

Her2-targeting Antigen Binding Molecules comprising 4-1BBL

FIELD OF THE INVENTION

The invention relates to Her2 targeting 4-1BB agonists, in particular 4-1BBL trimer-containing antigen binding molecules comprising an antigen binding domain capable of specific binding to Her2 and their use in the treatment of cancer. The invention further relates to
5 methods of producing these molecules and to methods of using the same.

BACKGROUND

4-1BB (CD137), a member of the TNF receptor superfamily, was first identified as an inducible molecule expressed by activated T cells (Kwon and Weissman, 1989, *Proc Natl Acad Sci USA* 86, 1963-1967). Subsequent studies demonstrated that many other immune cells
10 also express 4-1BB, including NK cells, B cells, NKT cells, monocytes, neutrophils, mast cells, dendritic cells (DCs) and cells of non-hematopoietic origin such as endothelial and smooth muscle cells (Vinay and Kwon, 2011, *Cell Mol Immunol* 8, 281-284). Expression of 4-1BB in different cell types is mostly inducible and driven by various stimulatory signals, such as T-cell receptor (TCR) or B-cell receptor triggering, as well as signaling induced through co-stimulatory
15 molecules or receptors of pro-inflammatory cytokines (Diehl et al., 2002, *J Immunol* 168, 3755-3762; Zhang et al., 2010, *Clin Cancer Res* 13, 2758-2767).

4-1BB ligand (4-1BBL or CD137L) was identified in 1993 (Goodwin et al., 1993, *Eur J Immunol* 23, 2631-2641). It has been shown that expression of 4-1BBL was restricted on professional antigen presenting cells (APC) such as B-cells, DCs and macrophages. Inducible
20 expression of 4-1BBL is characteristic for T-cells, including both $\alpha\beta$ and $\gamma\delta$ T-cell subsets, and endothelial cells (Shao and Schwarz, 2011, *J Leukoc Biol* 89, 21-29).

Co-stimulation through the 4-1BB receptor (for example by 4-1BBL ligation) activates multiple signaling cascades within the T cell (both CD4⁺ and CD8⁺ subsets), powerfully augmenting T cell activation (Bartkowiak and Curran, 2015). In combination with TCR
25 triggering, agonistic 4-1BB-specific antibodies enhance proliferation of T-cells, stimulate lymphokine secretion and decrease sensitivity of T-lymphocytes to activation-induced cell death (Snell et al., 2011, *Immunol Rev* 244, 197-217). This mechanism was further advanced as the first proof of concept in cancer immunotherapy. In a preclinical model administration of an

agonistic antibody against 4-1BB in tumor bearing mice led to potent anti-tumor effect (Melero et al., 1997, Nat Med 3, 682-685). Later, accumulating evidence indicated that 4-1BB usually exhibits its potency as an anti-tumor agent only when administered in combination with other immunomodulatory compounds, chemotherapeutic reagents, tumor-specific vaccination or
5 radiotherapy (Bartkowiak and Curran, 2015, Front Oncol 5, 117).

Signaling of the TNFR-superfamily needs cross-linking of the trimerized ligands to engage with the receptors, so does the 4-1BB agonistic antibodies which require wild type Fc-binding (Li and Ravetch, 2011, Science 333, 1030-1034). However, systemic administration of 4-1BB-specific agonistic antibodies with the functionally active Fc domain resulted in influx of CD8⁺ T-
10 cells associated with liver toxicity (Dubrot et al., 2010, Cancer Immunol Immunother 59, 1223-1233) that is diminished or significantly ameliorated in the absence of functional Fc-receptors in mice. In the clinic, an Fc-competent 4-1BB agonistic Ab (BMS-663513) (NCT00612664) caused a grade 4 hepatitis leading to termination of the trial (Simeone and Ascierto, 2012, J Immunotoxicol 9, 241-247). Therefore, there is a need for effective and safer 4-1BB agonists.

15 The human epidermal growth factor receptor-2 (Her2; ErbB2) is a receptor tyrosine kinase and a member of the epidermal growth factor receptor (EGFR) family of transmembrane receptors. Her2 is overexpressed in a range of tumor types and it has been implicated in disease initiation and progression. It is associated with poor prognosis. For example, overexpression of Her2 is observed in approximately 30% of human breast cancers and it is implicated in the
20 aggressive growth and poor clinical outcomes associated with these tumors (Slamon et al (1987) Science 235:177-182).

The humanized anti-Her2 monoclonal antibody trastuzumab (CAS 180288-69-1, HERCEPTIN®, huMAb4D5-8, rhuMAb Her2, Genentech) targets the extracellular domain of HER-2 (US 5677171; US 5821337; US 6054297; US 6165464; US 6339142; US 6407213; US
25 6639055; US 6719971; US 6800738; US 7074404; Coussens et al (1985) Science 230:1 132-9; Slamon et al (1989) Science 244:707-12; Slamon et al (2001) New Engl. J. Med. 344:783-792). Trastuzumab has been shown to inhibit the proliferation of human tumor cells that overexpress HER-2 and is a mediator of antibody-dependent cellular cytotoxicity, ADCC (Hudziak et al (1989) Mol Cell Biol 9:1 165-72; Lewis et al (1993) Cancer Immunol Immunother; 37:255-63;
30 Baselga et al (1998) Cancer Res. 58:2825-2831; Hotaling et al (1996) [abstract]. Proc. Annual Meeting Am Assoc Cancer Res; 37:471; Pegram MD, et al (1997) [abstract]. Proc Am Assoc Cancer Res; 38:602; Sliwkowski et al (1999) Seminars in Oncology 26(4), Suppl 12:60- 70; Yarden Y. and Sliwkowski, M. (2001) Nature Reviews: Molecular Cell Biology, Macmillan Magazines, Ltd., Vol. 2:127-137).

HERCEPTIN® (trastuzumab, Genentech Inc.) was approved in 1998 for the treatment of patients with Her2-overexpressing metastatic breast cancers (Baselga et al, (1996) J. Clin. Oncol. 14:737-744). In 2006, the FDA approved HERCEPTIN® as part of a treatment regimen containing doxorubicin, cyclophosphamide and paclitaxel for the adjuvant treatment of patients with Her2-positive, node-positive breast cancer.

Pertuzumab (also known as recombinant humanized monoclonal antibody 2C4, rhuMab 2C4, PERJETA®, Genentech, Inc, South San Francisco) is another antibody treatment targeting Her2. Pertuzumab is a Her dimerization inhibitor (HDI) and functions to inhibit the ability of Her2 to form active heterodimers or homodimers with other Her receptors (such as EGFR/Her 1, Her2, Her3 and Her4). See, for example, Harari and Yarden Oncogene 19:6102-14 (2000); Yarden and Sliwkowski. Nat Rev Mol Cell Biol 2:127-37 (2001); Sliwkowski, Nat Struct Biol 10:158-9 (2003); Cho et al. Nature 421:756-60 (2003); and Malik et al., Pro Am Soc Cancer Res 44:176-7 (2003); US 7560111. PERJETA® was first approved in 2012 in combination with trastuzumab and docetaxel for the treatment of patients with advanced or late-stage (metastatic) Her2-positive breast cancer. The combination therapy using trastuzumab and pertuzumab is meanwhile also approved for the neoadjuvant (before surgery) treatment of f Her2-positive, locally advanced, inflammatory, or early stage breast cancer and for adjuvant (after surgery) treatment of Her2-positive early breast cancer (EBC) at high risk of recurrence. The mechanisms of action of Perjeta and Herceptin are believed to complement each other, as both bind to the Her2 receptor, but to different places. The combination of Perjeta and Herceptin is thought to provide a more comprehensive, dual blockade of HER signaling pathways, thus preventing tumor cell growth and survival.

Bispecific, bivalent Her2 antibodies that are directed against domains II, III and IV of human ErbB2 are disclosed in WO 2012/143523. Bispecific HER-2 antibodies comprising optimized variants of the antibodies rhuMab 2C4 and hu4D5, called Herceptarg, have been described in WO 2015/091738.

Although the therapeutic efficacy of trastuzumab in breast carcinoma is well demonstrated, there are many patients who do not benefit from trastuzumab because of resistance. Given the lack of an effective anti-Her2 therapy in specific cancers expressing low levels of Her2, the resistance to the current therapies, and the prevalence of Her2 related cancers, new therapies are required to treat such cancers.

The new antigen binding molecules of the present invention combine an anti-Her2 antigen binding domain with a moiety that is capable of forming a costimulatory 4-1BB ligand trimer and that is sufficiently stable to be pharmaceutically useful. Fusion proteins composed of a binding specificity for CD137 and a binding specificity for Her2/neu are disclosed in WO

2016/177802. These molecules are antibody-lipocalin mutein fusion polypeptides, meaning that a lipocalin mutein with binding specificity for CD137 is fused to an anti-Her2 antibody.

Lipocalin muteins (anticalins) are non-antibody scaffolds and the conversion of such modalities into differentiated drugs has been challenging (Vazquez-Lombardi et al. 2015, Drug Discovery

- 5 Today 20, 1271-1283). Compared to antibodies challenges could arise in view of different serum half-life, tissue penetration and immunogenicity. Thus, there is still a need for drug candidates with improved properties that are based on antibody technology or human-like proteins.

SUMMARY OF THE INVENTION

- The new antigen binding molecules of the present invention combine an anti-Her2 antigen
10 binding domain with a moiety that is capable of forming a costimulatory 4-1BBL trimer and that is sufficiently stable to be pharmaceutically useful. Surprisingly, antigen binding molecules of the invention provide a trimeric and thus biologically active human 4-1BB ligand, although one of the trimerizing 4-1BBL ectodomains is located on another polypeptide than the other two 4-1BBL ectodomains of the molecule. Targeted by the anti-Her2 antigen binding domain the
15 antigen binding molecules of the present invention have an increased activity on the tumor site, comprise the natural human 4-1BB ligand and should thus impose less safety issues compared to conventional 4-1BB agonistic antibodies or more artificial fusion proteins.

In one aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule comprising

- 20 (a) an antigen binding domain capable of specific binding to Her2,
(b) a first and a second polypeptide that are linked to each other by a disulfide bond, wherein the antigen binding molecule is characterized in that the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other by a peptide linker and in that the second polypeptide comprises one ectodomain of 4-1BBL or a fragment
25 thereof, and
(c) an Fc domain composed of a first and a second subunit capable of stable association.

In a particular aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule, wherein the ectodomain of 4-1BBL or a fragment thereof comprises the amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO: 2, SEQ ID NO:3,
30 SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO: 6, SEQ ID NO:7 and SEQ ID NO:8, particularly the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:5.

In a further aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule, comprising

- (a) an antigen binding domain capable of specific binding to Her2,
35 (b) a first and a second polypeptide that are linked to each other by a disulfide bond,

wherein the antigen binding molecule is characterized in that the first polypeptide comprises the amino acid sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12 and in that the second polypeptide comprises the amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:3 and
5 SEQ ID NO:4, and
(c) an Fc domain composed of a first and a second subunit capable of stable association.

In one aspect, the Fc domain is an IgG, particularly an IgG1 Fc domain or an IgG4 Fc domain. More particularly, the Fc domain is an IgG1 Fc domain. In a particular aspect, the Fc domain comprises a modification promoting the association of the first and second subunit of the
10 Fc domain. In a particular aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule, wherein the Fc domain comprises knob-into-hole modifications promoting association of the first and the second subunit of the Fc domain. In a specific aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule, wherein the first subunit of the Fc domain comprises the amino acid substitutions S354C and T366W (numbering
15 according to Kabat EU index) and the second subunit of the Fc domain comprises the amino acid substitutions Y349C, T366S, L368A and Y407V (numbering according to Kabat EU index).

In another aspect, the invention is concerned with a 4-1BBL trimer-containing antigen binding molecule as defined herein before, comprising (c) an Fc domain composed of a first and a second subunit capable of stable association, wherein the Fc domain comprises one or more
20 amino acid substitution that reduces binding to an Fc receptor, in particular towards Fcγ receptor. In particular, the Fc domain comprises amino acid substitutions at positions 234 and 235 (EU numbering according to Kabat) and/or 329 (EU numbering according to Kabat) of the IgG heavy chains. Particularly, provided is a 4-1BBL trimer-containing antigen binding molecule, wherein the Fc domain is an IgG1 Fc domain comprising the amino acid substitutions the amino acid
25 substitutions L234A, L235A and P329G (numbering according to Kabat EU index).

In one aspect, the 4-1BBL trimer-containing antigen binding molecule is one, wherein wherein the antigen binding domain capable of specific binding to Her2 is a Fab molecule capable of specific binding to Her2. In another aspect, the antigen binding domain capable of specific binding to Her2 is a cross-over Fab molecule or a scFV molecule capable of specific
30 binding to Her2.

In one aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule as described herein before, wherein the 4-1BBL trimer-containing antigen binding molecule comprises one Fab domain capable of specific binding to Her2, meaning that it comprises monovalent binding towards Her2.

- In a further aspect, provided is a 4-1BBL trimer-containing antigen binding molecule, wherein wherein the antigen binding domain capable of specific binding to Her2 comprises
- (a) a VH domain comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:13, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:14, and (iii) CDR-H3 comprising the amino acid sequence of SEQ ID NO:15, and a VL domain comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:16, (v) CDR-L2 comprising the amino acid sequence of SEQ ID NO:17, and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:18, or
- (b) a VH domain comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:21, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:22, and (iii) CDR-H3 comprising the amino acid sequence of SEQ ID NO:23, and a VL domain comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:24, (v) CDR-L2 comprising the amino acid sequence of SEQ ID NO:25, and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:26, or
- (c) a VH domain comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:29, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:30, and (iii) CDR-H3 comprising the amino acid sequence of SEQ ID NO:31, and a VL domain comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:32, (v) CDR-L2 comprising the amino acid sequence of SEQ ID NO:33, and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:34.

- In a further aspect, the 4-1BBL trimer-containing antigen binding molecule of the invention comprises
- (a) a VH domain comprising an amino acid sequence of SEQ ID NO:19 and a VL domain comprising an amino acid sequence of SEQ ID NO:20, or
- (b) a VH domain comprising an amino acid sequence of SEQ ID NO:27 and a VL domain comprising an amino acid sequence of SEQ ID NO:28, or
- (c) a VH domain comprising an amino acid sequence of SEQ ID NO:35 and a VL domain comprising an amino acid sequence of SEQ ID NO:36.

- In a further aspect, provided is a 4-1BBL trimer-containing antigen binding molecule, wherein the antigen binding molecule comprises
- a first heavy chain and a first light chain, both comprising a Fab molecule capable of specific binding to Her2,
- a second heavy chain comprising the constant domains and two ectodomains of a 4-1BBL or a fragment thereof connected to each other by a first peptide linker fused at its C-terminus by a second peptide linker to a second heavy or light chain,
- and a second light chain comprising a constant domain and one ectodomain of 4-1BBL or a fragment thereof fused at its C-terminus by a third peptide linker to a second light or heavy

chain, respectively. More particularly, provided is a 4-1BBL trimer-containing antigen binding molecule, wherein the first peptide comprising two ectodomains of 4-1BBL or a fragment thereof connected to each other by a first peptide linker is fused at its C-terminus by a second peptide linker to a CL domain that is part of a heavy chain, and the second peptide comprising one ectodomain of said 4-1BBL or a fragment thereof is fused at its C-terminus by a third peptide linker to a CH1 domain that is part of a light chain.

In a particular aspect, the invention relates to a 4-1BBL trimer-containing antigen binding molecule as defined above, wherein the peptide linker is (G4S)₂, i.e. a peptide linker of SEQ ID NO:68. In one aspect, the peptide linker in all instances is (G4S)₂.

10 Provided is further a 4-1BBL trimer-containing antigen binding molecule, wherein in the CL domain adjacent to the TNF ligand family member the amino acid at position 123 (EU numbering) has been replaced by arginine (R) and the amino acid at position 124 (EU numbering) has been substituted by lysine (K), and wherein in the CH1 domain adjacent to the TNF ligand family member the amino acids at position 147 (EU numbering) and at position 213
15 (EU numbering) have been substituted by glutamic acid (E).

In another aspect, provided is a 4-1BBL trimer-containing antigen binding molecule, wherein the antigen binding molecule comprises

- (i) a first heavy chain comprising the VH domain comprising the amino acid sequence of SEQ ID NO:19 and a first light chain comprising the VL domain comprising the amino acid sequence
20 of SEQ ID NO:20 or
a first heavy chain comprising the VH domain comprising the amino acid sequence of SEQ ID NO:27 and a first light chain comprising the VL domain comprising the amino acid sequence of SEQ ID NO:28, or
a first heavy chain comprising the VH domain comprising the amino acid sequence of SEQ ID
25 NO:35 and a first light chain comprising the VL domain comprising the amino acid sequence of SEQ ID NO:36,
(ii) a second heavy chain comprising the amino acid sequence selected from the group consisting of SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41 and SEQ ID NO:43, and
(iii) a second light chain comprising the amino acid sequence selected from the group consisting
30 of SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42 and SEQ ID NO:44.

In a particular aspect, provided is a 4-1BBL trimer-containing antigen binding molecule comprising

- (a) a first heavy chain comprising the amino acid sequence of SEQ ID NO:45, a first light chain comprising the amino acid sequence of SEQ ID NO:46, a second heavy chain comprising the
35 amino acid sequence of SEQ ID NO:37 and a second light chain comprising the amino acid

sequence of SEQ ID NO:38, or

(b) a first heavy chain comprising the amino acid sequence of SEQ ID NO:47, a first light chain comprising the amino acid sequence of SEQ ID NO:48, a second heavy chain comprising the amino acid sequence of SEQ ID NO:37 and a second light chain comprising the amino acid

5 sequence of SEQ ID NO:38, or

(c) a first heavy chain comprising the amino acid sequence of SEQ ID NO:49, a first light chain comprising the amino acid sequence of SEQ ID NO:50, a second heavy chain comprising the amino acid sequence of SEQ ID NO:37 and a second light chain comprising the amino acid sequence of SEQ ID NO:38.

10 According to another aspect of the invention, there is provided an isolated nucleic acid molecule encoding a 4-1BBL trimer-containing antigen binding molecule as defined herein before. The invention further provides a vector, particularly an expression vector, comprising the isolated nucleic acid molecule of the invention and a host cell comprising the isolated nucleic acid or the vector of the invention. In some embodiments the host cell is an eukaryotic cell,
15 particularly a mammalian cell.

In another aspect, provided is a method for producing the 4-1BBL trimer-containing antigen binding molecule of the invention, comprising culturing the host cell of the invention under conditions suitable for expression of the 4-1BBL trimer-containing antigen binding molecule, and isolating the 4-1BBL trimer-containing antigen binding molecule. The invention
20 also encompasses a 4-1BBL trimer-containing antigen binding molecule produced by the method of the invention.

The invention further provides a pharmaceutical composition comprising the 4-1BBL trimer-containing antigen binding molecule of the invention and at least one pharmaceutically acceptable excipient. In another aspect, a pharmaceutical composition is provided comprising the
25 4-1BBL trimer-containing antigen binding molecule of the invention and at least one pharmaceutically acceptable excipient, further comprising an additional therapeutic agent, e.g. a chemotherapeutic agent and/ or other agents for use in cancer immunotherapy. In a further aspect, provided is a pharmaceutical composition further comprising a T-cell activating anti-CD3 bispecific antibody, in particular an anti-Her2/anti-CD3 bispecific antibody.

30 Also encompassed by the invention is the 4-1BBL trimer-containing antigen binding molecule of the invention, or the pharmaceutical composition of the invention, for use as a medicament. In one aspect is provided the 4-1BBL trimer-containing antigen binding molecule of the invention, or the pharmaceutical composition of the invention, for use in the treatment of a disease in an individual in need thereof. In a specific embodiment, provided is the 4-1BBL
35 trimer-containing antigen binding molecule of the invention, or the pharmaceutical composition

of the invention, for use in the treatment of cancer. In another aspect, provided is the 4-1BBL trimer-containing antigen binding molecule of the invention, or the pharmaceutical composition of the invention, for use in up-regulating or prolonging cytotoxic T cell activity. In another aspect, provided is the 4-1BBL trimer-containing antigen binding molecule of the invention, or the pharmaceutical composition of the invention, for use in the treatment of cancer, wherein the 4-1BBL trimer-containing antigen binding molecule is used in combination with another therapeutic agent, in particular a T-cell activating anti-CD3 bispecific antibody. In one aspect, the T-cell activating anti-CD3 bispecific antibody is administered concurrently with, prior to, or subsequently to the 4-1BBL trimer-containing antigen binding molecule.

Also provided is the use of the 4-1BBL trimer-containing antigen binding molecule of the invention for the manufacture of a medicament for the treatment of a disease in an individual in need thereof, in particular for the manufacture of a medicament for the treatment of cancer, as well as a method of treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of a composition comprising the 4-1BBL trimer-containing antigen binding molecule as disclosed herein in a pharmaceutically acceptable form. In a specific aspect, the disease is cancer. Further provided is the use of the 4-1BBL trimer-containing antigen binding molecule of the invention for the manufacture of a medicament for the treatment of cancer, wherein the 4-1BBL trimer-containing antigen binding molecule is used in combination with a T-cell activating anti-CD3 bispecific antibody, in particular an anti-Her2/anti-CD3 antibody. Furthermore, provided is a method for treating an individual having cancer comprising administering to the subject an effective amount of the 4-1BBL trimer-containing antigen binding molecule of the invention, or a pharmaceutical composition thereof, and an effective amount of a T-cell activating anti-CD3 bispecific antibody, in particular an anti-Her2/anti-CD3 antibody. Also provided is a method of up-regulating or prolonging cytotoxic T cell activity in an individual having cancer, comprising administering to the individual an effective amount of the 4-1BBL trimer-containing antigen binding molecule of the invention, or the pharmaceutical composition of the invention. In any of the above embodiments the individual is preferably a mammal, particularly a human.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the components for the assembly of the monovalent Her2-targeting split trimeric 4-1BB ligand Fc fusion antigen binding molecules. **Fig. 1A** shows the dimeric 4-1BB ligand that is fused at the C-terminus to a human IgG1-CL domain with mutations E123R and Q124K (charged variant) and **Fig. 1B** shows the monomeric 4-1BB ligand fused at its C-terminus to a human IgG1-CH1 domain with mutations K147E and K213E (charged variant).

Figure 2A illustrates schematically the structure of the monovalent Her2-targeting split trimeric 4-1BB ligand Fc (kih) fusion antigen binding molecule comprising CH-CL cross with charged residues. The thick black point stands for the knob-into-hole modification. * symbolizes amino acid modifications in the CH1 and CL domain (so-called charged variant). **Figure 2B** illustrates the mouse surrogate, i.e. a bispecific 4-1BB antibody with bivalent binding for mouse 4-1BB and monovalent binding for Her2 (anti -4-1BB/anti-Her2 moIgG1 DDKK DAPG, termed mu4-1BB-Her2). The thick black point stands for the DD/KK knob-into-hole modification. The DAPG mutations abolish the crosslinking of the fusion protein via mouse Fcγ receptors or the binding of complement but allow binding to FcRn, so that the molecule remains its antibody like pharmacokinetics..

Figure 3A shows the setup of the SPR experiments for simultaneous binding of the Her2-targeting split trimeric 4-1BB ligand-containing antigen binding molecules of the invention. The simultaneous binding of Her2(PER)-4-1BBL (Analyte 1) to immobilized human 4-1BB and human Her2 (analyte 2) is shown in **Fig. 3B**. Simultaneous binding to human 4-1BB and human Her2 of Her2(aff PER)-4-1BBL is shown in **Fig. 3C**.

Figures 4A to 4D show the binding of Her2-targeting 4-1BB split trimeric ligand Fc fusion antigen binding molecules to Her2 expressed on the cell surface by human breast cancer cell line SK-Br3 (Fig. 4A and 4C) or human gastric carcinoma cell line NCI-N87 (Fig. 4B and 4D). Her2-targeting split 4-1BBL antigen binding molecules displaying the Her2 binders pertuzumab (PER) or affinity-matured pertuzumab (aff-PER) or the fusion protein Her2 (TRAS)-anticalin 4-1BB huIgG4 (as described in patent WO2016/177802) or previously described agonistic anti-human 4-1BB antibodies anti-human 4-1BB clone 20H4.9 huIgG4 (described in US7659384 B2) or anti-human 4-1BB clone MOR-7480 huIgG2 (described in WO 2012/032433) or control molecules as indicated in the legend were incubated with Her2 expressing cell lines SK-Br3 (Fig. 4A and 4C) or NCI-N87 (Fig. 4B and 4D) at different concentrations as indicated in the X-axis. Afterwards excessive and not bound molecules were washed off and bound molecules were detected with a secondary binding PE-conjugated anti-human Fc-fragment specific goat IgG F(ab')₂ fragment. The median of fluorescence intensity (MFI) was measured by flow cytometry and indicates the affinity (monovalent binders) or avidity (bivalent binders) of the tested molecules in a dose dependent manner. Values are baseline corrected by subtracting the blank control (e.g. staining with 2nd detection fragment only), shown is the mean +/- SEM.

Figures 5A and 5B illustrate the binding of Her2-targeting split trimeric 4-1BB ligand Fc fusion antigen binding molecules to Her2 expressed by human breast cancer cell line KPL-4 the cell surface. Her2-targeting split 4-1BBL antigen binding molecules comprising the Her2 binders PER, aff-PER or trastuzumab (TRAS), the fusion protein Her2 (TRAS)-anticalin 4-1BB huIgG4

(as described in patent WO2016/177802) or previously described agonistic anti-human 4-1BB antibodies 20H4.9 huIgG4 or MOR-7480 huIgG2 or control molecules as indicated in the legend were incubated with Her2 expressing cell lines KL-4 at different concentrations as indicated in the X-axis. Afterwards excessive and not bound molecules were washed of and bound molecules
5 were detected with a secondary binding PE-conjugated anti-human Fc-fragment specific goat IgG F(ab')₂ fragment. The median of fluorescence intensity (MFI) was measured by flow cytometry and indicates the affinity (monovalent binders) or avidity (bivalent binders) of the tested molecules in a dose dependent manner. Values are baseline corrected by subtracting the blank control (e.g. staining with 2nd detection fragment only), shown is the mean +/- SEM.

10 **Figure 6** shows a scheme that illustrates the general principal of the NFκB activation assay with human 4-1BB expressing Jurkat reporter cell line. Crosslinking of human 4-1BB, expressed on the reporter cells, induces NFκB activation and NFκB-mediated Luciferase expression. After lysis of the cells, luciferase can catalyze the oxidation of Luciferin to Oxyluciferin. This chemical reaction correlates positively with the strength of NFκB-mediated luciferase expression
15 and can be measured by the strength of light emission (units of released light).

The NFκB-mediated luciferase activity in a Jurkat-hu4-1BB-NFκB-luc2 reporter cell line is shown in **Figures 7A to 7F**. In 96-well plates Jurkat-hu4-1BB-NFκB-luc2 reporter cells were incubated with different concentrations (indicated in the x-axis) of Her2 (PER)-4-1BBL or Her2 (PER)-4-1BBL molecules or the fusion protein Her2 (TRAS)-anticalin 4-1BB huIgG4 or
20 agonistic anti-human 4-1BB antibodies 20H4.9 huIgG4 or MOR-7480 huIgG2 or the control molecules as indicated in the legend. The results in the absence of Her2+ cells are shown in Fig 7A and 7D, in the presence of human Her2+ breast cancer cell line SK-Br3 in Fig. 7B and 7E or in the presence of Her2+ human gastric cancer cell line NCI-N87 in Fig. 7C and 7F. Reporter cells were incubated with the Her2-expressing tumor cells in an 1:5 ratio for 6 h. Afterwards
25 cells were washed, lysed and incubated with Luciferin in a detection buffer. Luciferase-catalyzed oxidation of luciferin was detected via light emission as units of released light (y-axis). Shown is the mean +/- SEM. All values are baseline corrected by subtracting the baseline light emission.

The results of a second experiment comparing Her2 (PER)-4-1BBL with Her2 (TRAS)-4-1BBL are shown in **Figures 8A to 8H**. In 384-well plates Jurkat-hu4-1BB-NFκB-luc2 reporter
30 cells were incubated with different concentrations (indicated in the x-axis) of Her2 (PER)-4-1BBL or Her2 (TRAS)-4-1BBL or the fusion protein Her2 (TRAS)-anticalin 4-1BB huIgG4 or agonistic anti-human 4-1BB antibodies 20H4.9 huIgG4 or MOR-7480 huIgG2 or control molecules as indicated in the legend. Shown is the NFκB-mediated luciferase expression in a Jurkat-hu4-1BB-NFκB-luc2 reporter cell line in the absence (Fig. 8A and 8E) or the presence of
35 human Her2+ breast cancer cell line SK-Br3 (Fig. 8B and 8F), in the presence of human breast cancer cell line KPL-4 (Fig. 8C and 8G) or Her2+ human gastric cancer cell line NCI-N87 (FIG.

8D and 8H) when given in a reporter cell line to tumor cell line 1:5 ratio for 6 h. Cells were washed, lysed and incubated with Luciferin in a detection buffer. Luciferase-catalyzed oxidation of luciferin was detected via light emission as units of released light (y-axis). Shown is the mean $\pm$ SEM. All values are baseline corrected by subtracting the baseline light emission.

5 **Figure 9** shows a scheme that illustrates the general principal of the activation assay with human PBMCs as described in Example 3.2.2. T cells are activated by 2 nM agonistic CD3 antibody and co-stimulated with different concentrations of agonistic 4-1BB molecules in the presence of Her2-expressing gastric carcinoma NCI-N87 cells. The content/well comprised 50 Gy irradiated 2×10^4 NCI-N87 cells, 7.5×10^4 CFSE-labelled human PBMCs, 2 nM agonistic anti-
10 human CD3 human IgG wt (clone V9) and different concentrations of Her2-targeting 4-1BB agonistic molecules (here shown as Her2-4-1BBL). Cells were incubated for 4 days and then T cell activation was determined by flow cytometry.

The results are shown as activation of CD8⁺ T cells in **Figures 10A to 10F**. Resting PBMCs isolated from a buffy coat of a healthy donor were activated with 2 nM agonistic CD3
15 antibody and co-stimulated with different concentrations of agonistic 4-1BB molecules as indicated in the x-axis and in the legend in presence of Her2-expressing gastric carcinoma NCI-N87 cells for 4 days. Cells were gated on living CD8⁺ T cells and analyzed for their frequency of CD25⁺ (**Fig. 10A and 10D**), Granzyme B^{high} (**Fig. 10B and 10E**) or proliferating (low CFSE MFI) CD8⁺ T cells (**Fig. 10C and 10F**). Shown is the mean $\pm$ SD.

20 The activation of CD4⁺ T cells is shown in **Figures 11A to 11F**. Resting PBMCs isolated from a buffy coat of a healthy donor were activated with 2 nM agonistic CD3 antibody and co-stimulated with different concentrations of agonistic 4-1BB molecules as indicated in the x-axis in the presence of Her2-expressing gastric carcinoma NCI-N87 cells for 4 days. Cells were gated on living CD4⁺ T cells and analyzed for their frequency of CD25⁺ (**Fig. 11A and 11D**),
25 Granzyme B^{high} (**Fig. 11B and 11E**) or proliferating (low CFSE MFI) CD4⁺ T cells (**Fig. 11C and 11F**). Shown is the mean $\pm$ SD.

Figure 12 shows a scheme that illustrates the general principal of the activation assay with mouse splenocytes as described in Example 3.2.3. T cells are activated by 0.5 μ g/mL (~ 3.6 nM) agonistic anti-mouse CD3 Armenian hamster IgG antibody (clone 1452C11) and co-stimulated
30 with different concentrations of agonistic mouse surrogate mu4-1BB-Her2 in the presence of Her2-expressing human breast cancer cell line KPL-4. The content/well comprised 50 Gy irradiated 2×10^4 KPL-4 cells, 15×10^4 violet proliferation dye-labelled mouse splenocytes, 0.5 μ g/mL (~ 3.6 nM) agonistic anti-mouse CD3 Armenian hamster IgG antibody (clone 1452C11) and different concentrations of mouse surrogate mu4-1BB-Her2 or an untargeted control mu4-

1BB muIgG1 DAPG. Cells were incubated for 3 days and then T cell activation was determined by flow cytometry.

The results are shown as activation of mouse CD8⁺ and CD4⁺ T cells in **Figures 13A to 13D**. Resting mouse splenocytes isolated from C57BL/6 spleens were activated with 0.5 µg/mL (~3.6 nM) agonistic anti-mouse CD3 Armenian hamster IgG antibody (clone 1452C11) and co-stimulated with different concentrations of mouse surrogate mu4-1BB-Her2 or untargeted control as indicated in the x-axis and in the legend in presence of Her2-expressing human breast cancer KPL-4 cells for 3 days. Cells were gated on living CD8⁺ or CD4⁺ T cells and analyzed for their frequency of CD25⁺ expression (**Fig. 13A and 13C**) or proliferating (low violet proliferation dye MFI) (**Fig. 13B and 13D**). Shown is the mean +/- SD of technical triplicates per point.

