InvivoGen insight

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NLRP3: a sophisticated drug target

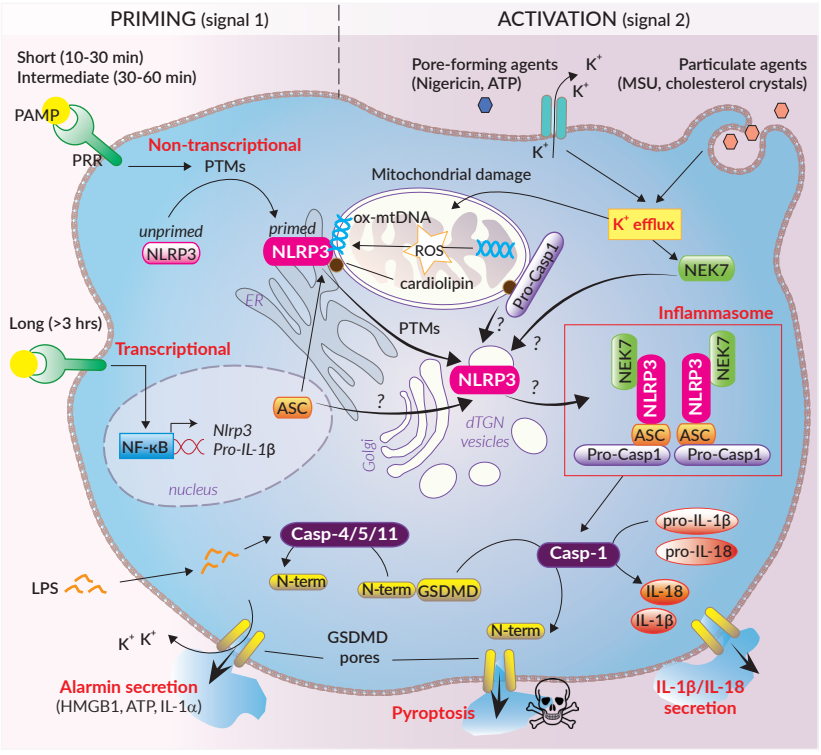
NLRP3 (NOD-like receptor pyrin domain-containing protein 3, cryopyrin or NALP3) is the best described inflammasome sensor and an attractive drug target. NLRP3 assembles into a multiprotein inflammasome complex to induce the secretion of IL-1β/IL-18 and pyroptosis in response to infections and cellular damage[1,2]. However, NLRP3 inflammasome functions can also be detrimental to the host, as its abberant 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)[3]. 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 and promotes NLRP3 activation and inflammasome assembly. This model is much more sophisticated than previously thought. Indeed, different priming mechanisms that depend on the stimulus' nature and duration have been described. Short and intermediate priming triggers post-translational modifications (PTMs) such as phosphorylation and (de-)ubiquitylation of NLRP3 expressed at basal level. Longer priming triggers NF-κB-mediated transcription of NLRP3, pro-caspase-1, and pro-IL-1β. Primed NLRP3 remains in an auto-repressed state until activation[2,4]. 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 mitochodrial (mt) cardiolipin and oxidized mtDNA, the two latter being proposed as NLRP3 ligands[2-5]. Yet, the exact mechanisms underlying NRLP3 activation remain a controversial topic[4,6].

Two major NLRP3 inflammasome responses, canonical and non-canonical, have been described and differ in their caspase (Casp) recruitment[1,2,6]. 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/pro-IL-18, and cleavage of the pore-forming gasdermin D (GSDMD), leading to secretion of IL-1β/-18 and pyroptosis[1,6]. The non-canonical response relies on the activation of Casp-4/5 (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[1,6].

Organelles play a key role in NLRP3 inflammasome assembly[2,4-6]. 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 occurs at mitochondria-associated ER membranes (MAMs), a recent report indicates that activation and inflammasome assembly rather occur around dispersed trans-Gogli network (dTGN) vesicles, without mitochondrial trafficking[7]. 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[2,4,6]. Research on the nature, timing and location of regulatory PTMs will help in identifying new targets for fine-tuned therapeutics with minimal side-effect to replace existing NLRP3 inhibitors and IL-1β/-18 blockade strategies[3,4].



