InvivoGen Insight

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Viral dsRNA Signaling Through TLRs and RLRs

Viral infections induce a strong innate immune response characterized by the rapid production of type I interferons (IFN α/β) leading to the inhibition of virus replication. This antiviral response is initiated through the recognition of viral products, such as double-stranded RNA (dsRNA), by two types of pathogen recognition receptors (PRRs): the Toll-like receptors (TLRs) and the RIG-I-like receptors (RLRs). The TLR family consists of more than 10 members expressed on the cell surface membrane or endosomes. The RLRs is a family of cytoplasmic RNA helicases that includes RIG-I and MDA-5[1].

Double-stranded RNA, which is synthesized during the replication of many viruses, is recognized by TLR3 and RIG-I/MDA-5 in a cell-type- and pathogen-type-specific manner. Studies of RIG-I- and MDA-5-deficient mice have revealed that conventional dendritic cells (DCs), macrophages and fibroblasts isolated from these mice have impaired IFN induction after RNA virus infection, while production of IFN is still observed in plasmacytoid DCs (pDCs)[2]. The TLR system is required for pDCs to induce the antiviral response but is dispensable for cDCs, macrophages and fibroblasts. For these cell types, RLRs are critical to sense viruses.

RIG-I and MDA-5 contain a DExD/H box RNA helicase and two caspase recruiting domain (CARD)-like domains. The helicase domain interacts with dsRNA, whereas the CARD domains are required to relay the signal. Despite the overall structural similarity between these two sensors, they detect distinct viral species. RIG-I participates in the recognition of Paramyxoviruses (Newcastle disease virus (NDV), Sendai virus (SeV)), Rhabdoviruses (vesicular stomatitis virus (VSV)), Flaviviruses (hepatitis C (HCV)) and Orthomyxoviruses (Influenza), whereas MDA-5 is essential for the recognition of Picornaviruses (encephalomyocarditis virus (EMCV)) and poly(I:C), a synthetic analog of viral dsRNA[1]. Notably, RIG-I binds specifically to RNA containing 5'-triphosphate such as viral RNA and *in vitro*-transcribed long dsRNA[3]. Mammalian RNA is either capped or contains base modifications suggesting that RIG-I is able to discriminate between self and non-self RNA.

Although they recognize dsRNA, RIG-I/MDA-5 and TLR3 differ in their downstream signaling pathways. TLR3 recruits the adapter protein TRIF leading to the activation of several transcription factors including IRF3 and NF-κB[4]. IRF3 controls the expression of type I IFNs, while NF-κB regulates the production of inflammatory cytokines. RIG-I and MDA-5 bind to the CARD-containing adaptor protein IPS-1 (also known as MAVS, CARDIF or VISA), which activates IRF3 and IRF7 through TRAF3, NAP1 and TBK1/IKKε[5-7]. IPS-1 interacts also with FADD, a death domain-containing adapter involved in death receptor signaling, and RIP1 which induces the activation of the NF-κB pathway[5-8].

A third RLR has been described: laboratory of genetics and physiology 2 (LGP2). LGP2 contains a RNA binding domain but since it lacks the CARD domains acts as a negative feedback regulator of RIG-I and MDA-5. LGP2 appears to exert this activity at multiple levels by i) competitively sequestering dsRNA, ii) forming a protein complex with IPS-1, and/or iii) binding directly to RIG-I through a repressor domain[9-11]. LGP2 is not the only molecule involved in the negative control of dsRNA-induced IFN production. Various endogenous and viral inhibitors appear to target the RIG-I/MDA-5 pathway.

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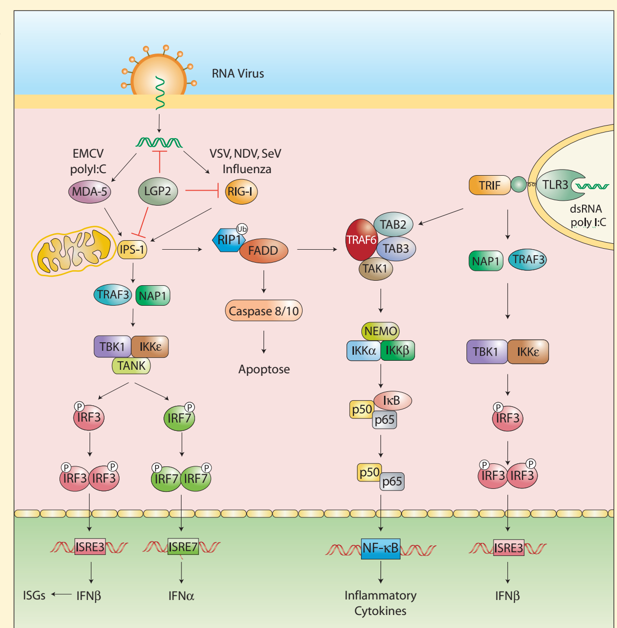

