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(54) Title: CELL ENGAGING BINDING MOLECULES

(57) Abstract: The present disclosure is broadly concerned with the field of cancer immunotherapy. For example, the present disclosure generally related to a binding molecule comprising antibody variable light (VL) regions, variable heavy (VH) regions, constant heavy 1 (CH1) regions, and light chain constant (CL) regions that are configured to form two antigen binding Fab regions and an antigen binding Fv region so that the binding molecule binds to two different antigens.

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CELL ENGAGING BINDING MOLECULES

CROSS REFERENCE

[0001] This application claims the benefit of priority from U.S. Provisional Application No. 62/655,762 filed April 10, 2018, and U.S. Provisional Application No. 62/719,484 filed August 17, 2018. This application further claims the benefit of priority from U.S. Application No. 16/372,172 filed April 1, 2019, U.S. Application No. 16/372,190 filed April 1, 2019, and U.S. Application No. 16/372,196 filed April 1, 2019. Each of the foregoing applications, in their entirety, are incorporated herein by reference.

SEQUENCE LISTING

[0002] This application incorporates by reference in its entirety the Computer Readable Form (CRF) of a Sequence Listing in ASCII text format submitted via EFS-Web. The Sequence Listing text file submitted via EFS-Web, entitled 14489-004-228_SEQ_LISTING.txt, was created on April 2, 2019, and is 140,862 bytes in size.

FIELD

[0003] The present disclosure generally relates to cell engaging binding molecules, methods of making the binding molecules, compositions comprising the binding molecules, and uses thereof.

BACKGROUND

[0004] Antibodies and/or antibody-based agents are now therapeutic options for a wide variety of diseases and disorders. Currently there are at least 70 antibodies approved in the United States and/or European Union, with large numbers of new molecules in preclinical studies and clinical trials. However, there is a continual search for new, better, and safer therapeutic agents within the research and clinical communities.

[0005] A naturally occurring antibody is monospecific and binds to one epitope or antigen. Multispecific antibodies combine specificities of multiple antibodies and have the capability to bind different antigens or epitopes. Many technical hurdles, however, have hampered development of multispecific antibodies; as such, few multispecific antibodies have been

approved as therapeutics. Thus, there is still a need for better multispecific antibodies and methods to efficiently produce functional and stable multispecific antibodies.

SUMMARY

[0006] The present disclosure provides, in part, cell engaging binding molecules having multiple binding domains, methods of making the binding molecules, and pharmaceutical compositions comprising the binding molecules. Also provided herein are methods of treatment comprising administering the binding molecules.

[0007] In one aspect, provided herein are cell engaging binding molecules. In some embodiments, provided is a binding molecule comprising:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
- (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

- (c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen.

[0008] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous binding molecules, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous binding molecules, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0009] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous binding molecules, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous binding molecules, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0010] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous binding molecules, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous binding molecules, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0011] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0012] In some embodiments, with regard to any of the previous binding molecules, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous binding molecules, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous binding molecules, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0013] In some embodiments, provided herein are binding molecules wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via fusion.

[0014] In some embodiments, the flexible peptide region comprises an antibody hinge region. In some embodiments, the antibody hinge region is an Immunoglobulin G (IgG) hinge region. In some embodiments, the antibody hinge region is selected from the group consisting of IgG1, IgG2, IgG3, and IgG4 hinge regions. In some embodiments, the antibody hinge region

comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide. In some embodiments, the flexible peptide region further comprises a linker. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S). In some embodiments, the linker comprises two tandem copies of the amino acid sequence of GGGGS (G4S).

[0015] In some embodiments, the first Fab region and the second Fab region bind to the same epitope of the first antigen. In some embodiments, the second antigen is expressed on an immune cell. In some embodiments, the immune cell is selected from the group consisting of lymphocytes and monocytes. In some embodiments, the immune cell is an effector cell. In some embodiments, the immune cell is selected from the group consisting of a T cell, a B cell, a dendritic cell, a granulocyte, an innate lymphoid cell, a megakaryocyte, a monocyte, a myeloid-derived suppressor cell, and a natural killer (NK) cell.

[0016] In some embodiments, the first antigen is a cancer antigen. In some embodiments, the cancer antigen is a tumor associated antigen (TAA) or a tumor specific antigen (TSA). In some embodiments, the first antigen is selected from a group consisting of CD19, CD20, EGFR, Her2, and PD-L1.

[0017] In some embodiments, the second antigen is CD3 or TNF alpha. In some embodiments, the first antigen is a cancer antigen and the second antigen is CD3. In some embodiments, the cancer antigen is selected from a group consisting of CD19, CD20, EGFR, Her2, and PD-L1.

[0018] In another aspect, provided herein are methods of making a binding molecule. In some embodiments, provided is a method of making a binding molecule, comprising:

- (i) expressing the binding molecule from one or more vectors in a host cell, wherein the one or more vectors comprise
 - (a) a first nucleic acid encoding a first polypeptide and a second nucleic acid encoding a second polypeptide, wherein each of the first polypeptide and the second polypeptide is an antibody light chain,
 - (b) a third nucleic acid encoding a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region and a second VH region; and
 - (c) a fourth nucleic acid encoding a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide can form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide can form a second antigen binding Fab region; and

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen, and

(ii) purifying the binding molecule.

[0019] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0020] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0021] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0022] In some embodiments, with regard to any of the previous methods, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0023] In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0024] In some embodiments, provided herein are methods wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region comprising an antibody hinge region. In some embodiments, the antibody hinge region comprises an interchain disulfide bond formed between the third polypeptide and the fourth polypeptide. In some embodiments, the flexible peptide region further comprises a linker. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S).

[0025] In some embodiments, the first antigen is a cancer antigen and the second antigen is CD3. In some embodiments, the first antigen is selected from a group consisting of CD19, CD20, EGFR, Her2, and PD-L1.

[0026] In yet another aspect, provided herein are pharmaceutical compositions comprising a binding molecule. In some embodiments, provided is a pharmaceutical composition comprising a binding molecule and a pharmaceutically acceptable carrier, wherein the binding molecule comprises:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
 - (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region, and a second VH region; and
 - (c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a VL region,
- wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;
- wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen Fab region;
- wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen.

[0027] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous pharmaceutical compositions, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous pharmaceutical compositions, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0028] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous pharmaceutical compositions, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous pharmaceutical compositions, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0029] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous pharmaceutical compositions, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous pharmaceutical compositions, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0030] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0031] In some embodiments, with regard to any of the previous pharmaceutical compositions, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous pharmaceutical compositions, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with

regard to any of the previous pharmaceutical compositions, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0032] In yet another aspect, provided herein are methods of treating a disease or condition comprising administering a binding molecule. In some embodiments, provided is a method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of a binding molecule to the subject, wherein the binding molecule comprises:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
- (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region, and a second VH region; and
- (c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen.

[0033] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0034] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0035] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0036] In some embodiments, with regard to any of the previous methods, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0037] In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0038] In one aspect, provided herein are cell engaging binding molecules. In some embodiments, provided is a binding molecule comprising:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
- (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

- (c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region binds to Programmed Death-Ligand 1 (PD-L1), and the Fv region binds to Cluster of Differentiation 3 (CD3).

[0039] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous binding molecules, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous binding molecules, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0040] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous binding molecules, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous binding molecules, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0041] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0042] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous binding molecules, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous binding molecules, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0043] In some embodiments, with regard to any of the previous binding molecules, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous binding molecules, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the

previous binding molecules, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0044] In some embodiments, provided is a binding molecule, wherein:

- (a) the antibody light chains of the first and the second polypeptide each comprise three Complementarity Determining Regions (CDRs) having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11;
- (b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and
- (c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[0045] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via fusion.

[0046] In some embodiments, the flexible peptide region comprises an antibody hinge region. In some embodiments, the antibody hinge region is an Immunoglobulin G (IgG) hinge region. In some embodiments, the antibody hinge region is selected from the group consisting of IgG1, IgG2, IgG3, and IgG4 hinge regions. In some embodiments, the antibody hinge region comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide.

[0047] In some embodiments, the flexible peptide region further comprises a linker. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S). In some embodiments, the linker comprises an amino acid sequence of GGGSGGGGS. In some embodiments, the linker comprises an amino acid sequence of GSGGGGSG.

[0048] In some embodiments, provided is a binding molecule, wherein:

- (a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 8;

(b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 4, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and

(c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 4, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.

[0049] In some embodiments, the first polypeptide and the second polypeptide each comprise the amino acid sequence of SEQ ID NO.: 3; the third polypeptide comprises the amino acid sequence of SEQ ID NO.: 1; and the fourth polypeptide comprises the amino acid sequence of SEQ ID NO.: 2.

[0050] In some embodiments, the first polypeptide and the second polypeptide each comprise the amino acid sequence of SEQ ID NO.: 95; the third polypeptide comprises the amino acid sequence of SEQ ID NO.: 96; and the fourth polypeptide comprises the amino acid sequence of SEQ ID NO.: 97.

[0051] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 95; the third polypeptide has the amino acid sequence of SEQ ID NO.: 98; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 99.

[0052] In another aspect, provided herein are methods of making a binding molecule. In some embodiments, provided is a method of making a binding molecule, comprising:

(i) expressing the binding molecule from one or more vectors in a host cell, wherein the one or more vectors comprise

(a) a first nucleic acid encoding a first polypeptide and a second nucleic acid encoding a second polypeptide, wherein each polypeptide comprises an antibody light chain,

(b) a third nucleic acid encoding a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth nucleic acid encoding a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to PD-L1, and the Fv region binds to CD3, and

(ii) purifying the binding molecule.

[0053] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0054] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0055] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0056] In some embodiments, with regard to any of the previous methods, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0057] In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the

previous methods, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0058] In some embodiments, provided is a method, wherein:

- (a) the antibody light chains of the first and the second polypeptide each comprise three Complementarity Determining Regions (CDRs) having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11;
- (b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and
- (c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[0059] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some embodiments, the antibody hinge region is an Immunoglobulin G (IgG) hinge region. In some embodiments, the antibody hinge region comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide.

[0060] In some embodiments, the flexible peptide region further comprises a linker. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S). In some embodiments, the linker comprises an amino acid sequence of GGGSGGGGS. In some embodiments, the linker comprises an amino acid sequence of GSGGGGSG.

[0061] In some embodiments, provided is a method, wherein the VH region of each of the first and second Fab regions comprises an amino acid sequence of SEQ ID NO.: 4; wherein the VL region of each of the first and second Fab regions comprises an amino acid sequence of SEQ ID NO.: 8;

wherein the VH region of the Fv region comprises an amino acid sequence of SEQ ID NO.: 12;
and

wherein the VL region of the Fv region comprises an amino acid sequence of SEQ ID NO.: 16.

[0062] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 3; the third polypeptide has the amino acid sequence of SEQ ID NO.: 1; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 2.

[0063] In yet another aspect, provided herein are pharmaceutical compositions comprising a binding molecule. In some embodiments, provided is a pharmaceutical composition comprising a binding molecule and a pharmaceutically acceptable carrier, wherein the binding molecule comprises:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
- (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region;
and

- (c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region binds to PD-L1, and the Fv region binds to CD3.

[0064] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous pharmaceutical compositions, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous pharmaceutical compositions, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0065] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous pharmaceutical compositions, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous pharmaceutical compositions, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0066] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous pharmaceutical compositions, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous pharmaceutical compositions, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0067] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0068] In some embodiments, with regard to any of the previous pharmaceutical compositions, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous pharmaceutical compositions, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous pharmaceutical compositions, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0069] In yet another aspect, provided herein are methods of treating a disease or condition comprising administering a binding molecule. In some embodiments, provided is a method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of a binding molecule to the subject, wherein the binding molecule comprises:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
 - (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region;
- and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region, wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region; wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region; wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and wherein the first Fab region and the second Fab region binds to PD-L1, and the Fv region binds to CD3.

[0070] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0071] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0072] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0073] In some embodiments, with regard to any of the previous methods, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0074] In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0075] In one aspect, provided herein are cell engaging binding molecules. In some embodiments, provided is a binding molecule comprising:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
- (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

- (c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region each binds to CD20 or epidermal growth factor receptor (EGFR), and the Fv region binds to CD3.

[0076] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous binding molecules, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous binding molecules, both the third and the fourth polypeptides

further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0077] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous binding molecules, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous binding molecules, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0078] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous binding molecules, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous binding molecules, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0079] In some embodiments, with regard to any of the previous binding molecules, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0080] In some embodiments, with regard to any of the previous binding molecules, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous binding molecules, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous binding molecules, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0081] In some embodiments, the first Fab region and the second Fab region bind to CD20, and

(a) the antibody light chains of the first and the second polypeptide each comprise three Complementarity Determining Regions (CDRs) having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33;

(b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

(c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[0082] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some embodiments, the antibody hinge region comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide. In some embodiments, the flexible peptide region further comprises a linker.

[0083] In some embodiments, provided is a binding molecule, wherein:

(a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 30;

(b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 26, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and

(c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 26, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.

[0084] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.

[0085] In some embodiments, the first Fab region and the second Fab region bind to EGFR, and

(a) the antibody light chains of the first and the second polypeptide each comprise three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47;

(b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43, and the second VH

region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

(c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[0086] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some embodiments, the antibody hinge region comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide. In some embodiments, the flexible peptide region further comprises a linker.

[0087] In some embodiments, provided is a binding molecule, wherein:

(a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 44;

(b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 40, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and

(c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 40, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.

[0088] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.

[0089] In another aspect, provided herein are methods of making a binding molecule. In some embodiments, provided is a method of making a binding molecule, comprising:

(i) expressing the binding molecule from one or more vectors in a host cell, wherein the one or more vectors comprise

(a) a first nucleic acid encoding a first polypeptide and a second nucleic acid encoding a second polypeptide, wherein each of the first polypeptide and the second polypeptide is an antibody light chain,

(b) a third nucleic acid encoding a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region, and a second VH region; and

- (c) a fourth nucleic acid encoding a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a VL region, wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region; wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region; wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and wherein the first Fab region and the second Fab region each binds to CD20 or EGFR, and the Fv region binds to CD3, and
- (ii) purifying the binding molecule.

[0090] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[0091] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[0092] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[0093] In some embodiments, with regard to any of the previous methods, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[0094] In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[0095] In some embodiments, the first Fab region and the second Fab region bind to CD20, and

- (a) the antibody light chains of the first and the second polypeptide each comprise three Complementarity Determining Regions (CDRs) having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33;
- (b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and
- (c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[0096] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some embodiments, the flexible peptide region further comprises a linker.

[0097] In some embodiment, provided is a method, wherein:

- (a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 30;

(b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 26, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and

(c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 26, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.

[0098] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.

[0099] In some embodiments, the first Fab region and the second Fab region bind to CD20, and

(a) the antibody light chains of the first and the second polypeptide each comprise three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47;

(b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

(c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00100] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some embodiments, the flexible peptide region further comprises a linker.

[00101] In some embodiment, provided is a method, wherein:

(a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 44;

(b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 40, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and

(c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 40, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.

[00102] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.

[00103] In yet another aspect, provided herein are pharmaceutical compositions comprising a binding molecule. In some embodiments, provided is a pharmaceutical composition comprising a therapeutically effective amount of a binding molecule and a pharmaceutically acceptable carrier, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region each binds to CD20 or EGFR, and the Fv region binds to CD3.

[00104] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous pharmaceutical compositions, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous pharmaceutical compositions, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[00105] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous pharmaceutical compositions, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous pharmaceutical compositions, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[00106] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous pharmaceutical compositions, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous pharmaceutical compositions, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[00107] In some embodiments, with regard to any of the previous pharmaceutical compositions, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[00108] In some embodiments, with regard to any of the previous pharmaceutical compositions, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous pharmaceutical compositions, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous pharmaceutical compositions, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[00109] In yet another aspect, provided herein are methods of treating a disease or condition comprising administering a binding molecule. In some embodiments, provided is a method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of a binding molecule to the subject, wherein the binding molecule comprises:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
 - (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region;
- and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region, wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region; wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region; wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and wherein the first Fab region and the second Fab region each binds to CD20 or EGFR, and the Fv region binds to CD3.

[00110] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions C-terminal to the second VH region and the VL region, respectively.

[00111] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises an albumin binding domain or site (ABS) C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise ABS C-terminal to the second VH region and the VL region, respectively.

[00112] In some embodiments, with regard to any of the previous methods, the third polypeptide further comprises a constant heavy 3 (CH3) region and an ABS C-terminal to the second VH region. In some embodiments, with regard to any of the previous methods, the fourth polypeptide further comprises a CH3 region and an ABS C-terminal to the VL region. In some embodiments, with regard to any of the previous methods, both the third and the fourth polypeptides further comprise CH3 regions and ABSs C-terminal to the second VH region and the VL region, respectively.

[00113] In some embodiments, with regard to any of the previous methods, the third polypeptide and/or the fourth polypeptide further comprise both a CH1 region and an ABS C-terminal to the second VH region and/or to the VL region, respectively.

[00114] In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS. In some embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS C-terminal to the antibody light chain. In specific embodiments, with regard to any of the previous methods, the first and/or second polypeptides further comprise an ABS at the C-terminus of the antibody light chain.

[00115] The present disclosure provides, in part, cell engaging binding molecules having multiple binding domains. In one aspect, provided herein is a binding molecule comprising:

- (a) a first antigen binding domain comprising two antibody Fab regions, each comprising:
 - (i) a first portion comprising an antibody variable heavy (VH) region and an antibody CH1 region, wherein the first portion does not contain an antibody CH2 region and an antibody CH3 region; and
 - (ii) a second portion comprising an antibody light chain (LC) comprising an antibody variable light (VL) region and an antibody light chain constant region (CL),

wherein the two antibody Fab regions each bind to an antigen, and

- (b) a second antigen binding domain comprising an antibody Fv region comprising a VH region and an antibody variable light (VL) region,

wherein the second antigen binding domain binds to an antigen present on an immune cell; and

wherein the first antigen binding domain and the second antigen binding domain are linked.

[00116] In certain embodiments, the first portion and the second portion of each Fab region of the first antigen binding domain are on the same polypeptide. In some embodiments, at least one Fab region is oriented from N-terminus to C-terminus in the following order: VH-CH1-VL-CL. In other embodiments, at least one Fab region is oriented from N-terminus to C-terminus in the following order: VL-CL-VH-CH1.

[00117] In certain embodiments, the first portion and the second portion of each Fab region are present on separate polypeptides.

[00118] In certain embodiments, the VH region and the VL region of the Fv region are on the same polypeptide. In some embodiments, the Fv region is oriented from N-terminus to C-terminus in the following order: VH-VL. In other embodiments, the Fv region is oriented from N-terminus to C-terminus in the following order: VL-VH.

[00119] In certain embodiments, the VH region and the VL region of the Fv region are on separate polypeptides.

[00120] In some embodiments, the first antigen binding domain and the second antigen binding domain are linked by a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some specific embodiments, the antibody hinge region is a IgG hinge region. In some more specific embodiments, the IgG hinge region is of IgG1 subtype. In other more specific embodiments, the IgG hinge region is of IgG2 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG3 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG4 subtype.

[00121] In certain embodiments, the flexible peptide region comprise additional amino acids. For example, in some embodiments, the flexible peptide region further comprises a linker between the antibody hinge region and the second antigen binding domain. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S).

[00122] In some embodiments, the second antigen binding domain further comprises a first CH3 region linked to the VH region of the Fv region and a second CH3 region linked to the VL region of the Fv region.

[00123] In some embodiments, the binding molecule further comprises one or more albumin binding domain or site (ABS). In some embodiments, the ABS is linked to the C-terminus of the VH region of the Fv region. In other embodiments, the ABS is linked to the C-terminus of the VL region of the Fv region. In yet other embodiments, the C-terminus of each of the VL and VH regions of the Fv region is linked to ABS. In other embodiments, the ABS is linked to the CL region of at least one of the Fab regions. In yet other embodiments, the binding molecule further comprises one or more albumin domain.

[00124] In some embodiments, the two Fab regions bind to different antigens. In other embodiments, the two Fab regions bind to the same antigen. In some embodiments, the two Fab

regions bind to the same epitope of the same antigen. In other embodiments, the two Fab regions bind to different epitopes of the same antigen.

[00125] In some embodiments, the first antigen binding domain and the second antigen binding domain bind to the same antigen. In some embodiments, the second antigen binding domain binds to the same epitope as at least one of the epitopes bound by the first antigen binding domain.

[00126] In other embodiments, the first antigen binding domain and the second antigen binding domain bind to different antigens, and wherein the first antigen binding domain binds to a first antigen and the second antigen binding domain binds to a second antigen.

[00127] In some embodiments, the first antigen is a cancer antigen. In other embodiments, the first antigen is not a cancer antigen.

[00128] In some embodiments, the second antigen is expressed on an immune cells including lymphocytes and monocytes. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a B cell. In other embodiments, the second antigen is expressed on a dendritic cell. In other embodiments, the second antigen is expressed on a granulocyte. In yet other embodiments, the second antigen is expressed on an innate lymphoid cell. In yet other embodiments, the second antigen is expressed on a megakaryocyte. In yet other embodiments, the second antigen is expressed on a monocyte. In yet other embodiments, the second antigen is expressed on a myeloid-derived suppressor cell. In yet other embodiments, the second antigen is expressed on a NK cell.

[00129] In some embodiments, the second antigen is expressed on an effector cell. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a T helper cell, a regulatory T cell, or a cytotoxic T cell. In some embodiments, the second antigen is expressed on a T helper cell. In some embodiments, the second antigen is expressed on a regulatory T cell. In some embodiments, the second antigen is expressed on a cytotoxic T cell. In some embodiments, the second antigen is expressed on a CD8+ T cell. In some embodiments, the second antigen is expressed on a CD4+ T cell. In some embodiments, the second antigen comprises an extracellular domain.

[00130] In some embodiments, the VH region of the first portion of each Fab region comprise the same CDR amino acid sequences. In other embodiments, the VH region of the first portion of each Fab region comprise different CDR amino acid sequences.

[00131] In some embodiments, the VL region of the second portion of each Fab region comprise the same CDR amino acid sequences. In other embodiments, the VL region of the second portion of each Fab region comprise different CDR amino acid sequence. In some embodiments, the VH region of the first portion of each Fab region comprise the same CDR amino acid sequences, and the VL region of the second portion of each Fab region comprise the same CDR amino acid sequences. In some embodiments, the VH region of the first portion of each Fab region comprise the same CDR amino acid sequences, and the VL region of the second portion of each Fab region comprise different CDR amino acid sequences. In some embodiments, the VH region of the first portion of each Fab region comprise different CDR amino acid sequences, and the VL region of the second portion of each Fab region comprise different CDR amino acid sequences. In some embodiments, the VH region of the first portion of each Fab region comprise different CDR amino acid sequences, and the VL region of the second portion of each Fab region comprise different CDR amino acid sequences.

[00132] In some specific embodiments, the second antigen is CD3. In some embodiments, the first antigen is a cancer antigen and the second antigen is CD3.

[00133] In some more specific embodiments, the first antigen is PD-L1 and the second antigen is CD3.

[00134] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00135] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00136] In other more specific embodiments, the first antigen is CD20 and the second antigen is CD3.

[00137] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, SEQ ID NO.: 29; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00138] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00139] In other more specific embodiments, the first antigen is EGFR and the second antigen is CD3.

[00140] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15 and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00141] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00142] In other more specific embodiments, the first antigen is Her2 and the second antigen is TNF alpha. In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 51; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 52; the VH region of the Fv region has an amino

acid sequence of SEQ ID NO.: 53; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 54.

[00143] In another aspect, provide herein is a binding molecule, comprising:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
- (b) a third polypeptide comprising a first VH region and a first CH1 region, and a second VH region; and
- (c) a fourth polypeptide comprising a third VH region and a second CH1, and a VL region, wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region; wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen Fab region; and wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region.

[00144] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some specific embodiments, the antibody hinge region is a IgG hinge region. In some more specific embodiments, the IgG hinge region is of IgG1 subtype. In other more specific embodiments, the IgG hinge region is of IgG2 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG3 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG4 subtype. In some embodiments, the flexible peptide region further comprises a linker between the antibody hinge region and the second antigen binding domain. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S).

[00145] In some embodiments, the first Fab region and the second Fab region bind to different antigens. In other embodiments, the first Fab region and the second Fab region bind to the same antigen. In some embodiments, the first Fab region and the second Fab region bind to the same epitope of the same antigen. In other embodiments, the first Fab region and the second Fab region bind to different epitopes of the same antigen.

[00146] In certain embodiments, the first Fab region and the second Fab region form a first antigen binding domain, and the Fv region forms a second antigen binding domain.

[00147] In some embodiments, the first antigen binding domain and the second antigen binding domain bind to the same antigen. In some embodiments, the second antigen binding domain binds to the same epitope as at least one of the epitopes bound by the first antigen binding domain.

[00148] In other embodiments, the first antigen binding domain and the second antigen binding domain bind to different antigens, and wherein the first antigen binding domain binds to a first antigen and the second antigen binding domain binds to a second antigen.

[00149] In some embodiments, the first antigen is a cancer antigen. In other embodiments, the first antigen is not a cancer antigen.

[00150] In some embodiments, the second antigen is expressed on an immune cells including lymphocytes and monocytes. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a B cell. In other embodiments, the second antigen is expressed on a dendritic cell. In other embodiments, the second antigen is expressed on a granulocyte. In yet other embodiments, the second antigen is expressed on an innate lymphoid cell. In yet other embodiments, the second antigen is expressed on a megakaryocyte. In yet other embodiments, the second antigen is expressed on a monocyte. In yet other embodiments, the second antigen is expressed on a myeloid-derived suppressor cell. In yet other embodiments, the second antigen is expressed on a NK cell.

[00151] In some embodiments, the second antigen is expressed on an effector cell. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a T helper cell, a regulatory T cell, or a cytotoxic T cell. In some embodiments, the second antigen is expressed on a T helper cell. In some embodiments, the second antigen is expressed on a regulatory T cell. In some embodiments, the second antigen is expressed on a cytotoxic T cell. In some embodiments, the second antigen is expressed on a CD8+ T cell. In some embodiments, the second antigen is expressed on a CD4+ T cell. In some embodiments, the second antigen comprises an extracellular domain.

[00152] In some specific embodiments, the second antigen is CD3. In some embodiments, the first antigen is a cancer antigen and the second antigen is CD3.

[00153] In some more specific embodiments, the first antigen is PD-L1 and the second antigen is CD3.

[00154] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 16, SEQ ID NO.: 17, and SEQ ID NO.: 19.

[00155] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00156] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 3; the third polypeptide has the amino acid sequence of SEQ ID NO.:1; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.:2.

[00157] In other more specific embodiments, the first antigen is CD20 and the second antigen is CD3.

[00158] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00159] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00160] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.

[00161] In other more specific embodiments, the first antigen is EGFR and the second antigen is CD3.

[00162] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, SEQ ID NO.: 43; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00163] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00164] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.

[00165] In other more specific embodiments, the first antigen is Her2 and the second antigen is TNF alpha. In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 51; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 52; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 53; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 54.

[00166] In yet another aspect, provided herein are methods for making the binding molecules provided herein. In some embodiments, provided herein is a method of making a binding molecule comprising transfecting one or more vectors into a host cell, wherein the one or more vectors comprise:

- (a) a first nucleic acid encoding a first polypeptide and a second polypeptide, each being an antibody light chain,
- (b) a second nucleic acid encoding a third polypeptide comprising a first VH region and a first CH1 region and a second VH region; and
- (c) a third nucleic acid encoding a fourth polypeptide comprising a third VH region and a second CH1 and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide can form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide can form a second antigen binding Fab region; and

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide can form an antigen binding Fv region.

[00167] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some specific embodiments, the antibody hinge region is a IgG hinge region. In some more specific embodiments, the IgG hinge region is of IgG1 subtype. In other more specific embodiments, the IgG hinge region is of IgG2 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG3 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG4 subtype. In some embodiments, the flexible peptide region further comprises a linker between the antibody hinge region and the second antigen binding domain. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S).

[00168] In some embodiments, the first Fab region and the second Fab region bind to different antigens. In other embodiments, the first Fab region and the second Fab region bind to the same antigen. In some embodiments, the first Fab region and the second Fab region bind to the same epitope of the same antigen. In other embodiments, the first Fab region and the second Fab region bind to different epitopes of the same antigen.

[00169] In certain embodiments, the first Fab region and the second Fab region form a first antigen binding domain, and the Fv region forms a second antigen binding domain.

[00170] In some embodiments, the first antigen binding domain and the second antigen binding domain bind to the same antigen. In some embodiments, the second antigen binding domain

binds to the same epitope as at least one of the epitopes bound by the first antigen binding domain.

[00171] In other embodiments, the first antigen binding domain and the second antigen binding domain bind to different antigens, and wherein the first antigen binding domain binds to a first antigen and the second antigen binding domain binds to a second antigen.

[00172] In some embodiments, the first antigen is a cancer antigen. In other embodiments, the first antigen is not a cancer antigen.

[00173] In some embodiments, the second antigen is expressed on an immune cells including lymphocytes and monocytes. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a B cell. In other embodiments, the second antigen is expressed on a dendritic cell. In other embodiments, the second antigen is expressed on a granulocyte. In yet other embodiments, the second antigen is expressed on an innate lymphoid cell. In yet other embodiments, the second antigen is expressed on a megakaryocyte. In yet other embodiments, the second antigen is expressed on a monocyte. In yet other embodiments, the second antigen is expressed on a myeloid-derived suppressor cell. In yet other embodiments, the second antigen is expressed on a NK cell.

[00174] In some embodiments, the second antigen is expressed on an effector cell. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a T helper cell, a regulatory T cell, or a cytotoxic T cell. In some embodiments, the second antigen is expressed on a T helper cell. In some embodiments, the second antigen is expressed on a regulatory T cell. In some embodiments, the second antigen is expressed on a cytotoxic T cell. In some embodiments, the second antigen is expressed on a CD8+ T cell. In some embodiments, the second antigen is expressed on a CD4+ T cell. In some embodiments, the second antigen comprises an extracellular domain.

[00175] In some specific embodiments, the second antigen is CD3. In some embodiments, the first antigen is a cancer antigen and the second antigen is CD3.

[00176] In some more specific embodiments, the first antigen is PD-L1 and the second antigen is CD3.

[00177] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7; the VL region of the second portion of each Fab region comprises three CDRs having amino

acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00178] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00179] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 3; the third polypeptide has the amino acid sequence of SEQ ID NO.: 1; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 2.

[00180] In some embodiments, the first nucleic acid has a nucleotide sequence of SEQ ID NO.: 22; the second nucleic acid has a nucleotide sequence of SEQ ID NO.: 20; and the third nucleic acid has a nucleotide sequence of SEQ ID NO.: 21.

[00181] In other more specific embodiments, the first antigen is CD20 and the second antigen is CD3.

[00182] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, SEQ ID NO.: 29; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00183] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00184] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.

[00185] In some embodiments, the first nucleic acid has a nucleotide sequence of SEQ ID NO.: 36; the second nucleic acid has a nucleotide sequence of SEQ ID NO.: 34; and the third nucleic acid has a nucleotide sequence of SEQ ID NO.: 35.

[00186] In other more specific embodiments, the first antigen is EGFR and the second antigen is CD3.

[00187] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00188] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00189] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.

[00190] In some embodiments, the first nucleic acid has a nucleotide sequence of SEQ ID NO.: 50; the second nucleic acid has a nucleotide sequence of SEQ ID NO.: 48; and the third nucleic acid has a nucleotide sequence of SEQ ID NO.: 49.

[00191] In other more specific embodiments, the first antigen is Her2 and the second antigen is TNF alpha. In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 51; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 52; the VH region of the Fv region has an amino

acid sequence of SEQ ID NO.: 53; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 54.

[00192] In yet another aspect, provided herein is a pharmaceutical composition comprising a therapeutically effective amount of the binding molecule provided herein and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition is for use in treating a disease or condition in a subject. In some embodiments, the disease or condition is a cancer. In other embodiments, cancer is lung cancer. In some embodiments, the cancer is Non Small Cell Lung Carcinoma (NSCLC). In some embodiments, the cancer is a Diffuse Large B cell Lymphoma (DLBCL). In other embodiments, the disease or condition is a PD-L1 positive cancer.

[00193] In yet another aspect, provided herein is a method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of the binding molecule provided herein to the subject. In some embodiments, the disease or condition is a cancer. In other embodiments, cancer is lung cancer. In some embodiments, the cancer is Non Small Cell Lung Carcinoma (NSCLC). In some embodiments, the cancer is a Diffuse Large B cell Lymphoma (DLBCL). In other embodiments, the disease or condition is a PD-L1 positive cancer.

[00194] Where aspects or embodiments of the disclosure are described in terms of a Markush group or other grouping of alternatives, the present disclosure encompasses not only the entire group listed as a whole, but also each member of the group individually and all possible subgroups of the main group, and also the main group absent one or more of the group members. The present disclosure also envisages the explicit exclusion of one or more of any of the group members in the claimed disclosure.

[00195] ILLUSTRATIVE EMBODIMENTS

1. A binding molecule, comprising:
 - (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
 - (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region;and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen.

2. The binding molecule of embodiment 1, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region.

3. The binding molecule of embodiment 2, wherein the first Fab region and the second Fab region are linked to the Fv region via fusion.

4. The binding molecule of embodiment 2, wherein the flexible peptide region comprises an antibody hinge region.

5. The binding molecule of embodiment 4, wherein the antibody hinge region is an Immunoglobulin G (IgG) hinge region.

6. The binding molecule of embodiment 5, wherein the antibody hinge region is selected from the group consisting of IgG1, IgG2, IgG3, and IgG4 hinge regions.

7. The binding molecule of embodiment 4, wherein the antibody hinge region comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide.

8. The binding molecule of embodiment 4, wherein the flexible peptide region further comprises a linker.

9. The binding molecule of embodiment 8, wherein the linker comprises an amino acid sequence of GGGGS (G4S).

10. The binding molecule of embodiment 9, wherein the linker comprises two tandem copies of the amino acid sequence of GGGGS (G4S).

11. The binding molecule of embodiment 1, wherein the first Fab region and the second Fab region bind to the same epitope of the first antigen.

12. The binding molecule of embodiment 1, wherein the second antigen is expressed on an immune cell.
13. The binding molecule of embodiment 12, wherein the immune cell is selected from the group consisting of lymphocytes and monocytes.
14. The binding molecule of embodiment 12, wherein the immune cell is an effector cell.
15. The binding molecule of embodiment 12, wherein the immune cell is selected from the group consisting of a T cell, a B cell, a dendritic cell, a granulocyte, an innate lymphoid cell, a megakaryocyte, a monocyte, a myeloid-derived suppressor cell, and a natural killer (NK) cell.
16. The binding molecule of embodiment 1, wherein the first antigen is a cancer antigen.
17. The binding molecule of embodiment 16, wherein the cancer antigen is a tumor associated antigen (TAA) or a tumor specific antigen (TSA).
18. The binding molecule of embodiment 1, wherein the first antigen is selected from a group consisting of CD19, CD20, EGFR, Her2, and PD-L1.
19. The binding molecule of embodiment 12, wherein the second antigen is CD3 or TNF alpha.
20. The binding molecule of embodiment 16, wherein the first antigen is a cancer antigen and the second antigen is CD3.
21. The binding molecule of embodiment 20, wherein the cancer antigen is selected from a group consisting of CD19, CD20, EGFR, Her2, and PD-L1.
22. A method of making a binding molecule, comprising:
 - (i) expressing the binding molecule from one or more vectors in a host cell, wherein the one or more vectors comprise
 - (a) a first nucleic acid encoding a first polypeptide and a second nucleic acid encoding a second polypeptide, wherein each of the first polypeptide and the second polypeptide is an antibody light chain,
 - (b) a third nucleic acid encoding a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region and a second VH region; and
 - (c) a fourth nucleic acid encoding a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region and a VL region,
 wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide can form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide can form a second antigen binding Fab region; and

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen, and

23. The method of embodiment 22, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region comprising an antibody hinge region.

24. The method of embodiment 23, wherein the antibody hinge region comprises an interchain disulfide bond formed between the third polypeptide and the fourth polypeptide.

25. The method of embodiment 23, wherein the flexible peptide region further comprises a linker.

26. The method of embodiment 25, wherein the linker comprises an amino acid sequence of GGGGS (G4S).

27. The method of embodiment 22, wherein the first antigen is a cancer antigen and the second antigen is CD3.

28. The method of embodiment 22, wherein the first antigen is selected from a group consisting of CD19, CD20, EGFR, Her2, and PD-L1.

29. A pharmaceutical composition comprising a binding molecule and a pharmaceutically acceptable carrier, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen.

30. A method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of a binding molecule to the subject, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen.

31. A binding molecule, comprising:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region binds to Programmed Death-Ligand 1 (PD-L1), and the Fv region binds to Cluster of Differentiation 3 (CD3).

32. The binding molecule of embodiment 31, wherein:

(a) the antibody light chains of the first and the second polypeptide each comprise three Complementarity Determining Regions (CDRs) having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11;

(b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

(c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

33. The binding molecule of embodiment 32, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region.

34. The binding molecule of embodiment 33, wherein the first Fab region and the second Fab region are linked to the Fv region via fusion.

35. The binding molecule of embodiment 33, wherein the flexible peptide region comprises an antibody hinge region.

36. The binding molecule of embodiment 35, wherein the antibody hinge region is an Immunoglobulin G (IgG) hinge region.

37. The binding molecule of embodiment 36, wherein the antibody hinge region is selected from the group consisting of IgG1, IgG2, IgG3, and IgG4 hinge regions.

38. The binding molecule of embodiment 35, wherein the antibody hinge region comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide.

39. The binding molecule of embodiment 35, wherein the flexible peptide region further comprises a linker.
40. The binding molecule of embodiment 39, wherein the linker comprises an amino acid sequence of GGGGS (G4S).
41. The binding molecule of embodiment 40, wherein the linker comprises an amino acid sequence of GGGGSGGGGS.
42. The binding molecule of embodiment 40, wherein the linker comprises an amino acid sequence of GGS GGGGSG.
43. The binding molecule of embodiment 32, wherein:
- (a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 8;
 - (b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 4, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and
 - (c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 4, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.
44. The binding molecule of embodiment 32, wherein the first polypeptide and the second polypeptide each comprise the amino acid sequence of SEQ ID NO.: 3; the third polypeptide comprises the amino acid sequence of SEQ ID NO.: 1; and the fourth polypeptide comprises the amino acid sequence of SEQ ID NO.: 2.
45. The binding molecule of embodiment 32, wherein the first polypeptide and the second polypeptide each comprise the amino acid sequence of SEQ ID NO.: 95; the third polypeptide comprises the amino acid sequence of SEQ ID NO.: 96; and the fourth polypeptide comprises the amino acid sequence of SEQ ID NO.: 97.
46. The binding molecule of embodiment 32, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 95; the third polypeptide has the amino acid sequence of SEQ ID NO.: 98; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 99.
47. A method of making a binding molecule, comprising:
- (i) expressing the binding molecule from one or more vectors in a host cell, wherein the one or more vectors comprise

- (a) a first nucleic acid encoding a first polypeptide and a second nucleic acid encoding a second polypeptide, wherein each polypeptide comprises an antibody light chain,
 - (b) a third nucleic acid encoding a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and
 - (c) a fourth nucleic acid encoding a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,
- wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;
- wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;
- wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and
- wherein the first Fab region and the second Fab region bind to PD-L1, and the Fv region binds to CD3, and
- (ii) purifying the binding molecule.

48. A method of embodiment 47, wherein:

- (a) the antibody light chains of the first and the second polypeptide each comprise three Complementarity Determining Regions (CDRs) having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11;
- (b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and
- (c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

49. The method of embodiment 48, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region.

50. The method of embodiment 49, wherein the flexible peptide region comprises an antibody hinge region.
51. The method of embodiment 50, wherein the antibody hinge region comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide.
52. The method of embodiment 50, wherein the antibody hinge region is an Immunoglobulin G (IgG) hinge region.
53. The method of embodiment 20, wherein the flexible peptide region further comprises a linker.
54. The method of embodiment 53, wherein the linker comprises an amino acid sequence of GGGGS (G4S).
55. The method of embodiment 54, wherein the linker comprises an amino acid sequence of GGGSGGGGS.
56. The method of embodiment 54, wherein the linker comprises an amino acid sequence of GGSGGGGSG.
57. The method of embodiment 48,
wherein the VH region of each of the first and second Fab regions comprises an amino acid sequence of SEQ ID NO.: 4;
wherein the VL region of each of the first and second Fab regions comprises an amino acid sequence of SEQ ID NO.: 8;
wherein the VH region of the Fv region comprises an amino acid sequence of SEQ ID NO.: 12; and
wherein the VL region of the Fv region comprises an amino acid sequence of SEQ ID NO.: 16.
58. The method of embodiment 48, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 3; the third polypeptide has the amino acid sequence of SEQ ID NO.: 1; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 2.
59. A pharmaceutical composition comprising a binding molecule and a pharmaceutically acceptable carrier, wherein the binding molecule comprises:
- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region binds to PD-L1, and the Fv region binds to CD3.

60. A method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of a binding molecule to the subject, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region binds to PD-L1, and the Fv region binds to CD3.

61. A binding molecule, comprising:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region each binds to CD20 or epidermal growth factor receptor (EGFR), and the Fv region binds to CD3.

62. The binding molecule of embodiment 61, wherein the first Fab region and the second Fab region bind to CD20, and

(a) the antibody light chains of the first and the second polypeptide each comprise three Complementarity Determining Regions (CDRs) having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33;

(b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

(c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

63. The binding molecule of embodiment 62, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region.

64. The binding molecule of embodiment 63, wherein the flexible peptide region comprises an antibody hinge region.
65. The binding molecule of embodiment 64, wherein the antibody hinge region comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide.
66. The binding molecule of embodiment 64, wherein the flexible peptide region further comprises a linker.
67. The binding molecule of embodiment 62, wherein:
- (a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 30;
 - (b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 26, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and
 - (c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 26, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.
68. The binding molecule of embodiment 62, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.
69. The binding molecule of embodiment 61, wherein the first Fab region and the second Fab region bind to EGFR, and
- (a) the antibody light chains of the first and the second polypeptide each comprise three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47;
 - (b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and
 - (c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

70. The binding molecule of embodiment 69, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region.
71. The binding molecule of embodiment 70, wherein the flexible peptide region comprises an antibody hinge region.
72. The binding molecule of embodiment 71, wherein the antibody hinge region comprises an interchain disulfide bond between the third polypeptide and the fourth polypeptide.
73. The binding molecule of embodiment 70, wherein the flexible peptide region further comprises a linker.
74. The binding molecule of embodiment 69, wherein:
- (a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 44;
 - (b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 40, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and
 - (c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 40, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.
75. The binding molecule of embodiment 69, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.
76. A method of making a binding molecule comprising:
- (i) expressing the binding molecule from one or more vectors in a host cell, wherein the one or more vectors comprise
 - (a) a first nucleic acid encoding a first polypeptide and a second nucleic acid encoding a second polypeptide, wherein each of the first polypeptide and the second polypeptide is an antibody light chain,
 - (b) a third nucleic acid encoding a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region, and a second VH region; and
 - (c) a fourth nucleic acid encoding a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region each binds to CD20 or EGFR, and the Fv region binds to CD3, and

(ii) purifying the binding molecule.

77. The method of embodiment 76, wherein the first Fab region and the second Fab region bind to CD20, and

(a) the antibody light chains of the first and the second polypeptide each comprise three Complementarity Determining Regions (CDRs) having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33;

(b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

(c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

78. The method of embodiment 77, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region.

79. The binding molecule of embodiment 78, wherein the flexible peptide region comprises an antibody hinge region.

80. The binding molecule of embodiment 79, wherein the flexible peptide region further comprises a linker.

81. The method of embodiment 77, wherein:

(a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 30;

(b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 26, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and

(c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 26, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.

