NLRP3: a sophisticated drug target

NLRP3 (NOD-like receptor pyrin domain-containing protein 3, cryopyrin or NALP3) is the best described inflammasome sensor and an attractive drug target. NLRP3 assembles into a multiprotein inflammasome complex to induce the secretion of IL-1β/IL-18 and pyroptosis in response to infections and cellular damage. However, NLRP3 inflammasome functions can also be detrimental to the host, as its aberrant or chronic activation is linked with pathologies such as type-2 diabetes, gouty arthritis, cardiovascular and Alzheimer’s diseases, and rare genetic disorders such as CAPS (cryopyrin-associated periodic syndrome). Fast-paced research now aims at filling the gaps in the comprehension of NLRP3 inflammasome activation and regulation to develop novel therapeutics.

Activation of the NLRP3 inflammasome relies on a 2-signal model. Signal 1 is provided by microbial components or endogenous cytokines and primes NLRP3. Signal 2 is triggered by a plethora of stimuli such as ion imbalances (e.g., K+ efflux), externalized mitochondrial (mt) cardiolipin and oxidized mtDNA, the two latter being proposed as PTMs governing NLRP3 activation, but their binding sites are not yet elucidated. NLRP3 can be activated by a wide range of stimuli that are structurally and chemically unrelated (e.g., pore-forming toxins, activators of ion channels, uric acid crystals, β-amyloid proteins) suggesting that NLRP3 doesn’t bind directly to these molecules. NLRP3 rather senses downstream cytosolic stress signals such as ion imbalances (e.g., K+ efflux), externalized mitochondrial (mt) cardiolipin and oxidized mtDNA, the two latter being proposed as NLRP3 ligands. Yet, the exact mechanisms underlying NLRP3 activation remain a controversial topic.

Two major NLRP3 inflammasome responses, canonical and non-canonical, have been described and differ in their caspase (Casp) recruitment. Upon activation, the canonical response is driven by aggregation of NLRP3 with the ASC adaptor and pro-Casp-1 (Fig. 2 inside). Self-cleaved active Casp-1 induces the maturation of pro-IL-1β/IL-18, and cleavage of the pore-forming gasdermin D (GSDMD), leading to secretion of IL-1β/IL-18 and pyroptosis. The non-canonical response relies on the activation of Casp-4/11 (human) or Casp-11 (mouse) upon the sensing of cytosolic LPS (Fig. 2 inside). Casp-4/5/11 cannot cleave pro-IL-1β/18, but they trigger stress signals through GSDMD pore formation at the plasma membrane. Thus, this ultimately induces NLRP3 inflammasome assembly and Casp-1-mediated IL-1β/18 maturation and secretion.

Organelles play a key role in NLRP3 inflammasome assembly. Coordinated priming and activation signals seem to orchestrate trafficking of NLRP3 and other proteins to mitochondria, endoplasmic reticulum (ER), and Golgi. NEK7 (NIMA-related protein kinase 7) was recently identified as an integral component of the NLRP3 inflammasome, but timing and location for its association with NLRP3 is not fully elucidated yet. While some studies suggest that the first steps of NLRP3 inflammasome assembly occur at mitochondria-associated ER membranes (MAMs), a recent report indicates that activation and inflammasome assembly rather occur around dispersed trans-Golgi network (dTGN) vesicles, without mitochondrial trafficking. Whether multiple organelle-linked mechanisms can rule NLRP3 inflammasome assembly requires further work.

Intricate activating and inhibiting PTMs of NLRP3 inflammasome components have emerged as important regulatory means during priming and activation steps. Research on the nature, timing and location of regulatory PTMs will help in identifying new targets for fine-tuned therapeutic strategies with minimal side-effect to replace existing NLRP3 inhibitors and IL-1β/18 blockade strategies.


