InvivoGen Insight
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Activation of the NLRP3 Inflammasome

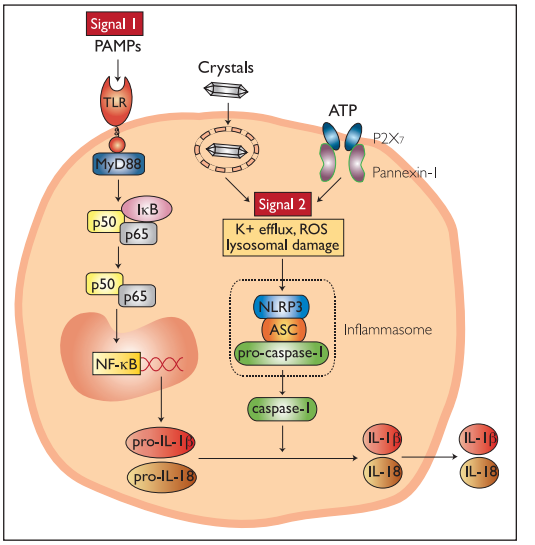
The inflammasome is a large multiprotein complex which plays a key role in innate immunity by participating in the production of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18. These related cytokines cause a wide variety of biological effects associated with infection, inflammation and autoimmune processes. They are both produced as inactive precursors, prol-IL-1β and pro-IL-18, and share a common maturation mechanism that requires caspase-1. Caspase-1 itself is synthesized as a zymogen, pro-caspase-1, that undergoes autocatalytic processing resulting in two subunits that form the active caspase-1. Activation of caspase-1 occurs within the inflammasome following its assembly. The best characterized inflammasome is the NLRP3 (also known as NALP3 and cryopyrin) inflammasome. It comprises the NLR protein NLRP3, the adapter ASC and pro-caspase-1. The general consensus is that maturation and release of IL-1β requires two distinct signals: the first signal leads to synthesis of pro-IL-1β and other components of the inflammasome, such as NLRP3 itself; the second signal results in the assembly of the NLRP3 inflammasome, caspase-1 activation and IL-1β secretion (see figure).

Activation of the NRLP3 inflammasome can be triggered by numerous stimuli, chemically and structurally highly different. Microbial molecules (pathogen associated molecular patterns, PAMPs), such as bacterial lipopolysaccharide and fungal zymosan, can activate the NLRP3 inflammasome and induce IL-1β secretion in the presence of ATP[1]. External ATP, considered as a danger signal, causes the opening of the $P2X_7$ receptor and the subsequent recruitment of the channel pannexin-1 leading to the release of intracellular potassium. The bacterial toxin nigericin has also been reported to induce the activation of NLRP3 by causing potassium efflux in a pannexin-1-dependent manner[2].

Besides PAMPs, the NLRP3 inflammasome can be activated by molecules associated with stress or danger, including crystalline and particulate substances. Crystals of uric acid and calcium pyrophosphate dihydrate, the aetiological agents of gout and pseudogout respectively, were the first crystals shown to engage the NLRP3 inflammasome[3]. Then, asbestos and silica were demonstrated to cause inflammatory lung diseases through a similar mechanism[4]. This mechanism is also responsible for the adjuvant properties of alum[5] and the pathogenicity of fibrillar amyloid-β, a particulate substance associated with Alzheimer's disease[6]. Recently, malarial hemozoin, a heme crystal, was shown to act as a danger signal that activates the NLRP3 inflammasome[7].

Considering the differences in the structure and function of the molecules reported to activate the NLRP3 inflammasome, it is unlikely that they directly interact with NLRP3. Several mechanisms seem to play a role in the assembly of the NLRP3 inflammasome. Membrane damage appears to be a common step in NLRP3 activation shared by a number of stimuli including ATP, nigericin and crystals. ATP and nigericin cause membrane damage by inducing the formation of a pore[2], while crystals create lysosomal membrane damage following their phagocytosis[5]. One hypothesis to explain how membrane damage can trigger NLRP3 activation is that this damage causes the modification or release of an endogenous molecule that is recognized by NLRP3. Another event that appears to be required for the activation of NLRP3 is the efflux of intracellular potassium. Indeed, glyburide (also known as glybenclamide), an inhibitor of ATP-sensitive K+ channels, was shown to block the activation of the NLRP3 inflammasome in reponse to ATP, nigericin and crystals[8]. Lastly, the generation of reactive oxygen species (ROS) seems also critical for the activation of the NLRP3 inflammasome[7].