1. Broz P. and Dixit V.M., 2016. Inflammasomes: mechanism of assembly, regulation and signalling. Nat Rev Immunol. 16:407. **2. Groslambert M. & Py B. 2018.** Spotlight on the NLRP3 inflammasome pathway. J. Inflamm. Res. 11:359. **3. Mangan M.S.J. *et al.*, 2018.** Targeting the NLRP3 inflammasome in inflammatory diseases. Nat. Rev. Drug. Discov. 17:558. **4. Swanson K.V. *et al.*, 2019.** The NLRP3 inflammasome: molecular activation and regulation to therapeutics. Nat. Rev. Immunol. 19:477. **5. Hamilton C. & Anand P.K. 2019.** Right place, right time: localisation and assembly of the NLRP3 inflammasome. F1000Research. 8:676. **6. Yang Y., *et al.*, 2019.** Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors. Cell Death Dis. 10:128. **7. Chen J. & Chen Z.J., 2018.** PtdIns4P on dispersed trans-Golgi network mediates NLRP3 inflammasome activation. Nature. 564:71.

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-1[1].

THP1-KO-NLRP3, -KO-ASC, and -KO-CASP4 cells feature a knockout (KO) of the *NLRP3*, *ASC*, and *CASP-4* gene, respectively. 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.**

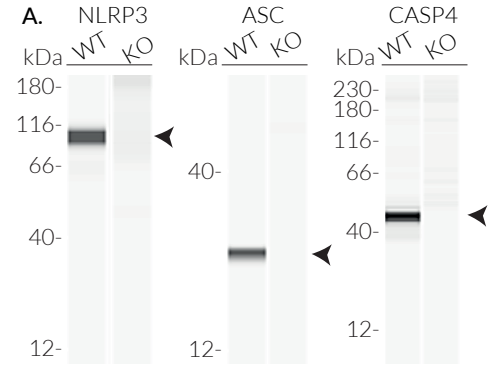
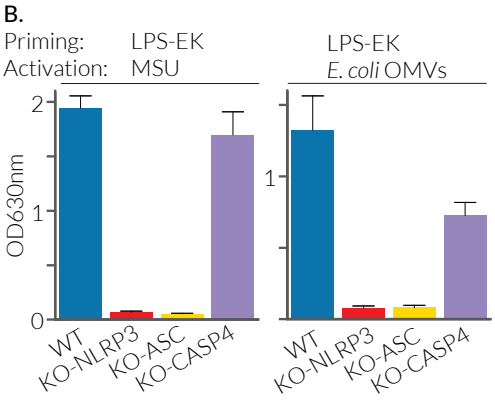


Figure 1: Validation of THP1-KO-NLRP3, -KO-ASC, and -KO-CASP4 cells.

(A) Western-Blot by WES™. 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) for 3 h at 37°C, before the addition of MSU crystals (250 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™ IL-1β sensor cells which express an NF-κB SEAP reporter gene. QUANTI-Blue™ Solution was used to measure SEAP activity. Optical density was read at 630 nm.