In **Figure 14A** T cell activation of the combination of anti-Her2/anti-CD3 bispecific antibody (Her2 TDB) with Her2(PER)-4-1BBL and the T cell activation of the single agents is shown. A robust T cell activation was induced by Her2 TDB alone as well as by the combination of both agents. Target cell killing of the combination of anti-Her2/anti-CD3 bispecific antibody (Her2 TDB) with Her2(PER)-4-1BBL and the single agents is shown in **Figure 14B**.

The results of the T cell proliferation assay for the combination of anti-Her2/anti-CD3 bispecific antibody (Her2 TDB) with Her2(PER)-4-1BBL are shown in **Figures 15A and 15B**. Addition of HER2-4-1BBL substantially enhanced anti-HER2/CD3-TDB induced T cell proliferation/survival *in vitro*.

Figures 16A to 16D show the tumor growth kinetics (linear scale) as observed in immune-competent mice that were implanted with human HER2 expressing Fo5 tumor allografts and treated with vehicle (Fig. 16A), Her2 TDB alone (Fig. 14B), mu 4-1BB-Her2 mouse surrogate alone (Fig. 14C) and the combination of Her2 TDB and mu 4-1BB-Her2 (Fig. 14D). The individual tumor growth kinetics of each animal for all treatment groups are shown. CR means no detectable tumor at the end of the study, PR means that at least 50% of tumor shrinkage is observed compared to day 0. Four of seven mice (57%) treated in combination with mu4-1BB-Her2 agonist demonstrated complete responses without detectable tumors in the end of study (CR = 57%).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless defined otherwise, technical and scientific terms used herein have the same meaning as generally used in the art to which this invention belongs. For purposes of interpreting

this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa.

As used herein, the term "**antigen binding molecule**" refers in its broadest sense to a molecule that specifically binds an antigenic determinant. Examples of antigen binding
5 molecules are antibodies, antibody fragments and scaffold antigen binding proteins.

The term "**antigen binding domain**" refers to the part of an antigen binding molecule that comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antigen binding molecule may only bind to a particular part of the antigen, which part is termed an epitope. An antigen binding domain may be provided by, for
10 example, one or more variable domains (also called variable regions). Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH).

As used herein, the term "**antigen binding domain capable of specific binding to Her2**" or "moiety capable of specific binding to Her2" refers to a polypeptide molecule that specifically
15 binds to Her2. In one aspect, the antigen binding domain is able to activate or inhibit signaling through Her2. In a particular aspect, the antigen binding domain is able to direct the entity to which it is attached (e.g. the 4-1BBL trimer) to a target site, for example to a specific type of tumor cell bearing Her2. Antigen binding domains capable of specific binding to Her2 include antibodies and fragments thereof as further defined herein. In relation to an antibody or fragment
20 thereof, the term "moiety capable of specific binding to a target cell antigen" refers to the part of the molecule that comprises the area which specifically binds to and is complementary to part or all of an antigen. A moiety capable of specific antigen binding may be provided, for example, by one or more antibody variable domains (also called antibody variable regions). Particularly, a moiety capable of specific antigen binding comprises an antibody light chain variable region
25 (VL) and an antibody heavy chain variable region (VH).

The term "**antibody**" herein is used in the broadest sense and encompasses various antibody structures, including but not limited to monoclonal antibodies, polyclonal antibodies, monospecific and multispecific antibodies (e.g., bispecific antibodies), and antibody fragments so long as they exhibit the desired antigen-binding activity.

The term "**monoclonal antibody**" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variant antibodies, e.g. containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to
35 polyclonal antibody preparations, which typically include different antibodies directed against

different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen.

The term “**monospecific**” antibody as used herein denotes an antibody that has one or more binding sites each of which bind to the same epitope of the same antigen. The term “**bispecific**” means that the antigen binding molecule is able to specifically bind to at least two distinct antigenic determinants. Typically, a bispecific antigen binding molecule comprises two antigen binding sites, each of which is specific for a different antigenic determinant. In certain embodiments the bispecific antigen binding molecule is capable of simultaneously binding two antigenic determinants, particularly two antigenic determinants expressed on two distinct cells.

The term “**valent**” as used within the current application denotes the presence of a specified number of binding sites in an antigen binding molecule. As such, the terms “monovalent”, “bivalent”, “tetravalent”, and “hexavalent” denote the presence of one binding site, two binding sites, four binding sites, and six binding sites, respectively, in an antigen binding molecule.

The terms “full length antibody”, “intact antibody”, and “whole antibody” are used herein interchangeably to refer to an antibody having a structure substantially similar to a native antibody structure. “**Native antibodies**” refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG-class antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two light chains and two heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3), also called a heavy chain constant region. Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a light chain constant domain (CL), also called a light chain constant region. The heavy chain of an antibody may be assigned to one of five types, called α (IgA), δ (IgD), ϵ (IgE), γ (IgG), or μ (IgM), some of which may be further divided into subtypes, e.g. $\gamma 1$ (IgG1), $\gamma 2$ (IgG2), $\gamma 3$ (IgG3), $\gamma 4$ (IgG4), $\alpha 1$ (IgA1) and $\alpha 2$ (IgA2). The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

An “**antibody fragment**” refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')₂; diabodies, triabodies, tetrabodies, cross-Fab fragments; linear antibodies; single-chain antibody molecules (e.g. scFv); and single domain antibodies. For a review of certain antibody fragments, see Hudson et al., Nat Med 9, 129-134 (2003). For a review of scFv fragments, see e.g.

Plückthun, in *The Pharmacology of Monoclonal Antibodies*, vol. 113, Rosenberg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994); see also WO 93/16185; and U.S. Patent Nos. 5,571,894 and 5,587,458. For discussion of Fab and F(ab')₂ fragments comprising salvage receptor binding epitope residues and having increased in vivo half-life, see U.S. Patent No.

5 5,869,046. Diabodies are antibody fragments with two antigen-binding sites that may be bivalent or bispecific, see, for example, EP 404,097; WO 1993/01161; Hudson et al., *Nat Med* 9, 129-134 (2003); and Hollinger et al., *Proc Natl Acad Sci USA* 90, 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., *Nat Med* 9, 129-134 (2003). Single-domain antibodies are antibody fragments comprising all or a portion of the heavy chain variable domain
10 or all or a portion of the light chain variable domain of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody (Domantis, Inc., Waltham, MA; see e.g. U.S. Patent No. 6,248,516 B1). Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells (e.g. *E. coli* or phage), as described herein.

15 Papain digestion of intact antibodies produces two identical antigen-binding fragments, called "Fab" fragments containing each the heavy- and light-chain variable domains and also the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. As used herein, Thus, the term "**Fab fragment**" refers to an antibody fragment comprising a light chain fragment comprising a VL domain and a constant domain of a light chain (CL), and a VH
20 domain and a first constant domain (CH1) of a heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteins from the antibody hinge region. Fab'-SH are Fab' fragments in which the cysteine residue(s) of the constant domains bear a free thiol group. Pepsin treatment yields an F(ab')₂ fragment that has two antigen-combining sites (two Fab
25 fragments) and a part of the Fc region.

 The term "**cross-Fab fragment**" or "xFab fragment" or "crossover Fab fragment" refers to a Fab fragment, wherein either the variable regions or the constant regions of the heavy and light chain are exchanged. Two different chain compositions of a crossover Fab molecule are possible and comprised in the bispecific antibodies of the invention: On the one hand, the variable regions
30 of the Fab heavy and light chain are exchanged, i.e. the crossover Fab molecule comprises a peptide chain composed of the light chain variable region (VL) and the heavy chain constant region (CH1), and a peptide chain composed of the heavy chain variable region (VH) and the light chain constant region (CL). This crossover Fab molecule is also referred to as CrossFab (VLVH). On the other hand, when the constant regions of the Fab heavy and light chain are
35 exchanged, the crossover Fab molecule comprises a peptide chain composed of the heavy chain variable region (VH) and the light chain constant region (CL), and a peptide chain composed of

the light chain variable region (VL) and the heavy chain constant region (CH1). This crossover Fab molecule is also referred to as CrossFab_(CLCH1).

A “single chain Fab fragment” or “**scFab**” is a polypeptide consisting of an antibody heavy chain variable domain (VH), an antibody constant domain 1 (CH1), an antibody light chain variable domain (VL), an antibody light chain constant domain (CL) and a linker, wherein said antibody domains and said linker have one of the following orders in N-terminal to C-terminal direction: a) VH-CH1-linker-VL-CL, b) VL-CL-linker-VH-CH1, c) VH-CL-linker-VL-CH1 or d) VL-CH1-linker-VH-CL; and wherein said linker is a polypeptide of at least 30 amino acids, preferably between 32 and 50 amino acids. Said single chain Fab fragments are stabilized via the natural disulfide bond between the CL domain and the CH1 domain. In addition, these single chain Fab molecules might be further stabilized by generation of interchain disulfide bonds via insertion of cysteine residues (e.g. position 44 in the variable heavy chain and position 100 in the variable light chain according to Kabat numbering).

A “crossover single chain Fab fragment” or “**x-scFab**” is a polypeptide consisting of an antibody heavy chain variable domain (VH), an antibody constant domain 1 (CH1), an antibody light chain variable domain (VL), an antibody light chain constant domain (CL) and a linker, wherein said antibody domains and said linker have one of the following orders in N-terminal to C-terminal direction: a) VH-CL-linker-VL-CH1 and b) VL-CH1-linker-VH-CL; wherein VH and VL form together an antigen-binding site which binds specifically to an antigen and wherein said linker is a polypeptide of at least 30 amino acids. In addition, these x-scFab molecules might be further stabilized by generation of interchain disulfide bonds via insertion of cysteine residues (e.g. position 44 in the variable heavy chain and position 100 in the variable light chain according to Kabat numbering).

A “**single-chain variable fragment (scFv)**” is a fusion protein of the variable regions of the heavy (V_H) and light chains (V_L) of an antibody, connected with a short linker peptide of ten to about 25 amino acids. The linker is usually rich in glycine for flexibility, as well as serine or threonine for solubility, and can either connect the N-terminus of the V_H with the C-terminus of the V_L, or *vice versa*. This protein retains the specificity of the original antibody, despite removal of the constant regions and the introduction of the linker. scFv antibodies are, e.g. described in Houston, J.S., Methods in Enzymol. 203 (1991) 46-96). In addition, antibody fragments comprise single chain polypeptides having the characteristics of a VH domain, namely being able to assemble together with a VL domain, or of a VL domain, namely being able to assemble together with a VH domain to a functional antigen binding site and thereby providing the antigen binding property of full length antibodies.

“**Scaffold antigen binding proteins**” are known in the art, for example, fibronectin and designed ankyrin repeat proteins (DARPs) have been used as alternative scaffolds for antigen-binding domains, see, e.g., Gebauer and Skerra, Engineered protein scaffolds as next-generation antibody therapeutics. *Curr Opin Chem Biol* 13:245-255 (2009) and Stumpp et al., Darpins: A new generation of protein therapeutics. *Drug Discovery Today* 13: 695-701 (2008). In one aspect of the invention, a scaffold antigen binding protein is selected from the group consisting of CTLA-4 (Evibody), Lipocalins (Anticalin), a Protein A-derived molecule such as Z-domain of Protein A (Affibody), an A-domain (Avimer/Maxibody), a serum transferrin (*trans*-body); a designed ankyrin repeat protein (DARPin), a variable domain of antibody light chain or heavy chain (single-domain antibody, sdAb), a variable domain of antibody heavy chain (nanobody, aVH), V_{NAR} fragments, a fibronectin (AdNectin), a C-type lectin domain (Tetranectin); a variable domain of a new antigen receptor beta-lactamase (V_{NAR} fragments), a human gamma-crystallin or ubiquitin (Affilin molecules); a kunitz type domain of human protease inhibitors, microbodies such as the proteins from the knottin family, peptide aptamers and fibronectin (adnectin). CTLA-4 (Cytotoxic T Lymphocyte-associated Antigen 4) is a CD28-family receptor expressed on mainly CD4⁺ T-cells. Its extracellular domain has a variable domain- like Ig fold. Loops corresponding to CDRs of antibodies can be substituted with heterologous sequence to confer different binding properties. CTLA-4 molecules engineered to have different binding specificities are also known as Evibodies (e.g. US7166697B1). Evibodies are around the same size as the isolated variable region of an antibody (e.g. a domain antibody). For further details see *Journal of Immunological Methods* 248 (1-2), 31-45 (2001). Lipocalins are a family of extracellular proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids and lipids. They have a rigid beta-sheet secondary structure with a number of loops at the open end of the conical structure which can be engineered to bind to different target antigens. Anticalins are between 160-180 amino acids in size, and are derived from lipocalins. For further details see *Biochim Biophys Acta* 1482: 337-350 (2000), US7250297B1 and US20070224633. An affibody is a scaffold derived from Protein A of *Staphylococcus aureus* which can be engineered to bind to antigen. The domain consists of a three-helical bundle of approximately 58 amino acids. Libraries have been generated by randomization of surface residues. For further details see *Protein Eng. Des. Sel.* 2004, 17, 455-462 and EP 1641818A1. Avimers are multidomain proteins derived from the A-domain scaffold family. The native domains of approximately 35 amino acids adopt a defined disulfide bonded structure. Diversity is generated by shuffling of the natural variation exhibited by the family of A-domains. For further details see *Nature Biotechnology* 23(12), 1556 - 1561 (2005) and *Expert Opinion on Investigational Drugs* 16(6), 909-917 (June 2007). A transferrin is a monomeric serum transport glycoprotein. Transferrins can be engineered to bind different target antigens by insertion of peptide sequences in a permissive surface loop. Examples of engineered transferrin scaffolds include the Trans-body. For further details see *J. Biol. Chem* 274, 24066-24073 (1999). Designed Ankyrin Repeat

Proteins (DARPin) are derived from Ankyrin which is a family of proteins that mediate attachment of integral membrane proteins to the cytoskeleton. A single ankyrin repeat is a 33 residue motif consisting of two alpha-helices and a beta-turn. They can be engineered to bind different target antigens by randomizing residues in the first alpha-helix and a beta-turn of each repeat. Their binding interface can be increased by increasing the number of modules (a method of affinity maturation). For further details see J. Mol. Biol. 332, 489-503 (2003), PNAS 100(4), 1700-1705 (2003) and J. Mol. Biol. 369, 1015-1028 (2007) and US20040132028A1. A single-domain antibody is an antibody fragment consisting of a single monomeric variable antibody domain. The first single domains were derived from the variable domain of the antibody heavy chain from camelids (nanobodies or V_HH fragments). Furthermore, the term single-domain antibody includes an autonomous human heavy chain variable domain (aVH) or V_{NAR} fragments derived from sharks. Fibronectin is a scaffold which can be engineered to bind to antigen. Adnectins consists of a backbone of the natural amino acid sequence of the 10th domain of the 15 repeating units of human fibronectin type III (FN3). Three loops at one end of the .beta.- sandwich can be engineered to enable an Adnectin to specifically recognize a therapeutic target of interest. For further details see Protein Eng. Des. Sel. 18, 435- 444 (2005), US20080139791, WO2005056764 and US6818418B1. Peptide aptamers are combinatorial recognition molecules that consist of a constant scaffold protein, typically thioredoxin (TrxA) which contains a constrained variable peptide loop inserted at the active site. For further details see Expert Opin. Biol. Ther. 5, 783-797 (2005). Microbodies are derived from naturally occurring microproteins of 25-50 amino acids in length which contain 3-4 cysteine bridges - examples of microproteins include KalataBI and conotoxin and knottins. The microproteins have a loop which can be engineered to include upto 25 amino acids without affecting the overall fold of the microprotein. For further details of engineered knottin domains, see WO2008098796.

Lipocalins are a family of extracellular proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids and lipids. They have a rigid beta-sheet secondary structure with a number of loops at the open end of the conical structure which can be engineered to bind to different target antigens. Anticalins are between 160-180 amino acids in size, and are derived from lipocalins. For further details see Biochim Biophys Acta 1482: 337-350 (2000), Biodrugs 19(5), 279-288 (2005), US7250297B1 and US20070224633.

An “**antigen binding molecule that binds to the same epitope**” as a reference molecule refers to an antigen binding molecule that blocks binding of the reference molecule to its antigen in a competition assay by 50% or more, and conversely, the reference molecule blocks binding of the antigen binding molecule to its antigen in a competition assay by 50% or more.

As used herein, the term “**antigenic determinant**” is synonymous with “antigen” and “epitope,” and refers to a site (e.g. a contiguous stretch of amino acids or a conformational

configuration made up of different regions of non-contiguous amino acids) on a polypeptide macromolecule to which an antigen binding moiety binds, forming an antigen binding moiety-antigen complex. Useful antigenic determinants can be found, for example, on the surfaces of tumor cells, on the surfaces of virus-infected cells, on the surfaces of other diseased cells, on the surface of immune cells, free in blood serum, and/or in the extracellular matrix (ECM). The proteins useful as antigens herein can be any native form the proteins from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g. mice and rats), unless otherwise indicated. In a particular embodiment the antigen is a human protein. Where reference is made to a specific protein herein, the term encompasses the “full-length”, unprocessed protein as well as any form of the protein that results from processing in the cell. The term also encompasses naturally occurring variants of the protein, e.g. splice variants or allelic variants.

The term “**capable of specific binding to Her2**” refers to an antigen binding molecule that is capable of binding to Her2 with sufficient affinity such that the antigen binding molecule is useful as a diagnostic and/or therapeutic agent in targeting Her2. The antigen binding molecule includes but is not limited to, antibodies, Fab molecules, crossover Fab molecules, single chain Fab molecules, Fv molecules, scFv molecules, single domain antibodies, and VH and scaffold antigen binding protein. In one aspect, the extent of binding of an anti-Her2 antigen binding molecule to an unrelated, non-Her2 protein is less than about 10% of the binding of the antigen binding molecule to Her2 as measured, e.g., by surface plasmon resonance (SPR). In particular, an antigen binding molecule that is capable of specific binding to Her2 has a dissociation constant (K_d) of $\leq 1 \mu\text{M}$, $\leq 100 \text{ nM}$, $\leq 10 \text{ nM}$, $\leq 1 \text{ nM}$, $\leq 0.1 \text{ nM}$, $\leq 0.01 \text{ nM}$, or $\leq 0.001 \text{ nM}$ (e.g. 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M , e.g., from 10^{-9} M to 10^{-13} M). In certain aspects, an anti-Her2 antigen binding molecule binds to Her2 from different species. In particular, the anti-Her2 antigen binding molecule binds to human and cynomolgus Her2.

The term “epitope” denotes the site on an antigen, either proteinaceous or non-proteinaceous, to which an anti-[[PRO]] antibody binds. Epitopes can be formed from contiguous amino acid stretches (linear epitope) or comprise non-contiguous amino acids (conformational epitope), e.g., coming in spatial proximity due to the folding of the antigen, i.e. by the tertiary folding of a proteinaceous antigen. Linear epitopes are typically still bound by an antibody after exposure of the proteinaceous antigen to denaturing agents, whereas conformational epitopes are typically destroyed upon treatment with denaturing agents. An epitope comprises at least 3, at least 4, at least 5, at least 6, at least 7, or 8-10 amino acids in a unique spatial conformation.

The “epitope 4D5” or “4D5 epitope” or “4D5” is the region in the extracellular domain of HER2 to which the antibody 4D5 (ATCC CRL 10463) and trastuzumab bind. This epitope is close to the transmembrane domain of HER2, and within domain IV of HER2. To screen for

antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of HER2 (e.g. any one or more residues in the region from about residue 550 to about residue 610, inclusive, of human HER2 (SEQ ID NO: 54)).

The “epitope 2C4” or “2C4 epitope” is the region in the extracellular domain of HER2 to which the antibody 2C4 binds. In order to screen for antibodies which bind to the 2C4 epitope, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 2C4 epitope of HER2. Epitope 2C4 comprises residues from domain II in the extracellular domain of HER2. The 2C4 antibody and pertuzumab bind to the extracellular domain of HER2 at the junction of domains I, II and III (Franklin et al. *Cancer Cell* 5:317-328 (2004)).

By “**specific binding**” is meant that the binding is selective for the antigen and can be discriminated from unwanted or non-specific interactions. The ability of an antigen binding molecule to bind to a specific antigen can be measured either through an enzyme-linked immunosorbent assay (ELISA) or other techniques familiar to one of skill in the art, e.g. Surface Plasmon Resonance (SPR) technique (analyzed on a BIAcore instrument) (Liljeblad et al., *Glyco J* 17, 323-329 (2000)), and traditional binding assays (Heeley, *Endocr Res* 28, 217-229 (2002)). In one embodiment, the extent of binding of an antigen binding molecule to an unrelated protein is less than about 10% of the binding of the antigen binding molecule to the antigen as measured, e.g. by SPR. In certain embodiments, an molecule that binds to the antigen has a dissociation constant (K_d) of $\leq 1 \mu\text{M}$, $\leq 100 \text{ nM}$, $\leq 10 \text{ nM}$, $\leq 1 \text{ nM}$, $\leq 0.1 \text{ nM}$, $\leq 0.01 \text{ nM}$, or $\leq 0.001 \text{ nM}$ (e.g. 10^{-8} M or less, e.g. from 10^{-8} M to 10^{-13} M , e.g. from 10^{-9} M to 10^{-13} M).

“**Affinity**” or “binding affinity” refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (e.g. an antibody) and its binding partner (e.g. an antigen). Unless indicated otherwise, as used herein, “binding affinity” refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g. antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (K_d), which is the ratio of dissociation and association rate constants (k_{off} and k_{on} , respectively). Thus, equivalent affinities may comprise different rate constants, as long as the ratio of the rate constants remains the same. Affinity can be measured by common methods known in the art, including those described herein. A particular method for measuring affinity is Surface Plasmon Resonance (SPR).

A “**target cell antigen**” as used herein refers to an antigenic determinant presented on the surface of a target cell, for example a T-cell or B-cell, a cell in a tumor such as a cancer cell or a cell of the tumor stroma. In certain aspects, the target cell antigen is an antigen on the surface of cancer cell. In one aspect, the target cell antigen is Her2.

5 The term “**Her2**”, also known as “ErbB2”, “ErbB2 receptor”, or “c-Erb-B2”, refers to any native, mature HER2 which results from processing of a HER2 precursor protein in a cell. The term includes HER2 from any vertebrate source, including mammals such as primates (e.g. humans and cynomolgus monkeys) and rodents (e.g., mice and rats), unless otherwise indicated. The term also includes naturally occurring variants of HER2, e.g., splice variants or allelic
10 variants. The amino acid sequence of an exemplary human HER2 protein is shown in SEQ ID NO:54.

A “**T-cell antigen**” as used herein refers to an antigenic determinant presented on the surface of a T lymphocyte, particularly a cytotoxic T lymphocyte.

A “**T cell activating therapeutic agent**” as used herein refers to a therapeutic agent
15 capable of inducing T cell activation in a subject, particularly a therapeutic agent designed for inducing T-cell activation in a subject. Examples of T cell activating therapeutic agents include bispecific antibodies that specifically bind an activating T cell antigen, such as CD3, and a target cell antigen, such as CEA or Folate Receptor.

An “**activating T cell antigen**” as used herein refers to an antigenic determinant expressed
20 by a T lymphocyte, particularly a cytotoxic T lymphocyte, which is capable of inducing or enhancing T cell activation upon interaction with an antigen binding molecule. Specifically, interaction of an antigen binding molecule with an activating T cell antigen may induce T cell activation by triggering the signaling cascade of the T cell receptor complex. An exemplary activating T cell antigen is CD3.

25 The term “**CD3**” refers to any native CD3 from any vertebrate source, including mammals such as primates (e.g. humans), non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The term encompasses “full-length,” unprocessed CD3 as well as any form of CD3 that results from processing in the cell. The term also encompasses naturally occurring variants of CD3, e.g., splice variants or allelic variants. In
30 one embodiment, CD3 is human CD3, particularly the epsilon subunit of human CD3 (CD3ε). The amino acid sequence of human CD3ε is shown in UniProt (www.uniprot.org) accession no. P07766 (version 144), or NCBI (www.ncbi.nlm.nih.gov/) RefSeq NP_000724.1. See also SEQ ID NO: 85. The amino acid sequence of cynomolgus [*Macaca fascicularis*] CD3ε is shown in NCBI GenBank no. BAB71849.1. See also SEQ ID NO: 86.

The term “**variable domain**” or “variable region” refers to the domain of an antibody heavy or light chain that is involved in binding the antigen binding molecule to antigen. The variable domains of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved
5 framework regions (FRs) and three hypervariable regions (HVRs). See, e.g., Kindt et al., Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007). A single VH or VL domain may be sufficient to confer antigen-binding specificity.

The term “**hypervariable region**” or “HVR” as used herein refers to each of the regions of an antigen binding variable domain which are hypervariable in sequence and which determine
10 antigen binding specificity, for example “**complementarity determining regions**” (“CDRs”). Generally, antigen binding domains comprise six CDRs: three in the VH (CDR-H1, CDR-H2, CDR-H3), and three in the VL (CDR-L1, CDR-L2, CDR-L3). Exemplary CDRs herein include:

(a) hypervariable loops occurring at amino acid residues 26-32 (L1), 50-52 (L2), 91-96 (L3), 26-32 (H1), 53-55 (H2), and 96-101 (H3) (Chothia and Lesk, *J. Mol. Biol.* 196:901-917
15 (1987));

(b) CDRs occurring at amino acid residues 24-34 (L1), 50-56 (L2), 89-97 (L3), 31-35b (H1), 50-65 (H2), and 95-102 (H3) (Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991)); and

(c) antigen contacts occurring at amino acid residues 27c-36 (L1), 46-55 (L2), 89-96 (L3),
20 30-35b (H1), 47-58 (H2), and 93-101 (H3) (MacCallum et al. *J. Mol. Biol.* 262: 732-745 (1996)).

Unless otherwise indicated, the CDRs are determined according to Kabat et al., *supra*. One of skill in the art will understand that the CDR designations can also be determined according to Chothia, *supra*, McCallum, *supra*, or any other scientifically accepted nomenclature. Kabat *et al.* also defined a numbering system for variable region sequences that is applicable to any antibody.
25 One of ordinary skill in the art can unambiguously assign this system of “Kabat numbering” to any variable region sequence, without reliance on any experimental data beyond the sequence itself. As used herein, “Kabat numbering” refers to the numbering system set forth by Kabat et al., U.S. Dept. of Health and Human Services, “Sequence of Proteins of Immunological Interest” (1983). Unless otherwise specified, references to the numbering of specific amino acid residue
30 positions in an antibody variable region are according to the Kabat numbering system.

As used herein, the term “**affinity matured**” in the context of antigen binding molecules (e.g., antibodies) refers to an antigen binding molecule that is derived from a reference antigen binding molecule, e.g., by mutation, binds to the same antigen, preferably binds to the same epitope, as the reference antibody; and has a higher affinity for the antigen than that of the

reference antigen binding molecule. Affinity maturation generally involves modification of one or more amino acid residues in one or more CDRs of the antigen binding molecule. Typically, the affinity matured antigen binding molecule binds to the same epitope as the initial reference antigen binding molecule.

- 5 **"Framework"** or "FR" refers to variable domain residues other than hypervariable region (HVR) residues. The FR of a variable domain generally consists of four FR domains: FR1, FR2, FR3, and FR4. Accordingly, the HVR and FR sequences generally appear in the following sequence in VH (or VL): FR1-H1(L1)-FR2-H2(L2)-FR3-H3(L3)-FR4.

- 10 An **"acceptor human framework"** for the purposes herein is a framework comprising the amino acid sequence of a light chain variable domain (VL) framework or a heavy chain variable domain (VH) framework derived from a human immunoglobulin framework or a human consensus framework, as defined below. An acceptor human framework "derived from" a human immunoglobulin framework or a human consensus framework may comprise the same amino acid sequence thereof, or it may contain amino acid sequence changes. In some embodiments,
15 the number of amino acid changes are 10 or less, 9 or less, 8 or less, 7 or less, 6 or less, 5 or less, 4 or less, 3 or less, or 2 or less. In some embodiments, the VL acceptor human framework is identical in sequence to the VL human immunoglobulin framework sequence or human consensus framework sequence.

- 20 The term **"chimeric"** antibody refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

- The **"class"** of an antibody refers to the type of constant domain or constant region possessed by its heavy chain. There are five major classes of antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g. IgG₁, IgG₂,
25 IgG₃, IgG₄, IgA₁, and IgA₂. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ respectively.

- The terms **"constant region derived from human origin"** or **"human constant region"** denote a constant heavy chain region of a human antibody of the subclass IgG₁, IgG₂, IgG₃, or IgG₄ and/or a constant light chain kappa or lambda region. Such constant regions are well
30 known in the state of the art and e.g. described by Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991) (see also e.g. Johnson, G., and Wu, T.T., Nucleic Acids Res. 28 (2000) 214-218; Kabat, E.A., et al., Proc. Natl. Acad. Sci. USA 72 (1975) 2785-2788). Unless otherwise specified herein, numbering of amino acid residues in the constant region is according to the EU
35 numbering system, also called the EU index of Kabat, as described in Kabat, E.A. et al.,

Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991), NIH Publication 91-3242.

A “**humanized**” antibody refers to a chimeric antibody comprising amino acid residues from non-human HVRs and amino acid residues from human FRs. In certain embodiments, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the HVRs (e.g., CDRs) correspond to those of a non-human antibody, and all or substantially all of the FRs correspond to those of a human antibody. A humanized antibody optionally may comprise at least a portion of an antibody constant region derived from a human antibody. A “**humanized form**” of an antibody, e.g., a non-human antibody, refers to an antibody that has undergone humanization. Other forms of “humanized antibodies” encompassed by the present invention are those in which the constant region has been additionally modified or changed from that of the original antibody to generate the properties according to the invention, especially in regard to C1q binding and/or Fc receptor (FcR) binding.

A “**human**” antibody is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human or a human cell or derived from a non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

The term “Fc domain” or “**Fc region**” herein is used to define a C-terminal region of an antibody heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, antibodies produced by host cells may undergo post-translational cleavage of one or more, particularly one or two, amino acids from the C-terminus of the heavy chain. Therefore an antibody produced by a host cell by expression of a specific nucleic acid molecule encoding a full-length heavy chain may include the full-length heavy chain, or it may include a cleaved variant of the full-length heavy chain. This may be the case where the final two C-terminal amino acids of the heavy chain are glycine (G446) and lysine (K447, numbering according to Kabat EU index). Therefore, the C-terminal lysine (Lys447), or the C-terminal glycine (Gly446) and lysine (Lys447), of the Fc region may or may not be present. Amino acid sequences of heavy chains including an Fc region are denoted herein without C-terminal glycine-lysine dipeptide if not indicated otherwise. In one embodiment, a heavy chain including an Fc region as specified herein, comprised in an antibody according to the invention, comprises an additional C-terminal glycine-lysine dipeptide (G446 and K447, numbering according to EU index of Kabat). In one embodiment, a heavy chain including an Fc region as specified herein,

comprised in an antibody according to the invention, comprises an additional C-terminal glycine residue (G446, numbering according to EU index of Kabat). Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991. An IgG Fc region comprises an IgG CH2 and an IgG CH3 domain. The “CH2 domain” of a human IgG Fc region usually extends from an amino acid residue at about position 231 to an amino acid residue at about position 340. In one embodiment, a carbohydrate chain is attached to the CH2 domain. The CH2 domain herein may be a native sequence CH2 domain or variant CH2 domain. The “CH3 domain” comprises the stretch of residues C-terminal to a CH2 domain in an Fc region (i.e. from an amino acid residue at about position 341 to an amino acid residue at about position 447 of an IgG). The CH3 region herein may be a native sequence CH3 domain or a variant CH3 domain (e.g. a CH3 domain with an introduced “protuberance” (“knob”) in one chain thereof and a corresponding introduced “cavity” (“hole”) in the other chain thereof; see US Patent No. 5,821,333, expressly incorporated herein by reference). Such variant CH3 domains may be used to promote heterodimerization of two non-identical antibody heavy chains as herein described.

The “**knob-into-hole**” technology is described e.g. in US 5,731,168; US 7,695,936; Ridgway et al., *Prot Eng* 9, 617-621 (1996) and Carter, *J Immunol Meth* 248, 7-15 (2001). Generally, the method involves introducing a protuberance (“knob”) at the interface of a first polypeptide and a corresponding cavity (“hole”) in the interface of a second polypeptide, such that the protuberance can be positioned in the cavity so as to promote heterodimer formation and hinder homodimer formation. Protuberances are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). The protuberance and cavity can be made by altering the nucleic acid encoding the polypeptides, e.g. by site-specific mutagenesis, or by peptide synthesis. In a specific embodiment a knob modification comprises the amino acid substitution T366W in one of the two subunits of the Fc domain, and the hole modification comprises the amino acid substitutions T366S, L368A and Y407V in the other one of the two subunits of the Fc domain. In a further specific embodiment, the subunit of the Fc domain comprising the knob modification additionally comprises the amino acid substitution S354C, and the subunit of the Fc domain comprising the hole modification additionally comprises the amino acid substitution Y349C. Introduction of these two cysteine residues results in the formation of a disulfide bridge between the two subunits of the Fc region, thus further stabilizing the dimer (Carter, *J Immunol Methods* 248, 7-15 (2001)). The numbering is according to EU index of Kabat et al, *Sequences of Proteins*

of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

A "region equivalent to the Fc region of an immunoglobulin" is intended to include naturally occurring allelic variants of the Fc region of an immunoglobulin as well as variants
5 having alterations which produce substitutions, additions, or deletions but which do not decrease substantially the ability of the immunoglobulin to mediate effector functions (such as antibody-dependent cellular cytotoxicity). For example, one or more amino acids can be deleted from the N-terminus or C-terminus of the Fc region of an immunoglobulin without substantial loss of biological function. Such variants can be selected according to general rules known in the art so
10 as to have minimal effect on activity (see, e.g., Bowie, J. U. et al., Science 247:1306-10 (1990)).