82. The method of embodiment 77, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.

83. The method of embodiment 87, wherein the first Fab region and the second Fab region bind to CD20, and

(a) the antibody light chains of the first and the second polypeptide each comprise three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47;

(b) in the third polypeptide, the first VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43, and the second VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

(c) in the fourth polypeptide, the third VH region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43, and the VL region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

84. The method of embodiment 83, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region.

85. The method of embodiment 84, wherein the flexible peptide region comprises an antibody hinge region.

86. The method of embodiment 85, wherein the flexible peptide region further comprises a linker.

87. The method of embodiment 83, wherein:

(a) the antibody light chains of the first and the second polypeptide each comprise a VL region that comprises the amino acid sequence of SEQ ID NO.: 44;

(b) in the third polypeptide, the first VH region comprises the amino acid sequence of SEQ ID NO.: 40, and the second VH region comprises the amino acid sequence of SEQ ID NO.: 12; and

(c) in the fourth polypeptide, the third VH region comprises the amino acid sequence of SEQ ID NO.: 40, and the VL region comprises the amino acid sequence of SEQ ID NO.: 16.

88. The method of embodiment 83, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.

89. A pharmaceutical composition comprising a therapeutically effective amount of a binding molecule and a pharmaceutically acceptable carrier, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region each binds to CD20 or EGFR, and the Fv region binds to CD3.

90. A method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of a binding molecule to the subject, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region each binds to CD20 or EGFR, and the Fv region binds to CD3.

BRIEF DESCRIPTION OF THE FIGURES

[00196] FIG. 1A illustrates a binding molecule (ALiCE) provided herein. FIG. 1B illustrates an exemplary binding molecule provided herein that contains CH3 regions. FIG. 1C illustrates an exemplary binding molecule provided herein that contains albumin binding sites (ABS). FIG. 1D illustrates an exemplary binding molecule provided herein that has a binding domain targeting a cancer antigen and also illustrates exemplary options for the flexible peptide region provided herein. FIG. 1E illustrates an exemplary binding molecule provided herein that has a binding domain targeting a cancer antigen and a binding domain targeting CD3.

[00197] FIG. 2A illustrates the assembly pattern of ACE-00. “BiP” illustrates the binding immunoglobulin protein (BiP), which binds CH1 or VH domains of ACE-00 that are exposed. FIG. 2B shows the results of the SDS-PAGE performed to identify the assembly pattern of ACE-00. The arrows indicate the band of ACE-00-VL in the “ACE-00+ Light chain” sample and the two bands of ACE-00-VH and ACE-00-VL in the “ACE-00-VH+ACE-00-VL+ Light chain” samples under the reducing condition. FIG. 2C shows the co-immunoprecipitation (co-IP) results of wild-type heavy chain (HC), CH1-truncated heavy chain (Δ CH1) and VH-CH1-truncated heavy chain (Δ VH-CH1) of adalimumab. Fig. 2D illustrates that BiP can regulate the assembly and secretion of heavy chain by interaction with VH and/or CH1 domain of heavy chain. “ERAD” represents endoplasmic-reticulum-associated protein degradation. FIG. 2E

illustrates contribution of the antibody VH domain to antibody assembly and contribution of the ACE-00-VH chain to the proper assembly of ACE-00 molecule. “X” represents no assembly; “O” represents assembly. FIG. 2F illustrates the structure of ACE-00. FIG. 2G shows the results of affinity chromatography for ACE-00 and ACE-00-VL2 proteins using Hitrap™ KappaSelect (GE healthcare, USA). FIG. 2H shows the results of capillary electrophoresis performed to identify the molecular size differentiation between ACE-00-VL2 and ACE-00. FIG. 2I shows the results of capillary electrophoresis showing the conformation of ACE-00 and ACE-00-VL2 molecules. Solid arrows indicate results of ACE-00; dashed arrows indicate results of ACE-00-VL2. FIG. 2J shows the results of capillary isoelectric focusing performed to corroborate the heterodimerization between ACE-00-VH chain and ACE-00-VL chain. FIG. 2K shows the results of SDS-PAGE and capillary electrophoresis performed to identify the assembly pattern of ACE-00. “R” represents reducing; “NR” represents non reducing. FIG. 2L shows the results of size exclusion chromatography of ACE-00.

[00198] FIG. 3 shows the result of SDS-PAGE (under reducing (left) and non-reducing (right) conditions) performed to identify the expression and assembly of ACE-02, ACE-02-VL2, ACE-03, ACE-03-VL2, ACE-00 and ACE-01. ACE-02 contains the second antigen as humanized 12F6 (h12F6, an anti-CD3 antibody), and ACE-03 contains the second antigen as humanized OKT3 (hOKT3, an anti-CD3 antibody). The arrows indicate the bands of assembled ACE-02, ACE-03 and ACE-03-VL2 respectively under non-reducing condition.

[00199] FIG. 4A illustrates the structure of ACE-04. “A” refers to anti-PD-L1. UCHT1 is an anti-CD3 antibody. FIG. 4B illustrates the structure of ACE-05. “A” refers to anti-PD-L1. OKT3 is an anti-CD3 antibody. FIG. 4C show the results of SDS-PAGE performed to identify the assembly pattern of ACE-04, ACE-04-VL2, ACE-05, and ACE-05-VL2. The arrows indicate the bands of ACE-04 and ACE-05 under reducing and non-reducing conditions. FIG. 4D shows the results of the SDS-PAGE performed to identify the assembly pattern of ACE-05 (top) and illustrates the potential regulatory mechanisms in ACE-05 assembly (bottom). FIGs. 4E-4F show the results of SDS-PAGE and capillary electrophoresis performed to identify the conformations of ACE-05 as well as the heterodimerization efficiency between ACE-05-VH and ACE-05-VL chains. “M” represents marker; “R” represents reducing; “NR” represents non reducing; “IN” represents input; “FT” represents flow through; “W” represents washing; “Elu.” represents elution. FIG. 4G shows the size exclusion chromatography performed to identify the

purity of ACE-05. FIG. 4H shows the results of size exclusion chromatography for gel filtration analysis of ACE-05. FIG. 4I shows the results of cationic exchange chromatography (CEX) performed to identify the structure conformations of ACE-05.

[00200] FIG. 5 shows the results of SDS-PAGE performed to identify the assembly pattern of ACE-09 and ACE-05 at 37 °C and 32 °C (top) and summarizes features of ACE-09 and ACE-05 (bottom). In the figures on top, lanes that are labeled with “ACE-05” show results of ACE-05; the rest of the lanes show the results of ACE-09; “R” represents reducing; “N.R” represents “non reducing”; “IP” represents input; “F.T” represents flow through; “W” represents washing; “OP” represents output.

[00201] FIG. 6A illustrates the structure of ACE-10. FIGs. 6B-6C show the expression and assembly analysis of the ACE-10 molecule (under reducing (left) and non-reducing (right) conditions). In FIG. 6B, the arrows indicate the bands of ACE-10 under reducing and non-reducing conditions. “ACE-10 dialysis” represents ACE-10 generated via transfection with dialyzed DNA. In FIG. 6C, the arrow in the results from the anti-kappa condition indicates ACE-10-VL/ACE-10-LC dimer and ACE-10-VH/ACE-10-LC dimer complex under non reducing condition; the arrow in the results from the anti-CH1 condition indicates the assembled ACE-10 under non reducing condition.

[00202] FIG. 7A illustrates the structure of ACE-11. FIG. 7B shows the results of SDS-PAGE performed to identify the expression and assembly pattern of ACE-11 and ACE-11-VL2 (analyzed by Coomassie Blue staining (left) and Western blot (right)). “M” represents marker. The arrows indicate the bands of assembled ACE-11 under non reducing condition.

[00203] FIG. 8 shows the results of SDS-PAGE performed to identify the assembly pattern of ACE-12, ACE-05 and ACE-09. The arrows indicate the bands of ACE-05-VH + LC dimer and ACE-05-VL + LC dimer under non-reducing condition using anti-kappa (left) and anti-CH1 (right) antibodies in Western blot.

[00204] FIG. 9 shows the results of the Enzyme-Linked Immunosorbent Assay (ELISA) for determining the affinity of ACE-00 and ACE-00-VL2 to TNF alpha.

[00205] FIGs. 10A-10C show the analysis of binding affinity of ACE-05 to PD-L1 (10A) and CD3 (10B-10C) using ELISA.

[00206] FIG. 11 shows the analysis of binding affinity of ACE-05 and ACE-09 to CD3 using ELISA.

[00207] FIGs. 12A-12C show the analysis of binding kinetics of ACE-05 to PD-L1 (12A, 12C) and CD3 (12B, 12C) using Surface Plasmon Resonance (SPR). FIG. 12D shows the kinetics analysis of ACE-05 binding simultaneously to PD-L1 and CD3 using Surface Plasmon Resonance (SPR).

[00208] FIG. 13A shows PD-L1 expression levels in HEK293E-PD-L1 cells and the parental HEK293E cells (right panel) and CD3 expression levels in Jurkat luciferase reporter cells as measured by various CD3 antibodies (left panel). FIGs. 13B-13E show the results of the T cell redirecting (activity) assay for ACE-05 and BiTE-05. FIG. 13F shows T cell activation in the presence of ACE-05, BiTE-05, or YBL-007 in different T cell stages. FIGs. 13G-13H show the results of the PD-1/PD-L1 blockage assay for ACE-05. FIGs. 13I-13J show T cell activation by ACE-05, BiTE-05, UCHT1 (an anti-CD3 antibody from BioLegend, USA), or OKT3 (an anti-CD3 antibody from BioLegend, USA). FIG. 13K shows the results of T cell cytotoxicity assay for determining ACE-05 mediated T cell cytotoxicity. FIG. 13L shows the results of T cell cytotoxicity mediated by ACE-05, BiTE-05, YBL-007, or UCHT1. "IgG" represents normal human IgG used as a negative control. FIGs. 13M shows the T cell cytotoxicity on tumor cells when in direct contact with PBMC cells in the presence of ACE-05 or YBL-007. Arrows indicate target HCC827 cancer cells. FIG. 13N shows IL-2 and INF- γ levels in the presence of ACE-05, BiTE-05 or YBL-007 in co-cultivated PBMC and HCC827 cells. "IgG" represents normal human IgG used as a negative control. FIG. 13O shows the thermodynamic stability results of ACE-05, BiTE-05, YBL-007, and UCHT1.

[00209] FIGs. 14A-14B show the results of T cell redirecting assay performed for determining ACE-10 mediated T cell activation. "hIgG" represents normal human IgG used as a control.

[00210] FIG. 15 shows the results of T cell cytotoxicity assay for determining ACE-11 mediated T cell cytotoxicity.

[00211] FIG. 16A-16B show the results of ACE-05 pharmacokinetic study in Sprague-Dawley (SD) rat.

[00212] FIG. 17A shows the results of the HCC827 (PD-L1 positive tumor) xenograft study in a humanized mouse model. FIG. 17B shows the anti-tumor effects of ACE-05 and other test articles in PBMC donor A and donor B. FIG. 17C shows body weight changes of donor A and donor B. FIG. 17D shows individual anti-tumor efficacy responses. FIG. 17E shows individual

body weight loss (%) (side-effect). FIG. 17F shows the comparison of anti-tumor efficacy of BiTE and ACE-05 and the comparison of the parental PD-L1 antibody and ACE-05.

[00213] FIGs. 18A-18D show the results of the dose limit study showing the anti-tumor effects of ACE-05 and other test articles in PBMC donor A and donor B in a humanized mouse model inoculated with HCC827 (PD-L1 positive tumor) xenograft. FIG. 18A shows dose response of anti-tumor efficacy for ACE-05 and BiTE-05. FIG. 18B and FIG. 18C show anti-tumor efficacy of individual mice in each dose group for ACE-05 and BiTE-05, respectively. FIG. 18D shows individual body weight loss (%) (side-effect) of ACE-05 and BiTE-05 treated group.

DETAILED DESCRIPTION

[00214] The present disclosure provides novel cell engaging binding molecules having multiple binding domains. These binding molecules are referred to herein as “antibody like cell engagers” (ALiCE). ALiCE molecules provided herein have two antigen binding domains. The first antigen binding domain has two Fab regions. The second antigen binding domain has a Fv region. A typical ALiCE molecule is depicted in FIG. 1A. Generally, in such a molecule the first antigen binding domain comprises Fab regions, and the second antigen binding domain is attached (directly or indirectly) to the first antigen binding domain generally at the position where CH2 and CH3 domains would generally be located in a native antibody structure. For example, in the depicted embodiment, the C terminus of heavy chain comprises a VH domain rather than a CH2 domain and the C-terminus of the second heavy chain comprises a VL domain rather than a domain.

[00215] The binding molecules disclosed herein provide many advantages over conventional antibodies and existing multispecific antibodies (e.g., bispecific antibodies). Due to its multiple antigen binding domains and overall configuration design, the binding molecules provided herein can be used as a cell engager to bring multiple cells together. For example, the first antigen binding domain can bind to an antigen expressed on a first cell and the second antigen binding domain can bind to an antigen expressed on a second cell, and thereby bring the two cells together.

[00216] In certain embodiments, one of the engaged cells is an immune cell, e.g., a cytotoxic T cell. In these embodiments, the binding molecules provided herein are particularly useful for directing and activating an immune cell. For example, in certain embodiments, while the

bivalent Fab portion of ALiCE molecule retains the functionality of conventional antibodies, the second Fc-less monovalent antigen-binding region (i.e., the Fv region) can recognize, engage, redirect, and/or activate effector cells of the immune system, such as T cells. For example, as demonstrated in the Example section below, ACE-05, an ALiCE molecule composed of anti-PD-L1 and anti-CD3 domains, shows synergistic effects for both PD-L1-dependent (mediated) T cell activation and PD-1 and PD-L1 blockade efficacy.

[00217] In certain embodiments, the absence of a fully functional Fc region, or the absence of complete CH2 and/or CH3 region abolishes or reduces certain undesirable Fc-mediated effector cytotoxicity. In certain embodiment, the native interaction between the VH and VL chains of the Fv portion facilitates heterodimerization of the ALiCE molecule without imparting undesirable immunogenicity through artificial engineering.

[00218] Pharmacokinetic (PK) studies presented herein indicate higher stability of ALiCE molecules than other formats such as BiTE (bispecific T-cell engager) or DART (dual-affinity re-targeting) (Campagne O. et al. Integrated Pharmacokinetic/Pharmacodynamic Model of a Bispecific CD3xCD123 DART Molecule in Nonhuman Primates: Evaluation of Activity and Impact of Immunogenicity. *Clin Cancer Res.* 2018 Jun 1;24(11):2631-2641; Moore P. A. et al. Application of dual affinity retargeting molecules to achieve optimal redirected T-cell killing of B-cell lymphoma. *Blood.* 2011 Apr 28;117(17):4542-51; Moore P. A. et al. Development of MGD007, a gpA33 x CD3-Bispecific DART Protein for T-Cell Immunotherapy of Metastatic Colorectal Cancer. *Mol Cancer Ther.* 2018 Aug;17(8):1761-1772; Yuraszeck T. et al. Translation and Clinical Development of Bispecific T-cell Engaging Antibodies for Cancer Treatment. *Clin Pharmacol Ther.* 2017 May;101(5):634-645. Each of these is incorporated herein by reference in its entirety). Further, *in vivo* efficacy study of an exemplary ALiCE molecule shows significant anti-cancer effects. These results demonstrate that ALiCE is an advantageous platform technology in antibody engineering, e.g., for cancer therapy.

I. Definitions

[00219] Techniques and procedures described or referenced herein include those that are generally well understood and/or commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in Sambrook et al., Molecular Cloning: A Laboratory Manual (3d ed. 2001); Current Protocols in Molecular

Biology (Ausubel et al. eds., 2003); Therapeutic Monoclonal Antibodies: From Bench to Clinic (An ed. 2009); Monoclonal Antibodies: Methods and Protocols (Albitar ed. 2010); and Antibody Engineering Vols 1 and 2 (Kontermann and Dübel eds., 2d ed. 2010).

[00220] Unless otherwise defined herein, technical and scientific terms used in the present description have the meanings that are commonly understood by those of ordinary skill in the art. For purposes of interpreting this specification, the following description of terms will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. In the event that any description of a term set forth conflicts with any document incorporated herein by reference, the description of the term set forth below shall control.

[00221] The term “binding molecule” refers to a protein comprising a portion (*e.g.*, one or more binding regions such as CDRs) that binds to a target or an antigen and, optionally, a scaffold or framework portion (*e.g.*, one or more scaffold or framework regions) that allows the binding portion to adopt a conformation that promotes binding of the binding protein to a polypeptide, fragment, or epitope. In the context of the present disclosure, a binding molecule is said to specifically bind or selectively bind to an antigen, for example, when the dissociation constant (K_D) is $\leq 10^{-7}$ M. In some embodiments, the binding molecule may specifically bind to an antigen with a K_D of from about 10^{-7} M to about 10^{-12} M. In certain embodiments, the binding molecule may specifically bind to an antigen with high affinity when the K_D is $\leq 10^{-8}$ M or K_D is $\leq 10^{-9}$ M. In one embodiment, the binding molecule may specifically bind to a purified human antigen with a K_D of from 1×10^{-9} M to 10×10^{-9} M as measured by OCTET®. In yet another embodiment, the binding molecule specifically binds to a human antigen expressed on cells with a K_D of from 0.1×10^{-9} M to 10×10^{-9} M. In certain embodiments, the binding molecule specifically binds to a human antigen expressed on cells with a K_D of about 0.1×10^{-9} M, about 0.5×10^{-9} M, about 1×10^{-9} M, about 5×10^{-9} M, about 10×10^{-9} M, or any range or interval thereof. The term “binding molecule” includes antibodies and molecules derived from antibodies.

[00222] The term “antibody,” “immunoglobulin,” or “Ig” is used interchangeably herein, and is used in the broadest sense and specifically covers, for example, monoclonal antibodies (including agonist, antagonist, neutralizing antibodies, full length or intact monoclonal antibodies), antibody compositions with polyeptopic or monoepitopic specificity, polyclonal or monovalent antibodies, multivalent antibodies, multispecific antibodies (*e.g.*, bispecific

antibodies so long as they exhibit the desired biological activity), formed from at least two intact antibodies, single chain antibodies, and fragments thereof, as described below. An antibody can be human, humanized, chimeric and/or affinity matured, as well as an antibody from other species, for example, mouse and rabbit, *etc.* The term “antibody” is intended to include a polypeptide product of B cells within the immunoglobulin class of polypeptides that is able to bind to a specific molecular antigen and is composed of two identical pairs of polypeptide chains, wherein each pair has one heavy chain (about 50-70 kDa) and one light chain (about 25 kDa), each amino-terminal portion of each chain includes a variable region of about 100 to about 130 or more amino acids, and each carboxy-terminal portion of each chain includes a constant region. *See, e.g., Antibody Engineering* (Borrebaeck ed., 2d ed. 1995); and Kuby, *Immunology* (3d ed. 1997). In specific embodiments, the specific molecular antigen can be bound by an antibody provided herein, including a polypeptide or an epitope. Antibodies also include, but are not limited to, synthetic antibodies, recombinantly produced antibodies, camelized antibodies, intrabodies, anti-idiotypic (anti-Id) antibodies, and functional fragments (*e.g.*, antigen-binding fragments) of any of the above, which refers to a portion of an antibody heavy or light chain polypeptide that retains some or all of the binding activity of the antibody from which the fragment was derived. Non-limiting examples of functional fragments (*e.g.*, antigen-binding fragments) include single-chain Fvs (scFv) (*e.g.*, including monospecific, bispecific, *etc.*), Fab fragments, F(ab') fragments, F(ab)₂ fragments, F(ab')₂ fragments, disulfide-linked Fvs (dsFv), Fd fragments, Fv fragments, diabody, triabody, tetrabody, and minibody. In particular, antibodies provided herein include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, for example, antigen-binding domains or molecules that contain an antigen-binding site that binds to an antigen (*e.g.*, one or more CDRs of an antibody). Such antibody fragments can be found in, for example, Harlow and Lane, *Antibodies: A Laboratory Manual* (1989); *Mol. Biology and Biotechnology: A Comprehensive Desk Reference* (Myers ed., 1995); Huston *et al.*, 1993, *Cell Biophysics* 22:189-224; Plückthun and Skerra, 1989, *Meth. Enzymol.* 178:497-515; and Day, *Advanced Immunochemistry* (2d ed. 1990). The antibodies provided herein can be of any class (*e.g.*, IgG, IgE, IgM, IgD, and IgA) or any subclass (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2) of immunoglobulin molecule. Antibodies may be agonistic antibodies or antagonistic antibodies.

[00223] An “antigen” is a structure to which an antibody can selectively bind. A target antigen may be a polypeptide, carbohydrate, nucleic acid, lipid, hapten, or other naturally occurring or synthetic compound. In some embodiments, the target antigen is a polypeptide. In certain embodiments, an antigen is associated with a cell, for example, is present on or in a cell, for example, an immune cell.

[00224] The terms “antigen-binding fragment,” “antigen-binding domain,” “antigen-binding region,” and similar terms refer to that portion of a binding molecule, which comprises the amino acid residues that interact with an antigen and confer on the binding agent its specificity and affinity for the antigen (*e.g.*, the CDRs).

[00225] The terms “binds” or “binding” refer to an interaction between molecules including, for example, to form a complex. Interactions can be, for example, non-covalent interactions including hydrogen bonds, ionic bonds, hydrophobic interactions, and/or van der Waals interactions. A complex can also include the binding of two or more molecules held together by covalent or non-covalent bonds, interactions, or forces. The strength of the total non-covalent interactions between a single antigen-binding site on an antibody and a single epitope of a target molecule, such as an antigen, is the affinity of the antibody or functional fragment for that epitope. The ratio of dissociation rate (k_{off}) to association rate (k_{on}) of a binding molecule (*e.g.*, an antibody) to a monovalent antigen ($k_{\text{off}}/k_{\text{on}}$) is the dissociation constant K_D , which is inversely related to affinity. The lower the K_D value, the higher the affinity of the antibody. The value of K_D varies for different complexes of antibody and antigen and depends on both k_{on} and k_{off} . The dissociation constant K_D for an antibody provided herein can be determined using any method provided herein or any other method well known to those skilled in the art. The affinity at one binding site does not always reflect the true strength of the interaction between an antibody and an antigen. When complex antigens containing multiple, repeating antigenic determinants, such as a polyvalent antigen, come in contact with antibodies containing multiple binding sites, the interaction of antibody with antigen at one site will increase the probability of a reaction at a second site. The strength of such multiple interactions between a multivalent antibody and antigen is called the avidity.

[00226] In connection with the binding molecules described herein terms such as “bind to,” “that specifically bind to,” and analogous terms are also used interchangeably herein and refer to binding molecules of antigen binding domains that specifically bind to an antigen, such as a

polypeptide. A binding molecule or antigen binding domain that binds to or specifically binds to an antigen may be cross-reactive with related antigens. In certain embodiments, a binding molecule or antigen binding domain that binds to or specifically binds to an antigen does not cross-react with other antigens. A binding molecule or antigen binding domain that binds to or specifically binds to an antigen can be identified, for example, by immunoassays, Octet[®], Biacore[®], or other techniques known to those of skill in the art. A binding molecule or antigen binding domain binds to or specifically binds to an antigen when it binds to an antigen with higher affinity than to any cross-reactive antigen as determined using experimental techniques, such as radioimmunoassays (RIA) and enzyme linked immunosorbent assays (ELISAs). Typically, a specific or selective reaction will be at least twice background signal or noise and may be more than 10 times background. *See, e.g., Fundamental Immunology* 332-36 (Paul ed., 2d ed. 1989) for a discussion regarding binding specificity. In certain embodiments, the extent of binding of a binding molecule or antigen binding domain to a “non-target” protein is less than about 10% of the binding of the binding molecule or antigen binding domain to its particular target antigen, for example, as determined by fluorescence activated cell sorting (FACS) analysis or RIA. With regard terms such as “specific binding,” “specifically binds to,” or “is specific for” means binding that is measurably different from a non-specific interaction. Specific binding can be measured, for example, by determining binding of a molecule compared to binding of a control molecule, which generally is a molecule of similar structure that does not have binding activity. For example, specific binding can be determined by competition with a control molecule that is similar to the target, for example, an excess of non-labeled target. In this case, specific binding is indicated if the binding of the labeled target to a probe is competitively inhibited by excess unlabeled target. A binding molecule or antigen binding domain that binds to an antigen includes one that is capable of binding the antigen with sufficient affinity such that the binding molecule is useful, for example, as a diagnostic agent in targeting the antigen. In certain embodiments, a binding molecule or antigen binding domain that binds to an antigen has a dissociation constant (K_D) of less than or equal to 10 nM, 5 nM, 4 nM, 3 nM, 2 nM, 1 nM, 0.9 nM, 0.8 nM, 0.7 nM, 0.6 nM, 0.5 nM, 0.4 nM, 0.3 nM, 0.2 nM, or 0.1 nM. In certain embodiments, a binding molecule or antigen binding domain binds to an epitope of an antigen that is conserved among the antigen from different species (*e.g.*, between human and cyno species).

[00227] “Binding affinity” generally refers to the strength of the sum total of noncovalent interactions between a single binding site of a molecule (e.g., a binding protein such as 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 term “B_{max}” refers to the maximum binding affinity extrapolated from experimental results. B_{max} can be calculated using known curve fitting methods in the art, for example, curve fitting methods provided in GraphPad Prism software 7. The affinity of a binding molecule X for its binding partner Y can generally be represented by the dissociation constant (K_D). Affinity can be measured by common methods known in the art, including those described herein. Low-affinity antibodies generally bind antigen slowly and tend to dissociate readily, whereas high-affinity antibodies generally bind antigen faster and tend to remain bound longer. A variety of methods of measuring binding affinity are known in the art, any of which can be used for purposes of the present disclosure. Specific illustrative embodiments include the following. In one embodiment, the “K_D” or “K_D value” may be measured by assays known in the art, for example by a binding assay. The K_D may be measured in a RIA, for example, performed with the Fab version of an antibody of interest and its antigen (Chen et al., 1999, J. Mol Biol 293:865-81). The K_D or K_D value may also be measured by using biolayer interferometry (BLI) or surface plasmon resonance (SPR) assays by Octet®, using, for example, a Octet® QK384 system, or by Biacore®, using, for example, a Biacore® TM-2000 or a Biacore® TM-3000. An “on-rate” or “rate of association” or “association rate” or “k_{on}” may also be determined with the same biolayer interferometry (BLI) or surface plasmon resonance (SPR) techniques described above using, for example, the Octet® QK384, the Biacore® TM-2000, or the Biacore® TM-3000 system.

[00228] The term “reducing” used herein refers to a condition in which interchain or intrachain disulfide (S-S) bridges within a protein are denatured or reduced, for example, by the addition of 2-Mercaptoethanol (2-ME) or dithiothreitol (DTT), resulting in multiple polypeptide chains. The term “non reducing” used herein refers to a condition in which interchain or intrachain disulfide (S-S) bridges within a protein remain intact in the absence of denaturing or reducing agents such as 2-Mercaptoethanol (2-ME) or dithiothreitol (DTT).

[00229] In certain embodiments, the binding molecules or antigen binding domains can comprise “chimeric” sequences in which a portion of the heavy and/or light chain is identical

with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (*see* U.S. Pat. No. 4,816,567; and Morrison *et al.*, 1984, Proc. Natl. Acad. Sci. USA 81:6851-55).

[00230] In certain embodiments, the binding molecules or antigen binding domains can comprise portions of “humanized” forms of nonhuman (*e.g.*, murine) antibodies that are chimeric antibodies that include human immunoglobulins (*e.g.*, recipient antibody) in which the native CDR residues are replaced by residues from the corresponding CDR of a nonhuman species (*e.g.*, donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, one or more FR region residues of the human immunoglobulin are replaced by corresponding nonhuman residues. Furthermore, humanized antibodies can comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. A humanized antibody heavy or light chain can comprise substantially all of at least one or more variable regions, in which all or substantially all of the CDRs correspond to those of a nonhuman immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. In certain embodiments, the humanized antibody will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see, Jones *et al.*, 1986, Nature 321:522-25; Riechmann *et al.*, 1988, Nature 332:323-29; Presta, 1992, Curr. Op. Struct. Biol. 2:593-96; Carter *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:4285-89; U.S. Pat. Nos: 6,800,738; 6,719,971; 6,639,055; 6,407,213; and 6,054,297.

[00231] In certain embodiments, the binding molecules or antigen binding domains can comprise portions of a “fully human antibody” or “human antibody,” wherein the terms are used interchangeably herein and refer to an antibody that comprises a human variable region and, for example, a human constant region. In specific embodiments, the terms refer to an antibody that comprises a variable region and constant region of human origin. “Fully human” antibodies, in certain embodiments, can also encompass antibodies which bind polypeptides and are encoded by nucleic acid sequences which are naturally occurring somatic variants of human germline immunoglobulin nucleic acid sequence. The term “fully human antibody” includes antibodies

having variable and constant regions corresponding to human germline immunoglobulin sequences as described by Kabat *et al.* (see Kabat *et al.* (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). A “human antibody” is one that possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues. Human antibodies can be produced using various techniques known in the art, including phage-display libraries (Hoogenboom and Winter, 1991, J. Mol. Biol. 227:381; Marks *et al.*, 1991, J. Mol. Biol. 222:581) and yeast display libraries (Chao *et al.*, 2006, Nature Protocols 1: 755-68). Also available for the preparation of human monoclonal antibodies are methods described in Cole *et al.*, Monoclonal Antibodies and Cancer Therapy 77 (1985); Boerner *et al.*, 1991, J. Immunol. 147(1):86-95; and van Dijk and van de Winkel, 2001, Curr. Opin. Pharmacol. 5: 368-74. Human antibodies can be prepared by administering the antigen to a transgenic animal that has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled, *e.g.*, mice (see, *e.g.*, Jakobovits, 1995, Curr. Opin. Biotechnol. 6(5):561-66; Brüggemann and Taussing, 1997, Curr. Opin. Biotechnol. 8(4):455-58; and U.S. Pat. Nos. 6,075,181 and 6,150,584 regarding XENOMOUSE™ technology). See also, for example, Li *et al.*, 2006, Proc. Natl. Acad. Sci. USA 103:3557-62 regarding human antibodies generated via a human B-cell hybridoma technology.

[00232] In certain embodiments, the binding molecules or antigen binding domains can comprise portions of a “recombinant human antibody,” wherein the phrase includes human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial human antibody library, antibodies isolated from an animal (*e.g.*, a mouse or cow) that is transgenic and/or transchromosomal for human immunoglobulin genes (see, *e.g.*, Taylor, L. D. *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies can have variable and constant regions derived from human germline immunoglobulin sequences (see Kabat, E. A. *et al.* (1991) Sequences of Proteins of

Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

[00233] In certain embodiments, the binding molecules or antigen binding domains can comprise a portion of a “monoclonal antibody,” wherein the term as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *e.g.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts, and each monoclonal antibody will typically recognize a single epitope on the antigen. In specific embodiments, a “monoclonal antibody,” as used herein, is an antibody produced by a single hybridoma or other cell. The term “monoclonal” is not limited to any particular method for making the antibody. For example, the monoclonal antibodies useful in the present disclosure may be prepared by the hybridoma methodology first described by Kohler *et al.*, 1975, Nature 256:495, or may be made using recombinant DNA methods in bacterial or eukaryotic animal or plant cells (*see, e.g.*, U.S. Pat. No. 4,816,567). The “monoclonal antibodies” may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, 1991, Nature 352:624-28 and Marks *et al.*, 1991, J. Mol. Biol. 222:581-97, for example. Other methods for the preparation of clonal cell lines and of monoclonal antibodies expressed thereby are well known in the art. *See, e.g.*, Short Protocols in Molecular Biology (Ausubel *et al.* eds., 5th ed. 2002).

[00234] A typical 4-chain antibody unit is a heterotetrameric glycoprotein composed of two identical light (L) chains and two identical heavy (H) chains. In the case of IgGs, the 4-chain unit is generally about 150,000 daltons. Each L chain is linked to an H chain by one covalent disulfide bond, while the two H chains are linked to each other by one or more disulfide bonds depending on the H chain isotype. Each H and L chain also has regularly spaced intrachain disulfide bridges. Each H chain has at the N-terminus, a variable domain (VH) followed by three constant domains (CH) for each of the α and γ chains and four CH domains for μ and ϵ isotypes. Each L chain has at the N-terminus, a variable domain (VL) followed by a constant domain (CL)

at its other end. The VL is aligned with the VH, and the CL is aligned with the first constant domain of the heavy chain (CH1). Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains. The pairing of a VH and VL together forms a single antigen-binding site. For the structure and properties of the different classes of antibodies, *see*, for example, Basic and Clinical Immunology 71 (Stites *et al.* eds., 8th ed. 1994); and Immunobiology (Janeway *et al.* eds., 5th ed. 2001).

[00235] The term “Fab” or “Fab region” refers to an antibody region that binds to antigens. A conventional IgG usually comprises two Fab regions, each residing on one of the two arms of the Y-shaped IgG structure. Each Fab region is typically composed of one variable region and one constant region of each of the heavy and the light chain. More specifically, the variable region and the constant region of the heavy chain in a Fab region are VH and CH1 regions, and the variable region and the constant region of the light chain in a Fab region are VL and CL regions. The VH, CH1, VL, and CL in a Fab region can be arranged in various ways to confer an antigen binding capability according to the present disclosure. For example, VH and CH1 regions can be on one polypeptide, and VL and CL regions can be on a separate polypeptide, similarly to a Fab region of a conventional IgG. Alternatively, VH, CH1, VL and CL regions can all be on the same polypeptide and oriented in different orders as described in more detail the sections below.

[00236] The term “variable region,” “variable domain,” “V region,” or “V domain” refers to a portion of the light or heavy chains of an antibody that is generally located at the amino-terminal of the light or heavy chain and has a length of about 120 to 130 amino acids in the heavy chain and about 100 to 110 amino acids in the light chain, and are used in the binding and specificity of each particular antibody for its particular antigen. The variable region of the heavy chain may be referred to as “VH.” The variable region of the light chain may be referred to as “VL.” The term “variable” refers to the fact that certain segments of the variable regions differ extensively in sequence among antibodies. The V region mediates antigen binding and defines specificity of a particular antibody for its particular antigen. However, the variability is not evenly distributed across the 110-amino acid span of the variable regions. Instead, the V regions consist of less variable (*e.g.*, relatively invariant) stretches called framework regions (FRs) of about 15-30 amino acids separated by shorter regions of greater variability (*e.g.*, extreme variability) called “hypervariable regions” that are each about 9-12 amino acids long. The variable regions of heavy and light chains each comprise four FRs, largely adopting a β sheet configuration,

connected by three hypervariable regions, which form loops connecting, and in some cases form part of, the β sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (*see, e.g., Kabat et al., Sequences of Proteins of Immunological Interest* (5th ed. 1991)). The constant regions are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). The variable regions differ extensively in sequence between different antibodies. In specific embodiments, the variable region is a human variable region.

[00237] The term “variable region residue numbering according to Kabat” or “amino acid position numbering as in Kabat”, and variations thereof, refer to the numbering system used for heavy chain variable regions or light chain variable regions of the compilation of antibodies in Kabat *et al., supra*. Using this numbering system, the actual linear amino acid sequence may contain fewer or additional amino acids corresponding to a shortening of, or insertion into, an FR or CDR of the variable domain. For example, a heavy chain variable domain may include a single amino acid insert (residue 52a according to Kabat) after residue 52 and three inserted residues (*e.g., residues 82a, 82b, and 82c, etc. according to Kabat*) after residue 82. The Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a “standard” Kabat numbered sequence. The Kabat numbering system is generally used when referring to a residue in the variable domain (approximately residues 1-107 of the light chain and residues 1-113 of the heavy chain) (*e.g., Kabat et al., supra*). The “EU numbering system” or “EU index” is generally used when referring to a residue in an immunoglobulin heavy chain constant region (*e.g., the EU index reported in Kabat et al., supra*). The “EU index as in Kabat” refers to the residue numbering of the human IgG 1 EU antibody. Other numbering systems have been described, for example, by AbM, Chothia, Contact, IMGT, and AHon.

[00238] An “intact” antibody is one comprising an antigen-binding site as well as a CL and at least heavy chain constant regions, CH1, CH2 and CH3. The constant regions may include human constant regions or amino acid sequence variants thereof. In certain embodiments, an intact antibody has one or more effector functions.

[00239] “Antibody fragments” comprise a portion of an intact antibody, such as the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include, without limitation, Fab, Fab’, F(ab’)₂, and Fv fragments; diabodies and di-diabodies (*see, e.g.,* Holliger *et al.*, 1993, Proc. Natl. Acad. Sci. 90:6444-48; Lu *et al.*, 2005, J. Biol. Chem. 280:19665-72; Hudson *et al.*, 2003, Nat. Med. 9:129-34; WO 93/11161; and U.S. Pat. Nos. 5,837,242 and 6,492,123); single-chain antibody molecules (*see, e.g.,* U.S. Pat. Nos. 4,946,778; 5,260,203; 5,482,858; and 5,476,786); dual variable domain antibodies (*see, e.g.,* U.S. Pat. No. 7,612,181); single variable domain antibodies (sdAbs) (*see, e.g.,* Woolven *et al.*, 1999, Immunogenetics 50: 98-101; and Streltsov *et al.*, 2004, Proc Natl Acad Sci USA. 101:12444-49); and multispecific antibodies formed from antibody fragments.

[00240] The term “heavy chain” when used in reference to an antibody refers to a polypeptide chain of about 50-70 kDa, wherein the amino-terminal portion includes a variable region of about 120 to 130 or more amino acids, and a carboxy-terminal portion includes a constant region. The constant region can be one of five distinct types, (*e.g.,* isotypes) referred to as alpha (α), delta (δ), epsilon (ε), gamma (γ), and mu (μ), based on the amino acid sequence of the heavy chain constant region. The distinct heavy chains differ in size: α, δ, and γ contain approximately 450 amino acids, while μ and ε contain approximately 550 amino acids. When combined with a light chain, these distinct types of heavy chains give rise to five well known classes (*e.g.,* isotypes) of antibodies, IgA, IgD, IgE, IgG, and IgM, respectively, including four subclasses of IgG, namely IgG1, IgG2, IgG3, and IgG4.

[00241] The term “light chain” when used in reference to an antibody refers to a polypeptide chain of about 25 kDa, wherein the amino-terminal portion includes a variable region of about 100 to about 110 or more amino acids, and a carboxy-terminal portion includes a constant region. The approximate length of a light chain is 211 to 217 amino acids. There are two distinct types, referred to as kappa (κ) or lambda (λ) based on the amino acid sequence of the constant domains.

[00242] As used herein, the terms “hypervariable region,” “HVR,” “Complementarity Determining Region,” and “CDR” are used interchangeably. A “CDR” refers to one of three hypervariable regions (H1, H2 or H3) within the non-framework region of the immunoglobulin (Ig or antibody) VH β-sheet framework, or one of three hypervariable regions (L1, L2 or L3)

within the non-framework region of the antibody VL β -sheet framework. Accordingly, CDRs are variable region sequences interspersed within the framework region sequences.

[00243] CDR regions are well known to those skilled in the art and have been defined by well-known numbering systems. For example, the Kabat Complementarity Determining Regions (CDRs) are based on sequence variability and are the most commonly used (*see, e.g., Kabat et al., supra*). Chothia refers instead to the location of the structural loops (*see, e.g., Chothia and Lesk, 1987, J. Mol. Biol. 196:901-17*). The end of the Chothia CDR-H1 loop when numbered using the Kabat numbering convention varies between H32 and H34 depending on the length of the loop (this is because the Kabat numbering scheme places the insertions at H35A and H35B; if neither 35A nor 35B is present, the loop ends at 32; if only 35A is present, the loop ends at 33; if both 35A and 35B are present, the loop ends at 34). The AbM hypervariable regions represent a compromise between the Kabat CDRs and Chothia structural loops, and are used by Oxford Molecular's AbM antibody modeling software (*see, e.g., Antibody Engineering Vol. 2* (Kontermann and Dübel eds., 2d ed. 2010)). The "contact" hypervariable regions are based on an analysis of the available complex crystal structures. Another universal numbering system that has been developed and widely adopted is ImMunoGeneTics (IMGT) Information System[®] (Lafranc *et al.*, 2003, *Dev. Comp. Immunol.* 27(1):55-77). IMGT is an integrated information system specializing in immunoglobulins (Ig), T cell receptors (TCR), and major histocompatibility complex (MHC) of human and other vertebrates. Herein, the CDRs are referred to in terms of both the amino acid sequence and the location within the light or heavy chain. As the "location" of the CDRs within the structure of the immunoglobulin variable domain is conserved between species and present in structures called loops, by using numbering systems that align variable domain sequences according to structural features, CDR and framework residues are readily identified. This information can be used in grafting and replacement of CDR residues from immunoglobulins of one species into an acceptor framework from, typically, a human antibody. An additional numbering system (AHon) has been developed by Honegger and Plückthun, 2001, *J. Mol. Biol.* 309: 657-70. Correspondence between the numbering system, including, for example, the Kabat numbering and the IMGT unique numbering system, is well known to one skilled in the art (*see, e.g., Kabat, supra; Chothia and Lesk, supra; Martin, supra; Lefranc et al., supra*). The residues from each of these hypervariable regions or CDRs are noted below.

Table 1: Residues of hypervariable regions or CDRs

Loop	Kabat	AbM	Chothia	Contact	IMGT
CDR L1	L24--L34	L24--L34	L24--L34	L30--L36	L27--L38
CDR L2	L50--L56	L50--L56	L50--L56	L46--L55	L56--L65
CDR L3	L89--L97	L89--L97	L89--L97	L89--L96	L105- L117
CDR H1	H31--H35B (Kabat Numbering)	H26-- H35B	H26-- H32..34	H30-- H35B	H27--H38
CDR H1	H31--H35 (Chothia Numbering)	H26--H35	H26--H32	H30--H35	
CDR H2	H50--H65	H50--H58	H52--H56	H47--H58	H56--H65
CDR H3	H95--H102	H95-- H102	H95-- H102	H93-- H101	H105- H117

[00244] The boundaries of a given CDR may vary depending on the scheme used for identification. Thus, unless otherwise specified, the terms “CDR” and “complementary determining region” of a given antibody or region thereof, such as a variable region, as well as individual CDRs (e.g., “CDR-H1, CDR-H2) of the antibody or region thereof, should be understood to encompass the complementary determining region as defined by any of the known schemes described herein above. In some instances, the scheme for identification of a particular CDR or CDRs is specified, such as the CDR as defined by the Kabat, Chothia, or Contact method. In other cases, the particular amino acid sequence of a CDR is given.

[00245] Hypervariable regions may comprise “extended hypervariable regions” as follows: 24-36 or 24-34 (L1), 46-56 or 50-56 (L2), and 89-97 or 89-96 (L3) in the VL, and 26-35 or 26-35A (H1), 50-65 or 49-65 (H2), and 93-102, 94-102, or 95-102 (H3) in the VH.

[00246] The term “constant region” or “constant domain” refers to a carboxy terminal portion of the light and heavy chain which is not directly involved in binding of the antibody to antigen but exhibits various effector function, such as interaction with the Fc receptor. The term refers to

the portion of an immunoglobulin molecule having a more conserved amino acid sequence relative to the other portion of the immunoglobulin, the variable region, which contains the antigen binding site. The constant region may contain the CH1, CH2, and CH3 regions of the heavy chain and the CL region of the light chain.

[00247] The term “framework” or “FR” refers to those variable region residues flanking the CDRs. FR residues are present, for example, in chimeric, humanized, human, domain antibodies, diabodies, linear antibodies, and bispecific antibodies. FR residues are those variable domain residues other than the hypervariable region residues or CDR residues.