The increasing number of publications on the inflammasome highlight its importance in the immune response to microbial molecules and danger signals. The inflammasome is becoming an attractive target for therapeutic intervention in a wide range of inflammatory diseases, including autoimmune diseases. However, more studies need to be carried out to better understand the activation and regulation of the inflammasome. In an effort to assist scientists in their studies on the inflammasome, InvivoGen provides a set of tools comprising a cell line specifically engineered to detect mature IL-1β, and molecules that act as inducers or inhibitors of the inflammasome.



1. Lamkanfi M. *et al.*, 2009. Fungal zymosan and mannan activate the cryopyrin inflammasome. J Biol Chem. 284(31):20574-81. **2. Pelegrin P, Surprenant A., 2007.** Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1beta release through a dye uptake-independent pathway. J Biol Chem. 282(4):2386-94. **3. Martinon F. *et al.*, 2006.** Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature. 440(7081):237-41. **4. Dostert C. *et al.*, 2008.** Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica. Science. 320(5876):674-7. **5. Hornung V, *et al.*, 2008.** Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat Immunol. 9(8):847-56. **6. Halle A. *et al.*, 2008.** The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat Immunol. 9(8):857-65. **7. Dostert C. *et al.*, 2009.** Malarial hemozoin is a Nalp3 inflammasome activating danger signal. PLoS One. 4(8):e6510. **8. Lamkanfi M. *et al.*, 2009.** Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. J Cell Biol. 187(1):61-70.

Inflammasome Inducers & Inhibitors

The NLRP3 inflammasome is a multi-protein complex involved in the production of mature IL-1β. It is activated by a large variety of microbial molecules, danger signals and crystalline substances. InvivoGen provides a selection of these molecules known to induce the assembly of the NLRP3 inflammasome. We also offer some inhibitors to confirm the ability of a given molecule to activate the inflammasome or to test the importance of phagocytosis or potassium efflux in this process. These inflammasome inducers and inhibitors have been validated using the THP-1/HEK-Blue™-IL-1β Assay (see next page).

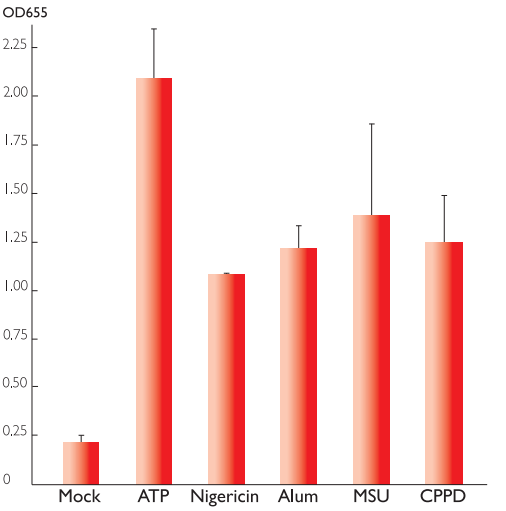
Inducers

- ATP
- Nigericin

Extracellular ATP is a stress-associated danger signal that activates NRLP3 and caspase-1[1]. ATP triggers the opening of the $P2X_7$ receptor resulting in potassium efflux which is necessary for the maturation of IL-1β[2]. ATP-induced caspase-1 activation requires another channel, pannexin-1, which is recruited upon $P2X_7$ activation[3]. Like ATP, the microbial toxin nigericin engages the NLRP3 inflammasome[1]. It acts as a cation ionophore that induces a net decrease in intracellular levels of potassium. Nigericin has been shown to signal independently of the $P2X_7$ receptor[4], but to require pannexin-1[3].

- Alum Crystals
- Monosodium Urate (MSU) Crystals **NEW**
- Calcium Pyrophosphate Dihydrate (CPPD) Crystals **NEW**

The NLRP3 inflammasome is activated by crystalline stimuli such as the vaccine adjuvant alum[5] and the pathogenic crystals MSU and CPPD[6], the aetiological agents of gout and pseudogout, respectively. Alum salts exert their adjuvancy by increasing the secretion of IL-1β in a NRLP3-dependent manner[5, 7]. MSU- and CPPD-induced activation of caspase-1 also requires the NRLP3 inflammasome as macrophages from mice deficient in various components of the inflammasome are defective in crystal-induced IL-1β induction[6]. NLRP3 activation requires the phagocytosis of crystals that leads to lysosomal damage that appears to be the signal recognized by the inflammasome resulting in its activation[8].



