Description

Title of Invention: CLAUDIN-6 BINDING MOLECULES AND USES THEREOF

Technical Field

[0001] The present disclosure relates to Claudin 6 binding molecules, including antigen-binding molecules and antibodies, uses thereof, and such.

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 (NPL 1).

[0003] In addition to the antibodies that adopt 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 (NPL 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 (NPL 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 administrating catumaxomab at such a low dose.

[0005] 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 (NPL 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 21). 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.

[0006] Claudin family is the family of cell membrane proteins of approximately 23 kD in molecular weight which have four transmembrane domains and constitute tight junctions. The Claudin family includes 24 members in humans and mice, and each member of the Claudin family is known to exhibit a very unique expression pattern depending on each epithelial cell type (NPL 11 to 14). In the sheet of epithelial cells, a mechanism works to prevent substances from leaking (diffusing) in the intercellular spaces, and cell-cell adhesion systems called tight junctions have been shown to really play a central role as a "barrier" in the mechanism to prevent leakage.

[0007] A tight junction molecule Claudin 6 (CLDN6), a member of Claudin family proteins, shows transcriptionally silent expression in normal adult tissues (NPL 15 and 16), while showing up-regulation in several kind of cancers such as ovarian cancer, NSCLC, and gastric cancers (NPL 17 to 19).

Regarding anti-CLDN6 antibodies, monospecific antibodies against CLDN6 have been reported to have ADCC activity or internalization activity against CLDN6 positive cancer lines (PTL 1 to 5). So far, CLDN6 targeting T cell-redirecting bispecific antibodies, named as 6PHU3, has been engineered using bispecific sc(Fv)$_2$ format with anti-CD3/anti-CLDN6 specificities (PTL 6 to 7). In preclinical evaluation, 6PHU3 has been reported to show a potent killing of cancer cells in vitro and in vivo

(NPL 20).

Citation List

Patent Literature

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[PTL 3] WO2012/003956
[PTL 4] WO2012/156018
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[PTL 6] WO2014/075697
[PTL 7] WO2014/075788

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[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.
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[NPL 9] Cancer Immunol Immunother. (2006) 55 (5), 503-14
[NPL 10] Cancer Immunol Immunother. (2009) 58 (1), 95-109
[NPL 11] Furuse and Tsukita, TRENDS in Cell Biology 2006, 16: 181
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[NPL 14] Morita, et al., Proc. Natl. Acad. Sci. USA 1999, 96: 511
[NPL 15] Dev Dyn. 2004 Oct;231(2):425-31.
[NPL 16] Am J Physiol Renal Physiol. 2006 Dec;291(6):F1132-41.
[NPL 17] Int J Cancer. 2014 Nov 1;135(9):2206-14.
[NPL 18] Histopathology. 2012 Dec;61(6):1043-56.
[NPL 19] J Gastrointest Cancer. 2010 Mar;41(1):52-9.
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[NPL 21] Nat Rev Drug Discov. 2014 Nov;13(11):799-801.

Summary of Invention

Technical Problem

[0010] As mentioned above, bispecific sc(Fv)$_2$ is a modified low-molecular-weight antibody molecule without an Fc region and shows significantly shorter blood half-life compared with IgG-type antibodies, which would cause inconvenience for their use as

a therapeutic drug. In the case of bispecific sc(Fv)$_2$ that binds to CLDN6, named 6PHU3, it has been reported that plasma protein levels of 6PHU3 dropped sharply within 6 hours and cytotoxic activity was barely detectable after 24 hours (Nat Med. 2017 Jul;23(7):815-817.). Furthermore, the format of sc(Fv)$_2$ faces manufacturing challenges including poor stability during long-term storage.

[0011] In one aspect, objective of the present disclosure is to provide antigen-binding molecules (including antibodies) that shows binding activity towards CLDN6 and T cell receptor complex that enable cancer treatment by having T cells close to CLDN6-expressing cells and using the cytotoxicity of T cells against CLDN6-expressing cancer cells, especially antigen-binding molecules with high stability and long serum half-life. Provision of methods for producing the antigen-binding molecules, and pharmaceutical composition comprising such an antigen-binding molecule as an active ingredient are also objectives of the present disclosure.

[0012] In another aspect, objective of the present disclosure is to provide antigen-binding molecules that shows binding activity towards CLDN6 that enable cancer treatment by targeting CLDN6-expressing cells, methods for producing the antigen-binding molecules, and pharmaceutical composition comprising such an antigen-binding molecule, or immunoconjugate comprising such an antigen-binding molecule, as an active ingredient. Use of the antigen-binding molecule in detecting the presence of CLDN6 in biological sample, and use of the antigen-binding molecule in diagnosis of various cancers, are also provided in the present disclosure.

[0013] The present disclosure also provides pharmaceutical compositions for use in treating or preventing various cancers, which comprise one of the above-mentioned antigen-binding molecules, or immunoconjugate, as an active ingredient, and therapeutic methods using the pharmaceutical compositions.

Solution to Problem

[0014] The inventors found that antigen-binding molecules that comprise specific heavy chain variable region (VH) together with specific light chain variable region (VL) which shows binding activity towards CLDN6, and a second VH together with a second VL which shows binding activity towards T-cell receptor complex can damage cells expressing CLDN6, and exert a superior cytotoxic/antitumor activity. The antigen-binding molecules comprising specific sequences also show superior stability, safety and manufacturability. The present disclosure provides the antigen-binding molecules and pharmaceutical compositions that can treat various cancers, especially those associated with CLDN6 such as CLDN6-positive tumors, by comprising the antigen-binding molecule as an active ingredient.

[0015] The inventors also found that antigen-binding molecules that comprise specific VH

together with specific VL show binding activity towards CLDN6 and superior specificity towards human CLDN6, as a result the antigen-binding molecules and immunoconjugates thereof can be used for targeting cells expressing CLDN6, detecting the presence of CLDN6 in biological sample, and diagnosis of various cancers.

[0016] More specifically, the present disclosure provides the following:

[1] An isolated antibody that comprises:

a first heavy chain variable region comprising HVR-H1, HVR-H2 and HVR-H3 amino acid sequences of SEQ ID NOs: 113, 117, and 118, respectively;

a first light chain variable region comprising HVR-L1, HVR-L2 and HVR-L3 amino acid sequences of SEQ ID NOs: 119, 120, and 121, respectively;

a second heavy chain variable region comprising HVR-H1, HVR-H2 and HVR-H3 amino acid sequences of SEQ ID NOs: 122, 123, and 124, respectively; and

a second light chain variable region comprising HVR-L1, HVR-L2 and HVR-L3 amino acid sequences of SEQ ID NOs: 114, 115, and 116, respectively.

[2] An isolated antibody that comprises:

a first heavy chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 85, 1, 33 and 84; and a first light chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 87, 2, 43, 86 and 88;

a second heavy chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 60 and 70; and a second light chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 61 and 71.

[3] The antibody of [1] or [2], wherein the first heavy chain variable region is linked to a first CH1 domain shown in SEQ ID NO: 95, the first light chain variable region is linked to a first CL domain shown in SEQ ID NO: 63, the second heavy chain variable region is linked to a second CH1 domain shown in SEQ ID NO: 97, and the second light chain variable region is linked to a second CL domain shown in SEQ ID NO: 62.

[4] The antibody of any one of [1] to [3], wherein the antibody further comprises any one of the Fc region combinations selected from:

(1) a first Fc region shown in SEQ ID NO: 72 and a second Fc region shown in SEQ ID NO: 73;

(2) a first Fc region shown in SEQ ID NO: 74 and a second Fc region shown in SEQ ID NO: 75;

(3) a first Fc region shown in SEQ ID NO: 76 and a second Fc region shown in SEQ ID NO: 77; and

(4) a first Fc region shown in SEQ ID NO: 78 and a second Fc region shown in SEQ ID NO: 79.

[5] The antibody of [4], wherein the first Fc region is in the same polypeptide chain with the first heavy chain variable region, and the second Fc region is in the same

polypeptide chain with the second heavy chain variable region.

[6] The antibody of any one of [1] to [5], wherein the first heavy chain variable region and the first light chain variable region form a first antigen-binding site, and the second heavy chain variable region and the second light chain variable region form a second antigen-binding site.

[7] An isolated antibody that comprises:

a first heavy chain comprising an amino acid sequence selected from SEQ ID NOs: 104, 105, 106, and 107 and a first light chain comprising an amino acid sequence shown in SEQ ID NO: 112;

a second heavy chain comprising an amino acid sequence selected from SEQ ID NOs: 108, 109, 110, and 111 and a second light chain comprising an amino acid sequence shown in SEQ ID NO: 98.

[8] The antibody of [7], wherein the variable regions of the first heavy chain and the first light chain form a first antigen-binding site, and the variable regions of the second heavy chain and the second light chain form a second antigen-binding site.

[9] The antibody of [6] or [8], wherein the first antigen-binding site has binding activity towards CLDN6.

[10] The antibody of any one of [6], [8] and [9], wherein the first antigen-binding site has binding activity towards human CLDN6.

[11] The antibody of any one of [6] and [8] to [10], wherein the first antigen-binding site has binding activity towards human CLDN6 as defined in SEQ ID NO: 125 or 126.

[12] The antibody of any one of [6] and [8] to [11], wherein the first antigen-binding site does not substantially bind to human CLDN9.

[13] The antibody of any one of [6] and [8] to [12], wherein the first antigen-binding site does not substantially bind to human CLDN4.

[14] The antibody of any one of [6] and [8] to [13], wherein the first antigen-binding site does not substantially bind to human CLDN3.

[15] The antibody of any one of [6] and [8] to [14], wherein the first antigen-binding site does not substantially bind to a CLDN6 mutant as defined in SEQ ID NO: 134.

[16] The antibody of any one of [6] and [8] to [15], wherein the second antigen-binding site has binding activity towards CD3.

[17] The antibody of any one of [6] and [8] to [16], wherein the second antigen-binding site has binding activity towards CD3 epsilon chain.

[18] The antibody of any one of [6] and [8] to [17], wherein the second antigen-binding site has binding activity towards human CD3 as defined in SEQ ID NO: 142.

[19] An isolated nucleic acid encoding the antibody of any one of [1] to [18].

[20] A host cell comprising the nucleic acid of [19].

[21] A method of producing an antibody comprising culturing the host cell of [20] so

that the antibody is produced.

[22] The method of [21], further comprising recovering the antibody from the culture of the host cell.

[23] A pharmaceutical composition comprising the antibody of any one of [1] to [18], and a pharmaceutically acceptable carrier.

[24] The pharmaceutical composition of [23], which induces T-cell-dependent cytotoxicity.

[25] The composition of [23] or [24], which is a pharmaceutical composition used for treatment and/or prevention of cancer.

[26] The composition of any one of [23] to [25], which is a pharmaceutical composition used for treatment and/or prevention of ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer.

[27] Use of the antibody of any one of [1] to [18] in the manufacture of a medicament.

[28] Use of the antibody of any one of [1] to [18] in the manufacture of a medicament for treatment and/or prevention of cancer.

[29] Use of the antibody of any one of [1] to [18] in the manufacture of a medicament for treatment and/or prevention of ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer.

[30] A method of treating an individual having cancer comprising administering to the individual an effective amount of the antibody of any one of [1] to [18].

[31] A method of treating an individual having ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer comprising administering to the individual an effective amount of the antibody of any one of [1] to [18].

[32] A kit for use in the treatment and/or prevention of cancer, which comprises at least the antibody of any one of [1] to [18], and instructions for use.

[33] A kit for use in the treatment and/or prevention of ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer, which comprises at least the antibody of any one of [1] to [18], and instructions for use.

[0017] Furthermore, the present disclosure also provides the following:

[34] An antigen-binding molecule comprising:

(a) a heavy chain variable region comprising any one of the HVR combinations selected from:

(a1) HVR-H1, HVR-H2, and HVR-H3 amino acid sequences shown in SEQ ID NOs: 113, 117, and 118, respectively; and

(a2) HVR-H1, HVR-H2, and HVR-H3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83, 84, 57 and 85;

and

(b) a light chain variable region comprising any one of the HVR combinations selected from:

(b1) HVR-L1, HVR-L2, and HVR-L3 amino acid sequences shown in SEQ ID NOs: 119, 120, and 121, respectively; and

(b2) HVR-L1, HVR-L2, and HVR-L3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 92, 41, 42, 43, 46, 51, 52, 53, 54, 82, 86, 88 and 58.

[35] The antigen-binding molecule of [34], wherein the heavy chain variable region and light chain variable region comprise mouse, rabbit, humanized or human frameworks.

[36] An antigen-binding molecule comprising a heavy chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 85, 1, 33, 84, 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83 and 57, and a light chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 87, 2, 43, 86, 88, 92, 41, 42, 46, 51, 52, 53, 54, 82 and 58.

[37] The antigen-binding molecule of any one of [34] to [36], wherein the heavy chain variable region and the light chain variable region form an antigen-binding site.

[38] The antigen-binding molecule of [37], wherein the antigen-binding site has binding activity towards CLDN6.

[39] The antigen-binding molecule of [37] or [38], wherein the antigen-binding site has binding activity towards human CLDN6 as defined in SEQ ID NO: 125 or 126.

[40] The antigen-binding molecule of any one of [37] to [39], wherein the antigen-binding site does not substantially bind to human CLDN9.

[41] The antigen-binding molecule of any one of [37] to [40], wherein the antigen-binding site does not substantially bind to human CLDN4.

[42] The antigen-binding molecule of any one of [37] to [41], wherein the antigen-binding site does not substantially bind to human CLDN3.

[43] The antigen-binding molecule of any one of [37] to [42], wherein the antigen-binding site does not substantially bind to a CLDN6 mutant as defined in SEQ ID NO: 134.

[44] The antigen-binding molecule of any one of [34] to [43] wherein the antigen-binding molecule further comprises an antibody Fc region.

[45] The antigen-binding molecule of [44], wherein the Fc region is an Fc region with at least one amino acid mutation at any of the Fc region constituting amino acids of SEQ ID NOs: 143 to 146 (IgG1 to IgG4).

[46] The antigen-binding molecule of [44] or [45], wherein the Fc region is an Fc region to which sugar-chain is attached, and the percentage of fucose-deficient sugar-chain-attached to the Fc region is higher than that to a native IgG Fc region, or the

percentage of bisecting N-acetylglucosamine added to the Fc region is higher than that to a native IgG Fc region.

[47] The antigen-binding molecule of any one of [34] to [46], wherein the antigen-binding molecule has cytotoxic activity.

[48] The antigen-binding molecule of [47], wherein the cytotoxic activity is ADCC or CDC.

[49] The antigen-binding molecule of any one of [34] to [46], wherein the antigen-binding molecule has internalizing activity.

[50] The antigen-binding molecule of any one of [34] to [49] which is conjugated with a cytotoxic agent.

[51] An immunoconjugate comprising the antigen-binding molecule of any one of [34] to [50] and a cytotoxic agent.

[52] A pharmaceutical composition comprising the antigen-binding molecule of any one of [34] to [50] or the immunoconjugate of [51], and a pharmaceutically acceptable carrier.

[53] The composition of [52], which is a pharmaceutical composition used for treatment and/or prevention of cancer.

[54] The composition of [52] or [53], which is a pharmaceutical composition used for treatment and/or prevention of ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer.

[55] Use of the antigen-binding molecule of any one of [34] to [50] or the immunoconjugate of [51] in the manufacture of a medicament.

[56] Use of the antigen-binding molecule of any one of [34] to [50] or the immunoconjugate of [51] in the manufacture of a medicament for treatment and/or prevention of cancer.

[57] Use of the antigen-binding molecule of any one of [34] to [50] or the immunoconjugate of [51] in the manufacture of a medicament for treatment and/or prevention of ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer.

[58] A method of treating an individual having cancer comprising administering to the individual an effective amount of the antigen-binding molecule of any one of [34] to [50] or the immunoconjugate of [51].

[59] A method of treating an individual having ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer comprising administering to the individual an effective amount of the antigen-binding molecule of any one of [34] to [50] or the immunoconjugate of [51].

[60] A kit for use in the treatment and/or prevention of cancer, which comprises at least the antigen-binding molecule of any one of [34] to [50] or the immunoconjugate of [51], and instructions for use.

[61] A kit for use in the treatment and/or prevention of ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer, which comprises at least the antigen-binding molecule of any one of [34] to [50] or the immunoconjugate of [51], and instructions for use.

[62] A method of detecting the presence of CLDN6 in a biological sample, which comprises contacting the biological sample with the antigen-binding molecule of any one of [34] to [41].

[63] The method of [62], wherein the biological sample is contacted with the antigen-binding molecule under conditions permissive for binding of the antigen-binding molecule to CLDN6, and detecting whether a complex is formed between the antigen-binding molecule and CLDN6.

[64] A method of diagnosing whether a subject have cancer or not, which comprises contacting a biological sample from the subject with the antigen-binding molecule of any one of [34] to [41].

[65] The method of [64], wherein the biological sample is contacted with the antigen-binding molecule under conditions permissive for binding of the antigen-binding molecule to CLDN6, and detecting whether a complex is formed between the antigen-binding molecule and CLDN6.

[66] The method of [64] or [65], wherein the cancer is ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer.

[67] An isolated nucleic acid encoding the antigen-binding molecule of any one of [34] to [41].

[68] A host cell comprising the nucleic acid of [67].

[69] A method of producing an antigen-binding molecule comprising culturing the host cell of [68] so that the antigen-binding molecule is produced.

[70] The method of [69], further comprising recovering the antigen-binding molecule from the culture of the host cell.

[0018] Furthermore, the present disclosure also provides the following:

[71] An antigen-binding molecule that comprises:

a first antigen-binding site that has binding activity towards CLDN6, and

a second antigen-binding site that has binding activity towards T-cell receptor complex,

wherein the first antigen-binding site is formed by

(a) a heavy chain variable region comprising any one of the HVR combinations selected from:

(a1) HVR-H1, HVR-H2, and HVR-H3 amino acid sequences shown in SEQ ID NOs: 113, 117, and 118, respectively; and

(a2) HVR-H1, HVR-H2, and HVR-H3 same with those of any one of the heavy

chain variable regions shown in SEQ ID NOs: 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83, 84, 57 and 85;
and
(b) a light chain variable region comprising any one of the HVR combinations selected from:
(b1) HVR-L1, HVR-L2, and HVR-L3 amino acid sequences shown in SEQ ID NOs: 119, 120, and 121, respectively; and
(b2) HVR-L1, HVR-L2, and HVR-L3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 92, 41, 42, 43, 46, 51, 52, 53, 54, 82, 86, 88 and 58.

[72] The antigen-binding molecule of [71], wherein the heavy chain variable region and the light chain variable region that form the first antigen-binding site further comprise mouse, rabbit, humanized or human frameworks.

[73] An antigen-binding molecule that comprises:
a first antigen-binding site that has binding activity towards CLDN6, and
a second antigen-binding site that has binding activity towards T-cell receptor complex,
wherein the first antigen-binding site comprises a heavy chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 85, 1, 33, 84, 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83 and 57 and a light chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 87, 2, 43, 86, 88, 92, 41, 42, 46, 51, 52, 53, 54, 82 and 58.

[74] The antigen-binding molecule of any one of [71] to [73], wherein the first antigen-binding site has binding activity towards human CLDN6.

[75] The antigen-binding molecule of any one of [71] to [74], wherein the first antigen-binding site has binding activity towards human CLDN6 as defined in SEQ ID NO: 125 or 126.

[76] The antigen-binding molecule of any one of [71] to [75], wherein the first antigen-binding site does not substantially bind to human CLDN9.

[77] The antigen-binding molecule of any one of [71] to [76], wherein the first antigen-binding site does not substantially bind to human CLDN4.