TLR9 Ligands

Stimulatory CpG ODNs - Specific and potent activators of TLR9

Toll-like receptor 9 (TLR9) detects unmethylated CpG dinucleotides in bacterial or viral DNA inducing strong immunostimulatory effects. TLR9 activation can be mimicked by synthetic phosphorothioate-stabilized oligodeoxynucleotides (ODN) containing immune stimulatory "CpG motifs". Three types of stimulatory CpG ODNs have been identified, types A, B and C, which differ in their immune-stimulatory activities (see below). They induce differentially the stimulation of human and murine immune cells *in vitro*, a species-specificity that is also observed with nonresponsive cells such as HEK293 cells transfected with human or mouse TLR9. InvivoGen offers a comprehensive collection of stimulatory CpG ODNs and control CpG ODNs that provide useful tools for studying TLR9-mediated activation. InvivoGen's CpG ODNs are endotoxin-free and tested for activity in various cell lines expressing human or mouse TLR9 (figure 1).

Control CpG ODNs that do not stimulate TLR9 have been designed for each stimulatory CpG ODN. They feature the same sequence as their stimulatory counterparts but contain GpC dinucleotides in place of CpG dinucleotides. Sequences and catalog codes of the control CpG ODNs are available on our website.

Stimulatory and control CpG ODNs are provided as 200 µg of lyophilized ultrapure DNA with 2 ml of sterile endotoxin-free water.

	Product	Type & Specificity	Sequence*	Refs	Cat. Code
NEW	ODN 1585	A - Mouse	5'-ggGGTCAACGTTGAgggggg-3'	1	tlrl-modna
NEW	ODN 1668	B - Mouse	5'-tccatgacgttcctgatgct-3'	2	tlrl-modnb
	ODN 1826	B - Mouse	5'-tccatgacgttcctgacgtt-3'	3	tlrl-modn
	ODN 2006	B - Human	5'-tcgtcgttttgtcgttttgtcgtt-3'	4, 6	tlrl-hodnb
	ODN 2216	A - Human	5'-ggGGGACGA:TCGTCgggggg-3'	5	tlrl-hodna
NEW	ODN 2336	A - Human	5'-gggGACGAC:GTCGTGgggggg-3'	6	tlrl-hodna2
NEW	ODN 2395	C - Human/Mouse	5'-tcgtcgttttcggcgc:gcgccg-3'	4, 6	tlrl-odnc
	ODN M362	C - Human/Mouse	5'-tcgtcgtcgttc:gaacgacgttgat-3'	7	tlrl-hodnc

* Bases in capital letters are phosphodiester, bases in lower case are phosphorothioate. Palindrome is underlined.

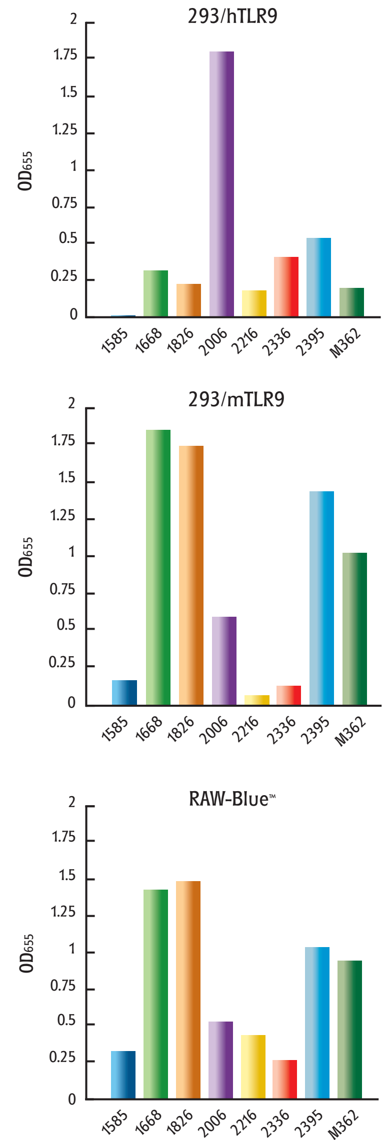

Figure 1: 293/hTLR9, 293/mTLR9 and RAW-Blue™ cells, all 3 cell lines stably expressing an NF-κB-inducible SEAP construct, were incubated with 10 µg/ml of CpG ODN type A, B or C. After 24h incubation, TLR9-induced NF-κB activation was assessed by measuring the levels of SEAP using QUANTI-Blue™.

