

Description

Title of Invention: IMMUNE ACTIVATING MULTISPECIFIC ANTIGEN-BINDING MOLECULES AND USES THEREOF

Technical Field

[0001] The present invention relates to multispecific antigen-binding molecules for cancer immunotherapy, and methods of using the same.

Background Art

[0002] Antibodies are drawing attention as pharmaceuticals since they are highly stable in plasma and have few side effects. Among multiple therapeutic antibodies, some types of antibodies require effector cells to exert an anti-tumor response. Antibody dependent cell-mediated cytotoxicity (ADCC) is a cytotoxicity exhibited by effector cells against antibody-bound cells via binding of the Fc region of the antibody to Fc receptors present on NK cells and macrophages. To date, multiple therapeutic antibodies that can induce ADCC to exert anti-tumor efficacy have been developed as pharmaceuticals for treating cancer (Nat. Biotechnol. (2005) 23, 1073-1078).

[0003] In addition to the antibodies that induce ADCC by recruiting NK cells or macrophages as effector cells, T cell-recruiting antibodies (TR antibodies) that adopt cytotoxicity by recruiting T cells as effector cells have been known since the 1980s (NPLs 2 to 4). A TR antibody is a bispecific antibody that recognizes and binds to any one of the subunits forming a T-cell receptor complex on T-cells, in particular the CD3 epsilon chain, and an antigen on cancer cells. Several TR antibodies are currently being developed. Catumaxomab, which is a TR antibody against EpCAM, has been approved in the EU for the treatment of malignant ascites. Furthermore, a type of TR antibody called "bispecific T-cell engager (BiTE)" has been recently found to exhibit a strong anti-tumor activity (NPLs 5 and 6). Blinatumomab, which is a BiTE molecule against CD19, received FDA approval first in 2014. Blinatumomab has been proved to exhibit a much stronger cytotoxic activity against CD19/CD20-positive cancer cells in vitro compared with Rituximab, which induces antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (NPL 7).

[0004] However, it is known that a trifunctional antibody binds to both a T-cell and a cell such as an NK cell or macrophage at the same time in a cancer antigen-independent manner, and as a result receptors expressed on the cells are cross-linked, and expression of various cytokines is induced in an antigen-independent manner. Systemic administration of a trifunctional antibody is thought to cause cytokine storm-like side effects as a result of such induction of cytokine expression. In fact, it has been reported that, in the phase I clinical trial, a very low dose of 5 micro g/body was the maximum

tolerance dose for systemic administration of catumaxomab to patients with non-small cell lung cancer, and that administration of a higher dose causes various severe side effects (NPL 8). When administered at such a low dose, catumaxomab can never reach the effective blood level. That is, the expected anti-tumor effect cannot be achieved by administering catumaxomab at such a low dose.

- [0005] In recent years, a modified antibody that causes cytotoxic activity mediated by T cells while circumventing adverse reactions has been provided by use of an Fc region having reduced binding activity against Fc gammaR (PTL 1). Even such an antibody, however, fails to act on two immunoreceptors, i.e., CD3epsilon and Fc gammaR, while binding to the cancer antigen, in view of its molecular structure.

An antibody that exerts both of cytotoxic activity mediated by T cells and cytotoxic activity mediated by cells other than the T cells in a cancer antigen-specific manner while circumventing adverse reactions has not yet been known.

- [0006] Meanwhile, unlike catumaxomab, a bispecific sc(Fv)₂ format molecule (BiTE) which has no Fc gamma receptor-binding site, and therefore it does not cross-link the receptors expressed on T-cells and cells such as NK cells and macrophages in a cancer antigen-independent manner. However, since bispecific sc(Fv)₂ is a modified low-molecular-weight antibody molecule without an Fc region, the problem is that its blood half-life after administration to a patient is significantly shorter than IgG-type antibodies conventionally used as therapeutic antibodies. In fact, the blood half-life of bispecific sc(Fv)₂ administered in vivo has been reported to be about several hours (NPLs 9 and 10). Blinatumomab, a sc(Fv)₂ molecule that binds to CD19 and CD3, has been approved for treatment of acute lymphoblastic leukemia. The serum half-life of blinatumomab has been revealed to be less than 2 hours in patients (NPL 11). In the clinical trials of blinatumomab, it was administered by continuous intravenous infusion using a minipump. This administration method is not only extremely inconvenient for patients but also has the potential risk of medical accidents due to device malfunction or the like. Thus, it cannot be said that such an administration method is desirable.

- [0007] T cells play important roles in tumor immunity, and are known to be activated by two signals: 1) binding of a T cell receptor (TCR) to an antigenic peptide presented by major histocompatibility complex (MHC) class I molecules and activation of TCR; and 2) binding of a costimulator on the surface of T cells to the ligands on antigen-presenting cells and activation of the costimulator. Furthermore, activation of molecules belonging to the tumor necrosis factor (TNF) superfamily and the TNF receptor superfamily, such as CD137(4-1BB) on the surface of T cells, has been described as important for T cell activation (NPL 12). In this regard, CD137 agonist antibodies have already been demonstrated to show anti-tumor effects, and this has been shown experimentally to be mainly due to activation of CD8-positive T cells and

NK cells (NPL 13). It is also understood that T cells engineered to have chimeric antigen receptor molecules (CAR-T cells) which consist of a tumor antigen-binding domain as an extracellular domain and the CD3 and CD137 signal transducing domains as intracellular domains can enhance the persistence of the efficacy (Porter, N ENGL J MED, 2011, 365;725-733 (NPL 14)).

[0008] However, side effects of such CD137 agonist antibodies due to their non-specific hepatotoxicity have been a problem clinically and non-clinically, and development of pharmaceutical agents has not advanced (Dubrot, Cancer Immunol. Immunother., 2010, 28, 512-22 (NPL 15)). The main cause of the side effects has been suggested to involve binding of the antibody to the Fc gamma receptor via the antibody constant region (Schabowsky, Vaccine, 2009, 28, 512-22 (NPL 16)). Furthermore, it has been reported that for agonist antibodies targeting receptors that belong to the TNF receptor superfamily to exert an agonist activity in vivo, antibody crosslinking by Fc gamma receptor-expressing cells (Fc gammaRII-expressing cells) is necessary (Li, Proc Natl Acad Sci USA. 2013, 110(48), 19501-6 (NPL 17)). WO2015/156268 (PTL 2) describes that a bispecific antibody which has a binding domain with CD137 agonistic activity and a binding domain to a tumor specific antigen can exert CD137 agonistic activity and activate immune cells only in the presence of cells expressing the tumor specific antigen.

[0009] Tri-specific antibodies comprising a tumor-specific antigen (EGFR)-binding domain, a CD137-binding domain, and a CD3-binding domain were already reported (WO2014116846). However, since antibodies with such a molecular format can bind to three different antigens at the same time, it was speculated that those tri-specific antibodies could result in cross-linking between CD3 epsilon-expressing T cells and CD137-expressing cells (e.g. T cells, B cells, NK cells, DCs etc.) by binding to CD3 and CD137 at the same time. In this context, an antibody that exerts both cytotoxic activity mediated by T cells and activation activity of T cells and other immune cells via CD137 in a cancer antigen-specific manner while circumventing adverse reactions has not yet been known.

[0010] Glypican-3 (GPC3) is an extracellular matrix protein that is expressed in embryonic tissues, particularly in the liver and kidney, and is involved in organogenesis. Although GPC3 is not expressed in normal tissue cells other than placenta in adult tissues, it is expressed in various cancer tissues, and thus is useful as a target molecule for cancer treatment, a tumor marker, and a diagnostic marker. One therapeutic mAb recognizing residues 524 to 563 of GPC3 has recently been described (NPLs 18 and 19). The monospecific mAb, designated GC33, induced antibody-dependent cellular cytotoxicity (ADCC) and exhibited tumor growth inhibition of subcutaneous transplanted HepG2 and HuH-7 ectopic xenografts in mice. WO2016/047722 (PTL 4) discloses a

bispecific antibody which binds to CD3 and GPC3, and exhibits cytotoxic activity towards cancer cells expressing GPC3.

Citation List

Patent Literature

- [0011] PTL 1: WO2012/073985
 PTL 2: WO2015/156268
 PTL 3: WO2014116846
 PTL 4: WO2016/047722

Non-patent Literature

- [0012] NPL 1: Nat. Biotechnol. (2005) 23, 1073-1078
 NPL 2: Nature. 1985 Apr 18-24;314(6012):628-31.
 NPL 3: Int J Cancer. 1988 Apr 15;41(4):609-15.
 NPL 4: Proc Natl Acad Sci U S A. 1986 Mar;83(5):1453-7.
 NPL 5: Proc Natl Acad Sci U S A. 1995 Jul 18;92(15):7021-5.
 NPL 6: Drug Discov Today. 2005 Sep 15;10(18):1237-44.
 NPL 7: Int J Cancer. 2002 Aug 20;100(6):690-7.
 NPL 8: Cancer Immunol Immunother (2007) 56 (10), 1637-44
 NPL 9: Cancer Immunol Immunother. (2006) 55 (5), 503-14
 NPL 10: Cancer Immunol Immunother. (2009) 58 (1), 95-109
 NPL 11: Nat Rev Drug Discov. 2014 Nov;13(11):799-801.
 NPL 12: Vinay, 2011, Cellular & Molecular Immunology, 8, 281-284
 NPL 13: Houot, 2009, Blood, 114, 3431-8
 NPL 14: Porter, N ENGL J MED, 2011, 365;725-733
 NPL 15: Dubrot, Cancer Immunol. Immunother., 2010, 28, 512-22
 NPL 16: Schabowsky, Vaccine, 2009, 28, 512-22
 NPL 17: Li, Proc Natl Acad Sci USA. 2013, 110(48), 19501-6
 NPL 18: Ishiguro, T. et al., (2008). Cancer research 68, 9832-9838
 NPL 19: Nakano, K. et al., (2009). Biochemical and biophysical research communications 378, 279-284

Summary of Invention

Technical Problem

- [0013] An objective of the present invention is to provide multispecific antigen-binding molecules that can recruit T cells efficiently and specifically to the target cancer cells especially glypican 3 (GPC3)-expressing cells such as cancer cells, and can treat cancer through the cytotoxic activity of T cells against target cancer tissues containing GPC3-expressing cells; methods for producing the antigen-binding molecules; and pharmaceutical compositions comprising the antigen-binding molecules as active in-

gradient. The invention also provides methods to obtain multispecific antigen binding molecules which induce T-cell dependent cytotoxicity more efficiently whilst circumventing adverse toxicity concerns or side effects prior art multispecific antigen-binding molecules that may have.

Solution to Problem

- [0014] Specifically, the present invention provides an antigen-binding molecule comprising: a first antigen-binding moiety that is capable of binding to CD3 and CD137 (4-1BB), but does not bind to CD3 and CD137 at the same time (i.e. dual-binding to CD3 and CD137 but not simultaneously); and a second antigen-binding moiety capable of binding to a molecule specifically expressed in a cancer tissue, specifically Glypican-3 (GPC3).
- [0015] Advantageously, by having a Dual binding capability to CD137 in addition to binding capability to CD3, the multispecific antigen-binding molecule of the present invention exhibits enhanced T-cell dependent cytotoxicity activity contributed by the synergistic co-stimulator CD137 signaling on the CD3 signaling, compared to a T-cell recruiting bispecific antibody which binds to CD3 alone. In addition, since the binding of the antigen-binding molecule to CD3 and CD137 is non-simultaneous (i.e. not binding to CD3 and CD137 at the same time), the simultaneous binding of CD3 and/or CD137 expressed on different immune cells (e.g. T cells) by the same antigen-binding molecule will not occur, thereby circumventing systemic toxicity concerns due to undesirable cross-linking between different immune cells which is considered to be responsible for adverse reactions when a conventional multispecific antigen-binding molecule capable of simultaneously binding to CD3 and a second molecule expressed on T cells (e.g. CD137) is administered in vivo.
- [0016] Additionally, by engineering and improving the binding activity to CD137 without adversely affecting the Dual-binding activity CD3 of the antigen-binding molecules of the present invention, the inventors have selected, out of more than 1000 variants, antigen binding molecules comprising specific heavy chain complementarity determining region (HCDRs) or heavy chain variable region (VH) together with specific light chain complementarity determining region (LCDRs) or light chain variable region (VL), that exhibit superior T-cell dependent cytotoxicity activity to tumors in a cancer antigen (GPC3)-dependent manner. In one aspect, the inventors surprisingly found that, by engineering the optimal CD3 and CD137 binding profile, the selected antigen binding molecules exhibit strong T-cell dependent cytotoxicity activity with low toxicity.
- [0017] Finally, a common challenge in the development of multispecific antibodies has been the production of multispecific antibody constructs at a clinically sufficient quantity

and purity, due to the mispairing of antibody heavy and light chains of different specificities upon co-expression, which decreases the yield of the correctly assembled construct and results in a number of non-functional side products from which the desired multispecific antibody may be difficult to separate. In one aspect, by way of careful antibody engineering and molecular format design (including charged mutations in the framework region and/or constant region, VH/VL exchanged, and Fc region selection), the present invention provides multispecific antigen-binding molecules designed for T cell activation and re-direction that combine good anticancer efficacy and low toxicity with favorable stability, manufacturability/produceability and structural homogeneity.

[0018] As a results of all the above efforts, the antigen-binding molecules and pharmaceutical compositions thereof can be used for targeting cells expressing GPC3, for use in immunotherapy for treating various cancers, especially those associated with GPC3 such as GPC3-positive tumors.

[0019] More specifically, the present disclosure provides the followings.

[1] A multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);

wherein the first antigen-binding moiety comprises any one selected from (a1) to (a15) below:

(a1) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 17, the heavy chain CDR 2 of SEQ ID NO: 31, the heavy chain CDR 3 of SEQ ID NO: 45, the light chain CDR 1 of SEQ ID NO: 64, the light chain CDR 2 of SEQ ID NO: 69 and the light chain CDR 3 of SEQ ID NO: 74;

(a2) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 18, the heavy chain CDR 2 of SEQ ID NO: 32, the heavy chain CDR 3 of SEQ ID NO: 46, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a3) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 19, the heavy chain CDR 2 of SEQ ID NO: 33, the heavy chain CDR 3 of SEQ ID NO: 47, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a4) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 19, the heavy chain CDR 2 of SEQ ID NO: 33, the heavy chain CDR 3 of SEQ ID NO: 47, the light chain CDR 1 of SEQ ID NO: 65, the light chain CDR 2 of SEQ ID NO: 70 and the light chain CDR 3 of SEQ ID NO: 75;

(a5) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO:

20, the heavy chain CDR 2 of SEQ ID NO: 34, the heavy chain CDR 3 of SEQ ID NO: 48, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a6) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 22, the heavy chain CDR 2 of SEQ ID NO: 36, the heavy chain CDR 3 of SEQ ID NO: 50, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a7) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 23, the heavy chain CDR 2 of SEQ ID NO: 37, the heavy chain CDR 3 of SEQ ID NO: 51, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a8) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 23, the heavy chain CDR 2 of SEQ ID NO: 37, the heavy chain CDR 3 of SEQ ID NO: 51, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a9) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 24, the heavy chain CDR 2 of SEQ ID NO: 38, the heavy chain CDR 3 of SEQ ID NO: 52, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a10) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 25, the heavy chain CDR 2 of SEQ ID NO: 39, the heavy chain CDR 3 of SEQ ID NO: 53, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a11) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 26, the heavy chain CDR 2 of SEQ ID NO: 40, the heavy chain CDR 3 of SEQ ID NO: 54, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a12) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 26, the heavy chain CDR 2 of SEQ ID NO: 40, the heavy chain CDR 3 of SEQ ID NO: 54, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a13) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 27, the heavy chain CDR 2 of SEQ ID NO: 41, the heavy chain CDR 3 of SEQ ID NO: 55, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a14) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 28, the heavy chain CDR 2 of SEQ ID NO: 42, the heavy chain CDR 3 of SEQ ID NO: 56, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO:

68 and the light chain CDR 3 of SEQ ID NO: 73; and

(a15) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 82, the heavy chain CDR 2 of SEQ ID NO: 83, the heavy chain CDR 3 of SEQ ID NO: 84, the light chain CDR 1 of SEQ ID NO: 65, the light chain CDR 2 of SEQ ID NO: 70 and the light chain CDR 3 of SEQ ID NO: 75.

[1A] The multispecific antigen-binding molecule of [1], wherein the second antigen-binding moiety capable of binding to glypican-3 (GPC3) comprises the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 235, the heavy chain CDR 2 of SEQ ID NO: 244, the heavy chain CDR 3 of SEQ ID NO: 253, the light chain CDR 1 of SEQ ID NO: 268, the light chain CDR 2 of SEQ ID NO: 274 and the light chain CDR 3 of SEQ ID NO: 280.

[1B] The multispecific antigen-binding molecule of any one of [1] or [1A], further comprises a Fc domain.

[1C] The multispecific antigen-binding molecule of [1B], wherein the Fc domain is composed of a first and a second Fc region subunits capable of stable association, and wherein the Fc domain exhibits reduced binding affinity to human Fc gamma receptor, as compared to a native human IgG1 Fc domain.

[1D] The multispecific antigen-binding molecule of [1C], wherein the first Fc-region subunit is selected from the group comprising:

(c1) a Fc region polypeptide comprising Ala at position 234 and Ala at position 235;

(c2) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, and Ala at position 297; and

(c3) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, Ala at position 297, Cys at position 354 and Trp at position 366; and

wherein the second Fc-region polypeptide is selected from the group comprising:

(c4) a Fc region polypeptide comprising Ala at position 234 and Ala at position 235;

(c5) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, and Ala at position 297; and

(c6) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, Ala at position 297, Cys at position 349, Ser at position 366, Ala at position 368 and Val at position 407; and

wherein the amino acid positions are numbered using EU index numbering.

[1E] The multispecific antigen-binding molecule of any one of [1B] to [1D], wherein the Fc domain is a IgG Fc domain, preferably a human IgG Fc domain, more preferably a human IgG1 Fc domain.

[2] The multispecific antigen-binding molecule of any one of [1] to [1E], wherein the first antigen binding moiety comprises any one selected from (a1) to (a15) below:

(a1) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO:

3, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 59;

(a2) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 4, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a3) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 5, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a4) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 5, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 60;

(a5) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 6, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a6) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 8, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a7) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 9, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a8) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 9, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;

(a9) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 10, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a10) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 11, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;

(a11) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 12, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;

(a12) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 12, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a13) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 13, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a14) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 14, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58; and

(a15) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 81, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 60.

[3] The multispecific antigen-binding molecule of any one of [1] to [2], wherein the second antigen binding moiety comprises a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 226 and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 262.

[4] The multispecific antigen-binding molecule of any one of [1B] to [3], wherein the Fc domain comprises a first Fc subunit shown in SEQ ID NO: 317 and a second Fc subunit shown in SEQ ID NO: 323.

[5] The multispecific antigen-binding molecule of any one of [1] to [4], wherein each of the first and the second antigen binding moiety is a Fab molecule.

[6] The multispecific antigen-binding molecule of [5], wherein the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of either one of the first or second subunit of the Fc domain, and the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the remaining subunit of the Fc domain.

[7] The multispecific antigen-binding molecule of [5] or [6], wherein the second antigen binding moiety is a crossover Fab molecule in which the variable regions of the Fab light chain and the Fab heavy chain are exchanged and which comprises a heavy chain variable region and a light chain variable region, and wherein the first antigen binding moiety is a conventional Fab molecule which comprises a heavy chain variable region (VH) and a light chain variable region (VL).

[8] The multispecific antigen-binding molecule of [7], wherein, in the constant domain CL of the light chain of the first antigen binding moiety, the amino acid(s) at position 123 and/or 124 is/are substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat), and wherein in the constant domain CH1 of the heavy chain of the first antigen binding moiety, the amino acid at position 147 and/or the amino acid at position 213 is substituted independently by glutamic acid (E) or aspartic acid (D) (numbering according to Kabat EU index).

[8A] The multispecific antigen-binding molecule of [8], wherein, in the constant domain CL of the light chain of the first antigen binding moiety, the amino acids at position 123 and 124 are arginine (R) and lysine (K) respectively (numbering according to Kabat), and wherein in the constant domain CH1 of the heavy chain of the first antigen binding moiety, the amino acids at position 147 and 213 are glutamic acid

(E) (numbering according to Kabat EU index).

[9] The multispecific antigen-binding molecule of any one of [8] to [8A], which comprises four polypeptides in any one of the combination selected from (a1) to (a6) below:

(a1) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 219 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a2) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 220 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a3) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 291 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a4) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 292 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a5) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 293 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4); and

(a6) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 294 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

and wherein, preferably the four polypeptide chains (chain 1 to chain 4) connect and/or associate with each other according to the orientation shown in Figure 2.

[10] An isolated polynucleotide or plurality of polynucleotides encoding the multi-specific antigen-binding molecule of any one of [1] to [9].

[11] A vector encoding the polynucleotide or plurality of polynucleotides of [10].

[12] A host cell comprising the polynucleotide or plurality of polynucleotides of [10], or the vector of [11].

[13] A method of producing the multispecific antigen-binding molecule of any one of [1] to [9C], comprising the steps of:

a) culturing the host cell of [12] under conditions suitable for the expression of the antigen-binding molecule and

b) recovering the antigen-binding molecule.

[13A] A multispecific antigen-binding molecule produced by the method of [13].

[14] A pharmaceutical composition comprising the multispecific antigen-binding molecule of any one of [1] to [9] and a pharmaceutically acceptable carrier.

[15] The multispecific antigen-binding molecule of any one of [1] to [9] or the pharmaceutical composition of [14], which induces cytotoxicity, preferably T-cell-dependent cytotoxicity.

[16] The multispecific antigen-binding molecule of any one of [1] to [9] or the pharmaceutical composition of [14], for use as a medicament.

[17] The multispecific antigen-binding molecule of any one of [1] to [9] or the pharmaceutical composition of [14], for use in the treatment of a disease in an individual in need thereof.

[18] The multispecific antigen-binding molecule or the pharmaceutical composition of [17], wherein the disease is cancer, preferably GPC3-expressing cancer or GPC3-positive cancer.

[19] Use of the multispecific antigen binding molecule of any one of [1] to [9] or the pharmaceutical composition of [14], for the manufacture of a medicament for the treatment of a disease in an individual in need thereof.

[20] A method of treating a disease in an individual, comprising administering to said individual a therapeutically effective amount of the multispecific antigen binding molecule of any one of [1] to [9] or the pharmaceutical composition of [14].

[21] The use of [19] or the method of [20], wherein said disease is cancer, preferably GPC3-positive cancer or GPC3-expressing cancer.

[22] A method for inducing lysis of a target cell, comprising contacting a target cell with the multispecific antigen binding molecule of any one of [1] to [9] or the pharmaceutical composition of [14] in the presence of a T cell.

[23] A kit comprising the pharmaceutical composition of [14]; and a package insert comprising instructions for administering to a subject to treat or delay progression of cancer, preferably GPC3-positive cancer or GPC3-expressing cancer.

Another aspect of the present invention relates to:

[24] A multispecific antigen-binding molecule comprising four polypeptides with any

one combination selected from (a1) to (a6) below:

(a1) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 219 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a2) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 220 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a3) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 291 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a4) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 292 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a5) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 293 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4); and

(a6) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 294 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

and wherein, preferably the four polypeptide chains (chain 1 to chain 4) connect and/or associate with each other according to the orientation shown in Figure 2.

Yet another aspect of the present invention relates to:

[25] An antigen-binding molecule comprising the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 82, the heavy chain CDR 2 of SEQ ID NO: 83, the heavy chain CDR 3 of SEQ ID NO: 84, the light chain CDR 1 of SEQ ID NO: 65, the light chain CDR 2 of SEQ ID NO: 70 and the light chain CDR 3 of SEQ ID

NO: 75.

[26] An antigen-binding molecule comprising a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 81, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 60.

Yet another aspect of the present invention relates to:

[27] A multispecific antigen-binding molecule comprising:

(i) a first antigen-binding moiety that binds to human CD3; and

(ii) a second antigen-binding moiety that binds to human glypican-3 (GPC3);

wherein the first antigen-binding moiety comprises any one selected from (a1) to (a15) below:

(a1) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 17, the heavy chain CDR 2 of SEQ ID NO: 31, the heavy chain CDR 3 of SEQ ID NO: 45, the light chain CDR 1 of SEQ ID NO: 64, the light chain CDR 2 of SEQ ID NO: 69 and the light chain CDR 3 of SEQ ID NO: 74;

(a2) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 18, the heavy chain CDR 2 of SEQ ID NO: 32, the heavy chain CDR 3 of SEQ ID NO: 46, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a3) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 19, the heavy chain CDR 2 of SEQ ID NO: 33, the heavy chain CDR 3 of SEQ ID NO: 47, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a4) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 19, the heavy chain CDR 2 of SEQ ID NO: 33, the heavy chain CDR 3 of SEQ ID NO: 47, the light chain CDR 1 of SEQ ID NO: 65, the light chain CDR 2 of SEQ ID NO: 70 and the light chain CDR 3 of SEQ ID NO: 75;

(a5) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 20, the heavy chain CDR 2 of SEQ ID NO: 34, the heavy chain CDR 3 of SEQ ID NO: 48, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a6) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 22, the heavy chain CDR 2 of SEQ ID NO: 36, the heavy chain CDR 3 of SEQ ID NO: 50, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a7) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 23, the heavy chain CDR 2 of SEQ ID NO: 37, the heavy chain CDR 3 of SEQ ID NO: 51, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a8) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 23, the heavy chain CDR 2 of SEQ ID NO: 37, the heavy chain CDR 3 of SEQ ID NO: 51, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a9) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 24, the heavy chain CDR 2 of SEQ ID NO: 38, the heavy chain CDR 3 of SEQ ID NO: 52, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a10) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 25, the heavy chain CDR 2 of SEQ ID NO: 39, the heavy chain CDR 3 of SEQ ID NO: 53, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a11) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 26, the heavy chain CDR 2 of SEQ ID NO: 40, the heavy chain CDR 3 of SEQ ID NO: 54, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a12) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 26, the heavy chain CDR 2 of SEQ ID NO: 40, the heavy chain CDR 3 of SEQ ID NO: 54, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a13) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 27, the heavy chain CDR 2 of SEQ ID NO: 41, the heavy chain CDR 3 of SEQ ID NO: 55, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a14) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 28, the heavy chain CDR 2 of SEQ ID NO: 42, the heavy chain CDR 3 of SEQ ID NO: 56, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73; and

(a15) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 82, the heavy chain CDR 2 of SEQ ID NO: 83, the heavy chain CDR 3 of SEQ ID NO: 84, the light chain CDR 1 of SEQ ID NO: 65, the light chain CDR 2 of SEQ ID NO: 70 and the light chain CDR 3 of SEQ ID NO: 75;

and (iii) further comprises a Fc domain composed of a first and a second Fc region subunits capable of stable association, and wherein the Fc domain exhibits reduced binding affinity to human Fc gamma receptor, as compared to a native human IgG1 Fc domain;

wherein the first Fc region subunit is a Fc region polypeptide comprising Ala at position 234, Ala at position 235, Ala at position 297, Cys at position 354 and Trp at

position 366; and

wherein the second Fc-region polypeptide is a Fc region polypeptide comprising Ala at position 234, Ala at position 235, Ala at position 297, Cys at position 349, Ser at position 366, Ala at position 368 and Val at position 407; and

wherein the amino acid positions are numbered using EU index numbering.

[28] The multispecific antigen-binding molecule of [27], wherein the first antigen binding moiety comprises any one selected from (a1) to (a15) below:

(a1) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 3, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 59;

(a2) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 4, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a3) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 5, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a4) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 5, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 60;

(a5) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 6, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a6) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 8, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a7) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 9, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a8) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 9, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;

(a9) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 10, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a10) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 11, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;

(a11) a heavy chain variable region comprising an amino acid sequence of SEQ ID

NO: 12, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;

(a12) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 12, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a13) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 13, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a14) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 14, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58; and

(a15) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 81, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 60.

[29] The multispecific antigen-binding molecule of [27] or [28], wherein the second antigen-binding moiety comprises a heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 235, the heavy chain CDR 2 of SEQ ID NO: 244, the heavy chain CDR 3 of SEQ ID NO: 253, the light chain CDR 1 of SEQ ID NO: 268, the light chain CDR 2 of SEQ ID NO: 274 and the light chain CDR 3 of SEQ ID NO: 280.

[30] The multispecific antigen-binding molecule of any one of [27] to [29], wherein the second antigen binding moiety comprise a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 226 and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 262.

[31] The multispecific antigen-binding molecule of any one of [27] to [30], wherein the Fc domain comprises a first Fc region subunit shown in SEQ ID NO: 317 and a second Fc region subunit shown in SEQ ID NO: 323.

[32] The multispecific antigen-binding molecule of any one of [27] to [31], wherein each of the first and the second antigen binding moiety is a Fab molecule.

[33] The multispecific antigen-binding molecule of [32], wherein the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of either one of the first or second Fc region subunit of the Fc domain, and the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the remaining Fc region subunit of the Fc domain.

[34] The multispecific antigen-binding molecule of [32] or [33], wherein the second antigen binding moiety is a crossover Fab molecule in which the variable regions of the Fab light chain and the Fab heavy chain are exchanged and which comprises a heavy chain variable region (VH) and a light chain variable region (VL), and wherein the first antigen binding moiety is a conventional Fab molecule which comprises a

heavy chain variable region (VH) and a light chain variable region (VL).

[35] The multispecific antigen-binding molecule of [34], wherein in the constant domain CL of the light chain of the first antigen binding moiety, the amino acids at position 123 and 124 are arginine (R) and lysine (K) respectively (numbering according to Kabat), and wherein in the constant domain CH1 of the heavy chain of the first antigen binding moiety, the amino acids at position 147 and 213 are glutamic acid (E) (numbering according to EU numbering).

[36] The multispecific antigen-binding molecule of any one of [27] to [35], comprises four polypeptides in any one of the combination selected from (a1) to (a6) below:

(a1) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 219 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a2) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 220 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a3) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 291 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a4) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 292 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a5) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 293 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4); and

(a6) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 294 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225

(chain 4).

[37] An isolated polynucleotide or plurality of polynucleotides encoding the multi-specific antigen-binding molecule of any one of [27] to [36].

[38] A vector encoding the polynucleotide or plurality of polynucleotides of [37].

[39] A host cell comprising the polynucleotide or plurality of polynucleotides of [37], or the vector of [38].

[40] A method of producing the multispecific antigen-binding molecule of any one of [27] to [36], comprising the steps of:

a) culturing the host cell of [39] under conditions suitable for the expression of the antigen-binding molecule, and

b) recovering the antigen-binding molecule.

[41] A pharmaceutical composition comprising the multispecific antigen-binding molecule of any one of [27] to [36], and a pharmaceutically acceptable carrier.

[42] The multispecific antigen-binding molecule of any one of [27] to [36], or the pharmaceutical composition of [41], which induces cytotoxicity, preferably T-cell-dependent cytotoxicity.

[43] The multispecific antigen-binding molecule of any one of [27] to [36], or the pharmaceutical composition of [41] or [42], for use as a medicament.

[44] The multispecific antigen-binding molecule of any one of [27] to [36], or the pharmaceutical composition of [41] or [42], for use in the treatment of cancer, preferably GPC3-expressing cancer or GPC3-positive cancer.

Brief Description of Drawings

[0020] [Fig.1]A drawing showing results of Biacore in-tandem blocking assay assessing non-simultaneous binding towards CD3 and CD137 with AE05 and AE15.

[Fig.2]A drawing schematically depicting various antibody formats with annotation of each component. Diagram (a) depicts 1+1 Bispecific antibodies by utilizing FAST-Ig, and diagram (b) depicts 1+1 Bispecific antibodies by utilizing CrossMab technology.

[Fig.3]A drawing showing results of the measurement of CD3 agonistic activity of affinity matured GPC3/Dual-Ig variants trispecific antibodies. Each graph shows mean Luminescence units +/- standard deviation (s.d.) detected by SK-pca60 cell line co-cultured with NFAT-luc2 Jurkat reporter cells by selected antibodies divided into plate 1 (left) and plate 2 (right). E:T ratio 5 for 24 hours. Antibodies were added at 0.02, 0.2 and 2 nM.

[Fig.4]A drawing showing results of the measurement of CD137 agonistic activity of affinity matured GPC3/Dual-Ig variants trispecific antibodies. Each graph shows mean Luminescence units +/- standard deviation (s.d.) detected by SK-pca60 cell line co-cultured with Jurkat NFkappaB reporter cells overexpressing CD137 by selected an-

tibodies divided into plate 1 (left) and plate 2 (right). E:T ratio 5 for 5 hours. Antibodies were added at 0.5, 2.5 and 5 nM.

