



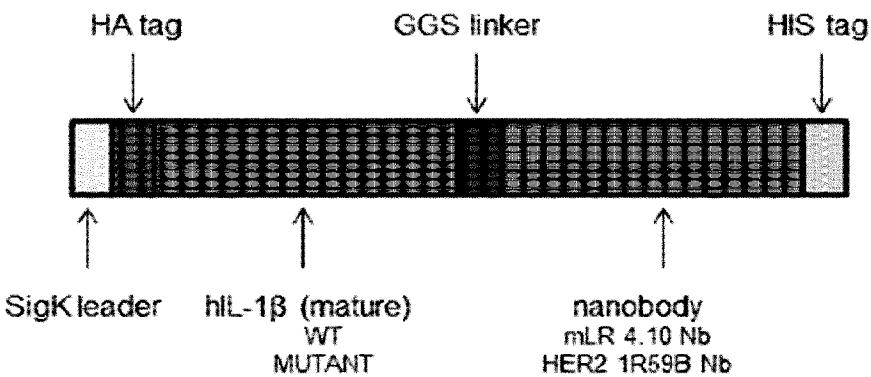
(86) Date de dépôt PCT/PCT Filing Date: 2014/07/04
(87) Date publication PCT/PCT Publication Date: 2015/01/22
(45) Date de délivrance/Issue Date: 2022/08/16
(85) Entrée phase nationale/National Entry: 2016/01/18
(86) N° demande PCT/PCT Application No.: EP 2014/064283
(87) N° publication PCT/PCT Publication No.: 2015/007542
(30) Priorité/Priority: 2013/07/19 (EP13306047.5)

(51) Cl.Int./Int.Cl. C07K 16/28 (2006.01),
C07K 14/545 (2006.01), C07K 16/32 (2006.01)

(72) Inventeurs/Inventors:
TAVERNIER, JAN, BE;
GERLO, SARAH, BE;
PEELMAN, FRANK, BE;
UZE, GILLES, FR

(73) Propriétaires/Owners:
UNIVERSITEIT GENT, BE;
CENTRE HOSPITALIER REGIONAL UNIVERSITAIRE
DE MONTPELLIER, FR;
VIB VZW, BE;

(54) Titre : MEMBRES DE LA FAMILLE IL-1 MODIFIES CIBLES
(54) Title: TARGETED MODIFIED IL-1 FAMILY MEMBERS



(57) Abrégé/Abstract:

The present disclosure relates to a modified Interleukin-1 (IL-1) family member cytokine, with reduced activity via its cytokine receptor, wherein said interleukin-1 family member cytokine is specifically delivered to target cells. Preferably, the IL-1 family member cytokine is a mutant, more preferably it is a mutant IL-1 with low affinity to the IL-1 receptor, wherein said mutant IL-1 is specifically delivered to target cells. The targeting is preferably realized by fusion of the modified IL-1 family member cytokine to a targeting moiety, preferably an antibody or antibody-like molecule. The disclosure relates further to the use of such targeted modified IL-1 family member cytokine to treat diseases.

(73) Propriétaires(suite)/Owners(continued):CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE, FR;
UNIVERSITE DE MONTPELLIER, FR

(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(10) International Publication Number
WO 2015/007542 A1

(43) International Publication Date
22 January 2015 (22.01.2015)

(51) International Patent Classification:
C07K 16/28 (2006.01) **C07K 16/32** (2006.01)
C07K 14/545 (2006.01)

(21) International Application Number:
PCT/EP2014/064283

(22) International Filing Date:
4 July 2014 (04.07.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
13306047.5 19 July 2013 (19.07.2013) EP

(71) Applicants: **VIB VZW** [BE/BE]; Rijvisschestraat 120, B-9052 Gent (BE). **UNIVERSITEIT GENT** [BE/BE]; Sint-Pietersnieuwstraat 25, B-9000 Gent (BE). **CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE** [FR/FR]; 3, rue Michel-Ange, F-75794 Paris (FR). **UNIVERSITÉ MONTPELLIER 2** [FR/FR]; Place Eugène Bataillon, F-34095 Montpellier (FR). **CENTRE HOSPITALIER REGIONAL UNIVERSITAIRE DE MONTPELLIER** [FR/FR]; 191 avenue du Doyen Gaston Giraud, F-34295 Montpellier (FR).

(72) Inventors: **TAVERNIER, Jan**; Bottelweg 2, B-9860 Balegem (BE). **GERLO, Sarah**; Winterstraat 15, B-9000 Gent (BE). **PEELMAN, Frank**; Désiré Mercierlaan 46, B-9050 Gentbrugge (BE). **UZÉ, Gilles**; 4 Enclos Laffoux, 11 avenue de Castelnau, F-34090 Montpellier (FR).

(74) Common Representative: **VIB VZW**; Rijvisschestraat 120, B-9052 Gent (BE).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

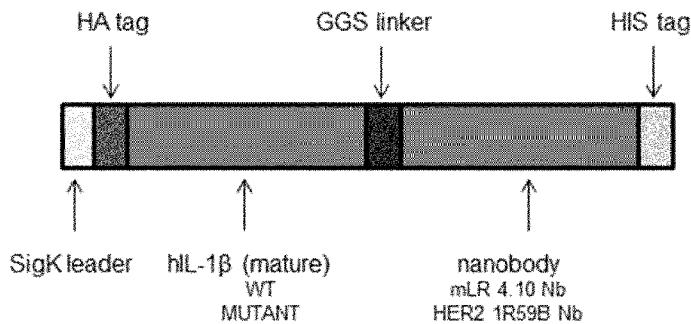
(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: TARGETED MODIFIED IL-1 FAMILY MEMBERS

Figure 1



(57) Abstract: The present disclosure relates to a modified Interleukin-1 (IL-1) family member cytokine, with reduced activity via its cytokine receptor, wherein said interleukin-1 family member cytokine is specifically delivered to target cells. Preferably, the IL-1 family member cytokine is a mutant, more preferably it is a mutant IL-1 with low affinity to the IL-1 receptor, wherein said mutant IL-1 is specifically delivered to target cells. The targeting is preferably realized by fusion of the modified IL-1 family member cytokine to a targeting moiety, preferably an antibody or antibody-like molecule. The disclosure relates further to the use of such targeted modified IL-1 family member cytokine to treat diseases.

WO 2015/007542 A1

TARGETED MODIFIED IL-1 FAMILY MEMBERS

The present invention relates to a modified Interleukin-1 (IL-1) family member cytokine, with reduced activity via its cytokine receptor, wherein said Interleukin-1 family member cytokine is specifically delivered to target cells. Preferably, the IL-1 family member cytokine is a mutant,

5 more preferably it is a mutant IL-1 with low affinity to the IL-1 receptor, wherein said mutant IL-1 is specifically delivered to target cells. The targeting is preferably realized by fusion of the modified IL-1 family member cytokine to a targeting moiety, preferably an antibody or antibody-like molecule. The invention relates further to the use of such targeted modified IL-1 family member cytokine to treat diseases.

10 The Interleukin-1 (IL-1) family consists of 11 structurally related family members (IL-1 α , IL-1 β , IL-1Ra, IL-18, IL-33 and IL-1F5 to IL-1F10), that are among the most potent immune system 15 signalling molecules, acting through a group of closely related receptors. All IL-1 receptors have a similar mode of activation: upon binding of ligand to the primary receptor subunit (i.e. IL-1R1 for IL-1 α and β , IL-18R for IL-18 and ST2 for IL-33), a second receptor subunit is recruited (i.e. IL-1RAP for IL-1 α and β , IL-18RAP for IL-18 and IL-1RAP for IL-33) and signalling is initiated via juxtaposition of the receptor subunits' cytoplasmic Toll/IL-1 receptor (TIR) domains. The dimerized TIR domains provide a docking platform for the MYD88 adaptor protein, which via recruitment of other intermediates leads to activation of the pro-inflammatory nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) pathways. The IL-1 20 family members are primarily produced by innate immune cells and act on a variety of cell types during the immune response (for review see Sims and Smith, 2010).

T lymphocytes are one of the main IL-1 family target cells and the potentiating effects of in particular IL-1 α and IL-1 β on the expansion and differentiation of different T cell subsets, in particular CD8+ T cells (Ben-Sasson, 2011; Ben-Sasson, 2013) and Th17 cells (Sutton et al., 25 2006; Acosta-Rodriguez et al., 2007; Dunne et al., 2010; Shaw et al., 2012) have been firmly established. Th17 cells are characterized by the production of IL-17 and play an important role in auto-immune disease and chronic inflammation (reviewed in Wilke et al., 2011). Among T cell subsets, Th17 cells express the highest levels of the IL-1R and IL-1 plays an important role in Th17 priming.

30 IL-18 is best known as an IFNy-inducing cytokine with a potent action on Th1 cells and natural killer (NK) cells, on (Okamura et al., 1995; Takeda et al. 1998). In addition, IL-18 enhances neutrophil function (Leung et al., 2001). Several reports demonstrate IL-18 anti-tumour action in animal models (Micallef et al., 1997; Loeffler et al., 2008; Wigginton et al., 2002; Zaki et al., 2010) and recombinant human IL-18 therapy recently entered clinical trials to evaluate its 35 efficacy for treatment of advanced cancer (Robertson et al., 2008). As opposed to IL-18, IL-33

acts primarily on Th2 cells (Schmitz et al., 2005) and mast cells (Allakhverdi et al., 2007), and recently was shown to act on CD8 + T cells to drive antiviral responses (Bonilla et al., 2012). The other IL-1 family members are less well characterized, but in summary different IL-1 family members have specificities for different T-cell subsets or other cell types and hence different 5 therapeutic applications.

Besides having indirect anti-tumour activity, via activation of T and NK cells, IL-1 family members were shown to have direct cytostatic properties, which were most convincingly demonstrated on human melanoma cells (Morinaga et al., 1990; Usui et al., 1991; Rangnekar et al., 1992).

10 In view of the contribution of several IL-1 family members to inflammatory processes, clinical interest has been mainly oriented towards the development of IL-1-antagonizing strategies (Dinarello et al., 2012). Nevertheless, exploitation of controlled agonistic IL-1 activity could have applications in different physiological/pathological processes, where immunostimulatory effects would be desirable. One of the main concerns regarding the use of IL-1 in 15 immunostimulatory therapies, is its severe toxicity when administered systemically. However, when IL-1 action could be confined to a selected cellular population, the toxicity issue might be resolved, which opens up therapeutic perspectives.

For instance, although there has been a lot of interest on blocking Th17 responses in view of their pathogenic role in auto-immune conditions such as multiple sclerosis, rheumatoid arthritis 20 and inflammatory bowel disease (Wilke et al., 2011), normal Th17 function is indispensable for protective immunity against a range of pathogens, including *Mycobacterium tuberculosis* (Khader et al., 2007), *Klebsiella pneumoniae* (Ye et al., 2001) and *Bordetella pertussis* (Higgins et al., 2006). As IL-1[3 stimulates Th17 function, the idea has been raised to use IL-1[3 as a T-cell adjuvant to enhance the response to weak vaccines (Ben-Sasson et al., 2011). 25 Other applications could be the targeting of IL-1[3 or IL-33 to the CD8+ T-cell population to enhance antiviral responses or targeting IL-18 to Th1 cells or NK cells to promote anti-tumor activity.

