InvivoGen Insight
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Inside this issue:

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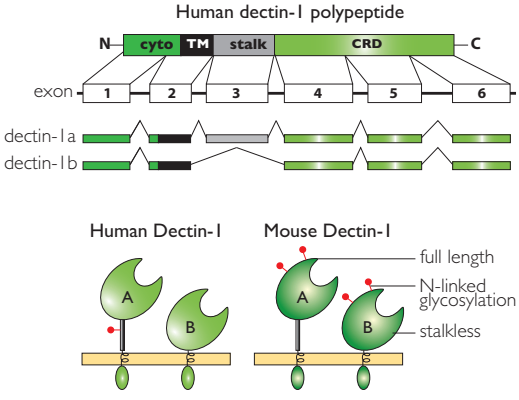
β-Glucans: bittersweet ligands of Dectin-1

β-Glucans have been consumed for many centuries for their healing properties. Since the discovery of their immunomodulating capabilities, about five decades ago, β-glucans have attracted a great deal of attention in the biomedical arena. Numerous articles have reported the biological activities of β-glucans including anti-infective, anti-cancer and wound repair activities. Unfortunately, many inconsistencies and contradictions remain unresolved.

β-Glucans are carbohydrates consisting of a backbone of glucose residues joined by β-(1→3) linkages with β(1→6) linked glucose side-chain residues. These polysaccharides are major cell wall structural components in fungi and are also found in plants and some bacteria. Depending on the source, β-glucans vary in the type of linkage, the degree of branching, molecular weight and tertiary structure (see pages 2&3). β-Glucans are not synthesized by animals and thus are recognized by the innate immune system as pathogen-associated molecular patterns. This recognition is mediated by pattern recognition receptors and, among them, Dectin-1 has emerged as the primary receptor for these carbohydrates[1].

Dectin-1 is a C-type lectin receptor expressed primarily by cells of myeloid origin, including macrophages, dendritic cells and neutrophils. In human and mouse, Dectin-1 is alternatively spliced into two major isoforms, a full-length A isoform and a 'stalkless' B isoform (fig. 1). Human Dectin-1 (hDectin-1) is structurally similar to mouse Dectin-1 (mDectin-1) with 60% identity in amino acid sequence, but display differences in the number and position of N-linked glycosylation (fig. 1). N-linked glycosylation has been shown to affect the cell surface expression and ligand binding of Dectin-1[2] and may explain some of the contradictory results published in the literature.



Schematic representation of the Dectin-1 major isoforms

Binding of β-glucans to Dectin-1 triggers a variety of cellular responses via the Syk/CARD9 signaling pathway, including phagocytosis, respiratory burst and secretion of cytokines. Although not yet fully understood, the ability of β-glucans to induce these cellular responses is influenced by their macromolecular structure. Dectin-1 is usually described as a β(1-3)-linked glucan specific receptor. However, we along with others[3] have found that linear β(1-6)-linked glucans, such as the lichen β-glucan pustulan, also bind and activate Dectin-1 (see pages 2, 4,&5). It is well accepted that particulate β-glucans, such as the widely used yeast cell-wall fraction zymosan, bind to and activate Dectin-1 inducing cellular responses. In contrast, the interaction of soluble β-glucans with Dectin-1 is subject to debate. The general consensus, though, is that soluble β-glucans, such as laminarin, bind to Dectin-1 but are unable to initiate signaling[4]. In accordance with published studies on mDectin-1, we found that a soluble mDectin-1 receptor was able to bind particulate as well as soluble β-glucans and that cells expressing the murine dectin-1 gene could respond to particulate but not soluble β-glucans (see pages 2&5). However, we obtained different responses with hDectin-1. Cells expressing the human stalkless dectin-1b isoform behaved similarly to mDectin-1-expressing cells, whereas cells expressing the human full-length dectin-1a isoform responded to both particulate and soluble β-glucans (see page 4). We found no data in the literature confirming or contradicting this latter result.

Most published studies focus exclusively on the responses of mDectin-1 to β-glucans, which may differ considerably from the response mediated by hDectin-1. In addition to discrepancies between mDectin-1 and hDectin-1, many inconsistencies exist due to the use of very different, and often impure, β-glucans, and the analysis of different cell types and model systems. Recognition of β-glucans by the immune system appears very complex and further studies are required to fully understand the immunomodulating properties of these molecules.

1. Tsoni SV & Brown GD.. **2008.** β-Glucans and dectin-1. Ann NY Acad Sci. 1143:45-60. **2.** Kato Y. et al., **2006.** Contribution of N-linked oligosaccharides to the expression and functions of beta-glucan receptor, Dectin-1. Biol Pharm Bull. 29(8):1580-6. **3.** Adachi Y. *et al.*, **2004.** Characterization of beta-glucan recognition site on C-type lectin, dectin 1. Infect Immun. 72(7):4159-71. **4.** Goodridge HS. *et al.* **2011.** Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse'. Nature. 472(7344):471-5.

CLR Ligands

➢ Dectin-1 Ligands

Dectin-1 is a major receptor for β-glucans, a diverse class of glucose polymers found in fungi, plants and some bacteria. InvivoGen provides an extensive collection of β-1,3 and/or β-1,6 glucans validated for their ability to activate Dectin-1 in RAW-Blue™ cells, a murine macrophage-derived reporter cell line, and in HEK-Blue™ Dectin-1 reporter cells, which stably express different isoforms of the Dectin-1 gene (see page 4).

Beta-glucan peptide (BGP) - β(1→4,1→3, 1→6)-glucan

Beta-glucan peptide (BGP) is a high molecular weight (~100 kDa) polysaccharide extracted from the fungus *Trametes versicolor*. BGP consists of a highly ramified glucan portion, comprising a beta 1-4 main chain and beta 1-3 side chain, with beta 1-6 side chains (fig. 2) covalently linked to a polypeptide portion rich in aspartic, glutamic and other amino acids. BGP activates murine macrophages and HEK-Blue™ Dectin-1 cells (figs. 1&4).

Laminarin - β(1→3, 1→6)-glucan

Laminarin from the brown seaweed *Laminaria digitata* is a linear β(1-3)-glucan with β(1-6)-linkages (fig. 2). Laminarin is a low molecular weight (6 kDa), water-soluble β-glucan. It can bind to Dectin-1 without stimulating downstream signaling[1] (fig. 1) and is able to block binding to Dectin-1 of particulate β(1-3)-glucans, such as zymosan[2] (fig. 4). The activity of laminarin on Dectin-1 seems to vary depending on the isoform (figs. 1&4).

