InvivoGen Insight
SPRING 2013

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Multi-TLR Agonists: Potential for Therapeutic Success

Toll-like receptors (TLRs) are the best studied pattern recognition receptors (PRRs) and their importance in stimulating innate and adaptive immunity is now well established. TLRs are sensors of microbial components as well as host-derived endogenous molecules released by injured tissues. TLRs play a critical role in defense against invading pathogens but are also involved in other serious pathological processes, such as tumorigenesis[1]. In view of this, TLR agonists have great potential as immunotherapeutics or vaccine adjuvants for the treatment of infectious diseases, cancer and autoimmune diseases.

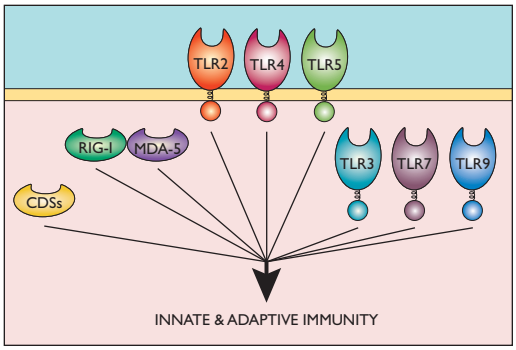
One of the most successful empiric vaccines ever developed, the live attenuated yellow fever vaccine YF-17D, activates immune cells via multiple TLRs[2]. In the same line, microbial products, such as Coley's toxin (a mixture of killed *Streptococcus pyogenes* and *Serratia marcescens* bacteria) and Bacille Calmette-Guérin (BCG, an attenuated strain of *Mycobacterium bovis*), which have been used as anticancer agents with some success, induce the host immune system through the activation of several TLRs. Studies have demonstrated that YF-17D and BCG efficacy requires a Th1 cytokine response which promotes antigen-specific cytotoxic T cells[2, 3].

Today, very few TLR agonists are approved for clinical use as stand-alone agents or adjuvants[4]. One of the major challenges of cancer immunotherapy is the reversal of tumor-driven immune suppression. Tumor cells release soluble factors leading to tumor infiltration by immune cells and their convertion into potent immuno-suppressive cells. Among the immune suppressor cells, myeloid-derived suppressor cells (MDSCs) are the focus of extensive studies. MDSCs release additional soluble factors that stimulate tumor growth, induce regulatory T cells and suppress $CD8^+$ T cells, thus impairing tumor surveillance and antitumor responses[5]. TLR3 or TLR9 agonists, which elicit strong IFN-α responses, have been shown to induce MDSC maturation and loss of suppressive functions, in contrast to TLR4 agonists which support MDSC suppressive functions[6].

Given that TLRs are expressed in different cellular compartments, by different cell types and that they trigger different signaling pathways, combining TLR agonists can act in synergy to promote Th1-type immunity. Encouraging clinical results have been reported with the combination of BCG and the TLR7 agonist Imiquimod in melanoma patients[7]. Furthermore, vaccination studies have demonstrated that the combination of Imiquimod and MPL, a TLR4 ligand, elicits synergistic increases in antigen-specific neutralizing antibodies compared to a single TLR ligand[8].

The combination of TLR agonists with agonists for other PRRs, such as the sensors of nucleic acids RIG-I/MDA-5, IFI16 and cGAS, may further enhance the immune response against cancer and infectious diseases. With this perspective, InvivoGen has developed multi-PRR agonists that activate TLR2 and/or TLR7 and can complex nucleic acids, such as double-stranded DNA,, leading to additional recognition by cytosolic DNA sensors (CDSs). These molecules have been shown to induce a strong immune response in a murine melanoma cancer model and in vaccination studies.

By mimicking the potent natural immuno-stimuli that are viruses and bacteria, multi-PRR ligands represent an interesting new class of agents in cancer immunotherapy or vaccination. However, further studies are needed to develop the best PRR agonist combinations for a given application.



1. Rakoff-Nahoum S. & Medzhitov R., 2007. Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88. Science. 317(5834):124-7. **2. Querec T. *et al.*, 2006.** Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. J Exp Med. 203(2):413-24. **3. Saint F. *et al.*, 2001.** T helper 1/2 lymphocyte urinary cytokine profiles in responding and nonresponding patients after 1 and 2 courses of bacillus Calmette-Guerin for superficial bladder cancer. J Urol. 166(6):2142-7. **4. Galluzzi L. *et al.*, 2012.** Trial Watch: Experimental Toll-like receptor agonists for cancer therapy. Oncoimmunology. 1(5): 699–716. 3. **5. Gabrilovich DI. *et al.*, 2012.** Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 12(4):253-68. **5. Lindau D. *et al.*, 2013.** The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells. Immunology. 138(2):105-15. **7. Kidner TB. *et al.*, 2012.** Combined intralesional Bacille Calmette-Guérin (BCG) and topical imiquimod for in-transit melanoma. J Immunother. 35(9):716-20. **8. Kasturi SP. *et al.*, 2011.** Programming the magnitude and persistence of antibody responses with innate immunity. Nature. 470(7335):543-7.

Multi-PRR Agonists

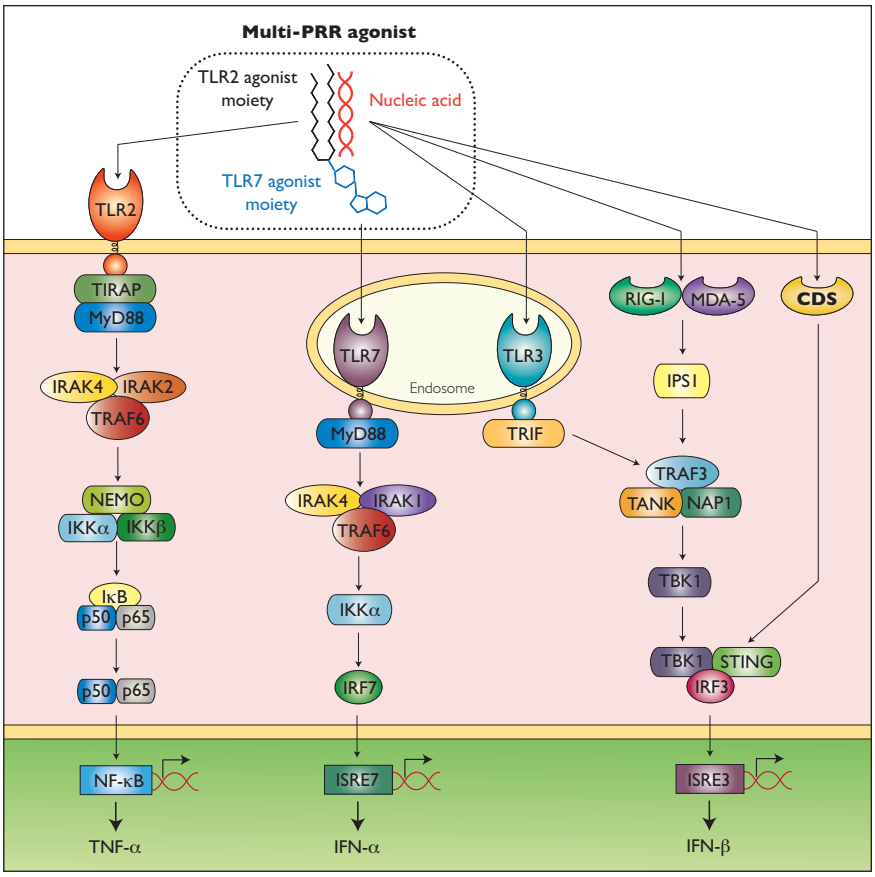
InvivoGen has developed a series of novel molecules designed to induce potent immune responses through the combined activation of several pattern recognition receptors (PRRs) that trigger different innate immune signaling pathways. These molecules are agonists for TLR2, TLR7 or both. In addition, some of these ligands have the ability to form complexes with nucleic acids (for example, double-stranded DNA, such as short oligonucleotides or plasmid DNA, or single-stranded RNA) and facilitate their penetration into the cell resulting in their recognition by additional PRRs that sense nucleic acids (e.g. the cytosolic DNA sensors DDX41 and IFI16 and the dsRNA receptors TLR3 and RIG-I/MDA-5).