IL-1β secretion by inflammasome inducers. THP1 cells pretreated with PMA and primed with LPS (1 μg/ml) were stimulated with ATP (5 mM), nigericin (1 μM), alum (200 μg/ml), MSU (200 μg/ml) or CPPD (200 μg/ml). After 24h incubation, THP-1 supernatants were added to HEK-Blue™ IL-1β cells. IL-1β-induced NF-κB activation was assessed by measuring the levels of SEAP in the supernatant of HEK-Blue™ IL-1β cells using the QUANTI-Blue™ assay.

Inhibitors

- Colchicine: **crystal phagocytosis inhibitor** **NEW**

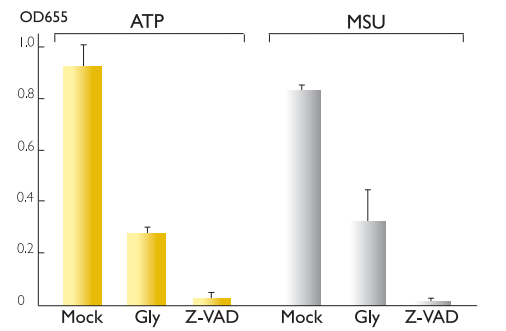
Colchicine is a drug frequently used for the treatment of autoinflammatory diseases, such as gout and pseudogout[9]. It works by inhibiting the microtubule assembly. Colchicine has been shown to block crystal-induced IL-1β generation upstream of the NLRP3 inflammasome suggesting that the drug acts at the level of crystal endocytosis and/or presentation to the inflammasome[6].

- Glybenclamide (glyburide): **proton pump inhibitor** **NEW**

Glybenclamide, also known as glyburide, blocks the maturation of caspase-1 and pro-IL-1β by inhibiting the K+ efflux[10]. Glybenclamide was shown to potently block the activation of the NRLP3 inflammasome induced by PAMPs, DAMPs and crystalline substances[11, 12]. Recent data suggest that glybenclamide works downstream of the $P2X_7$ receptor but upstream of NRLP3[11].

- Z-VAD-FMK: **caspase inhibitors** **NEW**

Z-VAD-FMK is as a cell-permeable irreversible pan-caspase inhibitor. It potently inhibits caspase-1 activation in NLRP3-induced cells[12].



Inhibition of ATP- or MSU-induced IL-1β secretion. THP-1 cells primed with LPS (1 μg/ml) were stimulated with ATP (5 mM) or MSU (200 μg/ml) in the presence or not of glyburide (25 μg/ml) or Z-VAD-FMK. After 24h incubation, THP-1 supernatants were added to HEK-Blue™ IL-1β cells. IL-1β-induced NF-κB activation was assessed by measuring the levels of SEAP in the supernatant of HEK-Blue™ IL-1β cells using the QUANTI-Blue™ assay.

Product	Quantity	Cat. Code
ATP	1 g	tlrl-atp
Nigericin	10 mg	tlrl-nig
Alum crystals	1 g	tlrl-alk
MSU crystals	5 mg	tlrl-msu
CPPD crystals	5 mg	tlrl-cppd
Colchicine	1 g	tlrl-col
Glybenclamide	1 g	tlrl-gly
Z-VAD-FMK	1 mg	tlrl-vad

1. Mariathasan S. *et al.*, 2006. Nature 440;228-32. **2. Ferrari D. *et al.*, 2006.** J Immunol. 176(7):3877-83. **3. Pelegrin P. & Surprenant A., 2007.** J Biol Chem. 282(4):2386-94. **4. Solle M. *et al.*, 2001.** J Biol Chem. 276(1):125-32. **5. Li H. *et al.*, 2008.** J Immunol. 181:17-21. **6. Martinon F. *et al.*, 2006.** Nature. 440(7081):237-41. **7. Eisenbarth SC. *et al.*, 2008.** Nature. 453(7198):1122-6. **8. Hornung V. *et al.*, 2008.** Nature Immunol. 9:847-856. **9. Molad Y., 2002.** Curr. Rheumatol. Rep. 4:252-256. **10. Laliberte RE. *et al.*, 1999.** J Biol Chem. 274(52):36944-51. **11. Lamkanfi M. *et al.*, 2009.** J. Cell Biol., 187: 61 - 70. **12. Dostert C. *et al.*, 2009.** PLoS One. 4(8):e6510.

