InvivoGen Insight
INNOVATION WITHIN REACH
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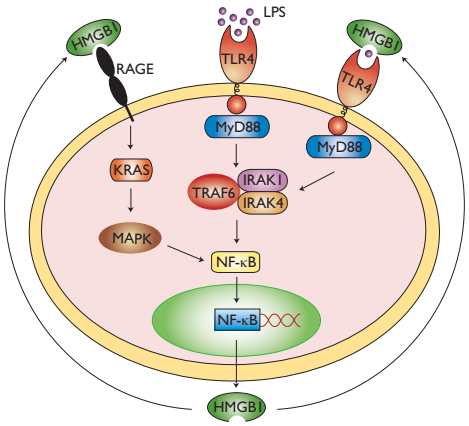
DAMPs: Endogenous Ligands of TLRs?

The Third International DAMPs and Alarmins Symposium was recently held in Pittsburgh, USA. The aim of this meeting was to introduce the emergent understanding of the danger signals also called alarmins or damage associated molecular patterns (DAMPs) by analogy to the pathogen associated molecular patterns (PAMPs). What brought our attention to this meeting is the recent discovery that TLRs play an important role in the immune response initiated by the DAMPs. The major DAMPs are HMGB1 (high mobility group box protein-1), S100A8/S100A9, heat-shock proteins, uric acid and DNA. Among these DAMPs, HMGB1 is the most studied as it has been associated with several diseases, including cancer, sepsis, rheumatoid arthritis, stroke and atherosclerosis. HMGB1 is a very abundant nuclear protein expressed in nearly all cell types. In normal conditions, HMGB1 binds to DNA and bends it to facilitate gene transcription. Under stress conditions, such as injury or infection, HMGB1 is released and promotes inflammation. HMGB1 is passively released by necrotic but not apoptotic death of normal cells and actively secreted by a variety of activated immune and non-immune cells. Contrary to many reports, HMGB1 is not a pro-inflammatory cytokine *per se*. HMGB1 by itself has little or no pro-inflammatory activity but it binds to mediators of inflammation such as LPS, DNA or IL-1β and induces signaling pathways leading to NF-κB activation thereby potentiating inflammatory responses. Although the signaling pathways elicited by HMGB1 are not fully defined, there is evidence that the triggering occurs via several receptors including RAGE (receptor for advanced glycation end-products), TLR2, TLR4 and TLR9[1]. HMGB1 binds RAGE to regulate migratory responses, but the use of ultrapure recombinant HMGB1 has demonstrated that it does not bind TLR4 (M. Bianchi, oral communication). However, HMGB1 which is released upon LPS-induced TLR4 activation, binds LPS even if present in very small amounts and carries it to TLR4 therefore perpetuating NF-κB activation and inflammation (see Figure). A similar mechanism was reported for DNA, which is released into the systemic circulation after traumatic shock or injury, and presented to TLR9 by HMGB1[2]. Thus, HMGB1 is not an endogenous ligand for TLRs but an amplifier of TLR-mediated inflammatory responses.

S100A8 (also known as Myeloid-related protein-8, MRP-8) and S100A9 (MRP14) are highly up-regulated in various diseases, such as sepsis, rheumatoid arthritis, inflammatory bowel disease and cancer. These calcium-binding proteins are the most abundant cytoplasmic proteins of neutrophils and monocytes. They are specifically released at sites of inflammation during the activation of phagocytes. S100A8 and S100A9 form complexes in which S100A8 appears to be the active component, while S100A9 modulates the activity of its binding partner. Although the biological functions of these proteins are not completely understood, they seem to depend on interactions with RAGE and TLR4. Similarly to HMGB1, S100A8-S100A9 complexes amplify the LPS-triggered inflammatory responses of phagocytes. But unlike HMGB1 and according to current knowledge, they can bind to both RAGE and TLR4[3].



