InvivoGen Insight

InvivoGen is dedicated to develop innovative tools to help scientists unravel the complex pathways involved in innate immunity in order to generate new effective therapeutic drugs. This newsletter features products that should prove useful to study the activation of TLR4, the LPS receptor, or Nod1/Nod2, intracellular receptors for PGN. These products comprise a new set of TLR4-reporter cells derived from murine embryonic fibroblasts or HEK293 cells, novel TLR4 agonist and antagonist molecules and the first commercially available Nod1-specific ligands. Furthermore, InvivoGen is happy to offer to its customer a substantial savings on its high quality antibiotics for gene selection in mammalian cells.

Inside this issue:

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Agonistic and Antagonistic Effects of LPS on TLR4

Bacterial lipopolysaccharide (LPS) is the major structural component of the outer wall of all Gram-negative bacteria and a potent activator of the immune system. LPS can lead to pathological reactions such as the induction of septic shock. LPS is recognized by Toll-like receptor 4 (TLR4) which interacts with three different extracellular proteins: LPS binding protein (LBP), CD14 and, myeloid differentiation protein 2 (MD-2), to induce a signaling cascade leading to the activation of NF-κB and the production of proinflammatory cytokines.

LPS consists of a polysaccharide region that is anchored in the outer bacterial membrane by a specific carbohydrate lipid moiety termed lipid A. Lipid A, also known as endotoxin, is responsible for the immunostimulatory activity of LPS. Lipid A is a glucosamine disaccharide linked to hydroxy fatty acids that are further substituted by nonhydroxylated fatty acids. The number of fatty acids is a major determinant of the immunogenicity of endotoxin. The most active form of lipid A contains six fatty acyl groups and is found in pathogenic bacteria such as *Escherichia coli* and *Salmonella* species. Underacylated lipid A structures, containing four or five fatty acids, induce markedly less host defense responses and can inhibit in a dose-dependent manner the strong endotoxic response triggered by hexa-acylated LPS. Such antagonist LPS have been isolated from *Rhodobacter sphaeroides*, *Porphyromonas gingivalis* and an *E. coli* strain bearing a mutation in the *msbB* gene[1].

LPS antagonists have received significant attention as potential therapeutic agents to treat septic shock long before their antagonist mechanism was known. According to the current model, LPS is delivered to CD14 by LBP and transferred to MD-2 to form a monomeric endotoxin:MD-2 complex that binds and activates TLR4[2, 3]. TLR4 activation can occur without LPB and CD14 but requires several orders of magnitude more endotoxin. Canonical lipid A binds MD-2 and induces conformational changes that trigger TLR4 oligomerization and signaling. Underacylated lipid A seem to utilize at least two distinct mechanisms to block LPS-dependent activation of TLR4. The main mechanism consists of direct competition between under-acylated LPS and hexa-acylated LPS for the same binding site on MD-2, while the secondary mechanism involves the ability of under-acylated LPS:MD-2 complexes to inhibit hexa-acylated endotoxin:MD-2 complexes function at TLR4[1-4].

Understanding the molecular mechanism of LPS-induced TLR4 activation is key for the development of therapeutic lipid A antagonists. HEK293 cells transfected to stably express TLR4, MD-2 and CD14 is the model of choice to study TLR4 activation. InvivoGen provides such cell line with or without a convenient reporter system to monitor TLR4-induced NF-κB activation, as well as a large collection of TLR4 agonists and antagonists.

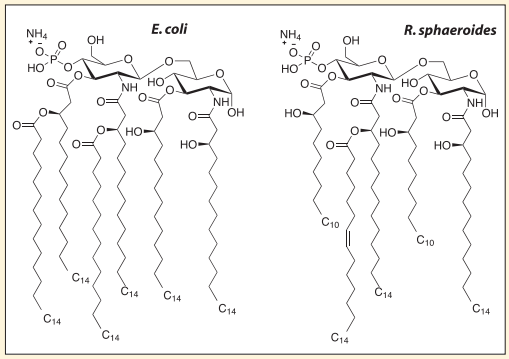


Figure 1: Structures of agonist hexa-acylated lipid A from *E. coli* and antagonist penta-acylated lipid A from *R. sphaeroides*