Lichenan - β(1→3, 1→4)-glucan

Lichenan (or lichenin) is a median molecular weight (22 kDa), linear glucan of (1-3, 1-4)-β-glycosidic bonds (fig. 2) that originates from the lichen *Cetraria islandica*. The proportion of 1-4 to 1-3 linkage is approximately 2:1. Lichenan binds to Dectin-1[3] and initiates downstream signaling leading to NF-κB activation (figs. 1&4).

Pustulan - β(1→6)-glucan

Pustulan is a median molecular weight (20 kDa), linear (1-6) linked β-D-glucan from lichen *Lasallia pustulata* (fig. 2). Pustulan is recognized by Dectin-1[4] and activates HEK-Blue™ Dectin-1 and RAW-Blue™ cells (figs. 1&4).

Schizophyllan - β(1→3, 1→6)-glucan

Schizophyllan (SPG) is a gel-forming β-glucan from the fungus *Schizophyllum commune*. SPG is a high molecular weight (450 kDa) (1-3)-β-D-glucan that has a 1,6-β-monoglucosyl branch in every three 1,3-β-glucosyl residues on the main chain (fig. 2). SPG binds to Dectin-1[5] and triggers a signaling cascade leading to NF-κB activation (figs. 1&4).

Scleroglucan - β(1→3, 1→6)-glucan

Scleroglucan is a high molecular weight (>1000 kDa) polysaccharide produced by fermentation of the filamentous fungus *Sclerotium rolfsii*. Scleroglucan consists of a linear β(1-3) D-glucose backbone with one β(1-6) D-glucose side chain every three main residues (fig. 2). Scleroglucan is recognized by Dectin-1[6] and strongly activates HEK-Blue™ Dectin-1 and RAW-Blue™ cells (figs. 1&4).

1. Gantner BN. *et al.*, 2005. Dectin-1 mediates macrophage recognition of *Candida albicans* yeast but not filaments. EMBO J. 24(6):1277-86. **2. Brown GD. *et al.*, 2002.** Dectin-1 is a major beta-glucan receptor on macrophages. J Exp Med. 196(3):407-12. **3. Ujita M. *et al.*, 2009.** Carbohydrate binding specificity of recombinant human macrophage beta-glucan receptor dectin-1. Biosci Biotechnol Biochem. 73(1):237-40. **4. Willment JA. *et al.*, 2001.** Characterization of the human beta -glucan receptor and its alternatively spliced isoforms. J Biol Chem. 276(47):43818-23. **5. Adachi Y. *et al.*, 2004.** Characterization of beta-glucan recognition site on C-type lectin, dectin 1. Infect Immun. 72(7):4159-71. **6. Adams EL. *et al.*, 2008.** Differential high-affinity interaction of dectin-1 with natural or synthetic glucans is dependent upon primary structure and is influenced by polymer chain length and side-chain branching. J Pharmacol Exp Ther. 325(1):115-23.

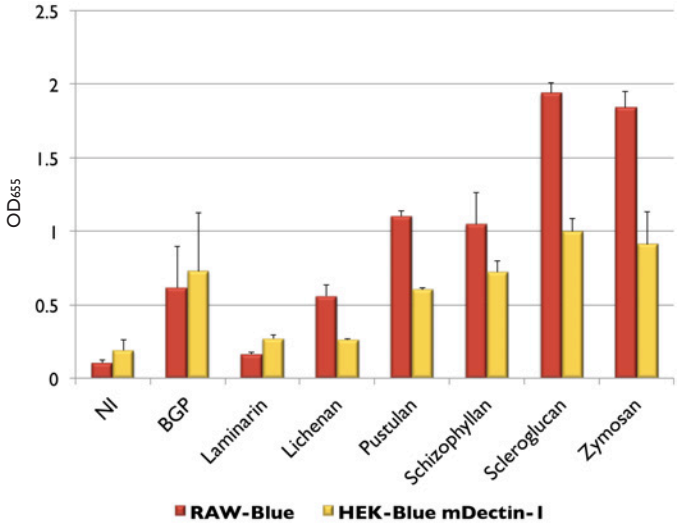


Figure 1: Responses to β-glucans. RAW-Blue™ cells and HEK-Blue™ mDectin-1 cells, which express the mouse dectin-1a gene, were stimulated with 100 µg/ml of various β-glucans. After 24h incubation, NF-κB activation was assessed by measuring the levels of SEAP (secreted embryonic alkaline phosphatase) using the QUANTI-Blue™ assay.

The TLR2 and TLR4 activities of Dectin-1 ligands are also tested using HEK-Blue™ TLR2 and HEK-Blue™ TLR4 cell lines. Data are available online.

PRODUCT		QUANTITY	CAT. CODE
Beta-glucan peptide	**NEW**	50 mg	tlrl-bgp
Laminarin	**NEW**	100 mg	tlrl-lam
Lichenan	**NEW**	100 mg	tlrl-lich
Pustulan	**NEW**	100 mg	tlrl-pst
Schizophyllan	**NEW**	100 mg	tlrl-spg
Scleroglucan	**NEW**	100 mg	tlrl-scg

Also Available

Dectin-1 ligands

Curdlan AL- Bacterial β(1-3)-glucan (tlrl-cura)
HKCA - Heat-killed *Candida albicans* (tlrl-hkca)
HKSC - Heat-killed *Saccharomyces cerevisiae* (tlrl-hksc)
WGP Dispersible - Yeast particulate β(1-3, 1-6)-glucan (tlrl-wgp)
WGP Soluble - Yeast soluble β(1-3, 1-6)-glucan (tlrl-wgps)
Zymosan - Yeast β(1-3, 1-6)-glucan (tlrl-zyn)
Zymosan Depleted - Yeast TLR2/TLR4-depleted β(1-3, 1-6)-glucan (tlrl-dzn)

Neutralizing antibodies

Anti-hDectin-1-IgG (mabg-hdect)
Anti-hMincle-IgG (mabg-hmcl)
MAb-mDectin-1 (mab-mdect)
Anti-mMincle-IgG (mabg-mmcl)

Structures of β-glucans

Depending on the source, there are clear differences in macromolecular structure between β-glucans. Fungal β-glucans consist of 1,3 β-linked glycopyranosyl residues with small numbers of 1,6 β-linked branches. In contrast, some plants, such as lichen, contain unbranched β-glucans with 1,3 and 1,4 β-linked glycopyranosyl residues, whereas bacterial β-glucans are unbranched 1,3 β-linked glycopyranosyl residues.