➢ **Dual TLR Agonists**

- TLR2 & TLR7 Ligands

➢ **TLR Agonists & Nucleic Acid Carriers**

- TLR2 Ligand
- TLR7 Ligand
- TLR2 & TLR7 Ligand



Schematic representation of innate immune signaling pathways activated by PamadiFectin™ (CL553), a multi-PRR agonist that activates TLR2, TLR7 and nucleic acid sensors when complexed with dsDNA, for example.

Description

Agonists that activate TLR2 are derived from the well-established TLR2 ligand, Pam2CSK4, and those recognized by TLR7 are derived from the 8-hydroxyadenine derivative CL264, a TLR7 agonist recently developed by InvivoGen. The ability to complex nucleic acids is conferred by the addition of a cationic lipid. TLR2 and TLR7 are two PRRs with distinct characteristics. TLR2 is a cell surface receptor expressed by many cell types, while TLR7 is an endosomal receptor expressed predominantly in plasmacytoid dendritic cells (pDC) and to a lesser extent in B cells. TLR2 signaling triggers the NF-κB pathway and the production of pro-inflammatory cytokines, such as TNF-α, whereas TLR7 signaling induces mainly the IRF pathway and the production of IFN-α. Sensors of nucleic acids are numerous and their signaling pathways, although not fully understood, lead mainly to the production of type I IFNs. Combined activation of these different pathways results in robust immune responses with potential therapeutic effects. InvivoGen's multi-PRR agonists are promising candidates for antitumor and vaccine applications.

In vitro Evaluation

All InvivoGen's multi-PRR agonists have been evaluated *in vitro*. TLR2- or TLR7-induced NF-κB activation has been determined using HEK-Blue™ TLR reporter cells which express TLR2 or TLR7 and an NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene, as well as the murine macrophage-derived RAW-Blue™ reporter cell line. Activation of the IRF pathway has been monitored in the RAW-Lucia™ ISG cell line, a mouse macrophage cell line expressing an IRF-inducible secreted luciferase (Lucia®) reporter gene.

In vivo Testing

A selection of InvivoGen's multi-PRR agonists has been tested *in vivo* using the B16 melanoma model in syngeneic C57/BL6 mice. The antitumor activity of these molecules has been studied after intratumoral administration by following tumor growth and mice survival. B16 melanoma cells express TLR2 but not TLR7.

Product		TLR Specificity	MW	Solubility	Working concentration
TLR Agonists					
CL401	**NEW**	TLR2 + TLR7	981	DMSO	1 ng - 10 µg/ml (~1 nM - 10 µM)
Adilipoline™ (CL413)	**NEW**	TLR2 + TLR7	1581	H_2O	50 pg - 10 µg/ml (~30 pM - 10 µM)
CL531	**NEW**	TLR2 + TLR7	1610	H_2O	5 pg - 10 µg/ml (~3 pM - 10 µM)
CL572	**NEW**	TLR2 (human only) + TLR7	828	DMSO	0.5 ng - 1 µg/ml (~1 nM - 10 µM)
CL307	**NEW**	TLR7	597	H_2O	5 ng - 1 µg/ml (~10 nM - 2 µM)
TLR Agonists & Nucleic Acid Carriers					
CL419	**NEW**	TLR2	856	H_2O	1 ng - 100 µg/ml (~1 nM - 100 µM)
AdiFectin™ (CL347)	**NEW**	TLR7	1280	Ethanol	300 ng - 3 µg/ml (~200 nM - 2 µM)
PamadiFectin™ (CL553)	**NEW**	TLR2 + TLR7	1251	Ethanol	100 ng - 1 µg/ml (~100 nM - 1 µM)

➤ Dual TLR Agonists

CL401 - TLR2 & TLR7 Ligand

CL401 (S-(2,3-bis(palmitoyloxy)-(2RS)propyl)-(R)-cysteinyl 4-((6-amino-2(butyl amino)-8-hydroxy-9H-purin-9-yl)methyl) aniline) is a small lipophilic molecule comprising an 8-hydroxyadenine compound conjugated with a Pam2C group. This bipartite structure confers to CL401 the ability to efficiently stimulate both TLR7 and TLR2, respectively (Figures 1, 2, 3). Intratumoral injection of CL401 leads to a significant antitumor activity (Figures 6A & 6B).

Adilipoline™ (CL413) - TLR2 & TLR7 Ligand

Adilipoline™ (S-(2,3-bis(palmitoyloxy)-(2RS)propyl)-(R)-cysteinyl-(S)-seryl-(S)-lysyl-(S)-lysyl-(S)-lysyl-(S)-lysyl 4-((6-amino-2-(butylamino)-8-hydroxy-9H-purin-9-yl)methyl) aniline) was generated by conjugation of an 8-hydroxyadenine moiety to the terminal acid function of Pam2CSK4. Adilipoline™ is a good ligand for both TLR2 and TLR7 (Figures 1, 2, 3). *In vivo* tumor studies have demonstrated that Adilipoline™ is a potent antitumor agent (Figures 6A & 6B). Intratumoral injection of Adilipoline™ in established B16 tumors resulted in tumor regression. However, in contrast to AdiFectin™ (CL347), no protection after tumor rechallenge was observed.

CL531 - TLR2 & TLR7 Ligand

CL531 (S-(2,3-bis(palmitoyloxy)-(2RS)propyl)-(R)-cysteinyl-(S)-seryl-(S)-lysyl-Ne-(4-(((6-amino-2-(butylamino)-8-hydroxy-9H-purin-9-yl)methyl) benzylamido)(S)-lysyl-(S)-lysyl-(S)-lysine) is an 8-hydroxyadenine derivative conjugated to the lateral chain of the second lysine of Pam2CSK4. CL531 is a very potent TLR2 agonist and a good TLR7 agonist (Figures 1 & 2). TLR2-mediated activation of NF-κB is achieved with concentrations as low as 5 pM (0.01 ng/ml).

