[DESCRIPTION]

[Title of Invention] CD3-TARGETING ANTIGEN-BINDING MOLECULE WITH IMPROVED STABILITY

[Technical Field]

[0001]

The present disclosure relates to antigen-binding molecules comprising a CD3-binding domain that has improved stability, pharmaceutical compositions comprising the antigen-binding molecule, and uses thereof.

[0002]

Antibodies are drawing attention as pharmaceuticals because of their high stability in plasma and few adverse reactions (NPLs 1 and 2). Antibodies are known to induce not only an antigen-binding action, an agonistic action, and an antagonistic action, but also effector-mediated cytotoxic activities (also called effector functions) such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cell phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC), and exhibit antitumor effects against cancer cells (Non-Patent Document 3). ADCC is a cytotoxicity exhibited by effector cells against antibody-bound target cancer cells via binding of the antibody Fc region to an Fc receptor present on effector cells such as NK cells and macrophages.

[0003]

Naturally-occurring immunoglobulins bind to antigens at their variable regions, and bind to receptors such as FcγR, FcRn, FcαR, and FcεR, and complement at their constant regions. Furthermore, a common naturally-occurring IgG-type antibody recognizes and binds a single epitope via its variable region (Fab); therefore, it can bind to only one antigen. On the other hand, many types of proteins are known to be involved in cancer and inflammation, and there may be crosstalk among the proteins.

[0004]

Antibodies (bispecific antibodies) that bind to two or more types of antigens with one molecule are being studied as molecules that inhibit multiple targets. It is possible to confer binding activities to two different antigens (a first antigen and a second antigen) by modifying naturally-occurring IgG-type antibodies (NPL 4). Therefore, it is possible to increase the antitumor activity by neutralizing two or more types of antigens with one molecule or by crosslinking a cell having cytotoxic activity with a cancer cell.

[0005]

A T-cell redirecting antibody that employs cytotoxicity mobilizing T cells as effector cells as the mechanism for its antitumor effect has been known from the 1980s as a bispecific antibody (NPLs 5, 6, and 7). Unlike antibodies that employ ADCC mobilizing NK cells or macrophages as effector cells as the mechanism for their antitumor effects, a T-cell redirecting antibody is an antibody against any one of the subunits constituting the T-cell receptor (TCR) complex on T cells, and is specifically a bispecific antibody comprising an antibody that binds to the CD3ε chain and an antibody that binds to an antigen on the target cancer cell. T cells come close to cancer cells via simultaneous binding of the CD3ε chain and a cancer antigen by a T-cell redirecting antibody. As a result, antitumor effects against cancer cells are considered to be exerted through the cytotoxic activity possessed by T cells.

[0006]

Catumaxomab, which is known as a T-cell redirecting antibody, binds at two Fabs each to a cancer antigen (EpCAM) and to a CD3ε (CD3 epsilon) chain expressed on T cells. Catumaxomab is a trifunctional antibody which induces T cell-mediated cytotoxic activity by binding to the cancer antigen and the CD3ε at the same time, and induces cytotoxic activity mediated by antigen-presenting cells such as NK cells and macrophages, by binding to the cancer antigen and FcγR at the same time. Trifunctional antibodies, however, bind to CD3ε and FcγR at the same time even in the absence of a cancer antigen and therefore crosslink CD3ε-expressing T cells with FcγR-expressing cells even in a cancer cell-absent environment, leading to production of various cytokines in large amounts. Such cancer antigen-independent induction of production of various cytokines restricts the current administration of trifunctional antibodies to an intraperitoneal route (NPL 8). Trifunctional antibodies are very difficult to administer systemically due to severe cytokine storm-like adverse reactions.

[0007]

Meanwhile, unlike catumaxomab, a bispecific T-cell engager (BiTE) has no Fcγ 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. Thus, it has been demonstrated that BiTE does not cause cancer antigen-independent cytokine induction which is observed when catumaxomab is administered. However, since BiTE 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.

[0008]

Recently, the use of an Fc region having reduced binding activity towards FcγR has provided a novel polypeptide complex that exhibits these features altogether, which are: antitumor activity exerted by keeping T cells in proximity to target cancer cells, outstanding

safety characteristics of not inducing cancer antigen-independent cytokine storm and the like, and a long blood half-life (PTL 1). Novel T cell redirecting antibodies having these beneficial effects were reported to be useful not only to target cancer cells but also to target regulatory T cells or exhausted T cells, which are cells present in cancer microenvironments and having functions to suppress immune responses (PTLs 2 and 3).

[Citation List]
[Patent Literature]
[0009]
[PTL 1] WO2012/073985
[PTL 2] WO2016/047722
[PTL 3] WO2015/174439
[Non-Patent Literature]
[0010]
[NPL 1] Nat. Biotechnol. (2005) 23, 1073-1078
[NPL 2] Eur J Pharm Biopharm. (2005) 59 (3), 389-396
[NPL 3] Drug Des Devel Ther (2009) 3, 7-16
[NPL 4] MAbs. (2012) Mar 1, 4(2)
[NPL 5] Nature (1985) 314 (6012), 628-31
[NPL 6] Int J Cancer (1988) 41 (4), 609-15.
[NPL 7] Proc Natl Acad Sci USA (1986) 83 (5), 1453-7
[NPL 8] Cancer Immunol Immunother. (2007) 56(9), 1397-406

[Summary of Invention]
[Technical Problem]
[0011]

The present invention was accomplished in view of the circumstances described above, with a specific focus on the CD3-binding domain used in T-cell redirecting antibodies. An objective of the present invention is to provide a novel CD3-binding domain with superior stability to CD3-binding domains that have been used in conventional T cell redirecting antibodies, antigen-binding molecules comprising the CD3-binding domain, methods for producing the antigen-binding molecules, pharmaceutical compositions comprising the antigen-binding molecules as an active ingredient, and uses thereof.

[Solution to Problem]
[0012]

The present inventors introduced various modifications to the CDRs of a CD3-binding Fab included in a bispecific antibody described in WO2015174439 and succeeded in obtaining variants having lower degree of decrease in CD3 binding activity when stored in environments at or above room temperature compared to the parent Fab. The present inventors further discovered that the degree of decrease in T cell-dependent cytotoxicity upon storage in environments at or above room temperature is also lower in bispecific antigen-binding molecules prepared by combining the modified CD3-binding domains with other antigen-binding domains such as cancer-specific antigen-binding domains. Based on these findings, the present inventors revealed that the multispecific antigen-binding molecules of the present invention can damage tissues comprising target cells expressing a target antigen such as a cancer-specific antigen.

[0013]

The present disclosure is based on such findings and specifically encompasses the embodiments described below as examples:

[1] an antigen-binding molecule comprising a first antigen-binding domain and a second antigen-binding domain, wherein the first antigen-binding domain comprises an antibody heavy chain (H chain) variable region and an antibody light chain (L chain) variable region having binding activity towards CD3,

wherein the H chain variable region comprises:

H chain CDR1 comprising the amino acid sequence NAWMH (SEQ ID NO: 1);

H chain CDR2 comprising the amino acid sequence QIX$_1$DKSQNYATX$_2$VAESVKG (SEQ ID NO: 2), wherein X$_1$ is K or R, and X$_2$ is Y or F; and

H chain CDR3 comprising the amino acid sequence VHYX$_3$AGYGVDX$_4$ (SEQ ID NO: 3), wherein X$_3$ is A or P, and X$_4$ is I, M, or L; and

wherein the L chain variable region comprises:

L chain CDR1 comprising the amino acid sequence RSX$_5$X$_6$X$_7$VVHENRX$_8$TYLH (SEQ ID NO: 4), wherein X$_5$ is S or T, X$_6$ is Q or M, X$_7$ is S or T, and X$_8$ is Q or N;

L chain CDR2 comprising the amino acid sequence KVSNRFS (SEQ ID NO: 5); and

L chain CDR3 comprising the amino acid sequence GQGTQVPYT (SEQ ID NO: 6);

[2] the antigen-binding molecule of [1], wherein the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise any one of the combinations of H chain CDR1, CDR2, and CDR3 and L chain CDR1, CDR2, and CDR3 selected from following (a1) to (a8):

(a1) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 7; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 10; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the

amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a2) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a3) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a4) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a5) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a6) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 17; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a7) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 18; L chain CDR2 comprising the

amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a8) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 19; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

[3] the antigen-binding molecule of [1] or [2], wherein the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise any one of the combinations of an H chain variable region and an L chain variable region selected from following (a1) to (a8):

(a1) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 20 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a2) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a3) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 26;

(a4) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a5) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 23 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a6) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 27;

(a7) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 28; and

(a8) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 29;

[4-1] the antigen-binding molecule of any one of [1] to [3], further comprising an Fc region;

[4-2] the antigen-binding molecule of any one of [1] to [3], further comprising an Fc region with reduced binding activity towards an Fcγ receptor;

[4-3] the antigen-binding molecule of [4-1] or [4-2], comprising any one of combinations of a heavy chain and a light chain selected from following (a1) to (a8):

(a1) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 30 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a2) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 31 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a3) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 31 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 36;

(a4) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a5) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 33 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a6) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 37;

(a7) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 38; and

(a8) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 39;

[5-1] the antigen-binding molecule of any one of [1] to [4], wherein the antigen-binding molecule is a monospecific antigen-binding molecule;

[5-2] the antigen-binding molecule of any one of [1] to [4], wherein the first antigen-binding domain and the second antigen-binding domain comprise the same amino acid sequence;

[5-3] the antigen-binding molecule of any one of [1] to [4], wherein the first antigen-binding domain and the second antigen-binding domain comprise amino acid sequences different from each other;

[6-1] the antigen-binding molecule of any one of [1] to [4], wherein the antigen-binding molecule is a multispecific antigen-binding molecule;

[6-2] the antigen-binding molecule of any one of [1] to [4], wherein the antigen-binding molecule is a bispecific antigen-binding molecule;

[6-3] the antigen-binding molecule of any one of [1] to [4], wherein the antigen-binding molecule has three or more specificities;

[7-1] the antigen-binding molecule of [6], wherein the second antigen-binding domain comprises an antibody variable region having binding activity towards a cancer antigen;

[7-2] the antigen-binding molecule of [6], wherein the second antigen-binding domain comprises an antibody variable region having binding activity towards a molecule that is expressed on the surface of a cell having a function to suppress an immune response;
[8] the antigen-binding molecule of any one of [1] to [7], wherein the antigen-binding molecule is an antibody;
[9] a nucleic acid encoding the antigen-binding molecule of any one of [1] to [8];
[10] a vector into which the nucleic acid of [9] has been introduced;
[11] a cell comprising the nucleic acid of [9] or the vector of [10];
[12] a method for producing the antigen-binding molecule of any one of [1] to [8], comprising culturing the cell of [11];
[13] an antigen-binding molecule produced by the method of [12];
[14] a pharmaceutical composition, comprising the antigen-binding molecule of any one of [1] to [8] and a pharmaceutically acceptable carrier;
[15-1] the pharmaceutical composition of [14], for inducing cytotoxicity;
[15-2] the pharmaceutical composition of [14], for inducing T-cell dependent cytotoxicity;
[15-3] the pharmaceutical composition of [14], for use in treatment or prevention of cancer;
[16-1] a method for inducing cytotoxicity, comprising administering the antigen-binding molecule of any one of [1] to [8] or the pharmaceutical composition of [14];
[16-2] a method for inducing T-cell dependent cytotoxicity, comprising administering the antigen-binding molecule of any one of [1] to [8] or the pharmaceutical composition of [14];
[16-3] a method for treating or preventing cancer, comprising administering the antigen-binding molecule of any one of [1] to [8] or the pharmaceutical composition of [14];
[17-1] a kit for inducing cytotoxicity, comprising the antigen-binding molecule of any one of [1] to [8] or the pharmaceutical composition of [14] and instructions for use;
[17-2] a kit for inducing T-cell dependent cytotoxicity, comprising the antigen-binding molecule of any one of [1] to [8] or the pharmaceutical composition of [14] and instructions for use;
[17-3] a kit for treating or preventing cancer, comprising the antigen-binding molecule of any one of [1] to [8] or the pharmaceutical composition of [14] and instructions for use;
[18-1] the antigen-binding molecule of any one of [1] to [8], for use in inducing cytotoxicity;
[18-2] the antigen-binding molecule of any one of [1] to [8], for use in inducing T-cell dependent cytotoxicity;
[18-3] the antigen-binding molecule of any one of [1] to [8], for use in treatment or prevention of cancer;
[19-1] use of the antigen-binding molecule of any one of [1] to [8] in the manufacture of an agent for inducing cytotoxicity;

[19-2] use of the antigen-binding molecule of any one of [1] to [8] in the manufacture of an agent for inducing T-cell dependent cytotoxicity;

[19-3] use of the antigen-binding molecule of any one of [1] to [8] in the manufacture of an agent for treatment or prevention of cancer;

[A1] a monovalent antigen-binding molecule comprising a single antigen-binding domain, wherein the single antigen-binding domain comprises an antibody H chain variable region and an antibody L chain variable region having binding activity towards CD3,

wherein the H chain variable region comprises:

H chain CDR1 comprising the amino acid sequence NAWMH (SEQ ID NO: 1);

H chain CDR2 comprising the amino acid sequence QIX$_1$DKSQNYATX$_2$VAESVKG (SEQ ID NO: 2), wherein X_1 is K or R, and X_2 is Y or F; and

H chain CDR3 comprising the amino acid sequence VHYX$_3$AGYGVDX$_4$ (SEQ ID NO: 3), wherein X_3 is A or P, and X_4 is I, M, or L; and

wherein the L chain variable region comprises:

L chain CDR1 comprising the amino acid sequence RSX$_5$X$_6$X$_7$VVHENRX$_8$TYLH (SEQ ID NO: 4), wherein X_5 is S or T, X_6 is Q or M, X_7 is S or T, and X_8 is Q or N;

L chain CDR2 comprising the amino acid sequence KVSNRFS (SEQ ID NO: 5); and

L chain CDR3 comprising the amino acid sequence GQGTQVPYT (SEQ ID NO: 6);

[A2] the antigen-binding molecule of [A1], wherein the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise any one of the combinations of H chain CDR1, CDR2, and CDR3 and L chain CDR1, CDR2, and CDR 3 selected from following (a1) to (a8):

(a1) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 7; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 10; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a2) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a3) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8; H chain

CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a4) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a5) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a6) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 17; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a7) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 18; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a8) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 19; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

[A3] the antigen-binding molecule of [A1] or [A2], wherein the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise

any one of the combinations of an H chain variable region and an L chain variable region selected from following (a1) to (a8):

(a1) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 20 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a2) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a3) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 26;

(a4) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a5) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 23 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a6) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 27;

(a7) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 28; and

(a8) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 29;

[A4-1] the antigen-binding molecule of any one of [A1] to [A3], further comprising an Fc region;

[A4-2] the antigen-binding molecule of any one of [A1] to [A3], further comprising an Fc region with reduced binding activity towards an Fcγ receptor;

[A4-3] the antigen-binding molecule of [A4-1] or [A4-2], comprising any one of combinations of a heavy chain and a light chain selected from following (a1) to (a8):

(a1) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 30 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a2) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 31 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a3) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 31 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 36;
(a4) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;
(a5) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 33 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;
(a6) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 37;
(a7) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 38; and
(a8) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 39;
[A5] a nucleic acid encoding the antigen-binding molecule of any one of [A1] to [A4];
[A6] a vector into which the nucleic acid of [A5] has been introduced;
[A7] a cell comprising the nucleic acid of [A5] or the vector of [A6];
[A8] a method for producing the antigen-binding molecule of any one of [A1] to [A4], comprising culturing the cell of [A7]; and
[A9] an antigen-binding molecule produced by the method of [A8].

[Description of Embodiments]
[0014]
I. Definitions

The definitions below are provided to help understanding of the present invention illustrated herein.

[0015]

The term “antigen binding molecule” refers to a molecule that is capable of binding to its antigen with sufficient affinity. In one embodiment, the extent of binding of an antigen binding molecule to a protein unrelated to its antigen is less than about 10% of the binding of the antigen binding molecule to the antigen as measured, e.g., by a radioimmunoassay (RIA). In certain embodiments, an antigen binding molecule has a dissociation constant (Kd) of 1 μM or less, 100 nM or less, 10 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, e.g. from 10^{-8} M to 10^{-13} M, e.g., from 10^{-9} M to 10^{-13} M) to its antigen. In certain embodiments, an antigen binding molecule binds to an epitope of the antigen that is conserved among the antigen from different species. In some embodiments, the antigen binding molecule is an antibody.

[0016]

Herein, “antibody” refers to a natural immunoglobulin or an immunoglobulin produced by partial or complete synthesis. Antibodies can be isolated from natural sources such as naturally-occurring plasma and serum, or culture supernatants of antibody-producing hybridomas. Alternatively, antibodies can be partially or completely synthesized using techniques such as genetic recombination. Preferred antibodies include, for example, antibodies of an immunoglobulin isotype or subclass belonging thereto. Known human immunoglobulins include antibodies of the following nine classes (isotypes): IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, and IgM. Of these isotypes, antibodies of the present invention include IgG1, IgG2, IgG3, and IgG4.

[0017]

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., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively.

[0018]

“Native antibodies” refer to naturally occurring immunoglobulin molecules with varying structures. For example, native IgG antibodies are heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light chains and two identical heavy chains that are disulfide-bonded. From N- to C-terminus, each heavy chain has a variable region (VH), also called a variable heavy domain or a heavy chain variable domain, followed by three constant domains (CH1, CH2, and CH3). Similarly, from N- to C-terminus, each light chain has a variable region (VL), also called a variable light domain or a light chain variable domain, followed by a constant light (CL) domain. The light chain of an antibody may be assigned to one of two types, called kappa (κ) and lambda (λ), based on the amino acid sequence of its constant domain.

[0019]

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).

[0020]

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).

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*.

[0021]

"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.

[0022]

The term "Fc region" 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 Gly446-Lys447) of the Fc region may or may not be present. In one embodiment, the C-terminal lysine (Lys447) or glycine-lysine (Gly446-Lys447) of the Fc region may be removed by degradation. 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.

[0023]

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof.

[0024]

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one amino acid modification, preferably one or more amino acid substitution(s). Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, e.g. from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% sequence identity with a native sequence Fc region and/or with an Fc region of a parent polypeptide, more preferably at least about 90% sequence identity therewith, and most preferably at least about 95% sequence identity therewith.

[0025]

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 FcγRI, FcγRII, and FcγRIII subclasses, including allelic variants and alternatively spliced forms of those receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB 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.

[0026]

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*.).

[0027]

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. A blood half life or serum half life may be measured in place of the plasma half life. 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).

[0028]

The term “Fc region-comprising antibody” refers to an antibody that comprises an Fc region. The C-terminal lysine (residue 447 according to the EU numbering system) or C-terminal glycine-lysine (residues 446-447) of the Fc region may be removed, for example, during purification of the antibody or by recombinant engineering of the nucleic acid encoding the antibody. Accordingly, a composition comprising an antibody having an Fc region according to this invention can comprise an antibody with G446-K447, with G446 and without K447, with all G446-K447 removed (in the case of an IgG, an antibody in which the C-terminus is P445 or an antibody in which the C-terminus is prolinamide (Pro-NH_2) in which an amide group (NH_2) is added to P445), or a mixture of three types of antibodies described above.

[0029]

“Effector functions” refer to those biological activities attributable to the Fc region of an antibody, which vary with the antibody isotype. Examples of antibody effector functions include: C1q binding and complement dependent cytotoxicity (CDC); Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down-regulation of cell surface receptors (e.g. B cell receptor); and B cell activation.

[0030]

“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 FcγRIII only, whereas monocytes

express FcγRI, FcγRII, and FcγRIII. 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).

[0031]

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies composing the population are identical and/or bind the same epitope (determinant), except for possible variant antibodies, e.g., containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different epitopes, each monoclonal antibody comprised in a monoclonal antibody preparation is directed against a single epitope on an antigen. Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including but not limited to the hybridoma method, recombinant DNA methods, phage-display methods, and methods utilizing transgenic animals containing all or part of the human immunoglobulin loci.

[0032]

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.

[0033]

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.

[0034]

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.

[0035]

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.

[0036]

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 antigen-binding molecules formed from antibody fragments.

[0037]

Herein, the term “variable fragment (Fv)” refers to the minimum unit of an antibody-derived antigen-binding domain 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 Fv 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.

[0038]

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, while lacking the constant regions. 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.

[0039]

scFv is an antigen-binding domain 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.

[0040]

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.

[0041]

Herein, the form of an antigen-binding domain forming an sc(Fv)2 include an antigen-binding molecule in which the two VH units and two VL units 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. Example order of the form is 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]

[0042]

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 antigen-binding molecules disclosed herein.

[0043]

Furthermore, the antigen-binding molecules of the present invention may be conjugated with a carrier polymer such as PEG or an organic compound such as an anticancer agent. Alternatively, a sugar chain may be preferably added to the antigen-binding molecules by insertion of a sugar chain addition sequence to the amino acid sequences of the antigen-binding molecules of the present invention in order to obtain a desired effect from the sugar chain.

[0044]

As linkers for linking the variable regions of an antibody, it is possible to use any peptide linkers that can be introduced by genetic engineering, or synthetic compound linkers (linkers disclosed in, for example, Protein Engineering, 9(3), 299-305, 1996). However, peptide

linkers are preferred in the present invention. 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. As an example of a peptide linker, flexible polypeptide linkers formed of glycine and serine are widely known. The length or sequences of peptide linkers can be selected accordingly by those skilled in the art depending on the purpose.

[0045]

Synthetic chemical linkers (chemical crosslinking agents) are crosslinking agents routinely used for crosslinking peptides, and are for example:
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.

[0046]

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.

[0047]

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

[0048]

“F(ab’)2” and “Fab” are produced by treating an immunoglobulin (monoclonal antibody) with a protease such as pepsin and papain, and refer to an antibody fragment generated by digesting an immunoglobulin (monoclonal antibody) around 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 CHγ1 (γ1 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'.

[0049]

"F(ab')2" consists of two light chains and two heavy chains comprising the constant regions of a CH1 domain and a portion of CH2 domain so that disulfide bonds are formed between the two heavy chains. The F(ab')2 forming an antigen-binding molecule disclosed herein can be preferably produced as follows. A whole monoclonal antibody or the like comprising a desired antigen-binding domain 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.