Surprisingly we found that it is possible to design IL-1 family modifications that are defective in activating their receptor, but, when fused to a targeting moiety, regain their activity on selected 30 cell types by a concentration effect at the cell surface. The IL-1 mutants have a reduced affinity for their cognate receptors, and hence are unable to efficiently bind and activate their receptors. However, by fusing them to a targeting moiety (such as a nanobodyTM) the activity of the mutant IL-1 family member is restored on cells expressing the cell surface target, recognized by the targeting moiety. Because the activation is confined to the selected targeted 35 cell types only, no major systemic toxicity occurs.

A first aspect of the invention is a targeting construct, comprising a modified IL-1 family member cytokine, characterized by a reduced affinity for its cytokine receptor, and a targeting moiety. IL-1 family member cytokines are known to the person skilled in the art, and include, but are not limited to IL-1 α , IL-1 β , IL-1Ra, IL18, IL-36Ra, IL-36 α , IL-37, IL-36 β , IL-36 γ , IL-38 and IL-33 (also indicated as IL-1F1, IL-1F2, IL-1F3, IL-1F4, IL-1F5, IL-1F6, IL-1F7, IL-1F8, IL-1F9, IL-1F10 and IL-1F11, respectively). For a review on the IL-1 family, see Dinarello (2011). A modified IL-1 family cytokine means that the IL-1 family cytokine has been changed to alter the affinity to its receptor, with as final result that the modified IL-1 family cytokine has a reduced affinity for the receptor and a consequent reduced biological activity, as compared to the endogenous wild type cytokine that binds normally to the receptor. Such a modification can be a modification that decreases the activity of the normal wild type cytokine, or it can be a modification that increases the affinity of a homologous, non-endogenous IL-1 family cytokine (such as, but not limited to a IL-1 family cytokine of another species that is not active on a human IL-1 family cytokine receptor). Modifications can be any modification reducing or increasing the activity, known to the person skilled in the art, including but not limited to chemical and/or enzymatic modifications such as pegylation and glycosylation, fusion to other proteins and mutations. Preferably said modification is a mutation, even more preferably it is a mutation decreasing the affinity of the IL-1 family cytokine. A reduced affinity and a consequent reduced biological activity as used here means that the modified IL-1 family cytokine has a biological activity of less than 70% of the biological activity of the IL-1 family cytokine, even more preferably less than 60% of the biological activity of the IL-1 family cytokine, more preferably less than 50% of the biological activity of the IL-1 family cytokine, more preferably less than 40% of the biological activity of the IL-1 family cytokine, more preferably less than 30% of the biological activity of the IL-1 family cytokine, more preferably less than 20% of the biological activity of the IL-1 family cytokine, more preferably less than 10% of the biological activity of the IL-1 family cytokine, most preferably less than 1% of the biological activity of the IL-1 family cytokine as compared to the IL-1 family cytokine that normally binds to the receptor. Preferably, the modified IL-1 family cytokine is a mutant of the wild type IL-1 family cytokine and the activity is compared with the wild type IL-1 family cytokine. The affinity and/or the activity can be measured by any method known to the person skilled in the art.

A preferred embodiment of the invention is a targeting construct, comprising a mutant IL-1 β characterized by reduced affinity for the Interleukin-1 receptor type I (IL-1RI) and/or the interleukin-1 receptor accessory protein (IL-1RAcP) receptor, and a targeting moiety. A mutant IL-1 β as used here can be any mutant form that has a lower affinity for the receptor and as a consequence a reduced activation of the proinflammatory transcription factor NF κ B. The affinity of the mutant IL-1 β to the receptor, in comparison to the affinity of the wild type IL-1 β to

the receptor can be measured by Scatchard plot analysis and computer-fitting of binding data (e.g. Scatchard, 1949) or by reflectometric interference spectroscopy under flow through conditions, as described by Brecht et al. (1993). The activity of the mutant IL-1 β is typically measured using a bioassay (for example by the induction of cell death) or by measuring 5 signaling events downstream of the receptor. Such signaling events can be the modification or nuclear translocation of NF- κ B, or the induction of a selected reporter gene. The mutant may be a point mutant, a deletion or an insertion mutant, or a combination thereof; several mutations may be present in one protein. Preferably, said mutant IL-1 β is obtained by active 10 mutagenesis, such as, but not limited to site directed mutagenesis by polymerase chain reaction amplification. Preferably, said mutant IL-1 β has a biological activity of less than 70% of the biological activity of the wild type IL-1 β , even more preferably less than 60% of the biological activity of the wild type IL-1 β , more preferably less than 50% of the biological activity of the wild IL-1 β , more preferably less than 40% of the biological activity of the wild IL-1 β , more 15 preferably less than 30% of the biological activity of the wild IL-1 β , more preferably less than 20% of the biological activity of the wild IL-1 β , more preferably less than 10% of the biological activity of the wild type, most preferably less than 1% of the wild type of which it is deduced (i.e. the wild type IL-1 β of which the coding sequence has been mutated to obtain the mutant IL-1 β). Preferably, said mutant is a mutant selected from the group consisting of 20 A117G/P118G, R120X, L122A, T125G/L126G, R127G, Q130X, Q131G, K132A, S137G/Q138Y, L145G, H146X, L145A/L147A, Q148X, Q148G/Q150G, Q150G/D151A, M152G, F162A, F162A/Q164E, F166A, Q164E/E167K, N169G/D170G, I172A, V174A, K208E, K209X, K209A/K210A, K219X, E221X, E221S/N224A, N224S/K225S, E244K, N245Q (wherein X can be any change in amino acid, preferably a non-conservative change). Even 25 more preferably said mutation is selected from the group consisting of R120A, R120G, Q130A, Q130W, H146A, H146G, H146E, H146N, H146R, Q148E, Q148G, Q148L, K209A, K209D, K219S, K219Q, E221S and E221K. Most preferably said mutation is selected from the group consisting of R120G, H146N, H146R, Q148E, Q148G and K209A. (numbering base on the human IL-1 β sequence, genbank accession number NP_000567, version NP-000567.1, GI: 10835145).

30 Preferred regions for mutations for IL-18 are Y37-K44, R49-Q54, D59-R63, E67-C74, R80, M87-A97, N127-K129, Q139-M149, K165-K171, R183 and Q190-N191. Most preferred are the regions E67-C74 and M87-A97 (numbering based on the human sequence, genbank accession number AAV38697, version AAV38697.1, GI: 54696650).

Preferred regions for mutations for IL-33 are I113-Y122, S127-E139, E144-D157, Y163-M183, E200, Q215, L220-C227 and T260-E269 (numbering based on the human sequence, genbank accession number NP_254274, version NP_254274.1, GI:15559209)

Preferably, said targeting moiety is targeting to a marker expressed on an IL-1 β receptor expressing cell, preferably a cell expressing IL1-RI. In one preferred embodiment, said targeting moiety is directed to a tissue specific marker.

The modified IL-1 family member is linked to a targeting moiety. "Linked" as used here may be by a covalent binding, or it may be by an affinity binding. A "targeting moiety" as used here is a binding molecule that can direct the fusion protein towards a binding site on a cell that is expressing a receptor for the IL-1 family member, by specific interaction between the binding site and the binding molecule. In one preferred embodiment, said binding molecule is a small compound, specifically binding to a molecule situated on the outside of the cell. In another preferred embodiment, said molecule is a sugar structure, directed towards a lectin-like molecule expressed on the cell wall. In another preferred embodiment said binding molecule is a peptide, targeting the tumor or inflammation environment. Such peptides are known to the person skilled in the art, and include, but are not limited to NGR and RGD peptides (Yang et al., 2011; WO2005054293). In still another preferred embodiment, said binding molecule is a protein comprising a binding domain. This includes, but is not limited to carbohydrate binding domains (CBD) (Blake et al, 2006), lectin binding proteins, heavy chain antibodies (hcAb), single domain antibodies (sdAb), minibodies (Tramontano et al., 1994), the variable domain of camelid heavy chain antibodies (VHH), the variable domain of the new antigen receptors (VNAR), affibodies (Nygren et al., 2008), alphabodies (WO2010066740), designed ankyrin-repeat domains (DARPins) (Stumpp et al., 2008), anticalins (Skerra et al., 2008), knottins (Kolmar et al., 2008) and engineered CH2 domains (nanoantibodies; Dimitrov, 2009). Preferably, said targeting moiety consists of a single polypeptide chain and is not post-translationally modified. Even more preferably, said targeting moiety is a nanobody.

The targeting moiety can be any targeting moiety known to the person skilled in the art. In a non-limiting example, said targeting moiety may be a bispecific antibody, directed to a binding site on the target cell for one specificity, and to the targeted cytokine, or to a tag fused to said cytokine for the other specificity. In another non-limiting example, the targeting moiety may be chemically linked to the mutant Interleukin-1, or it may be a recombinant fusion protein. Preferably, said targeting construct is a recombinant fusion protein. The targeting moiety may be fused directly to the mutant IL-1 β , or it may be fused with the help of a linker fragment, preferably a GGS linker. The targeting moiety may be fused at the aminoterminal or at the carboxyterminal end of the mutated IL-1 β ; preferably said targeting moiety is fused at the

carboxyterminal extremity of the mutated IL-1 β molecule. The targeting construct may further comprise other domains such as, but not limited to a tag sequence, a signal sequence, another cytokine or an antibody.

Another aspect of the invention is a targeting construct according to the invention for use as a medicament. One preferred embodiment is a targeting construct according to the invention for use in stimulation of the immune response. Indeed, it is known that IL-1 treatment can induce antigen expression on B-cells (Killar et al., 1989); likewise, IL-18 treatment is augmenting cellular and humoral immunities (Kinoshita et al., 2011). In a similar way, it has been demonstrated that IL-1 acts on T-cells to enhance the magnitude of in vivo immune responses (Ben-Sasson et al., 2011; Ben Sasson et al., 2013). Therefore, one preferred aspect of the invention is the targeting construct according to the invention for use as an adjuvant in vaccination. The targeting construct according to the invention is especially interesting in this respect, as the pro-inflammatory effect of normal wild type IL-1 makes the application of IL-1 as such impossible.

Still another aspect of the invention is a targeting construct according to the invention for use in treatment of cancer. Indeed, Morinaga et al., 1990, Usui et al., 1991 and Rangnekar et al., 1992 have shown that IL-1 family members do have direct cytostatic properties, which were most convincingly demonstrated on human melanoma cells.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Schematic representation of the IL-1 β -nanobody fusion proteins

Figure 2: Concentration dependency of the induction of the NF κ B activity by wild type and mutant Q148G IL-1 Her2 nanobody fusions (A) and other selected mutants (B), in mock transfected cells, or cells transfected with signaling deficient Her2.

Figure 3: Effect of wild type and mutant (Q148G, L145A/L147A, F162A/Q164E) IL-1 Her2 nanobody fusions on nuclear translocation of endogenous NF- κ B p65 in mock transfected cells, or cells transfected with signaling deficient Her2.

