InvivoGen Insight

To further augment its comprehensive TLR product line, InvivoGen is now providing TLR antibodies. These monoclonal or polyclonal antibodies can be used for detection or neutralization to determine whether a TLR is involved in the signaling of a given ligand such as lipomannan and lipoarabinomannan, two newly introduced TLR2 ligands. Furthermore, InvivoGen is expanding the pFUSE-Fc plasmid family by offering Fc regions from more IgG isotypes and also engineered Fc with altered properties to better suit your needs.

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Review

IgG-Fc Engineering for Therapeutic Use

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IgG-Fc Engineering For Therapeutic Use

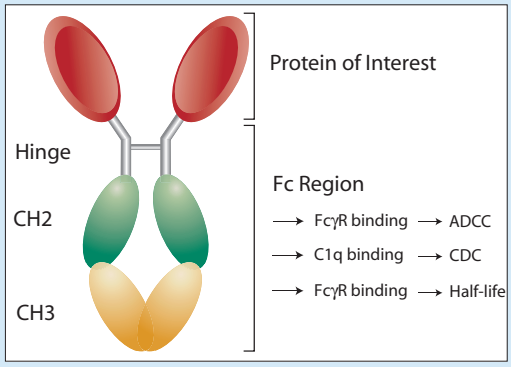
Recombinant fusion proteins consisting of the extracellular domain of immunoregulatory proteins and the constant (Fc) domain of immunoglobulin G (IgG) represent a growing class of human therapeutics.

The IgG class is divided in four isotypes: IgG1, IgG2, IgG3 and IgG4 in humans, and IgG1, IgG2a, IgG2b and IgG3 in mice. They share more than 95% homology in the amino acid sequences of the Fc regions but show major differences in the amino acid composition and structure of the hinge region. The Fc region mediates effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC, the Fc region of an antibody binds to Fc receptors (FcγRs) on the surface of immune effector cells such as natural killers and macrophages, leading to the phagocytosis or lysis of the targeted cells. In CDC, the antibodies kill the targeted cells by triggering the complement cascade at the cell surface. IgG isoforms exert different levels of effector functions increasing in the order of IgG4<IgG2<IgG1≤IgG3. Human IgG1 displays high ADCC and CDC, and is the most suitable for therapeutic use against pathogens and cancer cells.

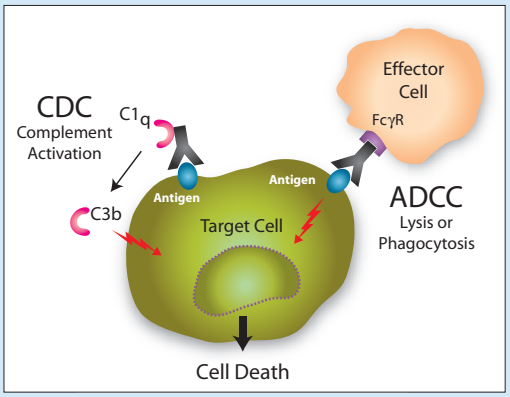
Under certain circumstances, for example when depletion of the target cell is undesirable, abrogating effector functions is required. On the contrary, in the case of antibodies intended for oncology use, increasing effector functions may improve their therapeutic activity[1]. Modifying effector functions can be achieved by engineering the Fc regions to either improve or reduce their binding to FcγRs or the complement factors. The binding of IgG to the activating (FcγRI, FcγRIIa, FcγRIIIa and FcγRIIIb) and inhibitory (FcγRIIb) FcγRs or the first component of complement (C1q) depends on residues located in the hinge region and the CH2 domain. Two regions of the CH2 domain are critical for FcγRs and complement C1q binding, and have unique sequences in IgG2 and IgG4. Substitution into human IgG1 of IgG2 residues at positions 233-236 and IgG4 residues at positions 327, 330 and 331 greatly reduced ADCC and CDC[2, 3]. Numerous mutations have been made in the CH2 domain of IgG and their effect on ADCC and CDC tested *in vitro*[3-6]. In particular, a mutation to alanine at E333 was reported to increase both ADCC and CDC[4, 5].

