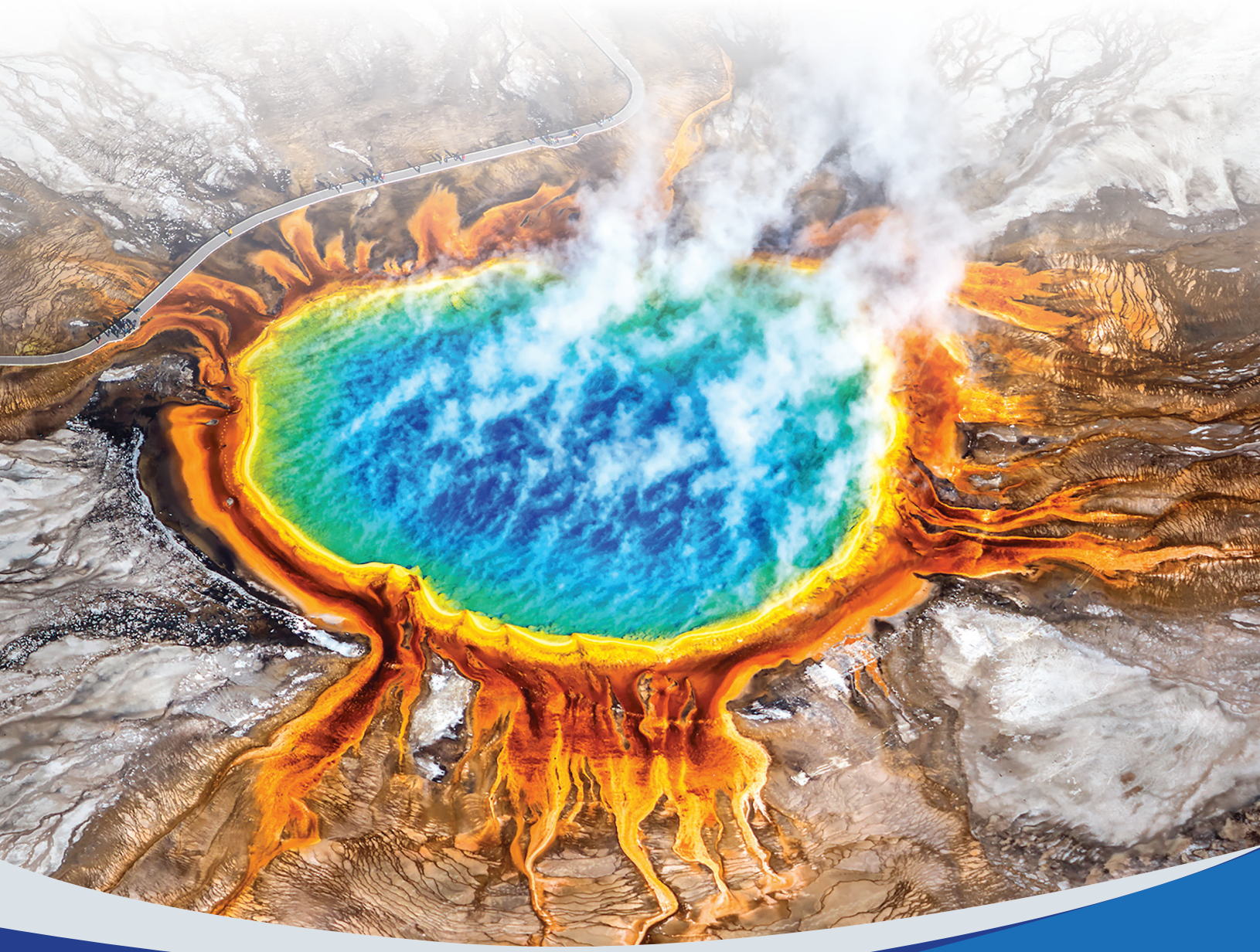
INFLAMMASOMES

InvivoGen *in focus*

PRACTICAL GUIDE



UNDERSTANDING INFLAMMASOMES

In 2002, the discovery of inflammasomes was a breakthrough in the understanding of how inflammation is initiated. InvivoGen offers a comprehensive collection of tools to assist your research on these inflammatory signaling hubs.

- Mini reviews
- Pathway illustrations
- Double-page poster
- Cell lines & Reagents
- Validation data

InvivoGen *infocus*

A PRACTICAL GUIDE TO UNDERSTANDING INFLAMMASOMES

ABBREVIATIONS

AIM2 : Absent-in-melanoma-2
ATP : Adenosine tri-phosphate
ASC : Apoptosis-associated speck-like protein
CARD : Caspase recruitment domain
CASP : Caspase
DAMPs : Danger-associated molecular patterns
GBPs : Guanylate binding proteins
GSDM : Gasdermin
HMGB1 : High mobility group B1 protein
IFN : Interferon
IL-1 : Interleukin 1
IRF : Interferon regulatory factor
IRGB10 : Interferon response gene B10 protein
LDH : Lactate dehydrogenase
LRR : Leucin-rich repeat
LPS : Lipopolysaccharide
NAIP : NLR family apoptosis inhibitory protein
NEK7 : NimA-related protein kinase 7
NF-κB : Nuclear factor kappa-light-chain enhancer of activated B cells
NBD : Nucleotide-binding domain
NLR : Nucleotide-binding domain and leucin-rich repeat
NLRP3 : NLR family PYD domain containing protein 3
NLRC4 : NLR family CARD domain containing protein 4
NOD : Nucleotide-binding oligomerization domain
OMVs : Outer membrane vesicles
PAMPs : Pathogen-associated molecular patterns
PRRs : Pattern recognition receptors
PTM : Post-translational modification
PYD : Pyrin domain
TLRs : Toll-like receptors

Inflammation is an innate immune reaction that aims to resolve various types of threats, including microbial infections, allergies, and auto-immune conditions. One major inflammation effector is the IL-1β cytokine, identified in the 1980's. It took two decades to characterize and assemble the different puzzle pieces that lead to this cytokine's secretion. The discovery of inflammasomes was a major breakthrough in the understanding of inflammation initiation. Inflammasomes are cytosolic multiprotein complexes, whose formation is triggered by sensors of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). Inflammatory caspases may fulfill the function of a sensor and/or mediator of downstream effectors. While inflammasomes are beneficial in clearing microbes, their aberant or excessive stimulation contributes to various pathologies, such as auto-inflammatory disorders, cardiometabolic and neurodegenerative diseases, as well as cancer. InvivoGen has developed a large collection of cellular assays, ligands, and inhibitors, to assist your research in this limitless field.

A BRIEF HISTORY OF INFLAMMASOME BREAKTHROUGHS

1992 — IDENTIFICATION OF CASPASE-I & CHARACTERIZED FUNCTION IN IL-1β PROCESSING
Thornberry N.A. *et al.*, Nature. 356:768.

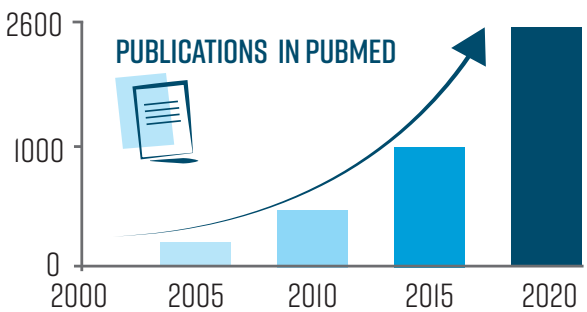
2002 — DISCOVERY OF THE NLRP1 INFLAMMASOME
Martinon F. *et al.*, Mol Cell. 10:417.

2004 — DISCOVERY OF THE NLRP3 INFLAMMASOME
Agostini L. *et al.*, Immunity. 20:319.

2004 / 2006 — DISCOVERY OF NLRC4/NAIP INFLAMMASOMES
Mariathasan S. *et al.*, Nature. 430:213.
Franchi L *et al.*, Nat. Immunol. 7:576.
Miao EA. *et al.*, Nat. Immunol. 7:569.

2011 / 2013 — DISCOVERY OF NON-CANONICAL INFLAMMASOMES
Kayagaki N. *et al.*, Nature. 479:117.
Kayagaki N. *et al.*, Science. 341:1246.
Hagar J.A. *et al.*, Science. 341:1250.

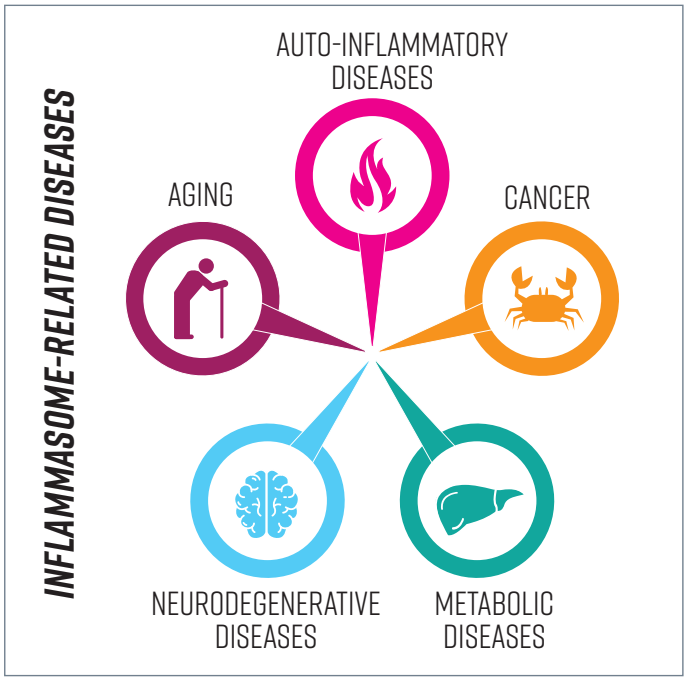
2016 — CHARACTERIZATION OF GASDERMIN-D PORE-FORMING FUNCTION
Ding J. *et al.*, Nature. 535:111.



6 (AT LEAST) INFLAMMASOME SENSORS
NLRP1 · NLRC4 · CASPASE II-4/5
NLRP3 · PYRIN · AIM2

3 PROTEINS IN CANONICAL ASSSEMBLY
SENSOR + ADAPTOR + CASPASE-I

2 SIGNALS FOR INFLAMMASOME FORMATION
PRIMING + ACTIVATION



70 STUDIES IN clinicaltrials.gov

InvivoGen OFFERS>60
CELLULAR ASSAYS, REAGENTS, AND GENE TOOLS TO ACCOMPANY YOUR SUCCESSFUL RESEARCH

INFLAMMASOME ACTORS

Inflammasomes are cytosolic multiprotein complexes generally comprised of a sensor and an inflammatory caspase connected to an adaptor protein. Their assembly can be triggered by a multitude of microbial and host-derived stimuli. Once activated, inflammasomes drive the formation of Gasdermin-D pores at the cell surface, allowing the non-conventional secretion of IL-1β and IL-18 pro-inflammatory cytokines, IL-1α and HMGB1 alarmins, and ultimately, pyroptotic cell death. Inflammasomes are defined as 'canonical' when their assembly requires caspase-1, and as 'non-canonical' when their assembly depends on human caspase-4 or caspase-5 (or their murine ortholog caspase-11).

Inflammasomes serve as cytosolic signaling hubs to promote inflammatory responses.

Inflammasome sensors

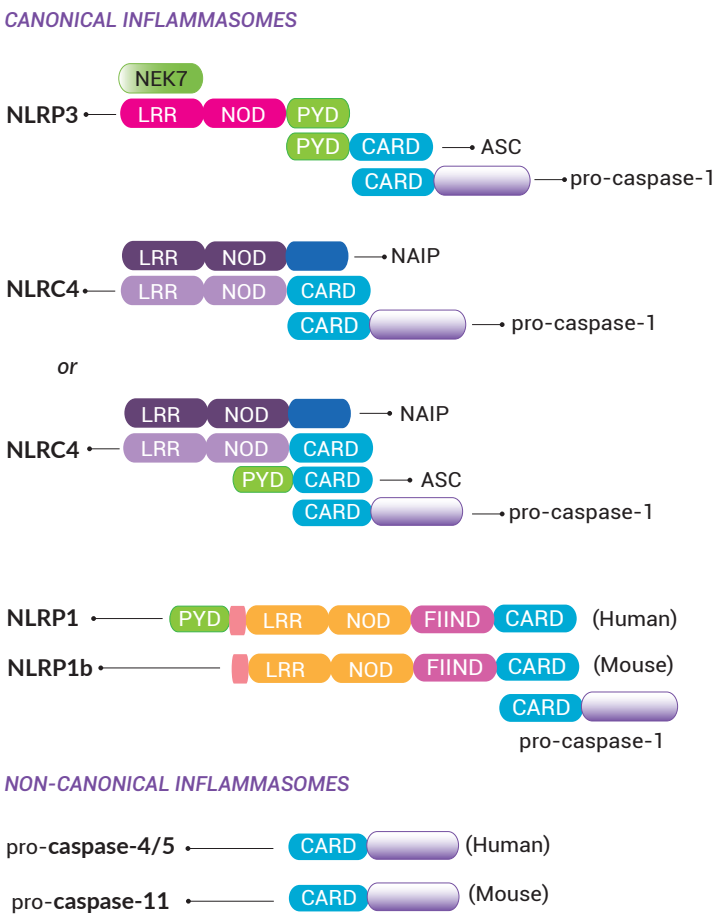
Inflammasomes are named after their sensor. These sensors are mainly cytoplasmic pattern-recognition receptors (PRRs) that are classified by their protein domain structures. Most of them belong to the NLR (Nucleotide-binding domain (NBD) and Leucine-rich repeat (LRR) Receptor) family, and in particular the NLRP and NLRC subgroups. NLRPs differ from NLRCs in their N-termini with a pyrin domain (PYD) and a caspase recruitment domains (CARD), respectively. NLR sensors include NLRP3, NLRP1 and NLRC4, which are functionally related in their ability to form inflammasomes. Other non-NLR sensors can also form inflammasomes, such as AIM2 and Pyrin. These sensors initiate the assembly of canonical inflammasomes by recruiting caspase-1 (CASP-1), with or without the ASC adaptor. The assembly of non-canonical inflammasomes involves human CASP-4/5 or murine CASP-11, which exhibit both sensor and effector functions.