[00248] The term “Fc region” herein is used to define a C-terminal region of an immunoglobulin heavy chain, including, for example, native sequence Fc regions, recombinant Fc regions, and variant Fc regions. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is often defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The C-terminal lysine (residue 447 according to the EU numbering system) of the Fc region may be removed, for example, during production or purification of the antibody, or by recombinantly engineering the nucleic acid encoding a heavy chain of the antibody.

Accordingly, a composition of intact antibodies may comprise antibody populations with all K447 residues removed, antibody populations with no K447 residues removed, and antibody populations having a mixture of antibodies with and without the K447 residue. A “functional Fc region” possesses an “effector function” of a native sequence Fc region. Exemplary “effector functions” include C1q binding; CDC; Fc receptor binding; ADCC; phagocytosis; downregulation of cell surface receptors (*e.g.*, B cell receptor), *etc.* Such effector functions generally require the Fc region to be combined with a binding region or binding domain (*e.g.*, an antibody variable region or domain) and can be assessed using various assays known to those skilled in the art. 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 (*e.g.*, substituting, addition, or deletion). In certain embodiments, 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, for example, from about one to about ten amino acid substitutions, or from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of a parent polypeptide. The variant Fc region herein can possess at least about 80% homology with

a native sequence Fc region and/or with an Fc region of a parent polypeptide, or at least about 90% homology therewith, for example, at least about 95% homology therewith.

[00249] A polypeptide “extracellular domain” or “ECD” refers to a form or a portion of the polypeptide that is essentially free of the transmembrane and cytoplasmic domains. For example, an ECD may have less than 1% of such transmembrane and/or cytoplasmic domains and can have less than 0.5% of such domains.

[00250] As used herein, an “epitope” is a term in the art and refers to a localized region of an antigen to which a binding molecule (e.g., an antibody) can specifically bind. An epitope can be a linear epitope or a conformational, non-linear, or discontinuous epitope. In the case of a polypeptide antigen, for example, an epitope can be contiguous amino acids of the polypeptide (a “linear” epitope) or an epitope can comprise amino acids from two or more non-contiguous regions of the polypeptide (a “conformational,” “non-linear” or “discontinuous” epitope). It will be appreciated by one of skill in the art that, in general, a linear epitope may or may not be dependent on secondary, tertiary, or quaternary structure. For example, in some embodiments, a binding molecule binds to a group of amino acids regardless of whether they are folded in a natural three dimensional protein structure. In other embodiments, a binding molecule requires amino acid residues making up the epitope to exhibit a particular conformation (*e.g.*, bend, twist, turn or fold) in order to recognize and bind the epitope.

[00251] The terms “polypeptide” and “peptide” and “protein” are used interchangeably herein and refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid, including but not limited to, unnatural amino acids, as well as other modifications known in the art. It is understood that, because the polypeptides of this disclosure may be based upon antibodies or other members of the immunoglobulin superfamily, in certain embodiments, a “polypeptide” can occur as a single chain or as two or more associated chains.

[00252] The term “vector” refers to a substance that is used to carry or include a nucleic acid sequence, including for example, a nucleic acid sequence encoding a binding molecule (e.g., an

antibody) as described herein, in order to introduce a nucleic acid sequence into a host cell. Vectors applicable for use include, for example, expression vectors, plasmids, phage vectors, viral vectors, episomes, and artificial chromosomes, which can include selection sequences or markers operable for stable integration into a host cell's chromosome. Additionally, the vectors can include one or more selectable marker genes and appropriate expression control sequences. Selectable marker genes that can be included, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media. Expression control sequences can include constitutive and inducible promoters, transcription enhancers, transcription terminators, and the like, which are well known in the art. When two or more nucleic acid molecules are to be co-expressed (*e.g.*, both an antibody heavy and light chain or an antibody VH and VL), both nucleic acid molecules can be inserted, for example, into a single expression vector or in separate expression vectors. For single vector expression, the encoding nucleic acids can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one constitutive promoter. The introduction of nucleic acid molecules into a host cell can be confirmed using methods well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid sequence or its corresponding gene product. It is understood by those skilled in the art that the nucleic acid molecules are expressed in a sufficient amount to produce a desired product and it is further understood that expression levels can be optimized to obtain sufficient expression using methods well known in the art.

[00253] The term “host” as used herein refers to an animal, such as a mammal (*e.g.*, a human).

[00254] The term “host cell” as used herein refers to a particular subject cell that may be transfected with a nucleic acid molecule and the progeny or potential progeny of such a cell. Progeny of such a cell may not be identical to the parent cell transfected with the nucleic acid molecule due to mutations or environmental influences that may occur in succeeding generations or integration of the nucleic acid molecule into the host cell genome.

[00255] An “isolated nucleic acid” is a nucleic acid, for example, an RNA, DNA, or a mixed nucleic acids, which is substantially separated from other genome DNA sequences as well as proteins or complexes such as ribosomes and polymerases, which naturally accompany a native

sequence. An “isolated” nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. Moreover, an “isolated” nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. In a specific embodiment, one or more nucleic acid molecules encoding an antibody as described herein are isolated or purified. The term embraces nucleic acid sequences that have been removed from their naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogues or analogues biologically synthesized by heterologous systems. A substantially pure molecule may include isolated forms of the molecule.

[00256] “Polynucleotide” or “nucleic acid,” as used interchangeably herein, refers to polymers of nucleotides of any length and includes DNA and RNA. The nucleotides can be deoxyribonucleotides, ribonucleotides, modified nucleotides or bases, and/or their analogs, or any substrate that can be incorporated into a polymer by DNA or RNA polymerase or by a synthetic reaction. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and their analogs. “Oligonucleotide,” as used herein, refers to short, generally single-stranded, synthetic polynucleotides that are generally, but not necessarily, fewer than about 200 nucleotides in length. The terms “oligonucleotide” and “polynucleotide” are not mutually exclusive. The description above for polynucleotides is equally and fully applicable to oligonucleotides. A cell that produces a binding molecule of the present disclosure may include a parent hybridoma cell, as well as bacterial and eukaryotic host cells into which nucleic acids encoding the antibodies have been introduced. Unless specified otherwise, the left-hand end of any single-stranded polynucleotide sequence disclosed herein is the 5' end; the left-hand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA transcript that are 5' to the 5' end of the RNA transcript are referred to as “upstream sequences”; sequence regions on the DNA strand having the same sequence as the RNA transcript that are 3' to the 3' end of the RNA transcript are referred to as “downstream sequences.”

[00257] “Carriers” as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and

concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers, such as phosphate, citrate, and other organic acids; antioxidants, including ascorbic acid; low molecular weight (*e.g.*, fewer than about 10 amino acid residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers, such as polyvinylpyrrolidone; amino acids, such as glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates, including glucose, mannose, or dextrins; chelating agents, such as EDTA; sugar alcohols, such as mannitol or sorbitol; salt-forming counterions, such as sodium; and/or nonionic surfactants, such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™. The term “carrier” can also refer to a diluent, adjuvant (*e.g.*, Freund’s adjuvant (complete or incomplete)), excipient, or vehicle. Such carriers, including pharmaceutical carriers, can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable, or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. Water is an exemplary carrier when a composition (*e.g.*, a pharmaceutical composition) is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients (*e.g.*, pharmaceutical excipients) include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol, and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. Compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations, and the like. Oral compositions, including formulations, can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc.* Examples of suitable pharmaceutical carriers are described in Remington and Gennaro, Remington’s Pharmaceutical Sciences (18th ed. 1990). Compositions, including pharmaceutical compounds, may contain a binding molecule (*e.g.*, an antibody), for example, in isolated or purified form, together with a suitable amount of carriers.

[00258] The term “pharmaceutically acceptable” as used herein means being approved by a regulatory agency of the Federal or a state government, or listed in United States Pharmacopeia,

European Pharmacopeia, or other generally recognized Pharmacopeia for use in animals, and more particularly in humans.

[00259] The term “effective amount” as used herein refers to the amount of binding molecule (e.g., an antibody) or pharmaceutical composition provided herein which is sufficient to result in the desired outcome.

[00260] The terms “subject” and “patient” may be used interchangeably. As used herein, in certain embodiments, a subject is a mammal, such as a non-primate (e.g., cow, pig, horse, cat, dog, rat, *etc.*) or a primate (e.g., monkey and human). In specific embodiments, the subject is a human. In one embodiment, the subject is a mammal, e.g., a human, diagnosed with a condition or disorder. In another embodiment, the subject is a mammal, e.g., a human, at risk of developing a condition or disorder.

[00261] “Administer” or “administration” refers to the act of injecting or otherwise physically delivering a substance as it exists outside the body into a patient, such as by mucosal, intradermal, intravenous, intramuscular delivery, and/or any other method of physical delivery described herein or known in the art.

[00262] As used herein, the terms “treat,” “treatment” and “treating” refer to the reduction or amelioration of the progression, severity, and/or duration of a disease or condition resulting from the administration of one or more therapies.

[00263] The terms “about” and “approximately” mean within 20%, within 15%, within 10%, within 9%, within 8%, within 7%, within 6%, within 5%, within 4%, within 3%, within 2%, within 1%, or less of a given value or range.

[00264] As used in the present disclosure and claims, the singular forms “a”, “an” and “the” include plural forms unless the context clearly dictates otherwise.

[00265] It is understood that wherever embodiments are described herein with the term “comprising” otherwise analogous embodiments described in terms of “consisting of” and/or “consisting essentially of” are also provided. It is also understood that wherever embodiments are described herein with the phrase “consisting essentially of” otherwise analogous embodiments described in terms of “consisting of” are also provided.

[00266] The term “and/or” as used in a phrase such as “A and/or B” herein is intended to include both A and B; A or B; A (alone); and B (alone). Likewise, the term “and/or” as used in a phrase such as “A, B, and/or C” is intended to encompass each of the following embodiments: A,

B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

II. Binding Molecules

[00267] Provided herein are binding molecules (ALiCE) comprising multiple antigen binding domains (e.g., two antigen binding domains). In some embodiments, the multiple antigen binding domains of the binding molecules provided herein are useful for engaging cells, bringing a cell to an immune cell, or redirecting an immune cell.

[00268] In certain embodiments, the binding molecules provided herein comprises two antigen binding domains, wherein the first antigen binding domain comprises two antibody Fab regions and the second antigen binding domain comprises an antibody Fv region. Each of the two Fab regions contains two portions: a first portion having an antibody variable heavy (VH) region and an antibody CH1 region; and a second portion having a second portion comprising an antibody light chain (LC) comprising an antibody variable light (VL) region and an antibody light chain constant region (CL). Each of the two Fab regions binds to an antigen. The Fv region in the second antigen binding domain comprises a VH region and an antibody variable light (VL) region. The two Fab regions are linked to the Fv region.

[00269] Thus, in one aspect, the present disclosure provides a binding molecule comprising:

- (a) a first antigen binding domain comprising two antibody Fab regions, each comprising:
 - (i) a first portion comprising an antibody variable heavy (VH) region and an antibody CH1 region, wherein the first portion does not contain an antibody CH2 region and an antibody CH3 region; and
 - (ii) a second portion comprising an antibody light chain (LC) comprising an antibody variable light (VL) region and an antibody light chain constant region (CL),

wherein the two antibody Fab regions each bind to an antigen, and

- (b) a second antigen binding domain comprising an antibody Fv region comprising a VH region and an antibody variable light (VL) region,

wherein the second antigen binding domain binds to an antigen present on an immune cell; and wherein the first antigen binding domain and the second antigen binding domain are linked.

[00270] A Fab region (i.e., antigen-binding fragment) is an antibody region that binds to antigens. A conventional IgG usually comprises two Fab regions, each residing on one of the two arms of the Y-shaped IgG structure. Each Fab region is typically composed of one variable region and one constant region of each of the heavy and the light chain. More specifically, the variable region and the constant region of the heavy chain in a Fab region are VH and CH1 regions, and the variable region and the constant region of the light chain in a Fab region are VL and CL regions. The VH, CH1, VL, and CL in a Fab region can be arranged in various ways to confer an antigen binding capability according to the present disclosure. For example, VH and CH1 regions can be on one polypeptide, and VL and CL regions can be on a separate polypeptide, similarly to a Fab region of a conventional IgG. Alternatively, VH, CH1, VL and CL regions can all be on the same polypeptide and oriented in different orders as described in more detail below.

[00271] A Fv region is an antigen binding region that comprises a VH region and a VL region. The VH and VL regions in a Fv region can be arranged in various ways to confer an antigen binding capability according to the present disclosure. For example, VH and VL region can be on the same or separate polypeptides. If the VH and VL regions are on the same polypeptide, they can be oriented in different orders as described in more detail below.

[00272] As explained in Section I above, the term “variable region” refers to a portion of the light or heavy chains of an antibody that is generally located at the amino-terminal of the light or heavy chain and has a length of about 120 to 130 amino acids in the heavy chain and about 100 to 110 amino acids in the light chain, and are used in the binding and specificity of each particular antibody for its particular antigen. The variable region of the heavy chain may be referred to as “VH.” The variable region of the light chain may be referred to as “VL.”

[00273] The term “constant region” refers to a carboxy terminal portion of the light and heavy chain which is not directly involved in binding of the antibody to antigen but exhibits various effector function, such as interaction with the Fc receptor. The term refers to the portion of an immunoglobulin molecule having a more conserved amino acid sequence relative to the other portion of the immunoglobulin, the variable region, which contains the antigen binding site. The constant region can determine the mechanism used to destroy antigen. Antibodies are divided into five major classes, IgM, IgG, IgA, IgD, and IgE, based on their constant region structure and immune function. IgG is the class of immunoglobulin characterized by γ heavy

chains. It is the most abundant class of immunoglobulin found in the plasma. The constant region of a light chain is referred to as “CL.” The multiple heavy-chain C domains (CH domains) are numbered from the amino-terminal end to the carboxy terminus, for example CH1, CH2, CH3 and so on. Any CL and CH1 regions of these antibody classes can be used in the present disclosure. In a specific embodiment, CL and CH1 regions provided herein are of IgG type (e.g., IgG1). A representative CL region of the Fab region provided herein has the following amino acid sequence:

TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD
SKDSTYSLSSSTLTLSKADYEEKHKVYACEVTHQGLSSPVTKSFNRGEC (SEQ ID NO.: 59).

A representative CH1 region of the Fab region provided herein has the following amino acid sequence:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKV (SEQ ID NO.: 60)

[00274] The language that “the first portion does not contain an antibody CH2 region and an antibody CH3 region” is used herein to mean that the first portion does not contain a complete antibody CH2 region or a complete CH3 region. However, this language does not exclude the embodiments wherein a part of a CH2 region and/or a CH3 region is included in the first portion. In addition, in certain embodiments, CH2 and/or CH3 variants or truncations that do not exhibit full CH2 and/or CH3 activity (e.g., effector function) may be included. Assays such as Fc receptor binding assays or ADCC activity assays or other well-known assays for determining Fc region related functions may be used herein to determine if CH2 and/or CH3 activities (or Fc region activities) are fully retained.

[00275] In some embodiments, the first portion and the second portion of each Fab region are present on separate polypeptides. Each of the two Fab regions can also be optionally a single chain Fab region. Thus, in other embodiments, the first portion and the second portion of both Fab regions of the first antigen binding domain are on the same polypeptide. In other embodiments, the first portion and the second portion of one of the two Fab regions are on the same polypeptide. In those embodiments wherein a Fab region is a single chain Fab (i.e., the first portion and the second portion of the Fab region are on the same polypeptide), the Fab region can be oriented from N-terminus to C-terminus in the following order: VH-CH1-VL-CL.

Alternatively, a single chain Fab region can be oriented from N-terminus to C-terminus in the following order: VL-CL-VH-CH1.

[00276] Similarly, in certain embodiments, the VH region and the VL region of the Fv region are on separate polypeptides. In other embodiments, the Fv region of the second antigen binding domain is a single chain Fv (i.e., the VH region and the VL region of the Fv region are on the same polypeptide). In such single chain Fv embodiments, the Fv region can be oriented from N-terminus to C-terminus in the following order: VH-VL, or can be oriented from N-terminus to C-terminus in the following order: VL-VH.

[00277] In some specific embodiments, the two portions of each Fab region are on separate polypeptides and the VH and VL regions of the Fv region are also on separate polypeptides.

[00278] In some embodiments, the first antigen binding domain and the second antigen binding domain are linked by a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some specific embodiments, the antibody hinge region is an IgG hinge region. The IgG hinge region provided herein may be selected from antibody hinge regions of various IgG subtypes. Table 2 below lists exemplary IgG subtypes with core hinge sequences that may be included in the flexible peptide region provided herein.

Table 2: Exemplary IgG subtypes

IgG subtype	Core hinge sequence	SEQ ID NO:
IgG1	EPKSCDKTHTCPPCP	55
IgG2	ERKCCVECPPCP	56
IgG3	ELKTPLDITHTCPRCP (EPKSCDTTPPCPRCP) ₃	57
IgG4	ESKYGPPCPSCP	58

[00279] Thus, in some more specific embodiments, the IgG hinge region is of IgG1 subtype. In other more specific embodiments, the IgG hinge region is of IgG2 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG3 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG4 subtype. In some specific embodiments, the

flexible peptide region provided herein comprises an amino acid sequence of SEQ ID NO: 55. In some specific embodiments, the flexible peptide region provided herein comprises an amino acid sequence of SEQ ID NO: 56. In other specific embodiments, the flexible peptide region provided herein comprises an amino acid sequence of SEQ ID NO: 57. In some specific embodiments, the flexible peptide region provided herein comprises an amino acid sequence of SEQ ID NO: 58.

[00280] In certain embodiments, the flexible peptide region comprise additional amino acids. For example, in some embodiments, the flexible peptide region further comprises a linker (*e.g.*, G4S) between the antibody hinge region and the second Fv antigen binding domain. Flexible linker between antibody hinge region and second Fv domain may influence binding affinity of the second Fv domain. Improved binding affinity of second Fv domain can lead to increased redirection efficiency of immune cells (*e.g.*, effector cells including T cells) to target cells (*e.g.*, cancer cells). The second Fv domain need to bend to be able to interact with and bind to a surface antigen presented on immune cells (*e.g.*, effector cells including T cells), because the paratope of the second Fv domain of ALiCE is structurally masked by the first Fab domain of ALiCE. Therefore, to reduce steric hindrance and optimize the binding of the second Fv domain to immune cells (*e.g.*, effector cells including T cells), flexible linker such as G4S can be introduced between the antibody hinge region and the second Fv domain. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S). In some embodiments, the linker comprises an amino acid sequence of (G4S)*n* where *n* is an integer. In some specific embodiments, the linker comprises an amino acid sequence of (G4S)₁. In some more specific embodiments, the linker comprises an amino acid sequence of (G4S)₂. In other more specific embodiments, the linker comprises an amino acid sequence of (G4S)₃. In yet other more specific embodiments, the linker comprises an amino acid sequence of (G4S)₄. Other methods for designing and constructing linkers with different flexibilities are described in more detail in, *e.g.*, Klein et al., Protein Engineering, Design & Selection, 2014, 27(10): 325-330, and DiGiammarino et al., Landes Bioscience, 2011, 3(5): 487-494, each of which is incorporated herein by reference in its entirety.

[00281] The binding molecules provided herein can optionally comprise a CH3 domain. FIG. 1B illustrates such an exemplary binding molecule. In some embodiments, the second antigen binding domain further comprise a first CH3 region linked to the VH region of the Fv region and

a second CH3 region linked to the VL region of the Fv region. In some embodiments, the CH regions are linked to the C-terminus of the VH and VL regions of the Fv region. The presence of the CH3 regions provides the Fc receptor binding capability of the binding molecules provided herein. In some embodiments, the CH3 regions linked to the Fv region are engineered to facilitate or enforce the association between the two CH3 regions using existing technologies such as knobs-into-holes (KiH) technology or electrostatic steering. For example, knobs-into-holes was originally proposed as a model for the packing of amino acid side chains between adjacent α -helices, and was later demonstrated to be an effective design strategy for engineering antibody heavy chain homodimers for heterodimerization. Briefly, in certain embodiments of this approach, a 'knob' variant can be first obtained by replacement of a small amino acid with a larger one in one IgG CH3 domain (e.g., T to Y substitution). The knob was designed to insert into a 'hole' in another IgG CH3 domain created by replacement of a large residue with a smaller one (e.g., Y to T substitution). The knob-into-holes technology is described in detail with several examples in e.g., WO 96/027011, Ridgway et al., *Protein Eng* 9 (1996) 617-621, and Merchant et al., *Nat Biotechnol* 16 (1998) 677-681, each of which is incorporated herein by reference in its entirety. Other well-known technologies for modifying CH3 regions to facilitate or enforce the association between the two CH3 regions are also contemplated in the present disclosure.

[00282] Albumin (e.g., human serum albumin) has been used to increase the serum half-life of biological drugs. See Dennis *et al.*, *The Journal of Biological Chemistry*, 2002, 277 (38): 35035-35043; Adams *et al.*, *MABS*, 2016, 8(7): 1336-1346. For example, human serum albumin (HSA) has been utilized. HSA is the most abundant protein in blood, and is widely distributed in tissues and has a non-acute function. It has a half life of 19 days. Therefore, in some embodiments, albumin (e.g., HSA) can be used herein to increase half-life of the binding molecules provided herein. Albumin can be used in a few ways. One exemplary approach is to directly couple an albumin domain (e.g., HSA) to the binding molecule provided herein, either genetically or chemically. Another exemplary approach is to use an albumin binding domain or site (ABD or ABS).

[00283] Thus, the binding molecules provided herein may also optionally include one or more albumin binding domain or albumin binding site (ABD or ABS). FIG. 1C illustrates such exemplary binding molecules. In some embodiments, the ABS of the binding molecules

provided herein mediates binding with endogenous albumin, thereby helping prolong the half-life and/or enhancing the therapeutic effects of the binding molecules provided herein. In some embodiments, the ABS of the binding molecules provided herein may also help improve the pharmacokinetics, through the non-covalent association to albumin. In some embodiments, the ABS is linked to the C-terminus of the VH region of the Fv region. In other embodiments, the ABS is linked to the C-terminus of the VL region of the Fv region. In yet other embodiments, the C-terminus of each of the VL and VH regions of the Fv region is linked to ABS. In other embodiments, the ABS is linked to the CL region of at least one of the Fab regions.

[00284] In certain embodiments, the binding molecule can further optionally comprises one or more albumin domain (e.g., HSA). In some embodiments, an albumin domain is linked to the C-terminus of the VH region of the Fv region. In other embodiments, an albumin domain is linked to the C-terminus of the VL region of the Fv region. In yet other embodiments, the C-terminus of each of the VL and VH regions of the Fv region is linked to an albumin domain. In other embodiments, an albumin domain is linked to the CL region of at least one of the Fab regions.

[00285] The two Fab regions and Fv region of the binding molecules provided herein can each bind to an antigen. In some embodiments, the two Fab regions bind to different antigens.

[00286] In other embodiments, the two Fab regions bind to the same antigen. In some embodiments, the two Fab regions bind to the same epitope of the same antigen. In other embodiments, the two Fab regions bind to different epitopes of the same antigen.

[00287] When two Fab regions bind to the same antigen—a first antigen, the first antigen can be the same or different from the antigen (a second antigen) bound by the Fv region. Thus, in some embodiments, the first antigen binding domain and the second antigen binding domain bind to the same antigen. In some embodiments, the second antigen binding domain binds to the same epitope as at least one of the epitopes bound by the first antigen binding domain.

[00288] In other embodiments, the first antigen binding domain and the second antigen binding domain bind to different antigens, and wherein the first antigen binding domain binds to a first antigen and the second antigen binding domain binds to a second antigen. FIG. 1E provides an illustration of such an ALiCE molecule wherein the first antigen binding domain (the two Fab regions) binds to a cancer antigen, and the second antigen binding domain binds to an immune cell such as a T cell through an antigen like CD3. Such ALiCE molecules can engage an immune cell (e.g., T cell) to a cancer cell and thus be used as a therapeutic for cancer treatment.

[00289] Thus, in some embodiments, the binding molecules provided herein are bispecific binding molecules which comprises (a) two Fab regions (in the first antigen binding domain) that provides the binding affinity to the first antigen and (b) a Fv region (in the second antigen binding domain) that provides the binding affinity to the second antigen. The first antigen binding domain can bind to an extracellular domain of a surface protein on one cell, and the second antigen binding domain can bind to an extracellular domain of a surface protein on an immune cell, and thereby bring the two cells together.

[00290] The first antigen binding domain (with the two Fab regions) can bind to a cancer cell. It can also bind to a non-cancer cell. Thus, in some embodiments, the first antigen is a cancer antigen (e.g., PD-L1). In other embodiments, the first antigen is not a cancer antigen.

[00291] In some embodiments, the second antigen is expressed on an immune cells including lymphocytes and monocytes. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a B cell. In other embodiments, the second antigen is expressed on a dendritic cell. In other embodiments, the second antigen is expressed on a granulocyte. In yet other embodiments, the second antigen is expressed on an innate lymphoid cell. In yet other embodiments, the second antigen is expressed on a megakaryocyte. In yet other embodiments, the second antigen is expressed on a monocyte. In yet other embodiments, the second antigen is expressed on a myeloid-derived suppressor cell. In yet other embodiments, the second antigen is expressed on a NK cell.

[00292] In some embodiments, the second antigen is expressed on an effector cell. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a T helper cell, a regulatory T cell, or a cytotoxic T cell. In some embodiments, the second antigen is expressed on a T helper cell. In some embodiments, the second antigen is expressed on a regulatory T cell. In some embodiments, the second antigen is expressed on a cytotoxic T cell. In some embodiments, the second antigen is expressed on a CD8+ T cell. In some embodiments, the second antigen is expressed on a CD4+ T cell. In some embodiments, the second antigen comprises an extracellular domain.

[00293] In some specific embodiments, the second antigen is CD3. In some embodiments, the first antigen is a cancer antigen and the second antigen is CD3.

[00294] In some more specific embodiments, the first antigen is PD-L1 and the second antigen is CD3.

[00295] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00296] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00297] In other more specific embodiments, the first antigen is CD20 and the second antigen is CD3.

[00298] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, SEQ ID NO.: 29; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00299] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00300] In other more specific embodiments, the first antigen is EGFR and the second antigen is CD3.

[00301] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43; the VL region of the second portion of each Fab region comprises three CDRs having

amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15 and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00302] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00303] In other more specific embodiments, the first antigen is Her2 and the second antigen is TNF alpha. In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 51; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 52; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 53; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 54.

[00304] In some specific embodiment, the first antigen binding domain is at the N-terminus and maintains the native antibody structure at the N-terminus, while the second antigen binding domain is at the C-terminus and C-terminal CH2 and CH3 domains of both heavy chains are each substituted with a single VH and VL domain, respectively. FIG. 1A is an illustration of such an exemplary ALiCE molecule. More specifically, the first portion and the second portion of each Fab region are on separate polypeptides, the first antigen binding domain binds to a first antigen, the VH region and the VL region of the Fv region are on separate polypeptides, the second antigen binding domain binds to a second antigen present on an immune cell, and the first antigen and the second antigen are different antigens.

[00305] Thus, in one specific embodiment, the binding molecule provided herein comprises:

- (a) a first antigen binding domain comprising two antibody Fab regions, each comprising:
 - (i) a first portion comprising an antibody variable heavy (VH) region and an antibody CH1 region, wherein the first portion does not contain an antibody CH2 region and an antibody CH3 region; and

- (ii) a second portion comprising an antibody light chain (LC) comprising an antibody variable light (VL) region and an antibody light chain constant region (CL),

wherein the first portion and the second portion are on separate polypeptides; and

wherein the first antigen binding domain binds to a first antigen.

- (b) a second antigen binding domain comprising an antibody Fv region comprising a VH region and an antibody variable light (VL) region, wherein the VH region and the VL region are on separate polypeptides; wherein the second antigen binding domain binds to a second antigen present on an immune cell,

wherein the first antigen and the second antigen are different antigens.

[00306] In some embodiments, the first portion of one Fab region and the VH region of the Fv region are on the same polypeptide, and the portion of the other Fab region and the VL region of the Fv region are on the same polypeptide. Thus, in some specific embodiments, the binding molecule provided herein comprises:

- (a) a first antigen binding domain comprising a first antibody Fab region and a second antibody Fab region, each comprising:

- (i) a first portion comprising an antibody variable heavy (VH) region and an antibody CH1 region, wherein the first portion does not contain an antibody CH2 region and an antibody CH3 region; and
- (ii) a second portion comprising an antibody light chain (LC) comprising an antibody variable light (VL) region and an antibody light chain constant region (CL),

wherein the first antigen binding domain binds to a first antigen.

- (b) a second antigen binding domain comprising an antibody Fv region comprising a VH region and an antibody variable light (VL) region, wherein the second antigen binding domain binds to a second antigen present on an immune cell,

wherein the first antigen and the second antigen are different antigens; and

wherein the first portion of the first Fab region and the VH region of the Fv region are on the same polypeptide; and the first portion of the second Fab region and the VL region of the Fv region are on the same polypeptide.

[00307] In some embodiments, the first antigen is a cancer antigen (e.g., PD-L1). In other embodiments, the first antigen is not a cancer antigen.

[00308] In some embodiments, the second antigen is expressed on an immune cells including lymphocytes and monocytes. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a B cell. In other embodiments, the second antigen is expressed on a dendritic cell. In other embodiments, the second antigen is expressed on a granulocyte. In yet other embodiments, the second antigen is expressed on an innate lymphoid cell. In yet other embodiments, the second antigen is expressed on a megakaryocyte. In yet other embodiments, the second antigen is expressed on a monocyte. In yet other embodiments, the second antigen is expressed on a myeloid-derived suppressor cell. In yet other embodiments, the second antigen is expressed on a NK cell.

[00309] In some embodiments, the second antigen is expressed on an effector cell. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a T helper cell, a regulatory T cell, or a cytotoxic T cell. In some embodiments, the second antigen is expressed on a T helper cell. In some embodiments, the second antigen is expressed on a regulatory T cell. In some embodiments, the second antigen is expressed on a cytotoxic T cell. In some embodiments, the second antigen is expressed on a CD8+ T cell. In some embodiments, the second antigen is expressed on a CD4+ T cell. In some embodiments, the second antigen comprises an extracellular domain.

[00310] In some specific embodiments, the second antigen is CD3. In some embodiments, the first antigen is a cancer antigen and the second antigen is CD3.

[00311] In some more specific embodiments, the first antigen is PD-L1 and the second antigen is CD3.

[00312] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00313] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00314] In other more specific embodiments, the first antigen is CD20 and the second antigen is CD3.

[00315] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, SEQ ID NO.: 29; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00316] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00317] In other more specific embodiments, the first antigen is EGFR and the second antigen is CD3.

[00318] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15 and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00319] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44; the VH region of the Fv region has an amino

acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00320] In other more specific embodiments, the first antigen is Her2 and the second antigen is TNF alpha. In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 51; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 52; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 53; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 54.

[00321] In another aspect, the binding molecules provided herein include four peptides (two antibody light chains and two heavy chain like chains), and their overall structure is similar to a traditional IgG except that the Fc region of the IgG is replaced with a Fv region. This structure can be further modified to generate variations that confer various properties. More specifically, in some embodiments, the binding molecule provided herein comprises:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
 - (b) a third polypeptide comprising a first VH region and a first CH1 region, and a second VH region; and
 - (c) a fourth polypeptide comprising a third VH region and a second CH1, and a VL region,
- wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;
- wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen Fab region; and
- wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region.

[00322] In some embodiments, the first polypeptide and the second polypeptide have the same amino acid sequence. In these embodiments, the binding molecules provided herein comprise two identical light chains (the first and second polypeptides) and two different heavy chain like chains (the third and fourth polypeptides).

[00323] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the flexible peptide region comprises an antibody hinge region. In some specific embodiments, the antibody is an IgG hinge region. In some more specific embodiments, the IgG hinge region is of IgG1 subtype. In

other more specific embodiments, the IgG hinge region is of IgG2 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG3 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG4 subtype. In some specific embodiments, the flexible peptide region provided herein comprises an amino acid sequence of SEQ ID NO: 55. In some specific embodiments, the flexible peptide region provided herein comprises an amino acid sequence of SEQ ID NO: 56. In other specific embodiments, the flexible peptide region provided herein comprises an amino acid sequence of SEQ ID NO: 57. In some specific embodiments, the flexible peptide region provided herein comprises an amino acid sequence of SEQ ID NO: 58.

[00324] In certain embodiments, the flexible peptide region comprise additional amino acids. For example, in some embodiments, the flexible peptide region further comprises a linker (*e.g.*, G4S) between the antibody hinge region and the second Fv antigen binding domain. Flexible linker between antibody hinge region and second Fv domain may influence binding affinity of the second Fv domain. Improved binding affinity of second Fv domain can lead to increased redirection efficiency of immune cells (*e.g.*, effector cells including T cells) to target cells (*e.g.*, cancer cells). The second Fv domain need to bend to be able to interact with and bind to a surface antigen presented on immune cells (*e.g.*, effector cells including T cells), because the paratope of the second Fv domain of ALiCE is structurally masked by the first Fab domain of ALiCE. Therefore, to reduce steric hindrance and optimize the binding of the second Fv domain to immune cells (*e.g.*, effector cells including T cells), flexible linker such as G4S can be introduced between the antibody hinge region and the second Fv domain. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S). In some embodiments, the linker comprises an amino acid sequence of (G4S)_n where n is an integer. In some specific embodiments, the linker comprises an amino acid sequence of (G4S)₁. In some more specific embodiments, the linker comprises an amino acid sequence of (G4S)₂. In other more specific embodiments, the linker comprises an amino acid sequence of (G4S)₃. In yet other more specific embodiments, the linker comprises an amino acid sequence of (G4S)₄.

[00325] In some embodiments, the first Fab region and the second Fab region bind to different antigens. In other embodiments, the first Fab region and the second Fab region bind to the same antigen. In some embodiments, the first Fab region and the second Fab region bind to the same

epitope of the same antigen. In other embodiments, the first Fab region and the second Fab region bind to different epitopes of the same antigen.

[00326] In certain embodiments, the first Fab region and the second Fab region form a first antigen binding domain, and the Fv region forms a second antigen binding domain.

[00327] In some embodiments, the first antigen binding domain and the second antigen binding domain bind to the same antigen. In some embodiments, the second antigen binding domain binds to the same epitope as at least one of the epitopes bound by the first antigen binding domain.

[00328] In other embodiments, the first antigen binding domain and the second antigen binding domain bind to different antigens, and wherein the first antigen binding domain binds to a first antigen and the second antigen binding domain binds to a second antigen.

[00329] In some embodiments, the first antigen is a cancer antigen. In other embodiments, the first antigen is not a cancer antigen.

[00330] In some embodiments, the second antigen is expressed on an immune cells including lymphocytes and monocytes. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a B cell. In other embodiments, the second antigen is expressed on a dendritic cell. In other embodiments, the second antigen is expressed on a granulocyte. In yet other embodiments, the second antigen is expressed on an innate lymphoid cell. In yet other embodiments, the second antigen is expressed on a megakaryocyte. In yet other embodiments, the second antigen is expressed on a monocyte. In yet other embodiments, the second antigen is expressed on a myeloid-derived suppressor cell. In yet other embodiments, the second antigen is expressed on a NK cell.

[00331] In some embodiments, the second antigen is expressed on an effector cell. In some embodiments, the second antigen is expressed on a T cell. In some embodiments, the second antigen is expressed on a T helper cell, a regulatory T cell, or a cytotoxic T cell. In some embodiments, the second antigen is expressed on a T helper cell. In some embodiments, the second antigen is expressed on a regulatory T cell. In some embodiments, the second antigen is expressed on a cytotoxic T cell. In some embodiments, the second antigen is expressed on a CD8⁺ T cell. In some embodiments, the second antigen is expressed on a CD4⁺ T cell. In some embodiments, the second antigen comprises an extracellular domain.

[00332] In some specific embodiments, the second antigen is CD3. In some embodiments, the first antigen is a cancer antigen and the second antigen is CD3.

[00333] In some more specific embodiments, the first antigen is PD-L1 and the second antigen is CD3.

[00334] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 16, SEQ ID NO.: 17, and SEQ ID NO.: 19.

[00335] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00336] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 3; the third polypeptide has the amino acid sequence of SEQ ID NO.: 1; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 2.

[00337] In other more specific embodiments, the first antigen is CD20 and the second antigen is CD3.

[00338] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00339] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30; the VH region of the Fv region has an amino

acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00340] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.

[00341] In other more specific embodiments, the first antigen is EGFR and the second antigen is CD3.

[00342] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, SEQ ID NO.: 43; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00343] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00344] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.

[00345] In other more specific embodiments, the first antigen is Her2 and the second antigen is TNF alpha. In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 51; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 52; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 53; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 54.

[00346] As described above, in certain embodiments, the binding molecules provided herein comprise two identical light chains and two different heavy chain like chains. To make recombinant protein in mammalian cells like CHO or HEK293, understanding antibody

assembly and quality control system happened in ER is very important. Antibody is assembled and secreted as a tetramer H_2L_2 and the quality control machinery is very tightly regulated in ER by BiP and PDI. It was known that unfolded CH1 domain of heavy chain has a role of regulation of antibody assembly in BiP dependent manner. As described below in the Example section, the present disclosure demonstrates that antibody VH domain also has a role of antibody assembly, and shows that the heavy chain like chain of the binding molecule provided herein that contains two VH regions (one in a Fab region and one in a Fv region) contributes to the proper assembly of the binding molecule.

[00347] C-terminal Fv also has an important role of heterodimerization of two different heavy chain like chains (the third and fourth polypeptides). Because the interaction between VH and VL regions is much stronger than VL-VL interaction, the VH-VL interaction was selected to make heterodimerization between the two different heavy chain like chains (the third and fourth polypeptides). The efficiency of heterodimerization was found very high and most of the binding molecules expressed and purified in mammalian cells were heterodimerized form (close to 99% heterodimerization efficiency).

[00348] In addition, this structure provides for the optimal synaptic distance between target and effector cells. The distance of N-terminal two Fab region and the C-terminal Fv region was estimated to be 40 Å. Furthermore, the binding molecule provided herein has more folding complexity (molecular size) than other known bispecific antibodies such as BiTE, DART and other ScFv based bispecific antibody formats and thus are expected to have improved thermodynamic stability.

[00349] Furthermore, in certain embodiments, the binding molecules provided herein are bispecific binding molecules, in which the two Fab regions (N-terminal $F(ab')_2$) bind to the first antigen (e.g., a cancer antigen) and the Fv region binds to an immune cell (e.g., T cell). In certain embodiments, the binding molecules provided herein are designed and constructed in Y-shape to provide synergistic effect of antibody function and immune redirecting (e.g., T cell redirecting), for example, for the maximum anti-tumor activity. The configuration of ALiCE molecules (predominantly reside in Y-shape) is designed to confer optimal immunological synaptic distance between the two antigen binding domains (two target paratopes) and maximize functional redirection of a cell (e.g., T cell) to other cells (e.g., tumor cells). In addition, high affinity and bivalent N-terminal (two Fab regions) is provided and, at the same time, unwanted

target independent T cell activation is reduced due to the monovalent and low affinity of the Fv region to an immune cell antigen.

[00350] It has been reported that a binding molecule can have different configurations, which may affect the distance between the domains in the binding molecule (Zhang X. et al. 3D Structural Fluctuation of IgG1 Antibody Revealed by Individual Particle Electron Tomography. *Scientific Reports* 5, Article number: 9803 (2015); Klein J. S. et al. Examination of the contributions of size and avidity to the neutralization mechanisms of the anti-HIV antibodies b12 and 4E10. *Proc Natl Acad Sci U S A*. 2009 May 5;106(18):7385-90; both references incorporated by reference in their entirety). In certain embodiments, ALiCE molecules may have different configurations, for example ALiCE molecules may reside in a Y-shape, or in a T-shape. In certain embodiments, the different configurations of ALiCE molecules may contribute to different distance between N-terminal two Fab region and the C-terminal Fv region in the ALiCE molecules. In certain embodiments, the distance between N-terminal two Fab region and the C-terminal Fv region in the binding molecules provided herein may be estimated to be in a range of between around 40 Å and around 70 Å. In certain embodiments, the distance between N-terminal two Fab region and the C-terminal Fv region in the binding molecules provided herein may be estimated to be around 42 Å. In some other embodiments, the distance between N-terminal two Fab region and the C-terminal Fv region in the binding molecules provided herein may be estimated to be around 60 Å.

[00351] Thus, in the various bispecific binding molecules provided herein, the binding affinity of the first antigen binding domain to the first antigen is higher than the binding affinity of the second antigen binding domain to the second antigen. For example, as shown in Example 3 below, the binding kinetics of ACE-05 to human PD-L1 was comparable to the parental anti-PD-L1 antibody (i.e., YBL-007 from Y-Biologics, Inc.) (see FIGs. 12A-12C). In contrast, the binding affinity of ACE-05 to CD3 was much lower than the parental anti-CD3 antibody (UCHT1 from BioLegend, USA) (see FIGs. 12A-12C).

[00352] Generally speaking, antigen-antibody interactions are non-covalent and reversible, formed by a combination of hydrogen bonds, hydrophobic interactions, electrostatic and van der Waals forces. When describing the strength of an antigen-antibody complex, affinity and/or avidity are usually mentioned. As mentioned above, the binding of an antibody to its antigen is a reversible process, and the affinity of the binding is typically reported as an equilibrium

dissociation constant (K_D). K_D is the ratio of an antibody dissociation rate (k_{off} or k_d) (how quickly it dissociates from its antigen) to the antibody association rate (k_{on} or k_a) (how quickly it binds to its antigen). In some embodiments, K_D values are determined by measuring the k_{on} and k_{off} rates of a specific antibody/antigen interaction and then using a ratio of these values to calculate the K_D value. K_D values may be used to evaluate and rank order the strength of individual antibody/antigen interactions. The lower the K_D of an antibody, the higher the affinity of the antibody for its target. Avidity gives a measure of the overall strength of an antibody-antigen complex. It is dependent on three major parameters: (i) affinity of the antibody for the epitope, (ii) valency of both the antibody and antigen, and (iii) structural arrangement of the parts that interact.

[00353] In certain embodiments, the binding molecule provided herein binds one or more targets, antigens, or epitopes with a dissociation constant (K_D) of about 1 μM or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, about 1 nM or less, about 0.1 nM or less, 50 pM or less, 10 pM or less, or 1 pM or less. In some embodiments, the binding molecule provided herein binds a target, antigen, or epitope with a K_D of about 20 nM or less. In some embodiments, the binding molecule binds a target, antigen, or epitope with a K_D of about 10 nM or less. In some embodiments, the binding molecule binds a target, antigen, or epitope with a K_D of about 1 nM or less. In some embodiments, the binding molecule binds a target, antigen, or epitope with a K_D of about 0.5 nM or less. In some embodiments, the binding molecule provided herein binds a target, antigen, or epitope with a K_D of about 0.1 nM or less. In some embodiments, the binding molecule provided herein binds a target, antigen, or epitope with a K_D of about 50 pM or less. In some embodiments, the binding molecule provided herein binds a target, antigen, or epitope with a K_D of about 25 pM or less. In some embodiments, the binding molecule provided herein binds a target, antigen, or epitope with a K_D of about 10 pM or less. In some embodiments, the binding molecule provided herein binds a target, antigen, or epitope with a K_D of about 1 pM or less. In some embodiments, the dissociation constant of a binding molecule provided herein to a target or an antigen is the dissociation constant determined using a fusion protein comprising at least a portion of the target protein immobilized on an Octet[®] chip. In some embodiments, the dissociation constant of a binding molecule provided herein to a target or an antigen is the dissociation constant determined using the binding agent captured by an anti-human IgG antibody on an Octet[®] chip and a soluble target protein.