Increasing the serum persistence of a therapeutic antibody is another way to improve its efficacy, allowing higher circulating levels, less frequent administration and reduced doses. This can be achieved by enhancing the binding of the Fc region to neonatal FcR (FcRn). FcRn, which is expressed on the surface of endothelial cells, binds the IgG in a pH-dependent manner and protects it from degradation. Several mutations located at the interface between the CH2 and CH3 domains have been shown to increase the half-life of IgG1[7, 8].

InvivoGen provides many of the engineered Fc regions mentioned in this short review. They are available in pFUSE-Fc, a plasmid specifically designed for the production of high levels of Fc fusion proteins in mammalian cells.

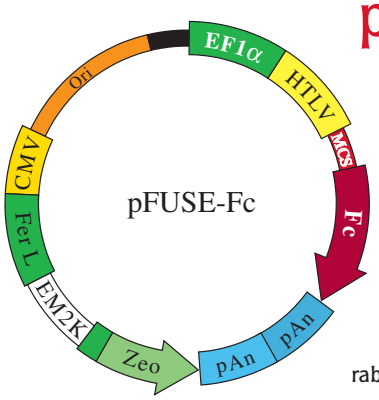


Therapeutic antibody architecture and structural features



Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)

1. Carter PJ., 2006. Potent antibody therapeutics by design. Nature Reviews Immunology. Advance online publication.
2. Armour KL. *et al.*, 1999. Recombinant human IgG molecules lacking Fcgamma receptor I binding and monocyte triggering activities. Eur J Immunol. 29(8):2613-24.
3. Shields RL. *et al.*, 2001. High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R. J Biol Chem. 276(9):6591-604.
4. Idusogie EE. *et al.*, 2001. Engineered antibodies with increased activity to recruit complement. J Immunol. 166(4):2571-5.
5. Idusogie EE. *et al.*, 2000. Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc. J Immunol. 164(8):4178-84.
6. Steurer W. *et al.*, 1995. Ex vivo coating of islet cell allografts with murine CTLA4/Fc promotes graft tolerance. J Immunol. 155(3):1165-74.
7. Hinton PR. *et al.*, 2004. Engineered human IgG antibodies with longer serum half-lives in primates. J Biol Chem. 279(8):6213-6.
8. Vaccaro C. *et al.*, 2005. Engineering the Fc region of immunoglobulin G to modulate in vivo antibody levels. Nat Biotechnol. 23(10):1283-8.



pFUSE-Fc

Fc Fusions Made Easy

Fc-fusion proteins are chimeric proteins featuring the Fc region of an immunoglobulin fused to their C terminus. These soluble chimera retain the activity of the native protein and present the advantages of a long half-life in the circulatory system, efficient mammalian expression and ease of purification. Fc-fusion proteins are useful research tools for many applications and hold promise as therapeutics. Furthermore, they can be used as antigens for vaccination applications. InvivoGen provides pFUSE-Fc, a family of plasmids featuring several Fc regions from various species: human (IgG1, IgG2 and IgG4), mouse (IgG1, IgG2a and IgG3), rabbit (IgG), rat (IgG2b), and engineered Fc from human IgG1, IgG2 and mouse IgG2a.

Choose a pFUSE-Fc plasmid accordingly to your application:

- Protein purification - All pFUSE-Fc can be used for Protein A or Protein G affinity chromatography.
- Long term expression *in vivo* - Choose a pFUSE-Fc with an Fc engineered to display an increased half-life.
- Therapeutic use with cell depletion activity - Choose a pFUSE-Fc with an Fc engineered to display increased ADCC and CDC.
- Therapeutic use without cell depletion activity - Choose a pFUSE-Fc with an Fc engineered to display reduced ADDC and CDC.

pFUSE-Fc plasmids feature a very innovative backbone with two unique promoters: EF1 prom/HTLV 5'UTR and CMV enh/FerL prom

- Strong - High levels of expression. Production of Fc-Fusions is usually in the µg/ml range.
- Constitutive - Expression independent of the cell cycle.
- Ubiquitous - Transfectable in a variety of mammalian cells, including cells commonly used in protein purification systems (CHO, COS, HEK293).

pFUSE-Fc plasmids allow the secretion of Fc-Fusion proteins. Two versions are available for each pFUSE-Fc:

- pFUSE-Fc1 is recommended when the protein of interest contains a native signal sequence.
- pFUSE-Fc2 contains an IL2 signal sequence (IL2ss) for the generation of Fc-Fusions derived from proteins that are not naturally secreted.