1. Schmid-Burgk, J.L. *et al.* 2015. Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. Eur. J. Immunol. 45:2911.

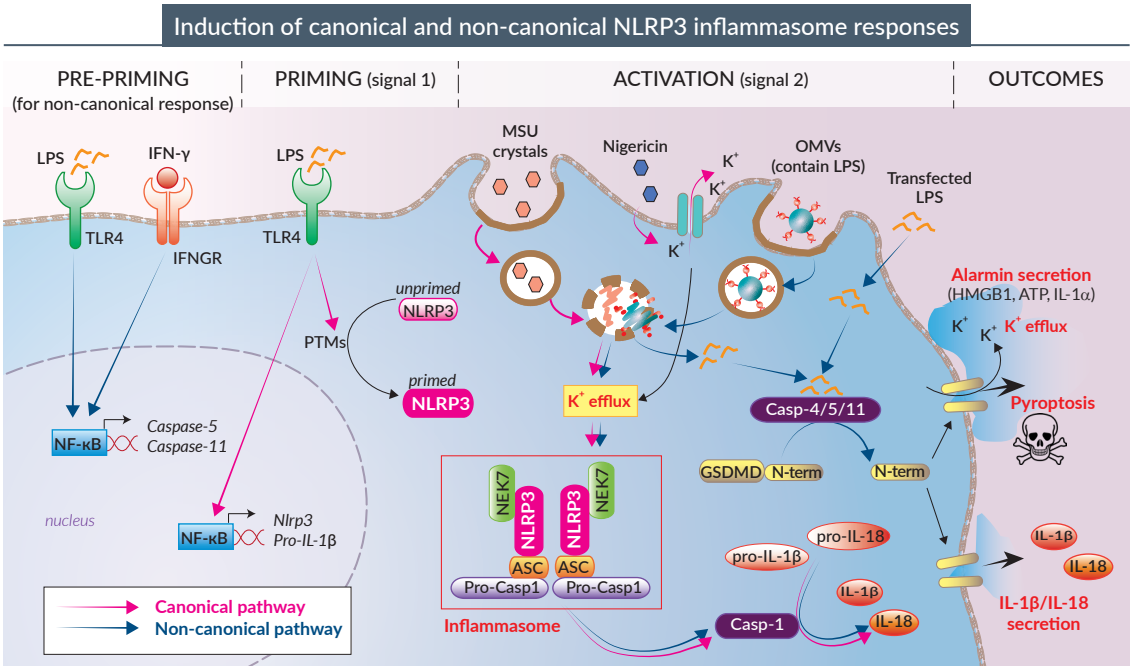


Figure 2: Canonical and non-canonical NLRP3 inflammasome pathways require a priming step (signal 1) inducing post-translational modifications (PTMs) of pre-existing cytosolic NLRP3, and *de novo* NLRP3 expression. Canonical NLRP3 activation is triggered by stress signals downstream of pore-forming toxins, or lysosomal rupture. These stress signals include cytosolic ion imbalances such as decreased K^+ concentration after K^+ efflux. Non-canonical NLRP3 activation relies on the sensing of intracellular LPS by Casp-4/5 (human) and Casp-11 (mice) which trigger GSDMD pore formation at the plasma membrane. The resulting K^+ efflux and ATP release activate the NLRP3 inflammasome. Of note, a pre-priming step is required to induce the expression of Casp-5 and Casp-11. Casp-4 is constitutively expressed in many cell types[1].

1. Man S.M & Kanneganti T.D. 2015. Converging roles of caspases in inflammasome activation, cell death and innate immunity. Nat. Rev. Immunol. 16:7.

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 macophage RAW 264.7 cell line which does not express ASC[1]. To allow the study of ASC-dependent inflammasomes[2] 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 blasticidin.

As expected, activation of the canonical and non-canonical NLRP3 inflammasomes by nigericin and *E. coli* OMVs, respectively, abrogates mature IL-1β secretion in RAW-ASC-KO-GSDMD cells when compared to RAW-ASC cells. Of note, nigericin is cytotoxic, and damaged/necrotic RAW-ASC-KO-GSDMD cells release some IL-1β.

1. Pelegrin P. *et al.* 2008. P2X7 receptor differentially couples to distinct release pathways for IL-1β in mouse macrophage. J. Immunol. 180:7147. **2. Hoss F. *et al.*, 2017.** Assembly and regulation of ASC specks. Cell. Mol. Life Sci. 74:1211.

