation of pores in the plasma membrane by pannexin-1 allowing delivery of microbial products to the cytosol for detection and activation of the inflammasome.

The bacterial toxin nigericin is a K^+ ionophore and activates NLRP3 (Mariathasan *et al.*, 2006). Extracellular ATP engages the ATP-gated cation channel P2X7 receptor, whereas bacterial toxins cause membrane pore formation to trigger K^+ efflux (Kahlenberg & Dubyak, 2004; Mariathasan *et al.*, 2006). Dropping intracellular levels of K^+ is sufficient to induce NLRP3 activation *in vitro* (Petrilli *et al.*, 2007a). Similarly, blocking K^+ efflux by the K^+ channel inhibitor glybencamide or the addition of high extracellular concentrations of K^+ inhibits NLRP3 inflammasome activation (Muruve *et al.*, 2008; Lamkanfi *et al.*, 2009; Masters *et al.*, 2010). Similar to NLRP3, oligomerization of NLRP1 is also inhibited by high K^+ concentrations (Petrilli *et al.*, 2007b).

There appears to be a requirement for K^+ efflux for the activity of certain NLRP3 stimuli, including crystalline activators and pore-forming toxins (Perregaux & Gabel, 1994; Walev *et al.*, 1995; Petrilli *et al.*, 2007b; Dostert *et al.*, 2008). In part, the inflammatory activities of adjuvants such as alum has been attributed to the fact that it activates NLRP3 via multiple mechanisms now known to include damage and rupture of the phagolysosomes, generating ROS and inducing K^+ efflux. The mechanism by which K^+ levels activate the inflammasome remains unknown. Some suggest it may be related to the mitochondria as they have several K^+ channels important to their function (Heinen *et al.*, 2007). However, ATP induced activation of NLRP3 is blocked when Na^+ in the cell culture medium was iso-osmotically substituted with Li^+ or choline or when Cl^- was replaced by SCN^- or I^- (Perregaux *et al.*, 1996). These studies suggest that perhaps a general change in the intracellular ionic state may be involved. Perhaps K^+ is the most potent or detectable as studies continually use it as a benchmark for activation of the NLRP3 inflammasome (Nakahira *et al.*, 2011; Lee *et al.*, 2011). Certainly, the dimerization of ASC is driven by subphysiological concentrations of potassium as shown *in vitro* (Fernandes-Alnemri *et al.*, 2007). The ionic environment of the cytosol therefore appears to be important for the inflammasome complex to associate. This environment possibly affects surface charge of the proteins involved, allowing them to associate in a complex and become activated.

Other regulatory mechanisms for NLRP3

In addition to the tight and complex biochemical regulation discussed, activation of NLRP3 appears to be regulated at other levels. The tyrosine kinase, Syk has recently been implicated in NLRP3 activation during fungal and malarial infections. Recent work highlights a link between phagosomal damage and Syk activity to NLRP3-mediated necrotic death (Wong & Jacobs, 2011). But the exact involvement of Syk in NLRP3 activity remains unknown. NLRP3 is also known to interact with CARD8 (CARDINAL, TUCAN) however, the relevance of this remains unclear as knockdown of CARD8 in monocytic cells does not alter inflammasome activation (Allen *et al.*, 2009). As a possible component of the NLRP3 inflammasome and inhibitor of NF-κB recent studies highlight the potential of CARD8 as a risk gene for inflammatory bowel disease (IBD) and ulcerative colitis (Schoultz *et al.*, 2009; Roberts *et al.*, 2010). Previous studies suggest NLRP3 is sequestered by heat shock protein 90 (HSP90) and ubiquitin ligase-associated protein, suppressor of G2 allele of skp1 (SGT1) in an auto-inhibitory but responsive conformation in the steady state (Shirasu, 2009). Most recently, the expression of caspase-4 which is located on the same locus as the caspase-1 gene was found to play a role in the regulation the NLRP3 inflammasome.

Specifically, caspase-4 was required for UVB-induced activation of pro-IL-1β in keratinocytes and for the NLRP3- and AIM2-dependent processing of pro-IL-1β in macrophages (Sollberger *et al.*, 2012). A direct role for NLRP3 in adipocytes has also been suggested as differentiation of preadipocytes from NLRP3–/– and caspase-1–/– mice leads to the production of fat cells with a higher metabolic activity than wild types. Deletion of NLRP3 in mice prevents lipid induced inflammasome activation and decreases fat deposits in the liver (Stienstra *et al.*, 2010; Vandanmagsar *et al.*, 2011). Therefore, NLRP3 may also sense obesity related danger signals and contribute to metabolic changes and inflammation associated with the disease.

Although our understanding of the biochemical regulation of the NLRP3 inflammasome has greatly expanded, a unifying molecular mechanism responsible for its activation remains largely unknown. Currently, there is difficulty reconciling the requirement for ROS, K^+ efflux and lysosomal damage into such a unifying mechanism. For example, while K^+ efflux is required for the activation of NLRP3, NLRP1 and to a lesser extent NLRC4 also require K^+ efflux. Yet these inflammasomes all respond to different stimuli.

A recent study by Shimada *et al.* (2012) linking ROS and K^+ efflux to the production of oxidized mitochondrial DNA (mtDNA) provides the most appealing mechanism to date. Shimada and colleagues demonstrate that NLRP3 secondary signal activators including ATP and nigericin induce mitochondrial dysfunction and apoptosis. These events result in the oxidation of mtDNA and its release into the cytosol where it was demonstrated to bind to NLRP3 and may be the underlying mechanism responsible for NLRP3 inflammasome activation. Importantly, mitochondrial dysfunction was eliminated by compensating K^+ efflux with the addition of extracellular K^+.

Certainly, several studies have demonstrated a central role for mitochondria in the activation of NLRP3. The activation of mitochondrial ROS is a requirement for NLRP3 activation. Also following activation, NLRP3 has been shown to localize with the mitochondrial associated membrane (MAM), suggesting that it is critically located to receive signals from mitochondria (Hayashi *et al.*, 2009). Furthermore, NLRP3 activation is extremely impaired when the mitochondrial anion channel VDAC has been inactivated or mitochondrial activity dampened via the removal of mitochondrial DNA (Zhou *et al.*, 2011; Nakahira *et al.*, 2011).