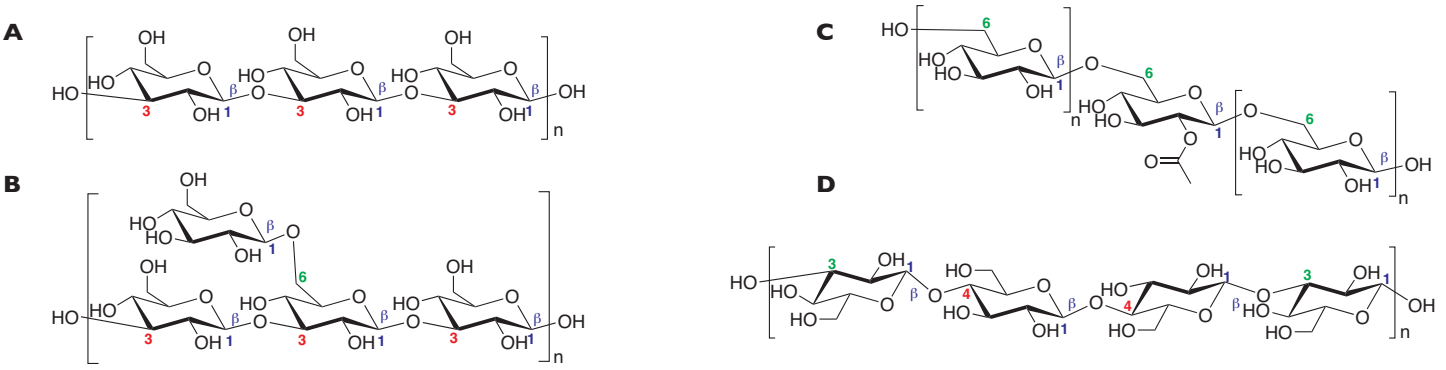


Figure 2: Partial structures of β-glucans. A - β(1→3)-linkages found in curdlan, B - β(1→3, 1→6)-linkages found in beta-glucan peptide, laminarin, schizophyllan and scleroglucan, C - β(1→6)-linkage found in pustulan, D - β(1→3,1→4)-linkages found in lichenan

➤ Mincle Ligands

The C-type lectin receptor Mincle is involved in the recognition of mycobacteria, including *Mycobacterium tuberculosis*. Mincle recognizes trehalose-6'6'-dimycolate (TDM), also known as 'cord factor', the major virulence factor of *M. tuberculosis* and signals through the Syk-Card9 pathway leading to the activation of NF -κB. Activation of Mincle is assayed in Mincle reporter cells, RAW-Blue™ cells and HEK-Blue™ Mincle cells (see page 4).

HKMT - Heat-killed *Mycobacterium tuberculosis*

HKMT is a heat-killed preparation of the avirulent strain of *Mycobacterium tuberculosis* H37 Ra. HKMT is sensed by Mincle which recognizes the mycobacterial cell wall glycolipid TDM[1]. HKMT also possesses a large repertoire of TLR2 ligands, such as lipoproteins and lipomannan[2]. Upon HKMT sensing, both Mincle and TLR2 lead to the activation of NF-κB (fig. 3).

TDB-HS15 - Synthetic cord factor analog

Trehalose-6,6-dibehenate (TDB) is a non-toxic synthetic analogue of TDM and an effective adjuvant for Th1/Th17 vaccination. TDB was found to rely on Mincle, Syk and Card9 for its adjuvant activity[3]. TDB is a poorly soluble compound and thus was formulated with Kolliphor® HS 15, a potent low-toxicity non-ionic solubilizer, to generate TDB-HS15, which is particularly suitable for *in vivo* studies. TDB-HS15 is a strong activator of NF-κB in a Mincle-dependent manner (fig. 3). TDB-HS15 is available in a standard grade and VacciGrade™ (sterility and absence of endotoxin guaranteed).

Vaccine Adjuvant

CFA - Complete Freund's Adjuvant

Complete Freund's adjuvant (CFA) consists of heat-killed *Mycobacterium tuberculosis* in a water-in-oil emulsion. CFA contains ligands for Mincle, TLR2, TLR4, and TLR9. Injection of antigen in CFA induces a Th1-dominated response, while injection in Incomplete Freund's Adjuvant (IFA), which lacks mycobacterial components, induces a Th2-dominated response.

1. Ishikawa E. *et al.*, 2009. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. 206(13):2879-88. **2. Bhatt K & Salgame P., 2007.** Host innate immune response to *Mycobacterium tuberculosis*. J Clin Immunol 27(4): 347–362. **3. Schoenen H, *et al.*, 2010.** Cutting edge: Mincle is essential for recognition and adjuvanticity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate. J Immunol. ;184(6):2756-60.

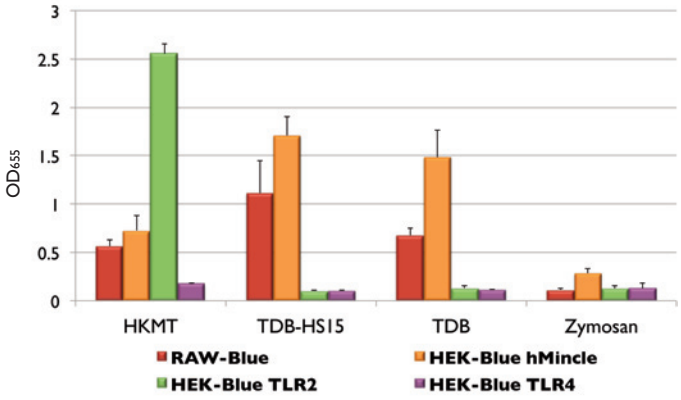


Figure 3: Responses to *M. tuberculosis*-derived ligands. RAW-Blue™ cells, and HEK-Blue™ hMincle, HEK-Blue™ TLR2 or HEK-Blue™ TLR4 cells, which express the human mincle, TLR2 or TLR4 gene, respectively, were stimulated with 10 µg/ml of *M. tuberculosis*-derived ligands. After 24h incubation, NF-κB activation was assessed by measuring the levels of SEAP using the QUANTI-Blue™ assay.