Figure 4: Induction of the NF κ B activity by wild type and 5 different IL-1 mutants, fused to an anti-murine leptin receptor nanobody, on cells expressing the murine leptin receptor (mLR) or not (no mLR).

Figure 5: Concentration dependency of the induction of the NF κ B activity by IL1 double mutants fused to the Her2 nanobody in mock transfected cells, or cells transfected with signaling deficient Her2.

EXAMPLES.**Materials and methods to the examples*****Cloning of IL-1-nanobody fusion proteins.***

5 The 4-10 nanobody directed against the murine leptin receptor is described in Zabeau et al. (2012) and in the patent WO 2006/053883. The anti-Her2 nanobody 1R59B is described in Vaneycken et al. (2011). Both nanobodies were cloned with a C-terminal His tag in the pMET7 eukaryotic expression vector. A codon-optimized sequence encoding the mature IL-1 β protein, preceded by the SigK leader peptide, and equipped with an N-terminal HA tag, was generated

10 via gene synthesis (Invitrogen Gene Art). To generate the IL-1 β -nanobody fusion proteins, the IL-1 β sequence was cloned 5' to the nanobody sequence in pMet7, with a 13 x GGS linker separating the cytokine and nanobody moieties. (Fig. 1)

IL-1 β mutants.

15 IL-1 β mutants expected to have reduced binding affinity for the IL-1R were selected based on literature and analysis of published crystal structures of human IL-1 β complexed with its receptor. Mutations in the hIL-1 β moiety were created via site-directed mutagenesis (QuickChange, Stratagene) using the mutagenesis primers as indicated in table I:

Table I: mutants and primers used

		Fw primer	Rev primer
1	A117G/ P118G	CCGACTACGCTGGCGGCAGTGACGGTGCA GAAGCCTGAACCTGC	GCAGTTCAGGCTCTGACACCGTCACTG CCGCCAGCGTAGTCGG
2	R120A	CTGGCGGCAGCGCCCCCTGTCGCTAGCCTGA ACTGCACCCCTGCG	CGCAGGGTGCAGTTCAGGCTAGCGACA GGGGCGCTGCCGCCAG
3	R120G	GCGGCAGCGCCCCCTGTCGGAAGCTTGAAC GCACCCCTGC	GCAGGGTGCAGTTCAAGCTTCCGACAG GGGCGCTGCCGC
4	L122A	CGCTGGCGGCAGTCCCCCTGTCAGAACGGC GAACCTGCACCCCTGCGGGACAGC	GCTGTCGGCAGGGTGCAGTTCGCGCT TCTGACAGGGGACTGCCGCCAGCG
5	T125G/ L126G	CGCCCCCTGTCAGAACGCTGAACCTGCGGGCG CCGGGACAGCCAGCAGAAAAGC	GCTTTCTGCTGGCTGCCCCGGCGCC GCAGTTCAGGCTCTGACAGGGGCG
6	R127G	AGAACGCTGAACCTGCACACTGGGGGACAGC CAGCAGAAAAGCCTGGC	GACCAGGCTTTCTGCTGGCTGTCCCC AGTGTGCAGTTCAGGTTCT
7	Q130A	CCCTGCGGGACAGCGCGCAGAAAAGCCTGG	CCAGGCTTTCTGCGCGCTGTCCCCCA GGG
8	Q130W	CTGCACCCCTGCGGGACAGCTGGCAGAAAAG CCTGGTCATGAGC	GCTCATGACCAGGCTTTCTGCCAGCTG TCCCGCAGGGTGCAG
9	Q131G	CTGCGGGACAGCCAGGGGAAGAGCCTGGTC	CGCTCATGACCAGGCTTTCCCCCTGGCT

		Fw primer	Rev primer
		ATGAGCG	GTCCCGCAG
10	K132A	GCACCCCTGCAGGACAGCCAGCAGGCTAGCC TGGTCATGAGCGGCC	GGCCGCTCATGACCAGGCTAGCCTGCT GGCTGTCCCGCAGGGTGC
11	S137G/ Q138Y	CAGCAGAAAAGCCTGGTCATGGGTACCCCT ACGAGCTGAAGGCAGTGC	GCAGTGCCTTCAGCTCGTAGGGGTAC CCATGACCAGGCTTTCTGCTG
12	L145G	GCCCCTACAGAGCTGAAGGCAGGTCATCTGCA GGGCCAGGACATGG	CCATGTCCCTGGCCCTGCAGATGACCTG CCTTCAGCTCGTAGGGC
13	H146A	CGAGCTGAAGGCAGTGGCTCTTCAGGGCCA GGACATGG	CCATGTCCCTGGCCCTGAAGAGGCCAGTG CCTTCAGCTCG
14	H146G	CCTACGAGCTGAAGGCAGTGGCTCGAGG GCCAGGACATGG	CCATGTCCCTGGCCCTGCAGACCCAGTG CCTTCAGCTCGTAGG
15	H146E	GCTGAAGGCAGTGGAGCTGCAGGGCCAGG	CCTGGCCCTGCAGCTCCAGTGCCTTCAGC GC
16	H146N	AGCTGAAGGCAGTGAATCTGCAGGGCCAG	CTGGCCCTGCAGATTCACTGCCTTCAGC T
17	H146R	CTGAAGGCAGTGCCTGCAGGGCCAG	CTGGCCCTGCAGACGCAGTGCCTTCAG
18	L145A/ L147A	CGGGCCCCCTACGAGCTGAAGGCAGCGCATG CGCAGGGCCAGGACATGG	CCATGTCCCTGGCCCTGCAGCTGCCTG CCTTCAGCTCGTAGGGGCCGC
19	Q148E	GGCACTGCATCTGGAGGGCCAGGACAT	ATGTCCCTGGCCCTCCAGATGCAGTGC
20	Q148G	GAAGGCAGTGCATCTGGGTGGCCAGGACAT GGAACAGC	GCTGTTCCATGTCCTGGCCACCCAGATG CAGTGCCTTC
21	Q148L	GCAC TGCACTGCTGGGCCAGGACATG	CATGTCCCTGGCCAGCAGATGCAGTGC
22	Q148G/ Q150G	CGAGCTGAAGGCAGTGCATCTGGGGGGCGG GGACATGGAACAGCAGG	CCTGCTGTTCCATGTCCTGGCCACCCAGATG GATGCAGTGCCTTCAGCTCG
23	Q150G/ D151A	GCAC TGCACTGCAGGGCGGGGCCATGGAA CAGCAGGTCGTGTTCA	GCTGAACACGACCTGCTGTTCCATGGCC CCGCCCTGCAGATGCAGTGC
24	M152G	GCAC TGCACTGCAGGGCCAGGACGGGGAA CAGCAGGTTGGTGGCATGAGC	GCTCATGCTGAACACCACTGCTGTTCC CCGTCCTGGCCCTGCAGATGCAGTGC
25	F162A	CATGGAACACGAGGTGGTGGCATGAGC GCCGTGCAGGGCGAGGAAGCAACGAC	GTCGTTGCTTCCCTGCAGG GCTCATGCTGAACACCACTGCTGTTCC ATG
26	F162A/ Q164E	GCAGGTCGTGTTCAAGCATGAGCGCCGTGGA GGCGAGGAAAGCAATGACAAGATCC	GGATCTTGTCAATTGCTTCCCTGCAGG CACGGCGCTCATGCTGAACACGACCTG C
27	F166A	CCGACTTCACCATGCAGGCCGCTCCAGCGG CGGCAGCAGATCTGG	CCAGATCTGCTGCCGCCGCTGGAGACG GCCTGCATGGTGAAGTCGG
28	Q164E/ E167K	GCATGAGCTTCGTGGGGGCAAGGAAAGCA ATGACAAGATCCCCGTGGC	GGCCACGGGATCTTGTCAATTGCTTCC TTGCCCTTACGAAGCTCATGC
29	N169G/ D170G	GCAGGGCGAGGAAAGCGGGCGCAAGATCCC CGTGGCCCTAGGCCTGAAAGAGAAG	CTTCTTTCAAGGCCTAGGGCCACGGG GATCTTGCCGCCGCTTCCCTGCCCTGC
30	I172A	GAAAGCAACGACAAGGCCCGTGGCCCTG GG	CCCAGGGCCACGGGGCCCTTGTGTT CTTTC
31	V174A	GCAACGACAAGATCCCCGCGGCCCTGGCC	CTTTCAGGCCAGGGCCGCGGGATCT

		Fw primer	Rev primer
		TGAAAG	TGTCGTTGC
32	K208E	GCAGCTGAAAGCGTGGATCCAAAGAACTAC CCCGAGAAAAAGATGGAAAAACGC	GCGTTTCCATCTTTCTGGGTAGT TCTTGGGATCCACGCTTCCAGCTGC
33	K209A	CCCCAAGAACTACCCCAAGGCAAAGATGGAA AAGCGCTCGTGTCAAC	GTTAACACGAAGCGCTTCCATCTT GCCTTGGGTAGTTCTGGGG
34	K209D	GCAGCTGAAAGCGTGGATCCAAAGAACTAC CCCAAGGACAAGATGGAAAAACGC	GCGTTTCCATCTGTCTTGGGTAG TTCTTGGGATCCACGCTTCCAGCTGC
35	K209A/ K210A	CCCCAAGAACTACCCCAAGGCAGCGATGGAA AAACGCTTCGTGTTC	GAACACGAAGCGTTTCCATCGCTGCC TTGGGGTAGTTCTGGGG
36	K219S	AAAAACGCTTCGTGTCAACACAGCATCGAGAT CAACAACAAGCTC	GAGCTTGTGTTGATCTCGATGCTGTTG AACACGAAGCGTTTT
37	K219Q	AAAAACGCTTCGTGTCAACCAGATCGAGAT CAACAACAAG	CTTGTGTTGATCTCGATCTGGTTGAAC ACGAAGCGTTTT
38	E221S	GCTTCGTGTTCAACAAGATCTCGATCAACAAAC AAGCTCGAGT	ACTCGAGCTTGTGTTGATCGAGATCTT GTTAACACGAAGC
39	E221K	CTTCGTGTTCAACAAGATCAAGATCAACAAACA AGCTCGA	TCGAGCTTGTGTTGATCTTGATCTTGT GAACACGAAG
40	K219S/ E221S	GGAAAAACGCTTCGTCTTCAACACAGCATCTG ATCAACAAACAAGCTCGAGTTCG	CGAACTCGAGCTTGTGTTGATCGAGAT GCTGTTGAAGACGAAGCGTTTTCC
41	E221S/ N224A	CGCTTCGTGTTCAACAAGATCTCGATCAACG CCAAGCTCGAGTTCGAG	CTCGAACACTCGAGCTTGGCGTTGATCGAG ATCTTGTGAACACGAAGCG
42	N224S/ K225S	CAACAAGATCGAGATCAACACAGCAGCCTCGAA TTCGAGAGCGCCAG	CTGGGCGCTCTGAATTGAGGCTGCT GTTGATCTCGATCTTGTG
43	E244K	CCCCAACTGGTACATCAGTACTAGTCAGGCC AAGAATATGCCGTGTTCC	GGAACACGGGCATATTCTGGCCTGACT AGTACTGATGTACCAAGTTGGGG
44	N245Q	CAGCACTAGTCAGGCCAGCAGATGCCGT CTTCCTGGCGCCACC	GGTCCGCCAGGAAGACGGGCATCTG CTCGGCCTGACTAGTGCTG
45	E244K/ N245Q	CATCAGCACTAGTCAGGCCAAGCAGATGCC GTCTTCCTGGCGGCACC	GGTCCGCCAGGAAGACGGGCATCTG CTTGGCCTGACTAGTGCTGATG
46	R120G/ * Q131G	GCAGGCAGCGCCCTGCGGAAGCTTGAAC GCACCCCTGC	GCAGGGTGCAAGCTTCAAGCTCCGACAG GGGCGCTGCCGC
47	R120G/ * H146A	CGAGCTGAAGGCAGTGGCTTCAAGGCCA GGACATGG	CCATGTCCTGGCCCTGAAGAGCCAGTG CCTTCAGCTCG
49	R120G/ * L145A/ L147A	GCAGGCCCTACGAGCTGAAGGCAGCGCATG CGCAGGGCCAGGACATGG	CCATGTCCTGGCCCTGCGCATGCGCTG CCTTCAGCTCGTAGGGGCCGC
48	R120G/ ** Q148G	GCAGGCAGCGCCCTGCGGAAGCTTGAAC GCACCCCTGC	GCAGGGTGCAAGCTTCAAGCTCCGACAG GGGCGCTGCCGC
50	R120G/ * F162A/ Q164E	GCAGGTGTTCAACGATGAGCGCCGTGGA GGCGAGGAAAGCAATGACAAGATCC	GGATCTTGTCAATTGCTTCCCTGCCCTC CACGGCGCTCATGCTGAACACGACCTG C
51	R120G/ * K208E	GCAGCTGAAAGCGTGGATCCAAAGAACTAC CCCGAGAAAAAGATGGAAAAACGC	GCGTTTCCATCTTTCTGGGTAGT TCTTGGGATCCACGCTTCCAGCTGC