Canonical inflammasome sensors

NLRP3 (aka cryopyrin, or NALP3) is the most studied sensor since Tschopp's team reported its inflammasome assembly function in 2004 [1]. This LRR-NOD-PYD sensor can be activated by a wide range of structurally and chemically unrelated stimuli (e.g. pore-forming toxins, activators of ion channels, uric acid crystals, β-amyloid proteins) [2-4], suggesting that NLRP3 does not directly bind to these molecules. It rather senses downstream cytosolic stress signals such as ion imbalances, in particular K^+ efflux which could be the convergent sensing point of disturbances in cellular homeostasis [5]. Importantly, NLRP3 is involved in numerous inflammatory pathologies, including Alzheimer disease, type-2 diabetes, and COVID-19 (see p. 8 & 19).

NLRC4 (aka Ipaf) was first reported to drive inflammasome responses by Dixit's team in 2004 [6]. Later, seminal work from Vance's and Shao's teams demonstrated that NLRC4 is an indirect sensor that interacts with NAIPs (NLR family apoptosis inhibitory proteins). These bind directly to PAMPs produced by intracellular bacteria (e.g. Flagellin) and components of bacterial secretion systems (e.g. Needle and Inner Rod). While a single NAIP operates upstream of NLRC4 in humans and recognizes each of these activators [7], multiple NAIPs have been described in mice with different affinities for each molecule [8-11]. The NLRC4 inflammasome appears to protect mucosal barriers, such as the lung, stomach, and intestine, from invading bacteria [12] (see p. 9 & 19).

NLRP1 (aka NALP1) was the first described inflammasome sensor of the NLR family, and later characterized by Tschopp's team in 2001-2 [13, 14]. Human NLRP1 features a CARD and a PYD domain. Murine NLRP1 paralogs (a, b, c) all lack the PYD domain, with NLRP1b being the best characterized. Human and murine NLRP1 feature a unique FIIND domain (function to find domain) upstream of CARD. NLRP1 remains without an identified cognate ligand [15, 16]. Yet, NLRP1b activation is induced by pathogenic enzymes (e.g. *B. anthracis* lethal protein or *S. flexneri* IpaH7.8) which trigger auto-proteolysis of FIIND and proteasomal degradation of the N-terminal domain [16].



AIM2 (Absent-in-melanoma 2) is a non-NLR protein that contains a PYD domain and an oligonucleotide-binding domain. AIM2 is a receptor for cytosolic double-stranded (ds)DNA of microbial or host origin (following cell damage) in a sequence-independent, but length-dependent manner [17-19]. While the inflammasome-forming function of AIM2 is well-established in murine myeloid cells, it is less clear in human cells [19-21].

Pyrin was initially identified in patients with Mediterranean fever (MEFV) who exhibit a mutated *MEFV* gene. Its activation is not completely understood yet, except that it involves its PYD domain. Furthermore, its cognate ligand remains unidentified. Though it has been shown to sense aberrant cytoskeleton dynamics, and can be activated by Rho-modifying proteins such as *C. difficile* toxin B and *C. botulinum* toxin C3 [15].

NLRP6 and NLRP9b sensors have recently gained interest with several reports suggesting that they mediate inflammasome responses in mice, and more specifically in intestinal epithelial cells [15].

Non-canonical sensors

Caspases-4 and -5 (CASP-4/5) in humans and caspase-11 (CASP-11) in mice are bi-functional molecules. They act both as direct sensors of cytosolic lipopolysaccharide (LPS) and direct inflammasome effectors. More precisely, CASP-11/4/5 form non-canonical inflammasomes leading to CASP-1-dependent IL-1β and IL-18 secretion and CASP-1-independent cell death [22, 23] (see p. 9 &13).

KEY POINT

CANONICAL AND NON-CANONICAL

'Canonical' and 'non-canonical' terms not only refer to the type of inflammasome, but also to the type of pathway leading to their activation. NLRP3 and CASP-11 form canonical and non-canonical inflammasomes, respectively. However, NLRP3 can be activated either through a canonical or a non-canoncial pathway. Learn more on p. 8-9.

Adaptors

ASC (aka PYCARD) is a bipartite adaptor with one CARD and one PYD domain, allowing the interaction between the sensor and pro-caspase-1. This protein is recruited by inflammasome sensors that do not contain a CARD domain, such as NLRP3 and AIM2. It is not required for the formation of the NLRP1, NLRC4, nor CASP-11/4/5 inflammasomes [15, 23]. In resting cells, ASC is present in a soluble and diffuse form, both in the cytoplasm and nucleus. Upon inflammasome activation, ASC molecules form a single large, micrometer-sized, 'speck' per cell, thus concentrating CASP-1 activation sites [24] (see page 16).

NEK7 (NIMA-related protein kinase 7), a serine-threonine kinase previously linked to mitosis, was recently identified as essential for NLRP3 activation to all known activators, downstream of K^+ efflux. In the absence of NEK7, CASP-1 activation and IL-1β release are abrogated *in vitro* and *in vivo* in response to NLRP3 activators [25]. A structural study has demonstrated that NEK7 bridges adjacent NLRP3 molecules through binding to their LRR domain, thereby exerting a scaffolding function [26].

Effectors

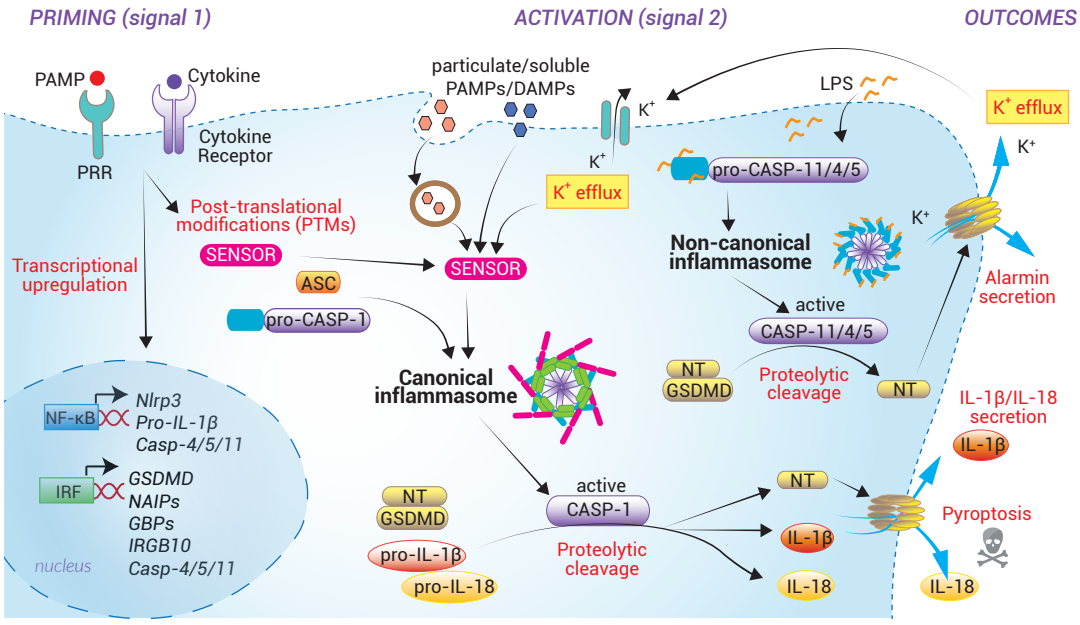
Caspase-1 (CASP-1) is a cysteine protease that was first identified as Interleukin-1β-converting-enzyme (ICE) [27]. CASP-1 is synthesized as an inactive pro-caspase-1 zymogen composed of a N-terminal CARD domain and two catalytic subunits, p20 and p10. CASP-1 is recruited to canonical inflammasomes via its CARD domain. Tetrameric forms of CASP-1 (and CASP-4 or -11) have been reported to allow proximity-induced autolytic activation and release of p20 and p10 [28]. Active CASP-1 cleaves pro-IL-1β and pro-IL-18 cytokines as well as Gasdermin D into their biologically active forms (see below) [28, 29]. Structural studies are needed to further understand its precise mode of action.

Gasdermin D (GSDMD, aka DFNA5L, or FKSG10) was first identified in 2004 but its biological function remained unclear for almost 15 years. This cytosolic protein features two distinct domains separated by a linker. The C-terminal domain (GSDMD-CT) exerts an inhibitory function on the N-terminal domain (GSDMD-NT). The release of the GSDMD-NT fragment requires proteolytic cleavage within the linker by activated CASP-1 or CASP-11/4/5. GSDMD-NT then binds to phospholipids in the plasma membrane, leading to its oligomerization and the formation of pores with a ~18 nm inner-diameter [30]. GSDMD pores allow the secretion of mature IL-1β and IL-18, and alarmins (see below). Ultimately, their accumulation causes pyroptotic cell death (see p.14).

Interleukin-1β (IL-1β) and IL-18 are gatekeeper cytokines, critically involved in many events related to activation and regulation of inflammation. IL-1β induces the expression of genes that control fever, vasodilatation, and infiltration of immune cells to infected or damaged tissues. IL-18 is necessary for interferon-gamma (IFN-γ) production and is a co-stimulatory cytokine that mediates adaptive immunity. These two cytokines are synthesized as pro-IL-1β and pro-IL-18 zymogens that must be cleaved to generate their mature forms [31]. These cytokines are secreted in an non-conventional manner (i.e. through GSDMD pores), as they lack N-terminal signal sequences necessary for entry into the vesicle-mediated biosynthetic pathway.

Alarmins are DAMPs that are released by damaged or dying cells in response to infection or injury. These molecules are present at low levels in the cytosol at homeostasis, ready for early secretion during the inflammasome response. Their expression is then upregulated, allowing propagation of the "danger" signal. IL-1α [32] and HMGB1 (High Mobility Group Box-1 protein) [33] are described as two major alarmins released upon inflammasome activation.

'Priming' and 'Activation' are two steps required by all inflammasomes for their assembly and mediated responses.



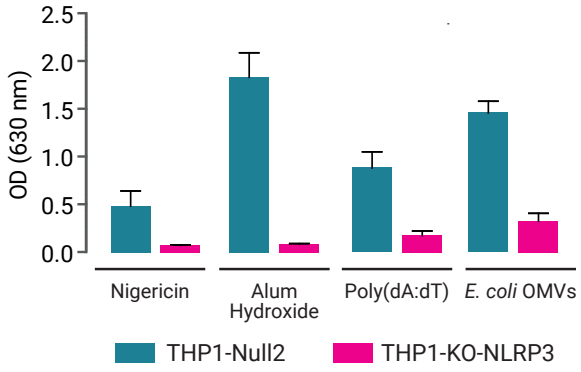
INFLAMMASOME CELLULAR ASSAYS

InvivoGen offers an expanding collection of inflammasome cell-based assays. THP-1 human monocytes are widely used for inflammasome studies, as they express high levels of NLRP3, ASC, and pro-caspase-1. To help you assess the role of specific inflammasome actors in the canonical and non-canonical responses, we engineered THP1- or RAW264.7-derived cells that are knockout (KO) or knockdown (def) for the genes encoding NLRP3, ASC, CASP-1, CASP-4, CASP-11, NLRC4, and GSDMD. In addition, these cells can be used as controls in the screening of novel therapeutics. Our cell lines are functionally validated by monitoring the release of bioactive IL-1β using the HEK-Blue™ IL-1β sensor cell line (see p.17).

Monitoring NLRP3-dependent responses

THP1-KO-NLRP3 Cells

THP1-KO-NLRP3 cells feature a KO of the N-terminal region of the *NLRP3* gene verified by DNA sequencing, PCR, and Western blot. These cells express an inactive NLRP3 C-terminal fragment. Thus, IL-1β secretion and pyroptosis are abrogated upon incubation with Nigericin or Alum Hydroxide (see p. 12). Of note, IL-1β secretion is severely impaired upon cGAS-STING-NLRP3 or CASP-4/5 inflammasome activation with Poly(dA:dT) (see p. 12) or *E. coli* outer membrane vesicles (OMVs, see p. 13), respectively. Therefore, THP1-KO-NLRP3 cells can be used to discriminate between the activation of NLRP3 and the other inflammasome sensors.



Functional characterization of THP1-KO-NLRP3 Cells.
THP1-KO-NLRP3 cells and their parental cell line, THP1-Null2, were primed with LPS-EK (1 µg/ml) then stimulated with Nigericin (5 µM), Alum Hydroxide (150 µg/ml), transfected Poly(dA:dT) (1 µg/ml), or E. coli OMVs (100 µg/ml). After overnight incubation, IL-1β secretion was assessed using HEK-Blue™ IL-1β sensor cells and the SEAP detection reagent QUANTI-Blue™ Solution. The optical density (OD) was read at 630 nm.

PRODUCT	QTY	CAT. CODE
THP1-KO-NLRP3 Cells	3-7 x 10^6 cells	thp-konlrp3z
THP1-Null2 Cells	3-7 x 10^6 cells	thp-nullz

KEY POINT

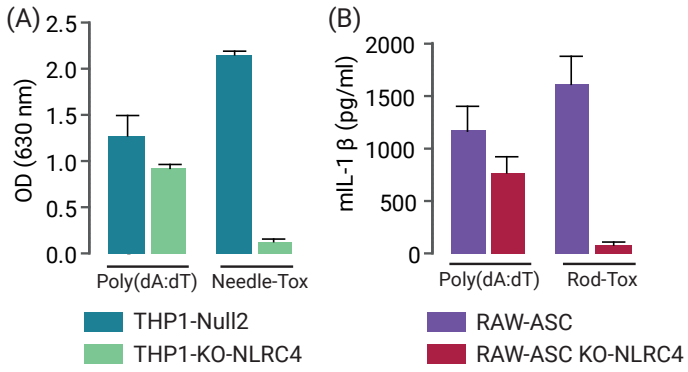
NLRP3: AN ATTRACTIVE DRUG TARGET

NLRP3 activation can be mediated by a wide range of stimuli. Yet, this evolutionary benefit for combating infections can also be a liability. Indeed, NLRP3 is associated with various diseases, such as type-2 diabetes, non-alcoholic steatohepatitis (NASH), gouty arthritis, Alzheimer's diseases and cancer, and thus has become an attractive drug target [34, 35].