PRODUCT		QUANTITY	CAT. CODE
CFA	**NEW**	6 x 10 ml	vac-cfa-60
HKMT	**NEW**	5 x 10 mg	tlrl-hkmt
TDB-HS15	**NEW**	2 mg	tlrl-stdb
TDB-HS15 VacciGrade	**NEW**	2 mg	vac-stdb

CLR Ligand Screening Service

InvivoGen has developed novel cellular assays to detect compounds that activate or block the C-type lectins, Dectin-1 and Mincle. These sensitive assays feature engineered HEK293 cells, which utilize an NF-κB-inducible SEAP (secreted embryonic alkaline phosphatase) reporter gene as the read-out. Dectin-1- or Mincle-triggered NF-κB activation is monitored using proprietary detection assays designed to provide rapid and reliable results.

Dectin-1 Ligand Screening

➢ Screening for Dectin-1 agonists

The Dectin-1 ligand screening service can be performed on three different HEK-Blue™ Dectin-1 cells, which express different isoforms of the dectin-1 gene:

- HEK-Blue™ mDectin-1a
- HEK-Blue™ hDectin-1a
- HEK-Blue™ hDectin-1b

HEK-Blue™ Dectin-1 cell lines express the murine dectin-1a, human dectin-1a or human dectin-1b gene, respectively. They also express genes of the Dectin-1-NF-κB signaling pathway, in addition to an NF-κB-inducible SEAP reporter gene. These reporter cell lines are activated specifically by Dectin-1 ligands. They do not respond to other CLR ligands.

The Dectin-1 activity of a test compound is determined by incubating HEK-Blue™ Dectin-1 cells with increasing concentrations of this compound and controls (positive and negative). After 24h incubation, activation of Dectin-1 is assessed by measuring the levels of NF-κB-induced SEAP in the supernatant using the QUANTI-Blue™ assay.

The three Dectin-1 reporter cell lines respond specifically to β(1-3) and/or (1-6)-glucans, but display differences in their response profile. In particular, HEK-Blue™ hDectin-1b cells do not respond to soluble β-glucans, such as laminarin and WGP soluble, in accordance with the literature, whereas HEK-Blue™ hDectin-1a cells are highly responsive to these ligands (fig. 4).

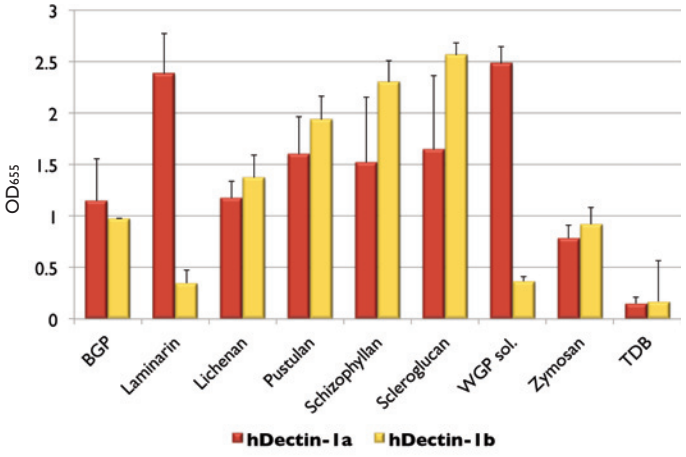


Figure 4. Stimulatory activity of Dectin-1 ligands: HEK-Blue™ hDectin-1a and HEK-Blue™ hDectin-1b cells were stimulated with 1 μg/ml or 10 μg/ml Dectin-1 ligands, respectively, and 10 μg/ml TDB. After 24h incubation, Dectin-1-induced NF-κB activation was assessed by measuring the levels of SEAP using the QUANTI-Blue™ assay.

➢ Screening for Dectin-1 antagonists

Some β-glucans, due to their biophysical properties, bind to Dectin-1 but are unable to induce Dectin-1 signaling of certain isoforms (fig. 4). In addition, these β-glucans can act as antagonists, such as laminarin and WGP soluble (fig. 5).

Screening for Dectin-1 antagonists can be performed using the HEK-Blue™ Dectin-1 cell lines. Increasing concentrations of a test compound are pre-incubated with HEK-Blue™ Dectin-1 cells prior to the addition of Dectin-1 agonists. After 24h incubation, inhibition of Dectin-1 activation is determined by measuring the levels of NF-κB-induced SEAP using the QUANTI-Blue™ assay.

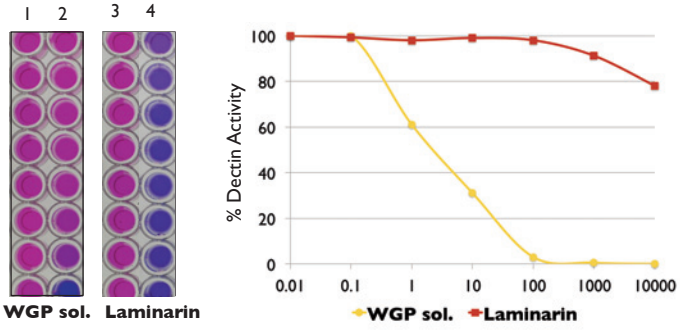


Figure 5. Inhibitory activity of Dectin-1 ligands: HEK-Blue™ hDectin-1b cells were incubated with increasing concentrations of WGP soluble or laminarin (up to 10 μg/ml), either alone (lanes 1 and 3) or with 100 μg/ml zymosan (lanes 2 and 4). After 24h, Dectin-1-activity was determined by measuring the levels of NF-κB-induced SEAP using the QUANTI-Blue™ assay.

Mincle Ligand Screening

➢ Screening for Mincle agonists

The Mincle ligand screening service utilizes the **HEK-Blue™ hMincle** reporter cell line, which co-expresses the human mincle gene, genes of the Mincle-NF-κB signaling pathway and an NF-κB-inducible SEAP reporter gene. This cell line responds specifically to Mincle ligands, such as trehalose-6,6-dibehenate (TDB) and heat-killed *M. tuberculosis* (HKMT) (fig. 6).

Screening for Mincle agonists is performed similarly to the screening for Dectin-1 agonists.

Screening for Mincle antagonists can also be performed, although as of today no ligand with inhibitory activity on Mincle has been identified.

