

# InvivoGen Insight

An Insightful Look At InvivoGen's Innovative Products

As the number of publications on Toll-like Receptors (TLRs) steadily increases, so does InvivoGen's TLR product line.

TLRs play important roles in innate immunity by recognizing specific molecular patterns associated with microbial pathogens. Individual TLRs induce distinct cellular responses that involve different adaptor proteins. Five adaptors have been described so far including the recent **TRAM** and **SARM1** proteins. InvivoGen provides these five adaptor genes in the expression vector **pUNO**. pUNO plasmids feature genes involved in the TLR pathways such

as the TLR genes, genes involved in TLR signaling and collaborative genes known to interact in TLR-induced responses. This last family of genes contains four members: **Dectin1**, **NOD1 & 2**, and **DC-SIGN**. To complete the list of tools to study the TLRs, InvivoGen has added new **TLR ligands** as well as **293/TLR clones**, HEK293 cells stably transfected with a pUNO-TLR plasmid.

Finally, InvivoGen introduces **Primocin™**, a new antibiotic formulation, designed to protect primary cell lines from all cell culture contaminants.

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- The Antimicrobial Shield For Your Primary Cells

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## The Toll/IL-1 Receptor Adaptor Family

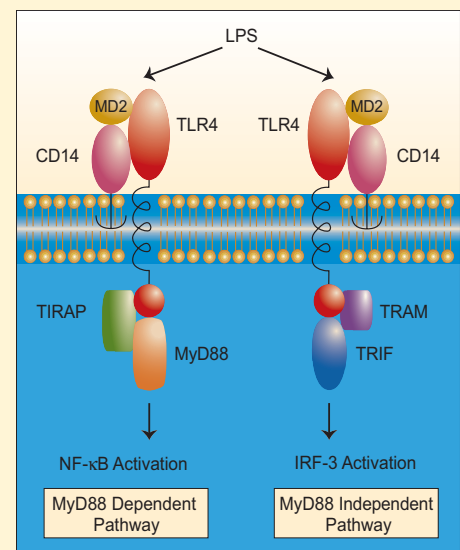
Recognition of pathogens by Toll-like receptors (TLRs) triggers innate immune responses through signaling pathways mediated by Toll/interleukin 1 receptor (TIR) domain containing adaptors. To date, four adaptor proteins have been well characterized, and have been named MyD88, TIRAP/Mal (TIR domain-containing adaptor protein, or MyD88 adaptor-like), TRIF/TICAM1 (TIR-domain-containing adaptor inducing interferon-beta, or TIR-containing adaptor molecule 1) and TRAM/TICAM2/TIRP (TRIF-related adaptor molecule, TIR-containing adaptor molecule 2, or TIR-containing protein). A fifth adaptor has been recently identified, termed SARM1 for sterile alpha motif (SAM) and Armadillo motif (ARM) domain-containing protein<sup>1</sup>. However its function in TLR signaling remains unclear. TLR signaling consists of at least two distinct pathways: a MyD88-dependent pathway that leads to the production of inflammatory cytokines, and a MyD88-independent pathway associated with the stimulation of IFN- $\beta$  and the maturation of dendritic cells. The MyD88-dependent pathway is common to all TLRs as MyD88-deficient mice are unable to produce inflammatory cytokines in response to all TLR ligands<sup>2</sup>. However, differences between signaling pathways induced by individual TLRs are emerging, with both TLR4 and TLR2 signaling requiring the adaptor TIRAP/Mal, which may account for the specificity of MyD88-dependent pathways<sup>3</sup>. Although cytokine production in response to the TLR4 ligand LPS is completely abolished in MyD88- or TIRAP-deficient mice, production of IFN- $\beta$  is still observed. This response is mediated mainly by the adaptor TRIF/TICAM-1 which also plays a critical role in TLR3 signaling<sup>4</sup>. TLR3 recognizes double-stranded RNA (dsRNA) and induces the production of IFN- $\beta$  through the MyD88-independent pathway. A dominant-negative form of TRIF inhibits TLR3-dependent activation of the IFN- $\beta$  promoter suggesting that TRIF is a key adaptor in the MyD88-independent pathway.