HMGB1 signaling pathways in sepsis

The list of DAMPs is rapidly increasing with new additions such as granulysin, eosinophil-derived neurotoxin and serum amyloid A (SAA). A recent report suggests that SAA induces inflammation in a TLR2-dependent manner while another report claims that SAA is an endogenous agonist for TLR4[4,5].

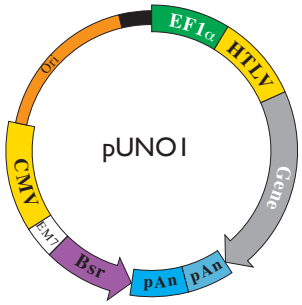
It is clear that DAMPs interact with TLRs, however it is less clear whether they bind to them. Recombinant DAMP proteins may contain traces of lipoproteins or endotoxins that may be sufficent to skew the results. Before this meeting on DAMPs and Alarmins, HMGB1 was thought to be a TLR4 ligand. This highlights how crucial it is to work with DAMP preparations that are totally free of microbial contaminants in order to understand the role of DAMPs in the numerous cellular processes in which they are involved.

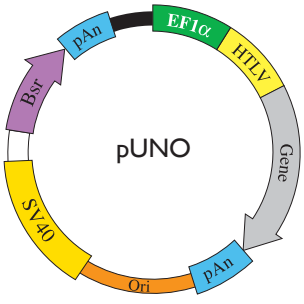
1. Klune JR. *et al.*, 2008. HMGB1: endogenous danger signaling. Mol Med. 14(7-8):476-84. **2. Tian J, *et al.*, 2007.** Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. Nat Immunol. 2007;8:487–96. **3. Foell D. *et al.*, 2007.** S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. J Leukoc Biol. 81(1):28-37. **4. Cheng N. *et al.*, 2008.** Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. J Immunol. 181(1):22-6. **5. Sandri S. *et al.*, 2008.** Is serum amyloid A an endogenous TLR4 agonist? J Leukoc Biol. 83(5):1174-80.

DAMPs & DAMP Receptors

Expression of DAMPs & DAMP Receptors

pUNO plasmids that express DAMPs or DAMP receptors can be useful to develop cellular models to study DAMP signaling. The genes can also be subcloned in a system appropriate for protein purification. pUNO plasmids contain the strong and ubiquitous EF1α/HTLV promoter and are selectable with blasticidin in both *E. coli* and mammalian cells. DAMPs genes are cloned downstream of a signal sequence to allow their secretion. Each pUNO plasmid is provided as a lyophilized transformed *E. coli* strain on paper disk.





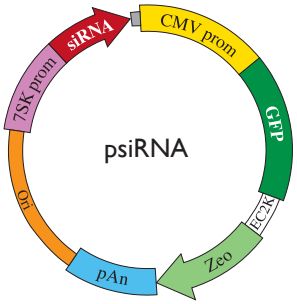
Product	Cat. Code (human)	Cat. Code (mouse)
DAMPs		
pUNO1-ssHMGB1	puno1-hhmgb1-s NEW	*
pUNO1-ssS100A8	puno1-hs100a8-s NEW	puno1-ms100a8-s NEW
pUNO1-ssS100A9	puno1-hs100a9-s NEW	puno1-ms100a9-s NEW
DAMP Receptors		
pUNO1-AGER (RAGE)	puno1-hager	puno1-mager
pUNO-TLR2	puno-htlr2	puno-mtlr2
pUNO-TLR4	puno-htlr4	puno-mtlr4

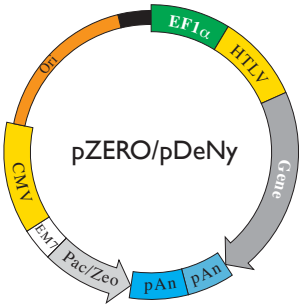
* Murine HMGB1 differs from the human form by only two amino acids.