		Fw primer	Rev primer
52	Q131G/ ** Q148G	CTGCGGGACAGCCAGGGAAAGAGCCTGGTC ATGAGCG	CGCTCATGACCAGGCTCTCCCTGGCT GTCCCGCAG
53	Q148G/ ** F162A/ Q164E	GCAGGGTCGTGTTCAGCATGAGGCCGTGGA GGCGAGGAAGCAATGACAAGATCC	GGATCTTGTCAATTGCTTCCTCGCCCTC CACGGCGCTCATGCTGAACACGACCTG C
54	Q148G/ ** K208E	GCAGCTGAAAGCGTGGATCCAAAGAACTAC CCCGAGAAAAAGATGGAAAAACGC	GCGTTTTCCATCTTTCTCGGGGTAGT TCTTGGGATCCACGCTTCCAGCTGC

* double/triple-mutants were created using R120G as template. ** double/triple-mutants were created using Q148G as template.

Production of IL-1/3 fusion proteins.

IL-1[3 fusion proteins were produced in HEK293T cells. For small-scale production, HEK293T
5 cells were seeded in 6-well plates at 400000 cells/well in DMEM supplemented with 10% FCS.
After 24 hours, culture medium was replaced by medium with reduced serum (DMEM/5%FCS)
and cells were transfected using linear PEI. Briefly, PEI transfection mix was prepared by
combining 1 pg expression vector with 5 pg PEI in 160 pl DMEM, incubated for 10 minutes at RT
and added to the wells dropwise. After 24 hours, transfected cells were washed with
10 DMEM and layered with 1.5 ml OptiMem™/well for protein production. Conditioned media were
recuperated after 48 hours, filtered through 0.45 p filters and stored at -20°C. IL-1[3 content in the
conditioned media was determined by Elisa according to the manufacturer's instructions (R&D
Systems).

NF-KB reporter gene assay.

15 To assess IL-1R activation, we used HEK-BlueTM IL-1[3 cells that stably express the IL-1R
(Invivogen) and transfected them transiently with an NF-KB luciferase reporter gene. Briefly,
HEK-BlueTM IL-16 cells were seeded in culture medium (DMEM/10%FCS) in 96-well plates
(10000 cells/well) and transfected the next day using the calciumphosphate precipitation
method with the indicated amounts of expression plasmids and 5 ng/well of the 3KB-Luc
20 reporter gene plasmid (Vanden Berghe et al., 1998). 24 hours post-transfection, culture
medium was replaced by starvation medium (DMEM) and 48 hours post-transfection, cells were
induced for 6 hours with fusion proteins. After induction, cells were lysed and luciferase activity in
lysates was determined using the Promega Firefly Luciferase Assay System on a Berthold centro
LB960 luminometer.

25 *Analysis of NF-KB nuclear translocation via confocal microscopy.*

For confocal imaging, 10^5 HEK293-T cells/well (in 6-well plate) were seeded on glass
coverslips (Zeiss), coated with poly-L-lysine (Sigma). The next day, cells were transfected with
200 ng/well of empty vector or HER2Acyt expression plasmid using the calcium phosphate

precipitation method. After 48 hours, cells were treated for 30 minutes with vehicle (medium) or IL1-Her2 nanobody fusion protein (10 ng/ml). Next, cells were rinsed with 1x PBS and fixed for 15 minutes at room temperature in 4% paraformaldehyde. After three washes with 1xPBS, cells were permeabilized with 0.1% Triton™ X-100 in 1xPBS for 10 minutes and blocked in 1%
5 BSA in 1xPBS for another 10 minutes at room temperature. Samples were then incubated for 1 hour at 37°C with rabbit anti-p65 antibody (Santa Cruz C20, diluted 1:800) and mouse anti-Flag Antibody (Sigma M2, 1:2000). After four washes in 1x PBS, cells were incubated for 1 hour at room temperature with anti-rabbit Alexa 488 and anti-mouse Alexa 594 fluorochrome-conjugated secondary antibodies (both diluted 1:800). After secondary antibody incubation,
10 cells were washed four times in 1xPBS and nuclei were stained with DAPI (2 pg/ml). After a final wash step in 1xPBS, coverslips were mounted using propyl gallate. Images were acquired using a 60x 1.35 NA objective on an Olympus IX-81 laser scanning confocal microscope and analyzed using Fluoview 1000 software.

Example 1: IL-113-ligand and IL-113-nanobody fusion proteins.

15 Fig. 1 shows a scheme of the IL-18-nanobody fusion proteins constructed with either WT hIL-1[3 or the hIL1[3 mutants described in table I.

Example 2: IL-113 activity of selected mutant IL-113-nanobody fusions is restored on cells expressing the Nb targets.

Wild type IL-1[3 and 45 IL-1[3 mutants (Table I) were fused to a well-characterized nanobody
20 recognizing Her2 (1 R59B). The IL-18-nanobody fusion proteins were tested on HEK-Blue™ IL-1[3 cells, transiently transfected with an NF-KB reporter gene plasmid (5 ng/well) and a Her2Acyt (signalling-deficient) expression plasmid (2 ng/well). Cells were treated for 6 hours with IL-18-Her2 nanobody fusions (dose response ranging from 0,4 to 250 ng/ml). As demonstrated in Fig. 2A, the IL-18-Q148G-Her2 nanobody fusion displayed a reduced ability to
25 activate NF-KB as compared to the WT IL1-8-Her2 nanobody fusion. Importantly, targeting of the Q148G mutant to Her2Acyt-expressing cells restored its activity and produced a dose-response curve for NF-KB activation that perfectly parallels that of the WT IL-18 on mock-transfected cells. Also evident from this figure is a strong targeting effect for the WT IL-1[3 Her2 nanobody fusion. Similar "activation by targeting" effects were observed for six other IL-1[3
30 mutants (R120G, Q131G, H146A, H145A/L147A, F162A/Q164E and K208E) fused to the Her2 nanobody (Fig. 2B).

To obtain further proof for the "activation by targeting" concept, we next explored whether we could visualize the selective activation of NF-KB in Her2-expressing cells by the IL-18-Her2 nanobody fusions via confocal microscopy. We measured activation of endogenous NF-KB by
35 assaying its nuclear translocation. As evident from Fig. 3, only the WT IL-18-Her2 nanobody

fusion promoted translocation of endogenous NF- κ B in cells that do not express Her2. Whereas they did not promote detectable NF- κ B translocation in mock-transfected cells, the three tested mutant IL1- β -Her2 nanobody fusions triggered NF- κ B nuclear translocation in cells that also stained positive for Her2, indicating they only act on targeted cells.

5 To evaluate whether the "activation by targeting" concept also works using a nanobody to an unrelated membrane protein, we fused WT IL-1 β and five of the disabled IL-1 β mutants (R120G, Q131G, H146A, Q148G, K209A) to a previously characterized nanobody recognizing the mLR (4-10). An experiment similar to that reported for the IL-1 β -Her2 nanobody fusion 10 (Fig. 2) was performed using HEK-BlueTM IL-1 β cells, transiently transfected with a mLR expression plasmid (10 ng/well). Similar to the results obtained with the Her2 nanobody fusion proteins, all investigated mutant IL-1 β nanobody fusions (tested at 12.5 ng/ml) showed a reduced ability, as compared to the WT fusion, to activate NF- κ B on cells that do not express mLRs. However, targeting by the mLR nanobody moiety partially restored the activity of the selected mutants (Fig. 4).

15 Because the IL-1 β mutants described above retained significant residual biological activity, we combined different mutations to obtain double/triple mutants with reduced basal activity. Nine double/triple mutants were tested (cf. table I mutants 46 to 54) and from these, six mutant proteins (Q131G/Q148G, Q148G/K208E, R120G/Q131G, R120G/Q131G, R120G/H146A, R120G/K208E, R120G/F162A/Q164E) displayed no residual activity (using the same assay for 20 measuring NF- κ B as in Fig. 2) on Her2-negative cells, whilst partially restored activity was apparent on cells overexpressing Her2 Δ cyt (Fig. 5).

These data altogether indicate that targeting partially inactive mutant IL-1 β , by fusing it to a nanobody recognizing a cell surface receptor, can restore its activity on nanobody target cells, probably by forced receptor interaction through a membrane concentration effect. The fact 25 that activation by targeting can be accomplished using nanobodies recognizing different classes of membrane proteins indicates broad applicability of the "activation by targeting" concept.

Because these data provide proof of concept for the ability of targeting mutant IL-1 family members to selected cell types, restoring their activity on these target cells only, nanobodies 30 are produced that allow targeting IL-1 family members to physiologically relevant IL-1 β target cells. In view of the important role of IL-1 family members as T- and NK-cell activators, the nanobodies are designed to specifically target IL-1 to T- and NK-cell subsets. More specifically nanobodies targeting CCR6, which are predominantly expressed on Th17 cells as well as 35 nanobodies targeting CD8 on cytotoxic T cells are developed and fused to the members of the IL1-family, preferably IL-1 β .

Example 3: Effect of IL-1 β -nanobody fusions on IL-17 production by primary human T cells.