In line with this, the study by Shimada and colleagues demonstrates that mtDNA is required for NLRP3-dependent IL-1β secretion. Furthermore, this work showed that oxidized DNA induced IL-1β secretion via preferential activation of NLRP3 and not AIM2. In addition, the inhibition of autophagy prevents the disposal of damaged mitochondria, thereby boosting NLRP3 inflammasome activity (Nakahira *et al.*, 2011). Shimada and colleagues demonstrate the endogenous binding of oxidized mtDNA with NLRP3 following the addition of NLRP3 activators in the presence of inhibitors of autophagy. This suggests that under such conditions, sufficient amounts of DNA reach the cytosol to form a complex with NLRP3. It remains unclear as to what controls the release of the oxidized mtDNA and whether it is part of the active inflammasome or only initiates oligomerization. Future work looking specifically at the characterization of this interaction will aid efforts to elucidate the unifying mechanism of NLRP3 inflammasome activation.

NLRP1 inflammasome

NLRP1 was the first NLR family member characterized with respect to inflammasome assembly and caspase-1 activation (Martinon *et al.*, 2002). It also has a unique combination of domains compared to other NLRs. While it has an N-terminal PYD and centrally located NACHT and LRR domains, it has a C-terminal extension consisting of an internal pair of ZU5- and UPA- like domains (previously known as the FIIND domain) and a CARD domain (Figure 1). The ZU5- and UPA- like domains have been shown to confer intra-proteolytic activity causing cleavage of NLRP1 (D'Osualdo *et al.*, 2011). This structure and particularly the presence of both a PYD and CARD suggest NLRP1 may have multiple signaling roles. Certainly, NLRP1 is expressed not only in immune cells and tissues but non-hematopoietic tissues, unlike NLRP3 and NLRC4 (Kummer *et al.*, 2007). Interestingly, variants in the genomic region of human NLRP1 are attributed to the risk of several autoimmune diseases including; vitiligo, vitiligo-associated type-I diabetes and Addisons disease (Jin *et al.*, 2007a,b). NLRP1 is encoded by a single locus in humans, whereas mice have several paralogs located on chromosome 11 (NLRP1a, NLRP1b and NLRP1c) (Boyden & Dietrich, 2006). Furthermore, murine NLRP1 lacks an N-terminal PYD. For these reasons NLRP1 is believed to have different roles in man and mouse. For example, murine NLRP1 could perform CARD8 (CARDINAL)-like functions in mice (Martinon *et al.*, 2009). CARD8 is a regulatory CARD-containing protein and shares high homology with the CARD of caspase-1 and the C-terminal region of NLRP1 but is not present in the mouse genome (Bouchier-Hayes & Martin, 2004). CARD8 has been shown to interact with NLRP3 (Agostini *et al.*, 2004; Fernandes-Alnemri *et al.*, 2007), however results indicate the interaction is not a requirement for the activation of the NLRP3 inflammasome (Allen *et al.*, 2009).

The human NLRP1 inflammasome was initially described to comprise NLRP1, ASC, caspase-1, and caspase-5. The interaction between NLRP1 and ASC facilitated the binding and processing of caspase-1 while the interaction between NLRP1 and caspase-5 induced processing of caspase-5 (Martinon *et al.*, 2002). To date there are two well characterized activators of the NLRP1 inflammasome: anthrax lethal toxin (LeTx) derived from spore forming bacterium *Bacillus antracis*, and the peptidoglycan component muramyl dipeptide (MDP)

of bacterial cell walls (Boyden & Dietrich, 2006; Faustin *et al.*, 2007).

Faustin and colleagues first showed that in human cell free lysates NLRP1 and caspase-1 were the minimum components required for inflammasome assembly and activation via interaction of their CARD domains. While MDP was sufficient for the activation of NLRP1 and cleavage of caspase-1, robust NLRP1 inflammasome activation required the addition of small amounts of ASC (Faustin *et al.*, 2007). This work proposed a two-step mechanism for NLRP1 activation. Firstly, the binding of MDP to the LRR of NLRP1 induced a conformational change, which in turn allowed subsequent binding of nucleotide triphosphates (NTPs) and self oligomerization via the NACHT domain. This was supported by earlier work demonstrating that deletion of the LRRs of NLRP1 facilitated the binding of ATP making NLRP1 constitutively active (Martinon *et al.*, 2002). Ultimately, direct binding of MDP to NLRP1 has never been adequately demonstrated and raises the possibility that the activation of NLRP1 by MDP is indirect. It has been demonstrated that the Bcl-2 family members Bcl-2 and Bcl-X(L) are bound to NLRP1 basally and following activation of macrophages, NLRP1 is released from this complex possibly allowing the binding of ATP (Bruey *et al.*, 2007). The inhibitory effect of Bcl-2 proteins was mapped to a 10-mer loop peptide, which bound and inhibited NLRP1 with high affinity. Furthermore, MDP-mediated IL-1β production is elevated in mice deficient for Bcl-2 (Bruey *et al.*, 2007; Faustin *et al.*, 2009).

B. antracis secretes a lethal toxin (LeTx) comprised of two subunits, protective antigen (PA) and lethal factor (LF). PA works by transporting LF into the cytosol of host cells giving it access to NLRP1. In mouse macrophages, LF causes rapid necrosis and is the causative agent of systemic *B. antracis* infection (Banks *et al.*, 2006). The binding, uptake, and endosome acidification of *B. antracis* are required to mediate translocation of LF into the cytosol of the host. LF cleaves cytosolic substrates by mechanisms involving Ca^{2+} ion flux and proteasome activity promoting the activation of caspase-1 by LeTx. Indeed catalytically inactive but structurally similar mutant LF fails to activate caspase-1 making direct recognition of LF by NLRP1 unlikely. It is thought LF may cleave as yet unknown inhibitors of NLRP1 and/or facilitate the production of activating factors that trigger NLRP1 inflammasome activation (Squires *et al.*, 2007; Fink *et al.*, 2008; Wickliffe *et al.*, 2008). Genetic studies have mapped variable sensitivity to LeTx-induced necrosis in macrophages of inbred mouse strains to variations in the polymorphic gene NLRP1b on chromosome 11 (Banks *et al.*, 2006). The NLRP1 paralog NLRP1b has been identified as the primary mediator of mouse macrophage susceptibility to LeTx. Reports demonstrate that LeTx-induced macrophage death requires caspase-1, which was activated in susceptible macrophages after *in vitro* treatment with LeTx. On the other hand, caspase-1 was not activated in resistant macrophages in response to LeTx (Nour *et al.*, 2009).