[Fig.5]A drawing showing results of measurement of CD137 agonistic activity of affinity matured GPC3/Dual-Ig variants trispecific antibodies. (a) Mean Luminescence units $\pm$ standard deviation (s.d.) detected by SK-pca60 cell line co-cultured with Jurkat NFkappaB reporter cells overexpressing CD137 by a group of the selected antibodies. (b) Similar to (a), mean Luminescence units $\pm$ standard deviation (s.d.) detected by SK-pca60 cell line co-cultured with Jurkat NFkappaB reporter cells overexpressing CD137 by other group of antibodies were analysed in a second plate.

[Fig.6]A drawing showing results of the measurement of cytotoxicity of GPC3/Dual-Ig variants. SK-pca60 was co-cultured with PBMC in the presence of selected GPC3/Dual-Ig trispecific molecules starting at 5 nM of 3-fold serial dilutions. E:T ratio 0.5. Analysed using real-time xCELLigence system. Mean Cell Growth Inhibition (%) values $\pm$ s.d. obtained at approximately 120 h was plotted in each graph shown.

[Fig.7]A drawing showing mean cytotoxicity (cell growth Inhibition (%) values $\pm$ s.d.) of GPC3/Dual-Ig variants. SK-pca60 was co-cultured with PBMC in the presence of selected GPC3/Dual-Ig trispecific molecules at 5 nM and 10 nM, E:T 0.5 and analysed using real-time xCELLigence system. Mean Cell Growth Inhibition (%) values $\pm$ s.d. obtained at 120 h was plotted in graph shown.

[Fig.8]A drawing showing results of the measurement of antigen independent cytokine (IFN gamma) release in PBMC solution. SK-pca60 was co-cultured with PBMC in the presence of selected GPC3/Dual-Ig trispecific molecules starting at 5 nM of 3-fold serial dilutions. E:T ratio 0.5. Supernatant of co-culture was analyzed at 48h timepoint. Graph shows mean concentration $\pm$ s.d. of IFN gamma. Antibodies were divided into plate 1 (upper panel) and plate 2 (lower panel) for evaluation.

[Fig.9]A drawing showing results of the measurement of antigen independent cytokine (IL-2) release in PBMC solution. SK-pca60 was co-cultured with PBMC in the presence of selected GPC3/Dual-Ig trispecific molecules starting at 5 nM of 3-fold serial dilutions. E:T ratio 0.5. Supernatant of co-culture was analyzed at 48h timepoint. Graph shows mean concentration $\pm$ s.d. of IL-2. Antibodies were divided into plate 1 (upper panel) and plate 2 (lower panel) for evaluation.

[Fig.10]A drawing showing results of the measurement of antigen independent cytokine (IL-6) release in PBMC solution. SK-pca60 was co-cultured with PBMC in the presence of selected GPC3/Dual-Ig trispecific molecules starting at 5 nM of 3-fold serial dilutions. E:T ratio 0.5. Supernatant of co-culture was analyzed at 48h timepoint. Graph shows mean concentration $\pm$ s.d. of IL-6. Antibodies were divided into plate 1 (upper panel) and plate 2 (lower panel) for evaluation.

[Fig.11]A drawing showing results of the measurement of TDCC activity of AE05 and

AE15 CrossMab antibodies against SK-pca60 cell line. Cell Growth Inhibition (%). E:T ratio 5. Antibodies were added at 0.008, 0.04, 0.2, 1.0, and 5 nM.

[Fig.12]A drawing schematically depicting design and construction of trispecific antibody, Antibody AB (mAb AB), relative to Antibody A (mAb A) and Antibody B (mAb B).

[Fig.13]A drawing schematically depicting naming rule of the trispecific antibody, Antibody AB (mAb AB).

[Fig.14]A drawing showing results of the measurement of antigen independent Jurkat activation on GPC3 negative cells. Parental CHO were co-cultured with NFAT-luc2 Jurkat reporter cells, E:T 5 for 24h and analysed using LDH assay. Graph depicting Mean Luminescence units +/- standard deviation (s.d.) of different antibody formats incubated at 0.5, 5 and 50 nM.

[Fig.15]A drawing showing results of the measurement of antigen independent Jurkat activation on GPC3 negative cells. CHO cells overexpressing CD137 were co-cultured with NFAT-luc2 Jurkat reporter cells, E:T 5 for 24h. Graph depicting Mean Luminescence units +/- standard deviation (s.d.) of different antibody formats incubated at 0.5, 5 and 50 nM.

[Fig.16]A drawing showing results of the measurement of antigen independent cytokine (IFN gamma) release in PBMC solution. Supernatant of affinity matured GPC3/Dual-Ig variants or GPC3/CD137xCD3 tri-specific antibodies that were added at 3.2, 16 and 80 nM to PBMC solution was analyzed at 48h timepoint. Graph shows mean concentration +/- s.d. of IFN gamma. Antibodies were divided into plate 1 (upper panel) and plate 2 (lower panel) for evaluation.

[Fig.17]A drawing showing results of the measurement of antigen independent cytokine (TNF alpha) release in PBMC solution. Supernatant of affinity matured GPC3/Dual-Ig variants or GPC3/CD137xCD3 tri-specific antibodies that were added at 3.2, 16 and 80 nM to PBMC solution was analyzed at 48h timepoint. Graph shows mean concentration +/- s.d. of TNF alpha. Antibodies were divided into plate 1 (upper panel) and plate 2 (lower panel) for evaluation.

[Fig.18]A drawing showing results of the measurement of antigen independent cytokine (IL-6) release in PBMC solution. Supernatant of affinity matured GPC3/Dual-Ig variants or GPC3/CD137xCD3 tri-specific antibodies that were added at 3.2, 16 and 80 nM to PBMC solution was analyzed at 48h timepoint. Graph shows mean concentration +/- s.d. of IL-6. Antibodies were divided into plate 1 (upper panel) and plate 2 (lower panel) for evaluation.

[Fig.19]A drawing showing results of the measurement of in vivo efficacy of antibodies against sk-pca-13a xenograft in huNOG mice model. Y-axis means the tumor volume (mm³) and X-axis means the days after tumor implantation.

[Fig.20]A drawing showing results of the measurement of in vivo efficacy of antibodies against LLC1/hGPC3 cancer cell line in humanised CD3/CD137 mice model. Y-axis means the tumor volume (mm^3) and X-axis means the days after tumor implantation.

[Fig.21]A drawing showing results of the measurement of plasma IL-6 concentration in mice that were administered each antibody. Mice were bled at 2h after antibody injection and plasma IL-6 concentration was measured using Bio-Plex Pro Mouse Cytokine Th1 Panel.

[Fig.22]A drawing showing results of the measurement of in vivo efficacy of antibodies against LLC1/hGPC3 xenograft in humanised CD3/CD137 mice model. Y-axis means the tumor volume (mm^3) and X-axis means the days after tumor implantation.

[Fig.23]A drawing showing results of the measurement of plasma IL-6 concentration in mice that were administered each antibody. Mice were bled at 2h after antibody injection and plasma IL-6 concentration was measured using Bio-Plex Pro Mouse Cytokine Th1 Panel.

[Fig.24]A drawing showing results of the measurement of in vivo efficacy of antibodies against LLC1/hGPC3 cancer cell line in humanised CD3/CD137 mice model. Y-axis means the tumor volume (mm^3) and X-axis means the days after tumor implantation.

[Fig.25]A drawing showing results of the measurement of in vivo efficacy of antibodies against Hepa1-6/hGPC3 cancer cell line in humanised CD3/CD137 mice model. Y-axis means the tumor volume (mm^3) and X-axis means the days after tumor implantation.

[Fig.26]A drawing showing results of the measurement of plasma concentration of Anti-GPC3/Dual-Fab antibodies on day 4 post injection in efficacy study against Hepa1-6/hGPC3 cancer cell line in humanised CD3/CD137 mice model.

[Fig.27]A drawing showing results of cIEF analysis for FAST-Ig and CrossMab.

Description of Embodiments

- [0021] The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook et al., *Molecular Cloning: A Laboratory Manual* 3d edition (2001) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *Current Protocols in Molecular Biology* (F.M. Ausubel, et al. eds., (2003)); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)), Harlow and Lane, eds. (1988) *Antibodies, A Laboratory Manual*,

and Animal Cell Culture (R.I. Freshney, ed. (1987)); Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Methods in Molecular Biology, Humana Press; Cell Biology: A Laboratory Notebook (J.E. Cellis, ed., 1998) Academic Press; Animal Cell Culture (R.I. Freshney), ed., 1987); Introduction to Cell and Tissue Culture (J. P. Mather and P.E. Roberts, 1998) Plenum Press; Cell and Tissue Culture: Laboratory Procedures (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., 1993-8) J. Wiley and Sons; Handbook of Experimental Immunology (D.M. Weir and C.C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller and M.P. Calos, eds., 1987); PCR: The Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols in Immunology (J.E. Coligan et al., eds., 1991); Short Protocols in Molecular Biology (Wiley and Sons, 1999); Immunobiology (C.A. Janeway and P. Travers, 1997); Antibodies (P. Finch, 1997); Antibodies: A Practical Approach (D. Catty., ed., IRL Press, 1988-1989); Monoclonal Antibodies: A Practical Approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); Using Antibodies: A Laboratory Manual (E. Harlow and D. Lane (Cold Spring Harbor Laboratory Press, 1999); The Antibodies (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and Cancer: Principles and Practice of Oncology (V.T. DeVita et al., eds., J.B. Lippincott Company, 1993).

[0022] The definitions and detailed description below are provided to facilitate understanding of the present disclosure illustrated herein.

[0023] Definitions

Amino acids

Herein, amino acids are described by one- or three-letter codes or both, for example, Ala/A, Leu/L, Arg/R, Lys/K, Asn/N, Met/M, Asp/D, Phe/F, Cys/C, Pro/P, Gln/Q, Ser/S, Glu/E, Thr/T, Gly/G, Trp/W, His/H, Tyr/Y, Ile/I, or Val/V.

[0024] Alteration of Amino Acids

For amino acid alteration (also described as "amino acid substitution" or "amino acid mutation" within this description) in the amino acid sequence of an antigen-binding molecule, known methods such as site-directed mutagenesis methods (Kunkel et al. (Proc. Natl. Acad. Sci. USA (1985) 82, 488-492)) and overlap extension PCR may be appropriately employed. Furthermore, several known methods may also be employed as amino acid alteration methods for substitution to non-natural amino acids (Annu Rev. Biophys. Biomol. Struct. (2006) 35, 225-249; and Proc. Natl. Acad. Sci. U.S.A. (2003) 100 (11), 6353-6357). For example, it is suitable to use a cell-free translation system (Clover Direct (Protein Express)) containing a tRNA which has a non-natural amino acid bound to a complementary amber suppressor tRNA of one of the stop codons, the UAG codon (amber codon).

[0025] In the present specification, the meaning of the term "and/or" when describing the

site of amino acid alteration includes every combination where "and" and "or" are suitably combined. Specifically, for example, "the amino acids at positions 33, 55, and/or 96 are substituted" includes the following variation of amino acid alterations: amino acid(s) at (a) position 33, (b) position 55, (c) position 96, (d) positions 33 and 55, (e) positions 33 and 96, (f) positions 55 and 96, and (g) positions 33, 55, and 96.

[0026] Furthermore, herein, as an expression showing alteration of amino acids, an expression that shows before and after a number indicating a specific position, one-letter or three-letter codes for amino acids before and after alteration, respectively, may be used appropriately. For example, the alteration N100bL or Asn100bLeu used when substituting an amino acid contained in an antibody variable region indicates substitution of Asn at position 100b (according to Kabat numbering) with Leu. That is, the number shows the amino acid position according to Kabat numbering, the one-letter or three-letter amino-acid code written before the number shows the amino acid before substitution, and the one-letter or three-letter amino-acid code written after the number shows the amino acid after substitution. Similarly the alteration P238D or Pro238Asp used when substituting an amino acid of the Fc region contained in an antibody constant region indicates substitution of Pro at position 238 (according to EU numbering) with Asp. That is, the number shows the amino acid position according to EU numbering, the one-letter or three-letter amino-acid code written before the number shows the amino acid before substitution, and the one-letter or three-letter amino-acid code written after the number shows the amino acid after substitution.

[0027] Polypeptides

As used herein, term "polypeptide" refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). The term "polypeptide" refers to any chain of two or more amino acids, and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides, "protein," "amino acid chain," or any other term used to refer to a chain of two or more amino acids, are included within the definition of "polypeptide," and the term "polypeptide" may be used instead of, or interchangeably with any of these terms. The term "polypeptide" is also intended to refer to the products of post-expression modifications of the polypeptide, including without limitation glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, or modification by non-naturally occurring amino acids. A polypeptide may be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including by chemical synthesis. A polypeptide as described herein may be of a size of about 3 or more, 5 or more, 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500

or more, 1,000 or more, or 2,000 or more amino acids. Polypeptides may have a defined three-dimensional structure, although they do not necessarily have such structure. Polypeptides with a defined three-dimensional structure are referred to as folded, and polypeptides which do not possess a defined three-dimensional structure, but rather can adopt a large number of different conformations, and are referred to as unfolded.

[0028] Percent (%) amino acid sequence identity

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide 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 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.

[0029] Recombinant Methods and Compositions

Antibodies and antigen-binding molecules may be produced using recombinant methods and compositions, e.g., as described in U.S. Patent No. 4,816,567. In one embodiment, isolated nucleic acid encoding an antibody as described herein is provided. Such nucleic acid may encode an amino acid sequence comprising the VL and/or an amino acid sequence comprising the VH of the antibody (e.g., the light and/or heavy chains of the antibody). In a further embodiment, one or more vectors (e.g., expression vectors) comprising such nucleic acid are provided. In a further embodiment, a host cell comprising such nucleic acid is provided. In one such embodiment, a host cell comprises (e.g., has been transformed with): (1) a vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and an amino acid sequence comprising the VH of the antibody, or (2) a first vector comprising a nucleic acid that encodes an amino acid sequence comprising the VL of the antibody and a second vector comprising a nucleic acid that encodes an amino acid sequence comprising the VH of the antibody. In one embodiment, the host cell is eukaryotic, e.g. a Chinese Hamster Ovary (CHO) cell or lymphoid cell (e.g., Y0, NS0, Sp2/0 cell). In one embodiment, a method of making the multispecific antigen-binding molecule of the present invention is provided, wherein the method comprises culturing a host cell comprising a nucleic acid encoding the antibody, as provided above, under conditions suitable for expression of the antibody, and optionally recovering the antibody from the host cell (or host cell culture medium).

For recombinant production of an antibody described herein, nucleic acid encoding an antibody, 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 nucleic acid may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody).

[0030] Suitable host cells for cloning or expression of antibody-encoding vectors include prokaryotic or eukaryotic cells described herein. For example, antibodies may be produced in bacteria, in particular when glycosylation and Fc effector function are not needed. For expression of antibody fragments and polypeptides in bacteria, see, e.g., U.S. Patent Nos. 5,648,237, 5,789,199, and 5,840,523. (See also Charlton, *Methods in Molecular Biology*, Vol. 248 (B.K.C. Lo, ed., Humana Press, Totowa, NJ, 2003), pp. 245-254, describing expression of antibody fragments in *E. coli*.) After expression, the

antibody may be isolated from the bacterial cell paste in a soluble fraction and can be further purified.

- [0031] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for antibody-encoding vectors, including fungi and yeast strains whose glycosylation pathways have been "humanized," resulting in the production of an antibody 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).
- [0032] Suitable host cells for the expression of glycosylated antibody 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.
- [0033] 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).
- [0034] 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 293 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 (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 Y0, NS0 and Sp2/0. For a review of certain mammalian host cell lines suitable for antibody 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).
- [0035] Recombinant production of an antigen-binding molecule described herein could be done with methods similar to those described above, by using a host cell comprises (e.g., has been transformed with) one or plural vectors comprising nucleic acid that encodes an amino acid sequence comprising the whole antigen-binding molecule or part of the antigen-binding molecule.
- [0036] Antigen-binding molecules and multispecific antigen-binding molecules

The term "antigen-binding molecule", as used herein, refers to any molecule that comprises an antigen-binding site or any molecule that has binding activity to an antigen, and may further refers to molecules such as a peptide or protein having a length of about five amino acids or more. The peptide and protein are not limited to those derived from a living organism, and for example, they may be a polypeptide produced from an artificially designed sequence. They may also be any of a naturally-occurring polypeptide, synthetic polypeptide, recombinant polypeptide, and such. Scaffold molecules comprising known stable conformational structure such as alpha/beta barrel as scaffold, and in which part of the molecule is made into antigen-binding site, is also one embodiment of the antigen binding molecule described herein.

- [0037] "Multispecific antigen-binding molecules" refers to antigen-binding molecules that bind specifically to more than one antigen. The term "bispecific" means that the antigen binding molecule is able to specifically bind to at least two distinct antigenic determinants. The term "trispecific" means that the antigen binding molecule is able to specifically bind to at least three distinct antigenic determinants.
- [0038] In certain embodiments, the multispecific antigen binding molecule of the present application is a trispecific antigen binding molecule, i.e. capable of specifically binding to three different antigens - capable of binding to either one of CD3 or CD137 but does not bind to both antigens simultaneously, and is capable of specifically binding to GPC3.
- [0039] In a first aspect the present disclosure provides a multispecific antigen binding molecule comprising
- (i) a first antigen binding moiety capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and
 - (ii) a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3), preferably human GPC3.
- [0040] One aspect the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3); wherein the first antigen-binding moiety comprises any one selected from (a1) to (a15) below:
- (a1) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 17, the heavy chain CDR 2 of SEQ ID NO: 31, the heavy chain CDR 3 of SEQ ID NO: 45, the light chain CDR 1 of SEQ ID NO: 64, the light chain CDR 2 of SEQ ID NO: 69 and the light chain CDR 3 of SEQ ID NO: 74;
 - (a2) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 18, the heavy chain CDR 2 of SEQ ID NO: 32, the heavy chain CDR 3 of SEQ ID NO: 46, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO:

68 and the light chain CDR 3 of SEQ ID NO: 73;

(a3) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 19, the heavy chain CDR 2 of SEQ ID NO: 33, the heavy chain CDR 3 of SEQ ID NO: 47, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a4) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 19, the heavy chain CDR 2 of SEQ ID NO: 33, the heavy chain CDR 3 of SEQ ID NO: 47, the light chain CDR 1 of SEQ ID NO: 65, the light chain CDR 2 of SEQ ID NO: 70 and the light chain CDR 3 of SEQ ID NO: 75;

(a5) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 20, the heavy chain CDR 2 of SEQ ID NO: 34, the heavy chain CDR 3 of SEQ ID NO: 48, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a6) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 22, the heavy chain CDR 2 of SEQ ID NO: 36, the heavy chain CDR 3 of SEQ ID NO: 50, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a7) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 23, the heavy chain CDR 2 of SEQ ID NO: 37, the heavy chain CDR 3 of SEQ ID NO: 51, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a8) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 23, the heavy chain CDR 2 of SEQ ID NO: 37, the heavy chain CDR 3 of SEQ ID NO: 51, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a9) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 24, the heavy chain CDR 2 of SEQ ID NO: 38, the heavy chain CDR 3 of SEQ ID NO: 52, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a10) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 25, the heavy chain CDR 2 of SEQ ID NO: 39, the heavy chain CDR 3 of SEQ ID NO: 53, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a11) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 26, the heavy chain CDR 2 of SEQ ID NO: 40, the heavy chain CDR 3 of SEQ ID NO: 54, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a12) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO:

26, the heavy chain CDR 2 of SEQ ID NO: 40, the heavy chain CDR 3 of SEQ ID NO: 54, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a13) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 27, the heavy chain CDR 2 of SEQ ID NO: 41, the heavy chain CDR 3 of SEQ ID NO: 55, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a14) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 28, the heavy chain CDR 2 of SEQ ID NO: 42, the heavy chain CDR 3 of SEQ ID NO: 56, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73; and

(a15) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 82, the heavy chain CDR 2 of SEQ ID NO: 83, the heavy chain CDR 3 of SEQ ID NO: 84, the light chain CDR 1 of SEQ ID NO: 65, the light chain CDR 2 of SEQ ID NO: 70 and the light chain CDR 3 of SEQ ID NO: 75.

[0041] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);

wherein the second antigen-binding moiety capable of binding to glypican-3 (GPC3) comprises the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 235, the heavy chain CDR 2 of SEQ ID NO: 244, the heavy chain CDR 3 of SEQ ID NO: 253, the light chain CDR 1 of SEQ ID NO: 268, the light chain CDR 2 of SEQ ID NO: 274 and the light chain CDR 3 of SEQ ID NO: 280.

[0042] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);

wherein said multispecific antigen-binding molecule further comprises a Fc domain.

[0043] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);

wherein said multispecific antigen-binding molecule further comprises a Fc domain, and

wherein said Fc domain is composed of a first and a second Fc region subunits capable of stable association, and wherein the Fc domain exhibits reduced binding affinity to human Fc gamma receptor, as compared to a native human IgG1 Fc domain.

- [0044] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);
 wherein said multispecific antigen-binding molecule further comprises a Fc domain, wherein said Fc domain is composed of a first and a second Fc region subunits capable of stable association, and wherein the Fc domain exhibits reduced binding affinity to human Fc gamma receptor, as compared to a native human IgG1 Fc domain, and
 wherein the first Fc region subunit is selected from the group comprising:
 (c1) a Fc region polypeptide comprising Ala at position 234 and Ala at position 235;
 (c2) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, and Ala at position 297;
 (c3) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, Ala at position 297, Cys at position 354 and Trp at position 366; and
 wherein the second Fc-region polypeptide is selected from the group comprising:
 (c4) a Fc region polypeptide comprising Ala at position 234 and Ala at position 235;
 (c5) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, and Ala at position 297;
 (c6) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, Ala at position 297, Cys at position 349, Ser at position 366, Ala at position 368 and Val at position 407; and
 wherein the amino acid positions are numbered using EU index numbering.
- [0045] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);
 wherein said multispecific antigen-binding molecule further comprises a Fc domain, and
 wherein the Fc domain is a IgG Fc domain, preferably a human IgG Fc domain, more preferably a human IgG1 Fc domain.
- [0046] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);
 wherein the first antigen binding moiety comprises any one selected from (a1) to (a15) below:
 (a1) a heavy chain variable region comprising an amino acid sequence of SEQ ID

NO: 3, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 59;

(a2) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 4, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a3) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 5, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a4) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 5, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 60;

(a5) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 6, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a6) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 8, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a7) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 9, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a8) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 9, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;

(a9) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 10, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a10) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 11, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;

(a11) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 12, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;

(a12) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 12, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a13) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 13, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;

(a14) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 14, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58; and

(a15) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 81, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 60.

- [0047] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);
 wherein the second antigen binding moiety comprises a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 226 and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 262.
- [0048] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);
 wherein said multispecific antigen-binding molecule further comprises a Fc domain, and
 wherein the Fc domain comprises a first Fc region subunit shown in SEQ ID NO: 317 and a second Fc region subunit shown in SEQ ID NO: 323.
- [0049] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);
 wherein each of the first and the second antigen binding moiety is a Fab molecule.
- [0050] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);
 wherein said multispecific antigen-binding molecule further comprises a Fc domain, wherein said Fc domain is composed of a first and a second Fc region subunits capable of stable association, and wherein the Fc domain exhibits reduced binding affinity to human Fc gamma receptor, as compared to a native human IgG1 Fc domain, and
 wherein the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of either one of the first or second Fc region subunit of the Fc domain, and the second antigen binding moiety is fused at the C-terminus of the Fab

heavy chain to the N-terminus of the remaining Fc region subunit of the Fc domain.

[0051] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);

wherein each of the first and the second antigen binding moiety is a Fab molecule, and

wherein the second antigen binding moiety is a crossover Fab molecule in which the variable regions of the Fab light chain and the Fab heavy chain are exchanged and which comprises a heavy chain variable region and a light chain variable region, and wherein the first antigen binding moiety is a conventional Fab molecule which comprises a heavy chain variable region (VH) and a light chain variable region (VL).

[0052] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);

wherein each of the first and the second antigen binding moiety is a Fab molecule,

wherein the second antigen binding moiety is a crossover Fab molecule in which the variable regions of the Fab light chain and the Fab heavy chain are exchanged and which comprises a heavy chain variable region and a light chain variable region, and wherein the first antigen binding moiety is a conventional Fab molecule which comprises a heavy chain variable region (VH) and a light chain variable region (VL), and

wherein, in the constant domain CL of the light chain of the first antigen binding moiety, the amino acid(s) at position 123 and/or 124 is/are substituted independently by lysine (K), arginine (R) or histidine (H) (numbering according to Kabat), and wherein in the constant domain CH1 of the heavy chain of the first antigen binding moiety, the amino acid at position 147 and/or the amino acid at position 213 is substituted independently by glutamic acid (E) or aspartic acid (D) (numbering according to Kabat EU index).

[0053] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);

wherein each of the first and the second antigen binding moiety is a Fab molecule,

wherein the second antigen binding moiety is a crossover Fab molecule in which the variable regions of the Fab light chain and the Fab heavy chain are exchanged and which comprises a heavy chain variable region and a light chain variable region, and

wherein the first antigen binding moiety is a conventional Fab molecule which comprises a heavy chain variable region (VH) and a light chain variable region (VL) and

wherein, in the constant domain CL of the light chain of the first antigen binding moiety, the amino acids at position 123 and 124 are arginine (R) and lysine (K) respectively (numbering according to Kabat), and wherein in the constant domain CH1 of the heavy chain of the first antigen binding moiety, the amino acids at position 147 and 213 are glutamic acid (E) (numbering according to Kabat EU index).

[0054] One aspect of the present disclosure provides a multispecific antigen-binding molecule comprising a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);

which comprises four polypeptides combination of any one selected from (a1) to (a6) below:

(a1) a heavy chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a light chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a heavy chain comprising amino acid sequence of SEQ ID NOs: 219 (chain 3) and a light chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a2) a heavy chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a light chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a heavy chain comprising amino acid sequence of SEQ ID NOs: 220 (chain 3) and a light chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a3) a heavy chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a light chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a heavy chain comprising amino acid sequence of SEQ ID NOs: 291 (chain 3) and a light chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a4) a heavy chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a light chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a heavy chain comprising amino acid sequence of SEQ ID NOs: 292 (chain 3) and a light chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a5) a heavy chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a light chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a heavy chain comprising amino acid sequence of SEQ ID NOs: 293 (chain 3) and a light chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4); and

(a6) a heavy chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a light chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a heavy chain comprising amino acid sequence of SEQ ID NOs: 294 (chain 3) and a light chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4).

- [0055] The components of the multispecific antigen binding molecules of the present invention can be fused to each other in a variety of configurations. Exemplary configurations are depicted in Figure 2. In particular embodiments, the multispecific antigen binding molecules comprises an Fc domain composed of a first and a second subunit capable of stable association.
- [0056] According to any of the above embodiments, components of the multispecific antigen binding molecules (e.g. antigen binding moiety, Fc domain) may be fused directly or through various linkers, particularly peptide linkers comprising one or more amino acids, typically about 2-20 amino acids, that are described herein or are known in the art. Suitable, non-immunogenic peptide linkers include, for example, (G4S)_n, (SG4)_n, (G4S)_n or G4(SG4)_n peptide linkers, wherein n is generally a number between 1 and 10, typically between 2 and 4.
- [0057] Pyroglutamylation
It is known that when an antibody is expressed in cells, the antibody is modified after translation. Examples of the posttranslational modification include cleavage of lysine at the C terminal of the heavy chain by a carboxypeptidase; modification of glutamine or glutamic acid at the N terminal of the heavy chain and the light chain to pyroglutamic acid by pyroglutamylation; glycosylation; oxidation; deamidation; and glycation, and it is known that such posttranslational modifications occur in various antibodies (Journal of Pharmaceutical Sciences, 2008, Vol. 97, p. 2426-2447).
The multispecific antigen binding molecules of the present invention also includes a multispecific antibody which has undergone posttranslational modification. Examples of the multispecific antigen binding molecules thereof of the present invention, which undergoes posttranslational modification, include multispecific antibodies which have undergone pyroglutamylation at the N terminal of the heavy chain variable region and/or deletion of lysine at the C terminal of the heavy chain. It is known in the field that such posttranslational modification due to pyroglutamylation at the N terminal and deletion of lysine at the C terminal does not have any influence on the activity of the antibody (Analytical Biochemistry, 2006, Vol. 348, p. 24-39).
- [0058] Antigen binding moiety
As used herein, the term "antigen binding moiety" refers to a polypeptide molecule that specifically binds to an antigen. In one embodiment, an antigen binding moiety is able to direct the entity to which it is attached (e.g. a second antigen binding moiety) to a target site, for example to a specific type of tumor cell expressing the cancer antigen (GPC3). In another embodiment an antigen binding moiety is able to activate signaling through its target antigen, for example a T cell receptor complex antigen (CD3) or co-stimulatory molecule CD137. Antigen binding moieties include antibodies and fragments thereof as further defined herein. Particular antigen binding moieties include

an antigen binding domain or an antibody variable region of an antibody, comprising an antibody heavy chain variable region and an antibody light chain variable region. In certain embodiments, the antigen binding moieties may comprise antibody constant regions as further defined herein and known in the art. Useful heavy chain constant regions include any of the five isotypes: alpha, delta, epsilon, gamma, or mu. Useful light chain constant regions include any of the two isotypes: kappa and lambda. As used herein, the terms "first", "second", "third", and "fourth" with respect to antigen binding moieties etc., are used for convenience of distinguishing when there is more than one of each type of moiety. Use of these terms is not intended to confer a specific order or orientation of the multispecific antigen binding molecule unless explicitly so stated.

[0059] Antigen-binding moiety capable of binding to CD3 and CD137 but not at the same time

The multispecific antigen binding molecule described herein comprises at least one antigen binding moiety capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time (also referred to herein as "Dual antigen binding moiety" or "first antigen binding moiety" or "Dual-Ig" or "Dual-Fab"). In a particular embodiment, the multispecific antigen binding molecule comprises not more than two antigen binding moiety capable of specific binding to CD3 and CD137 but does not bind to CD3 and CD137 at the same time. In one embodiment the multispecific antigen binding molecule provides monovalent binding to CD3 or CD137, but does not bind to CD3 and CD137 at the same time.

[0060] In certain embodiments, the Dual antigen binding moiety ("first antigen binding moiety") is generally a Fab molecule, particularly a conventional Fab molecule. In certain embodiments, the Dual antigen binding moiety ("first antigen binding moiety") is a domain comprising antibody light-chain and heavy-chain variable regions (VL and VH). Suitable examples of such domains comprising antibody light-chain and heavy-chain variable regions include "single chain Fv (scFv)", "single chain antibody", "Fv", "single chain Fv 2 (scFv2)", "Fab", "F(ab')₂", etc.

[0061] In certain embodiments, the Dual antigen binding moiety ("first antigen binding moiety") specifically binds to the whole or a portion of a partial peptide of CD3. In a particular embodiment CD3 is human CD3 or cynomolgus CD3, most particularly human CD3. In a particular embodiment the first antigen binding moiety is cross-reactive for (i.e. specifically binds to) human and cynomolgus CD3. In some embodiments, the first antigen binding moiety is capable of specific binding to the epsilon subunit of CD3, in particular the human CD3 epsilon subunit of CD3 which is shown in SEQ ID NOs: 7 (NP_000724.1) (RefSeq registration numbers are shown within the parentheses). In some embodiments, the first antigen binding moiety is capable of

specific binding to the CD3 epsilon chain expressed on the surface of eukaryotic cells. In some embodiments, the first antigen binding moiety binds to the CD3 epsilon chain expressed on the surface of T cells.

- [0062] In certain embodiments, the CD137 is human CD137. In some embodiments, favorable examples of an antigen-binding molecule of the present invention include antigen-binding molecules that bind to the same epitope as the human CD137 epitope bound by the antibody selected from the group consisting of:

antibody that recognize a region comprising the SPCPPNSFSSAGGQRTCD
ICRQCKGVFRTRKECSSTSNAECDCTPGFHCLGAGCSMCEQDCKQGQELTK
KGC

sequence (SEQ ID NO: 21),

antibody that recognize a region comprising the DCTPGFHCLGAGCSMCEQDC
KQGQELTKKGC sequence (SEQ ID NO: 35),

antibody that recognize a region comprising the LQDPCSNC
PAGTFCDNNRNQICSPCPPNSFSSAGGQRTCDICRQCKGVFRTRKECSSTSNA
EC

sequence (SEQ ID NO: 49), and

antibody that recognize a region comprising the LQDPCSNC
PAGTFCDNNRNQIC sequence (SEQ ID NO: 105) in the human CD137 protein.