PRODUCT		CAT. CODE
CLR Ligand Screening Service	**NEW**	tlrl-test2

Contact us for more information on the Dectin-1 & Mincle Ligand Screening Service

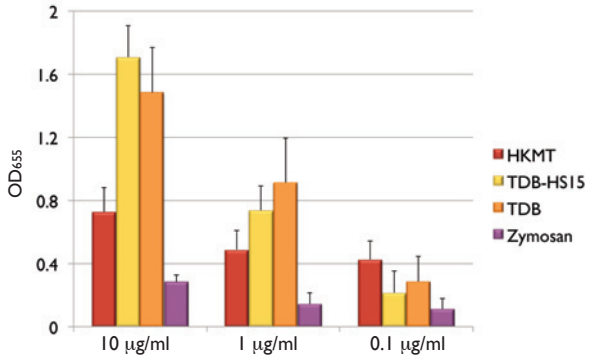
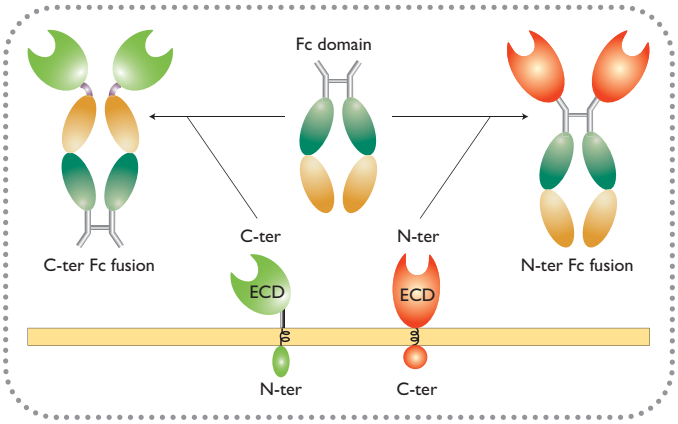


Figure 6. Stimulatory activity of Mincle ligands: HEK-Blue™ hMincle cells were stimulated with 0.1, 1 or 10 μg/ml Mincle ligands or zymosan. After 24h incubation, Mincle-induced NF-κB activation was assessed by measuring the levels of SEAP using the QUANTI-Blue™ assay.

Soluble Receptor:Fc Fusion Proteins

InvivoGen provides soluble forms of the pattern recognition receptors, Dectin-1 and TLR5, that consist of the extracellular domain (ECD) of each receptor fused to an IgG1 Fc domain, engineered to reduce ADCC and enhance half-life (see last page). Dectin-1, which is a type-II transmembrane receptor, is fused to the C-terminus of the Fc domain while TLR5, which is a type-I transmembrane receptor is fused to the N-terminus of the Fc domain. The Fc fusion proteins are expressed in CHO cells and purified by protein G affinity chromatography. The soluble Dectin-1 and TLR5 receptors can be used for receptor binding assays or neutralization studies.



➢ Soluble Dectin-1 Receptor

InvivoGen provides soluble forms of the human and mouse Dectin-1 receptors, Fc-hDectin-1a and Fc-mDectin-1a, respectively. Human Dectin-1 shares 60% sequence identity with the mouse homologue, which may lead to differences in protein folding and β-glucans recognition of the Fc-Dectin-1 fusion proteins.

Fc-hDectin-1a

Fc-hDectin-1a is a soluble human Dectin-1 receptor constructed by fusing the C-terminal extracellular domain of human Dectin-1a (aa 67-247) to the C-terminus of an engineered human IgG1 Fc domain with a 10 amino acid linker. Fc-hDectin-1a has an apparent molecular weight of ~55 kDa on SDS-PAGE.
Applications: Receptor binding assays, neutralization

Fc-mDectin-1a

Fc-mDectin-1a is a soluble murine Dectin-1 receptor constructed by fusing the C-terminal extracellular domain of mouse Dectin-1a (aa 67-244) to the C-terminus of an engineered human IgG1 Fc domain with a 10 amino acid linker. Fc-mDectin-1a has an apparent molecular weight of ~55 kDa on SDS-PAGE.
Applications: Receptor binding assays, neutralization

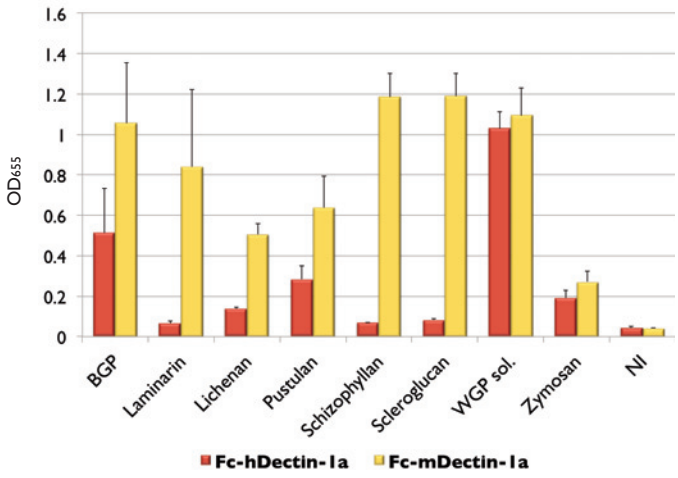


Figure 1. β-Glucan receptor binding assay: 96-well plates were coated with 0.5 µg of various β-glucans and incubated with 1 µg/ml Fc-hDectin-1a or Fc-mDectin-1a. After 2 hours, an anti-IgG secondary antibody conjugated to alkaline phosphatase was added. β-Glucan-Dectin-1 binding was assessed using the QUANTI-Blue™ assay.

➢ Soluble TLR5 Receptor

Toll-like receptor 5 (TLR5) is a type-I transmembrane receptor comprising an N-terminal extracellular leucine rich repeat domain and a C-terminal intracellular TIR signaling domain.

hTLR5-Fc - Soluble ectodomain of TLR5

The soluble TLR5 receptor, hTLR5-Fc, was generated by fusing the N-terminal extracellular domain of human TLR5 (aa 21-639) to the N-terminus of an engineered Fc region of human IgG1 with a 2 amino acid linker. The hTLR5-hFc fusion has an apparent molecular weight of 110 kDa on SDS-PAGE.
Applications: Neutralization, receptor binding assays

PRODUCT		QUANTITY	CAT. CODE
Fc-hDectin-1a	**NEW**	50 µg	fc-hdec1a
Fc-mDectin-1a	**NEW**	50 µg	fc-mdec1a
hTLR5-Fc	**NEW**	50 µg	fc-htlr5