CL572 - TLR2 (human) & TLR7 Ligand

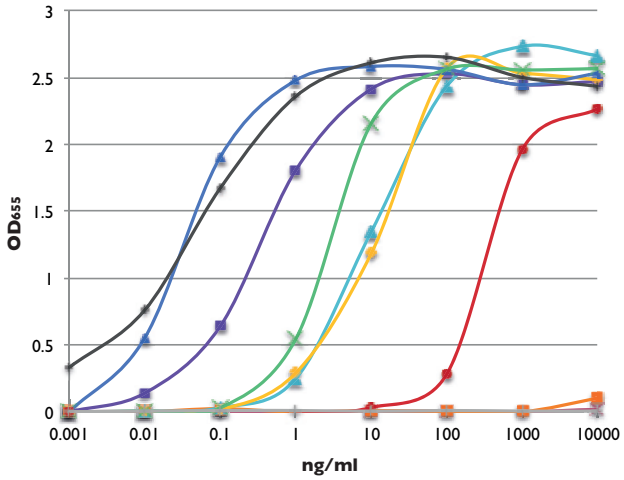
CL572 (S-(2-myristoyloxy ethyl)-(R)-cysteinyl 4-((6-amino-2-(butylamino)-8-hydroxy-9H-purin-9-yl)methyl) aniline) is a 8-hydroxy-adenine compound conjugated to a monoacyl-ethyl-cystein group via a glutamic acid derivative. Monoacy-ethyl-cystein-containing dipeptides have been recently shown to specifically activate human TLR2 (Agnihotri G. *et al.,* 2011). Indeed, CL572 is a robust inducer of human TLR2 (Figure 1) but is unable to stimulate mouse TLR2 (data not shown). CL572 is also a potent inducer of TLR7 (Figure 2).

Agnihotri G. *et al.,* 2011. Structure-activity relationships in toll-like receptor 2-agonists leading to simplified monoacyl lipopeptides. J Med Chem. 54(23):8148-60.

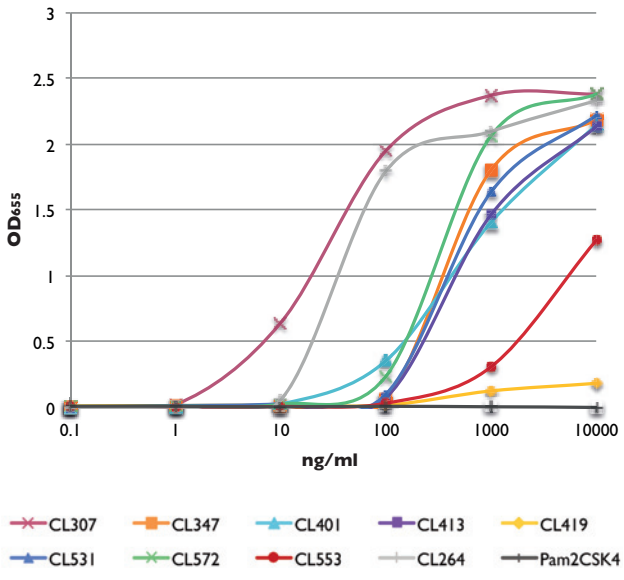
CL307 - TLR7 Ligand

CL307 (N1-glycinyl[4-((6-amino-2-(butylamino)-8-hydroxy-9H-purin-9-yl)methyl) benzoyl] spermine) was generated by covalently linking a spermine to the hydroxyadenine compound CL264. CL307 is a very potent TLR7 agonist. Titration experiments have showed that CL307 induces robust NF-κB activation even at concentrations as low as 20 nM (10 ng/ml) (Figures 2 & 3).

1- TLR2 response



2- TLR7 response



Figures 1 & 2. HEK-Blue™ hTLR2 cells (1) and HEK-Blue™ hTLR7 cells (2), which stably express an NF-κB-inducible SEAP reporter gene and human TLR2 or TLR7, respectively, were incubated in HEK-Blue™ Detection (a SEAP detection growth medium) and stimulated with increasing concentrations of the agonists indicated in the graph. After 24h incubation, the levels of NF-κB-induced SEAP were determined by reading the OD at 655 nm.

➢ TLR Agonists & Nucleic Acid Carriers

CL419 - TLR2 Ligand

CL419 (S-(2,3-bis(palmitoyloxy)-(2RS)propyl)-(R)-cysteinyl spermine) is a polyamine TLR2 agonist derived from Pam2CSK4 by replacement of Ser-(Lys)4 by a cationic sperminyl group. CL419 forms positively charged liposomes which allows it to complex nucleic acids and transport them into the cytosol and the nucleus. CL419 / nucleic acid complexes are recognized by TLR2 and nucleic acid sensors leading to the significant activation of the NF-κB and IRF pathways (Figures 1, 3, 4). *In vivo*, CL419 complexed with a plasmid DNA (pDNA) and injected intratumorally induces a modest reduction of the tumor growth (Figure 7C).

AdiFectin™ (CL347) - TLR7 Ligand

AdiFectin™ (bis(phytanyl) N4-{N1-[(4-((6-amino-2-(butylamino)-8-hydroxy-9H-purin-9-yl)methyl)benzoyl)glycinyl]sperminyl}propyl phosphonate) is derived from CL307 by conjugation with a bis(phytanyl) phosphonate group. Addition of this lipid confers to the molecule the ability to form positively charged liposomes, which can encapsulate DNA (or RNA). AdiFectin™ is a weaker TLR7 agonist than CL307 (Figures 2 & 3), but in contrast to CL307, is able to efficiently complex nucleic acids resulting in a strong IFN response (Figure 4) and transgene expression when the nucleic acid is a plasmid DNA carrying an expression cassette (Figure 5). Repeated *in vivo* studies have showed that pDNA/AdiFectin™ complexes display robust anti-tumor activity (Figures 7C & 7D). Tumor growth was markedly reduced resulting in a 50% survival rate. Notably, mice that achieved long-term clearance of tumor following AdiFectin™ treatment were protected from subsequent tumor rechallenge suggesting the generation of a tumor-specific memory immune response (data not shown).

PamadiFectin™ (CL553) - TLR2 & TLR7 Ligand

PamadiFectin™ (N4-(S-((2,3-bis(palmitoyloxy))-(2RS)propyl)-(R)-cysteinyl) N1-(4-(((6-amino-2-(butylamino)-8-hydroxy-9H-purin-9-yl)methyl)benzoyl) glycinyl) spermine) was generated by conjugation of CL307 to a Pam2C group. PamadiFectin™ induces NF-κB activation through stimulation of both TLR2 and TLR7 (Figures 1, 2, 3). In addition, at physiological pH, PamadiFectin™ is able to form complexes with nucleic acids and carry them in the cytosol and nucleus leading to a strong induction of the IRF pathway (Figure 4). Intratumoral administration of pDNA/PamadiFectin™ complexes leads to spectacular reduction of tumor growth and improved long-term survival in B16-F1 tumor-bearing mice (Figures 7C & 7D). Tumor rechallenge experiments have not yet been performed.