SUMMARY:

REVIEW

NLRP3: a sophisticated drug target
Inflammasome test cells

InvivoGen offers an expanding collection of human monocyte and mouse macrophage cell lines for the study of inflammasome signaling. Our new inflammasome test cells were engineered to assess the role of specific actors of the canonical and non-canonical NLRP3 inflammasome responses. These cells are knockout (KO) for the genes encoding NLRP3, ASC, caspase-4 (CASP4), or gasdermin D (GSDMD). Our inflammasome test cells are validated by monitoring the release of bioactive IL-1β and western-blot.

Human monocyte-derived cell lines

- THP1-KO-NLRP3 Cells NEW
- THP1-KO-ASC Cells NEW
- THP1-KO-CASP4 Cells NEW

THP1-KO-NLRP3, -KO-ASC, and -KO-CASP4 cells are derived from the human monocytic THP1 (WT) cell line which is widely used for inflammasome studies, as it expresses high levels of NLRP3, ASC, and pro-caspase-11. THP1-KO-NLRP3, -KO-ASC, and -KO-CASP4 cells feature a knockout (KO) of the genes encoding NLRP3, ASC, caspase-4 (CASP4), or gasdermin D (GSDMD). Their KO status has been confirmed by PCR, sequencing, western-blot (Fig. 1A), and functional assays (Fig. 1B).

As expected, THP1-KO-NLRP3 and -KO-ASC cells are unable to secrete mature IL-1β upon incubation with MSU crystals and E. coli OMVs (see next page for OMV description). In THP1-KO-CASP-4, IL-1β secretion is highly reduced but not abrogated. This is partly explained by the residual activity of Casp-5.

InvivoGen also offers THP1-derived inflammasome reporter cells: THP1-HMGB1-Lucia cells allow a bioluminescence monitoring of pyroptosis, and THP1-ASC-GFP cells allow the visualization of fluorescent ASC speck formation upon inflammasome activation. Learn more on our website.

Induction of canonical and non-canonical NLRP3 inflammasome responses


Figure 1: Validation of THP1-KO-NLRP3, -KO-ASC, and -KO-CASP4 cells.

(A) Western-Blot by WES\textsuperscript{TM}. Anti-human NLRP3, anti-human ASC, and anti-human caspase-4 antibodies were used on lysates from THP1-WT, -KO-NLRP3, -KO-ASC, and -KO-CASP4 cells. Arrows indicate the expected size (kDa) for the proteins tested: NLRP3 (110 kDa), ASC (27 kDa), Casp-4 (48 kDa).

(B) Mature IL-1β secretion upon NLRP3 inflammasome activation. Cells were primed with LPS-EK (1 µg/ml) or E. coli OMVs (100 µg/ml). After 6 or 24h incubation, the secretion of IL-1β was assessed in the supernatants using HEK-Blue\textsuperscript{TM}-IL-1β sensor cells which express an NF-κB SEAP reporter gene. QUANTI-Blue\textsuperscript{TM} Solution was used to measure SEAP activity. Optical density was read at 630 nm.
Mouse macrophage-derived cell lines

- RAW-ASC Cells  **NEW**
- RAW-ASC-KO-GSDMD Cells  **NEW**

RAW-ASC and RAW-ASC-KO-GSDMD cells are derived from the mouse macrophage RAW 264.7 cell line which does not express ASC. To allow the study of ASC-dependent inflammasomes in RAW cells, we have generated RAW-ASC cells by stable transfection of the murine ASC gene. RAW-ASC-KO-GSDMD cells were then engineered by knockout of the GSDMD gene. The stable expression of ASC and KO status of these cell lines have been confirmed by PCR, western blot (WES™) (Fig. 3A), and functional assays (Fig. 3B). RAW-ASC and RAW-ASC-KO-GSDMD cells are resistant to nigericin and damaged/necrotic RAW-ASC-inflammasomes by nigericin and blasticidin. As expected, activation of the canonical and non-canonical NLRP3 inflammasome by nigericin and P2X7 receptor differentially couples to distinct release pathways for IL-1β in mouse macrophage. J. Immunol. 180:7147.