[00354] In certain embodiments, the binding molecule provided herein binds a target, antigen, or epitope with a half maximal effective concentration (EC_{50}) of about 1 μ M or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, about 1 nM or less, or about 0.1 nM or less. In certain embodiments, a binding molecule provided herein binds a target, antigen, or epitope with an EC_{50} of about 1 μ M or less, about 100 nM or less, about 40 nM or less, about 20 nM or less, about 10 nM or less, about 1 nM or less, or about 0.1 nM or less.

[00355] In certain embodiments, the K_D for the binding molecule to the first antigen is about 2 fold, 3 fold, 4 fold, 5 fold, 6 fold, 7 fold, 8 fold, 9 fold, 10 fold, 15 fold, 20 fold, 50 fold or more of the K_D for the binding molecule to the second antigen. In some embodiments, the K_D for the binding molecule to the first antigen is about 10, 10^2 , 10^3 , or 10^4 fold of the K_D for the binding molecule to the second antigen.

[00356] In certain embodiments, the binding molecule provided herein (*e.g.*, a bispecific binding molecule) comprises at least a portion of one or more “parental” antibodies. In some embodiments, a parental antibody is a recombinant antibody. In some embodiments, a parental antibody is a monoclonal antibody. In some embodiments, the parental antibody is a polyclonal antibody. In some embodiments, a parental antibody is a chimeric antibody. In some embodiments, a parental antibody is a humanized antibody. In some embodiments, a parental antibody is a human antibody or fully human antibody. In some embodiments, a parental antibody is an IgA, IgD, IgE, IgG, or IgM antibody. In certain embodiments, a parental antibody is an IgG1 antibody. In certain embodiments, a parental antibody is an IgG2 antibody. In some embodiments, the parental antibody is an IgG3 antibody. In some embodiments, a parental antibody is an IgG4 antibody.

[00357] In some embodiments, the binding molecule provided herein (*e.g.*, a bispecific binding molecule) is isolated. In some embodiments, the binding molecule provided herein (*e.g.*, a bispecific binding molecule) is substantially pure.

[00358] In some embodiments, the binding molecule provided herein (*e.g.*, a bispecific binding molecule) or a portion thereof is derived from at least one monoclonal antibody. In some embodiments, a monoclonal antibody is prepared using hybridoma methods known to one of skill in the art. For example, using the hybridoma method, a mouse, rat, rabbit, hamster, or other appropriate host animal, is immunized as described above to elicit the production of antibodies that specifically bind the immunizing antigen. In some embodiments, lymphocytes are

immunized *in vitro*. In some embodiments, the immunizing antigen is a human protein or a fragment thereof. In some embodiments, the immunizing antigen is a mouse protein or a fragment thereof.

[00359] Following immunization, lymphocytes are isolated and fused with a suitable myeloma cell line using, for example, polyethylene glycol. The hybridoma cells are selected using specialized media as known in the art and unfused lymphocytes and myeloma cells do not survive the selection process. Hybridomas that produce monoclonal antibodies directed specifically against a chosen antigen can be identified by a variety of methods including, but not limited to, immunoprecipitation, immunoblotting, and *in vitro* binding assays (*e.g.*, flow cytometry, FACS, ELISA, and radioimmunoassay). Once hybridoma cells that produce antibodies of the desired specificity, affinity, and/or activity are identified, the clones may be subcloned by limiting dilution techniques. The hybridomas can be propagated either in *in vitro* culture using standard methods or *in vivo* as ascites tumors in an animal. The monoclonal antibodies can be purified from the culture medium or ascites fluid according to standard methods in the art including, but not limited to, affinity chromatography, ion-exchange chromatography, gel electrophoresis, and dialysis.

[00360] In certain embodiments, monoclonal antibodies can be made using recombinant DNA techniques as known to one skilled in the art. For example, in certain examples, polynucleotides encoding a monoclonal antibody are isolated from mature B-cells or hybridoma cells, such as by RT-PCR using oligonucleotide primers that specifically amplify the genes encoding the heavy and light chains of the antibody, and their sequence is determined using standard techniques. The isolated polynucleotides encoding the heavy and light chains are then cloned into suitable expression vectors which produce the monoclonal antibodies when transfected into host cells such as *E. coli*, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin proteins.

[00361] In certain other embodiments, recombinant monoclonal antibodies, or fragments thereof, can be isolated from phage display libraries expressing variable domains or CDRs of a desired species. Screening of phage libraries can be accomplished by various techniques known in the art.

[00362] In some embodiments, a monoclonal antibody is modified, for example, by using recombinant DNA technology to generate alternative antibodies. In some embodiments, the

constant domains of the light chain and heavy chain of, for example, a mouse monoclonal antibody can be substituted for constant regions of, for example, a human antibody to generate a chimeric antibody, or for a non-immunoglobulin polypeptide to generate a fusion antibody. In some embodiments, the constant regions are truncated or removed to generate a desired antibody fragment of a monoclonal antibody. In some embodiments, site-directed or high-density mutagenesis of the variable region(s) is used to optimize specificity and/or affinity of a monoclonal antibody.

[00363] In some embodiments, the binding molecule provided herein (e.g., a bispecific binding molecule) or a portion thereof is derived from a humanized antibody. Various methods for generating humanized antibodies are known in the art. In some embodiments, humanization is performed by substituting one or more non-human CDR sequences for the corresponding CDR sequences of a human antibody. In some embodiments, humanized antibodies are generated by substituting all six CDRs of a parent non-human antibody (e.g., rodent) for the corresponding CDR sequences of a human antibody.

[00364] The choice of which human heavy chain variable region and light chain variable region to be used in generating humanized antibodies can be made based on a variety of factors and by a variety of methods. In some embodiments, the “best-fit” method is used where the sequence of the variable region of a non-human (e.g., rodent) antibody is screened against the entire library of known human variable region sequences. The human sequence that is most similar to that of the non-human sequence is selected as the human variable region backbone for the humanized antibody. In some embodiments, a method is used wherein a particular variable region backbone derived from a consensus sequence of all human antibodies of a particular subgroup of light or heavy chains is selected. In some embodiments, the framework is derived from the consensus sequences of the most abundant human subclasses. In some embodiments, human germline genes are used as the source of the variable region framework sequences.

[00365] Other methods for humanization include, but are not limited to, a method called “superhumanization” which is described as the direct transfer of CDRs to a human germline framework, a method called Human String Content (HSC) which is based on a metric of antibody “humanness”, methods based on generation of large libraries of humanized variants (including phage, ribosomal, and yeast display libraries), and methods based on framework region shuffling.

[00366] In certain embodiments, the binding molecule provided herein (e.g., a bispecific binding molecule) or a portion thereof is derived from a human antibody. Human antibodies can be directly prepared using various techniques known in the art. In some embodiments, human antibodies are generated from immortalized human B lymphocytes immunized *in vitro*. In some embodiments, human antibodies are generated from lymphocytes isolated from an immunized individual. In any case, cells that produce an antibody directed against a target antigen can be generated and isolated. In some embodiments, a human antibody is selected from a phage library, where that phage library expresses human antibodies. Alternatively, phage display technology may be used to produce human antibodies and antibody fragments *in vitro*, from immunoglobulin variable region gene repertoires from unimmunized donors. Techniques for the generation and use of antibody phage libraries are well known in the art. Once antibodies are identified, affinity maturation strategies known in the art, including but not limited to, chain shuffling and site-directed mutagenesis, may be employed to generate higher affinity human antibodies.

[00367] In some embodiments, human antibodies are produced in transgenic mice that contain human immunoglobulin loci. Upon immunization, these mice are capable of producing the full repertoire of human antibodies in the absence of endogenous immunoglobulin production.

[00368] In some embodiments, the binding molecule provided herein (e.g., a bispecific binding molecule) or a portion thereof described herein are derived from antibodies (e.g., full-length antibodies or fragments thereof) that comprise modifications in at least one or more of the constant regions. In some embodiments, the antibodies comprise modifications to one or more of the three heavy chain constant regions (e.g., CH1) and/or to the light chain constant region (CL). In some embodiments, the heavy chain constant region of the modified antibodies comprises at least one human constant region. In some embodiments, the heavy chain constant region of the modified antibodies comprises more than one human constant region. In some embodiments, modifications to the constant region comprise additions, deletions, or substitutions of one or more amino acids in one or more regions. In some embodiments, one or more regions are partially or entirely deleted from the constant regions of the modified antibodies. In some embodiments, the entire CH2 domain has been removed from an antibody (Δ CH2 constructs). In some embodiments, the entire CH3 domain has been removed from an antibody (Δ CH3 constructs). In some embodiments, an omitted constant region is replaced by a short amino acid

spacer (*e.g.*, 10 amino acid residues) that provides some of the molecular flexibility typically imparted by the absent constant region.

[00369] Modifications to the constant region of antibodies (*e.g.*, parental antibody) and/or the binding molecule provided herein (*e.g.*, a bispecific antibody) described herein may be made using well known biochemical or molecular engineering techniques. In some embodiments, variants can be prepared by introducing appropriate nucleotide changes into the encoding DNA, and/or by direct synthesis of the desired polypeptide or agent. In this respect it may be possible to disrupt the activity or effector function provided by a specific sequence or region while substantially maintaining the structure, binding activity, and other desired characteristics of the modified binding agent.

[00370] The present disclosure further embraces additional variants and equivalents which are substantially homologous to the binding molecules described herein. In some embodiments, it is desirable to improve the binding affinity and/or other biological properties of the binding molecules, including but not limited to, specificity, thermostability, expression level, effector functions, glycosylation, reduced immunogenicity, or solubility. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of a polypeptide.

[00371] Variations may be a substitution, deletion, or insertion of one or more nucleotides encoding a multispecific binding agent that results in a change in the amino acid sequence as compared with the sequence of the parental binding agent. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, *e.g.*, conservative amino acid replacements. In some embodiments, insertions or deletions are in the range of about 1 to 5 amino acids. In certain embodiments, the substitution, deletion, or insertion includes less than 25 amino acid substitutions, less than 20 amino acid substitutions, less than 15 amino acid substitutions, less than 10 amino acid substitutions, less than 5 amino acid substitutions, less than 4 amino acid substitutions, less than 3 amino acid substitutions, or less than 2 amino acid substitutions relative to the parent molecule. Variations in the amino acid sequence that are biologically useful and/or relevant may be determined by systematically making insertions, deletions, or substitutions in the sequence and testing the resulting variant proteins for activity as compared to the parental protein.

[00372] In some embodiments, variants may include the addition of amino acid residues at the amino- and/or carboxyl-terminal end of one or more polypeptides that make up the binding molecules provided herein. The length of additional amino acids residues may range from one residue to a hundred or more residues. In some embodiments, a variant comprises an N-terminal methionyl residue. In some embodiments, the variant comprising an additional polypeptide/protein, i.e., a fusion protein. In certain embodiments, a variant is engineered to be detectable and may comprise a detectable label and/or protein (*e.g.*, an enzyme).

[00373] In some embodiments, a cysteine residue not involved in maintaining the proper conformation of the binding molecule provided herein is substituted or deleted to modulate the agent's characteristics, for example, to improve oxidative stability and/or prevent aberrant disulfide crosslinking. Conversely, in some embodiments, one or more cysteine residues are added to create disulfide bond(s) to improve stability.

[00374] In some embodiments, the binding molecule of the present disclosure is "deimmunized". The deimmunization of agents such as antibodies generally consists of introducing specific mutations to remove T cell epitopes without significantly reducing the binding affinity or other desired activities of the agent.

[00375] The variant binding molecules or polypeptides described herein may be generated using methods known in the art, including but not limited to, site-directed mutagenesis, alanine scanning mutagenesis, and PCR mutagenesis.

[00376] In some embodiments, a binding molecule described herein is chemically modified. In some embodiments, a binding molecule is a bispecific antibody that has been chemically modified by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, and/or linkage to a cellular ligand or other protein. Any of numerous chemical modifications may be carried out by known techniques.

[00377] The polypeptides that make up the multispecific binding agents described herein can be produced by any suitable method known in the art and described in more detail in Section III and Section IV below.

[00378] The present disclosure also provides conjugates comprising any one of the binding molecules (*e.g.*, bispecific antibodies) described herein. In some embodiments, the binding molecule provided herein is attached to an additional molecule. In some embodiments, the

binding molecule provided herein is conjugated to a cytotoxic agent or moiety. In some embodiments, the binding molecule provided herein is conjugated to a cytotoxic agent to form an ADC (antibody-drug conjugate). In some embodiments, the cytotoxic moiety is a chemotherapeutic agent including, but not limited to, methotrexate, adriamycin/doxorubicin, melphalan, mitomycin C, chlorambucil, duocarmycin, daunorubicin, pyrrolbenzodiazepines (PBDs), or other intercalating agents. In some embodiments, the cytotoxic moiety is a microtubule inhibitor including, but not limited to, auristatins, maytansinoids (*e.g.*, DMI and DM4), and tubulysins. In some embodiments, the cytotoxic moiety is an enzymatically active toxin of bacterial, fungal, plant, or animal origin, or fragments thereof, including, but not limited to, diphtheria A chain, non-binding active fragments of diphtheria toxin, exotoxin A chain, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), *Momordica charantia* inhibitor, curcin, crotin, *Sapaonaria officinalis* inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin, and tricothecenes. In some embodiments, the binding molecule provided herein is conjugated to one or more small molecule toxins, such as calicheamicins, maytansinoids, trichothenes, and CC1065. The derivatives of any one of these toxins can be used in a conjugate as long as the derivative retains the cytotoxic activity.

[00379] Conjugates comprising the binding molecule provided herein may be made using any suitable methods as known in the art. In some embodiments, conjugates are made using a variety of bifunctional protein-coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCl), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds (such as bis(p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as toluene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene).

[00380] In some embodiments, the binding molecule (*e.g.*, a bispecific antibody) described herein is conjugated to detectable substances or molecules that allow the antibodies to be used for diagnosis and/or detection. The detectable substances may include but not limited to, enzymes, such as horseradish peroxidase, alkaline phosphatase, beta-galactosidase, and acetylcholinesterase; prosthetic groups, such as biotin and flavine(s); fluorescent materials, such

as, umbelliferone, fluorescein, fluorescein isothiocyanate (FITC), rhodamine, tetramethylrhodamine isothiocyanate (TRITC), dichlorotriazinylamine fluorescein, dansyl chloride, cyanine (Cy3), and phycoerythrin; bioluminescent materials, such as luciferase; radioactive materials, such as ^{212}Bi , ^{14}C , ^{57}Co , ^{51}Cr , ^{67}Cu , ^{18}F , ^{68}Ga , ^{67}Ga , ^{153}Gd , ^{159}Gd , ^{68}Ge , ^3H , ^{166}Ho , ^{131}I , ^{125}I , ^{123}I , ^{121}I , ^{115}In , ^{113}In , ^{112}In , ^{111}In , ^{140}La , ^{177}Lu , ^{54}Mn , ^{99}Mo , ^{32}P , ^{103}Pd , ^{149}Pm , ^{142}Pr , ^{186}Re , ^{188}Re , ^{105}Rh , ^{97}Ru , ^{35}S , ^{47}Sc , ^{75}Se , ^{153}Sm , ^{113}Sn , ^{117}Sn , ^{85}Sr , $^{99\text{m}}\text{Tc}$, ^{201}Ti , ^{133}Xe , ^{90}Y , ^{69}Yb , ^{175}Yb , ^{65}Zn ; positron emitting metals; and magnetic metal ions.

[00381] In some embodiments, the binding molecule provided herein described herein is attached to a solid support, that are particularly useful for immunoassays or purification of a target antigen(s). Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride, or polypropylene.

[00382] In some embodiments, the binding molecules provided herein is formulated in a pharmaceutical composition. Therefore, in yet another aspect, provided herein is a pharmaceutical composition comprising a therapeutically effective amount of the binding molecule provided herein and a pharmaceutically acceptable carrier. The pharmaceutical composition provided herein is described in more detail in Section V below. In some embodiments, the pharmaceutical composition is for use in treating a disease or condition in a subject. In some embodiments, the disease or condition is a cancer. In other embodiments, the cancer is a PD-L1 positive cancer. In some embodiments, the cancer is a lung cancer. In some embodiments, the cancer is a Non Small Cell Lung Carcinoma (NSCLC). In some embodiments, the cancer is a Diffuse Large B cell Lymphoma (DLBCL). In some embodiments, the cancer is a colorectal cancer. In some embodiments, the cancer is a breast cancer. In some embodiments, the cancer is a lymphoma. In some embodiments, the cancer is a melanoma. In some embodiments, the cancer is ovarian cancer.

[00383] In some embodiments, the binding molecules provided herein is used for treating a disease or condition. Therefore, in yet another aspect, provided herein is a method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of the binding molecule provided herein to the subject. In some embodiments, the disease or condition is a cancer. In other embodiments, the cancer is a PD-L1 positive cancer. In some embodiments, the cancer is a lung cancer. In some embodiments, the cancer is a Non Small Cell Lung Carcinoma (NSCLC). In some embodiments, the cancer is a Diffuse Large B cell

Lymphoma (DLBCL). In some embodiments, the cancer is a colorectal cancer. In some embodiments, the cancer is a breast cancer. In some embodiments, the cancer is a lymphoma. In some embodiments, the cancer is a melanoma. In some embodiments, the cancer is ovarian cancer. A more detail description of methods for administering the present binding molecules is in Section VI below.

III. Polynucleotides

[00384] In certain embodiments, the disclosure encompasses polynucleotides that encode the binding molecule described herein. The term “polynucleotides that encode a polypeptide” encompasses a polynucleotide that includes only coding sequences for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequences. The polynucleotides of the disclosure can be in the form of RNA or in the form of DNA. DNA includes cDNA, genomic DNA, and synthetic DNA; and can be double-stranded or single-stranded, and if single stranded can be the coding strand or non-coding (anti-sense) strand.

[00385] In certain embodiments, a polynucleotide comprises the coding sequence for a polypeptide fused in the same reading frame to a polynucleotide which aids, for example, in expression and secretion of a polypeptide from a host cell (*e.g.*, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide). The polypeptide can have the leader sequence cleaved by the host cell to form a “mature” form of the polypeptide.

[00386] In certain embodiments, a polynucleotide comprises the coding sequence for a polypeptide fused in the same reading frame to a marker or tag sequence. For example, in some embodiments, a marker sequence is a hexa-histidine tag supplied by a vector that allows efficient purification of the polypeptide fused to the marker in the case of a bacterial host. In some embodiments, a marker is used in conjunction with other affinity tags.

[00387] The present disclosure further relates to variants of the polynucleotides described herein, wherein the variant encodes, for example, fragments, analogs, and/or derivatives of a polypeptide. In certain embodiments, the present disclosure provides a polynucleotide comprising a polynucleotide having a nucleotide sequence at least about 80% identical, at least about 85% identical, at least about 90% identical, at least about 95% identical, and in some

embodiments, at least about 96%, 97%, 98% or 99% identical to a polynucleotide encoding a polypeptide comprising a binding molecule described herein.

[00388] As used herein, the phrase “a polynucleotide having a nucleotide sequence at least, for example, 95% “identical” to a reference nucleotide sequence” is intended to mean that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence can include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence can be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence can be inserted into the reference sequence. These mutations of the reference sequence can occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

[00389] The polynucleotide variants can contain alterations in the coding regions, non-coding regions, or both. In some embodiments, a polynucleotide variant contains alterations which produce silent substitutions, additions, or deletions, but does not alter the properties or activities of the encoded polypeptide. In some embodiments, a polynucleotide variant comprises silent substitutions that results in no change to the amino acid sequence of the polypeptide (due to the degeneracy of the genetic code). Polynucleotide variants can be produced for a variety of reasons, for example, to optimize codon expression for a particular host (i.e., change codons in the human mRNA to those preferred by a bacterial host such as *E. coli*). In some embodiments, a polynucleotide variant comprises at least one silent mutation in a non-coding or a coding region of the sequence.

[00390] In some embodiments, a polynucleotide variant is produced to modulate or alter expression (or expression levels) of the encoded polypeptide. In some embodiments, a polynucleotide variant is produced to increase expression of the encoded polypeptide. In some embodiments, a polynucleotide variant is produced to decrease expression of the encoded polypeptide. In some embodiments, a polynucleotide variant has increased expression of the encoded polypeptide as compared to a parental polynucleotide sequence. In some embodiments,

a polynucleotide variant has decreased expression of the encoded polypeptide as compared to a parental polynucleotide sequence.

[00391] In certain embodiments, a polynucleotide is isolated. In certain embodiments, a polynucleotide is substantially pure.

[00392] Vectors and cells comprising the polynucleotides described herein are also provided. In some embodiments, an expression vector comprises a polynucleotide molecule. In some embodiments, a host cell comprises an expression vector comprising the polynucleotide molecule. In some embodiments, a host cell comprises one or more expression vectors comprising polynucleotide molecules. In some embodiments, a host cell comprises a polynucleotide molecule. In some embodiments, a host cell comprises one or more polynucleotide molecules.

IV. Methods of Making the Binding Molecules

[00393] In yet another aspect, provided herein are methods for making the various binding molecules provided herein. In some embodiments, provided herein is a method of making a binding molecule comprising transfecting one or more vectors into a host cell, wherein the one or more vectors comprise:

- (a) a first nucleic acid encoding a first polypeptide and a second polypeptide, each being an antibody light chain,
- (b) a second nucleic acid encoding a third polypeptide comprising a first VH region and a first CH1 region and a second VH region; and
- (c) a third nucleic acid encoding a fourth polypeptide comprising a third VH region and a second CH1 and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide can form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide can form a second antigen binding Fab region; and

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide can form an antigen binding Fv region.

[00394] In some embodiments, the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region. In some embodiments, the flexible peptide region

comprises an antibody hinge region. In some specific embodiments, the antibody hinge region is an IgG hinge region. In some more specific embodiments, the IgG hinge region is of IgG1 subtype. In other more specific embodiments, the IgG hinge region is of IgG2 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG3 subtype. In yet other more specific embodiments, the IgG hinge region is of IgG4 subtype. In some embodiments, the flexible peptide region further comprises a linker between the antibody hinge region and the second antigen binding domain. In some embodiments, the linker comprises an amino acid sequence of GGGGS (G4S). In some embodiments, the linker comprises an amino acid sequence of (G4S)_n where n is an integer. In some specific embodiments, the linker comprises an amino acid sequence of (G4S)₁. In some more specific embodiments, the linker comprises an amino acid sequence of (G4S)₂. In other more specific embodiments, the linker comprises an amino acid sequence of (G4S)₃. In yet other more specific embodiments, the linker comprises an amino acid sequence of (G4S)₄.

[00395] In some embodiments, the first Fab region and the second Fab region bind to different antigens. In other embodiments, the first Fab region and the second Fab region bind to the same antigen. In some embodiments, the first Fab region and the second Fab region bind to the same epitope of the same antigen. In other embodiments, the first Fab region and the second Fab region bind to different epitopes of the same antigen.

[00396] In certain embodiments, the first Fab region and the second Fab region form a first antigen binding domain, and the Fv region forms a second antigen binding domain.

[00397] In some embodiments, the first antigen binding domain and the second antigen binding domain bind to the same antigen. In some embodiments, the second antigen binding domain binds to the same epitope as at least one of the epitopes bound by the first antigen binding domain.

[00398] In other embodiments, the first antigen binding domain and the second antigen binding domain bind to different antigens, and wherein the first antigen binding domain binds to a first antigen and the second antigen binding domain binds to a second antigen.

[00399] In some embodiments, the first antigen is a cancer antigen. In other embodiments, the first antigen is not a cancer antigen.

[00400] In some embodiments, the second antigen is expressed on an immune cells including lymphocytes and monocytes. In some embodiments, the second antigen is expressed on a T cell.

In some embodiments, the second antigen is expressed on a B cell. In other embodiments, the second antigen is expressed on a dendritic cell. In other embodiments, the second antigen is expressed on a granulocyte. In yet other embodiments, the second antigen is expressed on an innate lymphoid cell. In yet other embodiments, the second antigen is expressed on a megakaryocyte. In yet other embodiments, the second antigen is expressed on a monocyte. In yet other embodiments, the second antigen is expressed on a myeloid-derived suppressor cell. In yet other embodiments, the second antigen is expressed on a NK cell.

[00401] In some specific embodiments, the second antigen is CD3. In some embodiments, the first antigen is a cancer antigen and the second antigen is CD3.

[00402] In some more specific embodiments, the first antigen is PD-L1 and the second antigen is CD3.

[00403] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00404] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00405] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 3; the third polypeptide has the amino acid sequence of SEQ ID NO.: 1; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 2.

[00406] In some embodiments, the first nucleic acid has a nucleotide sequence of SEQ ID NO.: 22; the second nucleic acid has a nucleotide sequence of SEQ ID NO.: 20; and the third nucleic acid has a nucleotide sequence of SEQ ID NO.: 21.

[00407] In other more specific embodiments, the first antigen is CD20 and the second antigen is CD3.

[00408] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, SEQ ID NO.: 29; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00409] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00410] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.

[00411] In some embodiments, the first nucleic acid has a nucleotide sequence of SEQ ID NO.: 36; the second nucleic acid has a nucleotide sequence of SEQ ID NO.: 34; and the third nucleic acid has a nucleotide sequence of SEQ ID NO.: 35.

[00412] In other more specific embodiments, the first antigen is EGFR and the second antigen is CD3.

[00413] In some embodiments, the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43; the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47; the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

[00414] In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44; the VH region of the Fv region has an amino

acid sequence of SEQ ID NO.: 12; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

[00415] In some embodiments, the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.

[00416] In some embodiments, the first nucleic acid has a nucleotide sequence of SEQ ID NO.: 50; the second nucleic acid has a nucleotide sequence of SEQ ID NO.: 48; and the third nucleic acid has a nucleotide sequence of SEQ ID NO.: 49.

[00417] In other more specific embodiments, the first antigen is Her2 and the second antigen is TNF alpha. In some embodiments, the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 51; the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 52; the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 53; and the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 54.

[00418] Recombinant expression of a binding molecule provided herein may require construction of an expression vector containing a polynucleotide that encodes the binding molecule or a fragment thereof. Once a polynucleotide encoding a binding molecule, an antibody heavy or light chain, or fragment thereof (such as, but not necessarily, containing the heavy and/or light chain variable domain) provided herein has been obtained, the vector for the production of the binding molecule may be produced by recombinant DNA technology using techniques well-known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Also provided are replicable vectors comprising a nucleotide sequence encoding a binding molecule provided herein, or a fragment thereof, or a heavy or light chain CDR, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of an antibody molecule (see, *e.g.*, International Publication Nos. WO 86/05807 and WO 89/01036; and U.S. Patent No. 5,122,464)

and the variable domain of an antibody may be cloned into such a vector for expression of the entire heavy, the entire light chain, or both the entire heavy and light chains.

[00419] The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce a binding molecule provided herein. Thus, also provided herein are host cells containing a polynucleotide encoding a binding molecule provided herein or fragments thereof, or a heavy or light chain thereof, or fragment thereof, operably linked to a heterologous promoter. In certain embodiments, multiple vectors comprising polynucleotides encoding different portions of a binding molecule provided herein may be co-expressed in the host cell for expression of the entire binding molecule, as detailed below.

[00420] A variety of host-expression vector systems may be utilized to express the binding molecules provided herein (see, *e.g.*, U.S. Patent No. 5,807,715). Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express a binding molecule provided herein *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing coding sequences; yeast (*e.g.*, *Saccharomyces Pichia*) transformed with recombinant yeast expression vectors containing coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV, tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, NS0, and 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). Bacterial cells such as *Escherichia coli*, or, eukaryotic cells, especially for the expression of whole recombinant antibody molecule, can be used for the expression of a recombinant binding molecule. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies or variants thereof

(Foecking *et al.*, 1986, Gene 45:101; and Cockett *et al.*, 1990, Bio/Technology 8:2). In some embodiments, antibodies provided herein are produced in CHO cells. In a specific embodiment, the expression of nucleotide sequences encoding binding molecules provided herein is regulated by a constitutive promoter, inducible promoter or tissue specific promoter.

[00421] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the binding molecule being expressed. For example, when a large quantity of such a binding molecule is to be produced, for the generation of pharmaceutical compositions of a binding molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, EMBO 12:1791), in which the coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione 5-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

[00422] In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

[00423] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the binding molecule in infected hosts (*e.g.*, see Logan & Shenk, 1984, Proc. Natl.

Acad. Sci. USA 8 1:355-359). Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, *etc.* (see, *e.g.*, Bittner *et al.*, 1987, Methods in Enzymol. 153:51-544).

[00424] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, 293, 3T3, W138, BT483, Hs578T, HTB2, BT20 and T47D, NS0 (a murine myeloma cell line that does not endogenously produce any immunoglobulin chains), CRL7030 and HsS78Bst cells.

[00425] For long-term, high-yield production of recombinant proteins, stable expression can be utilized. For example, cell lines which stably express the binding molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, *etc.*), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the binding molecule. Such engineered cell lines may be particularly

useful in screening and evaluation of compositions that interact directly or indirectly with the binding molecule.

[00426] A number of selection systems may be used, including but not limited to, the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthineguanine phosphoribosyltransferase (Szybalska & Szybalski, 1992, Proc. Natl. Acad. Sci. USA 48:202), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:8-17) genes can be employed in tk-, hgprt- or apt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: *dhfr*, which confers resistance to methotrexate (Wigler *et al.*, 1980, Natl. Acad. Sci. USA 77:357; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); *gpt*, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIB TECH 11(5):155-2 15); and *hygro*, which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30:147). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli *et al.* (eds.), Current Protocols in Human Genetics, John Wiley & Sons, NY (1994); Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1, which are incorporated by reference herein in their entireties.

[00427] The expression levels of a binding molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning, Vol. 3 (Academic Press, New York, 1987)). When a marker in the vector system expressing a binding molecule is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the binding molecule gene, production of the binding molecule will also increase (Crouse *et al.*, 1983, Mol. Cell. Biol. 3:257).

[00428] The host cell may be co-transfected with multiple expression vectors provided herein. The vectors may contain identical selectable markers which enable equal expression of

respective encoding polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing multiple polypeptides. The coding sequences may comprise cDNA or genomic DNA.

[00429] Once a binding molecule provided herein has been produced by recombinant expression, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (*e.g.*, ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, sizing column chromatography, and kappa-select affinity chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In a specific embodiment, kappa-select (*e.g.*, KappaSelect developed by GE Healthcare Life Science) is used for the purification of Fab (kappa) fragments or binding molecules that contain the Fab fragments. Further, the binding molecules provided herein can be fused to heterologous polypeptide sequences described herein or otherwise known in the art to facilitate purification.

V. Pharmaceutical Compositions

[00430] In one aspect, the present disclosure further provides pharmaceutical compositions comprising at least one binding molecule of the present disclosure. In some embodiments, a pharmaceutical composition comprises therapeutically effective amount of a binding molecule provided herein and a pharmaceutically acceptable carrier.

[00431] Pharmaceutical compositions comprising a binding molecule are prepared for storage by mixing the binding molecule having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (*see, e.g.*, Remington, Remington's Pharmaceutical Sciences (18th ed. 1980)) in the form of aqueous solutions or lyophilized or other dried forms.

[00432] The binding molecule of the present disclosure may be formulated in any suitable form for delivery to a target cell/tissue, *e.g.*, as microcapsules or macroemulsions (Remington, *supra*; Park *et al.*, 2005, *Molecules* 10:146-61; Malik *et al.*, 2007, *Curr. Drug. Deliv.* 4:141-51), as sustained release formulations (Putney and Burke, 1998, *Nature Biotechnol.* 16:153-57), or in liposomes (Maclean *et al.*, 1997, *Int. J. Oncol.* 11:325-32; Kontermann, 2006, *Curr. Opin. Mol. Ther.* 8:39-45).

[00433] A binding molecule provided herein can also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example,

hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacrylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules) or in macroemulsions. Such techniques are disclosed, for example, in Remington, *supra*.

[00434] Various compositions and delivery systems are known and can be used with a binding molecule as described herein, including, but not limited to, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the binding molecule, receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-32), construction of a nucleic acid as part of a retroviral or other vector, *etc.* In another embodiment, a composition can be provided as a controlled release or sustained release system. In one embodiment, a pump may be used to achieve controlled or sustained release (*see, e.g.*, Langer, *supra*; Sefton, 1987, Crit. Ref. Biomed. Eng. 14:201-40; Buchwald *et al.*, 1980, Surgery 88:507-16; and Saudek *et al.*, 1989, N. Engl. J. Med. 321:569-74). In another embodiment, polymeric materials can be used to achieve controlled or sustained release of a prophylactic or therapeutic agent (*e.g.*, a binding molecule as described herein) or a composition provided herein (*see, e.g.*, Medical Applications of Controlled Release (Langer and Wise eds., 1974); Controlled Drug Bioavailability, Drug Product Design and Performance (Smolen and Ball eds., 1984); Ranger and Peppas, 1983, J. Macromol. Sci. Rev. Macromol. Chem. 23:61-126; Levy *et al.*, 1985, Science 228:190-92; During *et al.*, 1989, Ann. Neurol. 25:351-56; Howard *et al.*, 1989, J. Neurosurg. 71:105-12; U.S. Pat. Nos. 5,679,377; 5,916,597; 5,912,015; 5,989,463; and 5,128,326; PCT Publication Nos. WO 99/15154 and WO 99/20253). Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. In one embodiment, the polymer used in a sustained release formulation is inert, free of leachable impurities, stable on storage, sterile, and biodegradable.

[00435] In yet another embodiment, a controlled or sustained release system can be placed in proximity of a particular target tissue, for example, the nasal passages or lungs, thus requiring only a fraction of the systemic dose (*see, e.g.*, Goodson, Medical Applications of Controlled

Release Vol. 2, 115-38 (1984)). Controlled release systems are discussed, for example, by Langer, 1990, Science 249:1527-33. Any technique known to one of skill in the art can be used to produce sustained release formulations comprising one or more binding molecule as described herein (*see, e.g.*, U.S. Pat. No. 4,526,938, PCT publication Nos. WO 91/05548 and WO 96/20698, Ning *et al.*, 1996, Radiotherapy & Oncology 39:179-89; Song *et al.*, 1995, PDA J. of Pharma. Sci. & Tech. 50:372-97; Cleek *et al.*, 1997, Pro. Int'l. Symp. Control. Rel. Bioact. Mater. 24:853-54; and Lam *et al.*, 1997, Proc. Int'l. Symp. Control Rel. Bioact. Mater. 24:759-60).

VI. Methods of Administration

[00436] In a specific embodiment, provided herein is a composition for use in the prevention, management, treatment and/or amelioration of a disease or condition comprising a binding molecule provided herein. In one embodiment, provided herein is a composition for use in the prevention of a disease or condition, wherein the composition comprises a binding molecule provided herein. In one embodiment, provided herein is a composition for use in the management of a disease or condition, wherein the composition comprises a binding molecule provided herein. In one embodiment, provided herein is a composition for use in the treatment of a disease or condition, wherein the composition comprises a binding molecule provided herein. In one embodiment, provided herein is a composition for use in the amelioration of a disease or condition, wherein the composition comprises a binding molecule provided herein. In some embodiments, the disease or condition is a cancer. In other embodiments, the cancer is a PD-L1 positive cancer. In some embodiments, the cancer is a lung cancer. In some embodiments, the cancer is a Non Small Cell Lung Carcinoma (NSCLC). In some embodiments, the cancer is a Diffuse Large B cell Lymphoma (DLBCL). In some embodiments, the cancer is a colorectal cancer. In some embodiments, the cancer is a breast cancer. In some embodiments, the cancer is a lymphoma. In some embodiments, the cancer is a melanoma. In some embodiments, the cancer is ovarian cancer. In certain embodiments, the subject is a subject in need thereof. In some embodiments, the subject has the disease or condition. In other embodiments, the subject is at risk of having the disease or condition. In some embodiments, the administration results in the prevention, management, treatment or amelioration of the disease or condition.

[00437] In one embodiment, provided herein is a composition for use in the prevention, management, treatment and/or amelioration of a symptom of a disease or condition, wherein the composition comprises a binding molecule provided herein. In one embodiment, provided herein is a composition for use in the prevention of a symptom of a disease or condition, wherein the composition comprises a binding molecule provided herein. In one embodiment, provided herein is a composition for use in the management of a symptom of a disease or condition, wherein the composition comprises a binding molecule provided herein. In one embodiment, provided herein is a composition for use in the treatment of a symptom of a disease or condition, wherein the composition comprises an a binding molecule provided herein. In one embodiment, provided herein is a composition for use in the amelioration of a symptom of a disease or condition, wherein the composition comprises a binding molecule provided herein. In one embodiment, the disease is cancer. In certain embodiments, the subject is a subject in need thereof. In some embodiments, the subject has the disease or condition. In other embodiments, the subject is at risk of having the disease or condition. In some embodiments, the administration results in the prevention, management, treatment or amelioration of the symptom of the disease or condition.

[00438] In another embodiment, provided herein is a method of preventing, managing, treating and/or ameliorating a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, provided herein is a method of preventing a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, provided herein is a method of managing a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, provided herein is a method of treating a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, provided herein is a method of ameliorating a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, the disease or condition is cancer. In certain embodiments, the subject is a subject in need thereof. In some embodiments, the subject has the disease or condition. In other embodiments, the subject is at risk of having the disease or condition. In some embodiments, the administration results in the prevention, management, treatment or amelioration of the disease or condition.

[00439] In another embodiment, provided herein is a method of preventing, managing, treating and/or ameliorating a symptom of a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, provided herein is a method of preventing a symptom of a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, provided herein is a method of managing a symptom of a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, provided herein is a method of treating a symptom of a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, provided herein is a method of ameliorating a disease or condition in a subject, comprising administering an effective amount of a binding molecule provided herein. In one embodiment, the disease or condition is cancer. In certain embodiments, the subject is a subject in need thereof. In some embodiments, the subject has the disease or condition. In other embodiments, the subject is at risk of having the disease or condition. In some embodiments, the administration results in the prevention, management, treatment or amelioration of the symptom of the disease or condition.

[00440] Also provided herein are methods of preventing, managing, treating and/or ameliorating a disease or condition by administering to a subject of an effective amount of a binding molecule provided herein, or pharmaceutical composition comprising a binding molecule provided herein. In one aspect, the binding molecule is substantially purified (*i.e.*, substantially free from substances that limit its effect or produce undesired side effects). In certain embodiments, the binding molecule is derived from one or more fully human monoclonal antibodies. The subject administered a therapy can be a mammal such as non-primate (*e.g.*, cows, pigs, horses, cats, dogs, rats *etc.*) or a primate (*e.g.*, a monkey, such as a cynomolgous monkey, or a human). In a one embodiment, the subject is a human. In another embodiment, the subject is a human with a disease or condition, *e.g.*, cancer.

[00441] Various delivery systems are known and can be used to administer a prophylactic or therapeutic agent (*e.g.*, a binding molecule provided herein), including, but not limited to, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the binding molecule, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, *J. Biol. Chem.* 262:4429-4432 (1987)), construction of a nucleic acid as part of a retroviral or other

vector, *etc.* Methods of administering a prophylactic or therapeutic agent (*e.g.*, a binding molecule provided herein), or pharmaceutical composition include, but are not limited to, parenteral administration (*e.g.*, intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous), epidural, and mucosal (*e.g.*, intranasal and oral routes). In a specific embodiment, a prophylactic or therapeutic agent (*e.g.*, a binding molecule provided herein), or a pharmaceutical composition is administered intranasally, intramuscularly, intravenously, or subcutaneously. The prophylactic or therapeutic agents, or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, intranasal mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents.

Administration can be systemic or local. In addition, pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent. See, *e.g.*, U.S. Patent Nos. 6,019,968, 5,985,320, 5,985,309, 5,934,272, 5,874,064, 5,855,913, 5,290,540, and 4,880,078; and PCT Publication Nos. WO 92/19244, WO 97/32572, WO 97/44013, WO 98/31346, and WO 99/66903, each of which is incorporated herein by reference their entirety.

[00442] In a specific embodiment, it may be desirable to administer a prophylactic or therapeutic agent, or a pharmaceutical composition provided herein locally to the area in need of treatment. This may be achieved by, for example, and not by way of limitation, local infusion, by topical administration (*e.g.*, by intranasal spray), by injection, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In some embodiments, when administering an antibody provided herein, care must be taken to use materials to which the antibody does not absorb.

[00443] In another embodiment, a prophylactic or therapeutic agent, or a composition provided herein can be delivered in a vesicle, in particular a liposome (see Langer, 1990, *Science* 249:1527-1533; Treat *et al.*, in *Liposomes in the Therapy of Infectious Disease and Cancer*, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*).

[00444] In another embodiment, a prophylactic or therapeutic agent, or a composition provided herein can be delivered in a controlled release or sustained release system. In one embodiment, a pump may be used to achieve controlled or sustained release (see Langer, *supra*; Sefton, 1987,

CRC Crit. Ref. Biomed. Eng. 14:20; Buchwald *et al.*, 1980, Surgery 88:507; Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used to achieve controlled or sustained release of a prophylactic or therapeutic agent (*e.g.*, an antibody provided herein) or a composition provided herein (see *e.g.*, Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, 1983, J., Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy *et al.*, 1985, Science 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105); U.S. Patent No. 5,679,377; U.S. Patent No. 5,916,597; U.S. Patent No. 5,912,015; U.S. Patent No. 5,989,463; U.S. Patent No. 5,128,326; PCT Publication No. WO 99/15154; and PCT Publication No. WO 99/20253. Examples of polymers used in sustained release formulations include, but are not limited to, poly(2-hydroxy ethyl methacrylate), poly(methyl methacrylate), poly(acrylic acid), poly(ethylene-co-vinyl acetate), poly(methacrylic acid), polyglycolides (PLG), polyanhydrides, poly(N-vinyl pyrrolidone), poly(vinyl alcohol), polyacrylamide, poly(ethylene glycol), polylactides (PLA), poly(lactide-co-glycolides) (PLGA), and polyorthoesters. In an embodiment, the polymer used in a sustained release formulation is inert, free of leachable impurities, stable on storage, sterile, and biodegradable. In yet another embodiment, a controlled or sustained release system can be placed in proximity of the therapeutic target, *i.e.*, the nasal passages or lungs, thus requiring only a fraction of the systemic dose (see, *e.g.*, Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 115-138 (1984)). Controlled release systems are discussed in the review by Langer (1990, Science 249:1527-1533). Any technique known to one of skill in the art can be used to produce sustained release formulations comprising one or more binding molecule provided herein. See, *e.g.*, U.S. Patent No. 4,526,938, PCT publication WO 91/05548, PCT publication WO 96/20698, Ning *et al.*, 1996, "Intratumoral Radioimmunotherapy of a Human Colon Cancer Xenograft Using a Sustained-Release Gel," Radiotherapy & Oncology 39:179-189, Song *et al.*, 1995, "Antibody Mediated Lung Targeting of Long-Circulating Emulsions," PDA Journal of Pharmaceutical Science & Technology 50:372-397, Cleek *et al.*, 1997, "Biodegradable Polymeric Carriers for a bFGF Antibody for Cardiovascular Application," Pro. Int'l. Symp. Control. Rel. Bioact. Mater. 24:853-854, and Lam *et al.*, 1997, "Microencapsulation of Recombinant Humanized Monoclonal

Antibody for Local Delivery,” Proc. Int’l. Symp. Control Rel. Bioact. Mater. 24:759-760, each of which is incorporated herein by reference in their entirety.

[00445] In a specific embodiment, where the composition provided herein is a nucleic acid encoding a prophylactic or therapeutic agent (*e.g.*, a binding molecule provided herein), the nucleic acid can be administered *in vivo* to promote expression of its encoded prophylactic or therapeutic agent, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see, *e.g.*, Joliot *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), *etc.* Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination.

[00446] In a specific embodiment, a composition provided herein comprises one, two or more binding molecules provided herein. In another embodiment, a composition provided herein comprises one, two or more binding molecules provided herein and a prophylactic or therapeutic agent other than a binding molecule provided herein. In one embodiment, the agents are known to be useful for or have been or are currently used for the prevention, management, treatment and/or amelioration of a disease or condition. In addition to prophylactic or therapeutic agents, the compositions provided herein may also comprise a carrier.