Primary human T cells were isolated from buffy coats. First, PBMC's were isolated by lymphoprep density gradient centrifugation and incubated O/N with 0.5 ng/ml rhIL-2 for 5 recovery. Next, T-cells were isolated using the pan-T cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, T cells were resuspended (1×10^6 /ml) in RPMI-1640 supplemented with 10 %FCS and CD3/CD28 activating microbeads (Miltenyi Biotec). Next, cells (100 μ l/well) were plated in U-bottom 96-well plates and stimulated for 96 hours with the indicated concentrations of IL-1 β variants. After an additional 6 hours 10 stimulation with PMA/ionomycin (both at 100 nM), supernatants were recovered and IL-17 levels were determined by Elisa (R&D Systems). Additional cytokines are evaluated via 15 Luminex technology.

For selected mutant IL-1 β -nanobody fusions (e.g. with a nanobody targeting CCR6) target cell-specific IL-17 and IFNy production are evaluated by intracellular staining using a flow 15 cytometric approach.

Also, to corroborate selectivity for the Th17 population, binding to PBMC subpopulations is measured via double staining using the Flag tag and selected CD markers, followed by flow cytometric analysis.

Finally, in a clinically relevant *in vitro* model of human Th17 cell function, the adjuvant activity 20 of the IL-1 β -nanobody fusions is assessed. In view of the need for more efficacious vaccines against *Bordetella pertussis* (or adjuvants for the existing vaccines), we determined whether the selected fusion proteins enhance the human Th17 response in a coculture model of naïve T cells with *B. pertussis*-treated monocyte-derived dendritic cells (MDDCs). Human MDDCs are isolated from buffy coats (using the monocyte isolation kit II, Miltenyi Biotec), treated with 25 different ratios of *B. pertussis* for 48 hours and then cocultured with naïve allogeneic T cells for 12 days. After restimulation with anti-CD3/anti-CD28, the cytokine profiles in supernatants are determined using Elisa/Luminex technology (cfr. supra).

Example 4: Effect of IL-1 β -nanobody fusions on CTLs

To assess whether IL-1 β -CD8 nanobody fusions can specifically enhance the function of CD8+ 30 T cells, human PBMC's are isolated by lymphoprep density gradient centrifugation from buffy coats and stimulated for 24 hours with CD3/CD28 activating microbeads (Miltenyi Biotec) in combination with wt or mutant IL1 β -CD8 Nb fusions. The effect of these fusion proteins on CD8+ T cell activation is evaluated by performing intracellular staining for active (phosphorylated) NF- κ B and IFNy. In addition, to investigate whether the IL-1 β -nanobody

fusions affect CTL degranulation, PBMC's (2×10^6 cells/ml) are differentiated for 48 hours in the presence of phytohaemagglutinin (PHA, 1 μ g/ml) and IL-2 (100 IU/ml) in combination with increasing doses of the IL-1 β fusion proteins. Next, to induce degranulation, cells are stimulated for 3 hours with CD3/CD28 dynabeads and analysed by flow cytometry.

5 Degranulation is measured via detection of cell surface CD107a, a well-established marker for natural killer activity. In all flow cytometric analyses on leukocyte pools, anti-CD8 staining is included to allow monitoring of the cell-type specificity of the IL-1 β -CD8 Nb effects.

Finally to assess whether the IL-1 β -CD8 nanobody fusions promote anti-tumor activity *in vivo*, C57BL/6 mice are injected subcutaneously with TC1 tumor cells, which produce the E6 and E7 10 antigenic oncoproteins from HPV16. This model was previously used to demonstrate that IL-1 β promotes CD8+ T cell-mediated, antigen-specific, anti-tumor responses (Ben-Sasson, 2013). Briefly, mice are immunized four days after tumor injection with a vaccine containing the HPV16E7₄₉₋₅₇ peptide, combined with DOTAP and LPS, and with our without WT or mutant IL-1 β -CD8 Nb fusions or IL-1 β -GFP Nb fusions. Tumor size is monitored for 18 days post-15 immunization.

Example 5: In vivo experiments - Vaccine adjuvans effect.

In a first series of experiments C57BL/6 mice are treated iv/ip with different doses of WT and mutant IL-1 β -nanobody fusions and unfused IL-1 β , to monitor acute toxicity. Venous blood is collected at different times post treatment by tail venopuncture and the cytokine profile in 20 serum is determined by Luminex assay. In addition, via flow cytometric analysis intracellular cytokine levels (IL-17, IFN γ) and activation of IL-1R (as assessed by measuring phospho-NF- κ B levels) are determined in selected leukocyte subsets.

When optimal doses have been established, their adjuvant activity is assessed in a murine vaccination protocol. Briefly, C57BL/6 mice are immunized ip with acellular pertussis vaccine 25 (Pa). The Pa vaccine is composed of 5 μ g/mouse of purified recombinant detoxified pertussis toxin (PT9K/129G) + filamentous hemagglutinin (FHA) (composition according to Brereton et al., 2011). 24 hours after immunization, selected mutant IL1 β -Nb or PBS are administered ip or iv. Animals are boosted after 28 days. One set of animals is sacrificed 14 days after the second immunization and splenocytes are isolated and restimulated *in vitro* with medium or 30 FHA for 3 days. Cytokine levels in culture supernatants (IL-17, IFN γ , IL-2, IL-10, IL-5, IL-4, etc.) are determined via Luminex technology. A second set of mice is challenged with *B. pertussis* on day 14 post-boost and sacrificed 2h and 5 and 10 days post-challenge. Lungs are isolated and CFU in lung homogenates will be quantified on Bordet-Gengou agar plates. Cytokine levels in lung homogenates are determined as in splenocyte supernatants.

In addition, blood is sampled (from the tail vein) before immunization and then every 14 days for determination of B. pertussis-specific IgG levels in serum.

Example 6: Direct antitumor effect of IL-1 β -nanobody fusions

To investigate the direct anti-tumour activity of selected IL1-nanobody fusions, we use human 5 A375 melanoma cells, which were shown to be highly susceptible to IL-1-induced cytostatic effects (Morinaga et al., 1990). To allow targeting of mutant IL-1 family members to the A375 cells, a stable A375 clone expressing a cell surface marker to which high-affinity nanobodies are already available (i.e. CD20) is generated. The sensitivity of this cell line, as compared to the parental A375 cells, to the antiproliferative effect of the mutant IL1-nanobody fusion, is 10 investigated in vitro using the XTT proliferation assay. In vivo anti-tumour activity of the mutant IL-1-nanobody fusions is investigated using an A375 xenotransplant model. Briefly, athymic nude mice are inoculated subcutaneously with A375 cells (parental or expressing a surface marker for targeting) and tumor growth is monitored for four weeks in animals treated with PBS or mutant IL1-nanobody fusions.

15 **Example 7: Extension of principle to IL18: application in tumor models**

To assess the indirect anti-tumour activity of IL1 family members, experiments are conducted to address the efficacy of selected mutant IL-18-nanobody fusions using the Meth A syngeneic mouse sarcoma model according to the protocol that was used previously to demonstrate anti-tumour activity of IL-18 (Micallef et al., 1997). IL18 variants used in these experiments consist 20 of mutant IL-18s fused to nanobodies targeting immune cells with tumoricidal properties (i.e. CTLs, NK-cells). The mice are treated with the construct, and a significant reduction of the tumor is noted when compared to the mock treated control.

REFERENCES

Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. (2007) Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol.* 8:942-9.

5 Allakhverdi Z, Smith DE, Comeau MR, Delespesse G. (2007) Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *J Immunol.* 179:2051-4.

Ben-Sasson SZ, Caucheteux S, Crank M, Hu-Li J, Paul WE. (2011) IL-1 acts on T cells to enhance the magnitude of in vivo immune responses. *Cytokine.* 56:122-5.

10 Ben-Sasson SZ, Hogg A, Hu-Li J, Wingfield P, Chen X, Crank M, Caucheteux S, Ratner-Hurevich M, Berzofsky JA, Nir-Paz R, Paul WE. (2013) IL-1 enhances expansion, effector function, tissue localization, and memory response of antigen-specific CD8 T cells. *J Exp Med.* 210:491-502.

15 Blake, A.W., McCartney, L., Flint, J., Bolam, D.N., Boraston, A.B., Gilbert, H.J. and Knox, J.P. (2006) Understanding the biological rationale for the diversity of cellulose-directed carbohydrate-binding molecules in prokaryotic enzymes. *J. Biol. Chem.* 281, 29321-29329.

Bonilla WV, Fröhlich A, Senn K, Kallert S, Fernandez M, Johnson S, Kreutzfeldt M, Hegazy AN, Schrick C, Fallon PG, Klemenz R, Nakae S, Adler H, Merkler D, Löhning M, Pinschewer DD. (2012). The alarmin interleukin-33 drives protective antiviral CD8⁺ T cell responses. *Science.* 335:984-9.

20 Brecht A., Gauglitz G., Polster J. (1993). Interferometric immunoassay in a FIA-system - A sensitive and rapid approach in label-free immunosensing. , *Biosens Bioelectron* 8 : 387-392.

Brereton CF, Sutton CE, Ross PJ, Iwakura Y, Pizza M, Rappuoli R, Lavelle EC, Mills KH. (2011). *Escherichia coli* heat-labile enterotoxin promotes protective Th17 responses against infection by driving innate IL-1 and IL-23 production. *J Immunol.* 2011 May 15;186(10):5896-906.

25 Dimitrov, D.S. (2009) Engineered CH2 domains (nanoantibodies). *mAbs* 1, 26-28.

Dinarello CA, Simon A, van der Meer JW. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat Rev Drug Discov.* 11:633-52.

Dunne A, Ross PJ, Pospisilova E, Masin J, Meaney A, Sutton CE, Iwakura Y, Tschopp J, Sebo P, Mills KH. (2010). Inflammasome activation by adenylate cyclase toxin directs Th17 responses and protection against *Bordetella pertussis*. *J Immunol.* 185:1711-9.

Higgins SC, Jarnicki AG, Lavelle EC, Mills KH. TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. *J Immunol*. 2006 Dec 1;177(11):7980-9.

Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, Shen F, Eaton SM, Gaffen SL, Swain SL, Locksley RM, Haynes L, Randall TD, Cooper AM. (2007). IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. *Nat Immunol*. 2007 8:369-77.

Killar, L.M., Hatfield, C.A., Carding, S.R., Pan, M., Winterrowd, G.E. and Bottomly, K. (1989) In vivo administration of interleukin 1 elicits an increased Ia antigen expression on B cells through the production of interleukin 4. *Eur. J. Immunol.* 19, 2205-2210.

Kinoshita, M., Miyazaki, H., Ono, S., Inatsu, A., Nakashima, H., Tsujimoto, H., Shinomiya, N., Saitoh, D. and Seki, S. (2011). Enhancement of neutrophil function by interleukin 18 therapy protects burn-injured mice from methicillin-resistant *Staphylococcus aureus*. *Infect. Immun.* 79, 2670-2680.

Kolmar, H. (2008) Alternative binding proteins: biological activity and therapeutic potential of cysteine-knot miniproteins. *FEBS J.* 275, 2684-2690.