- [0063] In specific embodiments, the Dual antigen binding moiety ("first antigen binding moiety") comprises any one of the antibody variable region sequences shown in Tables 1 below. In specific embodiments, the Dual antigen binding moiety ("first antigen binding moiety") comprises any one of the combinations of the heavy chain variable region and light chain variable region shown in Table 1.

- [0064] (Table 1)

SEQ ID NOs of the variable regions of the Dual antigen binding moiety ("first antigen binding moiety" or "Dual-Fab")

Name	SEQ ID NOs	
	Heavy chain variable region (VH)	Light chain variable region (VL)
DualAE08	3	59
DualAE06	4	58
DualAE17	5	58
DualAE10	5	60
DualAE05	6	58
DualAE19	8	58
DualAE20	9	58
DualAE21	9	61
DualAE22	10	58
DualAE23	11	61
DualAE09	12	61
DualAE18	12	58
DualAE14	13	58
DualAE15	14	58
DualAE16	81	60

[0065] In one embodiment the Dual antigen binding moiety ("first antigen binding moiety") comprises a heavy chain variable region sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 6 and a light chain variable region sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 58. In one embodiment the Dual antigen binding moiety ("first antigen binding moiety") comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 6 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 58.

[0066] In one embodiment the Dual antigen binding moiety ("first antigen binding moiety") comprises a heavy chain variable region sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 14 and a light chain variable region sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 58. In one embodiment the Dual antigen binding moiety ("first antigen binding moiety") comprises a heavy chain variable region comprising the amino acid sequence

of SEQ ID NO: 14 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 58.

[0067] In one embodiment the Dual antigen binding moiety ("first antigen binding moiety") comprises a heavy chain variable region sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 81 and a light chain variable region sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 58. In one embodiment the Dual antigen binding moiety ("first antigen binding moiety") comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 81 and a light chain variable region comprising the amino acid sequence of SEQ ID NO: 58.

[0068] In specific embodiments, the Dual antigen binding moiety ("first antigen binding moiety" or "Dual-Fab") comprises any one of the combinations of HVR sequences shown in Table 2 below.

[0069] (Table 2)
SEQ ID NOs of the HVR (CDR) sequences of the Dual antigen binding moiety ("first antigen binding moiety" or "Dual-Fab")

Name	SEQ ID NOs					
	HVR-H1	HVR-H2	HVR-H3	HVR-L1	HVR-L2	HVR-L3
DualAE08	17	31	45	64	69	74
DualAE06	18	32	46	63	68	73
DualAE17	19	33	47	63	68	73
DualAE10	19	33	47	65	70	75
DualAE05	20	34	48	63	68	73
DualAE19	22	36	50	63	68	73
DualAE20	23	37	51	63	68	73
DualAE21	23	37	51	66	71	76
DualAE22	24	38	52	63	68	73
DualAE23	25	39	53	66	71	76
DualAE09	26	40	54	66	71	76
DualAE18	26	40	54	63	68	73
DualAE14	27	41	55	63	68	73
DualAE15	28	42	56	63	68	73
DualAE16	82	83	84	65	70	75

[0070] The multispecific antigen binding molecules of the present invention also includes a multispecific antibody which has undergone posttranslational modification. Examples of the multispecific antigen binding molecules thereof of the present invention, which undergoes posttranslational modification, include multispecific antibodies which have undergone pyroglutamylation at the N terminal of the heavy chain variable region and/or deletion of lysine at the C terminal of the heavy chain. It is known in the field that such posttranslational modification due to pyroglutamylation at the N terminal and deletion of lysine at the C terminal does not have any influence on the activity of the antibody (Analytical Biochemistry, 2006, Vol. 348, p. 24-39).

[0071] Antigen-binding moiety capable of binding to GPC3

The multispecific antigen binding molecule described herein comprises at least one antigen binding moiety capable of binding to GPC3 (also referred to herein as a "GPC3 antigen binding moiety" or "second antigen binding moiety"). In certain embodiments, the multispecific antigen binding molecule comprises one antigen binding moiety

capable of binding to GPC3.

In certain embodiments, the multispecific antigen binding molecule comprises two antigen binding moieties capable of binding to GPC3. In a particular such embodiment, each of these antigen binding moieties specifically binds to the same epitope of GPC3. In an even more particular embodiment, all of these antigen binding moieties are identical. In one embodiment, the multispecific antigen binding molecule comprises an immunoglobulin molecule capable of specific binding to GPC3. In one embodiment the multispecific antigen binding molecule comprises not more than two antigen binding moieties capable of binding to GPC3.

In certain embodiments, the GPC3 antigen binding is a crossover Fab molecule, i.e. a Fab molecule wherein either the variable or the constant regions of the Fab heavy and light chains are exchanged.

- [0072] In certain embodiments, the GPC3 antigen binding moiety is a crossover Fab molecule in which the variable regions of the Fab light chain and the Fab heavy chain are exchanged and which comprises a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 226 and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 262.

In particular embodiments the multispecific antigen binding molecule comprises at least one antigen binding moiety that is specific for Glypican 3 (GPC3). In one embodiment, the antigen binding moiety that is specific for GPC3 comprises at least one heavy chain complementarity determining region (CDR) selected from the group consisting of SEQ ID NO: 235, SEQ ID NO: 244 and SEQ ID NO: 253 and at least one light chain CDR selected from the group of SEQ ID NO: 268, SEQ ID NO: 274 and SEQ ID NO: 280.

- [0073] In one embodiment, the antigen binding moiety that is specific for GPC3 comprises the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 235, the heavy chain CDR 2 of SEQ ID NO: 244, the heavy chain CDR 3 of SEQ ID NO: 253, the light chain CDR 1 of SEQ ID NO: 268, the light chain CDR 2 of SEQ ID NO: 274 and the light chain CDR 3 of SEQ ID NO: 280.

- [0074] In a further embodiment, the antigen binding moiety that is specific for GPC3 comprises a heavy chain variable region sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 226 and a light chain variable region sequence that is at least about 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 262, or variants thereof that retain functionality.

- [0075] In one embodiment, the antigen binding moiety that is specific for GPC3 comprises a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 226 and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 262.

[0076] The multispecific antigen binding molecules of the present invention also includes a multispecific antibody which has undergone posttranslational modification. Examples of the multispecific antigen binding molecules thereof of the present invention, which undergoes posttranslational modification, include multispecific antibodies which have undergone pyroglutamylation at the N terminal of the heavy chain variable region and/or deletion of lysine at the C terminal of the heavy chain. It is known in the field that such posttranslational modification due to pyroglutamylation at the N terminal and deletion of lysine at the C terminal does not have any influence on the activity of the antibody (Analytical Biochemistry, 2006, Vol. 348, p. 24-39).

[0077] Antigen

As used herein, the term "antigen" 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 referred to as antigens herein (e.g. CD3, CD137, GPC3) 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 CD3, human CD137 or human GPC3. 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.

[0078] In certain embodiments the multispecific antigen binding molecule described herein binds to an epitope of CD3, CD137 or GPC3 that is conserved among the CD3, CD137 or GPC3 from different species. In certain embodiments the multispecific antigen binding molecule of the present application is a trispecific antigen binding molecule, i.e. it is capable of specifically binding to three different antigens - capable of binding to either one of CD3 or CD137 but does not bind to both antigens simultaneously, and is capable of specifically binding to GPC3.

[0079] In certain embodiments, the multispecific antigen binding molecule specifically binds to the whole or a portion of a partial peptide of CD3. In a particular embodiment CD3 is human CD3 or cynomolgus CD3, most particularly human CD3. In a particular embodiment the multispecific antigen binding molecule is cross-reactive for (i.e. specifically binds to) human and cynomolgus CD3. In some embodiments, the multispecific antigen binding molecule is capable of specific binding to the epsilon subunit

of CD3, in particular the human CD3 epsilon subunit of CD3 which is shown in SEQ ID NOs: 7 (NP_000724.1) (RefSeq registration numbers are shown within the parentheses). In some embodiments, the multispecific antigen binding molecule is capable of specific binding to the CD3 epsilon chain expressed on the surface of eukaryotic cells. In some embodiments, the multispecific antigen binding molecule binds to the CD3 epsilon chain expressed on the surface of T cells.

- [0080] In certain embodiments, the CD137 is human CD137. In some embodiments, favorable examples of an antigen-binding molecule of the present invention include antigen-binding molecules that bind to the same epitope as the human CD137 epitope bound by the antibody selected from the group consisting of:
- antibody that recognize a region comprising the SPCPPNSFSSAGGQRTCD ICRQCKGVFRTRKECSSTSNAECDCTPGFHCLGAGCSMCEQDCKQGQELTK KGC sequence (SEQ ID NO: 21),
 - antibody that recognize a region comprising the DCTPGFHCLGAGCSMCEQDC KQGQELTKKGC sequence (SEQ ID NO: 35),
 - antibody that recognize a region comprising the LQDPCSNC PAGTFCDNNRNQICSPCPPNSFSSAGGQRTCDICRQCKGVFRTRKECSSTSNA EC sequence (SEQ ID NO: 49), and
 - antibody that recognize a region comprising the LQDPCSNC PAGTFCDNNRN QIC sequence (SEQ ID NO: 105) in the human CD137 protein.

[0081] Antigen binding domain

The term "antigen binding domain" refers to the part of an antibody that comprises the area which specifically binds to and is complementary to part or all of an antigen. An antigen binding domain may be provided by, for example, one or more antibody variable domains (also called antibody variable regions). Preferably, the antigen-binding domains contain both the antibody light chain variable region (VL) and antibody heavy chain variable region (VH). Such preferable antigen-binding domains include, for example, "single-chain Fv (scFv)", "single-chain antibody", "Fv", "single-domain antibody or VHH", "single-chain Fv2 (scFv2)", "Fab", and "F(ab)₂".

[0082] Variable region

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody 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 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. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. See, e.g., Portolano et al., J. Immunol. 150:880-887 (1993); Clarkson et al., Nature 352:624-628 (1991).

[0083] HVR or CDR

The term "hypervariable region" or "HVR" as used herein refers to each of the regions of an antibody variable domain which are hypervariable in sequence ("complementarity determining regions" or "CDRs") and/or form structurally defined loops ("hypervariable loops") and/or contain the antigen-contacting residues ("antigen contacts"). Hypervariable regions (HVRs) are also referred to as "complementarity determining regions" (CDRs), and these terms are used herein interchangeably in reference to portions of the variable region that form the antigen binding regions. Generally, antibodies comprise six HVRs: three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3).

[0084] Exemplary HVRs 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 (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));

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

(d) combinations of (a), (b), and/or (c), including HVR amino acid residues 46-56 (L2), 47-56 (L2), 48-56 (L2), 49-56 (L2), 26-35 (H1), 26-35b (H1), 49-65 (H2), 93-102 (H3), and 94-102 (H3).

Unless otherwise indicated, HVR residues and other residues in the variable domain (e.g., FR residues) are numbered herein according to Kabat et al., supra.

HVR-H1, HVR-H2, HVR-H3, HVR-L1, HVR-L2, and HVR-L3 are also mentioned as "H-CDR1", "H-CDR2", "H-CDR3", "L-CDR1", "L-CDR2", and "L-CDR3", respectively.

[0085] Capable of binding to CD3 and CD137

Whether the antibody variable region of the present invention is "capable of binding to CD3 and CD137" can be determined by a method known in the art.

This can be determined by, for example, an electrochemiluminescence method (ECL

method) (BMC Research Notes 2011, 4: 281).

Specifically, for example, a low-molecular antibody composed of a region capable of binding to CD3 and CD137, for example, a Fab region, of a biotin-labeled antigen-binding molecule to be tested, or a monovalent antibody (antibody lacking one of the two Fab regions carried by a usual antibody) thereof is mixed with CD3 or CD137 labeled with sulfo-tag (Ru complex), and the mixture is added onto a streptavidin-immobilized plate. In this operation, the biotin-labeled antigen-binding molecule to be tested binds to streptavidin on the plate. Light is developed from the sulfo-tag, and the luminescence signal can be detected using Sector Imager 600 or 2400 (MSD K.K.) or the like to thereby confirm the binding of the aforementioned region of the antigen-binding molecule to be tested to CD3 or CD137.

Alternatively, this assay may be conducted by ELISA, FACS (fluorescence activated cell sorting), ALPHAScreen (amplified luminescent proximity homogeneous assay screen), the BIACORE method based on a surface plasmon resonance (SPR) phenomenon, etc. (Proc. Natl. Acad. Sci. USA (2006) 103 (11), 4005-4010).

[0086] Specifically, the assay can be conducted using, for example, an interaction analyzer Biacore (GE Healthcare Japan Corp.) based on a surface plasmon resonance (SPR) phenomenon. The Biacore analyzer includes any model such as Biacore T100, T200, X100, A100, 4000, 3000, 2000, 1000, 8K or C. Any sensor chip for Biacore, such as a CM7, CM5, CM4, CM3, C1, SA, NTA, L1, HPA, or Au chip, can be used as a sensor chip. Proteins for capturing the antigen-binding molecule of the present invention, such as protein A, protein G, protein L, anti-human IgG antibodies, anti-human IgG-Fab, anti-human L chain antibodies, anti-human Fc antibodies, antigenic proteins, or antigenic peptides, are immobilized onto the sensor chip by a coupling method such as amine coupling, disulfide coupling, or aldehyde coupling. CD3 or CD137 is injected thereon as an analyte, and the interaction is measured to obtain a sensorgram. In this operation, the concentration of CD3 or CD137 can be selected within the range of a few micro M to a few pM according to the interaction strength (e.g., KD) of the assay sample.

[0087] Alternatively, CD3 or CD137 may be immobilized instead of the antigen-binding molecule onto the sensor chip, with which the antibody sample to be evaluated is in turn allowed to interact. Whether the antibody variable region of the antigen-binding molecule of the present invention has binding activity against CD3 or CD137 can be confirmed on the basis of a dissociation constant (KD) value calculated from the sensorgram of the interaction or on the basis of the degree of increase in the sensorgram after the action of the antigen-binding molecule sample over the level before the action.

[0088] In some embodiments, binding activity or affinity of the antibody variable region of

the present invention to the antigen of interest (i.e. CD3 or CD137) are assessed at 37 degrees C (for CD137) or 25 degrees C (for CD3) using e.g., Biacore T200 instrument (GE Healthcare) or Biacore 8K instrument (GE Healthcare). Anti-human Fc (e.g., GE Healthcare) is immobilized onto all flow cells of a CM4 sensor chip using amine coupling kit (e.g, GE Healthcare). The antigen binding molecules or antibody variable regions are captured onto the anti-Fc sensor surfaces, then the antigen (CD3 or CD137) is injected over the flow cell. The capture levels of the antigen binding molecules or antibody variable regions may be aimed at 200 resonance unit (RU). Recombinant human CD3 or CD137 may be injected at 2000 to 125 nM prepared by two-fold serial dilution, followed by dissociation. All antigen binding molecules or antibody variable regions and analytes are prepared in ACES pH 7.4 containing 20 mM ACES, 150 mM NaCl, 0.05% Tween 20, 0.005% NaN₃. Sensor surface is regenerated each cycle with 3M MgCl₂. Binding affinity are determined by processing and fitting the data to 1:1 binding model using e.g., Biacore Insight Evaluation software, version 2.0 (GE Healthcare) or Biacore 8K Evaluation software (GE Healthcare). The KD values are calculated for assessing the specific binding activity or affinity of the antigen binding domains of the present invention.

[0089] The ALPHAScreen is carried out by the ALPHA technology using two types of beads (donor and acceptor) on the basis of the following principle: luminescence signals are detected only when these two beads are located in proximity through the biological interaction between a molecule bound with the donor bead and a molecule bound with the acceptor bead. A laser-excited photosensitizer in the donor bead converts ambient oxygen to singlet oxygen having an excited state. The singlet oxygen diffuses around the donor bead and reaches the acceptor bead located in proximity thereto to thereby cause chemiluminescent reaction in the bead, which finally emits light. In the absence of the interaction between the molecule bound with the donor bead and the molecule bound with the acceptor bead, singlet oxygen produced by the donor bead does not reach the acceptor bead. Thus, no chemiluminescent reaction occurs.

[0090] One (ligand) of the substances between which the interaction is to be observed is immobilized onto a thin gold film of a sensor chip. The sensor chip is irradiated with light from the back such that total reflection occurs at the interface between the thin gold film and glass. As a result, a site having a drop in reflection intensity (SPR signal) is formed in a portion of reflected light. The other (analyte) of the substances between which the interaction is to be observed is injected on the surface of the sensor chip. Upon binding of the analyte to the ligand, the mass of the immobilized ligand molecule is increased to change the refractive index of the solvent on the sensor chip surface. This change in the refractive index shifts the position of the SPR signal (on the

contrary, the dissociation of the bound molecules gets the signal back to the original position). The Biacore system plots on the ordinate the amount of the shift, i.e., change in mass on the sensor chip surface, and displays time-dependent change in mass as assay data (sensorgram). The amount of the analyte bound to the ligand captured on the sensor chip surface (amount of change in response on the sensorgram between before and after the interaction of the analyte) can be determined from the sensorgram. However, since the amount bound also depends on the amount of the ligand, the comparison must be performed under conditions where substantially the same amounts of the ligand are used. Kinetics, i.e., an association rate constant (k_a) and a dissociation rate constant (k_d), can be determined from the curve of the sensorgram, while affinity (K_D) can be determined from the ratio between these constants. Inhibition assay is also preferably used in the BIACORE method. Examples of the inhibition assay are described in Proc. Natl. Acad. Sci. USA (2006) 103 (11), 4005-4010.

[0091] Does not bind to CD3 and CD137 (4-1BB) at the same time

The term "does not bind to CD3 and CD137 (4-1BB) at the same time" or "does not bind to CD3 and CD137 (4-1BB) simultaneously" means that the antigen-binding moiety or antibody variable region of the present invention cannot bind to CD137 in a state bound with CD3 whereas the antigen-binding moiety or antibody variable region cannot bind to CD3 in a state bound with CD137. In this context, the phrase "not bind to CD3 and CD137 at the same time" also includes not cross-linking a cell expressing CD3 to a cell expressing CD137, or not binding to CD3 and CD137 each expressed on a different cell, at the same time. This phrase further includes the case where the variable region is capable of binding to both CD3 and CD137 at the same time when CD3 and CD137 are not expressed on cell membranes, as with soluble proteins, or both reside on the same cell, but cannot bind to CD3 and CD137 each expressed on a different cell, at the same time. Such an antibody variable region is not particularly limited as long as the antibody variable region has these functions. Examples thereof can include variable regions derived from an IgG-type antibody variable region by the alteration of a portion of its amino acids so as to bind to the desired antigen. The amino acid to be altered is selected from, for example, amino acids whose alteration does not cancel the binding to the antigen, in an antibody variable region binding to CD3 or CD137.

In this context, the phrase "expressed on different cells" merely means that the antigens are expressed on separate cells. The combination of such cells may be, for example, the same types of cells such as a T cell and another T cell, or may be different types of cells such as a T cell and an NK cell.

[0092] Whether the antigen-binding molecule of the present invention does "not bind to CD3 and CD137 at the same time" can be confirmed by: confirming the antigen-

binding molecule to have binding activity against both CD3 and CD137; then allowing either CD3 or CD137 to bind in advance to the antigen-binding molecule comprising the variable region having this binding activity; and then determining the presence or absence of its binding activity against the other one by the method mentioned above. Alternatively, this can also be confirmed by determining whether the binding of the antigen-binding molecule to either CD3 or CD137 immobilized on an ELISA plate or a sensor chip is inhibited by the addition of the other one into the solution. In some embodiments, the binding of the antigen-binding molecule of the present invention to either CD3 or CD137 is inhibited by binding of the antigen-binding molecule to the other by at least 50%, preferably 60% or more, more preferably 70% or more, more preferably 80% or more, further preferably 90% or more, or even more preferably 95% or more.

[0093] In one aspect, while one antigen (e.g. CD3) is immobilized, the inhibition of the binding of the antigen-binding molecule to CD3 can be determined in the presence of the other antigen (e.g. CD137) by methods known in prior art (i.e. ELISA, BIACORE, and so on). In another aspect, while CD137 is immobilized, the inhibition of the binding of the antigen-binding molecule to CD137 also can be determined in the presence of CD3. When either one of two aspects mentioned above is conducted, the antigen-binding molecule of the present invention is determined not to bind to CD3 and CD137 at the same time if the binding is inhibited by at least 50%, preferably 60% or more, preferably 70% or more, further preferably 80% or more, further preferably 90% or more, or even more preferably 95% or more.

[0094] In some embodiments, the concentration of the antigen injected as an analyte is at least 1-fold, 2-fold, 5-fold, 10-fold, 30-fold, 50-fold, or 100-fold higher than the concentration of the other antigen to be immobilized.

In preferable manner, the concentration of the antigen injected as an analyte is 100-fold higher than the concentration of the other antigen to be immobilized and the binding is inhibited by at least 80%.

[0095] In one embodiment, the ratio of the KD value for the CD3 (analyte)-binding activity of the antigen-binding molecule to the CD137 (immobilized)-binding activity of the antigen-binding molecule ($KD(CD3)/KD(CD137)$) is calculated and the CD3 (analyte) concentration which is 10-fold, 50-fold, 100-fold, or 200-fold of the ratio of the KD value ($KD(CD3)/KD(CD137)$) higher than the CD137 (immobilized) concentration can be used for the competition measurement above. (e.g. 1-fold, 5-fold, 10-fold, or 20-fold higher concentration can be selected when the ratio of the KD value is 0.1. Furthermore, 100-fold, 500-fold, 1000-fold, or 2000-fold higher concentration can be selected when the ratio of the KD value is 10.)

[0096] In one aspect, while one antigen (e.g. CD3) is immobilized, the attenuation of the

binding signal of the antigen-binding molecule to CD3 can be determined in the presence of the other antigen (e.g. CD137) by methods known in prior art (i.e. ELISA, ECL and so on). In another aspect, while CD137 is immobilized, the attenuation of the binding signal of the antigen-binding molecule to CD137 also can be determined in the presence of CD3. When either one of two aspects mentioned above is conducted, the antigen-binding molecule of the present invention is determined not to bind to CD3 and CD137 at the same time if the binding signal is attenuated by at least 50%, preferably 60% or more, preferably 70% or more, further preferably 80% or more, further preferably 90% or more, or even more preferably 95% or more.

In some embodiments, the concentration of the antigen injected as an analyte is at least 1-fold, 2-fold, 5-fold, 10-fold, 30-fold, 50-fold, or 100-fold higher than the concentration of the other antigen to be immobilized.

In preferable manner, the concentration of the antigen injected as an analyte is 100-fold higher than the concentration of the other antigen to be immobilized and the binding is inhibited by at least 80%.

[0097] In one embodiment, the ratio of the KD value for the CD3 (analyte)-binding activity of the antigen-binding molecule to the CD137 (immobilized)-binding activity of the antigen-binding molecule ($KD(CD3)/KD(CD137)$) is calculated and the CD3 (analyte) concentration which is 10-fold, 50-fold, 100-fold, or 200-fold of the ratio of the KD value ($KD(CD3)/KD(CD137)$) higher than the CD137 (immobilized) concentration can be used for the measurement above. (e.g. 1-fold, 5-fold, 10-fold, or 20-fold higher concentration can be selected when the ratio of the KD value is 0.1. Furthermore, 100-fold, 500-fold, 1000-fold, or 2000-fold higher concentration can be selected when the ratio of the KD value is 10.)

[0098] Specifically, in the case of using, for example, the ECL method, a biotin-labeled antigen-binding molecule to be tested, CD3 labeled with sulfo-tag (Ru complex), and an unlabeled CD137 are prepared. When the antigen-binding molecule to be tested is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time, the luminescence signal of the sulfo-tag is detected in the absence of the unlabeled CD137 by adding the mixture of the antigen-binding molecule to be tested and labeled CD3 onto a streptavidin-immobilized plate, followed by light development. By contrast, the luminescence signal is decreased in the presence of unlabeled CD137. This decrease in luminescence signal can be quantified to determine relative binding activity. This analysis may be similarly conducted using the labeled CD137 and the unlabeled CD3.

[0099] In the case of the ALPHAScreen, the antigen-binding molecule to be tested interacts with CD3 in the absence of the competing CD137 to generate signals of 520 to 620 nm. The untagged CD137 competes with CD3 for the interaction with the antigen-

binding molecule to be tested. Decrease in fluorescence caused as a result of the competition can be quantified to thereby determine relative binding activity. The polypeptide biotinylation using sulfo-NHS-biotin or the like is known in the art. CD3 can be tagged with GST by an appropriately adopted method which involves, for example: fusing a polynucleotide encoding CD3 in frame with a polynucleotide encoding GST; and allowing the resulting fusion gene to be expressed by cells or the like harboring vectors capable of expression thereof, followed by purification using a glutathione column. The obtained signals are preferably analyzed using, for example, software GRAPHPAD PRISM (GraphPad Software, Inc., San Diego) adapted to a one-site competition model based on nonlinear regression analysis. This analysis may be similarly conducted using the tagged CD137 and the untagged CD3.

[0100] Alternatively, a method using fluorescence resonance energy transfer (FRET) may be used. FRET is a phenomenon in which excitation energy is transferred directly between two fluorescent molecules located in proximity to each other by electron resonance. When FRET occurs, the excitation energy of a donor (fluorescent molecule having an excited state) is transferred to an acceptor (another fluorescent molecule located near the donor) so that the fluorescence emitted from the donor disappears (to be precise, the lifetime of the fluorescence is shortened) and instead, the fluorescence is emitted from the acceptor. By use of this phenomenon, whether or not bind to CD3 and CD137 at the same time can be analyzed. For example, when CD3 carrying a fluorescence donor and CD137 carrying a fluorescence acceptor bind to the antigen-binding molecule to be tested at the same time, the fluorescence of the donor disappears while the fluorescence is emitted from the acceptor. Therefore, change in fluorescence wavelength is observed. Such an antibody is confirmed to bind to CD3 and CD137 at the same time. On the other hand, if the mixing of CD3, CD137, and the antigen-binding molecule to be tested does not change the fluorescence wavelength of the fluorescence donor bound with CD3, this antigen-binding molecule to be tested can be regarded as antigen binding domain that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time.

[0101] For example, a biotin-labeled antigen-binding molecule to be tested is allowed to bind to streptavidin on the donor bead, while CD3 tagged with glutathione S transferase (GST) is allowed to bind to the acceptor bead. The antigen-binding molecule to be tested interacts with CD3 in the absence of the competing second antigen to generate signals of 520 to 620 nm. The untagged second antigen competes with CD3 for the interaction with the antigen-binding molecule to be tested. Decrease in fluorescence caused as a result of the competition can be quantified to thereby determine relative binding activity. The polypeptide biotinylation using sulfo-NHS-biotin or the like is known in the art. CD3 can be tagged with GST by an appro-

priately adopted method which involves, for example: fusing a polynucleotide encoding CD3 in frame with a polynucleotide encoding GST; and allowing the resulting fusion gene to be expressed by cells or the like harboring vectors capable of expression thereof, followed by purification using a glutathione column. The obtained signals are preferably analyzed using, for example, software GRAPHPAD PRISM (GraphPad Software, Inc., San Diego) adapted to a one-site competition model based on nonlinear regression analysis.

[0102] The tagging is not limited to the GST tagging and may be carried out with any tag such as, but not limited to, a histidine tag, MBP, CBP, a Flag tag, an HA tag, a V5 tag, or a c-myc tag. The binding of the antigen-binding molecule to be tested to the donor bead is not limited to the binding using biotin-streptavidin reaction. Particularly, when the antigen-binding molecule to be tested comprises Fc, a possible method involves allowing the antigen-binding molecule to be tested to bind via an Fc-recognizing protein such as protein A or protein G on the donor bead.

[0103] Also, the case where the variable region is capable of binding to CD3 and CD137 at the same time when CD3 and CD137 are not expressed on cell membranes, as with soluble proteins, or both reside on the same cell, but cannot bind to CD3 and CD137 each expressed on a different cell, at the same time can also be assayed by a method known in the art.

Specifically, the antigen-binding molecule to be tested has been confirmed to be positive in ECL-ELISA for detecting binding to CD3 and CD137 at the same time is also mixed with a cell expressing CD3 and a cell expressing CD137. The antigen-binding molecule to be tested can be shown to be incapable of binding to CD3 and CD137 expressed on different cells, at the same time unless the antigen-binding molecule and these cells bind to each other at the same time. This assay can be conducted by, for example, cell-based ECL-ELISA. The cell expressing CD3 is immobilized onto a plate in advance. After binding of the antigen-binding molecule to be tested thereto, the cell expressing CD137 is added to the plate. A different antigen expressed only on the cell expressing CD137 is detected using a sulfo-tag-labeled antibody against this antigen. A signal is observed when the antigen-binding molecule binds to the two antigens respectively expressed on the two cells, at the same time. No signal is observed when the antigen-binding molecule does not bind to these antigens at the same time.

[0104] Alternatively, this assay may be conducted by the ALPHAScreen method. The antigen-binding molecule to be tested is mixed with a cell expressing CD3 bound with the donor bead and a cell expressing CD137 bound with the acceptor bead. A signal is observed when the antigen-binding molecule binds to the two antigens expressed on the two cells respectively, at the same time. No signal is observed when the antigen-

binding molecule does not bind to these antigens at the same time.

[0105] Alternatively, this assay may also be conducted by an Octet interaction analysis method. First, a cell expressing CD3 tagged with a peptide tag is allowed to bind to a biosensor that recognizes the peptide tag. A cell expressing CD137 and the antigen-binding molecule to be tested are placed in wells and analyzed for interaction. A large wavelength shift caused by the binding of the antigen-binding molecule to be tested and the cell expressing CD137 to the biosensor is observed when the antigen-binding molecule binds to the two antigens expressed on the two cells respectively, at the same time. A small wavelength shift caused by the binding of only the antigen-binding molecule to be tested to the biosensor is observed when the antigen-binding molecule does not bind to these antigens at the same time.

[0106] Instead of these methods based on the binding activity, assay based on biological activity may be conducted. For example, a cell expressing CD3 and a cell expressing CD137 are mixed with the antigen-binding molecule to be tested, and cultured. The two antigens expressed on the two cells respectively are mutually activated via the antigen-binding molecule to be tested when the antigen-binding molecule binds to these two antigens at the same time. Therefore, change in activation signal, such as increase in the respective downstream phosphorylation levels of the antigens, can be detected. Alternatively, cytokine production is induced as a result of the activation. Therefore, the amount of cytokines produced can be measured to thereby confirm whether or not to bind to the two cells at the same time. Alternatively, cytotoxicity against a cell expressing CD137 is induced as a result of the activation. Alternatively, the expression of a reporter gene is induced by a promoter which is activated at the downstream of the signal transduction pathway of CD137 or CD3 as a result of the activation. Therefore, the cytotoxicity or the amount of reporter proteins produced can be measured to thereby confirm whether or not to bind to the two cells at the same time.

[0107] Fab molecule

A "Fab molecule" refers to a protein consisting of the VH and CH1 domain of the heavy chain (the "Fab heavy chain") and the VL and CL domain of the light chain (the "Fab light chain") of an immunoglobulin.

[0108] Fused

By "fused" is meant that the components (e.g. a Fab molecule and an Fc domain subunit) are linked by peptide bonds, either directly or via one or more peptide linkers.

[0109] "Crossover" Fab

By a "crossover" Fab molecule (also termed "Crossfab") is meant a Fab molecule wherein either the variable regions or the constant regions 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 and the heavy chain constant region, and a

peptide chain composed of the heavy chain variable region and the light chain constant region. For clarity, in a crossover Fab molecule wherein the variable regions of the Fab light chain and the Fab heavy chain are exchanged, the peptide chain comprising the heavy chain constant region is referred to herein as the "heavy chain" of the crossover Fab molecule. Conversely, in a crossover Fab molecule wherein the constant regions of the Fab light chain and the Fab heavy chain are exchanged, the peptide chain comprising the heavy chain variable region is referred to herein as the "heavy chain" of the crossover Fab molecule.