[0050]

"Affinity" refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., an antibody) and its binding partner (e.g., an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair (e.g., antibody and antigen). The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd). Affinity can be measured by common methods known in the art, including those described herein. Specific illustrative and exemplary embodiments for measuring binding affinity are described in the following.

[0051]

The term "specific binding" means that one of the molecules involved in specific binding does not show any significant binding to molecules other than a single or a number of binding partner molecules. Furthermore, the term is also used when a domain containing an antibody variable region is specific to a particular epitope among multiple epitopes in an antigen. When an epitope bound by a domain containing an antibody variable region is included in a number of different antigens, antigen-binding molecules comprising the antibody variable region-containing domain can bind to various antigens that have the epitope.

[0052]

The term "epitope" denotes the site on an antigen, either proteinaceous or non-proteinaceous, to which an antigen-binding molecule (for example, an antibody) binds. For example, the epitope can be defined by its structure. Alternatively, the epitope may be defined by the antigen-binding activity of the antigen-binding molecule (for example, an antibody)

recognizing the epitope. When the antigen is a peptide or a polypeptide, the epitope may be identified by amino acid residues constituting the epitope. When the epitope is a sugar chain, the epitope may be identified by a particular sugar chain structure.

[0053]

A linear epitope refers to an epitope comprising an epitope that is recognized by its primary structure of amino acids. The linear epitope contains typically at least 3 and most commonly at least 5, for example, about 8 to about 10 or 6 to 20 amino acids, in its unique sequence.

[0054]

In contrast to the linear epitope, a conformational epitope refers to an epitope that is composed of discontinuous amino acid residues (also referred to as a discontinuous epitope). Amino acids that constitute an epitope typically do not exist as a continuous stretch of amino acids in a primary structure. The conformational epitope may contain an increased number of amino acids, as compared with the linear epitope. As for the recognition of the conformational epitope, the antigen-binding molecule (for example, an antibody) recognizes the three-dimensional structure of the peptide or the protein. For example, when a protein molecule is folded to form a three-dimensional structure, certain amino acids and/or polypeptide backbone constituting the conformational epitope are arranged in parallel to allow the antigen-binding molecule (for example, an antibody) to recognize the epitope. Examples of the method for determining the conformation of the epitope include, but are not limited to, X-ray crystallography, two-dimensional nuclear magnetic resonance spectroscopy, and site-specific spin labeling and electron paramagnetic resonance spectroscopy. See, for example, Epitope Mapping Protocols in Methods in Molecular Biology (1996), Vol. 66, Morris ed.

[0055]

The structure of the antigen-binding molecule (for example, an antibody) binding to the epitope is called a “paratope”, “antigen-binding site”, “antigen-binding region”, or “antigen-binding domain”. The paratope stably binds to the epitope through a hydrogen bond, electrostatic force, van der Waals forces, a hydrophobic bond, or the like acting between the epitope and the paratope. This binding force between the epitope and the paratope is called affinity. The total binding force when a plurality of antigen-binding molecules (for example, antibodies) bind to a plurality of antigens is called avidity. The affinity works synergistically when, for example, an antigen-binding molecule comprising a plurality of antigen binding domains (i.e., a polyvalent antigen-binding molecule) binds to a plurality of epitopes. Therefore, the avidity is higher than the affinity.

[0056]

A "competing antigen-binding molecule" for a reference antigen-binding molecule refers to an antigen-binding molecule that blocks binding of the reference antigen-binding molecule to its antigen in a competition assay by 50% or more, and conversely, the reference antigen-binding molecule blocks binding of the antigen-binding molecule to its antigen in a competition assay by 50% or more. Competing antigen-binding molecules include antigen-binding molecules binding to the same or an overlapping epitope as the reference antigen-binding molecule and antigen-binding molecules binding to an adjacent epitope sufficiently proximal to the epitope bound by the reference antigen-binding molecule for steric hindrance to occur. An exemplary competition assay is provided herein.

[0057]

An "isolated" antibody is one which has been separated from a component of its natural environment. In some embodiments, an antibody is purified to greater than 95% or 99% purity as determined by, for example, electrophoretic (e.g., SDS-PAGE, isoelectric focusing (IEF), capillary electrophoresis) or chromatographic (e.g., ion exchange or reverse phase HPLC). For review of methods for assessment of antibody purity, see, e.g., Flatman et al., *J. Chromatogr. B* 848:79-87 (2007).

[0058]

An "isolated" nucleic acid refers to a nucleic acid molecule that has been separated from a component of its natural environment. An isolated nucleic acid 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.

[0059]

"Isolated nucleic acid encoding an antigen-binding molecule" refers to one or more nucleic acid molecules encoding antigen-binding molecules, which preferably comprises antibody heavy and light chain variable regions, including such nucleic acid molecule(s) in a single vector or separate vectors, and such nucleic acid molecule(s) present at one or more locations in a host cell.

[0060]

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." The vector can be introduced into a host cell by a method using a virus, an

electroporation method, or the like, but the introduction of a vector is not limited to *in vitro* introduction, and it is also possible to directly introduce a vector into a body.

[0061]

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

[0062]

The terms "pharmaceutical formulation" and "pharmaceutical composition" refer 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.

[0063]

A "pharmaceutically acceptable carrier" refers to an ingredient in a pharmaceutical formulation or in a pharmaceutical composition, 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.

[0064]

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

[0065]

An "effective amount" of an agent, e.g., a pharmaceutical formulation, refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result.

[0066]

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.

[0067]

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, antibodies of the invention are used to delay development of a disease or to slow the progression of a disease.

[0068]

The terms “cell proliferative disorder” and “proliferative disorder” refer to disorders that are associated with some degree of abnormal cell proliferation. In one embodiment, the cell proliferative disorder is cancer.

[0069]

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.

[0070]

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.

[0071]

“Inhibiting cell growth or proliferation” means decreasing a cell’s growth or proliferation by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100%, and includes inducing cell death.

[0072]

“Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, Megalign (DNASTAR) software, or GENETYX (registered trademark) (Genetyx Co., Ltd.). Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

[0073]

The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, California, or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, including digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

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

[0074]

The term "and/or" herein is used to indicate any one of the subjects shown before and after "and/or", or any combination thereof. For example, "A, B, and/or C" includes the individual

subjects "A", "B", and "C", and also combinations "A and B", "A and C", "B and C", and "A and B and C".

[0075]

II. Detailed Description of the Invention

The present disclosure relates generally to antigen-binding molecules comprising a CD3-binding domain with excellent stability, pharmaceutical compositions comprising them, and uses thereof. More specifically, the antigen-binding molecules of the present invention comprise a CD3-binding domain having improved stability compared to a CD3-binding domain included in a conventional T-cell redirecting antibody (such as, the bispecific antibodies described in WO2015174439). The antigen-binding molecules of the present invention are not particularly limited in terms of their structures, as long as they are molecules comprising the CD3-binding domain of the present invention which has improved stability. They may be molecules having naturally occurring antibody structures or may be polypeptides having other artificially designed structures.

[0076]

In some embodiments, the antigen-binding molecules of the present invention are antibodies. In some embodiments, the antigen-binding molecules of the present invention are monoclonal antibodies, including chimeric, humanized or human antibodies. In one embodiment, the antigen-binding molecules of the present invention are antibody fragments, e.g., a Fv, Fab, Fab', scFv, diabody, single domain antibody, or $F(ab')_2$ fragment. In another embodiment, the antibodies are full length antibodies, e.g., intact IgG1 antibodies, intact IgG2 antibodies, intact IgG3 antibodies, or intact IgG4 antibodies.

[0077]

Antigen-binding molecules comprising CD3-binding domains with excellent stability

In one aspect, the present invention provides antigen-binding molecules comprising a first antigen-binding domain having binding activity towards CD3 (a CD3-binding domain) and a second antigen-binding domain. In another aspect, the present invention provides monovalent antigen-binding molecules comprising a single antigen-binding domain having binding activity towards CD3 (a CD3-binding domain). The CD3-binding domains included in the antigen-binding molecules of the present invention have excellent stability and are characterized in that decreases in their CD3 binding activity after storage at or above room temperature are small. In one embodiment, the CD3-binding domains included in the antigen-binding molecules of the present invention maintain their CD3-binding activity after being stored at or above room temperature (for example, at 50°C) for a certain period of time (for example, for three days) at a level of 65% or more, 70% or more, or 75% or more of the CD3 binding activity observed after storage at a lower temperature (for example, 4°C). In some embodiments, the CD3-binding

domains included in the antigen-binding molecules of the present invention have binding activity towards CD3ε chain. In some embodiments, CD3 is mammalian CD3. In certain embodiments, CD3 is human CD3. In some embodiments, the antigen-binding domains included in the antigen-binding molecules of the present invention are Fv, Fab, Fab', or scFv.

[0078]

CD3-binding domains

In one embodiment, the CD3-binding domains included in the antigen-binding molecules of the present invention comprise an antibody H chain variable region and an antibody L chain variable region having binding activity towards CD3,

the H chain variable region comprising:

H chain CDR1 comprising the amino acid sequence NAWMH (SEQ ID NO: 1);

H chain CDR2 comprising the amino acid sequence QIX_1DKSQNYATX_2VAESVKG (SEQ ID NO: 2), wherein X_1 is K or R, and X_2 is Y or F; and

H chain CDR3 comprising the amino acid sequence VHYX_3AGYGVDX_4 (SEQ ID NO: 3), wherein X_3 is A or P, and X_4 is I, M, or L; and

the L chain variable region comprising:

L chain CDR1 comprising the amino acid sequence RS$X_5X_6X_7$VVHENRX_8TYLH (SEQ ID NO: 4), wherein X_5 is S or T, X_6 is Q or M, X_7 is S or T, and X_8 is Q or N;

L chain CDR2 comprising the amino acid sequence KVSNRFS (SEQ ID NO: 5); and

L chain CDR3 comprising the amino acid sequence GQGTQVPYT (SEQ ID NO: 6).

In the present disclosure, the terms "H chain" and "heavy chain" are used interchangeably, and the terms "L chain" and "light chain" are used interchangeably.

[0079]

In certain embodiments, the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise any one of the combinations of H chain CDR1, CDR2, and CDR3 and L chain CDR1, CDR2, and CDR3, selected from following (a1) to (a8):

(a1) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 7; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 10; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a2) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; L chain CDR1

comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a3) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a4) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a5) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a6) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 17; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a7) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 18; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6; and

(a8) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1

comprising the amino acid sequence set forth in SEQ ID NO: 19; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6.

[0080]

In one embodiment, the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise any one of the combinations of an H chain variable region and an L chain variable region, selected from following (a1) to (a8):

(a1) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 20 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 25;

(a2) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 25;

(a3) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 26;

(a4) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 25;

(a5) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 23 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 25;

(a6) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 27;

(a7) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 28; and

(a8) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 29.

[0081]

In one embodiment, the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise any one of the combinations of an H chain variable region and an L chain variable region, selected from following (a1) to (a8):

(a1) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 20 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 7, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 10 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 25 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a2) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 21 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 25 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a3) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 21 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 26 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a4) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 22 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 25 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a5) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 23 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 25 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a6) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 22 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 27 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 17, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a7) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 22 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 28 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 18, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6; and

(a8) the combination of an H chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 22 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12 and an L chain variable region having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 29 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 19, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6.

[0082]

In one embodiment, the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise any one of the combinations of an H chain variable region and an L chain variable region, selected from following (a1) to (a8):

(a1) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 20 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a2) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a3) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 26;

(a4) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a5) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 23 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a6) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 27;

(a7) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 28; and

(a8) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 29.

[0083]

In one embodiment, the antigen-binding molecules of the present invention comprise any one of the combinations of a heavy chain and a light chain, selected from following (a1) to (a8), as a heavy chain and a light chain having binding activity towards CD3:

(a1) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 30 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 35;

(a2) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 31 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 35;

(a3) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 31 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 36;

(a4) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 32 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 35;

(a5) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 33 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 35;

(a6) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 32 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 37;

(a7) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 32 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 38; and

(a8) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 32 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 39.

[0084]

In one embodiment, the antigen-binding molecules of the present invention comprise any one of the combinations of a heavy chain and a light chain, selected from following (a1) to (a8), as a heavy chain and a light chain having binding activity towards CD3:

(a1) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 30 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 7, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 10 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 35 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a2) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 31 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 35 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a3) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 31 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 36 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a4) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 32 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 35 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a5) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 33 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 35 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a6) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 32 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 37 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 17, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a7) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 32 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 38 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 18, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6; and

(a8) the combination of a heavy chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 32 and comprising H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12 and a light chain having at least 90% or at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO: 39 and comprising L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 19, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6.

[0085]

In a certain embodiment, the antigen-binding molecules of the present invention comprise any one of the combinations of a heavy chain and a light chain, selected from following (a1) to (a8), as a heavy chain and a light chain having binding activity towards CD3:

(a1) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 30 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a2) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 31 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a3) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 31 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 36;

(a4) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a5) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 33 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 35;

(a6) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 37;

(a7) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 38; and

(a8) the combination of a heavy chain comprising the amino acid sequence set forth in SEQ ID NO: 32 and a light chain comprising the amino acid sequence set forth in SEQ ID NO: 39.

[0086]

Second antigen-binding domain

In the antigen-binding molecules of the present invention, the second antigen-binding domain is not particularly limited in terms of its structure, as long as it has binding activity towards its target antigen. In some embodiments, the second antigen-binding domain comprises an antibody H chain variable region and an antibody L chain variable region.

[0087]

Monospecific antigen-binding molecules

In some embodiments, the antigen-binding molecules of the present invention are monospecific antigen-binding molecules, and their first antigen-binding domain and second antigen-binding domain both have binding activity towards CD3 (for example, human CD3). In one embodiment, the first antigen-binding domain and the second antigen-binding domain have binding activity towards the same epitope in CD3. In certain embodiments, the first antigen-binding domain and the second antigen-binding domain both have binding activity towards CD3ε chain (for example, human CD3ε chain).

In certain embodiments, the first antigen-binding domain and the second antigen-binding domain comprise the same amino acid sequence. In other certain embodiments, the first antigen-binding domain and the second antigen-binding domain comprise amino acid sequences different from each other.

[0088]

Multispecific antigen-binding molecules

In some embodiments, the antigen-binding molecules of the present invention are multispecific antigen-binding molecules having binding specificities towards at least two different sites. In certain embodiments, the multispecific antigen-binding molecules of the present invention are bispecific antigen-binding molecules. In other certain embodiments, the multispecific antigen-binding molecules of the present invention are antigen-binding molecules having three or more specificities and, for example, are trispecific antigen-binding molecules, tetraspecific antigen-binding molecules, pentaspecific antigen-binding molecules, or hexaspecific antigen-binding molecules.

[0089]

In one embodiment of the multispecific antigen-binding molecules of the present invention, the first antigen-binding domain has binding activity towards CD3 and the second antigen-binding domain has binding activity towards an antigen other than CD3. In such an embodiment, the "antigen other than CD3" that the second antigen-binding domain recognizes and binds to is preferably a molecule that is expressed on the surface of a cell that is a target of cytotoxicity by T cells (T cell dependent cytotoxicity). Examples are molecules specifically expressed on the surface of cancer cells (referred to as cancer antigens or cancer specific antigens herein) and molecules expressed on the surfaces of cells that have functions to suppress immune responses (referred to as immunosuppressive cell surface antigens herein).

[0090]

In one embodiment, the second antigen-binding domain has binding activity towards a cancer antigen. In such embodiments, the multispecific antigen-binding molecules of the present invention bind to CD3 via the first antigen-binding domain and to a cancer antigen via the second antigen-binding domain, thereby allowing T cells to be brought into close proximity with

the target cancer cells. This enables the treatment or prevention of cancer through cytotoxicity by T cells against tissues containing the target cancer cells.

In different embodiments, the second antigen-binding domain has binding activity towards an immunosuppressive cell surface antigen. In such embodiments, the multispecific antigen-binding molecules of the present invention bind to CD3 via the first antigen-binding domain and to an immunosuppressive cell surface antigen via the second antigen-binding domain, thereby allowing T cells to be brought into close proximity with cells having functions to suppress immune responses (referred to as immunosuppressive cells herein). This enables the treatment or prevention of cancer through cytotoxicity by T cells against immune response suppressing cells.

In the multispecific antigen-binding molecules of the present invention, antigen-binding domains that have binding activity towards a cancer antigen or an immune suppressor cell surface antigen - including known domains and those that may be identified in the future - can be used as a second antigen-binding domain.

[0091]

In preferred embodiments, the multispecific antigen-binding molecules of the present invention have comparable or higher cytotoxicity, compared to the cytotoxicity of a control multispecific antigen-binding molecule that comprises, as its first antigen-binding domain having binding activity towards CD3, an antibody H chain variable region that comprises H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1, H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 7, and H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 10 (for example, an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 20) and an antibody L chain variable region that comprises L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 14, L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5, and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6 (for example, an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 24). Examples of such multispecific antigen-binding molecules include multispecific antigen-binding molecules (for example, bispecific antigen-binding molecules, or bispecific antibodies) comprising heavy and light chain CDRs, heavy and light chain variable regions, or heavy and light chains of antibody Nos. 2, 14, 25, 29, 30-32, and 34 shown in Table 5 described later as their first antigen-binding domains having binding activity towards CD3.

[0092]

Domain comprising an antibody variable region that has CD3-binding activity

Herein, the phrases “an antibody variable region that has CD3-binding activity” and “a domain comprising an antibody variable region that has CD3-binding activity” are used mutually

interchangeably, and refer to an anti-CD3 antibody portion that comprises a region that specifically binds to all or a portion of CD3, which is an adapter molecule constituting the T cell receptor complex along with the T cell receptor, and is also complementary thereto. Preferably, the domain comprises the light-chain and heavy-chain variable regions (VL and VH) of an anti-CD3 antibody. Suitable examples of such a domain include "single chain Fv (scFv)", "single chain antibody", "Fv", "single chain Fv 2 (scFv2)", "Fab", "F(ab')2", etc.

[0093]

The CD3-binding domain (the domain comprising an antibody variable region that has CD3-binding activity) of the present invention may be any epitope-binding domain as long as the epitope exists in the γ-chain, δ-chain, or ε-chain sequence that constitutes CD3. In the present invention, preferably, a domain comprising an anti-CD3 antibody light-chain variable region (VL) and heavy-chain variable region (VH) that bind to an epitope present in the extracellular region of the ε chain of the human CD3 complex is suitably used. The anti-CD3 antibody light chain variable region (VL) and anti-CD3 antibody heavy chain variable region (VH) described in the Examples are suitably used as such domains. Human antibodies and properly humanized antibodies as described above may be appropriately used as the source anti-CD3 antibody for the domain containing the antibody variable region having CD3-binding activity. Regarding the structure of the γ-chain, δ-chain, and ε-chain constituting CD3, their polynucleotide sequences are respectively registered as RefSeq accession numbers NM_000073.2, NM_000732.4, and NM_000733.3, and their polypeptide sequences are respectively registered as RefSeq accession numbers NP_000064.1, NP_000723.1, and NP_000724.1.

[0094]

<u>Domain comprising an antibody variable region having binding activity toward an antigen other than CD3</u>

Herein, the phrases "an antibody variable region having binding activity toward an antigen other than CD3" and "a domain comprising an antibody variable region having binding activity toward an antigen other than CD3" are used mutually interchangeably, and refer to an antibody portion that comprises a region that specifically binds to all or a portion of the antigen (e.g., a cancer antigen - also referred to herein as a cancer-specific antigen), and is also complementary thereto. Domains comprising an antibody variable region may be provided from variable domains of one or a plurality of antibodies. Preferably, domains comprising an antibody variable region comprise antibody light-chain and heavy-chain variable regions (VL and VH). Suitable examples of such domains comprising antibody variable regions include "single chain Fv (scFv)", "single chain antibody", "Fv", "single chain Fv 2 (scFv2)", "Fab", "F(ab')2", etc.

[0095]

Antigen

In the present specification, the “antigen” is limited only by containing an epitope to which the antigen binding domain binds. Preferred examples of the antigen include, but are not limited to, animal- or human-derived peptides, polypeptides, and proteins. Preferred examples of the antigen for use in the treatment of a disease caused by a target tissue include, but are not limited to, molecules expressed on the surface of target cells (e.g., cancer cells and inflammatory cells), molecules expressed on the surface of other cells in tissues containing target cells, molecules expressed on the surface of cells having an immunological role against target cells and tissues containing target cells, and large molecules present in the stromata of tissues containing target cells.

[0096]

In one embodiment, antigens may be derived from any animal species (for example, human; or nonhuman animals such as mouse, rat, hamster, guinea pig, rabbit, monkey, cynomolgus monkey, Rhesus monkey, hamadryas baboon, chimpanzee, goat, sheep, dog, horse, pig, bovine, or camel), or any birds; and the antigens are preferably derived from human, rabbit, monkey, rat, or mouse.

[0097]

The “antigen” which is recognized and bound by the antigen-binding molecule of the present invention (e.g., CD3) refers to any native “antigen” (e.g., CD3) from any vertebrate source, including mammals such as primates (e.g. humans) and rodents (e.g., mice and rats), unless otherwise indicated. The term encompasses “full-length” unprocessed “antigen” (e.g., CD3) as well as any form of “antigen” (e.g., CD3) that results from processing in the cell. These terms also encompass naturally occurring variants of the “antigen” (e.g., CD3), e.g., splice variants or allelic variants.