Inhibitory ODNs - Potent inhibitors of TLR9 signaling

Recent studies suggest the existence of DNA sequences that can neutralize the stimulatory effect of CpG ODNs (figure 2). The most potent inhibitory sequences are (TTAGGG)4 found in mammalian telomeres and ODN2088 which derives from a murine stimulatory CpG ODN by replacement of 3 bases. Inhibitory ODNs seem to act by disrupting the colocalization of CpG ODNs with TLR9 in endosomal vesicles without affecting cellular binding and uptake. Inhibitory ODNs are often utilized to demonstrate a TLR9 dependence in murine systems.

Control ODNs are available for each inhibitory ODN. For more information on these control ODNs, check our website.

Inhibitory and control ODNs are provided as 200 µg of lyophilized ultrapure DNA with 2 ml of sterile endotoxin-free water.

OD655

TTAGGG:2006

2088:1826

0:1 1:1 3:1 10:1 1:0

Inhibitor ODN:stimulatory ODN ratio

Figure 2: Inhibitory ODNs, TTAGGG and 2088, were added to 293/TLR9-SEAP cells together with stimulatory CpG ODNs, 2006 and 1826 respectively, at different ratios. After 24h incubation, TLR9-induced NF-κB activation was assessed by measuring the levels of SEAP using QUANTI-Blue™.

Product	Preferred Species	Sequence*	Refs	Cat. Code
ODN 2088	Mouse	5'-tcctggcggggaagt-3'	8	tlrl-minhodn
ODN TTAGGG	Human	5'-tttagggttagggttagggttaggg-3'	9	tlrl-hinhodn

Types of immune stimulatory CpG ODNs:

- Type A CpG ODNs are characterized by a phosphodiester central CpG-containing palindromic motif and a phosphorothioate 3' poly-G string. They induce high IFN-α production from plasmacytoid dendritic cells (pDC) but are weak stimulators of TLR9-dependent NF-κB signaling.

- Type B CpG ODNs contain a full phosphorothioate backbone with one or more CpG dinucleotides. They strongly activate B cells but stimulate weakly IFN-α secretion.

- Type C CpG ODNs combine features of both types A and B. They contain a complete phosphorothioate backbone and a CpG-containing palindromic motif. Type C CpG ODNs induce strong IFN-α production from pDC and B cell stimulation.

References

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Mouse Macrophage Reporter Cells

RAW-Blue™ Cells NEW

Macrophages are major players in the innate immune defense. They express a large repertoire of different classes of pattern recognition receptors (PRRs), such as the TLRs, RLRs and NLRs. RAW-Blue™ Cells are murine macrophages designed for the study of these PRRs. They stably express an NF-κB-inducible SEAP reporter gene and in combination with QUANTI-Blue™, a SEAP detection medium, provide a rapid and powerful method to monitor the activation of these PRRs.

Description

RAW-Blue™ Cells are derived from RAW 264.7 macrophages with chromosomal integration of a secreted embryonic alkaline phosphatase (SEAP) reporter construct inducible by NF-κB and AP-1. RAW-Blue™ Cells are resistant to the selectable marker Zeocin™.

RAW-Blue™ Cells express all TLRs (with the exception of TLR5) as well as RIG-I, MDA-5, NOD1 and NOD2; expression of TLR3 and NOD1 being very low. The presence of specific agonists of these PRRs induces signaling pathways leading to the activation of NF-κB and AP-1 and subsequently to the secretion of SEAP. The reporter is easily detectable when using **QUANTI-Blue™**, a medium that turns purple/blue in the presence of SEAP, and measurable by reading the OD at 620-655 nm (figure 2).

Contents

RAW-Blue™ Cells are grown in DMEM medium with 10% FBS and 3 mM L-glutamine supplemented with 200 µg/ml Zeocin™. Each vial contains 3-5 x 10^6 cells and is supplied with 10 mg Zeocin™. Cells are shipped on dry ice.

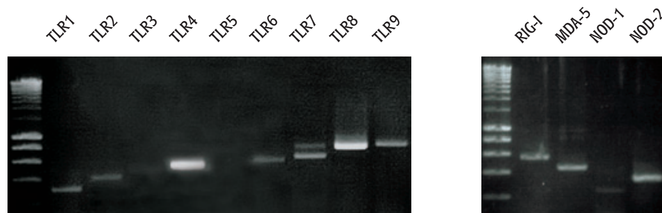

Figure 1: Expression of TLR, RLR and NOD mRNAs in RAW-Blue™ cells determined by RT-PCR. RT-PCR on TLR and RLR mRNAs were performed using the InvivoGen's Mouse TLR RT-Primer Set and RLR RT-Primers (see next page).