Inhibition of DAMP Recognition & Signaling

psiRNA plasmids express short hairpin RNA (shRNA) that efficiently silence DAMP receptors. They feature the human 7SK RNA Pol III promoter that generates high amounts of shRNAs. They also feature a GFP::Zeo fusion gene which confers both reporter and antibiotic resistance activities allowing simple monitoring of transfection efficiency and selection in both *E. coli* and mammalian cells. psiRNA plasmids are provided as 20 µg of lyophilized DNA.

pZERO-TLR and pDeNy plasmids express dominant negative variants of TLRs or TLR signaling genes. They were created by insertion of a mutation and/or deletion in a key region. pZERO-TLR and pDeNy plasmids are selectable with puromycin or Zeocin™, respectively. They are provided as a lyophilized transformed *E. coli* strain on paper disk.





Product	Cat. Code (human)	Cat. Code (mouse)
RAGE Pathway		
psiRNA-AGER (RAGE)	psirna42-hager NEW	psirna42-mager NEW
psiRNA-KRAS	psirna42-hkras NEW	psirna42-mkras NEW
TLR Pathway		
psiRNA-TLR2	psirna42-htlr2	psirna42-mtlr2
psiRNA-TLR4	psirna42-htlr4	psirna42-mtlr4
psiRNA-MyD88	psirna42-hmyd88	psirna42-mmyd88
pZERO-TLR2	pzero-htlr2	pzero-mtlr2
pZERO-TLR4	pzero-htlr4a	pzero-mtlr4
pDeNy-MyD88	pdn-hmyd88	pdn-mmyd88

Reporter Cell Lines

HEK-Blue™-2 Cells - TLR2 Reporter Cell Line

HEK-Blue™-4 Cells - TLR4 Reporter Cell Line

HEK-Blue™ cells are engineered HEK293 cells that stably express an NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene. They were further transfected with either TLR2 and CD14 genes to generate HEK-Blue™-2 cells, or TLR4, MD2 and CD14 to generate HEK-Blue™-4 cells. Stimulation with a TLR2 or TLR4 agonist, induces the activation of NF-κB and the subsequent release of SEAP in the supernatant. The activation of TLR2 or TLR4 can be assessed by monitoring the amount of SEAP produced using a SEAP detection assay such as Quanti-Blue™ (for more information go to our website). HEK-Blue™-2 and HEK-Blue™-4 cells are useful and convenient cellular models to determine whether a DAMP is a TLR2 or TLR4 ligand.

HEK-Blue™-2 and HEK-Blue™-4 Cells are grown in DMEM medium with 10% FBS supplemented with 1X HEK-Blue™ Selection, an antibiotic mix. Each vial contains 3-5 x 10^6 cells and is supplied with 1 vial of 250X HEK-Blue™ Selection. Cells are shipped on dry ice.

Product	Quantity	Cat. Code
HEK-Blue™-2 Cells	3-5 x 10^6 cells	hb2-cells
HEK-Blue™-4 Cells	3-5 x 10^6 cells	hb4-cells
HEK-Blue™ Selection	4 x 2 ml	hb-sel

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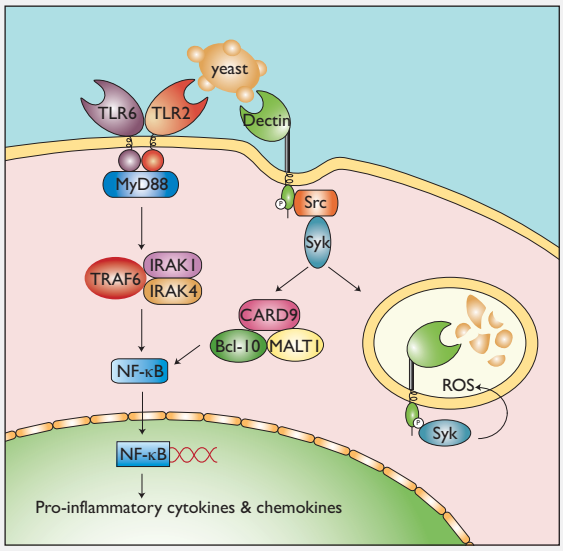
Dectin-1