TRAM/TICAM-2 is another adaptor molecule involved in the MyD88-independent pathway that has been recently characterized<sup>5</sup>. In response to LPS stimulation, TRAM physically bridges TLR4 and TRIF transmitting

LPS-induced signaling to TRIF which in turn triggers the production of IFN- $\beta$ . TRAM function is restricted to the TLR4 pathway, as TRAM-deficient mice are defective in cytokine production in response to the TLR4 ligand LPS but not to other TLR ligands<sup>7</sup>.

The diagram below depicts the interactions between TLR4 and the different adaptors.

1. O'Neill LA. *et al.*, 2003. The Toll-IL-1 receptor adaptor family grows to five members. *Trends Immunol.* 24(6):286-90. Review.
2. Adachi O. *et al.*, 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity.* 9(1):143-50.
3. Homg T. *et al.*, 2002. The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. *Nature.* 420(6913):329-33.
4. Yamamoto M. *et al.*, 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adaptor that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol.* 169(12):6668-72. Sep 30 (Ahead of print).
5. Fitzgerald KA. *et al.*, 2003. LPS-TLR4 Signaling to IRF-3/7 and NF- $\kappa$ B Involves the Toll Adaptors TRAM and TRIF. *J Exp Med.* 198(7):1043-1055.
6. Oshiumi H. *et al.*, 2003. TICAM-2: a bridging adaptor recruiting to Toll-like receptor 4 TICAM-1 that induces interferon-beta. *J Biol Chem.* Sep 30 (Ahead of print).
7. Yamamoto M. *et al.*, 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol.* Oct 1 (Ahead of print).



# Toll-Like Receptors

Toll-like receptors (TLRs) mediate the recognition of a wide range of microbial products and induce innate immune responses that are tightly controlled. Regulation is achieved mainly by collaborations between TLRs and other receptors involved in pathogen recognition. To help in understanding the mechanisms underlying the interaction between receptors, InvivoGen provides a comprehensive and expanding TLR product line that includes TLR ligands, 293/TLR clones and TLR or TLR associated genes.

## TLR Ligands

**Flagellin** - Flagellin is the major component of the bacterial flagellar filament, which confers motility on a wide range of bacterial species. Flagellin is recognized by TLR5 and induces the activation of NF- $\kappa$ B leading to the production of cytokines and nitric oxide depending on the nature of the TLR5 signaling complex.

**LTA** - Lipoteichoic acid (LTA) is a cell wall component of Gram+ bacteria with potent immunostimulatory activity. LTA is recognized by TLR2 leading to the activation of NF- $\kappa$ B and the production of inflammatory cytokines. LTA preparations from *B. subtilis* also induce the activation of NF- $\kappa$ B through TLR5 suggesting that these preparations are contaminated by flagellin. InvivoGen provides purified LTA preparations that only stimulate TLR2.

**Pam3CSK4** is a synthetic tripalmitoylated lipopeptide that mimics the acylated amino terminus of bacterial lipoproteins. Pam3CSK4 is a potent activator of NF- $\kappa$ B and inflammatory cytokines, in a TLR2-dependent manner.

**Ultra-Pure LPS** - Most lipopolysaccharide (LPS) preparations on the market are contaminated by other bacterial components, such as lipoproteins, thus activating TLR2 signaling as well as TLR4 signaling. InvivoGen provides two new ultra-pure LPS from *E. coli* and *Salmonella*. These preparations only activate the TLR4 pathway as shown in the figure.

**Stimulatory ODNs** - To date, two types of CpG ODNs have been described: CpG-A, a potent inducer of IFN- $\alpha$  in plasmacytoid dendritic cells (PDC), and CpG-B, a weak inducer of IFN- $\alpha$  but a potent activator of B cells. Recently, a new type of CpG ODN has been identified, termed CpG-C, with both high induction of PDC and activation of B cells. The sequence of CpG-C combines elements of both CpG-A and CpG-B. The most potent sequence is called M362<sup>1</sup>.