[0098]

In some embodiments, examples of the antigen include the following molecules: 17-IA, 4-1BB, 4Dc, 6-keto-PGF1a, 8-iso-PGF2a, 8-oxo-dG, A1 adenosine receptor, A33, ACE, ACE-2, activin, activin A, activin AB, activin B, activin C, activin RIA, activin RIA ALK-2, activin RIB ALK-4, activin RIIA, activin RIIB, ADAM, ADAM10, ADAM12, ADAM15, ADAM17/TACE, ADAM8, ADAM9, ADAMTS, ADAMTS4, ADAMTS5, addressin, aFGF, ALCAM, ALK, ALK-1, ALK-7, alpha-1-antitrypsin, alpha-V/beta-1 antagonist, ANG, Ang, APAF-1, APE, APJ, APP, APRIL, AR, ARC, ART, artemin, anti-Id, ASPARTIC, atrial natriuretic factor, av/b3 integrin, Axl, b2M, B7-1, B7-2, B7-H, B-lymphocyte stimulator (BlyS), BACE, BACE-1, Bad, BAFF, BAFF-R, Bag-1, BAK, Bax, BCA-1, BCAM, Bcl, BCMA, BDNF, b-ECGF, bFGF, BID, Bik, BIM, BLC, BL-CAM, BLK, BMP, BMP-2 BMP-2a, BMP-3/osteogenin, BMP-4, BMP-2b, BMP-5, BMP-6 Vgr-1, BMP-7 (OP-1), BMP-8 (BMP-8a, OP-2), BMPR, BMPR-IA (ALK-3),

BMPR-IB (ALK-6), BRK-2, RPK-1, BMPR-II (BRK-3), BMP, b-NGF, BOK, bombesin, bone-derived neurotrophic factor, BPDE, BPDE-DNA, BTC, complement factor 3 (C3), C3a, C4, C5, C5a, C10, CA125, CAD-8, calcitonin, cAMP, carcinoembryonic antigen (CEA), cancer-associated antigens, cathepsin A, cathepsin B, cathepsin C/DPPI, cathepsin D, cathepsin E, cathepsin H, cathepsin L, cathepsin O, cathepsin S, cathepsin V, cathepsin X/Z/P, CBL, CCI, CCK2, CCL, CCL1, CCL11, CCL12, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL2, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27, CCL28, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9/10, CCR, CCR1, CCR10, CCR10, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CD1, CD2, CD3, CD3E, CD4, CD5, CD6, CD7, CD8, CD10, CD11a, CD11b, CD11c, CD13, CD14, CD15, CD16, CD18, CD19, CD20, CD21, CD22, CD23, CD25, CD27L, CD28, CD29, CD30, CD30L, CD32, CD33 (p67 protein), CD34, CD38, CD40, CD40L, CD44, CD45, CD46, CD49a, CD52, CD54, CD55, CD56, CD61, CD64, CD66e, CD74, CD80 (B7-1), CD89, CD95, CD123, CD137, CD138, CD140a, CD146, CD147, CD148, CD152, CD164, CEACAM5, CFTR, cGMP, CINC, botulinum toxin, *Clostridium perfringens* toxin, CKb8-1, Claudin-6, CLC, CMV, CMV UL, CNTF, CNTN-1, COX, C-Ret, CRG-2, CT-1, CTACK, CTGF, CTLA-4, PD1, PDL1, LAG3, TIM3, galectin-9, CX3CL1, CX3CR1, CXCL, CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL15, CXCL16, CXCR, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, cytokeratin tumor-associated antigens, DAN, DCC, DcR3, DC-SIGN, decay accelerating factor, des(1-3)-IGF-I (brain IGF-1), Dhh, digoxin, DLL3, DNAM-1, Dnase, Dpp, DPPIV/CD26, Dtk, ECAD, EDA, EDA-A1, EDA-A2, EDAR, EGF, EGFR (ErbB-1), EMA, EMMPRIN, ENA, endothelin receptor, enkephalinase, eNOS, Eot, eotaxin 1, EpCAM, ephrin B2/EphB4, EPO, ERCC, E-selectin, ET-1, factor IIa, factor VII, factor VIIIc, factor IX, fibroblast-activating protein (FAP), Fas, FcR1, FEN-1, ferritin, FGF, FGF-19, FGF-2, FGF-3, FGF-8, FGFR, FGFR-3, fibrin, FL, FLIP, Flt-3, Flt-4, follicle-stimulating hormone, fractalkine, FZD1, FZD2, FZD3, FZD4, FZD5, FZD6, FZD7, FZD8, FZD9, FZD10, G250, Gas6, GCP-2, GCSF, GD2, GD3, GDF, GDF-1, GDF-3 (Vgr-2), GDF-5 (BMP-14, CDMP-1), GDF-6 (BMP-13, CDMP-2), GDF-7 (BMP-12, CDMP-3), GDF-8 (myostatin), GDF-9, GDF-15 (MIC-1), GDNF, GDNF, GFAP, GFRa-1, GFR-alpha 1, GFR-alpha 2, GFR-alpha 3, GITR, glucagon, Glut4, glycoprotein IIb/IIIa (GPIIb/IIIa), GM-CSF, gp130, gp72, GRO, growth hormone-releasing factor, hapten (NP-cap or NIP-cap), HB-EGF, HCC, HCMV gB envelope glycoprotein, HCMV gH envelope glycoprotein, HCMV UL, hematopoietic growth factor (HGF), Hep B gp120, heparanase, Her2, Her2/neu (ErbB-2), Her3 (ErbB-3), Her4 (ErbB-4), herpes simplex virus (HSV) gB glycoprotein, HSV gD glycoprotein, HGFA, high-molecular-weight melanoma-associated antigen (HMW-MAA), HIV gp120, HIV IIIB gp 120 V3 loop, HLA, HLA-DR, HM1.24, HMFG PEM, HRG, Hrk, human heart myosin,

human cytomegalovirus (HCMV), human growth hormone (HGH), HVEM, I-309, IAP, ICAM, ICAM-1, ICAM-3, ICE, ICOS, IFNg, Ig, IgA receptor, IgE, IGF, IGF binding protein, IGF-1R, IGFBP, IGF-I, IGF-II, IL, IL-1, IL-1R, IL-2, IL-2R, IL-4, IL-4R, IL-5, IL-5R, IL-6, IL-6R, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-18, IL-18R, IL-21, IL-23, IL-27, interferon (INF)-alpha, INF-beta, INF-gamma, inhibin, iNOS, insulin chain A, insulin chain B, insulin-like growth factor 1, integrin alpha 2, integrin alpha 3, integrin alpha 4, integrin alpha 4/beta 1, integrin alpha 4/beta 7, integrin alpha 5 (alpha V), integrin alpha 5/beta 1, integrin alpha 5/beta 3, integrin alpha 6, integrin beta 1, integrin beta 2, interferon gamma, IP-10, I-TAC, JE, kallikrein 2, kallikrein 5, kallikrein 6, kallikrein 11, kallikrein 12, kallikrein 14, kallikrein 15, kallikrein L1, kallikrein L2, kallikrein L3, kallikrein L4, KC, KDR, keratinocyte growth factor (KGF), laminin 5, LAMP, LAP, LAP (TGF-1), latent TGF-1, latent TGF-1 bp1, LBP, LDGF, LECT2, lefty, Lewis-Y antigen, Lewis-Y-related antigen, LFA-1, LFA-3, Lfo, LIF, LIGHT, lipoprotein, LIX, LKN, Lptn, L-selectin, LT-a, LT-b, LTB4, LTBP-1, lung surface, luteinizing hormone, lymphotoxin beta receptor, Mac-1, MAdCAM, MAG, MAP2, MARC, MCAM, MCAM, MCK-2, MCP, M-CSF, MDC, Mer, metalloproteinases, MGDF receptor, MGMT, MHC (HLA-DR), MIF, MIG, MIP, MIP-1-alpha, MK, MMAC1, MMP, MMP-1, MMP-10, MMP-11, MMP-12, MMP-13, MMP-14, MMP-15, MMP-2, MMP-24, MMP-3, MMP-7, MMP-8, MMP-9, MPIF, Mpo, MSK, MSP, mucin (Muc1), MUC18, mullerian-inhibiting substance, Mug, MuSK, NAIP, NAP, NCAD, N-C adherin, NCA 90, NCAM, NCAM, neprilysin, neurotrophin-3, -4, or -6, neurturin, nerve growth factor (NGF), NGFR, NGF-beta, nNOS, NO, NOS, Npn, NRG-3, NT, NTN, OB, OGG1, OPG, OPN, OSM, OX40L, OX40R, p150, p95, PADPr, parathyroid hormone, PARC, PARP, PBR, PBSF, PCAD, P-cadherin, PCNA, PDGF, PDGF, PDK-1, PECAM, PEM, PF4, PGE, PGF, PGI2, PGJ2, PIN, PLA2, placental alkaline phosphatase (PLAP), PlGF, PLP, PP14, proinsulin, prorelaxin, protein C, PS, PSA, PSCA, prostate-specific membrane antigen (PSMA), PTEN, PTHrp, Ptk, PTN, R51, RANK, RANKL, RANTES, RANTES, relaxin A chain, relaxin B chain, renin, respiratory syncytial virus (RSV) F, RSV Fgp, Ret, rheumatoid factor, RLIP76, RPA2, RSK, S100, SCF/KL, SDF-1, SERINE, serum albumin, sFRP-3, Shh, SIGIRR, SK-1, SLAM, SLPI, SMAC, SMDF, SMOH, SOD, SPARC, Stat, STEAP, STEAP-II, TACE, TACI, TAG-72 (tumor-associated glycoprotein-72), TARC, TCA-3, T cell receptor (e.g., T cell receptor alpha/beta), TdT, TECK, TEM1, TEM5, TEM7, TEM8, TERT, testicular PLAP-like alkaline phosphatase, TfR, TGF, TGF-alpha, TGF-beta, TGF-beta Pan Specific, TGF-beta RI (ALK-5), TGF-beta RII, TGF-beta RIIb, TGF-beta RIII, TGF-beta 1, TGF-beta 2, TGF-beta 3, TGF-beta 4, TGF-beta 5, thrombin, thymus Ck-1, thyroid stimulating hormone, Tie, TIMP, TIQ, tissue factor, TMEFF2, Tmpo, TMPRSS2, TNF, TNF-alpha, TNF-alpha/beta, TNF-beta 2, TNFc, TNF-RI, TNF-RII, TNFRSF10A (TRAIL R1 Apo-2, DR4), TNFRSF10B (TRAIL R2 DR5, KILLER, TRICK-2A, TRICK-B), TNFRSF10C (TRAIL R3

DcR1, LIT, TRID), TNFRSF10D (TRAIL R4 DcR2, TRUNDD), TNFRSF11A (RANK ODF R, TRANCE R), TNFRSF11B (OPG OCIF, TR1), TNFRSF12 (TWEAK R FN14), TNFRSF13B (TACI), TNFRSF13C (BAFF R), TNFRSF14 (HVEM ATAR, HveA, LIGHT R, TR2), TNFRSF16 (NGFR p75NTR), TNFRSF17 (BCMA), TNFRSF18 (GITR AITR), TNFRSF19 (TROY TAJ, TRADE), TNFRSF19L (RELT), TNFRSF1A (TNF RI CD120a, p55-60), TNFRSF1B (TNF RII CD120b, p75-80), TNFRSF26 (TNFRH3), TNFRSF3 (LTbR TNF RIII, TNFC R), TNFRSF4 (OX40 ACT35, TXGP1 R), TNFRSF5 (CD40 p50), TNFRSF6 (Fas Apo-1, APT1, CD95), TNFRSF6B (DcR3 M68, TR6), TNFRSF7 (CD27), TNFRSF8 (CD30), TNFRSF9 (4-1BB CD137, ILA), TNFRSF21 (DR6), TNFRSF22 (DcTRAIL R2 TNFRH2), TNFRST23 (DcTRAIL R1 TNFRH1), TNFRSF25 (DR3 Apo-3, LARD, TR-3, TRAMP, WSL-1), TNFSF10 (TRAIL Apo-2 ligand, TL2), TNFSF11 (TRANCE/RANK ligand ODF, OPG ligand), TNFSF12 (TWEAK Apo-3 ligand, DR3 ligand), TNFSF13 (APRIL TALL2), TNFSF13B (BAFF BLYS, TALL1, THANK, TNFSF20), TNFSF14 (LIGHT HVEM ligand, LTg), TNFSF15 (TL1A/VEGI), TNFSF18 (GITR ligand AITR ligand, TL6), TNFSF1A (TNF-a Conectin, DIF, TNFSF2), TNFSF1B (TNF-b LTa, TNFSF1), TNFSF3 (LTb TNFC, p33), TNFSF4 (OX40 ligand gp34, TXGP1), TNFSF5 (CD40 ligand CD154, gp39, HIGM1, IMD3, TRAP), TNFSF6 (Fas ligand Apo-1 ligand, APT1 ligand), TNFSF7 (CD27 ligand CD70), TNFSF8 (CD30 ligand CD153), TNFSF9 (4-1BB ligand CD137 ligand), TP-1, t-PA, Tpo, TRAIL, TRAIL R, TRAIL-R1, TRAIL-R2, TRANCE, transferrin receptor, TRF, Trk, TROP-2, TLR (toll-like receptor) 1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, TSG, TSLP, tumor-associated antigen CA125, tumor-associated antigen-expressing Lewis-Y-related carbohydrate, TWEAK, TXB2, Ung, uPAR, uPAR-1, urokinase, VCAM, VCAM-1, VECAD, VE-cadherin, VE-cadherin-2, VEFGR-1 (flt-1), VEGF, VEGFR, VEGFR-3 (flt-4), VEGI, VIM, viral antigens, VLA, VLA-1, VLA-4, VNR integrin, von Willebrand factor, WIF-1, WNT1, WNT2, WNT2B/13, WNT3, WNT3A, WNT4, WNT5A, WNT5B, WNT6, WNT7A, WNT7B, WNT8A, WNT8B, WNT9A, WNT9A, WNT9B, WNT10A, WNT10B, WNT11, WNT16, XCL1, XCL2, XCR1, XCR1, XEDAR, XIAP, XPD, HMGB1, IgA, A beta, CD81, CD97, CD98, DDR1, DKK1, EREG, Hsp90, IL-17/IL-17R, IL-20/IL-20R, oxidized LDL, PCSK9, prekallikrein, RON, TMEM16F, SOD1, chromogranin A, chromogranin B, tau, VAP1, high-molecular-weight kininogen, IL-31, IL-31R, Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.5, Nav1.6, Nav1.7, Nav1.8, Nav1.9, EPCR, C1, C1q, C1r, C1s, C2, C2a, C2b, C3, C3a, C3b, C4, C4a, C4b, C5, C5a, C5b, C6, C7, C8, C9, factor B, factor D, factor H, properdin, sclerostin, fibrinogen, fibrin, prothrombin, thrombin, tissue factor, factor V, factor Va, factor VII, factor VIIa, factor VIII, factor VIIIa, factor IX, factor IXa, factor X, factor Xa, factor XI, factor XIa, factor XII, factor XIIa, factor XIII, factor XIIIa, TFPI, antithrombin III, EPCR, thrombomodulin, TAPI, tPA, plasminogen, plasmin, PAI-1, PAI-2, GPC3, syndecan-1, syndecan-2, syndecan-3,

syndecan-4, LPA, and S1P. In some embodiments, examples of antigens include receptors for hormone and growth factors. In certain embodiments, antigens are antigen molecules that are expressed in or secreted from cells present in tumor tissues (e.g., tumor cells, immune cells, stromal cells), in particular, antigens specifically expressed in cancer cells (cancer-specific antigens). In certain embodiments, antigens are antigen molecules expressed on the surface of cells having a function of suppressing immune responses.

[0099]

Cancer specific antigen

"Cancer-specific antigen" as a non-limiting example of an antigen on a target cell means an antigen expressed by a cancer cell, which allows distinguishing between a cancer cell and a healthy cell; for example, it includes antigens that are expressed as cells become malignant and abnormal sugar chains that appear on the cell surface or on protein molecules when the cells become cancerous. Specific examples thereof include ALK, pleiotrophin (PTN), EpCAM,CA125, Prostatic acid phosphatase (PAP), Prostate-specific antigen (PSA), TYRP1, HMW-MAA, Prostate-specific membrane antigen (PSMA), CEA, MUC1, HMFG1, TAG-72, GICA (CA19-9), NY-ESO-1, LEA, CD15, CD17, CD19, CD20, CD22, CD30, CD33, CD38, CD77, CD79b, CD147, CD228, GD2, GD3, GM2, GM3, TSTA, virus-induced tumor antigens (e.g. envelope antigens of DNA and RNA tumor virus), alpha-fetoprotein (AFP), 5T4, differentiation antigens (e.g. L6 and L20 antigens), CD165, EGFR, ANKRD17, ErbB2, APO-1, SSEA-1, SCP-1, LeY, oligosaccharide antigens, SSEA-3, SSEA-4, CTAGE1, MART-1, sialyl Tn (STn), NY-CO-45, NY-LU-12, ART1, MA2, NOVA2, TSPAN8, MAGE-C1, MAGE-B1, MAGE-B2, MAGE-4A, MAGE-X2, YKL-40, EREG, CA15-3, CLEC12A, Nectin4, Trop2, BCMA, Tissue factor, FRα (FOLR1), ErbB3, Claudin18 (Claudin18.2), B7-H3 (CD276), MET, PSCA, PTK7, MSLN (Mesothelin), CCR4, CDH6, IL13RA2, GPC3, XPR1, NOX1, MARVELD3 isoform 1, MARVELD3 isoform 2, SPINT2, MANSC1, SLC12A2, CDCP1, SEZ6L2, FLVCR1, SLC7A5, STEAP1, MMP14, TNFRSF21, TMPRSS4, Claudin-6, DLL3 and any fragment of these polypeptides, and modified structures thereof.

[0100]

Antigen expressed on the surface of a cell having immune-response suppressing function

Here, "a cell having immune response-suppressing function" is not particularly limited as long as it has a function of suppressing an immune response, and examples include regulatory T cells (Treg), exhausted T cells, myeloma-derived stromal cells (MDSC), tumor-associated macrophages (TAM), induced regulatory T cells (Tr1), tumor-associated dendritic cells (TADC), tumor-associated neutrophils (TAN), cancer-associated fibroblasts (CAF), and regulatory B cells (Breg). In particular, regulatory T cells and exhausted T cells are preferable as the cells of interest. Specific examples of the molecules expressed on the surface of such cells having

immune response-suppressing functions include CTLA4, PD1, TIM3, LAG-3, CD244 (2B4), CD160, GARP, OX40, CD137 (4-1BB), CD25, VISTA, VISATA, BTLA, TNFR25, CD57, KLRG1, CCR2, CCR5, CCR6, CD39, CD73, CD4, CD18, CD49b, CD1d, CD5, CD21, TIM1, CD19, CD20, CD23, CD24, CD38, CD93, IgM, B220(CD45R), CD317, PD-L1, CD11b, Ly6G, ICAM-1, FAP, PDGFR, Podoplanin, and TIGIT. Among these molecules, examples of favorable molecules for targets of the binding domains of the present invention include CTLA4, TIM3, LAG3, CD137 (4-1BB), CD25, CCR5, CCR6, CD38, and TIGIT, which are cell surface molecules specifically expressed in cell fractions ($CD4^+$, $CD25^{high}$, and $CD45RA^-$) that have been reported to have high immune response-suppressing functions. Examples of preferred molecules for targets of the binding domains of the present invention include CTLA4, LAG3, and OX40 in particular.

[0101]

Binding activity toward an antigen

A method for confirming binding to an epitope by a test antigen-binding molecule having a domain that comprises an antibody variable region having CD3-binding activity is exemplified below, and a method for confirming binding to an epitope by a test antigen-binding molecule comprising a domain that contains an antibody variable region having binding activity toward other antigens may also be performed suitably in accordance with the examples below.

[0102]

For example, recognition of a linear epitope present in the CD3 molecule by a test antigen-binding molecule comprising a domain that contains an antibody variable region having CD3-binding activity can be confirmed as follows. A linear peptide comprising the amino acid sequence constituting the extracellular domain of CD3 is synthesized for the above-mentioned objective. The peptide may be synthesized chemically. Alternatively, it can be obtained by genetic engineering methods using a region in the cDNA of CD3 that encodes an amino acid sequence corresponding to the extracellular domain. Next, the binding activity between a linear peptide comprising the amino acid sequence constituting the extracellular domain and the test antigen-binding molecule comprising a domain that contains an antibody variable region having CD3-binding activity is evaluated. For example, ELISA which uses an immobilized linear peptide as the antigen may enable evaluation of the binding activity of the antigen-binding molecule towards the peptide. Alternatively, binding activity towards the linear peptide may be elucidated based on the level of inhibition caused by the linear peptide in the binding of the antigen-binding molecule to CD3-expressing cells. These tests may elucidate the binding activity of the antigen-binding molecules toward the linear peptide.

[0103]

Furthermore, recognition of the conformational epitope by a test antigen-binding molecule comprising a domain that contains an antibody variable region having CD3-binding activity can be confirmed as follows. CD3-expressing cells are prepared for the above-mentioned objective. For example, when the test antigen-binding molecule comprising a domain that contains an antibody variable region having CD3-binding activity contacts CD3-expressing cells, it binds strongly to the cells, but on the other hand, there are cases when the antigen-binding molecule does not substantially bind to the immobilized linear peptide comprising the amino acid sequence constituting the extracellular domain of CD3. In these cases, "does not substantially bind" refers to a binding activity of 80% or less, generally 50% or less, preferably 30% or less, and particularly preferably 15% or less relative to the binding activity towards human CD3-expressing cells.

[0104]

Methods for assaying the binding activity of a test antigen-binding molecule containing a CD3 antigen-binding domain towards CD3-expressing cells include, for example, the methods described in Antibodies: A Laboratory Manual (Ed Harlow, David Lane, Cold Spring Harbor Laboratory (1988) 359-420). Specifically, the assessment can be performed based on the principle of ELISA or fluorescence activated cell sorting (FACS) using CD3-expressing cells as antigen.

[0105]

In the ELISA format, the binding activity of a test antigen-binding molecule containing a CD3 antigen-binding domain towards CD3-expressing cells can be assessed quantitatively by comparing the levels of signal generated by enzymatic reaction. Specifically, a test antigen-binding molecule is added to an ELISA plate onto which CD3-expressing cells are immobilized. Then, the test antigen-binding molecule bound to the cells is detected using an enzyme-labeled antibody that recognizes the test antigen-binding molecule. Alternatively, in FACS, a dilution series of a test antigen-binding molecule is prepared, and the antibody binding titer for CD3-expressing cells can be determined to compare the binding activity of the test antigen-binding molecule towards CD3-expressing cells.