Monitoring NLRC4-dependent responses

THP1-KO-NLRC4 Cells & RAW-ASC KO-NLRC4 Cells

THP1-KO-NLRC4 cells exhibit a KO of the NLRC4 nucleotide-binding domain (NDB) rendering the NLRC4 protein unable to polymerize for inflammasome assembly (see p. 9).
RAW-ASC KO-NLRC4 cells stably express the murine *ASC* gene (otherwise absent in RAW264.7 cells [36]) and feature a biallelic *NLRC4* KO. The NLRC4 deletion has been verified in both cell lines by DNA sequencing, PCR, and Western blot. THP1-KO-NLRC4 and RAW-ASC KO-NLRC4 cells display impaired IL-1β secretion and pyroptosis upon incubation with Needle-Tox and Rod-Tox (see p. 13), respectively. Importantly, the response of both KO cell lines to other inflammasome inducers (e.g. Poly(dA:dT)) is only slightly affected.



Functional characterization of THP1-KO-NLRC4 and RAW-ASC KO-NLRC4 Cells.
(A) THP1-KO-NLRC4 cells and their parental cell line, THP1-Null2, were primed with LPS-EK (1 µg/ml). (B) RAW-ASC KO-NLRC4 cells and their parental cell line RAW-ASC, were primed with Pam3CSK4 (100 ng/ml). (A and B) After priming, the cells were stimulated with Poly(dA:dT) (1µg/ml), Needle-Tox (4 ng/ml) or Rod-Tox (2 µg/ml). After overnight incubation, the secretion of human or mouse IL-1β was assessed using (A) the HEK-Blue™ IL-1β cell based assay or (B) an ELISA assay, respectively.

PRODUCT	QTY	CAT. CODE
THP1-KO-NLRC4 Cells	3-7 x 10^6 cells	thp-konlrc4z
RAW-ASC KO-NLRC4 Cells	3-7 x 10^6 cells	raw-konlrc4
RAW-ASC Cells	3-7 x 10^6 cells	raw-asc

They trust InvivoGen

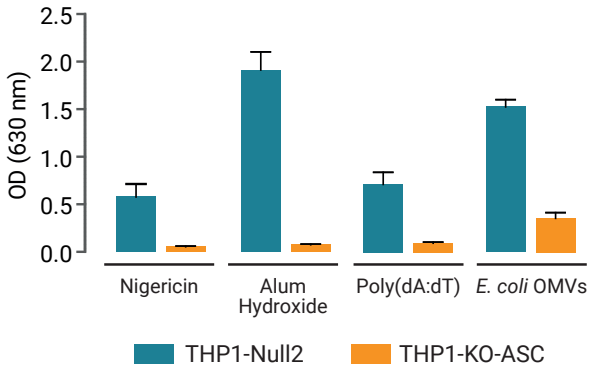
Sharma B. et al. 2019. Methods Mol. Biol. 1682:135-47. Assessing NLRP3 inflammasome activation by nanoparticles.

Jäger E. et al. 2020.. Nat. Commun. 11(1):4243. Calcium-sensing receptor-mediated NLRP3 inflammasome response to calciprotein particles drives inflammation in rheumatoid arthritis.

Monitoring ASC-dependent responses

THP1-KO-ASC Cells

THP1-KO-ASC cells feature a biallelic KO of the *ASC* gene, as confirmed by DNA sequencing, PCR, and Western blot. They can be used to determine whether an inflammasome requires ASC for its activation. Notably, their response to NLRP3 or CASP-4 inducers is severely impaired.



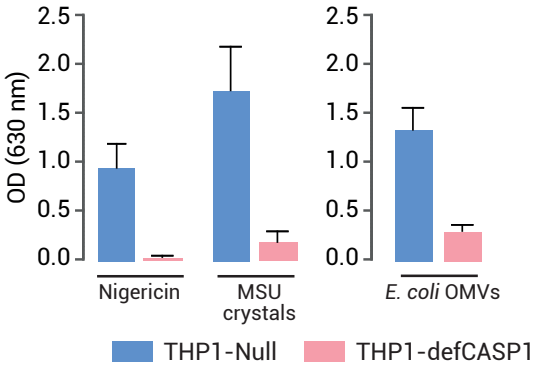
Functional characterization of THP1-KO-ASC Cells.
THP1-KO-ASC cells and their parental cell line, THP1-Null2, were primed with LPS-EK (1 µg/ml) and then stimulated with Nigericin (5 µM), Alum Hydroxide (150 µg/ml), transfected Poly(dA:dT) (1 µg/ml), or E. coli OMVs (100 µg/ml). After 24h activation, IL-1β secretion was assessed in the culture supernatant using HEK-Blue™ IL-1β sensor cells and the SEAP detection reagent QUANTI-Blue™ Solution. The optical density (OD) was read at 630 nm.

PRODUCT	QTY	CAT. CODE
THP1-KO-ASC Cells	3-7 x 10^6 cells	thp-koascz

Monitoring CASP-1-dependent responses

THP1-defCASP1 Cells

THP1-defCASP1 cells exhibit a ~7-fold reduction in caspase-1 expression and a strong deficiency in capase-1 activity as compared to their THP1-Null parental cell line. These cells represent a useful tool for screening novel inflammasome inducers.



Functional characterization of THP1-defCASP1 Cells.
THP1-defCASP1 cells and their parental cell line, THP1-Null, were primed with LPS-EK (1 µg/ml) prior to stimulation with Nigericin (5 µM), MSU crystals (250 µg/ml), or E. coli OMVs (100 µg/ml). After 24h activation, IL-1β secretion was assessed in the culture supernatant using HEK-Blue™ IL-1β sensor cells and the SEAP detection reagent QUANTI-Blue™ Solution. The optical density (OD) was read at 630 nm.

PRODUCT	QTY	CAT. CODE
THP1-defCASP1 Cells	3-7 x 10^6 cells	thp-dcasp1
THP1-Null Cells	3-7 x 10^6 cells	thp-null

TIPS

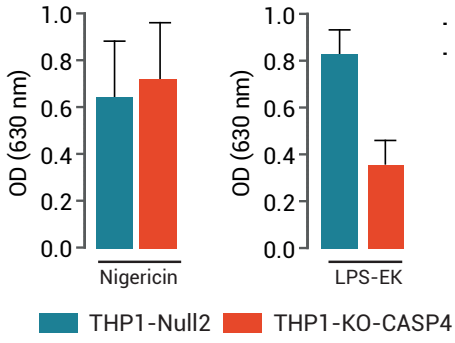
STUDYING SPECIFIC INFLAMMASOME ACTORS

- **Monocytes** and **macrophages** are innate cells at the frontline of immune defenses, and thus, are the most widely used cell types for studying inflammasome signaling.
- **Bone-marrow derived macrophages (BMDMs)** are a source of primary cells further differentiated *in vitro*.
- The **THP-1 cell line** is a human monocytic cell line that expresses high levels of NLRP3, ASC, and pro-caspase-1 [37].
- **Knockout (KO)** and **knockdown (KD)** are used for studying specific gene functions, and whether they can be compensated by another gene.
- **Chemical inhibitors** can complement KO/KD tools. These small molecules block the function of a protein, while still possibly allowing its assembly into macromolecular complexes.

Monitoring CASP-4-dependent responses

THP1-KO-CASP4 Cells

THP1-KO-CASP4 cells feature a biallelic KO of the *CASP4* gene, validated by DNA sequencing, PCR, and Western blot. As expected, the response to NLRP3 inducers (e.g. Nigericin) is unaffected, whereas the response to CASP-4 inducers (e.g. transfected LPS) is hindered. Despite having a complete KO of the *CASP4* gene, these cells still secrete detectable levels of IL-1β upon transfection of LPS, which may be attributed to a partial rescue by CASP-5.



Functional characterization of THP1-KO-CASP4 Cells.
THP1-KO-CASP4 cells and their parental cell line, THP1-Null2, were primed with LPS-EK (1 µg/ml) then stimulated with Nigericin (5 µM; canonical inducer) or transfected LPS-EK (5 µg/ml, non-canonical inducer). After 6h incubation, IL-1β secretion was assessed using HEK-Blue™ IL-1β sensor cells and and the SEAP detection reagent QUANTI-Blue™ Solution. The optical density (OD) was read at 630 nm.

InvivoGen also offers **RAW-ASC KO-CASP11** cells generated from the RAW-ASC cell line. These cells feature a biallelic KO for the *CASP-11* gene and their response to non-canonical inflammasome inducers, such as transfected LPS or *E. coli* OMVs, is impaired.

PRODUCT	QTY	CAT. CODE
THP1-KO-CASP4 Cells	3-7 x 10^6 cells	thp-kocasp4z
RAW-ASC KO-CASP11 Cells	3-7 x 10^6 cells	raw-kocasp11

INFLAMMASOME ACTIVATION AND ASSEMBLY

Inflammasomes rely on a 'two-step' activation model. However, each inflammasome has unique sensor activation and platform association mechanisms. The sensor can either bind to its agonist(s) directly or it relies on another molecule. For some inflammasomes, the ASC adaptor is mandatory for the assembly, while it is facultative or absent for others. Interaction between the different actors occurs through specific conserved domains. The various activation and assembly strategies may have evolved to allow a timely response, commensurate to the threat.

Each inflammasome has unique sensor activation and platform assembly mechanisms.

A 'two-step' activation model

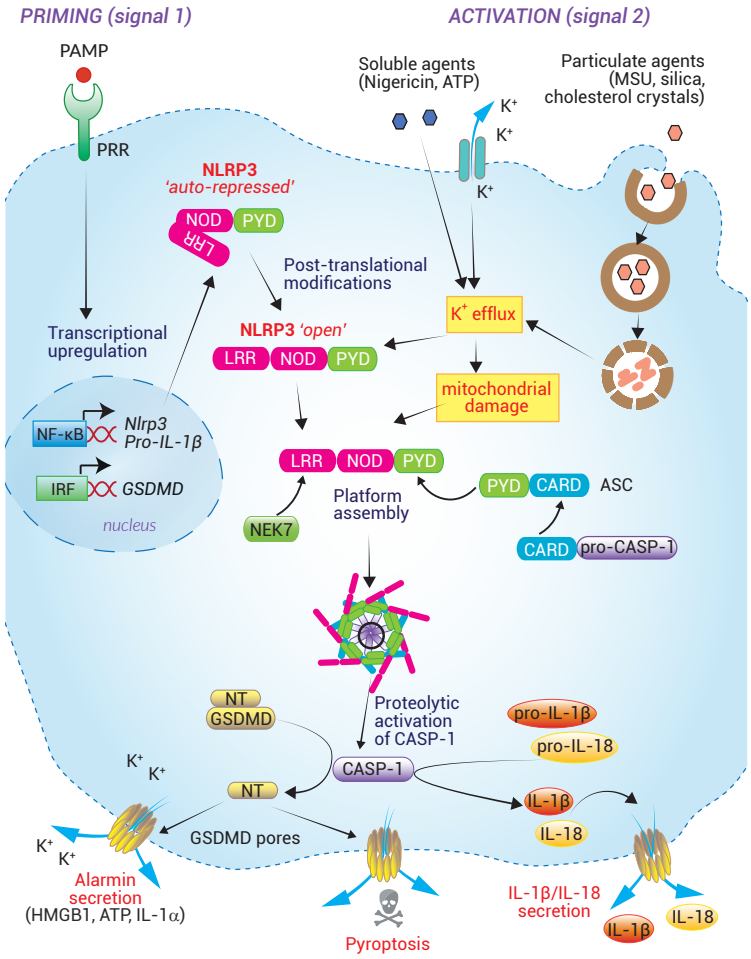
Priming and activation are two steps required by all inflammasomes. First, the priming step can be seen as a form of necessary regulation to avoid unwanted activation (see p. 15). It allows the transcriptional upregulation of proteins required for inflammasome assembly and downstream signaling. Once primed, the sensors remain in an auto-repressed but signal-sensitive state. They are activated by different PAMPs or DAMPs, either through direct-ligand binding or indirect intracellular events.
Activation, the second step, induces post-translational modifications (PTMs) of the primed sensor, resulting in a de-repressed conformational change. This is the starting point for sensor oligomerization and inflammasome platform assembly. Below we detail the unique activation and assembly mechanisms of the NLRP3, NLRC4, and CASP-11/4/5 inflammasomes.

NLRP3 inflammasome

The NLRP3 inflammasome is the prototypical and best characterized inflammasome. Yet, the precise mechanisms underlying NLRP3 activation remain a controversial topic [5, 38]. NLRP3 can be activated by a wide range of stimuli that are structurally and chemically unrelated (see p. 12). These can be either sterile (e.g. uric acid crystals, β-amyloid proteins, cholesterol crystals), microbial (e.g. pore-forming toxins, activators of ion channels), or environmental (e.g. asbestos, silica) [5]. The current paradigm is that NLRP3 does not bind directly to these molecules. Rather it senses downstream cytosolic stress signals such as K^+ efflux [5, 38], externalized mitochondrial (mt) cardiolipin, and oxidized mtDNA, with the latter two being suggested as the 'ultimate' NLRP3 ligands in some reports [39-41].