3- NF-κB response

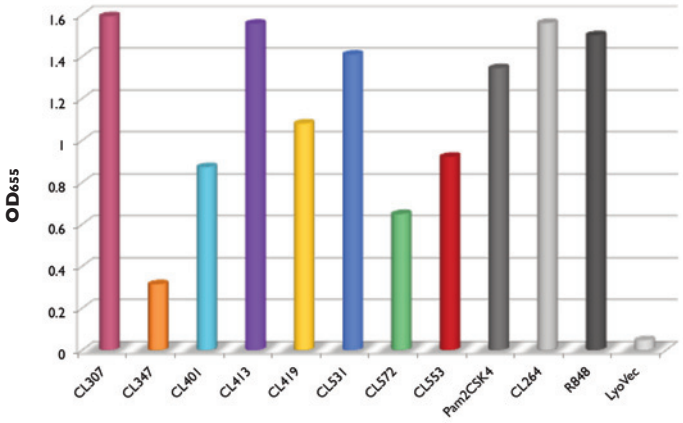


Figure 3. RAW-Blue™ cells, which stably express an NF-κB-inducible SEAP reporter gene, were stimulated with 0.6 µg/ml of InvivoGen's multi-PRR ligands complexed with 0.1 µg/ml HSV-60 (synthetic dsDNA). After 24h incubation, the levels of NF-κB-induced SEAP were determined using QUANTI-Blue™, a SEAP detection reagent.

4- IRF response

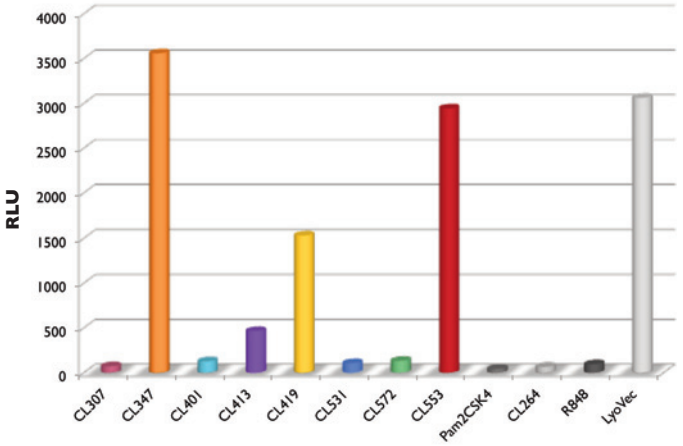
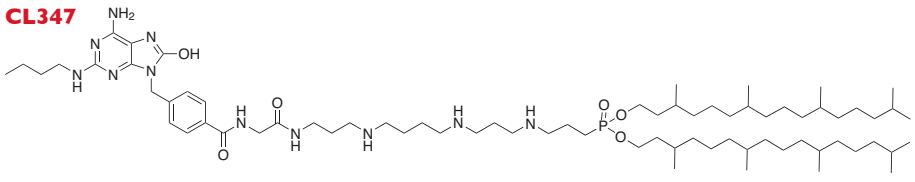


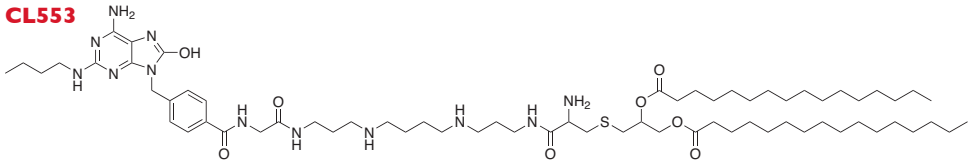
Figure 4. RAW-Lucia™ cells, which stably express an IRF-inducible Lucia® (a secreted luciferase) reporter gene, were stimulated with 6 µg/ml of InvivoGen's multi-PRR ligands complexed with 1 µg/ml HSV-60 (synthetic dsDNA). After 24h incubation, the levels of IRF-induced Lucia™ were determined using QUANTI-Luc™, a Lucia™ detection reagent.

Structures of AdiFectin™ and PamadiFectin™

CL347



CL553



Structures of all multi-PRR ligands are available online
www.invivogen.com/multi-prr-ligands

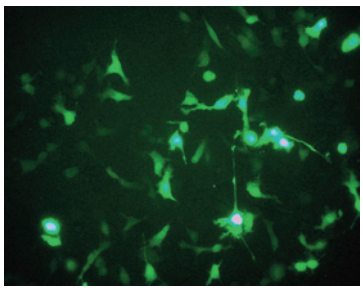
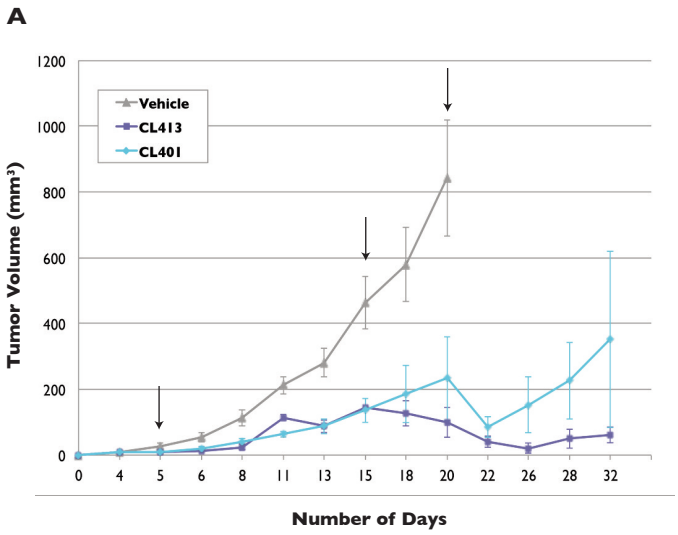
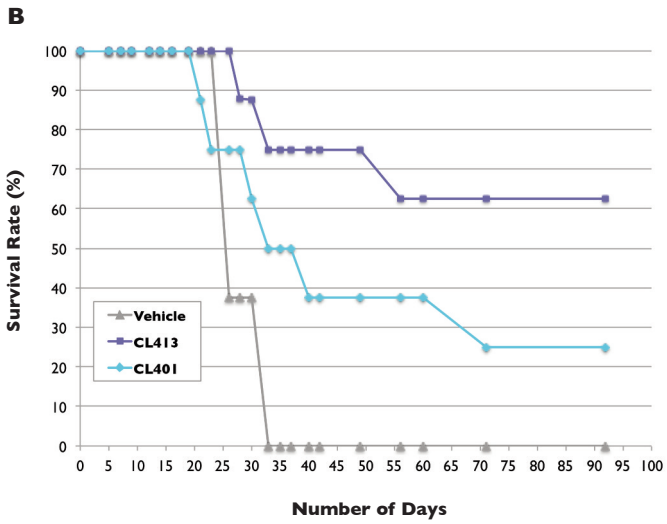