[0106]

The binding of a test antigen-binding molecule towards an antigen expressed on the surface of cells suspended in buffer or the like can be detected using a flow cytometer. Known flow cytometers include, for example, the following devices:

FACSCanto™ II

FACSAria™

FACSArray™

FACSVantage™ SE

FACSCalibur™ (all are trade names of BD Biosciences)
EPICS ALTRA HyPerSort
Cytomics FC 500
EPICS XL-MCL ADC EPICS XL ADC
Cell Lab Quanta/Cell Lab Quanta SC (all are trade names of Beckman Coulter)

[0107]

Preferable methods for assaying the binding activity of a test antigen-binding molecule containing a CD3 antigen-binding domain towards an antigen include, for example, the following method. First, CD3-expressing cells are reacted with a test antigen-binding molecule, and then this is stained with an FITC-labeled secondary antibody that recognizes the test antigen-binding molecule. The test antigen-binding molecule is appropriately diluted with a suitable buffer to prepare the complex at a desired concentration. For example, the complex can be used at a concentration within the range of 10 μg/ml to 10 ng/ml. Then, the fluorescence intensity and cell count are determined using FACSCalibur (BD). The fluorescence intensity obtained by analysis using the CELL QUEST Software (BD), i.e., the Geometric Mean value, reflects the quantity of antibody bound to cells. That is, the binding activity of a test antigen-binding molecule, which is represented by the quantity of the test antigen-binding molecule bound, can be determined by measuring the Geometric Mean value.

[0108]

Whether a test antigen-binding molecule comprising a CD3 antigen-binding domain shares a common epitope with another antigen-binding molecule can be assessed based on the competition between the two molecules for the same epitope. The competition between antigen-binding molecules can be detected by cross-blocking assay or the like. For example, the competitive ELISA assay is a preferred cross-blocking assay.

[0109]

Specifically, in cross-blocking assay, the CD3 protein immobilized to the wells of a microtiter plate is pre-incubated in the presence or absence of a candidate competitor antigen-binding molecule, and then a test antigen-binding molecule is added thereto. The quantity of test antigen-binding molecule bound to the CD3 protein in the wells is indirectly correlated with the binding ability of a candidate competitor antigen-binding molecule that competes for the binding to the same epitope. That is, the greater the affinity of the competitor antigen-binding molecule for the same epitope, the lower the binding activity of the test antigen-binding molecule towards the CD3 protein-coated wells.

[0110]

The quantity of the test antigen-binding molecule bound to the wells via the CD3 protein can be readily determined by labeling the antigen-binding molecule in advance. For

example, a biotin-labeled antigen-binding molecule is measured using an avidin/peroxidase conjugate and appropriate substrate. In particular, cross-blocking assay that uses enzyme labels such as peroxidase is called “competitive ELISA assay”. The antigen-binding molecule can also be labeled with other labeling substances that enable detection or measurement. Specifically, radiolabels, fluorescent labels, and such are known.

[0111]

When the candidate competitor antigen-binding molecule can block the binding by a test antigen-binding molecule containing a CD3 antigen-binding domain by at least 20%, preferably at least 20 to 50%, and more preferably at least 50% compared to the binding activity in a control experiment conducted in the absence of the competitor antigen-binding molecule, the test antigen-binding molecule is considered to bind to substantially the same epitope bound by the competitor antigen-binding molecule, or compete for the binding to the same epitope.

[0112]

When the structure of an epitope bound by a test antigen-binding molecule containing a CD3 antigen-binding domain has already been identified, whether the test and control antigen-binding molecules share a common epitope can be assessed by comparing the binding activities of the two antigen-binding molecules towards a peptide prepared by introducing amino acid mutations into the peptide forming the epitope.

[0113]

To measure the above binding activities, for example, the binding activities of test and control antigen-binding molecules towards a linear peptide into which a mutation is introduced are compared in the above ELISA format. Besides the ELISA methods, the binding activity towards the mutant peptide bound to a column can be determined by flowing test and control antigen-binding molecules through the column, and then quantifying the antigen-binding molecule eluted in the elution solution. Methods for adsorbing a mutant peptide to a column, for example, in the form of a GST fusion peptide, are known.

[0114]

Alternatively, when the identified epitope is a conformational epitope, whether test and control antigen-binding molecules share a common epitope can be assessed by the following method. First, CD3-expressing cells and cells expressing CD3 with a mutation introduced into the epitope are prepared. The test and control antigen-binding molecules are added to a cell suspension prepared by suspending these cells in an appropriate buffer such as PBS. Then, the cell suspensions are appropriately washed with a buffer, and an FITC-labeled antibody that recognizes the test and control antigen-binding molecules is added thereto. The fluorescence intensity and number of cells stained with the labeled antibody are determined using FACSCalibur (BD). The test and control antigen-binding molecules are appropriately diluted

using a suitable buffer, and used at desired concentrations. For example, they may be used at a concentration within the range of 10 μg/ml to 10 ng/ml. The fluorescence intensity determined by analysis using the CELL QUEST Software (BD), i.e., the Geometric Mean value, reflects the quantity of labeled antibody bound to cells. That is, the binding activities of the test and control antigen-binding molecules, which are represented by the quantity of labeled antibody bound, can be determined by measuring the Geometric Mean value.

[0115]

In the above method, whether an antigen-binding molecule does "not substantially bind to cells expressing mutant CD3" can be assessed, for example, by the following method. First, the test and control antigen-binding molecules bound to cells expressing mutant CD3 are stained with a labeled antibody. Then, the fluorescence intensity of the cells is determined. When FACSCalibur is used for fluorescence detection by flow cytometry, the determined fluorescence intensity can be analyzed using the CELL QUEST Software. From the Geometric Mean values in the presence and absence of the antigen-binding molecule, the comparison value (ΔGeo-Mean) can be calculated according to the following formula to determine the ratio of increase in fluorescence intensity as a result of the binding by the antigen-binding molecule.

[0116]

ΔGeo-Mean = Geo-Mean (in the presence of the antigen-binding molecule)/Geo-Mean (in the absence of the antigen-binding molecule)

[0117]

The Geometric Mean comparison value (ΔGeo-Mean value for the mutant CD3 molecule) determined by the above analysis, which reflects the quantity of a test antigen-binding molecule bound to cells expressing mutant CD3, is compared to the ΔGeo-Mean comparison value that reflects the quantity of the test antigen-binding molecule bound to CD3-expressing cells. In this case, the concentrations of the test antigen-binding molecule used to determine the ΔGeo-Mean comparison values for CD3-expressing cells and cells expressing mutant CD3 are particularly preferably adjusted to be equal or substantially equal. An antigen-binding molecule that has been confirmed to recognize an epitope in CD3 is used as a control antigen-binding molecule.

[0118]

If the ΔGeo-Mean comparison value for a test antigen-binding molecule against mutant CD3-expressing cells is at least less than 80%, (preferably less than 50%, more preferably less than 30%, and particularly preferably less than 15%) of the ΔGeo-Mean comparison value for that molecule against CD3-expressing cells, the test antigen-binding molecule is considered to "not substantially bind to mutant CD3-expressing cells". The formula for determining the Geo-Mean (Geometric Mean) value is described in the CELL QUEST Software User's Guide (BD

biosciences). When the comparison shows that the comparison values are substantially equivalent, the epitope for the test and control antigen-binding molecules can be determined to be the same.

[0119]

Antigen-binding molecule that comprises an FcRn-binding domain contained in an Fc region

A preferred example of an antigen-binding molecule of the present invention is an antigen-binding molecule that comprises an FcRn-binding domain comprised in an antibody Fc region. As a method for extending the blood half-life of a protein administered to a living body, the method of adding an FcRn-binding domain of an antibody to the protein of interest and utilizing the function of FcRn-mediated recycling is well known.

[0120]

In the present invention, the "FcRn-binding domain" is not particularly limited as long as it has binding activity to FcRn, and it may be a domain that directly or indirectly binds to FcRn. Examples of the domain that directly binds to FcRn include antibody variable regions, Fab and antibody Fc regions whose antigens are FcRn, fragments thereof, albumin, albumin domain 3, human serum albumin (HSA), and transferrin. Furthermore, an example of the domain that indirectly binds to FcRn includes a domain that has binding activity toward the aforementioned domain that directly binds to FcRn. In connection with the present invention, an embodiment of the FcRn-binding domain includes antibody Fc regions or fragments containing an FcRn-binding region of an Fc region. Herein, for example, IgA1, IgA2, IgD, IgE, IgG1, IgG2, IgG3, IgG4, and IgM type Fc regions exist as the "Fc region" and, for example, an Fc region derived from a naturally-occurring IgG may be used. A naturally-occurring IgG means a polypeptide that comprises the same amino acid sequence as an IgG found in nature, and belongs to a class of antibodies substantially encoded by immunoglobulin gamma genes. A naturally-occurring human IgG means, for example, a naturally-occurring human IgG1, a naturally-occurring human IgG2, a naturally-occurring human IgG3, or a naturally-occurring human IgG4. Naturally-occurring IgGs also include mutants and the like that naturally generate therefrom. A plurality of allotype sequences that result from genetic polymorphism have been described in Sequences of Proteins of Immunological Interest, NIH Publication No. 91-3242 for the human IgG1, human IgG2, human IgG3, and human IgG4 antibody constant region, and any of the sequences may be used in the present invention. In particular, the amino acid sequence of positions 356 to 358 according to EU numbering may be DEL or EEM for the human IgG1 sequence.

[0121]

In some embodiments, the antigen-binding molecules of the present invention comprise an antibody Fc region. When the antigen-binding molecules of the present invention comprise a

single antigen-binding domain, that antigen-binding domain may be linked to one out of the two polypeptides that constitute a dimeric Fc region. When the antigen-binding molecules of the present invention comprise a first antigen-binding domain and a second antigen-binding domain, the first antigen-binding domain may be linked to one of the two polypeptides that constitute a dimeric Fc region and the second antigen-binding domain may be linked to the other, but the molecules are not limited to this embodiment. In certain embodiments, the antigen-binding molecules of the present invention comprise an antibody Fc region with a reduced binding activity towards an Fcγ receptor. When the multispecific antigen-binding molecules of the present invention comprise an antibody Fc region, it is preferable to use an antibody Fc region with reduced Fcγ receptor binding activity, in order to avoid the occurrence of target antigen-independent induction of various cytokine production, which is observed with trifunctional antibodies such as catumaxomab.

Various amino acid mutations (modifications) to reduce the binding activity of the Fc region of antibodies to Fcγ receptors are known. Fc regions having these amino acid mutations (modifications) – known mutations (modifications) or those that may be identified in the future – may be used in the antigen-binding molecules of the present invention. The intensities of effector functions, which are exerted via the binding between antibody Fc regions and Fcγ receptors, differ among IgG subclasses, and are known to be higher in IgG1 and IgG3 and lower in IgG2 and IgG4 in humans. Thus, as the antibody Fc region with a reduced binding activity towards an Fcγ receptor comprised in an antigen-binding molecule of the present invention, naturally-occurring human IgG2 or IgG4 Fc regions may be used.

[0122]

Fcγ receptor

Fcγ receptor refers to a receptor capable of binding to the Fc region of monoclonal IgG1, IgG2, IgG3, or IgG4 antibodies, and includes all members belonging to the family of proteins substantially encoded by an Fcγ receptor gene. In human, the family includes FcγRI (CD64) including isoforms FcγRIa, FcγRIb and FcγRIc; FcγRII (CD32) including isoforms FcγRIIa (including allotype H131 and R131), FcγRIIb (including FcγRIIb-1 and FcγRIIb-2), and FcγRIIc; and FcγRIII (CD16) including isoform FcγRIIIa (including allotype V158 and F158) and FcγRIIIb (including allotype FcγRIIIb-NA1 and FcγRIIIb-NA2); as well as any undiscovered human FcγRs or FcγR isoforms or allotypes. However, Fcγ receptor is not limited to these examples. FcγR may be derived from any organisms, including, but not limited to, humans, mice, rats, rabbits, and monkeys. Mouse FcγR includes, without being limited to, FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIII-2 (CD16-2), as well as all unidentified mouse FcγRs, FcγR isoforms, and allotypes thereof. Such preferred Fcγ receptors include, for example, human FcγRI (CD64), FcγRIIA (CD32), FcγRIIB (CD32), FcγRIIIA (CD16), and/or

FcγRIIIB (CD16). The polynucleotide sequence and amino acid sequence of FcγRI are registered as RefSeq accession numbers NM_000566.3 and NP_000557.1, respectively; the polynucleotide sequence and amino acid sequence of FcγRIIA are registered as RefSeq accession numbers BC020823.1 and AAH20823.1, respectively; the polynucleotide sequence and amino acid sequence of FcγRIIB are registered as RefSeq accession numbers BC146678.1 and AAI46679.1, respectively; the polynucleotide sequence and amino acid sequence of FcγRIIIA are registered as RefSeq accession numbers BC033678.1 and AAH33678.1, respectively; and the polynucleotide sequence and amino acid sequence of FcγRIIIB are registered as RefSeq accession numbers BC128562.1 and AAI28563.1, respectively. Whether an Fcγ receptor has binding activity to the Fc region 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.

[0123]

Fcγ receptor-binding activity

The reduced binding activity of the Fc region to any of the Fcγ receptors FcγI, FcγIIA, FcγIIB, FcγIIIA, and/or FcγIIIB 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).

[0124]

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.

[0125]

For example, a biotin-labeled antigen-binding molecule is immobilized to the donor beads and glutathione S-transferase (GST)-tagged Fcγ receptor is immobilized to the acceptor beads. In the absence of an antigen-binding molecule comprising a competitive mutant Fc region, Fcγ receptor interacts with an antigen-binding molecule comprising a wild-type Fc

region, inducing a signal of 520 to 620 nm as a result. The antigen-binding molecule having an untagged mutant Fc region competes with the antigen-binding molecule comprising a wild-type Fc region for the interaction with Fcγ receptor. The relative binding affinity can be determined by quantifying the reduction of fluorescence as a result of competition. Methods for biotinylating antigen-binding molecules such as antibodies using Sulfo-NHS-biotin or the like are known. Appropriate methods for adding a GST tag to an Fcγ receptor include methods that involve fusing a polynucleotide encoding an Fcγ receptor and a polynucleotide encoding GST in-frame, expressing the fused gene in cells introduced with a vector capable of expressing 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).

[0126]

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.

[0127]

Herein, "Fcγ receptor-binding activity is reduced" means, for example, that based on the above-described analysis method the competitive activity of a test antigen-binding molecule 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 binding activity of a control antigen-binding molecule comprising an Fc region.

[0128]

Antigen-binding molecules comprising the Fc region of a monoclonal IgG1, IgG2, IgG3, or IgG4 antibody can be appropriately used as control antigen-binding molecules. Structures in which A is added to the N terminus of each of the amino acid sequences registered as RefSeq accession numbers AAC82527.1, AAB59393.1, CAA27268.1, and AAB59394.1 may be used as these Fc regions. In an embodiment, when an antigen-binding molecule comprising an Fc region mutant of an antibody of a particular isotype is used as a test substance, the effect of the mutation of the mutant on the Fcγ receptor-binding activity can be assessed using as a control an antigen-binding molecule comprising an Fc region of the same isotype. As described above, antigen-binding molecules comprising an Fc region mutant whose Fcγ receptor-binding activity has been judged to be reduced are appropriately prepared. In different embodiments, regarding the Fcγ receptor-binding activity, an Fc region of an antibody of a certain isotype may be compared with the Fc region of an antibody of another certain isotype, and the one having a lower Fcγ receptor-binding activity may be used in the antigen-binding molecules of the present invention. By introducing amino acid mutations into such an Fc region having a lower Fcγ receptor-binding activity, Fc regions having even lower Fcγ receptor-binding activity may be obtained.

[0129]

Such known mutations in the Fc region include, for example, deletion of amino acids 231A-238S (EU numbering) (WO 2009/011941), as well as mutations 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).

[0130]

Specifically, the preferred antigen-binding molecules include those comprising an Fc region having a substitution of the amino acid at any of the following position according to EU numbering: 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 in the amino acids forming the Fc region of an antibody of a particular isotype. The isotype of antibody from which the Fc region originates is not particularly limited, and it is possible to use an appropriate Fc region derived from a monoclonal IgG1, IgG2, IgG3, or IgG4 antibody.

[0131]

The preferred antigen-binding molecules include, for example, those comprising an Fc region 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 before the substitution) in the amino acids forming the Fc region of IgG1 antibody:

(a) L234F, L235E, P331S;

(b) C226S, C229S, P238S;

(c) C226S, C229S;

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

(e) L234A, L235A or L235R, N297A;

(f) L235A or L235R, S239K, N297A

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

[0132]

Furthermore, the preferred antigen-binding molecules also include those comprising an Fc region that has any one of the substitutions shown below, whose positions are specified according to EU numbering in the amino acids forming the Fc region of an IgG2 antibody:

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

(h) V234A;

(i) G237A;

(j) V234A and G237A;

(k) A235E and G237A;

(l) 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 before the substitution.

[0133]

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

(m) F241A;

(n) D265A;

(o) 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 before the substitution.

[0134]

Furthermore, the preferred antigen-binding molecules also include those comprising an Fc region that has any one of the substitutions shown below, whose positions are specified

according to EU numbering in the amino acids forming the Fc region of an IgG4 antibody:
(p) L235A, G237A, and E318A;
(q) L235E;
(r) 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 before the substitution.

[0135]

Other preferred antigen-binding molecules include, for example, those comprising an Fc region 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 region of an IgG1 antibody is substituted with an amino acid of the corresponding position in EU numbering in the corresponding IgG2 or IgG4.

[0136]

The preferred antigen-binding molecules also include, for example, those comprising an Fc region 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 region 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 comprising an Fc region in which any one or more of the amino acids at positions 234, 235, and 297 are substituted with alanine are particularly preferred.

[0137]

The preferred antigen-binding molecules also include, for example, those comprising an Fc region in which an amino acid at position 265 (EU numbering) in the amino acids forming the Fc region 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 comprising an Fc region in which an amino acid at position 265 is substituted with alanine are particularly preferred.

[0138]

In certain embodiments, the antigen-binding molecules of the present invention comprise an Fc region with reduced binding activity towards an Fcγ receptor. Examples of such embodiments include antibodies having a reduced effector function attributable to an Fc region. These antibodies are desirable when a certain effector function (complement or ADCC) is not necessary or harmful. Examples of antibodies with reduced effector function include those with substitution of one or more of Fc region residues 238, 265, 269, 270, 297, 327 and 329 (U.S. Patent No. 6,737,056). Such Fc mutants include Fc mutants with substitutions at two or more of amino acid positions 265, 269, 270, 297 and 327, including the so-called “DANA” Fc mutant with substitution of residues 265 and 297 to alanine (U.S. Patent No. 7,332,581).

[0139]

See also U.S. Patent No. 5,648,260; U.S. Patent No. 5,624,821; and WO 94/29351 concerning other examples of Fc region variants.

[0140]

Methods for producing antibodies

A preferred embodiment of the "antigen-binding molecule" of the present invention includes an antibody comprising an antibody variable region of the present invention.

[0141]

Methods for producing antibodies having the desired binding activity are known to those skilled in the art, and the antibodies may be obtained as polyclonal or monoclonal antibodies. Monoclonal antibodies derived from mammals may be suitably produced as the antibodies of the present invention. Such mammalian-derived monoclonal antibodies include antibodies produced by hybridomas and antibodies produced by host cells transformed with an expression vector carrying an antibody gene by genetic engineering techniques.

[0142]

There is no particular limitation on the mammal to be immunized for obtaining antibodies. It is preferable to select the mammal by considering its compatibility with the parent cells to be used in cell fusion for hybridoma production. In general, rabbits, monkeys, and rodents such as mice, rats, and hamsters are suitably used.

[0143]

The above animals are immunized with a sensitizing antigen by known methods. A general immunization method involves, for example, intraperitoneal or subcutaneous injection of a sensitizing antigen into mammals. Specifically, a sensitizing antigen is appropriately diluted with Phosphate-Buffered Saline (PBS), physiological saline, or the like. If desired, a conventional adjuvant such as Freund's complete adjuvant is mixed with the antigen, and the mixture is emulsified. Then, the sensitizing antigen is administered to a mammal several times at 4- to 21-day intervals. Appropriate carriers may be used in immunization with the sensitizing antigen. In particular, when a low-molecular-weight partial peptide is used as the sensitizing antigen, it is sometimes desirable to couple the sensitizing antigen peptide to a carrier protein such as albumin or keyhole limpet hemocyanin for immunization.

[0144]

Alternatively, hybridomas producing a desired antibody can be prepared using DNA immunization as mentioned below. DNA immunization is an immunization method that confers immunostimulation by expressing a sensitizing antigen in an animal immunized as a result of administering a vector DNA constructed to allow expression of an antigen protein-encoding gene

in the animal. As compared to conventional immunization methods in which a protein antigen is administered to animals to be immunized, DNA immunization is expected to be superior in that:
- immunostimulation can be provided while retaining the structure of a membrane protein; and
- there is no need to purify the antigen for immunization.

[0145]

In order to prepare a monoclonal antibody of the present invention using DNA immunization, first, a DNA expressing an antigen protein is administered to an animal to be immunized. The antigen protein-encoding DNA can be synthesized by known methods such as PCR. The obtained DNA is inserted into an appropriate expression vector, and then the resulting vector is administered to an animal to be immunized. Commercially available expression vectors, such as pcDNA3.1, can be suitably used as the expression vector. Vectors can be administered to an organism using conventional methods. For example, DNA immunization is performed by using a gene gun to introduce expression vector-coated gold particles into cells in the body of an animal to be immunized.

[0146]

After immunizing a mammal as described above, an increase in the titer of an antigen-binding antibody is confirmed in the serum. Then, immune cells are collected from the mammal, and then subjected to cell fusion. In particular, splenocytes are preferably used as immune cells.

[0147]

A mammalian myeloma cell is used as a cell to be fused with the above-mentioned immune cells. The myeloma cells preferably comprise a suitable selection marker for screening. A selection marker confers characteristics to cells for their survival (or death) under a specific culture condition. Hypoxanthine-guanine phosphoribosyltransferase deficiency (hereinafter abbreviated as HGPRT deficiency) and thymidine kinase deficiency (hereinafter abbreviated as TK deficiency) are known as selection markers. Cells with HGPRT or TK deficiency have hypoxanthine-aminopterin-thymidine sensitivity (hereinafter abbreviated as HAT sensitivity). HAT-sensitive cells cannot synthesize DNA in a HAT selection medium, and are thus killed. However, when the cells are fused with normal cells, they can continue DNA synthesis using the salvage pathway of the normal cells, and therefore they can grow even in the HAT selection medium.