E. coli outer membrane vesicles (OMVs)  **NEW**

**Caspase 11-4/5 inflammasome activator**

- **Cost effective:** Stronger activation potency than transfected LPS
- **Reliable:** Reproducible results

Outer membrane vesicles (OMVs) are small, immunogenic, bilayer spherical bodies produced by Gram-negative bacteria. They contain many pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS) from the outer membrane of the parent bacterium. OMVs initiate a myriad of immune responses, through Toll-like receptors (TLRs) and intracellular activation of the caspase 11-4/5 inflammasomes. As OMVs are naturally endocytosed, they act as a vehicle in the effective delivery of LPS across the host cell membrane and into the cytosol, without the need for a transfection reagent.

InvivoGen’s OMVs are purified from *Escherichia coli* BL21, thoroughly characterized, and functionally validated. Our *E. coli* OMVs are available in a standard grade, and a sterile InvivoFit™ grade (recommended for in vivo studies).

**RELATED PRODUCTS**

- Blasticidin: Selection antibiotic (# ant-bl-05)
- HEK-Blue™ IL-1β Cells: Human IL-1β sensor cells (# hkb-il1b)
- MSU Crystals: Monosodium Urate Crystals (# tlr1-msu)
- Nigericin: Potassium ionophore (# tlr1-nig)
- Pam3CSK4: TLR1/2 synthetic agonist (# tlr1-pms)
- QUANTI-Blue™: SEAP detection reagent (# rep-qbs)

---

**FLYER**

**OMVs InvivoFit™ 500 µg tlrl-omv**

**THP1-KO-ASC Cells 3-7 x 10⁶**

**THP1-KO-NLRP3 Cells 3-7 x 10⁶**

**THP1-KO-CASP4 Cells 3-7 x 10⁶**

**RAW-ASC Cells 3-7 x 10⁶**

**RAW-ASC-KO-GSDMD Cells 3-7 x 10⁶**

**RAW-ASC-KO-GSDMD Cells**

**THP1-KO-ASC Cells**

**THP1-KO-NLRP3 Cells**

**THP1-KO-CASP4 Cells**

**RAW-ASC Cells**

**RAW-ASC-KO-GSDMD Cells**

---

**Figure 3: Validation of the RAW-ASC and RAW-ASC-KO-GSDMD cells.**

(A) Western-Blot by WES™. Anti-mouse ASC and anti-mouse GSDMD were used on lysates from RAW-ASC and -KO-GSDMD cells. Arrows indicate the expected size (kDa) for the proteins tested: ASC (27 kDa), GSDMD (black arrow; full-length, 53 kDa, grey arrow: N-term cleaved region, 33 kDa). (B) Mature IL-1β secretion upon NLRP3 inflammasome activation. Cells were primed for 3 h at 37°C with Pam3CSK4 (100 ng/ml), and then incubated with Nigericin (10 µM) or E. coli OMVs (20 µg/ml) for 6h at 37°C. The secretion of IL-1β was assessed by ELISA. For non-canonical inflammasome activation with E. coli OMVs, cells were pre-primed (see Fig. 2) with murine recombinant IFN-γ (10 ng/ml) overnight at 37°C before the priming step.

---

**Related Products**

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>QUANTITY</th>
<th>CAT. CODE</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli OMVs</td>
<td>100 µg</td>
<td>tlr-omv-1</td>
</tr>
<tr>
<td>E. coli OMVs InvivoFit™</td>
<td>500 µg</td>
<td>tlr-omv</td>
</tr>
</tbody>
</table>
The good and the bad of LPS

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria, and elicits a potent innate immune response through Toll-like receptor 4 (TLR4). The resulting signaling triggers the release of pro-inflammatory cytokines, which can lead to both acute and chronic inflammatory diseases. But it is all about balance – small controlled amounts of LPS can be protective, but large uncontrolled amounts of LPS can lead to disastrous outcomes, such as septic shock\(^1\). Despite its highly inflammatory nature, LPS has remarkable therapeutic potential.

Activity of LPS? It’s all in the structure.