[78] The antigen-binding molecule of any one of [71] to [77], wherein the first antigen-binding site does not substantially bind to human CLDN3.

[79] The antigen-binding molecule of any one of [71] to [78], wherein the first antigen-binding site does not substantially bind to a CLDN6 mutant as defined in SEQ ID NO: 134.

[80] The antigen-binding molecule of any one of [71] to [73], wherein the first antigen-binding site is included in a single-chain Fv (scFv), Fv or Fab.

[81] The antigen-binding molecule of any one of [71] to [80], wherein the second antigen-binding site has binding activity towards CD3.
[82] The antigen-binding molecule of any one of [71] to [80], wherein the second antigen-binding site has binding activity towards CD3 epsilon chain.
[83] The antigen-binding molecule of any one of [71] to [80], wherein the second antigen-binding site has binding activity towards T-cell receptor.
[84] The antigen-binding molecule of any one of [71] to [82], wherein the second antigen-binding site is formed by a heavy chain variable region comprising HVR-H1, HVR-H2 and HVR-H3 amino acid sequences of SEQ ID NOs: 122, 123, and 124, respectively; and a light chain variable region comprising HVR-L1, HVR-L2 and HVR-L3 amino acid sequences of SEQ ID NOs: 114, 115, and 116, respectively.
[85] The antigen-binding molecule of [84], wherein the heavy chain variable region and light chain variable region comprise mouse, rabbit, humanized or human frameworks.
[86] The antigen-binding molecule of any one of [71] to [82], wherein the second antigen-binding site is formed by a heavy chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 60 and 70 and a light chain variable region comprising an amino acid sequence selected from SEQ ID NOs: 61 and 71.
[87] The antigen-binding molecule of any one of [71] to [86], wherein the second antigen-binding site is included in a single-chain Fv (scFv), Fv or Fab.
[88] The antigen-binding molecule of any one of [71] to [87], wherein the antigen-binding molecule further comprises an antibody Fc region.
[89] The antigen-binding molecule of [88], wherein the Fc region is an Fc region with reduced binding activity towards an Fc gamma receptor.
[90] The antigen-binding molecule of [88] or [89], wherein the Fc region is an Fc region with at least one amino acid mutation at any of the Fc region constituting amino acids of SEQ ID NOs: 143 to 146 (IgG1 to IgG4).
[91] The antigen-binding molecule of any one of [88] to [90], wherein the Fc region is an Fc region with mutation of at least one amino acid selected from the following amino acid positions specified by EU numbering:
position 220, position 226, position 229, position 231, position 232, position 233, position 234, position 235, position 236, position 237, position 238, position 239, position 240, position 264, position 265, position 266, position 267, position 269, position 270, position 295, position 296, position 297, position 298, position 299, position 300, position 325, position 327, position 328, position 329, position 330, position 331, and position 332.
[92] The antigen-binding molecule of any one of [88] to [91], wherein the Fc region is an Fc region comprising at least one amino acid selected from the following amino

acids specified by EU numbering:
Arg at amino acid position 234, Ala or Arg at amino acid position 235, Lys at amino acid position 239, and Ala at amino acid position 297.
[93] The antigen-binding molecule of any one of [88] to [92], wherein
the heavy chain variable domain that forms the first antigen-binding site is linked to the Fc region via a first CH1 domain, the light chain variable domain that forms the first antigen-binding site is linked to a first CL domain; and
the heavy chain variable domain that forms the second antigen-binding site is linked to the Fc region via a second CH1 domain, the light chain variable domain that forms the second antigen-binding site is linked to a second CL domain.
[94] The antigen-binding molecule of [93], wherein the electric charges of the CH1 and CL domains are controlled so that the heavy chain variable region that forms the first antigen-binding domain assembles with the light chain variable region that forms the first antigen-binding domain, and/or the heavy chain variable region that forms the second antigen-binding domain assembles with the light chain variable region that forms the second antigen-binding domain.
[95] The antigen-binding molecule of any one of [71] to [94], wherein the antigen-binding molecule has cytotoxic activity.
[96] The antigen-binding molecule of [95], wherein the cytotoxic activity is T-cell-dependent cytotoxic activity.
[97] An isolated polynucleotide encoding the antigen-binding molecule of any one of [71] to [96].
[98] A host cell comprising the polynucleotide of [97].
[99] A method of producing an antibody comprising culturing the host cell of [98] so that the antigen-binding molecule is produced.
[100] The method of [99], further comprising recovering the antigen-binding molecule from the culture of the host cell.
[101] A pharmaceutical composition comprising the antigen-binding molecule of any one of [71] to [96], and a pharmaceutically acceptable carrier.
[102] The pharmaceutical composition of [101], which induces T-cell-dependent cytotoxicity.
[103] The composition of [101] or [102], which is a pharmaceutical composition used for treatment and/or prevention of cancer.
[104] The composition of any one of [101] to [103], which is a pharmaceutical composition used for treatment and/or prevention of ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer.
[105] Use of the antigen-binding molecule of any one of [71] to [96] in the manufacture of a medicament.

[106] Use of the antigen-binding molecule of any one of [71] to [96] in the manufacture of a medicament for treatment and/or prevention of cancer.

[107] Use of the antigen-binding molecule of any one of [71] to [96] in the manufacture of a medicament for treatment and/or prevention of ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer.

[108] A method of treating an individual having cancer comprising administering to the individual an effective amount of the antigen-binding molecule of any one of [71] to [96].

[109] A method of treating an individual having ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer comprising administering to the individual an effective amount of the antigen-binding molecule of any one of [71] to [96].

[110] A kit for use in the treatment and/or prevention of cancer, which comprises at least the antigen-binding molecule of any one of [71] to [96], and instructions for use.

[111] A kit for use in the treatment and/or prevention of ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer, which comprises at least the antigen-binding molecule of any one of [71] to [96], and instructions for use.

Advantageous Effects of the Invention

[0019] The present disclosure provides multispecific antigen-binding molecules that enable cancer treatment by having T-cells close to CLDN6-expressing cells and using the cytotoxicity of T-cells against the CLDN6-expressing cancer cells, methods for producing the multispecific antigen-binding molecules, and pharmaceutical compositions containing such a multispecific antigen-binding molecule as an active ingredient for inducing cellular cytotoxicity. Multispecific antigen-binding molecules of the present disclosure have strong anti-tumor activity, inducing cellular cytotoxicity, and can target and damage CLDN6-expressing cells, thus enable treatment and prevention of various cancers. Furthermore, the antigen-binding molecules comprise specific sequences also have superior specificity towards CLDN6, long half-life in blood, as well as improved safety properties, stability and manufacturability.

[0020] The present disclosure also provides antigen-binding molecules that shows superior specificity towards CLDN6, which enable accurate detection of the CLDN6 presence in biological sample, and diagnosis of various cancers. Cancer treatment by targeting CLDN6-expressing cells are also enabled.

Brief Description of Drawings

[0021] [fig.1]T cell activation activity of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01, AH05/TR01, CDA0013/TR01 and 6PHU3/TR01). T cell activation activity of each antibody was examined in the presence of hCLDN6/BaF (A), hCLDN9/BaF (B), and hCLDN6 (Q156L)/BaF (C). The vertical axis indicates the lu-

minescence fold of antibody containing well compared to that of the well without antibody and the horizontal axis indicates the concentration of the anti-CLDN6/CD3 bispecific antibodies.

[fig.2-1]Binding specificity of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01. AH05/TR01, CDA0013/TR01 and 6PHU3/TR01) to human and mouse Claudin family proteins. Relative binding activity of anti-CLDN6/CD3 bispecific antibodies was examined by flow cytometer in a concentration of 10 micro g/ml. The vertical axis indicates the MFI values of an antibody binding to FreeStyle™ 293-F transfectants, which are transiently expressing human or mouse CLDN3, CLDN4, CLDN6, and CLDN9. KLH/TR01 was used as a negative control. Commercially available human CLDN6 antibody (MAB3656), human CLDN3 antibody (MAB4620), and human CLDN4 antibody (MAB4219) were tested as a control.

[fig.2-2]Continuation of Fig. 2-1.

[fig.3]In vivo anti-tumor efficacy of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01, AH05/TR01, and 6PHU3/TR01). Fig. 3a shows the tumor volume change of HuH-7 T cell injection model, and Fig. 3b shows the tumor volume change of OV-90 T cell injection model. In this study, each group (n=5) were administered 5mg/kg of antibodies or vehicle intravenously on day 14 after tumor inoculation.

[fig.4]The body weight change of mice treated with 5 mg/kg of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01, AH05/TR01, and 6PHU3/TR01) as described in Fig. 3. The body weight change of HuH-7 bearing T cell injection model (a) and OV-90 bearing T cell injection model (b) are shown respectively. The vertical axis indicates body weight and the horizontal axis indicates days after tumor inoculation.

[fig.5]Plasma ALT, GGT, TBIL, ALP, GLDH, and TBA levels in OV-90 T cell injection model after administration of 5 mg/kg of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01, AH05/TR01, and 6PHU3/TR01) or vehicle as described in Fig. 3. The vertical axis indicates concentration of ALT (Alanine aminotransferase), ALP (alkaline phosphatase), GGT (gamma-glutamyl transpeptidase), GLDH (glutamate dehydrogenase), TBIL (total bilirubin) and TBA (total bile acid), respectively.

[fig.6]The temporal change of the antibody concentration in mouse treated with 5mg/kg of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01, AH05/TR01, and 6PHU3/TR01) as described in Fig. 3. Plasma concentration of antibody in HuH-7 T cell injection model (upper) and OV-90 T cell injection model (lower) are shown respectively. The vertical axis indicates the antibody concentration (micro g/mL) in the mouse plasma and the horizontal axis indicates days after treatment.

[fig.7]In vivo anti-tumor efficacy and the body weight change of the anti-CLDN6/CD3 bispecific antibodies in OVCAR-3 T cell injection model. Mouse were treated with

5mg/kg of AE3-20/TR01, CDA0013/TR01, or vehicle intravenously on day 29 after tumor transplantation. The tumor volume (upper) and the body weight (lower) were examined after treatment.

[fig.8]Plasma ALT, GGT, TBIL, ALP, GLDH, and TBA levels after treatment of 5 mg/kg of CDA0013/TR01 in OVCAR-3 T cell injection model as described in Fig. 7. The vertical axis indicates concentration of ALT, GGT, TBIL, ALP, GLDH, and TBA respectively, after administration of CDA0013/TR01 or no treatment.

[fig.9]The temporal change of the concentration of AE3-20/TR01 and CDA0013/TR01 in OVCAR-3 T cell injection model as described in Fig. 7. The vertical axis indicates the antibody concentration (micro g/mL) in the mouse plasma and the horizontal axis indicates days after treatment.

[fig.10]Binding activities of various anti-CLDN6/CD3 bispecific antibodies to hCLDN6/BaF. The vertical axis indicates the MFI values of antibody to hCLDN6/BaF, in a linear manner, taken as binding activity.

[fig.11]T cell activation activity of various anti-CLDN6/CD3 bispecific antibodies in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF, or hCLDN9/BaF. The horizontal axis indicates the luminescence fold of antibody containing well compared with that of the well without antibody. KLH/TR01 was used as a negative control. The bars are shown in the following order from the bottom: CLDN6, CLDN3, CLDN4, and CLDN9.

[fig.12]Continuation of Fig. 11.

[fig.13]T cell activation activity of various anti-CLDN6/CD3 bispecific antibodies in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF, or hCLDN9/BaF. The horizontal axis indicates the luminescence fold of antibody containing well compared with that of the well without antibody. 1 nM and 0.1 nM of KLH/TR01 were not tested in the presence of hCLDN6/BaF. KLH/TR01 was used as a negative control. The bars are shown in the following order from the bottom: CLDN6 (antibody concentration 10 nM), CLDN6 (antibody concentration 1 nM), CLDN6 (antibody concentration 0.1 nM), CLDN3 (antibody concentration 10 nM), CLDN4 (antibody concentration 10 nM), and CLDN9 (antibody concentration 10 nM).

[fig.14]T cell activation activity of various anti-CLDN6/CD3 bispecific antibodies in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF, or hCLDN9/BaF. The horizontal axis indicates the luminescence fold of antibody containing well compared with that of the well without antibody. 1 nM and 0.1 nM of KLH/TR01 were not tested in the presence of hCLDN6/BaF. KLH/TR01 was used as a negative control. The bars are shown in the following order from the bottom: CLDN6 (antibody concentration 10 nM), CLDN6 (antibody concentration 1 nM), CLDN6 (antibody concentration 0.1 nM), CLDN3 (antibody concentration 10 nM), CLDN4 (antibody concentration 10

nM), and CLDN9 (antibody concentration 10 nM).

[fig.15]T cell activation activity of various anti-CLDN6/CD3 bispecific antibodies in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF, or hCLDN9/BaF. The horizontal axis indicates the luminescence fold of antibody containing well compared with that of the well without antibody. 1 nM and 0.1 nM of KLH/TR01 were not tested in the presence of hCLDN6/BaF. KLH/TR01 was used as a negative control. The bars are shown in the following order from the bottom: CLDN6 (antibody concentration 10 nM), CLDN6 (antibody concentration 1 nM), CLDN6 (antibody concentration 0.1 nM), CLDN3 (antibody concentration 10 nM), CLDN4 (antibody concentration 10 nM), and CLDN9 (antibody concentration 10 nM).

[fig.16]Charge heterogeneity of 65H0671Q39E-SG1189v14k/54L0571Q38K-SK1018//TR01H(Q39K)-SG1191v14h/TR01L(Q38E)-SK1017 (A) and 65H0671Q39E-SG1189v14k/54L0571Q38K-SK1021//TR01H(Q39K)-SG1191v14h/TR01L(Q38E)-SK1017 (B). The charge heterogeneity and the pI values were evaluated using the Maurice (ProteinSimple) with UV absorbance at 280 nm. The associated software Compass for iCE (2.0.10) was used for data analysis.

[fig.17]In vivo anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibodies AE3-20/TR01 and CS2201 in OV-90 T cell injection model. A single dose of each anti-CLDN6/CD3 bispecific antibody at 0.1 mg/kg on day 14 after tumor inoculation was administered intravenously. The tumor volume changes (upper) and body weight (lower) are shown respectively.

[fig.18]In vivo anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibodies AE3-20/TR01 and CS2201 in OV-90 T cell injection model. A single dose of each anti-CLDN6/CD3 bispecific antibody at 5 mg/kg on day 15 after tumor inoculation was administered intravenously. The tumor volume changes (upper) and body weight (lower) are shown respectively.

[fig.19]Plasma ALT, GLDH, TBA, ALP, and TBIL levels after treatment of 5 mg/kg of AE3-20/TR01 or CS2201 in OV-90 T cell injection model as described in Figs. 17 and 18. The vertical axis indicates concentration of ALT, GLDH, TBA, ALP, and TBIL after administration of AE3-20/TR01, CS2201, and vehicle.

[fig.20]The temporal change of the concentration of AE3-20/TR01 and CS2201 in OV-90 T cell injection model as described in Figs. 17 and 18. The vertical axis indicates the antibody concentration (micro g/mL) in the mouse plasma and the horizontal axis indicates days after treatment.

[fig.21A]In vivo anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibody CS2425 in OV-90 T cell injection model. A single dose of 0.1 mg/kg of CS2425 was administered intravenously on day 15 after the tumor inoculation. The tumor volume

changes (upper) and body weight changes (lower) are shown respectively.

[fig.21B]In vivo anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibody CS2425 in OV-90 T cell injection model. A single dose of 5 mg/kg of CS2425 was administered intravenously on day 15 after the tumor inoculation. The tumor volume changes (upper) and body weight changes (lower) are shown respectively.

[fig.22]Plasma ALT, GGT, TBA, ALP, and TBIL levels after treatment of CS2425 in OV-90 T cell injection model as described in Figs. 21A and 21B. The vertical axis indicates concentration of ALT, GGT, TBA, ALP, and TBIL after administration of 0.1 mg/kg and 5 mg/kg of CS2425, or vehicle.

[fig.23]The temporal change of the concentration of CS2425 in OV-90 T cell injection model as described in Figs. 21A and 21B. The vertical axis indicates the antibody concentration (micro g/mL) in the mouse plasma and the horizontal axis indicates days after treatment.

[fig.24]A chart showing the maximum value of the proportion of IL-2-secreting CD4+ T cells in CD8-CD25low PBMCs cultured for 67 hours in the presence of each of the test substances including negative control and positive control. The data shows the maximum values of the proportions of IL-2-secreting CD4+ T cells for the respective donors from whom the used PBMCs are derived. No antigen shows the result from the study control when culturing was performed in the absence of a test substance. The dotted line shows positive threshold.

[fig.25]A chart showing heatmaps of the number of the peptides per amino acid position identified for a part of the light chain of CS2425 by MAPPs. The number of the identified peptides per amino acid position for the region are shown in heatmaps in which the cell colors represent the number of identified peptides (see key). Peptide-MHC II complexes were obtained from CS2425-pulsed DCs differentiated from monocytes.

[fig.26]A chart showing the maximum value of the proportion of IL-2-secreting CD4+ T cells in CD8-CD25low PBMCs cultured for 67 hours in the presence of each of the test substances including negative control and positive control. The data shows the maximum values of the proportions of IL-2-secreting CD4+ T cells for the respective donors from whom the used PBMCs are derived. No antigen shows the result from the study control when culturing was performed in the absence of a test substance. The dotted line shows positive threshold.

[fig.27]In vivo anti-tumor efficacy in mice treated with the anti-CLDN6/CD3 bispecific antibodies CS2201, CS2425, CS2884, CS2885, and CS2886 in OV-90 T cell injection model. A single dose of 5 mg/kg of each anti-CLDN6/CD3 bispecific antibody was administered intravenously on day 15 after the tumor inoculation. The tumor volume (upper) and body weight (lower) were examined in mice after single ad-

ministration of 5 mg/kg of each antibodies.

[fig.28]Plasma level of liver injury markers in mice treated with the anti-CLDN6/CD3 bispecific antibodies CS2201, CS2425, CS2884, CS2885, and CS2886 in OV-90 T cell injection model as shown in Fig. 27. Plasma ALT, GGT, TBIL, ALP, GLDH, and TBA levels were evaluated in mice after treatment of each anti-CLDN6/CD3 bispecific antibodies. The vertical axis indicates concentration of ALT, GGT, TBIL, ALP, GLDH, and TBA in mouse plasma after administration of 5 mg/kg of each anti-CLDN6/CD3 bispecific antibody or vehicle.

[fig.29]In vivo anti-tumor efficacy in mice treated with the anti-CLDN6/CD3 bispecific antibodies CS2201, CS2425, CS2958, CS2959, CS2960, and CS2961 in OV-90 T cell injection model. A single dose of 5 mg/kg of each anti-CLDN6/CD3 bispecific antibody was administered intravenously on day 15 after the tumor inoculation. The tumor volume (upper) and body weight (lower) were examined in mice after single administration of 5 mg/kg of each antibodies

[fig.30]Plasma level of liver injury markers in mice treated with the anti-CLDN6/CD3 bispecific antibodies CS2201, CS2425, CS2958, CS2959, CS2960, and CS2961 in OV-90 T cell injection model as shown in Fig. 29. Plasma ALT, GGT, TBIL, ALP, GLDH, and TBA levels were evaluated in mice after treatment of each anti-CLDN6/CD3 bispecific antibodies. The vertical axis indicates concentration of ALT, GGT, TBIL, ALP, GLDH, and TBA in mouse plasma after administration of 5 mg/kg of each anti-CLDN6/CD3 bispecific antibody or vehicle.

[fig.31]The temporal change of the concentration of the anti-CLDN6/CD3 bispecific antibodies CS2201, CS2425, CS2958, CS2959, CS2960, and CS2961 in OV-90 T cell injection model as described in Fig. 29. The vertical axis indicates the antibody concentration (micro g/mL) in the mouse plasma and the horizontal axis indicates days after treatment.