[0148]

HGPRT-deficient and TK-deficient cells can be selected in a medium containing 6-thioguanine, 8-azaguanine (hereinafter abbreviated as 8AG), or 5'-bromodeoxyuridine. Normal cells are killed because they incorporate these pyrimidine analogs into their DNA. Meanwhile, cells that are deficient in these enzymes can survive in the selection medium, since they cannot incorporate these pyrimidine analogs. In addition, a selection marker referred to as G418

resistance provided by the neomycin resistance gene confers resistance to 2-deoxystreptamine antibiotics (gentamycin analogs). Various types of myeloma cells that are suitable for cell fusion are known.

[0149]

For example, myeloma cells including the following cells can be preferably used:

P3(P3x63Ag8.653) (J. Immunol. (1979) 123 (4), 1548-1550);

P3x63Ag8U.1 (Current Topics in Microbiology and Immunology (1978)81, 1-7);

NS-1 (C. Eur. J. Immunol. (1976)6 (7), 511-519);

MPC-11 (Cell (1976) 8 (3), 405-415);

SP2/0 (Nature (1978) 276 (5685), 269-270);

FO (J. Immunol. Methods (1980) 35 (1-2), 1-21);

S194/5.XX0.BU.1 (J. Exp. Med. (1978) 148 (1), 313-323);

R210 (Nature (1979) 277 (5692), 131-133).

[0150]

Cell fusions between the immune cells and myeloma cells are essentially carried out using known methods, for example, the method of Kohler and Milstein *et al.* (Methods Enzymol. (1981) 73: 3-46).

[0151]

More specifically, cell fusion can be carried out, for example, in a conventional culture medium in the presence of a cell fusion-promoting agent. The fusion-promoting agents include, for example, polyethylene glycol (PEG) and Sendai virus (HVJ). If required, an auxiliary substance such as dimethyl sulfoxide is also added to improve fusion efficiency.

[0152]

The ratio of immune cells to myeloma cells may be arbitrarily set, preferably, for example, one myeloma cell for every one to ten immunocytes. Culture media to be used for cell fusions include, for example, media that are suitable for the growth of myeloma cell lines, such as RPMI1640 medium and MEM medium, and other conventional culture medium used for this type of cell culture. In addition, serum supplements such as fetal calf serum (FCS) may be preferably added to the culture medium.

[0153]

For cell fusion, predetermined amounts of the above immune cells and myeloma cells are mixed well in the above culture medium. Then, a PEG solution (for example, the average molecular weight is about 1,000 to 6,000) prewarmed to about 37°C is added thereto at a concentration of generally 30% to 60% (w/v). The mixed solution is gently mixed to produce desired fusion cells (hybridomas). Then, an appropriate culture medium mentioned above is successively added, and the resulting mixture is repeatedly centrifuged to remove the

supernatant. Thus, cell fusion agents and the like which are unfavorable to hybridoma growth can be removed.

[0154]

The hybridomas thus obtained can be selected by culture using a conventional selective medium, for example, HAT medium (a culture medium containing hypoxanthine, aminopterin, and thymidine). The culture in the HAT medium is continued for a period of time sufficient to kill cells other than the desired hybridomas (non-fused cells). Typically, this sufficient period is several days to several weeks. Then, hybridomas producing the desired antibody are screened and subjected to single cloning by conventional limiting dilution methods.

[0155]

The hybridomas thus obtained can be selected using a selection medium based on the selection marker possessed by the myeloma used for cell fusion. For example, HGPRT- or TK-deficient cells can be selected by culture using the HAT medium (a culture medium containing hypoxanthine, aminopterin, and thymidine). Specifically, when HAT-sensitive myeloma cells are used for cell fusion, cells successfully fused with normal cells can selectively proliferate in the HAT medium. The culture in the HAT medium is continued for a period of time sufficient to kill cells other than the desired hybridomas (non-fused cells). Specifically, desired hybridomas can be selected by culture for generally several days to several weeks. Then, hybridomas producing the desired antibody can be screened and subjected to single cloning by conventional limiting dilution methods.

[0156]

Screening and single cloning of desired antibodies can be suitably performed by screening methods based on known antigen-antibody reaction. For example, a desired antibody can be selected by screening using fluorescence activated cell sorting (FACS). FACS is a system that enables measurement of the binding of an antibody to the cell surface by analyzing cells contacted with a fluorescent antibody using a laser beam, and measuring the fluorescence emitted from individual cells.

[0157]

To screen for hybridomas that produce a monoclonal antibody of the present invention by FACS, cells that express the antigen bound by the produced antibody are first prepared. Preferred cells used for screening are mammalian cells that are forced to express the antigen. By using untransformed mammalian cells used as host cells as a control, the binding activity of an antibody to the cell-surface antigen can be selectively detected. Specifically, hybridomas producing a desired monoclonal antibody can be obtained by selecting hybridomas that produce an antibody which binds to cells forced to express the antigen but not to the host cell.

[0158]

Alternatively, cells expressing the antigen of interest are immobilized and the binding activity of an antibody to the antigen-expressing cells can be assessed based on the principle of ELISA. For example, antigen-expressing cells are immobilized to the wells of an ELISA plate. Culture supernatants of hybridomas are contacted with the immobilized cells in the wells, and antibodies that bind to the immobilized cells are detected. When the monoclonal antibodies are derived from mice, antibodies bound to the cells can be detected using an anti-mouse immunoglobulin antibody. Hybridomas producing a desired antibody having the antigen-binding ability are selected by the above screening, and they can be cloned by a limiting dilution method or the like.

Monoclonal antibody-producing hybridomas thus prepared can be passaged in a conventional culture medium. The hybridomas can be stored in liquid nitrogen for a long period.

[0159]

The above hybridomas are cultured by a conventional method, and desired monoclonal antibodies can be obtained from the culture supernatants. Alternatively, the hybridomas are administered to and grown in compatible mammals, and monoclonal antibodies can be obtained from the ascites. The former method is suitable for obtaining antibodies with high purity.

[0160]

Antibodies that are encoded by antibody genes cloned from antibody-producing cells such as the above hybridomas can also be preferably used. A cloned antibody gene is inserted into an appropriate vector, and this is introduced into a host to express the antibody encoded by the gene. Methods for isolating antibody genes, inserting the genes into vectors, and transforming host cells have already been established, for example, by Vandamme *et al*. (Eur. J. Biochem. (1990) 192(3), 767-775). Methods for producing recombinant antibodies are also known as described below.

[0161]

Generally, to obtain a cDNA encoding the antibody variable region (V region), total RNA is first extracted from hybridomas. For example, the following methods can be used for extracting mRNAs from cells:

- the guanidine ultracentrifugation method (Biochemistry (1979) 18(24), 5294-5299), and
- the AGPC method (Anal. Biochem. (1987) 162(1), 156-159).

[0162]

Extracted mRNAs can be purified using the mRNA Purification Kit (GE Healthcare Bioscience) or the like. Alternatively, kits for extracting total mRNA directly from cells, such as the QuickPrep mRNA Purification Kit (GE Healthcare Bioscience), are also commercially available. mRNAs can be obtained from hybridomas using such kits. cDNAs encoding the antibody V region can be synthesized from the prepared mRNAs using a reverse transcriptase.

cDNAs can be synthesized using the AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (Seikagaku Corporation) or the like. Furthermore, the SMART RACE cDNA amplification kit (Clontech) and the PCR-based 5'-RACE method (Proc. Natl. Acad. Sci. USA (1988) 85(23), 8998-9002; Nucleic Acids Res. (1989) 17(8), 2919-2932) can be appropriately used to synthesize and amplify cDNAs. In such a cDNA synthesis process, appropriate restriction enzyme sites described below may be introduced into both ends of the cDNA.

[0163]

The cDNA fragment of interest is purified from the resulting PCR product, and then ligated to a vector DNA. A recombinant vector is thus constructed, and introduced into *E. coli* or such. After colony selection, the desired recombinant vector can be prepared from the colony-forming *E. coli*. Then, whether the recombinant vector has the cDNA nucleotide sequence of interest is confirmed by a known method such as the dideoxy nucleotide chain termination method.

[0164]

To isolate the gene encoding the variable region, it is convenient to use the 5'-RACE method with primers for amplifying the variable region gene. First, a 5'-RACE cDNA library is constructed by cDNA synthesis using RNAs extracted from hybridoma cells as a template. A commercially available kit such as the SMART RACE cDNA amplification kit is appropriately used to synthesize the 5'-RACE cDNA library.

[0165]

The antibody gene is amplified by PCR using the prepared 5'-RACE cDNA library as a template. Primers for amplifying the mouse antibody gene can be designed based on known antibody gene sequences. The nucleotide sequences of the primers vary depending on the immunoglobulin subclass. Therefore, it is preferable that the subclass is determined in advance using a commercially available kit such as the Iso Strip mouse monoclonal antibody isotyping kit (Roche Diagnostics).

[0166]

Specifically, for example, primers that allow amplification of genes encoding γ1, γ2a, γ2b, and γ3 heavy chains and κ and λ light chains can be used to isolate mouse IgG-encoding genes. In general, a primer that anneals to a constant region site close to the variable region is used as a 3'-side primer to amplify an IgG variable region gene. Meanwhile, a primer included in a 5' RACE cDNA library construction kit is used as a 5'-side primer.

[0167]

Immunoglobulins composed of a combination of heavy and light chains may be reconstituted using the thus amplified PCR products. A desired antibody can be selected by

screening using the antigen-binding activity of a reconstituted immunoglobulin as an indicator. The screening can be carried out, for example, by the following steps:

(1) contacting a desired antigen-expressing cell with an antibody comprising the V region encoded by a cDNA obtained from a hybridoma;

(2) detecting the binding of the antibody to the antigen-expressing cell; and

(3) selecting an antibody that binds to the antigen-expressing cell.

[0168]

Methods for detecting the binding of an antibody to the antigen-expressing cells are known. Specifically, the binding of an antibody to the antigen-expressing cells can be detected by the above-described techniques such as FACS. Fixed samples of the antigen-expressing cells may be appropriately used to assess the binding activity of an antibody.

[0169]

For antibody screening methods that use the binding activity as an indicator, panning methods that use phage vectors can also be used suitably. Screening methods using phage vectors are advantageous when the antibody genes are obtained from a polyclonal antibody-expressing cell population as heavy-chain and light-chain subclass libraries. Genes encoding the heavy-chain and light-chain variable regions can be linked by an appropriate linker sequence to form a single-chain Fv (scFv). Phages expressing scFv on their surface can be produced by inserting a scFv-encoding gene into a phage vector. The phages are contacted with an antigen of interest. Then, a DNA encoding scFv having the binding activity of interest can be isolated by collecting phages bound to the antigen. This process can be repeated as necessary to enrich scFv having the binding activity of interest.

[0170]

After isolation of the cDNA encoding the V region of the antibody of interest, the cDNA is digested with restriction enzymes that recognize the restriction sites introduced into both ends of the cDNA. Preferred restriction enzymes recognize and cleave a nucleotide sequence that occurs in the nucleotide sequence of the antibody gene at a low frequency. Furthermore, a restriction site for an enzyme that produces a sticky end is preferably introduced into a vector to insert a single-copy digested fragment in the correct orientation. The cDNA encoding the V region of the antibody is digested as described above, and this is inserted into an appropriate expression vector to construct an antibody expression vector. In this case, if a gene encoding the antibody constant region (C region) and a gene encoding the above V region are fused in-frame, a chimeric antibody is obtained. Herein, a “chimeric antibody” means that the origin of the constant region is different from that of the variable region. Thus, in addition to mouse/human heterochimeric antibodies, human/human allochimeric antibodies are included in the chimeric antibodies of the present invention. A chimeric antibody expression vector can be

constructed by inserting the above V region gene into an expression vector that already has the constant region. Specifically, for example, a recognition sequence for a restriction enzyme that excises the above V region gene can be appropriately placed on the 5' side of an expression vector carrying a DNA that encodes a desired antibody constant region (C region). A chimeric antibody expression vector is constructed by fusing in-frame two genes digested with the same combination of restriction enzymes.

[0171]

To produce a monoclonal antibody, antibody genes are inserted into an expression vector so that the genes are expressed under the control of an expression regulatory region. The expression regulatory region for antibody expression includes, for example, enhancers and promoters. Furthermore, an appropriate signal sequence may be attached to the amino terminus so that the expressed antibody is secreted to the outside of cells. The signal sequence is cleaved from the carboxyl terminus of the expressed polypeptide, and the resulting antibody can be secreted to the outside of cells. Then, appropriate host cells are transformed with the expression vector, and recombinant cells expressing the antibody-encoding DNA can be obtained.

[0172]

DNAs encoding the antibody heavy chain (H chain) and light chain (L chain) are separately inserted into different expression vectors to express the antibody gene. An antibody molecule having the H and L chains can be expressed by co-transfecting the same host cell with vectors into which the H chain and L chain are inserted. Alternatively, host cells can be transformed with a single expression vector into which DNAs encoding the H and L chains are inserted (see WO 94/11523).

[0173]

There are many known combinations of host cells and expression vectors for antibody preparation by introducing isolated antibody genes into appropriate hosts. All these expression systems are applicable to the isolation of the antigen-binding domains comprised in the antigen-binding molecules of the present invention.

[0174]

Appropriate eukaryotic cells used as host cells include animal cells, plant cells, and fungal cells. Specifically, the animal cells include, for example, the following cells:

(1) mammalian cells: CHO, COS, myeloma, baby hamster kidney (BHK), HeLa, Vero, or such;

(2) amphibian cells: Xenopus oocytes, or such; and

(3) insect cells: sf9, sf21, Tn5, or such.

[0175]

In addition, as for plant cells, an antibody gene expression system using cells derived from the *Nicotiana* genus such as *Nicotiana tabacum* is known. Callus cultured cells can be appropriately used to transform plant cells.

[0176]

Furthermore, the following cells can be used as fungal cells:

yeasts: the *Saccharomyces* genus such as *Saccharomyces cerevisiae*, and the *Pichia* genus such as the methylotrophic yeast *Pichia pastoris*; and

filamentous fungi: the *Aspergillus* genus such as *Aspergillus niger*.

[0177]

Furthermore, antibody gene expression systems that utilize prokaryotic cells are also known. For example, when using bacterial cells, *E. coli* cells, *Bacillus subtilis* cells, and such can suitably be utilized. Expression vectors carrying the antibody genes of interest are introduced into these cells by transformation. The transformed cells are cultured *in vitro*, and the desired antibody can be prepared from the culture of transformed cells.

[0178]

In addition to the above-described host cells, transgenic animals can also be used to produce a recombinant antibody. That is, the antibody can be obtained from an animal into which the gene encoding the antibody of interest is introduced. For example, the antibody gene can be constructed as a fusion gene by inserting in frame into a gene that encodes a protein produced specifically in milk. Goat β-casein or such can be used, for example, as the protein secreted in milk. DNA fragments containing the fusion gene into which the antibody gene is inserted are injected into a goat embryo, and then this embryo is introduced into a female goat. Desired antibodies can be obtained as a protein fused with the milk protein from milk produced by the transgenic goat born from the embryo-recipient goat (or progeny thereof). In addition, to increase the volume of milk containing the desired antibody produced by the transgenic goat, hormones can be administered to the transgenic goat (Bio/Technology (1994) 12 (7), 699-702).

[0179]

When an antigen-binding molecule described herein is administered to humans, an antigen-binding domain derived from a genetically recombinant antibody that has been artificially modified, for example, to reduce the heterologous antigenicity in humans, can be appropriately used as the various binding domains in the molecule when domains comprising an antibody variable region are used. Such genetically recombinant antibodies include, for example, humanized antibodies. These modified antibodies are appropriately produced by known methods.

[0180]

An antibody variable region used to produce the various binding domains of the antigen-binding molecules described herein is generally formed by three complementarity-determining regions (CDRs) that are separated by four framework regions (FRs). The CDRs are regions that substantially determine the binding specificity of an antibody. The amino acid sequences of CDRs are highly diverse. On the other hand, the FR-forming amino acid sequences often have high identity even among antibodies with different binding specificities. Therefore, generally, the binding specificity of a certain antibody can be introduced into another antibody by CDR grafting.

[0181]

A humanized antibody is also called a reshaped human antibody. Specifically, humanized antibodies, such as those prepared by grafting the CDR of a non-human animal antibody, for example a mouse antibody, onto a human antibody, are known. Common genetic engineering techniques for obtaining humanized antibodies are also known. Specifically, for example, overlap extension PCR is known as a method for grafting a mouse antibody CDR to a human FR. In overlap extension PCR, a nucleotide sequence encoding a mouse antibody CDR to be grafted is added to primers for synthesizing a human antibody FR. Primers are prepared for each of the four FRs. It is generally considered that when grafting a mouse CDR to a human FR, selecting a human FR that has high identity to a mouse FR is advantageous for maintaining the CDR function. That is, it is generally preferable to use a human FR comprising an amino acid sequence which has high identity to the amino acid sequence of the FR adjacent to the mouse CDR to be grafted.

[0182]

Nucleotide sequences to be ligated are designed so that they will be connected to each other in frame. Human FRs are individually synthesized using the respective primers. As a result, products in which the mouse CDR-encoding DNA is attached to the individual FR-encoding DNAs are obtained. Nucleotide sequences encoding the mouse CDR of each product are designed so that they overlap with each other. Then, the overlapping CDR regions of the products synthesized using a human antibody gene as a template are annealed to each other, and a complementary strand synthesis reaction is conducted. Human FRs are ligated *via* the mouse CDR sequences by this reaction.

[0183]

The full length V region gene, in which three CDRs and four FRs are ultimately ligated, is amplified using primers that anneal to its 5'- and 3'-ends, which are added with suitable restriction enzyme recognition sequences. An expression vector for a humanized antibody can be produced by inserting the DNA obtained as described above and a DNA that encodes a human antibody C region into an expression vector so that they will be ligated in frame. After

the recombinant vector is transfected into a host to establish recombinant cells, the recombinant cells are cultured, and the DNA encoding the humanized antibody is expressed to produce the humanized antibody in the cell culture (see European Patent Publication No. EP 239400 and International Patent Publication No. WO 1996/002576).

[0184]

By qualitatively or quantitatively measuring and evaluating the antigen-binding activity of the humanized antibody produced as described above, one can suitably select human antibody FRs that allow CDRs to form a favorable antigen-binding site when ligated through the CDRs. Amino acid residues in FRs may be substituted as necessary, so that the CDRs of a reshaped human antibody form an appropriate antigen-binding site. For example, amino acid sequence mutations can be introduced into FRs by applying the PCR method used for grafting a mouse CDR into a human FR. More specifically, partial nucleotide sequence mutations can be introduced into primers that anneal to the FR. Nucleotide sequence mutations are introduced into the FRs synthesized by using such primers. Mutant FR sequences having the desired characteristics can be selected by measuring and evaluating the activity of the amino acid-substituted mutant antibody to bind to the antigen by the above-mentioned method (Sato, K. *et al.*, Cancer Res. (1993) 53: 851-856).

[0185]

Alternatively, desired human antibodies can be obtained by immunizing transgenic animals having the entire repertoire of human antibody genes (see WO 1993/012227; WO 1992/003918; WO 1994/002602; WO 1994/025585; WO 1996/034096; WO 1996/033735) by DNA immunization.

[0186]

Furthermore, techniques for obtaining human antibodies by panning using human antibody libraries are also known. For example, the V region of a human antibody is expressed as a single-chain antibody (scFv) on the surface of phages by the phage display method. Phages expressing a scFv that binds to the antigen can be selected. The DNA sequence encoding the human antibody V region that binds to the antigen can be determined by analyzing the genes of selected phages. After the DNA sequence of the scFv that binds to the antigen is determined, an expression vector is prepared by fusing the V region sequence in frame with the C region sequence of a desired human antibody, and inserting this into an appropriate expression vector. The expression vector is introduced into cells appropriate for expression such as those described above. The human antibody can be produced by expressing the human antibody-encoding gene in the cells. These methods are already known (see WO 1992/001047; WO 1992/020791; WO 1993/006213; WO 1993/011236; WO 1993/019172; WO 1995/001438; WO 1995/015388).

[0187]

In addition to the phage display method, techniques that use a cell-free translation system, techniques for displaying antigen-binding molecules on the surface of viruses or cells, and techniques that use emulsions are also known as techniques for obtaining human antibodies by panning using human antibody libraries. For example, the ribosome display method where a complex is formed between the translated protein and mRNA *via* the ribosome by removing the stop codon and such, the cDNA display method or the mRNA display method where a genetic sequence and the translated protein are covalently linked using a compound such as puromycin, the CIS display method where a complex is formed between the gene and the translated protein using a nucleic acid-binding protein, or such may be used as techniques of using a cell-free translation system. For the technique of presenting antigen-binding molecules on the surface of cells or viruses, besides the phage display method, the *E. coli* display method, Gram-positive bacteria display method, yeast display method, mammalian cell display method, virus display method, and such may be used. As a technique that uses emulsions, the *in vitro* virus display method which involves incorporating genes and translation-related molecules into an emulsion, and such may be used. These methods are already publicly known (Nat Biotechnol. 2000 Dec;18(12):1287-92; Nucleic Acids Res. 2006;34(19): e127; Proc Natl Acad Sci U S A. 2004 Mar 2;101(9):2806-10; Proc Natl Acad Sci U S A. 2004 Jun 22;101(25):9193-8; Protein Eng Des Sel. 2008 Apr;21(4):247-55; Proc Natl Acad Sci U S A. 2000 Sep 26;97(20):10701-5; MAbs. 2010 Sep-Oct;2(5):508-18; and Methods Mol Biol. 2012, 911:183-98).

[0188]

Methods for producing multispecific antibodies

A preferred embodiment of the antigen-binding molecule of the present invention includes a multispecific antibody. When using an Fc region with decreased Fcγ receptor-binding activity as the Fc region of a multispecific antibody of the present invention, an Fc region derived from a known multispecific antibody may also be used appropriately. For the multispecific antibodies of the present invention, in particular, bispecific antibodies are preferred.

[0189]

For association of multispecific antibodies, one can apply the technique of introducing charge repulsion at the interface of the second constant region of the antibody H chain (CH2) or the third constant region of the H chain (CH3) to suppress undesired associations between H chains (WO2006/106905).