A Major Receptor in Antifungal Immunity

Dectin-1 is a recently discovered pattern-recognition receptor that plays an important role in antifungal innate immunity. Dectin-1, which is expressed on phagocytes, is a specific receptor for β-glucans[1]. β-Glucans are glucose polymers found in the cell walls of fungi, such as zymosan (a cell wall preparation of *Saccharomyces cerevisiae*) and *Candida albicans*. Dectin-1 binds and internalizes β-glucans and mediates the production of reactive oxygen species (ROS), activation of NF-κB and subsequent secretion of pro-inflammatory cytokines. Zymosan, which is composed primarily of β-glucan, mannan, mannoprotein and chitin, induces immune responses that are both Dectin-1 and TLR2-dependent[2]. However, it is now clear that its β-glucan moiety triggers NF-κB activation only through Dectin-1 as treatment with hot alkali or organic solvents abrogates the TLR2-dependent response[2, 3].

Dectin-1 is a type II transmembrane protein with a C-type lectin-like carbohydrate recognition domain (CRD) connected by a stalk to the transmembrane region, followed by a cytoplasmic tail containing an immunoreceptor tyrosinase-based activation motif (ITAM). Dectin-1 binds specifically to β-1,3 glucans and induces its own signaling pathway[4, 5]. After binding to its ligand, Dectin-1 is phosphorylated by a non-receptor tyrosinase kinase Src. Syk is then activated and induces the CARD9-Bcl10-Malt1 complex. This complex mediates the activation of NF-κB and the production of pro-inflammatory cytokines. Recent data suggest that Dectin-1 and TLR2/TLR6 signalings combine to enhance the responses triggered by each receptor[2, 5].

β-Glucans display various biological activities, including anti-tumor and anti-infective activities, that depend on their physicochemical properties. Further studies are needed to clarify the specificity of both Dectin-1 and β-glucans, thereby allowing to elucidate the immunomodulatory activities of β-Glucans.



1. Brown GD. *et al.*, 2003. Dectin-1 mediates the biological effects of beta-glucans. J Exp Med. 197(9):1119-24. 2. **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. **3. Ikeda Y. *et al.*, 2008.** Dissociation of Toll-like receptor 2-mediated innate immune response to Zymosan by organic solvent-treatment without loss of Dectin-1 reactivity. Biol Pharm Bull. 31(1):13-8. **4. Gross O. *et al.*, 2006.** Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. Nature. 442(7103):651-6. **5. Dennehy KM & Brown GD., 2007.** The role of the beta-glucan receptor Dectin-1 in control of fungal infection. J Leukoc Biol.;82(2):253-8.

Dectin-1 Reporter Cell Line

RAW-Blue™ cells derived from the murine monocytic cell line RAW 264.7. They express many pattern-recognition receptors, including TLRs, NLRs and RLRs. RAW-Blue™ cells also express high levels of endogenous Dectin-1 and therefore can be used as a Dectin-1 reporter cell line in particular when combined with a neutralizing anti-Dectin-1 antibody. Stimulation of RAW-Blue™ cells with zymosan or heat-killed preparations of yeast induces the activation of NF-κB in a Dectin-dependent manner (see graph). NF-κB activation can be readily monitored as RAW-Blue™ cells stably express an NF-κB-inducible SEAP reporter gene.

RAW-Blue™ cells are grown in DMEM medium with 10% FBS 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.