[fig.32]T cell activation activity of various concentrations of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01, CS2201, 6PHU3/TR01, CS2425 and CS2961) in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF, hCLDN9/BaF, or hCLDN6(Q156L)/BaF determined by T Cell Activation Bioassay using GloResponse ™ NFAT-luc2 Jurkat cells. The vertical axis indicates the luminescence fold of antibody containing well compared with well without antibody. KLH/TR01 was used as a negative control.

[fig.33]T cell activation activity of various concentrations of anti-CLDN6/CD3 bispecific antibodies (CS2201, CS2961, CS3346, CS3347, and CS3348) in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF or hCLDN9/BaF determined by T Cell Activation Bioassay using GloResponse™ NFAT-luc2 Jurkat cells. The vertical axis indicates the luminescence fold of antibody containing well compared

with well without antibody. KLH/TR01 was used as a negative control.

[fig.34]T-cell-dependent cell cytotoxicity of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01, CS2201, 6PHU3/TR01, CS2425, CS2961, CS3346, CS3347, and CS3348) against OVCAR3 ovarian cancer cell line expressing CLDN6 determined by LDH assay. The vertical axis indicates the percentage of cell lysis and the horizontal axis indicates the concentration of anti-CLDN6/CD3 bispecific antibodies. KLH/TR01 was used as a negative control.

[fig.35-1]Binding activity of anti-CLDN6/CD3 bispecific antibodies (CS2201, CS2961, AH05/TR01, and 6PHU3/TR01) to human CLDN family proteins (CLDN3, CLDN4, CLDN6, and CLDN9). Binding activity of anti-CLDN6/CD3 bispecific antibodies to BaF3 transfectants (hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF, and hCLDN9/BaF) was examined by flow cytometer in a concentration of 15 micro g/ml, and plotted as histogram. KLH/TR01 was used as a negative control. The specificity of CS2961 to human CLDN6 was highest among the tested antibodies.

[fig.35-2]Continuation of Fig. 35-1.

[fig.36]HMW species formation of anti-CLDN6/CD3 bispecific antibodies after storage at 40 degrees C. The vertical axis indicates percentage of HMW species in total peak area of each samples.

[fig.37]In vivo dose-dependent anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibody in OV-90 T cell injection model. A single dose ranging from 0.001 to 5 mg/kg of AE3-20/TR01 was administered intravenously 14 days after the tumor inoculation. The tumor volume changes (upper) and body weight changes (lower) are shown respectively.

[fig.38]In vivo dose-dependent anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibody in OV-90 T cell injection model. A single dose ranging from 0.0016 to 5 mg/kg of CS3348 was administered intravenously 15 days after the tumor inoculation. The tumor volume changes (upper) and body weight changes (lower) are shown respectively.

[fig.39]Amino acid sequence alignment of human CLDN9 and human CLDN6. Human CLDN9 and human CLDN6 comprises almost the same sequence in extracellular domain 1, except the N-terminus residue (Met/Leu at position 29). Two amino acids in extracellular domain 2 are different in human CLDN9 and human CLDN6 (Arg/Leu at position 145 and Gln/Leu at position 156).

[fig.40]Anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibody CS3348 in OVCAR-3 bearing T cell injection model (TK220001)

[fig.41]Anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibody CS3348 in NCI-H1435 bearing T cell injection model (TK219005)

[fig.42]Anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibody CS3348 in

NUGC-3 bearing T cell injection model (TK220003)

Description of Embodiments

[0022] 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).

[0023] The definitions and detailed description below are provided to facilitate understanding of the present disclosure illustrated herein.

[0024] 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.

[0025] Alteration of Amino Acids

For amino acid alteration (also described as "amino acid substitution" within this de-

scription) 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).

[0026] 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.

[0027] 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.

[0028] Antigen-binding molecule

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.

[0029] Claudin-6 (CLDN6) and other Claudin family proteins

The term "CLDN6", as used herein, refers to any native Claudin-6 from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice and rats), unless otherwise indicated. The amino acid sequence of human CLDN6 (hCLDN6) is shown in SEQ ID NO: 125 or 126, and the amino acid sequence of mouse CLDN6 (mCLDN6) is shown in SEQ ID NO: 130.

[0030] There are many other proteins within Claudin family other than CLDN6, such as CLDN3, CLDN4, and CLDN9. The amino acid sequences of human CLDN3 (hCLDN3), human CLDN4 (hCLDN4) and human CLDN9 (hCLDN9) are shown in SEQ ID NOs: 128, 129 and 127, respectively. The amino acid sequences of mouse CLDN3 (mCLDN3), mouse CLDN4 (mCLDN4) and mouse CLDN9 (mCLDN9) are shown in SEQ ID NOs: 132, 133 and 131, respectively.

[0031] Binding activity towards CLDN6

The binding activity towards CLDN6 described herein refers to a specific binding activity towards the CLDN6 protein described herein, or the whole or a portion of a partial peptide of the CLDN6 protein.

[0032] In certain embodiments, binding activity towards CLDN6 is the binding activity towards the first extracellular domain of CLDN6 (amino acids 29-81 of SEQ ID NO: 125 or 126) or the second extracellular domain of CLDN6 (amino acids 138-159 of SEQ ID NO: 125 or 126). In certain embodiments, binding activity towards CLDN6 is the binding activity towards human CLDN6 expressed on the surface of eukaryotic cells. In certain embodiments, binding activity towards CLDN6 is the binding activity towards the CLDN6 protein expressed on the surface of cancer cells.

[0033] Binding activity towards T cell receptor complex

The binding activity towards T cell receptor complex as used herein refers to the binding activity towards the whole or a portion of a partial peptide of a T cell receptor complex. The T cell receptor complex may be a T cell receptor itself, or an adaptor molecule constituting a T cell receptor complex along with a T cell receptor. CD3 is suitable as an adaptor molecule.

[0034] The binding activity towards T cell receptor as used herein refers to the binding activity towards the whole or a portion of a partial peptide of a T cell receptor. The portion of a T cell receptor may be a variable region of the T cell receptor or a constant region of the T cell receptor; however, an epitope present in the constant region is

preferred. Examples of the constant region sequence include the T cell receptor alpha chain of RefSeq Accession No. CAA26636.1 (SEQ ID NO: 135), the T cell receptor beta chain of RefSeq Accession No. C25777 (SEQ ID NO: 136), the T cell receptor gamma 1 chain of RefSeq Accession No. A26659 (SEQ ID NO: 137), the T cell receptor gamma 2 chain of RefSeq Accession No. AAB63312.1 (SEQ ID NO: 138), and the T cell receptor delta chain of RefSeq Accession No. AAA61033.1 (SEQ ID NO: 139).

[0035] The binding activity towards CD3 as used herein refers to the binding activity towards to the whole or a portion of a partial peptide of CD3. The portion of CD3 may be any epitope exists in the gamma-chain, delta-chain, or epsilon-chain sequence that constitutes human CD3. Regarding the structure of the gamma chain, delta chain, or epsilon chain constituting CD3, their polynucleotide sequences are disclosed in RefSeq Accession NOs. NM_000073.2, NM_000732.4 and NM_000733.3, and their polypeptide sequences are shown in SEQ ID NOs: 140 (NP_000064.1), 141 (NP_000723.1), and 142 (NP_000724.1), wherein the RefSeq accession numbers are shown in parentheses.

[0036] 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). Affinity can be measured by common methods known in the art, including those as described herein. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

[0037] 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 CLDN6 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.

[0038] 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.

[0039] 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 (Kd) 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 M^{-1} 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.

[0040] 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.

[0041] 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.

[0042] 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.

[0043] 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.

[0044] 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.

[0045] 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.

[0046] HVR

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"). Generally, antibodies comprise six HVRs: three in the VH (H1, H2, H3), and three in the VL (L1, L2, L3). 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).

[0047] 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.

[0048] 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.

[0049] 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).

[0050] 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.

[0051] 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.

[0052] 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.

[0053] 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 deoxyribonucleotides, 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). 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)NR_2 ("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.

[0054] 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.

[0055] 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.

[0056] Host cell

The terms "host cell," "host cell line," and "host cell culture" are used inter-changeably 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.

[0057] 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.

[0058] 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.

[0059] 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.

[0060] 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.

[0061] 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.

[0062] 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.

[0063] 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.

[0064] 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]

[0065] The molecular form of $sc(Fv)_2$ is also described in detail in WO 2006/132352. According to these descriptions, those skilled in the art can appropriately prepare desired $sc(Fv)_2$ to produce the polypeptide complexes disclosed herein.

[0066] 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.

[0067] 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 $sc(Fv)_2$ contains three peptide linkers, their length may be all the same or different.

[0068] For example, such peptide linkers include:
Ser
Gly Ser
Gly Gly Ser
Ser Gly Gly
Gly Gly Gly Ser (SEQ ID NO: 147)
Ser Gly Gly Gly (SEQ ID NO: 148)
Gly Gly Gly Gly Ser (SEQ ID NO: 149)
Ser Gly Gly Gly Gly (SEQ ID NO: 150)
Gly Gly Gly Gly Gly Ser (SEQ ID NO: 151)
Ser Gly Gly Gly Gly Gly (SEQ ID NO: 152)
Gly Gly Gly Gly Gly Gly Ser (SEQ ID NO: 153)
Ser Gly Gly Gly Gly Gly Gly (SEQ ID NO: 154)
(Gly Gly Gly Gly Ser (SEQ ID NO: 149))n
(Ser Gly Gly Gly Gly (SEQ ID NO: 150))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.

[0069] 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.

[0070] 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.

[0071] 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.

[0072] "$F(ab')_2$" 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'.

[0073] "$F(ab')_2$" 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 particularly 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.

[0074] Fc region

The term "Fc region" or "Fc domain" herein is used to define a C-terminal region of an immunoglobulin heavy chain that contains at least a portion of the constant region. The term includes native sequence Fc regions and variant Fc regions. In one embodiment, a human IgG heavy chain Fc region extends from Cys226, or from Pro230, to the carboxyl-terminus of the heavy chain. However, the C-terminal lysine (Lys447) or glycine-lysine (residues 446-447) of the Fc region may or may not be present. Unless otherwise specified herein, numbering of amino acid residues in the Fc region or constant region is according to the EU numbering system, also called the EU index, as described in Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD, 1991.

[0075] 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.

[0076] 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.).

[0077] 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).

[0078] 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 RIIB (CD32), Fc gamma RIIIA (CD16), and/or Fc gamma RIIIB (CD16). 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 RIIB 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.

[0079] 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.

[0080] 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 RIIB, 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).

[0081] 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.

[0082] 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).

[0083] 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 (ka) and dissociation rate constant (kd)) 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.

[0084] Fc region with a reduced Fc gamma receptor-binding activity

Herein, "a reduced Fc gamma receptor-binding activity" means, for example, that based on the above-described analysis method the competitive activity of a test antigen-binding molecule or antibody is 50% or less, preferably 45% or less, 40% or less, 35% or less, 30% or less, 20% or less, or 15% or less, and particularly preferably 10% or less, 9% or less, 8% or less, 7% or less, 6% or less, 5% or less, 4% or less, 3% or less, 2% or less, or 1% or less than the competitive activity of a control antigen-binding molecule or antibody.

[0085] Antigen-binding molecules or antibodies comprising the Fc domain of a monoclonal IgG1, IgG2, IgG3, or IgG4 antibody can be appropriately used as control antigen-binding molecules or antibodies. The Fc domain structures are shown in SEQ ID NOs: 143 (A is added to the N terminus of RefSeq accession number AAC82527.1), 144 (A

is added to the N terminus of RefSeq accession number AAB59393.1), 145 (A is added to the N terminus of RefSeq accession number CAA27268.1), and 146 (A is added to the N terminus of RefSeq accession number AAB59394.1). Furthermore, when an antigen-binding molecule or antibody comprising an Fc domain mutant of an antibody of a particular isotype is used as a test substance, the effect of the mutation of the mutant on the Fc gamma receptor-binding activity is assessed using as a control an antigen-binding molecule or antibody comprising an Fc domain of the same isotype. As described above, antigen-binding molecules or antibodies comprising an Fc domain mutant whose Fc gamma receptor-binding activity has been judged to be reduced are appropriately prepared.

[0086] Such known mutants include, for example, mutants having a deletion of amino acids 231A-238S (EU numbering) (WO 2009/011941), as well as mutants C226S, C229S, P238S, (C220S) (J. Rheumatol (2007) 34, 11); C226S and C229S (Hum. Antibod. Hybridomas (1990) 1(1), 47-54); C226S, C229S, E233P, L234V, and L235A (Blood (2007) 109, 1185-1192).

[0087] Specifically, the preferred antigen-binding molecules or antibodies include those comprising an Fc domain with a mutation (such as substitution) of at least one amino acid selected from the following amino acid positions: 220, 226, 229, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 264, 265, 266, 267, 269, 270, 295, 296, 297, 298, 299, 300, 325, 327, 328, 329, 330, 331, or 332 (EU numbering), in the amino acids forming the Fc domain of an antibody of a particular isotype. The isotype of antibody from which the Fc domain originates is not particularly limited, and it is possible to use an appropriate Fc domain derived from a monoclonal IgG1, IgG2, IgG3, or IgG4 antibody. It is preferable to use Fc domains derived from IgG1 antibodies.

[0088] The preferred antigen-binding molecules or antibodies include, for example, those comprising an Fc domain which has any one of the substitutions shown below, whose positions are specified according to EU numbering (each number represents the position of an amino acid residue in the EU numbering; and the one-letter amino acid symbol before the number represents the amino acid residue before substitution, while the one-letter amino acid symbol after the number represents the amino acid residue after the substitution) in the amino acids forming the Fc domain of IgG1 antibody:

(a) L234F, L235E, P331S;

(b) C226S, C229S, P238S;

(c) C226S, C229S; or

(d) C226S, C229S, E233P, L234V, L235A;

as well as those having an Fc domain which has a deletion of the amino acid sequence at positions 231 to 238.

[0089] Furthermore, the preferred antigen-binding molecules or antibodies also include

those comprising an Fc domain that has any one of the substitutions shown below, whose positions are specified according to EU numbering in the amino acids forming the Fc domain of an IgG2 antibody:

(e) H268Q, V309L, A330S, and P331S;

(f) V234A;

(g) G237A;

(h) V234A and G237A;

(i) A235E and G237A; or

(j) V234A, A235E, and G237A. Each number represents the position of an amino acid residue in EU numbering; and the one-letter amino acid symbol before the number represents the amino acid residue before substitution, while the one-letter amino acid symbol after the number represents the amino acid residue after the substitution.

[0090] Furthermore, the preferred antigen-binding molecules or antibodies also include those comprising an Fc domain that has any one of the substitutions shown below, whose positions are specified according to EU numbering in the amino acids forming the Fc domain of an IgG3 antibody:

(k) F241A;

(l) D265A; or

(m) V264A. Each number represents the position of an amino acid residue in EU numbering; and the one-letter amino acid symbol before the number represents the amino acid residue before substitution, while the one-letter amino acid symbol after the number represents the amino acid residue after the substitution.

[0091] Furthermore, the preferred antigen-binding molecules or antibodies also include those comprising an Fc domain that has any one of the substitutions shown below, whose positions are specified according to EU numbering in the amino acids forming the Fc domain of an IgG4 antibody:

(n) L235A, G237A, and E318A;

(o) L235E; or

(p) F234A and L235A. Each number represents the position of an amino acid residue in EU numbering; and the one-letter amino acid symbol before the number represents the amino acid residue before substitution, while the one-letter amino acid symbol after the number represents the amino acid residue after the substitution.

[0092] The other preferred antigen-binding molecules or antibodies include, for example, those comprising an Fc domain in which any amino acid at position 233, 234, 235, 236, 237, 327, 330, or 331 (EU numbering) in the amino acids forming the Fc domain of an IgG1 antibody is substituted with an amino acid of the corresponding position in EU numbering in the corresponding IgG2 or IgG4.

[0093] The preferred antigen-binding molecules or antibodies also include, for example,

those comprising an Fc domain in which any one or more of the amino acids at positions 234, 235, and 297 (EU numbering) in the amino acids forming the Fc domain of an IgG1 antibody is substituted with other amino acids. The type of amino acid after substitution is not particularly limited; however, the antigen-binding molecules or antibodies comprising an Fc domain in which any one or more of the amino acids at positions 234, 235, and 297 are substituted with alanine are particularly preferred.

[0094] The preferred antigen-binding molecules or antibodies also include, for example, those comprising an Fc domain in which an amino acid at position 265 (EU numbering) in the amino acids forming the Fc domain of an IgG1 antibody is substituted with another amino acid. The type of amino acid after substitution is not particularly limited; however, antigen-binding molecules or antibodies comprising an Fc domain in which an amino acid at position 265 is substituted with alanine are particularly preferred.

[0095] 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).

[0096] 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).

[0097] 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.

[0098] Isoelectric point (pI)

Unless otherwise specified and unless there are inconsistencies in the context, it is understood that the isoelectric point (pI) may be either a theoretical or an experimentally determined isoelectric point, and it is also referred to as "pI". The pI value can be determined experimentally, for example, by isoelectric focusing electrophoresis. Meanwhile, the theoretical pI value can be calculated using gene and amino acid sequence analysis software (Genetyx, etc.).

[0099] Cancer

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth/proliferation. Examples of cancer include, but are not limited to, carcinoma, lymphoma (e.g., Hodgkin's and non-Hodgkin's lymphoma), blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma, leukemia and other lymphoproliferative disorders, and various types of head and neck cancer.

[0100] 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.

[0101] Ovarian cancer

"Ovarian cancer" refers to a heterogeneous group of malignant tumors derived from the ovary. Approximately 90% of malignant ovarian tumors are epithelial in origin; the remainder are germ cell and stromal tumors. Epithelial ovarian tumors are classified into the following histological subtypes: serous adenocarcinomas (constituting about

50% of epithelial ovarian tumors); endometrioid adenocarcinomas (~20%); mucinous adenocarcinomas (~10%); clear cell carcinomas (~5-10%); Brenner (transitional cell) tumors (relatively uncommon). The prognosis for ovarian cancer, which is the sixth most common cancer in women, is usually poor, with five year survival rates ranging from 5-30%. For reviews of ovarian cancer, see Fox et al. (2002) "Pathology of epithelial ovarian cancer," in Ovarian Cancer ch. 9 (Jacobs et al., eds., Oxford University Press, New York); Morin et al. (2001) "Ovarian Cancer," in Encyclopedic Reference of Cancer, pp.654-656 (Schwab, ed., Springer-Verlag, New York). The present disclosure contemplates methods of diagnosing or treating any of the epithelial ovarian tumor subtypes described above, and in particular, the serous adenocarcinoma subtype.

[0102] Gastric cancer

The term "gastric cancer", or "gastric tumor", or "stomach tumor", or "stomach cancer" refers to any tumor or cancer of the stomach, including, e.g., adenocarcinomas (such as diffuse type and intestinal type), and less prevalent forms such as lymphomas, leiomyosarcomas, and squamous cell carcinomas.

[0103] 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.

[0104] 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.

[0105] 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.

[0106] 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.

[0107] Antibody of the disclosure

In one aspect, the disclosure provides an isolated antibody that comprises: a first heavy chain variable region comprising HVR-H1, HVR-H2 and HVR-H3 amino acid sequences of SEQ ID NOs: 113, 117, and 118, respectively; a first light chain variable region comprising HVR-L1, HVR-L2 and HVR-L3 amino acid sequences of SEQ ID NOs: 119, 120, and 121, respectively; a second heavy chain variable region comprising HVR-H1, HVR-H2 and HVR-H3 amino acid sequences of SEQ ID NOs: 122, 123, and 124, respectively; and a second light chain variable region comprising HVR-L1, HVR-L2 and HVR-L3 amino acid sequences of SEQ ID NOs: 114, 115, and 116, respectively.