Leung BP, Culshaw S, Gracie JA, Hunter D, Canetti CA, Campbell C, Cunha F, Liew FY, McInnes IB. (2001). A role for IL-18 in neutrophil activation. *J Immunol*. 167:2879-86.

Loeffler M, Le'Negrat G, Krajewska M, Reed JC. (2008). IL-18-producing *Salmonella* inhibit tumor growth. *Cancer Gene Ther*. 15:787-94.

Micallef MJ, Tanimoto T, Kohno K, Ikeda M, Kurimoto M. (1997). Interleukin 18 induces the sequential activation of natural killer cells and cytotoxic T lymphocytes to protect syngeneic mice from transplantation with Meth A sarcoma. *Cancer Res.* ;57:4557-63.

Morinaga Y, Hayashi H, Takeuchi A, Onozaki K. (1990). Antiproliferative effect of interleukin 1 (IL-1) on tumor cells: G0-G1 arrest of a human melanoma cell line by IL-1. *Biochem Biophys Res Commun*. 173:186-92.

Nygren, P-A. (2008) Alternative binding proteins: affibody binding proteins developed from a small three-helix bundle scaffold. *FEBS J.* 275, 2668-2676.

Okamura H, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, Torigoe K, Okura T, Nukada Y, Hattori K, et al. (1995). Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature*. 378:88-91.

Rangnekar VV, Waheed S, Rangnekar VM. (1992). Interleukin-1-inducible tumor growth arrest is characterized by activation of cell type-specific "early" gene expression programs. *J Biol Chem.* 267:6240-8.

Robertson MJ, Kirkwood JM, Logan TF, Koch KM, Kathman S, Kirby LC, Bell WN, Thurmond LM, Weisenbach J, Dar MM. (2008). A dose-escalation study of recombinant human interleukin-18 using two different schedules of administration in patients with cancer. *Clin Cancer Res.* 14:3462-9

Scatchard G. (1949). *Ann New York Acad Sci* 51, 660-72.

Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA. (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity.* 23:479-90.

Shaw MH, Kamada N, Kim YG, Núñez G. (2012). Microbiota-induced IL-1 β , but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. *J Exp Med.* 209:251-8.

Sims JE, Smith DE. The IL-1 family: regulators of immunity. (2010). *Nat Rev Immunol.* 10:89-102.

Skerra, A. (2008) Alternative binding proteins: anticalins – harnessing the structural plasticity of the lipocalin ligand pocket to engineer novel binding activities. *FEBS J.* 275, 2677-2683.

Stump, M.T., Binz, H.K., Amstutz, P. (2008) DARPin^s: a new generation of protein therapeutics. *Drug iscov. Today* 13, 695-701.

Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC. (2006). A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med.* 203:1685-91.

Takeda K, Tsutsui H, Yoshimoto T, Adachi O, Yoshida N, Kishimoto T, Okamura H, Nakanishi K, Akira S. (1998). Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity.* 8:383-90.

Tramontano, A., Bianchi, E., Venturini, S., Martin, F., Pessi, A and Sollazzo, M. (1994) The making of the minibody: an engineered beta-protein for the display of conformationally constrained peptides. *J. Mol. Recognition* 7, 9-24.

Usui N, Mimnaugh EG, Sinha BK. (1991). A role for the interleukin 1 receptor in the synergistic antitumor effects of human interleukin 1 alpha and etoposide against human melanoma cells. *Cancer Res.* 1991 51:769-74.

5 Vanden Berghe W, Plaisance S, Boone E, De Bosscher K, Schmitz ML, Fiers W, Haegeman G. (1998). p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J Biol Chem.* 273:3285-90.

10 Vaneycken I, Devoogdt N, Van Gassen N, Vincke C, Xavier C, Wernery U, Muyldermans S, Lahoutte T, Caveliers V. (2011). Preclinical screening of anti-HER2 nanobodies for molecular imaging of breast cancer. *FASEB J.* 25:2433-46.

Wigginton JM, Lee JK, Wiltrot TA, Alvord WG, Hixon JA, Subleski J, Back TC, Wiltrot RH. (2002). Synergistic engagement of an ineffective endogenous anti-tumor immune response and induction of IFN-gamma and Fas-ligand-dependent tumor eradication by combined administration of IL-18 and IL-2. *J Immunol.* 169:4467-74.

15 Wilke CM, Bishop K, Fox D, Zou W. (2011). Deciphering the role of Th17 cells in human disease. *Trends Immunol.* 32:603-11.

Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, Oliver P, Huang W, Zhang P, Zhang J, Shellito JE, Bagby GJ, Nelson S, Charrier K, Peschon JJ, Kolls JK. (2001). Requirement of interleukin 17 receptor signaling for lung CXC chemokine and 20 granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med.* 194:519-27.

Zabeau L, Verhee A, Catteeuw D, Faes L, Seeuws S, Decruy T, Elewaut D, Peelman F, Tavernier J. (2012). Selection of non-competitive leptin antagonists using a random nanobody-based approach. *Biochem J.* 441:425-34.

25 Zaki MH, Vogel P, Body-Malapel M, Lamkanfi M, Kanneganti TD. (2010). IL-18 production downstream of the Nlrp3 inflammasome confers protection against colorectal tumor formation. *J Immunol.* 185:4912-20.

CLAIMS:

1. A targeting construct, comprising:

(i) a mutated human IL-1 β characterized by a reduced affinity for its receptor as compared to wild type human IL-1 β wherein the mutated human IL-1 β comprises one or more mutations selected from the group consisting of R120X, Q131G, L145G, H146X, Q148X, F162A, Q164E, E167K, and K208E with respect to wild type human IL-1 β having the amino acid sequence of Genbank accession number NP_000567, version NP-000567.1, GI:10835145, where X is a non-conservative amino acid change, and

(ii) a targeting moiety comprising a single domain antibody (sdAb).

10 2. The targeting construct, according to claim 1, wherein said targeting moiety is targeting to a marker expressed on an IL-1R1 and/or IL-1RacP expressing cell.

3. The targeting construct according to claim 1 or 2, wherein said targeting moiety is directed to a tissue specific marker.

15 4. The targeting construct according to any one of claims 1 to 3, wherein said targeting moiety is directed to Her2.

5. The targeting construct according to any one of claims 1 to 4, wherein the mutated human IL-1 β comprises one or more mutations selected from the group consisting of R120G, Q131G, L145G, H146G, Q148G, F162A, and K208E.

20 6. The targeting construct according to any one of claims 1 to 4, wherein the mutated human IL-1 β comprises one or more mutations selected from the group consisting of:

(i) Q131G and Q148G;

(ii) Q148G and K208E;

(iii) R120G and Q131G;

(iv) R120G and H146G;

25 (v) R120G and K208E;

(vi) R120G, F162A, and Q164E;

(vii) F162A and Q164E; and

(viii) Q164E and E167K.

7. The targeting construct according to claim 6, wherein the targeting moiety comprises a single domain antibody against Her2.

8. The targeting construct according to any one of claims 1 to 7, wherein the targeting moiety restores activity of the mutated human IL-1 β on target cells.

5 9. The targeting construct according to any one of claims 1 to 8 for use as a medicament.

10. The targeting construct according to any one of claims 1 to 9 for use in stimulation of an immune response.

10 11. The targeting construct according to any one of claims 1 to 10 for use in treatment of cancer.

12. A composition comprising the targeting construct according to any one of claims 1 to 11 and a suitable excipient.

15 13. The targeting construct, according to claim 1, wherein the single domain antibody comprises a variable domain of camelid heavy chain antibodies (VHH) or a variable domain of new antigen receptors (VNAR).

14. Use of the targeting construct according to any one of claims 1 to 8 for stimulation of an immune response.

15. Use of the targeting construct according to any one of claims 1 to 8 for the treatment of cancer.

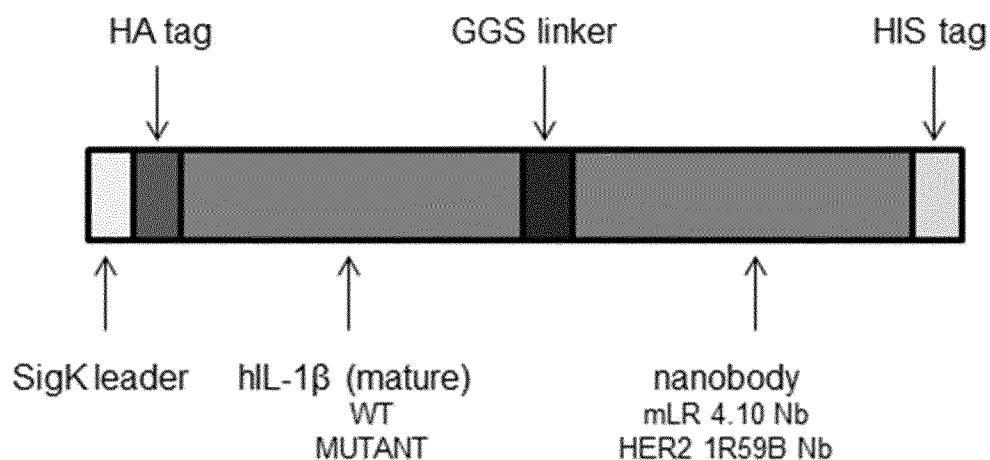
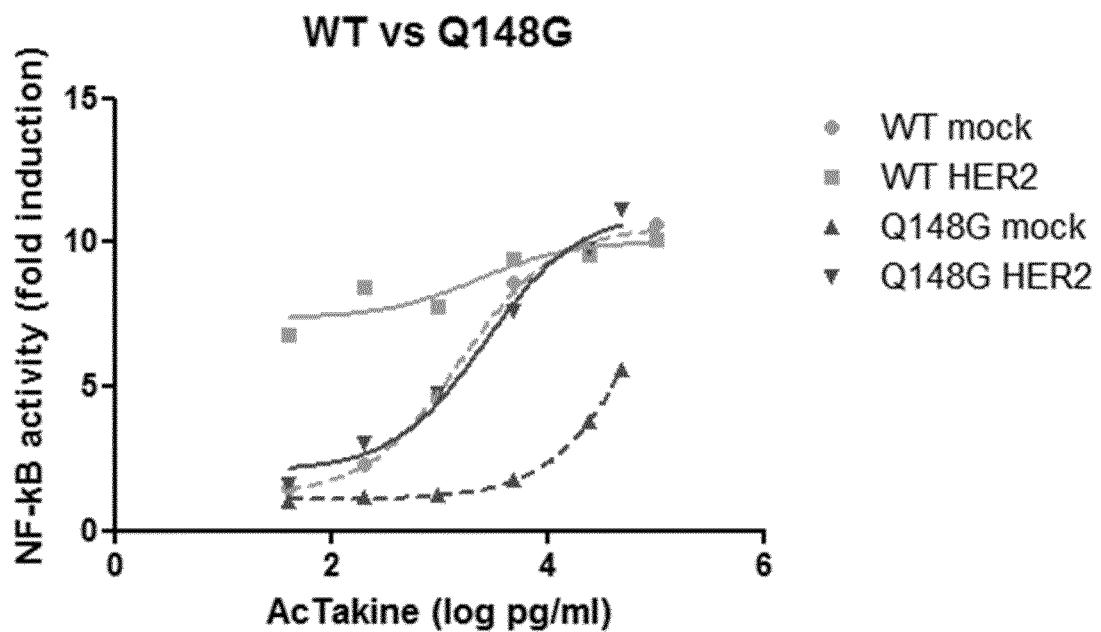
Figure 1**Figure 2****A**