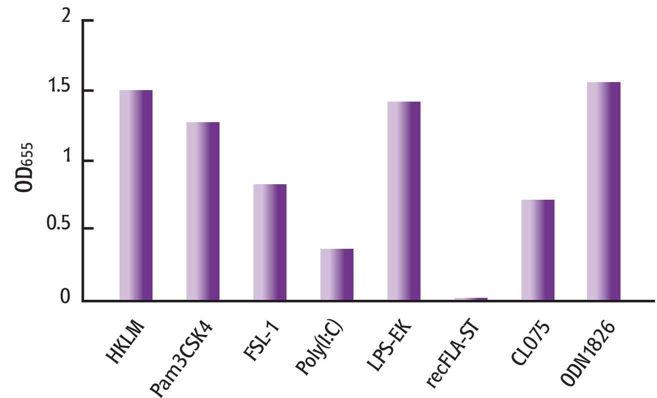

Figure 2: TLR stimulation profile in RAW-Blue Cells. RAW-Blue Cells were incubated with TLR agonists: TLR2 (HKLM, 1.10^8 cells/ml), TLR1/2 (Pam3CSK4, 100 ng/ml), TLR2/6 (FSL-1, 100 ng/ml), TLR3 (poly(I:C), 10 µg/ml), TLR4 (LPS-EK, 1 µg/ml), TLR5 (RecFLA-ST, 1 µg/ml), TLR7 (CL075, 300 ng/ml), TLR9 (ODN1826, 10 µg/ml). After 24h incubation, TLR stimulation was assessed by measuring the levels of SEAP using QUANTI-Blue™.

Product	Quantity	Cat. Code
RAW-Blue™	3-5 x 10^6 cells	raw-sp
Mouse TLR RT-Primer Set	20 x 2.5 nmol	rts-mtlrs
Zeocin™	1 g	ant-zn-1
QUANTI-Blue™	5 x 100 ml	rep-qb1

InvivoGen introduces InvivoGen Therapeutics, a new division dedicated to the research, development and manufacturing of gene therapy and immunotherapy products for the treatment of serious and life-threatening diseases. The initial focus of InvivoGen Therapeutics is the development of next generation plasmid vectors for the treatment of cancers and genetic diseases.

InvivoGen Therapeutics operates its own GMP facility for the production of clinical lots of plasmid DNA for gene therapy trials. With this GMP facility, we are able to perform all the operations necessary to go from research projects to pharmaceutical products. We offer a comprehensive package of services, from plasmid design to large scale production (>1 g).

Three plasmid DNA grades are available to better suit your needs:

- **Standard Research Grade** - *In vitro* studies
- **Pre-Clinical Grade** - *In vivo* studies
- **GMP Grade** - Phase I/II clinical trials

The GMP grade plasmid DNA meets all EU and FDA guidelines for the production of DNA pharmaceuticals products.

For more information, visit www.invivogen-therapeutics.com

Product	Quantity
Standard Research Grade	10-50 mg
Pre-Clinical Grade - Low Copy Plasmid	100 mg
Pre-Clinical Grade - Low Copy Plasmid	500 mg
Pre-Clinical Grade - High Copy Plasmid	100 mg
Pre-Clinical Grade - High Copy Plasmid	500 mg
GMP Grade	

RIG-I-Like Receptors (RLRs)

Tools for studying the RLR Pathway

C57/WT MEFs - RLR-Reporter Cell Line NEW

Murine embryonic fibroblasts (MEF) produce IFN-β in response to viral infection in a RLR-dependent manner[1]. Thus these cells are commonly used to study the RLR pathway. C57/WT MEFs were isolated from embryos under C57BL/6 background and immortalized with the SV40 large antigen. They stably express a SEAP reporter gene inducible by NF-κB and IRF3/7 providing a convenient method to monitor the activation of these transcription factors upon stimulation with a RIG-I/MDA-5 ligand.
Expression of RIG-I and MDA-5 was confirmed by RT-PCR using mouse RIG-I or MDA-5 RT-Primers (see below). Stimulation of C57/WT cells with poly(I:C)/LyoVec complexes induces the secretion of SEAP in a dose-dependent manner. In contrast, stimulation with naked poly(I:C) has no effect on SEAP secretion although TLR3 was also detected by RT-PCR. These data, that are available on our website, suggest that C57/WT MEFs respond to viral dsRNA primarily through the RLR pathway.
C57/WT MEFs are grown in DMEM medium with 10% FBS, 3 mM L-glutamine supplemented with 100 µg/ml Zeocin™ and 3 µg/ml blasticidin. Cells are shipped on dry ice.