NLRP3 is a tripartite protein containing a LRR domain, NOD (or NATCH) domain, and PYD domain (see p. 4). NLRP3 is auto-repressed in a closed conformation due to an internal interaction between the NOD and LRR domains [42]. ATP binding and hydrolysis at the NOD domain allows NLRP3 to undergo a change to an open conformation [43]. Moreover, PTMs are driven by different stimuli that can be mapped to specific residues in each NLRP3 domain [38, 44, 45]. As an example, stimulation with PRR agonists, such as LPS or Pam3CSK4, leads to JNK1-mediated phosphorylation of S198 in the NOD domain and subsequent NLRP3 deubiquitination [44]. Once the LRR domain is exposed, it binds to NEK7 (NimA-related protein kinase 7). NEK7 is necessary for bridging the gaps between adjacent NLRP3 subunits, however the timing and location for this interaction is not fully elucidated [25-27]. NLRP3 activation initiates the inflammasome assembly. The NLRP3 PYD domain recruits ASC, which then through its CARD domain binds pro-caspase-1. Proximity-induced autolytic activation of CASP-1 leads to the formation of GSDMD pores at the cell surface, allowing IL-1β/IL-18 and alarmin secretion, and ultimately, pyroptosis [5, 46].

Organelles play a key role in NLRP3 inflammasome assembly [5, 47]. Coordinated priming and activation signals orchestrate the subcellular localization of the NLRP3 inflammasome components to the mitochondria, endoplasmic reticulum (ER), and Golgi. Whether multiple organelle-linked mechanisms regulate NLRP3 inflammasome assembly is still being investigated [48].



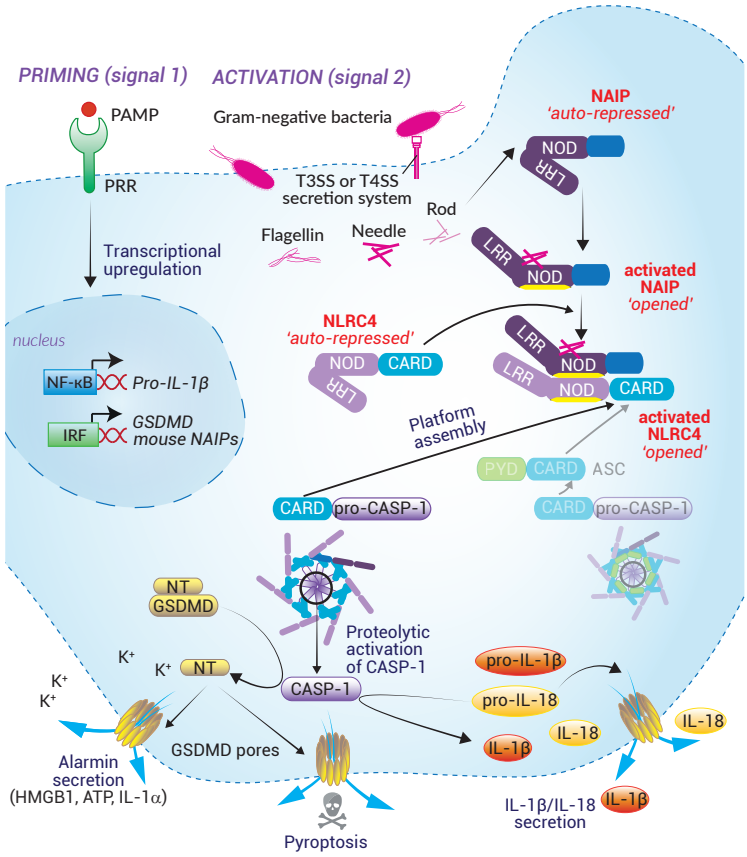
Different priming mechanisms have been described for the NLRP3 inflammasome. Prolonged TLR stimulation (>3 hours) triggers the NF-κB-mediated transcription of new NLRP3 molecules [44, 49]. Shorter TLR stimulation (<1 hour) more likely triggers MyD88- or TRIF-mediated PTM of the NLRP3 protein present at basal levels [44, 50]. NLRP3 PTM-mediated priming may have evolved to allow immediate inflammasome responses. Transcriptional-mediated priming potentiates the inflammasome response through the upregulated expression of NLRP3 and effector molecules. In line with this, a recent report showed that, at least *in vitro*, TLR-mediated priming is dispensable for NLRP3 inflammasome activation in human monocytes [51].

Importantly, the NLRP3 inflammasome activation and assembly can also be triggered by non-canonical CASP-11/4/5 inflammasome responses (next page).

NLRC4/NAIP inflammasome

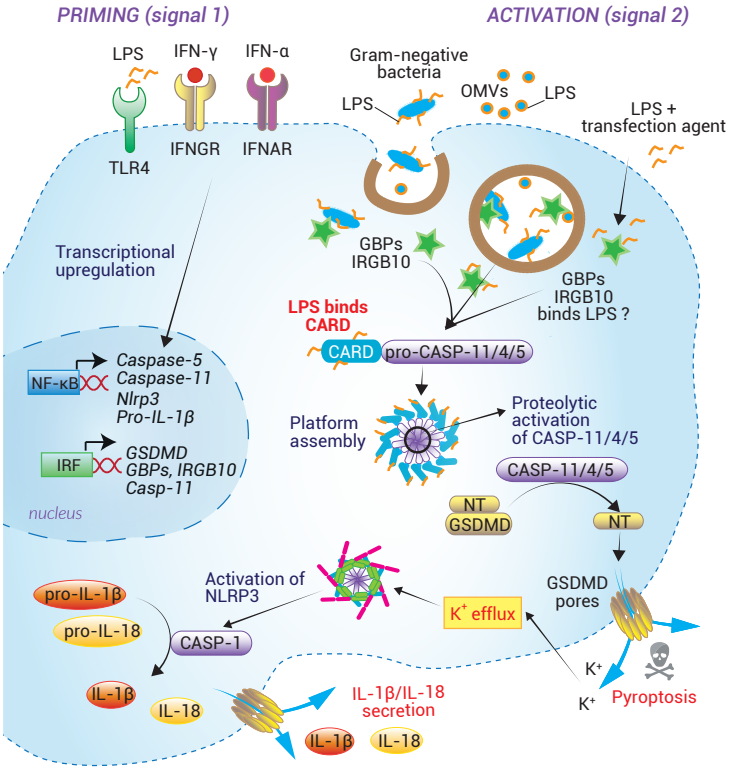
NLRC4 senses intracellular bacterial molecules such as Flagellin from the motility apparatus, or Inner Rod, and Needle proteins from the bacterial type III or IV secretion systems (T3SS or T4SS) (see p. 13). More specifically, NLRC4 associates with NAIP which directly binds to the ligands. There are differences between human and murine NAIP expression. In humans, a single NAIP operates upstream of NLRC4 and binds to each of the above-mentioned activators [7, 52]. Two isoforms of this unique NAIP have been described and reported to sense flagellin and T3SS proteins with different affinities [52]. Mice express multiple NAIPs which display different affinities for each ligand. NAIP1 exhibits a higher affinity for T3SS Needle, NAIP2 for T3SS Inner Rod, and NAIP5 and NAIP6 for Flagellin [8-11]. Although the transcriptional regulation of NAIPs and NLRC4 is still poorly documented, the basal expression of murine NAIPs has recently been found to depend, at least, on IRF8 [53].

NAIPs and NLRC4 share similar LRR and NOD domains with LRR being necessary for their auto-repressed state [8, 54-56]. NLRC4 also features a CARD domain (see p. 4). Ligand binding to NAIP on NOD regions releases NAIP auto-inhibition, allowing it to expose its NOD domain [55]. Activated NAIP then recruits one NLRC4 protomer through NOD-NOD interaction, forcing the opening of NLRC4. Activated NLRC4 uses its newly exposed NOD surface to associate with additional NLRC4 molecules in a domino-like reaction to assemble the inflammasome platform [56]. NLRC4 polymerization results from the clustering of NLRC4 CARD domains. The NLRC4/NAIP complex then associates with pro-caspase-1, either via direct CARD-CARD interactions, or through the ASC adaptor [15, 56].



CASP-11 & CASP-4/5 non-canonical inflammasomes

Mouse CASP-11 and human CASP-4/5 serve as both sensor and effector molecules and thus, form non-canonical inflammasomes. It was first shown in mice that upon infection with Gram-negative bacteria, CASP-11 senses intracellular LPS, independently of the cell surface LPS receptor, TLR4 [57, 58] (see p. 13). CASP-11, and CASP-4/5 drive GSDMD-mediated cell death and CASP-1-dependent IL-1β/IL-18 secretion [22].

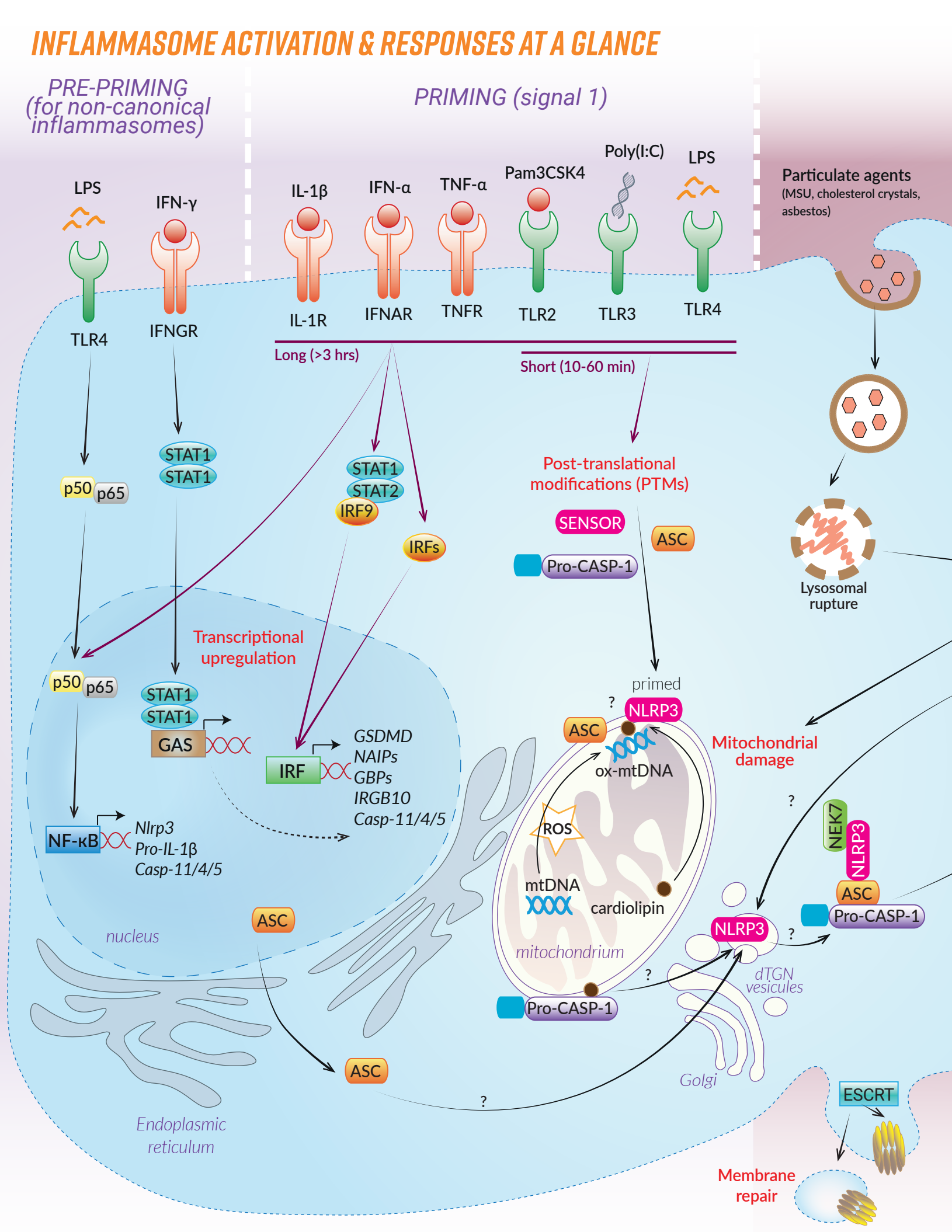


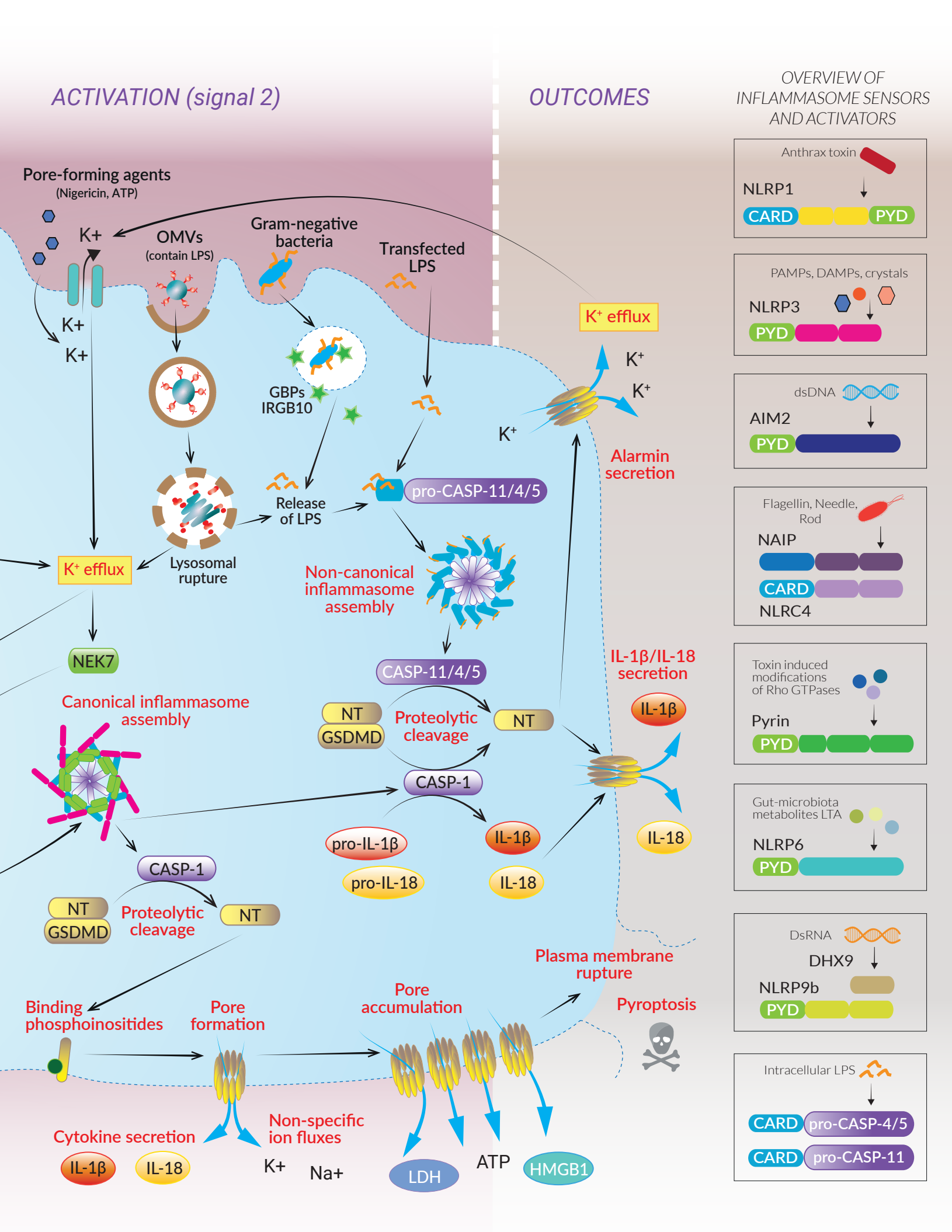
The CARD domain of CASP-11/4/5 mediates both LPS recognition and caspase oligomerization, a requisite for their auto-catalytic subunit activation [29]. These activated caspases cleave GSDMD, ultimately inducing pyroptosis. Unlike CASP-1, CASP-11/4/5 do not cleave pro-IL-1β and pro-IL-18 into their mature forms [59]. The formation of GSDMD pores at the plasma membrane triggers the release of cytosolic contents that act as stress signals. K^+ efflux induces NLRP3 inflammasome assembly and CASP-1-mediated IL-1β/IL-18 secretion [38, 60]. The exact mechanisms underlying CASP-11/4/5 activation still await structural studies.