**Inhibitory ODNs** - Recent studies suggest the existence of DNA sequences that can neutralize the stimulatory effect of CpG ODNs<sup>1</sup>. These sequences are characterized by three consecutive G downstream of a C or A, addition of a fourth G (G-tetrads) increases the inhibitory capability. The most potent inhibitory sequences are (TTAGGG)<sub>4</sub> found in mammalian telomeres<sup>2</sup> and ODN 2088 (TCCTGGCGGGGAAGT)<sup>3</sup>. Inhibitory ODNs act by disrupting the colocalization of CpG ODNs with TLR9 in endosomal vesicles without affecting cellular binding and uptake.

- Hartmann G. *et al.*, 2003. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN- $\alpha$  induction in plasmacytoid dendritic cells. *Eur J Immunol.* 33(6):1633-41.
- Gursel I. *et al.*, 2003. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. *J Immunol.* 171(3):1393-400.
- Stunz LL. *et al.*, 2002. Inhibitory oligonucleotides specifically block effects of stimulatory CpG oligonucleotides in B cells. *Eur J Immunol.* 32(5):1212-22.

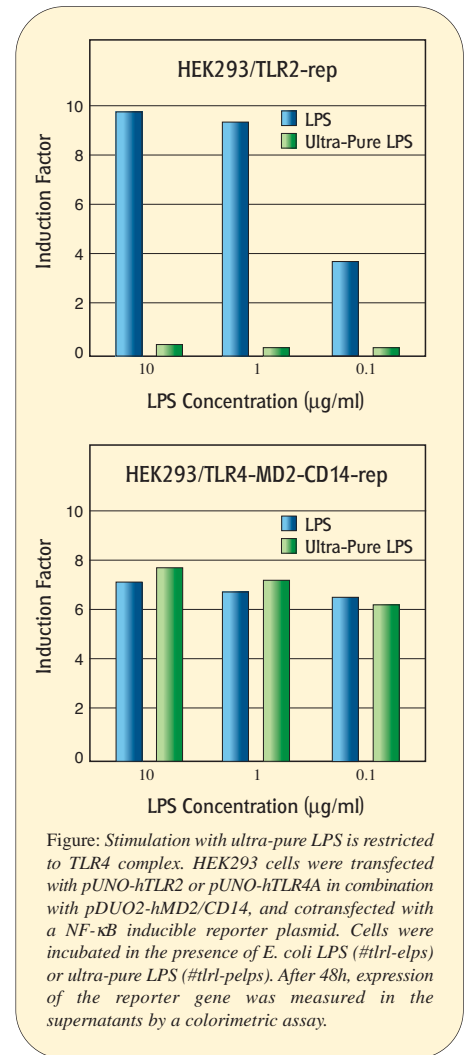


Figure: Stimulation with ultra-pure LPS is restricted to TLR4 complex. HEK293 cells were transfected with pUNO-hTLR2 or pUNO-hTLR4A in combination with pDUO2-hMD2/CD14, and cotransfected with a NF- $\kappa$ B inducible reporter plasmid. Cells were incubated in the presence of *E. coli* LPS (#tlrl-elps) or ultra-pure LPS (#tlrl-pelps). After 48h, expression of the reporter gene was measured in the supernatants by a colorimetric assay.

## 293/TLR Clones

293/TLR clones are HEK293 cells transfected with a pUNO-TLR or pDUO-TLR plasmid. Expression of the TLR gene(s) has been verified by Western Blot analysis when antibodies were available, or transient transfection assays with a NF- $\kappa$ B inducible reporter plasmid.

293/TLR clones are grown in standard DMEM medium with 10% FBS supplemented with blasticidin (10 µg/ml). Each vial of 293/TLR clone contains 1-5 x 10<sup>6</sup> cells.