[0108] In one aspect, the disclosure provides an isolated antibody that comprises: a first heavy chain variable region comprising amino acid sequence selected from SEQ ID NOs: 85, 1, 33 and 84; and a first light chain variable region comprising amino acid sequence selected from SEQ ID NOs: 87, 2, 43, 86 and 88; a second heavy chain variable region comprising amino acid sequence selected from SEQ ID NOs: 60 and 70; and a second light chain variable region comprising amino acid sequence selected from SEQ ID NOs: 61 and 71.

[0109] In certain embodiments, the first heavy chain variable region is linked to a first CH1 domain shown in SEQ ID NO: 95, the first light chain variable region is linked to a first CL domain shown in SEQ ID NO: 63, the second heavy chain variable region is linked to a second CH1 domain shown in SEQ ID NO: 97, and the second light chain variable region is linked to a second CL domain shown in SEQ ID NO: 62.

[0110] In certain embodiments, the antibody further comprises any one of the Fc region combinations selected from:

(1) a first Fc region shown in SEQ ID NO: 72 and a second Fc region shown in SEQ ID NO: 73;

(2) a first Fc region shown in SEQ ID NO: 74 and a second Fc region shown in SEQ ID NO: 75;

(3) a first Fc region shown in SEQ ID NO: 76 and a second Fc region shown in SEQ ID NO: 77; and

(4) a first Fc region shown in SEQ ID NO: 78 and a second Fc region shown in SEQ ID NO: 79.

The first Fc region could be in the same polypeptide chain with the first heavy chain variable region, and the second Fc region could be in the same polypeptide chain with

the second heavy chain variable region. In certain embodiments, the C-terminal lysine (Lys447) or glycine-lysine (residues 446-447) of the Fc region may not be present.

[0111] In certain embodiments, the first heavy chain variable region and the first light chain variable region form a first antigen-binding site, and the second heavy chain variable region and the second light chain variable region form a second antigen-binding site.

[0112] In one aspect, the disclosure provides an isolated antibody that comprises: a first heavy chain comprising amino acid sequence selected from SEQ ID NOs: 104, 105, 106, and 107 and a first light chain comprising amino acid sequence shown in SEQ ID NO: 112; a second heavy chain comprising amino acid sequence selected from SEQ ID NOs: 108, 109, 110, and 111 and a second light chain comprising amino acid sequence shown in SEQ ID NO: 98. In certain embodiments, the C-terminal lysine (Lys447) or glycine-lysine (residues 446-447) of the heavy chain may not be present.

The variable regions of the first heavy chain and the first light chain form a first antigen-binding site, and the variable regions of the second heavy chain and the second light chain form a second antigen-binding site.

[0113] In certain embodiments, the first antigen-binding site has binding activity towards CLDN6. In certain embodiments, the first antigen-binding site has binding activity towards human CLDN6. In certain embodiments, the first antigen-binding site has binding activity towards human CLDN6 as defined in SEQ ID NO: 125 or 126. In certain embodiments, the first antigen-binding site does not substantially bind to human CLDN9. In certain embodiments, the first antigen-binding site does not substantially bind to human CLDN4. In certain embodiments, the first antigen-binding site does not substantially bind to human CLDN3. In certain embodiments, the first antigen-binding site does not substantially bind to a CLDN6 mutant as defined in SEQ ID NO: 134.

[0114] In certain embodiments, the second antigen-binding site has binding activity towards CD3. In certain embodiments, the second antigen-binding site has binding activity towards CD3 epsilon chain. In certain embodiments, the second antigen-binding site has binding activity towards human CD3 as defined in SEQ ID NO: 142.

[0115] Antigen-binding molecule of the disclosure

In one aspect, the disclosure provides an antigen-binding molecule that comprises a heavy chain variable region comprising:

(a1) HVR-H1, HVR-H2, and HVR-H3 amino acid sequences shown in SEQ ID NOs: 113, 117, and 118, respectively; or

(a2) HVR-H1, HVR-H2, and HVR-H3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83, 84, 57 and 85.

[0116] In one aspect, the disclosure provides an antigen-binding molecule that comprises a

light chain variable region comprising

(b1) HVR-L1, HVR-L2, and HVR-L3 amino acid sequences shown in SEQ ID NOs: 119, 120, and 121, respectively; or

(b2) HVR-L1, HVR-L2, and HVR-L3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 92, 41, 42, 43, 46, 51, 52, 53, 54, 82, 86, 88 and 58.

[0117] In one aspect, the disclosure provides an antigen-binding molecule that comprises a heavy chain variable region and a light chain variable region, wherein:

the heavy chain variable region comprising any one of the HVR combinations selected from:

(a1) HVR-H1, HVR-H2, and HVR-H3 amino acid sequences shown in SEQ ID NOs: 113, 117, and 118, respectively; and

(a2) HVR-H1, HVR-H2, and HVR-H3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83, 84, 57 and 85;

and the light chain variable region comprising any one of the HVR combinations selected from:

(b1) HVR-L1, HVR-L2, and HVR-L3 amino acid sequences shown in SEQ ID NOs: 119, 120, and 121, respectively; and

(b2) HVR-L1, HVR-L2, and HVR-L3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 92, 41, 42, 43, 46, 51, 52, 53, 54, 82, 86, 88 and 58.

[0118] In certain embodiments, the heavy chain variable region and light chain variable region of the antigen-binding molecule comprise mouse, rabbit, humanized or human frameworks.

[0119] In one aspect, the disclosure provides an antigen-binding molecule that comprises a heavy chain variable region comprising amino acid sequence selected from SEQ ID NOs: 85, 1, 33, 84, 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83 and 57, and a light chain variable region comprising amino acid sequence selected from SEQ ID NOs: 87, 2, 43, 86, 88, 92, 41, 42, 46, 51, 52, 53, 54, 82 and 58.

[0120] One heavy chain variable region and one light chain variable region of the antigen-binding molecule form an antigen-binding site, which has binding activity towards CLDN6. In certain embodiments, the antigen-binding molecule of the disclosure has binding activity towards human CLDN6 as defined in SEQ ID NO: 125 or 126.

[0121] In certain embodiments, the antigen-binding molecule of the disclosure does not substantially bind to human CLDN9. In certain embodiments, the antigen-binding molecule of the disclosure does not substantially bind to human CLDN4. In certain embodiments, the antigen-binding molecule of the disclosure does not substantially

bind to human CLDN3. In certain embodiments, the antigen-binding molecule of the disclosure does not substantially bind to a CLDN6 mutant as defined in SEQ ID NO: 134.

[0122] Antigen-binding molecule of the disclosure is able to be used in combination with various known medical use technologies. In certain embodiments, the antigen-binding molecules of the disclosure could be used to make chimeric antigen binding receptor (CAR) and T-cell comprising chimeric antigen binding receptor (CAR-T). As an example of the method for making CAR, CLDN6-binding site of the antigen-binding molecules of the disclosure is linked with the extracellular domain of T cell receptor (TCR), and the costimulatory signaling domain such as CD28 is linked to the intra-cellular domain of the TCR. As an example of the method for making CAR-T, CAR is introduced into effector cells such as T cell using gene modification technology.

[0123] In one aspect, the disclosure provides a chimeric antigen binding receptor that comprises a heavy chain variable region comprising:

(a1) HVR-H1, HVR-H2, and HVR-H3 amino acid sequences shown in SEQ ID NOs: 113, 117, and 118, respectively; or

(a2) HVR-H1, HVR-H2, and HVR-H3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83, 84, 57 and 85.

[0124] In one aspect, the disclosure provides a chimeric antigen binding receptor that comprises a light chain variable region comprising

(b1) HVR-L1, HVR-L2, and HVR-L3 amino acid sequences shown in SEQ ID NOs: 119, 120, and 121, respectively; or

(b2) HVR-L1, HVR-L2, and HVR-L3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 92, 41, 42, 43, 46, 51, 52, 53, 54, 82, 86, 88 and 58.

[0125] In one aspect, the disclosure provides a chimeric antigen binding receptor that comprises a heavy chain variable region and a light chain variable region, wherein:

the heavy chain variable region comprising any one of the HVR combinations selected from:

(a1) HVR-H1, HVR-H2, and HVR-H3 amino acid sequences shown in SEQ ID NOs: 113, 117, and 118, respectively; and

(a2) HVR-H1, HVR-H2, and HVR-H3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83, 84, 57 and 85;

and the light chain variable region comprising any one of the HVR combinations selected from:

(b1) HVR-L1, HVR-L2, and HVR-L3 amino acid sequences shown in SEQ ID NOs:

119, 120, and 121, respectively; and

(b2) HVR-L1, HVR-L2, and HVR-L3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 92, 41, 42, 43, 46, 51, 52, 53, 54, 82, 86, 88 and 58.

[0126] In one aspect, the disclosure provides a chimeric antigen binding receptor that comprises a heavy chain variable region comprising amino acid sequence selected from SEQ ID NOs: 85, 1, 33, 84, 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83 and 57, and a light chain variable region comprising amino acid sequence selected from SEQ ID NOs: 87, 2, 43, 86, 88, 92, 41, 42, 46, 51, 52, 53, 54, 82 and 58.

[0127] In certain embodiments, the antigen-binding molecule of the disclosure is an antigen-binding molecule that comprises a plurality of antigen-binding sites.

[0128] In certain embodiments, the antigen-binding molecule of the disclosure comprises a first antigen-binding site that has binding activity towards CLDN6, and a second antigen-binding site that has binding activity towards T-cell receptor complex, wherein the first antigen-binding site comprises:

(a) a heavy chain variable region comprising any one of the HVR combinations selected from:

(a1) HVR-H1, HVR-H2, and HVR-H3 amino acid sequences shown in SEQ ID NOs: 113, 117, and 118, respectively; and

(a2) HVR-H1, HVR-H2, and HVR-H3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83, 84, 57 and 85;

and

(b) a light chain variable region comprising any one of the HVR combinations selected from:

(b1) HVR-L1, HVR-L2, and HVR-L3 amino acid sequences shown in SEQ ID NOs: 119, 120, and 121, respectively; and

(b2) HVR-L1, HVR-L2, and HVR-L3 same with those of any one of the heavy chain variable regions shown in SEQ ID NOs: 92, 41, 42, 43, 46, 51, 52, 53, 54, 82, 86, 88 and 58.

[0129] In certain embodiments, the heavy chain variable region and the light chain variable region that forms the first antigen-binding site of the antigen-binding molecule of the disclosure further comprises mouse, rabbit, humanized or human frameworks.

[0130] In certain embodiments, the antigen-binding molecule of the disclosure comprises a first antigen-binding site that has binding activity towards CLDN6, and a second antigen-binding site that has binding activity towards T-cell receptor complex,

wherein the first antigen-binding site comprises a heavy chain variable region

comprising amino acid sequence selected from SEQ ID NOs: 85, 1, 33, 84, 91, 34, 35, 36, 37, 38, 39, 40, 44, 45, 47, 48, 49, 50, 55, 56, 64, 65, 83 and 57 and a light chain variable region comprising amino acid sequence selected from SEQ ID NOs: 87, 2, 43, 86, 88, 92, 41, 42, 46, 51, 52, 53, 54, 82 and 58.

[0131] In certain embodiments, the first antigen-binding site of the antigen-binding molecule of the disclosure has binding activity towards human CLDN6. In certain embodiments, the first antigen-binding site of the antigen-binding molecule of the disclosure has binding activity towards human CLDN6 as defined in SEQ ID NO: 125 or 126. In certain embodiments, the first antigen-binding site of the antigen-binding molecule of the disclosure does not substantially bind to human CLDN9. In certain embodiments, the first antigen-binding site of the antigen-binding molecule of the disclosure does not substantially bind to human CLDN4. In certain embodiments, the first antigen-binding site of the antigen-binding molecule of the disclosure does not substantially bind to human CLDN3. In certain embodiments, the first antigen-binding site of the antigen-binding molecule of the disclosure does not substantially bind to a CLDN6 mutant as defined in SEQ ID NO: 134.

[0132] In certain embodiments, the first antigen-binding site of the antigen-binding molecule of the disclosure is included in a single-chain Fv (scFv), Fv or Fab.

[0133] In certain embodiments, the second antigen-binding site of the antigen-binding molecule of the disclosure has binding activity towards CD3. In certain embodiments, the second antigen-binding site of the antigen-binding molecule of the disclosure has binding activity towards CD3 epsilon chain. In certain embodiments, the second antigen-binding site of the antigen-binding molecule of the disclosure has binding activity towards T-cell receptor.

[0134] In certain embodiment, the second antigen-binding site of the antigen-binding molecule of the disclosure is formed by a heavy chain variable region comprising HVR-H1, HVR-H2 and HVR-H3 amino acid sequences of SEQ ID NOs: 122, 123, and 124, respectively; and a light chain variable region comprising HVR-L1, HVR-L2 and HVR-L3 amino acid sequences of SEQ ID NOs: 114, 115, and 116, respectively. In certain embodiment, the heavy chain variable region and the light chain variable region that form the second antigen-binding site of the antigen-binding molecule of the disclosure comprise mouse, rabbit, humanized or human frameworks.

[0135] In certain embodiment, the second antigen-binding site of the antigen-binding molecule of the disclosure is formed by a heavy chain variable region comprising amino acid sequence selected from SEQ ID NOs: 60 and 70 and a light chain variable region comprising amino acid sequence selected from SEQ ID NOs: 61 and 71.

[0136] In certain embodiments, the second antigen-binding site of the antigen-binding molecule of the disclosure is included in a single-chain Fv (scFv), Fv or Fab.

[0137] In certain embodiments, the antigen-binding molecule of the disclosure further comprises an antibody Fc region. In certain embodiments, the Fc region comprised in the antigen-binding molecule of the disclosure is an Fc region constituting amino acids of SEQ ID NOs: 143, 144, 145, or 146 (IgG1 to IgG4). In certain embodiments, the Fc region comprised in the antigen-binding molecule of the disclosure is an Fc region with at least one amino acid mutation at any of the Fc region constituting amino acids of SEQ ID NOs: 143 to 146 (IgG1 to IgG4). In certain embodiments, the Fc region comprised in the antigen-binding molecule of the disclosure is an Fc region to which sugar-chain is attached, and the percentage of fucose-deficient sugar-chain-attached to the Fc region is higher than that to a native IgG Fc region, or the percentage of bisecting N-acetylglucosamine added to the Fc region is higher than that to a native IgG Fc region.

[0138] In certain embodiments, especially in some embodiments that the antigen-binding molecule comprises a first antigen-binding site that has binding activity towards CLDN6 and a second antigen-binding site that has binding activity towards T-cell receptor complex, the Fc region comprised in the antigen-binding molecule is an Fc region with reduced binding activity towards an Fc gamma receptor.

[0139] In certain embodiments, the Fc region comprised in the antigen-binding molecule is an Fc region with mutation of at least one amino acid selected from the following amino acid positions specified by EU numbering: position 220, position 226, position 229, position 231, position 232, position 233, position 234, position 235, position 236, position 237, position 238, position 239, position 240, position 264, position 265, position 266, position 267, position 269, position 270, position 295, position 296, position 297, position 298, position 299, position 300, position 325, position 327, position 328, position 329, position 330, position 331, and position 332.

[0140] In certain embodiments, the Fc region comprised in the antigen-binding molecule is an Fc region comprising at least one amino acid selected from the following amino acids specified by EU numbering: Arg at amino acid position 234, Ala or Arg at amino acid position 235, Lys at amino acid position 239, and Ala at amino acid position 297.

[0141] In certain embodiments, the heavy chain variable domain that forms the first antigen-binding site is linked to the Fc region via a first CH1 domain, the light chain variable domain that forms the first antigen-binding site is linked to a first CL domain; and the heavy chain variable domain that forms the second antigen-binding site is linked to the Fc region via a second CH1 domain, the light chain variable domain that forms the second antigen-binding site is linked to a second CL domain. In certain embodiments, the electric charges of the CH1 and CL domains aforementioned are controlled so that the heavy chain variable region that forms the first antigen-binding domain assembles with the light chain variable region that forms the first antigen-binding domain, and/or

the heavy chain variable region that forms the second antigen-binding domain assembles with the light chain variable region that forms the second antigen-binding domain.

[0142] In certain embodiments, the antigen-binding molecule of the disclosure has cytotoxic activity. In certain embodiments, the cytotoxic activity of the antigen-binding molecule of the disclosure is ADCC or CDC. In certain embodiments, the cytotoxic activity of the antigen-binding molecule of the disclosure is T-cell-dependent cytotoxic activity.

[0143] In certain embodiments, the antigen-binding molecule of the disclosure has internalizing activity. In certain embodiments, the antigen-binding molecule of the disclosure is conjugated with a cytotoxic agent.

[0144] The present disclosure also provides immunoconjugates comprising an CLDN6 binding molecule, especially anti-CLDN6 antibody herein conjugated to one or more cytotoxic agents, such as chemotherapeutic agents or drugs, growth inhibitory agents, toxins (e.g., protein toxins, enzymatically active toxins of bacterial, fungal, plant, or animal origin, or fragments thereof), or radioactive isotopes.

[0145] In one embodiment, an immunoconjugate is an antibody-drug conjugate (ADC) in which an antibody is conjugated to one or more drugs, including but not limited to a maytansinoid (see U.S. Patent Nos. 5,208,020, 5,416,064 and European Patent EP 0 425 235 B1); an auristatin such as monomethylauristatin drug moieties DE and DF (MMAE and MMAF) (see U.S. Patent Nos. 5,635,483 and 5,780,588, and 7,498,298); a dolastatin; a calicheamicin or derivative thereof (see U.S. Patent Nos. 5,712,374, 5,714,586, 5,739,116, 5,767,285, 5,770,701, 5,770,710, 5,773,001, and 5,877,296; Hinman et al., Cancer Res. 53:3336-3342 (1993); and Lode et al., Cancer Res. 58:2925-2928 (1998)); an anthracycline such as daunomycin or doxorubicin (see Kratz et al., Current Med. Chem. 13:477-523 (2006); Jeffrey et al., Bioorganic & Med. Chem. Letters 16:358-362 (2006); Torgov et al., Bioconj. Chem. 16:717-721 (2005); Nagy et al., Proc. Natl. Acad. Sci. USA 97:829-834 (2000); Dubowchik et al., Bioorg. & Med. Chem. Letters 12:1529-1532 (2002); King et al., J. Med. Chem. 45:4336-4343 (2002); and U.S. Patent No. 6,630,579); methotrexate; vindesine; a taxane such as docetaxel, paclitaxel, larotaxel, tesetaxel, and ortataxel; a trichothecene; and CC1065.

[0146] In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to an enzymatically active toxin or fragment thereof, including but not limited to diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolacca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and the tricothecenes.

[0147] In another embodiment, an immunoconjugate comprises an antibody as described herein conjugated to a radioactive atom to form a radioconjugate. A variety of radioactive isotopes are available for the production of radioconjugates. Examples include ^{211}At, ^{131}I, ^{125}I, ^{90}Y, ^{186}Re, ^{188}Re, ^{153}Sm, ^{212}Bi, ^{32}P, ^{212}Pb and radioactive isotopes of Lu. When the radioconjugate is used for detection, it may comprise a radioactive atom for scintigraphic studies, for example Tc-99m or ^{123}I, or a spin label for nuclear magnetic resonance (NMR) imaging (also known as magnetic resonance imaging, MRI), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

[0148] Conjugates of an antibody and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta et al., Science 238:1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionuclide to the antibody. See WO94/11026. The linker may be a "cleavable linker" facilitating release of a cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, photolabile linker, dimethyl linker or disulfide-containing linker (Chari et al., Cancer Res. 52:127-131 (1992); U.S. Patent No. 5,208,020) may be used.