PRODUCT	QUANTITY	CAT. CODE
THP1-KO-NLRP3 Cells	3-7 x 10^6 cells	thp-konlrp3
THP1-KO-ASC Cells	3-7 x 10^6 cells	thp-koasc
THP1-KO-CASP4 Cells	3-7 x 10^6 cells	thp-kocasp4
RAW-ASC Cells	3-7 x 10^6 cells	raw-asc
RAW-ASC-KO-GSDMD Cells	3-7 x 10^6 cells	raw-kogsdmd

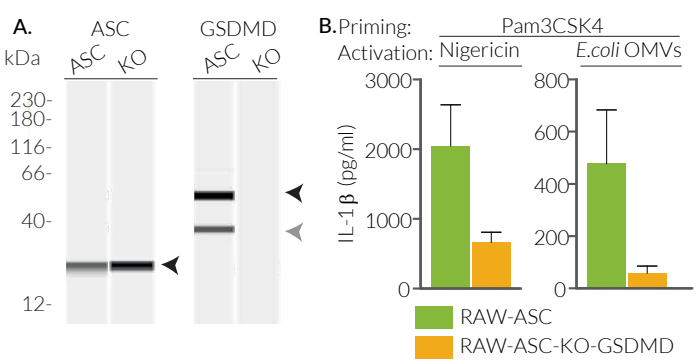


Figure 3: Validation of the RAW-ASC and RAW-ASC-KO-GSDMD cells.

(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

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

www.invivogen.com/inflammasomes

E. coli outer membrane vesicles (OMVs) NEW

Caspase 11-4/5 inflammasome activator

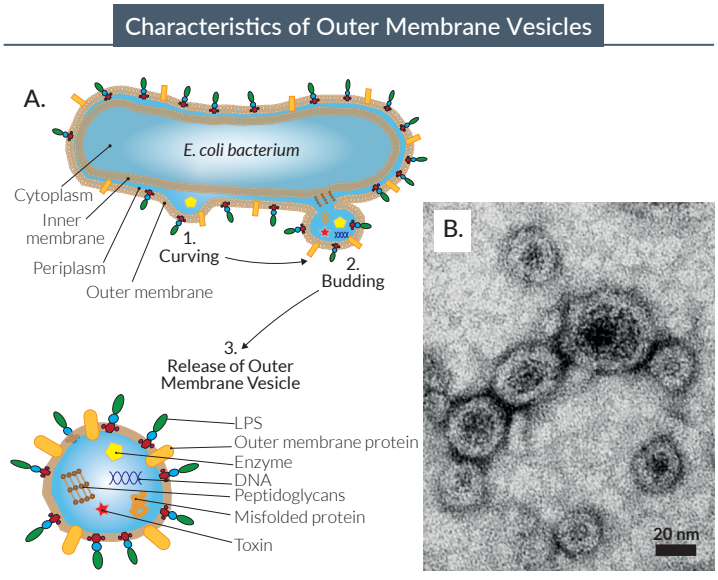
- **Cost effective:** Stronger activation potency than transfected LPS
- **Reliable:** Reproducible results

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)[1], and intracellular activation of the caspase 11-4/5 inflammasomes[2]. 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[2].

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).

1. Kaparakis-Liaskos, M. & Ferrero, R.L., 2015. Immune modulation by bacterial outer membrane vesicles. Nat. Rev. Immunol. 15:375. **2. Russo, A.J. *et al*, 2018.** Emerging insights into non-canonical inflammasome recognition of microbes. J. Mol. Biol. 430:207.

PRODUCT	QUANTITY	CAT. CODE
E. coli OMVs	100 µg	tlrl-omv-1
E. coli OMVs InvivoFit™	500 µg	tlrl-omv



(A) OMVs derive from the membrane of the parent bacterium and encapsulate a number of PAMPs including DNA, RNA, proteins, toxins, and LPS.
(B) Image of InvivoGen's *E. coli* OMVs by transmission electron microscope (80 kV).