[0110] "Conventional" Fab

In contrast thereto, by a "conventional" Fab molecule is meant a Fab molecule in its natural format, i.e. comprising a heavy chain composed of the heavy chain variable and constant regions (VH-CH1), and a light chain composed of the light chain variable and constant regions (VL-CL). The term "immunoglobulin molecule" refers to a protein having the structure of a naturally occurring antibody. For example, immunoglobulins of the IgG class 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 constant light (CL) domain, also called a light chain constant region. The heavy chain of an immunoglobulin may be assigned to one of five types, called alpha (IgA), delta (IgD), epsilon (IgE), gamma (IgG), or mu (IgM), some of which may be further divided into subtypes, e.g. gamma1 (IgG1), gamma2 (IgG2), gamma3 (IgG3), gamma4 (IgG4), alpha1 (IgA1) and alpha2 (IgA2). The light chain of an immunoglobulin may be assigned to one of two types, called kappa and lambda, based on the amino acid sequence of its constant domain. An immunoglobulin essentially consists of two Fab molecules and an Fc domain, linked via the immunoglobulin hinge region.

[0111] Affinity

"Affinity" refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antigen-binding molecule or 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., antigen-binding molecule and antigen, or antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (KD), which is the ratio of dissociation and association rate constants (koff and kon, 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 well-established methods known in the art, including those described herein. A particular method for measuring affinity is Surface Plasmon Resonance (SPR).

[0112] Methods to determine affinity

In certain embodiments, the antigen-binding molecule or antibody provided herein has a dissociation constant (KD) of 1 micro M or less, 120 nM or less, 100 nM or less, 80 nM or less, 70 nM or less, 50 nM or less, 40 nM or less, 30 nM or less, 20 nM or less, 10 nM or less, 2 nM or less, 1 nM or less, 0.1 nM or less, 0.01 nM or less, or 0.001 nM or less (e.g., 10^{-8} M or less, 10^{-8} M to 10^{-13} M, 10^{-9} M to 10^{-13} M) for its antigen. In certain embodiments, the KD value of the antibody/antigen-binding molecule for CD3, CD137 or GPC3 falls within the range of 1-40, 1-50, 1-70, 1-80, 30-50, 30-70, 30-80, 40-70, 40-80, or 60-80 nM.

[0113] In one embodiment, KD is measured by a radiolabeled antigen-binding assay (RIA). In one embodiment, an RIA is performed with the Fab version of an antibody of interest and its antigen. For example, solution binding affinity of Fabs for antigen is measured by equilibrating Fab with a minimal concentration of (^{125} I)-labeled antigen in the presence of a titration series of unlabeled antigen, then capturing bound antigen with an anti-Fab antibody-coated plate (see, e.g., Chen et al., J. Mol. Biol. 293:865-881(1999)). To establish conditions for the assay, MICROTITER (registered trademark) multi-well plates (Thermo Scientific) are coated overnight with 5 micro g/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23 degrees C). In a non-adsorbent plate (Nunc #269620), 100 pM or 26 pM [^{125} I]-antigen are mixed with serial dilutions of a Fab of interest (e.g., consistent with assessment of the anti-VEGF antibody, Fab-12, in Presta et al., Cancer Res. 57:4593-4599 (1997)). The Fab of interest is then incubated overnight; however, the incubation may continue for a longer period (e.g., about 65 hours) to ensure that equilibrium is reached. Thereafter, the mixtures are transferred to the capture plate for incubation at room temperature (e.g., for one hour). The solution is then removed and the plate washed eight times with 0.1% polysorbate 20 (TWEEN-20 (registered trademark)) in PBS. When the plates have dried, 150 micro L/well of scintillant (MICROSCINT-20™; Packard) is added, and the plates are counted on a TOPCOUNT™ gamma counter (Packard) for ten minutes. Concentrations of each Fab that give less than or equal to 20% of maximal binding are chosen for use in competitive binding assays.

[0114] According to another embodiment, Kd is measured using a BIACORE (registered trademark) surface plasmon resonance assay. For example, an assay using a BIACORE

(registered trademark)-2000 or a BIACORE(registered trademark)-3000 (BIAcore, Inc., Piscataway, NJ) is performed at 25 degrees C with immobilized antigen CM5 chips at ~10 response units (RU). In one embodiment, carboxymethylated dextran biosensor chips (CM5, BIACORE, Inc.) are activated with N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) according to the supplier's instructions. Antigen is diluted with 10 mM sodium acetate, pH 4.8, to 5 micro g/ml (~0.2 micro M) before injection at a flow rate of 5 micro L/minute to achieve approximately 10 response units (RU) of coupled protein. Following the injection of antigen, 1 M ethanolamine is injected to block unreacted groups. For kinetics measurements, two-fold serial dilutions of Fab (0.78 nM to 500 nM) are injected in PBS with 0.05% polysorbate 20 (TWEEN-20™) surfactant (PBST) at 25 degrees C at a flow rate of approximately 25 micro L/min. Association rates (k_{on}) and dissociation rates (k_{off}) are calculated using a simple one-to-one Langmuir binding model (BIACORE (registered trademark) Evaluation Software version 3.2) by simultaneously fitting the association and dissociation sensorgrams. The equilibrium dissociation constant (K_d) is calculated as the ratio k_{off}/k_{on} . See, e.g., Chen et al., J. Mol. Biol. 293:865-881 (1999). If the on-rate exceeds $10^6 \text{ M}^{-1} \text{ s}^{-1}$ by the surface plasmon resonance assay above, then the on-rate can be determined by using a fluorescent quenching technique that measures the increase or decrease in fluorescence emission intensity (excitation = 295 nm; emission = 340 nm, 16 nm band-pass) at 25 degrees C of a 20 nM anti-antigen antibody (Fab form) in PBS, pH 7.2, in the presence of increasing concentrations of antigen as measured in a spectrometer, such as a stop-flow equipped spectrophotometer (Aviv Instruments) or a 8000-series SLM-AMINCO™ spectrophotometer (ThermoSpectronic) with a stirred cuvette.

[0115] According to the methods for measuring the affinity of the antigen-binding molecule or the antibody described above, persons skilled in art can carry out affinity measurement for other antigen-binding molecules or antibodies, towards various kind of antigens.

[0116] Antibody

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

[0117] Antibody fragment

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, 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. Pluckthun, 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. 5,869,046 . 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 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.

[0118] Class of antibody

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, IgG3, IgG4, IgA1, and IgA2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively.

[0119] Unless otherwise indicated, amino acid residues in the light chain constant region are numbered herein according to Kabat et al., and numbering of amino acid residues in the heavy chain 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.

[0120] Framework

"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.

[0121] Human consensus framework

A "human consensus framework" is a framework which represents the most commonly occurring amino acid residues in a selection of human immunoglobulin VL or VH framework sequences. Generally, the selection of human immunoglobulin VL or VH sequences is from a subgroup of variable domain sequences. Generally, the subgroup of sequences is a subgroup as in Kabat et al., Sequences of Proteins of Immunological Interest, Fifth Edition, NIH Publication 91-3242, Bethesda MD (1991), vols. 1-3. In one embodiment, for the VL, the subgroup is subgroup kappa I as in Kabat et al., supra. In one embodiment, for the VH, the subgroup is subgroup III as in Kabat et al., supra.

[0122] Chimeric antibody

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. Similarly, the term "chimeric antibody variable domain" refers to an antibody variable region in which a portion of the heavy and/or light chain variable region is derived from a particular source or species, while the remainder of the heavy and/or light chain variable region is derived from a different source or species.

[0123] Humanized antibody

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. A "humanized antibody variable region" refers to the variable region of a humanized antibody.

[0124] Human antibody

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. A "human antibody variable region" refers to the variable region of a human antibody.

[0125] Polynucleotide (nucleic acid)

"Polynucleotide" or "nucleic acid" as used interchangeably herein, refers to polymers of nucleotides of any length, and include DNA and RNA. The nucleotides can be de-

oxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. A sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may comprise modification(s) made after synthesis, such as conjugation to a label. Other types of modifications include, for example, "caps," substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotides(s).

[0126] Further, any of the hydroxyl groups ordinarily present in the sugars may be replaced, for example, by phosphonate groups, phosphate groups, protected by standard protecting groups, or activated to prepare additional linkages to additional nucleotides, or may be conjugated to solid or semi-solid supports. The 5' and 3' terminal OH can be phosphorylated or substituted with amines or organic capping group moieties of from 1 to 20 carbon atoms. Other hydroxyls may also be derivatized to standard protecting groups. Polynucleotides can also contain analogous forms of ribose or deoxyribose sugars that are generally known in the art, including, for example, 2'-O-methyl-, 2'-O-allyl-, 2'-fluoro- or 2'-azido-ribose, carbocyclic sugar analogs, alpha-anomeric sugars, epimeric sugars such as arabinose, xyloses or lyxoses, pyranose sugars, furanose sugars, sedoheptuloses, acyclic analogs, and basic nucleoside analogs such as methyl riboside. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR₂ ("amidate"), P(O)R, P(O)OR', CO, or CH₂ ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (-O-) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. The preceding description applies to all polynucleotides referred to herein, including RNA and DNA.

[0127] Isolated (nucleic acid)

An "isolated" nucleic acid molecule is one which has been separated from a component of its natural environment. An isolated nucleic acid molecule further

includes a nucleic acid molecule contained in cells that ordinarily contain the nucleic acid molecule, but the nucleic acid molecule is present extrachromosomally or at a chromosomal location that is different from its natural chromosomal location.

[0128] Vector

The term "vector," as used herein, refers to a nucleic acid molecule capable of propagating another nucleic acid to which it is linked. 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. Certain vectors are capable of directing the expression of nucleic acids to which they are operatively linked. Such vectors are referred to herein as "expression vectors." Vectors could be introduced into host cells using virus or electroporation. However, introduction of vectors is not limited to in vitro method. For example, vectors could also be introduced into a subject using in vivo method directly.

[0129] Host cell

The terms "host cell," "host cell line," and "host cell culture" are used interchangeably and 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 selected for in the originally transformed cell are included herein.

[0130] Specificity

"Specific" means that a molecule that binds specifically to one or more binding partners does not show any significant binding to molecules other than the partners. Furthermore, "specific" is also used when an antigen-binding site is specific to a particular epitope of multiple epitopes contained in an antigen. If an antigen-binding molecule binds specifically to an antigen, it is also described as "the antigen-binding molecule has/shows specificity to/towards the antigen". When an epitope bound by an antigen-binding site is contained in multiple different antigens, an antigen-binding molecule containing the antigen-binding site can bind to various antigens that have the epitope.

[0131] Antibody fragment

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; linear antibodies; single-chain antibody molecules (e.g. scFv); and multispecific antibodies formed from antibody fragments.

- [0132] 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 or having heavy chains that contain an Fc region as defined herein.
- [0133] Variable fragment (Fv)
 Herein, the term "variable fragment (Fv)" refers to the minimum unit of an antibody-derived antigen-binding site that is composed of a pair of the antibody light chain variable region (VL) and antibody heavy chain variable region (VH). In 1988, Skerra and Pluckthun found that homogeneous and active antibodies can be prepared from the E. coli periplasm fraction by inserting an antibody gene downstream of a bacterial signal sequence and inducing expression of the gene in E. coli (Science (1988) 240(4855), 1038-1041). In the Fv prepared from the periplasm fraction, VH associates with VL in a manner so as to bind to an antigen.
- [0134] scFv, single-chain antibody, and sc(Fv)₂
 Herein, the terms "scFv", "single-chain antibody", and "sc(Fv)₂" all refer to an antibody fragment of a single polypeptide chain that contains variable regions derived from the heavy and light chains, but not the constant region. In general, a single-chain antibody also contains a polypeptide linker between the VH and VL domains, which enables formation of a desired structure that is thought to allow antigen-binding. The single-chain antibody is discussed in detail by Pluckthun in "The Pharmacology of Monoclonal Antibodies, Vol. 113, Rosenburg and Moore, eds., Springer-Verlag, New York, 269-315 (1994)". See also International Patent Publication WO 1988/001649; US Patent Nos. 4,946,778 and 5,260,203. In a particular embodiment, the single-chain antibody can be bispecific and/or humanized.
- [0135] scFv is an single chain low molecule weight antibody in which VH and VL forming Fv are linked together by a peptide linker (Proc. Natl. Acad. Sci. U.S.A. (1988) 85(16), 5879-5883). VH and VL can be retained in close proximity by the peptide linker.
- [0136] sc(Fv)₂ is a single chain antibody in which four variable regions of two VL and two VH are linked by linkers such as peptide linkers to form a single chain (J Immunol. Methods (1999) 231(1-2), 177-189). The two VH and two VL may be derived from different monoclonal antibodies. Such sc(Fv)₂ preferably includes, for example, a bispecific sc(Fv)₂ that recognizes two epitopes present in a single antigen as disclosed in the Journal of Immunology (1994) 152(11), 5368-5374. sc(Fv)₂ can be produced by methods known to those skilled in the art. For example, sc(Fv)₂ can be produced by linking scFv by a linker such as a peptide linker.
- [0137] Herein, an sc(Fv)₂ includes two VH units and two VL units which are arranged in the order of VH, VL, VH, and VL ([VH]-linker-[VL]-linker-[VH]-linker-[VL]) beginning from the N terminus of a single-chain polypeptide. The order of the two VH units and

two VL units is not limited to the above form, and they may be arranged in any order. Examples of the form are listed below.

[VL]-linker-[VH]-linker-[VH]-linker-[VL]

[VH]-linker-[VL]-linker-[VL]-linker-[VH]

[VH]-linker-[VH]-linker-[VL]-linker-[VL]

[VL]-linker-[VL]-linker-[VH]-linker-[VH]

[VL]-linker-[VH]-linker-[VL]-linker-[VH]

[0138] The molecular form of $\text{sc}(\text{Fv})_2$ is also described in detail in WO 2006/132352.

According to these descriptions, those skilled in the art can appropriately prepare desired $\text{sc}(\text{Fv})_2$ to produce the polypeptide complexes disclosed herein.

[0139] Furthermore, the antigen-binding molecules or antibodies of the present disclosure may be conjugated with a carrier polymer such as PEG or an organic compound such as an anticancer agent. Alternatively, a sugar chain addition sequence is preferably inserted into the antigen-binding molecules or antibodies such that the sugar chain produces a desired effect.

[0140] The linkers to be used for linking the variable regions of an antibody comprise arbitrary peptide linkers that can be introduced by genetic engineering, synthetic linkers, and linkers disclosed in, for example, Protein Engineering, 9(3), 299-305, 1996. However, peptide linkers are preferred in the present disclosure. The length of the peptide linkers is not particularly limited, and can be suitably selected by those skilled in the art according to the purpose. The length is preferably five amino acids or more (without particular limitation, the upper limit is generally 30 amino acids or less, preferably 20 amino acids or less), and particularly preferably 15 amino acids. When $\text{sc}(\text{Fv})_2$ contains three peptide linkers, their length may be all the same or different.

[0141] For example, such peptide linkers include:

Ser,

Gly-Ser,

Gly-Gly-Ser,

Ser-Gly-Gly,

Gly-Gly-Gly-Ser (SEQ ID NO: 91),

Ser-Gly-Gly-Gly (SEQ ID NO: 92),

Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 93),

Ser-Gly-Gly-Gly-Gly (SEQ ID NO: 94),

Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 95),

Ser-Gly-Gly-Gly-Gly-Gly (SEQ ID NO: 96),

Gly-Gly-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 97),

Ser-Gly-Gly-Gly-Gly-Gly-Gly (SEQ ID NO: 98),

(Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 93))_n, and

(Ser-Gly-Gly-Gly-Gly (SEQ ID NO: 94))_n,

where n is an integer of 1 or larger. The length or sequences of peptide linkers can be selected accordingly by those skilled in the art depending on the purpose.

[0142] Synthetic linkers (chemical crosslinking agents) are routinely used to crosslink peptides, and examples include:

N-hydroxy succinimide (NHS),

disuccinimidyl suberate (DSS),

bis(sulfosuccinimidyl) suberate (BS3),

dithiobis(succinimidyl propionate) (DSP),

dithiobis(sulfosuccinimidyl propionate) (DTSSP),

ethylene glycol bis(succinimidyl succinate) (EGS),

ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS),

disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST),

bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (BSOCOES), and

bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl] sulfone (sulfo-BSOCOES). These crosslinking agents are commercially available.

[0143] In general, three linkers are required to link four antibody variable regions together. The linkers to be used may be of the same type or different types.

[0144] Fab, F(ab')₂, and Fab'

"Fab" consists of a single light chain, and a CH1 domain and variable region from a single heavy chain. The heavy chain of Fab molecule cannot form disulfide bonds with another heavy chain molecule.

[0145] "F(ab')₂" or "Fab" is produced by treating an immunoglobulin (monoclonal antibody) with a protease such as pepsin and papain, and refers to an antibody fragment generated by digesting an immunoglobulin (monoclonal antibody) near the disulfide bonds present between the hinge regions in each of the two H chains. For example, papain cleaves IgG upstream of the disulfide bonds present between the hinge regions in each of the two H chains to generate two homologous antibody fragments, in which an L chain comprising VL (L-chain variable region) and CL (L-chain constant region) is linked to an H-chain fragment comprising VH (H-chain variable region) and CH gamma 1 (gamma 1 region in an H-chain constant region) via a disulfide bond at their C-terminal regions. Each of these two homologous antibody fragments is called Fab'.

[0146] "F(ab')₂" consists of two light chains and two heavy chains comprising the constant region of a CH1 domain and a portion of CH2 domains so that disulfide bonds are formed between the two heavy chains. The F(ab')₂ disclosed herein can be preferably produced as follows. A whole monoclonal antibody or such comprising a desired antigen-binding site is partially digested with a protease such as pepsin; and Fc fragments are removed by adsorption onto a Protein A column. The protease is not par-

ticularly limited, as long as it can cleave the whole antibody in a selective manner to produce F(ab')₂ under an appropriate setup enzyme reaction condition such as pH. Such proteases include, for example, pepsin and ficin.

[0147] Single-domain antibody

In the present specification, the term "single-domain antibody" is not limited by its structure as long as the domain can exert antigen binding activity by itself. It is known that a general antibody, for example, an IgG antibody, exhibits antigen binding activity in a state where a variable region is formed by the pairing of VH and VL, whereas the own domain structure of the single-domain antibody can exert antigen binding activity by itself without pairing with another domain. Usually, the single-domain antibody has a relatively low molecular weight and exists in the form of a monomer.

[0148] Examples of the single-domain antibody include, but are not limited to, antigen binding molecules congenitally lacking a light chain, such as VHH of an animal of the family Camelidae and shark VNAR, and antibody fragments containing the whole or a portion of an antibody VH domain or the whole or a portion of an antibody VL domain. Examples of the single-domain antibody which is an antibody fragment containing the whole or a portion of an antibody VH or VL domain include, but are not limited to, artificially prepared single-domain antibodies originating from human antibody VH or human antibody VL as described in U.S. Patent No. 6,248,516 B1, etc. In some embodiments of the present invention, one single-domain antibody has three CDRs (CDR1, CDR2 and CDR3).

[0149] The single-domain antibody can be obtained from an animal capable of producing the single-domain antibody or by the immunization of the animal capable of producing the single-domain antibody. Examples of the animal capable of producing the single-domain antibody include, but are not limited to, animals of the family Camelidae, and transgenic animals harboring a gene capable of raising the single-domain antibody. The animals of the family Camelidae include camels, lamas, alpacas, one-hump camels and guanacos, etc. Examples of the transgenic animals harboring a gene capable of raising the single-domain antibody include, but are not limited to, transgenic animals described in International Publication No. WO2015/143414 and U.S. Patent Publication No. US2011/0123527 A1. The framework sequences of the single-domain antibody obtained from the animal may be converted to human germline sequences or sequences similar thereto to obtain a humanized single-domain antibody. The humanized single-domain antibody (e.g., humanized VHH) is also one embodiment of the single-domain antibody of the present invention.

[0150] Alternatively, the single-domain antibody can be obtained by ELISA, panning, or the like from a polypeptide library containing single-domain antibodies. Examples of the polypeptide library containing single-domain antibodies include, but are not limited to,

naive antibody libraries obtained from various animals or humans (e.g., *Methods in Molecular Biology* 2012 911 (65-78); and *Biochimica et Biophysica Acta - Proteins and Proteomics* 2006 1764: 8 (1307-1319)), antibody libraries obtained by the immunization of various animals (e.g., *Journal of Applied Microbiology* 2014 117: 2 (528-536)), and synthetic antibody libraries prepared from antibody genes of various animals or humans (e.g., *Journal of Biomolecular Screening* 2016 21: 1 (35-43); *Journal of Biological Chemistry* 2016 291:24 (12641-12657); and *AIDS* 2016 30: 11 (1691-1701)).

[0151] Fc region

The term "Fc region" or "Fc domain" refers to a region comprising a fragment consisting of a hinge or a portion thereof and CH2 and CH3 domains in an antibody molecule. The Fc region of IgG class means, but is not limited to, a region from, for example, cysteine 226 (EU numbering (also referred to as EU index herein)) to the C terminus or proline 230 (EU numbering) to the C terminus. The Fc region can be preferably obtained by the partial digestion of, for example, an IgG1, IgG2, IgG3, or IgG4 monoclonal antibody with a proteolytic enzyme such as pepsin followed by the re-elution of a fraction adsorbed on a protein A column or a protein G column. Such a proteolytic enzyme is not particularly limited as long as the enzyme is capable of digesting a whole antibody to restrictively form Fab or F(ab')₂ under appropriately set reaction conditions (e.g., pH) of the enzyme. Examples thereof can include pepsin and papain.

[0152] An Fc region derived from, for example, naturally occurring IgG can be used as the "Fc region" of the present invention. In this context, the naturally occurring IgG means a polypeptide that contains an amino acid sequence identical to that of IgG found in nature and belongs to a class of an antibody substantially encoded by an immunoglobulin gamma gene. The naturally occurring human IgG means, for example, naturally occurring human IgG1, naturally occurring human IgG2, naturally occurring human IgG3, or naturally occurring human IgG4. The naturally occurring IgG also includes variants or the like spontaneously derived therefrom. A plurality of allotype sequences based on gene polymorphism are described as the constant regions of human IgG1, human IgG2, human IgG3, and human IgG4 antibodies in *Sequences of proteins of immunological interest*, NIH Publication No. 91-3242, any of which can be used in the present invention. Particularly, the sequence of human IgG1 may have DEL or EEM as an amino acid sequence of EU numbering positions 356 to 358.

[0153] In some embodiments, the Fc domain of the multispecific antigen binding molecule 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. In one embodiment the multispecific antigen binding molecule described herein comprises not more than one Fc domain.

[0154] In one embodiment described herein the Fc domain of the multispecific antigen binding molecule is an IgG Fc domain. In a particular embodiment the Fc domain is an IgG1 Fc domain. In another embodiment the Fc domain is an IgG1 Fc domain. In a further particular embodiment the Fc domain is a human IgG1 Fc region.

[0155] Fc region with a reduced Fc receptor (Fc gamma receptor)-binding activity

In certain embodiments, the Fc domain of the multispecific antigen binding molecules described herein exhibits reduced binding affinity to an Fc receptor, as compared to a native IgG1 Fc domain. In one such embodiment the Fc domain (or the multispecific antigen binding molecule comprising said Fc domain) exhibits less than 50%, preferably less than 20%, more preferably less than 10% and most preferably less than 5% of the binding affinity to an Fc receptor, as compared to a native IgG1 Fc domain (or a multispecific antigen binding molecule comprising a native IgG1 Fc domain). In one embodiment, the Fc domain (or the multispecific antigen binding molecule comprising said Fc domain) does not substantially bind to an Fc receptor. In a particular embodiment the Fc receptor is an Fc gamma receptor. In one embodiment the Fc receptor is a human Fc receptor. In one embodiment the Fc receptor is an activating Fc receptor. In a specific embodiment the Fc receptor is an activating human Fc gamma receptor, more specifically human Fc gammaRIIIa, Fc gammaRI or Fc gammaRIIa, most specifically human Fc gammaRIIIa.

[0156] In certain embodiments, the Fc domain of the multispecific antigen binding molecule comprises one or more amino acid mutation that reduces the binding affinity of the Fc domain to an Fc receptor. Typically, the same one or more amino acid mutation is present in each of the two subunits of the Fc domain. In one embodiment the amino acid mutation reduces the binding affinity of the Fc domain to an Fc receptor. In one embodiment the amino acid mutation reduces the binding affinity of the Fc domain to an Fc receptor by at least 2-fold, at least 5-fold, or at least 10-fold. In embodiments where there is more than one amino acid mutation that reduces the binding affinity of the Fc domain to the Fc receptor, the combination of these amino acid mutations may reduce the binding affinity of the Fc domain to an Fc receptor by at least 10-fold, at least 20-fold, or even at least 50-fold. In one embodiment the multispecific antigen binding molecule comprising an engineered Fc domain exhibits less than 20%, particularly less than 10%, more particularly less than 5% of the binding affinity to an Fc receptor as compared to a multispecific antigen binding molecule comprising a non-engineered Fc domain. In a particular embodiment the Fc receptor is an Fc gamma receptor. In some embodiments the Fc receptor is a human Fc receptor. In some em-

bodiments the Fc receptor is an activating Fc receptor. In a specific embodiment the Fc receptor is an activating human Fc gamma receptor, more specifically human Fc gammaRIIIa, Fc gammaRI or Fc gammaRIIa, most specifically human Fc gammaRIIIa. Preferably, binding to each of these receptors is reduced.

[0157] In one embodiment the amino acid mutation that reduces the binding affinity of the Fc domain to an Fc receptor is an amino acid substitution. In one embodiment the Fc domain comprises an amino acid substitution at a position selected from the group of E233, L234, L235, N297, P331 and P329. In a more specific embodiment the Fc domain comprises an amino acid substitution at a position selected from the group of L234, L235 and P329. In some embodiments the Fc domain comprises the amino acid substitutions L234A and L235A. In one such embodiment, the Fc domain is an IgG1 Fc domain, particularly a human IgG1 Fc domain. In one embodiment the Fc domain comprises an amino acid substitution at position P329. In a more specific embodiment the amino acid substitution is P329A or P329G, particularly P329G. In one embodiment the Fc domain comprises an amino acid substitution at position P329 and a further amino acid substitution at a position selected from E233, L234, L235, N297 and P331. In a more specific embodiment the further amino acid substitution is E233P, L234A, L235A, L235E, N297A, N297D or P331S. In particular embodiments the Fc domain comprises amino acid substitutions at positions P329, L234 and L235. In more particular embodiments the Fc domain comprises the amino acid mutations L234A, L235A and P329G ("P329G LALA"). In one such embodiment, the Fc domain is an IgG1 Fc domain, particularly a human IgG1 Fc domain. The "P329G LALA" combination of amino acid substitutions almost completely abolishes Fc gamma receptor (as well as complement) binding of a human IgG1 Fc domain, as described in PCT publication no. WO 2012/130831. WO 2012/130831 also describes methods of preparing such mutant Fc domains and methods for determining its properties such as Fc receptor binding or effector functions.

[0158] IgG4 antibodies exhibit reduced binding affinity to Fc receptors and reduced effector functions as compared to IgG1 antibodies. Hence, in some embodiments the Fc domain of the T cell activating bispecific antigen binding molecules described herein is an IgG4 Fc domain, particularly a human IgG4 Fc domain. In one embodiment the IgG4 Fc domain comprises amino acid substitutions at position S228, specifically the amino acid substitution S228P. To further reduce its binding affinity to an Fc receptor and/or its effector function, in one embodiment the IgG4 Fc domain comprises an amino acid substitution at position L235, specifically the amino acid substitution L235E. In another embodiment, the IgG4 Fc domain comprises an amino acid substitution at position P329, specifically the amino acid substitution P329G. In a particular embodiment, the IgG4 Fc domain comprises amino acid substitutions at positions S228,

L235 and P329, specifically amino acid substitutions S228P, L235E and P329G. Such IgG4 Fc domain mutants and their Fc gamma receptor binding properties are described in PCT publication no. WO 2012/130831.

- [0159] In certain embodiments N-glycosylation of the Fc domain has been eliminated. In one such embodiment the Fc domain comprises an amino acid mutation at position N297, particularly an amino acid substitution replacing asparagine by alanine (N297A) or aspartic acid (N297D).
- [0160] In a particular preferred embodiment the Fc domain exhibiting reduced binding affinity to an Fc receptor, as compared to a native IgG1 Fc domain, is a human IgG1 Fc domain comprising the amino acid substitutions L234A, L235A and N297A.
- [0161] 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 Fc gammaIIIa receptor.

- [0162] Fc receptor

The term "Fc receptor" or "FcR" refers to a receptor that binds to the Fc region of an antibody. In some embodiments, an FcR is a native human FcR. In some embodiments, an FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the Fc gamma RI, Fc gamma RII, and Fc gamma RIII subclasses, including allelic variants and alternatively spliced forms of those receptors. Fc gamma RII receptors include Fc gamma RIIA (an "activating receptor") and Fc gamma RIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor Fc gamma RIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor Fc gamma RIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see, e.g., Daeron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed, for example, in Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas et al., *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those

to be identified in the future, are encompassed by the term "FcR" herein.

- [0163] The term "Fc receptor" or "FcR" also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer et al., J. Immunol. 117:587 (1976) and Kim et al., J. Immunol. 24:249 (1994)) and regulation of homeostasis of immunoglobulins. Methods of measuring binding to FcRn are known (see, e.g., Ghetie and Ward., Immunol. Today 18(12):592-598 (1997); Ghetie et al., Nature Biotechnology, 15(7):637-640 (1997); Hinton et al., J. Biol. Chem. 279(8):6213-6216 (2004); WO 2004/92219 (Hinton et al.).
- [0164] Binding to human FcRn in vivo and plasma half life of human FcRn high affinity binding polypeptides can be assayed, e.g., in transgenic mice or transfected human cell lines expressing human FcRn, or in primates to which the polypeptides with a variant Fc region are administered. WO 2000/42072 (Presta) describes antibody variants with increased or decreased binding to FcRs. See also, e.g., Shields et al. J. Biol. Chem. 9(2):6591-6604 (2001).
- [0165] Fc gamma receptor
 Fc gamma receptor refers to a receptor capable of binding to the Fc domain of monoclonal IgG1, IgG2, IgG3, or IgG4 antibodies, and includes all members belonging to the family of proteins substantially encoded by an Fc gamma receptor gene. In human, the family includes Fc gamma RI (CD64) including isoforms Fc gamma RIa, Fc gamma RIb and Fc gamma RIc; Fc gamma RII (CD32) including isoforms Fc gamma RIIa (including allotype H131 and R131), Fc gamma RIIb (including Fc gamma RIIb-1 and Fc gamma RIIb-2), and Fc gamma RIIC; and Fc gamma RIII (CD16) including isoform Fc gamma RIIIA (including allotype V158 and F158) and Fc gamma RIIIB (including allotype Fc gamma RIIIB-NA1 and Fc gamma RIIIB-NA2); as well as all unidentified human Fc gamma receptors, Fc gamma receptor isoforms, and allotypes thereof. However, Fc gamma receptor is not limited to these examples. Without being limited thereto, Fc gamma receptor includes those derived from humans, mice, rats, rabbits, and monkeys. Fc gamma receptor may be derived from any organisms. Mouse Fc gamma receptor includes, without being limited to, Fc gamma RI (CD64), Fc gamma RII (CD32), Fc gamma RIII (CD16), and Fc gamma RIII-2 (CD16-2), as well as all unidentified mouse Fc gamma receptors, Fc gamma receptor isoforms, and allotypes thereof. Such preferred Fc gamma receptors include, for example, human Fc gamma RI (CD64), Fc gamma RIIA (CD32), Fc gamma RIIIB (CD32), Fc gamma RIIIA (CD16), and/or Fc gamma RIIIB (CD16).
- [0166] The polynucleotide sequence and amino acid sequence of Fc gamma RI are shown in RefSeq accession number NM_000566.3 and RefSeq accession number NP_000557.1, respectively; the polynucleotide sequence and amino acid sequence of Fc gamma RIIA are shown in RefSeq accession number BC020823.1 and RefSeq accession number

AAH20823.1, respectively; the polynucleotide sequence and amino acid sequence of Fc gamma RIIIB are shown in RefSeq accession number BC146678.1 and RefSeq accession number AAI46679.1, respectively; the polynucleotide sequence and amino acid sequence of Fc gamma RIIIA are shown in RefSeq accession number BC033678.1 and RefSeq accession number AAH33678.1, respectively; and the polynucleotide sequence and amino acid sequence of Fc gamma RIIIB are shown in RefSeq accession number BC128562.1 and RefSeq accession number AAI28563.1, respectively. Whether an Fc gamma receptor has binding activity to the Fc domain of a monoclonal IgG1, IgG2, IgG3, or IgG4 antibody can be assessed by ALPHA screen (Amplified Luminescent Proximity Homogeneous Assay), surface plasmon resonance (SPR)-based BIACORE method, and others (Proc. Natl. Acad. Sci. USA (2006) 103(11), 4005-4010), in addition to the above-described FACS and ELISA formats.