Extracellular LPS gains cytosolic access through cellular uptake of bacterial OMVs (see p.13) or lysis of intracellular bacteria [57, 61]. The later event involves guanylate binding proteins (GBPs) and the interferon response gene B10 protein (IRGB10). GBPs accumulate on phagosomal membranes to facilitate the release of bacterial products (e.g. DNA, LPS) into the cytosol [62]. They are involved in intracellular bacterial membrane permeabilization by recruiting IRGB10 [63]. GBPs have also been suggested to facilitate CASP-11 interaction with the hydrophobic lipid A moiety of LPS [64].

Basal expression of human CASP-4/5 and mouse CASP-11 differs among cell types, and can be upregulated upon pre-priming with various TLR agonists (e.g. LPS and Pam3CSK4) and interferons (IFN-β and IFN-γ) [22, 65-67]. Likewise, GBPs and IRGB10 are transcriptionally regulated by IFNs [67].

The existence of two CASP-11-like proteins in humans raises the question of whether CASP-4 and CASP-5 have redundant functions. *In vitro* experiments suggest that CASP-5 is minimally required in the response to transfected LPS, but is necessary in the context of infection by *S. typhimurium* [68]. The exact contribution of CASP-4 and CASP-5 during *in vivo* bacterial infection needs further research.


INFLAMMASOME ACTIVATION & RESPONSES AT A GLANCE
PRE-PRIMING (for non-canonical inflammasomes)
PRIMING (signal 1)
LPS
IFN-γ
IL-1β
IFN-α
TNF-α
Pam3CSK4
Poly(I:C)
LPS
Particulate agents (MSU, cholesterol crystals, asbestos)
TLR4
IFNGR
IL-1R
IFNAR
TNFR
TLR2
TLR3
TLR4
Long (>3 hrs)
Short (10-60 min)
STAT1
STAT1
p50
p65
STAT1
STAT2
IRF9
IRFs
Post-translational modifications (PTMs)
SENSOR
ASC
Pro-CASP-1
Lysosomal rupture
Transcriptional upregulation
p50
p65
STAT1
STAT1
GAS
IRF
GSDMD
NAIPs
GBPs
IRGB10
Casp-11/4/5
NF-κB
Nlrp3
Pro-IL-1β
Casp-11/4/5
primed
NLRP3
ASC
ox-mtDNA
Mitochondrial damage
ROS
mtDNA
cardiolipin
mitochondrium
NEK7
NLRP3
ASC
Pro-CASP-1
nucleus
ASC
NLRP3
dTGN vesicules
Pro-CASP-1
ASC
Golgi
Endoplasmic reticulum
ESCRT
Membrane repair


ACTIVATION (signal 2)
OUTCOMES
Pore-forming agents
(Nigericin, ATP)
K+
K+
K+
OMVs
(contain LPS)
Gram-negative
bacteria
Transfected
LPS
GBPs
IRGB10
Release
of LPS
Lysosomal
rupture
K⁺ efflux
NEK7
Canonical inflammasome
assembly
CASP-1
NT
GSDMD
Proteolytic
cleavage
NT
Binding
phosphoinositides
Pore
formation
Cytokine secretion
IL-1β
IL-18
Non-specific
ion fluxes
K+
Na+
K⁺ efflux
K⁺
K⁺
K⁺
Alarmin
secretion
pro-CASP-11/4/5
Non-canonical
inflammasome
assembly
CASP-11/4/5
NT
GSDMD
Proteolytic
cleavage
NT
CASP-1
pro-IL-1β
pro-IL-18
IL-1β
IL-18
IL-1β/IL-18
secretion
IL-1β
IL-18
Plasma membrane
rupture
Pyroptosis
Pore
accumulation
LDH
ATP
HMGB1
OVERVIEW OF
INFLAMMASOME SENSORS
AND ACTIVATORS
Anthrax toxin
NLRP1
CARD
PYD
PAMPs, DAMPs, crystals
NLRP3
PYD
dsDNA
AIM2
PYD
Flagellin, Needle,
Rod
NAIP
CARD
NLRC4
Toxin induced
modifications
of Rho GTPases
Pyrin
PYD
Gut-microbiota
metabolites LTA
NLRP6
PYD
DsRNA
DHX9
NLRP9b
PYD
Intracellular LPS
CARD
pro-CASP-4/5
CARD
pro-CASP-11

INFLAMMASOME INDUCERS

Inflammasomes are activated by a plethora of microbial or sterile danger signals. Microbes are a source of diverse PAMPs that are sensed by multiple PRRs which provide both priming and activating signals (e.g. Gram-negative bacteria can deliver LPS, dsDNA, flagellin, and toxins). Sterile danger signals are either of self-origin, such as ATP and the gout-causing MSU crystals, or non-self-origin, including aluminium salts or asbestos. InvivoGen provides a large collection of inflammasome inducers that are functionally validated and endotoxin-tested.

Microbial or sterile inflammasome inducers are of great interest in the development of vaccine adjuvants.

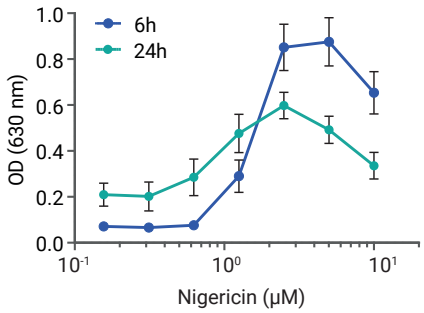
Canonical inflammasome inducers

ATP

Extracellular adenosine triphosphate (ATP) can trigger the activation of the **NLRP3** inflammasome upon binding to the P2X7R purinergic receptor. The rapid opening of this ATP-gated ion channel can result in intracellular K^+ efflux, which has been proposed as the minimal cellular event necessary to induce NLRP3 activation [2, 69-71] (see p. 8).

Nigericin

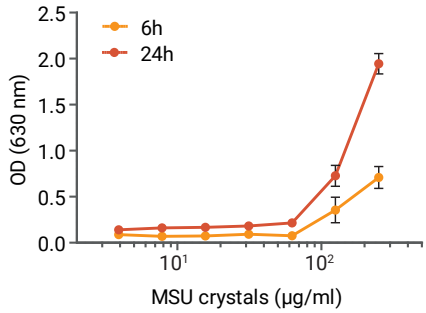
Nigericin is a microbial toxin derived from the Gram-positive bacteria *S. hygroscopicus*. It induces the release of IL-1β in response to the activation of the **NLRP3** inflammasome [2]. This toxin probably activates NLRP3 through its ionophore function which allows the intracellular K^+ efflux across the membrane [70]. Whether the formation of non-selective pannexin-1 pores also contributes to NLRP3 activation upon Nigericin treatment remains unclear [34].



Induction of IL-1β secretion by monocytes upon treatment with Nigericin.
Human THP-1 monocytes were primed with LPS-EK (1 µg/ml) prior to incubation with increasing concentrations of Nigericin. After 6h and 24h activation, IL-1β secretion was assessed in the culture supernatant using HEK-Blue™ IL-1β sensor cells and the SEAP detection reagent QUANTI-Blue™ Solution. The optical density (OD) was read at 630 nm.

MSU & CPPD crystals

MSU (monosodium urate) and **CPPD (calcium pyrophosphate dihydrate) crystals** deposited in joints and periarticular tissues can cause gout and pseudogout inflammatory conditions, respectively. MSU and CPPD crystals have been described as sterile endogenous inducers of the **NLRP3** inflammasome [3]. Lysosomal rupture after phagocytosis of particulate matter has been shown to trigger K^+ efflux, and subsequent activation of NLRP3 [70].



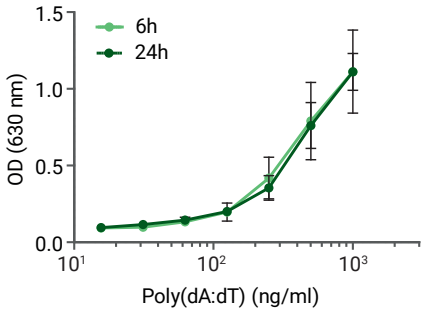
Induction of IL-1β secretion by monocytes upon treatment with MSU crystals.
Human THP-1 monocytes were primed with LPS-EK (1 µg/ml) prior to incubation with increasing concentrations of MSU crystals. After 6h and 24h activation, IL-1β secretion was assessed in the culture supernatant using HEK-Blue™ IL-1β sensor cells and the SEAP detection reagent QUANTI-Blue™ Solution. The optical density (OD) was read at 630 nm.

Alum Hydroxide

Alum hydroxide is a form of aluminum salts (or Alum), which are potent inducers of the **NLRP3** inflammasome [38]. Similarly to MSU and CPPD crystals, Alum hydroxide is thought to activate NLRP3 upon lysosomal destabilization and K^+ efflux [70].

Poly(dA:dT)

Poly(dA:dT) is a synthetic repetitive double-stranded DNA sequence of poly(dA-dT):poly(dT-dA). This multi-PRR agonist induces the **AIM2** inflammasome in murine macrophages [20]. In human myeloid cells, AIM2 appears to be redundant with a cGAS-STING-**NLRP3** pathway in response to cytosolic dsDNA [21] (see p. 6).



Induction of IL-1β secretion by monocytes upon transfection with Poly(dA:dT).
Human THP-1 monocytes were primed with LPS-EK (1 µg/ml) prior to transfection with increasing concentrations of Poly(dA:dT). After 6h and 24h activation, IL-1β secretion was assessed in the culture supernatant using HEK-Blue™ IL-1β sensor cells and the SEAP detection reagent QUANTI-Blue™ Solution. The optical density (OD) was read at 630 nm.

TIPS

INDUCING INFLAMMASOME ACTIVATION

- **Particulate inflammasome inducers** such as MSU crystals or Alum Hydroxide tend to aggregate. To ensure a homogenous distribution of the inducer on the cells, we recommend a short sonication (with either a bath or probe sonicator) to dissociate aggregates.
- The **optimal duration of the stimulation** depends on the inducer. For instance, Nigericin and ATP induce robust inflammasome activation within 1-6 hours. On the contrary, most particulate stimuli and pathogens require a longer incubation with the cells.
- For each inflammasome inducer, it is recommended to perform a **time-course experiment** in order to identify the minimal stimulation period to assess IL-1β or cell death.

LFn-Needle & LFn-Rod

Components of the type III or IV secretion systems (T3SS or T4SS) from intracellular bacteria are inducers of the **NLRC4/NAIP** inflammasome (see p. 9). InvivoGen offers Needle and Inner Rod proteins from the T3SS of *B. thailandensis* and *S. thyphimurium*, respectively. These proteins are fused to the N-terminal domain of the *B. anthracis* lethal factor (LFn). The combination of **LFn-Needle** or **LFn-Rod** with the anthrax protective antigen (PA) is named **Needle-Tox** or **Rod-Tox**, respectively [10]. The PA allows LFn-Needle and LFn-Rod translocation into the cytosol.

They trust InvivoGen

Tweedell R.E. *et al.*, 2020. Nat. Protocols. 15(10):3284-3333. A comprehensive guide to studying inflammasome activation and cell death.

Jain A. *et al.*, 2020. Nat. Immunol. 21:65-74. T cells instruct myeloid cells to produce inflammasome-independent IL-1β and cause autoimmunity.

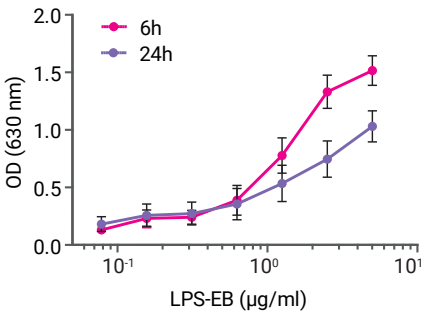
Zheng M. *et al.*, 2020. Cell. 181:674-687.e13. Caspase-6 Is a Key Regulator of Innate Immunity, Inflammasome Activation, and Host Defense.

Samir P. *et al.*, 2019. Nature. 573:590-594. DDX3X acts as a live-or-die checkpoint in stressed cells by regulating NLRP3 inflammasome.