[0190]

In the technique of suppressing unintended association between H chains by introducing charge repulsion at the interface of CH2 or CH3, examples of the amino acid residues that are contacted at the interface of other constant regions of the H chain include the region facing the

residue at position 356 (EU numbering), the residue at position 439 (EU numbering), the residue at position 357 (EU numbering), the residue at position 370 (EU numbering), the residue at position 399 (EU numbering), and the residue at position 409 (EU numbering) in the CH3 region.

[0191]

More specifically, for example, for an antibody comprising two types of H chain CH3 regions, the antibody can be designed such that one to three pairs of amino acid residues selected from the amino acid residue pairs shown below in (1) to (3) in the first H chain CH3 region have the same charge: (1) the amino acid residues at positions 356 and 439 (EU numbering) which are amino acid residues contained in the H chain CH3 region; (2) the amino acid residues at positions 357 and 370 (EU numbering) which are amino acid residues contained in the H chain CH3 region; and (3) the amino acid residues at positions 399 and 409 (EU numbering) which are amino acid residues contained in the H chain CH3 region.

[0192]

Furthermore, the antibody can be designed such that in the second H chain CH3 region, which is different from the first H chain CH3 region, one to three pairs of amino acid residues have a charge opposite to that of the corresponding amino acid residues in the first H chain CH3 region. These pairs are selected from the amino acid residue pairs shown in (1) to (3) above and correspond to the pairs in the first H chain CH3 region that have the same type of charge.

[0193]

The respective amino acid residues of (1) to (3) mentioned above are positioned close to each other when associated. For the desired H chain CH3 region or H chain constant region, those skilled in the art can find sites corresponding to the above-mentioned amino acid residues of (1) to (3) by homology modeling and such using commercially available software, and amino acid residues of these sites can be subjected to modifications as appropriate.

[0194]

In the above-mentioned antibodies, “amino acid residues having a charge” are preferably selected, for example, from amino acid residues contained in either one of groups (a) and (b) below:

(a) glutamic acid (E) and aspartic acid (D); and

(b) lysine (K), arginine (R), and histidine (H).

[0195]

Regarding the above-mentioned antibodies, “having the same type of charge” means, for example, that two or more amino acid residues all have amino acid residues included in either one of the above-mentioned groups (a) and (b). The phrase “having the opposite charge” means that, for example, when at least one of the two or more amino acid residues has an amino acid

residue included in either one of the above-mentioned groups (a) and (b), the remaining amino acid residue(s) will have an amino acid residue included in the other group.

[0196]

In a preferred embodiment of the above-mentioned antibody, the first H chain CH3 region and the second H chain CH3 region may be cross-linked by a disulfide bond.

[0197]

In the present invention, the amino acid residue to be subjected to modification is not limited to an amino acid residue of the constant region or variable region of the antibody described above. With regard to polypeptide mutants or heteromultimers, those skilled in the art can find amino acid residues that form the interface through homology modeling and such using commercially available software, and can subject the amino acid residues at those sites to modifications so that association is regulated.

[0198]

Other known techniques can also be used for the association of multispecific antibodies of the present invention. Polypeptides with different amino acids having an Fc region can be efficiently associated with each other by substituting an amino acid side chain present in one of the H chain variable regions of the antibody with a larger side chain (knob), and substituting an amino acid side chain present in the corresponding variable 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 AM *et al.* Nature Biotechnology (1998) 16, 677-681; and US20130336973).

[0199]

In addition, other known techniques can also be used to form multispecific antibodies of the present invention. Association of polypeptides having different sequences can be induced efficiently by complementary association of CH3s, using a strand-exchange engineered CH3 domain produced by changing part of CH3 in one of the H chains of an antibody into its corresponding IgA-derived sequence, and introducing into the complementary portion of the CH3 in the other H chain its corresponding IgA-derived sequence (Protein Engineering Design & Selection, 23; 195-202, 2010). This known technique can also be used to efficiently form multispecific antibodies of interest.

[0200]

In addition, the following techniques and such may be used for the formation of multispecific antibodies: techniques 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; techniques for producing bispecific antibodies using separately prepared monoclonal antibodies in combination (Fab Arm Exchange) as described in

WO2008/119353 and WO2011/131746; techniques for regulating association between antibody heavy chain CH3s as described in WO2012/058768 and WO2013/063702; techniques for producing bispecific antibodies composed of two types of light chains and one type of heavy chain as described in WO2012/023053; techniques for producing bispecific antibodies using two bacterial cell strains that individually express each 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)).

[0201]

An embodiment of multispecific antibody formation includes methods for obtaining bispecific antibodies by mixing two types of monoclonal antibodies in the presence of a reducing agent to cleave the disulfide bonds in the core hinge region, followed by re-association for heterodimerization (FAE) as described above. Furthermore, introduction of electrostatic interactions at the interacting interface of the CH3 region (WO2006/106905) can induce even more efficient heterodimerization during the re-association (WO2015/046467). In FAE using naturally-occurring IgG, re-association takes place randomly; and thus theoretically, bispecific antibodies can only be obtained at 50% efficiency; however, in this method, bispecific antibodies can be produced in high yield.

[0202]

Alternatively, even when a multispecific antibody of interest cannot be formed efficiently, a multispecific antibody of the present invention can be obtained by separating and purifying the multispecific antibody of interest from the produced antibodies. For example, a method has been reported that enables purification of two types of homologous forms and the heterologous antibody of interest by ion exchange chromatography, by conferring a difference in the isoelectric points by introducing amino acid substitutions into the variable regions of the two types of H chains (WO2007114325). To date, as a method for purifying heterologous forms, a method using Protein A to purify a heterodimerized 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 has been reported (WO98050431 and WO95033844). Furthermore, only the heterodimerized antibody can be purified efficiently using a Protein A column by changing the interaction between each of the H chains and Protein A, by using H chains in which amino acid residues at the IgG-Protein A binding site, positions 435 and 436 (EU numbering), are substituted with amino acids that yield a different binding strength to Protein A such as Tyr, His, or such.

[0203]

Alternatively, a common L chain that can confer binding ability to a plurality of different H chains can be obtained and used as the common L chain of a multispecific antibody. Efficient expression of a multispecific IgG can be achieved by introducing the genes of such a

common L chain and a plurality of different H chains into cells and expressing the IgG (Nature Biotechnology (1998) 16, 677-681). A method for selecting a common L chain that shows strong binding ability to any different H chains can also be used when selecting a common H chain (WO 2004/065611).

[0204]

Furthermore, an Fc region whose C-terminal heterogeneity has been improved can be appropriately used as an Fc region of the present invention. More specifically, Fc regions lacking glycine at position 446 and lysine at position 447, as specified by EU numbering, in the amino acid sequences of two polypeptides constituting an Fc region derived from IgG1, IgG2, IgG3, or IgG4, are provided.

[0205]

A plurality, such as two or more, of these techniques can be used in combination. Furthermore, these techniques can be appropriately and separately applied to the two H chains to be associated. Furthermore, these techniques can be used in combination with the above-mentioned Fc region of which Fcγ receptor-binding activity has been reduced. Furthermore, an antigen-binding molecule of the present invention may be a molecule produced separately based on an antigen-binding molecule subjected to the above-described modifications so as to have the same amino acid sequence.

[0206]

The multispecific antigen-binding molecules of the present invention may be any molecules that comprise (1) a first antigen-binding domain having binding activity towards CD3, which comprises the above-described antibody H chain variable region and L chain variable region, (2) a second antigen-binding domain comprising a different amino acid sequence from the first antigen-binding domain, and optionally (3) a domain comprising an Fc region with reduced binding activity towards an Fcγ receptor, and their structures are not limited.

In the present invention, each of the above-mentioned domains can be linked directly by peptide bonds. For example, when using $F(ab')_2$ as the antigen-binding domain of (1) and (2), and these Fc regions as the domain comprising an Fc region with reduced Fcγ receptor-binding activity of (3), the polypeptides formed by linking the antigen-binding domains of (1) and (2) and the Fc region-containing domain of (3) by peptide bonds will form an antibody structure. Such an antibody can be produced by purification from the above-mentioned hybridoma culture medium, and also by purifying the antibody from the culture medium of desired host cells that stably carry polynucleotides encoding the polypeptides constituting the antibody.

[0207]

Examples of a preferred antibody variable region having CD3-binding activity of the present invention include antibody variable regions having CD3ε-binding activity. Examples of

such antibody variable regions include: (i) the heavy chain variable regions of antibody Nos. 2, 14, 25, 29, 30-32, and 34 shown in Table 5 below, or antibody H-chain variable regions having the heavy chain CDR1, CDR2, and CDR3 amino acid sequences of said antibodies; (ii) the light chain variable regions of antibody Nos. 2, 14, 25, 29, 30-32, and 34 shown in Table 5, or antibody L-chain variable regions having the light chain CDR1, CDR2, and CDR3 amino acid sequences of said antibodies; and (iii) antibody H-chain and L-chain variable regions that are functionally equivalent to the aforementioned variable regions.

[0208]

In the present invention, the phrase "functionally equivalent" means that the binding affinities for an antigen are equivalent under given conditions, or alternatively, it means that the cytotoxic activities against target antigen-expressing cells or tissues containing these cells are equivalent when it is used as a multispecific antigen-binding molecule. The binding affinity and cytotoxic activity can be measured based on the description herein. The cells used for measurement of cytotoxic activity may be the desired target antigen-expressing cells or a desired tissue containing these cells, and for example, target antigen-expressing human cancer cell lines can be used. Regarding the antibody constant regions, the phrase may mean that the degree of reduction in Fcγ receptor-binding activity is equivalent.

[0209]

For example, an antibody H-chain variable region functionally equivalent to the antibody H chain variable region described herein (i.e., the original H chain variable region) means that this region has an equivalent binding affinity when it is combined with the antibody L-chain variable region described herein which forms a pair with the original H chain, or alternatively that the region has an equivalent cytotoxic activity towards target antigen-expressing cells or a tissue containing these cells when used for a multispecific antigen-binding molecule. Furthermore, an antibody L-chain variable region functionally equivalent to the antibody L-chain variable region described herein (i.e., the original L-chain variable region) means that this region has the same binding affinity when it is combined with the antibody H-chain variable region described herein which forms a pair with the original L chain, or alternatively that the region has the same cytotoxic activity towards target antigen-expressing cells or a tissue containing these cells when used for a multispecific antigen-binding molecule.

[0210]

The term "equivalent" does not necessarily have to mean the same degree of activity, and the activity may be enhanced; thus, the term may also be expressed as "equivalent or higher". Specifically, for antigen-binding affinity, examples of "equivalent" include the case where the value (KD value / parent KD value) obtained by comparison to the binding affinity of the antibody variable region serving as the control (parent KD value) is 2.0 or less. The value of

KD value / parent KD value is preferably 1.5 or less, more preferably 1.3 or less, 1.2 or less, 1.1 or less, 1.0 or less, 0.9 or less, 0.8 or less, 0.7 or less, 0.6 or less, or 0.5 or less. While there is no lower limit, examples include 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, or 10^{-6}. More specifically, in the present invention, the value of KD value / parent KD value is preferably 10^{-6} to 2.0, more preferably 10^{-3} to 1.5, even more preferably 10^{-1} to 1.3, even more preferably 0.8 to 1.2, yet even more preferably 0.9 to 1.1, and most preferably 1.0. For cytotoxic activity, examples of "equivalent" include the case where the value (cell growth inhibition rate / parent cell growth inhibition rate) obtained by comparison to the cell growth inhibition rate of the multispecific antigen-binding molecule serving as the control (parent cell growth inhibition rate) is 0.7 or more. The concentration of the added multispecific antigen-binding molecule can be determined appropriately, but is preferably, for example, 0.01 nM, 0.05 nM, 0.1 nM, 0.5 nM, or 1 nM; and preferably, measurements are taken at 0.05 nM or 0.1 nM. The value for cell growth inhibition rate / parent cell growth inhibition rate is preferably 0.8 or higher, more preferably 0.9 or higher, 1.0 or higher, 1.2 or higher, 1.5 or higher, 2 or higher, 3 or higher, 5 or higher, 10 or higher, or 20 or higher. While there is no upper limit, the value may be 10, 10^2, 10^3, 10^4, 10^5, or 10^6.

[0211]

Furthermore, for cytotoxic activity, examples include the case where the value (concentration for 50% inhibition of cell growth / parent concentration for 50% inhibition of cell growth) obtained by comparison to the concentration of the original multispecific antigen-binding molecule for 50% inhibition of cell growth (parent concentration for 50% inhibition of cell growth) is 1.5 or less. Concentration for 50% growth inhibition refers to the concentration of the multispecific antigen-binding molecule necessary for reducing the cell proliferation rate to one half compared to when the multispecific antigen-binding molecule is not added. The value of "concentration for 50% inhibition of cell growth / parent concentration for 50% inhibition of cell growth" is preferably 1.3 or less, more preferably 1.2 or less, 1.1 or less, 1.0 or less, 0.9 or less, 0.8 or less, 0.7 or less, 0.6 or less, or 0.5 or less. While there is no lower limit, the value may be, for example, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, or 10^{-6}. Specifically, the value is preferably 10^{-6} to 1.5×10^{-0}, more preferably 10^{-6} to 10^{-1}, even more preferably 10^{-6} to 10^{-2}, and yet even more preferably 10^{-6} to 10^{-3}.

[0212]

Cytotoxicity can be evaluated based on the *in vitro* TDCC activity as described in Example 4, and in such a case, "equivalent" can include the case where the value (TDCC activity / parent TDCC activity) obtained by comparison to the TDCC activity of a multispecific antigen-binding molecule serving as the control is 0.7 or higher. The value of TDCC activity / parent TDCC activity is preferably 0.8 or higher, more preferably 0.9 or higher, 1.0 or higher, 1.2 or higher, 1.5 or higher, 2 or higher, 3 or higher, 5 or higher, 10 or higher, or 20 or higher.

[0213]

Regarding the second antigen-binding domain having target antigen-binding activity, the KD value towards the target antigen may be, for example, 5 x 10^{-9} M or less, preferably 4 x 10^{-9} M or less, such as 3 x 10^{-9} M or less, 2 x 10^{-9} M or less, 1 x 10^{-9} M or less, 8 x 10^{-10} M or less, 5 x 10^{-10} M or less, 4 x 10^{-10} M or less, 3 x 10^{-10} M or less, 2 x 10^{-10} M or less, 1 x 10^{-10} M or less, 8 x 10^{-11} M or less, 5 x 10^{-11} M or less, 4 x 10^{-11} M or less, 3 x 10^{-11} M or less, 2 x 10^{-11} M or less, 1 x 10^{-11} M or less, 8 x 10^{-12} M or less, 5 x 10^{-12} M or less, 4 x 10^{-12} M or less, 3 x 10^{-12} M or less, 2 x 10^{-12} M or less, 1 x 10^{-12} M or less, 8 x 10^{-13} M or less, 5 x 10^{-13} M or less, 4 x 10^{-13} M or less, 3 x 10^{-13} M or less, 2 x 10^{-13} M or less, or 1 x 10^{-13} M or less.

[0214]

Regarding the antigen-binding domain comprising an antibody variable region having CD3-binding activity, the KD value towards CD3 such as human CD3, or more specifically for example human CD3ε chain may be, for example, 5 x 10^{-7} M or less, preferably 2 x 10^{-7} M or less, such as 1.5 x 10^{-7} M or less, 1.4 x 10^{-7} M or less, 1.3 x 10^{-7} M or less, 1.2 x 10^{-7} M or less, 1 x 10^{-7} M or less, 3 x 10^{-8} M or less, 2 x 10^{-8} M or less, 1 x 10^{-8} M or less, 8 x 10^{-9} M or less, 5 x 10^{-9} M or less, 4 x 10^{-9} M or less, 3 x 10^{-9} M or less, 2 x 10^{-9} M or less, 1 x 10^{-9} M or less, 8 x 10^{-10} M or less, 5 x 10^{-10} M or less, 4 x 10^{-10} M or less, 3 x 10^{-10} M or less, 2 x 10^{-10} M or less, 1 x 10^{-10} M or less, 8 x 10^{-11} M or less, 5 x 10^{-11} M or less, 4 x 10^{-11} M or less, 3 x 10^{-11} M or less, 2 x 10^{-11} M or less, 1 x 10^{-11} M or less, 8 x 10^{-12} M or less, 5 x 10^{-12} M or less, 4 x 10^{-12} M or less, 3 x 10^{-12} M or less, 2 x 10^{-12} M or less, or 1 x 10^{-12} M or less.

[0215]

The multispecific antigen-binding molecules of the present invention preferably have KD values toward the target antigen and human CD3 (for example, human CD3ε chain) that are 5 x 10^{-9} M or less and 5 x 10^{-7} M or less, respectively, and more preferably 1 x 10^{-9} M or less and 5 x 10^{-8} M or less, respectively.

[0216]

In the present invention, antibody variable regions that are “functionally equivalent” are not particularly limited as long as they are antibody H-chain and/or antibody L-chain variable regions that satisfy the above-described conditions. Examples of such antibody variable regions include regions that may have a substitution, deletion, addition, and/or insertion of one or more amino acids (for example, 1, 2, 3, 4, 5, or 10 amino acids) into the amino acid sequences of the variable regions of antibody Nos. 2, 14, 25, 29, 30-32, and 34 of Table 5. A method well known to those skilled in the art for introducing one or more amino-acid substitutions, deletions, additions, and/or insertions into an amino acid sequence is a method of introducing mutations into proteins. For example, those skilled in the art can prepare variable regions that are functionally equivalent to the antibody variable regions having the above-mentioned functions

by appropriately introducing mutations into amino acid sequences using methods such as site-directed mutagenesis (Hashimoto-Gotoh, T., Mizuno, T., Ogasahara, Y., and Nakagawa, M. (1995) An oligodeoxyribonucleotide-directed dual amber method for site-directed mutagenesis. Gene 152, 271-275; Zoller, M.J., and Smith, M. (1983) Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. 100, 468-500; Kramer, W., Drutsa, V., Jansen, H.W., Kramer, B., Pflugfelder, M., and Fritz, H.J. (1984) The gapped duplex DNA approach to oligonucleotide-directed mutation construction. Nucleic Acids Res. 12, 9441-9456; Kramer, W., and Fritz, H.J. (1987) Oligonucleotide-directed construction of mutations *via* gapped duplex DNA Methods. Enzymol. 154, 350-367; and Kunkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc Natl Acad. Sci. U S A. 82, 488-492).

[0217]

When an amino acid residue is altered, the amino acid is preferably mutated into a different amino acid that conserves the properties of the amino acid side-chain. Examples of amino-acid side chain properties are: hydrophobic amino acids (A, I, L, M, F, P, W, Y, and V), hydrophilic amino acids (R, D, N, C, E, Q, G, H, K, S, and T), amino acids containing aliphatic side chains (G, A, V, L, I, and P), amino acids containing hydroxyl group-containing side chains (S, T, and Y), amino acids containing sulfur atom-containing side chains (C and M), amino acids containing carboxylic acid- and amide-containing side chains (D, N, E, and Q), amino acids containing basic side chains (R, K, and H), and amino acids containing aromatic side chains (H, F, Y, and W) (amino acids are represented by one-letter codes in parentheses). Amino acid substitutions within each of these groups are called conservative substitutions. It is already known that a polypeptide containing a modified amino acid sequence in which one or more amino acid residues in a given amino acid sequence are deleted, added, and/or substituted with other amino acids can retain the original biological activity (Mark, D. F. *et al*., Proc. Natl. Acad. Sci. USA, (1984) 81: 5662-6; Zoller, M. J. and Smith, M., Nucleic Acids Res. (1982) 10: 6487-500; Wang, A. *et al*., Science (1984) 224: 1431-3; Dalbadie-McFarland, G. *et al*., Proc. Natl. Acad. Sci. USA (1982) 79: 6409-13). Variable regions of the present invention containing such amino acid modifications have an amino acid sequence identity of at least 70%, more preferably at least 75%, even more preferably at least 80%, still more preferably at least 85%, yet more preferably at least 90%, and most preferably at least 95%, with the amino acid sequence of the CDR sequences, FR sequences, or whole variable regions of the variable region prior to modification. Herein, sequence identity is defined as the percentage of residues identical to those in the original amino acid sequence of the H-chain variable region or L-chain variable region determined after the sequences are aligned, and gaps are appropriately introduced to

maximize the sequence identity as necessary. The identity of amino acid sequences can be determined by the method described below.

[0218]

Furthermore, a "functionally equivalent antibody variable region" can be obtained, for example, from nucleic acids that hybridize under stringent conditions with nucleic acids comprising a nucleotide sequence encoding the amino acid sequence of the variable region of antibody Nos. 2, 14, 25, 29, 30-32, or 34 of Table 5. Stringent hybridization conditions for isolating a nucleic acid that hybridizes under stringent conditions with a nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of a variable region include, for example, the conditions of 6 M urea, 0.4% SDS, 0.5x SSC, and 37°C, or hybridization conditions with a stringency equivalent thereto. Isolation of nucleic acids with a much higher homology can be expected with more stringent conditions, for example, the conditions of 6 M urea, 0.4% SDS, 0.1x SSC, and 42°C. The washing conditions following the hybridization are, for example, washing using 0.5x SSC (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate at pH7.0) and 0.1% SDS at 60°C, more preferably washing using 0.2x SSC and 0.1% SDS at 60°C, even more preferably washing using 0.2x SSC and 0.1% SDS at 62°C, yet even more preferably washing using 0.2x SSC and 0.1% SDS at 65°C, and still more preferably washing using 0.1x SSC and 0.1% SDS at 65°C. The sequences of the isolated nucleic acids can be determined by the known methods described below. The overall nucleotide sequence homology of the isolated nucleic acid is at least 50% or higher, preferably 70% or higher, and more preferably 90% or higher (for example, 95%, 96%, 97%, 98%, 99%, or higher) sequence identity.

Nucleic acids that hybridize under stringent conditions to a nucleic acid comprising a nucleotide sequence encoding the amino acid sequence of a variable region can also be isolated by using, instead of the above-described methods using hybridization techniques, gene amplification methods such as polymerase chain reaction (PCR) that uses primers synthesized based on information of the nucleotide sequence encoding the variable region amino acid sequence.