Recent work also suggests a role for NLRC2 (also known as nucleotide-binding oligomerization domain containing 2 (NOD2)) in the assembly of the NLRP1 inflammasome in response to both MDP and anthrax LeTx (Hsu *et al.*, 2008). Previous reports have identified NOD2 as an intracellular sensor for MDP. Furthermore, NOD2 contains two N-terminal CARD domains and initiates activation of NF-κB and MAPK kinases via RIP2 (Girardin *et al.*, 2003). NLRP1 is sufficient to activate caspase-1 in response to MDP in a cell free system, however NOD2 is required for *in vivo* detection of both MDP and LeTx. (Hsu *et al.*, 2008). NOD2 has been shown to interact directly with caspase-1 and NLRP1 and not with NLRP3. NOD2 may be able to generate both signals required for the activation of the NLRP1 inflammasome. The first signal is the activation of NF-κB and induction of pro-IL-1β and the second, recruitment and activation of caspase-1 through its N-terminal CARD facilitating the processing of mature IL-1β (Figure 4) (Hsu *et al.*, 2008; Ferwerda *et al.*, 2008).

Many questions remain concerning NLRP1. It appears both unique in its structure and potential signaling roles relative to other inflammasomes. NLRP1 interacts with caspase-2 and caspase-9 to facilitate cell death via the apoptosome (Hlaing *et al.*, 2001). The physiological relevance of this is unknown due to the lack of a NLRP1-deificient mice. Certainly, NLRP1 oligomers are arranged in a similar higher order structure to the APAF-1 apoptosome (Faustin *et al.*, 2007).

It is unclear whether MDP and anthrax LeTx are detected directly by NLRP1 or in combination with NOD2. NLRP1 cannot be dismissed as an actual MDP receptor as the role of MDP in NLRP1 inflammasome activation has been exclusively addressed in human cells, whereas the requirement of NOD2 has been concluded from mouse studies (Hsu *et al.*, 2008). The involvement of caspase-5 in the NLRP1 inflammasome remains unknown. Furthermore, the exact role of ASC has yet to be clarified. As murine NLRP1 lacks an N-terminal PYD, a direct interaction of murine ASC and NLRP1 seems to be unlikely. The secretion of IL-1β by the NLRP1 inflammasome is dependent on K^+ efflux and the lysosomal protein cathepsin B in a manner analogous to particulate uptake in NLRP3-dependent inflammasome activation (Squires *et al.*, 2007; Fink *et al.*, 2008; Wickliffe *et al.*, 2008).

NLRC4 inflammasome

NLRC4 (also known as Ipaf and Card12) consists of an N-terminal CARD, a central NACHT and a C-terminal LRR domain (Figure 1). Activation of NLRC4 is associated with a rapid form of cell death termed "pyroptosis" and the processing of IL-1β. Lacking a PYD, NLRC4 interacts with pro-caspase-1 directly via its CARD (Geddes *et al.*, 2001). ASC is not necessary for NLRC4-dependent cell death but has been shown to enhance IL-1β processing in an NLRC4-dependent manner (Mariathasan *et al.*, 2004). The activation of caspase-1 by NLRC4 is strongly

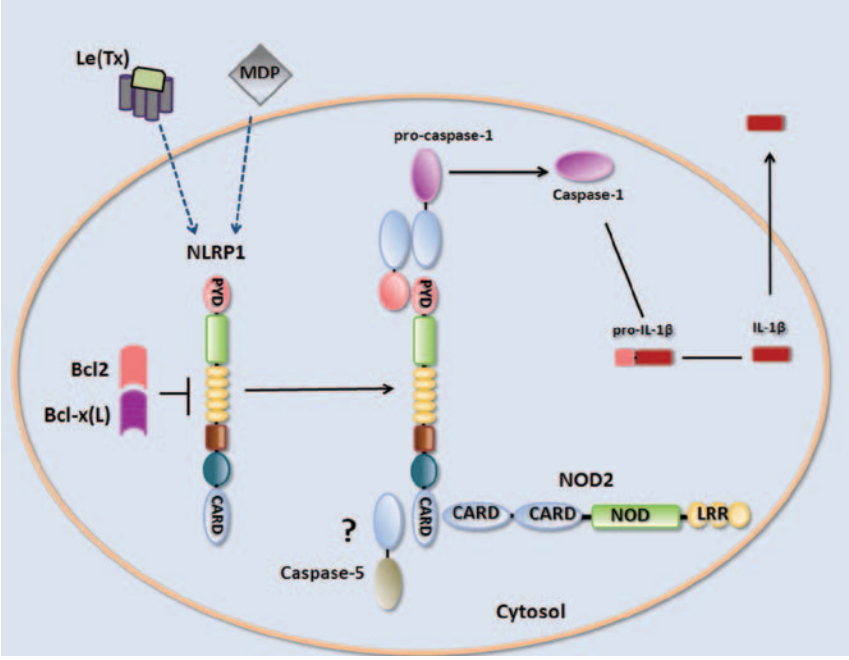

Figure 4. Activation and regulation of the NLRP1 inflammasome. NLRP1 was the first NLR family member characterized with respect to inflammasome assembly and caspase-1 activation. NLRP1 contains both a PYD and CARD and may have multiple signaling roles. Interactions between NLRP1 and ASC facilitate the binding and processing of caspase-1 while the interaction between NLRP1 and caspase-5 induces processing of caspase-5. The exact role of caspase-5 in this context is unknown. There are two well characterized activators of the NLRP1 inflammasome, anthrax lethal toxin (LeTx) derived from spore forming bacterium *Bacillus antracis* and the peptidoglycan component muramyl dipeptide (MDP) of bacterial cell. MDP is sufficient for the activation of NLRP1 and cleavage of caspase-1, the addition of small amounts of ASC is required for robust NLRP1 inflammasome activation. Direct binding of MDP to NLRP1 has never been demonstrated. Apoptotic proteins, Bcl-2 and Bcl-X(L) are bound to NLRP1 basally and following activation NLRP1 is released possibly allowing self oligomerization of NLRP1 molecules for the activation of the inflammasome. The binding, uptake, and endosome acidification of *B. antracis* is required to mediate activation of NLRP1 in response to Le(Tx). Recent work suggests a role for NLRC2 (also known as nucleotide-binding oligomerization domain containing 2 (NOD2)) in the assembly of the NLRP1 inflammasome. NOD2 has been shown to interact directly with caspase-1 and NLRP1 and not with NLRP3. The exact role of NOD2 in the NLRP1 inflammasome has to be elucidated.

associated with pryoptosis. On the other hand, stimuli that activate caspase-1 through NLRP3 predominantly result in the processing and secretion of IL-1β which does not necessarily result in cell death. This highlights the fact that alternate responses from multiple NLRs direct the activation of caspase-1 toward cytokine processing or pyroptosis, dictating the fate of the cell.