TLR Specificity	Product	Origin/Description	Code	Quantity	Price
TLR2 ligands	Pam3CSK4	Synthetic	tlrl-pms	1 mg	\$/€ 80
	Lipoteichoic acid	<i>L. casei</i>	tlrl-pclta	5 mg	\$/€ 140
		<i>S. aureus</i>	tlrl-pslta	5 mg	\$/€ 140
		Peptidoglycan	<i>S. aureus</i>	tlrl-pgnsa	5 mg
		<i>B. subtilis</i>	tlrl-pgnbs	5 mg	\$/€ 90
TLR4 ligand	Ultra-Pure LPS	<i>E. coli</i> K12	tlrl-pgnec	1 mg	\$/€ 140
		<i>E. coli</i> 0111:B4	tlrl-pelps	5 mg	\$/€ 140
		<i>S. minnesota</i>	tlrl-smlps	5 mg	\$/€ 140
TLR5 ligand	Flagellin	<i>S. thyphimurium</i>	tlrl-stfla	100 µg	\$/€ 240
TLR9 ligands	ODN M362	Stimulatory ODN-C	tlrl-hodnc	100 µg	\$/€ 140
	ODN TTAGGG	Inhibitory ODN	tlrl-hinhodn	100 µg	\$/€ 140
	ODN 2088	Inhibitory ODN	tlrl-minhodn	100 µg	\$/€ 140

Product	Code	Price
293/hTLR2	293-htr2	\$/€ 700
293/hTLR2/6	293-htr2/6	\$/€ 800
293/hTLR3	293-htr3	\$/€ 700
293/hTLR4A	293-htr4a	\$/€ 700
293/hTLR5	293-htr5	\$/€ 700
293/hTLR7	293-htr7	\$/€ 700
293/hTLR8	293-htr8	\$/€ 700
293/hTLR9	293-htr9	\$/€ 700

## Collaborative Recognition of Pathogens

In addition to TLRs, invading pathogens are recognized by many other receptors. Signaling through some of these receptors has been shown to interact with TLR signaling potentiating or inhibiting the inflammatory response.

- **Dectin-1** is a C-type lectin that is expressed by DCs as a phagocytic receptor for  $\beta$ -glucan containing particles such as Zymosan, a cell wall preparation of *S. cerevisiae*. Dectin-1 recognizes Zymosan and induces phagocytosis and the production of reactive oxygen. Zymosan is also recognized by TLR2/TLR6 heterodimers which signaling mediates the activation of NF- $\kappa$ B and the production of cytokines. Recent data suggest that Dectin-1 and TLR2/TLR6 signaling combine to enhance the responses triggered by each receptor<sup>1</sup>.

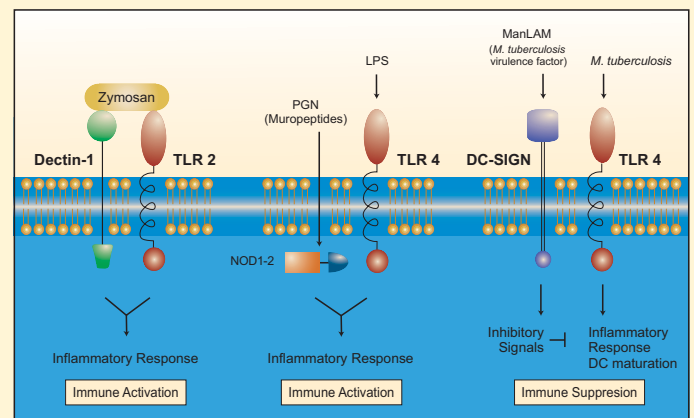
- **NOD1/CARD-4 and NOD2/CARD-15** are intracellular pattern-recognition proteins involved in bacterial detection. NOD1 is expressed ubiquitously, whereas the expression of NOD2 is restricted to monocytes. NOD1 and NOD2 recognize peptidoglycan (PGN) through the detection of distinct GlcNAc-MurNAc-peptides (known as muropeptides)<sup>2</sup>. NOD1 senses a unique diaminopimelate-containing GlcNAc-MurNAc-tripeptide muropeptide found mostly in Gram-negative bacterial PGNs, while NOD2 detects the muramyl dipeptide MurNAc-L Ala-D-isoGln (MDP), a motif found in all PGNs. Thus, NOD1 and NOD2 are likely to act as intracellular functional equivalents of TLRs. Their activation produces inflammatory responses independently from the TLR pathway that may contribute to amplify the TLR-induced signaling.