The structure of LPS is composed of 3 distinct regions, the O-antigen, the linking core oligosaccharide, and the lipid A tail\(^2\):

- **O-antigen** is the outermost domain of LPS, whose composition varies greatly amongst strains. LPS can be either a smooth (S) or rough (R) chemotype based on the presence or absence of the O-antigen, respectively\(^2\). There is clear evidence that they vary in their biological effects due to differing recognition by the TLR4 signaling co-receptors, for example, CD14 is required for S-LPS but not R-LPS signaling activation\(^3\).

- **Lipid A**, also known as *endotoxin*, is a carbohydrate lipid moiety anchoring LPS into the bacterial membrane and importantly defines the immune activation. The number of fatty acyl chains in lipid A causes a vast difference in the biological activity of LPS\(^1\). Hexa-acylated (6 chains) lipid A, found commonly on pathogenic bacteria such as *Escherichia coli* and *Salmonella* spp., is a highly potent agonist of TLR4. Under-acylated (4-5 chains) lipid A from bacteria such as *Porphyromonas gingivalis* and *Rhodobacter sphaeroides* (4-5 chains) lipid A from bacteria such as *Rhodobacter sphaeroides* and *Porphyromonas gingivalis* generally induces a lower or antagonistic immune response. This is due to the structural differences in lipid A inhibiting the necessary TLR4 dimerization for strong pro-inflammatory signaling\(^2\).

Are there really benefits to using LPS?

A number of beneficial LPS applications are currently being explored. Under-acylated lipid A outcompetes the potent hexa-acylated lipid A in a dose-dependent manner, making it a promising antagonistic treatment for bacterial-induced septic shock, although minimal clinical success has been reported to date\(^2\). LPS features many characteristics needed for an effective vaccine adjuvant. However, LPS is extremely toxic and can cause devastating diseases. Monophosphorylated lipid A (MPLA), a natural LPS derivative, displays lower toxicity while maintaining TLR4 immunostimulatory activity. A chemical derivative (MPL\(^3\)) of *Salmonella minnesota* MPLA is the first TLR ligand used in clinics in the human vaccine adjuvant AS04, and more recently in AS01\(^5\). Building from this, there are continuing efforts in the making of lipid A-based adjuvants.

Are sterile reagents LPS-free?

Bacteria are everywhere in the environment, and so is LPS. Contamination by LPS is a major threat for both research, with the misinterpretation of results, and industry, in the production of injectable drugs for use in both humans and animals. Reagents and products that are considered ‘sterile’, even after methods such as filtration, may still contain endotoxin, and thus its accurate detection is crucial. [https://www.invivogen.com/hek-blue-lps-detection-kit](https://www.invivogen.com/hek-blue-lps-detection-kit)

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>ORGANISM</th>
<th>QTY</th>
<th>CAT. CODE</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR4 agonists</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS-B5 UP(^*) (S)</td>
<td><em>E. coli</em> O55:B5</td>
<td>5 mg</td>
<td>tlrl-pb5lps</td>
</tr>
<tr>
<td>LPS-EK UP(^*) (R)</td>
<td><em>E. coli</em> K12</td>
<td>1 mg</td>
<td>tlrl-peklps</td>
</tr>
<tr>
<td>LPS-PG UP(^*) (P)</td>
<td><em>P. gingivalis</em></td>
<td>5 mg</td>
<td>tlrl-ppglps</td>
</tr>
<tr>
<td>MPLA-SM (R)</td>
<td><em>S. minnesota</em></td>
<td>1 mg</td>
<td>tlrl-mpla</td>
</tr>
<tr>
<td>CRX-527</td>
<td>Synthetic</td>
<td>1 mg</td>
<td>tlrl-crx527</td>
</tr>
<tr>
<td>TLR4 antagonist</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS-RS UP(^*) (S)</td>
<td><em>R. sphaeroides</em></td>
<td>1 mg</td>
<td>tlrl-prslps</td>
</tr>
</tbody>
</table>

\(^*\)UP - Ultrapure

[www.invivogen.com/tlr4-ligands](http://www.invivogen.com/tlr4-ligands)

---

**References**