[00447] The compositions provided herein include bulk drug compositions useful in the manufacture of pharmaceutical compositions (*e.g.*, compositions that are suitable for administration to a subject or patient) that can be used in the preparation of unit dosage forms. In an embodiment, a composition provided herein is a pharmaceutical composition. Such compositions comprise a prophylactically or therapeutically effective amount of one or more prophylactic or therapeutic agents (*e.g.*, a binding molecule provided herein or other prophylactic or therapeutic agent), and a pharmaceutically acceptable carrier. The pharmaceutical compositions can be formulated to be suitable for the route of administration to a subject.

[00448] In a specific embodiment, the term “carrier” refers to a diluent, adjuvant (*e.g.*, Freund’s adjuvant (complete and incomplete)), excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils,

including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is an exemplary carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc.* Examples of suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences (1990) Mack Publishing Co., Easton, PA. Such compositions will contain a prophylactically or therapeutically effective amount of the binding molecule provided herein, such as in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

[00449] In an embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Such compositions, however, may be administered by a route other than intravenous.

[00450] Generally, the ingredients of compositions provided herein are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[00451] A binding molecule provided herein can be packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of antibody. In one embodiment, the binding molecule is supplied as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water or saline to the appropriate concentration for administration to a subject. The lyophilized binding molecule can be stored at between 2 and 8° C in its original container and the binding molecule can be administered within 12 hours, such as within 6 hours, within 5 hours, within 3 hours, or within 1 hour after being reconstituted. In an alternative embodiment, a binding molecule provided herein is supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the antibody.

[00452] The compositions provided herein can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, *etc.*, and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, *etc.*

[00453] The amount of a prophylactic or therapeutic agent (*e.g.*, a binding molecule provided herein), or a composition provided herein that will be effective in the prevention, management, treatment and/or amelioration of a disease or condition can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of a disease or condition, and should be decided according to the judgment of the practitioner and each patient's circumstances.

[00454] Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

[00455] In certain embodiments, the route of administration for a dose of a binding molecule provided herein to a patient is intranasal, intramuscular, intravenous, or a combination thereof, but other routes described herein are also acceptable. Each dose may or may not be administered by an identical route of administration. In some embodiments, a binding molecule provided herein may be administered via multiple routes of administration simultaneously or subsequently to other doses of the same or a different binding molecule provided herein.

[00456] In certain embodiments, binding molecules provided herein are administered prophylactically or therapeutically to a subject. Antibodies provided herein can be prophylactically or therapeutically administered to a subject so as to prevent, lessen or ameliorate a disease or symptom thereof.

[00457] For the sake of conciseness, certain abbreviations are used herein. One example is the single letter abbreviation to represent amino acid residues. The amino acids and their corresponding three letter and single letter abbreviations are as follows:

alanine	Ala	(A)
arginine	Arg	(R)
asparagine	Asn	(N)
aspartic acid	Asp	(D)
cysteine	Cys	(C)
glutamic acid	Glu	(E)
glutamine	Gln	(Q)
glycine	Gly	(G)
histidine	His	(H)
isoleucine	Ile	(I)
leucine	Leu	(L)
lysine	Lys	(K)
methionine	Met	(M)
phenylalanine	Phe	(F)
proline	Pro	(P)
serine	Ser	(S)
threonine	Thr	(T)
tryptophan	Trp	(W)
tyrosine	Tyr	(Y)
valine	Val	(V)

[00458] The invention is generally disclosed herein using affirmative language to describe the numerous embodiments. The invention also specifically includes embodiments in which particular subject matter is excluded, in full or in part, such as substances or materials, method steps and conditions, protocols, procedures, assays or analysis. Thus, even though the invention

is generally not expressed herein in terms of what the invention does not include, aspects that are not expressly included in the invention are nevertheless disclosed herein.

[00459] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, the following examples are intended to illustrate but not limit the scope of invention described in the claims.

EXAMPLES

Example 1: Construction and Expression of Exemplary Binding Molecules

[00460] This example illustrates the construction and expression of exemplary binding molecules as provided herein (illustrated in FIGs. 1A-1E), in particular, binding molecules ACE-00, ACE-02, ACE-03, ACE-04, ACE-05, ACE-09, ACE-10, ACE-11, and ACE-12. The components targeting first and second antigens in each of the exemplary binding molecules are summarized in the table below.

Table 3: Components targeting first and second antigens in exemplary binding molecules

Binding molecule	Targeting component	
	First antigen	Second antigen
ACE-00	Trastuzumab	Adalimumab
ACE-02	Anti-CD19 Ab	Anti-CD3 humanized 12F6
ACE-03	Anti-CD19 Ab	Anti-CD3 humanized OKT3
ACE-04	Anti-PD-L1 Ab	Anti-CD3 chimetic (xi) OKT3 Fab
ACE-05	Anti-PD-L1 Ab	Anti-CD3 UCHT1

ACE-09	Anti-PD-L1 Ab	Anti-CD3 UCHT1
ACE-10	Anti-CD20	Anti-CD3
ACE-11	Anti-EGFR	Anti-CD3
ACE-12	Anti-PD-L1 Ab	Anti-CD3 UCHT1

[00461] Formats used in amino acid sequences:

BOLD: VH or VL;

BOLD and UNDERLINED: CDR;

ITALICIZED: antibody hinge region;

lower case: flexible linker;

[BRACKET]: CH1;

[BRACKET and UNDERLINED]: CL.

1.1. Construction and Expression of ACE-00

[00462] A HEK-293 transient expression system (Invitrogen, USA) was used for expressing ACE-00, whose second antigen binding Fv region binds to TNF alpha, and whose first antigen binding domain (Fab regions) binds to Her2 antigen (see FIG. 2F). ACE-00 has the same overall structure as the exemplary binding molecule illustrated in FIG. 1A. Briefly, ACE-00 contains two different heavy chain like chains (ACE-00-VH and ACE-00-VL) and two identical light chains (ACE-00-LC). The parental antibody used for constructing the anti-Her2 domain of ACE-00 is trastuzumab and the parental antibody used for constructing the anti-TNF alpha domain of ACE-00 is adalimumab. The amino acid sequences of these three type of polypeptides are as follows:

ACE-00-VH amino acid sequence:

EVQLVESGGGLVQP^{GGSLRL}SCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTN
GYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDY
WGQGT^{LVTVSS}[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVT^{SVNSGALT}
^{SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC]}*DKT*
^{HTCPPCPAPELLGGPEV}**QLVESGGGLVQPGRSLRLSCAASGFTEDDYAMHWVRQAP**
GKGLEWVSAITWNSGHIDYADSVEGRFTISRDNKNSLYLQMNSLRAEDTAVYYC
AKVSYLSTASSLDYWGQGTLVTVSS (SEQ ID NO: 115)

ACE-00-VL amino acid sequence:

**EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTN
GYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDY
WGQGT****LVTVSS**[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT
SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC]**DKT**
HTCPPCPAPELLGGPDIQMTQSPSSLSASVGDRVTITCRASQ**GIRNYLAWYQQKPGK**
APKLLIY**AAS****TLQSGVPSRFSGSGSGTDFTLT****ISSLQPEDVATYYC****QRYNRAPYTFG**
QGTKVEIKR (SEQ ID NO: 116)

ACE-00-LC amino acid sequence (anti-CD19 antibody light chain):

DIQMTQSPSSLSASVGDRVTITCRASQ**DVNTA****VAWYQQKPGKAPKLLIY****SAS****FLYSG**
VPSRFSGSRSGTDFTLT**ISSLQPEDFATYYC****QQHYTTPPTFGQGTKVEIK****[RSVAAPS**
VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTY
SLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC] (SEQ ID NO: 117)

[00463] The VH and VL amino acid sequences for the bivalent Fab region targeting Her2 and the monovalent Fv region targeting TNF alpha are listed in the table below:

Table 4: VHs and VLs of ACE-00

Fab region (Anti-Her2)	VH:	VL:
	EVQLVESGGGLVQPGGSLRLSCA ASGFNIKDTYIHWVRQAPGKGLE WVARIYPTNGYTRYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTA VYYCSRWGGDGFYAMDYWGQG TLVTVSS (SEQ ID NO: 51)	DIQMTQSPSSLSASVGDRVTITCR ASQDVNTA VAWYQQKPGKAPKL LIYSASFLYSGVPSRFSGSRSGTDF TLTISSLQPEDFATYYCQQHYTTP PTFGQGTKVEIKR (SEQ ID NO: 52)
	CDR H1: GFNIKDTY (SEQ ID NO: 118)	CDR L1: QDVNTA (SEQ ID NO: 121)
	CDR H2: IYPTNGYT (SEQ ID NO: 119)	CDR L2: SAS (SEQ ID NO: 122)

	CDR H3: SRWGGDGFYAMDY (SEQ ID NO: 120)	CDR L3: QQHYTTPPT (SEQ ID NO: 123)
Fv region (Anti-TNF alpha)	VH: EVQLVESGGGLVQPGRSLRLSCA ASGFTFDDYAMHWVRQAPGKGL EWVSAITWNSGHIDYADSVEGRF TISRDNAKNSLYLQMNSLRAEDT AVYYCAKVSYLSTASSLDYWGQ GTLVTVSS (SEQ ID NO: 53)	VL: DIQMTQSPSSLSASVGDRVTITCR ASQGIRNYLAWYQQKPGKAPKLL IYAASLTQSGVPSRFSGSGSGTDF TLTISSLQPEDVATYYCQRYNRAP YTFGQGTKVEIKR (SEQ ID NO: 54)
	CDR H1: GFTFDDYA (SEQ ID NO: 124)	CDR L1: QGIRNY (SEQ ID NO: 127)
	CDR H2: ITWNSGHI (SEQ ID NO: 125)	CDR L2: AAS (SEQ ID NO: 128)
	CDR H3: AKVSYLSTASSLDY (SEQ ID NO: 126)	CDR L3: QRYNRAPYT (SEQ ID NO: 129)

[00464] Tri-transfection of DNAs encoding ACE-00-VH, ACE-00-VL, and ACE-00-LC were performed as briefly described below. Polyethylenimine (PEI) was used as a transfection reagent (used at a ratio of DNA: PEI = 1:4 (w/w)). Six to seven days after the transfection when the cell survival rate was measured to be about 60% to 70%, the batch culture was discontinued, and the expression medium was collected and centrifuged (4,800 rpm, 30 min, 4°C) to remove the debris. The supernatant was then filtered by using a 0.22 μ m TOP-filter (Millipore, USA).

Subsequently, the filtered supernatant including ACE-00 molecules underwent an affinity chromatography purification process using Hitrap™ KappaSelect (GE healthcare, USA), followed by dialysis with pH 7.4 PBS using Slide-A Lyzer Dialysis Cassette (Thermo, USA) for elution buffer change. Purified proteins were analyzed by SDS-PAGE, capillary electrophoresis, and size exclusion chromatography (SEC). The purified ACE-00 molecules were also analyzed for their purity using Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) and SEC-HPLC (ThermoFisher, USA). The purity analysis was performed using the protocols provided by the manufactures.

[00465] As a control, bi-transfection of DNAs encoding ACE-00-VL and ACE-00-LC into HEK-293 cells was performed. SDS-PAGE was performed to identify the difference of assembly pattern of ACE-00 (containing two different heavy chain like chains ACE-00-VH and ACE-00-VL) and ACE-00-VL2 (containing two identical heavy chain like chains ACE-00-VL) (FIGs. 2A-2B).

[00466] Antibody is assembled and secreted as a tetramer H₂L₂ and the quality control machinery is very tightly regulated in Endoplasmic Reticulum (ER) by ER chaperones such as luminal binding protein (BiP) and protein disulfide isomerase (PDI). It was known that unfolded CH1 domain of heavy chain has a role of regulation of antibody assembly in BiP dependent manner. BiP may play a role in the heterodimer formation of CH1 and CL as well as in quality control mechanisms in antibody assembly by regulation of endoplasmic-reticulum-associated protein degradation (ERAD) (Lee Y. K. et al. BiP and immunoglobulin light chain cooperate to control the folding of heavy chain and ensure the fidelity of immunoglobulin assembly. *Mol Biol Cell*. 1999 Jul;10(7):2209-19; Feige M. J. et al. An unfolded CH1 domain controls the assembly and secretion of IgG antibodies. *Mol Cell*. 2009 Jun 12;34(5):569-79; Feige M. J. et al. How antibodies fold. *Trends Biochem Sci*. 2010 Apr;35(4):189-98. Each of these is incorporated herein by reference in its entirety). The possible interaction between VH domain and BiP was investigated to examine if two different ALiCE heavy chains can form heterodimer by specific interaction of VH-VL. CH1 or VH-CH1 truncated heavy chains, ΔCH1 or ΔVH-CH1, was cloned and delivered into HEK293 cells. Wild-type HC or truncated HC constructs was delivered into HEK293 cell. Cell lysates obtained from each transfectant were pulled down with protein A bead in order to identify antibody domains that may bind to BiP using anti-Fc-HRP (Thermo Fisher) and anti-BiP-HRP (R&D systems).

[00467] Without wishing to be bound by any particular mechanism or theory, the following results were obtained. Co-precipitated BiP was found by Western blot in ΔCH1 and WT-HC clone transfected lysate, indicating VH and BiP interaction (FIG. 2C). Furthermore, secreted polypeptide was detected in ΔVH-CH1 transfected expression medium, indicating that BiP can regulate the assembly and secretion of heavy chain by interaction with VH and/or CH1 domain of heavy chain (FIGs. 2C-2E). Potentially, antibody VH domain was also found to have a role of antibody assembly in BiP dependent manner in this study (see FIGs. 2C-2E). As shown in FIG. 2F, the heavy chain like chain of the binding molecule provided herein that contains two VH

regions (one in a Fab region and one in a Fv region), i.e., ACE-00-VH in this study, contributes to the proper assembly of the binding molecule provided herein in mammalian expression system. Without this quality control system, many unwanted different combinations of polypeptide chains will be found in expression medium.

[00468] KappaSelect was used for affinity chromatography for ACE-00 and ACE-00-VL2 proteins. As shown in FIG. 2G, no unbound ACE was detected in flow through (F.T) lanes.

[00469] Capillary electrophoresis was then performed to identify the molecular size differentiation between ACE-00-VL2 and ACE-00. In FIG. 2H, the size of each peak in the figure (left) is shown in the table (right). Peak 7 represents ACE-00-VL/ACE-00-LC dimer complex and peak 8 represents ACE-00-VH/ACE-00-LC dimer complex. The molecular weight of ACE-00 is 4 kDa higher than ACE-00-VL2 (peak 12).

[00470] Capillary electrophoresis results also showed the conformation of ACE-00 and ACE-00-VL2 molecules. As shown in FIG. 2I, most (almost 99%) of ACE-00 molecules were existed in a heterodimerized form. Capillary Isoelectric focusing was then performed and corroborated the heterodimerization between ACE-00-VH chain and ACE-00-VL chain. pI value was measured by cIEF for each of ACE-00 and ACE-00-VL2. The result is shown in FIG. 2J. The high efficiency of heterodimerization between ACE-00-VH and ACE-00-VL chains was corroborated in the SDS-PAGE and capillary electrophoresis results shown in FIG. 2K. As shown in FIG. 2L, a small portion of protein aggregates were found in size exclusion chromatography of ACE-00, and most ACE-00 was in soluble and uniform structure, which further corroborated the high efficiency of heterodimerization.

[00471] These results indicate that ACE-00 was properly expressed and assembled. These results also indicate that the heavy chain like chain of the binding molecule provided herein that contains two VH regions (one in a Fab region and one in a Fv region) contributes to the proper assembly of the binding molecule provided herein in mammalian expression system, and that VH-VL interaction in the Fv region is a major driving force to facilitate heterodimerization of two heavy chain like chains. Similar tests were performed for other exemplary binding molecules (e.g., ACE-05, ACE-10, and ACE-11) described below and same conclusions were arrived with for these molecules.

1.2. Construction and Expression of ACE-02

[00472] A HEK-293 transient expression system (Invitrogen, USA) was used for expressing ALiCE molecule ACE-02 provided herein. ACE-02 is composed of anti-CD19 and humanized anti-CD3 12F6 domains. ACE-02 contains two different heavy chain like chains (ACE-02-VH and ACE-02-VL) and two identical light chains (ACE-02-LC). The amino acid sequences of these three type of polypeptides are as follows:

ACE-02-VH amino acid sequence:

QVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWKQRPQGQGLEWIGQIWPG
DGDTNYNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYY
AMDYWGQGTTTVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
 SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKS
 C]**DKTHTCPPCPAPELLGGPQVQLVQSGGGVVQPGRSLRLSCKASGYTFTSYTMHWW**
RQAPGKGLEWIGYINPSSGYTKYNQKFKDRFTISADKSKSTAFLQMDSLRLPEDTGV
YFCARWQDYDYFDYWGQGTPTVTVSS (SEQ ID NO: 88)

ACE-02-VL amino acid sequence:

QVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWKQRPQGQGLEWIGQIWPG
DGDTNYNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCARRETTTVGRYYY
AMDYWGQGTTTVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
 SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKS
 C]**DKTHTCPPCPAPELLGGPDIDMTQSPSSLSASVGDRVTMTCRASSSVSYMHWWYQQT**
PGKAPKPWIYATSNLASGVPSRFSGSGSGTDYTLTISSLQPEDATYYCQQWSSNPPT
FGQGTKLQITR (SEQ ID NO: 89)

ACE-02-LC amino acid sequence (anti-CD19 antibody light chain):

DIQLTQSPASLAVSLGQRATISCKASQSVDYDGDSYLNWYQQIPGQPPKLLIYDASN
LVSGIPPRFSGSGSGTDFTLNHPVEKVDAAATYHCQQSTEDPWTFGGGKLEIK[**RT**
VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
KDSTYSLSSLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC] (SEQ ID NO: 90)

[00473] The VH and VL amino acid sequences and CDR sequences therein for the first antigen binding domain bivalent Fab region targeting CD19 and the second antigen binding domain monovalent Fv region of humanized 12F6 are listed in the table below:

Table 5: VHs, VLs and CDRs of ACE-02

Fab region (Anti-CD19)	VH: QVQLQQSGAELVRPGSSVKISCKA SGYAFSSYWMNWVKQRPGQGLE WIGQIWPGDGDTNYNGKFKGKAT LTADESSSTAYMQLSSLASEDSAV YFCARRETTTVGRYYYAMDYWG QGTTVTVSS (SEQ ID NO: 61)	VL: DIQLTQSPASLAVSLGQRATISCK ASQSVDDYDGDSYLNWYQQIPGQP PKLLIYDASNLVSGIPPRFSGSGSG TDFTLNHPVEKVDAAATYHCQQS TEDPWTFGGGKLEIK (SEQ ID NO: 65)
	CDR H1: SYWMN (SEQ ID NO: 62)	CDR L1: QSVDDYDGDSY (SEQ ID NO: 66)
	CDR H2: QIWPGDGDTNYNGKFKG (SEQ ID NO: 63)	CDR L2: DAS (SEQ ID NO: 67)
	CDR H3: RETTTVGRYYYAMDY (SEQ ID NO: 64)	CDR L3: QQSTEDPWT (SEQ ID NO: 68)
Fv region (Anti-CD3)	VH: QVQLVQSGGGVVQPGRSLRLSCK ASGYTFTSYTMHWVRQAPGKGLE WIGYINPSSGYTKYNQKFKDRFTIS ADKSKSTAFLQMDSLRPEDTGVYF CARWQDYDVYFDYWGQGTPVTV SS (SEQ ID NO: 69)	VL: DIQMTQSPSSLSASVGDRVTMTC RASSSVSYMHWYQQTPGKAPKP WIYATSNLASGVPSRFSGSGSGTD YTLTISSLQPEDIATYYCQQWSSN PPTFGQGTKLQITR (SEQ ID NO: 73)
	CDR H1: GYTFTSYT (SEQ ID NO: 70)	CDR L1: SSSVSY (SEQ ID NO: 74)
	CDR H2: INPSSGYT (SEQ ID NO: 71)	CDR L2: ATS (SEQ ID NO: 75)
	CDR H3: ARWQDYDVYFDY (SEQ ID NO: 72)	CDR L3: QQWSSNPPT (SEQ ID NO: 76)

[00474] DNA sequences encoding ACE-02-VH, ACE-02-VL and ACE-02-LC are as follows:

ACE-02-VH nucleotide sequence:

CAGGTTCAATTGCAGCAAAGCGGGGCTGAGTTGGTACGGCCTGGGTCCAGCGTGAA
GATATCATGTAAGGCTtctGGATATGCCTTCTCCTCTTACTGGATGAACTGGGTCAAGC

AACGGCCAGGACAAGGCCTGGAGTGGATTGGGCAAATATGGCCCCGGGGACGGAGA
TACTAATTATAATGGCAAGTTTAAGGGGAAAGCTACACTGACCGCAGACGAAAGCT
CCTCTACGGCCTATATGCAGCTCTCATCTCTTGCGTCCGAAGATAGTGCAGTATATTT
TTGTGCGCGCCGCGAGACCACCACGGTTGGGAGGTACTATTACGCGATGGATTACTG
GGGCCAGGGGACTACAGTTACGGTTTCATCAGCTAGCACCAAGGGCCCATCGGTCTT
CCCCCTGGCACCCCTCCTCCAAGAGCACCTCTGGGGGACAGCGGCCCTGGGCTGCCT
GGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGA
CCAGCGGCGTGACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCA
GCAGCGTGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAAC
GTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTG
TGACAAAACCTCACACATGCCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGAGcGCA
GGTGCAGCTGGTGCAGAGCGGCGGCGGCGTGGTGCAGCCCGGCCGAGCCTGCGCC
TGAGCTGCAAGGCCAGCGGCTACACCTTACCAGCTACACCATGCACTGGGTGCGC
CAGGCCCCCGGCAAGGGCCTGGAGTGGATCGGCTACATCAACCCAGCAGCGGCTA
CACCAAGTACAACCAGAAGTTCAAGGACCGCTTACCATCAGCGCCGACAAGAGCA
AGAGCACCGCCTTCTGTCAGATGGACAGCCTGCGCCCCGAGGACACCGGCGTGTAC
TTCTGCGCCCGCTGGCAGGACTACGACGTGTACTTCGACTACTGGGGCCAGGGCACC
CCCGTGACCGTGAGCAGCTAA (SEQ ID NO: 100)

ACE-02-VL nucleotide sequence:

CAGGTTCAATTGCAGCAAAGCGGGGCTGAGTTGGTACGGCCTGGGTCCAGCGTGAA
GATATCATGTAAGGCTTCTGGATATGCCTTCTCCTCTTACTGGATGAACTGGGTCAA
GCAACGGCCAGGACAAGGCCTGGAGTGGATTGGGCAAATATGGCCCCGGGGACGGA
GATACTAATTATAATGGCAAGTTTAAGGGGAAAGCTACACTGACCGCAGACGAAAG
CTCCTCTACGGCCTATATGCAGCTCTCATCTCTTGCGTCCGAAGATAGTGCAGTATAT
TTTTGTGCGCGCCGCGAGACCACCACGGTTGGGAGGTACTATTACGCGATGGATTAC
TGGGGCCAGGGGACTACAGTTACGGTTTCATCAGCTAGCACCAAGGGCCCATCGGT
CTTCCCCCTGGCACCCCTCCTCCAAGAGCACCTCTGGGGGACAGCGGCCCTGGGCTG
CCTGGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCT
GACCAGCGGCGTGACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCT
CAGCAGCGTGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCA
ACGTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCT

TGTGACAAAACCTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGGACC
 GGACATCCAGATGACCCAGAGCCCCAGCAGCCTGAGCGCCAGCGTGGGGCGACCGCG
 TGACCATGACCTGCCGCGCCAGCAGCAGCGTGAGCTACATGCACTGGTACCAGCAG
 ACCCCCGGCAAGGCCCCCAAGCCCTGGATCTACGCCACCAGCAACCTGGCCAGCGG
 CGTGCCCAGCCGCTTCAGCGGCAGCGGCAGCGGCACCGACTACACCCTGACCATCA
 GCAGCCTGCAGCCCGAGGACATCGCCACCTACTACTGCCAGCAGTGGAGCAGCAAC
 CCCCCACCTTCGGCCAGGGCACCAAGCTGCAGATCACCCGCTAA (SEQ ID NO: 101)

ACE-02-LC nucleotide sequence (anti-CD19 antibody light chain nucleotide sequence):

GATATTCAACTCACGCAATCTCCAGCAAGTCTCGCAGTTAGTTTGGGGCAGCGAGCT
 ACAATAAGTTGCAAGGCGAGCCAATCCGTGGATTATGATGGAGACAGCTATCTTAA
 CTGGTATCAGCAAATTCCAGGCCAGCCACCCAAGTTGCTGATCTACGACGCGTCAAA
 CCTGGTCTCAGGGATCCCTCCAAGATTTAGCGGCTCAGGTTACGGATTTTAC
 GCTCAATATCCATCCTGTAGAGAAGGTTGATGCAGCTACATACTGTCAACAGAG
 TACCGAGGATCCTTGGACCTTCGGAGGCGGTACAAAGCTGGAGATCAAGAGAACCG
 TGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA
 CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGT
 GGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAG
 GACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAGCAGA
 CTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGC
 CCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAA (SEQ ID NO: 102)

[00475] In FIG. 3, the SDS-PAGE results show the expression of ACE-02, ACE-03, ACE-00 and ACE-01. It also shows the difference in assembly patterns of ACE-02 and ACE-02-VL2 and the difference in assembly patterns of ACE-03 and ACE-03-VL2. ACE-01 has parent antibodies of anti-CD19 Ab targeting the first antigen and murine OKT3 targeting the second antigen. These results suggest that ACE-02 was properly expressed and assembled.

1.3. Construction and Expression of ACE-03

[00476] A HEK-293 transient expression system (Invitrogen, USA) was used for expressing ALiCE molecule ACE-03 provided herein. ACE-03 is composed of anti-CD19 and humanized anti-CD3 OKT3 domains. ACE-03 contains two different heavy chain like chains (ACE-03-VH

and ACE-03-VL) and two identical light chains (ACE-03-LC). The amino acid sequences of these three type of polypeptides are as follows:

ACE-03-VH amino acid sequence:

QVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPQGGLEWIGQIWPG**
DGDTNYNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCAR**RETTTVGRYYY**
AMDYWGQGTTVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKS
C]*DKTHTCPPCPAPELLGGPVQ***LVQSGGGVVQ**GRSLRL**SCKASGYTFTRY**TMHWVR
QAPGKGLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLRPEDTAVY
YCA**RYYYDDHYCLDYWGQGTPVT**VSS (SEQ ID NO: 91)**

ACE-03-VL amino acid sequence:

QVQLQQSGAELVRPGSSVKISCKASGYAFSSYWMNWVKQRPQGGLEWIGQIWPG**
DGDTNYNGKFKGKATLTADESSSTAYMQLSSLASEDSAVYFCAR**RETTTVGRYYY**
AMDYWGQGTTVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWN
SGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKS
C]*DKTHTCPPCPAPELLGGPDIQMTQSPSSLSASVGDRVTITCSASSSVSYMNWYQQTP*
GKAPKRWIYD**TSKL**ASGVPSR**FSGSGSGTDYFT**ISS**LQPED**ATYYC**QQWSSNPFT**
FGQGTKLQITR (SEQ ID NO: 92)**

ACE-03-LC amino acid sequence (anti-CD19 antibody light chain):

DIQLTQSPASLAVSLGQRATISCKASQSV**DYDGDSYLNWYQQIPGQPPKLLIYDASN**
LVSGIPPRFSGSGSGTDFTLNHPVEK**VDAATYHCQQSTEDPWTFGGG**TKLEIK**[RT**
VAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQ**WKVDNALQSGNSQESVTEQDS**
KDSTYSLSSLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC] (SEQ ID NO: 90)

[00477] The VH and VL amino acid sequences and CDR sequences therein for the first antigen binding domain bivalent Fab region targeting CD19 and the second antigen binding domain monovalent Fv region of humanized OKT3 are listed in the table below:

Table 6: VHs, VLs and CDRs of ACE-03

Fab region (Anti- CD19)	VH: QVQLQQSGAELVRPGSSVKISCKA SGYAFSSYWMNWKQRPQGGL	VL: DIQLTQSPASLAVSLGQRATISCK ASQSVDYDGDSYLNWYQQIPGQP
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	WIGQIWPGDGDNTNYNGKFKGKAT LTADESSSTAYMQLSSLASEDSAV YFCARRETTTVGRYYYAMDYWG QGTTVTVSS (SEQ ID NO: 61)	PKLLIYDASNLVSGIPPRFSGSGSG TDFTLNIHPVEKVDAATYHCQQS TEDPWTFGGGTKLEIK (SEQ ID NO: 65)
	CDR H1: SYWMN (SEQ ID NO: 62)	CDR L1: QSVDDYDGDSY (SEQ ID NO: 66)
	CDR H2: QIWPGDGDNTNYNGKFKG (SEQ ID NO: 63)	CDR L2: DAS (SEQ ID NO: 67)
	CDR H3: RETTTVGRYYYAMDY (SEQ ID NO: 64)	CDR L3: QQSTEDPWT (SEQ ID NO: 68)
Fv region (Anti-CD3)	VH: VQLVQSGGGVVQPGRSLRLSCKA SGYTFTRYTMHWVRQAPGKGLE WIGYINPSRGYTNYNQKVKDRFTI STDKSKSTAFLQMDSL RPEDTAVY YCARYYDDHYCLDYWGQGTPVT VSS (SEQ ID NO: 77)	VL: DIQMTQSPSSLSASVGDRVTITCS ASSSVSYMNWYQQTPGKAPKRW IYDTSKLASGVPSRFSGSGSGTDY TFTISSLQPEDIATYYCQQWSSNPFT TFGQGTKLQITR (SEQ ID NO: 81)
	CDR H1: GYTFTRYT (SEQ ID NO: 78)	CDR L1: SSVSY (SEQ ID NO: 82)
	CDR H2: INPSRGYT (SEQ ID NO: 79)	CDR L2: DTS (SEQ ID NO: 83)
	CDR H3: ARYYDDHYCLDY (SEQ ID NO: 80)	CDR L3: QQWSSNPFT (SEQ ID NO: 84)

[00478] DNA sequences encoding ACE-03-VH, ACE-03-VL and ACE-03-LC are as follows:

ACE-03-VH nucleotide sequence:

CAGGTTCAATTGCAGCAAAGCGGGGCTGAGTTGGTACGGCCTGGGTCCAGCGTGAA
GATATCATGTAAGGCTtctGGATATGCCTTCTCCTCTTACTGGATGAACTGGGTCAAGC
AACGGCCAGGACAAGGCCTGGAGTGGATTGGGCAAATATGGCCCGGGGACGGAGA
TACTAATTATAATGGCAAGTTTAAGGGGAAAGCTACACTGACCGCAGACGAAAGCT
CCTCTACGGCCTATATGCAGCTCTCATCTCTTGCGTCCGAAGATAGTGCAGTATATTT

TTGTGCGCGCCGCGAGACCACCACGGTTGGGAGGTACTATTACGCGATGGATTACTG
GGGCCAGGGGACTACAGTTACGGTTTCATCAGCTAGCACCAAGGGCCCATCGGTCTT
CCCCCTGGCACCCCTCCTCCAAGAGCACCTCTGGGGGACAGCGGCCCTGGGCTGCCT
GGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGA
CCAGCGGCGTGACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCA
GCAGCGTGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAAC
GTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTG
TGACAAAACCTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACcGGT
GCAGCTGGTGCAGAGCGGCGGCGGCGTGGTGCAGCCCGGCCGCAGCCTGCGCCTGA
GCTGCAAGGCCAGCGGCTACACCTTCACCCGCTACACCATGCACTGGGTGCGCCAG
GCCCCCGGCAAGGGCCTGGAGTGGATCGGCTACATCAACCCCAGCCGCGGCTACAC
CAACTACAACCAGAAGGTGAAGGACCGCTTCACCATCAGCACCGACAAGAGCAAGA
GCACCGCCTTCTGTCAGATGGACAGCCTGCGCCCCGAGGACACCGCCGTGTACTACT
GCGCCCGCTACTACGACGACCACTACTGCCTGGACTACTGGGGCCAGGGCACCCCC
GTGACCGTGAGCAGCTAA (SEQ ID NO: 103)

ACE-03-VL nucleotide sequence:

CAGGTTCAATTGCAGCAAAGCGGGGCTGAGTTGGTACGGCCTGGGTCCAGCGTGAA
GATATCATGTAAGGCTtctGGATATGCCTTCTCCTCTTACTGGATGAACTGGGTCAAGC
AACGGCCAGGACAAGGCCTGGAGTGGATTGGGCAAATATGGCCCCGGGGACGGAGA
TACTAATTATAATGGCAAGTTTAAGGGGAAAGCTACACTGACCGCAGACGAAAGCT
CCTCTACGGCCTATATGCAGCTCTCATCTCTTGCGTCCGAAGATAGTGCAGTATATTT
TTGTGCGCGCCGCGAGACCACCACGGTTGGGAGGTACTATTACGCGATGGATTACTG
GGGCCAGGGGACTACAGTTACGGTTTCATCAGCTAGCACCAAGGGCCCATCGGTCTT
CCCCCTGGCACCCCTCCTCCAAGAGCACCTCTGGGGGACAGCGGCCCTGGGCTGCCT
GGTCAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGA
CCAGCGGCGTGACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCA
GCAGCGTGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAAC
GTGAATCACAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTG
TGACAAAACCTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGGAccgGA
CATCCAGATGACCCAGAGCCCCAGCAGCCTGAGCGCCAGCGTGGGCGACCGCGTGA
CCATCACCTGCAGCGCCAGCAGCAGCGTGAGCTACATGAACTGGTACCAGCAGACC

CCCGGCAAGGCCCCCAAGCGCTGGATCTACGACACCAGCAAGCTGGCCAGCGGCGT
 GCCCAGCCGCTTCAGCGGCAGCGGCAGCGGCACCGACTACACCTTCACCATCAGCA
 GCCTGCAGCCCGAGGACATCGCCACCTACTACTGCCAGCAGTGGAGCAGCAACCCC
 TTCACCTTCGGCCAGGGCACCAAGCTGCAGATCACCCGCTAA (SEQ ID NO: 104)

ACE-03-LC nucleotide sequence (anti-CD19 antibody light chain nucleotide sequence):

GATATTCAACTCACGCAATCTCCAGCAAGTCTCGCAGTTAGTTTGGGGCAGCGAGCT
 ACAATAAGTTGCAAGGCGAGCCAATCCGTGGATTATGATGGAGACAGCTATCTTAA
 CTGGTATCAGCAAATTCCAGGCCAGCCACCCAAGTTGCTGATCTACGACGCGTCAAA
 CCTGGTCTCAGGGATCCCTCCAAGATTTAGCGGCTCAGGTTACGGATTTTAC
 GCTCAATATCCATCCTGTAGAGAAGGTTGATGCAGCTACATACTGTCAACAGAG
 TACCGAGGATCCTTGGACCTTCGGAGGCGGTACAAAGCTGGAGATCAAGAGAACCG
 TGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA
 CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGT
 GGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAG
 GACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAGCAGA
 CTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGC
 CCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAA (SEQ ID NO: 102)

[00479] In FIG. 3, the SDS-PAGE results show the expression of ACE-02, ACE-03, ACE-00 and ACE-01. It also shows the difference in assembly patterns of ACE-02 and ACE-02-VL2 and the difference in assembly patterns of ACE-03 and ACE-03-VL2. These results suggest that ACE-03 was properly expressed and assembled.

1.4. Construction and Expression of ACE-04

[00480] A HEK-293 transient expression system (Invitrogen, USA) was used for expressing ALiCE molecule ACE-04 provided herein. ACE-04 is composed of anti-PD-L1 and chimeric OKT3 Fab domains (see FIG. 4A). ACE-04 contains two different heavy chain like chains ACE-04-VH (VL-CL-VH-CH1) and ACE-04-VL (VH-CH1-VL-CL) and two identical light chains (ACE-04-LC). The amino acid sequences of these three type of polypeptides are as follows:

ACE-04-VH amino acid sequence:

QMQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGRIIPILG
 IANYAQKFQGRVTITADKSTSTAYMELSSLRSED~~TAVYYCAKPRDGYNLVAFDIWG~~
 QGTMVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
 VHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]*DKTHT*
*CPPCPAPELLGGP*ggggsQVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQ
 RPGQGLEWIGYINPSRGYTNYNQKF~~DKATLT~~TDKSSSTAYMQLSSLTSEDSAVYY
 CARYYDDHYCLDYWGQGTTVTVSA[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDY
 FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNT
 KVDKRVEPKSC] (SEQ ID NO: 93)

ACE-04-VL amino acid sequence:

QMQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGRIIPILG
 IANYAQKFQGRVTITADKSTSTAYMELSSLRSED~~TAVYYCAKPRDGYNLVAFDIWG~~
 QGTMVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
 VHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]*DKTHT*
*CPPCPAPELLGGP*ggggsQIVLTQSPA~~IMSASPGEKVTMTCSASSSVSYMNWYQQKSGT~~
 SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAE~~DAATYYCQQWSSNPFTF~~
 GSGTKLEIN[RTVAAPS~~VFIFPPSDEQLKSGTASVVCLLNNFY~~PREAKVQWKVDNALQS
 GNSQESVTEQDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC]
 (SEQ ID NO: 94)

ACE-04-LC amino acid sequence (anti-PD-L1 antibody light chain):

QLVLTQPPSVSGAPGQRVTISCTGSSNIGAGYDVHWYQQLPGAAPKLLIYGDINRP
 SGVPDRFSGSKSGISASLAITGLQAEDEADYYCQSYDSSLGGVFGGGTKLTVL[RSV
 AAPSVFIFPPSDEQLKSGTASVVCLLNNFY~~PREAKVQWKVDNALQSGNSQESVTEQDSK~~
 DSTYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC] (SEQ ID NO: 95)

[00481] The VH and VL amino acid sequences and CDR sequences therein for the first antigen binding domain bivalent Fab region targeting PD-L1 and the second antigen binding domain monovalent Fv region of chimeric OKT3 Fab region are listed in the table below:

Table 7: VHs, VLs and CDRs of ACE-04

Fab region (Anti-PD-L1)	VH: QMQLVQSGAEVKKPGSSVKV SCK ASGGTFSSYAISWVRQAPGQGLE WMGRIIPILGIANYAQKFQGRVTIT ADKSTSTAYMELSSLRSEDTAVYY CAKPRDGYNLVAFDIWGQGTMTVT VSS (SEQ ID NO: 4)	VL: QLVLTQPPSVSGAPGQRVTISCTG SSSNIGAGYDVHWYQQLPGAAPK LLIYGDINRPSGVPDRFSGSKSGIS ASLAITGLQAEDEADYYCQSYDS SLSGGVFGGGTKLTVLR (SEQ ID NO: 8)
	CDR H1: GGTFSSYA (SEQ ID NO: 5)	CDR L1: SSNIGAGYD (SEQ ID NO: 9)
	CDR H2: IIPILGIA (SEQ ID NO: 6)	CDR L2: GDI (SEQ ID NO: 10)
	CDR H3: AKPRDGYNLVAFDI (SEQ ID NO: 7)	CDR L3: QSYDSSLGGV (SEQ ID NO: 11)
Fv region (Anti-CD3)	VH: QVQLQQSGAELARPGASVKMSCK ASGYTFTRYTMHWVKQRPGQGLE WIGYINPSRGYTNYNQKFKDKATL TTDKSSSTAYMQLSSLTSEDSAVY YCARYYDDHYCLDYWGQGT TTVT VSA (SEQ ID NO: 85)	VL: QIVLTQSPA IMSASPGEKVTMTCS ASSSVSYMNWYQQKSGTSPKRWI YDTSK LASGVPAHFRGSGSGTSY SLTISGMEAEDAATYYCQQWSSN PFTFGSGTKLEINR (SEQ ID NO: 86)
	CDR H1: GYTFTRYT (SEQ ID NO: 78)	CDR L1: SSVSY (SEQ ID NO: 82)
	CDR H2: INPSRGYT (SEQ ID NO: 79)	CDR L2: DTS (SEQ ID NO: 83)
	CDR H3: ARYYDDHYCLDY (SEQ ID NO: 80)	CDR L3: QQWSSNPF (SEQ ID NO: 87)

[00482] DNA sequences encoding ACE-04-VH, ACE-04-VL and ACE-04-LC are as follows:

ACE-04-VH nucleotide sequence:

CAGATGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAA
GGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCG
ACAGGCCCTGGACAAGGGCTTGAGTGGATGGGAAGGATCATCCCTATCCTTGGTAT

AGCAAACCTACGCACAGAAGTTCCAGGGCAGAGTCACGATTACCGCGGACAAATCCA
CGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTAT
TACTGTGCGAAACCGAGAGATGGCTACAATTTGGTTGCTTTTGATATCTGGGGCCAA
GGGACGATGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTG
GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA
GGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCG
GCGTGACACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCG
TGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAATC
ACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA
ACTCACACATGCCACCGTGCCAGCACCTGAACTCCTGGGGGGACCGGGCGGAGG
TGGGAGTCAGGTCCAGTTGCAACAGTCTGGAGCCGAGCTCGCCAGGCCAGGAGCCT
CCGTCAAAATGTCATGCAAGGCCTCAGGGTACACATTTACGCGATATACCATGCACT
GGGTGAAACAAAGACCAGGTGAGGGACTTGAATGGATCGGTTACATTAACCCCTCT
AGAGGCTATACGAATTACAACCAGAAATTCAAAGACAAAGCAACACTTACGACTGA
CAAATCCAGTAGTACGGCTTACATGCAGCTCTCATCTTTGACTTCAGAAGACTCTGC
TGTATATTATTGTGCCCGCTATTACGATGACCATTACTGCCTTGATTACTGGGGCCAG
GGCACTACTGTTACCGTAAGTGCGGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTG
GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA
GGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCG
GCGTGACACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCG
TGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAATC
ACAAGCCCAGCAACACCAAGGTGGACAAGAGAGTTGAGCCCAAATCTTGTGTA

(SEQ ID NO: 105)

ACE-04-VL nucleotide sequence:

CAGATGCAGCTGGTGCACTCTGGGGCTGAGGTGAAGAAGCCTGGGTCTCTCGGTGAA
GGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCG
ACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGAAGGATCATCCCTATCCTTGGTAT
AGCAAACCTACGCACAGAAGTTCCAGGGCAGAGTCACGATTACCGCGGACAAATCCA
CGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTAT
TACTGTGCGAAACCGAGAGATGGCTACAATTTGGTTGCTTTTGATATCTGGGGCCAA
GGGACGATGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTG

GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA
GGACTACTTCCCCGAACCGGTGACGGTGTCTGTGGAACCTCAGGCGCCCTGACCAGCG
GCGTGACACACCTTCCC GGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCG
TGGTGACCGTGCCCTCCAGCAGCCTGGGACCCAGACCTACATCTGCAACGTGAATC
ACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA
ACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGGGCGGAGG
TGGGAGTCAGATCGTCCTCACTCAAAGTCCTGCTATTATGTCCGCAAGCCCTGGTGA
AAAGGTTACCATGACTTGCTCCGCATCTAGTTCTGTCTCTTACATGAACTGGTACCA
GCAAAAGTCTGGAACGTCCCCGAAAAGGTGGATATATGATACGAGCAAATTGGCAA
GCGGAGTACCCGCGCATTTTAGGGGTTTCAAGGAGCGGTACGTCATATAGCCTGACTA
TTAGCGGAATGGAGGCGGAGGATGCTGCAACATATTATTGCCAACAATGGTCATCA
AATCCTTTTACTTTCGGCTCAGGCACAAAACCTTGAAATAAATAGAACCGTGGCTGCA
CCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACCTGCCTCTG
TTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGG
ATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAG
GACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAA
ACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAA
AGAGcTTCAACAGGGGAGAGTGTTAA (SEQ ID NO: 106)

ACE-04-LC (anti-PD-L1 antibody light chain nucleotide sequence):

CAGCTCGTGCTGACTCAGCCGCCCTCAGTGTCTGGGGCCCCAGGGCAGAGGGTCAC
CATCTCCTGCACTGGGAGCAGCTCCAACATCGGGGCAGGTTATGATGTACACTGGTA
TCAGCAACTTCCAGGAGCAGCCCCCAAACCTCCTCATCTATGGCGACATCAATCGGCC
CTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCTGGCATCTCAGCCTCCCTGGC
TATCACTGGGCTCCAGGCTGAGGACGAGGCTGATTATTACTGCCAGTCCTATGACAG
CAGCCTGAGTGGGGGGGTGTTCTGGCGGAGGGACCAAGCTGACCGTCCTAAGATCTG
TGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA
CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGT
GGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAG
GACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGA
CTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGC
CCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAG (SEQ ID NO: 107)

[00483] In FIG. 4C, the SDS-PAGE results show the expression of ACE-04 and ACE-05, the difference in the assembly patterns of ACE-04 and ACE-04-VL2 and the difference in the assembly patterns of ACE-05 and ACE-05-VL2. The results suggest that ACE-04 was properly expressed and assembled.