All pFUSE-Fc plasmids are provided as 20 µg of lyophilized DNA.

	Product	Isotype	Species	Effector Activities	Protein A binding	Protein G binding	Catalog code (without IL2ss)	Catalog code (with IL2ss)
	Wild-type Fc							
NEW	pFUSE-hIgG1-Fc	IgG1	human	ADCC +++, CDC +++	++++	++++	pfuse-hg1fc1	pfuse-hg1fc2
	pFUSE-hIgG2-Fc	IgG2	human	ADCC +/-, CDC +	++++	++++	pfuse-hfc1	pfuse-hfc2
NEW	pFUSE-hIgG4-Fc	IgG4	human	ADCC +/-, CDC -	++++	++++	pfuse-hg4fc1	pfuse-hg4fc2
NEW	pFUSE-mIgG1-Fc	IgG1	mouse	ADCC -, CDC +/-	++++	++++	pfuse-mg1fc1	pfuse-mg1fc2
	pFUSE-mIgG2Aa-Fc	IgG2a	mouse	ADCC +++, CDC +++	+	++++	pfuse-mfc1	pfuse-mfc2
NEW	pFUSE-mIgG3-Fc	IgG3	mouse	ADCC +++, CDC +	++++	+++	pfuse-mg3fc1	pfuse-mg3fc2
	pFUSE-rIgG-Fc	IgG	rabbit	CDC +++	++	+++	pfuse-rfc1	pfuse-rfc2
NEW	pFUSE-rtIgG2B-Fc	IgG2b	rat	ADCC ++, CDC ++	-	++	pfuse-rtg2bfc1	pfuse-rtg2bfc2

	Product	Isotype	Mutations	Characteristics	Refs*	Catalog code (without IL2ss)	Catalog code (with IL2ss)
	Engineered Fc						
NEW	pFUSE-hIgG1e1-Fc	human IgG1	T250Q/M428L	Increased half-life	7	pfc1-hg1e1	pfc2-hg1e1
NEW	pFUSE-hIgG1e2-Fc	human IgG1	M252Y/S254T/T256E + H433K/N434F	Increased half-life	8	pfc1-hg1e2	pfc2-hg1e2
NEW	pFUSE-hIgG1e3-Fc	human IgG1	E233P/L234V/L235A/ΔG236 + A327G/A330S/P331S	Reduced ADCC and CDC	2, 3	pfc1-hg1e3	pfc2-hg1e3
NEW	pFUSE-hIgG1e4-Fc	human IgG1	E333A	Increased ADCC and CDC	3, 4	pfc1-hg1e4	pfc2-hg1e4
NEW	pFUSE-hIgG2e1-Fc	human IgG2	K322A	Reduced CDC	5	pfc1-hg2e1	pfc2-hg2e1
NEW	pFUSE-mIgG2Aae1-Fc	mouse IgG2a	L235E + E318A/K320A/K322A	Reduced ADCC and CDC	6	pfc1-mg2aae1	pfc2-mg2aae1

* See references on first page. Sequences are available on our website.

TLR Antibodies

Mouse Monoclonal Antibodies - MAb-TLRs

InvivoGen provides a selection of monoclonal anti-TLR antibodies (MAb-TLR). These antibodies can be used for various applications: detection by flow cytometry, immunoprecipitation, or Western blot, immuno assays, and neutralization by blocking the activation induced by the appropriate TLR ligand. MAb-TLRs are purified and lyophilized.