Contents and storage

Products are endotoxin-tested and validated using the THP-1/HEK-Blue™ IL-1β assay (see next page). Each product is provided as a solid and shipped at room temperature. Store at room temperature, 4°C or -20°C according to the product label.

HEK-Blue™ IL-1β Cells - Detection of Interleukin-1β in Inflammasome Studies

Many studies on the inflammasome use the human monocytic THP-1 cell line and Western blot or ELISA for the detection of mature IL-1β. InvivoGen has developed a new method to detect bioactive IL-1β that is simple, rapid and cost-effective. This method is based on HEK293 cells specifically engineered to selectively respond to IL-1β, named HEK-Blue IL-1β.

Principle

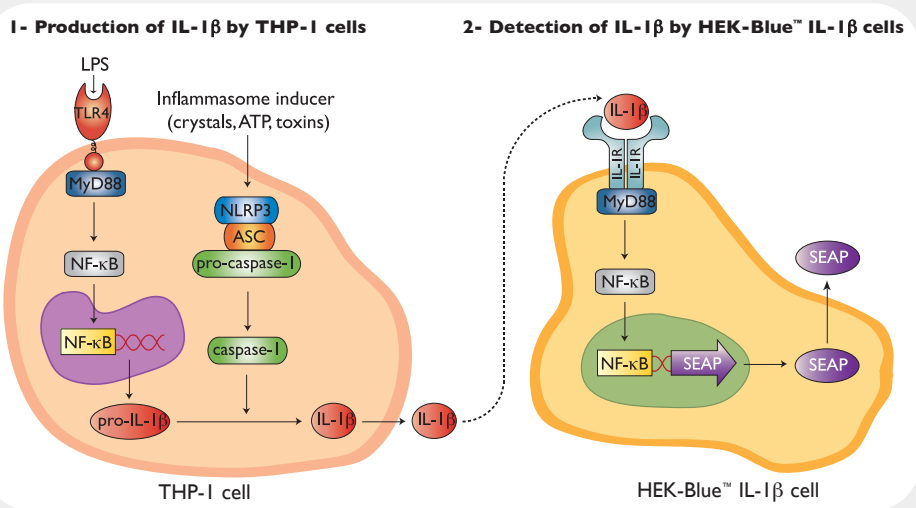
HEK-Blue™ IL-1β cells provide a convenient read-out to determine the amount of IL-1β secreted by THP-1 cells following stimulation by NLRP3 inflammasome inducers.

HEK-Blue™ IL-1β cells feature the SEAP (secreted embryonic alkaline phosphotase) reporter gene under the control of an NF-κB-inducible promoter. They naturally express the IL-1β receptor (IL-1R), and all the proteins involved in the MyD88-dependent IL-1R signaling pathway that leads to NF-κB activation. Thus upon IL-1β binding to IL-1R, a signaling cascade is initiated triggering NF-κB activation and the subsequent production of SEAP. Detection of SEAP in the supernatant of HEK-Blue™ IL-1β cells can be readily assessed using QUANTI-Blue™, a SEAP detection medium. QUANTI-Blue™ turns blue in the presence of SEAP which can be easily quantified using a spectrophotometer.

Contents

HEK-Blue™ IL-1β cells are grown in DMEM medium with 10% FBS, 100 µg/ml Zeocin™ and 200 µg/ml HygroGold™ (ultrapure Hygromycin). Each vial contains 3-5 x 10^6 cells and is supplied with 10 mg Zeocin™ and 10 mg HygroGold™. Cells are shipped on dry ice.

Product	Quantity	Cat. Code
HEK-Blue™ IL-1β Cells	3-5 x 10^6 cells	hkb-il1b
QUANTI-Blue™	5 pouches	rep-qb1
HygroGold™	1 g	ant-hg-1
Zeocin™	1 g	ant-zn-1



THP-1/HEK-Blue™ IL-1β Assay

1- Production of IL-1β by THP-1 cells: Typically, THP-1 cells are pretreated with phorbol 12-myristate acetate (PMA) to become more susceptible to inflammasome activators, then are primed with lipopolysaccharide (LPS). These treatments induce the production of pro-IL-1β, the immature form of IL-1β. Subsequent stimulation with inflammasome inducers, such as crystals or ATP, leads to NRLP3 and caspase-1 activation resulting in IL-1β maturation and secretion.