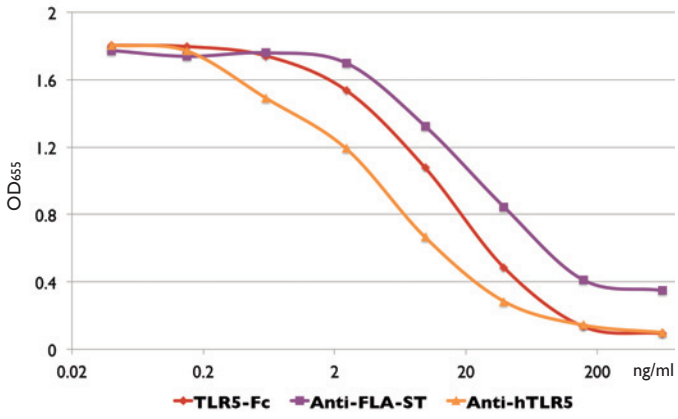


Figure 2. Neutralization activity of hTLR5-Fc: Increasing concentrations of hTLR5-Fc, anti-FLA-ST (antibody against *S. typhimurium* flagellin) or anti-hTLR5 (antibody against human TLR5) were pre-incubated with 5 µg FLA-ST (*S. typhimurium* flagellin) prior to the addition of HEK-Blue™ hTLR5 cells, which express the human TLR5 gene and an NF-kB-inducible SEAP gene. After 24h incubation, TLR5-induced NF-κB activation was assessed by measuring the levels of SEAP using the QUANTI-Blue™ assay.

Flagellins

➢ TLR5 and NLRC4 Ligands

InvivoGen provides an expanding collection of flagellins of diverse origins and purity grades that are ligands for TLR5 and/or NLRC4. In addition, various flagellin antibodies, genes and reporter cell lines to monitor TLR5 and NLRC4 activity are now available.

RecFLA-ST NQ - Flagellin mutant from *S. typhimurium*

RecFLA-ST NQ is a N-glycosylation mutant of the *Salmonella typhimurium* flagellin (FliC gene) where potential asparagine (N) glycosylation sites are substituted by glutamine (Q) residues (see explanation below). RecFLA-ST NQ migrates on SDS-PAGE at ~52 kDa, a similar molecular weight as the extracted FLA-ST Ultrapure (figure 1). This flagellin mutant is a potent activator of TLR5 (figure 2).

FLA-BS Ultrapure - Native flagellins from *B. subtilis*

FLA-BS Ultrapure is a high purity grade of flagellin isolated from the Gram$^+$ bacterium *Bacillus subtilis*. FLA-BS Ultrapure is extracted by violent agitation and purified by several different separation techniques resulting in the depolymerized protein. This flagellin is >95% pure and migrates on SDS-PAGE at ~30 kDa (figure 1). FLA-BS Ultrapure more strongly activates TLR5 compared to standard FLA-BS (figure 2).

FLA-PA Ultrapure - Native flagellin from *P. aeruginosa*

Pseudomonas aeruginosa is a virulent Gram$^-$ bacterial pathogen that infects the respiratory tracts. FLA-PA Ultrapure is a flagellin isolated from *P. aeruginosa* by acid hydrolysis and purified by ultrafiltration and chromatography with a purity of >95%. FLA-PA Ultrapure migrates on SDS-PAGE at ~52 kDa (figure 1) and strongly activates TLR5 (figure 2).

Glycosylation of recombinant flagellins

Production of recombinant flagellins in mammalian cells highly reduces the risk of endotoxin and other bacterial contaminations. These flagellins are altered by N-glycosylation, a post-translational modification rarely observed in bacteria, that may affect their immunostimulatory activity. To avoid this modification, mutations to generate N-Q substitutions were incorporated in the flagellin gene, resulting in recombinant flagellins with a similar molecular weight as their counterparts extracted from bacteria.

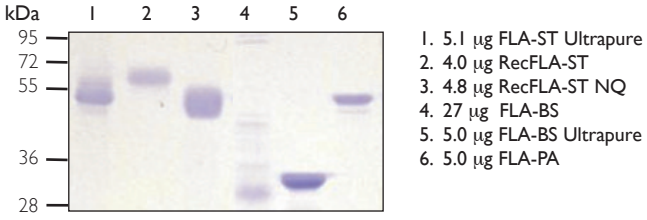


Figure 1. Analysis of the various flagellins using SDS-PAGE. The flagellins indicated were separated by SDS-PAGE and visualized by Coomassie Blue staining. The RecFLA-ST NQ protein migrates at a similar molecular weight as the native FLA-ST Ultrapure.

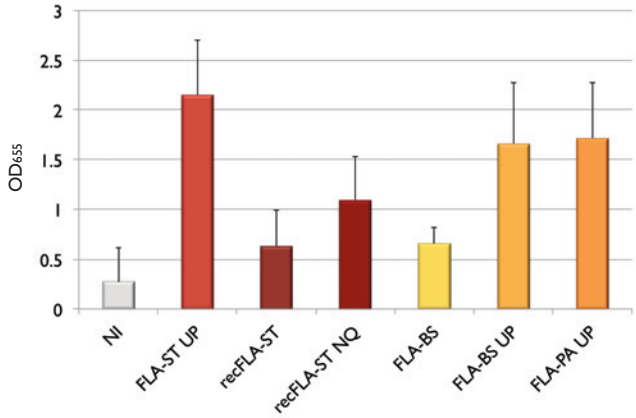


Figure 2. Response of HEK-Blue™ hTLR5 cells to various flagellins. HEK-Blue™ hTLR5 cells were incubated in HEK-Blue™ Detection (SEAP detection medium) and stimulated with 10 ng/ml of the flagellin indicated. After 24h incubation, levels of NF-κB-induced SEAP were determined by reading the OD at 655 nm.

➢ Anti-flagellin Antibodies

Anti-FLA-ST - Detection and neutralization antibody

Anti-FLA-ST is a mouse monoclonal antibody specific against flagellin from *Salmonella typhimurium*. The antibody has been screened for the ability to neutralize FLA-ST-induced TLR5 activity (see fig. 2 on page 5).

Anti-FLA-BS - Detection and neutralization antibody

Anti-FLA-BS is a mouse monoclonal antibody that specifically targets flagellin from *Bacillus subtilis*. The antibody has been screened for the ability to neutralize FLA-BS-induced TLR5 activity.