[0219]

The identity of one nucleotide sequence or amino acid sequence to another can be determined using the algorithm BLAST, by Karlin and Altschul (Proc. Natl. Acad. Sci. USA (1993) 90: 5873-7). Programs called BLASTN and BLASTX were developed based on this algorithm (Altschul *et al.*, J. Mol. Biol. (1990) 215: 403-10). To analyze nucleotide sequences according to BLASTN based on BLAST, the parameters are set, for example, as score = 100 and wordlength = 12. On the other hand, parameters used for the analysis of amino acid sequences by BLASTX based on BLAST include, for example, score = 50 and wordlength = 3. Default parameters for each program are used when using the BLAST and Gapped BLAST programs.

Specific techniques for such analyses are known in the art (see the website of the National Center for Biotechnology Information (NCBI), Basic Local Alignment Search Tool (BLAST); http://www.ncbi.nlm.nih.gov).

[0220]

Nucleic acids, vectors, and cells

In an aspect, the present invention also relates to nucleic acids (polynucleotides) encoding the antigen-binding molecules of the present invention. The nucleic acid can be inserted into any suitable expression vectors. Suitable hosts can be transformed with the expression vectors to produce cells that express the antigen-binding molecules. Antigen-binding molecules encoded by the nucleic acids (polynucleotides) can be obtained by culturing the cells that express the antigen-binding molecules, and collecting the expression products from culture supernatants. That is, the present invention relates to vectors comprising a nucleic acid (polynucleotide) encoding an antigen-binding molecule of the present invention, cells carrying such a vector, and methods for producing antigen-binding molecules, which comprise culturing the cells and collecting antigen-binding molecules from culture supernatants. These can be obtained by techniques similar to those for recombinant antibodies mentioned above.

[0221]

Pharmaceutical compositions

In an aspect, the present invention provides pharmaceutical compositions comprising as the active ingredient an antigen-binding molecule (in particular, a multispecific antigen-binding molecule) of the present invention. In an embodiment, the present invention provides pharmaceutical compositions that induce cell injury, which comprise the antigen-binding molecule as an active ingredient. Pharmaceutical compositions of the present invention which induce cell injury, particularly T-cell-dependent cellular cytotoxicity, are preferably administered to a subject suffering from a disease for which said cell injury is required for prevention or treatment, or to a subject who is at risk of relapse. In an embodiment, the pharmaceutical compositions of the present invention are for use in the treatment or prevention of cancer.

[0222]

The pharmaceutical composition comprising an antigen-binding molecule (in particular, a multispecific antigen-binding molecule) of the present invention as an active ingredient can be expressed as a cytotoxicity-inducing agent or cell growth-inhibiting agent comprising as an active ingredient the antigen-binding molecule, a method for inducing cell injury or method for inhibiting cell growth comprising the step of administering the antigen-binding molecule to a subject, the antigen-binding molecule for use in inducing cell injury or inhibiting cell growth, or use of the antigen-binding molecule in the manufacture of a cytotoxicity-inducing agent and a cell growth-inhibiting agent.

The pharmaceutical composition comprising an antigen-binding molecule (in particular, a multispecific antigen-binding molecule) of the present invention as an active ingredient may also be expressed as a therapeutic or preventive agent for cancer comprising the antigen-binding molecule as an active ingredient, a method for treating or preventing cancer comprising a step of administering the antigen-binding molecule to a subject, the antigen-binding molecule for use in the treatment or prevention of cancer, or use of the antigen-binding molecule in the manufacture of an agent for the treatment or prevention of cancer.

[0223]

In the present invention, "comprising an antigen-binding molecule as an active ingredient" means comprising the antigen-binding molecule as a major active component, without limitation to the content ratio of the antigen-binding molecule.

[0224]

In an embodiment, the antigen-binding molecules of the present invention can be administered by a method in which a nucleic acid encoding the antigen-binding molecule of the present invention is administered or incorporated into a body using a vector or the like, thereby allowing the antigen-binding molecule of the present invention to be directly expressed *in vivo*. However, administration may also occur without using a vector. As vectors, viral vectors, plasmid vectors are exemplified, and even adenoviral vectors and adeno-associated viral vectors are exemplified. The nucleic acid encoding the antigen-binding molecule of the present invention may be administered directly to the body, or cells into which the nucleic acid encoding the antigen-binding molecule of the present invention are introduced may be administered to the body. For example, the antigen-binding molecule of the present invention may be administered by a method in which mRNA encoding the antigen-binding molecule of the present invention is chemically modified to enhance the stability of mRNA *in vivo*, and this mRNA is then administered directly to humans to express the antigen-binding molecule of the present invention *in vivo* (see EP2101823B, WO2013/120629). Also, B cells into which the nucleic acid encoding the antigen-binding molecule of the present invention is introduced may be administered (Sci Immunol. (2019), 4(35), eaax0644). Bacteria into which the nucleic acid encoding the antigen-binding molecule of the present invention is introduced may also be administered (Nature Reviews Cancer (2018) 18, 727-743).

[0225]

Moreover, if necessary, the antigen-binding molecules of the present invention may be encapsulated in microcapsules (e.g., those made of hydroxymethylcellulose, gelatin, and poly(methylmetacrylate)), or incorporated as components of a colloidal drug delivery system (e.g., liposomes, albumin microspheres, microemulsion, nanoparticles, and nanocapsules) (see, for example, "Remington's Pharmaceutical Science 16th edition", Oslo Ed. (1980)). Methods

for preparing the pharmaceutical agents as controlled-release pharmaceutical agents are also well known, and such methods may be applied to the antigen-binding molecules of the present invention (J. Biomed. Mater. Res. (1981) 15: 267-277; Chemtech. (1982) 12: 98-105; U.S. Patent No. 3,773,719; European Patent Application Publication Nos. EP 58,481 and EP 133,988; Biopolymers (1983) 22: 547-556).

[0226]

The pharmaceutical compositions, cytotoxicity-inducing agents, cell growth-inhibiting agents, and agents for treating or agents for preventing cancer of the present invention (hereinafter collectively referred to as "the pharmaceutical compositions of the present invention") may be administered to patients by oral or parenteral administration, and parenteral administration is preferred. Specific examples of the administration method include administration by injection, transnasal administration, transpulmonary administration, and transdermal administration. Examples of administration by injection include intravenous injection, intramuscular injection, intraperitoneal injection, and subcutaneous injection. A pharmaceutical composition of the present invention can be administered systemically or locally, for example, through administration by injection. The method of administration can be selected appropriately according to the age and symptoms of the patient. The dose can be selected from the range of 0.0001 mg to 1000 mg per kilogram body weight for a single administration. Alternatively, for example, the dose may be selected from the range of 0.001 mg/body to 100000 mg/body per patient. However, the pharmaceutical compositions of the present invention are not limited to these doses.

[0227]

The pharmaceutical compositions of the present invention can be formulated according to conventional methods (for example, Remington's Pharmaceutical Science, latest edition, Mark Publishing Company, Easton, U.S.A), and may also contain pharmaceutically (pharmacologically) acceptable carriers and additives. Examples include, but are not limited to surfactants, excipients, coloring agents, perfumes, preservatives, stabilizers, buffers, suspending agents, isotonization agents, binders, disintegrants, lubricants, fluidity promoting agents, and flavoring agents; and other commonly used carriers can be suitably used. Specific examples of the carriers include light anhydrous silicic acid, lactose, crystalline cellulose, mannitol, starch, carmellose calcium, carmellose sodium, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyvinylacetal diethylaminoacetate, polyvinylpyrrolidone, gelatin, medium chain fatty acid triglyceride, polyoxyethylene hardened castor oil 60, saccharose, carboxymethyl cellulose, corn starch, inorganic salt, and such.

[0228]

As a non-limiting example of techniques that can be combined with the antigen-binding molecules of the present invention, the generation of T cells secreting T cell redirecting antibodies utilizing a CD3-binding domain is exemplified (Trends Immunol.(2019) 40(3) 243-257). One of the non-limiting techniques for the generation is the introduction of a nucleic acid encoding a bispecific antibody, comprising a CD3 binding domain described herein and a binding domain for a cancer antigen, into an effector cell such as a T cell by genetic modification techniques.

[0229]

Methods of treatment and such

In one aspect, the present invention provides methods for causing (inducing) injury to a target cell or methods for suppressing growth of a target cell, comprising a step of allowing the target cell to contact with a multispecific antigen-binding molecule of the present invention which binds to an antigen that is specifically expressed on the surface of the target cell. In one embodiment, the present invention provides methods for causing (inducing) injury to a cell that expresses a target cancer-specific antigen or to a tumor tissue comprising such cells, or methods for suppressing growth of the cell or the tumor tissue, comprising a step of allowing the cell that expresses the target cancer-specific antigen to contact with a multispecific antigen-binding molecule of the present invention that binds to the antigen. In another embodiment, the present invention provides methods for causing (inducing) injury to a cell that has a function to suppress an immune response or methods for suppressing growth of the cell, comprising a step of allowing the cell that has a function to suppress an immune response to contact with a multispecific antigen-binding molecule of the present invention that binds to an antigen expressed on the surface of the cell. The multispecific antigen-binding molecule that binds to the antigen is as described above for an antigen-binding molecule of the present invention that binds to the antigen, which is comprised in the cytotoxicity-inducing agents and cell growth-inhibiting agents of the present invention. The cells bound by a multispecific antigen-binding molecule of the present invention that binds to the antigen are not particularly limited as long as they are cells expressing the antigen.

[0230]

In the present invention, “contact” is carried out, for example, by adding a multispecific antigen-binding molecule of the present invention which binds to the antigen to the culture medium of target antigen-expressing cells cultured *in vitro*. In this case, a liquid or a solid obtained by freeze-drying or such may be suitably used as the form of the added antigen-binding molecule. When added as an aqueous solution, it may be an aqueous solution that simply contains only the multispecific antigen-binding molecule of the present invention, or it may be a solution containing also, for example, the above-mentioned surfactants, excipients, coloring

agents, perfumes, preservatives, stabilizers, buffers, suspending agents, isotonization agents, binders, disintegrants, lubricants, fluidity promoting agents, and flavoring agents. The concentration at which the addition is performed is not particularly limited, but a suitable final concentration in the culture solution is preferably in the range of 1 pg/ml to 1 g/ml, more preferably 1 ng/ml to 1 mg/ml, and even more preferably 1 μg/mL to 1 mg/mL.

[0231]

Furthermore, in another embodiment, "contact" of the present invention is also carried out by administering an antigen-binding molecule of the present invention to non-human animals with cells expressing the target antigen transplanted into their bodies, and to animals carrying cells that intrinsically express the antigen. The method of administration may be oral or parenteral, and parenteral administration is particularly preferred. Specific examples of the administration method include administration by injection, transnasal administration, transpulmonary administration, and transdermal administration. Examples of administration by injection include intravenous injection, intramuscular injection, intraperitoneal injection, and subcutaneous injection. A pharmaceutical composition of the present invention or a cytotoxicity-inducing agent and a cell growth-inhibiting agent can be administered systemically or locally, for example, through administration by injection. The method of administration can be selected appropriately according to the age and symptoms of the test animal. When administered as an aqueous solution, an aqueous solution containing simply only a multispecific antigen-binding molecule of the present invention may be used, or a solution containing also the above-mentioned surfactants, excipients, coloring agents, perfumes, preservatives, stabilizers, buffers, suspending agents, isotonization agents, binders, disintegrants, lubricants, fluidity promoting agents, flavoring agents, and such may be used. The dose can be selected from the range of 0.0001 mg to 1000 mg per kilogram body weight for a single administration. Alternatively, for example, the dose may be selected from the range of 0.001 mg/body to 100000 mg/body per subject. However, the amount of the multispecific antigen-binding molecule of the present invention administered is not limited to these doses.

[0232]

The following method is suitably used as a method for evaluating or measuring cell injury induced in target cells (in particular, cells expressing a target antigen other than CD3, which is bound by the second antigen-binding domain of the multispecific antigen-binding molecule of the present invention) as a result of contacting the cells with a multispecific antigen-binding molecule of the present invention. Examples of a method for evaluating or measuring the cytotoxic activity *in vitro* include methods for measuring cytotoxic T cell activity and such. Whether or not a multispecific antigen-binding molecule of the present invention has T cellular cytotoxicity can be measured by known methods (for example, Current protocols in

Immunology, Chapter 7. Immunologic studies in humans, Editor, John E. Coligan *et al.*, John Wiley & Sons, Inc., (1993) and the like). For activity measurements, an antigen-binding molecule that binds to an antigen different from the target antigen, which is an antigen not expressed in the cells used for the examination, can be used as a control in the same manner as a multispecific antigen-binding molecule of the present invention, and the activity can be determined to be present when the multispecific antigen-binding molecule of the present invention shows a stronger cytotoxic activity than the antigen-binding molecule used as a control.

[0233]

To evaluate or measure cytotoxic activity *in vivo*, cells expressing a target antigen are intradermally or subcutaneously transplanted into a non-human test animal. Then, a test antigen-binding molecule is intravenously or intraperitoneally administered daily or at intervals of a few days, starting from the day of transplantation or the following day. The cytotoxic activity can be determined by measuring the tumor size daily and observing the difference in the change of tumor size. In a similar manner to the *in vitro* evaluation, the cytotoxic activity of an antigen-binding molecule of the present invention can be determined to be present when administration of a control antigen-binding molecule shows that the tumor size in the group subjected to administration of an antigen-binding molecule of the present invention is significantly smaller than the tumor size in the group subjected to administration of the control antigen-binding molecule.

[0234]

As a method for evaluating or measuring the suppressive effect on proliferation of cells expressing a target antigen, a method of measuring the uptake of isotope-labeled thymidine into cells or the MTT method may be suitably used. As a method for evaluating or measuring the cell proliferation-suppressing activity *in vivo*, the same method described above for evaluating or measuring cytotoxic activity *in vivo* may be suitably used.

[0235]

Kits

The present invention also provides kits for use in the methods of the present invention, which comprise an antigen-binding molecule of the present invention or an antigen-binding molecule produced by a production method of the present invention. Additionally, the kit may include in its package, a pharmaceutically acceptable carrier, solvent, and instructions describing the method of use.

The present invention also relates to an antigen-binding molecule of the present invention or an antigen-binding molecule produced by a production method of the present invention for use in a method of the present invention.

[0236]

Although the foregoing invention has 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 invention. The disclosures of all patent and scientific literature cited herein are expressly incorporated in their entirety by reference.

Examples

[0237]

Herein below, the present invention will be specifically described with reference to the Examples, but it is not to be construed as being limited thereto.

[0238]

[Example 1] Preparation of CDR variants having elevated binding stability to CD3 antigen

To date, there have been reports of bispecific antibodies that recognize CD3 (a T cell marker) and an antigen on target cells, thereby damaging the target cells. However, there remains room for improving binding stability of the reported CD3-binding antibody variable regions. By improving this profile, effects such as sustained *in vivo* efficacy and ease of *in vitro* evaluation were expected. More specifically, the antibody variable region disclosed in WO2015174439 loses binding activity in a buffer over time, and further modifications to improve binding stability were considered necessary.

First, an antibody heavy chain gene TR01H113-F760mnP17 (SEQ ID NO: 30), having a heavy chain variable region of an antibody against human CD3 described in WO2015174439 and a human IgG1 constant region sequence, and an antibody light chain gene L0011-KT0 (SEQ ID NO: 34), having a light chain variable region of an antibody against human CD3 and a human κ chain constant region sequence, were prepared. Modifications were introduced to the nucleotides defining the CDR amino acid sequences in these antibody genes by methods known to a person skilled in the art, and plasmids defining CDR-altered antibodies were obtained. Plasmids defining the heavy and light chains were mixed and introduced into human embryonic kidney cell-derived HEK293 type cells. After four days of culturing, antibodies were obtained by purification from the supernatant using methods known to those skilled in the art. Absorbance at 280 nm was measured for each purified antibody solution using a spectrophotometer. The concentration of the purified antibody was calculated from the obtained measurement values using the extinction coefficient calculated by the PACE method (Protein Science (1995) 4, 2411-2423).

[0239]

[Table 1]

Generated CDR variants

Antibody No.	Abbreviated name of heavy chain variable region	Abbreviated name of light chain variable region	Antibody name
1	TR01H113	L0011	TR01H113-F760mnP17/L0011-KT0
2	TR01H113	L0011.71	TR01H113-F760mnP17/L0011.71-KT0
3	TR01H113.38	L0011.57	TR01H113.38-F760mnP17/L0011.57-KT0
4	TR01H113.38	L0011.71	TR01H113.38-F760mnP17/L0011.71-KT0
5	TR01H113.H96A	L0011	TR01H113.H96A-F760mnP17/L0011-KT0
6	TR01H113.412	L0011.71	TR01H113.412-F760mnP17/L0011.71-KT0
7	TR01H113.453	L0011.71	TR01H113.453-F760mnP17/L0011.71-KT0
8	TR01H113.412	L0011.273	TR01H113.412-F760mnP17/L0011.273-KT0

9	TR01H113.412	L0011.274	TR01H113.412-F760mnP17/L0011.274-KT0
10	TR01H113.412	L0011.57	TR01H113.412-F760mnP17/L0011.57-KT0
11	TR01H113.455	L0011.71	TR01H113.455-F760mnP17/L0011.71-KT0
12	TR01H113.456	L0011.71	TR01H113.456-F760mnP17/L0011.71-KT0
13	TR01H113.412	L0011.115	TR01H113.412-F760mnP17/L0011.115-KT0
14	TR01H113.457	L0011.71	TR01H113.457-F760mnP17/L0011.71-KT0
15	TR01H113.458.S	L0011.71	TR01H113.458.S-F760mnP17/L0011.71-KT0
16	TR01H113.412	L0011.275	TR01H113.412-F760mnP17/L0011.275-KT0
17	TR01H113.455	L0011.74	TR01H113.455-F760mnP17/L0011.74-KT0
18	TR01H113.456	L0011.74	TR01H113.456-F760mnP17/L0011.74-KT0
19	TR01H113.412	L0011.65	TR01H113.412-F760mnP17/L0011.65-KT0
20	TR01H113.412	L0011.277	TR01H113.412-F760mnP17/L0011.277-KT0
21	TR01H113.412	L0011.278	TR01H113.412-F760mnP17/L0011.278-KT0
22	TR01H113.412	L0011.279	TR01H113.412-F760mnP17/L0011.279-KT0
23	TR01H113.460	L0011.71	TR01H113.460-F760mnP17/L0011.71-KT0
24	TR01H113.412	L0011.280	TR01H113.412-F760mnP17/L0011.280-KT0
25	TR01H113.457	L0011.74	TR01H113.457-F760mnP17/L0011.74-KT0
26	TR01H113.412	L0011.281	TR01H113.412-F760mnP17/L0011.281-KT0
27	TR01H113.469	L0011.71	TR01H113.469-F760mnP17/L0011.71-KT0
28	TR01H113.412	L0011.283	TR01H113.412-F760mnP17/L0011.283-KT0
29	TR01H113.479	L0011.71	TR01H113.479-F760mnP17/L0011.71-KT0
30	TR01H113.124	L0011.71	TR01H113.124-F760mnP17/L0011.71-KT0
31	TR01H113.479	L0011.273	TR01H113.479-F760mnP17/L0011.273-KT0
32	TR01H113.479	L0011.274	TR01H113.479-F760mnP17/L0011.274-KT0
33	TR01H113.124	L0011.74	TR01H113.124-F760mnP17/L0011.74-KT0
34	TR01H113.479	L0011.57	TR01H113.479-F760mnP17/L0011.57-KT0
35	TR01H113.480	L0011.71	TR01H113.480-F760mnP17/L0011.71-KT0
36	TR01H113.412	L0011.74	TR01H113.412-F760mnP17/L0011.74-KT0

[0240]

[Example 2] Evaluation of antibodies having altered CDRs with respect to the binding to a CD3 antigen

A polynucleotide encoding an antigen consisting of a human CD3ε, CD3γ, and FLAG tag was introduced into HEK293 type cells. Culture supernatant was applied to ion exchange chromatography, affinity chromatography based on FLAG tag, and gel filtration chromatography for purification, and an antigen (herein below, CD3eg-linker) was obtained.

Analysis of interactions between the prepared antibodies and the CD3eg-linker was carried out by the following method using Octet HTX. Phosphate buffered saline (pH7.4) containing 0.05% TWEEN20 was used as a buffer. Protein A chip (sartorius) was used as a sensor chip. Each of the antibodies described in Table 1 was prepared at 10 μg/mL, and human CD3eg-linker was prepared in serial dilution at 900, 300, 100, and 33 nM in the buffer. After a baseline (30 seconds), antibodies were loaded onto sensor chips for 60 seconds, and then following a 30 second baseline step, antigen association/dissociation steps (each for 180 seconds) were carried out to measure antigen binding properties. The intensities of responses at 295 seconds of measurement were defined as the binding value. Equilibrium dissociation constant (KD) was determined by global fitting (Octet BLI Analysis Version: 12.2.2.4) to response curves at multiple concentrations. In this global fitting, calculation was made based on a 1:1 binding model over the entire regions of the association phase and dissociation phase.