2- Detection of IL-1β by HEK-Blue™ IL-1β cells: IL-1β-containing THP-1 supernatants are added to HEK-Blue™ IL-1β cells leading to NF-κB activation and the subsequent production of SEAP. The presence of SEAP in HEK-Blue™ IL-1β supernatants is assessed using QUANTI-Blue™, a SEAP detection medium.

Pepinh-MYD & Pepinh-TRIF - Inhibitory Peptides of TLR Signaling

Pepinh-MYD and pepinh-TRIF are cell-penetrating peptides that interfere with TLR signaling. They contain the BB loop sequences of the TIR domains of MyD88 or TRIF preceeded by a protein transduction sequence derived from antennapedia which enables the peptide to translocate through the cell membrane[1]. Pepinh-MYD blocks MyD88 signaling by inhibiting its homodimerization through binding[2]. Pepinh-TRIF inhibits TRIF signaling by interfering with TLR-TRIF interaction[3].

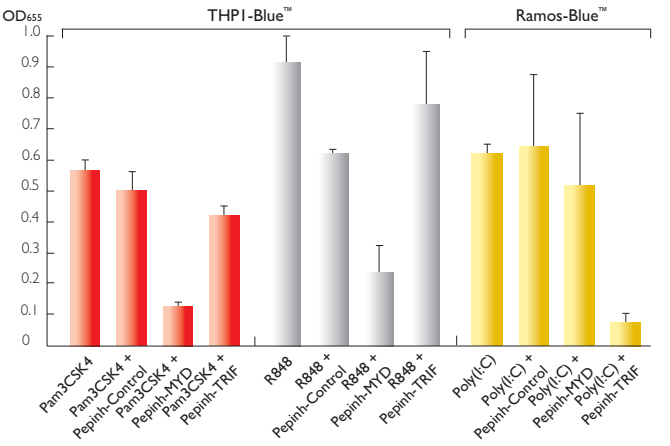
Pepinh-MYD was shown to significantly block TLR2- and TLR8-induced NF-κB activation in THP1-Blue™ cells while the efficacy of Pepinh-TRIF was confirmed in Ramos-Blue™ cells induced by the TLR3 ligand poly(I:C) (see graphs).

Contents

Both inhibitory peptides are provided lyophilized with a control peptide.

1. Derossi D. *et al.,* **1994**. J. Biol. Chem., 269: 10444-50. 2. Loiarro M. *et al.,* **2005**. J. Biol. Chem., 280: 15809-14. 3. Toshchakov VU. *et al.,* **2005**. J. Immunol., 175: 494 - 500.

Product		Quantity	Cat. Code
Pepinh-MYD	**NEW**	2 mg	tlrl-pimyd
Pepinh-TRIF	**NEW**	2 mg	tlrl-pitrif



Inhibitory activities of Pepinh-MYD and Pepinh-TRIF. THP1-Blue™ cells and Ramos-Blue™ cells were pretreated with 50 µM Pepinh-Control, Pepinh-MYD or Pepinh-TRIF for 6 hours. Then THP1-Blue™ cells were stimulated with Pam3CSK4 (100 ng/ml) or R848 (10 µg/ml) and Ramos-Blue™ cells with poly(I:C) (100 ng/ml). After 24h incubation, NF-κB activation was assessed by measuring the levels of SEAP in the supernatant using the QUANTI-Blue™ assay.

Ramos-Blue™ Cells

NF-κB/AP-1 reporter B lymphocytes

B lymphocytes are key players in the adaptative immune system but are also prominent in the innate immune response. Consistent with their dual role, they express Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) that allow them to discriminate among a wide spectrum of pathogen-associated molecules (PAMPs). Upon PRR stimulation by PAMPs, various signaling pathways are induced leading to the activation of transcription factors, such as NF-κB and AP-1, and the subsequent production of inflammatory cytokines.

Description

Ramos-Blue™ is a B lymphocyte cell line that stably expresses an NF-κB/AP-1-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene. Ramos-Blue™ cells derive from a human Burkitt's lymphoma which is negative for Epstein Barr virus. They have the characteristics of B lymphocytes and are routinely used as a model of B lymphocytes and for apoptosis studies. The Ramos-Blue™ cell line was isolated for its ability to respond to CpG ODNs (TLR9 ligands).