The term "**effector functions**" refers to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC), Fc receptor binding, antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular
15 phagocytosis (ADCP), cytokine secretion, immune complex-mediated antigen uptake by antigen presenting cells, down regulation of cell surface receptors (e.g. B cell receptor), and B cell activation.

An "**activating Fc receptor**" is an Fc receptor that following engagement by an Fc region of an antibody elicits signaling events that stimulate the receptor-bearing cell to perform effector
20 functions. Activating Fc receptors include FcγRIIIa (CD16a), FcγRI (CD64), FcγRIIa (CD32), and FcαRI (CD89). A particular activating Fc receptor is human FcγRIIIa (see UniProt accession no. P08637, version 141).

The term "**TNF ligand family member**" or "TNF family ligand" refers to a proinflammatory cytokine. Cytokines in general, and in particular the members of the TNF
25 ligand family, play a crucial role in the stimulation and coordination of the immune system. At present, nineteen cytokines have been identified as members of the TNF (tumour necrosis factor) ligand superfamily on the basis of sequence, functional, and structural similarities. All these ligands are type II transmembrane proteins with a C-terminal extracellular domain (ectodomain), N-terminal intracellular domain and a single transmembrane domain. The C-
30 terminal extracellular domain, known as TNF homology domain (THD), has 20-30% amino acid identity between the superfamily members and is responsible for binding to the receptor. The TNF ectodomain is also responsible for the TNF ligands to form trimeric complexes that are recognized by their specific receptors. Members of the TNF ligand family are selected from the group consisting of Lymphotoxin α (also known as LTA or TNFSF1), TNF (also known as
35 TNFSF2), LTβ (also known as TNFSF3), OX40L (also known as TNFSF4), CD40L (also

known as CD154 or TNFSF5), FasL (also known as CD95L, CD178 or TNFSF6), CD27L (also known as CD70 or TNFSF7), CD30L (also known as CD153 or TNFSF8), 4-1BBL (also known as TNFSF9), TRAIL (also known as APO2L, CD253 or TNFSF10), RANKL (also known as CD254 or TNFSF11), TWEAK (also known as TNFSF12), APRIL (also known as CD256 or TNFSF13), BAFF (also known as CD257 or TNFSF13B), LIGHT (also known as CD258 or TNFSF14), TL1A (also known as VEGI or TNFSF15), GITRL (also known as TNFSF18), EDA-A1 (also known as ectodysplasin A1) and EDA-A2 (also known as ectodysplasin A2). The term refers to any native TNF family ligand from any vertebrate source, including mammals such as primates (e.g. humans), non-human primates (e.g. cynomolgus monkeys) and rodents (e.g. mice and rats), unless otherwise indicated. The term “**costimulatory TNF ligand family member**” or “costimulatory TNF family ligand” refers to a subgroup of TNF ligand family members, which are able to costimulate proliferation and cytokine production of T-cells. These TNF family ligands can costimulate TCR signals upon interaction with their corresponding TNF receptors and the interaction with their receptors leads to recruitment of TNFR-associated factors (TRAF), which initiate signalling cascades that result in T-cell activation. Costimulatory TNF family ligands are selected from the group consisting of 4-1BBL, OX40L, GITRL, CD70, CD30L and LIGHT, more particularly the costimulatory TNF ligand family member is 4-1BBL.

As described herein before, 4-1BBL is a type II transmembrane protein and one member of the TNF ligand family. Complete or full length 4-1BBL having the amino acid sequence of SEQ ID NO:69 has been described to form trimers on the surface of cells. The formation of trimers is enabled by specific motives of the ectodomain of 4-1BBL. Said motives are designated herein as “trimerization region”. The amino acids 50-254 of the human 4-1BBL sequence (SEQ ID NO:70) form the extracellular domain of 4-1BBL, but even fragments thereof are able to form the trimers. In specific embodiments of the invention, the term “ectodomain of 4-1BBL or a fragment thereof” refers to a polypeptide having an amino acid sequence selected from SEQ ID NO:4 (amino acids 52-254 of human 4-1BBL), SEQ ID NO:1 (amino acids 71-254 of human 4-1BBL), SEQ ID NO:3 (amino acids 80-254 of human 4-1BBL) and SEQ ID NO:2 (amino acids 85-254 of human 4-1BBL) or a polypeptide having an amino acid sequence selected from SEQ ID NO:5 (amino acids 71-248 of human 4-1BBL), SEQ ID NO:8 (amino acids 52-248 of human 4-1BBL), SEQ ID NO:7 (amino acids 80-248 of human 4-1BBL) and SEQ ID NO:6 (amino acids 85-248 of human 4-1BBL), but also other fragments of the ectodomain capable of trimerization are included herein.

An “**ectodomain**” is the domain of a membrane protein that extends into the extracellular space (i.e. the space outside the target cell). Ectodomains are usually the parts of proteins that initiate contact with surfaces, which leads to signal transduction. The ectodomain of TNF ligand family member as defined herein thus refers to the part of the TNF ligand protein that extends into the extracellular space (the extracellular domain), but also includes shorter parts or

fragments thereof that are responsible for the trimerization and for the binding to the corresponding TNF receptor. The term “ectodomain of a TNF ligand family member or a fragment thereof” thus refers to the extracellular domain of the TNF ligand family member that forms the extracellular domain or to parts thereof that are still able to bind to the receptor
 5 (receptor binding domain).

The term “**peptide linker**” refers to a peptide comprising one or more amino acids, typically about 2 to 20 amino acids. Peptide linkers are known in the art or are described herein. Suitable, non-immunogenic linker peptides are, for example, $(G_4S)_n$, $(SG_4)_n$ or $G_4(SG_4)_n$ peptide linkers, wherein “n” is generally a number between 1 and 10, typically between 1 and 4, in
 10 particular 2, i.e. the peptides selected from the group consisting of GGGGS (SEQ ID NO:71), GGGGSGGGGS (SEQ ID NO:68), SGGGSGGGG (SEQ ID NO:72), $(G_4S)_3$ or GGGGSGGGGSGGGGS (SEQ ID NO:73), GGGGSGGGGSGGGG or $G_4(SG_4)_2$ (SEQ ID NO:74), and $(G_4S)_4$ or GGGGSGGGGSGGGGSGGGGS (SEQ ID NO:75), but also include the sequences GSPGSSSSGS (SEQ ID NO:76), GSGSGSGS (SEQ ID NO:77), GSGSGNGS (SEQ
 15 ID NO:78), GSGSGSGS (SEQ ID NO:79), GSGSGS (SEQ ID NO:80), GSGS (SEQ ID NO:81), GSGNGSGS (SEQ ID NO:82), GGNGSGSG (SEQ ID NO:83) and GGNGSG (SEQ ID NO:84). Peptide linkers of particular interest are $(G_4S)_1$ or GGGGS (SEQ ID NO:71), $(G_4S)_2$ or GGGGSGGGGS (SEQ ID NO:68) and $(G_4S)_3$ (SEQ ID NO:73).

The term “**amino acid**” as used within this application denotes the group of naturally
 20 occurring carboxy α -amino acids comprising alanine (three letter code: ala, one letter code: A), arginine (arg, R), asparagine (asn, N), aspartic acid (asp, D), cysteine (cys, C), glutamine (gln, Q), glutamic acid (glu, E), glycine (gly, G), histidine (his, H), isoleucine (ile, I), leucine (leu, L), lysine (lys, K), methionine (met, M), phenylalanine (phe, F), proline (pro, P), serine (ser, S), threonine (thr, T), tryptophan (trp, W), tyrosine (tyr, Y), and valine (val, V).

A “**fusion polypeptide**” or “fusion protein” as used herein refers to a single chain polypeptide composed of an antibody fragment and a peptide that is not derived from an antibody. In one aspect, a fusion polypeptide is composed of one or two ectodomains of 4-1BBL or a fragment thereof fused to a part of antigen binding domain or Fc part. The fusion may occur by directly linking the N or C-terminal amino acid of the antigen binding moiety via a peptide
 30 linker to the C- or N-terminal amino acid of the ectodomain of said 4-1BBL or fragment thereof.

By “fused” or “connected” is meant that the components (e.g. a polypeptide and an ectodomain of said TNF ligand family member) are linked by peptide bonds, either directly or via one or more peptide linkers.

“**Percent (%) amino acid sequence identity**” with respect to a reference polypeptide
 35 (protein) sequence is defined as the percentage of amino acid residues in a candidate sequence

that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, SAWI or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary. In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

In certain embodiments, **amino acid sequence variants** of the TNF ligand trimer-containing antigen binding molecules provided herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the TNF ligand trimer-containing antigen binding molecules. Amino acid sequence variants of the TNF ligand trimer-containing antigen binding molecules may be prepared by introducing appropriate modifications into the nucleotide sequence encoding the molecules, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or

substitutions of residues within the amino acid sequences of the antibody. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics, e.g., antigen-binding. Sites of interest for substitutional mutagenesis include the HVRs and Framework (FRs). Conservative substitutions are provided in Table B under the heading “Preferred Substitutions” and further described below in reference to amino acid side chain classes (1) to (6). Amino acid substitutions may be introduced into the molecule of interest and the products screened for a desired activity, e.g., retained/improved antigen binding, decreased immunogenicity, or improved ADCC or CDC.

TABLE A

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	Val; Leu; Ile	Val
Arg (R)	Lys; Gln; Asn	Lys
Asn (N)	Gln; His; Asp, Lys; Arg	Gln
Asp (D)	Glu; Asn	Glu
Cys (C)	Ser; Ala	Ser
Gln (Q)	Asn; Glu	Asn
Glu (E)	Asp; Gln	Asp
Gly (G)	Ala	Ala
His (H)	Asn; Gln; Lys; Arg	Arg
Ile (I)	Leu; Val; Met; Ala; Phe; Norleucine	Leu
Leu (L)	Norleucine; Ile; Val; Met; Ala; Phe	Ile
Lys (K)	Arg; Gln; Asn	Arg
Met (M)	Leu; Phe; Ile	Leu
Phe (F)	Trp; Leu; Val; Ile; Ala; Tyr	Tyr
Pro (P)	Ala	Ala
Ser (S)	Thr	Thr
Thr (T)	Val; Ser	Ser
Trp (W)	Tyr; Phe	Tyr
Tyr (Y)	Trp; Phe; Thr; Ser	Phe
Val (V)	Ile; Leu; Met; Phe; Ala; Norleucine	Leu

Amino acids may be grouped according to common side-chain properties:

- (1) hydrophobic: Norleucine, Met, Ala, Val, Leu, Ile;
- (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln;
- (3) acidic: Asp, Glu;
- (4) basic: His, Lys, Arg;
- 5 (5) residues that influence chain orientation: Gly, Pro;
- (6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

The term “**amino acid sequence variants**” includes substantial variants wherein there are amino acid substitutions in one or more hypervariable region residues of a parent antigen binding molecule (e.g. a humanized or human antibody). Generally, the resulting variant(s) selected for further study will have modifications (e.g., improvements) in certain biological properties (e.g., increased affinity, reduced immunogenicity) relative to the parent antigen binding molecule and/or will have substantially retained certain biological properties of the parent antigen binding molecule. An exemplary substitutional variant is an affinity matured antibody, which may be conveniently generated, e.g., using phage display-based affinity maturation techniques such as those described herein. Briefly, one or more CDR residues are mutated and the variant antigen binding molecules displayed on phage and screened for a particular biological activity (e.g. binding affinity). In certain embodiments, substitutions, insertions, or deletions may occur within one or more CDRs so long as such alterations do not substantially reduce the ability of the antigen binding molecule to bind antigen. For example, conservative alterations (e.g., conservative substitutions as provided herein) that do not substantially reduce binding affinity may be made in CDRs. A useful method for identification of residues or regions of an antibody that may be targeted for mutagenesis is called “alanine scanning mutagenesis” as described by Cunningham and Wells (1989) *Science*, 244:1081-1085. In this method, a residue or group of target residues (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) are identified and replaced by a neutral or negatively charged amino acid (e.g., alanine or polyalanine) to determine whether the interaction of the antibody with antigen is affected. Further substitutions may be introduced at the amino acid locations demonstrating functional sensitivity to the initial substitutions. Alternatively, or additionally, a crystal structure of an antigen-antigen binding molecule complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues may be targeted or eliminated as candidates for substitution. Variants may be screened to determine whether they contain the desired properties.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal

insertions include a 4-1BBL trimer-containing antigen binding molecule with an N-terminal methionyl residue. Other insertional variants of the molecule include the fusion to the N- or C-terminus to a polypeptide which increases the serum half-life of the 4-1BBL trimer-containing antigen binding molecule.

5 In certain embodiments, the 4-1BBL trimer-containing antigen binding molecules provided herein are altered to increase or decrease the extent to which the antibody is glycosylated. Glycosylation variants of the molecules may be conveniently obtained by altering the amino acid sequence such that one or more glycosylation sites is created or removed. Where the 4-1BBL trimer-containing antigen binding molecule comprises an Fc region, the carbohydrate attached
10 thereto may be altered. Native antibodies produced by mammalian cells typically comprise a branched, biantennary oligosaccharide that is generally attached by an N-linkage to Asn297 of the CH2 domain of the Fc region. See, e.g., Wright et al. *TIBTECH* 15:26-32 (1997). The oligosaccharide may include various carbohydrates, e.g., mannose, N-acetyl glucosamine (GlcNAc), galactose, and sialic acid, as well as a fucose attached to a GlcNAc in the “stem” of
15 the biantennary oligosaccharide structure. In some embodiments, modifications of the oligosaccharide in 4-1BBL ligand trimer-containing antigen binding molecule may be made in order to create variants with certain improved properties. In one aspect, variants of 4-1BBL trimer-containing antigen binding molecules are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. Such fucosylation variants may
20 have improved ADCC function, see e.g. US Patent Publication Nos. US 2003/0157108 (Presta, L.) or US 2004/0093621 (Kyowa Hakko Kogyo Co., Ltd). Further variants of the 4-1BBL trimer-containing antigen binding molecules of the invention include those with bisected oligosaccharides, e.g., in which a biantennary oligosaccharide attached to the Fc region is bisected by GlcNAc. Such variants may have reduced fucosylation and/or improved ADCC
25 function., see for example WO 2003/011878 (Jean-Mairet et al.); US Patent No. 6,602,684 (Umana et al.); and US 2005/0123546 (Umana *et al.*). Variants with at least one galactose residue in the oligosaccharide attached to the Fc region are also provided. Such antibody variants may have improved CDC function and are described, e.g., in WO 1997/30087 (Patel et al.); WO 1998/58964 (Raju, S.); and WO 1999/22764 (Raju, S.).

30 In certain embodiments, it may be desirable to create **cysteine engineered variants** of the 4-1BBL trimer-containing antigen binding molecule of the invention, e.g., “thioMAbs,” in which one or more residues of the molecule are substituted with cysteine residues. In particular embodiments, the substituted residues occur at accessible sites of the molecule. By substituting those residues with cysteine, reactive thiol groups are thereby positioned at accessible sites of the
35 antibody and may be used to conjugate the antibody to other moieties, such as drug moieties or linker-drug moieties, to create an immunoconjugate. In certain embodiments, any one or more of the following residues may be substituted with cysteine: V205 (Kabat numbering) of the light

chain; A118 (EU numbering) of the heavy chain; and S400 (EU numbering) of the heavy chain Fc region. Cysteine engineered antigen binding molecules may be generated as described, e.g., in U.S. Patent No. 7,521,541.

In certain aspects, the 4-1BBL trimer-containing antigen binding molecules provided
5 herein may be further modified to contain additional non-proteinaceous moieties that are known in the art and readily available. The moieties suitable for derivatization of the antibody include but are not limited to water soluble polymers. Non-limiting examples of water soluble polymers include, but are not limited to, polyethylene glycol (PEG), copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl
10 pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propylene glycol homopolymers, polypropylene oxide/ethylene oxide co-polymers, polyoxyethylated polyols (e.g., glycerol), polyvinyl alcohol, and mixtures thereof. Polyethylene glycol propionaldehyde may have advantages in
15 manufacturing due to its stability in water. The polymer may be of any molecular weight, and may be branched or unbranched. The number of polymers attached to the antibody may vary, and if more than one polymer is attached, they can be the same or different molecules. In general, the number and/or type of polymers used for derivatization can be determined based on considerations including, but not limited to, the particular properties or functions of the antibody
20 to be improved, whether the bispecific antibody derivative will be used in a therapy under defined conditions, etc. In another aspect, conjugates of an antibody and non-proteinaceous moiety that may be selectively heated by exposure to radiation are provided. In one embodiment, the non-proteinaceous moiety is a carbon nanotube (Kam, N.W. et al., Proc. Natl. Acad. Sci. USA 102 (2005) 11600-11605). The radiation may be of any wavelength, and includes, but is
25 not limited to, wavelengths that do not harm ordinary cells, but which heat the non-proteinaceous moiety to a temperature at which cells proximal to the antibody-non-proteinaceous moiety are killed.

In another aspect, immunoconjugates of the 4-1BBL trimer-containing antigen binding molecules provided herein may be obtained. An “**immunoconjugate**” is an antibody conjugated
30 to one or more heterologous molecule(s), including but not limited to a cytotoxic agent.

The term “**nucleic acid molecule**” or “polynucleotide” includes any compound and/or substance that comprises a polymer of nucleotides. Each nucleotide is composed of a base, specifically a purine- or pyrimidine base (i.e. cytosine (C), guanine (G), adenine (A), thymine (T) or uracil (U)), a sugar (i.e. deoxyribose or ribose), and a phosphate group. Often, the nucleic
35 acid molecule is described by the sequence of bases, whereby said bases represent the primary structure (linear structure) of a nucleic acid molecule. The sequence of bases is typically

represented from 5' to 3'. Herein, the term nucleic acid molecule encompasses deoxyribonucleic acid (DNA) including e.g., complementary DNA (cDNA) and genomic DNA, ribonucleic acid (RNA), in particular messenger RNA (mRNA), synthetic forms of DNA or RNA, and mixed polymers comprising two or more of these molecules. The nucleic acid molecule may be linear or circular. In addition, the term nucleic acid molecule includes both, sense and antisense strands, as well as single stranded and double stranded forms. Moreover, the herein described nucleic acid molecule can contain naturally occurring or non-naturally occurring nucleotides. Examples of non-naturally occurring nucleotides include modified nucleotide bases with derivatized sugars or phosphate backbone linkages or chemically modified residues. Nucleic acid molecules also encompass DNA and RNA molecules which are suitable as a vector for direct expression of an antibody of the invention in vitro and/or in vivo, e.g., in a host or patient. Such DNA (e.g., cDNA) or RNA (e.g., mRNA) vectors, can be unmodified or modified. For example, mRNA can be chemically modified to enhance the stability of the RNA vector and/or expression of the encoded molecule so that mRNA can be injected into a subject to generate the antibody in vivo (see e.g., Stadler et al, Nature Medicine 2017, published online 12 June 2017, doi:10.1038/nm.4356 or EP 2 101 823 B1).

By "**isolated**" nucleic acid molecule or polynucleotide is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, a recombinant polynucleotide encoding a polypeptide contained in a vector is considered isolated for the purposes of the present invention. Further examples of an isolated polynucleotide include recombinant polynucleotides maintained in heterologous host cells or purified (partially or substantially) polynucleotides in solution. An isolated polynucleotide includes a polynucleotide molecule contained in cells that ordinarily contain the polynucleotide molecule, but the polynucleotide molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location. Isolated RNA molecules include in vivo or in vitro RNA transcripts of the present invention, as well as positive and negative strand forms, and double-stranded forms. Isolated polynucleotides or nucleic acids according to the present invention further include such molecules produced synthetically. In addition, a polynucleotide or a nucleic acid may be or may include a regulatory element such as a promoter, ribosome binding site, or a transcription terminator.

By a nucleic acid or polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of

nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence. As a practical matter, whether any particular polynucleotide sequence is at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence of the present invention can be determined conventionally using known computer programs, such as the ones discussed above for polypeptides (e.g. ALIGN-2).

10 The term "**expression cassette**" refers to a polynucleotide generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among
15 other sequences, a nucleic acid sequence to be transcribed and a promoter. In certain embodiments, the expression cassette of the invention comprises polynucleotide sequences that encode bispecific antigen binding molecules of the invention or fragments thereof.

The term "**vector**" or "expression vector" is synonymous with "expression construct" and refers to a DNA molecule that is used to introduce and direct the expression of a specific gene to
20 which it is operably associated in a target cell. The term includes the vector as a self-replicating nucleic acid structure as well as the vector incorporated into the genome of a host cell into which it has been introduced. The expression vector of the present invention comprises an expression cassette. Expression vectors allow transcription of large amounts of stable mRNA. Once the expression vector is inside the target cell, the ribonucleic acid molecule or protein that is
25 encoded by the gene is produced by the cellular transcription and/or translation machinery. In one embodiment, the expression vector of the invention comprises an expression cassette that comprises polynucleotide sequences that encode bispecific antigen binding molecules of the invention or fragments thereof.

The terms "**host cell**", "host cell line," and "host cell culture" are used interchangeably and
30 refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells," which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or
35 selected for in the originally transformed cell are included herein. A host cell is any type of cellular system that can be used to generate the bispecific antigen binding molecules of the

present invention. Host cells include cultured cells, e.g. mammalian cultured cells, such as CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, insect cells, and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or
5 animal tissue.

An "**effective amount**" of an agent refers to the amount that is necessary to result in a physiological change in the cell or tissue to which it is administered.

A "**therapeutically effective amount**" of an agent, e.g. a pharmaceutical composition, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired
10 therapeutic or prophylactic result. A therapeutically effective amount of an agent for example eliminates, decreases, delays, minimizes or prevents adverse effects of a disease.

An "**individual**" or "subject" is a mammal. Mammals include, but are not limited to, domesticated animals (e.g. cows, sheep, cats, dogs, and horses), primates (e.g. humans and non-human primates such as monkeys), rabbits, and rodents (e.g. mice and rats). Particularly, the
15 individual or subject is a human.

The term "**pharmaceutical composition**" refers to a preparation which is in such form as to permit the biological activity of an active ingredient contained therein to be effective, and which contains no additional components which are unacceptably toxic to a subject to which the formulation would be administered.

20 A "**pharmaceutically acceptable excipient**" refers to an ingredient in a pharmaceutical composition, other than an active ingredient, which is nontoxic to a subject. A pharmaceutically acceptable excipient includes, but is not limited to, a buffer, a stabilizer, or a preservative.

The term "**package insert**" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications,
25 usage, dosage, administration, combination therapy, contraindications and/or warnings concerning the use of such therapeutic products.

As used herein, "**treatment**" (and grammatical variations thereof such as "treat" or "treating") refers to clinical intervention in an attempt to alter the natural course of the individual being treated, and can be performed either for prophylaxis or during the course of clinical
30 pathology. Desirable effects of treatment include, but are not limited to, preventing occurrence or recurrence of disease, alleviation of symptoms, diminishment of any direct or indirect pathological consequences of the disease, preventing metastasis, decreasing the rate of disease progression, amelioration or palliation of the disease state, and remission or improved prognosis.

In some embodiments, the molecules of the invention are used to delay development of a disease or to slow the progression of a disease.

The term “**cancer**” as used herein refers to proliferative diseases, such as lymphomas, carcinoma, lymphoma, blastoma, sarcoma, leukemia, lymphocytic leukemias, lung cancer, non-
5 small cell lung (NSCL) cancer, bronchioloalviolar cell lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, gastric cancer, colorectal cancer (CRC), pancreatic cancer, breast cancer, triple-negative breast cancer, uterine cancer, carcinoma of the fallopian tubes, carcinoma of the endometrium, carcinoma of the
10 cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, prostate cancer, cancer of the bladder, cancer of the kidney or ureter, renal cell carcinoma, carcinoma of the renal pelvis, mesothelioma, hepatocellular cancer,
15 biliary cancer, neoplasms of the central nervous system (CNS), spinal axis tumors, brain stem glioma, glioblastoma multiforme, astrocytomas, schwannomas, ependymomas, medulloblastomas, meningiomas, squamous cell carcinomas, pituitary adenoma and Ewings sarcoma, melanoma, multiple myeloma, B-cell cancer (lymphoma), chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL), hairy cell leukemia, chronic myeloblastic leukemia, including
20 refractory versions of any of the above cancers, or a combination of one or more of the above cancers.

A “Her2-positive” cancer comprises cancer cells which have higher than normal levels of Her2. Examples of Her2-positive cancer include Her2-positive breast cancer and Her2-positive gastric cancer. Optionally, Her2-positive cancer has an immunohistochemistry (IHC) score of 2+
25 or 3+ and/or an in situ hybridization (ISH) amplification ratio >2.0.

The term “early stage breast cancer (EBC)” or “early breast cancer” is used herein to refer to breast cancer that has not spread beyond the breast or the axillary lymph nodes. This includes ductal carcinoma in situ and stage I, stage IIA, stage IIB, and stage IIIA breast cancers.

Reference to a tumor or cancer as a “Stage 0”, “Stage I”, “Stage II”, “Stage III”, or “Stage
30 IV”, and various sub-stages within this classification, indicates classification of the tumor or cancer using the Overall Stage Grouping or Roman Numeral Staging methods known in the art. Although the actual stage of the cancer is dependent on the type of cancer, in general, a Stage 0 cancer is an in situ lesion, a Stage I cancer is small localized tumor, a Stage II and III cancer is a local advanced tumor which exhibits involvement of the local lymph nodes, and a Stage IV

cancer represents metastatic cancer. The specific stages for each type of tumor is known to the skilled clinician.

The term “metastatic breast cancer” means the state of breast cancer where the cancer cells are transmitted from the original site to one or more sites elsewhere in the body, by the blood
5 vessels or lymphatics, to form one or more secondary tumors in one or more organs besides the breast.

An “advanced” cancer is one which has spread outside the site or organ of origin, either by local invasion or metastasis. Accordingly, the term “advanced” cancer includes both locally advanced and metastatic disease.

10 A “recurrent” cancer is one which has regrown, either at the initial site or at a distant site, after a response to initial therapy, such as surgery. A “locally recurrent” cancer is cancer that returns after treatment in the same place as a previously treated cancer. An “operable” or “resectable” cancer is cancer which is confined to the primary organ and suitable for surgery (resection). A “non-resectable” or “unresectable” cancer is not able to be removed (resected) by
15 surgery.

4-1BBL trimer-containing antigen binding molecules of the invention

The invention provides novel 4-1BBL trimer-containing antigen binding molecules with particularly advantageous properties such as producibility, stability, binding affinity, biological activity, targeting efficiency, reduced toxicity and reduced immunogenicity.

20 In a first aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule comprising
(a) an antigen binding domain capable of specific binding to Her2,
(b) a first and a second polypeptide that are linked to each other by a disulfide bond,
wherein the antigen binding molecule is characterized in that the first polypeptide comprises two
25 ectodomains of 4-1BBL or a fragment thereof that are connected to each other by a peptide linker and in that the second polypeptide comprises one ectodomain of 4-1BBL or a fragment thereof, and
(c) an Fc domain composed of a first and a second subunit capable of stable association.

In a further aspect, provided is a 4-1BBL trimer-containing antigen binding molecule as
30 defined herein before, comprising
(a) an antigen binding domain capable of specific binding to Her2, and
(b) a first and a second polypeptide that are linked to each other by a disulfide bond,
wherein the antigen binding molecule is characterized in that

- (i) the first polypeptide contains a CH1 or CL domain and the second polypeptide contains a CL or CH1 domain, respectively, wherein the second polypeptide is linked to the first polypeptide by a disulfide bond between the CH1 and CL domain, and wherein the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other and to the CH1 or CL domain by a peptide linker and wherein the second polypeptide comprises one ectodomain of said 4-1BBL or a fragment thereof connected via a peptide linker to the CL or CH1 domain of said polypeptide, or
- (ii) the first polypeptide contains a CH3 domain and the second polypeptide contains a CH3 domain, respectively, and wherein the first polypeptide comprises two ectodomains of a 4-1BBL or a fragment thereof that are connected to each other and to the C-terminus of the CH3 domain by a peptide linker and wherein the second polypeptide comprises only one ectodomain of said 4-1BBL or a fragment thereof connected via a peptide linker to C-terminus of the CH3 domain of said polypeptide, or
- (iii) the first polypeptide contains a VH-CL or a VL-CH1 domain and the second polypeptide contains a VL-CH1 domain or a VH-CL domain, respectively, wherein the second polypeptide is linked to the first polypeptide by a disulfide bond between the CH1 and CL domain, and wherein the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other and to to VH or VL by a peptide linker and wherein the second polypeptide comprises one ectodomain of said TNF ligand family member or a fragment thereof connected via a peptide linker to VL or VH of said polypeptide, and
- (c) an Fc domain composed of a first and a second subunit capable of stable association.

In another aspect, provided is a 4-1BBL trimer-containing antigen binding molecule of as defined herein before, comprising

- (a) an antigen binding domain capable of specific binding to Her2, and
- (b) a first and a second polypeptide that are linked to each other by a disulfide bond, wherein the antigen binding molecule is characterized in that
- (i) the first polypeptide contains a CH1 or CL domain and the second polypeptide contains a CL or CH1 domain, respectively, wherein the second polypeptide is linked to the first polypeptide by a disulfide bond between the CH1 and CL domain, and wherein the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other and to the CH1 or CL domain by a peptide linker and wherein the second polypeptide comprises one ectodomain of said 4-1BBL or a fragment thereof connected via a peptide linker to the CL or CH1 domain of said polypeptide, or
- (ii) the first polypeptide contains a CH3 domain and the second polypeptide contains a CH3 domain, respectively, and wherein the first polypeptide comprises two

- ectodomains of 4-1BBL or a fragment thereof that are connected to each other and to the C-terminus of the CH3 domain by a peptide linker and wherein the second polypeptide comprises only one ectodomain of said 4-1BBL or a fragment thereof connected via a peptide linker to C-terminus of the CH3 domain of said polypeptide,
- 5 and
- (c) an Fc domain composed of a first and a second subunit capable of stable association.

In one aspect, the ectodomain of 4-1BBL comprises the amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO: 2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO: 6, SEQ ID NO:7 and SEQ ID NO:8, particularly the amino acid sequence of

10 SEQ ID NO:1 or SEQ ID NO:5. More particularly, the ectodomain of 4-1BBL comprises the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:5. Most particularly, the ectodomain of 4-1BBL comprises the amino acid sequence of SEQ ID NO:5. In particular, provided is a 4-1BBL trimer-containing antigen binding molecule of as defined herein before, wherein all three ectodomains of 4-1BBL or a fragment thereof are identical.

- 15 In a further aspect, the 4-1BBL trimer-containing antigen binding molecule of the invention comprises
- (a) an antigen binding domain capable of specific binding to Her2,
- (b) a first and a second polypeptide that are linked to each other by a disulfide bond, wherein the antigen binding molecule is characterized in that the first polypeptide comprises the
- 20 amino acid sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12 and in that the second polypeptide comprises the amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:5, SEQ ID NO:3 and SEQ ID NO:4, and
- (c) an Fc domain composed of a first and a second subunit capable of stable association.

- 25 In one aspect, the 4-1BBL trimer-containing antigen binding molecule of the invention comprises
- (a) an antigen binding domain capable of specific binding to Her2,
- (b) a first and a second polypeptide that are linked to each other by a disulfide bond, wherein the antigen binding molecule is characterized in that the first polypeptide comprises the
- 30 amino acid sequence of SEQ ID NO:10 and in that the second polypeptide comprises the amino acid sequence of SEQ ID NO:5, and
- (c) an Fc domain composed of a first and a second subunit capable of stable association.

- In a further aspect, the 4-1BBL trimer-containing antigen binding molecule of the invention comprises
- 35 (a) an antigen binding domain capable of specific binding to Her2,

(b) a first and a second polypeptide that are linked to each other by a disulfide bond, wherein the antigen binding molecule is characterized in that the first polypeptide comprises the amino acid sequence of SEQ ID NO:9 and in that the second polypeptide comprises the amino acid sequence of SEQ ID NO:1, and

- 5 (c) an Fc domain composed of a first and a second subunit capable of stable association.

In another aspect, the 4-1BBL trimer-containing antigen binding molecule of the invention comprises

- (a) an antigen binding domain capable of specific binding to Her2,
(b) a first polypeptide containing a CH1 or CL domain and a second polypeptide containing a CL
10 or CH1 domain, respectively, wherein the second polypeptide is linked to the first polypeptide by a disulfide bond between the CH1 and CL domain,
and wherein the antigen binding molecule is characterized in that the first polypeptide comprises two ectodomains of 4-1BBL or fragments thereof that are connected to each other and to the CH1 or CL domain by a peptide linker and in that the second polypeptide comprises only one
15 ectodomain of 4-1BBL or a fragment thereof connected by a peptide linker to the CL or CH1 domain of said polypeptide.