Intracellular flagellin was the first pathogenic component demonstrated to activate the NLRC4 inflammasome (Franchi *et al.*, 2006; Miao *et al.*, 2006). The idea of flagellin as a possible ligand for NLRC4 is appealing. In this sense, it could act as a single PAMP that could initiate both the priming of IL-1β via TLR5 (Hayashi *et al.*, 2001) and its subsequent processing by activating the NLRC4 inflammasome. NLRC4-deficient macrophages display markedly reduced secretion of IL-1β and pyroptosis after infection with gram-negative bacteria *Salmonella typhimurium*, *Legionella pneumophila*, *Shigella flexneri*, and *Pseudomonas aeruginosa* (Mariathasan *et al.*, 2004; Sutterwala & Flavell, 2009). Furthermore, *L. pneumophila* and *S. typhimurium* strains deficient in flagellin are unable to activate caspase-1 in macrophages (Miao *et al.*, 2006; Zamboni *et al.*, 2006). On the other hand, *S. flexneri* does not express flagellin yet caspase-1 activation following infection with the bacterium is NLRC4 dependent. Activation of NLRC4 by these gram-negative bacteria was later shown to be dependent on a functional type-III or type-IV secretion system, T3SS and T4SS, respectively. These systems work by delivering activating factors into the cytosol of host cells (Miao *et al.*, 2010). *S. typhimurium, P. aeruginosa* and *S. flexneri* use a T3SS to inject NLRC4 activating virulence factors, whilst *L. pneumophila* makes use of a T4SS.

The matter was clarified further when Miao and colleagues demonstrated that NLRC4 can detect rod proteins which make up the basal rod body component of the T3SS apparatus of several gram-negative bacteria. Indeed, rod proteins share a sequence motif very similar to the C-terminal part of flagellin and are essential for detection by NLRC4. *S. typhimurium* requires the translocase SipB from its SPI1 T3SS to induce IL-1β secretion in infected cells. SipB is a component of the transmembrane pore inserted into the host cell membrane that delivers proteins to the cytosol (Miao *et al.*, 2010). On the other hand, the gram-positive and flagellated bacterium *Listeria monocytogenes* triggers NLRC4 activation even though it does not express a secretion system. In this case, flagellin from *L. monocytogenes* gains access to the cytosol when the bacterium escapes from the phagolysosome in order to replicate within the host cell (Warren *et al.*,

2008). Infection of mouse macrophages with *S. flexneri* demonstrates both an NLRC4 and NLRP3-dependent activation of caspase-1, cell death and IL-1β processing (Willingham *et al.*, 2007; Suzuki *et al.*, 2007) with the NLRP3 inflammasome playing a prominent role at high doses of *S. flexneri* infection. Additionally, most recent studies reveal a tissue specific role for the NLRC4 inflammasome in innate immune responses against mucosal *C. albicans* (Tomalka *et al.*, 2011). NLRP3 is known to limit the severity of infection with *C. albicans* in hematopoietic or stromal compartments and NLRC4 was demonstrated to play a specific role in limiting mucosal responses to the infection. Furthermore, and quite interestingly only flagellin from specific bacteria induce inflammasome activation via NLRC4. Even when delivered to the host cytosol, flagellin of *Escherichia coli* fails to activate NLRC4 (Ren *et al.*, 2006). This demonstrates the ability of NLRC4 to distinguish different sources of flagellin that may represent a host strategy to discriminate pathogenic from commensal bacteria.

Similar to NLRP3, the exact mechanisms regulating NLRC4 activation are largely unknown. There is no direct evidence of flagellin or rod proteins binding NLRC4 (Franchi *et al.*, 2006). NLRC4 may act as a sensor to some cellular response or host factor induced by flagellin. Evidence suggests that NLRC4 is not activated in an exclusive manner to its stimuli. Recent work demonstrates that NAIP (NLR family, apoptosis inhibitory protein) paralogues determine the specificity of the NLRC4 inflammasome for distinct bacterial ligands. A homologue of NLRC4, NAIP5 has been implicated in activation of the NLRC4 inflammasome (Kofoed & Vance, 2011; Lightfield *et al.*, 2011). NAIP5 is required for the activation of caspase-1 in response to *L. pneumophila* and both NLRC4 and NAIP5 have been shown to interact in ectopic overexpression systems (Lightfield *et al.*, 2008). However, NAIP5 is not required for caspase-1 activation in response to *S. typhimurium, P. aeruginosa* and *L. monocytogenes* indicating that NAIP5 plays an auxiliary role in the context of NLRC4 activation in response to *L. pneumophila*. Additionally, the activation of the NLRC4 inflammasome by the protein, PrgJ of the T3SS of *Salmonella enterica* requires NAIP2 (Kofoed & Vance, 2011; Zhao *et al.*, 2011). NAIP6 was also found to activate the NLRC4 inflammasome in response to bacterial flagellin (Kofoed & Vance, 2011). A ligand dependent oligomerization and hence activation of NLRC4 was found to be the underlying mechanism explaining the interaction of NAIPs with NLRC4. The NLRC4-NAIP2 complex was shown to interact with PrgJ but not flagellin, whereas NLRC4-NAIP5 associated with flagellin but not PrgJ. The specificity with which these NAIP proteins facilitate the activation of the NLRC4 inflammasome suggests other NAIP family members may recognize as of yet unidentified microbial products to aid activation. The current model for activation of the NLRC4 inflammasome is illustrated in Figure 5.

In more general terms, several parameters have been investigated with regard to the biochemical regulation of the NLRC4 inflammasome. K^+ flux was the first to be investigated. Initial studies suggested that K^+ efflux was not a prerequisite for NLRC4 inflammasome activation (Petrilli *et al.*, 2007b). However, raising extracellular levels of K^+ was sufficient to block NLRC4 activation in response to the T3SS of a non-flagellated strain of *P. aeruginosa* (Arlehamn *et al.*, 2010). This indicated a requirement for a decrease in intracellular K^+ levels for the activation of the NLRC4 inflammasome similar to that seen for NLRP3. Albeit the concentration of extracellular K^+ used was much higher than that required for inhibition of the NLRP3 inflammasome. However it is worth noting that other groups did not observe activation of caspase-1 activation following infection with *P. aeruginosa* deficient in flagellin (Franchi *et al.*, 2007; Miao *et al.*, 2008). As a result, the possibility of a role for K^+ efflux in the NLRC4 inflammasome requires further clarification.