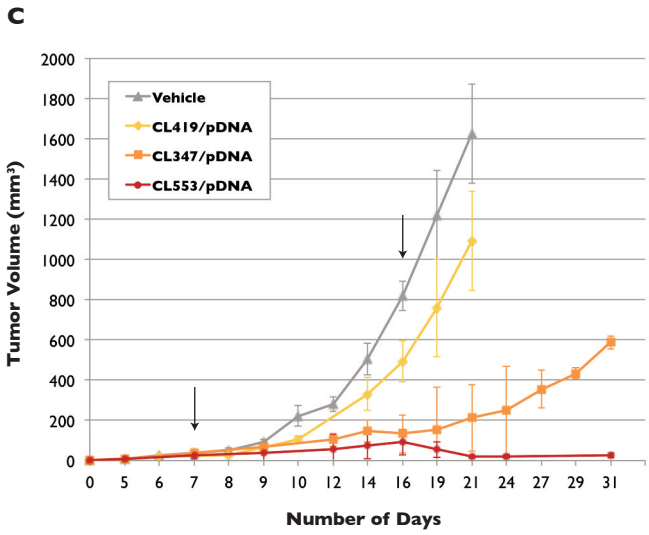
Figure 5. Transfection efficiency of AdiFectin™. B16-F1 cells were incubated with pDNA-GFP/CL347 complexes at a 1/6 ratio (w/w). After 48h incubation, GFP expression was detected using fluorescence microscopy. Similar results were obtained with pDNA-GFP complexed with CL419 or CL553. No fluorescence was observed with pDNA-GFP mixed with other multi-PRR ligands, such as CL307 or CL531.

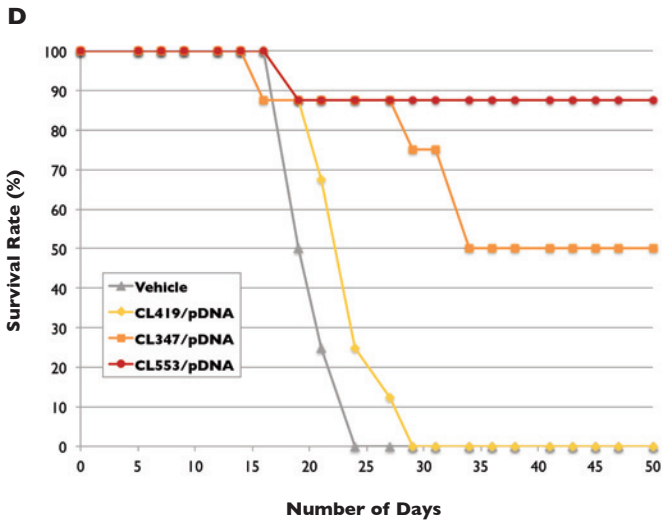
6- Antitumor effect of CL401 & CL413





7- Antitumor effect of CL419, CL347 & CL413 complexed to pDNA





Figures 6 & 7: (A) Tumor growth after CL413 or CL401 treatment. C57BL/6 mice were inoculated subcutaneously with $5x10^5$ B16-F1 mouse melanoma cells. After seven days, CL413, CL401 or vehicle were injected intratumorally (50 µg/mouse/50 µl) on days 5, 15 and 20. (B) Survival curves for untreated as well as CL413- or CL401-treated mice. (C) Tumor growth after treatment with CL419, CL347 or CL401complexed with plasmid DNA (pDNA). Seven days after C57BL/6 mice were inoculated subcutaneously with 5.10^5 B16-F1 cells, pDNA/CL419, pDNA/CL347 or pDNA/CL553 complexes were injected intratumorally at a 10:40 (w:w) ratio (10 µg:40 µg/mouse/100 µl) on days 7 and 16. A fourth group received intratumoral injections of the vehicle. (D) Survival curves for untreated as well as pDNA/CL419-, pDNA/CL347- or pDNA/CL553-treated mice.
Each group contained 8 mice. Black arrows represent the days of injection. Tumor growth was monitored and measured with calipers after day 5 of grafting tumor cells into mice and then every 2 days thereafter. Tumor volume in mm^3 was determined according to the formula $V = W^2 \times L/2$, where L = length (mm) and W = width (mm).

Contents and Storage

All multi-PRR ligands are provided lyophilized with 2 ml of sterile endotoxin-free water. Products are shipped at room temperature and should be stored at -20°C.

Related Products

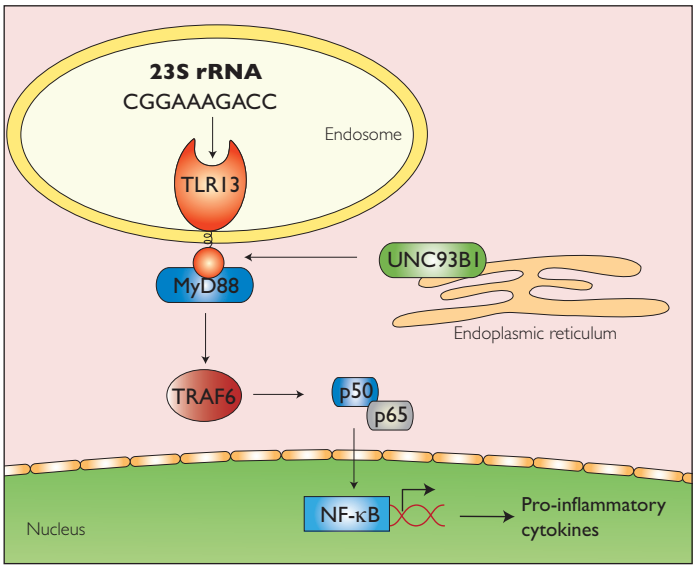
HEK-Blue™ hTLR2 (human), 3-7 10^6 cells (hkb-htlr2)
HEK-Blue™ mTLR2 (mouse), 3-7 10^6 cells (hkb-mtlr2)
HEK-Blue™ hTLR7 (human), 3-7 10^6 cells (hkb-htlr7)
HEK-Blue™ mTLR7 (mouse), 3-7 10^6 cells (hkb-mtlr7)
RAW-Blue™ (NF-κB), 3-7 10^6 cells (raw-sp)
RAW-Lucia™ ISG, 3-7 10^6 cells (rawl-isg)
Pam2CSK4, 100 µg (tlrl-pm2s)
CL264, 500 µg (tlrl-c264e)
R848, 500 µg (tlrl-r848)
HSV-60 Naked, 200 µg (tlrl-hsv60n)

PRODUCT		QTY	CAT. CODE
CL307	**NEW**	500 µg	tlrl-c307
AdiFectin™ (CL347)	**NEW**	500 µg	tlrl-c347
CL401	**NEW**	500 µg	tlrl-c401
Adilipoline™ (CL413)	**NEW**	500 µg	tlrl-c413
CL419	**NEW**	500 µg	tlrl-c419
CL531	**NEW**	500 µg	tlrl-c531
PamadiFectin™ (CL553)	**NEW**	500 µg	tlrl-c553
CL572	**NEW**	500 µg	tlrl-c572