1.5. Construction and Expression of ACE-05

[00484] A HEK-293 transient expression system (Invitrogen, USA) was used for expressing another ALiCE molecule provided herein ACE-05 (a binding molecule composed of anti-PD-L1 and anti-CD3 domains; see FIG. 4B). ACE-05 contains two different heavy chain like chains (ACE-05-VH and ACE-05-VL) and two identical light chains (ACE-05-LC). ACE-05 contains a G4S linker (amino acid sequence of GGGGS, SEQ ID NO: 112) in the flexible peptide region. The amino acid sequences of these three type of polypeptides are as follows:

ACE-05-VH amino acid sequence:

**QMQLVQSGAEVKKPGSSVKVSCKASGGTFESSYAISWVRQAPGQGLEWMGRIIPILG
IANYAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYCAKPRDGYNLVAFDIWG
QGTMTVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]DKTHT
CPPCPAPELLGGPggggsEVQLQQSGPELVKPGPSMKISCKASGYSFTGYTMNWVKQS
HGKNLEWMGLINPYKGVSTYNQKFQDKATLTVDKSSSTAYMELLSLTSEDSAVYY
CARSGYYGDSDWYFDVWGQGTTLTVFS (SEQ ID NO: 1)**

ACE-05-VL amino acid sequence:

**QMQLVQSGAEVKKPGSSVKVSCKASGGTFESSYAISWVRQAPGQGLEWMGRIIPILG
IANYAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYCAKPRDGYNLVAFDIWG
QGTMTVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]DKTHT
CPPCPAPELLGGPggggsDIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPD
GTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLPWTF
AGGTKLEIKR (SEQ ID NO: 2)**

ACE-05-LC amino acid sequence:

QLVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQLPGAAPKLLIYGDNRP

SGVPDRFSGSKSGISASLAITGLQAEDEADYYCQSYDSSLGGVFGGGTKLTVL[RTV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK
DSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC] (SEQ ID NO: 3)

[00485] The VH and VL amino acid sequences and CDR sequences therein for the first antigen binding domain bivalent Fab region targeting PD-L1 and the second antigen binding domain monovalent Fv region targeting CD3 are listed in the table below:

Table 8: VHs, VLs and CDRs of ACE-05

Fab region (Anti-PD-L1)	VH: QMQLVQSGAEVKKPGSSVKVSCK ASGGTFSSYAISWVRQAPGQGLE WMGRIIPILGIANYAQKFQGRVTIT ADKSTSTAYMELSSLRSEDTAVYY CAKPRDGYNLVAFDIWGQGTMTVT VSS (SEQ ID NO: 4)	VL: QLVLTQPPSVSGAPGQRVTISCTG SSSNIGAGYDVHWYQQLPGAAPK LLIYGDINRPSGVPDRFSGSKSGIS ASLAITGLQAEDEADYYCQSYDS SLSGGVFGGGTKLTVLR (SEQ ID NO: 8)
	CDR H1: GGTFSSYA (SEQ ID NO: 5)	CDR L1: SSNIGAGYD (SEQ ID NO: 9)
	CDR H2: IIPILGIA (SEQ ID NO: 6)	CDR L2: GDI (SEQ ID NO: 10)
	CDR H3: AKPRDGYNLVAFDI (SEQ ID NO: 7)	CDR L3: QSYDSSLGGV (SEQ ID NO: 11)
Fv region (Anti-CD3)	VH: EVQLQQSGPELVKPGPSMKISCKA SGYSFTGYTMNWVKQSHGKNLE WMGLINPYKGVSTYNQKFKDKAT LTVDKSSSTAYMELLSLTSEDSAV YYCARSGYYGDSDWYFDVWGQG TTLTVFS (SEQ ID NO: 12)	VL: DIQMTQTTSSLSASLGDRVTISCR ASQDIRNYLNWYQQKPDGTVKL LIYYTSRLHSGVPSKFSGSGSGTD YSLTISNLEQEDIATYFCQQGNTL PWTFAGGTKLEIKR (SEQ ID NO: 16)
	CDR H1: GYSFTGYTMN (SEQ ID NO: 13)	CDR L1: RASQDIRNYLN (SEQ ID NO: 17)

	CDR H2: LINPYKGVST (SEQ ID NO: 14)	CDR L2: YTSRLHS (SEQ ID NO: 18)
	CDR H3: SGYYGDSDWYFDV (SEQ ID NO: 15)	CDR L3: QQGNTLPWT (SEQ ID NO: 19)

[00486] A tri-transfection was performed to transfect the host cells with DNA of ACE-05-VH, ACE-05-VL and ACE-05-LC (at 0.5:0.5:1 w/w ratio). DNA sequences encoding ACE-05-VH, ACE-05-VL and ACE-05-LC are as follows:

ACE-05-VH nucleotide sequence:

CAGATGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAA
 GGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCG
 ACAGGCCCTGGACAAGGGCTTGAGTGGATGGGAAGGATCATCCCTATCCTTGGTAT
 AGCAAACCTACGCACAGAAGTTCCAGGGCAGAGTCACGATTACCGCGGACAAATCCA
 CGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTAT
 TACTGTGCGAAACCGAGAGATGGCTACAATTTGGTTGCTTTTGATATCTGGGGCCAA
 GGGACGATGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTG
 GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA
 GGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCG
 GCGTGACACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCG
 TGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAATC
 ACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA
 ACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGGGCGGAGG
 TGGGAGTGAGGTGCAGCTCCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGACCTT
 CAATGAAGATATCCTGCAAGGCTTCTGGTTACTCATTCACTGGCTACACCATGAACT
 GGGTGAAGCAGAGTCATGGAAAGAACCTTGAGTGGATGGGACTTATTAATCCTTAC
 AAAGGTGTTAGTACCTACAACCAGAAGTTCAAGGACAAGGCCACACTGACTGTAGA
 CAAGTCATCCAGCACAGCCTACATGGAACCTCCTCAGTCTGACATCTGAGGACTCTGC
 AGTCTATTACTGTGCAAGATCGGGGTACTACGGTGATAGTGACTGGTACTTCGATGT
 CTGGGGGCCAGGGGACCACGCTGACCGTCTTCTCATAA (SEQ ID NO: 20)

ACE-05-VL nucleotide sequence:

CAGATGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAA

GGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCG
ACAGGCCCTGGACAAGGGCTTGAGTGGATGGGAAGGATCATCCCTATCCTTGGTAT
AGCAAACCTACGCACAGAAGTTCCAGGGCAGAGTCACGATTACCGCGGACAAATCCA
CGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTAT
TACTGTGCGAAACCGAGAGATGGCTACAATTTGGTTGCTTTTGATATCTGGGGCCAA
GGGACGATGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTG
GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA
GGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCG
GCGTGACACACCTTCCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCG
TGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAATC
ACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA
ACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGGGCGGAGG
TGGGAGTGACATCCAGATGACCCAGACCACCTCCTCCCTGTCTGCCTCCCTGGGCGA
CAGAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGAAATTATTTAAACTGGT
ATCAACAGAAACCAGATGGAACCTGTTAACTCCTGATCTACTACACATCAAGATTAC
ACTCAGGAGTCCCATCAAAGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCA
CCATTAGCAACCTGGAGCAAGAGGATATTGCCACTTACTTTTGCCAACAGGGTAATA
CGCTTCCGTGGACGTTTCGCTGGAGGCACCAAGCTGGAAATCAAACGGTAA (SEQ ID
NO: 21)

ACE-05-LC nucleotide sequence:

CAGCTCGTGCTGACTCAGCCGCCCTCAGTGTCTGGGGCCCCAGGGCAGAGGGTCAC
CATCTCCTGCACTGGGAGCAGCTCCAACATCGGGGCAGGTTATGATGTACACTGGTA
TCAGCAACTTCCAGGAGCAGCCCCCAAACCTCCTCATCTATGGCGACATCAATCGGCC
CTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCTGGCATCTCAGCCTCCCTGGC
TATCACTGGGCTCCAGGCTGAGGACGAGGCTGATTATTACTGCCAGTCCTATGACAG
CAGCCTGAGTGGGGGGGTGTTTCGGCGGAGGGACCAAGCTGACCGTCCTAAGAA^{acc}GT
GGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAC
TGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTG
GAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGG
ACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGAC

TACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCC
CGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAA (SEQ ID NO: 22)

[00487] Transfection was performed as described above in Section 1.1. More specifically, polyethylenimine (PEI) was used as a transfection reagent (used at a ratio of DNA: PEI = 1:4 (w/w)). Six to seven days after the transfection when the cell survival rate was measured to be about 60% to 70%, the batch culture was discontinued, and the expression medium was collected and centrifuged (4,800 rpm, 30 min, 4°C) to remove the debris. The supernatant was then filtered by using a 0.22 µm TOP-filter (Millipore, USA). Subsequently, the filtered supernatant including ACE-05 molecules underwent an affinity chromatography purification process using Hitrap™ KappaSelect (GE healthcare, USA), followed by dialysis with pH 7.4 PBS using Slide-A Lyzer Dialysis Cassette (Thermo, USA) for elution buffer change. Purified proteins were analyzed by SDS-PAGE, capillary electrophoresis, and size exclusion chromatography (SEC). The level of expression using HEK-293F transient expression system was determined to be around 50 mg/L in this experiment. The purification assay using Hitrap™ KappaSelect showed that most of the molecules expressed in the medium were recovered. The purified ACE-05 molecules were also analyzed for their purity using Agilent 2100 Bioanalyzer (Agilent Technologies, Germany) and SEC-HPLC (ThermoFisher, USA). The purity analysis was performed using the protocols provided by the manufactures.

[00488] In FIG. 4C, the SDS-PAGE results show the expression of ACE-05 and ACE-04, the difference in the assembly patterns of ACE-05 and ACE-05-VL2, and the difference in the assembly patterns of ACE-04 and ACE-04-VL2. FIG. 4D shows the results of the SDS-PAGE performed to identify the assembly pattern of ACE-05 (top) and illustrates the potential regulatory mechanism in ACE-05 assembly.

[00489] FIGs. 4E-4F show the SDS-PAGE and capillary electrophoresis performed to identify the conformations of ACE-05 as well as heterodimerization efficiency between ACE-05-VH and ACE-05-VL chains. The left panels in FIGs. 4E-4F show SDS-PAGE results of purified ACE-05. The left panel in FIG. 4E also shows the results of affinity chromatography for ACE-05 and ACE-05-VL2 proteins using KappaSelect. The right four panels in FIGs. 4E-4F show almost the same amount of ACE-05-VH and ACE-05-VL chains, the amount of the ACE-05-LC, and high efficiency of heterodimerization between ACE-05-VH and ACE-05-VL chains. The capillary electrophoresis result also suggests that ACE-05 was properly expressed and assembled. FIG.

4G shows the size exclusion chromatography performed to identify purity of ACE-05. As shown, KappaSelect purified sample contains free light chains. A second gel filtration chromatography (GFC) step was then applied to remove free light chains, which generated pure and properly assembled ACE-05 molecules. As expected, ACE-05-VH and ACE-05-LC dimer and ACE-05-VL and ACE-05-LC dimer were observed in NR lane of the SDS-PAGE image. Hydrophobic interaction between ACE-05-VH and ACE-05-VL was broken by SDS detergent. [00490] FIG. 4H shows the results of size exclusion chromatography for gel filtration analysis of ACE-05. The gel filtration analysis was performed to examine ACE-05 conformations after kappa-select affinity purification. About 19% aggregation of ACE-05 was detected by Superdex 200A column chromatography. FIG. 4I shows the results of cationic exchange chromatography (CEX) performed to identify the structural conformations of ACE-05. The major peak isolated from gel filtration was analyzed and separated into 2 peaks through CEX column. The higher peak (67.87%) is the assembled ACE-05, and the lower peak (32%) is the ACE-05-VH chain that has free thiol groups in the hinge region.

1.6. Construction and Expression of ACE-09

[00491] A HEK-293 transient expression system (Invitrogen, USA) was also used for corroborating the proper expression and assembly of ACE-09 (a binding molecule composed of anti-PD-L1 and UCHT1 domains) using a similar method described in Sections above. ACE-09 contains two different heavy chain like chains (ACE-09-VH and ACE-09-VL) and two identical light chains (ACE-09-LC) (FIG. 5 bottom). In comparison to ACE-05, ACE-09 does not contain the G4S linker (amino acid sequence of GGGGS) that ACE-05 contains in the flexible peptide region (FIG. 5 bottom). The amino acid sequences of these three type of polypeptides are as follows:

ACE-09-VH amino acid sequence (without the G4S linker):

**QMQLVQSGAEVKKPGSSVKVSCKASGGTFESSYAISWVRQAPGQGLEWMGRIIPILG
 IANYAQKFQGRVTITADKSTSTAYMELSSLRSEDVAVYYCAKPRDGYNLVAFDIWG
 QGTMVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
 VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]DKTHT
 CPPCPAPELLGGPEVQLQQSGPELVKPGPSMKISCKASGYSETGYTMNWVKQSHGK**

NLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYCARS
GYYGDSWDWYFDVWGQGTTLTVFS (SEQ ID NO: 96)

ACE-09-VL amino acid sequence (without the G4S linker):

QMQLVQSGAEVKKPGSSVKVSCASGGTFSSYAISWVRQAPGQGLEWMGRIIPILG
IANYAQKFQGRVTITADKSTSTAYMELSSLRSED**TA****VYYCAKPRDGYNLVAFDIWG**
QGTMTVTVSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]*DKTHT*
*CPPCPAPELLGGPD***IQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDGTV**
KLLIYYTSRLHSGVPSKFS**SGSGTDYSLTISNLEQEDIATYFCQQGNTLPWTFAGG**
TKLEIKR (SEQ ID NO: 97)

ACE-09-LC amino acid sequence (anti-PD-L1 antibody light chain):

QLVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQLPGAAPKLLIYG**DINRP**
SGVPDRFSGSKSGISASLAITGLQAEDEADYYCQSYDSSLGGVFGGGTKLTVL**[RSV**
AAPSVFIFPPSDEQLKSGTASVVCLLNFPREAKVQWKVDNALQSGNSQESVTEQDSK
DSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC] (SEQ ID NO: 95)

[00492] The VH and VL amino acid sequences and CDR sequences therein for the bivalent Fab region targeting PD-L1 and the UCHT1 are listed in the table below:

Table 9: VHs, VLs and CDRs of ACE-09

Fab region	VH:	VL:
(Anti-PD-L1)	QMQLVQSGAEVKKPGSSVKVSCK ASGGTFSSYAISWVRQAPGQGLE WMGRIIPILGIANYAQKFQGRVTIT ADKSTSTAYMELSSLRSED TA VYYCAKPRDGYNLVAFDIWGQGTMTV VSS (SEQ ID NO: 4)	QLVLTQPPSVSGAPGQRVTISCTG SSSNIGAGYDVHWYQQLPGAAPK LLIYG DINRPSGVPDRFSGSKSGIS ASLAITGLQAEDEADYYCQSYDS SLSGGVFGGGTKLTVLR (SEQ ID NO: 8)

	CDR H1: GGTFSYA (SEQ ID NO: 5)	CDR L1: SSNIGAGYD (SEQ ID NO: 9)
	CDR H2: IIPILGIA (SEQ ID NO: 6)	CDR L2: GDI (SEQ ID NO: 10)
	CDR H3: AKPRDGYNLVAFDI (SEQ ID NO: 7)	CDR L3: QSYDSSLGGV (SEQ ID NO: 11)
Fv region (Anti-CD3)	VH: EVQLQQSGPELVKPGPSMKISCKA SGYSFTGYTMNWVKQSHGKNLE WMGLINPYKGVSTYNQKFKDKAT LTVDKSSSTAYMELLSLTSEDSAV YYCARSGYYGDSDWYFDVWGQG TTLTVFS (SEQ ID NO: 12)	VL: DIQMTQTTSSLSASLGDRVTISCR ASQDIRNYLNWYQQKPDGTVKL LIYYTSRLHSGVPSKFSGSGSGTD YSLTISNLEQEDIATYFCQQGNTL PWTFAGGTKLEIKR (SEQ ID NO: 16)
	CDR H1: GYSFTGYTMN (SEQ ID NO: 13)	CDR L1: RASQDIRNYLN (SEQ ID NO: 17)
	CDR H2: LINPYKGVST (SEQ ID NO: 14)	CDR L2: YTSRLHS (SEQ ID NO: 18)
	CDR H3: SGYYGDSDWYFDV (SEQ ID NO: 15)	CDR L3: QQGNTLPWT (SEQ ID NO: 19)

[00493] DNA sequences encoding ACE-09-VH, ACE-09-VL and ACE-09-LC are as follows:

ACE-09-VH nucleotide sequence (without G4S linker):

CAGATGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAA
GGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCG
ACAGGCCCTGGACAAGGGCTTGAGTGGATGGGAAGGATCATCCCTATCCTTGGTAT
AGCAAACCTACGCACAGAAGTTCCAGGGCAGAGTCACGATTACCGCGGACAAATCCA
CGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTAT
TACTGTGCGAAACCGAGAGATGGCTACAATTTGGTTGCTTTTGATATCTGGGGCCAA
GGGACGATGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTG
GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA
GGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCG
GCGTGACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCG

TGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAATC
ACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA
ACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGGACcGGAGGTGCA
GCTCCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGACCTTCAATGAAGATATCCTG
CAAGGCTTCTGGTTACTCATTCACTGGCTACACCATGAACTGGGTGAAGCAGAGTCA
TGGAAGAAGAACCTTGAGTGGATGGGACTTATTAATCCTTACAAAGGTGTTAGTACCTA
CAACCAGAAGTTCAAGGACAAGGCCACACTGACTGTAGACAAGTCATCCAGCACAG
CCTACATGGAACCTCCTCAGTCTGACATCTGAGGACTCTGCAGTCTATTACTGTGCAA
GATCGGGGTACTACGGTGATAGTGACTGGTACTTCGATGTCTGGGGCCAGGGGACC
ACGCTGACCGTCTTCTCATAA (SEQ ID NO: 108)

ACE-09-VL nucleotide sequence (without G4S linker):

CAGATGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCTCGGTGAA
GGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCG
ACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGAAGGATCATCCCTATCCTTGGTAT
AGCAAACCTACGCACAGAAGTTCCAGGGCAGAGTCACGATTACCGCGGACAAATCCA
CGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTAT
TACTGTGCGAAACCGAGAGATGGCTACAATTTGGTTGCTTTTGATATCTGGGGCCAA
GGGACGATGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTG
GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA
GGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCG
GCGTGACACCTTCCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCG
TGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAATC
ACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA
ACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGGAccgGACATCCAG
ATGACCCAGACCACCTCCTCCCTGTCTGCCTCCCTGGGCGACAGAGTCACCATCAGT
TGCAGGGCAAGTCAGGACATTAGAAATTATTTAAACTGGTATCAACAGAAACCAGA
TGGAACCTGTTAAACTCCTGATCTACTACACATCAAGATTACACTCAGGAGTCCCATC
AAAGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAGCAACCTGGA
GCAAGAGGATATTGCCACTTACTTTTGCCAACAGGGTAATACGCTTCCGTGGACGTT
CGCTGGAGGCACCAAGCTGGAAATCAAACGGTAA (SEQ ID NO: 109)

ACE-09-LC nucleotide sequence (anti-PD-L1 antibody light chain nucleotide sequence):

CAGCTCGTGCTGACTCAGCCGCCCTCAGTGTCTGGGGCCCCAGGGCAGAGGGTCAC
 CATCTCCTGCACTGGGAGCAGCTCCAACATCGGGGCAGGTTATGATGTACACTGGTA
 TCAGCAACTTCCAGGAGCAGCCCCAACTCCTCATCTATGGCGACATCAATCGGCC
 CTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCTGGCATCTCAGCCTCCCTGGC
 TATCACTGGGCTCCAGGCTGAGGACGAGGCTGATTATTACTGCCAGTCCTATGACAG
 CAGCCTGAGTGGGGGGGTGTTCTGGCGGAGGGACCAAGCTGACCGTCCTAAGATCTG
 TGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA
 CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGGCCAAAGTACAGT
 GGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAG
 GACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGA
 CTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGC
 CCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAG (SEQ ID NO: 107)

[00494] FIG. 5 shows the SDS-PAGE results of affinity chromatography for ACE-09 using KappaSelect and the SDS-PAGE result of ACE-09 and ACE-05 at 37 °C and 32 °C. The results indicate that ACE-09 was properly expressed and assembled. Furthermore, ACE-09 has the same VHs and VLs in the Fab and Fv regions as ACE-05 (shown in Tables 8 and 9), but ACE-09 does not contain the G4S linker (amino acid sequence of GGGGS) in ACE-05 between the antibody hinge region and the second Fv domain (FIG. 5 bottom). G4S flexible linkers may reduce steric hindrance and optimize the binding of the second Fv domain to immune cells (*e.g.*, effector cells including T cells), and thus lead to increased redirecting efficiency of immune cells to target cells (*e.g.*, cancer cells). Unexpectedly, ACE-05 and ACE-09 showed similar level of expression, suggesting that the G4S linker may offer flexibility that benefits the ALiCE molecules' activities without affecting their expression and assembly.

1.7. Construction and Expression of ACE-10

[00495] A HEK-293 transient expression system (Invitrogen, USA) was also used for corroborating the proper expression and assembly of ACE-10 (a binding molecule composed of anti-CD20 and anti-CD3 domains; see FIG. 6A) using a similar method described in Section 1.1 and Section 1.2 above. ACE-10 contains two different heavy chain like chains (ACE-10-VH and ACE-10-VL) and two identical light chains (ACE-10-LC). The amino acid sequences of these three type of polypeptides are as follows:

ACE-10-VH amino acid sequence:

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPG
NGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFN
VWGAGTTVTVSA[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]DK
*THTCPPCPAPELLGPP*ggggs**EVQLQQSGPELVKPGPSMKISCKASGYSFTGYTMNWV**
KQSHGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSA
VYYCARSGYYGDSDWYFDVWGQGTTTLTVFS (SEQ ID NO: 23)

ACE-10-VL amino acid sequence:

QVQLQQPGAELVKPGASVKMSCKASGYTFTSYNMHWVKQTPGRGLEWIGAIYPG
NGDTSYNQKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYYCARSTYYGGDWYFN
VWGAGTTVTVSA[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]DK
*THTCPPCPAPELLGPP*ggggs**DIQMTQTSSLSASLGDRVTISCRASQDIRNYLNWYQQ**
KPDGTVKLLIYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLP
WTFAGGTKLEIKR (SEQ ID NO: 24)

ACE-10-LC amino acid sequence:

QIVLSQSPAILSASPGEKVTMTTCRASSSVSYIHWFQQKPGSSPKPWIYATSNLASGVP
VRFSGSGSGTSYSLTISRVEAEDAATYYCQQWTSNPPTFGGGTKLEIK[RTVAAPSVE
IFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDYSL
SSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC] (SEQ ID NO: 25)

[00496] The VH and VL amino acid sequences and CDR sequences therein for the bivalent Fab region targeting CD20 and the monovalent Fv region targeting CD3 are listed in the table below:

Table 10: VHs, VLs and CDRs of ACE-10

Fab region	VH:	VL:
(Anti-CD20)	QVQLQQPGAELVKPGASVKMSCK ASGYTFTSYNMHWVKQTPGRGLE WIGAIYPGNGDTSYNQKFKGKATL TADKSSSTAYMQLSSLTSEDSAVY YCARSTYYGGDWYFNVWGAGTT VTVSA (SEQ ID NO: 26)	QIVLSQSPAILSASPGEKVTMTCR ASSSVSYIHWFQQKPGSSPKPWIY ATSNLASGVPVRFSGSGSGTSYSL TISRVEAEDAATYYCQQWTSNPP TFGGGTKLEIKR (SEQ ID NO: 30)

	CDR H1: GYTFTSYN (SEQ ID NO: 27)	CDR L1: SSVSY (SEQ ID NO: 31)
	CDR H2: IYPGNGDT (SEQ ID NO: 28)	CDR L2: ATS (SEQ ID NO: 32)
	CDR H3: ARSTYYGGDWYFNV (SEQ ID NO: 29)	CDR L3: QQWTSNPPT (SEQ ID NO: 33)
Fv region (Anti-CD3)	VH: EVQLQQSGPELVKPGPSMKISCKA SGYSFTGYTMNWVKQSHGKNLE WMGLINPYKGVSTYNQKFQDKAT LTVDKSSSTAYMELLSLTSEDSAV YYCARSGYYGDSDWYFDVWGQG TTLTVFS (SEQ ID NO: 12)	VL: DIQMTQTTSSLSASLGDRVTISCR ASQDIRNYLNWYQQKPDGTVKL LIYYTSRLHSGVPSKFSGSGSGTD YSLTISNLEQEDIATYFCQQGNTL PWTFAGGTKLEIKR (SEQ ID NO: 16)
	CDR H1: GYSFTGYTMN (SEQ ID NO: 13)	CDR L1: RASQDIRNYLN (SEQ ID NO: 17)
	CDR H2: LINPYKGVST (SEQ ID NO: 14)	CDR L2: YTSRLHS (SEQ ID NO: 18)
	CDR H3: SGYYGDSDWYFDV (SEQ ID NO: 15)	CDR L3: QQGNTLPWT (SEQ ID NO: 19)

[00497] DNA sequences encoding ACE-10-VH, ACE-10-VL and ACE-10-LC are as follows:

ACE-10-VH nucleotide sequence:

CAGGTGCAGCTGCAGCAGCCTGGAGCCGAGCTGGTGAAGCCCGGCCAGCGTGAA
GATGAGCTGCAAGGCCAGCGGCTACACCTTCACCAGCTACAACATGCACTGGGTGA
AGCAGACCCCTGGAAGAGGACTGGAGTGGATCGGCGCCATCTACCCCGGCAACGGC
GACACCAGCTACAACCAGAAGTTCAAGGGCAAGGCCACCCTGACCGCCGACAAGAG
CAGCAGCACCGCCTACATGCAGCTGAGCAGCCTGACCAGCGAGGACAGCGCCGTGT
ACTACTGCGCCCGCAGCACCTACTACGGCGGCGACTGGTACTTCAACGTGTGGGGA
GCTGGAACCACCGTGACCGTGAGCGCCGCTAGCACCAAGGGCCCATCGGTCTTCCC
CCTGGCACCCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGT
CAAGGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGACCA

GCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCA
GCGTGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTG
AATCACAAGCCCAGCAACACCAAGGTGGACAAGAAGGTTGAGCCCAAATCTTGTGA
CAAACTCACACATGCCACCGTGCCAGCACCTGAACTCCTGGGGGGACCGGGCG
GAGGTGGGAGTGAGGTGCAGCTCCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGA
CCTTCAATGAAGATATCCTGCAAGGCTTCTGGTTACTCATTCACTGGCTACACCATG
AACTGGGTGAAGCAGAGTCATGGAAAGAACCTTGAGTGGATGGGACTTATTAATCC
TTACAAAGGTGTTAGTACCTACAACCAGAAGTTCAAGGACAAGGCCACACTGACTG
TAGACAAGTCATCCAGCACAGCCTACATGGAACCTCAGTCTGACATCTGAGGACT
CTGCAGTCTATTACTGTGCAAGATCGGGGTACTACGGTGATAGTGACTGGTACTTCG
ATGTCTGGGGCCAGGGGACCACGCTGACCGTCTTCTCATAA (SEQ ID NO: 34)

ACE-10-VL nucleotide sequence:

CAGGTGCAGCTGCAGCAGCCTGGAGCCGAGCTGGTGAAGCCCGGCCAGCGTGAA
GATGAGCTGCAAGGCCAGCGGCTACACCTTACCAGCTACAACATGCACTGGGTGA
AGCAGACCCCTGGAAGAGGACTGGAGTGGATCGGCGCCATCTACCCCGGCAACGGC
GACACCAGCTACAACCAGAAGTTCAAGGGCAAGGCCACCCTGACCGCCGACAAGAG
CAGCAGCACCGCCTACATGCAGCTGAGCAGCCTGACCAGCGAGGACAGCGCCGTGT
ACTACTGCGCCCGCAGCACCTACTACGGCGGGCGACTGGTACTTCAACGTGTGGGGA
GCTGGAACCACCGTGACCGTGAGCGCCGCTAGCACCAAGGGCCCATCGGTCTTCCC
CCTGGCACCCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGT
CAAGGACTACTTCCCCGAACCGGTGACGGTGTCTGTGGAACCTCAGGCGCCCTGACCA
GCGGCGTGCACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCA
GCGTGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTG
AATCACAAGCCCAGCAACACCAAGGTGGACAAGAAGGTTGAGCCCAAATCTTGTGA
CAAACTCACACATGCCACCGTGCCAGCACCTGAACTCCTGGGGGGACCGGGCG
GAGGTGGGAGTGACATCCAGATGACCCAGACCACCTCCTCCCTGTCTGCCTCCCTGG
GCGACAGAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGAAATTATTAAAC
TGGTATCAACAGAAACCAGATGGAACCTGTAAACTCCTGATCTACTACACATCAAGA
TTACTCAGGAGTCCCATCAAAGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCT
CTCACCATTAGCAACCTGGAGCAAGAGGATATTGCCACTTACTTTTGCCAACAGGGT

AATACGCTTCCGTGGACGTTGCTGGAGGCACCAAGCTGGAAATCAAACGGTAA
(SEQ ID NO: 35)

ACE-10-LC nucleotide sequence:

CAGATCGTGCTGAGCCAGAGCCCtGctATCCTGAGCGCCAGCCCtGGCGAGAAGGTGA
CCATGACCTGCCGCGCCAGCAGCAGCGTGAGCTACATCCACTGGTTCCAGCAGAAG
CCCGGCAGCAGCCCCAAGCCCTGGATCTACGCCACCAGCAACCTGGCCAGCGGAGT
GCCTGTGCGCTTCAGCGGCAGCGGCAGCGGCACCAGCTACAGCCTGACCATCAGCA
GAGTGGAGGCTGAGGACGCCGCTACCTACTACTGCCAGCAGTGGACCAGCAACCCC
CCCACCTTCGGCGGCGGCACCAAGCTGGAGATCAAGAGAACCGTGGCTGCACCATC
TGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAACTGCCTCTGTTGTG
TGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAAGGTGGATAA
CGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTACAGAGCAGGACAGCAAGGACA
GCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAGCAGACTACGAGAAACAC
AAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAG
CTTCAACAGGGGAGAGTGTTAG (SEQ ID NO: 36)

[00498] FIGs. 6B-6C show the expression analysis of the ACE-10 molecule. As shown, ACE-10 was properly expressed and assembled. In addition, the results indicate that assembly of ACE-10 is regulated in a VH-BiP dependent manner.

1.8. Construction and Expression of ACE-11

[00499] A HEK-293 transient expression system (Invitrogen, USA) was also used for corroborating the proper expression and assembly of ACE-11 (a binding molecule composed of anti-EGFR and anti-CD3 domains; see FIG. 7A) using a similar method described in Section 1.1 and Section 1.2 above. ACE-11 has the same overall structure as ACE-05 and ACE-10, and contains two different heavy chain like chains (ACE-11-VH and ACE-11-VL) and two identical light chains (ACE-11-LC). The amino acid sequences of these three type of polypeptides are as follows:

ACE-11-VH amino acid sequence:

QVQLKQSGPGLVQPSSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSSG
NTDYNTPFTSRLSINKDNSKSQVFFKMNSLQSNDAIYYCARALTYDYEFAYWGQ
GTLVTVSA[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV

HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDDKKVEPKSC]*DKTHTC*
*PPCPAPELLGGP*ggggs**EVQLQQSGPELVKPGPSMKISCKAS****GYSTGYTMNWVKQSH**
GKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDSAVYYC
ARSGYYGDSDWYFDVWGQGTTLTVFS (SEQ ID NO: 37)

ACE-11-VL amino acid sequence:

QVQLKQSGPGLVQPSQSLSITCTV**SGFSLTNYGVHWVRQSPGKGLEWL****GVIWSSG**
NTDYNTPFTS**RLSINKDNSKSQVFFKMNSLQSN**DTAIYYC**ARALTYDYEFAYWGQ**
GTLVTVSA[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV
 HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDDKKVEPKSC]*DKTHTC*
*PPCPAPELLGGP*ggggs**DIQMTQT**TSSLSASLGDRVTISCRASQDIRNYLNWYQQKPDG
TVKLLIYYTSRLHSGVPSKFSSGSGSGTDYSLTISNLEQEDIA**TYFCQQGNTLPWTFA**
GGTKLEIKR (SEQ ID NO: 38)

ACE-11-LC amino acid sequence:

DILLTQSPVILSVSPGERVSFSCRASQ**SIGTNIHWYQ**QRTNGSPRLLIKY**ASESISGIPS**
RFSGSGSGTDFTLSINSVESEDIADYYC**QQNNNWPTTF**GAGTKLELK[RTVAAPSVEI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLS
STLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC] (SEQ ID NO: 39)

[00500] The VH and VL amino acid sequences and CDR sequences therein for the first antigen binding domain bivalent Fab region targeting EGFR and the second antigen binding domain monovalent Fv region targeting CD3 are listed in the table below:

Table 11: VHs, VLs and CDRs of ACE-11

Fab region	VH:	VL:
(Anti-CD20)	QVQLKQSGPGLVQPSQSL SITCTVS GFSLTNYGVHWVRQSPGKGLEWL GVIWSSGNTDYNTPTFTSRLSINKD NSKSQVFFKMNSLQSNDAIYYCA	DILLTQSPVILSVSPGERVSFSCRA S SIGTNIHWYQQRTNGSPRLLIK YASESISGIPSRFSGSGSGTDFTLSI NSVESEDIADYYCQQNNNWPTTF GAGTKLELKR (SEQ ID NO: 44)

	RALTYDYEFAYWGQGLTVSA (SEQ ID NO: 40)	
	CDR H1: GFSLTNYG (SEQ ID NO: 41)	CDR L1: QSIGTN (SEQ ID NO: 45)
	CDR H2: IWSGGNT (SEQ ID NO: 42)	CDR L2: YAS (SEQ ID NO: 46)
	CDR H3: ARALTYDYEFAY (SEQ ID NO: 43)	CDR L3: QQNNNWPTT (SEQ ID NO: 47)
Fv region (Anti-CD3)	VH: EVQLQQSGPELVKPGPSMKISCKA SGYSFTGYTMNWVKQSHGKNLE WMGLINPYKGVSTYNQKFKDKAT LTVDKSSSTAYMELLSLTSEDSAV YYCARSGYYGDSDWYFDVWGQG TTLTVFS (SEQ ID NO: 12)	VL: DIQMTQTTSSLSASLGDRVTISCR ASQDIRNYLNWYQQKPDGTVKL LIYYTSRLHSGVPSKFSGSGSGTD YSLTISNLEQEDIATYFCQQGNTL PWTFAGGTKLEIKR (SEQ ID NO: 16)
	CDR H1: GYSFTGYTMN (SEQ ID NO: 13)	CDR L1: RASQDIRNYLN (SEQ ID NO: 17)
	CDR H2: LINPYKGVST (SEQ ID NO: 14)	CDR L2: YTSRLHS (SEQ ID NO: 18)
	CDR H3: SGYYGDSDWYFDV (SEQ ID NO: 15)	CDR L3: QQGNTLPWT (SEQ ID NO: 19)

[00501] DNA sequences encoding ACE-11-VH, ACE-11-VL and ACE-11-LC are as follows:

ACE-11-VH nucleotide sequence:

CAAGTCCAACTGAAACAATCGGGTCCGGGTCTGGTCCAACCGTCCCAATCACTGAG
CATCACCTGTACCGTGTCTGGGCTTCTCGCTGACCAATTATGGTGTGCATTGGGTTCGT
CAGAGTCCGGGCAAAGGTCTGGAATGGCTGGGCGTTATTTGGTCCGGCGGTAATAC
CGATTACAACACCCCGTTTACGAGTCGCCTGTCCATCAATAAAGACAACCTCGAAAA
GCCAGGTGTTTTTCAAAATGAATTCAGTCAATCGAACGATACCGCGATTATTACT
GCGCACGTGCTCTGACGTATTACGACTATGAATTTGCCTACTGGGGCCAGGGTACCC
TGGTGACGGTTAGCGCGGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCCT

CCTCCAAGAGCACCTCTGGGGGACACAGCGGCCCTGGGCTGCCTGGTCAAGGACTAC
TTCCCCGAACCGGTGACGGTGTCTGTGGAAGTCAAGGCGCCCTGACCAGCGGCGTGCA
CACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGAC
CGTGCCCTCCAGCAGCCTGGGACCCAGACCTACATCTGCAACGTGAATCACAAGC
CCAGCAACACCAAGGTGGACAAGAAGGTTGAGCCCAAATCTTGTGACAAAACCTCAC
ACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGGGCGGAGGTGGGAG
TGAGGTGCAGCTCCAGCAGTCTGGACCTGAGCTGGTGAAGCCTGGACCTTCAATGA
AGATATCCTGCAAGGCTTCTGGTTACTCATTCACTGGCTACACCATGAACTGGGTGA
AGCAGAGTCATGGAAAGAACCTTGAGTGGATGGGACTTATTAATCCTTACAAAGGT
GTTAGTACCTACAACCAGAAGTTCAAGGACAAGGCCACACTGACTGTAGACAAGTC
ATCCAGCACAGCCTACATGGAACCTCCTCAGTCTGACATCTGAGGACTCTGCAGTCTA
TACTGTGCAAGATCGGGGTACTACGGTGATAGTGAAGTGGTACTTCGATGTCTGGGG
CCAGGGGACCACGCTGACCGTCTTCTCATAA (SEQ ID NO: 48)

ACE-11-VL nucleotide sequence:

CAAGTCCAAGTGAACAATCGGGTCCGGGTCTGGTCCAACCGTCCCAATCACTGAG
CATCACCTGTACCGTGTCTGGGCTTCTCGCTGACCAATTATGGTGTGCATTGGGTTCGT
CAGAGTCCGGGCAAAGGTCTGGAATGGCTGGGCGTTATTTGGTCCGGCGGTAATAC
CGATTACAACACCCCGTTTACGAGTCGCTGTCCATCAATAAAGACAACCTCGAAAA
GCCAGGTGTTTTTCAAATGAATTCAGTCAATCGAACGATACCGCGATTTATTACT
GCGCACGTGCTCTGACGTATTACGACTATGAATTTGCCTACTGGGGCCAGGGTACCC
TGGTGACGGTTAGCGCGGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCCT
CCTCCAAGAGCACCTCTGGGGGACACAGCGGCCCTGGGCTGCCTGGTCAAGGACTAC
TTCCCCGAACCGGTGACGGTGTCTGTGGAAGTCAAGGCGCCCTGACCAGCGGCGTGCA
CACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGAC
CGTGCCCTCCAGCAGCCTGGGACCCAGACCTACATCTGCAACGTGAATCACAAGC
CCAGCAACACCAAGGTGGACAAGAAGGTTGAGCCCAAATCTTGTGACAAAACCTCAC
ACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGGGCGGAGGTGGGAG
TGACATCCAGATGACCCAGACCACTCCTCCCTGTCTGCCTCCCTGGGCGACAGAGT
CACCATCAGTTGCAGGGCAAGTCAGGACATTAGAAATTATTTAAACTGGTATCAACA
GAAACCAGATGGAAGTGTAACTCCTGATCTACTACACATCAAGATTACACTCAGG
AGTCCCATCAAAGTTCAGTGGCAGTGGGTCTGGAACAGATTATTCTCTCACCATTAG

CAACCTGGAGCAAGAGGATATTGCCACTTACTTTTGCCAACAGGGTAATACGCTTCC
GTGGACGTTCGCTGGAGGCACCAAGCTGGAAATCAAACGGTAA (SEQ ID NO: 49)

ACE-11-LC nucleotide sequence:

GATATTCTGCTGACCCAGAGCCCGGTGATCCTGAGTGTTTCCCCGGGCGAACGTGTG
TCATTTTCGTGTCGCGCGAGCCAGTCTATTGGTACCAATATCCACTGGTATCAGCAA
CGTACGAACGGCTCTCCGCGCCTGCTGATTAAATACGCCAGTGAATCCATTTTCAGGC
ATCCCGAGCCGCTTTTCGGGCAGCGGTTCTGGCACCGATTTCACGCTGAGTATTAAC
TCCGTGGAATCAGAAGATATCGCAGACTATTACTGCCAGCAAAACAATAACTGGCC
GACCACGTTTGGTGCTGGCACCAAACTGGAAGTGAAGAACCCTGGGCTGCACCAT
CTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAAGTGCCTCTGTTGT
GTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGTGGAAGGTGGATA
ACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAGGACAGCAAGGAC
AGCACCTACAGCCTCAGCAGCACCTGACGCTGAGCAAAGCAGACTACGAGAAACA
CAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGA
GCTTCAACAGGGGAGAGTGTTAA (SEQ ID NO: 50).

[00502] FIG. 7B shows the expression and assembly of ACE-11 and ACE-11-VL2. The arrows indicate the bands of assembled ACE-11. The results suggest that ACE-11 was properly expressed and assembled.