Figure 2 continued

B

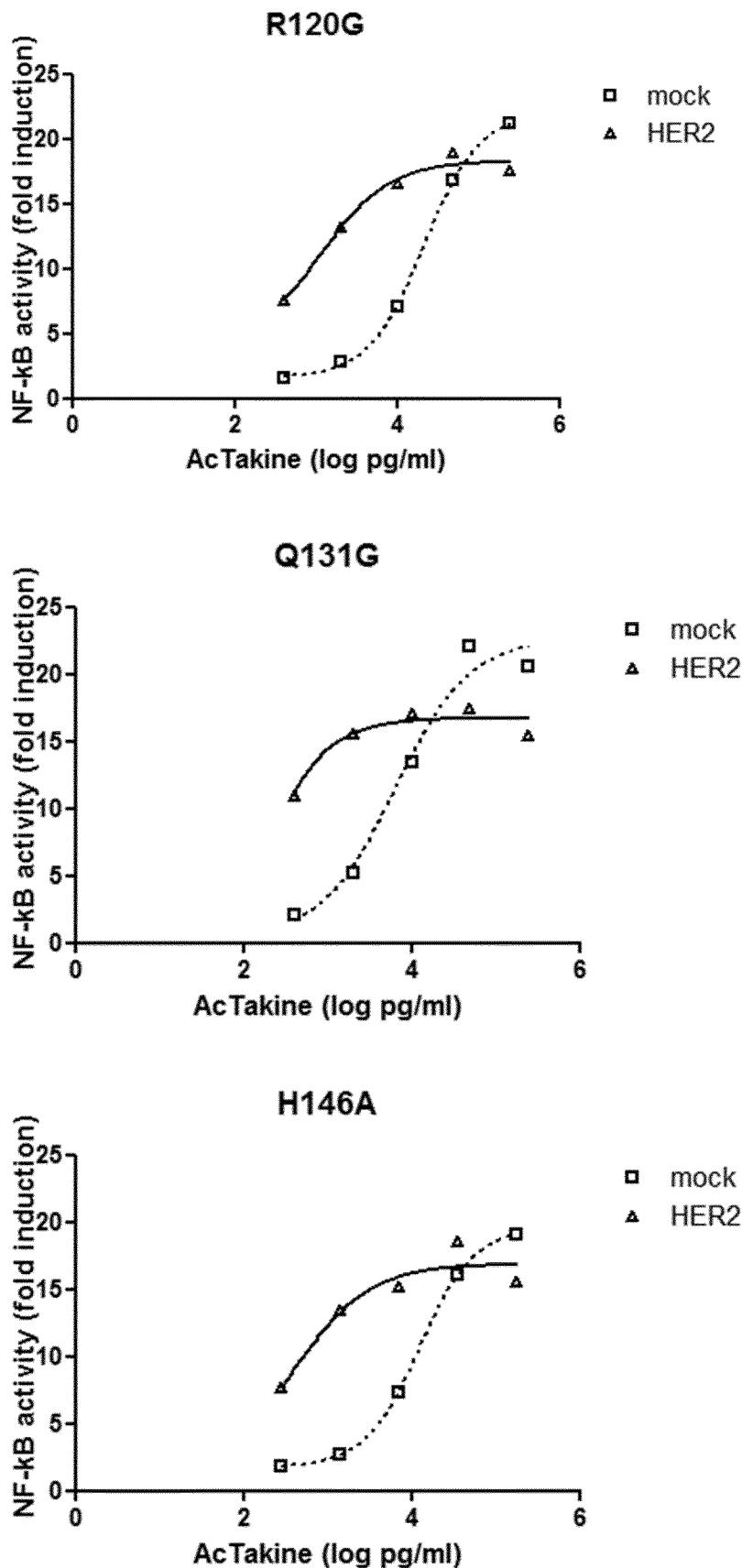


Figure 2 continued

B

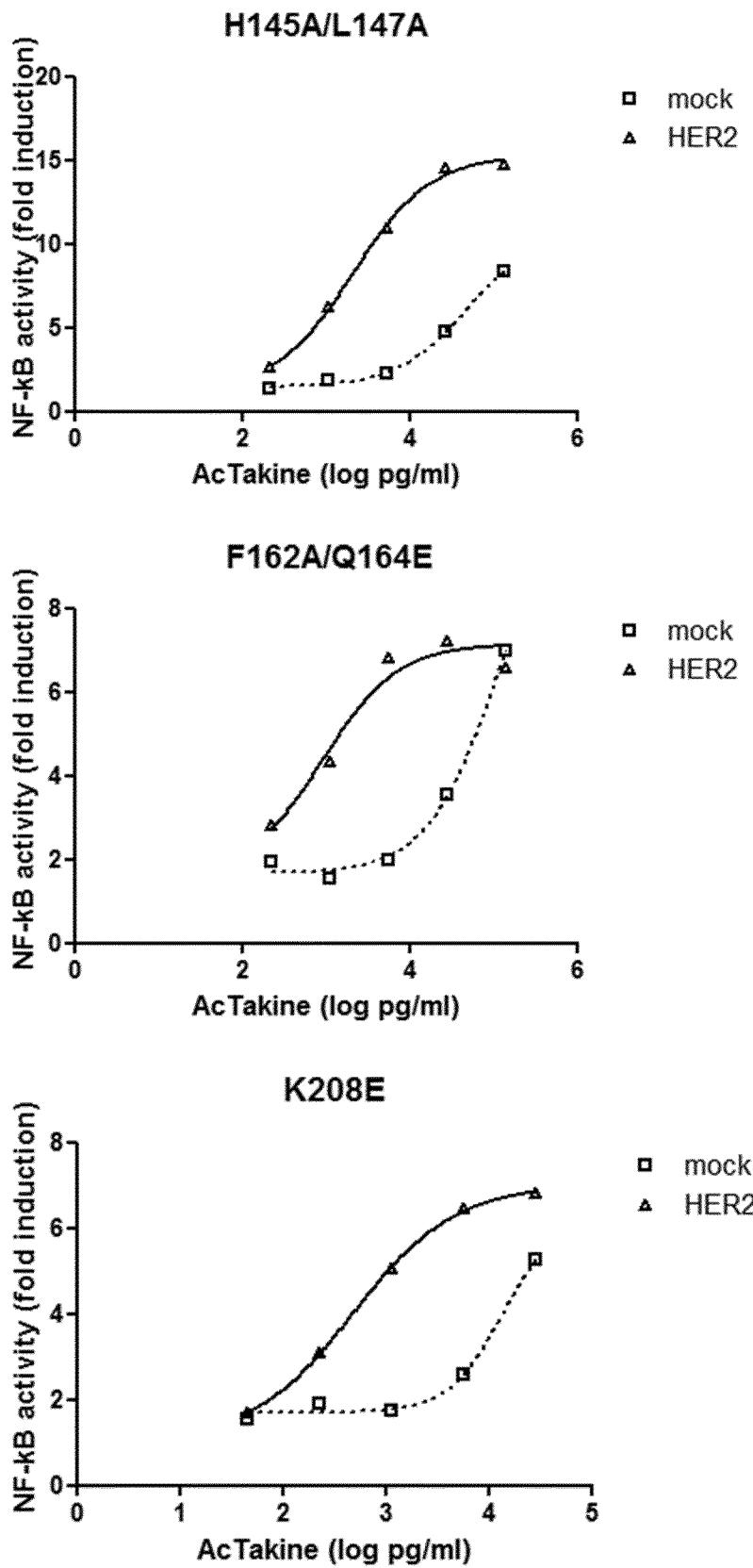


Figure 3

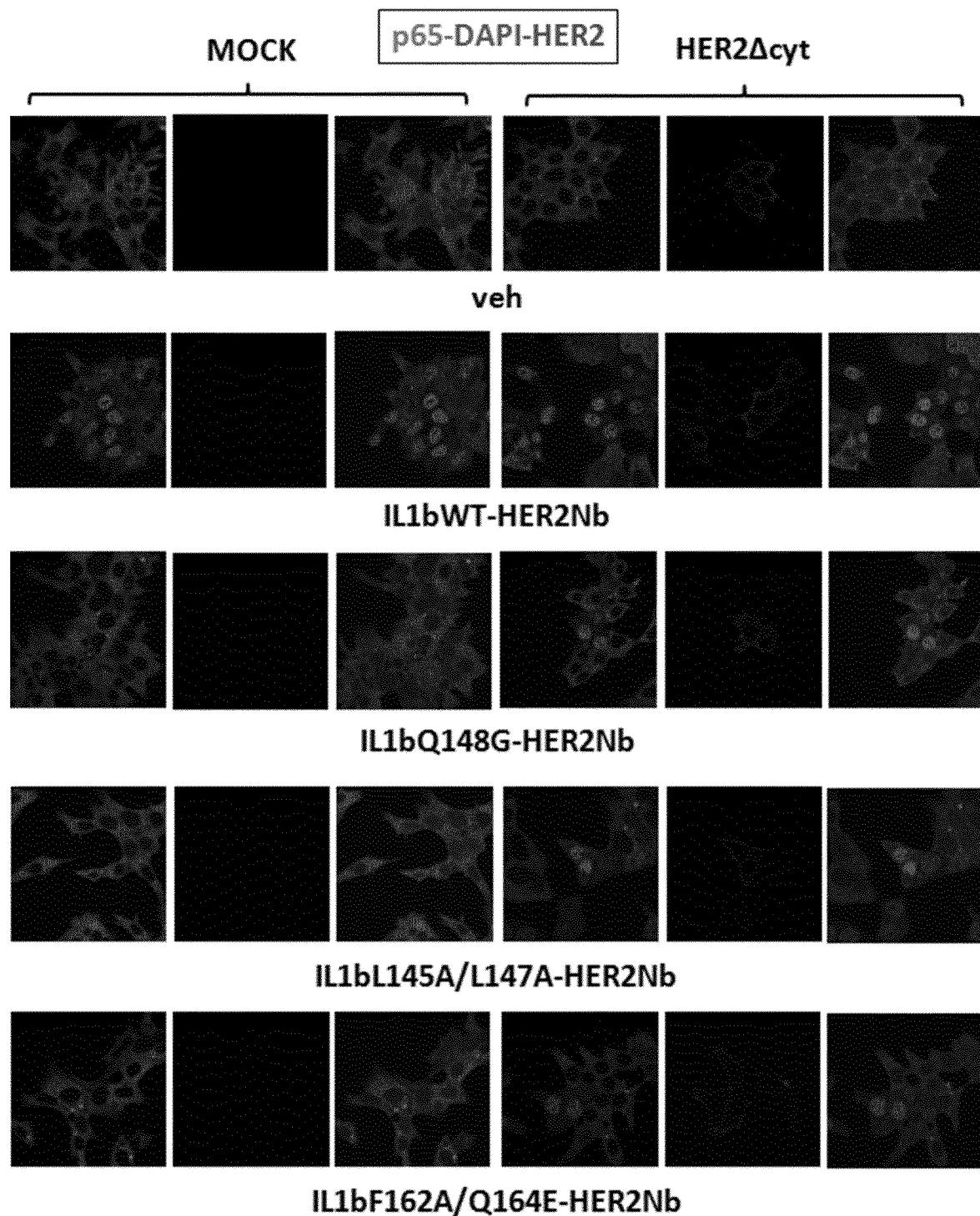


Figure 4

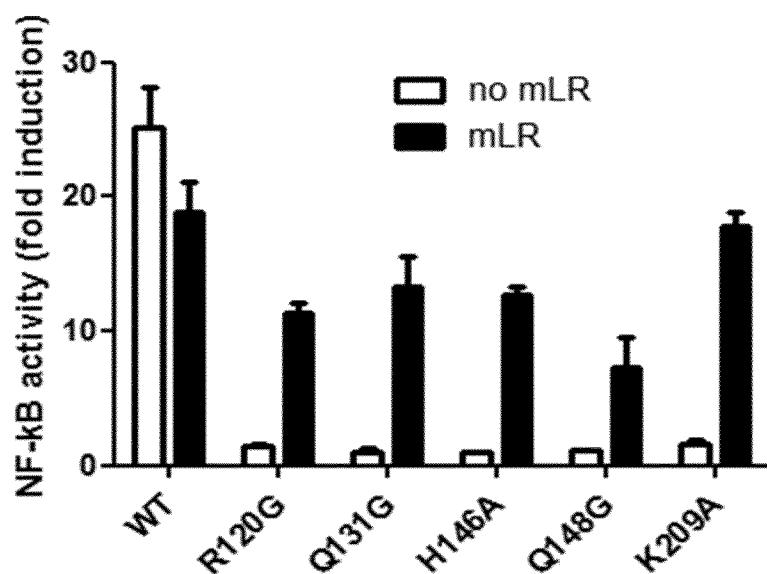


Figure 5

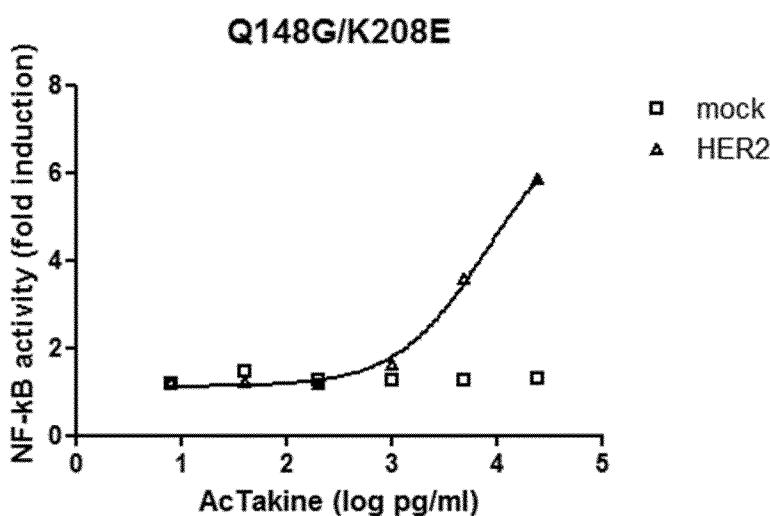