Product	Clone	Specificity	Reported Applications*	Refs	Quantity	Catalog Code
MAb hTLR1	GD2.F4	human TLR1	FC, Neutralization	1	100 µg	mab-htlr1
MAb hTLR2	TL2.1	human TLR2	FC, IHC, WB, Neutralization	2	100 µg	mab-htlr2
MAb mTLR2	T2.5	mouse/human TLR2	FC, IHC, Neutralization	3	100 µg	mab-mtlr2
MAb hTLR3	TLR3.7	human TLR3	FC, WB	4	100 µg	mab-htlr3
MAb hTLR4	HTA125	human/monkey TLR4	FC, IHC, Neutralization	5	100 µg	mab-htlr4
MAb mTLR4/MD2	MTS510	mouse TLR4/MD2	FC, IHC, Neutralization	6	100 µg	mab-mtlr4md2
MAb mTLR9	5G5	mouse/human TLR9	FC, IHC, WB	7	100 µg	mab-mtlr9

FC: flow cytometry, IHC: immunohistochemistry, WB: Western blot

Rat Polyclonal Antibodies - PAb-TLRs

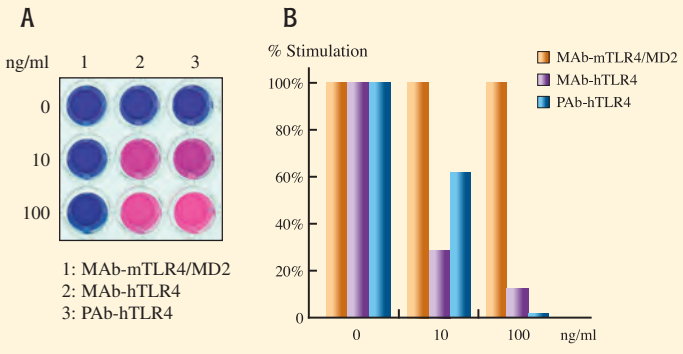
NEW

InvivoGen has developed new polyclonal anti-TLR antibodies (PAb-TLR). PAb-TLRs have been generated by DNA vaccination. Wistar rats have received four hydrodynamic injections of a pFUSE-hTLR-Fc plasmid, expressing the extracellular region of human TLRs fused to the Fc portion of human IgG2. The sera were harvested and the IgG fraction purified by Protein G affinity chromatography. PAb-TLRs are sterile, azide-free (contain Pen/Strep), endotoxin-tested (<0.125 EU/µg) and lyophilized.

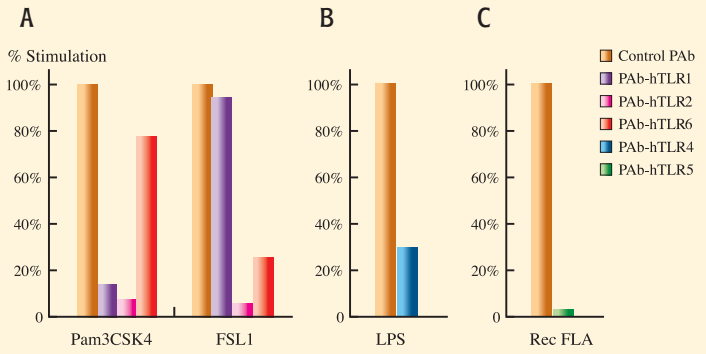
*** All TLR antibodies have been tested in house for neutralization.**

Product	Specificity	Application*	Quantity	Catalog Code
PAb hTLR1	human TLR1	Neutralization	200 µg	pab-htlr1
PAb hTLR2	human TLR2	Neutralization	200 µg	pab-htlr2
PAb hTLR4	human TLR4	Neutralization	200 µg	pab-htlr4
PAb hTLR5	human TLR5	Neutralization	200 µg	pab-htlr5
PAb hTLR6	human TLR6	Neutralization	200 µg	pab-htlr6