- **DC-SIGN** (DC-specific intercellular adhesion molecule-grabbing nonintegrin) is a C-type lectin expressed by immature DCs and involved in the capture of different pathogens. DC-SIGN recognizes distinct carbohydrate structures such as the viral envelope glycoproteins expressed by the HIV1, hepatitis C virus, and Ebola virus. Non-viral pathogens can also interact with DC-SIGN including yeast, parasites and bacteria, such as

*M. tuberculosis*<sup>5</sup>. Upon recognition, DC-SIGN internalizes the pathogens for processing and antigen presentation. The central feature of the pathogens recognized by DC-SIGN is that they misuse DC-SIGN by distinct mechanisms that either circumvent antigen processing or alter TLR-mediated signaling, skewing T-cell responses<sup>6</sup>.

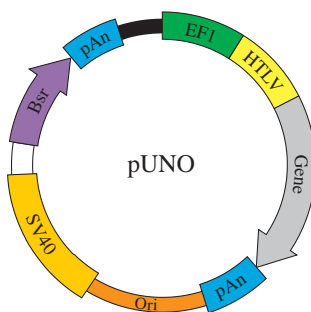
Determining how signaling pathways interact upon ligand stimulation should help understand the molecular mechanisms that govern the rich diversity of inflammatory responses.

1. Gantner BN. *et al.*, 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J.Exp. Med.* 197(9):1107-17.
2. Girardin SE. *et al.*, 2003. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J Biol Chem.* 278(43): 41702-8.
3. Yang S. *et al.*, 2001. Synergistic effect of muramyl dipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. *Infect Immun.* 69(4):2045-53.
5. Appelmek BJ. *et al.*, 2003. Cutting edge: carbohydrate profiling identifies new pathogens that interact with dendritic cell-specific ICAM-3-grabbing nonintegrin on dendritic cells. *J Immunol.* 170(4):1635-9.
6. Van Kooyk Y, Geijtenbeek TB. 2003. DC-SIGN: escape mechanism for pathogens. *Nat Rev Immunol.* 3(9):697-709.



## pUNO Plasmid Family

pUNO is a family of plasmids expressing TLR, TLR-related or TLR collaborative genes. pUNO plasmids are selectable with blasticidin in both *E. coli* and mammalian cells. Expression of the gene of interest is strong, constitutive and ubiquitous. Each gene is flanked by unique restriction sites to facilitate its subcloning into another vector.



### Gene Families Available in pUNO Plasmids

#### • TLR Genes

This family contains all TLR genes described, that is human TLR1-10 and murine TLR1-9.

#### • TLR-Related Genes

This family includes TLR co-receptor genes such as CD14 and MD2, genes involved in TLR signaling such as TRAF6 and RICK (that interacts with NOD1 and NOD2), and adaptor genes such as MyD88, TIRAP and the recently cloned TRAM and SARM1 genes.

#### • TLR Collaborative Genes

This new family features genes encoding receptors involved in pathogen recognition, known to collaborate in TLR-induced responses, such as Dectin-1 and NOD2.

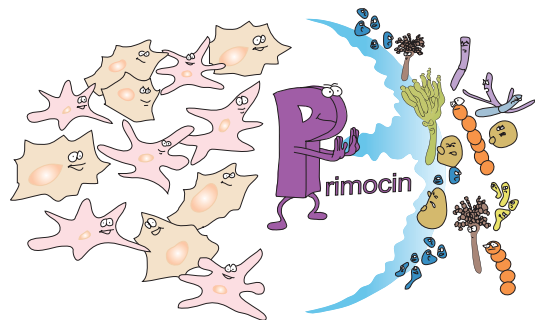
### TLR-Related Genes

Gene	Code (human)	Code (mouse)	Price
RICK	puno-hrick	puno-mrick	\$/€ 400
SARM1	puno-hsarm1a	puno-msarm1b	\$/€ 400
TRAF6	puno-htraf6		\$/€ 400
TRAM	puno-htram	puno-mtram	\$/€ 400