In one aspect, provided is a 4-1BBL trimer-containing antigen binding molecule comprising

- (a) an antigen binding domain capable of specific binding to Her2,
20 (b) a first polypeptide containing a CH1 domain and a second polypeptide containing a CL domain, wherein the second polypeptide is linked to the first polypeptide by a disulfide bond between the CH1 and CL domain,
and wherein the antigen binding molecule is characterized in that the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other and to the
25 CH1 domain by a peptide linker and in that the second polypeptide comprises one ectodomain of 4-1BBL or a fragment thereof connected via a peptide linker to the CL domain of said polypeptide.

In another aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule comprising

- 30 (a) one antigen binding domain capable of specific binding to Her2, and
(b) a first and a second polypeptide that are linked to each other by a disulfide bond, wherein the antigen binding molecule is characterized in that the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other by a peptide linker and in that the second polypeptide comprises one ectodomain of 4-1BBL or a fragment
35 thereof, and
(c) an Fc domain composed of a first and a second subunit capable of stable association

In yet another aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule comprising

- (a) more than one antigen binding domain capable of specific binding to Her2, and
- (b) a first and a second polypeptide that are linked to each other by a disulfide bond,
- 5 wherein the antigen binding molecule is characterized in that the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other by a peptide linker and in that the second polypeptide comprises one ectodomain of 4-1BBL or a fragment thereof, and
- (c) an Fc domain composed of a first and a second subunit capable of stable association.

10 In one aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule comprising

- (a) two antigen binding domains capable of specific binding to Her2, and
- (b) a first and a second polypeptide that are linked to each other by a disulfide bond,
- wherein the antigen binding molecule is characterized in that the first polypeptide comprises two
- 15 ectodomains of 4-1BBL or a fragment thereof that are connected to each other by a peptide linker and in that the second polypeptide comprises one ectodomain of 4-1BBL or a fragment thereof, and
- (c) an Fc domain composed of a first and a second subunit capable of stable association.

In a further aspect, the invention provides a 4-1BBL trimer-containing antigen binding
20 molecule as defined herein before, wherein antigen binding domain capable of specific binding to Her2 is selected from the group consisting of an antibody, an antibody fragment and a scaffold antigen binding protein.

In one aspect, provided is a 4-1BBL trimer-containing antigen binding molecule as described herein before, wherein the antigen binding domain capable of specific binding to Her2
25 is selected from the group consisting of an antibody fragment, a Fab molecule, a crossover Fab molecule, a single chain Fab molecule, a Fv molecule, a scFv molecule, a single domain antibody, or aVH and a scaffold antigen binding protein. In one aspect, the antigen binding domain capable of specific binding to Her2 is an aVH or a scaffold antigen binding protein.

In a particular aspect, provided is a 4-1BBL trimer-containing antigen binding molecule,
30 wherein the antigen binding domain capable of specific binding to Her2 is a Fab molecule or a crossover Fab molecule capable of specific binding to Her2. In particular, the antigen binding domain capable of specific binding to Her2 is a Fab capable of specific binding to Her2.

In a further aspect, provided is a 4-1BBL trimer-containing antigen binding molecule according to the invention, wherein a peptide comprising two ectodomains of 4-1BBL or a
35 fragment thereof connected to each other by a first peptide linker is fused at its C-terminus to the

CH1 domain of a heavy chain by a second peptide linker and wherein one ectodomain of said 4-1BBL or a fragment thereof is fused at the its C-terminus to the CL domain on a light chain by a third peptide linker.

5 In another aspect, provided is a 4-1BBL trimer-containing antigen binding molecule according to the invention, wherein a peptide comprising two ectodomains of 4-1BBL or a fragment thereof connected to each other by a first peptide linker is fused at its C-terminus to the CL domain of a heavy chain by a second peptide linker and wherein one ectodomain of said 4-1BBL or a fragment thereof is fused at the its C-terminus to the CH1 domain on a light chain by a third peptide linker.

10 In a further aspect, the invention is concerned with a 4-1BBL trimer-containing antigen binding molecule according to the invention, wherein a peptide comprising two ectodomains of a 4-1BBL or a fragment thereof connected to each other by a first peptide linker is fused at its C-terminus to the CL domain of a light chain by a second peptide linker and wherein one ectodomain of said 4-1BBL or a fragment thereof is fused at the its C-terminus to the CH1
15 domain of the heavy chain by a third peptide linker.

In a particular aspect, the invention relates to a 4-1BBL trimer-containing antigen binding molecule as defined above, wherein the peptide linker is (G4S)₂. In one aspect, the first peptide linker is (G4S)₂ (SEQ ID NO:68), the second peptide linker is (G4S)₂ (SEQ ID NO:68) and the third peptide linker is (G4S)₂ (SEQ ID NO:68).

20 In another aspect, the 4-1BBL trimer-containing antigen binding molecule as defined herein before comprises an Fc domain composed of a first and a second subunit capable of stable association.

In particular, the 4-1BBL trimer-containing antigen binding molecule of the invention comprises (a) a Fab molecule capable of specific binding to Her2, wherein the Fab heavy chain
25 is fused at the C-terminus to the N-terminus of a CH2 domain in the Fc domain and (c) an Fc domain composed of a first and a second subunit capable of stable association.

In a further aspect, the Fc domain is an IgG, particularly an IgG1 Fc domain or an IgG4 Fc domain. More particularly, the Fc domain is an IgG1 Fc domain. In a particular aspect, the Fc domain comprises a modification promoting the association of the first and second subunit of the
30 Fc domain.

Fc domain modifications reducing Fc receptor binding and/or effector function

The Fc domain of the 4-1BBL trimer-containing antigen binding molecules of the invention consists of a pair of polypeptide chains comprising heavy chain domains of an

immunoglobulin molecule. For example, the Fc domain of an immunoglobulin G (IgG) molecule is a dimer, each subunit of which comprises the CH2 and CH3 IgG heavy chain constant domains. The two subunits of the Fc domain are capable of stable association with each other.

The Fc domain confers favorable pharmacokinetic properties to the antigen binding molecules of the invention, including a long serum half-life which contributes to good accumulation in the target tissue and a favorable tissue-blood distribution ratio. At the same time it may, however, lead to undesirable targeting of the bispecific antibodies of the invention to cells expressing Fc receptors rather than to the preferred antigen-bearing cells. Accordingly, in particular aspects, the Fc domain of the 4-1BBL trimer-containing antigen binding molecule of the invention exhibits reduced binding affinity to an Fc receptor and/or reduced effector function, as compared to a native IgG1 Fc domain. In one aspect, the Fc does not substantially bind to an Fc receptor and/or does not induce effector function. In a particular aspect the Fc receptor is an Fc γ receptor. In one aspect, the Fc receptor is a human Fc receptor. In a specific aspect, the Fc receptor is an activating human Fc γ receptor, more specifically human Fc γ RIIIa, Fc γ RI or Fc γ RIIa, most specifically human Fc γ RIIIa. In one aspect, the Fc domain does not induce effector function. The reduced effector function can include, but is not limited to, one or more of the following: reduced complement dependent cytotoxicity (CDC), reduced antibody-dependent cell-mediated cytotoxicity (ADCC), reduced antibody-dependent cellular phagocytosis (ADCP), reduced cytokine secretion, reduced immune complex-mediated antigen uptake by antigen-presenting cells, reduced binding to NK cells, reduced binding to macrophages, reduced binding to monocytes, reduced binding to polymorphonuclear cells, reduced direct signaling inducing apoptosis, reduced dendritic cell maturation, or reduced T cell priming.

In certain aspects, one or more amino acid modifications may be introduced into the Fc region of a 4-1BBL trimer-containing antigen binding molecule provided herein, thereby generating an Fc region variant. The Fc region variant may comprise a human Fc region sequence (e.g., a human IgG1, IgG2, IgG3 or IgG4 Fc region) comprising an amino acid modification (e.g. a substitution) at one or more amino acid positions.

In a particular aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule comprising

- (a) an antigen binding domain capable of specific binding to Her2,
- (b) a first and a second polypeptide that are linked to each other by a disulfide bond,

wherein the antigen binding molecule is characterized in that the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other by a peptide linker and in that the second polypeptide comprises one ectodomain of 4-1BBL or a fragment thereof, and

(c) an Fc domain composed of a first and a second subunit capable of stable association, wherein the Fc domain comprises one or more amino acid substitution that reduces binding to an Fc receptor, in particular towards Fc γ receptor.

In one aspect, the Fc domain of the 4-1BBL trimer-containing antigen binding molecule of the invention comprises one or more amino acid mutation that reduces the binding affinity of the Fc domain to an Fc receptor and/or effector function. Typically, the same one or more amino acid mutation is present in each of the two subunits of the Fc domain. In particular, the Fc domain comprises an amino acid substitution at a position of E233, L234, L235, N297, P331 and P329 (EU numbering). In particular, the Fc domain comprises amino acid substitutions at positions 234 and 235 (EU numbering) and/or 329 (EU numbering) of the IgG heavy chains. More particularly, provided is a trimeric TNF family ligand-containing antigen binding molecule according to the invention which comprises an Fc domain with the amino acid substitutions L234A, L235A and P329G ("P329G LALA", EU numbering) in the IgG heavy chains. The amino acid substitutions L234A and L235A refer to the so-called LALA mutation. The "P329G LALA" combination of amino acid substitutions almost completely abolishes Fc γ receptor binding of a human IgG1 Fc domain and is described in International Patent Appl. Publ. No. WO 2012/130831 A1 which also describes methods of preparing such mutant Fc domains and methods for determining its properties such as Fc receptor binding or effector functions. "EU numbering" refers to the numbering according to EU index of Kabat et al, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

Fc domains with reduced Fc receptor binding and/or effector function also include those with substitution of one or more of Fc domain residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called "DANA" Fc mutant with substitution of residues 265 and 297 to alanine (US Patent No. 7,332,581).

In another aspect, the Fc domain is an IgG4 Fc domain. IgG4 antibodies exhibit reduced binding affinity to Fc receptors and reduced effector functions as compared to IgG1 antibodies. In a more specific aspect, the Fc domain is an IgG4 Fc domain comprising an amino acid substitution at position S228 (Kabat numbering), particularly the amino acid substitution S228P. In a more specific aspect, the Fc domain is an IgG4 Fc domain comprising amino acid substitutions L235E and S228P and P329G (EU numbering). Such IgG4 Fc domain mutants and their Fc γ receptor binding properties are also described in WO 2012/130831.

Mutant Fc domains can be prepared by amino acid deletion, substitution, insertion or modification using genetic or chemical methods well known in the art. Genetic methods may

include site-specific mutagenesis of the encoding DNA sequence, PCR, gene synthesis, and the like. The correct nucleotide changes can be verified for example by sequencing.

Binding to Fc receptors can be easily determined e.g. by ELISA, or by Surface Plasmon Resonance (SPR) using standard instrumentation such as a BIAcore instrument (GE Healthcare), and Fc receptors such as may be obtained by recombinant expression. A suitable such binding assay is described herein. Alternatively, binding affinity of Fc domains or cell activating bispecific antigen binding molecules comprising an Fc domain for Fc receptors may be evaluated using cell lines known to express particular Fc receptors, such as human NK cells expressing FcγIIIa receptor.

Effector function of an Fc domain, or bispecific antibodies of the invention comprising an Fc domain, can be measured by methods known in the art. A suitable assay for measuring ADCC is described herein. Other examples of in vitro assays to assess ADCC activity of a molecule of interest are described in U.S. Patent No. 5,500,362; Hellstrom et al. Proc Natl Acad Sci USA 83, 7059-7063 (1986) and Hellstrom et al., Proc Natl Acad Sci USA 82, 1499-1502 (1985); U.S. Patent No. 5,821,337; Bruggemann et al., J Exp Med 166, 1351-1361 (1987). Alternatively, non-radioactive assays methods may be employed (see, for example, ACTI™ non-radioactive cytotoxicity assay for flow cytometry (CellTechnology, Inc. Mountain View, CA); and CytoTox 96® non-radioactive cytotoxicity assay (Promega, Madison, WI)). Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g. in a animal model such as that disclosed in Clynes et al., Proc Natl Acad Sci USA 95, 652-656 (1998).

In some embodiments, binding of the Fc domain to a complement component, specifically to C1q, is reduced. Accordingly, in some embodiments wherein the Fc domain is engineered to have reduced effector function, said reduced effector function includes reduced CDC. C1q binding assays may be carried out to determine whether the bispecific antibodies of the invention is able to bind C1q and hence has CDC activity. See e.g., C1q and C3c binding ELISA in WO 2006/029879 and WO 2005/100402. To assess complement activation, a CDC assay may be performed (see, for example, Gazzano-Santoro et al., J Immunol Methods 202, 163 (1996); Cragg et al., Blood 101, 1045-1052 (2003); and Cragg and Glennie, Blood 103, 2738-2743 (2004)).

In a particular aspect, the Fc domain comprises a modification promoting the association of the first and second subunit of the Fc domain.

Fc domain modifications promoting heterodimerization

In one aspect, the 4-1BBL trimer-containing antigen binding molecules of the invention comprise (a) an antigen binding domain capable of specific binding to Her2, (b) a first and a second polypeptide that are linked to each other by a disulfide bond, wherein the antigen binding molecule is characterized in that the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other by a peptide linker and in that the second polypeptide comprises one ectodomain of 4-1BBL or a fragment thereof, and (c) an Fc domain composed of a first and a second subunit capable of stable association. Thus, they comprise different moieties, fused to one or the other of the two subunits of the Fc domain that are typically comprised in two non-identical polypeptide chains ("heavy chains"). Recombinant co-expression of these polypeptides and subsequent dimerization leads to several possible combinations of the two polypeptides. To improve the yield and purity of the 4-1BBL trimer-containing antigen binding molecules in recombinant production, it will thus be advantageous to introduce in the Fc domain of the 4-1BBL trimer-containing antigen binding molecules of the invention a modification promoting the association of the desired polypeptides.

Accordingly, the Fc domain of the 4-1BBL trimer-containing antigen binding molecules of the invention comprises a modification promoting the association of the first and the second subunit of the Fc domain. The site of most extensive protein-protein interaction between the two subunits of a human IgG Fc domain is in the CH3 domain of the Fc domain. Thus, said modification is particularly in the CH3 domain of the Fc domain.

In a specific aspect, said modification is a so-called "knob-into-hole" modification, comprising a "knob" modification in one of the two subunits of the Fc domain and a "hole" modification in the other one of the two subunits of the Fc domain. Thus, in a particular aspect, the invention relates to a 4-1BBL trimer-containing antigen binding molecule as described herein before which comprises an IgG molecule, wherein the Fc part of the first heavy chain comprises a first dimerization module and the Fc part of the second heavy chain comprises a second dimerization module allowing a heterodimerization of the two heavy chains of the IgG molecule and the first dimerization module comprises knobs and the second dimerization module comprises holes according to the knob into hole technology.

The knob-into-hole technology is described e.g. in US 5,731,168; US 7,695,936; Ridgway et al., Prot Eng 9, 617-621 (1996) and Carter, J Immunol Meth 248, 7-15 (2001). Generally, the method involves introducing a protuberance ("knob") at the interface of a first polypeptide and a corresponding cavity ("hole") in the interface of a second polypeptide, such that the protuberance can be positioned in the cavity so as to promote heterodimer formation and hinder homodimer formation. Protuberances are constructed by replacing small amino acid side chains

from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are created in the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine).

- 5 Accordingly, in a particular aspect, in the CH3 domain of the first subunit of the Fc domain of the 4-1BBL trimer-containing antigen binding molecules of the invention an amino acid residue is replaced with an amino acid residue having a larger side chain volume, thereby generating a protuberance within the CH3 domain of the first subunit which is positionable in a cavity within the CH3 domain of the second subunit, and in the CH3 domain of the second
10 subunit of the Fc domain an amino acid residue is replaced with an amino acid residue having a smaller side chain volume, thereby generating a cavity within the CH3 domain of the second subunit within which the protuberance within the CH3 domain of the first subunit is positionable.

The protuberance and cavity can be made by altering the nucleic acid encoding the polypeptides, e.g. by site-specific mutagenesis, or by peptide synthesis.

- 15 In a specific aspect, in the CH3 domain of the first subunit of the Fc domain the threonine residue at position 366 is replaced with a tryptophan residue (T366W), and in the CH3 domain of the second subunit of the Fc domain the tyrosine residue at position 407 is replaced with a valine residue (Y407V). More particularly, in the second subunit of the Fc domain additionally the threonine residue at position 366 is replaced with a serine residue (T366S) and the leucine
20 residue at position 368 is replaced with an alanine residue (L368A). More particularly, in the first subunit of the Fc domain additionally the serine residue at position 354 is replaced with a cysteine residue (S354C), and in the second subunit of the Fc domain additionally the tyrosine residue at position 349 is replaced by a cysteine residue (Y349C). The introduction of these two cysteine residues results in the formation of a disulfide bridge between the two subunits of the Fc
25 domain. The disulfide bridge further stabilizes the dimer (Carter, J Immunol Methods 248, 7-15 (2001)).

- In an alternative aspect, a modification promoting association of the first and the second subunit of the Fc domain comprises a modification mediating electrostatic steering effects, e.g. as described in PCT publication WO 2009/089004. Generally, this method involves replacement
30 of one or more amino acid residues at the interface of the two Fc domain subunits by charged amino acid residues so that homodimer formation becomes electrostatically unfavorable but heterodimerization electrostatically favorable.

Modifications in the CH1/CL domains

To further improve correct pairing, the 4-1BBL trimer-containing antigen binding molecules can contain different charged amino acid substitutions (so-called “charged residues”). These modifications are introduced in the crossed or non-crossed CH1 and CL domains. In a particular aspect, the invention relates to a 4-1BBL trimer-containing antigen binding molecule, wherein in one of CL domains the amino acid at position 123 (EU numbering) has been replaced by arginine (R) and the amino acid at position 124 (EU numbering) has been substituted by lysine (K) and wherein in one of the CH1 domains the amino acids at position 147 (EU numbering) and at position 213 (EU numbering) have been substituted by glutamic acid (E).

More particularly, the invention relates to a 4-1BBL trimer-containing antigen binding molecule, wherein in the CL domain adjacent to the TNF ligand family member the amino acid at position 123 (EU numbering) has been replaced by arginine (R) and the amino acid at position 124 (EU numbering) has been substituted by lysine (K), and wherein in the CH1 domain adjacent to the TNF ligand family member the amino acids at position 147 (EU numbering) and at position 213 (EU numbering) have been substituted by glutamic acid (E).

Thus, in a particular aspect, provided is a 4-1BBL trimer-containing antigen binding molecule comprising

- (a) an antigen binding domain capable of specific binding to Her2,
- (b) a first polypeptide containing a CL domain comprising the amino acid mutations E123R and Q124K and a second polypeptide containing a CH1 domain comprising the amino acid mutations K147E and K213E, wherein the second polypeptide is linked to the first polypeptide by a disulfide bond between the CH1 and CL domain, and wherein the antigen binding molecule is characterized in that the first polypeptide comprises two ectodomains of 4-1BBL or a fragment thereof that are connected to each other and to the CL domain by a peptide linker and in that the second polypeptide comprises one 4-1BBL or a fragment thereof connected via a peptide linker to the CH1 domain of said polypeptide; and
- (c) an Fc domain composed of a first and a second subunit capable of stable association.

In one aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule, wherein in the CL domain adjacent to the TNF ligand family member the amino acid at position 123 (EU numbering) has been replaced by arginine (R) and the amino acid at position 124 (EU numbering) has been substituted by lysine (K), and wherein in the CH1 domain adjacent to the TNF ligand family member the amino acids at position 147 (EU numbering) and at position 213 (EU numbering) have been substituted by glutamic acid (E). These modifications lead to so-called charged residues with advantageous properties that avoid undesired effects such as for example mispairing.

In particular, the CL domain comprises the amino acid mutations E123R and Q124K and the CH1 domain comprises the amino acid mutations K147E and K213E.

Particular 4-1BBL trimer-containing antigen binding molecules

The invention provides a 4-1BBL trimer-containing antigen binding molecule that
5 comprises an antigen binding domain capable of specific binding to Her2. In a particular aspect, the 4-1BBL trimer-containing antigen binding molecule comprises one moiety capable of specific binding to Her2, meaning the 4-1BBL trimer-containing antigen binding molecule is monovalent. In another aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule comprising two moieties capable of specific binding to Her2, meaning the 4-
10 1BBL trimer-containing antigen binding molecule is bivalent.

In one aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule, wherein the antigen binding domain capable of specific binding to Her2 comprises (a) a VH domain comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:13, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:14, and (iii) CDR-H3
15 comprising the amino acid sequence of SEQ ID NO:15, and a VL domain comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:16, (v) CDR-L2 comprising the amino acid sequence of SEQ ID NO:17, and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:18, or
(b) a VH domain comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID
20 NO:21, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:22, and (iii) CDR-H3 comprising the amino acid sequence of SEQ ID NO:23, and a VL domain comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:24, (v) CDR-L2 comprising the amino acid sequence of SEQ ID NO:25, and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:26, or
25 (c) a VH domain comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:29, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:30, and (iii) CDR-H3 comprising the amino acid sequence of SEQ ID NO:31, and a VL domain comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:32, (v) CDR-L2 comprising the amino acid sequence of SEQ ID NO:33, and (vi) CDR-L3 comprising the amino acid sequence of SEQ
30 ID NO:34.

In one aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule, wherein the antigen binding domain capable of specific binding to Her2 comprises a VH domain comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:13, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:14, and (iii) CDR-H3 comprising
35 the amino acid sequence of SEQ ID NO:15, and a VL domain comprising (iv) CDR-L1

comprising the amino acid sequence of SEQ ID NO:16, (v) CDR-L2 comprising the amino acid sequence of SEQ ID NO:17, and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:18.

In another aspect, provided is a 4-1BBL trimer-containing antigen binding molecule,
5 wherein the antigen binding domain capable of specific binding to Her2 comprises a VH domain comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:21, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:22, and (iii) CDR-H3 comprising the amino acid sequence of SEQ ID NO:23, and a VL domain comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:24, (v) CDR-L2 comprising the amino acid sequence of
10 SEQ ID NO:25, and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:26.

In a further aspect, provided is a 4-1BBL trimer-containing antigen binding molecule,
wherein the antigen binding domain capable of specific binding to Her2 comprises a VH domain comprising (i) CDR-H1 comprising the amino acid sequence of SEQ ID NO:29, (ii) CDR-H2 comprising the amino acid sequence of SEQ ID NO:30, and (iii) CDR-H3 comprising the amino
15 acid sequence of SEQ ID NO:31, and a VL domain comprising (iv) CDR-L1 comprising the amino acid sequence of SEQ ID NO:32, (v) CDR-L2 comprising the amino acid sequence of SEQ ID NO:33, and (vi) CDR-L3 comprising the amino acid sequence of SEQ ID NO:34.

In a further aspect, the antigen binding domain capable of specific binding to Her2
comprises a heavy chain variable region comprising an amino acid sequence that is at least about
20 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:19 and a light chain variable region comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:20.

In another aspect, the antigen binding domain capable of specific binding to Her2
comprises a heavy chain variable region comprising an amino acid sequence that is at least about
25 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:27 and a light chain variable region comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:28.

In a further aspect, the antigen binding domain capable of specific binding to Her2
comprises a heavy chain variable region comprising an amino acid sequence that is at least about
30 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:35 and a light chain variable region comprising an amino acid sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to the amino acid sequence of SEQ ID NO:36.

In a further aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule, wherein the antigen binding domain capable of specific binding to Her2 comprises

- (a) a VH domain comprising an amino acid sequence of SEQ ID NO:19 and a VL domain comprising an amino acid sequence of SEQ ID NO:20, or
- (b) a VH domain comprising an amino acid sequence of SEQ ID NO:27 and a VL domain comprising an amino acid sequence of SEQ ID NO:28, or
- 5 (c) a VH domain comprising an amino acid sequence of SEQ ID NO:35 and a VL domain comprising an amino acid sequence of SEQ ID NO:36.

In one aspect, provided is a 4-1BBL trimer-containing antigen binding molecule as defined herein before, wherein the antigen binding domain capable of specific binding to Her2 comprises a variable heavy chain comprising an amino acid sequence of SEQ ID NO:19 and a variable light chain comprising an amino acid sequence of SEQ ID NO:20 or wherein the antigen binding domain capable of specific binding to Her2 comprises a variable heavy chain comprising an amino acid sequence of SEQ ID NO:35 and a variable light chain comprising an amino acid sequence of SEQ ID NO:36.

In a particular aspect, the antigen binding domain capable of specific binding to Her2 comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:19 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:20. In another particular aspect, the antigen binding domain capable of specific binding to Her2 comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:35 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:36. In a specific aspect, the antigen binding domain capable of specific binding to Her2 comprises a VH domain consisting of amino acid sequence of SEQ ID NO:27 and a VL domain consisting of the amino acid sequence of SEQ ID NO:28.

In a further aspect, the 4-1BBL trimer-containing antigen binding molecule of the invention comprises

25 (i) a first heavy chain comprising the VH domain comprising the amino acid sequence of SEQ ID NO:19 and a first light chain comprising the VL domain comprising the amino acid sequence of SEQ ID NO:20 or

a first heavy chain comprising the VH domain comprising the amino acid sequence of SEQ ID NO:27 and a first light chain comprising the VL domain comprising the amino acid sequence of

30 SEQ ID NO:28, or

a first heavy chain comprising the VH domain comprising the amino acid sequence of SEQ ID NO:35 and a first light chain comprising the VL domain comprising the amino acid sequence of SEQ ID NO:36,

(ii) a second heavy chain comprising the amino acid sequence selected from the group consisting of SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41 and SEQ ID NO:43, and

35

(iii) a second light chain comprising the amino acid sequence selected from the group consisting of SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42 and SEQ ID NO:44.

In a particular aspect, the 4-1BBL trimer-containing antigen binding molecule of the invention comprises

- 5 (a) an antigen binding domain of specific binding to Her2 comprising a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:19 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:20 or a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:27 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:28 or a heavy chain variable region
10 comprising the amino acid sequence of SEQ ID NO:35 and a light chain variable region comprising the amino acid sequence of SEQ ID NO:36, and
(b) a first and a second polypeptide that are linked to each other by a disulfide bond, wherein the antigen binding molecule is characterized in that the first polypeptide comprises the amino acid sequence of SEQ ID NO:10 and the second polypeptide comprises the amino acid
15 sequence of SEQ ID NO:5.

In a particular aspect, provided is a 4-1BBL trimer-containing antigen binding molecule, wherein the antigen binding molecule comprises

- (i) a first heavy chain comprising the VH domain comprising the amino acid sequence of SEQ ID NO:19 and a first light chain comprising the VL domain comprising the amino acid sequence
20 of SEQ ID NO:20 or
a first heavy chain comprising the VH domain comprising the amino acid sequence of SEQ ID NO:27 and a first light chain comprising the VL domain comprising the amino acid sequence of SEQ ID NO:28, or
a first heavy chain comprising the VH domain comprising the amino acid sequence of SEQ ID
25 NO:35 and a first light chain comprising the VL domain comprising the amino acid sequence of SEQ ID NO:36,
(ii) a second heavy chain comprising the amino acid sequence selected from the group consisting of SEQ ID NO:37, SEQ ID NO:39, SEQ ID NO:41 and SEQ ID NO:43, and
(iii) a second light chain comprising the amino acid sequence selected from the group consisting
30 of SEQ ID NO:38, SEQ ID NO:40, SEQ ID NO:42 and SEQ ID NO:44.

In another aspect, the invention provides a 4-1BBL trimer-containing antigen binding molecule, wherein the antigen binding molecule comprises

- (a) a first heavy chain comprising the amino acid sequence of SEQ ID NO:45, a first light chain comprising the amino acid sequence of SEQ ID NO:46, a second heavy chain comprising the
35 amino acid sequence of SEQ ID NO:37 and a second light chain comprising the amino acid sequence of SEQ ID NO:38, or

- (b) a first heavy chain comprising the amino acid sequence of SEQ ID NO:47, a first light chain comprising the amino acid sequence of SEQ ID NO:48, a second heavy chain comprising the amino acid sequence of SEQ ID NO:37 and a second light chain comprising the amino acid sequence of SEQ ID NO:38, or
- 5 (c) a first heavy chain comprising the amino acid sequence of SEQ ID NO:49, a first light chain comprising the amino acid sequence of SEQ ID NO:50, a second heavy chain comprising the amino acid sequence of SEQ ID NO:37 and a second light chain comprising the amino acid sequence of SEQ ID NO:38.

Polynucleotides

- 10 The invention further provides isolated nucleic acid molecules encoding a 4-1BBL trimer-containing antigen binding molecule as described herein or a fragment thereof.

- The isolated polynucleotides encoding 4-1BBL trimer-containing antigen binding molecules of the invention may be expressed as a single polynucleotide that encodes the entire antigen binding molecule or as multiple (e.g., two or more) polynucleotides that are co-
- 15 expressed. Polypeptides encoded by polynucleotides that are co-expressed may associate through, e.g., disulfide bonds or other means to form a functional antigen binding molecule. For example, the light chain portion of an immunoglobulin may be encoded by a separate polynucleotide from the heavy chain portion of the immunoglobulin. When co-expressed, the heavy chain polypeptides will associate with the light chain polypeptides to form the
- 20 immunoglobulin.

In some aspects, the isolated nucleic acid molecule encodes the entire 4-1BBL trimer-containing antigen binding molecule according to the invention as described herein. In particular, the isolated polynucleotide encodes a polypeptide comprised in the 4-1BBL trimer-containing antigen binding molecule according to the invention as described herein.

- 25 In one aspect, the present invention is directed to isolated nucleic acid molecules encoding a 4-1BBL trimer-containing antigen binding molecule, wherein the nucleic acid molecule comprises (a) a sequence that encodes an antigen binding domain capable of specific binding to a Her2, (b) a sequence that encodes a polypeptide comprising two ectodomains of 4-1BBL or a fragment thereof that are connected to each other by a peptide linker and (c) a sequence that
- 30 encodes a polypeptide comprising one ectodomain of said 4-1BBL or a fragment thereof.

In another aspect, provided is an isolated polynucleotide encoding a 4-1BB ligand trimer-containing antigen binding molecule, wherein the polynucleotide comprises (a) a sequence that encodes a moiety capable of specific binding to Her2, (b) a sequence that encodes a polypeptide comprising two ectodomains of 4-1BBL or two fragments thereof that are connected to each

other by a peptide linker and (c) a sequence that encodes a polypeptide comprising one ectodomain of 4-1BBL or a fragment thereof.

In certain aspects, the polynucleotide or nucleic acid is DNA. In other embodiments, a polynucleotide of the present invention is RNA, for example, in the form of messenger RNA (mRNA). RNA of the present invention may be single stranded or double stranded.

Recombinant Methods

4-1BBL trimer-containing antigen binding molecules of the invention may be obtained, for example, by solid-state peptide synthesis (e.g. Merrifield solid phase synthesis) or recombinant production. For recombinant production one or more polynucleotide encoding the 4-1BBL trimer-containing antigen binding molecule or polypeptide fragments thereof, e.g., as described above, is isolated and inserted into one or more vectors for further cloning and/or expression in a host cell. Such polynucleotide may be readily isolated and sequenced using conventional procedures. In one aspect of the invention, a vector, preferably an expression vector, comprising one or more of the polynucleotides of the invention is provided. Methods which are well known to those skilled in the art can be used to construct expression vectors containing the coding sequence of the 4-1BBL trimer-containing antigen binding molecule (fragment) along with appropriate transcriptional/translational control signals. These methods include in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Maniatis et al., MOLECULAR CLONING: A LABORATORY MANUAL, Cold Spring Harbor Laboratory, N.Y. (1989); and Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates and Wiley Interscience, N.Y. (1989). The expression vector can be part of a plasmid, virus, or may be a nucleic acid fragment. The expression vector includes an expression cassette into which the polynucleotide encoding the 4-1BBL trimer-containing antigen binding molecule or polypeptide fragments thereof (i.e. the coding region) is cloned in operable association with a promoter and/or other transcription or translation control elements. As used herein, a "coding region" is a portion of nucleic acid which consists of codons translated into amino acids. Although a "stop codon" (TAG, TGA, or TAA) is not translated into an amino acid, it may be considered to be part of a coding region, if present, but any flanking sequences, for example promoters, ribosome binding sites, transcriptional terminators, introns, 5' and 3' untranslated regions, and the like, are not part of a coding region. Two or more coding regions can be present in a single polynucleotide construct, e.g. on a single vector, or in separate polynucleotide constructs, e.g. on separate (different) vectors. Furthermore, any vector may contain a single coding region, or may comprise two or more coding regions, e.g. a vector of the present invention may encode one or more polypeptides, which are post- or co-translationally separated into the final proteins via proteolytic cleavage. In addition, a vector, polynucleotide, or nucleic

acid of the invention may encode heterologous coding regions, either fused or unfused to a polynucleotide encoding the 4-1BBL trimer-containing antigen binding molecule of the invention or polypeptide fragments thereof, or variants or derivatives thereof. Heterologous coding regions include without limitation specialized elements or motifs, such as a secretory
5 signal peptide or a heterologous functional domain. An operable association is when a coding region for a gene product, e.g. a polypeptide, is associated with one or more regulatory sequences in such a way as to place expression of the gene product under the influence or control of the regulatory sequence(s). Two DNA fragments (such as a polypeptide coding region and a promoter associated therewith) are "operably associated" if induction of promoter function
10 results in the transcription of mRNA encoding the desired gene product and if the nature of the linkage between the two DNA fragments does not interfere with the ability of the expression regulatory sequences to direct the expression of the gene product or interfere with the ability of the DNA template to be transcribed. Thus, a promoter region would be operably associated with a nucleic acid encoding a polypeptide if the promoter was capable of effecting transcription of
15 that nucleic acid. The promoter may be a cell-specific promoter that directs substantial transcription of the DNA only in predetermined cells. Other transcription control elements, besides a promoter, for example enhancers, operators, repressors, and transcription termination signals, can be operably associated with the polynucleotide to direct cell-specific transcription.