1. Wyllie DH. *et al.*, 2000. Evidence for an accessory protein function for Toll-like receptor 1 in anti-bacterial responses. J Immunol. 165(12):7125-32. [Flow cytometry]
2. Flo TH. *et al.*, 2000. Human toll-like receptor 2 mediates monocyte activation by Listeria monocytogenes, but not by group B streptococci or lipopolysaccharide. J Immunol. 164(4):2064-9. [Flow cytometry]
3. Meng G. *et al.*, 2004. Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. J Clin Invest. 113(10):1473-81. [Flow cytometry]
4. Matsumoto M. *et al.*, 2003. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol. 171(6):3154-62. [Flow cytometry]
5. Tabeta K. *et al.*, 2000. Toll-like receptors confer responsiveness to lipopolysaccharide from Porphyromonas gingivalis in human gingival fibroblasts. Infect Immun. 68(6):3731-5. [Immunohistochemistry]
6. Akashi S. *et al.*, 2000. Cutting edge: cell surface expression and lipopolysaccharide signaling via the toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. J Immunol. 164(7):3471-5. [Flow cytometry, Neutralization]
7. Rutz M. et al., 2004. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. Eur J Immunol. 34(9):2541-50. [Western Blot]



TLR4 Neutralization: THP1 cells expressing an NF-κB-inducible SEAP plasmid were incubated with 0, 10 or 100 ng/ml MAb-mTLR4/MD2 (MTS510), MAb-hTLR4 (HTA125) or PAb-TLR4 for 10 min prior to the addition of 100 ng/ml *E. coli K12* LPS. After 24h, TLR stimulation was assessed by the naked eye **(A)** or by reading the OD at 655 nm **(B)** using HEK-Blue™ Detection, a SEAP detection cell culture medium (cat. code hb-det). HEK-Blue™ Detection turns blue following TLR stimulation but remains pink if neutralization occurs.



Neutralization with PAb-hTLRs: HEK293 cells expressing a given TLR and an NF-κB-inducible SEAP plasmid were incubated with 3 µg/ml PAb-hTLR for 10 min prior to the addition of the ligand. After 24h, TLR stimulation was assessed using HEK-Blue™ Detection. **A-** 293/hTLR2 cells were stimulated with 5 ng/ml Pam3CSK4 or FSL1. **B-** 293/hTLR4-MD2-CD14 cells were stimulated with 1 ng/ml *E. coli* K12 LPS. **C-** 293/hTLR5 were stimulated with 10 ng/ml recombinant flagellin (Rec FLA).

Lipoarabinomannan and Lipomannan

TLR2-Dependent Immune Modulators

Lipoarabinomannans (LAM) and lipomannans (LM) are lipoglycans restricted to the *Mycobacterium* genus that act as potent modulators of the host immune response. They are found in the envelope of all mycobacteria species, such as the pathogenic strains *M. tuberculosis* and *M. leprae*, the vaccine strain, *M. bovis BCG*, the opportunistic strains *M. avium* and *M. foruitum*, and the non-pathogenic strain *M. smegmatis*. LAM and LM, which induce different immune responses depending on the species they originate from, signal through TLR2. InvivoGen provides LAM and LM from *M. smegmatis*.

Lipoarabinomannan - LAM-MS

Lipoarabinomannans (LAM) display different immunomodulatory effects depending on their structure. PILAM, which are phosphoinositol-capped LAM and found in non-pathogenic species *(M. smegmatis)*, are proinflammatory molecules whereas ManLAM, which are mannose-capped LAM and found in pathogenic species *(M. tuberculosis)*, are anti-inflammatory molecules[1]. LAM-MS is a PILAM and is known to activate macrophages in a TLR2-dependent manner[2, 3]. However, this activation is weak compared to that of LM-MS, its biosynthetic precursor (Fig. 1).

Lipomannan - LM-MS

Lipomannans (LMs) are composed of a mannan core and a glycosyl-phosphoinositol anchor and have an average MW of 6 kDa. LMs display strong inflammatory activity regardless of the species of Mycobacterium from which they are isolated[2]. They induce the release of cytokines, such as TNF-α and IL-8, from differentiated cells[4] in a TLR2 and MyD88 dependent manner[3]. LM-MS is one of the most potent TLR2 ligand.