Poly(I:C)/LyoVec Complexes - RIG-I/MDA-5 Ligand NEW

Poly(I:C) is widely used as a synthetic analog of viral dsRNA. Poly(I:C)/LyoVec complexes are recognized by the cytoplasmic sensors RIG-I and MDA-5 while naked poly(I:C) is recognized by the endosomal sensor TLR3[2]. Poly(I:C)/LyoVec complexes stimulate C57/WT MEFs at concentrations ranging from 100 ng to 5 µg/ml. Poly(I:C)/LyoVec complexes are provided lyophilized

pUNO-RLR - RLR Genes

RLR genes, RIG-I, MDA-5 and LGP2, of human and mouse origins, are available in the pUNO expression plasmid, selectable with blasticidin.The genes are cloned from the ATG to the Stop codon downstream of the strong and ubiquitous EF1α/HTLV promoter. Each pUNO-RLR plasmid is provided as a lyophilized transformed *E. coli* strain on a paper disk.

psiRNA-RLR - RLR-Targeting shRNAs

shRNAs that target and silence the RLRs are produced by the psiRNA-h7SKGFPzeo plasmid from the human 7SK RNA polIII promoter.The plasmids feature a GFP::zeo fusion gene that allows simple monitoring of transfection efficiency and selection in *E. coli* and mammalian cells with the same antibiotic, Zeocin™. Each psiRNA-RLR is provided as 20 µg of lyophilized DNA.

RLR RT-Primers - RT-PCR Primers NEW

RLR RT-Primers are designed for the analysis of mRNA expression of RIG-I or MDA-5 (human or mouse) by RT-PCR. They are provided as pairs with a positive control double stranded DNA.

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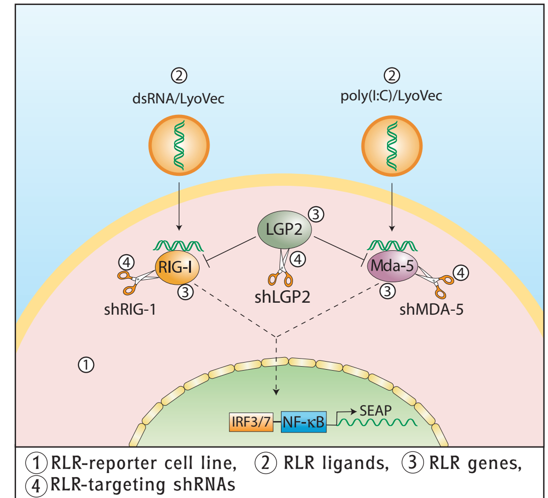

① RLR-reporter cell line, ② RLR ligands, ③ RLR genes, ④ RLR-targeting shRNAs

Product	Quantity	Cat. Code
RLR-Reporter Cell Line		
C57/WT MEFs	3-5 x 10^6 cells	mef-c57wt
RLR Ligand		
Poly(I:C)/LyoVec	100 µg	tlrl-piclv
RLR Genes (pUNO)		
RIG-I (human)	*E. coli*	puno-hrigi
RIG-I (mouse)	*E. coli*	puno-mrigi
MDA-5 (human)	*E. coli*	puno1-hmda5
MDA-5 (mouse)	*E. coli*	puno1-mmda5
LGP2 (human)	*E. coli*	puno-hlgp2
LGP2 (mouse)	*E. coli*	puno-mlgp2
RLR shRNAs (psiRNA)		
shRIG-I (human)	20 µg	psirna42-hrigi
shRIG-I (mouse)	20 µg	psirna42-mrigi
shMDA-5 (human)	20 µg	psirna42-hmda5
shMDA-5 (mouse)	20 µg	psirna42-mmda5
shLGP2 (human)	20 µg	psirna42-hlgp2
shLGP2 (mouse)	20 µg	psirna42-mlgp2
RLR RT-Primers		
RIG-I RT-Primers (human)	2 x 2.5 nmol	rtp-hrigi
RIG-I RT-Primers (mouse)	2 x 2.5 nmol	rtp-mrigi
MDA-5 RT-Primers (human)	2 x 2.5 nmol	rtp-hmda5
MDA-5 RT-Primers (mouse)	2 x 2.5 nmol	rtp-mmda5

For more RLR products, check our website:
www.invivogen.com