Ramos-Blue™ cells are responsive to NF-κB inducers, such as TNF-α and TLR agonists. When stimulated, they produce SEAP in the supernatant that can be readily monitored using QUANTI-Blue™. QUANTI-Blue™ is a SEAP detection medium that turns blue in the presence of SEAP. Levels of SEAP can be determined qualitatively with the naked eye or quantitatively using a spectrophotometer at 620-655 nm.

Ramos-Blue™ cells express all TLRs and NOD1 mRNAs as detected by RT-PCR (figure 1). However, activation of NF-κB/AP-1 was only observed following stimulation with TLR2, TLR3, TLR7, TLR9 and NOD1 agonists (Figures 2 & 3).

Ramos-Blue™ cells are resistant to Zeocin™ (100 µg/ml). The cells are guaranteed mycoplasma-free.

Applications

Ramos-Blue™ cells can be used to study the NF-κB and AP-1 signaling pathways in B lymphocytes and in particular the TLR3, TLR7, TLR9 and NOD1 signaling pathways.

Contents

Ramos-Blue™ cells are grown in IMDM medium, 2 mM L-glutamine, 10% FBS supplemented with 100 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.

Product	Quantity	Cat. Code
Ramos-Blue™ cells **NEW**	3-5 x 10^6 cells	rms-sp
QUANTI-Blue™	5 x 100 ml	rep-qb1
Zeocin™	1 g	ant-zn-1

Check our extensive list of TLR and NOD ligands on our website
www.invivogen.com/innate-immunity-pamps

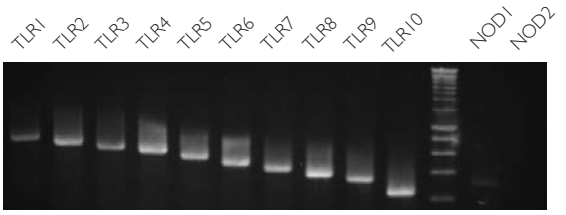


Figure 1: Expression of TLR and NOD mRNAs in Ramos-Blue™ cells determined by RT-PCR.

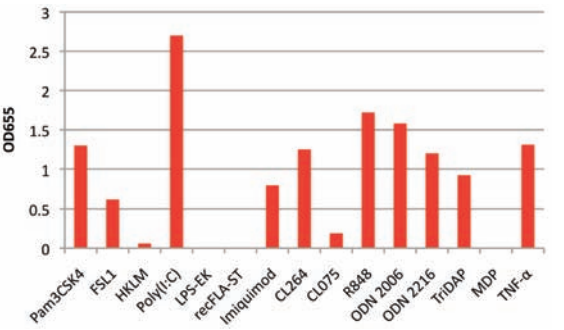


Figure 2: NF-κB/AP-1 activation in Ramos-Blue™ cells induced by various activators. Cells were incubated with 10 µg/ml each of Pam3CSK4 (TLR1/2), FSL-1 (TLR2/6), HKLM (TLR2, 1.10^9 cells/ml), poly(I:C) (TLR3), LPS-EK (TLR4), recFLA-ST (TLR5), imiquimod and CL264 (TLR7), CL075 (TLR8), R848 (TLR7/8), ODN2006 and ODN2216 (TLR9), TriDAP (NOD1), MDP (NOD2) and TNF-α. After 24h incubation, NF-κB/AP-1 activation was assessed by measuring the levels of SEAP in the supernatant using QUANTI-Blue™.

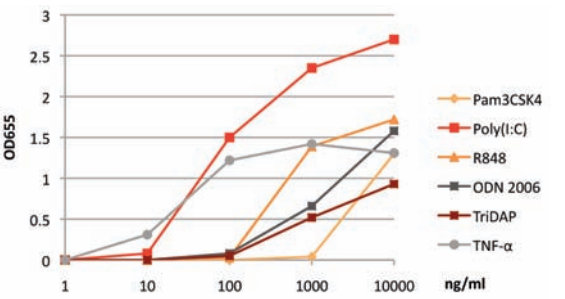


Figure 3: Dose responses to various NF-κB activators. Ramos™-Blue cells were incubated with increasing concentrations (1 ng/ml to 10 µg/ml) of Pam3CSK4, poly(I:C), R848, ODN2006, TriDAP and TNF-α. After 24h incubation, NF-κB/AP-1 activation was determined using the QUANTI-Blue™ assay.

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