Non-canonical inflammasome inducers

LPS-EK & LPS-EB

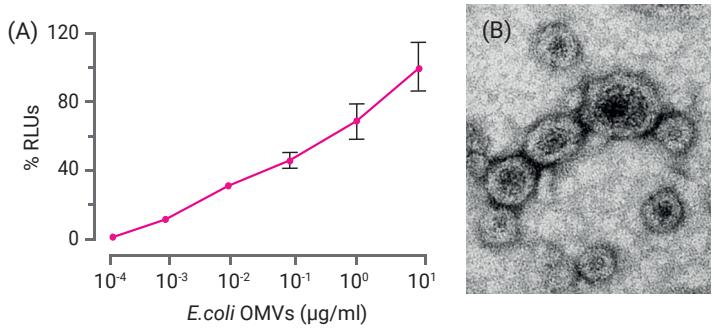
Lipopolysaccharides (LPS) from Gram-negative bacteria, when present in the cytosol of mammalian cells, induce the activation of the **CASP-11/4/5** inflammasomes (see p. 9). LPS consists of a polysaccharide region that is anchored to the outer bacterial membrane by a specific carbohydrate-lipid moiety termed lipid A (also known as endotoxin). Bacterial species exhibit variations in the number of fatty acyl chains in lipid A which can affect CASP-11/4/5 activation. **LPS-EK** (rough LPS) and **LPS-EB** (smooth LPS) are purified from *E. coli* and feature a conserved hexa-acylated (6 fatty acid chains) lipid A which potently activates both human CASP-4/5 and murine CASP-11 upon transfection.



Induction of IL-1β secretion by monocytes upon transfection with LPS-EB.
Human THP-1 monocytes were primed with LPS-EK (1 µg/ml) prior to transfection with increasing concentrations of LPS-EB. After 6h and 24h activation, IL-1β secretion was assessed in the culture supernatant using HEK-Blue™ IL-1β sensor cells and the SEAP detection reagent QUANTI-Blue™ Solution. The optical density (OD) was read at 630 nm.

E. coli outer membrane vesicles (OMVs)

Outer membrane vesicles (OMVs) are small, immunogenic, bilayer spherical bodies produced by Gram-negative bacteria including *E. coli*. They contain many PAMPs, including LPS. As they are naturally endocytosed by mammalian cells, *E. coli* OMVs can be used for effective delivery of LPS into the cytosol without the need for a transfection reagent. They efficiently trigger the **CASP-11/4/5** inflammasomes. InvivoGen's *E. coli* OMVs are purified from *E. coli* BL21.



(A) Functional characterization of E. coli OMVs.
THP1-HMGB1-Lucia™ cells (see page 16) were incubated with increasing concentrations of E. coli OMVs. After 24h activation, secretion of HMGB1 was assessed by measuring the Lucia luciferase activity in the culture supernatant using QUANTI-Luc™ detection reagent.
(B) Image of InvivoGen's E. coli OMVs by transmission electron microscopy (80 kV).

KEY POINT

DIFFERENTIAL CASPASE 11/4/5 ACTIVATION BY VARIOUS LPS STRUCTURES

CASP-11 is potently activated by LPS from *S. typhimurium* and *E. coli* which have a conserved hexa-acylated (6 fatty acid chains) lipid A, but is poorly activated by tetra-acylated (4 fatty acid chains) LPS from *H. pylori* and *R. sphaeroides* [22, 23, 57].

On the contrary, CASP-4 seems to display a broader activity than its murine counterpart as it has been reported to detect under-acylated lipid A from different bacterial species, in addition to hexa-acylated LPS [71].

Bacterial strains (of a same species) exhibit either smooth or rough LPS, which differ in the presence or absence of the O-antigen, respectively. There is currently no evidence that the O-antigen impacts non-canonical inflammasome responses [72].

PRODUCT	INFLAMMASOME	QTY	CAT. CODE
Alum Hydroxide	NLRP3	500 µl	tlrl-aloh
ATP		1 g	tlrl-atpl
MSU Crystals		5 mg	tlrl-msu
Nigericin		10 mg	tlrl-nig
Poly(dA:dT)	AIM2	200 µg	tlrl-patn
LFn-Needle	NLRC4	5 µg	tlrl-ndl
LFn-Rod		50 µg	tlrl-rod
LPS-EB Ultrapure (*E.coli* O111:B4)	CASP-11/4/5	5 x 10e6 EU	tlrl-3pelps
LPS-EK Ultrapure (*E.coli* K12)		1 mg	tlrl-peklps
E. coli OMVs		100 µg	tlrl-omv-1

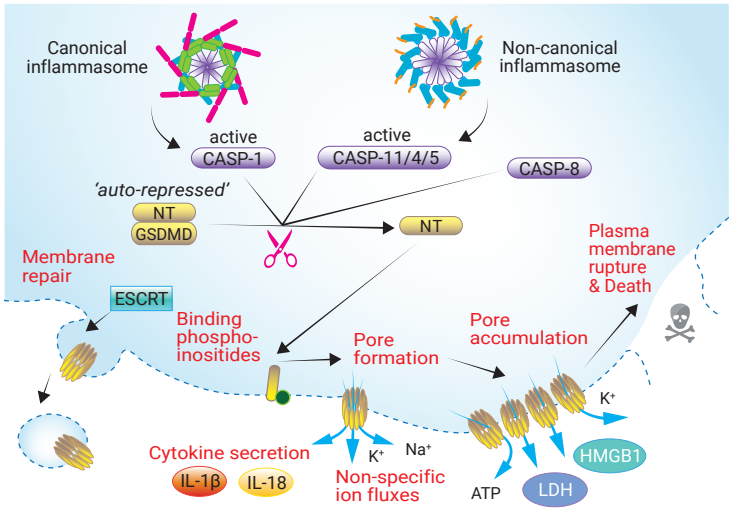
PYROPTOSIS AND GASDERMIN D

Gasdermin D (GSDMD) is a key downstream effector of both canonical and non-canonical inflammasomes. GSDMD is cleaved by CASP-1, -4, -5 and -11 releasing its N-terminal domain which forms pores in lipidic membranes, ultimately leading to pyroptotic cell death.

GSDMD (aka DFNA5L, or FKSG10) belongs to a family of pore-forming proteins consisting of 6 members in humans and 10 in mice. These include GSDMA, GSDMB, GSDMC, GSDMD, GSDME, and PJVK [73]. GSDMD is widely expressed among human tissues and in different immune cells. This cytosolic protein has two distinct domains with a central linker region. The C-terminal domain exerts an auto-inhibitory function through binding to the N-terminal domain (GSDMD-NT). Proteolytic cleavage within the linker by activated canonical or non-canonical inflammasome-associated caspases releases GSDMD-NT [29, 59, 74]. This functional domain translocates to the plasma membrane and oligomerizes to form pores with a ~18 nm inner-diameter [31]. These allow the passage of soluble cytosolic contents (e.g. IL-1β and IL-18), as well as non-specific ions (e.g. Na^+ influx, K^+ efflux) [31, 59, 75].

Accumulation of GSDMD pores at the plasma membrane causes a well-characterized form of regulated cell death named pyroptosis. Pyroptotic cells display characteristic morphological changes, such as cell swelling and formation of bubble-like herniations, ultimately leading to plasma membrane rupture (PMR). PMR allows the release of large cytosolic molecules, including Lactate Dehydrogenase (LDH, a standard marker of PMR) and HMGB1 (see p. 5), into the extracellular space [75].

GSDMD pore formation is a highly regulated process. Indeed, it is crucial to prevent inappropriate or exaggerated pyroptosis, which can cause pathological inflammation. GSDMD expression is low in the resting state and transcriptionally regulated at the priming stage [76]. It remains in an auto-inhibition state until it is cleaved by activated CASP-1 or CASP-11/4/5 [74]. Interestingly, CASP-8 has also been shown to cleave and activate GSDMD [77], whereas CASP-3 can restrict pore formation by cleaving GSDMD-NT into inactive fragments [78]. GSDMD-NT preferably targets lipids (i.e. phosphoinositides) that are only present in the inner leaflet of the plasma membrane, and can thus mediate cell death only from the inside [31].



Although GSDMD pore formation generally leads to pyroptosis, multiple cell types (e.g. macrophages, dendritic cells and neutrophils) are able to release bioactive IL-1β and remain viable [79, 80]. These cells use a membrane repair mechanism to prevent or delay pyroptosis. This mechanism, mediated by the endosomal sorting complexes required for transport (ESCRT)-III machinery, removes GSDMD pores through exocytosis of vesicles containing the damaged membrane [80]. It is still unclear whether additional mechanisms can disassemble or destroy GSDMD pores.

GSDMD has become an attractive target for the treatment of human inflammatory diseases. Other GSDM family members, such as GSDME and GSDMA3, may play roles in human diseases. A more comprehensive investigation into the mechanisms and regulation of these proteins will benefit the development of GSDM-targeting therapeutics.

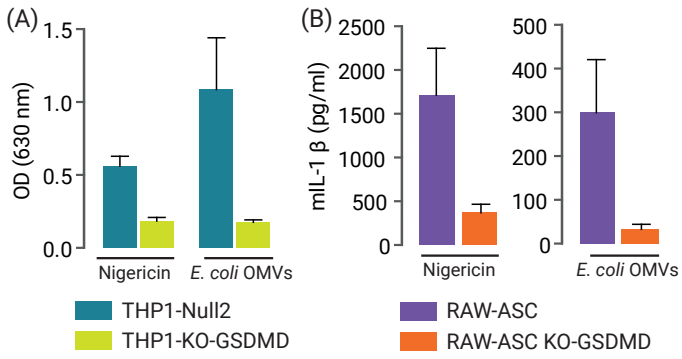
GASDERMIN D CELLULAR ASSAY

Monitoring GSDMD-dependent responses

THP1-KO-GSDMD Cells & RAW-ASC KO-GSDMD Cells

THP1-KO-GSDMD and **RAW-ASC KO-GSDMD** cells derive from the human monocytic THP-1 and mouse macrophagic RAW 264.7 with stable ASC expression, respectively. They feature a biallelic knockout of the *GSDMD* gene. These cells exhibit impaired IL-1β secretion and pyroptosis upon early canonical (e.g. with Nigericin) and non-canonical (e.g. with cytosolic LPS (OMVs)) inflammasome activation.

PRODUCT	QTY	CAT. CODE
THP1-KO-GSDMD Cells	3-7 x 10^6 cells	thp-kogsdmdz
RAW-ASC KO-GSDMD Cells	3-7 x 10^6 cells	raw-kogsdmd



Functional characterization of THP1-KO-GSDMD and RAW-ASC KO-GSDMD Cells. *THP1-Null2 parental and THP1-KO-GSDMD cells (A) or RAW-ASC parental and RAW-ASC KO-GSDMD cells (B) were primed with LPS-EK or Pam3CSK4, prior to stimulation with Nigericin (5 µM) or E. coli outer membrane vesicles (OMVs) (100 µg/ml). After 6h activation, human IL-1β secretion was assessed in the culture supernatant using HEK-Blue™ IL-1β sensor cells (see page 7) (A). Murine IL-1β secretion was assessed in the culture supernatant using an ELISA assay (B).*

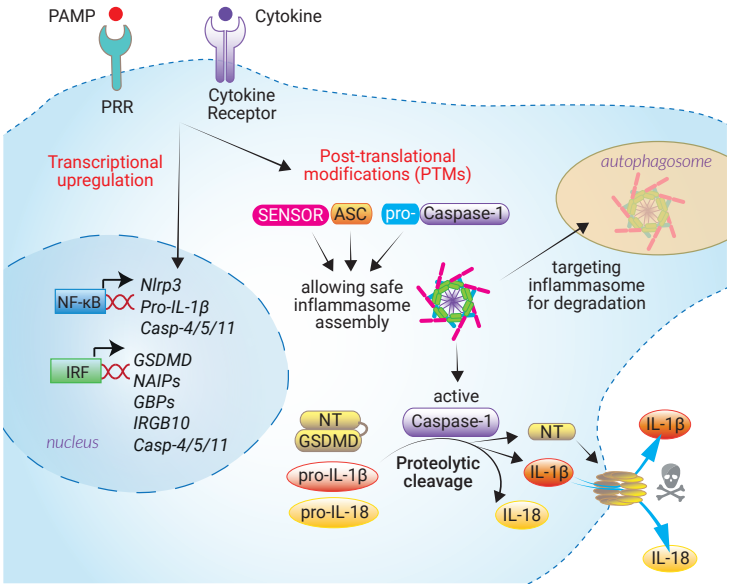
INFLAMMASOME REGULATION

Inflammasomes constitute rapid and powerful immune defenses, acting through the release of inflammatory cytokines and alarmins, as well as pyroptotic cell death. These events must be finely tuned to remain within the injury perimeter while shaping the ensuing adaptive immune responses. Hence, tight regulation of inflammasomes warrants adequate immune protection while preventing unwanted activation and limiting collateral tissue damage. Multi-layered safeguards exist at all steps of the inflammasome response.

The complexity of inflammasome regulation is largely built upon the diversity of molecules involved. Indeed, inflammasomes rely on sensor molecules (e.g. NLRP3, NLRC4, NAIPs), adaptors and scaffolding proteins (e.g. ASC and NEK7), inflammatory caspases (CASP-1 and CASP-11/4/5), pore-forming proteins (e.g. GSDMD), inflammatory cytokines (IL-1β and IL-18), and alarmins (e.g. IL-1α) (see p. 4-5). Each actor is controlled at a genomic and protein level, and to different degrees.

The priming step (see p. 8) allows transcriptional upregulation of inflammasome components and effectors which exhibit low basal expression levels [4, 67]. PRR agonists are major priming agents (see below) that trigger the activation of transcription factors, mainly NF-κB and interferon regulatory factors (IRFs) [67]. For example, expression of NLRP3 and Pyrin is mediated by NF-κB, and expression of the murine NAIPs depends on IRF8. Transcriptional regulation of the ASC adaptor and CASP-1 remains poorly understood and requires further research. The non-canonical inflammatory CASP-11/4/5 are differentially upregulated depending on the cell type (see p. 9). The expression of pore-forming GSDMD (see p. 14) and the permeabilization proteins GBPs and IRGB10 (see p. 9) depends on IRF1/2 and IRF1, respectively. Inducible expression of the IL-1α alarmin and the cytokine zymogen pro-IL-1β and pro-IL-18 cytokines seem to depend on multiple transcription factors, which are still under investigation. However, it is acknowledged that transcriptional upregulation is mandatory for pro-IL-1β.