1.9. Construction and Expression of ACE-12

[00503] A HEK-293 transient expression system (Invitrogen, USA) was also used for corroborating the proper expression and assembly of ACE-12 (a binding molecule composed of anti-PD-L1 and UCHT1 domains) using a similar method described in the Sections above. ACE-12 contains two different heavy chain like chains (ACE-12-VH and ACE-12-VL) and two identical light chains (ACE-12-LC). ACE-12 contains the G4S linker having amino acid sequences of GGGGSGGGGS (SEQ ID NO: 113) and GGS GGGGSG (SEQ ID NO: 114), whereas ACE-05 contains the G4S linker having amino acid sequence of GGGGS in the flexible peptide region. The amino acid sequences of these three type of polypeptides are as follows:

ACE-12-VH amino acid sequence (with 10 residues GGGGSGGGGS):

**QMQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGRIIPILG
IANYAQKFQGRVTITADKSTSTAYMELSSLRSEDVAVYYCAKPRDGYNLVAFDIWG**

**QGTMTVTSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]*DKTHT*
CPPCPAPELLGGPggggsggggsEVQLQQSGPELVKPGPSMKISCKASGYSTGYTMNW
VKQSHGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAYMELLSLTSEDS
AVYYCARSGYYGDSDWYFDVWGQGTTTLTVFS** (SEQ ID NO: 98)

ACE-12-VL amino acid sequence (with 9 residues GSGGGGSG):

**QMQLVQSGAEVKKPGSSVKVSCKASGGTFSSYAISWVRQAPGQGLEWMGRIIPILG
IANYAQKFQGRVTITADKSTSTAYMELSSLRSEDTAVYYCAKPRDGYNLVAFDIWG
QGTMTVTSS[ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC]*DKTHT*
CPPCPAPELLGGPggggsgggsgDIQMTQTSSLSASLGDRVTISCRASQDIRNYLNWYQQ
KPDGTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQEDIATYFCQQGNTLP
WTFAGGTKLEIKR** (SEQ ID NO: 99)

ACE-12-LC amino acid sequence (anti-PD-L1 antibody light chain):

**QLVLTQPPSVSGAPGQRVTISCTGSSSNIGAGYDVHWYQQLPGAAPKLLIYGDIINRP
SGVPDRFSGSKSGISASLAITGLQAEDEADYYCQSYDSSLGGVFGGGTKLTVL[RSV
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK
DSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC]** (SEQ ID NO: 95)

[00504] The VH and VL amino acid sequences and CDR sequences therein for the first antigen binding domain bivalent Fab region targeting PD-L1 and the second antigen binding domain monovalent Fv region of UCHT1 are listed in the table below:

Table 12: VHs, VLs and CDRs of ACE-12

Fab region (Anti-PD-L1)	VH: QMQLVQSGAEVKKPGSSVKVSCK ASGGTFSSYAISWVRQAPGQGLE WMGRIIPILGIANYAQKFQGRVTIT ADKSTSTAYMELSSLRSEDTAVYY	VL: QLVLTQPPSVSGAPGQRVTISCTG SSSNIGAGYDVHWYQQLPGAAPK LLIYGDIINRPSGVPDRFSGSKSGIS ASLAITGLQAEDEADYYCQSYDS
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	CAKPRDGYNLVAFDIWGQGTMTV VSS (SEQ ID NO: 4)	SLSGGVFGGGTKLTVLR (SEQ ID NO: 8)
	CDR H1: GGTFSSYA (SEQ ID NO: 5)	CDR L1: SSNIGAGYD (SEQ ID NO: 9)
	CDR H2: IIPILGIA (SEQ ID NO: 6)	CDR L2: GDI (SEQ ID NO: 10)
	CDR H3: AKPRDGYNLVAFDI (SEQ ID NO: 7)	CDR L3: QSYDSSLGGV (SEQ ID NO: 11)
Fv region (Anti-CD3)	VH: EVQLQQSGPELVKPGPSMKISCKA SGYSFTGYTMNWVKQSHGKNLE WMGLINPYKGVSTYNQKFKDKAT LTVDKSSSTAYMELLSLTSEDSAV YYCARSGYYGDSWDYFDVWGQG TTLTVFS (SEQ ID NO: 12)	VL: DIQMTQTTSSLSASLGDRVTISCR ASQDIRNYLNWYQQKPDGTVKL LIYYTSRLHSGVPSKFSGSGSGTD YSLTISNLEQEDIATYFCQQGNTL PWTFAGGTKLEIKR (SEQ ID NO: 16)
	CDR H1: GYSFTGYTMN (SEQ ID NO: 13)	CDR L1: RASQDIRNYLN (SEQ ID NO: 17)
	CDR H2: LINPYKGVST (SEQ ID NO: 14)	CDR L2: YTSRLHS (SEQ ID NO: 18)
	CDR H3: SGYYGDSWDYFDV (SEQ ID NO: 15)	CDR L3: QQGNTLPWT (SEQ ID NO: 19)

[00505] DNA sequences encoding ACE-12-VH, ACE-12-VL and ACE-12-LC are as follows:

ACE-12-VH nucleotide sequence (with 10 residues GGGGSGGGGS):

CAGATGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAA
GGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCG
ACAGGCCCTGGACAAGGGCTTGAGTGGATGGGAAGGATCATCCCTATCCTTGGTAT
AGCAAACACTACGCACAGAAGTTCCAGGGCAGAGTCACGATTACCGCGGACAAATCCA
CGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTAT
TACTGTGCGAAACCGAGAGATGGCTACAATTTGGTTGCTTTTGATATCTGGGGCCAA
GGGACGATGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTG
GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA

GGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCG
GCGTGACACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCG
TGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAATC
ACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA
ACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGGGCGGAGG
TGGGAGTGGAGGCGGAGGATCTGAGGTGCAGCTCCAGCAGTCTGGACCTGAGCTGG
TGAAGCCTGGACCTTCAATGAAGATATCCTGCAAGGCTTCTGGTTACTCATTCACTG
GCTACACCATGAACTGGGTGAAGCAGAGTCATGGAAAGAACCTTGAGTGGATGGGA
CTTATTAATCCTTACAAAGGTGTTAGTACCTACAACCAGAAGTTCAAGGACAAGGCC
ACACTGACTGTAGACAAGTCATCCAGCACAGCCTACATGGAACCTCCTCAGTCTGACA
TCTGAGGACTCTGCAGTCTATTACTGTGCAAGATCGGGGTACTACGGTGATAGTGAC
TGGTACTTCGATGTCTGGGGCCAGGGGACCACGCTGACCGTCTTCTCATAA (SEQ ID
NO: 110)

ACE-12-VL nucleotide sequence (with 9 residues GGS GGG GSG):

CAGATGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAA
GGTCTCCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCG
ACAGGCCCCTGGACAAGGGCTTGAGTGGATGGGAAGGATCATCCCTATCCTTGGTAT
AGCAAACCTACGCACAGAAGTTCCAGGGCAGAGTCACGATTACCGCGGACAAATCCA
CGAGCACAGCCTACATGGAGCTGAGCAGCCTGAGATCTGAGGACACGGCCGTGTAT
TACTGTGCGAAACCGAGAGATGGCTACAATTTGGTTGCTTTTGATATCTGGGGCCAA
GGGACGATGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTG
GCACCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAA
GGACTACTTCCCCGAACCGGTGACGGTGTCGTGGAACCTCAGGCGCCCTGACCAGCG
GCGTGACACACCTTCCCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCG
TGGTGACCGTGCCCTCCAGCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAATC
ACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA
ACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGGGGACCGGGCGGATC
CGGCGGAGGCGGCAGCGGAGACATCCAGATGACCCAGACCACCTCCTCCCTGTCTG
CCTCCCTGGGCGACAGAGTCACCATCAGTTGCAGGGCAAGTCAGGACATTAGAAAT
TATTTAAACTGGTATCAACAGAAACCAGATGGAACGTGTTAAACTCCTGATCTACTAC
ACATCAAGATTACACTCAGGAGTCCCATCAAAGTTCAGTGGCAGTGGGTCTGGAAC

AGATTATTCTCTCACCATTAGCAACCTGGAGCAAGAGGATATTGCCACTTACTTTTG
CCAACAGGGTAATACGCTTCCGTGGACGTTGCTGGAGGCACCAAGCTGGAAATCA
AACGGTAA (SEQ ID NO: 111)

ACE-12-LC nucleotide sequence (anti-PD-L1 antibody light chain nucleotide sequence):

CAGCTCGTGCTGACTCAGCCGCCCTCAGTGTCTGGGGCCCCAGGGCAGAGGGTCAC
CATCTCCTGCACTGGGAGCAGCTCCAACATCGGGGCAGGTTATGATGTACACTGGTA
TCAGCAACTTCCAGGAGCAGCCCCAACTCCTCATCTATGGCGACATCAATCGGCC
CTCAGGGGTCCCTGACCGATTCTCTGGCTCCAAGTCTGGCATCTCAGCCTCCCTGGC
TATCACTGGGCTCCAGGCTGAGGACGAGGCTGATTATTACTGCCAGTCCTATGACAG
CAGCCTGAGTGGGGGGGTGTTGCGGCGGAGGGACCAAGCTGACCGTCCTAAGATCTG
TGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA
CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACAGT
GGAAGGTGGATAACGCCCTCCAATCGGGTAACTCCCAGGAGAGTGTCACAGAGCAG
GACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAGCAGA
CTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAGCTCGC
CCGTCACAAAGAGCTTCAACAGGGGAGAGTGTTAG (SEQ ID NO: 107)

[00506] FIG. 8 shows the results of SDS-PAGE performed to identify the assembly of ACE-12, ACE-05 and ACE-09. The results indicate that ACE-12 was properly expressed and assembled. Furthermore, ACE-12, ACE-05, and ACE-09 have the same VHs and VLs in the Fab and Fv regions (shown in Tables 8, 9 and 12), and their structures differ in the lengths of the G4S linkers between the antibody hinge region and the second Fv domain (FIG.8 bottom). G4S flexible linkers may reduce steric hindrance and optimize the binding of the second Fv domain to immune cells (*e.g.*, effector cells including T cells), and thus lead to increased redirecting efficiency of immune cells to target cells (*e.g.*, cancer cells). Unexpectedly, ACE-12, ACE-05 and ACE-09 showed similar level of expression. Linkers in different lengths may offer different level of flexibility that benefits the ALiCE molecules' activities without affecting their expression and assembly.

[00507] In sum, the above experiments illustrate the successful construction and expression of exemplary binding molecules provided herein and show that these ALiCE molecules can be properly expressed and assembled.

Example 2: Analysis of Binding Affinity of Exemplary Binding Molecules Using Enzyme-Linked Immunosorbent Assay (ELISA)

[00508] Affinity of ACE-00 and ACE-00-VL2 to TNF alpha was measured by Enzyme-Linked Immunosorbent Assay (ELISA). Bmax is the maximum binding affinity extrapolated from experimental results (calculated using curve fitting methods provided in GraphPad Prism software 7). As shown in FIG. 9, K_D of the parent antibody adalimumab to TNF alpha was determined to be 1.125 nM; K_D of ACE-00, which has only a monovalent binding domain to TNF alpha, was determined to be 30.03 nM; and ACE-00-VL2, which is composed of a ACE-00-VL homodimer, was determined to have no affinity to TNF alpha.

[00509] The affinity of ACE-05 and control antibodies to each antigen (e.g., PD-L1 and CD3 for ACE-05) was measured using ELISA. More specifically, antigens such as human PD-L1 (YBL-007 made by Y-Biologics, Inc.) and CD3 (Sino Biological) were immobilized on an immune-plate (Thermo scientific, USA) at a concentration of 1 to 10 µg/ml (100 to 1000 ng/well) using pH 7.4 PBS as a coating buffer at 4 °C overnight. Next day, plate was washed once with 200 µl of PBST, and then a surface blocking was performed at room temperature with 5% skim milk for 1-2 hours. Subsequently, after washing each well twice with 200 µl of PBST, ACE-05 and control antibodies were diluted at a ratio of 1/2 to 1/5 and allowed to react at room temperature for 1-2 hours. Each well was then washed three times with 200 µl of PBST to remove unbound samples. Horseradish peroxidase (HRP)-conjugated anti-human IgG (Fab specific) antibodies (Sigma, USA) were added to wells at a ratio of 1:1000 for a reaction at room temperature. After three times of washing with 200 ul of PBST, a color reaction of HRP was induced using TMB solution (GEhealthcare, USA) in a volume of 100 µl/well. The reaction was terminated by using a stop solution (2.5 M H₂SO₄, 100 µl/well). A spectrophotometer was used to measure the absorbance at a wavelength of A450 to calculate the binding affinity. The GraphPad Prism software 7 was used to analyze the affinity of ACE-05 and other control antibodies to their respective targets. The results are shown in FIG. 10A, 10B and 10C.

[00510] The affinity of ACE-09, ACE-05 and a control antibody to CD3 was measured using similar methods described above. As shown in FIG. 11, ACE-09 showed a similar level of binding affinity as ACE-05.

Example 3: Analysis of Binding Kinetics of ACE-05 Using Surface Plasmon Resonance (SPR)

[00511] Binding kinetics of ACE-05 to human PD-L1 and CD3 were measured using Surface Plasmon Resonance (SPR) analysis. Measurement of label-free kinetics (protein-protein interaction) using OCTET® QKe system (ForteBio, USA) was performed by using an anti-human IgG capture (AHC) biosensor (ForteBio, USA) for human PD-L1 and amine reactive (ARG2) biosensor for CD3εδ chains. PD-L1 and CD3εδ were fixed on their respective biosensor surfaces and sequentially reacted with ACE-05 that had been diluted with kinetic buffers of different concentrations. Sensorgrams were collected over time. Parental antibodies which were used for making ACE-05 (i.e., anti-hPD-L1 antibody (YBL-007, produced in house using conventional methods known in the art) and anti-CD3 antibody (UCHT1 from BioLegend, USA)) were also tested and compared to ACE-05 in this experiment. As shown in FIGs. 12A-12C, the binding kinetics of ACE-05 to human PD-L1 were comparable to the parental anti-PD-L1 antibody (i.e., YBL-007 from Y-Biologics, Inc.) and the K_D was less than 1.09×10^{-11} (see FIGs. 12A-12C). In contrast and as expected, the binding affinity of ACE-05 to CD3 was much lower than the parental anti-CD3 antibody (UCHT1 from BioLegend, USA) and the K_D was determined to be 6.82×10^{-9} (see FIG. 5C). As shown in FIGs. 12A-12C, the binding kinetics of ACE-05 was also compared to a bispecific T cell engager (BiTE), i.e., BiTE-05. BiTE is an existing bispecific antibody technology, which generates fusion proteins consisting of two single-chain variable fragments (scFvs) of different antibodies, or amino acid sequences from four different genes, on a single peptide chain of about 55 kilodaltons. More detailed description of BiTE technology can be found, e.g., in Huehls *et al.*, *Immunol Cell Biol.*, 2015, 93(3): 290-296; Baeuerle *et al.*, *Drug Discovery Today*, 2005, 10: 1237-1244; and Kufer *et al.*, *Trends in Biotechnology*, 2004, 22(5): 238-244. ACE-05 was demonstrated to have higher affinity to human PD-L1 than BiTE-05. On the other hand, ACE-05 was demonstrated to have lower affinity to CD3 than BiTE-05, and thus expected to have less cytotoxicity than BiTE-05.

[00512] In FIG 12D, label-free kinetics OCTET® system (ForteBio, USA) was used to examine simultaneous binding of ACE-05 to hPD-L1 and hCD3. As ligands, histidine labeled recombinant ligand proteins (e.g., hPD-L1-his, hCD3εδ-his) and Fc-labeled recombinant ligand proteins (e.g., hPD-L1-Fc, hCD3εδ-Fc) were prepared. To capture (immobilize) first antigen on the biosensor chip, a histidine capturing NTA chip (ForteBio, USA) was used. After the first

ligand was fully captured on the NTA chip, ACE-05 diluted with kinetic buffer was analyzed. Subsequently, second ligand also diluted in kinetic buffer was analyzed to assess simultaneous binding of ACE-05 to the second ligand.

Example 4: T cell Redirecting (Activity) and T cell Cytotoxicity

[00513] ACE-05 was tested for its activity for redirecting and activating T cells. In the T cell redirecting (activity) assay, a HEK293E-PD-L1 cell line stably expressing PD-L1 was generated and used as an antigen donor. CD3 positive Jurkat cell line engineered to contain NFAT luciferase reporter system was used as an effector cell (Jurkat luciferase reporter cell, produced in house using conventional methods known in the art).

[00514] FACS analysis was then used to determine PD-L1 and CD3 expression levels in the above mentioned cell lines. PD-L1 expression levels in HEK293E-PD-L1 cell and the parental HEK293E cell are shown in FIG. 13A (right panel). CD3 expression level in the Jurkat luciferase reporter cell as measured by various CD3 antibodies is also shown in FIG. 13A (left panel).

[00515] The T cell redirecting (activity) assay was performed as follows: HEK293E cells and HEK293E-PD-L1 cells (7×10^4 cells/well) were seeded on Poly-L-Lysine (Sigma) coated white bottom plates and incubated for 24 hrs. The next day, Jurkat luciferase reporter cells (1.4×10^5 cells/well) were treated with serial dilutions of ACE-05, UCHT1 (parental anti-CD3 antibody from BioLegend, USA) and control molecules, and then incubated at 37 °C for 7 hrs. Bio-Glo Luciferase assay (Promega, USA) was performed to detect degrees of Jurkat activation. In the case of ACE-05, to identify synergistic effect of both PD-1/PD-L1 interaction blocking and PD-L1 targeted T cell redirecting, PD-1/PD-L1 blockade assay was performed using PD-1/PD-L1 Blockade Bioassay kit (Promega, USA). Data were processed and analyzed using GraphPad Prism 7 software.

[00516] As shown in FIG. 13B, when the target cells expressing PD-L1 (HEK293E-PD-L1 cell) were used, ACE-05 was able to activate the effector T cells (Jurkat luciferase reporter cells); whereas when the target cells not expressing PD-L1 (HEK293E) were used, the effector T cells could not be effectively activated by ACE-05. These results indicate that ACE-05 demonstrates target cell-dependent activation of T cells. ACE-05 was also compared with BiTE-05 in this study. As shown in FIG. 13B, ACE-05 demonstrated more efficient T cell activation than BiTE-

05 in the presence of PD-L1. However, in the absence of PD-L1, T cell activation in the presence of BiTE-05 was higher than ACE-05. Therefore, BiTE-05 shows higher cell-independent T cell activation than ACE-05.

[00517] FIG. 13C is the same data from FIG. 13B plotted separately for ACE-05 (left panel) and BiTE-05 (right panel) each with or without PD-L1, showing that the dynamic range of ACE-05 mediated T cell activation was much higher than BiTE-05 mediated T cell activation (indicated by vertical dotted arrows), consistent with observations from FIG. 13B. FIGs. 13D-13E show only target mediated (HEK expressing PD-L1) T cell activation of ACE-05 and BiTE-05, using the same data presented in FIG 13C.

[00518] T cell response against cancer varies in cancer immune cycle. HCC827 PD-L1 positive Non Small Cell Lung Carcinoma (NSCLC) and Jurkat luciferase reporter cells with or without PD-1 expression were used to measure T cell response against cancer. Priming and activation stage of T cell was represented as Jurkat-PD-1 [-], and resting and tolerance stage of T cell was represented as Jurkat-PD-1 [+] in FIG. 13F. As shown, the results indicate that the anti-cancer efficacy of ACE-05 is expected to be much higher than BiTE-05 in both developmental stages of T cell.

[00519] To identify synergistic effect from both blocking PD-1/PD-L1 interaction and PD-L1 targeted T cell redirecting, PD-1/PD-L1 Blockade Bioassay kit (Promega, USA) was used for measuring the biological ability of ACE-05. PD-1/PD-L1 blockade assay was performed by following the protocols provided by the manufacture. The result of the PD-1/PD-L1 blockage assay is shown in FIG. 13G, which demonstrates that ACE-05 could block PD-1/PD-L1 interaction as well as T cell redirecting. FIG. 13H further indicates that, when compared side by side with BiTE-05, ACE-05 shows the highest T cell activation signal because of the synergistic effects from simultaneous PD-L1 blockade and T cell redirecting. YBL-007 is anti-PD-L1 antibody and UCHT1 is anti-CD3 antibody.

[00520] In addition, as shown in FIG. 13I, ACE-05 shows less target independent T cell activation than the anti-CD3 antibody. This result is consistent with the observed lower affinity of ACE-05 to CD3 as compared with the parental anti-CD3 antibody. Thus, these results indicate that ACE-05 will exhibit less cytotoxicity than anti-CD3 antibody because monovalent anti-CD3 domain alone is inefficient to activate T cells. FIG. 13J shows data from the same assay comparing ACE-05 and BiTE-05 side by side, indicating that BiTE-05 shows highest T

cell activation signal in the absence of PD-L1 target because the affinity of BiTE-05 towards CD3 is much higher than that of ACE-05, implicating a higher level of cytotoxicity.

[00521] ACE-05 mediated T cell cytotoxicity was determined using a LDH (lactate dehydrogenase) assay. PBMC from healthy donors were used as effector cells and HCC827 non-small cell lung cancer cells (ATCC) having over-expressed PD-L1 were used as target cells. LDH released from dead tumor cells was measured by LDH assay system and different ratios of T:E (target:effector) were tested in the presence of ACE-05 (FIG. 13K). Furthermore, ACE-05 showed dose-dependent tumor killing ability against HCC827 cells (PD-L1 positive) that were co-incubated with PBMC isolated from healthy donor (FIG. 13L). FIGs. 13M shows the T cell cytotoxicity on tumor cells when in direct contact with PBMC in the presence of ACE-05 or YBL-007. Over a period of 24 hours, the target HCC827 cancer cells were killed in the presence of ACE-05, whereas the target HCC827 cells grew with no treatment or in the presence of YBL-007.

[00522] The levels of immune cytokines released by activated leukocytes may reflect adverse events related to off-target T cell activation. Thus, IL-2 and INF- γ levels in the presence of ACE-05 or BiTE-05 were monitored in co-cultivated PBMC from healthy donors and HCC827 cells using IL-2 or IFN- γ assay kit (BioLegend, USA). Lower level of IL-2 was observed in the presence of ACE-05 than that of BiTE-5, suggesting that it is less likely for ACE-05 to activate CD3+ T cells, which are major sources of IL-2 secretion. Thus, ACE-05 may induce less off-target activation of T cells than BiTE-05. In addition, the presence of ACE-05 and BiTE-05 led to similar levels of INF- γ that is released by NKT or NK cells (FIG. 13N).

[00523] The thermodynamic stability of ACE-05 was evaluated using C1000 Thermal Cycler with CFX 96TM ORM system (BioRad, USA). 3 μ M binding molecules were mixed with 10 μ l of 1/25 diluted CYPPO orange protein stain (Invitrogen, USA), and 50 μ l of the mixtures were allowed to incubate for 30 min at 25°C. Samples were denatured by heating at 1°C/min from room temperature to 99°C. The amount of CYPPO-stained denatured proteins were recorded and the melting temperature (TM) was calculated by CFX manager software. As shown in FIG. 13O, ACE-05 had higher thermodynamic stability than BiTE-05.

[00524] T cell redirecting assay was performed to determine ACE-10 mediated T cell activation via targeting CD20. CD20 positive Raji cells were used as antigen donor target cells and Karpas-299 cells were used as a CD20 negative control. ACE-10 shows more effective T cell

activation than that of BiTE-10. Comparison of the results in the CD20 positive Raji cells and the results in the CD20 negative Karpas-299 cells suggests that the T cell activation depends on CD20 expression in the target cells (see FIGs. 14A-14B).

[00525] ACE-11 mediated T cell cytotoxicity was also determined using the LDH assay. PBMC from healthy donors were used as effector cells and EGFR-positive SW48, HT29 and HCT116 colon cancer cells were used as target cells. LDH released from dead tumor cells was measured by the LDH assay system. HT29 and HCT116 cancer cells have Ras and Raf mutations that can lead to continuous activation of growth signal. ACE-11 showed cytotoxicity in all three cancer cell lines. Results are shown in FIG. 15.

Example 5: Pharmacokinetic Study in Sprague-Dawley (SD) Rats

[00526] A pharmacokinetic study of ACE-05 and control antibodies was performed in SD rats. The study was approved by the institutional animal care and use committee (Approval number: QBSIACUC-A17099).

[00527] SD rats received a single intravenous dose of 10 mg/kg of ACE-05 or control antibodies (YBL-007 or avelumab) via the tail vein (n=3 male rats/group). A total 500 µl of serum was collected from each animal at the following time points: 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 24 hours, 3 days, 5 days, and 9 days after administration of ACE-05 or control antibodies. Samples were centrifuged at 10,000~13,000 rpm for 2 min and 70 µl of plasma from each sample was separated and stored at -80 °C until further analysis. The concentration of each sample was determined by ELISA. 96-well immune-plate (Thermo Scientific, USA) was coated with PD-L1 (generated by Y-Biologics, Inc.). Rat plasma samples diluted at 1:1000, 1:4000, or 1:8000 were added to the wells. Bounded samples were detected with HRP conjugated anti-human IgG (Fab specific) (Sigma, USA). The wells were developed by TMB substrate (Sigma, USA) according to the manufacture's protocol at a ratio of 1:500. A450 was then measured. The concentration of ACE-05 and control antibodies were determined by comparing with standard curves using the sample proteins of known concentrations. The observance data was analyzed by BA Calc 2007 software. The pharmacokinetic study results are shown in FIG. 16A. As shown, the half-life of ACE-05 was measured to be 10.113 hrs. However, it is most likely that the actual half-life of ACE-05 is much longer than 10 hrs, because the half-life of the parental antibodies were determined to be 98 and 73 hrs in this experiment,

which were much shorter than typical half-life for such antibodies (7 or 10 days). As shown in FIG. 16B, ACE-05 exhibited longer half-life than BiTE-05, suggesting the ACE-05 has higher plasma stability than BiTE.

Example 6: Evaluation of Efficacy of ACE-05 in Treating Human Non-Small Cell Lung Cancer

[00528] The efficacy of ACE-05 in treating human non-small cell lung cancer was evaluated and compared with other antibodies using HCC827 cell line in NCG mice (CrownBio, USA). The study was designed as shown in the table below:

Table 13: Study design

Group	N*	HCC827 (day 0)	PBMC (day -3)	Treatment	Dose (mg/kg)	Dosing Route	Schedule
1	10	Day 0, s.c., HCC827 (5×10^6 / 100 μ L/mouse)	Day -3, i.v. PBMC (5×10^6 /100 μ L/mouse)	IgG	5mpk	i.v.	Q3d (day 4, 7, 10)
2	10			YBL-007	5mpk	i.v.	Q3d (day 4, 7, 10)
3	10			BiTE-05	0.5mpk	i.v.	Q2d (day 4, 6, 8)
4	10			ACE-05	0.5mpk	i.v.	Q2d (day 4, 6, 8)

Note: N: animal number; *2 donors will be used, n=5/group

Dosing volume: adjust dosing volume based on body weight (5 uL/g)

Treatment regimen may be changed per BW loss or other adverse effect according to rules set forth and/or client requests.

[00529] FIGs. 17A-17F show the results of the HCC827 (PD-L1 positive tumor) xenograft study in a humanized mice model. 6-8 weeks old female NCG mice were chosen for PBMC reconstitution. As shown in Table 13, there were 4 study groups (10 mice/group) and 2 PBMC donors (5 mice/donor). Test articles were treated with ACE-05, BiTE-05, IgG, and YBL-007 4 days after tumor inoculation (size of TV= 50 mm³) and PBMC isolated from each donors were reconstituted days before tumor inoculation. ACE-05 and BiTE-05 were injected on day 4, day 6, day 8 (total 3 times). IgG and YBL-007 were injected on day 4, day 7 and day 10 (total 3 times). On day 12 of this study, tumors of 9 out of 10 mice were completely gone after ACE-05 treatment. Body weight loss (BWL) (%) of BiTE treated group was much higher than ACE-05 treated group. Three mice out of 10 from the BiTE treated group were terminated because of over 20% of BWL during this study. The results show that ACE-05 is effective in treating the

observed lung cancer and also indicates that ACE-05 exhibits greater safety than the BiTE-05, the bispecific antibody generated using BiTE technology.

Example 7: Dose Limit Study on Anti-Cancer Efficacy of ACE-05 and BiTE-05

[00530] To find an effective dose of ACE-05, a dose limits study was performed in an HCC827 humanized xenograft model (FIGs. 18A-18D). NCG female mice (CrownBio, USA) 6-8 weeks old were chosen for PBMC reconstituted humanization and divided into 14 study groups (6 mice/group). Before grouping and treatment, all animals were weighed and the tumor volumes were measured using a caliper. Since the tumor volume can affect the effectiveness of any given treatment, tumor volume was used as numeric parameter to randomize selected animals into specified groups. The grouping was performed by using StudyDirector™ software (Studylog Systems, USA). “Matched distribution” randomization method was selected for group allocation, which showed minimal group to group variation in tumor volume.

[00531] Human PBMCs from 2 donors were implanted via i.v. 3 days before tumor cell inoculation. Each mouse was inoculated subcutaneously at the right flank region with HCC827 tumor cells (5×10^6) in 0.1 ml of PBS for tumor development. Five different concentration of ACE-05 and BiTE-05 from 0.5 mpk (mg/kg) to 0.0005 mpk (0.5, 0.1, 0.05, 0.005, and 0.0005 mpk) were treated day 4 post HCC827 inoculation. Test articles administration was scheduled on every 3 days, i.e., on day 4, day 7, day 10, and day 13 (Q3d, 4 doses total), as shown with 4 vertical arrows above the x-axis in FIG. 18A.

[00532] Tumor dimension (FIGs. 18A-18C) and body weight (FIG. 18D) were scored on every 2 or 3 days after the initial administration of test molecules. The standard error of the mean (SEM) of tumor volume and changes of body weight from each group were plotted against time in FIG. 18A, respectively. FIGs. 18B-18C show anti-tumor efficacy of individual mouse in each dose group treated with ACE-05 and BiTE-05, respectively. Four mice were terminated on day 15 in the BiTE-05 treated group, but only 1 mouse was terminated in ACE-05 treated group. In most animals treated with 0.5mpk, 0.1mpk, and 0.05mpk of ACE-05, tumors were completely disappeared (FIG. 18B), in contrast with the group treated with BiTE-05 (FIG. 18C).

[00533] Table 14 summarizes the anti-tumor activity of ACE-05 and BiTe-05. The mean tumor volume (TV) on day 15 is shown with the standard error of the mean. Percentage tumor growth inhibition (TGI%) is the difference between the mean tumor volume of a test group and control group, calculated using the following formula: $TGI (\%) = (\text{Mean TV of control} - \text{Mean TV of$

treated)/Mean TV of control $\times 100$. T/C (%) was calculated using the following formula: T/C (%) = mean TV of treated/mean TV of control $\times 100$. The results show that ACE-05 is more effective than BiTE-05 in treating the observed lung cancer over a range of concentrations.

Table 14: Anti-tumor activity of ACE-05 and BiTE-05

Group	Test article	Dose/schedule	TV (mm ³) on Day 15, mean	TGI (%)	T/C (%)	Mortality on Day 15
1a	IgG	5mpk / Q3d	79.99 $\pm$ 10.56	-	-	-
1b			69.21 $\pm$ 5.15	-	-	-
5a	BiTE-05	0.5mpk / Q3d	0.00 $\pm$ 0	100.00%	0.00%	Dead(I), TS(2)
5b			0.00 $\pm$ 0	100.00%	0.00%	Dead(I)
6a		0.1mpk / Q3d	9.70 $\pm$ 4.88	87.87%	12.13%	-
6b			5.71 $\pm$ 5.71	91.75%	8.25%	-
7a		0.05mpk / Q3d	22.22 $\pm$ 3.66	72.23%	27.77%	-
7b			4.51 $\pm$ 4.51	93.48%	6.52%	-
8a		0.005mpk / Q3d	28.52 $\pm$ 4.81	64.34%	35.66%	-
8b			12.08 $\pm$ 8.55	82.55%	17.45%	-
9a		0.0005mpk / Q3d	64.69 $\pm$ 15.97	19.12%	80.88%	-
9b			39.42 $\pm$ 2.62	43.04%	56.96%	-
10a	ACE-05	0.5mpk / Q3d	7.00 $\pm$ 7.00	91.24%	8.76%	-
10b			0.00 $\pm$ 0	100.00%	0.00%	TS(1)
11a		0.1mpk / Q3d	0.00 $\pm$ 0	100.00%	0.00%	-
11b			0.00 $\pm$ 0	100.00%	0.00%	-
12a		0.05mpk / Q3d	17.56 $\pm$ 8.85	78.04%	21.96%	-
12b			0.00 $\pm$ 0	100.00%	0.00%	-
13a		0.005mpk / Q3d	62.60 $\pm$ 1.55	21.74%	78.26%	-
13b			42.17 $\pm$ 6.57	39.07%	60.93%	-
14a		0.0005mpk / Q3d	40.96 $\pm$ 0.92	20.86%	79.14%	-
14b			30.27 $\pm$ 2.41	56.27%	43.73%	-

[00534] From the foregoing, it will be appreciated that, although specific embodiments have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of what is provided herein. All of the references referred to above are incorporated herein by reference in their entireties.

WHAT IS CLAIMED:

1. A binding molecule comprising:
 - (a) a first antigen binding domain comprising two antibody Fab regions, each comprising:
 - (i) a first portion comprising an antibody variable heavy (VH) region and an antibody CH1 region, wherein the first portion does not contain an antibody CH2 region and an antibody CH3 region; and
 - (ii) a second portion comprising an antibody light chain (LC) comprising an antibody variable light (VL) region and an antibody light chain constant region (CL),wherein the two antibody Fab regions each bind to an antigen, and
 - (b) a second antigen binding domain comprising an antibody Fv region comprising a VH region and a VL region,wherein the second antigen binding domain binds to an antigen present on an immune cell; andwherein the first antigen binding domain and the second antigen binding domain are linked.
2. The binding molecule of claim 1, wherein the first portion and the second portion of each Fab region of the first antigen binding domain are on the same polypeptide.
3. The binding molecule of claim 2, wherein at least one Fab region is oriented from N-terminus to C-terminus in the following order: VH-CH1-VL-CL.
4. The binding molecule of claim 2, wherein at least one Fab region is oriented from N-terminus to C-terminus in the following order: VL-CL-VH-CH1.
5. The binding molecule of claim 1, wherein the first portion and the second portion of each Fab region are present on separate polypeptides.
6. The binding molecule of any one of claims 1-5, wherein the VH region and the VL region of the Fv region are on the same polypeptide.

7. The binding molecule of claim 6, wherein the Fv region is oriented from N-terminus to C-terminus in the following order: VH-VL.
8. The binding molecule of claim 6, wherein the Fv region is oriented from N-terminus to C-terminus in the following order: VL-VH.
9. The binding molecule of any one of claims 1-5, wherein the VH region and the VL region of the Fv region are on separate polypeptides.
10. The binding molecule of any one of claims 1-9, wherein the first antigen binding domain and the second antigen binding domain are linked by a flexible peptide region.
11. The binding molecule of claim 10, wherein the flexible peptide region comprises an antibody hinge region.
12. The binding molecule of claim 11, wherein the antibody hinge region is a IgG hinge region.
13. The binding molecule of claim 12, wherein the IgG hinge region is of IgG1 subtype.
14. The binding molecule of claim 12, wherein the IgG hinge region is of IgG2 subtype.
15. The binding molecule of claim 12, wherein the IgG hinge region is of IgG3 subtype.
16. The binding molecule of claim 12, wherein the IgG hinge region is of IgG4 subtype.
17. The binding molecule of claim 11, wherein the flexible peptide region further comprises a linker between the antibody hinge region and the second antigen binding domain.
18. The binding molecule of claim 17, wherein the linker comprises an amino acid sequence of GGGGS (G4S).
19. The binding molecule of claim 1, wherein the second antigen binding domain further comprises a first CH3 region linked to the VH region of the Fv region and a second CH3 region linked to the VL region of the Fv region.

20. The binding molecule of claim 1, wherein the binding molecule further comprises one or more albumin binding domain or site (ABS).
21. The binding molecule of claim 20, wherein the ABS is linked to the C-terminus of the VH region of the Fv region.
22. The binding molecule of claim 20, wherein the ABS is linked to the C-terminus of the VL region of the Fv region.
23. The binding molecule of claim 20, wherein the ABS is linked to the CL region of at least one of the Fab regions.
24. The binding molecule of claim 1, wherein the binding molecule further comprises one or more albumin domain.
25. The binding molecule of any one of claims 1-18, wherein the two Fab regions bind to different antigens.
26. The binding molecule of any one of claims 1-18, wherein the two Fab regions bind to the same antigen.
27. The binding molecule of claim 26, wherein the two Fab regions bind to the same epitope of the same antigen.
28. The binding molecule of claim 26, wherein the two Fab regions bind to different epitopes of the same antigen.
29. The binding molecule of claim 26, wherein the first antigen binding domain and the second antigen binding domain bind to the same antigen.
30. The binding molecule of claim 26, wherein the second antigen binding domain binds to the same epitope as at least one of the epitopes bound by the first antigen binding domain.
31. The binding molecule of claim 26, wherein the first antigen binding domain and the second antigen binding domain bind to different antigens, and wherein the first antigen binding domain binds to a first antigen and the second antigen binding domain binds to a second antigen.

32. The binding molecule of claim 31, wherein the first antigen is a cancer antigen.
33. The binding molecule of claim 31, wherein the first antigen is not a cancer antigen.
34. The binding molecule of claim 31, wherein the second antigen is expressed on an immune cell selected from a group consisting of lymphocytes and monocytes.
35. The binding molecule of claim 31, wherein the second antigen is expressed on an effector cell.
36. The binding molecule of claim 34, wherein the second antigen is expressed on a T cell.
37. The binding molecule of claim 36, wherein the second antigen is expressed on a T helper cell, a regulatory T cell, or a cytotoxic T cell.
38. The binding molecule of claim 37, wherein the second antigen is expressed on a T helper cell.
39. The binding molecule of claim 37, wherein the second antigen is expressed on a regulatory T cell.
40. The binding molecule of claim 37, wherein the second antigen is expressed on a cytotoxic T cell.
41. The binding molecule of claim 36, wherein the second antigen is expressed on a CD8+ T cell.
42. The binding molecule of claim 36, wherein the second antigen is expressed on a CD4+ T cell.
43. The binding molecule of claim 34, wherein the second antigen comprises an extracellular domain.
44. The binding molecule of claim 35, wherein the second antigen is CD3.
45. The binding molecule of claim 31, wherein the first antigen is a cancer antigen and the second antigen is CD3.

46. The binding molecule of claim 45, wherein the first antigen is PD-L1 and the second antigen is CD3.

47. The binding molecule of claim 46,

wherein the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7;

wherein the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11;

wherein the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, SEQ ID NO.: 15; and

wherein the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

48. The binding molecule of claim 46,

wherein the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4;

wherein the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8;

wherein the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and

wherein the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

49. The binding molecule of claim 45, wherein the first antigen is CD20 and the second antigen is CD3.

50. The binding molecule of claim 49,

wherein the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, SEQ ID NO.: 29;

wherein the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33;

wherein the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, SEQ ID NO.: 15; and

wherein the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

51. The binding molecule of claim 49,

wherein the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26;

wherein the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30;

wherein the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and

wherein the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

52. The binding molecule of claim 45, wherein the first antigen is EGFR and the second antigen is CD3.

53. The binding molecule of claim 52,

wherein the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43;

wherein the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47;

wherein the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15 and

wherein the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

54. The binding molecule of claim 52,

wherein the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40;

wherein the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44;

wherein the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12;
and

wherein the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

55. A binding molecule, comprising:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising a first VH region and a first CH1 region, and a second VH region; and

(c) a fourth polypeptide comprising a third VH region and a second CH1, and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen Fab region; and

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region.

56. The binding molecule of claim 55, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region.

57. The binding molecule of claim 56, wherein the flexible peptide region comprises an antibody hinge region.

58. The binding molecule of claim 57, wherein the antibody hinge region is a IgG hinge region.

59. The binding molecule of claim 58, wherein the IgG hinge region is of IgG1 subtype.

60. The binding molecule of claim 58, wherein the IgG hinge region is of IgG2 subtype.
61. The binding molecule of claim 58, wherein the IgG hinge region is of IgG3 subtype.
62. The binding molecule of claim 58, wherein the IgG hinge region is of IgG4 subtype.
63. The binding molecule of claim 57, wherein the flexible peptide region further comprises a linker.
64. The binding molecule of claim 63, wherein the linker comprises an amino acid sequence of GGGGS (G4S).
65. The binding molecule of any one of claims 55-64, wherein the first Fab region and the second Fab region bind to different antigens.
66. The binding molecule of any one of claims 55-64, wherein the first Fab region and the second Fab region bind to the same antigen.
67. The binding molecule of claim 66, wherein the first Fab region and the second Fab region bind to the same epitope of the same antigen.
68. The binding molecule of claim 66, wherein the first Fab region and the second Fab region bind to different epitopes of the same antigen.
69. The binding molecule of claim 66, wherein the first Fab region and the second Fab region form a first antigen binding domain, and the Fv region is a second antigen binding domain.
70. The binding molecule of claim 69, wherein the first antigen binding domain and the second antigen binding domain bind to the same antigen.
71. The binding molecule of claim 69, wherein the second antigen binding domain binds to the same epitope as at least one of the epitopes bound by the first antigen binding domain.
72. The binding molecule of claim 69, wherein the first antigen binding domain and the second antigen binding domain bind to different antigens, and wherein the first antigen binding domain binds to a first antigen and the second antigen binding domain binds to a second antigen.

73. The binding molecule of claim 72, wherein the first antigen is a cancer antigen.
74. The binding molecule of claim 72, wherein the first antigen is not a cancer antigen.
75. The binding molecule of claim 72, wherein the second antigen is expressed on an immune cell selected from a group consisting of lymphocytes and monocytes.
76. The binding molecule of claim 72, wherein the second antigen is expressed on an effector cell.
77. The binding molecule of claim 75, wherein the second antigen is expressed on a T cell.
78. The binding molecule of claim 77, wherein the second antigen is expressed on a T helper cell, a regulatory T cell, or a cytotoxic T cell.
79. The binding molecule of claim 78, wherein the second antigen is expressed on a T helper cell.
80. The binding molecule of claim 78, wherein the second antigen is expressed on a regulatory T cell.
81. The binding molecule of claim 78, wherein the second antigen is expressed on a cytotoxic T cell.
82. The binding molecule of claim 77, wherein the second antigen is expressed on a CD8+ T cell.
83. The binding molecule of claim 77, wherein the second antigen is expressed on a CD4+ T cell.
84. The binding molecule of claim 75, wherein the second antigen comprises an extracellular domain.
85. The binding molecule of claim 75, wherein the second antigen is CD3.
86. The binding molecule of claim 72, wherein the first antigen is a cancer antigen and the second antigen is CD3.

87. The binding molecule of claim 86, wherein the first antigen is PD-L1 and the second antigen is CD3.

88. The binding molecule of claim 87,

wherein the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7;

wherein the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11;

wherein the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

wherein the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 16, SEQ ID NO.: 17, and SEQ ID NO.: 19.

89. The binding molecule of claim 87,

wherein the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4;

wherein the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8;

wherein the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and

wherein the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

90. The binding molecule of claim 87, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 3; the third polypeptide has the amino acid sequence of SEQ ID NO.:1; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.:2.

91. The binding molecule of claim 72, wherein the first antigen is CD20 and the second antigen is CD3.

92. The binding molecule of claim 91,

wherein the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, and SEQ ID NO.: 29;

wherein the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33;

wherein the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

wherein the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

93. The binding molecule of claim 91,

wherein the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26;

wherein the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30;

wherein the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and

wherein the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

94. The binding molecule of claim 91, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.

95. The binding molecule of claim 72, wherein the first antigen is EGFR and the second antigen is CD3.

96. The binding molecule of claim 95,

wherein the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, SEQ ID NO.: 43;

wherein the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47;

wherein the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

wherein the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

97. The binding molecule of claim 95,

wherein the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40;

wherein the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44;

wherein the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and

wherein the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

98. The binding molecule of claim 95, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.

99. A method of making a binding molecule comprising transfecting one or more vectors into a host cell, wherein the one or more vectors comprise:

(a) a first nucleic acid encoding a first polypeptide and a second polypeptide, each being an antibody light chain,

(b) a second nucleic acid encoding a third polypeptide comprising a first VH region and a first CH1 region and a second VH region; and

(c) a third nucleic acid encoding a fourth polypeptide comprising a third VH region and a second CH1 and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide can form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide can form a second antigen binding Fab region; and

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide can form an antigen binding Fv region.

100. The method of claim 99, wherein the first Fab region and the second Fab region are linked to the Fv region via a flexible peptide region.

101. The method of claim 100, wherein the flexible peptide region comprises an antibody hinge region.

102. The method of claim 101, wherein the antibody hinge region is a IgG hinge region.

103. The method of claim 102, wherein the IgG hinge region is of IgG1 subtype.

104. The method of claim 102, wherein the IgG hinge region is of IgG2 subtype.

105. The method of claim 102, wherein the IgG hinge region is of IgG3 subtype.

106. The method of claim 102, wherein the IgG hinge region is of IgG4 subtype.

107. The method of claim 101, wherein the flexible peptide region further comprises a linker.

108. The method of claim 107, wherein the linker comprises an amino acid sequence of GGGGS (G4S).

109. The method of any one of claims 100-108, wherein the first Fab region and the second Fab region bind to different antigens.