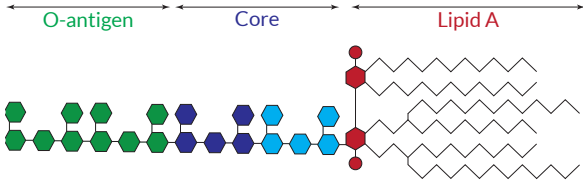
www.invivogen.com/ecoli-omvs

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].



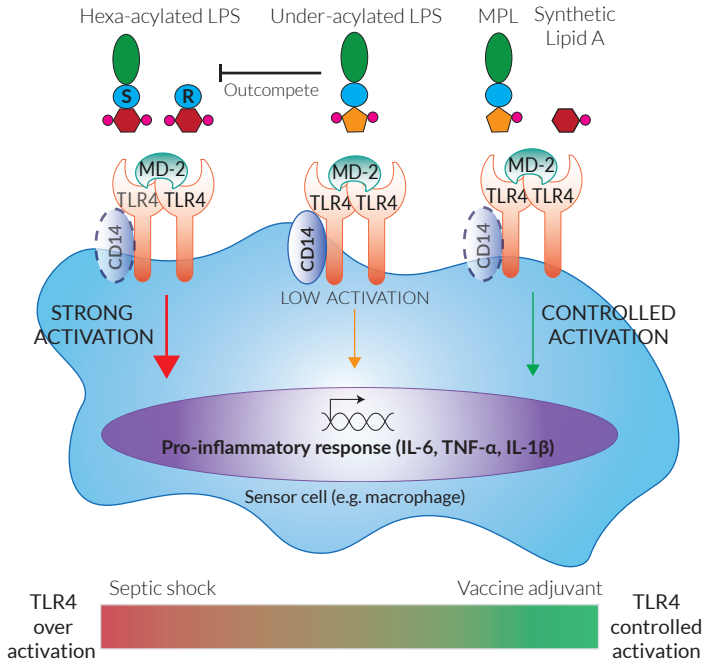
- **O-antigen** is the outermost domain of LPS, whose composition varies greatly amongst strains. LPS can be either a smooth (S) or rough (R) chemotype based on the presence or absence of the O-antigen, respectively[2]. There is clear evidence that they vary in their biological effects due to differing 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* generally induces a lower or antagonistic response. This is due to the structural differences in lipid A inhibiting the neccessary 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[4]. 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®) 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.

Balanced activation of TLR4 by different LPS types



1. Steimle, A. *et al.* 2016. Structure and function: Lipid A modifications in commensals and pathogens. Int J Med Microbiol 306, 290-301. **2. Cochet, F. & Peri, F. 2017.** The Role of Carbohydrates in the Lipopolysaccharide (LPS)/Toll-Like Receptor 4 (TLR4) Signalling. Int J Mol Sci 18. **3. Biedron, R. *et al.* 2016.** CD36 Differently Regulates Macrophage Responses to Smooth and Rough Lipopolysaccharide. PLoS One 11, e0153558. **4. Nijland, R. *et al.* 2014.** Recognition of LPS by TLR4: potential for anti-inflammatory therapies. Mar Drugs 12, 4260-4273. **5. Del Giudice, G. *et al.* 2018.** Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. Semin Immunol 39, 14-21.

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**

PRODUCT	ORGANISM	QTY	CAT. CODE
TLR4 agonists			
LPS-B5 UP* (S)	*E. coli* 055/B5	5 mg	tlrl-pb5lps
LPS-EK UP* (R)	*E. coli* K12	1 mg	tlrl-peklps
LPS-PG UP* (S)	*P. gingivalis*	5 mg	tlrl-ppglps
MPLA-SM (R)	*S. minnesota*	1 mg	tlrl-mpla
MPLA Synthetic	Synthetic	1 mg	tlrl-mpls
CRX-527	Synthetic	1 mg	tlrl-crx527
TLR4 antagonist			
LPS-RS UP* (S)	*R. sphaeroides*	1 mg	tlrl-prslps

**UP - Ultrapure*

www.invivogen.com/tlr4-ligands

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