Additionally, deletion of the LRR domain of NLRC4 results in a constitutively active form of the molecule in a similar manner to other NLRs that gain activity upon deletion of their LRR domain (Poyet, 2001). NLRC4 also interacts with HSP90 and SGT1 (Shirasu, 2009). HSP90 and SGT1 have been shown to be required for NLRP3 activation and are predicted to also be required for NLRC4 (Mayor *et al.*, 2007). The role of ASC in the NLRC4 inflammasome is also ambiguous. ASC is required for an NLRC4-dependent response to *S. typhimurium, P. aeruginosa* and *L. monocytogenes*. Certainly, lower levels of caspase-1 activation and IL-1β secretion have been reported amongst ASC-deficient macrophage in response to *S. typhimurium, P. aeruginosa* and *S. flexneri* (Schroder & Tschopp, 2010). On the other hand, ASC is not necessary for NLRC4-dependent cell death (Mariathasan *et al.*, 2004; Suzuki *et al.*, 2007; Sutterwala *et al.*, 2007). This indicates that NLRC4 may utilize ASC under certain cellular contexts including the processing of IL-1β. Furthermore, cell death associated with *S. flexneri* infection is independent of caspase-1, but dependent on cathepsin B (Willingham *et al.*, 2007). There are many questions to be answered regarding the exact biochemical regulation of the NLRC4 inflammasome.

AIM2 inflammasome

The PYHIN family of proteins (also known as the IFI-200/HIN-200 family) consists of proteins with an N-terminal PYD domain and one or two copies of a 200 amino acid repeat HIN (HIN-200) domain at their C-terminal (Figure 1). The family was originally classified based on these conserved structural domains, the ability to be induced by type-I interferons (IFN), nuclear localization and expression among hemopoietic cells. More recently, based solely on their structure, the PYHIN family of proteins is referred to as PYHIN proteins or AIM2-like receptors (ALRs). There are six known PHYIN proteins in mice p202a, p202b, p203, p204, MNDAL and AIM2

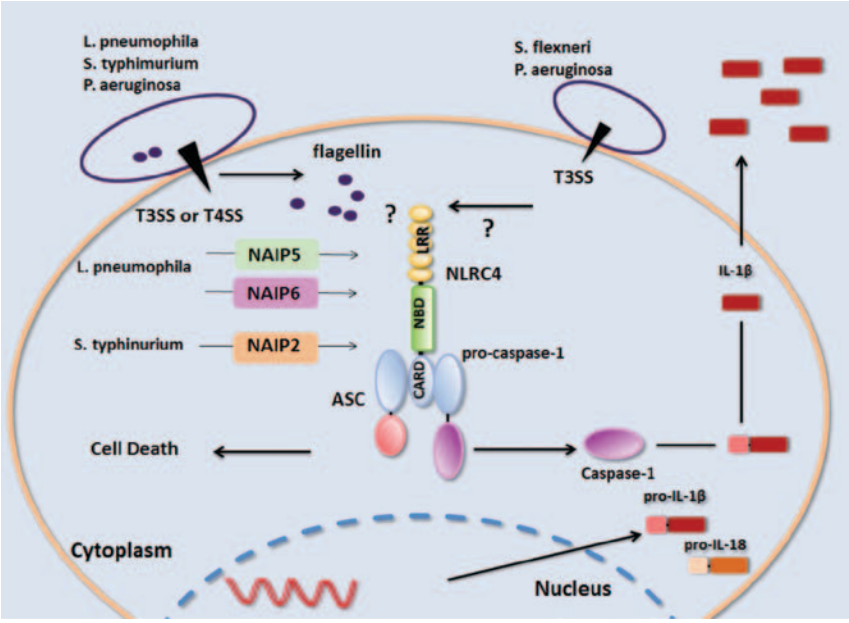

Figure 5. Biochemical regulation of the NLRC4 inflammasome. Activation is associated with a rapid form of cell death termed "pyroptosis" and the processing of IL-1β. Lacking a PYD, NLRC4 interacts with pro-caspase-1 directly via its CARD. ASC is not necessary for NLRC4-dependent cell death but has been shown to enhance IL-1β processing in an NLRC4-dependent manner. Intracellular flagellin was the first pathogenic component demonstrated to activate the NLRC4 inflammasome. Flagellin may act as a single PAMP initiating both the priming of IL-1β via TLR5 and its subsequent processing by activating the NLRC4 inflammasome. Activation of the NLRC4 inflammasome by various gram-negative bacteria including *Salmonella typhimurium*, *Legionella pneumophila*, *Shigella flexneri* and *Pseudomonas aeruginosa* is dependent on a functional type-III or type-IV secretion system (T3SS and T4SS). These systems work by delivering activating factors into the cytosol of host cells. There is no direct evidence of flagellin or rod proteins binding NLRC4. Recent work highlights that NLR family, apoptosis inhibitory protein paralogues (NAIPs) determine the specificity of the NLRC4 inflammasome. NAIP5 and NAIP6 have been implicated in activation of the NLRC4 inflammasome in response to *L. pneumophila* while NAIP2 has been shown to play a role in the activation of the NLRC4 inflammasome by the protein, PrgJ of the T3SS of *Salmonella enterica*. A role if any for K^+ flux remains unknown. Some studies demonstrate a requirement for a decrease in intracellular K^+ levels for the activation of the NLRC4 inflammasome. The possibility of a role for K^+ efflux requires further clarification.

and four in humans IFI16, AIM2, MNDA and IFIX (Asefa *et al.*, 2004; Ludlow *et al.*, 2005). PHYIN proteins play a role in proliferation and apoptosis (Ludlow *et al.*, 2005; Mondini *et al.*, 2010). Most recently, members of the family, namely AIM2 and IFI16 have been highlighted as molecular sensors of microbial DNA and activators of the inflammasome. Several DNA recognition receptors have been identified and include DNA-dependent activator of interferon (IFN)-regulatory factors (DAI), RNA polymerase III (Pol III), DExD/H box helicases (DHX9 and DHX36), Ku70 and of course AIM2 and IFI16 (Schattgen & Fitzgerald, 2011). Of the known sensors for microbial DNA, AIM2 and IFI16 are the only two that belong to the same family.

As a PYHIN protein, AIM2 contains an N-terminal PYD. This is followed by a HIN-200 domain of the subtype C (HIN C) (Figure 1). The conserved HIN motifs have been implicated in protein protein interactions yet the exact functional role for many remains unclear. On the other hand, they have been shown to bind DNA. In this context, viral and microbial DNA activate a number of innate immune pathways that result in the production of pro-inflammatory cytokines such as IFN and IL-1β. A study infecting macrophages with the DNA viruses adenovirus (AdV) or herpes simplex virus-1 (HSV-1) demonstrated the activation of caspase-1 in response to these stimuli. This raised the possibility that viral DNA was capable of triggering an inflammasome. Indeed, administration of purified viral DNA to the cytosol was a potent trigger of caspase-1 activation (Muruve *et al.*, 2008). Intriguingly, this response was dependent on the presence of ASC but independent of NLRP3. The dependence on ASC implied that an upstream sensor was responsible for recognizing the cytosolic DNA and as such activation would involve the interaction of the PYD of ASC with that of such a "sensor".