[0149] The immunoconjugates or ADCs herein expressly contemplate, but are not limited to such conjugates prepared with cross-linker reagents including, but not limited to, BMPS, EMCS, GMBS, HBVS, LC-SMCC, MBS, MPBH, SBAP, SIA, SIAB, SMCC, SMPB, SMPH, sulfo-EMCS, sulfo-GMBS, sulfo-KMUS, sulfo-MBS, sulfo-SIAB, sulfo-SMCC, and sulfo-SMPB, and SVSB (succinimidyl-(4-vinylsulfone)benzoate) which are commercially available (e.g., from Pierce Biotechnology, Inc., Rockford, IL., U.S.A).

[0150] In one aspect, the disclosure provides nucleic acid encoding an antibody or antigen-binding molecule described herein. 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 certain embodiments, nucleic acid encoding an antibody or antigen-binding molecule of the disclosure is able to be incorporated in vectors that are able to be ad-

ministered to a subject directly. It is also possible to administer cells which are genetically modified to express and secret an antibody or antigen-binding molecule of the disclosure to a subject, allowing continuous in vivo secretion of an antibody or antigen-binding molecule of the disclosure.

[0151] The disclosure also provides a method of producing an antibody or antigen-binding molecule described herein. In further embodiment, the method of producing an antibody or antigen-binding molecule described herein comprises culturing a host cell comprising nucleic acid that encodes the antigen or antigen-binding molecule. In further embodiments, the method of producing an antibody or antigen-binding molecule described herein comprises culturing a host cell comprising nucleic acid that encodes the antigen or antigen-binding molecule, and recovering the antibody or antigen-binding molecule from the culture of the hose cell.

[0152] Methods and assay

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 an anti-CLDN6 antibody 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).

[0153] 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).

[0154] 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.

[0155] 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).

[0156] 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.

[0157] 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).

[0158] 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).

[0159] 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.

[0160] Production and purification of bispecific antibodies

"Multispecific antigen-binding molecules" refers to antigen-binding molecules that bind specifically to more than one antigen. In certain embodiment, multispecific antigen-binding molecules described herein comprise two or more antigen-binding sites, and different antigen-binding sites are able to bind specifically to different antigens.

[0161] In certain embodiments, the antigen-binding molecule of the disclosure is bispecific IgG antibody. IgG-type bispecific antibodies can be secreted from a hybrid hybridoma (quadroma) produced by fusing two types of hybridomas that produce IgG antibodies (Milstein et al., Nature (1983) 305, 537-540).

[0162] Furthermore, IgG-type bispecific antibodies are secreted by introducing the genes of L chains and H chains constituting the two types of IgGs of interest, i.e., a total of four genes, into cells, and co-expressing them. However, the number of combinations of H and L chains of IgG that can be produced by these methods is theoretically ten combinations. Accordingly, it is difficult to purify an IgG comprising the desired combination of H and L chains from ten types of IgGs. Furthermore, theoretically, the amount of secretion of the IgG having the desired combination will decrease remarkably, and therefore large-scale culturing will be necessary, and production costs will increase further.

[0163] Therefore, 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.

[0164] 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 bispecific antibody association (WO2006/106905).

[0165] 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.

[0166] 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.

[0167] 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.

[0168] 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.

[0169] 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).

[0170] 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.

[0171] 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.

[0172] 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.

[0173] Other known techniques can also be used for the association of bispecific antibodies of the present invention. Fc region-containing polypeptides comprising different amino acids can be efficiently associated with each other by substituting an amino acid side chain present in one of the H-chain Fc regions of the antibody with a larger side chain (knob), and substituting an amino acid side chain present in the corresponding Fc region of the other H chain with a smaller side chain (hole) to allow placement of the knob within the hole (WO1996/027011; Ridgway JB et al., Protein Engineering (1996) 9, 617-621; Merchant A. M. et al. Nature Biotechnology (1998) 16, 677-681; and US20130336973).

[0174] In addition, other known techniques can also be used for formation of bispecific antibodies 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 bispecific antibodies of interest.

[0175] 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 bispecific antibodies composed of two types of light chains and one type of heavy chain as described in WO2012/023053; technologies for producing bispecific 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 bispecific antibodies.

[0176] Alternatively, even when a bispecific antibody of interest cannot be formed efficiently, a bispecific antibody of the present invention can be obtained by separating

and purifying the bispecific 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.

[0177] 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.

[0178] Measurement of T cell dependent cellular cytotoxicity

In the embodiment that the antigen-binding molecule binds to both CLDN6 and T cell receptor complex, 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 CLDN6-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 CLDN6 and which is not expressed in the cells, and T-cell receptor complex, 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.

[0179] 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.

[0180] 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.

[0181] 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. CLDN6-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.

[0182] Additionally or alternatively, for example, TDCC can also be measured by real-time cell growth inhibition assay. In this assay, target cells (e.g. CLDN6-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} x 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.

[0183] 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. CLDN6-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.

[0184] Methods and Compositions for Diagnostics and Detection

In certain embodiments, any of the antigen-binding molecules or antibodies provided herein is useful for detecting the presence of CLDN6 in a biological sample. The term "detecting" as used herein encompasses quantitative or qualitative detection. In certain embodiments, a biological sample comprises a cell or tissue, such as cancer tissues.

[0185] In one embodiment, an antigen-binding molecule or antibody for use in a method of diagnosis or detection is provided. In a further aspect, a method of detecting the presence of CLDN6 in a biological sample is provided. In certain embodiments, the method comprises contacting the biological sample with an antigen-binding molecule or antibody as described herein under conditions permissive for binding of the antigen-binding molecule or antibody to CLDN6, and detecting whether a complex is formed between the antigen-binding molecule or antibody and CLDN6. Such method may be an in vitro or in vivo method. In one embodiment, an antigen-binding molecule or antibody is used to select subjects eligible for therapy with antigen-binding molecules or antibodies as described herein, e.g. where CLDN6 is a biomarker for selection of patients.

[0186] Exemplary disorders that may be diagnosed using an antibody of the invention include cancer, especially ovarian tumor, non-small cell lung cancer, gastric cancer, and liver cancer.

[0187] In certain embodiments, labeled antigen-binding molecules or antibodies described herein are provided. Labels include, but are not limited to, labels or moieties that are

detected directly (such as fluorescent, chromophoric, electron-dense, chemiluminescent, and radioactive labels), as well as moieties, such as enzymes or ligands, that are detected indirectly, e.g., through an enzymatic reaction or molecular interaction. Exemplary labels include, but are not limited to, the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, beta-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, those coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels, stable free radicals, and the like.

[0188] 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 ovarian cancer, non-small cell lung cancer, gastric cancer, or liver cancer. In certain embodiments, the pharmaceutical composition of the disclosure is cell growth-suppressing agent. In certain embodiments, the pharmaceutical composition of the disclosure is anticancer agent.

[0189] 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.

[0190] 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. Pharma-

ceutically 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 dextrins; 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.

[0191] 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.

[0192] 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.

[0193] If necessary, the antigen-binding molecules or antibodies of the present disclosure may be encapsulated in microcapsules (microcapsules made from hydroxymethylcellulose, 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).

[0194] 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 cytotoxicity, 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.

[0195] 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 colorectal cancer or gastric 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

[0196] The present disclosure also provides methods for damaging cells expressing CLDN6 or for suppressing the cell growth by contacting the cells expressing CLDN6 with an antigen-binding molecule of the present disclosure that binds to CLDN6. Monoclonal antibodies that bind to CLDN6 are described above as an antigen-binding molecule of the present disclosure, which is included in the therapeutic agents for inducing cellular cytotoxicity, cell growth-suppressing agents, and anticancer agents of the present disclosure. Cells to which an antigen-binding molecule of the present disclosure binds are not particularly limited, as long as they express CLDN6. Specifically, in the present disclosure, the preferred cancer antigen-expressing cells include ovary cancer cells, prostate cancer cells, breast cancer cells, uterine cancer cells, liver cancer cells, lung cancer cells, pancreatic cancer cells, stomach cancer cells, urinary bladder cancer cells, and colon cancer cells.

[0197] 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 CLDN6 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, isotonizing 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.

[0198] In another embodiment of the present disclosure, "contact" can also be carried out by administration to nonhuman animals transplanted with CLDN6-expressing cells in vivo or to animals having cancer cells expressing CLDN6 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. 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, isotonizing 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.

[0199] 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.

[0200] 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. 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.

[0201] It is understood that any of the above articles of manufacture may include an immunoconjugate of the invention in place of or in addition to an antigen-binding molecule or antibody.

[0202] All documents cited herein are incorporated herein by reference.

Examples

[0203] 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.

[0204] EXAMPLE 1. Stability of sc(Fv)$_2$ molecule

Regarding the stability of blinatumomab, blinatumomab was purified at 3.8 mg/mL in 20mM His, 150mM NaCl, pH6.0 in-house. High molecular weight (HMW) species ratio was determined to be 6.4% by SEC analysis. For the purpose of physicochemical tests, it was dialyzed against PBS, pH7.4 and the concentration was adjusted to

1mg/mL. After the dialysis, HMW ratio was turned to 42.6%. Generally, the HMW ratio of IgG antibodies do not change during a dialysis. Therefore, Blinatumomab has an aggregation tendency in solution and it was thought to be less stable compared to IgG antibodies. SEC analysis was conducted using SWXL G2000 column (TOSOH) with 50 mM sodium phosphate, 300 mM NaCl, pH7.0 as a running buffer. Detection was done by UV detector (280 nm). Chromatograms were analyzed using Empower 3 software (Waters).

[0205] EXAMPLE 2. Selection of T cell redirecting antibody which binds claudin-6 (CLDN6)

EXAMPLE 2-1. Generation of anti-CLDN6 T cell-redirecting antibodies

To overcome the limitation of sc(Fv)$_2$ format, we generated T cell redirecting bispecific antibodies targeting CLDN6 (anti-CLDN6/CD3 bispecific antibodies) in IgG format, which enable the antibody to gain a better stability and a longer serum half-life.

[0206] The variable regions of three anti-CLDN6 antibodies (AE3-20, AH05, CDA0013), the CLDN6-binding variable regions of 6PHU3, and the variable regions of one anti-CD3 antibody (TR01) were used to generate anti-CLDN6/CD3 bispecific antibodies. CDA0013 was obtained from B cells of rabbits which were immunized with CLDN6 expression vector.

[0207] The abovementioned variable regions were used to generate anti-CLDN6/CD3 bispecific antibodies using Fab arm exchange technique reported by Igawa et al. (WO 2016159213). The variable regions comprised in the anti-CLDN6/CD3 antibodies arm are shown in Table 1, and the generated bispecific antibodies contain a silent Fc with attenuated affinity for the Fc gamma receptor. Four anti-CLDN6/CD3 bispecific antibodies, AE3-20/TR01, AH05/TR01, CDA0013/TR01, and 6PHU3/TR01 were generated (Table 1).

[0208] (Table 1) Generated anti-CLDN6/CD3 bispecific antibodies.

Antibody Name	SEQ ID NO of			
	CLDN6-binding heavy chain variable region (VH)	CLDN6-binding light chain variable region (VL)	CD3-binding heavy chain variable region (VH)	CD3-binding light chain variable region (VL)
AE3-20/TR01	1	2	70	71
AH05/TR01	89	90		
CDA0013/TR01	91	92		
6PHU3/TR01	93	94		

[0209] EXAMPLE 2-2. T cell activation activity of the anti-CLDN6/CD3 bispecific antibodies using Jurkat reporter gene assay

T cell activation activity of the anti-CLDN6/CD3 bispecific antibodies generated in Example 2-1, in the presence of hCLDN6/BaF, hCLDN9/BaF or hCLDN6(Q156L)/BaF was examined by the method described in Reference Example 3. T cell activation activity of each antibody under the concentration of 0.001, 0.01, 0.1, 1, and 10 nM, respectively was determined.

[0210] The results (Fig. 1A, Fig. 1B) showed that all the anti-CLDN6/CD3 bispecific antibodies induced strong T cell activation in presence of hCLDN6/BaF. In the presence of hCLDN9/BaF, slight T cell activation was observed in 6PHU3/TR01 while no activation in other AE3-20/TR01, AH05/TR01, and CDA0013/TR01. This result suggests that all the anti-CLDN6/CD3 bispecific antibodies showed relatively high specificity towards CLDN6, although 6PHU3/TR01 slightly binds to human CLDN9 expressing cells.

[0211] Fig. 1A and Fig. 1C show that T cell activation of all the anti-CLDN6/CD3 bispecific antibodies was lower in the presence of hCLDN6(Q156L)/BaF, compared with their T cell activation in the presence of hCLDN6/BaF, suggesting that all the anti-CLDN6/CD3 bispecific antibodies bind to epitopes that include the amino acid residue at position 156 in human CLDN6.

[0212] EXAMPLE 2-3. Antigen binding specificity of the anti-CLDN6/CD3 bispecific antibodies towards CLDN family proteins

FreeStyle™ 293-F cells transiently expressed human and mouse CLDN3, 4, 6, and 9 were generated as below. Expressing vector of human and mouse CLDNs (including CLDN6, CLDN9, CLDN3, and CLDN4) were established by inserting synthesized cDNA coding corresponding sequences into mammalian expression vector pCXND3 (WO2008/156083).

[0213] Each vector was introduced into FreeStyle™ 293-F cells (Invitrogen) using 293fectin (Invitrogen). Cells were cultured 2-4 days, and collected by centrifugation. Collected cells were immediately resuspended in 0.1% BSA containing D-PBS(-) in concentration of 3 x 10^6 cells/mL.

[0214] Evaluated antibodies:

1) anti-CLDN6/CD3 bispecific antibodies: AE3-20/TR01, AH05/TR01, CDA0013/TR01, 6PHU3/TR01;

2) Bispecific antibody of Anti- keyhole limpet hemocyan (KLH) antibody (VH shown in SEQ ID NO: 68, VL shown in SEQ ID NO: 69) paired with human CD3 antibody (VH shown in SEQ ID NO: 70, VL shown in SEQ ID NO: 71) by Fab arm exchange: KLH/TR01 (as a negative control);

3) Commercially available anti-CLDN bivalent antibodies (purchased from R&D systems): mouse anti-human CLDN6 antibody (MAB3656), mouse anti-human CLDN3 antibody (MAB4620), mouse anti-human CLDN4 antibody (MAB4219).

[0215] Antibodies were mixed with FreeStyle™ 293-F cells transiently expressed human and mouse CLDN3, 4, 6, and 9 at final concentration of 10 micro g/mL. After 1 hour of incubation, cells were washed by 0.1% BSA containing D-PBS(-), and incubated in 5 micro g/mL of either Goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Invitrogen, A21445) or Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Invitrogen, A21236), for 1 hour. Cells were then washed with 0.1% BSA containing D-PBS(-), resuspended in 5 micro L of 0.1% BSA containing D-PBS(-), and analyzed by flow cytometer (iQue Screener, intellicyte).

[0216] As shown in Figs. 2-1 and 2-2, strong binding towards human CLDN6 was observed in all the anti-CLDN6/CD3 bispecific antibodies. Binding to mouse CLDN6 was also observed in all the anti-CLDN6/CD3 bispecific antibodies, although they were weaker than that towards human CLDN6. All the anti-CLDN6/CD3 bispecific antibodies showed slight or no binding towards human CLDN9, human CLDN4, human CLDN3, mouse CLDN9, mouse CLDN4, or mouse CLDN3. Especially, CDA0013/TR01 showed the best specificity towards CLDN6.

[0217] EXAMPLE 2-4. In vivo efficacy analysis of the anti-CLDN6/CD3 bispecific antibodies

The in vivo anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibodies was evaluated using tumor bearing mice model. The human cancer cell lines expressing human CLDN6 (HuH-7 (Health Science Research Resources Bank), OV-90 (ATCC) or OVCAR-3 (ATCC)) was transplanted subcutaneously into NOD/ShiJic-scid mice, and human PBMC was injected into the mice (so-called T cell injection model). Tumor bearing mice were randomized to treatment groups to receive an administration of the antibody, or vehicle as a control.

[0218] More specifically, the following test was conducted in the efficacy test using HuH-7 or OV-90 or OVCAR-3 T cell injection model. Expansion of T cells was performed using human PBMC (AllCells) and T Cell Activation/Expansion Kit (Miltenyi Biotec). 1 x 10^7 cells of HuH-7, 5 x 10^6 cells of OV-90 or 1 x 10^7 cells of OVCAR-3 and Matrigel Basement Membrane Matrix (BD) were mixed and transplanted subcutaneously into the lateral region of the NOD/ShiJic-scid mice (CLEA Japan, Female, 6 to 7 weeks-old). The day of transplantation was defined as day 0. After randomization and grouping according to tumor size and body weight, anti-asialo GM1 antibody (FUJIFILM Wako Pure Chemical Corporation) was administered intraperitoneally at 0.2 mg / mouse. On the next day, the T cells were collected from the expansion culture, and used for implantation at 3 x 10^7 cells/mouse intraperitoneally. Approximately four hours after T cell transplantation, the antibodies were administered intravenously. The antibodies were administrated only once. The length (L) and width (W) of the tumor

mass and body weight of each mouse were measured, and tumor volume (TV) was calculated as: TV = (L x W x W) / 2.

[0219] Fig. 3 shows the in vivo anti-tumor efficacy of AE3-20/TR01, AH05/TR01, and 6PHU3/TR01 in OV-90 and HuH-7 T cell injection models. Infusion of these antibodies at 5mg/kg retarded tumor growth strongly. Especially, in HuH-7 T cell injection model, AE3-20/TR01 showed the best efficacy compared to other two antibodies, AH05/TR01 and 6PHU3/TR01 (Fig. 3a).

[0220] Toxicity and pharmacokinetics (PK) analysis of the anti-CLDN6/CD3 bispecific antibodies was conducted using same mouse models. Although AE3-20/TR01, AH05/TR01, and 6PHU3/TR01 all showed anti-tumor efficacy in OV-90 and HuH-7 T-cell injection models, drastic body weight loss was observed in mice treated with AH05/TR01 and 6PHU3/TR01. No body weight change was observed in mice treated with AE3-20/TR01 (Fig. 4). In addition, the mice with body weight loss showed worsening general condition and yellow discoloration of the skin and plasma.

[0221] After euthanasia, plasma was collected and blood biochemical test was conducted. Plasma level of hepatotoxicity markers, including ALT, ALP, GGT, GLDH, TBIL, and TBA were determined (TBA-120FR, Canon medical system). In mice treated with AH05/TR01 and 6PHU3/TR01, liver parameters were significantly elevated (Fig. 5). These data indicated that AH05/TR01 and 6PHU3/TR01 cause hepatotoxicity in mice.

[0222] PK of anti-CLDN6/CD3 bispecific antibodies was evaluated as follows. Antibodies were injected intravenously into mice at a dose of 5 mg/kg. Blood was collected from three mice on day 3, 7, 13 after the antibody injection in HuH-7 T-cell injection model and on day 3, 6, 14 after the antibody injection in OV-90 T-cell injection model. The plasma samples in heparin was obtained by centrifuging at 10,000 x g for 5 minutes at 4 degrees C. The concentration of the antibodies in mouse plasma was measured by ELISA as described below. Anti-human IgG antibody (Sigma) was dispensed onto a Nunc-ImmunoPlate MaxiSorp (Thermo Fisher) and kept for 1 hour at room temperature to prepare anti-human IgG immobilized plates. Sample antibodies in a concentrations of 8, 4, 2, 1, 0.5, 0.25 or 0.125 ng/mL were used for calibration, and mouse plasma samples were prepared by dilution with 400-fold or more for examination. Each sample was dispensed into the anti-human IgG immobilized plates, and kept at room temperature for 1 hour. Then, biotinylated anti-Fc specific antibody (CHUGAI) was added to react for 1 hour at room temperature. Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) was added to react for 1 hour at room temperature, and chromogenic reaction was carried out using ABTS (Roche) as a substrate. Then, the absorbance at 405 nm (650 nm as reference) was measured by a microplate reader. The antibody concentration in mouse plasma was calculated from the absorbance of the calibration curve using the analytical software SOFTmax PRO

(Molecular Devices).