Anti-FLA-PA - Detection antibody

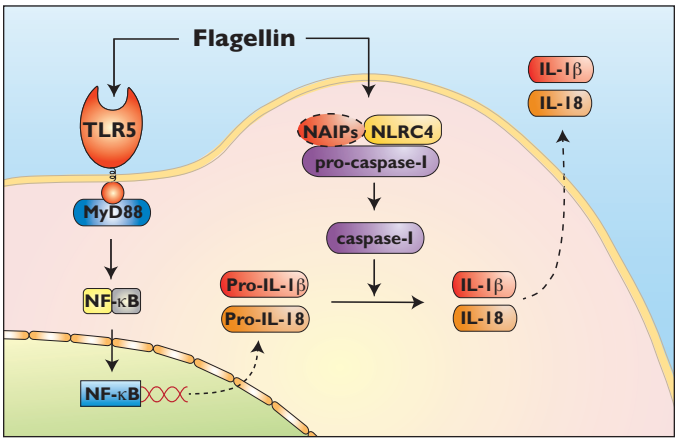
Anti-FLA-PA is a mouse monoclonal antibody specific against flagellin from *Pseudomonas aeruginosa*. The antibody is suitable only for detection proposes.

PRODUCT		QUANTITY	CAT. CODE
Flagellins			
FLA-BS Ultrapure	**NEW**	50 µg	tlrl-pbsfla
FLA-PA Ultrapure	**NEW**	50 µg	tlrl-pafla
recFLA-ST NQ	**NEW**	10 µg	tlrl-flicnq
Anti-flagellin antibodies			
Anti-FLA-BS	**NEW**	100 µg	mabg-flabs
Anti-FLA-PA	**NEW**	100 µg	mabg-flapa
Anti-FLA-ST	**NEW**	100 µg	mabg-flast

For more flagellin-related products, visit www.invivogen.com

Flagellin recognition

Flagellin is a globular protein and the principal component of the bacterial flagella present on motile Gram$^+$ and Gram$^-$ bacteria. Flagellin potently activates the innate immune system through the surface pattern recognition receptor, Toll-like receptor 5 (TLR5)[1]. Recognition by TLR5 induces a MyD88-dependent signaling pathway leading to the activation of NF-κB and the production of pro-inflammatory cytokines, including pro-IL1β and pro-IL-18. Flagellin can also be injected into the cytosol of mammalian cells by bacteria possessing a type III or type IV secretion systems, such as *Salmonella typhimurium* and *Pseudomonas aeruginosa*. Cytosolic flagellin is recognized by the NOD-like receptor (NLR) protein NLRC4 triggering the formation of inflammasomes, which leads to the activation of caspase-1 and the subsequent maturation and secretion of IL-1β and IL-18[2,3]. NAIPs, another subfamily of NLRs, act as adaptors of NLRC4, functioning as receptors for flagellins from certain bacteria. The exact mechanism of NLRC4 activation by flagellin is still under investigation to clarify if particular species and/or forms of flagellin preferentially activate NLRC4. The strong immunostimulatory activity of flagellin has been exploited in the development of vaccine adjuvants either mixed or fused to the target protein antigen[4].



1. Hayashi F. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 410(6832):1099-103. **2. ZhaoY. *et al.*, 2011.** The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. Nature. 477(7366):596-600. **3. Kofoed EM and Vance RE, 2011.** Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. Nature. 477(7366): p. 592-5. **4. Mizel SB and Bates JT, 2010.** Flagellin as an adjuvant: Cellular mechanisms and potential. J Immuno. 185(10):5677-5682.

➢ NLRC4 Reporter Assay

InvivoGen has developed a new cell-based assay to monitor the activation of the NLRC4 inflammasome by intracellular flagellin. This assay relies on two cell lines, an NLRC4 inflammasome test cell line, THP1-NLRC4, and a TLR5-deficient IL-1β reporter cell line, HEK-Blue KD-TLR5, which can be used sequentially or co-cultured to save time.

THP1-NLRC4 cells

NLRC4 inflammasome test cell line

THP1-NLRC4 cells are derived from the THP1 human monocytic cell line, which represents the most commonly used model cell line to study inflammasome activation. THP1-NLRC4 cells stably overexpress NLRC4 and naturally express TLR5. Stimulation of these cells with flagellin triggers TLR5 signaling leading to NF-κB activation and the production of pro-IL1β. Once in the cytosol, flagellin induces the formation of the NLRC4 inflammasome resulting in the activation of caspase-1 and the release of IL-1β. Levels of IL-1β secreted into the supernatant of THP-1 cells can be monitored using the HEK-Blue™ KD-TLR5 cell line.

HEK-Blue™ KD-TLR5 cells

TLR5 deficient, IL-1β reporter cells

HEK-Blue™ KD-TLR5 cells are designed to monitor bioactive IL-1β secreted by THP-1 cells upon flagellin-induced NLRC4 activation. HEK-Blue™ KD-TLR5 cells are derived from the HEK293 cell line, which endogenously expresses TLR5 and the IL-1β receptor (IL-1R). This cell line features an NF-κB-inducible SEAP reporter gene and was engineered to knock-down the expression of TLR5 to avoid activation of NF-κB upon flagellin-induced TLR5 stimulation. The knockdown of TLR5 permits the analysis of flagellin specifically for its NLRC4 stimulating activity.

Binding of IL-1β to IL-1R initiates a signaling cascade leading to the activation of NF-κB and the subsequent production of SEAP. Detection of SEAP in the supernatant of HEK-Blue™ KD-TLR5 cells can be readily assessed using the QUANTI-Blue™ assay.

THP1-NLRC4 cells are resistant to blasticidin and HEK-Blue™ KD-TLR5 cells are resistant to Zeocin™ and puromycin.

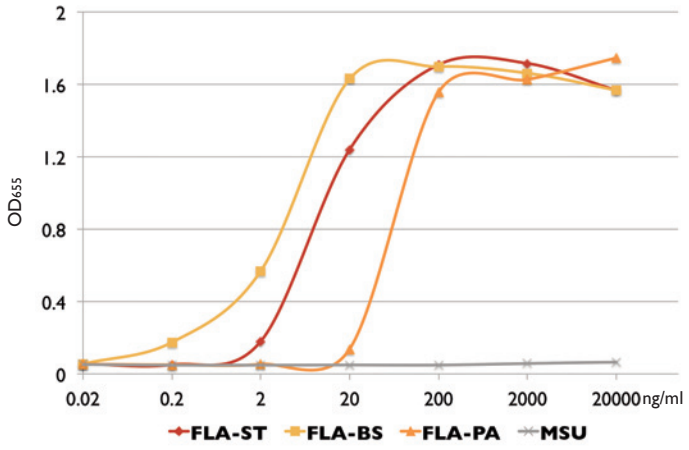


Figure 3. Detection of flagellin-induced IL-1β using HEK-Blue™KD-TLR5 cells. THP1-NLRC4 and HEK-Blue™ KD-TLR5 cells were co-cultured and stimulated with increasing concentrations of ultrapure flagellin from *S. typhimurium* (FLA-ST UP), *B. subtilis* (FLA-BS UP) or *P. aeruginosa* (FLA-PA UP), or monosodium urate (MSU, an NLRP3 inflammasome inducer which requires prior priming of THP-1 cells). After 24h incubation, IL-1β-induced NF-κB activation was assessed by measuring the levels of SEAP using the QUANTI-Blue™ assay. When cultured alone, HEK-Blue™ KD-TLR5 cells do not respond to flagellin.