At this time four independent studies classified AIM2 as the component responsible for the formation of a functional inflammasome and activation of caspase-1 in response to cytosolic DNA (Roberts *et al.*, 2009; Hornung *et al.*, 2009; Fernandes-Alnemri *et al.*, 2009; Burckstummer *et al.*, 2009). These findings were confirmed by studies in AIM2-deficient mice (Rathinam *et al.*, 2010; Fernandes-Alnemri *et al.*, 2010). Furthermore, ASC-dependent caspase-1 cleavage was demonstrated following exogenous expression of AIM2 in HEK293 cells and the HIN C domain of AIM2 was shown to directly bind cytosolic DNA. Upon binding DNA, AIM2 likely undergoes oligomerization and associates with ASC via PYD interactions initiating the recruitment of pro-caspase 1 and processing of IL-1β (Figure 6).

Before the discovery of the AIM2 inflammasome, it was noted that AIM2 was different to other PYHIN proteins. Its PYD was more closely related to that of the NLRPs and of ASC. Also in contrast to IFIX, IFI16, and MNDA, all of which were localized to the nucleus, AIM2 was cytosolic (Ludlow *et al.*, 2005). The AIM2 inflammasome became the first inflammasome described not to comprise an NLR and AIM2 the first PYHIN protein to have involvement in innate responses to infection. In addition to the processing of IL-1β, activation of the AIM2 inflammasome leads to the induction of pryoptosis similar to NLRC4. It is thought that AIM2-mediated pryoptosis plays an important role in the clearance of infection.

The source and sequence of cytoplasmic dsDNA does not appear important for activation of the AIM2 inflammasome. Viral, bacterial, mammalian and synthetic dsDNA have all been shown to activate caspase-1 when delivered to the cytosol (Muruve *et al.*, 2008; Hornung *et al.*, 2009; Schroder *et al.*, 2009). The only necessity here is that the DNA is double stranded. Furthermore, immunogenicity was found to increase with increasing DNA length. Therefore, the localization of dsDNA in the cytoplasm is sufficient to activate the AIM2 inflammasome. Additionally, the fact that AIM2 is responsive to mammalian DNA has led many to speculate that AIM2 may play a role in autoinflammatory responses. In this context, patients suffering from systemic lupus erythematodes (SLE) have been shown to develop autoantibodies against dsDNA that forms immunocomplexes which may be recognised by AIM2 (Baechler *et al.*, 2003). Furthermore, HIN-200 proteins lie within a susceptibility loci for SLE (Choubey & Panchanathan, 2008).

The AIM2 inflammasome plays an integral role in response to both viral and bacterial infection. Vaccinia virus (VACV), a poxvirus that replicates in the cytoplasm, induces caspase-1 activation and the processing of IL-1β and IL-18 in a manner that is heavily AIM2 dependent (Rathinam *et al.*, 2010). Upon infecting cells, viruses undergo a life cycle during which at certain points the viral genome must be exposed to the cytoplasm. At this point, dsDNA from these pathogens gains access to AIM2 (Figure 6). The AIM2 inflammasome is also activated in response to the DNA virus mouse cytomegalovirus (MCMV) which gains access to the cytosol. However, activation of the AIM2 inflammasome has yet to be confirmed for all other DNA viruses including those of the herpesviruses family (Schattgen & Fitzgerald, 2011). For example, during infection with HSV-1, DNA from the pathogen builds up within the cytosol and activates IFI16 (Kim *et al.*, 2010b). The differential recognition of pathogenic DNA by AIM2 is an important point of investigation

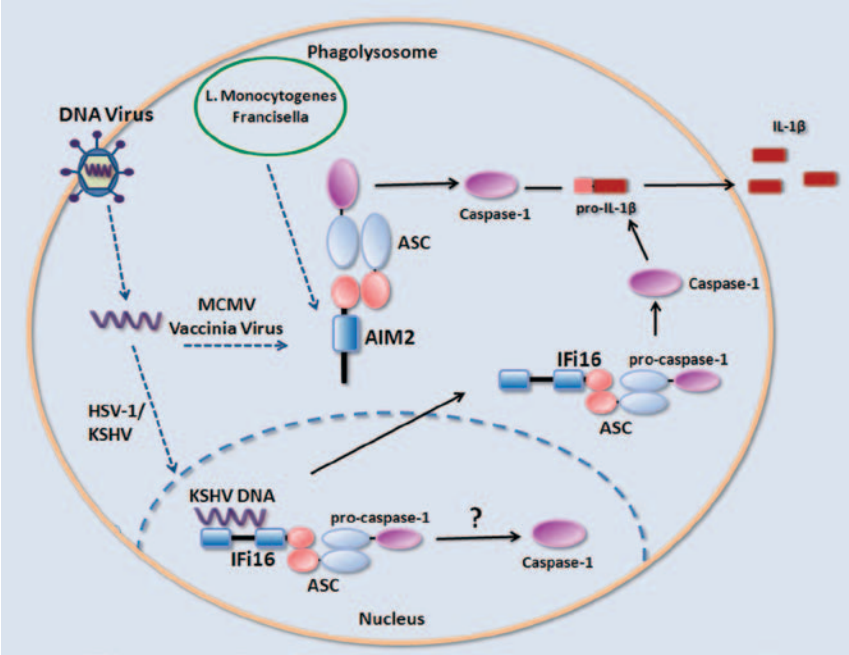

Figure 6. Schematic representation of AIM2 and IFI16 inflammasome activation. The AIM2 inflammasome is most notably unique as a direct ligand has been identified. Cytosolic dsDNA from invading viruses and bacteria directly bind to AIM2 resulting in the recruitment of ASC and activation of pro-caspase-1 which leads to the processing of IL-1β. dsDNA from vaccinia virus, mouse cytomegalovirus (MCMV), *L. monocytogenes* and *F. tularensis* have all been shown to activate the AIM2 inflammasome. Exactly how DNA from intact bacteria leaks into the cytosol to activate AIM2 remains unknown. Endogenous DNA complexes may also lead to activation of AIM2 (i.e. DNA antibody complexes, not shown). While IFI16 can directly bind viral DNA via its HIN-200 domains and induce interferon beta (IFNβ) in a stimulator of interferon genes (STING), TBK1, and IRF3-dependent manner (not shown), a role for IFI16 in the detection of DNA in the nucleus has also been demonstrated. IFI16 has the potential to bind both DNA via its HIN domains and to bind ASC via its PYD domain. IFI16 binds viral DNA of Kaposi's sarcoma-associated herpes virus (KSHV) in the nucleus and associates with ASC leading to the recruitment of pro-caspase-1 and processing of pro-IL-1β.

and the biochemical regulation of this process has yet to be determined.