Inflammasome actors are also regulated by post-translational modifications (PTMs) including phosphorylation, ubiquitination, deubiquitination, and proteolytic cleavage [44, 81]. PTMs are triggered by distinct stimuli (e.g. PRR agonists or inflammasome sensor inducers) and can be mapped to specific residues of the same actor, leading to distinct outcomes. This has been most extensively described for NLRP3 but other actors, including ASC, CASP-1, and pro-IL-1β, are subject to PTMs [44, 81]. Notably, regulatory functions driven by PTMs ensure safe conformational changes which are necessary for the inflammasome platform assembly. Furthermore, proteolytic cleavage is required for CASP-1 and CASP-11/4/5 auto-activation, conversion of pro-IL-1β/IL-18 to their bioactive forms, and release of the N-terminal pore-forming domain of GSDMD. Other regulatory functions of PTMS have been described in the response termination (e.g. inducing delivery of assembled inflammasomes to autophagosomes) [45, 84, 85]. Because PTMs govern multiple steps of the inflammasome response and are dependent on enzymatic activities, they emerge as attractive therapeutic targets for the modulation of inflammasomes involved in inflammatory pathologies.



PRIMING AGENTS

LPS

Lipopolysaccharide (LPS) is the major constituent of the outer membrane of Gram-negative bacteria, such as *E. coli*, and a potent inducer of the innate immune system. It is recognized at the cell surface by TLR4 which forms a complex with CD14 and MD-2 and triggers signaling cascades leading to the activation of the NF-κB and IRF3 transcription factors [83] and the production of pro-inflammatory cytokines and type I interferons, respectively. To avoid responses with high background, macrophages that were differentiated using phorbol 12-myristate 13-acetate (PMA) can be primed with LPS ~1 week after PMA treatment.

Poly(I:C)

Polyinosine-polycytidylic acid (Poly(I:C)) is a synthetic analog of double-stranded RNA (dsRNA), a PAMP associated with viral infection. Poly(I:C) activates TLR3 but also RIG-I/MDA5 and PKR, thereby inducing signaling via inflammatory NF-κB and IRF pathways [84, 85].

Pam3CSK4

Pam3CSK4 (or Pam3CysSerLys4) is a synthetic mimic of the acylated amino terminus of lipopeptides found in the bacterial cell wall. Pam3CSK4 is a potent activator of TLR2, which in cooperation with TLR1, induces the activation of NF-κB [86]. We recommend using Pam3CSK4 for priming RAW264.7-dervied macrophages to avoid high background responses.

Pre-priming with IFN-γ is highly recommended to induce expression of human CASP-4/5 and murine CASP-11 non-canonical inflammasomes (see p. 9)

PRODUCT	QTY	CAT. CODE
LPS-EK Ultrapure (*E.coli* K12)	5 mg	tlrl-peklps
Pam3CSK4	1 mg	tlrl-pms
Poly(I:C) HMW	10 mg	tlrl-pic

ASSESSING INFLAMMASOME ACTIVATION

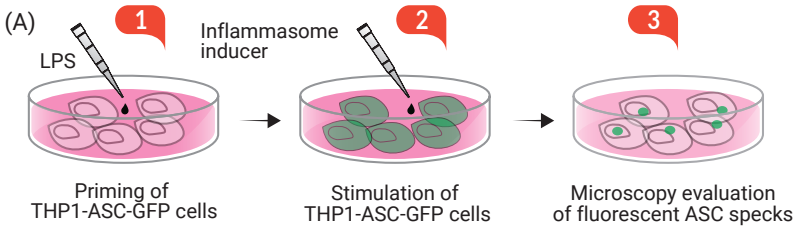
Inflammasome activation leads to signature events such as ASC speck formation, induction of pyroptotic cell death and secretion of bioactive IL-1β/18 cytokines, and the alarmin HMGB1. To assess these key events, InvivoGen has developed cellular tools derived from the human THP-1 monocytic cell line, the most commonly used model to study inflammasome activation *in vitro*, and the human HEK293 embryonic kidney cell line, widely used for reporter systems.

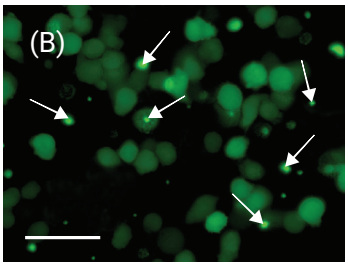
Monitoring ASC-specks in real-time

THP1-ASC-GFP Cells

PRODUCT	QTY	CAT. CODE
THP1-ASC-GFP Cells	3-7 x 10^6 cells	thp-ascgfp

THP1-ASC-GFP cells allow the visualization of ASC speck formation in live cells by fluorescent microscopy. They stably express an ASC::GFP fusion protein in an NF-κB-dependent manner. Inflammasome activation, upon stimulation with inducers such as Poly(dA:dT), can be analyzed by following the assembly of fluorescent ASC specks without altering the inflammasome response.





Visualization of ASC speck formation by fluorescence microscopy.
A. *Procedure for inducing ASC::GFP expression in THP1-ASC-GFP cells.* ***B.*** *ASC speck visualization in THP1-ASC-GFP cells after priming with 1 µg/ml LPS-EK for 3 h, and activation with 250 ng/ml Poly(dA:dT) for 1 to 3 h. In most cells, only one speck forms upon inflammasome activation (arrows). Scale bar: 50 µm.*

TIPS

EVALUATION OF INFLAMMASOME ACTIVATION

- **RT-qPCR** to measure NF-κB-induced upregulation of pro-IL-1β and NLRP3 during priming
- **Fluorescence** microscopy or flow cytometry to monitor ASC-speck formation in engineered cell lines
- **Western blot** to determine caspase-1 cleavage or pro-IL-1β/IL-18 maturation
- **ELISA** to assess the release of IL-1β, IL-18 or HMGB1
- **Lactate dehydrogenase (LDH) assay** or **propidium iodide staining** to measure pyroptotic cell death
- **Reporter cell lines** to detect IL-1β or IL-18 secretion

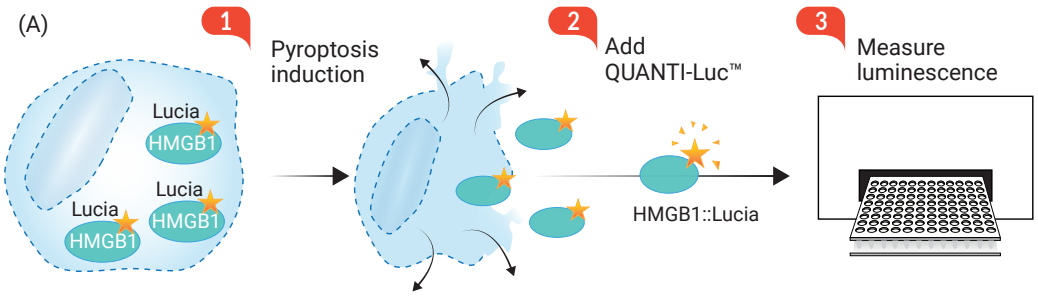
All of these methods present pros and cons. It is thus recommended to use a combination of these methods to appropriately assess inflammasome activation.

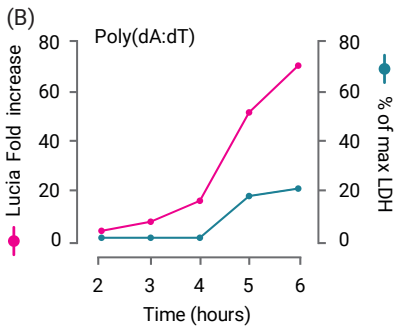
Monitoring pyroptotic cell death

THP1-HMGB1-Lucia™ Cells

PRODUCT	QTY	CAT. CODE
THP1-HMGB1-Lucia™ Cells	3-7 x 10^6 cells	thp-gb1lc

THP1-HMGB1-Lucia™ cells represent a robust and convenient tool for the quantification of pyroptosis, and an alternative to the classical LDH assay. It relies on the measurement of the cytolysis-mediated release of the HMGB1 alarmin fused to the luminescent reporter, Lucia luciferase. Following their priming with LPS and treatment with an inflammasome inducer such as Poly(dA:dT), THP1-HMGB1-Lucia™ cells undergo pyroptosis and release HMGB1::Lucia into the extracellular milieu. Levels of HMGB1::Lucia in the supernatant can be readily monitored by measuring the light signal produced using the QUANTI-Luc™ detection reagent.

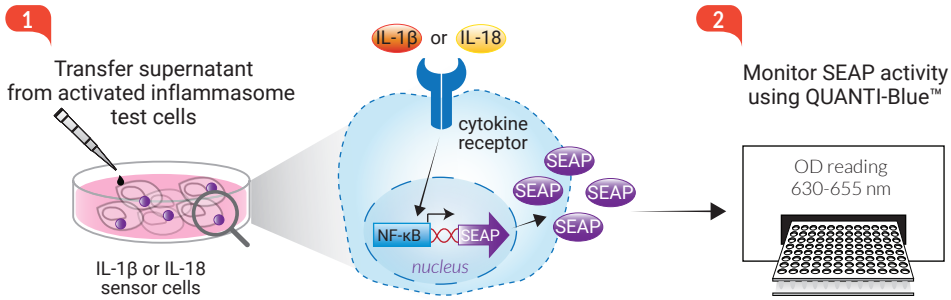




Luminescent quantification of inflammasome-induced pyroptosis.
A. *Assay principle for THP1-HMGB1-Lucia™ cells.* ***B.*** *THP1-HMGB1-Lucia™ cells were primed with LPS-EK (1 µg/ml) for 3h and then incubated with the inflammasome inducer Poly(dA:dT) (0.5 µg/ml). Lucia luciferase activity (pink curve, left axis) and LDH release (blue curve, right axis) in the supernatant were quantified at 2, 3, 4, 5 and 6 h post-induction.*

Monitoring IL-1β and IL-18 secretion

InvivoGen has developed reporter cell lines for the simple, rapid, and reliable detection and quantification of bioactive human (h) or murine (m) IL-1β and IL-18. These cells are derived from the human embryonic kidney HEK293 cell line. They express a secreted alkaline phosphatase (SEAP) reporter inducible by NF-κB/AP-1 upon activation of the cytokine signaling pathway. Levels of SEAP in the supernatant can be easily monitored using the QUANTI-Blue™ Solution detection reagent.



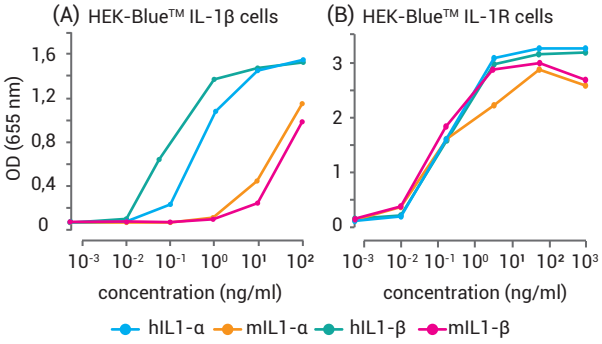
HEK-Blue™ IL-1β Cells & HEK-Blue™ IL-1R Cells

These cells allow the monitoring of hIL-1α/β, mIL-1α/β within a sample. Both cell lines express the endogenous human IL-1 receptor (IL-1R).

HEK-Blue™ IL-1β cells are more sensitive to human IL-1 isoforms than murine isoforms. We recommend using this cell line to test supernatants from human inflammasome cellular assays (e.g. THP1-derived assays, see p. 6-7).

HEK-Blue™ IL-1R cells are stably transfected to additionally express mIL-1R, conferring a higher sensitivity to mIL-1β. Therefore, this cell line is more suitable for the detection of IL-1β from mouse samples such as cell surpernatants or sera. However, their high sensitivity can lead to a high background with some cells, such as RAW264.7-derived cells.

Of note, both cell lines can be used to assess levels of the IL-1α alarmin within a sample. The specificity of HEK-Blue™ IL-1β and HEK-Blue™ IL-1R cells for the detection of one isoform can be assessed by including a neutralization antibody against the other isoform in the assay.



Dose response of HEK-Blue™ IL-1β and HEK-Blue™ IL-1R cells to human and murine IL-1.
(A) HEK-Blue™ IL-1β and (B) HEK-Blue™ IL-1R cells were incubated with increasing concentrations of recombinant hIL-1α, hIL-1β, mIL-1α, or mIL-1β. After overnight incubation, SEAP activity was measured in the supernatant using QUANTI-Blue™ Solution.

PRODUCT	QTY	CAT. CODE
HEK-Blue™ IL-1β Cells	3-7 x 10^6 cells	hkb-il1bv2
HEK-Blue™ IL-1R Cells	3-7 x 10^6 cells	hkb-il1r
HEK-Blue™ IL-18 Cells	3-7 x 10^6 cells	hkb-hmil18

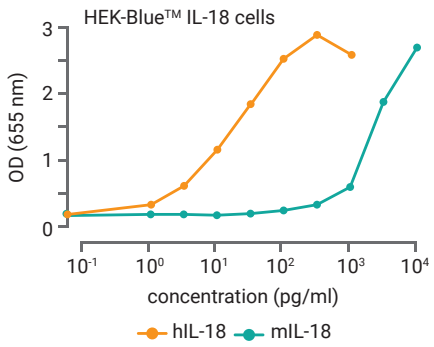
ADVANTAGES

USING INVIVOGEN'S CYTOKINE SENSOR CELLS

- Detection of bioactive cytokines
- Cost-efficiency
- Short hands-on-time experimentation
- Naked-eye visualization
- High sensitivity Wide dynamic range
- Suitable for drug screening (normal or HTS, kinetics)

HEK-Blue™ IL-18 Cells

These cells provide a robust cellular assay to monitor the concentration of human and murine IL-18 within a sample. **HEK-Blue™ IL-18** cells are stably transfected to express the human IL-18 receptor. These cells are more sensitive to hIL-18 than to mIL-18.