Suitable promoters and other transcription control regions are disclosed herein. A variety
20 of transcription control regions are known to those skilled in the art. These include, without limitation, transcription control regions, which function in vertebrate cells, such as, but not limited to, promoter and enhancer segments from cytomegaloviruses (e.g. the immediate early promoter, in conjunction with intron-A), simian virus 40 (e.g. the early promoter), and retroviruses (such as, e.g. Rous sarcoma virus). Other transcription control regions include those
25 derived from vertebrate genes such as actin, heat shock protein, bovine growth hormone and rabbit α -globin, as well as other sequences capable of controlling gene expression in eukaryotic cells. Additional suitable transcription control regions include tissue-specific promoters and enhancers as well as inducible promoters (e.g. promoters inducible tetracyclins). Similarly, a variety of translation control elements are known to those of ordinary skill in the art. These
30 include, but are not limited to ribosome binding sites, translation initiation and termination codons, and elements derived from viral systems (particularly an internal ribosome entry site, or IRES, also referred to as a CITE sequence). The expression cassette may also include other features such as an origin of replication, and/or chromosome integration elements such as retroviral long terminal repeats (LTRs), or adeno-associated viral (AAV) inverted terminal
35 repeats (ITRs).

Polynucleotide and nucleic acid coding regions of the present invention may be associated with additional coding regions which encode secretory or signal peptides, which direct the

secretion of a polypeptide encoded by a polynucleotide of the present invention. For example, if secretion of the 4-1BBL trimer-containing antigen binding molecule or polypeptide fragments thereof is desired, DNA encoding a signal sequence may be placed upstream of the nucleic acid encoding a 4-1BBL trimer-containing antigen binding molecule of the invention or polypeptide fragments thereof. According to the signal hypothesis, proteins secreted by mammalian cells have a signal peptide or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Those of ordinary skill in the art are aware that polypeptides secreted by vertebrate cells generally have a signal peptide fused to the N-terminus of the polypeptide, which is cleaved from the translated polypeptide to produce a secreted or "mature" form of the polypeptide. In certain embodiments, the native signal peptide, e.g. an immunoglobulin heavy chain or light chain signal peptide is used, or a functional derivative of that sequence that retains the ability to direct the secretion of the polypeptide that is operably associated with it. Alternatively, a heterologous mammalian signal peptide, or a functional derivative thereof, may be used. For example, the wild-type leader sequence may be substituted with the leader sequence of human tissue plasminogen activator (TPA) or mouse β -glucuronidase.

DNA encoding a short protein sequence that could be used to facilitate later purification (e.g. a histidine tag) or assist in labeling the fusion protein may be included within or at the ends of the polynucleotide encoding a 4-1BBL trimer-containing antigen binding molecule of the invention or polypeptide fragments thereof.

In a further aspect of the invention, a host cell comprising one or more polynucleotides of the invention is provided. In certain embodiments a host cell comprising one or more vectors of the invention is provided. The polynucleotides and vectors may incorporate any of the features, singly or in combination, described herein in relation to polynucleotides and vectors, respectively. In one aspect, a host cell comprises (e.g. has been transformed or transfected with) a vector comprising a polynucleotide that encodes (part of) a 4-1BBL trimer-containing antigen binding molecule of the invention of the invention. As used herein, the term "host cell" refers to any kind of cellular system which can be engineered to generate the fusion proteins of the invention or fragments thereof. Host cells suitable for replicating and for supporting expression of antigen binding molecules are well known in the art. Such cells may be transfected or transduced as appropriate with the particular expression vector and large quantities of vector containing cells can be grown for seeding large scale fermenters to obtain sufficient quantities of the antigen binding molecule for clinical applications. Suitable host cells include prokaryotic microorganisms, such as *E. coli*, or various eukaryotic cells, such as Chinese hamster ovary cells (CHO), insect cells, or the like. For example, polypeptides may be produced in bacteria in particular when glycosylation is not needed. After expression, the polypeptide may be isolated from the bacterial cell paste in a soluble fraction and can be further purified. In addition to

prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been “humanized”, resulting in the production of a polypeptide with a partially or fully human glycosylation pattern. See Gerngross, *Nat Biotech* 22, 1409-1414 (2004), and Li et al., *Nat Biotech* 24, 210-215 (2006).

Suitable host cells for the expression of (glycosylated) polypeptides are also derived from multicellular organisms (invertebrates and vertebrates). Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains have been identified which may be used in conjunction with insect cells, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures can also be utilized as hosts. See e.g. US Patent Nos. 5,959,177, 6,040,498, 6,420,548, 7,125,978, and 6,417,429 (describing PLANTIBODIES™ technology for producing antibodies in transgenic plants). Vertebrate cells may also be used as hosts. For example, mammalian cell lines that are adapted to grow in suspension may be useful. Other examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7); human embryonic kidney line (293 or 293T cells as described, e.g., in Graham et al., *J Gen Virol* 36, 59 (1977)), baby hamster kidney cells (BHK), mouse sertoli cells (TM4 cells as described, e.g., in Mather, *Biol Reprod* 23, 243-251 (1980)), monkey kidney cells (CV1), African green monkey kidney cells (VERO-76), human cervical carcinoma cells (HELA), canine kidney cells (MDCK), buffalo rat liver cells (BRL 3A), human lung cells (W138), human liver cells (Hep G2), mouse mammary tumor cells (MMT 060562), TRI cells (as described, e.g., in Mather et al., *Annals N.Y. Acad Sci* 383, 44-68 (1982)), MRC 5 cells, and FS4 cells. Other useful mammalian host cell lines include Chinese hamster ovary (CHO) cells, including dhfr- CHO cells (Urlaub et al., *Proc Natl Acad Sci USA* 77, 4216 (1980)); and myeloma cell lines such as YO, NS0, P3X63 and Sp2/0. For a review of certain mammalian host cell lines suitable for protein production, see, e.g., Yazaki and Wu, *Methods in Molecular Biology*, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ), pp. 255-268 (2003). Host cells include cultured cells, e.g., mammalian cultured cells, yeast cells, insect cells, bacterial cells and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue. In one embodiment, the host cell is a eukaryotic cell, preferably a mammalian cell, such as a Chinese Hamster Ovary (CHO) cell, a human embryonic kidney (HEK) cell or a lymphoid cell (e.g., YO, NS0, Sp20 cell). Standard technologies are known in the art to express foreign genes in these systems. Cells expressing a polypeptide comprising either the heavy or the light chain of an immunoglobulin, may be engineered so as to also express the other of the immunoglobulin chains such that the expressed product is an immunoglobulin that has both a heavy and a light chain.

In one aspect, a method of producing a 4-1BBL trimer-containing antigen binding molecule of the invention or polypeptide fragments thereof is provided, wherein the method

comprises culturing a host cell comprising polynucleotides encoding the 4-1BBL trimer-containing antigen binding molecule of the invention or polypeptide fragments thereof, as provided herein, under conditions suitable for expression of the 4-1BBL trimer-containing antigen binding molecule of the invention or polypeptide fragments thereof, and recovering the
5 4-1BBL trimer-containing antigen binding molecule of the invention or polypeptide fragments thereof from the host cell (or host cell culture medium).

In the 4-1BBL trimer-containing antigen binding molecule of the invention, the components (at least one moiety capable of specific binding to a target cell antigen, one polypeptide comprising two ectodomains of a TNF ligand family member or fragments thereof
10 and a polypeptide comprising one ectodomain of said 4-1BBL family member or a fragment thereof) are not genetically fused to each other. The polypeptides are designed such that its components (two ectodomains of a TNF ligand family member or fragments thereof and other components such as CH or CL) are fused to each other directly or through a linker sequence. The composition and length of the linker may be determined in accordance with methods well known
15 in the art and may be tested for efficacy. Examples of linker sequences between different components of the antigen binding molecules of the invention are found in the sequences provided herein. Additional sequences may also be included to incorporate a cleavage site to separate the individual components of the fusion protein if desired, for example an endopeptidase recognition sequence.

In certain embodiments the moieties capable of specific binding to a target cell antigen (e.g. Fab fragments) forming part of the antigen binding molecule comprise at least an immunoglobulin variable region capable of binding to an antigen. Variable regions can form part of and be derived from naturally or non-naturally occurring antibodies and fragments thereof. Methods to produce polyclonal antibodies and monoclonal antibodies are well known in the art
20 (see e.g. Harlow and Lane, "Antibodies, a laboratory manual", Cold Spring Harbor Laboratory, 1988). Non-naturally occurring antibodies can be constructed using solid phase-peptide synthesis, can be produced recombinantly (e.g. as described in U.S. patent No. 4,186,567) or can be obtained, for example, by screening combinatorial libraries comprising variable heavy chains and variable light chains (see e.g. U.S. Patent. No. 5,969,108 to McCafferty).

Any animal species of immunoglobulin can be used in the invention. Non-limiting immunoglobulins useful in the present invention can be of murine, primate, or human origin. If the fusion protein is intended for human use, a chimeric form of immunoglobulin may be used wherein the constant regions of the immunoglobulin are from a human. A humanized or fully human form of the immunoglobulin can also be prepared in accordance with methods well
30 known in the art (see e. g. U.S. Patent No. 5,565,332 to Winter). Humanization may be achieved by various methods including, but not limited to (a) grafting the non-human (e.g., donor

antibody) CDRs onto human (e.g. recipient antibody) framework and constant regions with or without retention of critical framework residues (e.g. those that are important for retaining good antigen binding affinity or antibody functions), (b) grafting only the non-human specificity-determining regions (SDRs or a-CDRs; the residues critical for the antibody-antigen interaction) onto human framework and constant regions, or (c) transplanting the entire non-human variable domains, but "cloaking" them with a human-like section by replacement of surface residues. Humanized antibodies and methods of making them are reviewed, e.g., in Almagro and Fransson, *Front Biosci* 13, 1619-1633 (2008), and are further described, e.g., in Riechmann et al., *Nature* 332, 323-329 (1988); Queen et al., *Proc Natl Acad Sci USA* 86, 10029-10033 (1989); US Patent Nos. 5,821,337, 7,527,791, 6,982,321, and 7,087,409; Jones et al., *Nature* 321, 522-525 (1986); Morrison et al., *Proc Natl Acad Sci* 81, 6851-6855 (1984); Morrison and Oi, *Adv Immunol* 44, 65-92 (1988); Verhoeven et al., *Science* 239, 1534-1536 (1988); Padlan, *Molec Immun* 31(3), 169-217 (1994); Kashmiri et al., *Methods* 36, 25-34 (2005) (describing SDR (a-CDR) grafting); Padlan, *Mol Immunol* 28, 489-498 (1991) (describing "resurfacing"); Dall'Acqua et al., *Methods* 36, 43-60 (2005) (describing "FR shuffling"); and Osbourn et al., *Methods* 36, 61-68 (2005) and Klimka et al., *Br J Cancer* 83, 252-260 (2000) (describing the "guided selection" approach to FR shuffling). Particular immunoglobulins according to the invention are human immunoglobulins. Human antibodies and human variable regions can be produced using various techniques known in the art. Human antibodies are described generally in van Dijk and van de Winkel, *Curr Opin Pharmacol* 5, 368-74 (2001) and Lonberg, *Curr Opin Immunol* 20, 450-459 (2008). Human variable regions can form part of and be derived from human monoclonal antibodies made by the hybridoma method (see e.g. *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)). Human antibodies and human variable regions may also be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge (see e.g. Lonberg, *Nat Biotech* 23, 1117-1125 (2005). Human antibodies and human variable regions may also be generated by isolating Fv clone variable region sequences selected from human-derived phage display libraries (see e.g., Hoogenboom et al. in *Methods in Molecular Biology* 178, 1-37 (O'Brien et al., ed., Human Press, Totowa, NJ, 2001); and McCafferty et al., *Nature* 348, 552-554; Clackson et al., *Nature* 352, 624-628 (1991)). Phage typically display antibody fragments, either as single-chain Fv (scFv) fragments or as Fab fragments.

In certain aspects, the moieties capable of specific binding to a target cell antigen (e.g. Fab fragments) comprised in the antigen binding molecules of the present invention are engineered to have enhanced binding affinity according to, for example, the methods disclosed in PCT publication WO 2012/020006 (see Examples relating to affinity maturation) or U.S. Pat. Appl. Publ. No. 2004/0132066. The ability of the antigen binding molecules of the invention to bind to

a specific antigenic determinant can be measured either through an enzyme-linked immunosorbent assay (ELISA) or other techniques familiar to one of skill in the art, e.g. surface plasmon resonance technique (Liljeblad, et al., Glyco J 17, 323-329 (2000)), and traditional binding assays (Heeley, Endocr Res 28, 217-229 (2002)). Competition assays may be used to
5 identify an antigen binding molecule that competes with a reference antibody for binding to a particular antigen. In certain embodiments, such a competing antigen binding molecule binds to the same epitope (e.g. a linear or a conformational epitope) that is bound by the reference antigen binding molecule. Detailed exemplary methods for mapping an epitope to which an antigen binding molecule binds are provided in Morris (1996) "Epitope Mapping Protocols", in Methods
10 in Molecular Biology vol. 66 (Humana Press, Totowa, NJ). In an exemplary competition assay, immobilized antigen is incubated in a solution comprising a first labeled antigen binding molecule that binds to the antigen and a second unlabeled antigen binding molecule that is being tested for its ability to compete with the first antigen binding molecule for binding to the antigen. The second antigen binding molecule may be present in a hybridoma supernatant. As a control,
15 immobilized antigen is incubated in a solution comprising the first labeled antigen binding molecule but not the second unlabeled antigen binding molecule. After incubation under conditions permissive for binding of the first antibody to the antigen, excess unbound antibody is removed, and the amount of label associated with immobilized antigen is measured. If the amount of label associated with immobilized antigen is substantially reduced in the test sample
20 relative to the control sample, then that indicates that the second antigen binding molecule is competing with the first antigen binding molecule for binding to the antigen. See Harlow and Lane (1988) Antibodies: A Laboratory Manual ch.14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

4-1BBL trimer-containing antigen binding molecules of the invention prepared as
25 described herein may be purified by art-known techniques such as high performance liquid chromatography, ion exchange chromatography, gel electrophoresis, affinity chromatography, size exclusion chromatography, and the like. The actual conditions used to purify a particular protein will depend, in part, on factors such as net charge, hydrophobicity, hydrophilicity etc., and will be apparent to those having skill in the art. For affinity chromatography purification an
30 antibody, ligand, receptor or antigen can be used to which the 4-1BBL trimer-containing antigen binding molecule binds. For example, for affinity chromatography purification of fusion proteins of the invention, a matrix with protein A or protein G may be used. Sequential Protein A or G affinity chromatography and size exclusion chromatography can be used to isolate an antigen binding molecule essentially as described in the Examples. The purity of the 4-1BBL trimer-
35 containing antigen binding molecule or fragments thereof can be determined by any of a variety of well-known analytical methods including gel electrophoresis, high pressure liquid chromatography, and the like. For example, the 4-1BBL trimer-containing antigen binding

molecules expressed as described in the Examples were shown to be intact and properly assembled as demonstrated by reducing and non-reducing SDS-PAGE.

Assays

The antigen binding molecules provided herein may be identified, screened for, or
5 characterized for their physical/chemical properties and/or biological activities by various assays known in the art. Biological activity may include, e.g., the ability to enhance the activation and/or proliferation of different immune cells especially T-cells. E.g. they enhance secretion of immunomodulating cytokines. Other immunomodulating cytokines which are or can be enhanced are e.g IL2, Granzyme B etc. Biological activity may also include, cynomolgus
10 binding crossreactivity, as well as binding to different cell types. Antigen binding molecules having such biological activity in vivo and/or in vitro are also provided.

1. Affinity assays

The affinity of the 4-1BBL trimer-containing antigen binding molecule provided herein for 4-1BB (CD137) can be determined in accordance with the methods set forth in the Examples by
15 surface plasmon resonance (SPR), using standard instrumentation such as a BIAcore instrument (GE Healthcare), and receptors or target proteins such as may be obtained by recombinant expression. The affinity of the 4-1BBL trimer-containing antigen binding molecule for HER2 can also be determined by surface plasmon resonance (SPR), using standard instrumentation such as a BIAcore instrument (GE Healthcare), and receptors or target proteins such as may be
20 obtained by recombinant expression. A specific illustrative and exemplary embodiment for measuring binding affinity is described in Example 4. According to one aspect, K_D is measured by surface plasmon resonance using a BIACORE® T100 machine (GE Healthcare) at 25 °C.

2. Binding assays and other assays

Binding of the 4-1BBL trimer-containing antigen binding molecule provided herein to the
25 corresponding receptor expressing cells may be evaluated using cell lines expressing the particular receptor or target antigen, for example by flow cytometry (FACS). In one aspect, fresh peripheral blood mononuclear cells (PBMCs) expressing 4-1BB can be used in the binding assay. These cells are used directly after isolation (naïve PMBCs) or after stimulation (activated PMBCs). In another aspect, activated mouse splenocytes (expressing 4-1BB) can be used to
30 demonstrate the binding of the 4-1BBL trimer-containing antigen binding molecule of the invention to 4-1BB expressing cells.

In a further aspect, cell lines expressing Her2 were used to demonstrate the binding of the antigen binding molecules to this target cell antigen.

In another aspect, competition assays may be used to identify an antigen binding molecule that competes with a specific antibody or antigen binding molecule for binding to Her2 or 4-1BB, respectively. In certain embodiments, such a competing antigen binding molecule binds to the same epitope (e.g., a linear or a conformational epitope) that is bound by a specific anti-Her2 antibody or a specific 4-1BB antibody. Detailed exemplary methods for mapping an epitope to which an antibody binds are provided in Morris (1996) "Epitope Mapping Protocols," in *Methods in Molecular Biology* vol. 66 (Humana Press, Totowa, NJ).

3. Activity assays

In one aspect, assays are provided for identifying 4-1BBL trimer-containing antigen binding molecules that bind to Her2 and to 4-1BB having biological activity. Biological activity may include, e.g., agonistic signalling through 4-1BB on cancer cells expressing Her2. 4-1BBL trimer-containing antigen binding molecules identified by the assays as having such biological activity in vitro are also provided.

In certain aspects, a 4-1BBL trimer-containing antigen binding molecule of the invention is tested for such biological activity. Assays for detecting the biological activity of the molecules of the invention are those described in Example 3. Furthermore, assays for detecting cell lysis (e.g. by measurement of LDH release), induced apoptosis kinetics (e.g. by measurement of Caspase 3/7 activity) or apoptosis (e.g. using the TUNEL assay) are well known in the art. In addition, the biological activity of such complexes can be assessed by evaluating their effects on survival, proliferation and lymphokine secretion of various lymphocyte subsets such as NK cells, NKT-cells or $\gamma\delta$ T-cells or assessing their capacity to modulate phenotype and function of antigen presenting cells such as dendritic cells, monocytes/macrophages or B-cells.

Pharmaceutical Compositions, Formulations and Routes of Administration

In a further aspect, the invention provides pharmaceutical compositions comprising any of the 4-1BBL trimer-containing antigen binding molecules provided herein, e.g., for use in any of the below therapeutic methods. In one embodiment, a pharmaceutical composition comprises any of the 4-1BBL trimer-containing antigen binding molecules provided herein and at least one pharmaceutically acceptable excipient. In another embodiment, a pharmaceutical composition comprises any of the 4-1BBL trimer-containing antigen binding molecules provided herein and at least one additional therapeutic agent, e.g., as described below.

Pharmaceutical compositions of the present invention comprise a therapeutically effective amount of one or more 4-1BBL trimer-containing antigen *binding* molecules dissolved or dispersed in a pharmaceutically acceptable excipient. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that are generally

non-toxic to recipients at the dosages and concentrations employed, i.e. do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of a pharmaceutical composition that contains at least one 4-1BBL trimer-containing antigen binding molecule and optionally an additional
5 active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. In particular, the compositions are lyophilized formulations or aqueous solutions. As used herein, "pharmaceutically acceptable excipient" includes any and all solvents, buffers, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g.
10 antibacterial agents, antifungal agents), isotonic agents, salts, stabilizers and combinations thereof, as would be known to one of ordinary skill in the art.

Parenteral compositions include those designed for administration by injection, e.g. subcutaneous, intradermal, intralesional, intravenous, intraarterial intramuscular, intrathecal or intraperitoneal injection. For injection, the 4-1BBL trimer-containing antigen binding molecules
15 of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the fusion proteins may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use. Sterile injectable solutions are prepared by
20 incorporating the fusion proteins of the invention in the required amount in the appropriate solvent with various of the other ingredients enumerated below, as required. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of
25 sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The
30 composition must be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein. Suitable pharmaceutically acceptable excipients include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including
35 ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol;

cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including
5 glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Aqueous injection suspensions may contain compounds which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, dextran, or the like. Optionally, the suspension may
10 also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleates or triglycerides, or liposomes.

15 Active ingredients may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in Remington's
20 Pharmaceutical Sciences (18th Ed. Mack Printing Company, 1990). Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide, which matrices are in the form of shaped articles, e.g. films, or microcapsules. In particular embodiments, prolonged absorption of an injectable composition can be brought about by the
25 use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin or combinations thereof.

Exemplary pharmaceutically acceptable excipients herein further include interstitial drug dispersion agents such as soluble neutral-active hyaluronidase glycoproteins (sHASEGP), for example, human soluble PH-20 hyaluronidase glycoproteins, such as rHuPH20 (HYLENEX®,
30 Baxter International, Inc.). Certain exemplary sHASEGPs and methods of use, including rHuPH20, are described in US Patent Publication Nos. 2005/0260186 and 2006/0104968. In one aspect, a sHASEGP is combined with one or more additional glycosaminoglycanases such as chondroitinases.

Exemplary lyophilized antibody formulations are described in US Patent No. 6,267,958.
35 Aqueous antibody formulations include those described in US Patent No. 6,171,586 and WO2006/044908, the latter formulations including a histidine-acetate buffer.

In addition to the compositions described previously, the fusion proteins may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the fusion proteins may be formulated with suitable polymeric or
5 hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Pharmaceutical compositions comprising the fusion proteins of the invention may be manufactured by means of conventional mixing, dissolving, emulsifying, encapsulating, entrapping or lyophilizing processes. Pharmaceutical compositions may be formulated in
10 conventional manner using one or more physiologically acceptable carriers, diluents, excipients or auxiliaries which facilitate processing of the proteins into preparations that can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

The 4-1BBL trimer-containing antigen binding molecules may be formulated into a composition in a free acid or base, neutral or salt form. Pharmaceutically acceptable salts are
15 salts that substantially retain the biological activity of the free acid or base. These include the acid addition salts, e.g. those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium,
20 potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Pharmaceutical salts tend to be more soluble in aqueous and other protic solvents than are the corresponding free base forms.

The composition herein may also contain more than one active ingredients as necessary for the particular indication being treated, preferably those with complementary activities that do not
25 adversely affect each other. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended.

In one aspect, the pharmaceutical compositions may comprise any of the 4-1BBL trimer-containing antigen binding molecules provided herein and at least one additional therapeutic agent. In one aspect, the pharmaceutical compositions may comprise any of the 4-1BBL trimer-
30 containing antigen binding molecules provided herein and a T-cell activating anti-CD3 bispecific antibody, in particular an anti-Her2/anti-CD3 antibody.

In one aspect, the anti-Her2/anti-CD3 antibody comprises a first antigen binding domain that binds to CD3, and a second antigen binding domain that binds to Her2. In a particular aspect the second binding domain binding to Her2 binds to a different epitope on Her2 than the 4-1BBL
35 trimer-containing antigen binding molecule.

In one aspect, the anti-Her2/anti-CD3 bispecific antibody as used herein comprises a first antigen binding domain comprising a heavy chain variable region (V_HCD3) comprising CDR-H1 sequence of SEQ ID NO:91, CDR-H2 sequence of SEQ ID NO:92, and CDR-H3 sequence of SEQ ID NO:93; and/or a light chain variable region (V_LCD3) comprising CDR-L1 sequence of SEQ ID NO:94, CDR-L2 sequence of SEQ ID NO:95, and CDR-L3 sequence of SEQ ID NO:96. More particularly, the anti-Her2/anti-CD3 bispecific antibody comprises a first antigen binding domain comprising a heavy chain variable region (V_HCD3) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:97 and/or a light chain variable region (V_LCD3) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:98. In a further aspect, the anti-Her2/anti-CD3 bispecific antibody comprises a heavy chain variable region (V_HCD3) comprising the amino acid sequence of SEQ ID NO:97 and/or a light chain variable region (V_LCD3) comprising the amino acid sequence of SEQ ID NO:98.

In one aspect, the anti-Her2/anti-CD3 bispecific antibody comprises a second antigen binding domain that binds to the same epitope as the antibody 4D5 (humanized version thereof known as trastuzumab). In another aspect, the anti-Her2/anti-CD3 bispecific antibody comprises a second antigen binding domain that binds to the same epitope as the antibody 2C4 (humanized version thereof known as pertuzumab). In yet another aspect, anti-Her2/anti-CD3 bispecific antibody comprises a second antigen binding domain that binds to the same epitope as the antibody 7C2 (U.S. Patent No. 9,518,118).

In another aspect, the anti-Her2/anti-CD3 bispecific antibody comprises a second antigen binding domain comprising

- (a) a heavy chain variable region (V_HHer2) comprising CDR-H1 sequence of SEQ ID NO:21, CDR-H2 sequence of SEQ ID NO:22, and CDR-H3 sequence of SEQ ID NO:23, and/or a light chain variable region (V_LHer2) comprising CDR-L1 sequence of SEQ ID NO:24, CDR-L2 sequence of SEQ ID NO:25, and CDR-L3 sequence of SEQ ID NO:26, or
- (b) a heavy chain variable region (V_HHer2) comprising CDR-H1 sequence of SEQ ID NO:13, CDR-H2 sequence of SEQ ID NO:14, and CDR-H3 sequence of SEQ ID NO:15, and/or a light chain variable region (V_LHer2) comprising CDR-L1 sequence of SEQ ID NO:16, CDR-L2 sequence of SEQ ID NO:17, and CDR-L3 sequence of SEQ ID NO:18, or
- (c) a heavy chain variable region (V_HHer2) comprising CDR-H1 sequence of SEQ ID NO:99, CDR-H2 sequence of SEQ ID NO:100, and CDR-H3 sequence of SEQ ID NO:101, and/or a light chain variable region (V_LHer2) comprising CDR-L1 sequence of SEQ ID NO:102, CDR-L2 sequence of SEQ ID NO:103, and CDR-L3 sequence of SEQ ID NO:104.

In one aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) that is at least 90%, 95%, 96%, 97%,

98%, or 99% identical to the amino acid sequence of SEQ ID NO:27 and/or a light chain variable region (V_LHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:28. In a further aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) comprising the amino acid sequence of SEQ ID NO:27 and/or a light chain variable region (V_LHer2) comprising the amino acid sequence of SEQ ID NO:28. In another aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:19 and/or a light chain variable region (V_LHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:20. In a further aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) comprising the amino acid sequence of SEQ ID NO:19 and/or a light chain variable region (V_LCEA) comprising the amino acid sequence of SEQ ID NO:20. In another aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:105 and/or a light chain variable region (V_LHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:106. In a further aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) comprising the amino acid sequence of SEQ ID NO:105 and/or a light chain variable region (V_LCEA) comprising the amino acid sequence of SEQ ID NO:106.

In one aspect, the anti-Her2/anti-CD3 bispecific antibody comprises a first antigen binding domain comprising a heavy chain variable region (V_HCD3) comprising the amino acid sequence of SEQ ID NO:97 and/or a light chain variable region (V_LCD3) comprising the amino acid sequence of SEQ ID NO:98 and a second antigen binding domain comprising a heavy chain variable region (V_HHer2) comprising the amino acid sequence of SEQ ID NO:27 and/or a light chain variable region (V_LHer2) comprising the amino acid sequence of SEQ ID NO:28.

The formulations to be used for in vivo administration are generally sterile. Sterility may be readily accomplished, e.g., by filtration through sterile filtration membranes.

30 **Therapeutic methods and compositions**

Any of the 4-1BBL trimer-containing antigen binding molecules provided herein may be used in therapeutic methods.

For use in therapeutic methods, 4-1BBL trimer-containing antigen binding molecules of the invention can be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being

treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

In one aspect, 4-1BBL trimer-containing antigen binding molecules of the invention for
5 use as a medicament are provided. In further aspects, 4-1BBL trimer-containing antigen binding
molecules of the invention for use in treating a disease, in particular for use in the treatment of
cancer, are provided. In certain aspects, 4-1BBL trimer-containing antigen binding molecules of
the invention for use in a method of treatment are provided. In one aspect, the invention provides
a 4-1BBL trimer-containing antigen binding molecule as described herein for use in the
10 treatment of a disease in an individual in need thereof. In certain aspects, the invention provides
a 4-1BBL trimer-containing antigen binding molecule for use in a method of treating an
individual having a disease comprising administering to the individual a therapeutically effective
amount of the fusion protein. In certain aspects, the disease to be treated is Her2-positive cancer.
Examples of Her2-positive cancers include breast cancer, ovarian cancer, gastric cancer, bladder
15 cancer, salivary gland, endometrial cancer, pancreatic cancer and non-small-cell lung cancer
(NSCLC). In one aspect, the Her2-positive cancer is Her2+ positive breast cancer, for instance
early or locally advanced Her2+ positive breast cancer. Thus, a 4-1BBL trimer-containing
antigen binding molecule as described herein for use in the treatment of these cancers is
provided. The subject, patient, or "individual" in need of treatment is typically a mammal, more
20 specifically a human.

In another aspect, provided is a 4-1BBL trimer-containing antigen binding molecule as
described herein for use in the treatment of infectious diseases, in particular for the treatment of
viral infections. In a further aspect, provided is a 4-1BBL trimer-containing antigen binding
molecule as described herein for use in the treatment of autoimmune diseases such as for
25 example Lupus disease.

In a further aspect, the invention relates to the use of a 4-1BBL trimer-containing antigen
binding molecule in the manufacture or preparation of a medicament for the treatment of a
disease in an individual in need thereof. In one aspect, the medicament is for use in a method of
treating a disease comprising administering to an individual having the disease a therapeutically
30 effective amount of the medicament. In certain embodiments the disease to be treated is a
proliferative disorder, particularly cancer. Thus, in one aspect, the invention relates to the use of
a 4-1BBL trimer-containing antigen binding molecule of the invention in the manufacture or
preparation of a medicament for the treatment of cancer, in particular Her2-positive cancers.
Examples of Her2-positive cancers include breast cancer, ovarian cancer, gastric cancer, bladder
35 cancer, salivary gland, endometrial cancer, pancreatic cancer and non-small-cell lung cancer
(NSCLC). In certain aspect, cancers to be treated are Her2-positive breast cancer, in particular

Her2-positive metastatic breast cancer. A skilled artisan may recognize that in some cases the 4-1BBL trimer-containing antigen binding molecule may not provide a cure but may only provide partial benefit. In some aspects, a physiological change having some benefit is also considered therapeutically beneficial. Thus, in some aspects, an amount of 4-1BBL trimer-containing
5 antigen binding molecule that provides a physiological change is considered an "effective amount" or a "therapeutically effective amount".

In a further aspect, the invention provides a method for treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of a 4-1BBL trimer-containing antigen binding molecule of the invention. In one aspect a composition is
10 administered to said individual, comprising a fusion protein of the invention in a pharmaceutically acceptable form. In certain aspects, the disease to be treated is a proliferative disorder. In a particular aspect, the disease is cancer. In certain aspects, the method further comprises administering to the individual a therapeutically effective amount of at least one additional therapeutic agent, e.g. an anti-cancer agent if the disease to be treated is cancer. An
15 "individual" according to any of the above embodiments may be a mammal, preferably a human.