Bacteria including *Francisella tularensis* and *L. monocytogenes* are also capable of activating AIM2 (Rathinam *et al.*, 2010; Fernandes-Alnemri *et al.*, 2010; Kim *et al.*, 2010a). *F. tularensis* is a facultative intracellular pathogen and while infection is mediated by the phagocytic pathway, the bacteria are known to quickly escape the phagosome and replicate in the cytosol. Subcutaneous *Francisella* infection of AIM2-deficient mice results in decreased IL-1β/IL-18 secretion, cell death and increased mortality. Furthermore, *Francisella novicida* infection is known to induce IL-1β and IL-18 independent of NLRP3 and NLRC4 but is dependent upon ASC. As a result it appears AIM2 is a sensor during *Francisella* infection.

As discussed, several reports demonstrate that both NLRP3 and NLRC4 are activated in response to *L. monocytogenes*. However, several studies have also confirmed that AIM2 acts as a molecular sensor for the pathogen, activating the inflammasome and initiating the processing of IL-1β. Exactly how DNA from intact bacteria leaks into the cytosol to activate AIM2 remains unknown. Phagosomal acidification during infection with *L. monocytogenes* is well documented (Sauer *et al.*, 2010). Following phagocytosis, *Listeria* quickly escapes the phagosome entering the cytosol to undergo replication. At this point, DNA from *Listeria* may gain access to AIM2. Certainly, in order to escape the phagosome *Listeria* requires listeriolysin O (LLO). Infection with a LLO-deficient strain of *Listeria* is unable to activate the AIM2 inflammasome (Warren *et al.*, 2010). Furthermore, several groups have confirmed a role for AIM2 in response to *L. monocytogenes* (Rathinam *et al.*, 2010; Fernandes-Alnemri *et al.*, 2010). Specifically, suppressed levels of caspase-1 cleavage and IL-1β secretion have been demonstrated in macrophages from AIM2-deficient mice in response to *L. monocytogenes*.

The AIM2 inflammasome is most notably unique among inflammasomes as a direct ligand has been identified. It has also been determined that there is a length dependency to the DNA that can activate AIM2. Short double-stranded DNA fails to activate the AIM2 inflammasome. While lacking a NACHT domain, it is hypothesized that in order to oligomerize multiple, AIM2 molecules may bind to a single molecule of DNA and induce oligomerization via a proximity induced environment in which the recruitment of ASC is facilitated by PYD PYD interactions in turn leading to the binding and activation of caspase-1 required for the processing of IL-1β. Furthermore, the AIM2 inflammasome is negatively regulated by the PHYIN family protein p202. p202 has been shown to bind dsDNA in the cytoplasm (Roberts *et al.*, 2009) and the cleavage of caspase-1 is enhanced upon transfection of DNA to the cytoplasm following RNAi mediated knock down of p202. It is highly unlikely that p202 form an active inflammasome as it does not contain a PYD domain for the requirement recruitment of ASC.

The recognition of nucleic acids is paramount to innate responses and the clearance of infection. Knowledge surrounding the PRRs and mechanisms involved in the recognition of both RNA and DNA molecules has increased greatly. However, the inflammasomes involved and the mechanisms regulating them are only beginning to be elucidated. Whether AIM2 can detect aberrant self DNA and impact on the pathology of autoimmune diseases awaits investigation in AIM2-deficient mice.

IFI16

Most recently, in attempts to delineate exactly how microbial DNA is sensed by the immune system, a role for IFI16 has been demonstrated. Consistent with being a PYHIN family protein, IFI16 is inducible by IFNs and contains an N-terminal PYD domain. This is followed by two HIN domains with characterized consensus motifs A and B, respectively (Figure 1) (Ludlow *et al.*, 2005). Unterholzner *et al.*'s work was the first discovery to allude to a role for IFI16 in innate responses to nucleic acids (Unterholzner *et al.*, 2010). The group demonstrates that IFI16 is critical for interferon-β responses upon exposure to intracellular cytoplasmic DNA and HSV-1 infection. Furthermore, IFI16 was shown to directly associate with IFN-β-inducing viral DNA motifs and the stimulator of interferon genes (STING).

Early studies were unsuccessful in attempts to implicate IFI16 in the inflammasome. Specifically, an interaction between ASC and IFI16 was not found (Hornung *et al.*, 2009). Light was shed on this in a study by Kerur *et al.*, demonstrating that infection of endothelial cells with Kaposi sarcoma-associated herpesvirus (KSHV) led to the activation of an ASC-containing inflammasome, with a concomitant proteolytic processing of pro-IL-1β that was shown to be dependent on IFI16. In addition, the nuclear localization of pro-caspase-1 and ASC prior to infection was demonstrated. Cleaved caspase-1 (p20) and ASC were also shown to be in the nucleus at early timepoints of infection followed by the movement of both to the cytoplasm at later times of infection with KSHV. Furthermore, by using short hairpin RNAs (shRNAs) targeting IFI16 or ASC, they demonstrated that both were required for KSHV induced processing of caspase-1(Figure 6) (Kerur *et al.*, 2011). IFI16 was found to be required for the induction of pro-IL-1β and IL-6 in response to HSV-1, highlighting a role for IFI16 in both transcriptional activation as well as the inflammasome (Unterholzner *et al.*, 2010).

KSHV is a DNA virus that does not undergo a productive cycle, but establishes a latent infection whereby the genome persists in the nucleus as an episome tethered to host chromatin. As a result, it becomes clear how earlier studies had difficulty examining a role if any for IFI16 in the inflammasome. These involved transfecting cytoplasmic DNA as a trigger to examine a possible interaction with ASC and importantly IFI16 may not play a prominent role in this setting as it is mainly localized

to the nucleus. IFI16 was observed to colocalize with the KSHV genome in the nuclei of infected cells. The requirement of a nuclear stimulus was further confirmed when IFI16 was expressed along with other inflammasome components in the absence or presence of KSHV. Significantly, elevated levels of IL-1β were observed only upon infection whereas in the absence of KSHV substantially lower levels of IL-1β were induced by overexpressing components.