### TLR Collaborative Genes

Gene	Code (human)	Code (mouse)	Price
DC-SIGN1	puno-hdcsign1a	puno-mdcsign1a	\$/€ 400
Dectin-1	puno-hdectin1b		\$/€ 400
NOD1	puno-hnod1	puno-mnod1	\$/€ 400
NOD2	puno-hnod2		\$/€ 400





# Primocin™

## The Antimicrobial Shield For Your Primary Cells

Among cultured cell lines, primary cell lines require the highest care and attention. Primary cells derive from animal and human tissues and have not been genetically modified. They represent very valuable models for scientific experimentation in many fields of basic research and therapeutic development, as they allow to reproduce *in vivo* situations *in vitro*. However their use is

limited by their short lifespan and the high risk of microbial contamination that they face due to potential infection by contaminants present in the host organism or introduced during the dissection process. InvivoGen has developed Primocin™, a new antibiotic formulation specifically designed for primary cells that offers complete protection against microbial contaminants.

### Protection Against All Cell Culture Contaminants

**Primocin™** is a new antibiotic formulation specifically designed to protect primary cell lines from cell culture contaminations. Primocin™ is active against both Gram+ and Gram- bacteria, mycoplasma and fungi. Primocin™ is the first formulation to offer complete protection against microbial contaminants. There is no need to add Pen/Strep.

### Potent Antimicrobial Combination

**Primocin™** combines antibacterial and antifungal compounds. The antibacterial compounds eliminate mycoplasma and a wide range of bacteria by blocking DNA and protein synthesis. The antifungal agent eliminates fungi, yeasts and molds by disrupting the cell membrane.

### No Cytotoxicity

**Primocin™** is non-toxic to primary cells. It acts on targets found only in micro-organisms. Bacterial targets are the DNA gyrase and the prokaryotic ribosomal subunits, 30S and 50S. The fungal target is ergosterol, a molecule only found in the cell membrane of fungi and yeasts.

### Cell Culture Ready

**Primocin™** is a sterile water soluble solution that can be added directly to the cell culture medium. The solution is at 50 mg/ml and the recommended working concentration is 100 µg/ml. Primocin™ is provided as 1 ml vials or a 20 ml bottle. One 1 ml vial is enough to treat a 500 ml-bottle of medium.

Product	Quantity	Code	Price
Primocin™	500 mg (10 x 1 ml)	ant-pm-1	\$/€ 110
	1 g (1 x 20 ml)	ant-pm-2	\$/€ 175

### Primocin™ Antimicrobial Activity

MICROBIAL STRAINS	Primocin™	Pen/Strep AmphoB
<b>Gram+ bacteria</b>		
<i>Bacillus subtilis</i>	<0.8	<0.8
<i>Enterococcus faecalis</i>	6.25	12.5
<i>Staphylococcus aureus</i>	<0.8	<0.8
<i>Staphylococcus capitis</i>	<0.8	1.5
<b>Gram- bacteria</b>		
<i>Acinetobacter baumannii</i>	1.5	25
<i>Escherichia coli</i>	<0.8	6.25-200
<i>Enterobacter cloacae</i>	0.8	3.12-6.25
<i>Pseudomonas aeruginosa</i>	3.12	25-50
<i>Serratia marcescens</i>	<0.8	50
<i>Stenotrophomonas matophilia</i>	12.5	50-200
<b>Mycoplasma</b>		
<i>Acholeplasma laidlawii</i>	3.12	<1.5
<i>Mycoplasma arginini</i>	6.25	>800
<i>Mycoplasma fermentans</i>	1.5	100
<i>Mycoplasma hyorhinis</i>	1.5	200-800
<i>Mycoplasma orale</i>	25	>800
<b>Molds / Yeasts</b>		
<i>Candida albicans</i>	25	100
<i>Candida parapsilosis</i>	25	100-200
<i>Penicillium sp.</i>	25	100-200
<i>Saccharomyces cerevisiae</i>	12.5	100-200

Values are in µg/ml. They represent the minimal inhibitory concentrations of Primocin™ and Pen/Strep/Ampho B solutions to common cell culture contaminants.



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