Toll Like Receptor 13

A receptor for bacterial ribosomal RNA

Toll-like receptor 13 (TLR13) is an endosomal TLR expressed in mice, which role and ligand remain unclear. Recently, three separate groups have identified 23S ribosomal RNA (rRNA) as a ligand for TLR13. This single-stranded rRNA is present in gram-positive and gram-negative bacteria but not in eukaryotic cells. A conserved sequence of 10 residues within the catalytic center of 23S rRNA, "CGGAAAGACC", was found to be both necessary and sufficient to trigger TLR13 signaling. Other forms of rRNA are unable to activate the TLR13 pathway. Thus, unlike other nucleic acid receptors, TLR13 appears to recognize a specific RNA sequence. Interestingly, Oldenburg *et al.* have shown that this sequence is the binding site of the macrolide- lincosamide-streptogramin (MLS) group antibiotics, and that methylation-mediated resistance to these antibiotics (which target A2085 in *S. aureus* 23S rRNA) abolishes the immunostimulatory activity of 23S rRNA. This study suggests that acquisition of antibiotic resistance is a mechanism developed by bacteria to evade host innate immune system. Humans lack TLR13 and probably rely on other pathogen receptors to detect pathogenic bacterial infection. The TLR13 signaling cascade clearly engages MyD88 and UNC93B1, but the details of the pathway require further investigation.



1. Oldenburg M. *et al.*, 2012. TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification. Science. 337(6098). **2. Hidmark A. *et al.*, 2012.** Cutting edge: TLR13 is a receptor for bacterial RNA. J Immunol. 189(6):2717-21. **3. Li XD & Chen ZJ. 2012.** Sequence specific detection of bacterial 23S ribosomal RNA by TLR13. elife. 1:e00102.

To assist you in your research on TLR13 function and signaling, InvivoGen provides a set of tools that have been validated in-house and include ligands, genes and a reporter cell line.

ORN Sa19 and ORN Sa19 Control — NEW

Mouse TLR13 Ligands

ORN Sa19 is a 19 mer *S. aureus* 23S rRNA derived oligoribonucleotide, ORN Sa19, which contains an A in its center to mirror *S. aureus* A2085, is highly stimulatory in TLR13-expressing cells in contrast to ORN Sa19 Control, which carries a G in place of the central A. ORN Sa19 and ORN Sa19 Control are stabilized by phosphorothioate modification.

ORN Sa19 5'-GGACGG**A**AAGACCCCGUGG-3'
ORN Sa19 Control 5'-GGACGG**G**AAGACCCCGUGG

pUNO-mTLR13

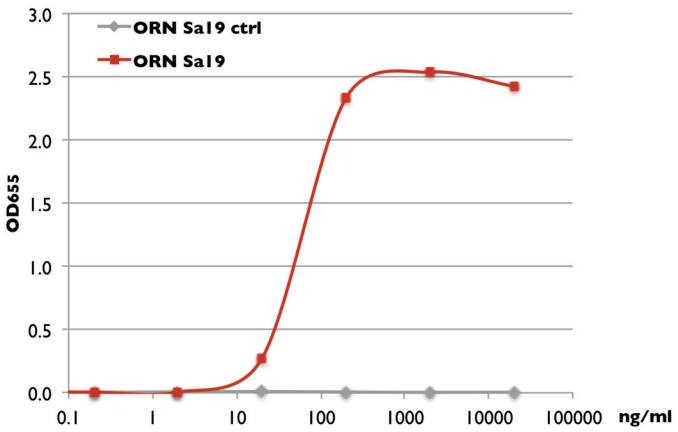
Mouse TLR13 gene

The pUNO-mTLR13 plasmid carries the full-length ORF of mouse TLR13 in an expression cassette featuring the strong and constitutive hEF1-HTLV promoter and the efficient SV40 late polyA signal. pUNO-mTLR13 is selectable with blasticidin in both *E. coli* and mammalian cells. The mTLR13 gene can be easily subcloned into another expression vector.
The mTLR13 gene is also available fused at the 3'end to the influenza hemaglutinine (HA) tag in the pUNO-mTLR13-HA plasmid.

HEK-Blue™ mTLR13 — NEW

Mouse TLR13 reporter cell line

HEK-Blue mTLR13 cells are HEK293-derived cells stably transfected with pUNO-mTLR13 and an NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter plasmid. Stimulation with a TLR13 ligand, such as ORN Sa19, activates NF-κB and leads to the production of SEAP. Levels of SEAP can be easily determined with HEK-Blue™ Detection, a growth & detection medium that turns purple/blue in the presence of alkaline phosphatase.



NF-κB response of HEK-Blue™ mTLR13 cells to TLR13 ligands. Cells were incubated in HEK-Blue™ Detection and stimulated with increasing concentrations of ORN Sa19 and ORN Sa19 Control. After 24h incubation, the levels of NF-κB-induced SEAP were determined by reading the OD at 655 nm.

PRODUCT	QUANTITY	CAT. CODE
ORN S19	200 µg	tlrl-orn19
ORN S19 Control	200 µg	tlrl-orn19c
pUNO-mTLR13	*E. coli* disk	puno-mtlr13
pUNO1-mTLR13-HA	*E. coli* disk	puno1ha-mtlr13
HEK-Blue™ mTLR13	3-7 x 10^6 cells	hkb-mtlr13

For more information on our TLR13 product line, please visit www.invivogen.com

STING Activation & Detection

A few weeks ago, cGAMP was discovered as the first endogenous cyclic dinucleotide in mammals. Similar to other cyclic dinucleotides, including c-di-AMP and c-di-GMP, cGAMP has been shown to bind to and activate STING leading to the production of type I IFNs. cGAMP, as well as c-di-IMP and c-di-UMP, are now available as STING ligands from InvivoGen. All STING ligands have been tested in IRF-reporter cell lines expressing high or low levels of STING.