Although IFI16 houses a bipartite nuclear localization signal, it should be noted that IFI16 can be found in the cytoplasm under certain circumstances. This was shown in a case of UV-mediated DNA damage in epithelial cells (Costa *et al.*, 2011) and the study by Kerur *et al.* in which at later timepoints of infection with KSHV nuclear export of IFI16 was observed. Relocalization of IFI16 with caspase-1 was observed following stimulation with KSHV (Figure 6) (Kerur *et al.*, 2011). Certainly, IFI16 plays a role during IFNβ responses to cytoplasmic DNA. Also of note is evidence to support nucleotide polymorphisms in the IFI16 gene that caused mislocalization of the protein to the cytoplasm which are proposed to render IFI16 inactive (Xin *et al.*, 2003).

In the context of a nuclear inflammasome, while AIM2 and NLRP3 reside in the cytosol and are known to activate inflammasomes in response to infection by bacteria, DNA and RNA viruses, it is tempting to speculate a role for IFI16 specifically in relation to detection of DNA in the nucleus. Indeed, IFI16 has the potential to bind both DNA via its HIN domains and to bind ASC via its PYD domain. Specifically, the IFI16–200 A domain was shown to have a higher affinity for single stranded DNA (ssDNA) compared with double-stranded DNA (dsDNA) and could wrap, stretch and form oligomers with ssDNA (Yan *et al.*, 2008). Unterholzner *et al.* confirmed this finding. However, in this case, they demonstrated that the IFI16 HIN-200B domain alone was able to bind to dsDNA with relatively high affinity. Binding was enhanced when both 200 domains were present. Also, at least in the case of IFI16, DNA sensing leading to innate immune activation is independent of the sequence composition of the DNA (Unterholzner *et al.*, 2010). To further support the concept of IFI16 regulating a nuclear inflammasome, IFI16 did not affect inflammasome activation after infection with vaccinia virus, a DNA virus that replicates in the cytosol. Vaccinia virus has previously been shown to activate the AIM2 inflammasome (Rathinam *et al.*, 2010).

Interestingly cellular stress and viral infection can lead to the induction of the DNA damage response (DDR) and cell death (Lilley *et al.*, 2007). It is hypothesized that IFI16 is strategically localized in the nucleus to detect damaged "self" and "non-self" DNA and may respond by inducing the inflammasome. In steady state, cellular DNA may be protected from recognition by IFI16 via its association with histones. Furthermore, IFI16 has been shown to localize with genomic sites of DNA damage and BRCA1 a component of the DDR (Aglipay *et al.*, 2003).

Overall recent work in the field has identified IFI16 as a nuclear pathogen sensing molecule analogous to cytoplasmic inflammasomes NLRP3 or AIM2. Additional studies are required to understand whether IFI16 interacts directly with the KSHV genome or acts downstream of the DDR. Furthermore, it will be of great interest as to whether IFI16-mediated caspase-1 activation is unique to KSHV or a common occurrence against nuclear replicating DNA viruses (Figure 6).

Research investigating the role of IFI16 in the inflammasome remains in its infancy and there is very little evidence regarding its biochemical regulation. Similar to NLRP3, a metabolic influence has been suggested by a recent study. This work demonstrated that IFI16 can be induced by restricting glucose and contributes to autophagy via the PI3K/AKT pathway (Duan *et al.*, 2011). This suggests a role for IFI16 in "energetic stress" induced regulation of autophagy. Depending on energy levels or "energetic stress," cells undergo either autophagy or cell death and the implications for IFI16 in this context are unclear. Autophagy has been shown to negatively regulate the NLRP3 inflammasome, at least in part, by sequestering pro-IL1β in autophagolysosomes targeting it for degradation (Harris *et al.*, 2011). This suggests a possible anti-inflammatory role for IFI16.

Furthermore, there is evidence that AIM2 and IFI16 can form heterodimers (Veeranki *et al.*, 2011). Veeranki *et al.* have demonstrated that expression of IFI16 protein (by IFNs I and II) in THP-1 cells suppressed activation of caspase-1 by the AIM2 and NLRP3 inflammasomes (Veeranki *et al.*, 2011). IFI16 was bound to AIM2 in the cytoplasm and increased levels of the IFI16 protein in transfected cells inhibited the activation of the AIM2 inflammasome. The group also demonstrates that constitutive knockdown of the IFI16 in THP-1 cells increased basal levels of activated caspase-1. Collectively, these observations demonstrate that IFI16 plays an anti-inflammatory role by suppressing activation of caspase-1 in the AIM2 and NLRP3 inflammasomes. Similar to NLRP3, findings in relation to IFI16 and its inflammasome imply a degree of complexity to its regulation. The study by Veeranki *et al.* is the latest to shed light on this. Future studies are required to decipher the exact mechanisms regulating this new and exciting inflammasome.

If IFI16 does indeed recognize the presence of viral DNA in the nucleus, the question arises as to how IFI16 distinguishes viral genomic DNA from the abundance of cellular DNA in this location. While it had previously been assumed that it is the presence of DNA in the cytosol that acts as a distinguishing feature between self and exogenous DNA, this has never been formally demonstrated. Also, at least in the case of IFI16, DNA sensing leading to innate immune activation is independent of the sequence composition of the DNA (Unterholzner *et al.*, 2010). It is possible that cellular DNA is protected from IFI16-mediated recognition due to its association with histones and other protective host proteins. An alternative and particularly tantalizing explanation may be that

IFI16 senses DNA damage before initiating its innate immune signaling functions. Many viruses, including herpesviruses such as KSHV, cause a DNA damage response during the replication of their genome (Lilley *et al*., 2007), and this may alert IFI16 to the presence of a DNA virus in the nucleus. In fact, IFI16 is known to associate with components of the DNA damage response, such as BRCA1, and has been shown to assemble on genomic sites of DNA damage in a BRCA1-dependent manner (Aglipay *et al*., 2003). Also, IFI16 can translocate out of the nucleus after UV-mediated DNA damage in epithelial cells (Costa *et al*., 2011), in analogy to the nuclear export of IFI16 and ASC in response to KSHV infection observed by Kerur *et al*. Thus, it is tempting to speculate that IFI16 might be involved in the detection of both "stranger"—namely, intracellular viral DNA—and "danger" such as damaged DNA. Overall, the idea of innate immune detection of viral DNA in the nucleus is intriguing, and this study should provoke further investigation in order to test this hypothesis.

Concluding remarks

The discovery of the inflammasome has provided new insights into the regulation of inflammation during host defense and tissue injury. The biochemical processes regulating inflammasome function are highly complex and are providing new insights into the regulation of multi-protein complexes in signal transduction. Ongoing analysis of biochemical regulation and the role of the inflammasome during inflammatory events may give insights which will ultimately yield new therapies for inflammatory diseases involving the cytokine IL-1β.

Declaration of interest

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