[0167] Meanwhile, "Fc ligand" or "effector ligand" refers to a molecule and preferably a polypeptide that binds to an antibody Fc domain, forming an Fc/Fc ligand complex. The molecule may be derived from any organisms. The binding of an Fc ligand to Fc preferably induces one or more effector functions. Such Fc ligands include, but are not limited to, Fc receptors, Fc gamma receptor, Fc alpha receptor, Fc beta receptor, FcRn, C1q, and C3, mannan-binding lectin, mannose receptor, Staphylococcus Protein A, Staphylococcus Protein G, and viral Fc gamma receptors. The Fc ligands also include Fc receptor homologs (FcRH) (Davis et al., (2002) Immunological Reviews 190, 123-136), which are a family of Fc receptors homologous to Fc gamma receptor. The Fc ligands also include unidentified molecules that bind to Fc.

[0168] Fc gamma receptor-binding activity

The impaired binding activity of Fc domain to any of the Fc gamma receptors Fc gamma RI, Fc gamma RIIA, Fc gamma RIIIB, Fc gamma RIIIA, and/or Fc gamma RIIIB can be assessed by using the above-described FACS and ELISA formats as well as ALPHA screen (Amplified Luminescent Proximity Homogeneous Assay) and surface plasmon resonance (SPR)-based BIACORE method (Proc. Natl. Acad. Sci. USA (2006) 103(11), 4005-4010).

[0169] ALPHA screen is performed by the ALPHA technology based on the principle described below using two types of beads: donor and acceptor beads. A luminescent signal is detected only when molecules linked to the donor beads interact biologically with molecules linked to the acceptor beads and when the two beads are located in close proximity. Excited by laser beam, the photosensitizer in a donor bead converts oxygen around the bead into excited singlet oxygen. When the singlet oxygen diffuses around the donor beads and reaches the acceptor beads located in close proximity, a chemiluminescent reaction within the acceptor beads is induced. This reaction ultimately results in light emission. If molecules linked to the donor beads do not interact

with molecules linked to the acceptor beads, the singlet oxygen produced by donor beads do not reach the acceptor beads and chemiluminescent reaction does not occur.

[0170] For example, a biotin-labeled antigen-binding molecule or antibody is immobilized to the donor beads and glutathione S-transferase (GST)-tagged Fc gamma receptor is immobilized to the acceptor beads. In the absence of an antigen-binding molecule or antibody comprising a competitive mutant Fc domain, Fc gamma receptor interacts with an antigen-binding molecule or antibody comprising a wild-type Fc domain, inducing a signal of 520 to 620 nm as a result. The antigen-binding molecule or antibody having a non-tagged mutant Fc domain competes with the antigen-binding molecule or antibody comprising a wild-type Fc domain for the interaction with Fc gamma receptor. The relative binding affinity can be determined by quantifying the reduction of fluorescence as a result of competition. Methods for biotinylating the antigen-binding molecules or antibodies such as antibodies using Sulfo-NHS-biotin or the like are known. Appropriate methods for adding the GST tag to an Fc gamma receptor include methods that involve fusing polypeptides encoding Fc gamma receptor and GST in-frame, expressing the fused gene using cells introduced with a vector carrying the gene, and then purifying using a glutathione column. The induced signal can be preferably analyzed, for example, by fitting to a one-site competition model based on nonlinear regression analysis using software such as GRAPHPAD PRISM (GraphPad; San Diego).

[0171] One of the substances for observing their interaction is immobilized as a ligand onto the gold thin layer of a sensor chip. When light is shed on the rear surface of the sensor chip so that total reflection occurs at the interface between the gold thin layer and glass, the intensity of reflected light is partially reduced at a certain site (SPR signal). The other substance for observing their interaction is injected as an analyte onto the surface of the sensor chip. The mass of immobilized ligand molecule increases when the analyte binds to the ligand. This alters the refraction index of solvent on the surface of the sensor chip. The change in refraction index causes a positional shift of SPR signal (conversely, the dissociation shifts the signal back to the original position). In the Biacore system, the amount of shift described above (i.e., the change of mass on the sensor chip surface) is plotted on the vertical axis, and thus the change of mass over time is shown as measured data (sensorgram). Kinetic parameters (association rate constant (k_a) and dissociation rate constant (k_d)) are determined from the curve of sensorgram, and affinity (KD) is determined from the ratio between these two constants. Inhibition assay is preferably used in the BIACORE methods. Examples of such inhibition assay are described in Proc. Natl. Acad. Sci. USA (2006) 103(11), 4005-4010.

[0172] Production and purification of multispecific antigen binding molecule

Multispecific antigen binding molecules described herein comprise two different antigen binding moieties (e.g. the "first antigen binding moiety" and the "second antigen binding moiety"), fused to one or the other of the two subunits of the Fc domain, thus the two subunits of the Fc domain are typically comprised in two non-identical polypeptide 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 multispecific antigen binding molecules in recombinant production, it will thus be advantageous to introduce in the Fc domain of the multispecific antigen binding molecule a modification promoting the association of the desired polypeptides.

[0173] Accordingly, in particular embodiments the Fc domain of the multispecific antigen binding molecule described herein 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, in one embodiment said modification is in the CH3 domain of the Fc domain.

In a specific embodiment 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.

[0174] 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).

[0175] Accordingly, in a particular embodiment, in the CH3 domain of the first subunit of the Fc domain of the multispecific antigen binding molecule 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 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.

- [0176] 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.
- [0177] In a specific embodiment, 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). In one embodiment, 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 residue at position 368 is replaced with an alanine residue (L368A).
- [0178] In yet a further embodiment, 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). Introduction of these two cysteine residues results in formation of a disulfide bridge between the two subunits of the Fc domain, further stabilizing the dimer (Carter, J Immunol Methods 248, 7-15 (2001)).
- [0179] In other embodiments, other techniques for promoting the association among H chains and between L and H chains having the desired combinations can be applied to the multispecific antigen-binding molecules of the present invention.
- [0180] For example, techniques for suppressing undesired H-chain association by introducing electrostatic repulsion at the interface of the second constant region or the third constant region of the antibody H chain (CH2 or CH3) can be applied to multispecific antibody association (WO2006/106905).
- [0181] In the technique of suppressing unintended H-chain association by introducing electrostatic repulsion at the interface of CH2 or CH3, examples of amino acid residues in contact at the interface of the other constant region of the H chain include regions corresponding to the residues at EU numbering positions 356, 439, 357, 370, 399, and 409 in the CH3 region.
- [0182] More specifically, examples include an antibody comprising two types of H-chain CH3 regions, in which one to three pairs of amino acid residues in the first H-chain CH3 region, selected from the pairs of amino acid residues indicated in (1) to (3) below, carry the same type of charge: (1) amino acid residues comprised in the H chain CH3 region at EU numbering positions 356 and 439; (2) amino acid residues comprised in the H-chain CH3 region at EU numbering positions 357 and 370; and (3) amino acid residues comprised in the H-chain CH3 region at EU numbering positions 399 and 409.
- [0183] Furthermore, the antibody may be an antibody in which pairs of the amino acid residues in the second H-chain CH3 region which is different from the first H-chain

CH3 region mentioned above, are selected from the aforementioned pairs of amino acid residues of (1) to (3), wherein the one to three pairs of amino acid residues that correspond to the aforementioned pairs of amino acid residues of (1) to (3) carrying the same type of charges in the first H-chain CH3 region mentioned above carry opposite charges from the corresponding amino acid residues in the first H-chain CH3 region mentioned above.

[0184] Each of the amino acid residues indicated in (1) to (3) above come close to each other during association. Those skilled in the art can find out positions that correspond to the above-mentioned amino acid residues of (1) to (3) in a desired H-chain CH3 region or H-chain constant region by homology modeling and such using commercially available software, and amino acid residues of these positions can be appropriately subjected to modification.

[0185] In the antibodies mentioned above, "charged amino acid residues" are preferably selected, for example, from amino acid residues included in either one of the following groups:

- (a) glutamic acid (E) and aspartic acid (D); and
- (b) lysine (K), arginine (R), and histidine (H).

[0186] In the above-mentioned antibodies, the phrase "carrying the same charge" means, for example, that all of the two or more amino acid residues are selected from the amino acid residues included in either one of groups (a) and (b) mentioned above. The phrase "carrying opposite charges" means, for example, that when at least one of the amino acid residues among two or more amino acid residues is selected from the amino acid residues included in either one of groups (a) and (b) mentioned above, the remaining amino acid residues are selected from the amino acid residues included in the other group.

[0187] In a preferred embodiment, the antibodies mentioned above may have their first H-chain CH3 region and second H-chain CH3 region crosslinked by disulfide bonds.

[0188] In the present invention, amino acid residues subjected to modification are not limited to the above-mentioned amino acid residues of the antibody variable regions or the antibody constant regions. Those skilled in the art can identify the amino acid residues that form an interface in mutant polypeptides or heteromultimers by homology modeling and such using commercially available software; and amino acid residues of these positions can then be subjected to modification so as to regulate the association.

[0189] In addition, other known techniques can also be used for formation of multispecific antigen binding molecule of the present invention. Association of polypeptides having different sequences can be induced efficiently by complementary association of CH3 using a strand-exchange engineered domain CH3 produced by changing part of one of

the H-chain CH3s of an antibody to a corresponding IgA-derived sequence and introducing a corresponding IgA-derived sequence into the complementary portion of the other H-chain CH3 (Protein Engineering Design & Selection, 23; 195-202, 2010). This known technique can also be used to efficiently form multispecific antigen binding molecule of interest.

- [0190] In addition, technologies for antibody production using association of antibody CH1 and CL and association of VH and VL as described in WO 2011/028952, WO2014/018572, and Nat Biotechnol. 2014 Feb; 32(2):191-8; technologies for producing bispecific antibodies using separately prepared monoclonal antibodies in combination (Fab Arm Exchange) as described in WO2008/119353 and WO2011/131746; technologies for regulating association between antibody heavy-chain CH3s as described in WO2012/058768 and WO2013/063702; technologies for producing multispecific antibodies composed of two types of light chains and one type of heavy chain as described in WO2012/023053; technologies for producing multispecific antibodies using two bacterial cell strains that individually express one of the chains of an antibody comprising a single H chain and a single L chain as described by Christoph et al. (Nature Biotechnology Vol. 31, p 753-758 (2013)); and such may be used for the formation of multispecific antibodies.
- [0191] Alternatively, even when a multispecific antibody of interest cannot be formed efficiently, a multispecific antibody of the present invention can be obtained by separating and purifying the multispecific antibody of interest from the produced antibodies. For example, a method for enabling purification of two types of homomeric forms and the heteromeric antibody of interest by ion-exchange chromatography by imparting a difference in isoelectric points by introducing amino acid substitutions into the variable regions of the two types of H chains has been reported (WO2007114325). To date, as a method for purifying heteromeric antibodies, methods using Protein A to purify a heterodimeric antibody comprising a mouse IgG2a H chain that binds to Protein A and a rat IgG2b H chain that does not bind to Protein A have been reported (WO98050431 and WO95033844). Furthermore, a heterodimeric antibody can be purified efficiently on its own by using H chains comprising substitution of amino acid residues at EU numbering positions 435 and 436, which is the IgG-Protein A binding site, with Tyr, His, or such which are amino acids that yield a different Protein A affinity, or using H chains with a different protein A affinity, to change the interaction of each of the H chains with Protein A, and then using a Protein A column.
- [0192] Furthermore, an Fc region whose Fc region C-terminal heterogeneity has been improved can be appropriately used as an Fc region of the present invention. More specifically, the present invention provides Fc regions produced by deleting glycine at position 446 and lysine at position 447 as specified by EU numbering from the amino

acid sequences of two polypeptides constituting an Fc region derived from IgG1, IgG2, IgG3, or IgG4.

[0193] Multispecific antigen binding molecules prepared as 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 antibody, ligand, receptor or antigen can be used to which the multispecific antigen binding molecule binds. For example, for affinity chromatography purification of multispecific antigen binding molecules 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 a multispecific antigen binding molecule. The purity of the multispecific antigen binding molecule can be determined by any of a variety of well known analytical methods including gel electrophoresis, high pressure liquid chromatography, and the like.

[0194] Antibody-dependent cell-mediated cytotoxicity

"Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g. NK cells, neutrophils, and macrophages) enable these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. The primary cells for mediating ADCC, NK cells, express Fc gamma RIII only, whereas monocytes express Fc gamma RI, Fc gamma RII, and Fc gamma RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an in vitro ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 or U.S. Patent No. 6,737,056 (Presta), may be performed. Useful effector cells for such assays include PBMC and NK cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be assessed in vivo, e.g., in an animal model such as that disclosed in Clynes et al. *PNAS (USA)* 95:652-656 (1998).

[0195] Complement dependent cytotoxicity

"Complement dependent cytotoxicity" or "CDC" refers to the lysis of a target cell in the presence of complement. Activation of the classical complement pathway is initiated by the binding of the first component of the complement system (C1q) to antibodies (of the appropriate subclass), which are bound to their cognate antigen. To assess complement activation, a CDC assay, e.g., as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed. Polypeptide variants

with altered Fc region amino acid sequences (polypeptides with a variant Fc region) and increased or decreased C1q binding capability are described, e.g., in US Patent No. 6,194,551 B1 and WO 1999/51642. See also, e.g., Idusogie et al. J. Immunol. 164: 4178-4184 (2000).

[0196] T cell dependent cellular cytotoxicity

"T cell dependent cellular cytotoxicity" or "TDCC" refers to a form of cytotoxicity in which an antigen-binding molecule binds to both an antigen expressed on the target cell, and another antigen expressed on T cell, that redirect T cell near to the target cell, as cytotoxicity against the target cell is induced due to the T cell. The method to assess T cell dependent cellular cytotoxicity, an in vitro TDCC assay, is also described in the "Measurement of T cell dependent cellular cytotoxicity" section of this description.

[0197] Measurement of T cell dependent cellular cytotoxicity

In the embodiment that the antigen-binding molecule binds to both GPC3 and CD3/CD137, the methods described below are preferably used as a method for assessing or determining T cell dependent cellular cytotoxicity (TDCC) caused by contacting an antigen-binding molecule of the present disclosure with GPC3-expressing cells to which the antigen-binding site in the antigen-binding molecules of the present disclosure binds. The methods for assessing or determining the cytotoxic activity in vitro include methods for determining the activity of cytotoxic T-cells or the like. Whether an antigen-binding molecule of the present disclosure has the activity of inducing T-cell mediated cellular cytotoxicity can be determined by known methods (see, for example, Current protocols in Immunology, Chapter 7. Immunologic studies in humans, Editor, John E, Coligan et al., John Wiley & Sons, Inc., (1993)). In the cytotoxicity assay, an antigen-binding molecule which is able to bind to an antigen different from GPC3 and which is not expressed in the cells, and CD3/CD137, is used as a control antigen-binding molecule. The control antigen-binding molecule is assayed in the same manner. Then, the activity is assessed by testing whether an antigen-binding molecule of the present disclosure exhibits a stronger cytotoxic activity than that of a control antigen-binding molecule.

[0198] Meanwhile, the in vivo anti-tumor efficacy is assessed or determined, for example, by the following procedure. Cells expressing the antigen to which the antigen-binding site in an antigen-binding molecule of the present disclosure binds are transplanted intracutaneously or subcutaneously to a nonhuman animal subject. Then, from the day of transplantation or thereafter, a test antigen-binding molecule is administered into vein or peritoneal cavity every day or at intervals of several days. The tumor size is measured over time. Difference in the change of tumor size can be defined as the cytotoxic activity. As in an in vitro assay, a control antigen-binding molecule is administered. The antigen-binding molecule of the present disclosure can be judged to

have cytotoxic activity when the tumor size is smaller in the group administered with the antigen-binding molecule of the present disclosure than in the group administered with the control antigen-binding molecule.

- [0199] An MTT method and measurement of isotope-labeled thymidine uptake into cells are preferably used to assess or determine the effect of contact with an antigen-binding molecule of the present disclosure to suppress the growth of cells expressing an antigen to which the antigen-binding site in the antigen-binding molecule binds. Meanwhile, the same methods described above for assessing or determining the *in vivo* cytotoxic activity can be used preferably to assess or determine the activity of suppressing cell growth *in vivo*.
- [0200] The TDCC of an antibody or antigen-binding molecule of the disclosure can be evaluated by any suitable method known in the art. For example, TDCC can be measured by lactate dehydrogenase (LDH) release assay. In this assay, target cells (e.g. GPC3-expressing cells) are incubated with T cells (e.g. PBMCs) in the presence of a test antibody or antigen-binding molecule, and the activity of LDH that has been released from target cells killed by T cells is measured using a suitable reagent. Typically, the cytotoxic activity is calculated as a percentage of the LDH activity resulting from the incubation with the antibody or antigen-binding molecule relative to the LDH activity resulting from 100% killing of target cells (e.g. lysed by treatment with Triton-X). If the cytotoxic activity calculated as mentioned above is higher, the test antibody or antigen-binding molecule is determined to have higher TDCC.
- [0201] Additionally or alternatively, for example, TDCC can also be measured by real-time cell growth inhibition assay. In this assay, target cells (e.g. GPC3-expressing cells) are incubated with T cells (e.g. PBMCs) in the presence of a test antibody or antigen-binding molecule on a 96-well plate, and the growth of the target cells is monitored by methods known in the art, for example, by using a suitable analyzing instrument (e.g. xCELLigence Real-Time Cell Analyzer). The rate of cell growth inhibition (CGI: %) is determined from the cell index value according to the formulation given as $CGI (\%) = 100 - (CI_{Ab} \times 100 / CI_{NoAb})$. "CI_{Ab}" represents the cell index value of wells with the antibody or antigen-binding molecule on a specific experimental time and "CI_{NoAb}" represents the average cell index value of wells without the antibody or antigen-binding molecule. If the CGI rate of the antibody or antigen-binding molecule is high, i.e., has a significantly positive value, it can be said that the antibody or antigen-binding molecule has TDCC activity. .
- [0202] In one aspect, an antibody or antigen-binding molecule of the disclosure has T cell activation activity. T cell activation can be assayed by methods known in the art, such as a method using an engineered T cell line that expresses a reporter gene (e.g. luciferase) in response to its activation (e.g. Jurkat / NFAT-RE Reporter Cell Line (T

Cell Activation Bioassay, Promega)). In this method, target cells (e.g.GPC3-expressing cells) are cultured with T cells in the presence of a test antibody or antigen-binding molecule, and then the level or activity of the expression product of the reporter gene is measured by appropriate methods as an index of T cell activation. When the reporter gene is a luciferase gene, luminescence arising from reaction between luciferase and its substrate may be measured as an index of T cell activation. If T cell activation measured as described above is higher, the test antibody or antigen-binding molecule is determined to have higher T cell activation activity.

[0203] Pharmaceutical composition

In one aspect, the present disclosure provides a pharmaceutical composition comprising the antigen-binding molecule or antibody of the disclosure. In certain embodiments, the pharmaceutical composition of the disclosure induces T-cell-dependent cytotoxicity, in another word, the pharmaceutical composition of the disclosure is a therapeutic agent for inducing cellular cytotoxicity. In certain embodiments, the pharmaceutical composition of the disclosure is a pharmaceutical composition used for treatment and/or prevention of cancer. In certain embodiments, the pharmaceutical composition of the disclosure is a pharmaceutical composition used for treatment and/or prevention of GPC3-positive cancer or GPC3-expressing cancer.

[0204] A pharmaceutical composition of the present disclosure, a therapeutic agent for inducing cellular cytotoxicity, a cell growth-suppressing agent, or an anticancer agent of the present disclosure may be formulated with different types of antigen-binding molecules or antibodies, if needed. For example, the cytotoxic action against cells expressing an antigen can be enhanced by a cocktail of multiple antigen-binding molecules or antibodies of the disclosure.

[0205] Pharmaceutical compositions of an antigen-binding molecule or antibody as described herein are prepared by mixing such antigen-binding molecule or antibody having the desired degree of purity with one or more optional pharmaceutically acceptable carriers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Pharmaceutically acceptable carriers are generally nontoxic to recipients at the dosages and concentrations employed, and include, but are not limited to: buffers such as phosphate, citrate, and other organic acids; antioxidants including 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 glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counterions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as polyethylene glycol (PEG). Exemplary pharmaceutically acceptable carriers 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 (registered trademark), 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.

- [0206] Exemplary lyophilized antibody formulations are described in US Patent No. 6,267,958. Aqueous antibody formulations include those described in US Patent No. 6,171,586 and WO2006/044908, the latter formulations including a histidine-acetate buffer.
- [0207] The formulation 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 adversely affect each other. Such active ingredients are suitably present in combination in amounts that are effective for the purpose intended.
- [0208] If necessary, the antigen-binding molecules or antibodies of the present disclosure may be encapsulated in microcapsules (microcapsules made from hydroxymethyl-cellulose, gelatin, poly[methylmethacrylate], and the like), and made into components of colloidal drug delivery systems (liposomes, albumin microspheres, microemulsions, nano-particles, and nano-capsules) (for example, see "Remington's Pharmaceutical Science 16th edition", Oslo Ed. (1980)). Moreover, methods for preparing agents as sustained-release agents are known, and these can be applied to the antigen-binding molecules of the present disclosure (J. Biomed. Mater. Res. (1981) 15, 267-277; Chemtech. (1982) 12, 98-105; US Patent No. 3773719; European Patent Application (EP) Nos. EP58481 and EP133988; Biopolymers (1983) 22, 547-556).
- [0209] The pharmaceutical compositions, cell growth-suppressing agents, or anticancer agents of the present disclosure may be administered either orally or parenterally to patients. Parental administration is preferred. Specifically, such administration methods include injection, nasal administration, transpulmonary administration, and percutaneous administration. Injections include, for example, intravenous injections, intramuscular injections, intraperitoneal injections, and subcutaneous injections. For example, pharmaceutical compositions, therapeutic agents for inducing cellular cyto-

toxicity, cell growth-suppressing agents, or anticancer agents of the present disclosure can be administered locally or systemically by injection. Furthermore, appropriate administration methods can be selected according to the patient's age and symptoms. The administered dose can be selected, for example, from the range of 0.0001 mg to 1,000 mg per kg of body weight for each administration. Alternatively, the dose can be selected, for example, from the range of 0.001 mg/body to 100,000 mg/body per patient. However, the dose of a pharmaceutical composition of the present disclosure is not limited to these doses.

- [0210] Preferably, a pharmaceutical composition of the present disclosure comprises an antigen-binding molecule or antibody as described herein. In one aspect, the composition is a pharmaceutical composition for use in inducing cellular cytotoxicity. In another aspect, the composition is a pharmaceutical composition for use in treating or preventing cancer. Preferably, the cancer is GPC3-positive cancer. The pharmaceutical composition of the present disclosure can be used for treating or preventing cancer. Thus, the present disclosure provides a method for treating or preventing cancer, in which the antigen-binding molecule or antibody as described herein is administered to a patient in need thereof
- [0211] The present disclosure also provides methods for damaging cells expressing GPC3 or GPC3-positive cancer, or for suppressing the cell growth by contacting the cells expressing GPC3 with an antigen-binding molecule of the present disclosure that binds to GPC3. Cells to which an antigen-binding molecule of the present disclosure binds are not particularly limited, as long as they express GPC3.
- [0212] In the present disclosure, "contact" can be carried out, for example, by adding an antigen-binding molecule of the present disclosure to culture media of cells expressing GPC3 cultured in vitro. In this case, an antigen-binding molecule to be added can be used in an appropriate form, such as a solution or solid prepared by lyophilization or the like. When the antigen-binding molecule of the present disclosure is added as an aqueous solution, the solution may be a pure aqueous solution containing the antigen-binding molecule alone or a solution containing, for example, an above-described surfactant, excipient, coloring agent, flavoring agent, preservative, stabilizer, buffering agent, suspending agent, isotonicizing agent, binder, disintegrator, lubricant, fluidity accelerator, and corrigent. The added concentration is not particularly limited; however, the final concentration in a culture medium is preferably in a range of 1 pg/ml to 1 g/ml, more preferably 1 ng/ml to 1 mg/ml, and still more preferably 1 micro g/ml to 1 mg/ml.
- [0213] In another embodiment of the present disclosure, "contact" can also be carried out by administration to nonhuman animals transplanted with GPC3-expressing cells in vivo or to animals having cancer cells expressing GPC3 endogenously. The administration

method may be oral or parenteral. Parenteral administration is particularly preferred. Specifically, the parenteral administration method includes injection, nasal administration, pulmonary administration, and percutaneous administration. Injections include, for example, intravenous injections, intramuscular injections, intraperitoneal injections, and subcutaneous injections. For example, pharmaceutical compositions, therapeutic agents for inducing cellular cytotoxicity, cell growth-suppressing agents, or anticancer agents of the present disclosure can be administered locally or systemically by injection. Furthermore, an appropriate administration method can be selected according to the age and symptoms of an animal subject.

- [0214] When the antigen-binding molecule is administered as an aqueous solution, the solution may be a pure aqueous solution containing the antigen-binding molecule alone or a solution containing, for example, an above-described surfactant, excipient, coloring agent, flavoring agent, preservative, stabilizer, buffering agent, suspending agent, isotonicizing agent, binder, disintegrator, lubricant, fluidity accelerator, and corrigent. The administered dose can be selected, for example, from the range of 0.0001 to 1,000 mg per kg of body weight for each administration. Alternatively, the dose can be selected, for example, from the range of 0.001 to 100,000 mg/body for each patient. However, the dose of an antigen-binding molecule of the present disclosure is not limited to these examples.
- [0215] The present disclosure also provides kits for use in a method of the present disclosure, which contain an antigen-binding molecule of the present disclosure or an antigen-binding molecule produced by a method of the present disclosure. The kits may be packaged with an additional pharmaceutically acceptable carrier or medium, or instruction manual describing how to use the kits, etc.
- [0216] 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 on or a package insert 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 pierceable by a hypodermic injection needle). At least one active ingredient in the composition is an antibody of the invention.
- [0217] 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 an

antibody 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 (BWFI), 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.

[0218] Package insert

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

[0219] Pharmaceutical formulation

The term "pharmaceutical formulation" or "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.

[0220] Pharmaceutically acceptable carrier

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

[0221] Treatment

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 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, antigen-binding molecules or antibodies of the present disclosure are used to delay development of a disease or to slow the progression of a disease.

[0222] Cancer

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation.

[0223] In certain embodiments, the cancer is a GPC3-expressing or GPC3-positive cancer.

[0224] Tumor

The term "tumor" refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. The terms "cancer," "cancerous," "cell proliferative disorder," "proliferative disorder" and "tumor" are not mutually exclusive as referred to herein.

[0225] Other Agents and Treatments

The multispecific antigen binding molecules described herein may be administered in combination with one or more other agents in therapy. For instance, a multispecific antigen binding molecules as described herein may be co-administered with at least one additional therapeutic agent. The term "therapeutic agent" encompasses any agent administered to treat 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 embodiments, an additional therapeutic agent is an immunomodulatory agent, a cytostatic agent, an inhibitor of cell adhesion, a cytotoxic agent, an activator of cell apoptosis, or an agent that increases the sensitivity of cells to apoptotic inducers. In a particular embodiment, the additional therapeutic agent is an anti-cancer agent, for example a microtubule disruptor, an antimetabolite, a topoisomerase inhibitor, a DNA intercalator, an alkylating agent, a hormonal therapy, a kinase inhibitor, a receptor antagonist, an activator of tumor cell apoptosis, or an anti-angiogenic agent.

[0226] 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 multispecific antigen binding molecules used, the type of disorder or treatment, and other factors discussed above. The multispecific antigen binding molecules are generally 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 administration, in which case, administration of the multispecific antigen binding molecules described herein can occur prior to, simultaneously, and/or following, administration of the additional therapeutic agent and/or adjuvant. Multispecific antigen binding molecules as described herein can also be used in combination with radiation therapy.

- [0227] All documents cited herein are incorporated herein by reference.
- [0228] The following are examples of methods and compositions of the present disclosure. It is understood that various other embodiments may be practiced, given the general description provided above.

Examples

- [0229] [Example 1] Screening of affinity matured variants of parental Dual-Fab H183L072 for improvement in in vitro cytotoxicity on tumor cells

- [0230] 1.1 Sequence of affinity matured variants

Concept of providing an immunoglobulin variable (Fab) region that binds CD3 and CD137, but does not bind to CD3 and CD137 at same time (Dual-Fab) is disclosed in WO2019111871 (incorporated herein by reference). To increase the binding affinity of parental Dual-Fab H183L072 (Heavy chain: SEQ ID NO: 1; Light chain: SEQ ID NO: 57) disclosed in WO2019111871, more than 1,000 Dual-Fab variants were generated using H183L072 as a template by introduce single or multiple mutations on variable region. Antibodies were expressed Expi293 (Invitrogen) and purified by Protein A purification followed by gel filtration, when gel filtration was necessary. The sequences of 22 represented Dual-Fab variants with multiple mutations are listed in Table 4 and Table 6-1 to 6-6 and binding affinity and kinetics towards CD3 and CD137 were evaluated in the Example 1.2.2 (Tables 7-1 and 7-2) at 25 degrees C and/or 37 degrees C using Biacore T200 instrument (GE Healthcare) described below.