Dose response of HEK-Blue™ IL-18 cells to human and murine IL-18.
HEK-Blue™ IL-18 cells were incubated with increasing concentrations of recombinant human or murine IL-18. After overnight incubation, levels of NF-κB/AP1-induced SEAP in the supernatant were determined using the SEAP detection reagent QUANTI-Blue™ Solution. The optical density (OD) was read at 655 nm.

They trust InvivoGen

Martine P. et al. 2019. Cell Death Dis. doi: 10.1038/s41419-019-1491-7. HSP70 is a negative regulator of NLRP3 inflammasome activation.

Metho S. et al. 2019. Mol. Cell. doi: 10.1016/j.molcel.2018.11.08. The Crohn's disease risk factor IRGM limits NLRP3 inflammasome activation by impeding its assembly and by mediating its selective autophagy.

INFLAMMASOME INHIBITORS

Inflammasome signaling can be pharmacologically inhibited at different levels. InvivoGen offers a selection of inhibitors to block inflammasome-induced responses. These include small molecule compounds that target a specifc sensor or inflammatory caspase, or multiple actors (e.g. NF-κB and a sensor). All of InvivoGen's inhibitors are tested to confirm the absence of bacterial contaminants, thereby preventing experimental bias.

Caspase inhibitors

Ac-YVAD-cmk

Ac-YVAD-cmk is a cell-permeable, selective and irreversible inhibitor of CASP-1, with little activity against CASP-4/5. This small peptide sequence is based on the CASP-1 target sequence in pro-IL-1β [87].

Z-VAD-FMK

Z-VAD-FMK is a cell-permeable pan-caspase inhibitor that irreversibly binds to the catalytic site of caspase proteases. It has been shown to be a potent inhibitor of CASP-1 activation in cells upon NLRP3 activation [87, 88].

VX-765

VX-765 is a pro-drug converted by plasma esterases into the VRT-043198 peptidomimetic metabolite, which potently inhibits CASP-1 and CASP-4. It acts by covalent modification of the caspase catalytic cysteine [89, 90].

Multi-target inhibitors

Parthenolide

Parthenolide has multiple targets including NF-κB, inflammasome sensors, and CASP-1. This drug inhibits the activity of multiple inflammasomes, including NLRP1, NLRP3, NLRC4, but not AIM2 [91-93]. Mechanistically, it has been reported to inhibit the IκB kinase function required for NF-κB activation [94, and to alkylate the cysteine residues in CASP-1 and the ATPase domain of NLRP3 [93].

BAY11-7082

BAY11-7082 is an NF-κB pathway inhibitor [96] that also exhibits direct inhibitory functions on NLRP3 by blocking the sensor's ATPase activity [91]. This compound does not affect NLRP1 activation, and may partially inhibit the Salmonella-induced NLRC4 inflammasome, albeit this effect could be due to a toxic effect on the bacteria itself [91].

Isoliquiritigenin

Isoliquiritigenin has multiple effects. This molecule inhibits TNF-α-induced NF-κB activation by blocking IκB kinase activity [97]. It also inhibits NLRP3-ASC oligomerization [93]. Of note, isoliquiritigenin is a more potent NLRP3 inhibitor than parthenolide and glybenclamide, and it has been reported to have no effect on the AIM2 inflammasome [93].

ODN TTAGGG (A151)

A151 is a synthetic oligonucleotide, a TLR9 antagonist, as well as a potent competitive inhibitor of double-stranded DNA binding to cGAS, and the AIM2 inflammasome [98].

They trust InvivoGen

Hafner-Bratkovič I. et al., 2018 Nat. Commun. 59:5182. NLRP3 lacking the leucine-rich repeat domain can be fully activated via the canonical inflammasome pathway.
Irmscher S. et al., 2019 Nat. Commun. 10:2961. Serum FHR1 binding to necrotic-type cells activates monocytic inflammasome and marks necrotic sites in vasculopathies.
da Costa L.S. et al., 2019 Cell Death Dis. 10:346. RNA viruses promote activation of the NLRP3 inflammasome through cytopathogenic effect-induced potassium efflux.

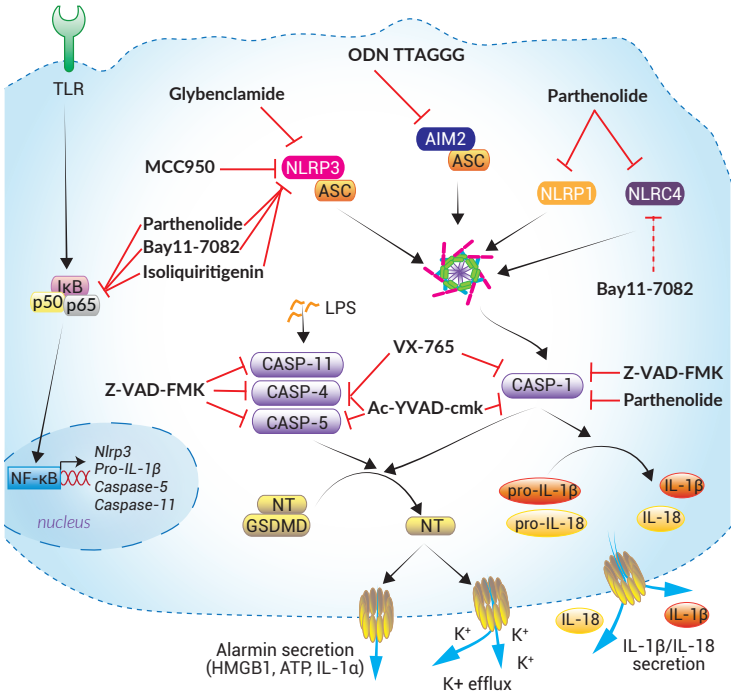
NLRP3 inhibitors

MCC950

MCC950 (aka CRID3 or CP-456,773) is a potent, reversible, and specific NLRP3 inhibitor that prevents NLRP3 inflammasome assembly, without affecting the AIM2, NLRC4, or NLRP1 inflammasomes. It directly targets the NLRP3 NATCH domain and interferes with ATP hydrolysis, which is required for NLRP3 conformational changes and oligomerization [99, 100].

Glybenclamide

Glybenclamide (or Glyburide) inhibits NLRP3 inflammasome activation indirectly by inducing the closure of ATP-sensitive K^+ channels, thereby raising the intracellular K^+ concentration. This drug works downstream of the ATP receptor P2X7, and upstream of NLRP3 [101]. Glybenclamide seems to be a specific inhibitor of NLRP3 since no impact on NLRC4 and NLRP1-mediated responses have been detected [101].



PRODUCT	QTY	CAT. CODE
Ac-YVAD-cmk	5 mg	inh-yvad
Z-VAD-FMK	1 mg	tlrl-vad
VX-765	10 mg	inh-vx765i-1
Parthenolide	50 mg	inh-ptd
BAY11-7082	10 mg	tlrl-b82
Isoliquiritigenin	10 mg	inh-ilg
ODN TTAGGG (A151)	200 µg	tlrl-ttag151
MCC950	10 mg	inh-mcc
Glybenclamide	1 g	tlrl-gly

INFLAMMASOMES IN DISEASES

In the past century, a drastic shift from infectious to non-communicable causes of death has occurred in Western industrialized societies. Most of them are linked to inflammasomes, with NLRP3 being implicated in many pathologies. Distinct approaches are used in the clinic to overcome the tremendous impact of inflammasome-linked diseases on public health.

Non-infectious diseases

The cryopyrin-associated-periodic-syndrome (CAPS) illustrates the liable gain-of-function mutations in NLRP3. Other inflammatory conditions including type-2 diabetes, gouty arthritis, cancer, cardiovascular and Alzheimer's diseases are linked to NLRP3-mediated chronic inflammation secondary to accumulation of sterile danger signals in tissues [35]. Auto-immune diseases such as vitiligo, Addison's disease, and type-1 diabetes are linked to single-nucleotide polymorphisms in NLRP1 [46]. The familial cold autoinflammatory syndrome (FCAS), enterocolitis and recurrent macrophage activation syndrome (MAS) are linked to mutations in NLRC4 [15]. Gain-of-function mutations in the gene encoding Pyrin are associated with familial Mediterranean fever (FMF)[15].

The cytokine storm

This excessive secretion of inflammatory cytokines can lead to organ failure and death. Dysregulation of NLRP3-induced inflammatory responses have been associated with toxic shock-like syndromes such as Streptococcal Toxic-Shock-like Syndrome (STSLS) [102], and acute respiratory distress syndromes such as COVID-19 caused by SARS-CoV-2 viral infection [103].

Therapeutic approaches

Most clinically-approved strategies are aimed at blocking the inflammatory IL-1β/IL-1R signaling. Anakinra, a recombinant human IL-1R antagonist, Canakinumab, a monoclonal anti-IL-1β antibody, and Rilonacept, a human IL-1R-Fc fusion protein, have proven successful in the treatment of CAPS. Moreover, these drugs may be relevant in metabolic and neurodegenerative inflammasomopathies [36]. Similar to IL-1 blocking agents, Tadekinig alfa and GSK1070806, target the IL-18 signaling and have entered clinal trials [36]. Yet, major caveats to these drugs include the absence of specific targeting and an increased risk of infection. Another promising approach consists in using pharmacological inhibitors of specific inflammasome sensors. One important example is MCC950, a specific NLRP3 inhibitor [99, 100]. Its efficiency was highlighted in murine inflammatory disease models and it was tested in phase II clinical trials for rheumatoid arthritis. Although MCC950 has been found to cause liver toxicity [35, 36], it has led the path to the design of more potent and safer molecules. Importantly, chronic inflammation can lead to cancer. But often, once established, tumors grow in a suppressive environment to escape the immune response. Thus pyroptosis-inducing compounds hold promises to kill cancer cells and improve anti-tumor T cell responses [104].

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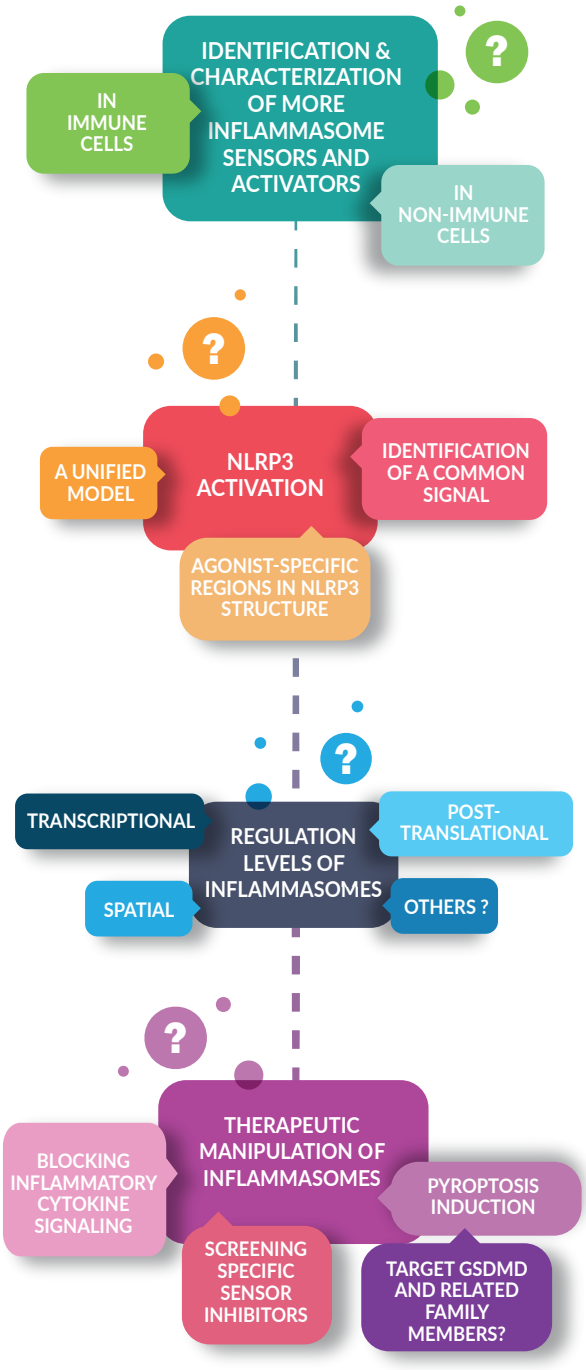

Inflammasomes are cytoplasmic protein complexes providing a rapid defense against microbial and non-infectious aggressions. They act as signaling hubs to induce the maturation and secretion of inflammatory cytokines, eventually leading to pyroptosis, a lytic immunogenic cell death.

Over the past two decades, multiple inflammasome sensors have been identified both in immune and non-immune cells. This indicates a remarkable sentinel system, with its activation beneficial for clearing pathogens. Yet, it can also contribute to sterile inflammation associated with autoinflammatory, neurodegenerative and metabolic diseases such as gouty arthritis, Alzheimer's disease, and type-2 diabetes. Additionally, inflammasomes have contrasting functions in cancer, either protecting the host or contributing to the tumor growth.

The NLRP3 inflammasome stands out as the prototypical, best characterized inflammasome, and has been linked to a long list of diseases and conditions. While the exact mechanisms underlying its activation remain a controversial topic, NLRP3 is the most targeted sensor by biotech companies developing inflammasome modulators. Inflazome, IFM Therapeutics, Nodthera, and Jecure focus on small-molecule antagonists of NLRP3. Such inhibitors may be the winning ticket for treating high-mortality diseases in industrialized societies.

While significant progress has contruibuted to our understanding of the inflammasome and its implications in health and disease, several questions remain. To what extent are the various inflammasome sensors participating in innate immune defenses? Are inflammasomes dedicated to a specific threat or can they function in a concerted manner? A great deal of work has already highlighted the complexity of inflammasome regulation, opening new avenues of therapeutic intervention.

The golden age of inflammasomes is yet to come...

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