➢ STING Ligands

cGAMP NEW

Cyclic guanosine monophosphate-adenosine monophosphate (cyclic GMP-AMP, cGAMP) has recently been identified as the first cyclic di-nucleotide in metazoa[1]. cGAMP has also been identified in the bacterium *Vibro cholerae* and shown to play a role in bacterial chemotaxis and colonization[2]. In human and mouse cells, cGAMP has been reported to function as a second messenger that triggers interferon production in response to cytosolic DNA. cGAMP is synthesized by cGAMP synthetase (cGAS) and binds to STING leading to the activation of IRF3 and the induction of interferon β (IFN-β)[3]. cGAMP is more potent in activating IRF3 than c-di-IMP, c-di-AMP and c-diGMP, other cyclic dinucleotides that also bind to STING[4] (figs. 1 & 2).

c-di-IMP NEW

Cyclic di-inosine monophosphate (c-di-IMP) is a synthetic analog of the bacterial second messengers c-di-AMP and c-di-GMP. Although chemically different, c-di-IMP exhibits similar conformational features and biological properties as c-di-GMP. *In vitro* studies have revealed that c-di-IMP displays comparable capacities to c-di-GMP in promoting the activation and maturation of antigen presenting cells[5]. Furthermore, c-di-IMP has been shown to possess strong adjuvant properties when co-administered with an antigen by the mucosal route[5]. The immunostimulatory activity of c-di-IMP depends on the signaling molecule STING[4]. Cells with reduced STING expression (e.g. THP1-Blue™ ISG KD-STING cells) respond weakly to c-di-IMP in contrast to cells expressing high levels of STING (e.g. THP1-Blue™ ISG cells and RAW-Lucia™ ISG cells) (figs. 1 & 2).

c-di-UMP NEW

Cyclic di-uridine monophosphate (c-di-UMP) is a synthetic cyclic dinucleotide containing a pyrimidine base. Unlike the purine-containing cyclic dinucleotides (c-di-AMP, c-di-GMP and c-di-IMP), c-di-UMP is not predicted to bind strongly to STING[6]. The smaller size of the pyrimidine base is expected to prevent the formation of stacking interactions that are crtitical for proper binding to STING. Indeed, STING-expressing cells, such as THP1-Blue™ ISG cells, do not respond to c-di-UMP, even at high concentrations (figs. 1 & 2).

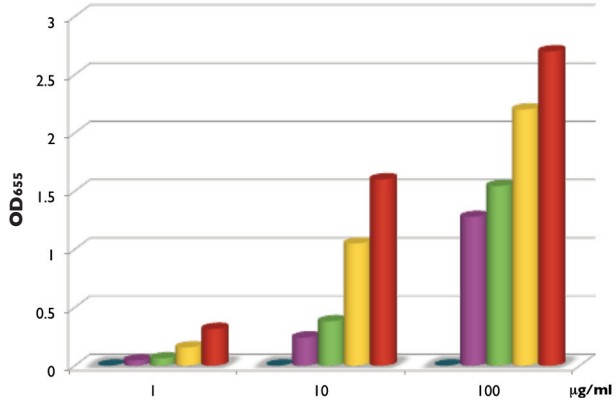
1.Wu J. *et al.*, 2012. Cyclic GMP-AMP Is an Endogenous Second Messenger in Innate Immune Signaling by Cytosolic DNA. Science. 339(6121):826-30. **2. Davies BW. *et al.*, 2012.** Coordinated regulation of accessory genetic elements produces cyclic di-nucleotides for V. cholerae virulence. Cell. 149(2):358-70. **3. Sun L. *et al.*, 2012.** Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. Science. 339(6121):786-91. **4. Burdette DL. *et al.*, 2011.** STING is a direct innate immune sensor of cyclic di-GMP. Nature. 478(7370):515-8. **5. Libanova R. *et al.*, 2010.** The member of the cyclic di-nucleotide family bis-(3', 5')-cyclic dimeric inosine monophosphate exerts potent activity as mucosal adjuvant. Vaccine. 28(10):2249-58. **6. Yin Q. *et al.*, 2012.** Cyclic di-GMP sensing via the innate immune signaling protein STING. Mol Cell. 46(6):735-45.

Also available

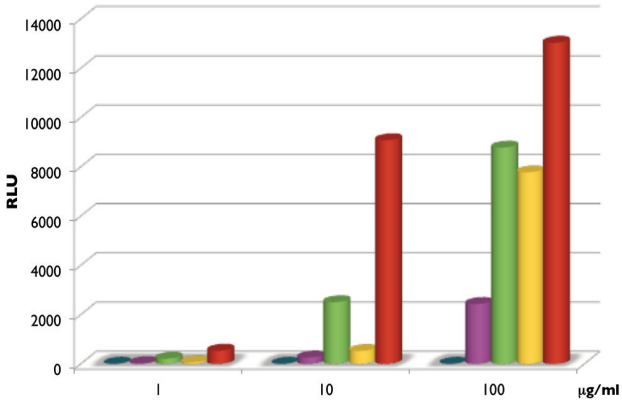
c-di-AMP, # tlrl-cda (1 mg) **c-di-GMP,** # tlrl-cdg (1 mg)
RAW-Blue™ ISG, # thp-isg (3-7 10^6 cells)

m-cGAS and h-cGAS genes, in a pUNO1 plasmid
shRNAs targeting m-cGAS and h-cGAS, in a psiRNA-7SKGFPzeo plasmid

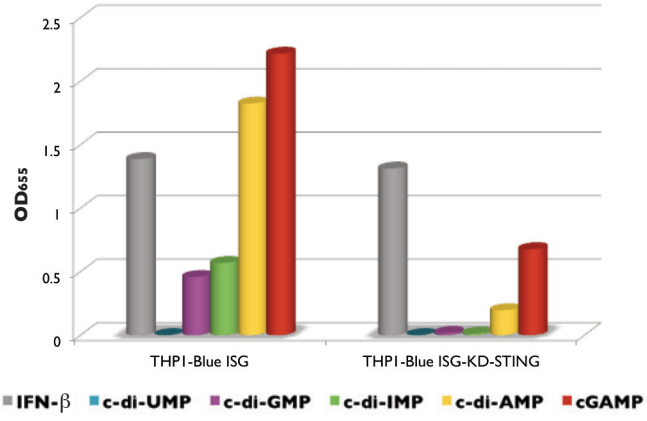
1- THP1-Blue™ ISG response



2- RAW-Lucia™ ISG response



3- THP1-Blue™ ISG & THP1-Blue™ ISG-KD-STING response



Figures 1, 2 & 3. THP1-Blue™ ISG cells (1) and RAW-Lucia™ ISG cells (2) were stimulated with increasing concentrations of cGAMP, c-di-AMP, c-di-IMP, c-di-GMP or c-di-UMP. Cells were not permeabilized. After 24h incubation, the levels of IRF-induced SEAP (1) or IRF-induced Lucia™ (2) were determined using QUANTI-Blue™ or QUANTI-Luc™, respectively. (3) THP1-Blue™ ISG cells and THP1-Blue™ ISG-KD-STING cells were stimulated with 50 µg/ml of cyclic dinucleotides or 10^4 UI/ml IFN-β. After 24h incubation, the levels of IRF-induced SEAP were determined using QUANTI-Blue™.

➢ KD-STING Reporter Cells

THP1-Blue™ ISG-KD-STING cells NEW

THP1-Blue™ ISG-KD-STING cells and **THP1-Blue™ ISG cells** are derived from human THP-1 monocytes, a cell line often used for the study of DNA sensing pathways as they express all the cytosolic DNA sensors identified so far (with the exception of DAI). THP1-Blue™ ISG-KD-STING cells were generated from THP1-Blue™ ISG cells through knockdown of the STING gene. As a result, THP1-Blue™ ISG-KD-STING cells display a considerable reduction of STING expression.