- [0231] (Table 4)

Dual/AE No.	Ab name	VHR name	VLR name	VHR	VHR_ CDR1	VHR_ CDR2	VHR_ CDR3	VLR	VLR_ CDR1	VLR_ CDR2	VLR_ CDR3
Parent	H183/L072	dBBDu183H	dBBDu072L	1	15	29	43	57	62	67	72
Dual/AE01	H0868L0581	dBBDu183H0868	dBBDu072L0581	2	16	30	44	58	63	68	73
Dual/AE08	H1550L0918	dBBDu183H1550	dBBDu072L0918	3	17	31	45	59	64	69	74
Dual/AE06	H1571L0581	dBBDu183H1571	dBBDu072L0581	4	18	32	46	58	63	68	73
Dual/AE17	H1610L0581	dBBDu183H1610	dBBDu072L0581	5	19	33	47	58	63	68	73
Dual/AE10	H1610L0939	dBBDu183H1610	dBBDu072L0939	5	19	33	47	60	65	70	75
Dual/AE05	H1643L0581	dBBDu183H1643	dBBDu072L0581	6	20	34	48	58	63	68	73
Dual/AE19	H1647L0581	dBBDu183H1647	dBBDu072L0581	8	22	36	50	58	63	68	73
Dual/AE20	H1649L0581	dBBDu183H1649	dBBDu072L0581	9	23	37	51	58	63	68	73
Dual/AE21	H1649L0943	dBBDu183H1649	dBBDu072L0943	9	23	37	51	61	66	71	76
Dual/AE22	H1651L0581	dBBDu183H1651	dBBDu072L0581	10	24	38	52	58	63	68	73
Dual/AE23	H1652L0943	dBBDu183H1652	dBBDu072L0943	11	25	39	53	61	66	71	76
Dual/AE09	H1673L0943	dBBDu183H1673	dBBDu072L0943	12	26	40	54	61	66	71	76
Dual/AE18	H1673L0581	dBBDu183H1673	dBBDu072L0581	12	26	40	54	58	63	68	73
Dual/AE14	H2591L0581	dBBDu183H2591	dBBDu072L0581	13	27	41	55	58	63	68	73
Dual/AE15	H2594L0581	dBBDu183H2594	dBBDu072L0581	14	28	42	56	58	63	68	73
Dual/AE16	H1644L0939	dBBDu183H1644	dBBDu072L0939	81	82	83	84	60	65	70	75
Dual/AE02	H0888L0581	dBBDu183H0888	dBBDu072L0581	101	114	127	140	58	63	68	73
Dual/AE24	H1595L0581	dBBDu183H1595	dBBDu072L0581	104	117	130	143	58	63	68	73
Dual/AE07	H1573L0581	dBBDu183H1573	dBBDu072L0581	106	119	132	145	58	63	68	73
Dual/AE25	H1579L0581	dBBDu183H1579	dBBDu072L0581	107	120	133	146	58	63	68	73
Dual/AE26	H1572L0581	dBBDu183H1572	dBBDu072L0581	110	123	136	149	58	63	68	73
Dual/AE27	H0883	dBBDu183H0883	dBBDu072L	113	126	139	152	57	62	67	72
	CD3ε	CD3εVH	CD3εVL	77				78			
	CD137	CD137VH	CD137VL	79				80			

[0232] (Table 6-1)

SEQ list	SEQ number	Amino Acid Sequence
dBBDu183H	1	QQLVESGGGLVQPGRSLRLSCAASGFTFSNAWMHWRQAPGKGLEWVAQIKDKGNAYAAAYA PSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTVLPAGVDAGWGQGTTVTVSS
dBBDu183H0868	2	QQLVESGGGLVQPGRSLRLSCAASGFKFSNVWMHWRQAPGKGLEWVAQIKDKYNAYAAAYA PSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAGVDAGWGQGTTVTVSS
dBBDu183H1550	3	QQLVESGGGLVQPGRSLRLSCAASGFKFSNVWMHWRQAPGKGLEWVAQIKDKYNAYAAAYA PSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAGVDAGWGQGTTVTVSS
dBBDu183H1571	4	QQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWRQAPGKGLEWVAQIKDKYNAYATYAP SVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAGVDAGWGQGTTVTVSS
dBBDu183H1610	5	QQLVESGGGLVQPGRSLRLSCAASGFKFSNVWMHWRQAPGKGLEWVAQIKDKWNAYAAAYA PSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAGIDAGWGQGTTVTVSS
dBBDu183H1643	6	QQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWRQAPGKGLEWVAQIKDYNNAYAAAYAP SVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAEGVDAGWGQGTTVTVSS
dBBDu183H1647	8	QQLVESGGGLVQPGRSLRLSCAASGFKFSNTWFHWRQAPGKGLEWVAQIKDYNDYAAAYAP SVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAEGVDAGWGQGTTVTVSS
dBBDu183H1649	9	QQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWRQAPGKGLEWVAQIKDKYNAYADYYAP SVKERFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAEGVDAGWGQGTTVTVSS
dBBDu183H1651	10	QQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWRQAPGKGLEWVAQIKDKYNAYADYYAP SVEGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAEGVDAGWGQGTTVTVSS
dBBDu183H1652	11	QQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWRQAPGKGLEWVAQIKDYNNAYADYYAP SVEGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAEGVDAGWGQGTTVTVSS
dBBDu183H1673	12	QQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWRQAPGKGLEWVAQIKDKWNAYADYYA PSVKERFTISRDDSKNSIYLQMNSLKTEDTAVYYCHVHYASASTLLPAEGIDAGWGQGTTVTVSS

[0233] (Table 6-2)

SEQ list	SEQ number	Amino Acid Sequence
dBBDu183H2591	13	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWRQAPGKGLEWVAQIKDYNNAYAGYYHP SVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYCHYVHYAAASTLLPAEGVDWGGGTTTVSS
dBBDu183H2594	14	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWRQAPGKGLEWVAQIKDYNNAYAGYYHP SVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYCHYVHYAAASQLLPAEGVDWGGGTTTVSS
dBBDu183H_VHR_CDR1	15	NAWMH
dBBDu183H0868_VHR_CDR1	16	NVWMH
dBBDu183H1550_VHR_CDR1	17	NVWMH
dBBDu183H1571_VHR_CDR1	18	NVWFH
dBBDu183H1610_VHR_CDR1	19	NVWMH
dBBDu183H1643_VHR_CDR1	20	NVWFH
dBBDu183H1647_VHR_CDR1	22	NTWFH
dBBDu183H1649_VHR_CDR1	23	NVWFH
dBBDu183H1651_VHR_CDR1	24	NVWFH
dBBDu183H1652_VHR_CDR1	25	NVWFH
dBBDu183H1673_VHR_CDR1	26	NVWFH
dBBDu183H2591_VHR_CDR1	27	NVWFH
dBBDu183H2594_VHR_CDR1	28	NVWFH
dBBDu183H_VHR_CDR2	29	QIKDKGNAYAAAYAPSVKG
dBBDu183H0868_VHR_CDR2	30	QIKDKYNAYAAAYAPSVKG
dBBDu183H1550_VHR_CDR2	31	QIKDKYNAYAAAYAPSVKG
dBBDu183H1571_VHR_CDR2	32	QIKDKYNAYATYAPSVKG

[0234] (Table 6-3)

SEQ list	SEQ number	Amino Acid Sequence
dBBDu183H1610_VHR_CDR2	33	QIKDKWNAYAAAYAPSVKG
dBBDu183H1643_VHR_CDR2	34	QIKDYNNAYAAAYAPSVKG
dBBDu183H1647_VHR_CDR2	36	QIKDYNDYAAAYAPSVKG
dBBDu183H1649_VHR_CDR2	37	QIKDKYNAYADYYAPSVKE
dBBDu183H1651_VHR_CDR2	38	QIKDKYNAYADYYAPSVGE
dBBDu183H1652_VHR_CDR2	39	QIKDYNNAYADYYAPSVGE
dBBDu183H1673_VHR_CDR2	40	QIKDKWNAYADYYAPSVKE
dBBDu183H2591_VHR_CDR2	41	QIKDYNNAYAGYYHPSVKG
dBBDu183H2594_VHR_CDR2	42	QIKDYNNAYAGYYHPSVKG
dBBDu183H_VHR_CDR3	43	VHYASASTVLPAFGVDA
dBBDu183H0868_VHR_CDR3	44	VHYASASTLLPAFGVDA
dBBDu183H1550_VHR_CDR3	45	IHYASASTLLPAFGVDA
dBBDu183H1571_VHR_CDR3	46	VHYASASTLLPAFGVDA
dBBDu183H1610_VHR_CDR3	47	IHYASASTLLPAEGIDA
dBBDu183H1643_VHR_CDR3	48	VHYASASTLLPAEGVDA
dBBDu183H1647_VHR_CDR3	50	VHYASASTLLPAEGVDA
dBBDu183H1649_VHR_CDR3	51	VHYASASTLLPAEGVDA
dBBDu183H1651_VHR_CDR3	52	VHYASASTLLPAEGVDA
dBBDu183H1652_VHR_CDR3	53	VHYASASTLLPAEGVDA
dBBDu183H1673_VHR_CDR3	54	IHYASASTLLPAEGIDA
dBBDu183H2591_VHR_CDR3	55	VHYAAASTLLPAEGVDA
dBBDu183H2594_VHR_CDR3	56	VHYAAASQLLPAEGVDA

[0235] (Table 6-4)

SEQ list	SEQ number	Amino Acid Sequence
dBBDu072L	57	DIVMTQSPVLPVTPGEPASISQASQELVHNMNRNTYLHWYQQKPGQAPRLLIKVSNRFPVDP RFGSGSGTDFTLKISRVEAEDVGVYCAQGTSPFTFGQGTKLEIK
dBBDu072L0581	58	DIVMTQSPVLPVTPGEPASISQPSQEVVHNMNRNTYLHWYQQKPGQAPRLLIKVSNRFPVDP RFGSGSGTDFTLKISRVEAEDVGVYCAQGTSPFTFGQGTKLEIK
dBBDu072L0918	59	DIVMTQSPVLPVTPGEPASISQPSQEVVHNMNRNTYLHWYQQKPGQAPRLLIKVSNRFPVDP RFGSGSGTDFTLKISRVEAEDVGVYCAQGTSPFTFGQGTKLEIK
dBBDu072L0939	60	DIVMTQSPVLPVTPGEPASISQPSQEVVHNMNRNTYLHWYQQKPGQAPRLLIKVSNRFPVDP RFGSGSGTDFTLKISRVEAEDVGVYCAQGTSPFTFGQGTKLEIK
dBBDu072L0943	61	DIVMTQSPVLPVTPGEPASISQPSQEVVHNMNRNTYLHWYQQKPGQAPRLLIKVSNRFPVDP RFGSGSGTDFTLKISRVEAEDVGVYCAQGTSPFTFGQGTKLEIK
dBBDu072L_VLR_CDR1	62	QASQELVHNMNRNTYLH
dBBDu072L0581_VLR_CDR1	63	QPSQEVVHNMNRNTYLH
dBBDu072L0918_VLR_CDR1	64	QPSQEVVHNMNRNTYLH
dBBDu072L0939_VLR_CDR1	65	QPSQEVVHNMNRNTYLH
dBBDu072L0943_VLR_CDR1	66	QPSQEVVHNMNRNTYLH
dBBDu072L_VLR_CDR2	67	KVSNRFP
dBBDu072L0581_VLR_CDR2	68	KVSNRFP
dBBDu072L0918_VLR_CDR2	69	KVSNRFP
dBBDu072L0939_VLR_CDR2	70	KVSNRFP
dBBDu072L0943_VLR_CDR2	71	KVSNRFP
dBBDu072L_VLR_CDR3	72	AQGTSPFT
dBBDu072L0581_VLR_CDR3	73	AQGTSPFT
dBBDu072L0918_VLR_CDR3	74	AQGTSPFT
dBBDu072L0939_VLR_CDR3	75	AQGTSPFT
dBBDu072L0943_VLR_CDR3	76	AQGTSPFT

[0236] (Table 6-5)

SEQ list	SEQ number	Amino Acid Sequence
CD3εVH	77	QVQLVESGGGVQPGGSLRLSCAASGFTFSNWMHWMVRQAPGKGLEWVAQIKDKSQNYATYV AESVKGRFTISRADSKNSIYQMNSLKTEDTAVYYCRVHYAAGYGVDIWGQGTTVTSS
CD3εVL	78	DIVMTQSPVLPVTPGEPASISCRSQPLVHSNRTYLVHYYQQKPGQAPRLIYKVSNRFSGVPDRF SGSGGTDFTLKISRVEAEDGVVYCGQGTQVPTFGGQTKLEIK
CD137VH	79	QVQLQWGGAGLLKPSETLSLTCAVYGGSGFYWSWIROSPEKGLEWIGEINHGGYVTYNPSLESR VTISVDTSKNQFSLKLSVTAADTAVYYCARDYGPNGVDWYFDLWGRGTLVTSS
CD137VL	80	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLIYDASNRATGIPARFSGSGSGT DFTLTISLLEPEDFAVYYCQQRSNWPPALTFGGGTKVEIK
dBBDu183H1644	81	QVQLVESGGGLVQPGRLSLRSCAASGFVFSNVWFHWMVRQAPGKGLEWVAQIKDYNYAAYAP SVKGRFTISRDDSKNSIYQMNSLKTEDTAVYYCHYVHYASASTLLPAEGVDWVGQGTTVTSS
dBBDu183H1644_VHR_CDR1	82	NVWFH
dBBDu183H1644_VHR_CDR2	83	QIKDYNYAAYAPSVKG
dBBDu183H1644_VHR_CDR3	84	VHYASASTLLPAEGVDA
dBBDu183H0888	101	QVQLVESGGGLVQPGRLSLRSCAASGKFNSVWMHWMVRQAPGKGLEWVAQIKDKWNAYAAYA PSVKGRFTISRDDSKNSIYQMNSLKTEDTAVYYCHYHYASASTLLPAFGIDAWGQGTTVTSS
dBBDu183H1595	104	QVQLVESGGGLVQPGRLSLRSCAASGKFNSVWMHWMVRQAPGKGLEWVAQIKDKYNAAYA PSVKGRFTISRDDSKNSIYQMNSLKTEDTAVYYCHYHYASASTLLPAFGVDWVGQGTTVTSS
dBBDu183H1573	106	QVQLVESGGGLVQPGRLSLRSCAASGKFNSVWFHWMVRQAPGKGLEWVAQIKDYNYAAYAP SVKGRFTISRDDSKNSIYQMNSLKTEDTAVYYCHYHYASASTLLPAFGVDWVGQGTTVTSS
dBBDu183H1579	107	QVQLVESGGGLVQPGRLSLRSCAASGKFNSVWFHWMVRQAPGKGLEWVAQIKDKYNAAYAP SVKGRFTISRDDSKNSIYQMNSLKTEDTAVYYCHYHYASASTLLPAFGVDWVGQGTTVTSS
dBBDu183H1572	110	QVQLVESGGGLVQPGRLSLRSCAASGKFNSVWFHWMVRQAPGKGLEWVAQIKDKYNAAYAP SVKGRFTISRDDSKNSIYQMNSLKTEDTAVYYCHYHYASASTLLPAEGVDWVGQGTTVTSS
dBBDu183H0883	113	QVQLVESGGGLVQPGRLSLRSCAASGFTFSNWMHWMVRQAPGKGLEWVAQIKDKGNAYAAYA PSVKGRFTISRDDSKNSIYQMNSLKTEDTAVYYCRVHYASASTLLPAFGVDWVGQGTTVTSS

[0237] (Table 6-6)

SEQ list	SEQ number	Amino Acid Sequence
dBBDu183H0888_VHR_CDR1	114	NVWMH
dBBDu183H1595_VHR_CDR1	117	NTWMH
dBBDu183H1573_VHR_CDR1	119	NVWFH
dBBDu183H1579_VHR_CDR1	120	HVWFH
dBBDu183H1572_VHR_CDR1	123	NVWFH
dBBDu183H0883_VHR_CDR1	126	NAWMH
dBBDu183H0888_VHR_CDR2	127	QIKDKWNAYAAAYAPSVKG
dBBDu183H1595_VHR_CDR2	130	QIKDKYNAYAAAYAPSVKG
dBBDu183H1573_VHR_CDR2	132	QIKDYNNAYAAAYAPSVKG
dBBDu183H1579_VHR_CDR2	133	QIKDKYNAYAAAYAPSVKG
dBBDu183H1572_VHR_CDR2	136	QIKDKYNAYAAAYAPSVKG
dBBDu183H0883_VHR_CDR2	139	QIKDKGNAYAAAYAPSVKG
dBBDu183H0888_VHR_CDR3	140	IHYASASTLLPAFGIDA
dBBDu183H1595_VHR_CDR3	143	IHYASASTLLPAFGVDA
dBBDu183H1573_VHR_CDR3	145	VHYASASTLLPAFGVDA
dBBDu183H1579_VHR_CDR3	146	VHYASASTLLPAFGVDA
dBBDu183H1572_VHR_CDR3	149	VHYASASTLLPAEGVDA
dBBDu183H0883_VHR_CDR3	152	VHYASASTLLPAFGVDA

[0238] 1.2. Binding kinetics information of affinity matured variants

[0239] 1.2.1 Expression and purification of human CD3 and CD137

The gamma and epsilon subunits of the human CD3 complex (human CD3eg linker) were linked by a 29-mer linker and a Flag-tag was fused to the C-terminal end of the gamma subunit (SEQ ID NO: 102, Tables 3 and 5). This construct was expressed

transiently using FreeStyle293F cell line (Thermo Fisher). Conditioned media expressing human CD3eg linker was concentrated using a column packed with Q HP resins (GE healthcare) then applied to FLAG-tag affinity chromatography. Fractions containing human CD3eg linker were collected and subsequently subjected to a Superdex 200 gel filtration column (GE healthcare) equilibrated with 1x D-PBS. Fractions containing human CD3eg linker were then pooled. Human CD137 extra-cellular domain (ECD) (SEQ ID NO: 103, Tables 3 and 5) with hexahistidine (His-tag) and biotin acceptor peptide (BAP) on its C-terminus was expressed transiently using FreeStyle293F cell line (Thermo Fisher). Conditioned media expressing human CD137 ECD was applied to a HisTrap HP column (GE healthcare) and eluted with buffer containing imidazole (Nacalai). Fractions containing human CD137 ECD were collected and subsequently subjected to a Superdex 200 gel filtration column (GE healthcare) equilibrated with 1x D-PBS. Fractions containing human CD137 ECD were then pooled and stored at -80 degrees C.

[0240] (Table 3)

Antigen name	SEQ List
Human CD3eg linker	102
Human CD137 ECD	103

[0241] (Table 5)

Antigen name	SEQ List	Amino Acid Sequence
Human CD3 ^{eg} linker	102	QDGNEMGGITQTPYKVSISGTTVILTCPPGSEILWQHNDKNIGGDEDDKNIGSDEDEHLSLKEFSELEQSG YVVCYPRGSKPEDANFYLYLRARVGSADDAKDAKKDDAKKDDAKKDGSGSIKGNHLLVKVYDYQEDGSVLL TCDAEAKNITWFKDGKMIGFLTEDKKWNLGNSNAKDPGRMYQCKGSKNKSPLQVYYRMDYKDDDDK
Human CD137 ECD	103	LQDPCSNCPAGTFCDDNNRNQICSPCPNPSFSSAGGQRTCDICRQCKGVFTRKECSSTSNAECDCTPGFHCL GAGCSMCEQDCKQGQELTKGCKDCCFGTFNDQKRGICRPWTNCSLDGKSVLVNGTKERDVVCGPSPADLS PGASSVTPPAPAREPGHSPQHSHHHHHGGGGGLNDIFEAQKIEWHE

[0242] 1.2.2 Affinity measurement towards human CD3 and CD137

Binding affinity of Dual-Fab antibodies (Dual-Ig) to human CD3 were assessed at 25 degrees C using Biacore 8K instrument (GE Healthcare). Anti-human Fc (GE Healthcare) was immobilized onto all flow cells of a CM4 sensor chip using amine coupling kit (GE Healthcare). Antibodies were captured onto the anti-Fc sensor

surfaces, then recombinant human CD3 or CD137 was injected over the flow cell. All antibodies and analytes were prepared in ACES pH 7.4 containing 20 mM ACES, 150 mM NaCl, 0.05% Tween 20, 0.005% NaN₃. Sensor surface was regenerated each cycle with 3M MgCl₂. Binding affinity was determined by processing and fitting the data to 1:1 binding model using Biacore Insight Evaluation software, version 2.0 (GE Healthcare). CD137 binding affinity assay was conducted in same condition except assay temperature was set at 37 degrees C. Binding affinity of Dual-Fab antibodies to recombinant human CD3 and CD137 are shown in Tables 7-1 and 7-2. As illustrated in Tables 7-1 and 7-2, the Dual Fab variants showed different binding kinetics towards CD3 and CD137 as compared H183/L072.

[0243] (Table 7-1)

Antibody name	CD3 (25°C)			CD137 (37°C)		
	ka (M-1s-1)	kd (s-1)	KD (M)	ka (M-1s-1)	kd (s-1)	KD (M)
H183L072	3.54E+04	1.20E-02	3.40E-07	3.47E+03	1.96E-02	5.66E-06
H0868L0581	1.23E+05	1.94E-02	1.57E-07	1.22E+04	1.36E-03	1.11E-07
H1550L0918	7.20E+04	3.16E-03	4.38E-08	1.09E+04	5.79E-03	5.30E-07
H1571L0581	1.42E+05	1.56E-02	1.10E-07	1.21E+04	1.05E-03	8.68E-08
H1610L0581	6.80E+04	1.42E-03	2.09E-08	1.07E+04	1.10E-03	1.03E-07
H1610L0939	5.00E+04	2.53E-03	5.07E-08	1.30E+04	8.01E-04	6.18E-08
H1643L0581	9.46E+04	2.51E-02	2.65E-07	1.23E+04	6.06E-04	4.94E-08
H1644L0939	5.58E+04	8.08E-02	1.45E-06	1.21E+04	4.44E-04	3.68E-08
H1647L0581	4.43E+04	1.01E-01	2.28E-06	9.98E+03	6.47E-04	6.48E-08
H1649L0581	7.50E+04	3.36E-02	4.49E-07	1.29E+04	5.53E-04	4.28E-08
H1649L0943	6.10E+04	4.81E-02	7.89E-07	1.43E+04	4.68E-04	3.28E-08
H1651L0581	7.18E+04	3.71E-02	5.17E-07	1.40E+04	6.03E-04	4.32E-08
H1652L0943	6.23E+04	6.36E-02	1.02E-06	1.29E+04	4.70E-04	3.64E-08
H1673L0581	7.96E+04	1.06E-03	1.33E-08	1.19E+04	9.60E-04	8.04E-08
H1673L0943	5.50E+04	1.16E-03	2.10E-08	1.22E+04	7.22E-04	5.91E-08
H2591L0581	1.02E+05	5.35E-02	5.25E-07	2.04E+04	7.42E-04	3.63E-08
H2594L0581	9.83E+04	5.84E-02	5.93E-07	2.09E+04	1.63E-03	7.81E-08

[0244] (Table 7-2)

Antibody Name	CD3 (25°C)			CD137 (37°C)		
	ka (1/Ms)	kd (1/s)	KD (M)	ka (1/Ms)	kd (1/s)	KD (M)
H0888L0581	9.50E+04	1.92E-03	2.02E-08	1.50E+04	3.11E-03	2.08E-07
H1595L0581	1.16E+05	6.58E-03	5.70E-08	1.38E+04	2.69E-03	1.95E-07
H1573L0581	1.21E+05	1.88E-02	1.56E-07	1.46E+04	1.03E-03	7.06E-08
H1579L0581	1.24E+05	3.40E-02	2.73E-07	1.48E+04	4.06E-03	2.75E-07
H1572L0581	9.77E+04	2.80E-02	2.86E-07	1.39E+04	7.22E-04	5.21E-08
H0883	9.07E+04	9.99E-03	1.10E-07	n.d.		

[0245] 1.2.3 Non-simultaneous binding towards CD3 and CD137 with Dual-Fab AE05 (H1643L0581) and AE15 (H2594L0581)

Biacore in-tandem blocking assay was performed to characterize non-simultaneous binding of Dual-Ig Ab for both CD3 and CD137. The assay was performed on Biacore T200 instrument (GE Healthcare) at 25 degrees C in ACES pH 7.4 buffer containing 20 mM ACES, 150 mM NaCl, 0.05% Tween 20, 0.005% NaN₃. Anti-human Fc (GE Healthcare) was immobilized onto all flow cells of a CM4 sensor chip using amine

coupling kit (GE Healthcare). Antibodies were captured onto the anti-Fc sensor surfaces, then 8micro M CD3 was injected over the flow cell followed by an identical injection of 8 micro M CD137 in the presence of 8 micro M CD3. An increased of binding response for second injection was indicative of binding to different paratopes therefore a simultaneous binding interactions; whereas no enhancement or decreased of binding response for the 2nd injection was indicative of binding to the same or overlapping or adjacent paratopes, therefore a non-simultaneous binding interactions.

[0246] Results of this assay are shown in Figure 1. Both xGPC3/DualAE05-xSG1350k1349hV11 and xGPC3DualAE15-xSG1350kSG1349hV11 showed decreased of binding response for the 2nd injection which is indicative of non-simultaneous binding to CD3 and CD137.

A binding switch from CD3 to CD137 was observed for both mAb as shown by slower off rate in the dissociation phase as compared to CD3 binding alone. Both xGPC3/DualAE05-xSG1350k1349hV11 and xGPC3DualAE15-xSG1350kSG1349hV11 showed slow off rate for CD137 binding and fast off rate for CD3 binding. It is known that antigens binding to overlapped epitope could replace each other (Abdiche YN, Yeung AY, Ni I, Stone D, Miles A, Morishige W, et al., 2017, Antibodies Targeting Closely Adjacent or Minimally Overlapping Epitopes Can Displace One Another. PLoS ONE 12(1): e0169535.).

[0247] 1.3. Generation and sequence of Tri-specific antibodies

Tri-specific antibodies with one arm targeting GPC3 and the other arm with dual targeting function to CD3 and CD137 (anti-GPC3/Dual-Fab Tri-specific antibodies) were generated by utilizing FAST-Ig (WO2013065708) or CrossMab technology (WO2017055539) (Figure 2). Fc region was Fc gammaR silent and deglycosylated. The target antigen of each Fv region in the tri-specific antibodies was shown in Table 8. The naming rule of each of binding domain is shown in Figure 2, and the corresponding SEQ ID NOs are shown in Tables 9 and 10 and the sequences are shown in Tables 11-1 to 11-12. All antibodies were expressed as tri-specific form by transient expression in Expi293 cells (Invitrogen) and purified according to Reference example 1.

[0248] (Table 8)

Ab name	Fv A	Fv B	Format	Fc (knob)	Fc (hole)
GPC3/DualAE05-SG1363k1364hV11	GPC3	DualAE05	FAST-Ig	SG1363kV11Fc	SG1364hV11Fc
GPC3KE/DualAE05EK-SG1363k1365hV11	GPC3KE	DualAE05EK	FAST-Ig	SG1363kV11Fc	SG1365hV11Fc
GPC3/DualAE05-SG1364k1363hV11	GPC3	DualAE05	FAST-Ig	SG1364kV11Fc	SG1363hV11Fc
GPC3EK/DualAE05KE-SG1365k1363hV11	GPC3EK	DualAE05KE	FAST-Ig	SG1365kV11Fc	SG1363hV11Fc
GPC3/DualAE15-SG1363k1364hV11	GPC3	DualAE15	FAST-Ig	SG1363kV11Fc	SG1364hV11Fc
GPC3KE/DualAE15EK-SG1363k1365hV11	GPC3KE	DualAE15EK	FAST-Ig	SG1363kV11Fc	SG1365hV11Fc
GPC3/DualAE15-SG1364k1363hV11	GPC3	DualAE15	FAST-Ig	SG1364kV11Fc	SG1363hV11Fc
GPC3EK/DualAE15KE-SG1365k1363hV11	GPC3EK	DualAE15KE	FAST-Ig	SG1365kV11Fc	SG1363hV11Fc
xGPC3/DualAE05-xSG1350k1349hV11	xGPC3	DualAE05	CrossMab	xSG1350kV11Fc	SG1349hV11Fc
xGPC3/DualAE15-xSG1350k1349hV11	xGPC3	DualAE15	CrossMab	xSG1350kV11Fc	SG1349hV11Fc
xGPC3/183H072L-xSG1350k1349hV11	xGPC3	183H072L	CrossMab	xSG1350kV11Fc	SG1349hV11Fc
xGPC3/DualAE15-xSG1356k1355hV11	xGPC3	DualAE15	CrossMab	xSG1356kV11Fc	SG1355hV11Fc
xGPC3/DualAE05-xSG1356k1355hV11	xGPC3	DualAE05	CrossMab	xSG1356kV11Fc	SG1355hV11Fc
xGPC3/DualAE05-xSG1386k1385hV11	xGPC3	DualAE05	CrossMab	xSG1386kV11Fc	SG1385hV11Fc
xGPC3/DualAE15-xSG1386k1385hV11	xGPC3	DualAE15	CrossMab	xSG1386kV11Fc	SG1385hV11Fc
xCtrl/DualAE05-xSG1350k1349hV11	xC17	DualAE05	CrossMab	xSG1350kV11Fc	SG1349hV11Fc

[0249] (Table 9)

Fc name	Sequence ID	FcR silent	deglycosylation	Knob into hole mutation	Act5
SG1363kV11Fc	312	L234A, L235A	N297A	S354C,T366W	
SG1364kV11Fc	313	L234A, L235A	N297A	S354C,T366W	
SG1365kV11Fc	314	L234A, L235A	N297A	S354C,T366W	
xSG1350kV11Fc	315	L234A, L235A	N297A	S354C,T366W	
xSG1356kV11Fc	316	L234A, L235A	N297A	S354C,T366W	M428L,N434A,Q438R,S440E
xSG1386kV11Fc	317	L234A, L235A	N297A	S354C,T366W	
SG1364hV11Fc	318	L234A, L235A	N297A	Y349C,T366S,L368A,Y407V	
SG1365hV11Fc	319	L234A, L235A	N297A	Y349C,T366S,L368A,Y407V	
SG1363hV11Fc	320	L234A, L235A	N297A	Y349C,T366S,L368A,Y407V	
SG1349hV11Fc	321	L234A, L235A	N297A	Y349C,T366S,L368A,Y407V	
SG1355hV11Fc	322	L234A, L235A	N297A	Y349C,T366S,L368A,Y407V	M428L,N434A,Q438R,S440E
SG1385hV11Fc	323	L234A, L235A	N297A	Y349C,T366S,L368A,Y407V	

[0250] (Table 10)

VR name	VHR name	VLR name	VHR	VHR_ CDR1	VHR_ CDR2	VHR_ CDR3	VLR	VLR_ CDR1	VLR_ CDR2	VLR_ CDR3
GPC3	GCH065H	TR01L0011	226	235	244	253	262	268	274	280
GPC3KE	GCH065H.Q39K	TR01L0011.Q38E	227	236	245	254	263	269	275	281
GPC3EK	GCH065H.Q39E	TR01L0011.Q38K	228	237	246	255	264	270	276	282
xGPC3	GCH065H	TR01L0011	226	235	244	253	262	268	274	280
DualAE05	dBBDu183H1643	dBBDu072L0581	229	238	247	256	265	271	277	283
DualAE05EK	dBBDu183H1643.Q39E	dBBDu072L0581.Q38K	230	239	248	257	266	272	278	284
DualAE05KE	dBBDu183H1643.Q39K	dBBDu072L0581.Q38E	231	240	249	258	267	273	279	285
DualAE15	dBBDu183H2594	dBBDu072L0581	232	241	250	259	265	271	277	283
DualAE15EK	dBBDu183H2594.Q39E	dBBDu072L0581.Q38K	233	242	251	260	266	272	278	284
DualAE15KE	dBBDu183H2594.Q39K	dBBDu072L0581.Q38E	234	243	252	261	267	273	279	285
183H072L	dBBDu183H.GS	dBBDu072L.GS	296	297	298	299	300	301	302	303
xCtrl	IC17HdK	IC17L	308	309	310	311	304	305	306	307

[0251] (Table 11-1)

SEQ number	Amino Acid Sequence
201	QVQLVQSGAEVKKPGASVTVSCKASGYTFTDYEMHWIRQPPGEGLEWIGAI DGTPDTA YSEKFKGRVTLTADKSTSTAYMELSSLTSED TAVYYCTRFYSYTYWGQGLTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLKSSGLYSLSSVVT VPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTV LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCL VKGFIYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQEGNVFSCSVM HEALHNHYTQKSLSLSP
202	QVQLVQSGAEVKKPGASVTVSCKASGYTFTDYEMHWIRKPPGEGLEWIGAI DGTPDTAY SEKFKGRVTLTADKSTSTAYMELSSLTSED TAVYYCTRFYSYTYWGQGLTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLKSSGLYSLSSVVT PSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKD TLTMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLV KGFYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQEGNVFSCSVMH EALHNHYTQKSLSLSP
203	QVQLVQSGAEVKKPGASVTVSCKASGYTFTDYEMHWIRQPPGEGLEWIGAI DGTPDTA YSEKFKGRVTLTADKSTSTAYMELSSLTSED TAVYYCTRFYSYTYWGQGLTVTVSSASTKGPS VFPLAPSSKSTSGGTAALGCLVEDYFPEPVT VSWNSGALTSGVHTFPAVLESSGLYSLSSVVT VPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTV LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCL VKGFIYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQEGNVFSCSVM HEALHNHYTQKSLSLSP
204	QVQLVQSGAEVKKPGASVTVSCKASGYTFTDYEMHWIREPPGEGLEWIGAI DGTPDTAY SEKFKGRVTLTADKSTSTAYMELSSLTSED TAVYYCTRFYSYTYWGQGLTVTVSSASTKGPSV FPLAPSSKSTSGGTAALGCLVEDYFPEPVT VSWNSGALTSGVHTFPAVLESSGLYSLSSVVT PSSSLGTQTYICNVNHKPSNTKVDREVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKD TLTMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVL HQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLV KGFYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQEGNVFSCSVMH EALHNHYTQKSLSLSP
205	DIVMTQSPLSLPVTPGEPASISCRSSQPLVHSNRNTYLHWYQQKPGQAPRL LIYKVSNRFS GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGGQTQVPYTFGQG TKLEIKSSASTKGPSV FPLAPSSKSTSGGTAALGCLVKDYFPEPVT VSWNSGALTSGVHTFPAVLQSSGLYSLSSVVT VPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPK DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTV LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCL VKGFIYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQEGNVFSCSVM HEALHNHYTQKSLSLSP

[0252] (Table 11-2)