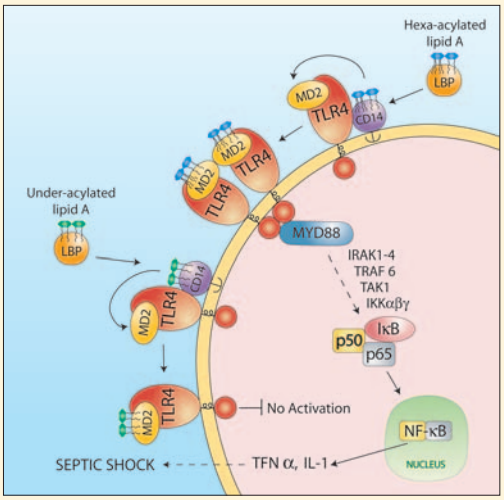


Figure 2: Agonist and antagonist activities of lipid A. Hexa-acylated but not under-acylated lipid A bound to MD-2 induce oligomerization of TLR4 leading to the production of inflammatory cytokines.

1. Coats SR. *et al.*, 2005. MD-2 mediates the ability of tetra-acylated and penta-acylated lipopolysaccharides to antagonize Escherichia coli lipopolysaccharide at the TLR4 signaling complex. J Immunol.;175(7):4490-8.
2. Teghanemt A. *et al.*, 2005. Molecular basis of reduced potency of underacylated endotoxins. J Immunol. 175(7):4669-76.
3. Visintin A. *et al.*, 2005. Pharmacological inhibition of endotoxin responses is achieved by targeting the TLR4 coreceptor, MD-2. J Immunol. 175(10):6465-72.
4. Saitoh S. *et al.*, 2004. Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. Int Immunol. 16(7):961-9.

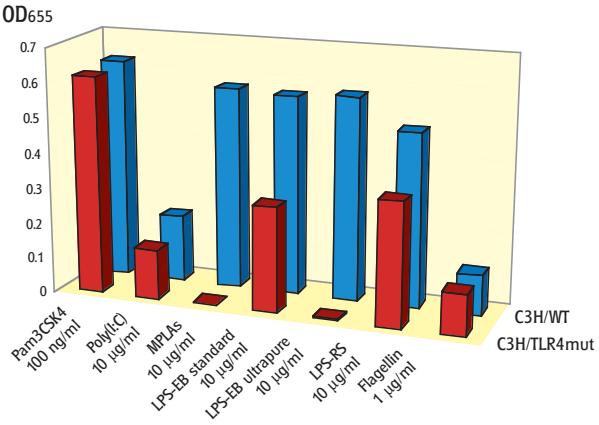
TLR4 Reporter Cell Lines NEW

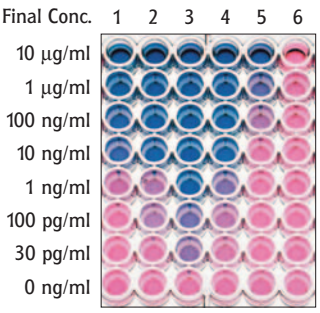
C3H/TLR4mut & C3H/WT - MEF Cell Lines

Primary murine embryonic fibroblasts (MEFs) have been the model of choice for numerous TLR studies. InvivoGen now provides immortalized MEFs isolated from C3H/HeJ (TLR4-deficient, C3H/TLR4mut) and C3H/HeN (wild-type, C3H/WT) mice. C3H/TLR4mut and C3H/WT MEF cell lines were established by stably transfecting the SV40 large antigen. Furthermore, these MEFs express an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) that allows to monitor in a simple and convenient manner the activation of NF-κB.

C3H/WT MEFs express high levels of TLR2 and TLR4 and low levels of TLR3 and TLR5. The presence of TLR2, TLR3, TLR4, or TLR5 agonists triggers a signaling cascade in C3H/WT MEFs leading to the activation of NF-κB and the subsequent induction of SEAP. The amount of SEAP secreted in the supernatant can be readily detected when using QUANTI-Blue™, a SEAP detection medium. In C3H/TLR4mut MEFs, TLR4 agonists do not induce the activation of NF-κb and the production of SEAP in contrast to TLR2, TLR3 and TLR5 ligands. Thus these two cell lines provide a useful tool to determine whether a given compound is a specific TLR4 agonist. They can be used to assess the purity of a given LPS by detecting the presence of contaminants that stimulate TLR2 (see graph, LPS-EB standard versus LPS-EB ultrapure).

C3H/TLR4mut and C3H/WT MEF cell lines are grown in DMEM medium with 10% FBS supplemented with 10 µg/ml blasticidin. Each vial contains 3-5 x 10^6 cells and is supplied with 1 mg blasticidin. Cells are shipped on dry ice.