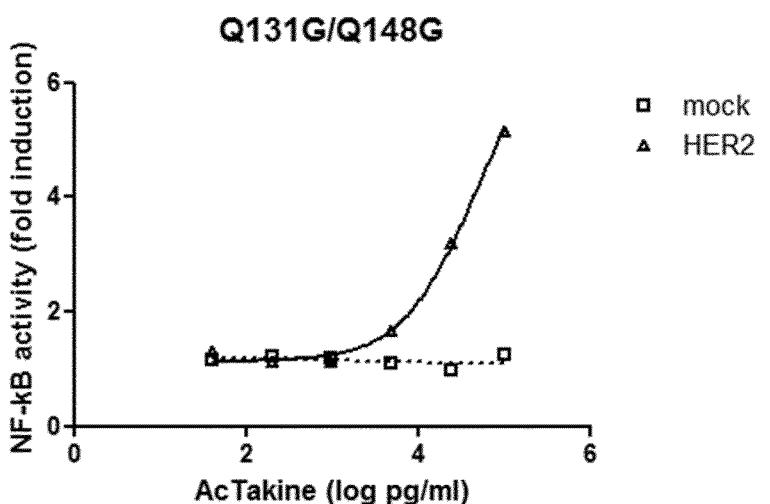


Figure 5 continued

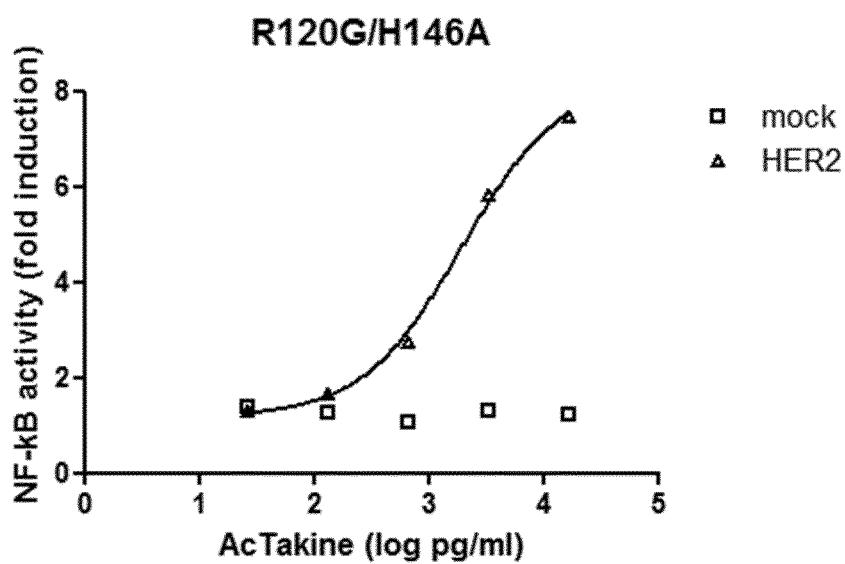
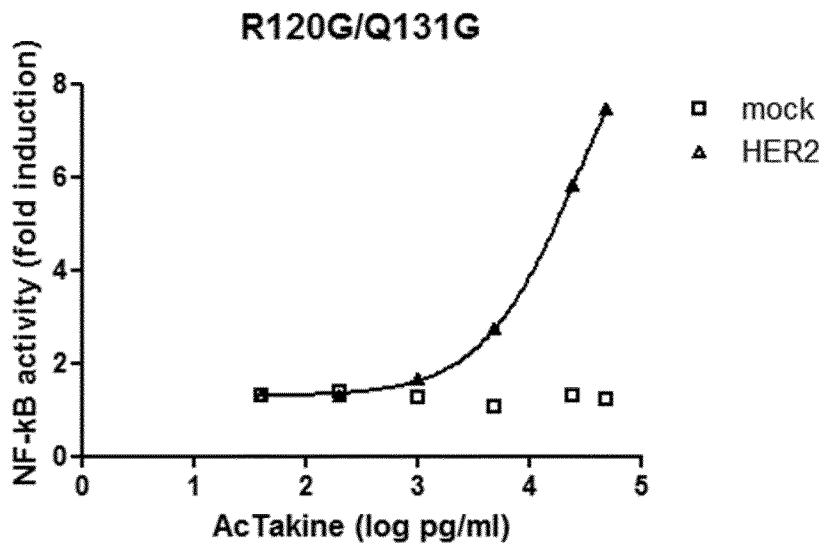
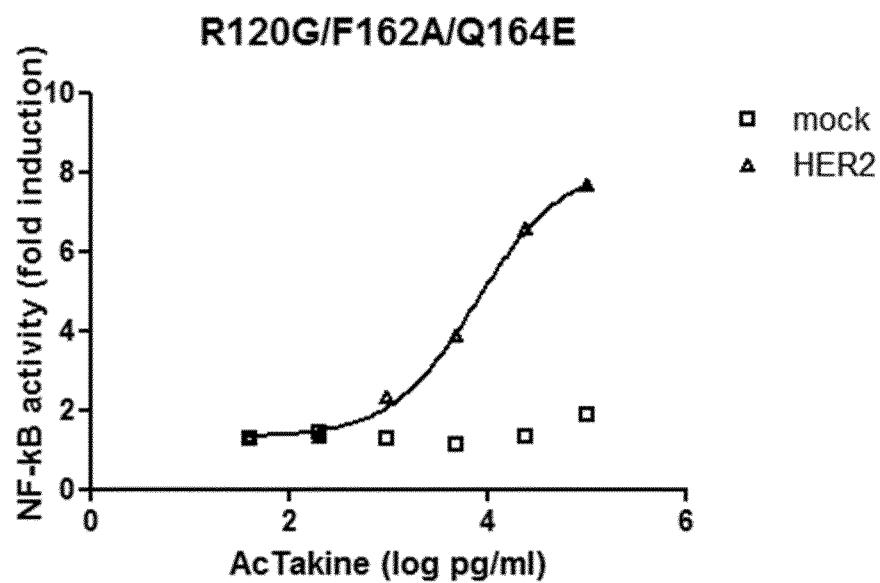
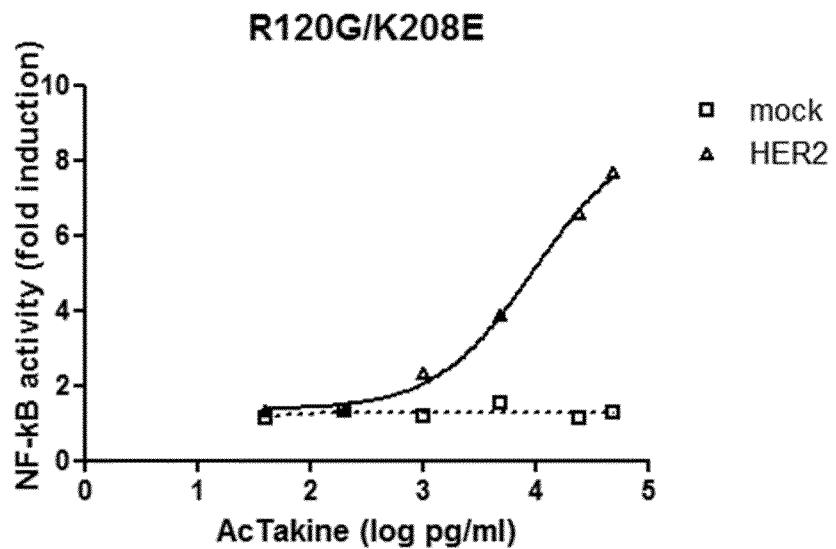


Figure 5 continued





SigKleader

hIL-1 β (mature)

WT

MUTANT

nanobody
mLR 4.10 Nb
HER2 1R59B Nb