SEQ number	Amino Acid Sequence
206	DIVMTQSPLSLPVTGPGEASISCRSSQPLVHSNRNTYLHWYQQKPGQAPRLLIYKVSNRFS GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQGTQVPYTFGQGTKLEIKRTVAAPSVFIF PPSDEQLKSGTAEEVCLLNFFYPREAKVQWKVDNALQSGNSEESVTEQDSKDSTYLSSTL TLKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
207	DIVMTQSPLSLPVTGPGEASISCRSSQPLVHSNRNTYLHWYQEKPGQAPRLLIYKVSNRFSG VPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQGTQVPYTFGQGTKLEIKRTVAAPSVFIFP PSDEQLKSGTAEEVCLLNFFYPREAKVQWKVDNALQSGNSEESVTEQDSKDSTYLSSTLE LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
208	DIVMTQSPLSLPVTGPGEASISCRSSQPLVHSNRNTYLHWYQQKPGQAPRLLIYKVSNRFS GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQGTQVPYTFGQGTKLEIKRTVAAPSVFIF PPSDEQLKSGTAKVVCLLNFFYPREAKVQWKVDNALQSGNSKESVTEQDSKDSTYLSSTL TLKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
209	DIVMTQSPLSLPVTGPGEASISCRSSQPLVHSNRNTYLHWYQKKPGQAPRLLIYKVSNRFSG VPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQGTQVPYTFGQGTKLEIKRTVAAPSVFIFP PSDKQLKSGTAKVVCLLNFFYPREAKVQWKVDNALQSGNSKESVTEQDSKDSTYLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
210	QVQLVQSGAEVKKPGASVTVSCKASGYFTDYEMHWIRQPPGEGLEWIGAIDGTPDPTAY SEKFKGRVTLTADKSTSTAYMELSSLTSEDVAVYYCTRFYSYTYWGQGTLTVTSSASVAAPSV FIFPPSDEQLKSGTASVCLLNFFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLS STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
211	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYNNAY AAYYAPSVKGRFTISRDDSKNSIYLQMNSLKTEDAVYYCHYVHYASASTLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLESSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVKPKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPISR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMHEALHNHYTQKSLSLSP
212	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVREAPGKGLEWVAQIKDYNNAYA AAYYAPSVKGRFTISRDDSKNSIYLQMNSLKTEDAVYYCHYVHYASASTLLPAEGVDAWGQ GTTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTFP AVLESSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDERVEPKSCDKTHTCPPCPAPEA AGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPISR EMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMHEALHNHYTQKSLSLSP

[0253] (Table 11-3)

SEQ number	Amino Acid Sequence
213	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AAYYAPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYASASTLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLKSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVKPSCKDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMEALHNHYTQKSLSLSP
214	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRKAPGKGLEWVAQIKDYINAYA AYYAPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYASASTLLPAEGVDAWGQ GTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLKSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVKPSCKDKTHTCPPCPAPEA AGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPRE EMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMEALHNHYTQKSLSLSP
215	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AGYYHPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLESSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVKPSCKDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMEALHNHYTQKSLSLSP
216	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVREAPGKGLEWVAQIKDYINAYA GYYHPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLESSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVKPSCKDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMEALHNHYTQKSLSLSP

[0254] (Table 11-4)

SEQ number	Amino Acid Sequence
217	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AGYYHPSVKGRFTISRDDSKNSIYLMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLKSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMEALHNHYTQKSLSLSP
218	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRKAPGKGLEWVAQIKDYINAYA GYYHPSVKGRFTISRDDSKNSIYLMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTF PAVLKSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMEALHNHYTQKSLSLSP
219	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AAYYAPSVKGRFTISRDDSKNSIYLMNSLKTEDTAVYYCHYVHYASASTLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDERVEPKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMEALHNHYTQKSLSLSP
220	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AGYYHPSVKGRFTISRDDSKNSIYLMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDERVEPKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMEALHNHYTQKSLSLSP

[0255] (Table 11-5)

SEQ number	Amino Acid Sequence
221	DIVMTQSPLSLPVTPGEPASISCQPSQEVVHMRNTYLHWYQQKPGQAPRLLIYKVSNRF PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSHPFTFGQGTKLEIKRTVAAPSVFI FPPSDEQLKSGTAKVVCLLNNFYPRKAVQWKVDNALQSGNSKESVTEQDSKDYSLST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
222	DIVMTQSPLSLPVTPGEPASISCQPSQEVVHMRNTYLHWYQQKPGQAPRLLIYKVSNRF PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSHPFTFGQGTKLEIKRTVAAPSVFI FPPSDKQLKSGTAKVVCLLNNFYPRKAVQWKVDNALQSGNSKESVTEQDSKDYSLST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
223	DIVMTQSPLSLPVTPGEPASISCQPSQEVVHMRNTYLHWYQQKPGQAPRLLIYKVSNRF PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSHPFTFGQGTKLEIKRTVAAPSVFI FPPSDEQLKSGTAEVCLLNNFYPRKAVQWKVDNALQSGNSEESVTEQDSKDYSLST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
224	DIVMTQSPLSLPVTPGEPASISCQPSQEVVHMRNTYLHWYQEKPGQAPRLLIYKVSNRF PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSHPFTFGQGTKLEIKRTVAAPSVFI FPPSDEQLKSGTAEVCLLNNFYPRKAVQWKVDNALQSGNSEESVTEQDSKDYSLST LELSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
225	DIVMTQSPLSLPVTPGEPASISCQPSQEVVHMRNTYLHWYQQKPGQAPRLLIYKVSNRF PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSHPFTFGQGTKLEIKRTVAAPSVFI FPPSDRKLKSGTASVCLLNNFYPRKAVQWKVDNALQSGNSQESVTEQDSKDYSLST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
226	QVQLVQSGAEVKKPGASVTVSCKASGYFTDYEMHWIRQPPGEGLEWIGRIDGTPDTAY SEKFKGRVTLTADKSTSTAYMELSSLTSEDVAVYYCTRFYSYTYWGQGLTVTVSS
227	QVQLVQSGAEVKKPGASVTVSCKASGYFTDYEMHWIRKPPGEGLEWIGRIDGTPDTAY SEKFKGRVTLTADKSTSTAYMELSSLTSEDVAVYYCTRFYSYTYWGQGLTVTVSS
228	QVQLVQSGAEVKKPGASVTVSCKASGYFTDYEMHWIREPPGEGLEWIGRIDGTPDTAY SEKFKGRVTLTADKSTSTAYMELSSLTSEDVAVYYCTRFYSYTYWGQGLTVTVSS
229	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AAYYAPSVKGRFTISRDDSKNSIYLMNSLKTEDVAVYYCHYVHYASASTLLPAEGVDAWG QGTITVTVSS
230	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVREAPGKGLEWVAQIKDYINAYA AAYYAPSVKGRFTISRDDSKNSIYLMNSLKTEDVAVYYCHYVHYASASTLLPAEGVDAWGQ GTTITVTVSS

[0256] (Table 11-6)

SEQ number	Amino Acid Sequence
231	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRKAPGKGLEWVAQIKDYYNAYA AAYAPS VKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYASASTLLPAEGVDAWGQ GTTTVTVSS
232	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYYNAY AGYYHPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTTVTVSS
233	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVREAPGKGLEWVAQIKDYYNAYA GYYHPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTTVTVSS
234	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRKAPGKGLEWVAQIKDYYNAYA GYYHPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTTVTVSS
235	DYEMH
236	DYEMH
237	DYEMH
238	NVWFH
239	NVWFH
240	NVWFH
241	NVWFH
242	NVWFH
243	NVWFH
244	AIDGTPDTAYSEKFKG
245	AIDGTPDTAYSEKFKG
246	AIDGTPDTAYSEKFKG
247	QIKDYYNAYAAYAPS VKG
248	QIKDYYNAYAAYAPS VKG

[0257] (Table 11-7)

SEQ number	Amino Acid Sequence
249	QIKDYYNAYAAYAPSVKG
250	QIKDYYNAYAGYYHPSVKG
251	QIKDYYNAYAGYYHPSVKG
252	QIKDYYNAYAGYYHPSVKG
253	FYSYTY
254	FYSYTY
255	FYSYTY
256	VHYASASTLLPAEGVDA
257	VHYASASTLLPAEGVDA
258	VHYASASTLLPAEGVDA
259	VHYAAASQLLPAEGVDA
260	VHYAAASQLLPAEGVDA
261	VHYAAASQLLPAEGVDA
262	DIVMTQSPLSLPVTGPGEPAISCRSSQPLVHSNRNTYLHWYQQKPGQAPRLLIYKVSNRFS GVDPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQGTQVPYTFGQGTKLEIK
263	DIVMTQSPLSLPVTGPGEPAISCRSSQPLVHSNRNTYLHWYQEKPGQAPRLLIYKVSNRFSG VPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQGTQVPYTFGQGTKLEIK
264	DIVMTQSPLSLPVTGPGEPAISCRSSQPLVHSNRNTYLHWYQKPGQAPRLLIYKVSNRFSG VPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQGTQVPYTFGQGTKLEIK
265	DIVMTQSPLSLPVTGPGEPAISCRSSQPLVHSNRNTYLHWYQKPGQAPRLLIYKVSNRFS PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSHPFTFGQGTKLEIK

[0258] (Table 11-8)

SEQ number	Amino Acid Sequence
266	DIVMTQSPLSLPVTGPGEPAISCRSSQPLVHSNRNTYLHWYQKPGQAPRLLIYKVSNRFS PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSHPFTFGQGTKLEIK
267	DIVMTQSPLSLPVTGPGEPAISCRSSQPLVHSNRNTYLHWYQEKPGQAPRLLIYKVSNRFS PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSHPFTFGQGTKLEIK
268	RSSQPLVHSNRNTYLH
269	RSSQPLVHSNRNTYLH
270	RSSQPLVHSNRNTYLH
271	QPSQEVVHMNRNTYLH
272	QPSQEVVHMNRNTYLH
273	QPSQEVVHMNRNTYLH
274	KVSNRFS
275	KVSNRFS
276	KVSNRFS
277	KVSNRFP
278	KVSNRFP
279	KVSNRFP
280	GQGTQVPYT
281	GQGTQVPYT
282	GQGTQVPYT

[0259] (Table 11-9)

SEQ number	Amino Acid Sequence
283	AQGTSHPFT
284	AQGTSHPFT
285	AQGTSHPFT
286	DIVMTQSPLSLPVTGPGEASISCRSSQPLVHSNRNTYLHWYQQKPGQAPRLLIYKVSNRFS GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQGTQVPYTFGGGTKLEIKSSASTKGPSVF PLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTL MISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGF YPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQEGNVFSCSVLHEALH AHYTRKELSLSP
287	DIVMTQSPLSLPVTGPGEASISCRSSQPLVHSNRNTYLHWYQQKPGQAPRLLIYKVSNRFS GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCGQGTQVPYTFGGGTKLEIKSSASTKGPSVF PLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTP SSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTL MISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGF YPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEAL HNHYTQKSLSLSP
288	DIQMTQSSSFVSLGDRVITITCKASEDIYNRLAWYQQKPGNAPRLLISGATSLETGVPSRFS GSGSGKDYLITSLQTEDVATYYCQYWSTPYTFGGGTKLEVKSSASTKGPSVFPLAPSSKS TSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQ TYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPE VTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYASTYRVVSVLTVLHQDWLNG KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIA VEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQEGNVFSCSVMHEALHNHYT QKSLSLSP
289	QVQLQQSGPQLVRPGASVKISCKASGYSTSYWMHWVNQRPGQGLEWIGMIDPSYSET RLNQKFKDKATLTVDKSSSTAYMQLSSPTSEDSAVYYCALYGNFYFDYWGQGTTLTVSSASV AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKD STYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
290	QVQLVESGGGLVQPGRSLRLSCAASGFTFSNAWMHWVRQAPGKGLEWVAQIKDKGNAY AAYYAPSVKGRFTISRDDSKNSIYLMNSLKTEDTAVYYCHYVHYASASTVLPAGVDWAG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKRVKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPPSR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVMHEALHNHYTQKSLSLSP

[0260] (Table 11-10)

SEQ number	Amino Acid Sequence
291	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AGYYHPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDERVEPKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVLHEALHAHYTRKELSLSP
292	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AAYYAPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYASASTLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDERVEPKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQEGNVFSCSVLHEALHAHYTRKELSLSP
293	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AAYYAPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYASASTLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDERVEPKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSP
294	QVQLVESGGGLVQPGRSLRLSCAASGFKFSNVWFHWVRQAPGKGLEWVAQIKDYINAY AGYYHPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYAAASQLLPAEGVDAWG QGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVEDYFPEPVTVSWNSGALTSGVHTF PAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDERVEPKSCDKTHTCPPCPAPE AAGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPRE EQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVCTLPSPR EEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLVSKLTVDKSR WQQGNVFSCSVMHEALHNHYTQKSLSLSP

[0261] (Table 11-11)

SEQ number	Amino Acid Sequence
295	DIVMTQSPLSLPVTPGEPASISCCQASQELVHMMNRNTYLHWYQQKPGQAPRLLIYKVSNRF PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSPFTFGQGTKLEIKRTVAAPSVFI FPPSDRKLKSGTASVVCLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKSTYLSST LTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
296	QVQLVESGGGLVQPGRSLRLSCAASGFTFSNAWMHWVRQAPGKGLEWVAQIKDKGNA YAAYYAPSVKGRFTISRDDSKNSIYLQMNSLKTEDTAVYYCHYVHYASASTVLPAGVDAW GQGTTVTVSS
297	NAWMH
298	QIKDKGNAYAAAYYAPSVKG
299	VHYASASTVLPAGVDA
300	DIVMTQSPLSLPVTPGEPASISCCQASQELVHMMNRNTYLHWYQQKPGQAPRLLIYKVSNRF PGVPDRFSGSGSGTDFTLKISRVEAEDVGVYYCAQGTSPFTFGQGTKLEIK
301	QASQELVHMMNRNTYLH
302	KVSNRF
303	AQGTSPFT
304	DIQMTQSSSFVSLGDRVITCKASEDIYNRLAWYQQKPGNAPRLLISGATSLETGVPSRF SGSGSGKDYTLISLTQTEDVATYYCQYWSTPYTFGGGTKLEVK
305	KASEDIYNRLA
306	GATSLET
307	QYWSTPYT
308	QVQLQQSGPQLVRPGASVKISCKASGYSFTSYWMHWVNQRPGQGLEWIGMIDPSYSET RLNQQKFKDQATLTVDKSSSTAYMQLSSPTSEDSAVYYCALYGNFYDYWGQGTTLTVSS
309	SYWMH
310	MIDPSYSETRLNQQKFD
311	YGNFYDY
312	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFF LYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
313	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVCTLPSPREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFL VSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
314	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP QVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFF LYSKLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSP

[0262] (Table 11-12)

SEQ number	Amino Acid Sequence
315	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLSP
316	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQEGNVFSCSVLHEALHAHYTRKELSLSP
317	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVYTLPPCREEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGS FFLYSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSP
318	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVCTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSF FLVSKLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLSP
319	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVCTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSF FLVSKLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLSP
320	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVCTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSF FLVSKLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLSP
321	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVCTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSF FLVSKLTVDKSRWQEGNVFSCSVMEALHNHYTQKSLSLSP
322	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVCTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSF FLVSKLTVDKSRWQEGNVFSCSVLHEALHAHYTRKELSLSP
323	CPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQASTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR EPQVCTLPPSREEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSF FLVSKLTVDKSRWQQGNVFSCSVMEALHNHYTQKSLSLSP

[0263] [Example 2] Evaluation of in vitro cytotoxicity by affinity matured Dual-Fab variants derived from parental Dual-Fab H183L072 on tumor cells

[0264] 2.1. Assessment of CD3 agonistic activity of affinity matured Dual-Fab variants in vitro

To evaluate CD3 agonistic activity as a result of affinity maturation, NFAT-luc2 Jurkat luciferase assay was conducted. Briefly, 4×10^3 cells/well SK-pca60 cells (Reference Example 2) which express human GPC3 on the cell membrane, was used as target cells and co-cultured with 2.0×10^4 cells/well of NFAT-luc2 Jurkat cells (E:T

ratio 5) for 24 hours in the presence of 0.02, 0.2 and 2 nM of tri-specific antibodies. Variants were divided into plate 1 in Figure 3 upper panel and plate 2 in Figure 3 lower panel. 24 hours later, luciferase activity was detected with Bio-Glo luciferase assay system (Promega, G7940) according to manufacturer's instructions. Luminescence (units) was detected using GloMax(registered trademark) Explorer System (Promega #GM3500) and captured values were plotted using Graphpad Prism 7. Parental tri-specific antibody GPC3/H183L072 and bi-specific antibody GPC3/CD3 epsilon were included at 2nM concentration. Figure 3 shows that most variants have similar CD3 agonist activity. Particularly at 2nM, variants had similar activity as parental H183L072. Figure 3 upper panel shows that all variants in Plate 1 has similar CD3 agonistic activity. Figure 3 lower panel shows that H1610L939 have slightly weaker CD3 agonist activity while H2591L581 has the strongest CD3 agonistic activity amongst the variants in plate 2.

[0265] 2.2. Assessment of CD137 agonistic activity of affinity matured Dual-Fab variants in vitro

To evaluate which antibody variant could result in strong CD137 agonistic activity as a result of affinity maturation, the GloResponse™ NF- kappa B-Luc2/CD137 Jurkat cell line (Promega #CS196004) as effector cells while similar to above, SK-pca60 cell line (Reference Example 2) was used as target cells. Both 4.0×10^3 cells/well SK-pca60 cells (target cells) and 2.0×10^4 cells/well NF- kappa B-Luc2/CD137 Jurkat (Effector cells) were added on the each well of white-bottomed, 96-well assay plate (Costar, 3917) at E:T ratio of 5. Antibodies were added to each well at 0.5nM, 2.5nM and 5nM concentration incubated at 37 degrees C, 5% CO₂ at 37 degrees C for 5 hours. The expressed Luciferase was detected with Bio-Glo luciferase assay system (Promega, G7940) according to Manufacturer's instructions. Luminescence (units) was detected using GloMax(registered trademark) Explorer System (Promega #GM3500) and captured values were plotted using Graphpad Prism 7.

[0266] As shown in Figure 4, antibody variants were divided into plate 1 and plate 2. All variants in both plates had detectable CD137 agonistic activity compared to GPC3/CD3 epsilon, which was used as a negative control. Parental antibody before affinity maturation, GPC3/H183L072 was also used as a control in both plates. As shown in Figure 4, all variants showed stronger CD137 agonistic antibody than the parental antibody GPC3/H183L072 after affinity maturation for CD137 binding. Accordingly, GPC3/H1643L581 and GPC3/H868L581 in plate 1 and GPC3/H2594L581 and GPC3/H2591L581 in plate 2 were the top variants that resulted in stronger CD137 agonistic activity. Whereas variants such as GPC3/H1550L918 in plate 1 and GPC3/H1610L581 and GPC3/H1610L939 in plate 2 showed weaker CD137 activity. Taken together, Figures 3 and Figure 4 show that GPC3/H1643L581,

GPC3/H2594L581, GPC3/H868L581 and GPC3/H2591L581 appear to have strong activity in Jurkat cells whereas GPC3/H1610L939 has weaker activity amongst the variants.

[0267] As shown in Figure 5, antibody variants were divided into plate 1 and plate 2 with GPC3/H0868L581 and GPC3/H1643L0581 variant as inter-plate controls. All variants in both plates had detectable CD137 agonistic activity compared to GPC3/CD3epsilon. Accordingly, GPC3/H1643L581, GPC3/H1571L581 and GPC3/H1573L581 were the top variants that resulted in stronger CD137 agonistic activity in plate 1 while GPC3/H1572L581, GPC3/H0868L581 and GPC3/H1595L0581 in plate 2 that resulted in stronger CD137 agonistic activity whereas variants such as GPC3/H888L581, and GPC3/H1673L581 showed weaker CD137 activity.

[0268] 2.3. Evaluation of in vitro cytotoxicity of affinity matured variants

In order to extend the observations of CD3 and/or CD137 activation to in vitro cytotoxicity, affinity matured variants described earlier were subjected to evaluation of T-cell dependent cytotoxicity (TDCC) activity on SK-pca60 cells using human peripheral blood mononuclear cells.

[0269] 2.3.1. Preparation of frozen human PBMC

Cryovials containing PBMCs purchased commercially (STEMCELL Technologies.) were placed in the water bath at 37 degrees C to thaw cells. Cells were then dispensed into a 15 mL falcon tube containing 9 mL of media (media used to culture target cells). Cell suspension was then subjected to centrifugation at 1,200 rpm for 5 minutes at room temperature. The supernatant was aspirated gently and fresh warmed medium was added for resuspension and used as the human PBMC solution.

[0270] 2.3.2. Measurement of TDCC activity induced by affinity matured anti-GPC3/Dual-Fab Tri-specific antibodies

Cytotoxic activity was assessed by observing the rate of tumor cell growth inhibition using xCELLigence Real-Time Cell Analyzer (Roche Diagnostics) in the presence of PBMCs. Figure 6 shows the TDCC activity of anti- GPC3 affinity matured Dual-Fab tri-specific antibodies. SK-pca60 cell line was used as target cells. Target cells were detached from the dish and cells were plated into E-plate 96 (Roche Diagnostics) in aliquots of 100 micro L/well by adjusting the cells to 3.5×10^3 cells/well, and measurement of cell growth was initiated using the xCELLigence Real-Time Cell Analyzer. 24 hours later, the plate was removed and 50 micro L of the respective antibodies prepared at each concentration (3-fold serial dilutions starting from 5nM i.e., 0.19, 0.56, 1.67 and 5nM) were added to the plate. After 15 minutes of reaction at room temperature, 50 micro L of the fresh human PBMC solution prepared in (Example 2.3.1) was added in effector: target ratio of 0.5 (i.e. 1.75×10^3 cells/well) and measurement of cell growth was resumed using xCELLigence Real-Time Cell

Analyzer. The reaction was carried out under the conditions of 5% carbon dioxide gas at 37 degrees C. As CD137 signaling enhances T-cell survival and prevents activation induced cell death, TDCC assay was conducted at a low E:T ratio. An extended period of time may be required to observe superior cytotoxicity contributed by CD137 activation. As such, approximately 120 hours after the addition of PBMCs, Cell Growth Inhibition (CGI) rate (%) was determined using the equation below. The Cell Index Value obtained from xCELLigence Real-Time Cell Analyzer used in the calculation was a normalized value where the Cell Index value immediately at the time point before antibody addition was defined as 1.

Cell Growth Inhibition rate (%) = $(A-B) \times 100 / (A-1)$

[0271] A represents the mean value of Cell Index values in wells without antibody addition (containing only target cells and human PBMCs), and B represents the mean value of the Cell Index values of target wells. The examinations were performed in triplicates.

Affinity matured variants were divided into 2 plates as with above examples with GPC3/H1643L581 as an internal plate control for reference in Figure 6. Although most variants showed similar TDCC activity, it can be observed that H1643L581 showed relatively stronger TDCC activity at lower concentration of 0.56nM and 1.67nM in both plates among the variants. Figure 6a shows that GPC3/H2591L581 is relatively weaker while Figure 6b shows that GPC3/H1610L939 is relatively weaker at 0.56nM concentration.

[0272] As shown in Figure 7, affinity matured variants with stronger cytotoxicity than GPC3/CD3epsilon included GPC3/H1643L581, GPC3/H1571L581 and GPC3/H1595L581 at both 5nM and 10nM antibody concentrations. This suggests that binding to CD137 contributes to improved cytotoxicity of these variants compared to GPC3/CD3epsilon. Variants such as GPC3/H0868L581, GPC3/H1572L581 showed weaker cytotoxicity than GPC3/CD3epsilon at 5nM.

[0273] 2.3.3. Measurement of cytokine release using affinity matured anti-GPC3/Dual-Fab Tri-specific antibodies

To further confirm in vitro potency of antibodies, they were also evaluated for cytokine release. Supernatant from TDCC assay similarly conducted in Example 2.3.2 at 48h were harvested and evaluated for the presence of cytokines. Since most antibodies showed similar CD3 agonistic activity as GPC3/CD3 epsilon as shown in Figure 3, GPC3/CD3 epsilon was added to this assay to evaluate cytokine release as a result of synergistic activity with CD137. Similarly, GPC3/H1643L581 was used as an internal plate control. Total cytokine release was evaluated using cytometric bead array (CBA) Human Th1/T2 Cytokine kit II (BD Biosciences #551809). IFN gamma (Figure 8), IL-2 (Figure 9) and IL-6 (Figure 10) were evaluated.

[0274] As shown in Figure 8 and 9, GPC3/H2591L581 and GPC3/H1643L581 are the top 2

variants that resulted in high IFN gamma and IL-2 at 5nM and 1.67nM in Plate 1. In plate 2, GPC3/H1610L939, GPC3/H2594L581 and GPC3/H1643L581 showed relatively strong cytokine release at 5nM. However, only GPC3/H1643L581 showed stronger cytokine release at 1.67nM. As for IL-6 levels shown in Figure 10, all variants showed similar levels to GPC3/CD3 epsilon in plate 1 except for GPC3/H2591L581 which showed lower levels of IL-6 at 0.56nM and 0.19nM. Similarly in plate 2, all variants showed similar cytokine release levels as GPC3/H1643L581. Taken together, Dual Fab variants can show improved IFN gamma and IL-2 levels compared to GPC3/CD3 epsilon without increasing IL-6 levels significantly.

[0275] Taken together, affinity matured variants showed stronger CD137 agonistic activity which can elicit TDCC activity corresponding to cytokine release. Particularly, variants showed improved IFN gamma and IL-2 levels relative to GPC3/CD3 epsilon.

[0276] 2.3.4. Measurement of TDCC activity using AE05 and AE15 CrossMab Ab

Cytotoxic activity was assessed by observing the rate of tumor cell growth inhibition using xCELLigence Real-Time Cell Analyzer (Roche Diagnostics) in the presence of PBMCs. Figure 11 shows the TDCC activity of AE05 and AE15 CrossMab antibodies prepared in Example 1.3. SK-pca60 cell line was used as target cells. Target cells were detached from the dish and cells were plated into E-plate 96 (Roche Diagnostics) in aliquots of 100 micro L/well by adjusting the cells to 3.5×10^3 cells/well, and measurement of cell growth was initiated using the xCELLigence Real-Time Cell Analyzer. 24 hours later, the plate was removed and 50 micro L of the respective antibodies prepared at each concentration (5-fold serial dilutions starting from 5nM i.e., 0.008, 0.04, 0.2, 1 and 5nM) were added to the plate. After 15 minutes of reaction at room temperature, 50 micro L of the fresh human PBMC solution prepared in (Example 2.3.1) was added in effector: target ratio of 0.5 (i.e. 1.75×10^3 cells/well) and measurement of cell growth was resumed using xCELLigence Real-Time Cell Analyzer. The reaction was carried out under the conditions of 5% carbon dioxide gas at 37 degrees C. As CD137 signaling enhances T-cell survival and prevents activation induced cell death, TDCC assay was conducted at a low E:T ratio. An extended period of time may be required to observe superior cytotoxicity contributed by CD137 activation. As such, approximately 140 hours after the addition of PBMCs, Cell Growth Inhibition (CGI) rate (%) was determined using the equation below. The Cell Index Value obtained from xCELLigence Real-Time Cell Analyzer used in the calculation was a normalized value where the Cell Index value immediately at the time point before antibody addition was defined as 1.

$$\text{Cell Growth Inhibition rate (\%)} = (A-B) \times 100 / (A-1)$$

A represents the mean value of Cell Index values in wells without antibody addition (containing only target cells and human PBMCs), and B represents the mean value of

the Cell Index values of target wells. The examinations were performed in duplicates. As shown in Figure 11, AE05 and AE15 CrossMab antibodies showed TDCC activity against SK-pca60 cell line in dose dependent manner. AE05 showed slightly stronger TDCC activity than that of AE15 at 0.2nM concentration.

[0277] [Example 3] Evaluation of off-target cytotoxicity of GPC3/CD3/human CD137 (2+1) Tri-specific antibodies and Anti-GPC3/Dual (1+1) Tri-specific antibodies

[0278] 3.1. Preparation of anti-GPC3/CD137xCD3 (2+1) Trispecific antibodies

To investigate target independent cytotoxicity and cytokine release, tri-specific antibodies were generated by utilizing CrossMab and FAE (Fab-arm exchange) technology (Figure 12 and 13). Tetravalent IgG-like molecule, Antibody A (mAb A) which of each arm has two binding domains resulting in four binding domains in one molecule was generated with CrossMab as mentioned above. Bivalent IgG, Antibody B (mAb B) is the same format as a conventional IgG. Fc region of both mAb A and mAb B is Fc gammaR silent with attenuated affinity for Fc gamma receptor, deglycosylated and applicable for FAE. Six tri-specific antibodies were constructed. The target antigen of each Fv region in six tri-specific antibodies is shown in Table 12. The naming rule of each of binding domain of mAb A, mAb B, and mAb AB are shown in Figure 13. The pair of mAb A and mAb B to generate the respective tri-specific antibodies, mAb AB, and their SEQ ID NOs are shown in Table 13 and Table 14. Antibody CD3D(2)_i121 described in WO2005/035584A1 (abbreviated as AN121) was used as anti-CD3 antibody. Tri-specific antibodies described in Tables 13 and 14 were expressed and purified by the method described above.

[0279] (Table 12)

Target of each arm of antibodies

Name of mAb AB	Fv A1	Fv A2	Fv B
GPC3/CD137xCD3	Anti-CD137	Anti-CD3e	Anti-GPC3
Ctrl/CD137xCD3	Anti-CD137	Anti-CD3e	Ctrl

[0280] (Table 13)

SEQ ID NO of each variable sequence of antibody described in Table 12

Name of mAb AB	Name of mAb A to generate mAb AB	VHA1 (SEQ ID NO.)	VLA1 (SEQ ID NO.)	VHA2 (SEQ ID NO.)	VLA2 (SEQ ID NO.)	Name of mAb B to generate mAb AB	VHB (SEQ ID NO.)	VLB (SEQ ID NO.)
GPC3/CD137xCD3	CD137xCD3	85	86	87	88	GPC3	89	90
Ctrl/CD137xCtrl	CD137xCtrl	85	86	Ctrl	Ctrl	Ctrl	Ctrl	Ctrl

[0281] (Table 14)

Amino acid sequence of variable region of antibody described in Tables 12 and 13

CD137VH SEQ ID NO: 85	QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWSWIRQSPEKG LEWIGEINHGGYVTYNPSLESRTISVDTSKNQFSLKLSSVTAADTA VYYCARDYGPNGYDWYFDLWGRGTLTVSS
CD137VL SEQ ID NO: 86	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLI YDASNRRATGIPARFSGSGSGTDFTLTISSELPEDFAVYYCQQRSNW PPALTFGGGKVEIK
CD3VH SEQ ID NO: 87	QVQLVESGGGLVQPGRSLRLSCAASGFTFSNAWMHWVRQAPG KGLEWVAQIKDRANSYNTYYAESVKGRFTISRDDSKNSIYLMNS LKTEDTAVYYCRYVHYTTYAGSSFSGYGVDAWGQGTTVTVSS
CD3VL SEQ ID NO: 88	DIVMTQSPLSLPVTPGEPASISCRSSQPLVHSNRNTYLHWYQQKP GQAPRLLIYKVSNRFGVDPDRFSGSGSGTDFTLKISRVEAEDVGVY YCGQGTQVPYTFGQGTKLEIK
GPC3VH SEQ ID NO: 89	QVQLVQSGAEVKKPGASVTVSCASGYTFTDYEMHWIRQPPGE GLEWIGAIIDGPTPDYAYSEKFKGRVTLTADKSTSTAYMELSSLTSED TAVYYCTRFYSYTWGQGTTLTVSS
GPV3VL SEQ ID NO: 90	DIVMTQSPLSLPVTPGEPASISCRSSQPLVHSNRNTYLHWYQQKP GQAPRLLIYKVSNRFGVDPDRFSGSGSGTDFTLKISRVEAEDVGVY YCGQGTQVPYTFGQGTKLEIK

[0282] 3.2. Evaluation of the binding of GPC3/CD137xCD3 (2+1) Trispecific antibodies

Binding affinity of trispecific antibodies to human CD3 and CD137 were assessed at 37 degrees C using Biacore T200 instrument (GE Healthcare). Anti-human Fc antibody (GE Healthcare) was immobilized onto all flow cells of a CM4 sensor chip using amine coupling kit (GE Healthcare). Antibodies were captured onto the anti-Fc sensor surfaces, then recombinant human CD3 or CD137 was injected over the flow cell. All antibodies and analytes were prepared in ACES pH 7.4 containing 20 mM ACES, 150 mM NaCl, 0.05% Tween 20, 0.005% NaN₃. Sensor surface was re-generated each cycle with 3M MgCl₂. Binding affinity was determined by processing and fitting the data to 1:1 binding model using Biacore T200 Evaluation software, version 2.0 (GE Healthcare).

[0283] Binding affinity of tri-specific antibodies to recombinant human CD3 and CD137 is shown in Table 15.