To test whether LAM-MS and LM-MS signal through TLR2 alone or require additional TLRs, such as TLR1 or TLR6, we performed TLR neutralizing experiments on HEK293 cells, that are known to express endogenous levels of TLR1 and TLR6, transfected with human TLR2 and an NF-κB-inducible SEAP plasmid. Polyclonal antibodies against human TLR1 and TLR2 (PAb-hTLR1 and PAb-hTLR2) blocked the responses induced by both LAM-MS and LM-MS whereas PAb-hTLR6 had no effect (Fig. 2). These data suggest that LAM-MS and LM-MS require TLR1 and TLR2 to induce an immune response.

1. Quesniaux VJ. *et al.*, 2004. Toll-like receptor 2 (TLR2)-dependent-positive and TLR2-independent-negative regulation of proinflammatory cytokines by mycobacterial lipomannans. J Immunol. 172(7):4425-34.
2. Elass E. *et al.*, 2005. Mycobacterial lipomannan induces matrix metalloproteinase-9 expression in human macrophagic cells through a Toll-like receptor 1 (TLR1)/TLR2- and CD14-dependent mechanism. Infect Immun. 73(10):7064-8.
3. Tapping RI & Tobias PS., 2003. Mycobacterial lipoarabinomannan mediates physical interactions between TLR1 and TLR2 to induce signaling. J Endotoxin Res. 9(4):264-8.
4. Vignal C. *et al.*, 2003. Lipomannans, but not lipoarabinomannans, purified from Mycobacterium chelonae and Mycobacterium kansasii induce TNF-alpha and IL-8 secretion by a CD14-toll-like receptor 2-dependent mechanism. J Immunol. 171(4):2014-23.

Product	Quantity	Code
LM-MS	500 µg	tlrl-lmms
LAM-MS	500 µg	tlrl-lams

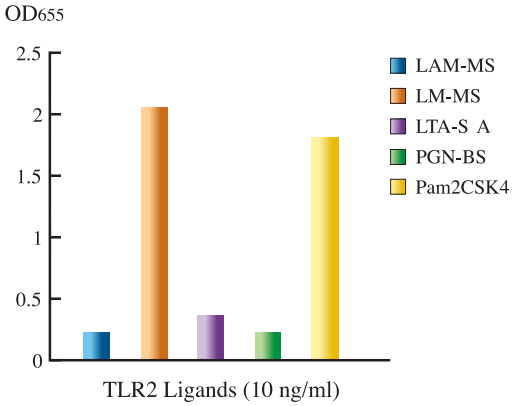


Fig. 1: 293/hTLR2 cells expressing an NF-κB-inducible SEAP plasmid were incubated with 10 ng/ml of various TLR2 ligands. After 24h, TLR2 stimulation was assessed by measuring the levels of SEAP secreted in the supernatant by using HEK-Blue™ Detection.

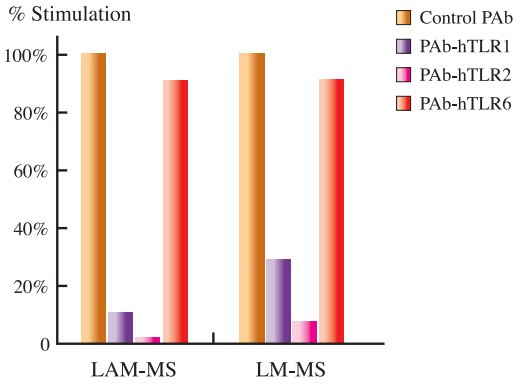


Fig. 2: 293/hTLR2 cells transfected with an NF-κB-inducible SEAP plasmid were incubated with 3 µg/ml PAb-hTLR1, -hTLR2 or -hTLR6 for 10 min prior to the addition of 10 ng/ml LAM-MS or LM-MS. After 24h, TLR stimulation was assessed by measuring the levels of SEAP secreted in the supernatant by using HEK-Blue™ Detection.