For the prevention or treatment of disease, the appropriate dosage of a 4-1BBL trimer-containing antigen binding molecule of the invention (when used alone or in combination with one or more other additional therapeutic agents) will depend on the type of disease to be treated, the route of administration, the body weight of the patient, the type of antigen binding molecule,
20 the severity and course of the disease, whether the fusion protein is administered for preventive or therapeutic purposes, previous or concurrent therapeutic interventions, the patient's clinical history and response to the fusion protein, and the discretion of the attending physician. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject. Various
25 dosing schedules including but not limited to single or multiple administrations over various time-points, bolus administration, and pulse infusion are contemplated herein.

The 4-1BBL trimer-containing antigen binding molecule is suitably administered to the patient at one time or over a series of treatments. Depending on the type and severity of the disease, about 1 $\mu\text{g/kg}$ to 15 mg/kg (e.g. 0.1 mg/kg – 10 mg/kg) of 4-1BBL trimer-containing
30 antigen binding molecule can be an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. One typical daily dosage might range from about 1 $\mu\text{g/kg}$ to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment would generally be sustained until a desired suppression of disease
35 symptoms occurs. One exemplary dosage of the fusion protein would be in the range from about 0.005 mg/kg to about 10 mg/kg. In other examples, a dose may also comprise from about 1

µg/kg body weight, about 5 µg/kg body weight, about 10 µg/kg body weight, about 50 µg/kg body weight, about 100 µg/kg body weight, about 200 µg/kg body weight, about 350 µg/kg body weight, about 500 µg/kg body weight, about 1 mg/kg body weight, about 5 mg/kg body weight, about 10 mg/kg body weight, about 50 mg/kg body weight, about 100 mg/kg body weight, about 200 mg/kg body weight, about 350 mg/kg body weight, about 500 mg/kg body weight, to about 1000 mg/kg body weight or more per administration, and any range derivable therein. In examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg body weight to about 100 mg/kg body weight, about 5 µg/kg body weight to about 500 mg/kg body weight etc., can be administered, based on the numbers described above. Thus, one or more doses of about 0.5 mg/kg, 2.0 mg/kg, 5.0 mg/kg or 10 mg/kg (or any combination thereof) may be administered to the patient. Such doses may be administered intermittently, e.g. every week or every three weeks (e.g. such that the patient receives from about two to about twenty, or e.g. about six doses of the fusion protein). An initial higher loading dose, followed by one or more lower doses may be administered. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

The 4-1BBL trimer-containing antigen binding molecules of the invention will generally be used in an amount effective to achieve the intended purpose. For use to treat or prevent a disease condition, the 4-1BBL trimer-containing antigen binding molecules of the invention, or pharmaceutical compositions thereof, are administered or applied in a therapeutically effective amount. Determination of a therapeutically effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure provided herein.

For systemic administration, a therapeutically effective dose can be estimated initially from in vitro assays, such as cell culture assays. A dose can then be formulated in animal models to achieve a circulating concentration range that includes the IC₅₀ as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

Initial dosages can also be estimated from in vivo data, e.g., animal models, using techniques that are well known in the art. One having ordinary skill in the art could readily optimize administration to humans based on animal data.

Dosage amount and interval may be adjusted individually to provide plasma levels of the 4-1BBL trimer-containing antigen binding molecules which are sufficient to maintain therapeutic effect. Usual patient dosages for administration by injection range from about 0.1 to 50 mg/kg/day, typically from about 0.5 to 1 mg/kg/day. Therapeutically effective plasma levels may be achieved by administering multiple doses each day. Levels in plasma may be measured, for example, by HPLC.

In cases of local administration or selective uptake, the effective local concentration of the 4-1BBL trimer-containing antigen binding molecule may not be related to plasma concentration. One skilled in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

5 A therapeutically effective dose of the 4-1BBL trimer-containing antigen binding molecules described herein will generally provide therapeutic benefit without causing substantial toxicity. Toxicity and therapeutic efficacy of a fusion protein can be determined by standard pharmaceutical procedures in cell culture or experimental animals. Cell culture assays and animal studies can be used to determine the LD₅₀ (the dose lethal to 50% of a population) and the
10 ED₅₀ (the dose therapeutically effective in 50% of a population). The dose ratio between toxic and therapeutic effects is the therapeutic index, which can be expressed as the ratio LD₅₀/ED₅₀. 4-1BBL trimer-containing antigen binding molecules that exhibit large therapeutic indices are preferred. In one embodiment, the 4-1BBL trimer-containing antigen binding molecule according to the present invention exhibits a high therapeutic index. The data obtained from cell
15 culture assays and animal studies can be used in formulating a range of dosages suitable for use in humans. The dosage lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon a variety of factors, e.g., the dosage form employed, the route of administration utilized, the condition of the subject, and the like. The exact formulation, route of administration and dosage
20 can be chosen by the individual physician in view of the patient's condition (see, e.g., Fingl et al., 1975, in: The Pharmacological Basis of Therapeutics, Ch. 1, p. 1, incorporated herein by reference in its entirety).

The attending physician for patients treated with fusion proteins of the invention would know how and when to terminate, interrupt, or adjust administration due to toxicity, organ
25 dysfunction, and the like. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated, with the route of administration, and the like. The severity of the condition may, for example, be evaluated, in part, by standard prognostic
30 evaluation methods. Further, the dose and perhaps dose frequency will also vary according to the age, body weight, and response of the individual patient.

Other agents and treatments

The 4-1BBL trimer-containing antigen binding molecules of the invention may be administered in combination with one or more other agents in therapy. For instance, a fusion
35 protein of the invention may be co-administered with at least one additional therapeutic agent.

The term “therapeutic agent” encompasses any agent that can be administered for treating a symptom or disease in an individual in need of such treatment. Such additional therapeutic agent may comprise any active ingredients suitable for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. In certain
5 embodiments, an additional therapeutic agent is another anti-cancer agent.

Such other agents are suitably present in combination in amounts that are effective for the purpose intended. The effective amount of such other agents depends on the amount of 4-1BBL trimer-containing antigen binding molecule used, the type of disorder or treatment, and other factors discussed above. The 4-1BBL trimer-containing antigen binding molecules are generally
10 used in the same dosages and with administration routes as described herein, or about from 1 to 99% of the dosages described herein, or in any dosage and by any route that is empirically/clinically determined to be appropriate.

Such combination therapies noted above encompass combined administration (where two or more therapeutic agents are included in the same or separate compositions), and separate
15 administration, in which case, administration of the 4-1BBL trimer-containing antigen binding molecule of the invention can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant.

Thus, in one aspect a 4-1BBL trimer-containing antigen binding molecule as described herein for use in the treatment of cancer, in particular Her2 positive cancer is provided, wherein
20 the 4-1BBL trimer-containing antigen binding molecule is used in combination with a T-cell activating anti-CD3 bispecific antibody, in particular anti-Her2/anti-CD3 bispecific antibody.

In one aspect, the anti-Her2/anti-CD3 antibody comprises a first antigen binding domain that binds to CD3, and a second antigen binding domain that binds to Her2. In a particular aspect the second binding domain binding to Her2 binds to a different epitope on Her2 than the 4-1BBL
25 trimer-containing antigen binding molecule.

In one aspect, the anti-Her2/anti-CD3 bispecific antibody as used herein comprises a first antigen binding domain comprising a heavy chain variable region (V_H CD3) comprising CDR-H1 sequence of SEQ ID NO:91, CDR-H2 sequence of SEQ ID NO:92, and CDR-H3 sequence of SEQ ID NO:93; and/or a light chain variable region (V_L CD3) comprising CDR-L1 sequence of
30 SEQ ID NO:94, CDR-L2 sequence of SEQ ID NO:95, and CDR-L3 sequence of SEQ ID NO:96. More particularly, the anti-Her2/anti-CD3 bispecific antibody comprises a first antigen binding domain comprising a heavy chain variable region (V_H CD3) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:97 and/or a light chain variable region (V_L CD3) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the
35 amino acid sequence of SEQ ID NO:98. In a further aspect, the anti-Her2/anti-CD3 bispecific

antibody comprises a heavy chain variable region (V_HCD3) comprising the amino acid sequence of SEQ ID NO:97 and/or a light chain variable region (V_LCD3) comprising the amino acid sequence of SEQ ID NO:98.

In one aspect, the anti-Her2/anti-CD3 bispecific antibody comprises a second antigen
5 binding domain that binds to the same epitope as the antibody 4D5 (humanized version thereof known as trastuzumab). In another aspect, the anti-Her2/anti-CD3 bispecific antibody comprises a second antigen binding domain that binds to the same epitope as the antibody 2C4 (humanized version thereof known as pertuzumab). In yet another aspect, anti-Her2/anti-CD3 bispecific antibody comprises a second antigen binding domain that binds to the same epitope as the
10 antibody 7C2 (U.S. Patent No. 9,518,118).

In another aspect, the anti-Her2/anti-CD3 bispecific antibody comprises a second antigen binding domain comprising
(a) a heavy chain variable region (V_HHer2) comprising CDR-H1 sequence of SEQ ID NO:21, CDR-H2 sequence of SEQ ID NO:22, and CDR-H3 sequence of SEQ ID NO:23, and/or a light
15 chain variable region (V_LHer2) comprising CDR-L1 sequence of SEQ ID NO:24, CDR-L2 sequence of SEQ ID NO:25, and CDR-L3 sequence of SEQ ID NO:26, or
(b) a heavy chain variable region (V_HHer2) comprising CDR-H1 sequence of SEQ ID NO:13, CDR-H2 sequence of SEQ ID NO:14, and CDR-H3 sequence of SEQ ID NO:15, and/or a light chain variable region (V_LHer2) comprising CDR-L1 sequence of SEQ ID NO:16, CDR-L2
20 sequence of SEQ ID NO:17, and CDR-L3 sequence of SEQ ID NO:18, or
(c) a heavy chain variable region (V_HHer2) comprising CDR-H1 sequence of SEQ ID NO:99, CDR-H2 sequence of SEQ ID NO:100, and CDR-H3 sequence of SEQ ID NO:101, and/or a light chain variable region (V_LHer2) comprising CDR-L1 sequence of SEQ ID NO:102, CDR-L2 sequence of SEQ ID NO:103, and CDR-L3 sequence of SEQ ID NO:104.

In one aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:27 and/or a light chain variable region (V_LHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:28. In a further aspect, the anti-Her2/anti-CD3 bispecific
30 comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) comprising the amino acid sequence of SEQ ID NO:27 and/or a light chain variable region (V_LHer2) comprising the amino acid sequence of SEQ ID NO:28. In another aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the
35 amino acid sequence of SEQ ID NO:19 and/or a light chain variable region (V_LHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID

NO:20. In a further aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain variable region (V_HHer2) comprising the amino acid sequence of SEQ ID NO:19 and/or a light chain variable region (V_LCEA) comprising the amino acid sequence of SEQ ID NO:20. In another aspect, the anti-Her2/anti-CD3 bispecific comprises a
5 second antigen binding domain comprising a heavy chain variable region (V_HHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:105 and/or a light chain variable region (V_LHer2) that is at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO:106. In a further aspect, the anti-Her2/anti-CD3 bispecific comprises a second antigen binding domain comprising a heavy chain
10 variable region (V_HHer2) comprising the amino acid sequence of SEQ ID NO:105 and/or a light chain variable region (V_LCEA) comprising the amino acid sequence of SEQ ID NO:106.

In one aspect, the anti-Her2/anti-CD3 bispecific antibody comprises a first antigen binding domain comprising a heavy chain variable region (V_HCD3) comprising the amino acid sequence of SEQ ID NO:97 and/or a light chain variable region (V_LCD3) comprising the amino acid
15 sequence of SEQ ID NO:98 and a second antigen binding domain comprising a heavy chain variable region (V_HHer2) comprising the amino acid sequence of SEQ ID NO:27 and/or a light chain variable region (V_LHer2) comprising the amino acid sequence of SEQ ID NO:28.

In a further aspect, the 4-1BBL trimer-containing antigen binding molecule is used in combination with a T-cell activating anti-CD3 bispecific antibody and the T-cell activating anti-
20 CD3 bispecific antibody is administered concurrently with, prior to, or subsequently to the 4-1BBL trimer-containing antigen binding molecule.

In a further aspect, provided is the use of the 4-1BBL trimer-containing antigen binding molecule for the manufacture of a medicament for the treatment of cancer, wherein the 4-1BBL trimer-containing antigen binding molecule is used in combination with a T-cell activating anti-
25 CD3 bispecific antibody, in particular an anti-Her2/anti-CD3 bispecific antibody. In certain aspects, the disease to be treated is Her2-positive cancers. Examples of Her2-positive cancers include breast cancer, ovarian cancer, gastric cancer, bladder cancer, salivary gland, endometrial cancer, pancreatic cancer and non-small-cell lung cancer (NSCLC). In certain aspects, cancers to be treated are Her2-positive breast cancer, in particular Her2-positive metastatic breast cancer.

In a further aspect, the invention provides a method for treating cancer in an individual, comprising administering to said individual a therapeutically effective amount of a 4-1BBL trimer-containing antigen binding molecule of the invention and an effective amount a T-cell activating anti-CD3 bispecific antibody, in particular an anti-Her2/anti-CD3 bispecific antibody as defined above. In certain aspects, the method is for Her2-positive cancers. Examples of Her2-
30 positive cancers include breast cancer, ovarian cancer, gastric cancer, bladder cancer, salivary
35

gland, endometrial cancer, pancreatic cancer and non-small-cell lung cancer (NSCLC). In one aspect, the method is for treating Her2-positive metastatic breast cancer.

Articles of Manufacture

In another aspect of the invention, an article of manufacture containing materials useful for the treatment, prevention and/or diagnosis of the disorders described above is provided. The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, IV solution bags, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is by itself or combined with another composition effective for treating, preventing and/or diagnosing the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper that is pierceable by a hypodermic injection needle). At least one active agent in the composition is a 4-1BBL trimer-containing antigen binding molecule of the invention.

The label or package insert indicates that the composition is used for treating the condition of choice. Moreover, the article of manufacture may comprise (a) a first container with a composition contained therein, wherein the composition comprises a 4-1BBL trimer-containing antigen binding molecule of the invention; and (b) a second container with a composition contained therein, wherein the composition comprises a further cytotoxic or otherwise therapeutic agent. The article of manufacture in this embodiment of the invention may further comprise a package insert indicating that the compositions can be used to treat a particular condition.

Alternatively, or additionally, the article of manufacture may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Table B (Sequences):

SEQ ID NO:	Description	Sequence
1	Human (hu) 4-1BBL (71-254)	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLLIDGPLS WYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYYVFFQ LELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVD LPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARA RHAWQLTQGATVLGLFRVTPEIPAGLPSRSE
2	hu 4-1BBL (85-254)	LDLRQGMFAQLVAQNVLLIDGPLSWYSDPGLAGVSLTG GLSYKEDTKELVVAAGVYYVFFQLELRRVVAGEGSGS VSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFG

SEQ ID NO:	Description	Sequence
		FQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLPSRSE
3	hu 4-1BBL (80-254)	DPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLPSRSE
4	hu 4-1BBL (52-254)	PWAVSGARASPGSAASPRIREGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLPSRSE
5	Human (hu) 4-1BBL (71-248)	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGL
6	hu 4-1BBL (85-248)	LDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGL
7	hu 4-1BBL (80-248)	DPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGL
8	hu 4-1BBL (52-248)	PWAVSGARASPGSAASPRIREGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGL
9	dimeric hu 4-1BBL (71-254) connected by (G4S) ₂ linker	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLPSRSEGGGSGGGGREGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLPSRSE
10	dimeric hu 4-1BBL (71-248) connected by (G4S) ₂ linker	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGLGGGGSGGGGSGREGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGL
11	dimeric hu 4-1BBL (80-254) connected by (G4S) ₂ linker	DPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVTPEIPAGL

SEQ ID NO:	Description	Sequence
		ATVLGLFRVTPEIPAGLPSRSEGGGGSGGGSDPAGL LDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTG GLSYKEDTKELVVAKAGVYVFFQLELRRVVAGEGSGS VSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFG FQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVLG LFRVTPEIPAGLPSRSE
12	dimeric hu 4-1BBL (52-254) connected by (G4S) ₂ linker	PWAVSGARASPGSAASPRREGPELSPDDPAGLLDLRQ GMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYK EDTKELVVAKAGVYVFFQLELRRVVAGEGSGSVSLAL HLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLL HLSAGQRLGVHLHTEARARHAWQLTQGATVLGLFRVT PEIPAGLPSRSEGGGGSGGGSPWAVSGARASPGSAA SPRLREGPELSPDDPAGLLDLRQGMFAQLVAQNVLID GPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYV VFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALA LTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHT EARARHAWQLTQGATVLGLFRVTPEIPAGLPSRSE
13	heavy chain CDR-H1, pertuzumab	GFTFTDYTMD
14	heavy chain CDR-H2, pertuzumab	DVNPNSGGSIYNQRFKG
15	heavy chain CDR-H3, pertuzumab	NLGPSFYFDY
16	light chain CDR-L1, pertuzumab	KASQDVSIGVA
17	light chain CDR-L2, pertuzumab	SASYRYT
18	light chain CDR-L3, pertuzumab	QQYYIYPYT
19	heavy chain variable domain VH, pertuzumab	EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVR QAPGKGLEWVADVNPNSGGSIYNQRFKGRFTLSVDRSK NTLYLQMNSLRAEDTAVYYCARNLGPSFYFDYWGQGT LTVSS
20	light chain variable domain VL, pertuzumab	DIQMTQSPSSLSASVGDRVTITCKASQDVSIQVAWYQQ KPGKAPKLLIYSASYRYTGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCQQYYIYPYTFTGQGTKEIK
21	heavy chain CDR-H1, trastuzumab	GFNIKDTYIH
22	heavy chain CDR-H2, trastuzumab	RIYPTNGYTRYADSVKG
23	heavy chain CDR-H3, trastuzumab	WGGDGFYAMDY
24	light chain CDR-L1, trastuzumab	RASQDVNTAVA
25	light chain CDR-L2, trastuzumab	SASFLYS
26	light chain CDR-L3, trastuzumab	QQHYTTPPT
27	heavy chain variable domain VH, trastuzumab	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR QAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LTVSS
28	light chain variable domain VL, trastuzumab	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQ KPGKAPKLLIYSASFYSGVPSRFSGSGSGTDFTLTIS SLQPEDFATYYCQQHYTTPPTFTGQGTKEIK
29	heavy chain CDR-H1, aff. pertuzumab	GFTFNDYTMD
30	heavy chain CDR-H2, aff. pertuzumab	DVNPNSGGSIYNRRFKG
31	heavy chain CDR-H3, aff. pertuzumab	NLGPFYFDY
32	light chain CDR-L1, aff. pertuzumab	KASQDVSTAVA

SEQ ID NO:	Description	Sequence
33	light chain CDR-L2, aff. pertuzumab	SASFRYT
34	light chain CDR-L3, aff. pertuzumab	QQHYTTPPT
35	heavy chain variable domain VH, aff. pertuzumab	EVQLVESGGGLVQPGGSLRLSCAASGFTFNDYTMDWVRQAPGKGLEWVADVNPNSGGSIIVNRRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARNLGPFYFDYWGQGTLVTVSS
36	light chain variable domain VL, aff. pertuzumab	DIQMTQSPSSLSASVGRVTITCKASQDVSTAVAWYQQKPGKAPKLLIYSASFRYTGVPSPRFGSGRSGTDFTLTISLTQPEDFATYYCQQHYTTPPTFGQGTKEIK
37	Dimeric 4-1BB ligand (71-248) – CL* Fc knob chain	see Table 1
38	Monomeric 4-1BB ligand (71-248)-CH1*	see Table 1
39	Dimeric 4-1BB ligand (71-248) – CL Fc knob chain	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARA RHAQWLTQGATVLGLFRVTPEIPAGLGGGSGGGGSRE GPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQWLTQGATVLGLFRVTPEIPAGLGGGSGGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCVLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSPVTKSFNRGECDKTHTCPPCPAPEAAGGPPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLVHQQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE PQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
40	Monomeric 4-1BB ligand (71-248)-CH1	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARA RHAQWLTQGATVLGLFRVTPEIPAGLGGGSGGGGSAS TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC
41	Dimeric 4-1BB ligand (71-254) – CL* Fc knob chain	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARA RHAQWLTQGATVLGLFRVTPEIPAGLPSRSEGGGSGGGSGREGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYVFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARA RHAQWLTQGATVLGLFRVTPEIPAGLPSRSEGGGSGGGSGGGSGRTVAAPSVFIFPPSDRKLKSGTASVVCVLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSPVTKSFNRGECDKTHTCPPCPAPEAAGGPPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLVHQQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPRE PQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP

SEQ ID NO:	Description	Sequence
		EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIE KTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSP
42	Monomeric 4-1BB ligand (71-254)-CH1*	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLS WYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYVFFQ LELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVD LPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARA RHAWQLTQGATVLGLFRVTPEIPAGLPSPRSEGGGGSG GGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFP EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDEKVEPKSC
43	Dimeric 4-1BB ligand (71-254) - CL Fc knob chain	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLS WYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYVFFQ LELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVD LPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARA RHAWQLTQGATVLGLFRVTPEIPAGLPSPRSEGGGGSG GGGSREGPELSPDDPAGLLDLRQGMFAQLVAQNVLID GPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYV VFFQLELRRVVAGEGSGSVSLALHLQPLRSAAGAAALA LTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHT EARARHAWQLTQGATVLGLFRVTPEIPAGLPSPRSEGG GGSGGGGSRTVAAPSVFIFPPSDEQLKSGTASVVCCLN NFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTSYSL SSTLTLSKADYEKKHVVACEVTHQGLSSPVTKSFNRGE CDKTHTCPPEAAGGGSVFLFPPKPKDTLMISRTPE EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALGAPIE KTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK LTVDKSRWQQGNVFCSCVMHEALHNHYTQKSLSLSP
44	Monomeric 4-1BB ligand (71-254) -CH1	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLIDGPLS WYSDPGLAGVSLTGGLSYKEDTKELVVAAGVYVFFQ LELRRVVAGEGSGSVSLALHLQPLRSAAGAAALALTVD LPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHTEARA RHAWQLTQGATVLGLFRVTPEIPAGLPSPRSEGGGGSG GGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFP EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP SSSLGTQTYICNVNHKPSNTKVDDKVEPKSC
45	anti-Her2 (PER) Fc hole chain	see Table 1
46	anti-Her2 (PER) light chain	see Table 1
47	anti-Her2 (TRAS) Fc hole chain	see Table 2
48	anti-Her2 (TRAS) light chain	see Table 2
49	anti-Her2 (aff-PER) Fc hole chain	see Table 3
50	anti-Her2 (aff-PER) light chain	see Table 3
51	human 4-1BB ECD, aa 24-186 of Q07011	LQDPCSNCPAGTFCDNNRNQICSPCPNFSFSSAGGQRT CDICRQCKGVFRTRKECSSTSNAECDCTPGFHCLGAGC SMCEQDCKQGQELTKKGCKDCCFGTFNDQKRGICRPWT NCSLDGKSVLVNGTKERDVVCGPSPADLSPGASSVTPP APAREPGHSPQ
52	Fc hole chain	see Table 5

SEQ ID NO:	Description	Sequence
53	human 4-1BB antigen Fc knob chain	see Table 5
54	human Her2, UniProt Acc. No. P04626-1	MELAALCRWG LLLALLPPGA ASTQVCTGTD MKLRLPASPE THLDMLRHLY QGCQVVQGNL ELTYLPTNAS LSFLQDIQEV QGYVLIHNNQ VRQVPLQRLR IVRGTLQFED NYALAVLDNG DPLNNTTPVT GASPGGLREL QLRSLTEILK GGVLIQRNPQ LCYQDTILWK DIFHKNNQLA LTLIDTNRSR ACHPCSPMCK GSRCWGESSE DCQSLTRTVC AGGCARCKGP LPTDCCHEQC AAGCTGPKHS DCLACLHFNH SGICELHCPA LVTYNTDTFE SMPNPEGRYT FGASCVTACP YNYLSTDVGS CTLVCPLHNQ EVTAEDGTQR CEKCSKPCAR VCYGLGMEHL REVRVTSAN IQEFAGCKKI FGSLAFLPES FDGDPASNTA ETLEEITGYL YISAWPDSL PDLVFNQLQV IRGRILHNGA YSLTLQGLGI SWLGLRSLRE LGSGLALIIH NTHLCFVHTV PWDQLFRNPH QALLHTANRP EDECVGEGLA CHQLCARGHC WGPPTQCVN CSQFLRGQEC VEECRVLQGL PREYVNAHRC LPCHPECQPQ NGSVTCFGPE ADQCVACAHY KDPPFCVARC PSGVKPDLSY MPIWKFPDEE GACQPCPINC THSCVDLDDK GCPAEQRASP LTSIIISAVVG ILLVVVLGVV FGILIKRRQQ KIRKYTMRR LQETELVEPL TSPGAMPNQA QMRILKETEL RKVKVLGSGA FGTVYKGIWI PDGENVKIPV AIKVLRENTS PKANKEILDE AYVMAGVGSP YVSRLLGICL TSTVQLVTQL MPYGCLLDHV RENRGRIGSQ DLLNWCMIQA KGMSYLEDVR LVHRDLAARN VLVKSPNHVK ITDFGLARLL DIDETEHAD GGVPIKWMA LESILRRRFT HQSDVWSYGV TVWELMTFGA KPYDGIPARE IPDLLEKGER LPQPPICTID VYMIMVKCWM IDSECRPRFR ELVSEFSRMA RDPQRFVVIQ NEDLGPASPL DSTFYRSLLE DDDMGDLVDA EEYLVPQQGF FCPDPAPGAG GMVHHRHRSS STRSGGGDLT LGLEPSEEEA PRSPLAPSEG AGSDVFDGDL GMGAAKGLQS LPTHDPSPLO RYSEDPTVPL PSETDGYVAP LTCSPQPEYV NQPDVVRQPP SPREGPLPAA RPAGATLERP KTLSPGKNGV VKDVFAFGGA VENPEYLTPQ GGAAPQPHPP PAFSPAFDNL YYWDQDPPER GAPPSTFKGT PTAENPEYLG LDVPV
55	4-1BB antibody 20H4.9 IgG4, Heavy chain	MKHLWFFLLL VAAPRWVLSQ VQLQQWGAGL LKPSETLSLT CAVYGGSFSG YYWSWIRQSP EGGLEWIGEI NHGGYVTYNP SLESRTISV DTSKNQFSLK LSSVTAADTA VYYCARDYGP GNYDWYFDLW GRGTLVTVSS ASTKGPSVFP LAPCSRSTSE STAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTKT YTCNVDHKPS NTKVDKRVES KYGPPCPPCP APEFLGGPSV FLFPKPKDIT LMISRTPEVT CVVVDVSQED PEVQFNWYVD GVEVHNAKTK PREEQFNSTY RWSVLTVLHQ DWLNGKEYKC KVSNGKLPSS IEKTISKAKG

SEQ ID NO:	Description	Sequence
		QPREPQVYTL PPSQEEMTKN QVSLTCLVKG FYPSDIAVEW ESNGQPENNY KTTTPVLDS GSFFLYSRLT VDKSRWQEGN VFSCSVMHEA LHNHYTQKSL SLSL
56	4-1BB antibody 20H4.9 IgG4, Light chain	EIVLTQSPAT LSLSPGERAT LSCRASQSVS SYLAWYQQKP GQAPRLLIYD ASNRATGIPA RFSGSGSGTD FTLTISSLEP EDFAVYQCQ RSNWPPALTF CGGKVEIKR TVAAPS VFIF PPSDEQLKSG TASVVCLLNN FYPREKQVQW KVDNALQSGN QESVTEQDS KDSTYLSST LTLKADYEK HKVYACEVTH QGLSSPVTKS FNRGEC
57	4-1BB antibody MOR7480 IgG2, Heavy chain	EVQLVQSGAEVKKPGESLRISCKGSGYSFSTYWISWVR QMPGKGLEWMGKIYPGDSYTNYSFQGVVTSADKSI STAYLQWSSLKASDTAMYYCARGYGFIDYWGQGTLLTV SSASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEP VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPS NFGTQTYTCNVDHKPSNTKVDKTVKCCVECPPCPAP PVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP EVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVS VLT VHQLDNLGKEYKCKVSNKGLPAPIEKTISKTKGQPREPQ VYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTTPMLDSGGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSP
58	4-1BB antibody MOR7480 IgG2, Light chain	SYELTQPPSVSPGQTASITCSGDNIGDQYAHWYQQK PGQSPVLVIYQDKNRPSGIPERFSGSNSGNTATLTISG TQAMDEADYYCATYTGFGLAVFGGGTKLTVLGQPKAA PSVTLFPPSSEELQANKATLYCLISDFYPGAVTVAWKA DSSPVKAGVETTTPSKQSNKYAASSYLSLTPEQWKSH RSYSCQVTHEGSTVEKTVAPTECS
59	Her2 (TRAS)-anticalin-4-1BB human IgG4 heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR QAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTV VPSSSLGKTYTCNVDHKPSNTKVDKRVESKYGPCCP CPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDV SQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVS VLTVLHQLDNLGKEYKCKVSNKGLPSSIEKTISKAKQ PREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTTPVLDSGGSFFLYSRLTVDKSRWQ EGNVFSCSVMHEALHNHYTQKSLSLGLKGGGSGGGG SGGGGSQDSTSLIPAPPLSKVPLQQNFQDNQFHGKWKY VVGQAGNIRLREDKDPKMMATIELKEDKSYDVTMVK FDDKKCMYDIWTFVPGSQPGEFTLGKIKSFPGHTSSLV RVVSTNYNQHAMVFFKVFQNRREEFYITLYGRTKELTS ELKENFIRFSKSLGLPENHIVFPVPIDQCIDG
60	Her2 (TRAS)-anticalin-4-1BB human IgG4 light chain	DIQMTQSPSSLSASVGDRTITCRASQDVNTAVAWYQQ KPGKAPKLLIYSASFYSGVPSRFSGSRSGTDFLTIS SLQPEDFATYYCQHYTTPPTFGQGTKVEIKRTVAAPS VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN ALQSGNSQESVTEQDSKSTYLSSTLTLSKADYEKHK VYACEVTHQGLSSPVTKSFNRGEC
61	DP47 Fc hole chain	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVR QAPGKGLEWVSAISGSGGSTYADSVKGRFTISRDNK NTLYLQMNSLRAEDTAVYYCAKSGGFDYWGQGTLLTVS SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPS

SEQ ID NO:	Description	Sequence
		LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPR EPQVCTLPSPSRDELTKNQVSLSCAVKGFYPSDIAVEWE SNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQQG NVFSCSVMEALHNHYTQKSLSLSP
62	DP47 light chain	EIVLTQSPGTLSSLSPGERATLSCRASQSVSSSYLAWYQ QKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTIT SRLEPEDFAVYYCQQYGSSPLTFGQGTKEIKRTVAAP SVFIFPPSDEQLKSGTASVCLLNNFYPREAKVQWKVD NALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHK KVYACEVTHQGLSSPVTKSFNRGEC
63	DP47-anticalin-4-1BB human IgG4 heavy chain	EVQLLESGLLVQPGGSLRLSCAASGFTFSSYAMSWVR QAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNISK NTLYLQMNSLRAEDTAVYYCAKSGGFDYWGQGLTVTVS SASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSS LGTQTYTCNVNHHKPSNTKVDKRVESKYGPCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHQEDP EVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQ VYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNG QPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVF SCSVMEALHNHYTQKSLSLSLGKGGGGSGGGSGGGG SQDSTSDLIAPPPLSKVPLQQNFQDNQFHGKWKYVVGQA GNIRLREDKDKPIKMMATIELKEDKSYDVTMVKFDDKK CMYDIWTFVPGSQPGEFTLGKIKSFPGHTSSLVRVST NYNQHAMVFFKVFQNRREEFYITLYGRTKELTSELKEN FIRFSKSLGLPENHIVFPVPIDQCIDG
64	DP47-anticalin-4-1BB human IgG4 light chain	EIVLTQSPGTLSSLSPGERATLSCRASQSVSSSYLAWYQ QKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTIT SRLEPEDFAVYYCQQYGSSPLTFGQGTKEIKRTVAAP SVFIFPPSDEQLKSGTASVCLLNNFYPREAKVQWKVD NALQSGNSQESVTEQDSKDYSLSTLTLSKADYEKHK KVYACEVTHQGLSSPVTKSFNRGEC
65	Her2 (TRAS) human IgG1 P329G LALA, Heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVR QAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSK NTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LTVVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC PPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKA KGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSR RWQQGNVFSCSVMEALHNHYTQKSLSLSP
66	Her2 (PER) human IgG1 P329G LALA, Heavy chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMWVR QAPGKGLEWVADVNPNSGGSIYNQRFKGRFTLSVDRSK NTLYLQMNSLRAEDTAVYYCARNLGPSFYFDYWGQGT LTVVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYF PEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTV PSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTC PPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVV VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKA

SEQ ID NO:	Description	Sequence
		GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSP
67	DP47 human IgG1 P329G LALA, Heavy chain	EVQLLESQGGGLVQPGGSLRLSCAASGFTFSSYAMSWVR QAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNK NTLYLQMNSLRAEDTAVYYCAKSGFDYWGQGLTVTVS SASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSS LGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP APEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSH EDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVSVL TVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPR EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE SNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSP
68	(G4S) ₂ peptide linker	GGGGSGGGGS
69	human 4-1BBL (UniProt no. P41273)	MEYASDASLD PEAPWPPAPR ARACRVLPWA LVAGLLLLLL LAAACAVFLA CPWAVSGARA SPGSAASPRL REGPELSPDD PAGLLDLRQG MFAQLVAQNV LLIDGPLSWY SDPGLAGVSL TGGLSYKEDT KELVVAAGV YVVFQLELR RVVAGEGSGS VSLALHLQPL RSAAGAAALA LTVDLPPASS EARNSAFGFQ GRLLHLSAGQ RLGVHLHTEA RARHAWQLTQ GATVGLGLFRV TPEIPAGLPS PRSE
70	human 4-1BBL(50-254)	ACPWAVSGARASPGSAASPRLREGPELSPDDPAGLLDL RQGMFAQLVAQNVLLIDGPLSWYSDPGLAGVSLTGGLS YKEDTKELVVAAGVYVVFQLELRRVVAGEGSGSVSL ALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQ GRLLHLSAGQRLGVHLHTEARARHAWQLTQGATVGLGLFR VTPEIPAGLPSPRSE
71	Peptide linker G4S	GGGGGS
72	Peptide linker (SG4) ₂	SGGGGSGGGG
73	Peptide linker (G4S) ₃	GGGGSGGGGSGGGGS
74	Peptide linker G4(SG4) ₂	GGGGSGGGGSGGGG
75	Peptide linker (G4S) ₄	GGGGSGGGGSGGGGSGGGGS
76	Peptide linker	GSPGSSSSGS
77	Peptide linker	SGSGSGSGS
78	Peptide linker	SGSGNGS
79	Peptide linker	GGSGSGSG
80	Peptide linker	GGSGSG
81	Peptide linker	GGSG
82	Peptide linker	GGSGNGSG
83	Peptide linker	GGNGSGSG
84	Peptide linker	GGNGSG
85	human CD3ε	UniProt No. P07766
86	cynomolgus CD3ε	NCBI GenBank no. BAB71849.1 Uniprot Q05LI5
87	VHCH1(EF) (MU137-1) Fc-KK (mu4- 1BB-Her2)	See Table 4A
88	VLCH1 (2C4) VHCH1(EF) (MU137- 1) Fc-DD (mu4-1BB-Her2)	See Table 4A
89	VLCL(RK)-Light chain (MU137-1) (mu4-1BB-Her2)	See Table 4A

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SEQ ID NO:	Description	Sequence
90	VHCL-Light chain (2C4)	See Table 4A
91	heavy chain CDR-H1, CD3	NYIIH
92	heavy chain CDR-H2, CD3	WIYPGDGNTK YNEKFKG
93	heavy chain CDR-H3, CD3	DSYSNYYFDY
94	light chain CDR-L1, CD3	KSSQSLLNSR TRKNYLA
95	light chain CDR-L2, CD3	WASTRES
96	light chain CDR-L3, CD3	TQSFILRT
97	heavy chain variable domain VH, CD3	EVQLVQSGAE VKKPGASVKV SCKASGYTFT NYIIHWVRQA PGQGLEWIGW IYPGDGNTKY NEKFKGRATL TADTSTSTAY LELSSLRSED TAVYYCARDS YSNYYFDYWG QGTLVTVSS
98	light chain variable domain VL, CD3	DIVMTQSPDS LAVSLGERAT INCKSSQSLL NSRTRKNYLA WYQQKPGQPP KLLIYWASTR ESGVPDRFSG SGSGTDFTLT ISSLQAEDVA VYYCTQSFIL RTFGQGTVKE IK
99	heavy chain CDR-H1, Her2 (7C2)	GYWMN
100	heavy chain CDR-H2, Her2 (7C2)	MIHPSDSEIR ANQKFRD
101	heavy chain CDR-H3, Her2 (7C2)	GTVDGGFEY
102	light chain CDR-L1, Her2 (7C2)	RASQSVSGSR FTYMH
103	light chain CDR-L2, Her2 (7C2)	YASILES
104	light chain CDR-L3, Her2 (7C2)	QHSWEIPPWT
105	heavy chain variable domain VH, Her2 (7C2)	QVQLQQPGAE LVRPGASVKL SCKASGYSFT GYWMNWLKQR PGQGLEWIGM IHPDSEIRA NQKFRDKATL TVDKSSTAY MQLSSPTSED SAVYYCARGT YDGGFEYWGQ GTTLTVSS
106	light chain variable domain VL, Her2 (7C2)	DIVLTQSPAS LVSLLGQRAT ISCRASQSVS GSRFTYMHY WYQQKPGQPPK LIKYASILES GVPARFSGGG SGTDFTLNIH PVEEDDTATY YCQHSWEIPP WTFGGGTVKE IK

General information regarding the nucleotide sequences of human immunoglobulins light and heavy chains is given in: Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991).