[0223] The temporal change of plasma concentration of AE3-20/TR01, AH05/TR01, and 6PHU3/TR01 after intravenous administration is shown in Fig. 6. The half-life ($T_{1/2}$) of AE3-20/TR01, AH05/TR01, and 6PHU3/TR01 was approximately 3.44 to 4.79 days in the plasma of HuH-7 T-cell injection model and approximately 2.77 to 3.22 days in OV-90 T-cell injection model.

[0224] Most notably, plasma half-life of 6PHU3/TR01 was prolonged to 3.44 days in HuH-7 model and 2.77 days in OV-90 model, compared to those of bispecific sc$(Fv)_2$ 6PHU3 described in previous report (6). This prolongation is most likely to be due to antibody conversion from bispecific sc$(Fv)_2$ to IgG format. However, 6PHU3/TR01 led to hepatotoxicity in mice, as confirmed by the significant decrease in body weight and increase in liver markers after infusion (Figs. 4 and 5). These findings suggest that prolongation of plasma half-life of anti-CLDN6/CD3 bispecific antibodies might led to liver toxicity.

[0225] In vivo anti-tumor efficacy and safety of AE3-20/TR01 and CDA0013/TR01 at a dose of 5 mg/kg was also evaluated using OVCAR-3 T cell injection mouse model. Experiment was conducted with same procedure as described above.

[0226] As shown in Fig. 7, treatment with AE3-20/TR01 led to significant tumor volume reduction, while treatment with CDA0013/TR01 showed moderate efficacy in OVCAR-3 bearing mice. After antibody treatment, drastic body weight loss was barely detectable as a whole, but obvious increases in liver toxicity markers were found in CDA0013/TR01 treatment group (Fig. 8). In fact, one out of five mice in CDA0013/TR01 treatment group showed weak body weight reduction.

[0227] PK of AE3-20/TR01 and CDA0013/TR01 were calculated using animals with intravenous injection at 5 mg/kg as follows. The plasma samples in heparin were prepared from blood collected from three animals on day 3, 7, 14 post injection, then subjected to measurement of the concentration of antibodies by ELISA as described previously in this Example.

[0228] As shown in Fig. 9, the PK feature of AE3-20/TR01 and CDA0013/TR01 was comparable. The plasma half-life ($T_{1/2}$) of AE3-20/TR01 and CDA0013/TR01 were 3.60 and 3.98 days and the area under the plasma concentration-time curve from zero to infinity (AUC_{inf}) was 138 and 161 day*micro g/mL, respectively.

[0229] According to the result of this Example, some anti-CLDN6/CD3 bispecific antibodies in IgG format tend to have a potential to cause liver injury, although all the antibodies showed relatively high specificity towards CLDN6 and similar pharmacokinetics profile. Among the tested anti-CLDN6/CD3 bispecific antibodies, AE3-20/TR01 was found to be the only anti-CLDN6/CD3 bispecific antibody which showed both efficacy and good safety profile in various tumor bearing mouse models.

[0230] EXAMPLE 3. Humanization of CLDN6 binding variable regions for anti-CLDN6/CD3 bispecific antibody

Although AE3-20/TR01 showed both efficacy and good safety profile in various tumor bearing mouse models, the CLDN6 binding variable regions of AE3-20/TR01 are mouse sequences, it is not applicable for administration of this antibody to patients due to its high immunogenicity. Humanization of the CLDN6 binding variable regions of AE3-20/TR01 was carried out. The CD3 binding variable regions of AE3-20/TR01 are humanized sequences.

[0231] Amino acid residues of the CLDN6 binding VH of AE3-20/TR01(AE3.20H, SEQ ID NO: 1) and the CLDN6-binding VL of AE3-20/TR01 (AE3.20L, SEQ ID NO: 2) are numbered according to Kabat (Kabat et al., Sequence of proteins of immunological interest, 5th Ed., Public Health Service, National Institutes of Health, Bethesda, MD (1991)).

[0232] 49 kinds of VHs were designed by substituting the frameworks of AE3.20H with human germline frameworks (SEQ ID NO: 3, 4, 5, 6, 7, 8 or 9 as FR1, SEQ ID NO: 10 as FR2, SEQ ID NO: 11, 12, 13, 14, 15, 16 or 17 as FR3, SEQ ID NO: 18 as FR4). 16 kinds of VLs were designed by substituting the frameworks of AE3.20L with human germline frameworks (SEQ ID NO: 19, 20, 21 or 22 as FR1, SEQ ID NO: 23 as FR2, SEQ ID NO: 24, 25, 26, or 27 as FR3, SEQ ID NO: 28 as FR4). The polynucleotides encoding AE3.20H and the designed VHs were cloned into expression vectors containing polynucleotides encoding heavy chain constant region SG1 (the amino acid sequence is shown in SEQ ID NO: 80), respectively. The polynucleotides encoding AE3.20L and the designed VLs were cloned into expression vectors containing polynucleotides encoding light chain constant region SK1 (the amino acid sequence is shown in SEQ ID NO: 81), respectively. Heavy chains comprising AE3.20H and the designed VHs were transiently expressed with the light chain comprising AE3.20L respectively, and light chains comprising the designed VLs were transiently expressed with the heavy chain comprising AE3.20H respectively, in Expi 293 cells.

[0233] Binding of the antibodies comprising designed VH and AE3.20L and the antibodies comprising designed VL and AE3.20H to hCLDN6/BaF was determined by FCM analysis. Binding activities of each antibody is represented by the Mean Fluorescence Intensity values (MFI). Antibodies were incubated with hCLDN6/BaF for 30 minutes at 4 degrees C and washed with HEPES-BSA washing buffer (Nacalai Tesque, Cat. L3P8406). Goat $F(ab')_2$ anti-Human IgG, Mouse ads-PE (Southern Biotech, Cat. 2043-09) secondary antibody was then added and incubated for 30 minutes in the dark at 4 degrees C and subsequently washed. Data acquisition was performed using LSR Fortessa X-20 (BD Biosciences) and analyzed with FlowJo software (Tree Star).

[0234] Among the 49 kinds of designed VHs, only two kinds of VH (shown in Table 2)

were able to improve the binding activity of the antibody towards human CLDN6, compared with the antibodies comprises AE3.20H. None of the 16 kinds of designed VLs showed contribute to the binding activity of the antibody towards human CLDN6 as much as AE3.20L. It seems to be difficult to humanize AE3.20L without compromising the CLDN6-binding activity of the antibody.

[0235] (Table 2)

Name of VH	CDRs	SEQ ID NO: of			
		FR1	FR2	FR3	FR4
22H	Same with those of AE3.20H	3	10	14	18
25H		6	10	14	18

[0236] To achieve enough binding activity towards human CLDN6, CLDN6-binding VLs were rationally designed (Human germline framework sequences which are similar to the corresponding framework of AE3.20L were selected, and introduction of back mutations in frameworks were also tried). The designed VLs (17L, 18L, 19L, and 20L) have same CDR sequences with AE3.20L, and the sequences of their frameworks are shown in Table 3. Back mutations were adopted in the frameworks of 18L, 19L and 20L, which are also shown in Table 3. Bivalent IgG antibodies comprising the VH 25H and VL selected from 17L, 18L, 19L and 20L were transiently expressed in Expi 293 cells. Fig. 10 shows the binding of various antibodies towards hCLDN6/BaF determined by FCM analysis. Binding activities of each antibody is represented by the MFI. The names on the horizontal axis in Fig. 10 show the VH and VL comprised in each antibody.

[0237] All the antibodies comprising designed VL (17L, 18L, 19L, and 20L) showed weaker binding activity towards human CLDN6 than the antibody comprises AE3.20L as VL, which also indicated the difficulty to humanize AE3.20L without compromising the CLDN6-binding activity. However, antibody comprises 20L, which harbors the G66R mutation, showed stronger binding activity to human CLDN6 compared with the antibody comprises 17L, which does not harbor the G66R mutation, suggesting that the substitution from Gly at amino acid position 66 to Arg in the light chain frameworks would help improving the antibody's binding activity to human CLDN6.

[0238] (Table 3)

Name of VL	CDRs	SEQ ID NO: of				Amino acid substitution compared with 17L (back mutation)
		FR1	FR2	FR3	FR4	
17L	Same with those of AE3.20L	19	23	24	29	-
18L		19	30	24	29	K45Q
19L		19	31	24	29	A43S, K45Q
20L		19	23	32	29	G66R

[0239] EXAMPLE 4. Affinity maturation of anti-CLDN6 humanized antibody

To improve the binding affinity of the humanized CLDN6 binding variable regions, exhaustive single or multiple mutations were introduced into the CDRs of AE3.20H and AE3.20L, and SEQ ID NO: 6 was used as heavy chain FR1, SEQ ID NO: 10 was used as heavy chain FR2, SEQ ID NO: 14 was used as heavy chain FR3, SEQ ID NO: 18 was used as heavy chain FR4, SEQ ID NO: 22 or 19 was used as light chain FR1, SEQ ID NO: 23 was used as light chain FR2, SEQ ID NO: 24 or 32 was used as light chain FR3, SEQ ID NO: 28 or 29 was used as light chain FR4, to make various antibody variants. The antibody variants were expressed Expi293 (Invitrogen) and purified by Protein A purification.

[0240] By checking CLDN6 binding activity of the antibody variants, it was suggested that the amino acid substitutions shown in Table 4 and Table 5 might be helpful for improving the binding activity towards human CLDN6.

[0241] (Table 4)

VH name	Amino acids substitution in CDRs compared with AE3.20H	Frameworks	SEQ ID NO
25H0021	Y32L	Same with those of 25H	35
25H0024	Y32V		36
25H0042	T33V		37
25H0176	G53K		38
25H0322	D61F		39
25H0562	Y102M		40
25H0581	Y32L, D61F		34

[0242] (Table 5)

VL name	Amino acids substitution in CDRs compared with AE3.20L	Frameworks	SEQ ID NO
20L0072	E27Y	Same with those of 20L	42
20L0532	E27Y, A50Y		43

[0243] The VHs descried in Table 4 and the VLs described in Table 5, together with a variable regions shows binding activity towards human CD3 (VH SEQ ID NO: 70; VL SEQ ID NO: 71) were used to generate anti-CLDN6/CD3 bispecific antibodies using Fab arm exchange technique reported by Igawa et al. (WO 2016159213). The variable regions comprised in the anti-CLDN6/CD3 antibodies arm are shown in Table 6, and the generated bispecific antibodies contain a silent Fc with attenuated affinity for the Fc gamma receptor.

[0244] (Table 6)

Antibody name	CLDN6 binding VH		CLDN6 binding VL		CD3 binding VH	CD3 binding VL
	SEQ ID NO	Name	SEQ ID NO	Name		
25H0021/20L0072//TR01	35	25H0021	42	20L0072	SEQ ID NO: 70	SEQ ID NO: 71
25H0024/20L0072//TR01	36	25H0024	42	20L0072		
25H0042/20L0072//TR01	37	25H0042	42	20L0072		
25H0176/20L0072//TR01	38	25H0176	42	20L0072		
25H0322/20L0072//TR01	39	25H0322	42	20L0072		
25H0562/20L0072//TR01	40	25H0562	42	20L0072		
25H0581/20L0072//TR01	34	25H0581	42	20L0072		
25H/20L0532//TR01	33	25H	43	20L0532		
25H0021/20L0532//TR01	35	25H0021	43	20L0532		
25H0024/20L0532//TR01	36	25H0024	43	20L0532		
25H0042/20L0532//TR01	37	25H0042	43	20L0532		
25H0176/20L0532//TR01	38	25H0176	43	20L0532		
25H0322/20L0532//TR01	39	25H0322	43	20L0532		
25H0562/20L0532//TR01	40	25H0562	43	20L0532		
25H0581/20L0532//TR01	34	25H0581	43	20L0532		
AE3-20/TR01	1	AE3.20H	2	AE3.20L		

[0245] Fig. 11 shows T cell activation activity of 10 nM of anti-CLDN6/CD3 bispecific antibodies as shown in Table 6 in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF, or hCLDN9/BaF determined by method as described in Reference Example 3. KLH/TR01 generated in Example 2-3 was used as a negative control.

[0246] Some of the bispecific antibodies, such as 25H0322/20L0072//TR01 and 25H0562/20L0072//TR01 showed lower T cell activation activity in the presence of hCLDN6/BaF compared with AE3-20/TR01.

[0247] Some of the bispecific antibodies, such as 25H0581/20L0072//TR01 and 25H0581/20L0532//TR01 showed higher T cell activation activity in the presence of hCLDN9/BaF and hCLDN4/BaF compared with AE3-20/TR01.

[0248] But several bispecific antibodies showed comparable similar T cell activation activity in the presence of hCLDN6/BaF without showing higher T cell activation in the presence of hCLDN3/BaF, hCLDN4/BaF, or hCLDN9/BaF compared with AE3-20/TR01.

[0249] ECM (extracellular matrix) is an extracellular constituent and resides at various sites in vivo. Therefore, an antibody strongly binds to ECM is known to have poorer kinetics (shorter half-life) in blood (WO2012093704 A1).

[0250] ECM binding of the bivalent IgG antibody comprising the VH and VL shown in Table 7 was evaluated. The ECM binding value (ECL reaction) of each antibody was divided by the ECM binding value of the antibody MRA (VH: SEQ ID NO: 66, VL: SEQ ID NO: 67) obtained in the same plate or at the same execution date, and the resulting value is shown in Table 7. As shown in Table 7, the ECM binding of the antibodies varies hugely.

[0251] (Table 7) ECM binding results

Antibody name	Luminescence	Luminescence ratio vs MRA	SEQ ID NO: of			
			VH	VL	CH	CL
MRA (negative control)	13043.5	1.00	66	67	80	81
25H0021/20L0072	32891.5	2.52	35	42		
25H0024/20L0072	29640.5	2.27	36			
25H0042/20L0072	40377.5	3.10	37			
25H0176/20L0072	77022	5.91	38			
25H0322/20L0072	70573.5	5.41	39			
25H0562/20L0072	32630.5	2.50	40			

[0252] The results of the above mentioned experiments indicated that it is very difficult to have all the preferable properties, including high binding activity towards CLDN6, high binding specificity towards CLDN6 (low binding activity towards CLDN3, CLDN4 and CLDN9), and low ECM binding at the same time. For example, although there are only few amino acid differences in the antibodies compared with each other, antibodies comprising 25H0322 or 25H0562 as VH showed poorer binding activity towards CLDN6 compared with those comprising AE3.20H as VH; antibodies comprising 25H0581 as VH showed poor binding specificity towards CLDN6; an-

tibodies comprising 25H0176 or 25H0322 as VH were not most preferable variants from ECM binding perspective.

[0253] Considering the binding activity and specificity towards human CLDN6, together with ECM binding, the antibody comprises 25H or 25H0042 as VH, 20L0532 as VL was used for further engineering.

[0254] EXAMPLE 5. Antibody optimization

Bispecific antibodies made by Fab arm exchanging technology is not suitable for the purification and production process to provide the compound in a large scale. To improve the manufacturability of the bispecific antibody, further optimization was carried out as follows.

[0255] EXAMPLE 5-1. Removal of degradation hotspot

To avoid chemical degradations such as cyclization, isomerization, cleavage, and methionine oxidation, in another word, to improve the stability of the antibody, some amino acid residues in corresponding sequence within the CDRs were substituted. Amino acid substitutions which were found to further improve the binding affinity and specificity of the CLDN6 binding variable regions were also introduced into the CDRs.

[0256] The name of the VH and amino acid substitutions compared with 25H0042 are shown in Table 8. The name of the VL and amino acid substitutions compared with 20L0532 are shown in Table 9. The VH descried in Table 8 and the VL described in Table 9, together with a variable regions shows binding activity towards human CD3 (VH SEQ ID NO: 70; VL SEQ ID NO: 71) were used to generate anti-CLDN6/CD3 bispecific antibodies using Fab arm exchange technique reported by Igawa et al. (WO2016159213). The variable regions comprised in the anti-CLDN6/CD3 antibodies arm are shown in Table 10, and the generated bispecific antibodies contain a silent Fc with attenuated affinity for the Fc gamma receptor.

[0257] (Table 8)

VH Name	Amino acid substitutions in CDRs compared with 25H0042	SEQ ID NO
25H0671	M34G, G54V, S62V, Y97F	45

[0258] (Table 9)

VL Name	Amino acid substitutions in CDRs compared with 20L0532	SEQ ID NO
20L0571	S31L, Y50L, D56I	46

[0259] (Table 10)

Antibody name	CLDN6 binding VH	CLDN6 binding VL	CD3 binding VH	CD3 binding VL
25H0671/20L0571//TR01	25H0671	20L0571	SEQ ID NO: 70	SEQ ID NO: 71
AE3-20/TR01	AE3.20H	AE3.20L		
CS2201	25H	20L0532		

[0260] Fig. 12 showed T cell activation activity of 10 nM of anti-CLDN6/CD3 bispecific antibodies in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF or hCLDN9/BaF determined by method as described in Reference Example 3. 25H0671/20L0571//TR01 induced comparable T cell activation in the presence of hCLDN6/BaF compared with CS2201, without showing higher T cell activation in the presence of hCLDN3/BaF, hCLDN4/BaF or hCLDN9/BaF compared with CS2201.

[0261] EXAMPLE 5-2. pI Engineering

In preparation of a bispecific antibody, when two types of Heavy chains and two type of Light chain are used for expression, theoretically ten types of Heavy chain and Light chain combinations are expressed. To enable easy purification of the bispecific antibody of interest by separating these ten types of antibodies, amino acid modifications were carried out to decrease the isoelectric point (pI) of the CLDN6 binding VH and VL.

[0262] To evaluate the pI reduction effect of various amino acid substitutions, Amino acid substitutions was introduced into the VH 25H0671 and the VL 20L0571, to reduce the number of positively charged amino acids (such as arginine and lysine) while increasing the negatively charged amino acids (such as aspartic acid and glutamic acid).

Table 11 shows the pI engineered VH variants, and Table 12 shows the pI engineered VL variants.

[0263] (Table 11)

VH Name	Amino acid substitutions in FR compared with 25H0671	SEQ ID NO
57H0671	R16D, Q43E, N73D	47
65H0671	R16G, A84D, Q105E	50

[0264] (Table 12)

VL Name	Amino acid substitutions in FR compared with 20L0571	SEQ ID NO
53L0571	K45E, S60D	53
54L0571	K45E, S60D, L104V, K107E	54

[0265] Bivalent IgG antibodies that comprises VH selected from Table 11 and VL selected from Table 12 were used for calculation of theoretical pI. The produced bivalent IgG

antibodies comprises same IgG constant regions. Theoretical pI of the antibodies were calculated using GENETYX-SV/RC Ver 14.0.0 (GENETYX CORPORATION) by a method similar to that described previously.

The calculated theoretical pI values are shown in Table 13.