PRODUCT		QUANTITY	CAT. CODE
THP1-NLRC4 cells	**NEW**	3-7 x 10^6 cells	thp-nlrc4
HEK-Blue™ KD-TLR5 cells	**NEW**	3-7 x 10^6 cells	hkb-kdtlr5

Fc Fusions Proteins

Fc fusion proteins are molecules consisting of an immunoglobulin Fc domain fused genetically to a protein of interest, such as an extracellular domain of a receptor, ligand, enzyme, or peptide. The Fc domain comprises the CH2 and CH3 regions of the IgG heavy chain and the hinge region. Fusion to an Fc domain endows the hybrid protein with additional biological and biophysical properties:

- **increased serum half-life,** owing to the binding of the Fc domain to the salvage neonatal Fc receptor (FcRn) and the larger size of the molecule which limits renal clearance
- **effector functions** (see below) through interaction with Fc receptors (FcγRs), a feature particularly important for oncology and vaccine applications
- **improved solubility and stability** of the partner molecule both *in vitro* and *in vivo*
- **easy, cost-effective purification** by protein G/A affinity chromatography

These beneficial antibody-like properties make Fc fusion proteins an attractive platform for the development of therapeutic drugs.

InvivoGen provides an extensive collection of **pFUSE-Fc** plasmids designed for the fusion the Fc domain of an immunoglobulin to the **C-terminus** of a target protein (see www.invivogen.com/fc-fusions for more information). Now, InvivoGen introduces **pFUSEN-Fc**, a new family of plasmids that allows the fusion of an Fc domain to the **N-terminus** of a protein of interest.

pFUSEN-Fc plasmids - N-terminal Fc fusions

pFUSEN-Fc plasmid features a secretion cassette comprising, in its 5' to 3' direction, the signal sequence of interleukin 2 (IL-2), an immunoglobulin Fc domain and cloning sites to insert the protein of interest.
pFUSEN-Fc plasmids are selectable with Zeocin™ in *E. coli* and mammalian cells. They can be used for transient or stable transfection.

Immunoglobulin Fc domains

- **Human IgG1-Fc** exhibits moderate to high affinity for FcγRs and complement receptors, thus triggering strong ADCC and CDC, respectively. Currently, all Fc-fusions licensed for clinical use contain the IgG1-Fc domain.
- **Human IgG1e2-Fc** contains mutations in the site of interaction of IgG1 with FcRn that enhance the plasma half-life by increasing the affinity of IgG1 for FcRn.
- **Human IgG2-Fc** displays low affinity towards FcγRs and complement receptors. This Fc domain is more suitable for applications for which ADCC and CDC are not desirable.
- **Mouse IgG2a-Fc** is the murine equivalent of human IgG1-Fc. It has moderate to high affinity for FcγRs and complement receptors, inducing strong ADCC and CDC.

Lucia®-Tagged Fc-Fusion Proteins

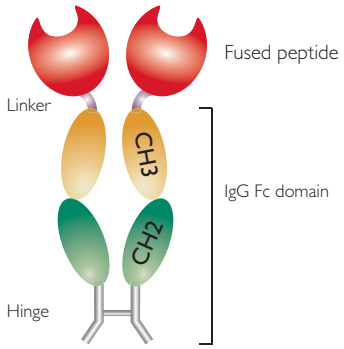
Lucia® is a novel secreted luciferase reporter protein with advantageous characteristics when associated with Fc-fusion proteins. It possesses superior carrier ability for excellent secretion of the chimeric protein. It provides a simple means to screen for recombinant clones and it minimally affects the activity of the protein of interest.

Examples of N-terminal Fc fusions

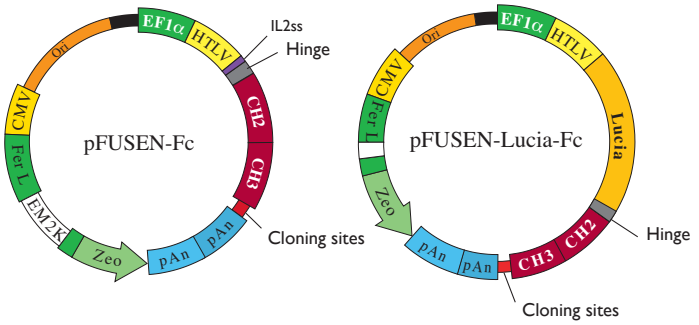
InvivoGen provides Dectin-1 soluble receptors fused to the human IgG1-Fc (see page 5). These N-terminal Fc fusions were generated using the pFUSEN-hG1Fc plasmid and purified by protein G affinity chromatography.

Fc effector functions

Fc effector functions include ADCC (antibody-dependent cell-mediated cytotoxicity), a cytotoxic reaction in which Fc-bearing killer cells recognize target cells via specific antibodies, and CDC (complement-mediated cytotoxicity), the interaction of surface antigen-bound antibodies with complement proteins that triggers cell death through the complement cascade.



Schematic representation of a N-terminal Fc fusion



PRODUCT		QTY	CAT. CODE
pFUSEN-hG1Fc	**NEW**	20 µg	pfcn-hg1
pFUSEN-hG1e2Fc	**NEW**	20 µg	pfcn-hg1e2
pFUSEN-hG2Fc	**NEW**	20 µg	pfcn-hg2
pFUSEN-mG2aFc	**NEW**	20 µg	pfcn-mg2
pFUSEN-Lucia-hG1Fc	**NEW**	20 µg	pfcn-lchg1
pFUSEN-Lucia-hG1e2Fc	**NEW**	20 µg	pfcn-lchg1e2
pFUSEN-Lucia-hG2Fc	**NEW**	20 µg	pfcn-lchg2
pFUSEN-Lucia-mG2aFc	**NEW**	20 µg	pfcn-lcmg2a

For more information on Fc fusions, visit www.invivogen.com/fc-fusions