- 5 Amino acids of antibody chains are numbered and referred to according to the EU numbering systems according to Kabat (Kabat, E.A., et al., Sequences of Proteins of Immunological Interest, 5th ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991)) as defined above.

EXAMPLES

The following are examples of methods and compositions of the invention. It is understood that various other embodiments may be practiced, given the general description provided above.

Recombinant DNA techniques

- 5 Standard methods were used to manipulate DNA as described in Sambrook et al., Molecular cloning: A laboratory manual; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The molecular biological reagents were used according to the manufacturer's instructions. General information regarding the nucleotide sequences of human immunoglobulin light and heavy chains is given in: Kabat, E.A. et al., (1991) Sequences of
10 Proteins of Immunological Interest, Fifth Ed., NIH Publication No 91-3242.

DNA sequencing

DNA sequences were determined by double strand sequencing.

Gene synthesis

- Desired gene segments were either generated by PCR using appropriate templates or were
15 synthesized by Geneart AG (Regensburg, Germany) from synthetic oligonucleotides and PCR products by automated gene synthesis. In cases where no exact gene sequence was available, oligonucleotide primers were designed based on sequences from closest homologues and the genes were isolated by RT-PCR from RNA originating from the appropriate tissue. The gene segments flanked by singular restriction endonuclease cleavage sites were cloned into standard
20 cloning / sequencing vectors. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The DNA sequence of the subcloned gene fragments was confirmed by DNA sequencing. Gene segments were designed with suitable restriction sites to allow sub-cloning into the respective expression vectors. All constructs were designed with a 5'-end DNA sequence coding for a leader peptide which targets proteins for
25 secretion in eukaryotic cells.

Cell culture techniques

Standard cell culture techniques were used as described in Current Protocols in Cell Biology (2000), Bonifacino, J.S., Dasso, M., Harford, J.B., Lippincott-Schwartz, J. and Yamada, K.M. (eds.), John Wiley & Sons, Inc.

Protein purification

Proteins were purified from filtered cell culture supernatants referring to standard protocols. In brief, antibodies were applied to a Protein A Sepharose column (GE healthcare) and washed with PBS. Elution of antibodies was achieved at pH 2.8 followed by immediate
5 neutralization of the sample. Aggregated protein was separated from monomeric antibodies by size exclusion chromatography (Superdex 200, GE Healthcare) in PBS or in 20 mM Histidine, 150 mM NaCl pH 6.0. Monomeric antibody fractions were pooled, concentrated (if required) using e.g., a MILLIPORE Amicon Ultra (30 MWCO) centrifugal concentrator, frozen and stored at -20°C or -80°C. Part of the samples were provided for subsequent protein analytics and
10 analytical characterization e.g. by SDS-PAGE, size exclusion chromatography (SEC) or mass spectrometry.

SDS-PAGE

The NuPAGE® Pre-Cast gel system (Invitrogen) was used according to the manufacturer's instruction. In particular, 10% or 4-12% NuPAGE® Novex® Bis-TRIS Pre-Cast gels (pH 6.4)
15 and a NuPAGE® MES (reduced gels, with NuPAGE® Antioxidant running buffer additive) or MOPS (non-reduced gels) running buffer was used.

Analytical size exclusion chromatography

Size exclusion chromatography (SEC) for the determination of the aggregation and oligomeric state of antibodies was performed by HPLC chromatography. Briefly, Protein A
20 purified antibodies were applied to a Tosoh TSKgel G3000SW column in 300 mM NaCl, 50 mM KH₂PO₄/K₂HPO₄, pH 7.5 on an Agilent HPLC 1100 system or to a Superdex 200 column (GE Healthcare) in 2 x PBS on a Dionex HPLC-System. The eluted protein was quantified by UV absorbance and integration of peak areas. BioRad Gel Filtration Standard 151–1901 served as a standard.

25

Example 1

Generation and Production of Her2-targeting 4-1BB agonistic antigen binding molecules

1.1. Generation and Production of Her2-targeting 4-1BB ligand trimer-containing Fc fusion antigen binding molecules

The Her2 binders used to target the trimeric 4-1BB ligand were pertuzumab (termed in the
30 text below PER), trastuzumab (TRAS) and affinity matured pertuzumab (aff-PER) as described in WO 2015/091738.

The variable region of heavy and light chain DNA sequences encoding a binder specific for Her2, were subcloned in frame with either the constant heavy chain of the hole or the constant light chain of human IgG1.

The DNA sequence encoding part of the ectodomain (amino acid 71-248) of human 4-1BB ligand was synthesized according to the P41273 sequence of Uniprot database.

A polypeptide containing two ectodomains of 4-1BB ligand, separated by (G4S)₂ linkers, and fused to the human IgG1-CL domain, was cloned as depicted in **Figure 1A**: human 4-1BB ligand, (G4S)₂ connector, human 4-1BB ligand, (G4S)₂ connector, human CL.

A polypeptide containing one ectodomain of 4-1BB ligand and fused to the human IgG1-CH domain, was cloned as described in **Figure 1B**: human 4-1BB ligand, (G4S)₂ connector, human CH.

To improve correct pairing the following mutations were introduced in the crossed CH-CL. In the dimeric 4-1BB ligand fused to human CL the mutations E123R and Q124K were introduced. In the monomeric 4-1BB ligand fused to human CH1, the mutations K147E and K213E were cloned into the human CH1 domain as described in International Patent Appl. Publ. No. WO 2015/150447.

The variable region of heavy and light chain DNA sequences encoding the binders specific for Her2 (PER, TRAS and aff-PER) were subcloned in frame with either the constant heavy chain of the hole or the constant light chain of human IgG1.

In the Fc domain the P329G, L234A and L235A mutations were introduced in the constant region of the knob and hole heavy chains to abrogate binding to Fc gamma receptors according to the method described in International Patent Appl. Publ. No. WO 2012/130831. Combination of the dimeric ligand-Fc knob chain containing the S354C/T366W mutations, the monomeric CH1 fusion, the targeted anti-Her2-Fc hole chain containing the Y349C/T366S/L368A/Y407V mutations and the anti-Her2 light chain allowed the generation of a heterodimer, which includes an assembled trimeric 4-1BB ligand and a Her2 binding Fab (**Figure 2A**).

Table 1 shows the amino acid sequences of the monovalent anti-Her2(PER) split trimeric 4-1BB ligand Fc (kih) fusion antigen binding molecule containing CH1-CL crossover and charged residues. The molecule is called Her2(PER)-4-1BBL.

Table 2 shows the amino acid sequences of the monovalent anti-Her2(TRAS) split trimeric 4-1BB ligand Fc (kih) fusion antigen binding molecule containing CH1-CL crossover and charged residues. The molecule is called Her2(TRAS)-4-1BBL

Table 3 shows the amino acid sequences of the monovalent Her2(aff-PER) split trimeric 4-1BB ligand Fc (kih) fusion antigen binding molecule containing CH1-CL crossover and charged residues. The molecule is called Her2(aff-PER)-4-1BBL

Table 1: Amino acid sequences of Her2(PER)-4-1BBL containing CH1-CL crossover and charged residues (*for charged residues)

SEQ ID NO:	Description	Sequences
37	Dimeric hu 4-1BBL (71-248)–CL* Fc knob chain	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLLIDGPLSWYSDPG LAGVSLTGGLSYKEDTKELVVAKAGVYVFFQLELRRVVAGEGS GSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGR LLHLSAGQRLGVHLHTEARARHAWQLTQGATVGLGLFRVTPEIPA GLGGGGSGGGGSREGPELSPDDPAGLLDLRQGMFAQLVAQNVLL IDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYVFFQ LELRRVVAGEGSVSLALHLQPLRSAAGAAALALTVDLPPASS EARNSAFGFQGRLLHLSAGQRLGVHLHTEARARHAWQLTQGATV LGLFRVTPEIPAGLGGGGSGGGGSRTVAAPSVFIFPPSDRKLKS GTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDS TYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC KTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVYTLPP CRDELTKNQVSLVCLVKGFYPSDIAVEWESNGQPENNYKTTTPV LDSGDSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSL SLSP
38	Monomeric hu 4-1BBL (71-248) –CH1*	REGPELSPDDPAGLLDLRQGMFAQLVAQNVLLIDGPLSWYSDPG LAGVSLTGGLSYKEDTKELVVAKAGVYVFFQLELRRVVAGEGS GSVSLALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGR LLHLSAGQRLGVHLHTEARARHAWQLTQGATVGLGLFRVTPEIPA GLGGGGSGGGGSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDY FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKVEPKSC
45	anti-Her2 (PER) Fc hole chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKG LEWVADVNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRA EDTAVYYCARNLGPSFYFDYWGQGLTVTVSSASTKGPSVFPLAP SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKVEPK SCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVCT LPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSGDSFFLVSKLTVDKSRWQQGNVFSQSVMEALHNHYTQ KSLSLSP
46	anti-Her2 (PER) light chain	DIQMTQSPSSLSASVGDRTITCKASQDVSIGVAWYQQKPGKAP KLLIYSASYRYTGVPSTRFSGSGSGTDFTLTISSLQPEDFATYYC QQYYIYPYTFGQGTKEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSSTLS STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Table 2: Amino acid sequences of Her2 (TRAS)-4-1BBL containing CH1-CL crossover and charged residues (*for charged residues)

SEQ ID NO:	Description	Sequences
37	Dimeric hu 4-1BBL (71-248)–CL* Fc knob chain	see Table 1
38	Monomeric hu 4-1BBL (71-248) – CH1*	see Table 1
47	anti-Her2 (TRAS) Fc hole chain	EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKG LEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRA EDTAVYYCSRWGGDGFYAMDYWGQGTTLTVSSASTKGPSVFPLA PSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP KSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVC TLPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKT TPPVLDSDGSFFLVSKLTVDKSRWQQGNVFCSCVMHEALHNHYT QKSLSLSP
48	anti-Her2 (TRAS) light chain	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAP KLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYC QQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYSL STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Table 3: Amino acid sequences of Her2 (aff-PER)-4-1BBL containing CH1-CL crossover and charged residues (*for charged residues)

SEQ ID NO:	Description	Sequences
37	Dimeric hu 4-1BBL (71-248)–CL* Fc knob chain	see Table 1
38	Monomeric hu 4-1BBL (71-248) – CH1*	see Table 1
49	anti-Her2 (aff-PER) Fc hole chain	EVQLVESGGGLVQPGGSLRLSCAASGFTFNDYTMDWVRQAPGKG LEWVADVNPNSGGSIIVNRRFKGRFTLSVDRSKNTLYLQMNSLRA EDTAVYYCARNLGPFFYFDYWGQGTTLTVSSASTKGPSVFPLAP SSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPK SCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCV

		VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALGAPIEKTISKAKGQPREPQVCT LPPSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLVSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQ KSLSLSP
50	anti-Her2 (aff- PER) light chain	DIQMTQSPSSLSASVGDRVTITCKASQDVSTAVAWYQQKPGKAP KLLIYSASFRYTGVPSRFSGSRSGTDFTLTISSLQPEDFATYYC QQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV VCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSL STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

The bispecific constructs were produced by co-transfecting HEK293-EBNA cells with the mammalian expression vectors using polyethylenimine. The cells were transfected with the corresponding expression vectors in a 1:1:1:1 (“vector 4-1BBL Fc-knob chain”: “vector 4-1BBL light chain” : “vector Fc-hole chain ”: “vector light chain”).

Production was performed in shake flasks using HEK293 EBNA cells. For transfection, cells were centrifuged and medium replaced by pre-warmed CD CHO medium. Expression vectors were mixed in CD CHO medium, PEI was added, the solution vortexed and incubated for 10 minutes at room temperature. Afterwards, cells were mixed with the DNA/PEI solution, transferred to shake flask and incubated for 3 hours at 37°C in an incubator with a 5% CO₂ atmosphere. After the incubation, Excell medium with supplements was added. One day after transfection 12% Feed was added. After culturing for 7 days, the cell supernatant was collected by centrifugation. The solution was sterile filtered, supplemented with sodium azide to a final concentration of 0.01 % (w/v), and kept at 4°C.

Secreted proteins were purified from cell culture supernatants by affinity chromatography using Protein A, followed by size exclusion chromatography. For affinity chromatography, the supernatant was loaded on a Protein A MabSelect SuRe column (GE Healthcare) equilibrated with 20 mM sodium phosphate, 20 mM sodium citrate pH 7.5. Unbound protein was removed by washing with equilibration buffer. The bound protein was eluted using a step elution created with 20 mM sodium citrate, 100 mM sodium chloride, 100 mM glycine, pH 3.0. The pH of collected fractions was adjusted by adding 1/10 (v/v) of 0.5 M sodium phosphate pH8.0. The protein was concentrated and filtered prior to loading on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with 20 mM histidine, 140 mM sodium chloride, pH 6.0.

The protein concentration of purified constructs was determined by measuring the OD at 280 nm, using the molar extinction coefficient calculated on the basis of the amino acid sequence. Purity and molecular weight of the bispecific constructs were analyzed by CE-SDS in the presence and absence of a reducing agent (Invitrogen, USA) using a LabChipGXII (Caliper). The aggregate content of bispecific constructs was analyzed using a TSKgel G3000 SW XL

analytical size-exclusion column (Tosoh) equilibrated in a 25 mM K₂HPO₄, 125 mM NaCl, 200mM L-Arginine Monohydrochloride, 0.02 % (w/v) NaN₃, pH 6.7 running buffer at 25°C.

Table 4 summarizes the yield and final monomer content of the HER2 targeted and untargeted 4-1BB ligand trimer-containing Fc (kih) fusion antigen binding molecules and control molecules.

Table 4: Biochemical analysis of Her2 targeted 4-1BB ligand trimer-containing Fc (kih) fusion antigen binding molecules

Molecule	Monomer [%] (SEC)	Yield [mg/l]	CE-SDS (non-red)
Her2 (PER) 4-1-BBL	98	3.5	89
Her2 (TRAS) 4-1BBL	100	2.8	97
Her2 (aff-PER) 4-1BBL	98	16.4	93

1.2. Generation and Production of Her2-targeting 4-1BB agonistic antigen binding molecule as mouse surrogate (mu4-1BB-Her2)

As the mu 4-1BBL naturally forms a dimer (*A. Brita et al. 2018*) and not a trimer as in humans, a bispecific agonistic 4-1BB antibody with bivalent binding for mouse 4-1BB and monovalent binding for Her2, also termed 2+1, has been prepared as illustrated in **Figure 2B**.

In this example the HC1 of the construct was comprised of the following components, VHCH1 of an anti-mouse 4-1BB (clone MU137-1) followed by CH2 and CH3. HC2 was comprised of VLCH1 of anti-Her2 (clone 2C4, Adams CW et al, Cancer Immunol Immunother, 55(6), 2006, pp. 717-727, cross Fab) followed by VHCH1 of an anti-mouse 4-1BB (clone MU137-1) and CH2 and CH3. The mutations promoting heterodimerization described by Gunasekaran et al., J. Biol. Chem. 2010,19637-19646, namely E356K and D399K (termed KK) and K392D and K409D (termed DD), were introduced in HC1 and HC2, respectively.

Furthermore, DAPG mutations were introduced in the constant regions of the heavy chains to abrogate binding to mouse Fc gamma receptors according to the method described e.g. in Baudino et al. J. Immunol. (2008), 181, 6664-6669, or in WO 2016/030350 A1. Briefly, the D265A and P329G mutations have been introduced in the constant region of the Fc-DD and Fc-KK heavy chains to abrogate binding to Fc gamma receptors (numbering according to Kabat EU index; i.e. D265A, P329G).

Combination of the Fc-DD with the Fc-KK chain allowed generation of a heterodimer, which includes one Her2 binding Fab and two 4-1BB binding Fabs. To improve correct pairing, the following mutations were introduced in the CH-CL of the anti-4-1BB Fab: E123R and Q124K in CL and K147E and K213E in CH1.

- 5 The amino acid sequences for the 2+1 anti-4-1BB(MU137-1), anti-Her2 (2C4) antibody can be found in **Table 4A**.

Table 4A: Amino acid sequences of 2+1 anti-mu 4-1BB anti-Her2 (2C4) antibody DAPG DDKK (mu4-1BB-Her2)

SEQ ID NO:	Description	Sequences
87	VHCH1(EE) (MU137-1) Fc-KK	DVQLVESGGGLVQPGRSLKLSAASGFIFSYFDMAWVRQAPTKG LEWVASISPSGDIPIYRDSVKGRFTVSRENAKSSLYLQMDSLRS EDTATYYCARRSYGGYSELDYWGQGMVTVSSAKTTPPSVYPLA PGSAAQTNSMVTLGCLVEGYFPEPVTVTWNSGSLSSGVHTFPAV LQSDLYTLSSSVTVPSSTWPSQTVTCNVAHPASSTKVDEKIVPR DCGCKPCICTVPEVSSVFI FPPKPKDVLITITLTPKVTCTVVAIS KDDPEVQFSWFVDDVEVHTAQTKPREEQINSTFRSVSELPIMHQ DWLNGKEFKCRVNSAAGAPIEKTISKTKGRPKAPQVYTIPPPK KQMAKDKVSLTCMITNFFPEDITVEWQWNGQPAENYKNTQPIMK TDGSYFVYSKLNQKSNWEAGNTFTCSVLHEGLHNHHTKSLSH SP
88	VLCH1 (2C4) VHCH1(EE) (MU137-1) Fc-DD	DTVMTQSHKIMSTSVGDRVSI TCKASQDVSI GVAWYQQRPGQSP KLLIYSASYRYTGVPDRFTGSGSGTDFTFTISSVQAEDLAVYYC QQYYIYPYTFGGGTKLEIKSSAKTTPPSVYPLAPGSAAQTNSMV TLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSS VTVPSSTWPSQTVTCNVAHPASSTKVDKIVPRDCGGGGSGGGG SDVQLVESGGGLVQPGRSLKLSAASGFIFSYFDMAWVRQAPTK GLEWVASISPSGDIPIYRDSVKGRFTVSRENAKSSLYLQMDSLR SEDATYYCARRSYGGYSELDYWGQGMVTVSSAKTTPPSVYPL APGSAAQTNSMVTLGCLVEGYFPEPVTVTWNSGSLSSGVHTFPA VLQSDLYTLSSSVTVPSSTWPSQTVTCNVAHPASSTKVDEKIVP RDCGCKPCICTVPEVSSVFI FPPKPKDVLITITLTPKVTCTVVAI SKDDPEVQFSWFVDDVEVHTAQTKPREEQINSTFRSVSELPIMH QDWLNGKEFKCRVNSAAGAPIEKTISKTKGRPKAPQVYTIPPP KEQMAKDKVSLTCMITNFFPEDITVEWQWNGQPAENYDNTQPIM DTDGSYFVYSDLNQKSNWEAGNTFTCSVLHEGLHNHHTKSLSH HSP
89	VLCL(RK)-Light chain (MU137-1)	DIQMTQSPASLSASLEEIVTITCQASQDIGNWLAWYHQPGKSP QLLIYGTSSLADGVPSRFSGSSSGSQYSLKISRLQVEDIGIYYC LQAYGAPWTFGGGTKLELKRADAAPT VSI FPPSSRKLTSGGASV VCFLNNFYPKDINVKWKIDGSRQNGVLNSWTDQDSKDYSTYSMS STLTTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC
90	VHCL-Light chain (2C4)	EVQLQQSGPELVKPGTSVKISCKASGFTFTDYTMDWVKQSHGKS LEWIGDVNPNSGGSIYNQRFKGKASLTVDRSSRIVYMELRSLTF EDTAVYYCARNLGPSFYFDYWGQGTTLTVSSASDAAPT VSI FPP SSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSRQNGVLNSWT

		DQDSKDSTYSMSSTLTTLTKDEYERHNSYTCEATHKTSTSPIVKS FNRNEC
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Table 4B: Biochemical analysis of 2+1 anti-mu 4-1BB anti-Her2 (2C4) antibody

Molecule	Monomer [%] (SEC)	Yield [mg/l]	CE-SDS (non-red)
2+1 H2H anti-mu 4-1BB anti-Her2 (2C4) muIgG1 DAPG DDKK (mu4-1BB-Her2)	100	2.5	100

Example 2

5 **Functional Characterization of Her2-targeting split trimeric 4-1BB ligand Fc fusion
antigen binding molecules by surface plasmon resonance**

Preparation of 4-1BB Fc (kih) fusion molecule

A DNA sequence encoding the ectodomain of human 4-1BB (amino acids 24 to 186 of human 4-1BB according to Q07011, SEQ ID NO:51) were subcloned in frame with the human
10 IgG1 heavy chain CH2 and CH3 domains on the knob. An AcTEV protease cleavage site was introduced between an antigen ectodomain and the Fc of human IgG1. An Avi tag for directed biotinylation was introduced at the C-terminus of the antigen-Fc knob. Combination of the antigen-Fc knob chain containing the S354C/T366W mutations, with a Fc hole chain containing the Y349C/T366S/L368A/Y407V mutations allows generation of a heterodimer which includes
15 a single copy of 4-1BB ectodomain containing chain, thus creating a monomeric form of Fc-linked antigen. Table 5 shows the amino acid sequences of the antigen Fc-fusion construct.

Table 5: Amino acid sequences of monomeric human 4-1BB Fc(kih) fusion molecule

SEQ ID NO:	Antigen	Sequence
52	Fc hole chain	DKTHTCPCPCAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVTV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLP PSRDELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTP VLDSGDGSFFLVSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKS LSLSP

53	human 4-1BB antigen Fc knob chain	LQDPCSNCPAGTFCDNNRNQICSPPNSFSSAGGQRTCDICRQ CKGVFRTRKECSSTSNAECDCTPGFHCLGAGCSMCEQDCKQGQE LTKKGCKDCCFGTFNDQKRGICRPWTNCSLDGKSVLVNGTKERD VVCGPSADLSPGASSVTPPAPAREPGHSPQVDEQLYFQGGSPK SADKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMSRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL TVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT LPPCRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQ KSLSLSPGKSGGLNDIFEAQKIEWHE
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All 4-1BB-Fc-fusion molecule encoding sequences were cloned into a plasmid vector, which drives expression of the insert from an MPSV promoter and contains a synthetic polyA signal sequence located at the 3' end of the CDS. In addition, the vector contains an EBV OriP
5 sequence for episomal maintenance of the plasmid.

For preparation of the biotinylated monomeric antigen/Fc fusion molecule, exponentially growing suspension HEK293 EBNA cells were co-transfected with three vectors encoding the two components of fusion protein (knob and hole chains) as well as BirA, an enzyme necessary for the biotinylation reaction. The corresponding vectors were used at a 2 : 1 : 0.05 ratio
10 ("antigen ECD-AcTEV- Fc knob" : "Fc hole" : "BirA").

For protein production in 500 ml shake flasks, 400 million HEK293 EBNA cells were seeded 24 hours before transfection. For transfection cells were centrifuged for 5 minutes at 210 g, and the supernatant was replaced by pre-warmed CD CHO medium. Expression vectors were resuspended in 20 mL of CD CHO medium containing 200 µg of vector DNA. After addition of
15 540 µL of polyethylenimine (PEI), the solution was vortexed for 15 seconds and incubated for 10 minutes at room temperature. Afterwards, cells were mixed with the DNA/PEI solution, transferred to a 500 mL shake flask and incubated for 3 hours at 37°C in an incubator with a 5 % CO₂ atmosphere. After the incubation, 160 mL of F17 medium was added and cells were cultured for 24 hours. One day after transfection, 1 mM valproic acid and 7 % Feed 1 with
20 supplements were added to the culture. After 7 days of culturing, the cell supernatant was collected by spinning down cells for 15 min at 210 g. The solution was sterile filtered (0.22 µm filter), supplemented with sodium azide to a final concentration of 0.01 % (w/v), and kept at 4°C.

Secreted proteins were purified from cell culture supernatants by affinity chromatography using Protein A, followed by size exclusion chromatography. For affinity chromatography, the
25 supernatant was loaded on a HiTrap ProteinA HP column (CV = 5 mL, GE Healthcare) equilibrated with 40 mL 20 mM sodium phosphate, 20 mM sodium citrate pH 7.5. Unbound protein was removed by washing with at least 10 column volumes of 20 mM sodium phosphate, 20 mM sodium citrate, 0.5 M sodium chloride containing buffer (pH 7.5). The bound protein was eluted using a linear pH-gradient of sodium chloride (from 0 to 500 mM) created over 20 column

volumes of 20 mM sodium citrate, 0.01 % (v/v) Tween-20, pH 3.0. The column was then washed with 10 column volumes of 20 mM sodium citrate, 500 mM sodium chloride, 0.01 % (v/v) Tween-20, pH 3.0.

The pH of collected fractions was adjusted by adding 1/40 (v/v) of 2M Tris, pH8.0. The protein was concentrated and filtered prior to loading on a HiLoad Superdex 200 column (GE Healthcare) equilibrated with 2mM MOPS, 150 mM sodium chloride, 0.02 % (w/v) sodium azide solution of pH 7.4.

Recombinant human Her2 (ECD of ErbB2 protein, amino acids 23 to 652 of SEQ ID NO:54, UniProt Acc. No. P04626-1) is commercially available (e.g. from abcam, Cat No. ab168896) and was used for the determination of binding to Her2.

Determination of simultaneous binding of Her2-targeting split trimeric 4-1BB ligand Fc fusion antigen binding molecules

The capacity of binding simultaneously human 4-1BB Fc(kih) and human Her2 was assessed by surface plasmon resonance (SPR). All SPR experiments were performed on a Biacore T200 at 25 °C with HBS-EP as running buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, Biacore, Freiburg/Germany). Biotinylated human 4-1BB Fc(kih) was directly coupled to a flow cell of a streptavidin (SA) sensor chip. Immobilization levels up to 450 resonance units (RU) were used.

The Her2-targeting split trimeric 4-1BB ligand Fc fusion constructs were passed at a concentration range of 200 nM with a flow of 30 µL/minute through the flow cells over 90 seconds and dissociation was set to zero sec. Human Her2 was injected as second analyte with a flow of 30 µL/minute through the flow cells over 90 seconds at a concentration of 500 nM (**Figure 3A**). The dissociation was monitored for 120 sec. Bulk refractive index differences were corrected for by subtracting the response obtained in a reference flow cell, where no protein was immobilized.

As can be seen in the graphs of **Figure 3B and 3C**, the bispecific constructs could bind simultaneously human 4-1BB and human Her2.

Example 3

Functional characterization of the Her2-targeting split trimeric 4-1BB ligand Fc fusion antigen binding molecules

For the functional assays we tested the above described monovalent Her2-targeting split trimeric 4-1BB ligand Fc fusion antigen binding molecules, namely Her2 (PER)-4-1BBL, Her2

(aff-PER)-4-1BBL and Her2 (TRAS)-4-1BBL against two previously described agonistic anti-human 4-1BB antibodies, i.e. anti-human 4-1BB clone 20H4.9 human IgG4 (described in patents EP1670828B1 and US7659384(B2), antibody with heavy chains of SEQ ID NO:55 and light chains of SEQ ID NO:56) and anti-human 4-1BB clone MOR7480 human IgG2 (described in
5 patent application WO2012/032433, antibody with heavy chains of SEQ ID NO:57 and light chains of SEQ ID NO:58), and a previously described fusion polypeptide Her2 (TRAS)-anticalin-4-1BB human IgG4 (fusion polypeptide of SEQ ID Nos 59 and 60 as described in patent application WO2016/177802).

Furthermore, different control molecules like untargeted DP47-4-1BBL (molecule
10 comprising the amino acid sequences of SEQ ID Nos:37, 28, 61 and 62 and described as Control D in WO 2016/075278 A1, a germline control, termed DP47, not binding to the antigen was used to replace the antigen binding domain), DP47-anticalin-4-1BB human IgG4 (fusion polypeptide of SEQ ID NO:63 and 64 prepared in analogy to the molecules described in WO2016/17780), Her2 (TRAS) human IgG1 P329G LALA (antibody with heavy chains of SEQ ID NO:65 and
15 light chains of SEQ ID NO:48), Her2 (PER) human IgG1 P329G LALA (antibody with heavy chains of SEQ ID NO:66 and light chains of SEQ ID NO:46) and a non-targeted DP47 human IgG1 P329G LALA (antibody with heavy chains of SEQ ID NO:67 and light chains of SEQ ID NO:62) were used. These IgG1 antibodies comprise the Pro329Gly, Leu234Ala and Leu235Ala mutations, to abrogate binding to Fc gamma receptors.