[0266] (Table 13) Theoretical pI

Antibody name	VH	VL	Theoretical pI
25H0671/20L0571_bivalent	25H0671	20L0571	8.04
57H0671/20L0571_bivalent	57H0671	20L0571	7.54
65H0671/20L0571_bivalent	65H0671	20L0571	7.69
25H0671/53L0571_bivalent	25H0671	53L0571	7.69
25H0671/54L0571_bivalent	25H0671	54L0571	7.37
65H0671/53L0571_bivalent	65H0671	53L0571	7.19
65H0671/54L0571_bivalent	65H0671	54L0571	6.83

[0267] The CLDN6 binding variable regions descried in Table 14, together with a variable regions shows binding activity towards human CD3 (VH SEQ ID NO: 70; VL SEQ ID NO: 71) were used to generate anti-CLDN6/CD3 bispecific antibodies using Fab arm exchange technique reported by Igawa et al. (WO2016159213). The generated bispecific antibodies contain a silent Fc with attenuated affinity for the Fc gamma receptor.

[0268] (Table 14)

Antibody name	CLDN6-binding VH	CLDN6-binding VL	CD3 binding VH	CD3 binding VL
AE3-20/TR01	AE3.20H	AE3.20L	SEQ ID NO: 70	SEQ ID NO: 71
57H0671/20L0571//TR01	57H0671	20L0571		
65H0671/20L0571//TR01	65H0671	20L0571		
25H0671/20L0571//TR01	25H0671	20L0571		
25H0671/53L0571//TR01	25H0671	53L0571		
25H0671/54L0571//TR01	25H0671	54L0571		

[0269] Fig. 13 shows T cell activation activity of anti-CLDN6/CD3 bispecific antibodies with pI engineering in CLDN6-binding VH (57H0671/20L0571//TR01 and 65H0671/20L0571//TR01) in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF or hCLDN9/BaF determined by method as described in Reference Example 3.

[0270] According to the theoretical pI, T cell activation activity and the sequences of the frameworks, frameworks of 65H0671 were selected for further evaluation.

[0271] Fig. 14 shows T cell activation activity of anti-CLDN6/CD3 bispecific antibodies

with pI engineering in CLDN6-binding VL (25H0671/53L0571//TR01 and 25H0671/54L0571//TR01) in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF or hCLDN9/BaF determined by method as described in Reference Example 3.

[0272] According to the theoretical pI and T cell activation activity, frameworks of 54L0571 was selected for further evaluation.

[0273] The CLDN6 binding variable regions descried in Table 15, together with a variable regions shows binding activity towards human CD3 (VH SEQ ID NO: 70; VL SEQ ID NO: 71) were used to generate anti-CLDN6/CD3 bispecific antibodies using Fab arm exchange technique reported by Igawa et al. (WO2016159213). The generated bispecific antibodies contain a silent Fc with attenuated affinity for the Fc gamma receptor.

[0274] (Table 15)

Antibody name	CLDN6-binding VH	CLDN6-binding VL
65H0671/54L0571//TR01	65H0671	54L0571
25H0671/20L0571//TR01	25H0671	20L0571

[0275] Fig. 15 shows T cell activation activity of anti-CLDN6/CD3 bispecific antibodies with pI engineering in both CLDN6-binding VH and CLDN6-binding VL in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF or hCLDN9/BaF determined by method as described in Reference Example 3. The result indicated that the binding activity and binding specificity towards human CLDN6 of 65H0671/54L0571//TR01 is comparable with that of 25H0671/20L0571//TR01.

[0276] The pI values of antibodies comprising the selected frameworks were also actually determined using the Maurice (ProteinSimple) with the cIEF cartridge (PS-MC02-C). The constant region in the antibodies were the same with those shown in Table 13. Sample load was done for 55 s. Subsequently, focusing was carried out at 1500 V for 1 min and 3000 V for 6 min. Fluorescent emission detection at 330 nm and 280 nm excitation in combination to the standard UV absorbance at 280 nm. Fluorescent exposure time were the 3sec, 5sec, 10sec and 20sec. For the anolyte, 80 mM phosphoric acid in 0.1% methyl cellulose, and for the catholyte, 100 mM sodium hydroxide in 0.1% methyl cellulose were used. Each sample solution contained constant amounts of 0.017 mg/ml sample, 5% Pharmalyte 3-10, 0.42% methyl cellulose, 3.6M urea, 10mM Arg, 1% pI marker 4.05 and 1% pI marker 9.99. The associated software Compass for iCE (2.0.10) was used for data analysis.

[0277] (Table 16)

Antibody name	pI
25H0671/20L0571_bivalent	9.12
65H0671/54L0571_bivalent	7.49

[0278] As shown in the Table 16, measured pI of 65H0671/54L0571_bivalent was 7.49, which was lower than the pI of 25H0671/20L0571_bivalent, the antibody without any pI engineering amino acid modification.

[0279] EXAMPLE 5-3. Control of heavy chain and light chain pairing

In preparation of a bispecific antibody, when two types of Heavy chains and two type of Light chain are used for expression, theoretically ten types of Heavy chain and Light chain combinations are expressed. Since only one of these combinations is the bispecific antibody of interest, obtaining a bispecific antibody of interest requires purifying one antibody of interest from the mixture of 10 different antibodies, which is highly inefficient and difficult. As means to solve this problem, "Knob" and "Hole" modifications (KiH: Knobs into Hole) were introduced into the CH3 domain of IgG H chains so that IgG with two different H chains combined is preferentially secreted, and, to further efficiently obtain a molecule of interest, amino acid substitutions and combinations thereof in the variable regions and the CH1-CL domain interface for promoting desired H chain-L chain association (Referring WO2013065708).

[0280] The amino acid substitution Q39K was introduced into the CD3 binding VH shown in SEQ ID NO: 70, and the amino acid substitution Q38E was introduced into the CD3 binding VL shown in SEQ ID NO: 71. The sequences of the amino acid substituted CD3 binding VH and VL were shown in SEQ ID NO: 60 and SEQ ID NO: 61, respectively.

[0281] The amino acid substitution Q39E was introduced into the CLDN6 binding VH 65H0671, and the amino acid substitution Q38K was introduced into the CLDN6 binding VL 54L0571. The amino acid substituted CLDN6 binding VH and VL were named 65H0671Q39E (SEQ ID NO: 57) and 54L0571Q38K (SEQ ID NO: 58) respectively.

[0282] The CLDN6 binding VH 65H0671Q39E was linked to a first CH1 domain region shown in SEQ ID NO: 95, the CLDN6 binding VL 54L0571Q38K was linked to a first CL domain shown in SEQ ID NO: 59 to make a first light chain shown in SEQ ID NO: 96. The CD3 binding VH shown in SEQ ID NO: 60 was linked to a second CH1 domain shown in SEQ ID NO: 97, the CD3 binding VL shown in SEQ ID NO: 61 was linked to a second CL domain shown in SEQ ID NO: 62 to make a second light chain shown in SEQ ID NO: 98. The CH1 domains and CL domains also contains amino acid substitutions compared with natural occurring ones, which are: Glu (E) at position

147, 175 and 213 in the first CH1 domain; Lys (K) at position 123, 131, 160 and 180 in the first CL domain; Lys (K) at amino acid position 175 in the second CH1 domain; and Glu (E) at amino acid position 131, 160 and 180 in the second CL domain. The first CH1 domain was further linked to a first hinge-CH2-CH3 region to make a first heavy chain shown in SEQ ID NO: 99, the second CH1 domain was further linked to a second hinge-CH2-CH3 region to make a second heavy chain shown in SEQ ID NO: 100. As a result, the antibody CS2425 (first heavy chain SEQ ID NO: 99, first light chain SEQ ID NO: 96, second heavy chain SEQ ID NO: 100, second light chain SEQ ID NO: 98) was generated.

[0283] The charge heterogeneity of CS2425 was evaluated using the Maurice (ProteinSimple) with the cIEF cartridge (PS-MC02-C). Sample load was done for 55 s. Subsequently, focusing was carried out at 1500 V for 1 min and 3000 V for 6 min. The detection was UV absorbance at 280 nm. For the anolyte, 80 mM phosphoric acid in 0.1% methyl cellulose, and for the catholyte, 100 mM sodium hydroxide in 0.1% methyl cellulose were used. Each sample solution contained constant amounts of 0.085 mg/ml sample, 2% Pharmalyte 5-8, 2% Pharmalyte 8-10.5, 0.42% methyl cellulose, 3.6M urea, 10mM Arg, 1% pI marker 5.85 and 1% pI marker 10.17. The associated software Compass for iCE (2.0.10) was used for data analysis.

[0284] Fig. 16(A) showed the charge heterogeneity of CS2425(65H0671Q39E-SG1189v14k/54L0571Q38K-SK1018//TR01H(Q39K)-SG1191v14h/TR01L(Q38E)-SK1017) when the first heavy chain, the first light chain, the second heavy chain, and the second light chain made above were expressed together. The electropherogram suggested that both mutations had a good HH and HL control, because there were no other obvious peaks other than the main peak around pI 9.0.

[0285] EXAMPLE 5-4. Anti-tumor efficacy and safety analysis of anti-CLDN6/CD3 bispecific antibodies

The in vivo anti-tumor efficacy of the anti-CLDN6/CD3 bispecific antibody CS2201 made in Example 5-1 was determined using OV-90 T cell injection model. AE3-20/TR01 was used as comparison. In mouse treated with bispecific antibodies at 0.1 mg/kg, CS2201 was more efficacious than AE3-20/TR01 against OV-90 T cell injection model (Fig. 17). On the other hand, CS2201 and AE3-20/TR01 showed comparable anti-tumor efficacy at 5 mg/kg (Fig. 18). With regard to hepatotoxicity of CS2201, no abnormality of hepatotoxic marker was observed from the result of blood test (Fig. 19). Furthermore, the PK feature of CS2201 and AE3-20/TR01 was found to be comparable (Fig. 20). The plasma half-life ($T_{1/2}$) of CS2201 and AE3-20/TR01 were 3.64 and 3.48 days in 0.1 mg/kg group, respectively. The area under the plasma concentration-time curve from zero to infinity (AUCinf) of CS2201 and AE3-20/TR01 were 1.83 and 2.35 day*micro g/mL in 0.1 mg/kg group, respectively.

[0286] Anti-tumor efficacy and toxicity of anti-CLDN6/CD3 bispecific antibody CS2425 were examined using OV-90 T-cell injection model. Mouse inoculated with OV-90 cells were treated with 0.1 mg/kg or 5 mg/kg of CS2425, and tumor volume and body weight were examined.

[0287] Significant tumor regression was observed in mouse given CS2425 at 0.1 mg/kg. In this treatment group, no toxic sign was noted based on the result of body weight change and general condition of mouse after antibody administration (Fig. 21A). However, mouse treated with CS2425 at 5 mg/kg showed significant body weight loss (Fig. 21B). Consistent with severe body weight change, blood markers of liver injury were drastically elevated in all animals received 5 mg/kg of CS2425 (Fig. 22).

[0288] Circulating levels of CS2425 were also calculated. The plasma samples prepared from three animals on day 4, 13 post injection for 0.1mg/kg group and day 4, 6 post injection for 5 mg/kg group were used for PK analysis as described in EXAMPLE 2-4. Fig. 23 represents the time course of plasma concentration of CS2425 after administration.

[0289] EXAMPLE 6: Re-engineering of the CLDN6 binding variable regions

EXAMPLE 6-1: In vitro immunogenicity studies using human PBMCs and re-choice of heavy chain and light chain pairing controlling amino acid substitutions

Bivalent IgG antibodies shown in Table 17 was generated. Antibody A was known to have low immunogenicity, and was used as negative control in the immunogenicity study. Anti-hA33 antibody, which was known to have high immunogenicity, was used as positive control in the immunogenicity study.

[0290] (Table 17)

Antibody name	Heavy chain	Light chain
CS2419	SEQ ID NO: 101 (comprising CLDN6 binding VH same with that of CS2425)	antibody-A light chain
CS2421	antibody-A heavy chain	CLDN6 binding light chain of CS2425, shown in SEQ ID NO: 96

[0291] The immunogenic potential of the antibodies were evaluated by using as an indicator the proportion of IL-2-secreting $CD4^+$ T cells before active proliferation is exhibited as described in WO/2018/124005 (Kubo C. et al). Specifically, $CD8^-CD25^{low}$ PBMCs were prepared from human PBMCs, and the cells were cultured for 67 hours in the presence of the antibodies. Fig. 24 shows the results of determining the proportions of IL-2-secreting cells in the cultured cell populations. As shown in Fig. 24, the frequency of positive donors for CS2419 was low compared to that for antibody-A. On the other

hand, the frequency of positive donors for CS2421 was similar to that for anti-hA33 antibody. Therefore, using the proportion of IL-2-secreting $CD4^+$ T cells as the indicator, CS2419 was considered not having high immunogenic potential, but CS2421 recombined antibody having high immunogenic potential. From the results, as the CLDN6 binding light chain of CS2425, which is shown in SEQ ID NO: 96, was considered involving potential T-cell epitopes.

[0292] To identify a potential T-cell epitope from the CLDN6 binding light chain of CS2425, MHC associated peptide proteomics (MAPPs) was performed with CS2425, as described in US20050202009A1 (Kropshofer H.et al) and mAbs 2018; 10: 1168-81 (Sekiguchi N. et al). Monocytes were prepared from human PBMCs, and the cells were differentiated to dendritic cells (DCs). The DCs were pulsed with antibodies in the presence of LPS, and lysed in detergent TX-100. Peptide-MHC II complexes were obtained from lysed DCs using magnetic beads coupled with the anti-HLA-DR antibody in the immunoprecipitation process, and HLA-DR associated peptides eluted with 0.1% TFA were analyzed by LC/Orbitrap MS/MS technology. Heatmaps of MAPPs in a part of the light chain of CS2425 are shown in Fig. 25. As shown in Fig. 25, many peptides were identified as potential T-cell epitopes in a region (Sequence No. 167~184) of the CLDN6 binding light chain of CS2425, which contains Lys at amino acid position 180. As a result, this amino acid substitution at position 180 was considered a possibility to generate a potential T-cell epitope in the CLDN6 binding light chain of CS2425.

[0293] According to the results of immunogenicity analysis, the amino acid substitution T180K in the CL linked to CLDN6-binding VL caused high risk of immunogenicity, thus this mutation was not adopted to make the anti-CLDN6/CD3 bispecific antibody.

[0294] The CLDN6 binding VH 65H0671Q39E was linked to a first CH1 domain region shown in SEQ ID NO: 95, the CLDN6 binding VL 54L0571Q38K was linked to a first CL domain shown in SEQ ID NO: 63 to make a first light chain shown in SEQ ID NO: 102. The CD3 binding VH shown in SEQ ID NO: 60 was linked to a second CH1 domain shown in SEQ ID NO: 97, the CD3 binding VL shown in SEQ ID NO: 61 was linked to a second CL domain shown in SEQ ID NO: 62 to make a second light chain shown in SEQ ID NO: 98. The CH1 domains and CL domains also contains amino acid substitutions compared with natural occurring ones, which are: Glu (E) at position 147, 175 and 213 in the first CH1 domain; Lys (K) at position 123, 131 and 160 in the first CL domain; Lys (K) at amino acid position 175 in the second CH1 domain; and Glu (E) at amino acid position 131, 160 and 180 in the second CL domain. The first CH1 domain was further linked to a first hinge-CH2-CH3 region to make a first heavy chain shown in SEQ ID NO: 99, the second CH1 domain was further linked to a second hinge-CH2-CH3 region to make a second heavy chain shown in SEQ ID NO:

100. As a result, the antibody 65H0671Q39E-SG1189v14k/54L0571Q38K-SK1021//TR01H(Q39K)-SG1191v14h/TR01L(Q38E)-SK1017 (first heavy chain SEQ ID NO: 99, first light chain SEQ ID NO:102, second heavy chain SEQ ID NO: 100, second light chain SEQ ID NO:98) was generated.

[0295] The charge heterogeneity was evaluated using the Maurice (ProteinSimple) with the cIEF cartridge (PS-MC02-C). Sample load was done for 55 s. Subsequently, focusing was carried out at 1500 V for 1 min and 3000 V for 6 min. The detection was UV absorbance at 280 nm. For the anolyte, 80 mM phosphoric acid in 0.1% methyl cellulose, and for the catholyte, 100 mM sodium hydroxide in 0.1% methyl cellulose were used. Each sample solution contained constant amounts of 0.085 mg/ml sample, 2% Pharmalyte 5-8, 2% Pharmalyte 8-10.5, 0.42% methyl cellulose, 3.6M urea, 10mM Arg, 1% pI marker 5.85 and 1% pI marker 10.17. The associated software Compass for iCE (2.0.10) was used for data analysis.

[0296] Fig. 16(B) showed the charge heterogeneity of resulted antibody when the first heavy chain, the first light chain, the second heavy chain, and the second light chain made above were expressed together. The electropherogram suggested that both mutations had a good HH and HL control, because there were no other obvious peaks other than the main peak around pI 9.0.

[0297] The bivalent IgG antibody shown in Table 18 was generated.

[0298] (Table 18)

Antibody name	Heavy chain	Light chain
CS2652	antibody-A heavy chain	54L0571Q38K-SK1021

[0299] In the light chain of CS2652, the CLDN6 binding VL 54L0571Q38K was linked to a CL domain shown in SEQ ID NO: 63 to make the light chain shown in SEQ ID NO: 102. The CL domain contains amino acid substitutions compared with natural occurring ones, which are: Lys (K) at position 123, 131 and 160.

[0300] The potential immunogenicity of the antibodies by using as an indicator the proportion of IL-2-secreting $CD4^+$ T cells. As shown in Fig. 26, the frequencies of positive donors for CS2652 was similar to that for antibody A. Therefore, this antibody was considered not to have high immunogenic potential.

[0301] EXAMPLE 6-2: Generation of CLDN6 binding variable region variants

Although AE3-20/TR01 and CS2201 shows strong efficacy without any toxicities in mouse, CS2425 unexpectedly cause serious liver damage in mouse. We speculated that the modifications of amino acid sequence in the CLDN6 binding variable regions described in Example 5 might have an impact on the character of antibody, and subsequently caused toxicity. To confirm which modifications affect the antibody to cause

liver toxicity, CLDN6 binding variable region variants which have less amino acid substitutions compared to that of CS2425 were constructed.

[0302] The constructed CLDN6 binding variable region variants, together with the variable regions shows binding activity towards human CD3 (VH SEQ ID NO: 70; VL SEQ ID NO: 71) were used to generate anti-CLDN6/CD3 bispecific antibodies using Fab arm exchange technique reported by Igawa et al. (WO 2016159213). The variable regions comprised in the anti-CLDN6/CD3 antibodies are shown in Table 19 and Table 20, and the generated bispecific antibodies shown in Table 21 contain a silent Fc with attenuated affinity for the Fc gamma receptor.

[0303] ECM binding of these antibodies was also evaluated by method as described in Example 4. As a results, all of these antibodies showed low ECM binding.

[0304] (Table 19)

CLDN6 binding VH Name	Amino acid substitutions in CDRs compared with 25H	Amino acid substitutions in FRs compared with 25H	SEQ ID NO
25H	-	-	33
65H	None	R16G, A84D, Q105E	84
65HQ39E	None	R16G, A84D, Q105E, Q39E	85
25HQ39E	None	Q39E	83
65H0671	T33V, M34G, G54V, S62V, Y97F	R16G, A84D, Q105E	50

[0305] (Table 20)

CLDN6 binding VL Name	Amino acid substitutions in CDRs compared with 20L0532	Amino acid substitutions in FRs compared with 20L0532	SEQ ID NO
20L0532	-	-	43
54L0532	None	K45E, S60D, L104V, K107E	86
54L0532Q38K	None	K45E, S60D, L104V, K107E, Q38K	87
20L0532Q38K	None	Q38K	88
54L0571	S31L, Y50L, D56I	K45E, S60D, L104V, K107E	54

[0306] (Table 21)

Antibody name	CLDN6 binding VH	CLDN6 binding VL	SEQ ID NO: of					
			CD3 binding VH	CD3 binding VL	CH1 linked to CLDN6 binding VH	CL linked to CLDN6 binding VL	CH1 linked to CD3 binding VH	CL linked to CD3 binding VL
CS2884	65H	54L0532	70	71	103	81	103	81
CS2885	25H	54L0532			103	81	103	81
CS2886	65H	20L0532			103	81	103	81
CS2958	65HQ39E	54L0532Q38K			95	63	97	62
CS2959	25HQ39E	20L0532Q38K			95	63	97	62
CS2960	65H0671	54L0571			103	81	103	81

[0307] EXAMPLE 6-3. Efficacy and toxicology evaluation of anti-CLDN6/CD3 bispecific antibodies

To evaluate the anti-tumor efficacy and safety of the generated anti-CLDN6/CD3 bispecific antibodies, in vivo mice studies were conducted by OV-90 T-cell injection model as follows. Mouse bearing OV-90 tumor were administered with CS2201, CS2425, CS2884, CS2885, CS2886, CS2958, CS2959 and CS2960 at 5 mg/kg, then tumor volume and body weight were calculated. Blood was also collected after treatment, then used to analyze the plasma level of liver injury markers.