Detection of TLR4 agonist LPS using HEK-Blue™-4 Cells

1 - MPLAs
2 - LPS-EB (*E. coli 0111:B4*)
3 - LPS-EK (*E. coli K12*)
4 - LPS-SM (*S. minnesota*)
5 - LPS (*P gingivalis*)
6 - LPS-RS (*R. sphaeroides*)

HEK-Blue™-4 Cells - TLR4-Expressing HEK293 Cells

HEK-Blue™-4 cells are designed for studying the stimulation of human TLR4 by monitoring the activation of NF-κB. HEK-Blue™-4 cells were obtained by co-transfection of the TLR4, MD2 and CD14 genes and an optimized SEAP reporter gene placed under the control of an NF-κB-inducible promoter into HEK293 cells.
HEK-Blue™-4 cells are highly sensitive to hexa-acylated LPS. They detect as low as 30 pg/ml LPS. LPS-induced TLR4 activation is readily detected by using QUANTI-Blue™ which turns blue in the presence of SEAP.

HEK-Blue™-4 cells are grown in DMEM medium with 10% FBS supplemented with 1X HEK-Blue™ Selection, an antibiotic mix. Each vial contains 3-5 x 10^6 cells and is supplied with 1 vial of 250X HEK-Blue™ Selection. Cells are shipped on dry ice.

Product	Quantity	Catalog Code
C3H/TLR4mut MEF	3-5 x 10^6 cells	mef-c3h4m
C3H/WT MEF	3-5 x 10^6 cells	mef-c3hwt
HEK-Blue™-4 Cells	3-5 x 10^6 cells	hb4-cells
QUANTI-Blue™	5 x 100 ml	rep-qb1

New TLR4 Ligands

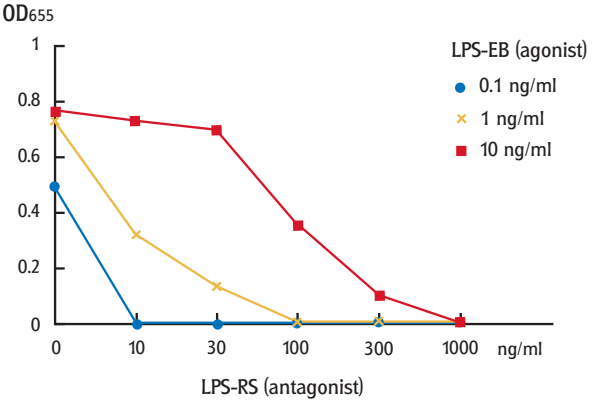
LPS from *R. sphaeroides* - TLR4 Antagonist

LPS from the photosynthetic bacterium *Rhodobacter sphaeroides* (LPS-RS) is a potent antagonist of LPS from pathogenic bacteria. Complete competitive inhibition of LPS activity is possible at a 100 fold excess of the antagonist (see graph). LPS-RS does not induce TLR4 signaling but is detected by the LAL assay, the standard endotoxin detection assay.

Synthetic Lipid A from *E. coli* - TLR4 Agonist

Synthetic lipid A from *E. coli* (MPLAs) is a monophosphoryl lipid A with 6 fatty acyl groups. It is structurally very similar to natural MPLA except that natural MPLA contains a mixture of 5, 6, and 7 acyl Lipid A. *E. coli* synthetic LPS activates TLR4 but does not activate TLR2 even at high concentrations reflecting its high purity.

Product	Working Conc.	Quantity	Catalog Code
LPS-RS	10 ng - 10 µg/ml	5 mg	tlrl-rslps
MPLAs (synthetic)	10 ng - 10 µg/ml	500 µg	tlrl-mpls
LPS-EB (0111:B4)	10 ng - 10 µg/ml	5 mg	tlrl-pelps



Antagonistic effect of LPS-RS on LPS-stimulated TLR4: HEK-Blue™-4 cells were incubated with increasing concentrations of LPS-EB (*E. coli* 0111:B4) and LPS-RS. After 24h incubation, TLR4 inhibition was assessed by measuring the levels of SEAP secreted in the supernatant by using QUANTI-Blue™, which turns blue in the presence of SEAP.