20 **3.1. Binding to Her2-expressing tumor cells**

To test binding to cell surfaced expressed Her2, different human Her2-expressing tumor cells were used: human breast cancer cell line SK-Br3 (ATCC HTB-30), human breast cancer cell line KPL-4 (Kawasaki Medical School) and human gastric cancer cell line NCI-N87 (ATCC CRL-5822).

25 0.2 x 10⁶ tumor cells resuspended in DPBS (Gibco by Life Technologies, Cat. No. 14190-326) were added to each well of a round-bottom suspension cell 96-well plates (greiner bio-one, cellstar, Cat.-No. 650185). Cells were washed once with 200 µL DPBS and pellets were resuspended. 100 µL/well of 4 °C cold DPBS buffer containing 1:5000 diluted Fixable Viability Dye eFluor 450 (eBioscience, Cat.-No. 65-0863-18) were added and plates were incubated for
30 30 minutes at 4°C. Cells were washed once with 200 µL 4 °C cold DPBS buffer and resuspended in 50 µL/well of 4 °C cold FACS buffer (DPBS supplied with 2 % FBS, 5 mM EDTA pH8 (Amresco, Cat.-No. E177) and 7.5 mM Sodium azide (Sigma-Aldrich S2002) containing titrated concentrations of the human split 4-1BB ligand molecules called Her2 (PER)-4-1BBL or Her2 (aff-PER)-4-1BBL or Her2 (TRAS)-4-1BBL as well as the control molecule, i.e. non-targeted
35 DP47-4-1BBL, or anti-human 4-1BB antibodies called anti-human 4-1BB clone 20H4.9 huIgG4

or anti-human 4-1BB clone MOR-7480 huIgG2 or fusion proteins Her2 (TRAS)-anticalin-4-1BB huIgG4 or its untargeted control DP47-anticalin-4-1BB huIgG4 or Her2 (TRAS) huIgG1 P329G LALA or Her2 (PER) huIgG1 P329G LALA or DP47 huIgG1 P329G LALA. The cells were incubated for 1 hour at 4 °C and afterwards washed several times with cold FACS buffer to
 5 remove non bound antibodies. Cells were further stained with 50 µL/well of 4 °C cold FACS buffer 5 µg/mL PE-conjugated AffiniPure anti-human IgG F(ab')₂-fragment-specific goat F(ab')₂ fragment (Jackson ImmunoResearch, Cat. No. 109 116 097) for 30 minutes at 4 °C. Cells were washed twice with 200 µL 4 °C FACS buffer and cells were fixed in 50 µL/well DPBS containing 1 % Formaldehyde (Sigma, HT501320-9.5L) for at least 10 min. Afterwards
 10 cells were resuspended in 100 µL 4 °C FACS buffer and acquired using the MACS Quant Analyzer 10 flow cytometer (Miltenyi Biotech) coupled to a Cytomat (ThermoFisher). Data was analyzed using FlowJo Version 10 (FlowJo LLC), Microsoft Excel and Graph Pad Prism Version 6 (Graph Pad Software Inc.).

As shown in **Figures 4A to 4D**, Her2 (PER)-4-1BBL and Her2 (aff-PER)-4-1BBL show a
 15 similar binding to human Her2 expressed by the human tumor cells SK-Br3 or NCI-N87, whereby the Her2 (aff-PER)-4-1BBL molecule shows slightly higher EC₅₀ values as listed in **Table 6**. The antibody Her2 (PER) huIgG P329G LALA binds – different to Her2 (PER)-4-1BBL – bivalent instead of monovalent. This is reflected by a slightly lower MFI (less antibodies/cell surface as the antibody can occupy two instead of one Her2 molecule) as shown
 20 in **Figure 4** and a slighter lower EC₅₀ value reflecting the avidity effect as listed in **Table 6**. The bivalent trastuzumab binding fusion protein Her2 (TRAS)-anticalin-4-1BB huIgG4 reflects a lower MFI than the bivalent binding Her2 (PER) huIgG P329G LALA and higher EC₅₀ values (shown in **Figure 4** and **Table 6**). This reflects the lower affinity and avidity of the trastuzumab (TRAS) binder compared to the pertuzumab (PER) binder.

25 **Table 6: EC₅₀ values of binding curves to Her2⁺ tumor cell lines shown in Figure 4**

	Her2 (aff-PER)-4-1BBL	Her2 (PER)-4-1BBL	Her2 (PER) huIgG1 PGLALA	Her2 (TRAS)-anticalin 4-1BB huIgG4
EC ₅₀ [nM] on SK-Br3 cells	7.3	2.4	1.3	4.1
EC ₅₀ [nM] on NCI-N87 cells	4.3	2.9	2.1	5.3

As shown in **Figures 5A and 5B**, Her2 (PER)-4-1BBL and Her2 (aff-PER)-4-1BBL show a similar binding to human Her2 expressed by the human breast cancer cell line KPL-4. Her2 (TRAS)-4-1BBL has a lower MFI (**Figure 5A**) and a slightly higher EC₅₀ value (**Table 7**) than
 30 Her2 (PER)-4-1BBL and Her2 (aff-PER)-4-1BBL, reflecting the lower affinity of trastuzumab (TRAS) compared to pertuzumab (PER). This is only shown for the monovalent Her2 binding constructs as the bivalent Her2-binding Her2 (TRAS) huIgG1 PG LALA and Her2 (PER)

huIgG1 PG LALA molecules (displaying avidity) binding similar (**Figure 5B** and **Table 7**). The bivalent Her2-binding molecule Her2 (TRAS)-anticalin-4-1BB huIgG4 showed a lower MFI than Her2 (Tras)-4-1BBL (Figure 5A) but lower EC₅₀ (Table 6) – this reflects the difference between affinity (monovalent binding) and avidity (bivalent binding) of the two Her2 (TRAS)-
 5 targeted molecules.

Table 7: EC₅₀ values of binding curves to Her2⁺ tumor cell line KPL-4 shown in Figure 5

	EC ₅₀ [nM] on KPL-4 cells
Her2 (PER)-4-1BBL	0.23
Her2 (aff-PER)-4-1BBL	0.35
Her2 (TRAS)-4-1BBL	0.54
Her2 (TRAS)-anticalin 4-1BB huIgG4	0.34
Her2 (PER) huIgG1 PGLALA	0.12
Her2 (TRAS) huIgG1 PGLALA	0.16

3.2 Biological Activity Assays

3.2.1 NFκB activation in Jurkat cells expressing human 4-1BB and a NFκB-luc 10 reporter cassette

Jurkat-hu4-1BB-NFκB-luc2 reporter cell line was ordered and received from Promega (CS196004) and cultured in RPMI 1640 (GIBCO by Life Technologies, Cat.-No. 42401-042) supplied with 10 % FCS (GIBCO by Life Technologies, Cat.-No. 16000-044), 2 mM GlutaMAX-I (GIBCO by Life Technologies, Cat.-No. 35050-038), 1 mM Sodium-Pyruvate
 15 (SIGMA-Aldrich, Cat.-No. S8636), 0.1 mM MEM-non essential amino acid solution (SIGMA-Aldrich, Cat.-No. M7145), 25 mM HEPES (Sigma Life Science, Cat.-No. H0887), 600 µg/ml G-418 (Roche, Cat.-No. 04727894001) and 400 µg/ml Hygromycin B (Roche, Cat.-No. 10843555001). To set up the activation assay, Jurkat-hu4-1BB-NFκB-luc2 were resuspended in RPMI 1640 supplied with 10 % FCS and 2 mM GlutaMAX-I (further referred as assay medium)
 20 and 20'000 cells in 100 µL were seeded in each well of a tissue-culture treated flat bottom white 96-well plate (Huber lab, greiner bio-one Cat.-No. 655083) or 2'000 cells in 10 µL were seeded in each well of a tissue-culture treated flat bottom white 384-well plate (Corning, Cat.-No. 3826). Afterwards 50 µL (96-well plate) or 10 µL (384-well plate) of either assay medium or

Her2-expressing tumor cells (either SK-Br3, KLP-4 or NCI-N87, 100'000 cells/well in 96-well plate or 10'000 cells/well in 384-well plate) were added. Finally 50 μ L (96-well plate) or 10 μ L (384-well plate) assay medium containing different titrated concentrations of monovalent Her2-targeting split trimeric 4-1BB ligand Fc fusion antigen binding molecules called Her2 (PER)-4-1BBL, Her2(aff-PER)-4-1BBL or Her2 (TRAS)-4-1BBL or non-targeted control DP47-4-1BBL or anti-human 4-1BB antibodies 20H4.9 huIgG4 or MOR-7480 huIgG2 or the fusion proteins Her2 (TRAS)-anticalin-4-1BB huIgG4 or its untargeted control DP47-anticalin-4-1BB huIgG4 or the control antibodies Her2 (TRAS) huIgG1 P329G LALA or Her2 (PER) huIgG1 P329G LALA or DP47 huIgG1 P329G LALA were added. Plates were incubated for 6 h at 37°C and 5% CO₂ in a cell culture incubator.

To detect luciferase activity using 96-well plates, plates were washed twice with 200 μ L/well DPBS. 40 μ L fresh prepared Reporter Lysis Buffer (Promega, Cat-No: E3971) were added to each well and the plate were stored over night at -20 °C. The next day frozen plates and detection buffer (Luciferase 1000 Assay System, Promega, Cat. No. E4550) were thawed to room temperature. 100 μ L of detection buffer were added to each well and plates were measured as fast as possible using a Tecan microplate reader (Tecan) with 500ms integration time, no filter, collecting all wave length and top reading.

To detect luciferase activity using 384-well plates, to each well freshly to RT thawed 6 μ L One-Glo Luciferase (Promega, Cat.-No. E6110) were added and plates were measured as fast as possible using a Tecan microplate reader (Tecan) with 500 ms integration time, no filter, collecting all wave length and top reading. Light emission emitting due to luciferase-mediated Luciferin oxidation was detected as units of released light (URLs). Data was analyzed using Microsoft Excel and Graph Pad Prism Version 6 (Graph Pad Software Inc.).

In **Figure 6**, the setup of the Jurkat-hu4-1BB-NF κ B-luc2 activation assay is illustrated. As shown in **Figures 7A to 7F**, Her2 (PER)-4-1BBL and Her2 (aff-PER)-4-1BBL display a similar activity in the presence of Her2-expressing tumor cells (**Figure 7B and 7C**), whereas the fusion protein Her2(TRAS)-anticalin 4-1BB huIgG1 shows less activity potential – displayed in less area under the curve as well as a higher EC₅₀ value listed in **Table 8**. All three Her2 targeted 4-1BB agonistic molecules do not induce any activation of the reporter cell line Jurkat-hu4-1BB-NF κ B-luc2 in the absence of Her2-expressing cells (**Figure 7A**). Only the agonistic anti-human 4-1BB clone 20H4.9 huIgG4 was able to induce Jurkat-hu4-1BB-NF κ B-luc2 activation – independent of the presence or absence of Her2-expressing tumor cells (**Figures 7D, 7E and 7F**).

Table 8: EC₅₀ values of NF κ B-activation-induced Luciferase activity-curves shown in Figure 7

	Her2(PER)-4-1BBL	Her2 (aff-PER)-4-1BBL	Her2 (TRAS)-anticalin-4-1BB huIgG4	Anti-hu 4-1BB clone 20H4.9 huIgG4
EC ₅₀ [nM] with no Her2 ⁺ cells	n.d.	n.d.	n.d.	0.16
EC ₅₀ [nM] on SK-Br3 cells	0.02	0.03	0.23	0.20
EC ₅₀ [nM] on NCI-N87 cells	0.03	0.05	0.32	0.14

In **Figures 8A to 8D**, Her2 (PER)-4-1BBL is compared with Her2 (TRAS)-4-1BBL. Due to the lower affinity binder trastuzumab (TRAS) the Her2 (TRAS)-4-1BBL displays less activity than Her2 (PER)-4-1BBL (**Figures 8B, 8C and 8D**). This is displayed in a lower area under the curve and a higher EC₅₀ value as displayed in **Table 9**. In the absence of Her2⁺ tumor cells both molecules did not activate the reporter cell line Jurkat-hu4-1BB-NFkB-luc2 (**Figure 8A**). Again the fusion protein Her2 (TRAS)-anticalin 4-1BB huIgG1 shows less activity than Her2 (PER)-4-1BBL or Her2 (TRAS)-4-1BBL shown by lower area under the curve, lower plateau values and a higher EC₅₀ (shown in **Figure 8B-8D** and **Table 9**). Similar as in Figures 7, also in this experiment only the agonistic anti-human 4-1BB clone 20H4.9 huIgG4 was able to induce Jurkat-hu4-1BB-NFkB-luc2 activation – independent of the presence or absence of Her2-expressing tumor cells (**Figures 8E-8H**).

Table 9: EC₅₀ values of NFkB-activation-induced Luciferase activity-curves shown in Figure 8

EC ₅₀ [nM]	Her2(PER)-4-1BBL	Her2 (TRAS)-4-1BBL	Her2 (TRAS)-anticalin-4-1BB huIgG4	Anti-hu 4-1BB clone 20H4.9 huIgG4
no Her-2 ⁺ cells	n.d.	0.02	0.02	0.07
SK-Br3	0.01	0.07	0.28	0.01
KPL-4	0.01	0.13	0.62	0.02
NCI-N87	0.02	0.16	0.43	0.02

3.2.2 Activation assay of human PBMCs in the presence of Her2-expressing tumor cell line NCI-N87

For Her2-binding-mediated crosslinking the Her2-expressing adherent human gastric carcinoma cell line NCI-N87 was used. NCI-N87 cells were washed with DPBS (Gibco by Life Technologies, Cat. No. 14190 326) and treated with enzyme-free, PBS-based Cell Dissociation Buffer (Gibco by Life Technologies, Cat.-No. 13151-014) for 15 minutes at 37 °C. Cells were

harvested and resuspended in T cell medium consisting of RPMI 1640 supplied with 10 % FCS, 2 mM GlutaMAX-I, 1 mM Sodium-Pyruvate (SIGMA-Aldrich, Cat.-No. S8636), 1% MEM-non essential Aminoacid Solution (SIGMA-Aldrich, Cat.-No. M7145) and 50 μ M β -Mercaptoethanol (Sigma-Aldrich, Cyt.-No. M3148) and irradiated with 50 Gy (X-Ray Irradiator RS 2000, Rad
5 source).

2 x 10⁴ NCI-N87 cells in 50 μ L T cell medium were seeded to each well of a round bottom tissue culture 96-well plate (TTP, Cat.-No. 92697). 50 μ L of T cell medium containing different titrated concentrations of human monovalent Her2-targeting split trimeric 4-1BB ligand Fc fusion antigen binding molecules Her2 (PER)-4-1BBL, Her2(aff-PER)-4-1BBL or Her2
10 (TRAS)-4-1BBL or non-targeted control molecule DP47-4-1BBL or agonistic anti-human 4-1BB antibodies anti-human 4-1BB clone 20H4.9 huIgG4 or anti-human 4-1BB clone MOR-7480 huIgG2 or fusion proteins Her2 (TRAS)-anticalin-4-1BB huIgG4 or its untargeted control DP47-anticalin-4-1BB huIgG4 or the control antibodies Her2 (TRAS) huIgG1 P329G LALA, Her2
15 (PER) huIgG1 P329G LALA or DP47 huIgG1 P329G LALA were added. Human PBMCs isolated from a buffy coat of a healthy donor were labeled in 37 °C warm DPBS containing 40 nM CFDA-SE (SIGMA-Aldrich, Cat.-No. 21888-25MG-F) for 15 min at 37°C. CFSE-labeling was stopped by adding fetal bovine serum (FBS), PBMCs were washed twice and resuspended in T cell medium to a final concentration of 1.5 x 10⁶ cells/mL. 50 μ L of this PBMC cell solution were seeded to each well to add 7.5 x 10⁴ CFSE-labeled PBMCs well. Finally a stock solution of
20 T cell medium containing 8 nM agonistic anti-human CD3 human IgG1 clone V9 was prepared and 50 μ L/well were added to each well giving a final concentration of 2 nM anti-human CD3 human IgG1 clone V9.

Plates were incubated for 4 days at 37°C and 5% CO₂ in a cell incubator. Cells were washed with DPBS and stained with 100 μ L/well DPBS containing 1:1000 diluted LIVE/DEAD
25 Fixable Aqua Dead Cell Stain Kit (Molecular Probes by Life Technology, Cat.-No. L34957) for 30 min at 4°C. Cells were washed once with 200 μ L/well DPBS and stained with 50 μ L FACS buffer (DPBS supplied with 2 % FBS, 5 mM EDTA pH8 (Amresco, Cat. No. E177) and 7.5 mM Sodium azide (Sigma-Aldrich S2002)) containing 0.1 μ g/mL PerCP-Cy5.5-conjugated anti-human CD137 mouse IgG1 κ (clone 4B4-1, BioLegend, Cat.-No. 309814), 0.1 μ g/mL PE/Cy7-
30 conjugated anti-human PD-1 mouse IgG1 κ (clone EH12.2H7, BioLegend, Cat.-No. 329918), 0.03 μ g/mL APC-conjugated anti-human CD25 mouse IgG1 κ (clone BC96, BioLegend, Cat.-No. 302610), 0.06 μ g/mL APC/Cy7-conjugated anti-human CD8 Mouse IgG1 κ (clone RPA-T8, BioLegend, Cat.-No. 3301016), BV421-conjugated anti-human CD4 Mouse IgG1 κ (clone RPA-T4, BioLegend, Cat.-No. 300532) for 30 min at 4°C. Cells were washed twice with 200 μ L/well
35 DPBS and incubated for 30 min at 4°C with 50 μ L/well freshly prepared FoxP3 Perm/Fix buffer (eBioscience Cat.-No. 00-5123). Cells were washed twice with 200 μ L/well DPBS, resuspended in 50 μ L/well freshly prepared Perm-buffer (eBioscience Cat.-No 00-8333) supplied with PE-

conjugated 1:250 diluted anti-human Granzyme B mouse IgG1 κ (clone GB11, Lot 4269803, BD Pharmingen, Cat.-No. 561142) and incubated for 1 h at 4°C. Plates were washed twice with 200 μ L/well DPBS and cells were fixed for 15 min with DPBS containing 1 % Formaldehyde (Sigma, HT501320-9.5L). Cells were resuspended in 100 μ L/well FACS-buffer and acquired using the MACS Quant Analyzer X (Miltenyi Biotech). Data was analyzed using FlowJo V10 (FlowJo, LLC), Microsoft Excel and GraphPad Prism 6 (GraphPad Software, Inc).

In **Figure 9**, the setup of the PBMC activation assay is illustrated. As shown in **Figures 10A to 10F** and **Figures 11A to 11F**, Her2 (PER)-4-1BBL induced the strongest activation of CD8 and CD4 T cells indicated by the upregulation of IL-2R α (CD25) (**Figure 10A** and **11A**), intracellular increase of granzyme B (**Figure 10B** and **11B**) and increased proliferation (**Figure 10C** and **11C**). Her2 (TRAS)-4-1BBL induced as well a strong activation of CD8 (**Figures 10A-10C**) and CD4 T cells (**Figures 11A-11C**), however with higher EC₅₀ values. The fusion polypeptide Her2(TRAS)-anticalin 4-1BB huIgG4 mediated less T cell activation displayed mainly in much lower frequency of CD25⁺, Granzyme B^{high} and proliferating CD8 (**Figures 10A-10C**) and CD4 T cells (**Figures 11A-11C**). The agonistic anti-human 4-1BB clone 20H4.9 huIgG4 antibody displays again some activation potential, however not as potent as the Her2-targeted 4-1BB agonist polypeptides (**Figures 10D-10F** and **Figures 11D-11F**). EC₅₀ values and area under the curve values above background are listed in **Table 10** and **Table 11**.

Table 10: EC₅₀ values of CD8 and CD4 T cell activation curves shown in Figure 10 and 11

EC ₅₀ [nM] PBMC activation assay	Her2(PER)- 4-1BBL	Her2 (TRAS)- 4-1BBL	Her2 (TRAS)- anticalin-4-1BB huIgG4	Anti-hu 4-1BB clone 20H4.9 huIgG4	Anti-hu 4-1BB clone MOR-7480 hu IgG2
% CD25 ⁺ CD8	0.01	0.06	n.d.	n.d.	n.d.
% GnzB ^{high} CD8	0.02	0.19	0.19	n.d.	n.d.
% proliferating CD8	0.01	0.04	0.05	~0.03	n.d.
% CD25 ⁺ CD4	0.02	0.08	n.d.	n.d.	n.d.
% GnzB ^{high} CD4	0.01	0.19	0.21	n.d.	n.d.
% proliferating CD4	0.02	0.04	0.05	0.01	0.13

20

Table 11: Area under the curve above background values of CD8 and CD4 T cell activation curves shown in Figure 10 and 11

AUC above background	Her2(PER)- 4-1BBL	Her2 (TRAS)- 4-1BBL	Her2 (TRAS)- anticalin-4-1BB huIgG4	Anti-hu 4-1BB clone 20H4.9 huIgG4	Anti-hu 4-1BB clone MOR-7480 hu IgG2
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% CD25 ⁺ CD8	10	8	4	n.d.	n.d.
% GnzB ^{high} CD8	122	63	58	n.d.	n.d.
% proliferating CD8	38	27	13	16	0.1
% CD25 ⁺ CD4	81	64	15	n.d.	n.d.
% GnzB ^{high} CD4	55	27	23	n.d.	n.d.
% proliferating CD4	25	20	12	10	12

3.2.3 Activation assay of mouse splenocytes in the presence of Her2-expressing tumor cell line KPL-4

For Her2-binding-mediated crosslinking the Her2-expressing adherent human breast cancer cell line KPL-4 was used. KPL-4 cells were washed with DPBS (Gibco by Life Technologies, Cat. No. 14190 326) and treated with enzyme-free, PBS-based Cell Dissociation Buffer (Gibco by Life Technologies, Cat.-No. 13151-014) for 15 minutes at 37 °C. Cells were harvested and resuspended in T cell medium consisting of RPMI 1640 supplied with 10 % FCS, 2 mM GlutaMAX-I, 1 mM Sodium-Pyruvate (SIGMA-Aldrich, Cat.-No. S8636), 1% MEM-non essential Aminoacid Solution (SIGMA-Aldrich, Cat.-No. M7145) and 50 µM β-Mercaptoethanol (Sigma-Aldrich, Cyt.-No. M3148) and irradiated with 50 Gy (X-Ray Irradiator RS 2000, Rad source).

2 x 10⁴ irradiated KPL-4 cells in 50 µL T cell medium were seeded to each well of a round bottom tissue culture 96-well plate (TTP, Cat.-No. 92697). 50 µL of T cell medium containing different titrated concentrations of mouse surrogate mu4-1BB-Her2 or the untargeted control mu4-1BB muIgG1 DAPG were added. Mouse splenocytes were isolated from freshly collected spleens of C57BL/6 mice (Octo Dissociator, Miltenyi Biotech following manufacture's protocol). Afterwards erythrocytes were lysed by an incubation for 10 min at room temperature in ACK lysis buffer (0.15M NH₄CL, 10 mM KHCO₃, 0.1mM EDTA in ddH₂O, pH 7.2). Lysis was stopped by adding T cell medium and cells were washed with DPBS. Mouse splenocytes were labeled by incubation in 37 °C warm DPBS containing 0.5 µM CellTrace violet proliferation dye (Molecular Probes by Life Technologies, Cat.-No. C34557) for 15 min at 37°C. Labeling was stopped by adding fetal bovine serum (FBS), mouse splenocytes were washed twice and resuspended in T cell medium to a final concentration of 3 x 10⁶ cells/mL. 50 µL of this mouse splenocyte cell solution were seeded to each well to add 15 x 10⁴ violet proliferation dye-labeled mouse splenocytes per well. Finally a stock solution of T cell medium containing 2 µg/mL agonistic anti-mouse CD3ε armenian hamster IgG1 clone 1452C11 (BioLegend Cat.-No. 100331) was prepared and 50 µL/well were added to each well giving a final concentration of 0.5 µg/mL agonistic anti-mouse CD3ε armenian hamster IgG1 clone 1452C11.

Plates were incubated for 3 days at 37°C and 5% CO₂ in a cell incubator. Cells were washed with DPBS and stained with 100 µL/well DPBS containing 1:1000 diluted LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Molecular Probes by Life Technology, Cat.-No. L34957) for 30 min at 4°C. Cells were washed once with 200 µL/well DPBS and stained with 50 µL FACS buffer (DPBS supplied with 2 % FBS, 5 mM EDTA pH8 (Amresco, Cat. No. E177) and 7.5 mM Sodium azide (Sigma-Aldrich S2002)) containing 0.67 µg/mL anti-mouse CD8a-APC-Cy7 (BioLegend, Cat.-No. 100714, clone 53-6.7, rat IgG2a κ), 0.67 µg/mL anti-mouse CD4-APC (BioLegend, Cat.-No. 100412 clone GK1.5, rat IgG2b, κ), 0.67 µg/mL anti-mouse CD137-PE (BioLegend, Cat.-No. 106106, clone 17B5, Syrian Hamster IgG), 0.67 µg/mL anti-mouse CD25-PerCP-Cy5.5 (BioLegend, Cat.-No. 101912, clone 3C7, Rat IgG2b, κ) for 30 min at 4°C. Cells were washed twice with 200 µL/well DPBS and incubated for 30 min at 4°C with 50 µL/well freshly prepared FoxP3 Perm/Fix buffer (eBioscience Cat.-No. 00-5123). Cells were washed twice with 200 µL/well DPBS, resuspended in 50 µL/well freshly prepared Perm-buffer (eBioscience Cat.-No. 00-8333) supplied with 10 µg/mL anti-mouse Eomes-AlexaFluor 488 (eBioscience, Cat.-No. 53-4875-82, clone Dan11mag, ratIgG2a) and incubated for 1 h at 4°C. Plates were washed twice with 200 µL/well DPBS and cells were fixed for 15 min with DPBS containing 1 % Formaldehyde (Sigma, HT501320-9.5L). Cells were resuspended in 100 µL/well FACS-buffer and acquired using the MACS Quant Analyzer X (Miltenyi Biotech). Data was analyzed using FlowJo V10 (FlowJo, LLC), Microsoft Excel and GraphPad Prism 6 (GraphPad Software, Inc).

In **Figure 12**, the setup of the mouse splenocyte activation assay is illustrated. As shown in **Figures 13A to 13D** mouse surrogate mu4-1BB-Her2 induced activation of CD8 and CD4 T cells indicated by the upregulation of IL-2Rα (CD25) (**Figure 13A** and **13C**) and increased proliferation (**Figure 13B** and **13D**). In contrast, non-targeted agonistic mu4-1BB muIgG1 DAPG did not induce such an activation. Therefore, this synergistic costimulatory effect is strongly dependent on Her2-crosslinking. EC₅₀ values and area under the curve values above background are listed in **Table 12** and **Table 13**.

Table 12: EC₅₀ values of mouse CD8 and CD4 T cell activation curves shown in Figure 13

EC ₅₀ [nM] Mouse splenocyte activation assay	mu4-1BB-Her2
% CD25 ⁺ CD8	0.35
% proliferating CD8	~0.03
% CD25 ⁺ CD4	0.36
% proliferating CD4	~0.1

Table 13: Area under the curve of mouse CD8 and CD4 T cell activation curves shown in Figure 14

AUC Mouse splenocyte activation assay	mu4-1BB muIgG1 DAPG	mu4-1BB-Her2
% CD25 ⁺ CD8	53	64
% proliferating CD8	354	380
% CD25 ⁺ CD4	70	80
% proliferating CD4	208	279

Example 4**5 *In vitro* testing of the combination of Her2(PER)-4-1BBL and Her2/CD3 bispecific antibody**

To test the combination of Her2(PER)-4-1BBL and Her2/CD3 bispecific antibody (Her2 TDB, Junttila et al., 2014), we generated a 4-1BBL bispecific molecule that binds to Her2 using pertuzumab (2C4) derived Fab (Figure 2A). Trastuzumab based Her2/CD3 bispecific antibody (Her2-TDB) binds to domain IV of Her2 (Cho et al., 2003), whereas Her2(PER)-4-1BBL binds to domain II of HER2 (Franklin et al., 2004) non-competitively. Her2 TDB was produced as described in WO 2015/095392 A1. The mouse surrogate is a muIgG2a HER2 TDB with the “knob” arm being murine anti-HER2(hu4D5) and the “hole” arm being chimeric anti-murine CD3(2C11) (Leo et al. Proc Natl Acad Sci USA. 84: 1374-1378, 1987).

15 Human Peripheral blood mononuclear cells (PBMC) and CD8⁺ T cell isolation:

Human PBMC were separated from the blood of healthy volunteers using Lymphoprep medium (STEMCELL Technologies). CD8⁺ cells were extracted from PBMC using human CD8⁺ Isolation Kit from Miltenyi Biotec (#130-094-156) by negative selection.

In vitro T cell activation:

20 All antibodies for flow cytometry cell staining were from BD Biosciences (San Jose, CA). Human CD8⁺ cells and human breast cancer cell line SKBR3 cells (in 3:1 ratio) were incubated in the presence of test article for 24 hours in flat-bottom 96 well plate (BD). After incubation, cells were transferred to a new V-bottom 96 well plate. Cells were stained with anti CD8-FITC, anti CD69-PE, and anti CD25-APC. CD69 and CD25 surface expression was detected on CD8⁺ T cells by flow cytometry. The percentage of CD8⁺CD69⁺CD25⁺ was reported as T cell activation.

In vitro target cell killing:

Effector cells (human CD8⁺ cells) and target cells (SKBR3, at density of 20,000 cells per well) were incubated in 3:1 ratio in the presence of test article for 48 hours in black, clear-bottomed 96 well plates. In the end of incubation, cell supernatant was discarded and plates were washed 2x with PBS. 100uL Cell Titer-Glow Luminescent Cell Viability reagent (Promega cat#G7570) was added and plates were read on luminometer as described in the instructions.

In vitro T cell proliferation & survival:

CD8⁺ T cell proliferation response was detected by measuring carboxyfluorescein succinimidyl ester (CFSE) fluorescence intensity dilution. In brief, Human CD8⁺ T cells were labeled with CFSE and co-cultured with SKBR3 target cells and tested articles. HER2 TDB was used at 100 ng/ml and 4-1BB agonist was used at 1000 ng/ml. An aliquot of the cells from day 0 (fresh isolated and labeled CD8⁺), day 3 and day 7 were analyzed by flow cytometry for CFSE fluorescence intensity. The survival of CD8⁺ T cells were measured by number of live CD8⁺ T cells in day 0, day 3 and day 7.

As expected, single agent Her2(PER)-4-1BBL did not induce T cell activation or target cell killing in vitro (Figures 14A and 14B, respectively). Anti-HER2/CD3-TDB induced robust T cell activation and tumor cell killing, but this was not substantially enhanced by co-treatment with Her2(PER)-4-1BBL. In contrast, addition of Her2(PER)-4-1BBL substantially enhanced anti-HER2/CD3-TDB induced T cell proliferation/survival in vitro (Figures 15A and 15B).

Example 5

***In vivo* anti-tumor efficacy of of the combination of mouse surrogate Her2(2C4)-4-1BB and Her2/CD3 bispecific antibody**

Anti-tumor activity of the murine 4-1BB agonists was tested in immune-competent mice that were implanted with human HER2 expressing Fo5 tumor allografts (Lewis Phillips et al., 2008). Single agent HER2 TDB treatment typically results in transient responses in treatment of this model and complete responses are rare even with high dose levels (Li et al., 2018). TDB co-treatment with mu4-1BB Her2 did not result in significant improvement of responses, but four of seven mice (57%) treated with the combination of Her2 TDB and mu4-1BB-Her2 demonstrated complete responses without detectable tumors in the end of study (Figure 16D).

Anti-tumor efficacy - Fo5 tumor allograft model:

A 1 cm incision was made in the skin just rostral to the third mammary fat pad on female FVB WT mice. A pocket for the tumor was made into the No. 2/3 mammary fat pad and a 2x2 mm MMTV-Her2-transgenic Founder #5 (Fo5) tumor section (Lewis Phillips et al., 2008) was placed into the pocket. The skin was closed using wound clips. Wound clips were removed at 7-

10 days post-surgery and mice were monitored for appearance of palpable tumors. When tumor volumes grew to an average of $\sim 190 \text{ mm}^3$, they were placed into treatment cohorts with an equivalent tumor volume average size.

Tumor-bearing mice were divided in groups of 7 animals per group (N=7). One group was treated with 0.5 mg/kg i.v. doses of HER2 TDB twice weekly (qwx2) on days 0, 7 and 14. Another group was treated with 10 mg/kg doses of mu4-1-BB-Her(2C4) (mouse surrogate) twice weekly (qwx2) injected i.v. on day 0 and i.p. on day 7 and a further group was treated with 0.5 mg/kg i.v. doses of Her2 TDB twice weekly (qwx2) on days 0, 7 and 14 and 10 mg/kg doses of mu4-1-BB Her2(2C4) (mouse surrogate) twice weekly (qwx2) injected i.v. on day 0 and i.p. on day 7. The control group was only treated with vehicle.

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