Dectin-1 Ligands

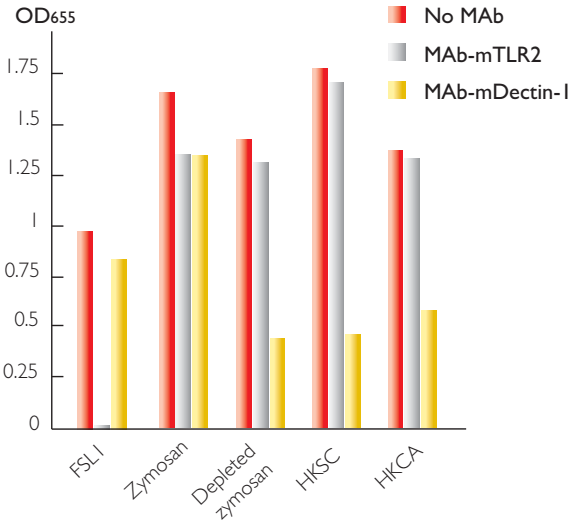
Dectin-1 recognizes a wide variety of fungal species, including the yeasts *Saccharomyces* and *Candida*. Although fungal β-glucans were orginally thought to be burried under a mannoprotein layer, they are actually displayed on the cell surface. InvivoGen provides, in addition to zymosan, new Dectin-1 agonists which unlike zymosan exhibit no TLR2 activity. All Dectin-1 ligands are provided lyophilized.

Zymosan is an insoluble cell wall preparation of *Saccharomyces cerevisiae* that is composed mainly of β-glucans, mannoproteins and chitin. Zymosan is often used as a representative fungal particle. Zymosan is a ligand for Dectin-1 and TLR2 which both induce the activation of NF-κB[1]. The response to zymosan can be reduced by using a neutralizing antibody against Dectin-1 or TLR2. Neither antibody can abrogate totally the response (see graph).

Depleted Zymosan was obtained by treating zymosan with hot alkali to remove all its TLR-stimulating properties. Depleted zymosan activates Dectin-1 but not TLR2.

HKSC is a heat-killed preparation of *S. cerevisiae*. This yeast is one of the most intensively studied eukaryotic model organisms in molecular and cell biology. HKSC derives from the strain FL200 (ATCC 32119). HKSC activity can be blocked by an anti-Dectin-1 antibody but not an anti-TLR2 antibody.

HKCA is a heat-killed preparation of *Candida albicans*. *C. albicans* is an opportunistic yeast that causes serious infections in immunocompromised patients. HKCA derives from the strain ATCC 10231. It activates Dectin-1 but not TLR2.



RAW-Blue™ cells were stimulated with FSL-1 (10 ng/ml), zymosan (10 μg/ml), depleted zymosan (100 μg/ml), HKSC (10^8 cells/ml) or HKCA (10^8 cells/ml) in the presence or absence of 10 μg/ml of an anti-mTLR2 (clone T2.5) or anti-mDectin-1 (clone 218820) monoclonal antibody. After 24h incubation, NF-κB activation was assessed by measuring the levels of SEAP in the supernatant by using QUANTI-Blue™, a SEAP detection medium.

Product	Quantity	Cat. Code
RAW-Blue™ Cells	3-5 x 10^6 cells	raw-sp
Zymosan	100 mg	tlrl-zyn
Depleted Zymosan NEW	10 mg	tlrl-dzn
HKSC NEW	10^9 cells	tlrl-hksc
HKCA NEW	10^9 cells	tlrl-hkca

TLR7 & TLR8 Activation

TLR7 and TLR8 play an important role in the innate antiviral immunity by recognizing viral RNA in endocytic compartments. TLR7/8 activation by viral RNA can be mimicked by short U-rich single-stranded RNAs (ssRNAs). According to Diebold *et al.*, this activation is sequence-independent as long as the ssRNAs contain several uridines in close proximity[1]. However, recent studies suggest sequence-dependent recognition of U-rich ssRNAs by TLR7 and TLR8. They demonstrate that AU-rich and GU-rich oligoribonucleotides (ORNs) are capable of activating TLR8, whereas only GU-rich ORNs are able to stimulate TLR7[2]. InvivoGen provides a selection of ssRNAs/ORNs described in the literature. All ssRNAs/ORNs have been tested in 293 cells stably transfected with TLR7 or TLR8 to confirm their specificity.