THP1-Blue™ ISG-KD-STING and THP1-Blue™ ISG cells express the SEAP (secreted embryonic alkaline phosphatase) reporter gene under the control of an IRF-inducible promoter. This composite promoter is comprised of five IFN-stimulated response elements (ISRE) fused to an ISG54 minimal promoter. Both cell lines allow the monitoring of IRF activation by determining the activity of SEAP. The levels of IRF-induced SEAP in the cell culture supernatant are readily assessed with QUANTI-Blue™, a SEAP detection reagent.

THP1-Blue™ ISG-KD-STING cells respond poorly to cytosolic DNA and cyclic dinucleotides compared to THP1-Blue™ ISG cells. Note, both cell lines exhibit comparable IFN response (Fig. 3).

THP1-Blue™ ISG-KD-STING and THP1-Blue™ ISG cells are resistant to Zeocin™.

PRODUCT	QUANTITY	CAT. CODE
cGAMP	500 µg	tlrl-cga-s
cGAMP	1 mg	tlrl-cga
c-di-IMP	1 mg	tlrl-cdi
c-di-UMP	1 mg	tlrl-cdu
THP1-Blue™ ISG-KD-STING	3-7 x 10^6 cells	thp-kdstg
THP1-Blue™ ISG	3-7 x 10^6 cells	thp-isg

Contents and Storage

cGAMP, c-di-IMP and c-di-UMP are provided lyophilized. Products are free of TLR2 and TLR4 activities. They are shipped at room temperature. Store at -20°C.

THP1-Blue™ ISG-KD-STING and THP1-Blue™ ISG cells are grown in RPMI medium, 2mM L-glutamine, 10% FBS supplemented with 100 µg/ml Normocin™ and 100 µg/ml Zeocin™. Cells are provided frozen in a cryotube containing 3-7 x 10^6 cells and supplied with 50 mg of Normocin™, 10 mg Zeocin™ and 1 pouch of QUANTI-Blue™. Cells are guaranteed mycoplasma-free.

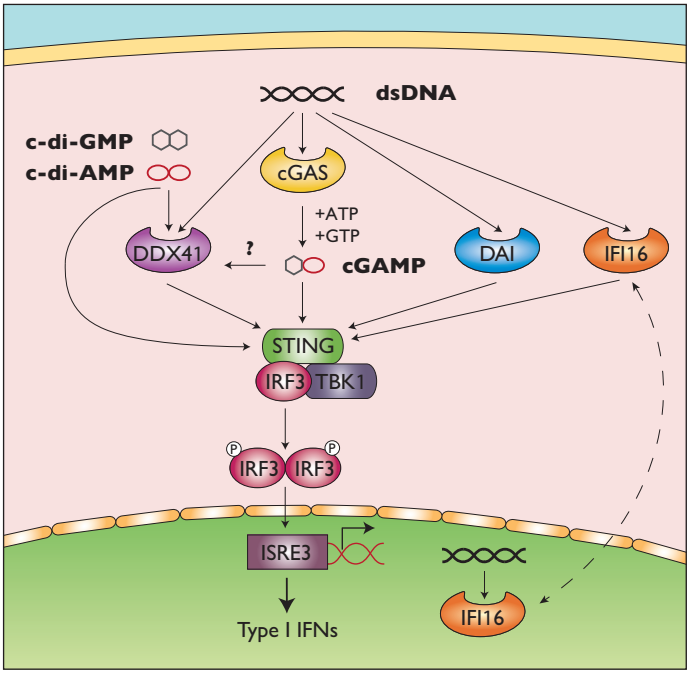
cGAS - The long awaited cytosolic DNA sensor?

The detection of viral and bacterial nucleic acids by the innate immune system has become an area of intense research. Cytosolic DNA is well-known to induce the production of type I interferons (IFNs) through the STING-TBK1-IRF3 axis but the mechanism whereby it is sensed remains elusive.

In two recent articles published in Science, Chen and colleagues describe the discovery of cGAMP, a second messenger that triggers IFN production in response to cytosolic DNA[1], and cGAS, an enzyme that catalyzes the synthesis of cGAMP and acts as a cytosolic DNA sensor[2]. In the first article, Wu *et al.* have found that cytosolic extracts of murine cells transfected with DNA, such as ISD or poly(dA:dT), activated IRF3 and that the activator present in these extracts was not a protein, DNA or RNA. Through mass spectrometry, the authors identified cyclic GMP-AMP (cGAMP), the first known cyclic dinucleotide in metazoa, as the activator of IRF3. Further, they have demonstrated that cGAMP binds to and activates STING leading to the dimerization of IRF3. Interestingly, two other cyclic dinucleotides, c-di-AMP and c-di-GMP, which are produced by bacteria, have recently been found to also bind STING[3].

In the second article, Sun *et al.*, describe the identification of the cGAMP synthase (cGAS), a new member of the nucleotidyltransferase family. Using biochemical fractionation and quantitative mass spectrometry, they purified and identified the mouse protein E330016A19 as m-cGAS and the human homolog C6orf150 (also known as MB21D1) as h-cGAS. Expression levels of m-cGAS and h-cGAS in different cell lines correlated with the ability of these cells to produce cGAMP and induce IFN-β in response to cytosolic DNA. cGAS was found to catalyze the synthesis cGAMP from ATP and GTP and activate IRF3 in a STING-dependent manner in response to DNA transfection or DNA virus infection. Further, cGAS was demonstrated to bind to DNA in the cytoplasm and therefore function as a cytosolic DNA sensor.

Wu *et al.* and Sun *et al.* reveal a novel mechanism by which cGAS senses cytosolic DNA and generates the second messenger cGAMP which binds to STING and activates the STING-TBK1-IRF3 signaling axis leading to the production of type I IFNs. How other cytosolic DNA sensors fit into this process is unclear. Recently, DDX41 was reported to bind c-di-AMP and c-di-GMP upstream of STING[4]. Although controversial, this finding raises the question whether the DNA sensor DDX41 can also bind cGAMP and thus acts between cGAS and STING. IFI16 is another DNA sensor that seems to be a key player in the innate immune response to DNA[5]. What is the role of IFI16 relative to cGAS and the other DNA sensors? Further studies are required and one can suspect that cell-type specificity will be found.



1. Wu J. *et al.*, 2012. Cyclic GMP-AMP Is an Endogenous Second Messenger in Innate Immune Signaling by Cytosolic DNA. Science. 339(6121):826-30. **2. Sun L. *et al.*, 2012.** Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. Science. 339(6121):786-91. **3. Burdette DL. *et al.*, 2011.** STING is a direct innate immune sensor of cyclic di-GMP. Nature. 478(7370):515-8. **4. Parvatiyar K. *et al.*, 2012** The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response. Nat. Immunol. 13(12):1155-61. **5. Veeranki S. & Choubey D., 2012.** Interferon-inducible p200-family protein IFI16, an innate immune sensor for cytosolic and nuclear double-stranded DNA: regulation of subcellular localization. Mol Immunol. 49(4):567-71.