	TLR2	TLR4	LAL Assay
LPS-RS	+	-	+
MPLAs (synthetic)	-	+	+
LPS-EB (0111:B4)	-	+	+

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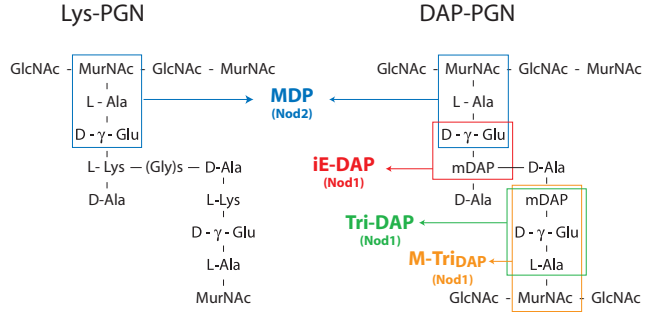
Nod1 and Nod2

Intracellular Receptors for Peptidoglycan-related Molecules

Peptidoglycan (PGN) is an essential constituent of the bacterial cell wall responsible for its rigidity and structure. In Gram-positive bacteria, PGN is embedded in a relatively thick cell wall and usually associated with lipoproteins and lipoteichoic acids, whereas in Gram-negative bacteria, it is present as a thin layer overlaid by a thick layer of LPS. PGN consists of glycan chains of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) that are cross-linked to each other by short peptides with alternating L- and D-amino acids. The nature of the third amino acid of these short peptides differs in Gram-positive and Gram-negative PGNs. In Gram-positive bacteria, this residue is commonly an L-lysine, and in Gram-negative bacteria it is primarily a meso-diaminopimelic acid (mDAP).

PGN is known to be a potent activator of NF-κB and was thought initially to be recognized by TLR2. However, a recent study demonstrated that TLR2 is not a sensor for PGN but is activated by the lipoproteins and lipoteichoic acids commonly present in many PGN preparations[1]. Other pattern recognition molecules are involved in PGN recognition, in particular Nod1 (CARD4) and Nod2 (CARD15), two members of the growing family of Nod-like receptors (NLRs, also known as CATERPILLER), characterized by a nucleotide-oligomerization domain (NOD) and ligand-recognizing leucine-rich repeats. Nod1 and Nod2 are intracellular sensors that detect specific motifs within the PGN. Nod1 is expressed in multiple tissues, including intestinal epithelia while Nod2 expression is restricted to immune cells and intestinal epithelial cells. Nod1 senses the iE-DAP dipeptide which is found in PGN of all Gram-negative and certain Gram-positive bacteria whereas Nod2 recognizes the muramyl dipeptide (MDP) structure found in almost all bacteria. Thus Nod2 acts as a general sensor of PGN and Nod1 is involved in the recognition of a specific subset of bacteria. Both Nod1 and Nod2 signal via the serine/threonine RIP2 (RICK, CARDIAK) kinase which interacts with IKK leading to the activation of NF-κB and the production of inflammatory cytokines such as TNFα and IL-6[2].

Studies of Nod1 have been hampered by the high dose of iE-DAP required for its activation. As Nod1 is located in the cytosol, Masumoto *et al.* suggested that hydrophobic acylation of iE-DAP may improve its membrane permeability and ability to stimulate Nod1[3]. As expected, addition of an acyl residue to the NH_2 terminus of iE-DAP increased the ability to stimulate Nod1 by improving transfer into the host cell. InvivoGen offers a new selection of known Nod1 ligands that have been chemically synthesized and tested in HEK293 overexpressing Nod1 (see below).



Schematic structure of Lys-PGN (found in Gram-positive bacteria) and DAP-PGN (found in Gram-negative bacteria).

1. Travassos LH. *et al.*, 2004. Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition. EMBO Rep. 5(10):1000-6.
2. Park JH. et *al.*, 2007. RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. J Immunol. 178(4):2380-6.
3. Masumoto J. *et al.*, 2006. Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment in vivo. J Exp Med. 203(1):203-13.

Nod1 Ligands NEW

• iE-DAP

D-γ-Glu-mDAP (iE-DAP) is a dipeptide present in the PGN of a subset of bacteria that include Gram-negative bacilli and particular Gram-positive bacteria such as *Bacillus subtilis* and *Listeria monocytogenes*[1]. iE-DAP is the minimal motif recognized by Nod1.

• Tri-DAP

L-Ala-D-γ-Glu-mDAP (Tri-DAP) comprises the iE-DAP dipeptide and an L-Ala residue. Similarly to iE-DAP, this tripeptide is specifically recognized by Nod1 but exhibits a ~3-fold higher ability to activate NF-κB than iE-DAP[2].