[0308] Fig. 27 indicates the result of in vivo experiment using CS2884, CS2885, and CS2886 in addition to CS2201 and CS2425. As described in Table 21, these partially optimized anti-CLDN6/CD3 bispecific antibodies comprise the amino acid substitutions described in Example 5-2. As shown in Fig. 27, CS2884, CS2885, and CS2886 showed growth inhibitory effect against OV-90 tumor. There were no changes of body weight and of liver injury markers among mouse received these anti-CLDN6/CD3 bispecific antibodies except for fully optimized CS2425. This result indicates that the amino acid substitutions described in Example 5-2 was not the cause of liver injury (Fig. 28).

[0309] Fig. 29 indicates the result of in vivo experiment using CS2958, CS2959 and CS2960 in addition to CS2201 and CS2425. As described in Table 21, CS2958 comprises the amino acid substitutions described in Example 5-2 and Example 5-3, CS2959 only comprises the variable region amino acid substitutions described in Example 5-3, CS2960 comprises the amino acid substitutions described in Example 5-1 and Example 5-2. As shown in Fig. 29, these anti-CLDN6/CD3 bispecific antibodies reduced tumor growth, however, significant decrease of body weight was observed in CS2425 and CS2960 treatment groups. Consistent with body weight loss, significant elevation of liver toxicity markers were also found in CS2425 and CS2960 treatment groups (Fig. 30).

[0310] The result showing that CS2425 and CS2960 caused liver damage, suggested that the amino acid substitutions described in Example 5-1 was most likely to be the cause of toxicity.

[0311] As shown in Fig. 31, the plasma half-life ($T_{1/2}$) of CS2201, CS2958, CS2959, and CS2960 were 3.26, 3.77, 3.68 and 3.45 days, respectively. The area under the plasma concentration-time curve from zero to infinity (AUCinf) of CS2201, CS2958, CS2959 and CS2960 were 90.0, 134, 106 and 117 day*micro g/mL, respectively.

[0312] EXAMPLE 6-4. Fc optimization

To select Fc regions, several Fc variants were generated as shown in Table 22. Amino acid mutations that reduce the binding activity of the Fc region to Fc gamma receptors (FcgR silent mutations), deglycosylation mutation, and mutations to promote heavy chain hetero dimerization were introduced (as described in WO2006106905).

[0313] (Table 22)

Fc name	FcgR silent mutation	Deglycosylation mutation	Stabilization mutation	Heavy chain Hetero	SEQ ID NO
SG1212	L234A, L235A, A327G, A330S, P331S	N297A	S239K	K439E	72
SG1213	L234A, L235A, A327G, A330S, P331S	N297A	S239K	E356K	73
SG1260	L234A, L235A, A327G, A330S, P331S	N297A		K439E	74
SG1261	L234A, L235A, A327G, A330S, P331S	N297A		E356K	75
SG1323	L234A, L235A	N297A	S239K	K439E	76
SG1324	L234A, L235A	N297A	S239K	E356K	77
SG1325	L234A, L235A	N297A		K439E	78
SG1326	L234A, L235A	N297A		E356K	79

[0314] EXAMPLE 6-5. Generation of CS2961, CS3346, CS3347 and CS3348

By using these Fc variants, four bispecific antibodies shown in Table 23 were constructed.

[0315] (Table 23)

	Antibody name			
	CS2961	CS3346	CS3347	CS3348
CLDN6 binding VH	65HQ39E			
1st CH1 linked to the CLDN6 binding VH	SEQ ID NO: 95			
Fc region linked to the 1st CH1 via hinge domain	SG1212	SG1260	SG1323	SG1325
CLDN6 binding heavy chain	SEQ ID NO: 104	SEQ ID NO: 105	SEQ ID NO: 106	SEQ ID NO: 107
CLDN6 binding VL	54L0532Q38K			
1st CL linked to the CLDN6 binding VL	SK1021	SK1021	SK1021	SK1021
CLDN6 binding light chain	SEQ ID NO: 112	SEQ ID NO: 112	SEQ ID NO: 112	SEQ ID NO: 112
CD3 binding VH	SEQ ID NO: 60			
2nd CH1 linked to the CD3 binding VH	SEQ ID NO: 97			
Fc region linked to the 2nd CH1 via hinge domain	SG1213	SG1261	SG1324	SG1326
CD3 binding heavy chain	SEQ ID NO: 108	SEQ ID NO: 109	SEQ ID NO: 110	SEQ ID NO: 111
CD3 binding VL	SEQ ID NO: 61			
2nd CL linked to the CLDN6 binding VL	SK1017	SK1017	SK1017	SK1017
CD3 binding light chain	SEQ ID NO: 98	SEQ ID NO: 98	SEQ ID NO: 98	SEQ ID NO: 98

[0316] EXAMPLE 7. Characterization of CS2961, CS3346, CS3347 and CS3348

EXAMPLE 7-1. T cell activation activity of anti-CLDN6/CD3 bispecific antibodies in presence of CLDN family protein expressing cells

Fig. 32 shows T cell activation activity of various concentrations of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01, CS2201, 6PHU3/TR01, CS2425 and CS2961) in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF, hCLDN9/BaF, or hCLDN6(Q156L)/BaF determined by method as described in Reference Example 3. All our anti-CLDN6/CD3 bispecific antibodies induced T cell activation in the presence of hCLDN6/BaF. In addition, 10 nM of AE3-20/TR01

induced a bit higher T cell activation in the presence of hCLDN4/BaF and hCLDN6(Q156L)/BaF than the other anti-CLDN6/CD3 bispecific antibodies and 10 nM of 6PHU3/TR01 induced a bit higher T cell activation in the presence of hCLDN9/BaF.

[0317] Fig. 33 shows T cell activation activity of various concentrations of anti-CLDN6/CD3 bispecific antibodies such as CS2201, CS2961, CS3346, CS3347, and CS3348 in the presence of hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF or hCLDN9/BaF determined by method as described in Reference Example 3. All our anti-CLDN6/CD3 bispecific antibodies induced T cell activation in the presence of hCLDN6/BaF and no T cell activation was observed by all our anti-CLDN6/CD3 bispecific antibodies in the presence of hCLDN3/BaF, hCLDN4/BaF and hCLDN9/BaF.

[0318] EXAMPLE 7-2. Measurement of T-cell-dependent cell cytotoxicity of anti-CLDN6/CD3 bispecific antibodies

Fig. 34 shows the T-cell-dependent cell cytotoxicity of anti-CLDN6/CD3 bispecific antibodies (AE3-20/TR01, CS2201, 6PHU3/TR01, CS2425, CS2961, CS3346, CS3347, and CS3348) against OVCAR3 ovarian cancer cell line expressing CLDN6. Cell cytotoxicity was evaluated by LDH assay using human PBMC (StemCell). 20,000 target cells and 200,000 human PBMC (E/T = 10) were seeded into each well of a 96-well U-bottom plate and incubated with various concentrations of antibody for 24 hours at 37 degrees C and 5% CO_2. Target cell killing was measured by LDH cytotoxicity detection kit (Takara Bio). The cytotoxic activity (%) of each antibody was calculated using the following formula.

Cytotoxic activity (%) = (A - B - C) x 100 / (D - C)

"A" represents the absorbance of wells treated with antibody and PBMC, "B" represents the average absorbance value of effector cells PBMC only, "C" represents the average absorbance value of untreated target cells only, and "D" represents the average values of wells lysed with Triton-X. Background absorbance have been accounted for and subtracted. All our anti-CLDN6/CD3 bispecific antibodies showed T-cell-dependent cell cytotoxicity against OVCAR3 cells.

[0319] EXAMPLE 7-3. FACS analysis of specificity of CS2961 against CLDN family

Amino acid sequence are highly conserved among CLDN3, CLDN4, CLDN6 and CLDN9. Thus we examined CLDN6 binding specificity by FACS analysis. hCLDN6/BaF, hCLDN3/BaF, hCLDN4/BaF, and hCLDN9/BaF were incubated with CS2201, CS2961, AH05/TR01 and 6PHU3/TR01 at 15 micro g/ml. KLH/TR01 generated in Example 2-3 was used as a staining control. Binding of each antibodies were detected with Alexa Fluor 488-conjugated anti human IgG (Invitrogen). Dead cells were separated by eFlour 780 (Invitrogen) staining.

[0320] As shown in Figs. 35-1 and 35-2, all anti-CLDN6/CD3 bispecific antibodies showed strong binding to hCLDN6/BaF. Among the evaluated anti-CLDN6/CD3 bispecific antibodies, CS2961 showed the best specificity towards CLDN6.

[0321] EXAMPLE 7-4. Stability

The stability of CS2961, CS3346, CS3347 and CS3348 was evaluated. Each of the antibodies were dialyzed against PBS, pH 7.4 and concentration were adjusted to 1 mg/mL upon recovery. The samples were stored at 5 degrees C and 40 degrees C for one week, and 40 degrees C for two weeks. After storage, SEC analysis was conducted using SWXL G3000 column (TOSOH) with 50 mM NaPhosphate, 300 mM NaCl, pH 7.0 as running buffer. Detection was done by UV detector (280 nm). Chromatograms were analyzed using Empower 3 software (Waters).

[0322] The result shown in Fig. 36 indicated that all the antibodies shows good stability, which is surely far better than that of bispecific $sc(Fv)_2$ molecule.

[0323] EXAMPLE 7-5. In vivo efficacy and safety

To evaluate the anti-tumor efficacy and safety of CS2961, in vivo mice studies were conducted as follows. Mouse bearing OV-90 tumor were administered with purified CS2961 at 5 mg/kg, then tumor volume and body weight were calculated. Blood was also collected after treatment, then used to analyze the plasma level of liver injury markers.

[0324] Fig. 29 shows the result of in vivo experiment using CS2961. As shown in Fig. 29, CS2961 reduced tumor volume without decreasing the body weight of treated mice and increasing liver toxicity markers (Fig. 30).

[0325] As shown in Fig. 31, the plasma half-life ($T_{1/2}$) of CS2961 was 11.2 days, the area under the plasma concentration-time curve from zero to infinity (AUCinf) of CS2961 was 486 day*micro g/mL. These results indicated that CS2961 showed slower elimination rate compared to anti-CLDN6/CD3 bispecific antibodies generated by Fab arm exchanging technology.

[0326] CS2961 showed a potent anti-tumor activity against OV-90 tumor and good safety and PK profile in mouse. Furthermore, this antibody showed improved stability, better heterodimer formation which are preferable for large scale production. CS2961 is also easier to be purified because of the amino acid substitutions described in Example 5-2.

[0327] EXAMPLE 7-6. Dose dependent in vivo efficacy

In vivo potency of CS3348 were compared with AE3-20/TR01 using OV-90 T cell injection model as described in Example 2-4.

[0328] After tumor volume reached around $200mm^3$ in the mice, animals were randomized and administered each bispecific antibodies or vehicle. Fig. 37 shows the tumor growth of mice treated with AE3-20/TR01, and Fig. 38 shows those with CS3348. In the experiment, the cancer cell OV-90 was transplanted into the mice at day 0, various

dosage of AE3-20/TR01 was administered into the mice at day 14, and various dosage of CS3348 was administered into the mice at day 15. All the mice received one administration.

[0329] Significant efficacy against OV-90 tumors were seen in both AE3-20/TR01 and CS3348 in dose dependent manner without body weight loss at all dosage.

[0330] Of note, the tumor regression was observed in mice treated with 0.04 mg/kg of CS3348, while those was observed in mice treated with 1 mg/kg of AE3-20/TR01. In another word, the anti-tumor efficacy of CS3348 is likely to be about 25 times as strong as that of AE3-20/TR01.

[0331] EXAMPLE 8. In vivo anti-tumor efficacy and toxicity study

The in vivo efficacy and toxicity of antibodies were evaluated using tumor bearing mice model. The human cancer cell lines OVCAR-3, NCI-H1435 or NUGC-3 which express human claudin-6 were transplanted subcutaneously into NOD/ShiJic-scid mice. Tumor bearing mice were randomized to treatment groups to receive vehicle control or Ab when tumor size reached approximately 200 mm^3 (called as T cell injection model).

That is, the following test was conducted in the efficacy test towards OVCAR-3, NCI-H1435 or NUGC-3 using T cell injection model. Expansion of T cells was performed using human PBMC (AllCells) and T Cell Activation/Expansion Kit (Miltenyi Biotec). 1 x 10^7 cells of cancer cells and Matrigel Basement Membrane Matrix (BD) were mixed and transplanted subcutaneously into the lateral region of the NOD/ShiJic-scid mice (CLEA Japan, Female, 6 - 7 weeks-old). The day of transplantation was defined as day 0. After randomization and grouping according to tumor size and body weight, anti-asialo GM1 antibody (FUJIFILM Wako Pure Chemical Corporation) was administered intraperitoneally at 0.2 mg / mouse. On the next day, the T cells which were obtained by the expansion culture were implanted intraperitoneally at 3 x 10^7 cells / mouse. Details of groups are shown in Table 24. Approximately four hours after T cell transplantation, CS3348 were administered intravenously. Administration of CS3348 was done only once. The length (L) and width (W) of the tumor mass and body weight were measured, and tumor volume (TV) was calculated as: TV = (L x W x W) / 2.

As shown in Figs. 40 to 42 (Fig. 40: OVCAR-3 bearing mice, Fig. 41: NCI-H1435 bearing mice, and Fig. 42: NUGC-3 bearing mice), CS3348 showed obvious anti-tumor activity against these 3 cancer cell lines as dose dependent manner without any toxicity including body weight loss.

[0332] (Table 24) Details of study groups for in vivo anti-tumor efficacy evaluation

a. Study group in TK220001 (OVCAR-3 bearing mice)

Group	Antibody	Dosage
1	vehicle	
2	CS3348	0.0008 mg/kg
3	CS3348	0.004 mg/kg
4	CS3348	0.02 mg/kg
5	CS3348	0.1 mg/kg
6	CS3348	0.5 mg/kg
7	CS3348	2.5 mg/kg

b. Study group in TK219005 (NCI-H1435 bearing mice)

Group	Antibody	Dosage
1	vehicle	
2	CS3348	0.02 mg/kg
3	CS3348	0.1 mg/kg
4	CS3348	0.5 mg/kg

c. Study group in TK220003 (NUGC-3 bearing mice)

Group	Antibody	Dosage
1	vehicle	
2	CS3348	0.004 mg/kg
3	CS3348	0.02 mg/kg
4	CS3348	0.1 mg/kg

[0333] 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.). In case of bispecific antibodies, ProteinL chromatography (Protenova) is necessary to remove homodimers (WO2018159615). 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).

[0334] Reference EXAMPLE 2. Generation of Claudin expressing cells

Ba/F3 cells expressing human CLDN6 (hCLDN6/BaF), Ba/F3 cells expressing human CLDN9 (hCLDN9/BaF), Ba/F3 cells expressing human CLDN3 (hCLDN3/BaF), Ba/F3 cells expressing human CLDN4 (hCLDN4/BaF), Ba/F3 cells expressing mouse CLDN6 (mCLDN6/BaF), Ba/F3 cells expressing mouse CLDN9 (mCLDN9/BaF), Ba/F3 cells expressing mouse CLDN3 (mCLDN3/BaF), and Ba/F3 cells expressing mouse CLDN4 (mCLDN4/BaF), were established by transfecting human CLDN6, human CLDN9, human CLDN3, human CLDN4, mouse CLDN6, mouse CLDN9, mouse CLDN3, and mouse CLDN4 expression vectors into mouse pro B cell line Ba/F3 respectively.

[0335] Claudin family proteins have two extracellular domains which are accessible to antibody. With regard to amino acid sequence similarity between the extracellular domains of human CLDN6 and human CLDN9, the first extracellular domain are almost the same, and there are only two different amino acids in the second extracellular domain (Fig. 39). The glutamine at position 156 of human Claudin 6 (position 156 in the sequence shown in SEQ ID NO: 125 or 126) was substituted to leucine to make a human CLDN6 mutant comprising the same amino acid as human Claudin 9 at position 156. This human CLDN6 mutant was named as hCLDN6(Q156L) (SEQ ID NO: 134). Ba/F3 transfectant stably express hCLDN6(Q156L) was generated using similar method described above. The established Ba/F3 transfectant was named hCLDN6(Q156L)/BaF.

[0336] FreeStyle™ 293-F transfectant cells transiently expressed human and mouse CLDN3, 4, 6, and 9 were generated by introducing expressing vector of human and mouse CLDNs (including CLDN6, CLDN9, CLDN3, and CLDN4) into FreeStyle™ 293-F cells (Invitrogen) using 293fectin (Invitrogen). The generated FreeStyle™ 293-F transfectant cells were named as hCLDN3/FS293, hCLDN4/FS293, hCLDN6/FS293, hCLDN9/FS293, mCLDN3/FS293, mCLDN4/FS293, mCLDN6/FS293, and mCLDN9/FS293, respectively.

[0337] Reference EXAMPLE 3. T cell activation activity assay using Jurkat report gene assay

T cell activation of the anti-CLDN6/CD3 bispecific antibodies in the presence of target cells was examined by T Cell Activation Bioassay using GloResponse™ NFAT-luc2 Jurkat cells (Promega). One kind of cells selected from the claudin expressing cells generated in Reference Example 2 and cancer cell lines were used as target cell in each assay.

[0338] Firstly, 2 x 10^4 of target cells were seeded on 96-wells flat bottom plate (Corning) at 25 micro L/well. Next 1 x 10^5 of Jurkat/ NFAT-RE Reporter Cell Line are added in

each well as effector cells, making the ratio of effector cell and target cells in each well to 5:1. Then 25 micro L of antibodies solutions with different concentrations (antibody concentration were determined assay by assay) were added to the wells, respectively. After overnight culture at 37 degrees C, 75 micro L of Bio-Glo reagent (Promega) was added to each well and the plate was incubated at room temperature for 10 minutes. Luminescence (RLU) arising from activating Jurkat cells in each well was measured by EnVision (PerkinElmer Japan) or GloMax (registered trademark) Explorer microplate reader. The luminescence ratio of each wells was calculated by comparing between the wells with and without antibody.

[0339] Although the foregoing inventions have been described in some detail by way of illustration and example for purposes of clarity of understanding, the descriptions and examples should not be construed as limiting the scope of the present disclosure. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.

Industrial Applicability

[0340] The antigen-binding molecules of the present disclosure are useful in treating and/or preventing cancers, detecting the presence of CLDN6 in biological sample, and diagnosis of various cancers. One of the embodiment of the antigen-binding molecule is antigen-binding molecules that shows binding activity towards CLDN6 and T cell receptor complex that enable cancer treatment by having T cells close to CLDN6-expressing cells and using the cytotoxicity of T cells against CLDN6-expressing cancer cells.