[0241]

[Table 2]

Measurement of binding between the generated variants and human CD3eg-linker

Anti-body No.	Abbreviated name of heavy chain variable region	Abbreviated name of light chain variable region	Antibody name	Binding value (nm)	KD (nM)	KD Error (nM)
1	TR01H113	L0011	TR01H113-F760mnP17/L0011-KT0	0.129	319.1	3.29
2	TR01H113	L0011.71	TR01H113-F760mnP17/L0011.71-KT0	0.105	491.4	15.33
3	TR01H113.38	L0011.57	TR01H113.38-F760mnP17/L0011.57-KT0	0.084	323	12.12
4	TR01H113.38	L0011.71	TR01H113.38-F760mnP17/L0011.71-KT0	0.081	383.3	13.85
5	TR01H113.H96A	L0011	TR01H113.H96A-F760mnP17/L0011-KT0	0.073	894.8	26.17
6	TR01H113.412	L0011.71	TR01H113.412-F760mnP17/L0011.71-KT0	0.089	241.9	5.844
7	TR01H113.453	L0011.71	TR01H113.453-F760mnP17/L0011.71-KT0	0.075	370.3	8.951
8	TR01H113.412	L0011.273	TR01H113.412-F760mnP17/L0011.273-KT0	0.077	703.8	42.25
9	TR01H113.412	L0011.274	TR01H113.412-F760mnP17/L0011.274-KT0	0.068	33570	78190
10	TR01H113.412	L0011.57	TR01H113.412-F760mnP17/L0011.57-KT0	0.073	1822	237
11	TR01H113.455	L0011.71	TR01H113.455-F760mnP17/L0011.71-KT0	0.082	618.1	23.67
12	TR01H113.456	L0011.71	TR01H113.456-F760mnP17/L0011.71-KT0	0.076	1230	71.42
13	TR01H113.412	L0011.115	TR01H113.412-F760mnP17/L0011.115-KT0	0.078	359.5	8.488
14	TR01H113.457	L0011.71	TR01H113.457-F760mnP17/L0011.71-KT0	0.086	276.8	6.39
15	TR01H113.458.S	L0011.71	TR01H113.458.S-F760mnP17/L0011.71-KT0	0.063	275.8	6.652
16	TR01H113.412	L0011.275	TR01H113.412-F760mnP17/L0011.275-KT0	0.075	264.3	4.955
17	TR01H113.455	L0011.74	TR01H113.455-F760mnP17/L0011.74-KT0	0.076	156.8	2.852
18	TR01H113.456	L0011.74	TR01H113.456-F760mnP17/L0011.74-KT0	0.077	235.9	5.222
19	TR01H113.412	L0011.65	TR01H113.412-F760mnP17/L0011.65-KT0	0.069	184.9	4.133
20	TR01H113.412	L0011.277	TR01H113.412-F760mnP17/L0011.277-KT0	0.064	141	4.026
21	TR01H113.412	L0011.278	TR01H113.412-F760mnP17/L0011.278-KT0	0.058	326.4	11.68
22	TR01H113.412	L0011.279	TR01H113.412-F760mnP17/L0011.279-KT0	0.064	392.4	10.43
23	TR01H113.460	L0011.71	TR01H113.460-F760mnP17/L0011.71-KT0	0.058	380.6	9.186
24	TR01H113.412	L0011.280	TR01H113.412-F760mnP17/L0011.280-KT0	0.071	362.5	7.948
25	TR01H113.457	L0011.74	TR01H113.457-F760mnP17/L0011.74-KT0	0.089	383	8.562
26	TR01H113.412	L0011.281	TR01H113.412-F760mnP17/L0011.281-KT0	0.053	193.8	6.432

27	TR01H113.469	L0011.71	TR01H113.469-F760mnP17/L0011.71-KT0	0.070	237.1	6.19
28	TR01H113.412	L0011.283	TR01H113.412-F760mnP17/L0011.283-KT0	0.062	175.3	6.35
29	TR01H113.479	L0011.71	TR01H113.479-F760mnP17/L0011.71-KT0	0.127	253.3	3.026
30	TR01H113.124	L0011.71	TR01H113.124-F760mnP17/L0011.71-KT0	0.108	413.4	8.33
31	TR01H113.479	L0011.273	TR01H113.479-F760mnP17/L0011.273-KT0	0.119	329.1	4.226
32	TR01H113.479	L0011.274	TR01H113.479-F760mnP17/L0011.274-KT0	0.117	419.3	5.239
33	TR01H113.124	L0011.74	TR01H113.124-F760mnP17/L0011.74-KT0	0.092	1250	51.8
34	TR01H113.479	L0011.57	TR01H113.479-F760mnP17/L0011.57-KT0	0.134	346.3	5.967
35	TR01H113.480	L0011.71	TR01H113.480-F760mnP17/L0011.71-KT0	0.085	1194	50.08
36	TR01H113.412	L0011.74	TR01H113.412-F760mnP17/L0011.74-KT0	0.066	1401	99.54

[0242]

[Example 3] Evaluation of antibodies having altered CDRs with respect to the stability of binding to CD3eg-linker

The prepared antibodies were diluted to 0.1 mg/mL and stored under conditions at 4°C for three days or at 50°C for three days. Antigen binding was evaluated using the same method as in Example 2.

[0243]

[Table 3]

Stability of binding between the generated variants and human CD3eg-linker

Antibody No.	Abbreviated name of heavy chain variable region	Abbreviated name of light chain variable region	Antibody name	Binding value (nm)		Binding H/I ratio
				When stored at 4°C	When stored at 50°C	
1	TR01H113	L0011	TR01H113-F760mnP17/L0011-KT0	0.129	0.078	61%
2	TR01H113	L0011.71	TR01H113-F760mnP17/L0011.71-KT0	0.105	0.080	76%
3	TR01H113.38	L0011.57	TR01H113.38-F760mnP17/L0011.57-KT0	0.084	0.063	74%
4	TR01H113.38	L0011.71	TR01H113.38-F760mnP17/L0011.71-KT0	0.081	0.066	81%
5	TR01H113.H96A	L0011	TR01H113.H96A-F760mnP17/L0011-KT0	0.073	0.031	42%
6	TR01H113.412	L0011.71	TR01H113.412-F760mnP17/L0011.71-KT0	0.089	0.066	75%
7	TR01H113.453	L0011.71	TR01H113.453-F760mnP17/L0011.71-KT0	0.075	0.049	65%
8	TR01H113.412	L0011.273	TR01H113.412-F760mnP17/L0011.273-KT0	0.077	0.065	84%
9	TR01H113.412	L0011.274	TR01H113.412-F760mnP17/L0011.274-KT0	0.068	0.055	80%
10	TR01H113.412	L0011.57	TR01H113.412-F760mnP17/L0011.57-KT0	0.073	0.056	77%
11	TR01H113.455	L0011.71	TR01H113.455-F760mnP17/L0011.71-KT0	0.082	0.066	80%
12	TR01H113.456	L0011.71	TR01H113.456-F760mnP17/L0011.71-KT0	0.076	0.059	78%
13	TR01H113.412	L0011.115	TR01H113.412-F760mnP17/L0011.115-KT0	0.078	0.058	75%
14	TR01H113.457	L0011.71	TR01H113.457-F760mnP17/L0011.71-KT0	0.086	0.078	90%
15	TR01H113.458.S	L0011.71	TR01H113.458.S-F760mnP17/L0011.71-KT0	0.063	0.050	79%
16	TR01H113.412	L0011.275	TR01H113.412-F760mnP17/L0011.275-KT0	0.075	0.064	85%
17	TR01H113.455	L0011.74	TR01H113.455-F760mnP17/L0011.74-KT0	0.076	0.057	75%

18	TR01H113.456	L0011.74	TR01H113.456-F760mnP17/L0011.74-KT0	0.077	0.064	83%
19	TR01H113.412	L0011.65	TR01H113.412-F760mnP17/L0011.65-KT0	0.069	0.053	77%
20	TR01H113.412	L0011.277	TR01H113.412-F760mnP17/L0011.277-KT0	0.064	0.059	92%
21	TR01H113.412	L0011.278	TR01H113.412-F760mnP17/L0011.278-KT0	0.058	0.053	92%
22	TR01H113.412	L0011.279	TR01H113.412-F760mnP17/L0011.279-KT0	0.064	0.055	86%
23	TR01H113.460	L0011.71	TR01H113.460-F760mnP17/L0011.71-KT0	0.058	0.050	86%
24	TR01H113.412	L0011.280	TR01H113.412-F760mnP17/L0011.280-KT0	0.071	0.061	86%
25	TR01H113.457	L0011.74	TR01H113.457-F760mnP17/L0011.74-KT0	0.089	0.078	87%
26	TR01H113.412	L0011.281	TR01H113.412-F760mnP17/L0011.281-KT0	0.053	0.046	86%
27	TR01H113.469	L0011.71	TR01H113.469-F760mnP17/L0011.71-KT0	0.070	0.061	88%
28	TR01H113.412	L0011.283	TR01H113.412-F760mnP17/L0011.283-KT0	0.062	0.054	87%
29	TR01H113.479	L0011.71	TR01H113.479-F760mnP17/L0011.71-KT0	0.127	0.104	81%
30	TR01H113.124	L0011.71	TR01H113.124-F760mnP17/L0011.71-KT0	0.108	0.081	75%
31	TR01H113.479	L0011.273	TR01H113.479-F760mnP17/L0011.273-KT0	0.119	0.097	82%
32	TR01H113.479	L0011.274	TR01H113.479-F760mnP17/L0011.274-KT0	0.117	0.103	88%
33	TR01H113.124	L0011.74	TR01H113.124-F760mnP17/L0011.74-KT0	0.092	0.075	81%
34	TR01H113.479	L0011.57	TR01H113.479-F760mnP17/L0011.57-KT0	0.134	0.114	85%
35	TR01H113.480	L0011.71	TR01H113.480-F760mnP17/L0011.71-KT0	0.085	0.074	87%
36	TR01H113.412	L0011.74	TR01H113.412-F760mnP17/L0011.74-KT0	0.066	0.061	92%

[0244]

The “binding H/I ratio” in the table represents a ratio of binding measurement values after storage under the storage conditions of 4°C and 50°C for each antibody. This ratio shows how much binding activity each antibody retains under the 50°C condition. The binding values under the 50°C storage condition for antibody numbers 2, 14, 25, 29, 30 to 32, and 34 prepared in the present invention were 0.078 or more of reported control antibody 1. For these antibodies, the binding H/I ratios were 75% or more, which were higher than the value of the reported control antibody 1 (61%). Thus, it was confirmed that the reduction of binding ability in a buffer under the 50°C condition was greatly improved for these variants.

[0245]

[Example 4] Evaluation of antibodies having altered CDRs by TDCC reporter bioassay

The antibodies prepared in the present invention and an anti-MarvelD3 antibody were used to prepare bispecific antibodies by methods known to those skilled in the art. Each of the prepared antibodies was diluted to 0.044 mg/mL and stored at 4°C or 50°C for three days as in Example 3. *In vitro* TDCC activities of these antibodies were measured and examined for difference in TDCC activity due to storage temperature. TCR/CD3 effector cells (NFAT) and

Bio-Glo luciferase assay system (Promega) were used for the *in vitro* TDCC activity measurement, where the TDCC activities were quantified based on luminescence from a reporter gene (luciferase). To each well of a 384-well plate, 10 μL each of NFAT-RE-luc2 Jurkat whose concentration was adjusted with culture medium to 3x 10^6 /mL, LS 174T cells (ATCC) whose concentration was adjusted to 5x 10^5 mL, and 10 nM or 100 nM antibody solution were added and mixed, to start reactions. It is noted that LS 174T is a cell line that expresses the MarvelD3 antigen. The reactions were allowed to continue overnight at 37°C. Reporter gene expression was quantified by adding luciferase substrate to each well after incubation and measuring luminescence with a plate reader. Here, a luciferase response (counts per second: CPS) shows the average of measurements for each of the 10 nM antibody solution and the 100 nM antibody solution.

[0246]

[Table 4]

Generated variants and TDCC activities

Anti-body No.	Abbreviated name of heavy chain variable region	Abbreviated name of light chain variable region	Antibody name	Luciferase response		Acti-vation H/I ratio
				When stored at 4°C	When stored at 50°C	
1	TR01H113	L0011	TR01H113-F760mnP17/L0011-KT0	6.4.E+05	4.3.E+05	67%
2	TR01H113	L0011.71	TR01H113-F760mnP17/L0011.71-KT0	5.9.E+05	3.5.E+05	60%
3	TR01H113.38	L0011.57	TR01H113.38-F760mnP17/L0011.57-KT0	4.2.E+05	2.9.E+05	68%
4	TR01H113.38	L0011.71	TR01H113.38-F760mnP17/L0011.71-KT0	4.3.E+05	2.9.E+05	66%
5	TR01H113.H96A	L0011	TR01H113.H96A-F760mnP17/L0011-KT0	5.0.E+05	3.3.E+05	66%
6	TR01H113.412	L0011.71	TR01H113.412-F760mnP17/L0011.71-KT0	4.7.E+05	3.7.E+05	78%
7	TR01H113.453	L0011.71	TR01H113.453-F760mnP17/L0011.71-KT0	4.7.E+05	3.1.E+05	66%
8	TR01H113.412	L0011.273	TR01H113.412-F760mnP17/L0011.273-KT0	5.2.E+05	3.4.E+05	66%
9	TR01H113.412	L0011.274	TR01H113.412-F760mnP17/L0011.274-KT0	5.0.E+05	3.7.E+05	74%
10	TR01H113.412	L0011.57	TR01H113.412-F760mnP17/L0011.57-KT0	4.6.E+05	3.0.E+05	64%
11	TR01H113.455	L0011.71	TR01H113.455-F760mnP17/L0011.71-KT0	5.3.E+05	4.0.E+05	75%
12	TR01H113.456	L0011.71	TR01H113.456-F760mnP17/L0011.71-KT0	4.5.E+05	3.0.E+05	67%
13	TR01H113.412	L0011.115	TR01H113.412-F760mnP17/L0011.115-KT0	5.7.E+05	3.9.E+05	69%
14	TR01H113.457	L0011.71	TR01H113.457-F760mnP17/L0011.71-KT0	5.6.E+05	3.9.E+05	70%
15	TR01H113.458.S	L0011.71	TR01H113.458.S-F760mnP17/L0011.71-KT0	4.5.E+05	3.3.E+05	73%
16	TR01H113.412	L0011.275	TR01H113.412-F760mnP17/L0011.275-KT0	5.8.E+05	4.0.E+05	69%
17	TR01H113.455	L0011.74	TR01H113.455-F760mnP17/L0011.74-KT0	5.2.E+05	3.7.E+05	71%
18	TR01H113.456	L0011.74	TR01H113.456-F760mnP17/L0011.74-KT0	4.7.E+05	3.2.E+05	68%
19	TR01H113.412	L0011.65	TR01H113.412-F760mnP17/L0011.65-KT0	4.6.E+05	3.3.E+05	72%
20	TR01H113.412	L0011.277	TR01H113.412-F760mnP17/L0011.277-KT0	5.0.E+05	3.3.E+05	66%
21	TR01H113.412	L0011.278	TR01H113.412-F760mnP17/L0011.278-KT0	5.2.E+05	3.8.E+05	73%
22	TR01H113.412	L0011.279	TR01H113.412-F760mnP17/L0011.279-KT0	4.8.E+05	3.3.E+05	69%
23	TR01H113.460	L0011.71	TR01H113.460-F760mnP17/L0011.71-KT0	5.1.E+05	3.0.E+05	58%
24	TR01H113.412	L0011.280	TR01H113.412-F760mnP17/L0011.280-KT0	5.7.E+05	3.8.E+05	65%
25	TR01H113.457	L0011.74	TR01H113.457-F760mnP17/L0011.74-KT0	5.7.E+05	4.2.E+05	74%

26	TR01H113.412	L0011.281	TR01H113.412-F760mnP17/L0011.281-KT0	3.9.E+05	3.2.E+05	82%
27	TR01H113.469	L0011.71	TR01H113.469-F760mnP17/L0011.71-KT0	4.7.E+05	3.1.E+05	67%
28	TR01H113.412	L0011.283	TR01H113.412-F760mnP17/L0011.283-KT0	4.5.E+05	2.8.E+05	62%
29	TR01H113.479	L0011.71	TR01H113.479-F760mnP17/L0011.71-KT0	6.4.E+05	5.5.E+05	86%
30	TR01H113.124	L0011.71	TR01H113.124-F760mnP17/L0011.71-KT0	6.8.E+05	6.1.E+05	90%
31	TR01H113.479	L0011.273	TR01H113.479-F760mnP17/L0011.273-KT0	7.6.E+05	5.5.E+05	72%
32	TR01H113.479	L0011.274	TR01H113.479-F760mnP17/L0011.274-KT0	7.5.E+05	5.7.E+05	76%
33	TR01H113.124	L0011.74	TR01H113.124-F760mnP17/L0011.74-KT0	7.2.E+05	6.8.E+05	95%
34	TR01H113.479	L0011.57	TR01H113.479-F760mnP17/L0011.57-KT0	6.2.E+05	4.9.E+05	79%
35	TR01H113.480	L0011.71	TR01H113.480-F760mnP17/L0011.71-KT0	NA	NA	NA
36	TR01H113.412	L0011.74	TR01H113.412-F760mnP17/L0011.74-KT0	3.5.E+05	2.7.E+05	78%

NA means that no measurement was carried out.

[0247]

The “activation H/I ratio” in the table represents a ratio of luciferase response values after storage under the storage conditions of 4°C and 50°C for each antibody. This ratio shows how much binding activity each antibody lost under the 50°C condition.

Antibody numbers 2, 14, 25, 29, 30 to 32, and 34 selected in Example 3, when stored under the 4°C condition, exhibited luciferase responses of values equal to or greater than 4.3.E+05 which corresponds to the response of reported control antibody 1 when stored at 50°C. Antibody numbers 14, 25, 29, 30 to 32, and 34 exhibited activation H/I TDCC activity ratios of 67% or more when stored under the 50°C condition. These values were superior to that of reported control antibody 1, and thus, these antibodies were recognized to be variants whose activation H/I ratios were improved while maintaining TDCC activities. The amino acid sequences of antibody numbers 1, 2, 14, 25, 29, 30 to 32, and 34 are shown in Table 5.

[0248]

[Table 5]

Anti-body No.	SEQ ID NOs of amino acid sequences									
	Heavy chain	Light chain	Heavy chain variable region	Light chain variable region	Heavy chain CDR1	Heavy chain CDR2	Heavy chain CDR3	Light chain CDR1	Light chain CDR2	Light chain CDR3
1	30	34	20	24	1	7	10	14	5	6
2	30	35	20	25	1	7	10	15	5	6
14	31	35	21	25	1	8	11	15	5	6
25	31	36	21	26	1	8	11	16	5	6
29	32	35	22	25	1	9	12	15	5	6
30	33	35	23	25	1	9	13	15	5	6
31	32	37	22	27	1	9	12	17	5	6
32	32	38	22	28	1	9	12	18	5	6
34	32	39	22	29	1	9	12	19	5	6

[Industrial Applicability]

[0249]

By the present invention, novel CD3-binding domains with superior stability compared to conventional CD3-binding domains used in T cell redirecting antibodies, as well as antigen-binding molecules comprising said CD3-binding domains were provided. Bispecific antigen-binding molecules prepared by combining the CD3-binding domains of the present invention with antigen-binding domains binding to antigens expressed on the surfaces of target cells (e.g., cancer cells) can induce T-cell-dependent cytotoxicity against the target cells and are useful for treating or preventing various types of cancer. Antigen-binding molecules comprising the CD3-binding domains of the present invention have lower degree of decrease in CD3 binding activity in environments at or above room temperature, and when administered to patients, their T-cell dependent cytotoxicity is expected to persist for a long period of time.

[CLAIMS]

[Claim 1]

An antigen-binding molecule comprising a first antigen-binding domain and a second antigen-binding domain, wherein the first antigen-binding domain comprises an antibody heavy chain (H chain) variable region and an antibody light chain (L chain) variable region having binding activity towards CD3,

wherein the H chain variable region comprises:

H chain CDR1 comprising the amino acid sequence NAWMH (SEQ ID NO: 1);

H chain CDR2 comprising the amino acid sequence QIX_1DKSQNYATX_2VAESVKG (SEQ ID NO: 2), wherein X_1 is K or R, and X_2 is Y or F; and

H chain CDR3 comprising the amino acid sequence VHYX_3AGYGVDX_4 (SEQ ID NO: 3), wherein X_3 is A or P, and X_4 is I, M, or L; and

wherein the L chain variable region comprises:

L chain CDR1 comprising the amino acid sequence RS$X_5X_6X_7$VVHENRX_8TYLH (SEQ ID NO: 4), wherein X_5 is S or T, X_6 is Q or M, X_7 is S or T, and X_8 is Q or N;

L chain CDR2 comprising the amino acid sequence KVSNRFS (SEQ ID NO: 5); and

L chain CDR3 comprising the amino acid sequence GQGTQVPYT (SEQ ID NO: 6).

[Claim 2]

The antigen-binding molecule of claim 1, wherein the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise any one of the combinations of H chain CDR1, CDR2, and CDR3 and L chain CDR1, CDR2, and CDR 3 selected from following (a1) to (a8):

(a1) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 7; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 10; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a2) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a3) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 8; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 11; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 16; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a4) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a5) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 13; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 15; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a6) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 17; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a7) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 18; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6;

(a8) the combination of H chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 1; H chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 9; H chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 12; L chain CDR1 comprising the amino acid sequence set forth in SEQ ID NO: 19; L chain CDR2 comprising the amino acid sequence set forth in SEQ ID NO: 5; and L chain CDR3 comprising the amino acid sequence set forth in SEQ ID NO: 6.

[Claim 3]

The antigen-binding molecule of claim 1 or 2, wherein the antibody H chain variable region and the antibody L chain variable region having binding activity towards CD3 comprise any one of the combinations of an H chain variable region and an L chain variable region selected from following (a1) to (a8):

(a1) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 20 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a2) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a3) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 21 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 26;

(a4) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a5) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 23 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 25;

(a6) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 27;

(a7) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 28; and

(a8) the combination of an H chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 22 and an L chain variable region comprising the amino acid sequence set forth in SEQ ID NO: 29.

[Claim 4]

The antigen-binding molecule of any one of claims 1 to 3, further comprising an Fc region.

[Claim 5]

The antigen-binding molecule of any one of claims 1 to 4, wherein the antigen-binding molecule is a monospecific antigen-binding molecule.

[Claim 6]

The antigen-binding molecule of any one of claims 1 to 4, wherein the antigen-binding molecule is a bispecific antigen-binding molecule.

[Claim 7]

The antigen-binding molecule of claim 6, wherein the second antigen-binding domain comprises an antibody variable region having binding activity towards a cancer antigen.

[Claim 8]

The antigen-binding molecule of any one of claims 1 to 7, wherein the antigen-binding molecule is an antibody.

[Claim 9]

A nucleic acid encoding the antigen-binding molecule of any one of claims 1 to 8.

[Claim 10]

A vector into which the nucleic acid of claim 9 has been introduced.

[Claim 11]

A cell comprising the vector of claim 10.

[Claim 12]

A method for producing the antigen-binding molecule of any one of claims 1 to 8, comprising culturing the cell of claim 11.

[Claim 13]

An antigen-binding molecule produced by the method of claim 12.

[Claim 14]

A pharmaceutical composition, comprising the antigen-binding molecule of any one of claims 1 to 8 and a pharmaceutically acceptable carrier.

[Claim 15]

The pharmaceutical composition of claim 14, for use in treatment or prevention of cancer.