TLR7/8 RNA Agonists

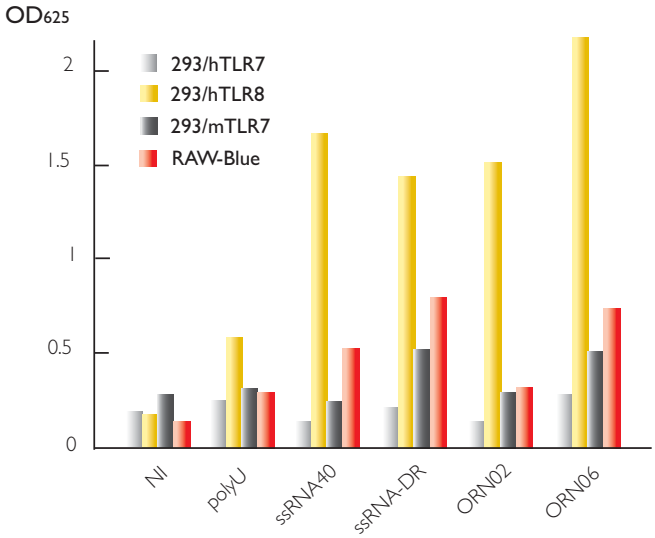
- **Poly(U)** is an RNA homopolymer of undefined length that activates mouse TLR7 and human pDC[1].
- **ssRNA40**, also known as R-1075, is a U-rich single-stranded RNA derived from the HIV-1 long terminal repeat[3].
- **ssRNA-DR** is a single-stranded RNA that contains 2 copies of the 9 mer sequence GUCCUUCAA, a motif responsible for the IFN-α induction of siRNA[4].
- **ORN06** contains 6 repeats of the UUGU sequence motif, identified as the minimal motif responsible for ssRNA40 immunoactivity[2].
- **ORN02** derives from ORN06 by substitution of G to A.[2]

ssRNA40 5'-GCCCGUCUGUUGUGUGACUC-3' (20 mer)
ssRNA-DR 5'-UGUCCUUCAAUGUCCUUCAA-3' (20 mer)
ORN02 5'-UUAUUAUUAUUAUUAUUAUU-3' (20 mer)
ORN06 5'-UUGUUGUUGUUGUUGUUGUU-3' (20 mer)

1. Diebold S. *et al.*, 2006. Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides. Eur. J. Immunol., 36:3256-67. **2. Forsbach A. *et al.*,** 2008. Identification of RNA Sequence Motifs Stimulating Sequence-Specific TLR8-Dependent Immune Responses. J. Immunol., 180: 3729-38. **3. Heil F. *et al.*, 2004.** Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8. Science, 303:1526-29. **4. Hornung V. *et al.*, 2005.** Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med., 11(3):263-70.

Contents and Storage

All ssRNAs/ORNs are phosphorothioate modified and complexed with the transfection reagent LyoVec to protect them from degradation and facilitate their cellular uptake. They are provided as 4 vials of 25 µg lyophilized complexes.



HEK293 cells stably expressing TLR7 or TLR8 and an NF-κB-inducible SEAP reporter gene, and RAW-Blue (mouse macrophage cell line) were stimulated with 5 µg/ml of various ssRNA agonists. After 24h incubation, NF-κB activation was assessed by measuring the levels of SEAP in the supernatant by using QUANTI-Blue™, a SEAP detection medium.

Product	Quantity	Cat. Code
ORN 02 NEW	4 x 25 µg	tlrl-orn2
ORN 06 NEW	4 x 25 µg	tlrl-orn6
Poly(U)	4 x 25 µg	tlrl-lpu
ssRNA40	4 x 25 µg	tlrl-lrna40
ssRNA-DR	4 x 25 µg	tlrl-ssdr

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