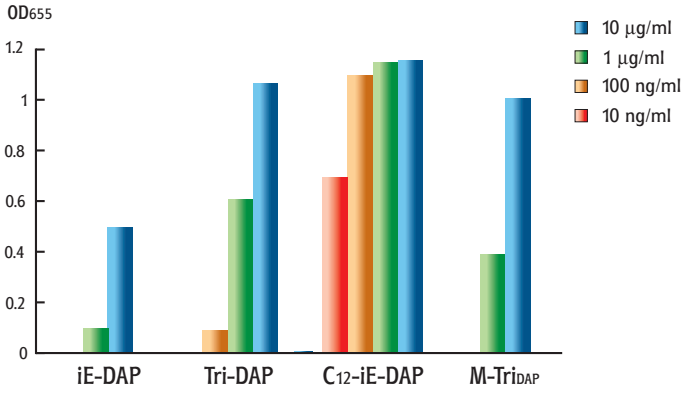
• C12-iE-DAP

C12-iE-DAP is an acylated derivative of iE-DAP. It was generated by addition of a lauroyl (C_{12}) group to the glutamic residue of iE-DAP. C12-iE-DAP stimulates specifically Nod1 at concentrations 100- to 1000-fold lower than the original iE-DAP.

• M-Tri$_{DAP}$

MurNAc-L-Ala-D-γ-Glu-mDAP (M-Tri$_{DAP}$), also called DAP-containing muramyl tripeptide is a PGN degradation product found mostly in Gram-negative bacteria. It is recognized by Nod1 and induces the activation of NF-κB at similar levels to Tri-DAP[2].

1. Chamaillard M. *et al.*, 2003. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat. Immunol.4(7):702-7
2. Girardin SE. *et al.*, 2003. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2.J Biol Chem. 278(43):41702-8.



Nod1 stimulation: HEK293 cells stably expressing Nod1 and an NF-κB-inducible SEAP reporter plasmid were incubated with iE-DAP, Tri-DAP ,C12-iE-DAP, and M-Tri$_{DAP}$ at various concentrations. After 24h incubation, Nod1 stimulation was assessed by measuring the levels of SEAP secreted in the supernatant by using HEK-Blue™ Detection medium, which turns blue in the presence of SEAP.

Product	Working Conc.	Quantity	Catalog Code
iE-DAP	1 - 100 µg/ml	5 mg	tlrl-dap
Tri-DAP	100 ng - 10 µg/ml	1 mg	tlrl-tdap
C12-iE-DAP	1 ng - 1 µg/ml	1 mg	tlrl-c12dap
M-Tri $_{DAP}$	1 - 100 µg/ml	5 mg	tlrl-mtd

Check our website for an extensive list of Nod2 ligands

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Product	Origin	Working Conc. in mammalian cells	Description	Quantity	Catalog Code
Blasticidin S	*Streptomyces griseochromogenes*	1 - 10 µg/ml	Solution @ 10 mg/ml Solution @ 10 mg/ml Powder	100 mg 500 mg 1 g	ant-bl-1 ant-bl-5 ant-bl-10p
G418	*Micromonospora rhodorangea*	400 - 1000 µg/ml	Solution @ 100 mg/ml Solution @ 100 mg/ml	1 g 5 g	ant-gn-1 ant-gn-5
Hygromycin B / HygroGold™	*Streptomyces hygroscopicus*	50 - 200 µg/ml	Solution @ 100 mg/ml Solution @ 100 mg/ml Powder	1 g 5 g 10 g	ant-hg-1 ant-hg-5 ant-hg-10p
Phleomycin	*Streptomyces verticillus*	10 µg/ml (yeast) 25 -150 µg/ml (filamentous fungi)	Solution @ 20 mg/ml Powder Powder	100 mg 250 mg 1 g	ant-ph-1 ant-ph-2p ant-ph-10p
Puromycin	*Streptomyces alboniger*	1 - 10 µg/ml	Solution @ 10 mg/ml Solution @ 10 mg/ml	100 mg 500 mg	ant-pr-1 ant-pr-5
Zeocin™	*Streptomyces CL990*	50 - 300 µg/ml	Solution @ 100 mg/ml Solution @ 100 mg/ml Powder	1 g 5 g 1 g	ant-zn-1 ant-zn-5 ant-zn-1p

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