[0284] (Table 15)

Binding affinity of trispecific antibodies described in Table 3.1 for human CD137 or CD3 measured by Biacore

Ab name	CD137			CD3		
	$k_a (M^{-1} s^{-1})$	$k_d (s^{-1})$	KD (M)	$k_a (M^{-1} s^{-1})$	$k_d (s^{-1})$	KD (M)
GPC3/CD137xCD3	5.47E+05	2.06E-02	3.77E-08	8.18E+04	1.61E-03	1.97E-08
Ctrl/CD137xCD3	5.48E+05	1.82E-02	3.31E-08	8.24E+04	1.52E-03	1.85E-08

[0285] 3.3. Assessment of off target cytotoxicity to human CD137 expression cells of GPC3/CD137xCD3 Tri-specific antibodies and Anti-GPC3/Dual-Fab Tri-specific antibodies

[0286] To investigate if affinity maturation of H183L072 may result in potential off-target cytotoxicity, affinity matured variants were subjected to the same evaluation, comparing against tri-specific 2+1 antibody format (GPC3/CD137xCD3, GPC3/CtrlxCD3) where hCD3 expressing Jurkat cells were co-cultured with hCD137 expressing CHO cells. 5.0×10^3 cells/well of hCD137 expressing CHO (Figure 15) or parental CHO (Figure 14) were co-cultured with 2.5×10^4 NFAT-luc2 Jurkat cells for 24 hours in the presence of 0.5, 5 and 50 nM of tri-specific antibodies. Figure 6a shows no non-specific activation of Jurkat cells by all tri-specific antibodies when co-cultured with parental CHO cells. However, it was observed that both trispecific 2+1 format antibodies GPC3/CD137xCD3 and Ctrl/CD137xCD3 can activate Jurkat cells in the presence of hCD137 expressing CHO cells. Affinity matured variants in 1+1 format did not result in activation of Jurkat cells when co-cultured with hCD137 expressing CHO cells. Taken together, this suggests that tri-specific format GPC3/CD137xCD3 can result in Jurkat cell activation independent of target or tumor antigen binding, giving rise to off-target cytotoxicity unlike that of GPC3/Dual (1+1) format even after affinity maturation of CD137 binding.

[0287] 3.4. Assessment of off target cytokine release of GPC3/CD137xCD3 Tri-specific antibodies and GPC3/Dual-Fab Trispecific antibodies from PBMCs

Comparison of tri-specific formats for off-target toxicity was also assessed using human PBMC solution. Briefly, 2.0×10^5 PBMCs prepared as described in Example 2.3.1 were incubated with 80, 16 and 3.2 nM of tri-specific antibodies in the absence of target cells for 48 hours. As IL-2 was not detected by any antibodies, IL-6, IFN gamma and TNFalpha levels in the supernatant are shown in Figures 16 to 18. Measurement of cytokine release was conducted similarly to that described in Example 2.3.3. Similar to Example 2, affinity matured variants were divided into 2 plates. As shown in Figures 16 to 18, GPC3/CD137xCD3 but not anti-GPC3/Dual-Fab resulted in IFN gamma (Figure 16), TNF alpha (Figure 17), and IL-6 (Figure 18) release from PBMCs. These results suggest that GPC3/CD137xCD3 tri-specific format resulted in non-specific ac-

tivation of PBMCs in the absence of target cells. Finally, the data showed that Dual-Fab tri-specific 1+1 format can confer target-specific effector cell activation without off-target toxicity.

[0288] [Example 4] Evaluation of in vivo efficacy of GPC3/CD3 epsilon Bispecific antibodies and Anti-GPC3/Dual-Fab (1+1) Trispecific antibodies

[0289] 4.1. Anti-GPC3/Dual-Fab, GPC3/CD3 epsilon and GPC3/CD137 bi-specific antibody preparation

Antibodies for in vivo efficacy studies were generated by either crossmab technology as described in Example 1.3 or by Fab-arm exchange (FAE) according to a method described in (Proc Natl Acad Sci USA. 2013 Mar 26; 110(13): 5145-5150). GPC3/CD3 epsilon. GPC3/H1643L0581, GPC3/H1644L0939 and GPC3/CD137 antibodies were generated by FAE and is comprised of mouse Fc with attenuated affinity for Fc gamma receptor. GPC3/CD3 epsilon is comprised of one arm targeting GPC3 while other arm targeting human CD3. GPC3/CD137 is comprised of one arm targeting GPC3 while other arm targeting human CD137. GPC3/H1643L0581 and GPC3/H1644L0939 is comprised of one arm targeting human GPC3 while other arm has dual targeting properties to human CD3 and CD137. GPC3/H1643L0581-BS11ab were generated by FAE and is comprised of human Fc with attenuated affinity for Fc gamma receptor, with one arm targeting GPC3 and the other arm has dual targeting properties to CD3 and CD137. Variable region sequences were showed in Table 10 and Table 4.

[0290] 4.2. Generation of CD137/CD3 double humanized mouse

Human CD137 knock-in (KI) mouse strain was generated by replacing mouse endogenous Cd137 genomic region with human CD137 genomic sequence using mouse embryonic stem cells. Human CD3 EDG-replaced mouse was established as a strain in which all three components of the CD3 complex -- CD3e, CD3d, and CD3g -- are replaced with their human counterparts, CD3E, CD3D, and CD3G (Scientific Rep. 2018; 8: 46960). CD137/CD3 double humanized mouse strain was established by crossbreeding the human CD137 KI mice with the human CD3 EDG-replaced mice.

[0291] 4.3. Preparation of LLC1/hGPC3 cell line

The mouse cancer cell line LL/2(LLC1) (ATCC) were transfected with pCXND3-hGPC3 and performed single cell clone isolation with 500 micro g/ml G418. Selected clone (LLC1/hGPC3) were confirmed the expression of hGPC3.

[0292] 4.4. Assessment of in vivo efficacy of Anti-GPC3/Dual-Fab Tri-specific antibodies with hCD3/hCD137 mice

[0293] 4.4.1. Assessment of in vivo efficacy of Anti-GPC3/Dual-Fab Tri-specific antibodies with hCD3/hCD137 mice

In drug efficacy tests of the GPC3/H1644L0939 using the LLC1/hGPC3 model, the

tests below were performed. LLC1/hGPC3 (1×10^6 cells) were transplanted into the inguinal subcutaneous region of hCD3/hCD137 mice. The day of transplantation was defined as day 0. On the days 9 after the transplantation, the mice were randomized into groups according to their body weight and tumor size. On the day of randomization, the GPC3/H1643L0581, GPC3/H1644L0939, or GPC3/CD3 epsilon antibody was administered intravenously through the caudate vein at 5 mg/kg. The antibodies were administered only once. Tumor volume and body weight was measured every 3-4 days. For IL-6 analysis, mice were bled at 2h after treatment. Plasma samples were analyzed with Bio-Plex Pro Mouse Cytokine Th1 Panel according to the manufacture's protocol.

[0294] As a result, anti-tumor activities were more clearly observed in GPC3/ H1643L0581 group and GPC3/H1644L0939 group than GPC3/CD3 epsilon group (Figure 20). As shown in Figure 21, GPC3/H1644L0939 group showed less IL-6 production compared to GPC3/ H1643L0581 group and GPC3/CD3 epsilon group.

[0295] In another in vivo efficacy evaluation, LLC/hGPC3 cells were transplanted into the right flank of hCD3/hCD137 mice. On day 9, the mice were randomized into groups on the basis of their tumor volume and body weight, and injected i.v. with vehicle or antibodies prepared in Example 4.1. Tumor volume was measured twice per week. For IL-6 analysis, mice were bled at 2h after treatment. Plasma samples were analyzed with Bio-Plex Pro Mouse Cytokine Th1 Panel according to the manufacture's protocol. As shown in Figure 22 and 23, GPC3/Dual group showed stronger anti-tumor activity and less IL-6 production compared to GPC3/CD3 epsilon group.

[0296] 4.4.2. Assessment of in vivo efficacy of Anti-GPC3/Dual-Fab Tri-specific antibodies with compared to parental antibody

The anti-tumor activity of anti-GPC3/Dual-Fab antibodies and parental antibody prepared in Example 4.1 was tested in a LLC1/hGPC3 cancer model. LLC1/hGPC3 (3×10^6 cells) were transplanted into the inguinal subcutaneous region of hCD3/hCD137 mice. The day of transplantation was defined as day 0. On the days 13 after the transplantation, the mice were randomized into groups according to their body weight and tumor volume, and injected i.v. with either vehicle (PBS containing 0.05% Tween), 5 mg/kg xGPC3/Dual183H072L-xSG1350kSG1349hV11, 5 mg/kg xGPC3/DualAE15-xSG1350kSG1349hV11, 5 mg/kg xGPC3/DualAE15-xSG1356kSG1355hV11, 5 mg/kg xGPC3/DualAE05-xSG1350kSG1349hV11 or 5 mg/kg xGPC3/DualAE05-xSG1356kSG1355hV11.

[0297] As a result, four anti-GPC3/Dual-Fab antibodies showed stronger efficacy than xGPC3/Dual183H072L-xSG1350kSG1349hV11 antibody (Figure 24).

[0298] 4.4.3. Assessment of in vivo efficacy of Anti-GPC3/Dual-Fab prepared with

CrossMab or with FAE

The anti-tumor activity of anti-GPC3/Dual-Fab antibodies prepared with CrossMab or with FAE in Example 4.1 was tested in a mouse hepatic Hepa1-6/hGPC3 cancer model. Specifically, xGPC3/DualAE05-xSG1350kSG1349hV11 was generated in CrossMab format, while GPC3/H1643L0581-BS11ab was generated by FAE technology with human Fc. In order to obtain the Hepa1-6/hGPC3 cell line, the human GPC3 gene was integrated into the chromosome of the mouse hepatoma cell line Hepa1-6 (ATCC No. CRL-1830) by a method well known to those skilled in the art. Hepa1-6/hGPC3 (1×10^7 cells) were transplanted into the inguinal subcutaneous region of hCD3/hCD137 mice. The day of transplantation was defined as day 0. On the days 7 after the transplantation, the mice were randomized into groups according to their body weight and tumor volume, and injected i.v. with either vehicle (PBS containing 0.05% Tween), 0.2 mg/kg xGPC3/DualAE05-xSG1350kSG1349hV11 or 0.2 mg/kg GPC3/H1643L0581-BS11ab.

[0299] As a result, xGPC3/DualAE05-xSG1350kSG1349hV11 showed stronger efficacy than GPC3/H1643L0581-BS11ab antibody, which suggested that Anti-GPC3/Dual-Fab in crossmab format showed better efficacy than that of in FAE format (Figure 25).

[0300] 4.4.3.1 Antibody plasma concentration in assessment of in vivo efficacy of Anti-GPC3/Dual-Fab prepared with CrossMab or with FAE

Circulating levels of xGPC3/DualAE05-xSG1350kSG1349hV11 and GPC3/H1643L0581-BS11ab were quantified as follows. Blood was collected from seven animals in each group on day 4 post injection. Heparinized plasma samples were obtained by centrifuging at $1,900 \times g$ for 10 min at 4 degrees C. The concentration of both Anti-GPC3/Dual-Fab antibodies in mouse plasma was measured by electrochemiluminescence (ECL) immunoassay. All procedures were done at room temperature. Human core Glypican-3 (hGPC3) recombinant protein (Prepared in house) was immobilized to an uncoated MULTI-ARRAY Standard plate (Meso Scale Discovery) for one hour. After that, plates were incubated with blocking solution containing 5% of bovine serum albumin for one hour. Calibration curve samples with 1.50, 0.500, 0.167, 0.0556, 0.0185, 0.00617, and 0.00206 micro g/mL and mouse plasma samples diluted 100-fold or more were prepared. Subsequently, the samples were dispensed into the hGPC3-immobilized plates, and allowed to stand for one hour. Then, biotinylated anti-human IgG specific antibody (Jackson ImmunoResearch) was added to the plates and incubated for one hour. Subsequently, SULFO-TAG labeled Streptavidin (Meso Scale Discovery) was added to react for 30min at room temperature. ECL measurement was carried out by SECTOR S 600 (Meso Scale Discovery). The concentration in mouse plasma was calculated using the analytical software SOFTmax PRO (Molecular

Devices).

[0301] As a result, the mean plasma concentration of xGPC3/DualAE05-xSG1350kSG1349hV11 on day 4 post dose was 1.46-fold higher than that of GPC3/H1643L0581-BS11ab, which suggested that crossmab format showed better PK profile than FAE format (Figure. 26).

[0302] 4.5. Assessment of in vivo efficacy of Anti-GPC3/Dual-Fab Tri-specific antibodies with HuNOG mice

The anti-tumor activity of anti-GPC3/Dual-Fab antibody, GPC3/CD3 epsilon bi-specific antibody and GPC3/CD137 bi-specific antibody prepared in Example 4.1 was tested in a human hepatic sk-pca-13a cancer model. The GPC3/CD3 epsilon bi-specific antibody was also tested in combination with the GPC3/CD137 bi-specific antibody. Sk-pca-13a cells were subcutaneously transplanted to NOG humanized mice.

In order to obtain the sk-pca-13a cell line, the human GPC3 gene was integrated into the chromosome of the human liver adenocarcinoma cell line SK-HEP-1 (ATCC No. HTB-52) by a method well known to those skilled in the art.

[0303] NOG female mice were purchased from In-Vivo Science. For humanization, mice were sub lethally irradiated followed 1 day later by injection of 100,000 human cord blood cells (ALLCELLS). Sixteen weeks later, sk-pca-13a cells (1×10^7 cells) were mixed with Matrigel™ Basement Membrane Matrix (Corning) and transplanted to the right flank of humanized NOG mice. The day of transplantation was defined as day 0. On day 19, the mice were randomized on the basis of tumor volume and body weight, and injected i.v. with either vehicle (PBS containing 0.05% Tween), 5 mg/kg GPC3/CD3 epsilon, 5 mg/kg GPC3/H1643L0581, or combination of 5 mg/kg GPC3/CD3 epsilon and 5 mg/kg GPC3/CD137.

[0304] As a result, anti-GPC3/Dual-Fab (GPC3/H1643L0581) showed greater anti-tumor activity than GPC3/CD3 epsilon (Figure 19).

[0305] [Example 5] Antibody format and Fc selection for anti-GPC3/Dual-Fab (1+1) Tri-specific antibodies

Anti-GPC3/Dual-Fab Tri-specific antibodies were generated as described in Example 1.3. Antibody purities, binding kinetics and expression levels were evaluated as described as Example 5.1, 5.2 and 5.3.

[0306] In order to control the correct pairing of antibody light chains, CrossMab (WO2017055539) or FAST-Ig (WO2013065708) technology is used as illustrated in Figure 2, Table 8 and Table 9. For CrossMab Ab, the VH and VL region of one arm is exchanged, while charged mutations are introduced into the CH1 and CL region of the other arm to generate electrostatic repulsion for the mis-paired light chains. For the FAST-Ig antibodies, mutations are introduced into Fab region of each arm to generate electrostatic repulsion for the mis-paired light chains.

[0307] 5.1. Assessment of purity of CrossMab and FAST-Ig mutation

Each sample was diluted to 0.085 mg/mL in a master mix containing 0.4375% methylcellulose solution; 2.5% Pharmalyte, pH 5-8; 2.5% Pharmalyte pH 8-10.5; 3.75M Urea; 12.5mM Arg; 12.5mM IDA; 0.625% pI marker 5.85, and 0.625% pI marker 9.99. The samples were then loaded onto a Maurice C. analyzer (Protein Simple, San Jose, CA) and focused at 1,500 V for 1 min, followed by 3,000 V for 6 min. The protein was detected under UV absorbance at 280 nm and native fluorescence (Ex. 280 nm, Em. 320-450 nm). The resulting electrophoresis profiles were analyzed with the use of Compass for iCE software version 2.1.0.

Electropherogram for FAST-Ig and CrossMab are shown in Figure 27. Impurities of mis-pairing product was clearly seen for GPC3/DualAE05-SG1364k1363hV11 (FAST-Ig format) as shown by arrow.

[0308] 5.2. Binding kinetics information of CrossMab and Fast-Ig

Binding affinity of Dual-Ig antibodies to human CD3 were assessed at 25 degrees C using Biacore 8K instrument (GE Healthcare). Anti-human Fc (GE Healthcare) was immobilized onto all flow cells of a CM4 sensor chip using amine coupling kit (GE Healthcare). Antibodies were captured onto the anti-Fc sensor surfaces, then recombinant human CD3 or CD137 was injected over the flow cell. All antibodies and analytes were prepared in ACES pH 7.4 containing 20 mM ACES, 150 mM NaCl, 0.05% Tween 20, 0.005% NaN₃. Sensor surface was regenerated each cycle with 3M MgCl₂. Binding affinity was determined by processing and fitting the data to 1:1 binding model using Biacore Insight Evaluation software, version 2.0 (GE Healthcare). CD137 binding affinity assay was conducted in same condition except assay temperature was set at 37 degrees C.

[0309] Binding affinity of Dual-Ig antibodies to recombinant human CD3 and CD137 are shown in Table 16. FAST-Ig antibody GPC3/DualAE05-SG1363k1364hV11 and GPC3/DualAE05-SG1364k1363hV11 showed about 2-fold weaker binding affinity to CD137 as compared to CrossMab prepared antibodies, suggesting that the charged mutations used in FAST-Ig antibodies affect/weaken the CD137 binding activity. This is caused by faster off rate in FAST-Ig construct. FAST-Ig antibodies GPC3KE/DualAE05EK-SG1363k1365hV11 and GPC3EK/DualAE05KE-SG1365k1363hV11 showed about 3-fold weaker binding affinity to CD3 as compared to CrossMab xSG1350, suggesting that the charged mutations used in FAST-Ig antibodies affect/weaken the CD137 binding activity. This is caused by faster off rate in FAST-Ig construct.

[0310] Taken together, the results suggest the selected CrossMab format (and mutations) is better than FAST-Ig format in controlling the correct pairing of antibody light chains with less impact on the antigen-binding activity.

[0311] (Table 16)

Antibody name	CD3 (25C)			CD137 (37C)		
	ka (M-1s-1)	kd (s-1)	KD (M)	ka (M-1s-1)	kd (s-1)	KD (M)
GPC3/DualAE05-SG1363k1364hV11	8.85E+04	2.78E-02	3.14E-07	1.28E+04	1.02E-03	7.95E-08
GPC3/DualAE05-SG1364k1363hV11	9.34E+04	2.63E-02	2.82E-07	1.36E+04	7.45E-04	5.47E-08
GPC3KE/DualAE05EK-SG1363k1365hV11	7.59E+04	6.11E-02	8.06E-07	1.33E+04	5.06E-04	3.80E-08
GPC3EK/DualAE05KE-SG1365k1363hV11	8.35E+04	6.28E-02	7.51E-07	1.45E+04	6.30E-04	4.35E-08
xGPC3/DualAE05-xSG1350kSG1349hV11	9.29E+04	2.75E-02	2.96E-07	1.30E+04	5.25E-04	4.05E-08

[0312] 5.3. Expression level of FAST-Ig and CrossMab

Fast-Ig or CrossMab antibodies were expressed as tri-specific form by transient expression in Expi293F cells (ThermoFisher Scientific) to express antibodies. Each antibody was purified from the obtained culture supernatant by Bravo Automated Liquid Handling Platform (Agilent) with Protein A (PA-W) Cartridges (Agilent). As for the concentration of the purified antibody, the absorbance was measured at 280 nm using a spectrophotometer, and the antibody concentration was calculated by use of an

extinction coefficient calculated from the obtained value by PACE (Protein Science 1995; 4: 2411-2423). Expression level is calculated by the [final concentration] x [elution volume]/[culture volume].

The expression level of each antibody was shown as Table 17. FAST-Ig antibodies GPC3KE/DualAE05EK-SG1363k1365hV11 and GPC3EK/DualAE05KE-SG1365k1363hV11 showed much lower expression level than CrossMab format (xGPC3/DualAE05xSG1350kSG1349hV11).

[0313] (Table 17)

Antibody name	Expression level
GPC3/DualAE05-SG1363k1364hV11	48 mg/L
GPC3/DualAE05-SG1364k1363hV11	45 mg/L
GPC3KE/DualAE05EK-SG1363k1365hV11	15 mg/L
GPC3EK/DualAE05KE-SG1365k1363hV11	7 mg/L
xGPC3/DualAE05-xSG1350kSG1349hV11	40 mg/L

[0314] [Reference EXAMPLE 1] Preparation of antibody expression vector and expression and purification of antibody

Amino acid substitution or IgG conversion was carried out by a method generally known to those skilled in the art using PCR, or In fusion Advantage PCR cloning kit (Takara Bio Inc.), etc., to construct expression vectors. The obtained expression vectors were sequenced by a method generally known to those skilled in the art. The prepared plasmids were transiently transferred to FreeStyle 293 cells (ThermoFisher Scientific) or Expi293F cells (ThermoFisher Scientific) to express antibodies. Each antibody was purified from the obtained culture supernatant by a method generally known to those skilled in the art using rProtein A Sepharose(TM) Fast Flow (GE Healthcare Japan Corp.). As for the concentration of the purified antibody, the absorbance was measured at 280 nm using a spectrophotometer, and the antibody concentration was calculated by use of an extinction coefficient calculated from the obtained value by PACE (Protein Science 1995; 4: 2411-2423).

[0315] [Reference Example 2] Experimental Cell Lines

The human GPC3 gene was integrated into the chromosome of the mouse colorectal cancer cell line CT-26 (ATCC No. CRL-2638) by a method well known to those skilled in the art to obtain the high expression CT26-GPC3 cell line. The expression level of human GPC3 (2.3×10^5 /cell) was determined using the QIFI kit (Dako) by the manufacturer's recommended method. To maintain the human GPC3 gene, these recombinant cell lines were cultured in ATCC-recommended media by adding Geneticin (GIBCO) at 200 micro g/ml for CT26-GPC3. After culturing, these cells were detached using 2.5 g/L trypsin-1 mM EDTA (nacalai tesque), and then used for each of the ex-

periments. The transfectant cell line is herein referred to as SKpca60a.

The human CD137 gene was integrated into the chromosome of the Chinese Hamster Ovary cell line CHO-DG44 by a method well known to those skilled in the art to obtain the high expression CHO-hCD137 cell line. The expression level of human CD137 was determined by FACS analysis using the PE anti-human CD137 (4-1BB) Antibody (BioLegend, Cat. No. 309803) by the manufacturer's instructions.

NCI-H446 and Huh7 cell lines were maintained in RPMI1640 (Gibco) and DMEM (low glucose) respectively. Both media were supplemented with 10% fetal bovine serum (Bovogen Biologicals), 100 units/mL of penicillin and 100 micro g/mL of streptomycin and cells were cultured at 37 degrees C with 5% CO₂.

Industrial Applicability

- [0316] The present invention provides multispecific antigen-binding molecules capable of binding to CD3 and CD137 (4-1BB) but not binding to CD3 and CD137 at the same time, and capable of binding to GPC3. The antigen-binding molecules of the present invention exhibit enhanced T-cell dependent cytotoxicity activity in a GPC3-dependent manner through binding to the CD3/CD37 and GPC3. The antigen-binding molecules and pharmaceutical compositions thereof can be used for targeting cells expressing GPC3, for use in immunotherapy for treating various cancers, especially those associated with GPC3 such as GPC3-positive cancers.

Claims

[Claim 1]

A multispecific antigen-binding molecule comprising

- (i) a first antigen-binding moiety that is capable of binding to CD3 and CD137, but does not bind to CD3 and CD137 at the same time; and
- (ii) a second antigen-binding moiety that is capable of binding to glypican-3 (GPC3);

wherein the first antigen-binding moiety comprises any one selected from (a1) to (a15) below:

(a1) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 17, the heavy chain CDR 2 of SEQ ID NO: 31, the heavy chain CDR 3 of SEQ ID NO: 45, the light chain CDR 1 of SEQ ID NO: 64, the light chain CDR 2 of SEQ ID NO: 69 and the light chain CDR 3 of SEQ ID NO: 74;

(a2) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 18, the heavy chain CDR 2 of SEQ ID NO: 32, the heavy chain CDR 3 of SEQ ID NO: 46, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a3) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 19, the heavy chain CDR 2 of SEQ ID NO: 33, the heavy chain CDR 3 of SEQ ID NO: 47, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a4) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 19, the heavy chain CDR 2 of SEQ ID NO: 33, the heavy chain CDR 3 of SEQ ID NO: 47, the light chain CDR 1 of SEQ ID NO: 65, the light chain CDR 2 of SEQ ID NO: 70 and the light chain CDR 3 of SEQ ID NO: 75;

(a5) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 20, the heavy chain CDR 2 of SEQ ID NO: 34, the heavy chain CDR 3 of SEQ ID NO: 48, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a6) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 22, the heavy chain CDR 2 of SEQ ID NO: 36, the heavy chain CDR 3 of SEQ ID NO: 50, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3

of SEQ ID NO: 73;

(a7) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 23, the heavy chain CDR 2 of SEQ ID NO: 37, the heavy chain CDR 3 of SEQ ID NO: 51, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a8) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 23, the heavy chain CDR 2 of SEQ ID NO: 37, the heavy chain CDR 3 of SEQ ID NO: 51, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a9) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 24, the heavy chain CDR 2 of SEQ ID NO: 38, the heavy chain CDR 3 of SEQ ID NO: 52, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a10) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 25, the heavy chain CDR 2 of SEQ ID NO: 39, the heavy chain CDR 3 of SEQ ID NO: 53, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a11) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 26, the heavy chain CDR 2 of SEQ ID NO: 40, the heavy chain CDR 3 of SEQ ID NO: 54, the light chain CDR 1 of SEQ ID NO: 66, the light chain CDR 2 of SEQ ID NO: 71 and the light chain CDR 3 of SEQ ID NO: 76;

(a12) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 26, the heavy chain CDR 2 of SEQ ID NO: 40, the heavy chain CDR 3 of SEQ ID NO: 54, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a13) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 27, the heavy chain CDR 2 of SEQ ID NO: 41, the heavy chain CDR 3 of SEQ ID NO: 55, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73;

(a14) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 28, the heavy chain CDR 2 of SEQ ID NO: 42, the heavy

chain CDR 3 of SEQ ID NO: 56, the light chain CDR 1 of SEQ ID NO: 63, the light chain CDR 2 of SEQ ID NO: 68 and the light chain CDR 3 of SEQ ID NO: 73; and

(a15) the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 82, the heavy chain CDR 2 of SEQ ID NO: 83, the heavy chain CDR 3 of SEQ ID NO: 84, the light chain CDR 1 of SEQ ID NO: 65, the light chain CDR 2 of SEQ ID NO: 70 and the light chain CDR 3 of SEQ ID NO: 75;

and (iii) further comprises a Fc domain composed of a first and a second Fc region subunits capable of stable association, and wherein the Fc domain exhibits reduced binding affinity to human Fc gamma receptor, as compared to a native human IgG1 Fc domain; wherein the first Fc region subunit is selected from the group consisting of:

(1) a Fc region polypeptide comprising Ala at position 234 and Ala at position 235;

(2) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, and Ala at position 297;

(3) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, Ala at position 297, Cys at position 354 and Trp at position 366; and

wherein the second Fc-region polypeptide is selected from the group comprising:

(4) a Fc region polypeptide comprising Ala at position 234 and Ala at position 235;

(5) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, and Ala at position 297; and

(6) a Fc region polypeptide comprising Ala at position 234, Ala at position 235, Ala at position 297, Cys at position 349, Ser at position 366, Ala at position 368 and Val at position 407; and

wherein the amino acid positions are numbered using EU index numbering.

[Claim 2]

The multispecific antigen-binding molecule of claim 1, wherein the first antigen binding moiety comprises any one selected from (a1) to (a15) below:

(a1) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 3, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 59;

- (a2) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 4, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;
- (a3) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 5, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;
- (a4) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 5, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 60;
- (a5) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 6, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;
- (a6) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 8, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;
- (a7) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 9, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;
- (a8) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 9, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;
- (a9) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 10, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;
- (a10) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 11, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;
- (a11) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 12, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 61;
- (a12) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 12, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;
- (a13) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 13, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 58;
- (a14) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 14, and a light chain variable region comprising an

amino acid sequence of SEQ ID NO: 58; and
 (a15) a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 81, and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 60.

- [Claim 3] The multispecific antigen-binding molecule of claim 1 or 2, wherein the second antigen-binding moiety capable of binding to glypican-3 (GPC3) comprises the heavy chain complementarity determining region (CDR) 1 of SEQ ID NO: 235, the heavy chain CDR 2 of SEQ ID NO: 244, the heavy chain CDR 3 of SEQ ID NO: 253, the light chain CDR 1 of SEQ ID NO: 268, the light chain CDR 2 of SEQ ID NO: 274 and the light chain CDR 3 of SEQ ID NO: 280.
- [Claim 4] The multispecific antigen-binding molecule of any one of claims 1 to 3, wherein the second antigen binding moiety comprise a heavy chain variable region comprising an amino acid sequence of SEQ ID NO: 226 and a light chain variable region comprising an amino acid sequence of SEQ ID NO: 262.
- [Claim 5] The multispecific antigen-binding molecule of any one of claims 1 to 4, wherein the Fc domain comprises a first Fc region subunit shown in SEQ ID NO: 317 and a second Fc region subunit shown in SEQ ID NO: 323.
- [Claim 6] The multispecific antigen-binding molecule of any one of claims 1 to 5, wherein each of the first and the second antigen binding moiety is a Fab molecule.
- [Claim 7] The multispecific antigen-binding molecule of claim 6, wherein the first antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of either one of the first or second Fc region subunit of the Fc domain, and the second antigen binding moiety is fused at the C-terminus of the Fab heavy chain to the N-terminus of the remaining Fc region subunit of the Fc domain.
- [Claim 8] The multispecific antigen-binding molecule of claim 6 or 7, wherein the second antigen binding moiety is a crossover Fab molecule in which the variable regions of the Fab light chain and the Fab heavy chain are exchanged and which comprises a heavy chain variable region (VH) and a light chain variable region (VL), and wherein the first antigen binding moiety is a conventional Fab molecule which comprises a heavy chain variable region (VH) and a light chain variable region (VL).
- [Claim 9] The multispecific antigen-binding molecule of claim 8, wherein in the

constant domain CL of the light chain of the first antigen binding moiety, the amino acids at position 123 and 124 are arginine (R) and lysine (K) respectively (numbering according to Kabat), and wherein in the constant domain CH1 of the heavy chain of the first antigen binding moiety, the amino acids at position 147 and 213 are glutamic acid (E) (numbering according to Kabat EU index).

[Claim 10]

The multispecific antigen-binding molecule of any one of claims 1 to 9, comprises four polypeptides in any one of the combination selected from (a1) to (a6) below:

(a1) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 219 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a2) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 205 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 220 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a3) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 291 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a4) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 286 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 292 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4);

(a5) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 293 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225

(chain 4); and

(a6) a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 287 (chain 1) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 210 (chain 2), and a polypeptide chain comprising amino acid sequence of SEQ ID NOs: 294 (chain 3) and a polypeptide chain comprising amino acid sequence of SEQ ID NO: 225 (chain 4).

- [Claim 11] An isolated polynucleotide or plurality of polynucleotides encoding the multispecific antigen-binding molecule of any one of claims 1 to 10.
- [Claim 12] A vector encoding the polynucleotide or plurality of polynucleotides of claim 11.
- [Claim 13] A host cell comprising the polynucleotide or plurality of polynucleotides of claim 11, or the vector of claim 12.
- [Claim 14] A method of producing the multispecific antigen-binding molecule of any one of claims 1 to 10, comprising the steps of:
 - a) culturing the host cell of claim 13 under conditions suitable for the expression of the antigen-binding molecule, and
 - b) recovering the antigen-binding molecule.
- [Claim 15] A pharmaceutical composition comprising the multispecific antigen-binding molecule of any one of claims 1 to 10 and a pharmaceutically acceptable carrier.
- [Claim 16] The multispecific antigen-binding molecule of any one of claims 1 to 10 or the pharmaceutical composition of claim 15, which induces cytotoxicity, preferably T-cell-dependent cytotoxicity.
- [Claim 17] The multispecific antigen-binding molecule of any one of claims 1 to 10 or the pharmaceutical composition of claim 15, for use as a medicament.
- [Claim 18] The multispecific antigen-binding molecule of any one of claims 1 to 10 or the pharmaceutical composition of claim 15, for use in the treatment of cancer, preferably GPC3-expressing cancer or GPC3-positive cancer.