110. The method of any one of claims 100-108, wherein the first Fab region and the second Fab region bind to the same antigen.

111. The method of claim 110, wherein the first Fab region and the second Fab region bind to the same epitope of the same antigen.
112. The method of claim 110, wherein the first Fab region and the second Fab region bind to different epitopes of the same antigen.
113. The method of claim 110, wherein the first Fab region and the second Fab region form a first antigen binding domain, and the Fv region is a second antigen binding domain.
114. The method of claim 113, wherein the first antigen binding domain and the second antigen binding domain bind to the same antigen.
115. The method of claim 113, wherein the second antigen binding domain binds to the same epitope as at least one of the epitopes bound by the first antigen binding domain.
116. The method of claim 113, wherein the first antigen binding domain and the second antigen binding domain bind to different antigens, and wherein the first antigen binding domain binds to a first antigen and the second antigen binding domain binds to a second antigen.
117. The method of claim 116, wherein the first antigen is a cancer antigen.
118. The method of claim 116, wherein the first antigen is not a cancer antigen.
119. The binding molecule of claim 116, wherein the second antigen is expressed on an immune cell selected from a group consisting of lymphocytes and monocytes.
120. The binding molecule of claim 116, wherein the second antigen is expressed on an effector cell.
121. The binding molecule of claim 119, wherein the second antigen is expressed on a T cell.
122. The binding molecule of claim 121, wherein the second antigen is expressed on a T helper cell, a regulatory T cell, or a cytotoxic T cell.
123. The binding molecule of claim 122, wherein the second antigen is expressed on a T helper cell.

124. The binding molecule of claim 122, wherein the second antigen is expressed on a regulatory T cell.
125. The binding molecule of claim 122, wherein the second antigen is expressed on a cytotoxic T cell.
126. The binding molecule of claim 121, wherein the second antigen is expressed on a CD8+ T cell.
127. The binding molecule of claim 121, wherein the second antigen is expressed on a CD4+ T cell.
128. The binding molecule of claim 119, wherein the second antigen comprises an extracellular domain.
129. The method of claim 119, wherein the second antigen is CD3.
130. The method of claim 116, wherein the first antigen is a cancer antigen and the second antigen is CD3.
131. The method of claim 130, wherein the first antigen is PD-L1 and the second antigen is CD3.
132. The method of claim 131,
- wherein the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 5, SEQ ID NO.: 6, and SEQ ID NO.: 7;
- wherein the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 9, SEQ ID NO.: 10, and SEQ ID NO.: 11;
- wherein the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and
- wherein the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

133. The method of claim 131,

wherein the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 4;

wherein the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 8;

wherein the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and

wherein the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

134. The method of claim 131, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 3; the third polypeptide has the amino acid sequence of SEQ ID NO.: 1; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 2.

135. The method of claim 131, wherein the first nucleic acid has a nucleotide sequence of SEQ ID NO.: 22; the second nucleic acid has a nucleotide sequence of SEQ ID NO.: 20; and the third nucleic acid has a nucleotide sequence of SEQ ID NO.: 21.

136. The method of claim 130, wherein the first antigen is CD20 and the second antigen is CD3.

137. The method of claim 136,

wherein the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 27, SEQ ID NO.: 28, SEQ ID NO.: 29;

wherein the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 31, SEQ ID NO.: 32, and SEQ ID NO.: 33;

wherein the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

wherein the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

138. The method of claim 136,

wherein the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 26;

wherein the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 30;

wherein the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and

wherein the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

139. The method of claim 136, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 25; the third polypeptide has the amino acid sequence of SEQ ID NO.: 23; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 24.

140. The method of claim 136, wherein the first nucleic acid has a nucleotide sequence of SEQ ID NO.: 36; the second nucleic acid has a nucleotide sequence of SEQ ID NO.: 34; and the third nucleic acid has a nucleotide sequence of SEQ ID NO.: 35.

141. The method of claim 131, wherein the first antigen is EGFR and the second antigen is CD3.

142. The method of claim 141,

wherein the VH region of the first portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 41, SEQ ID NO.: 42, and SEQ ID NO.: 43;

wherein the VL region of the second portion of each Fab region comprises three CDRs having amino acid sequences of SEQ ID NO.: 45, SEQ ID NO.: 46, and SEQ ID NO.: 47;

wherein the VH region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 13, SEQ ID NO.: 14, and SEQ ID NO.: 15; and

wherein the VL region of the Fv region comprises three CDRs having amino acid sequences of SEQ ID NO.: 17, SEQ ID NO.: 18, and SEQ ID NO.: 19.

143. The method of claim 141,

wherein the VH region of the first portion of each Fab region has an amino acid sequence of SEQ ID NO.: 40;

wherein the VL region of the second portion of each Fab region has an amino acid sequence of SEQ ID NO.: 44;

wherein the VH region of the Fv region has an amino acid sequence of SEQ ID NO.: 12; and

wherein the VL region of the Fv region has an amino acid sequence of SEQ ID NO.: 16.

144. The method of claim 141, wherein the first polypeptide and the second polypeptide each have the amino acid sequence of SEQ ID NO.: 39; the third polypeptide has the amino acid sequence of SEQ ID NO.: 37; and the fourth polypeptide has the amino acid sequence of SEQ ID NO.: 38.

145. The method of claim 141, wherein the first nucleic acid has a nucleotide sequence of SEQ ID NO.: 50; the second nucleic acid has a nucleotide sequence of SEQ ID NO.: 48; and the third nucleic acid has a nucleotide sequence of SEQ ID NO.: 49.

146. A pharmaceutical composition comprising a therapeutically effective amount of the binding molecule of anyone of claims 1-98 and a pharmaceutically acceptable carrier.

147. The pharmaceutical composition of claim 146 for use in treating a disease or condition in a subject.

148. The pharmaceutical composition of claim 147, wherein the disease or condition is a cancer.

149. The pharmaceutical composition of claim 148, wherein the cancer is lung cancer.

150. The pharmaceutical composition of claim 149, wherein the lung cancer is Non Small Cell Lung Carcinoma (NSCLC).

151. The pharmaceutical composition of claim 148, wherein the cancer is Diffuse Large B cell Lymphoma (DLBCL).
152. The pharmaceutical composition of claim 147, wherein the disease or condition is a PD-L1 positive cancer
153. A method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of the binding molecule of anyone of claims 1-98 to the subject.
154. The method of claim 151, wherein the disease or condition is a cancer.
155. The method of claim 151, wherein the cancer is lung cancer.
156. The method of claim 155, wherein the lung cancer is NSCLC.
157. The method of claim 154, wherein the cancer is DLBCL.
158. The method of claim 153, wherein the disease or condition is a PD-L1 positive cancer.
159. A binding molecule, comprising:
- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
 - (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and
 - (c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,
- wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;
- wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;
- wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and
- wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen.

160. A method of making a binding molecule, comprising:

(i) expressing the binding molecule from one or more vectors in a host cell, wherein the one or more vectors comprise

(a) a first nucleic acid encoding a first polypeptide and a second nucleic acid encoding a second polypeptide, wherein each of the first polypeptide and the second polypeptide is an antibody light chain,

(b) a third nucleic acid encoding a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region and a second VH region; and

(c) a fourth nucleic acid encoding a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide can form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide can form a second antigen binding Fab region; and

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen, and

(ii) purifying the binding molecule.

161. A pharmaceutical composition comprising a binding molecule and a pharmaceutically acceptable carrier, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen.

162. A method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of a binding molecule to the subject, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a VL region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to a first antigen, the Fv region binds to a second antigen, and the first antigen is different from the second antigen.

163. A binding molecule, comprising:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region binds to Programmed Death-Ligand 1 (PD-L1), and the Fv region binds to Cluster of Differentiation 3 (CD3).

164. A method of making a binding molecule, comprising:

(i) expressing the binding molecule from one or more vectors in a host cell, wherein the one or more vectors comprise

(a) a first nucleic acid encoding a first polypeptide and a second nucleic acid encoding a second polypeptide, wherein each polypeptide comprises an antibody light chain,

(b) a third nucleic acid encoding a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth nucleic acid encoding a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region bind to PD-L1, and the Fv region binds to CD3, and

(ii) purifying the binding molecule.

165. A pharmaceutical composition comprising a binding molecule and a pharmaceutically acceptable carrier, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region binds to PD-L1, and the Fv region binds to CD3.

166. A method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of a binding molecule to the subject, wherein the binding molecule comprises:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region binds to PD-L1, and the Fv region binds to CD3.

167. A binding molecule, comprising:

(a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,

(b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and

(c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region each binds to CD20 or epidermal growth factor receptor (EGFR), and the Fv region binds to CD3.

168. A method of making a binding molecule comprising:

(i) expressing the binding molecule from one or more vectors in a host cell, wherein the one or more vectors comprise

(a) a first nucleic acid encoding a first polypeptide and a second nucleic acid encoding a second polypeptide, wherein each of the first polypeptide and the second polypeptide is an antibody light chain,

- (b) a third nucleic acid encoding a third polypeptide comprising, in the order from N-terminus to C-terminus, a first VH region and a first CH1 region, and a second VH region; and
 - (c) a fourth nucleic acid encoding a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a VL region,
- wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;
- wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;
- wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and
- wherein the first Fab region and the second Fab region each binds to CD20 or EGFR, and the Fv region binds to CD3, and
- (ii) purifying the binding molecule.

169. A pharmaceutical composition comprising a therapeutically effective amount of a binding molecule and a pharmaceutically acceptable carrier, wherein the binding molecule comprises:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
 - (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and
 - (c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,
- wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;
- wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;
- wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and
- wherein the first Fab region and the second Fab region each binds to CD20 or EGFR, and the Fv region binds to CD3.

170. A method of treating a disease or condition in a subject comprising administering a therapeutically effective amount of a binding molecule to the subject, wherein the binding molecule comprises:

- (a) a first polypeptide and a second polypeptide, each comprising an antibody light chain,
- (b) a third polypeptide comprising, in the order from N-terminus to C-terminus, a first variable heavy (VH) region and a first constant heavy 1 (CH1) region, and a second VH region; and
- (c) a fourth polypeptide comprising, in the order from N-terminus to C-terminus, a third VH region and a second CH1 region, and a variable light (VL) region,

wherein the first polypeptide and the first VH region and the first CH1 region of the third polypeptide form a first antigen binding Fab region;

wherein the second polypeptide and the third VH region and the second CH1 region of the fourth polypeptide form a second antigen binding Fab region;

wherein the second VH region of the third polypeptide and the VL region of the fourth polypeptide form an antigen binding Fv region; and

wherein the first Fab region and the second Fab region each binds to CD20 or EGFR, and the Fv region binds to CD3.

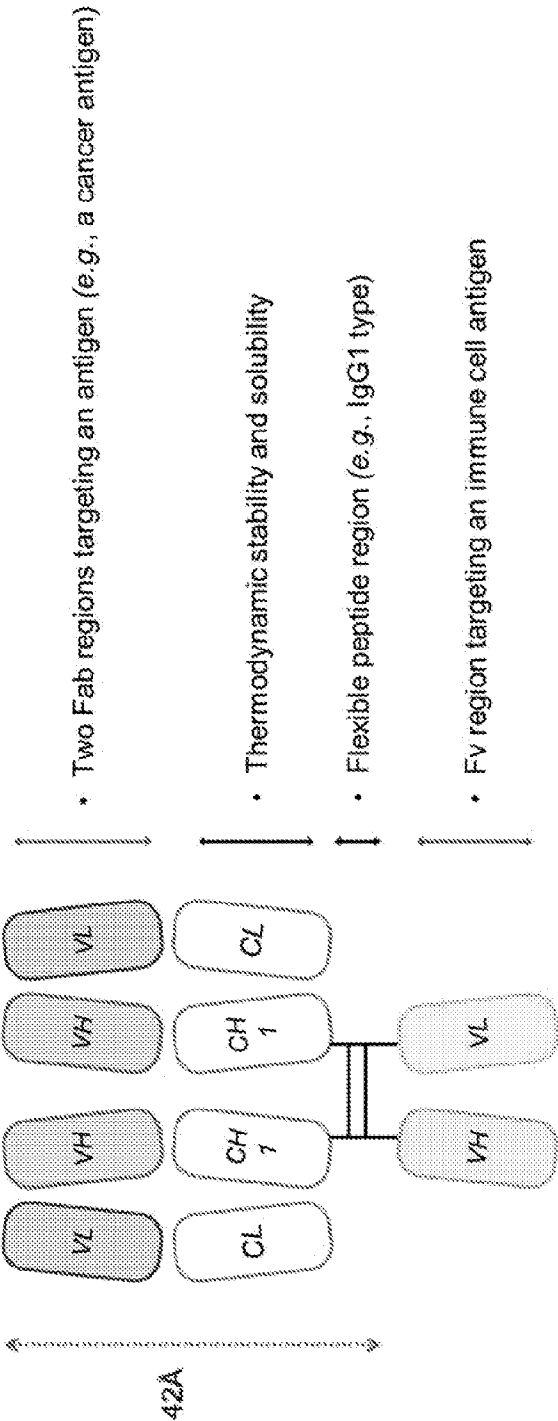


FIG. 1A

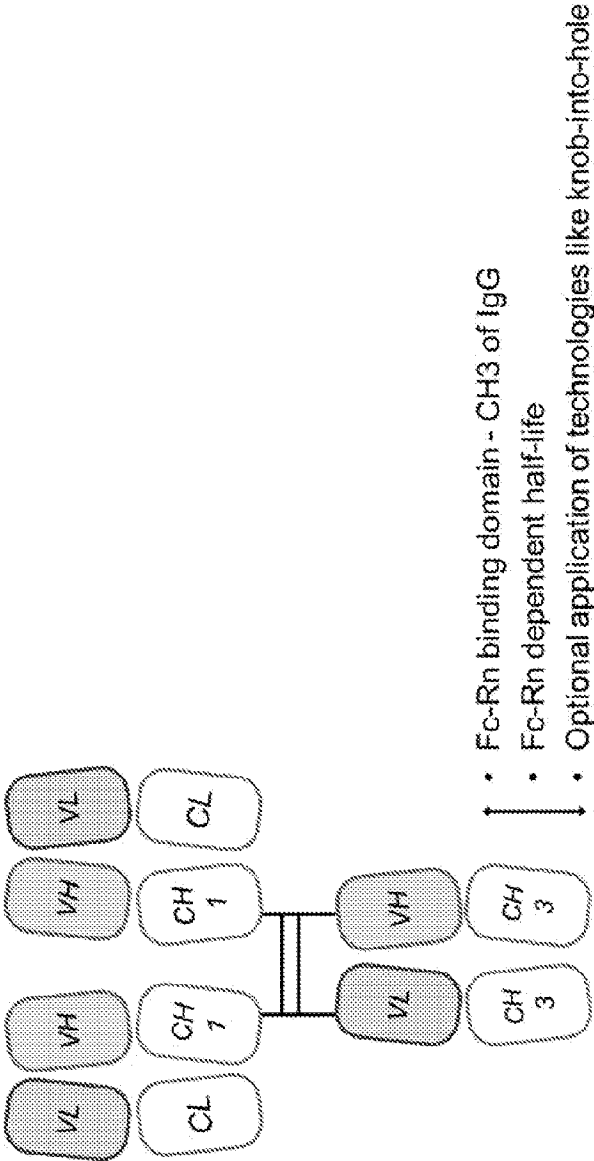


FIG. 1B

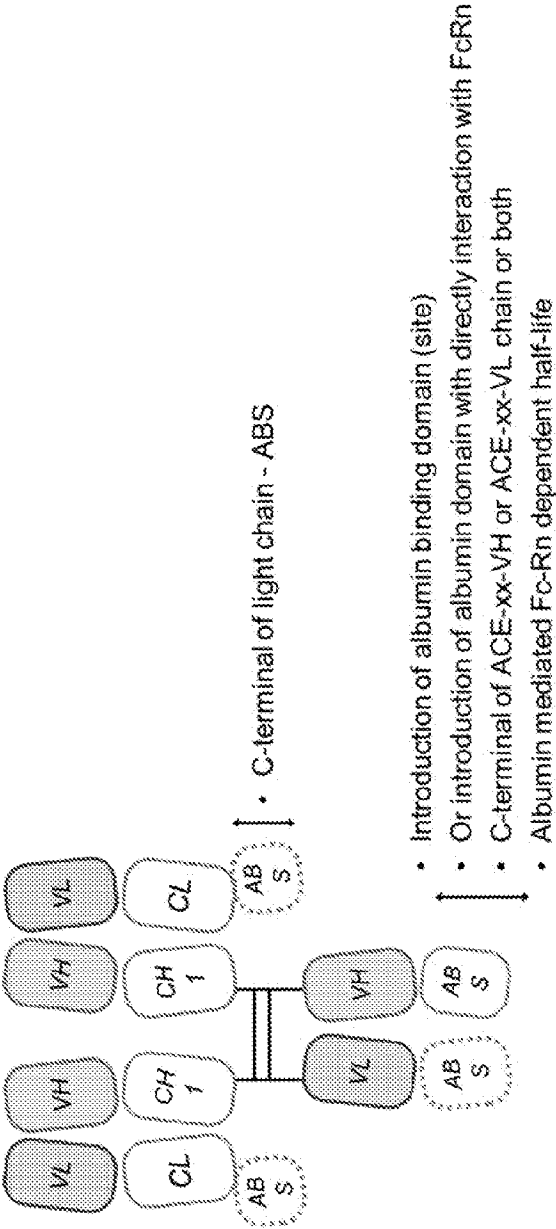


FIG. 1C

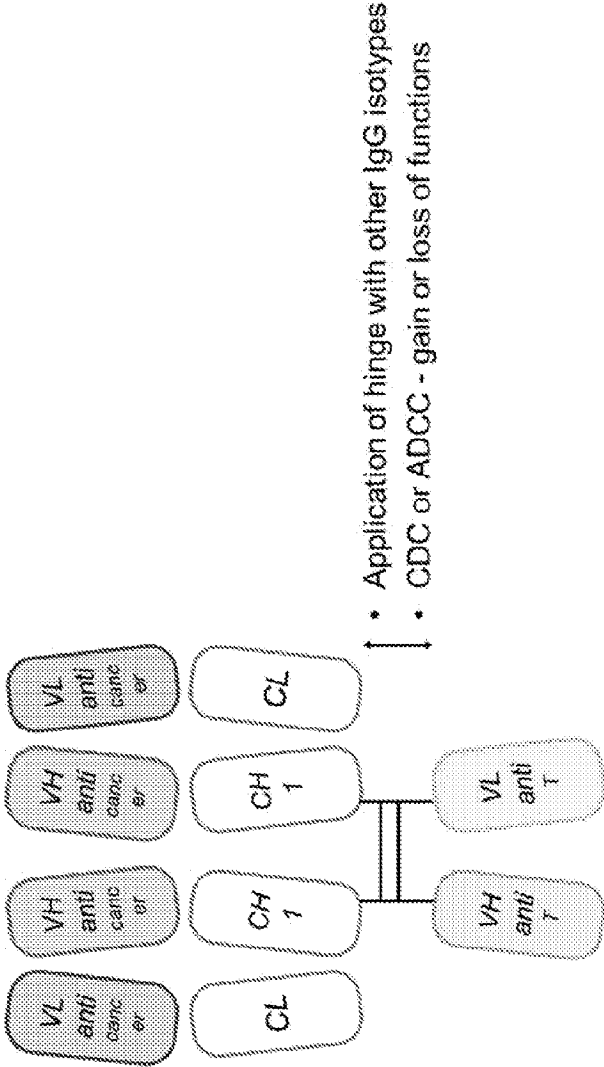


FIG. 1D

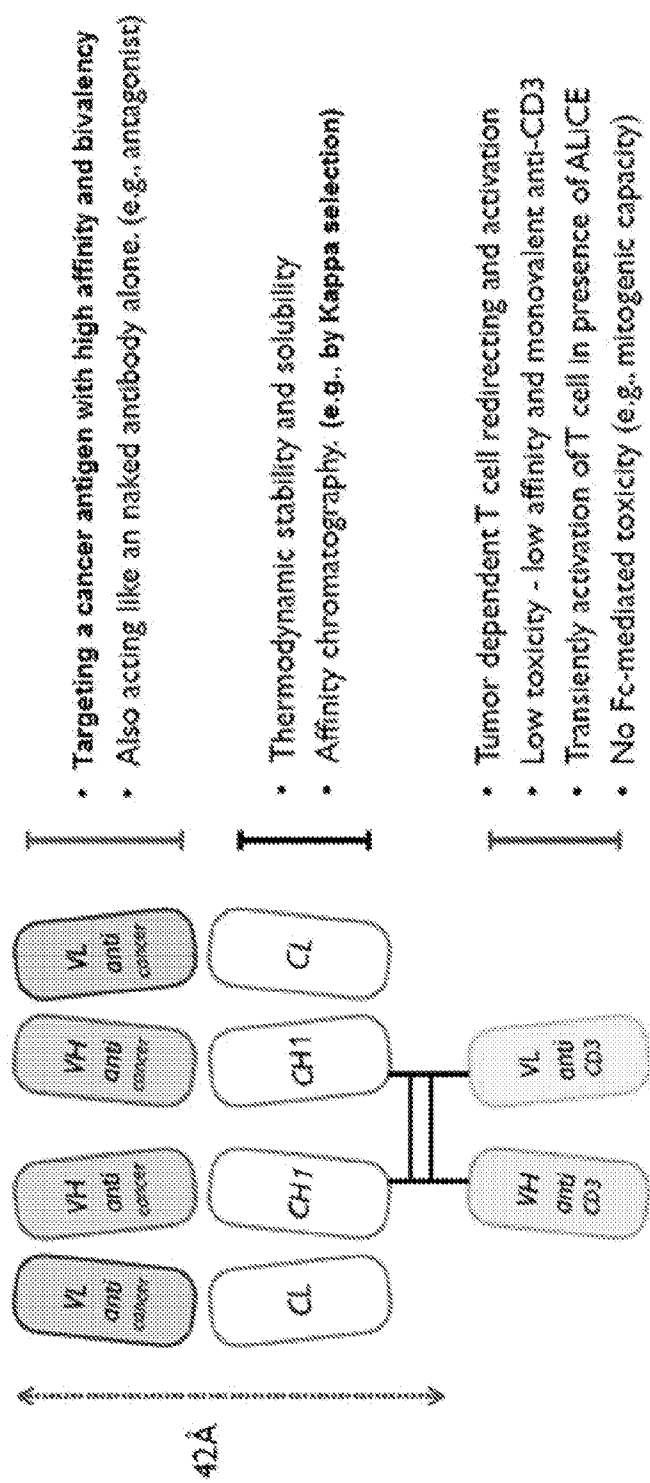


FIG. 1E

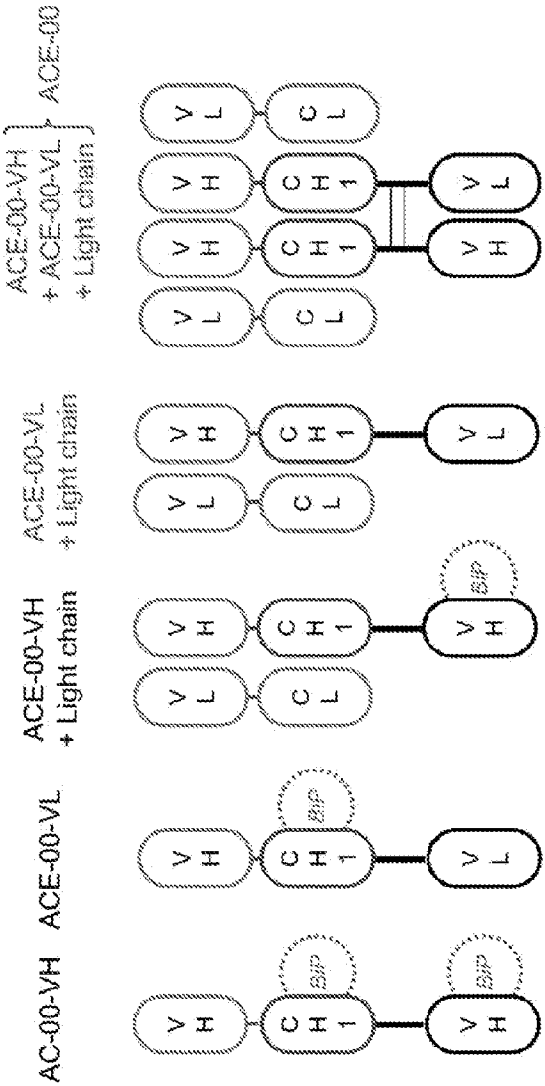


FIG. 2A

ACE-00-VH ACE-00-VL ACE-00-VH + ACE-00-VL
ACE-00-VH + Light chain ACE-00-VL + Light chain ACE-00-VH + ACE-00-VL + Light chain

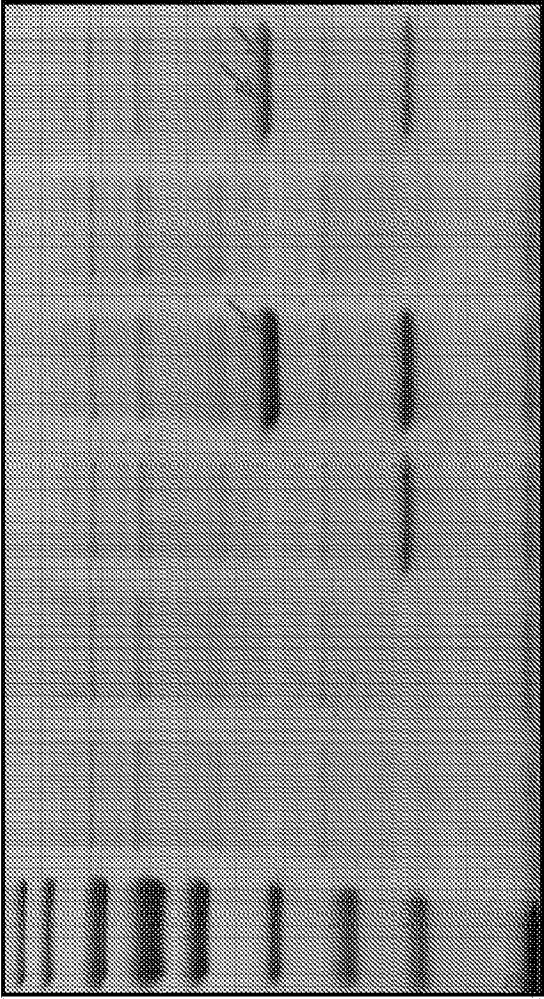


FIG. 2B

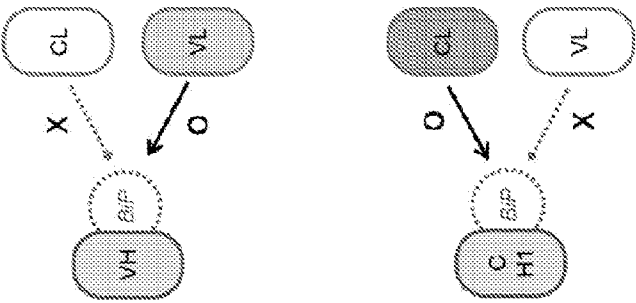


FIG. 2E

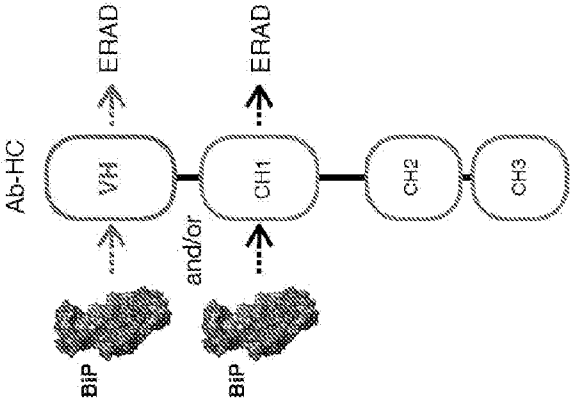


FIG. 2D

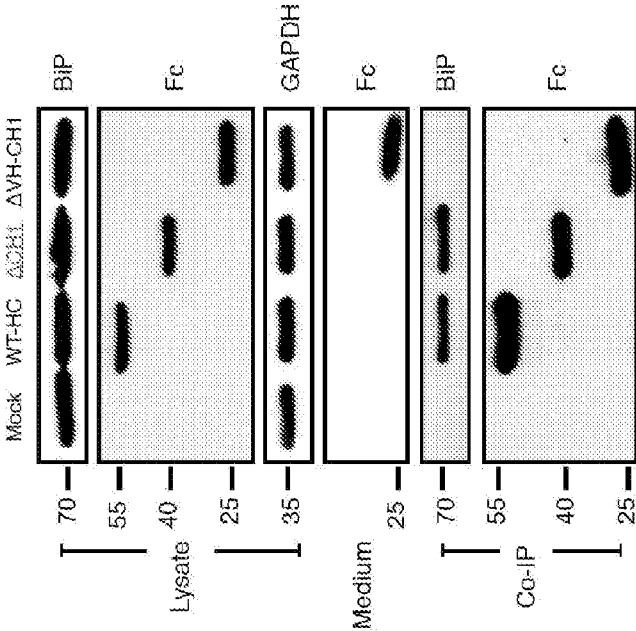


FIG. 2C

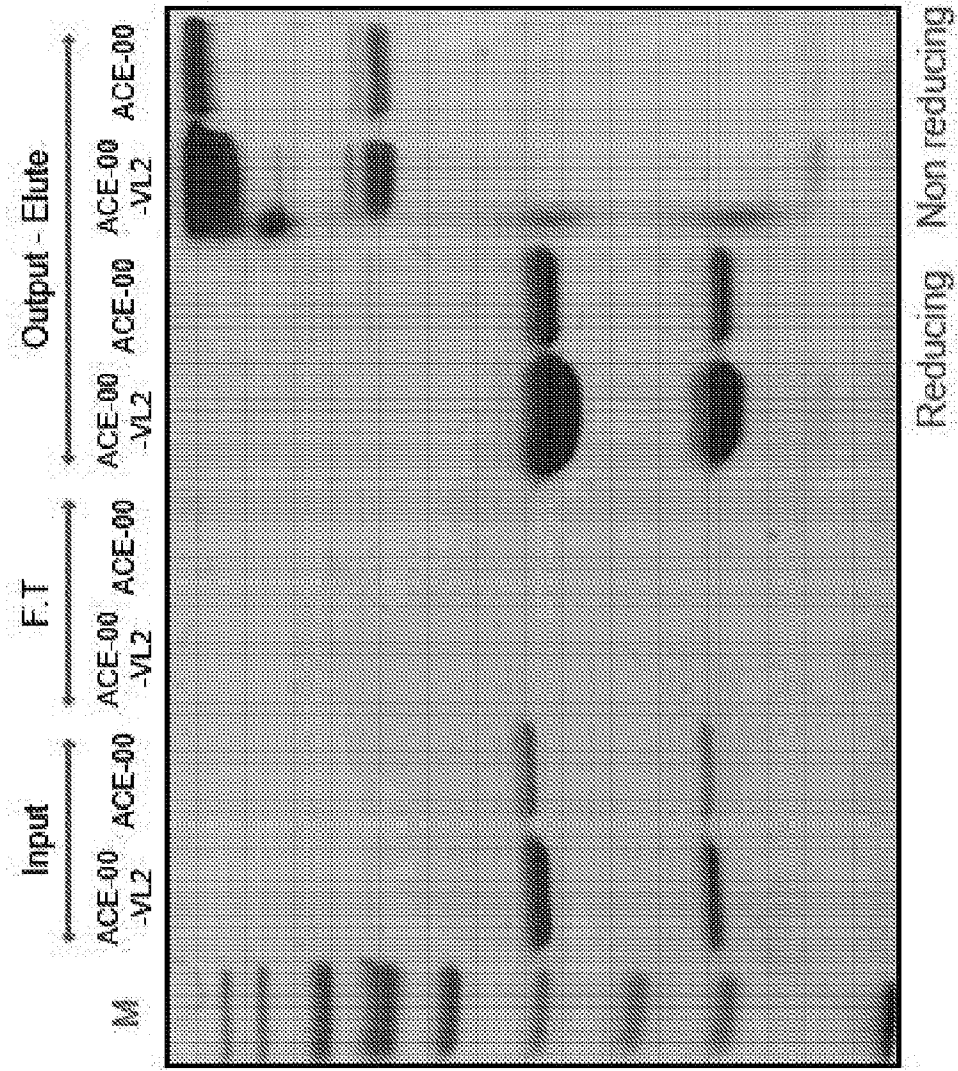


FIG. 2G

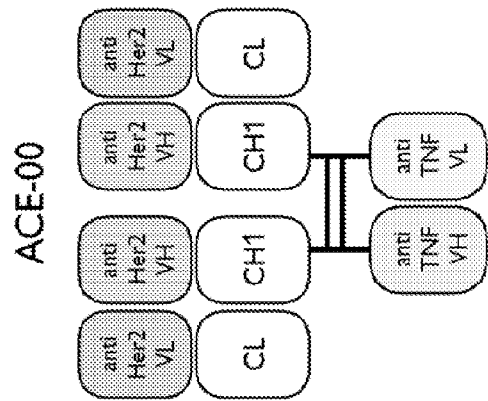


FIG. 2F

ACE-00-VL2

Peak	Size [kDa]	Rel. Conc. [ng/μl]	Calib. Conc. [ng/μl]	% of Total	Observations
1	0.1	0.0	0.0	0.0	
2	4	0.0	0.0	0.0	Lower Marker
3	7.6	0.0	0.0	0.0	System Peak
4	27.0	10.6	0.0	0.8	
5	29.4	26.3	0.0	1.9	
6	40.8	25.4	0.0	1.8	
7	59.4	185.1	0.0	13.1	
8	67.4	57.2	0.0	4.1	
9	84.0	13.0	0.0	0.9	
10	108.1	33.0	0.0	2.3	
11	119.2	47.5	0.0	3.4	
12	122.7	1,006.9	0.0	71.5	
13	219.1	4.1	0.0	0.2	Upper Marker
14	240.0	60.0	0.0	0.0	

ACE-00

Peak	Size [kDa]	Rel. Conc. [ng/μl]	Calib. Conc. [ng/μl]	% of Total	Observations
1	0.2	0.0	0.0	0.0	
2	4.5	0.0	0.0	0.0	Lower Marker
3	7.1	0.0	0.0	0.0	System Peak
4	27.0	7.6	0.0	0.6	
5	29.4	39.6	0.0	3.0	
6	40.9	12.4	0.0	0.9	
7	59.5	138.1	0.0	10.5	
8	67.1	242.8	0.0	18.5	
9	84.7	5.0	0.0	0.4	
10	108.8	35.0	0.0	2.7	
11	120.8	14.6	0.0	1.1	
12	126.1	818.2	0.0	62.3	
13	240.0	60.0	0.0	0.0	Upper Marker

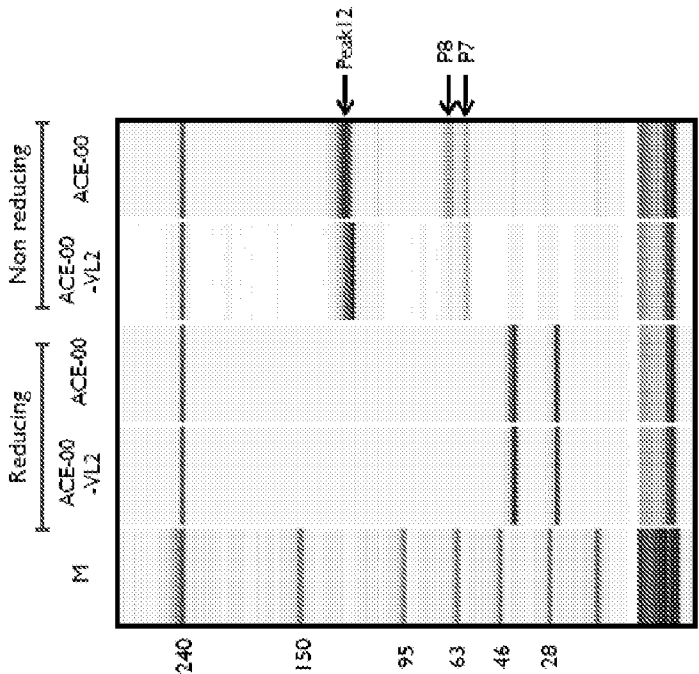


FIG. 2H

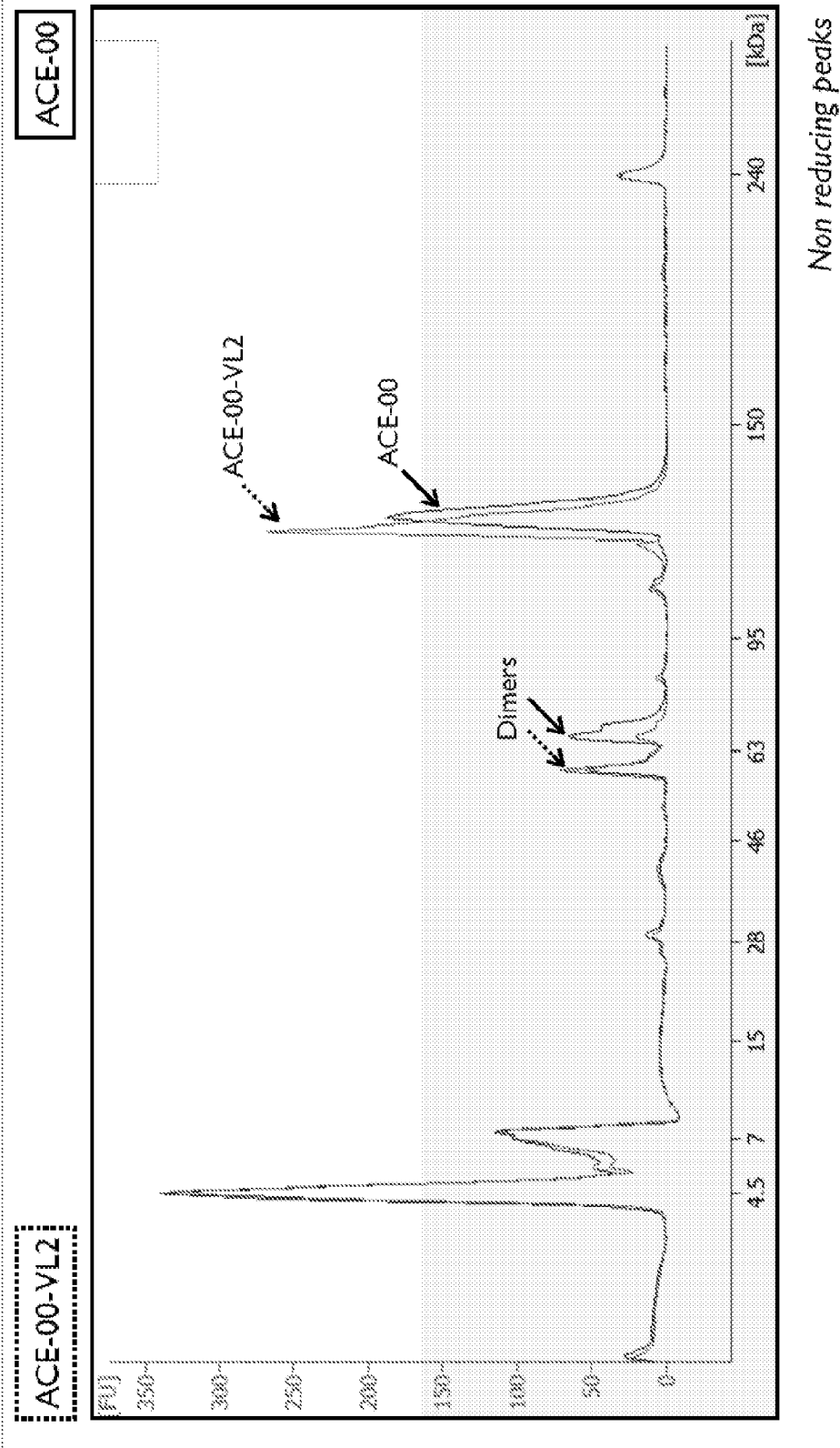
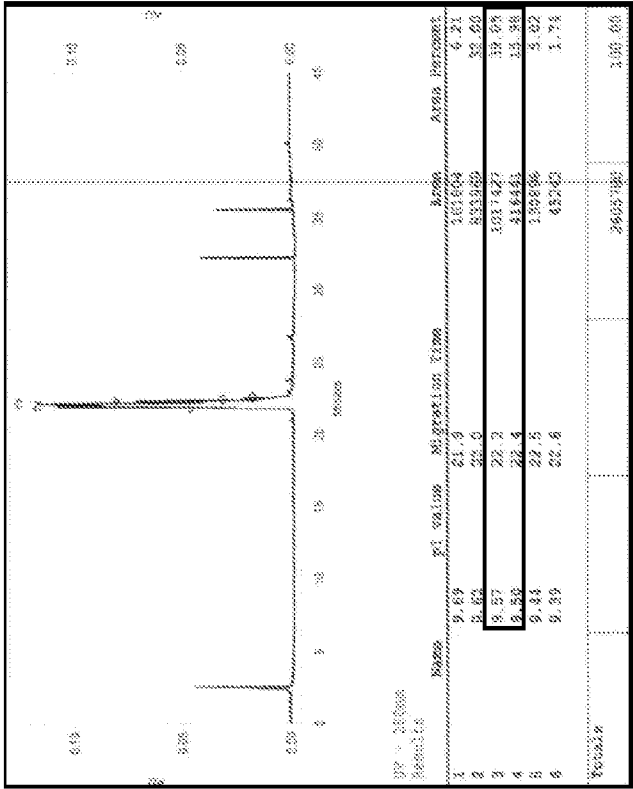
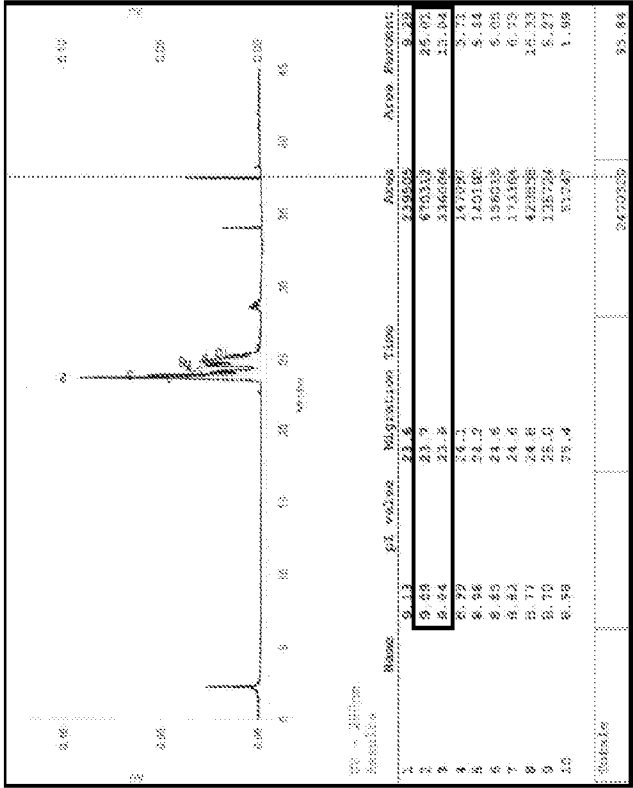


FIG. 2I

ACE-00-VL2



ACE-00



Chains	M.W	pI	Charge at pH 7.0
ACE-00-VH	38.8 kDa	6.66	-2.97
ACE-00-VL	37.3 kDa	8.44	+5.46
ACE-00-LC	23.4 kDa	6.71	-1.37

Proteins	M.W	pI	Charge at pH 7.0
ACE-00-VL2	121.5 kDa	7.48	+8.68
ACE-00	123 kDa	7.01	+0.25

FIG. 2J

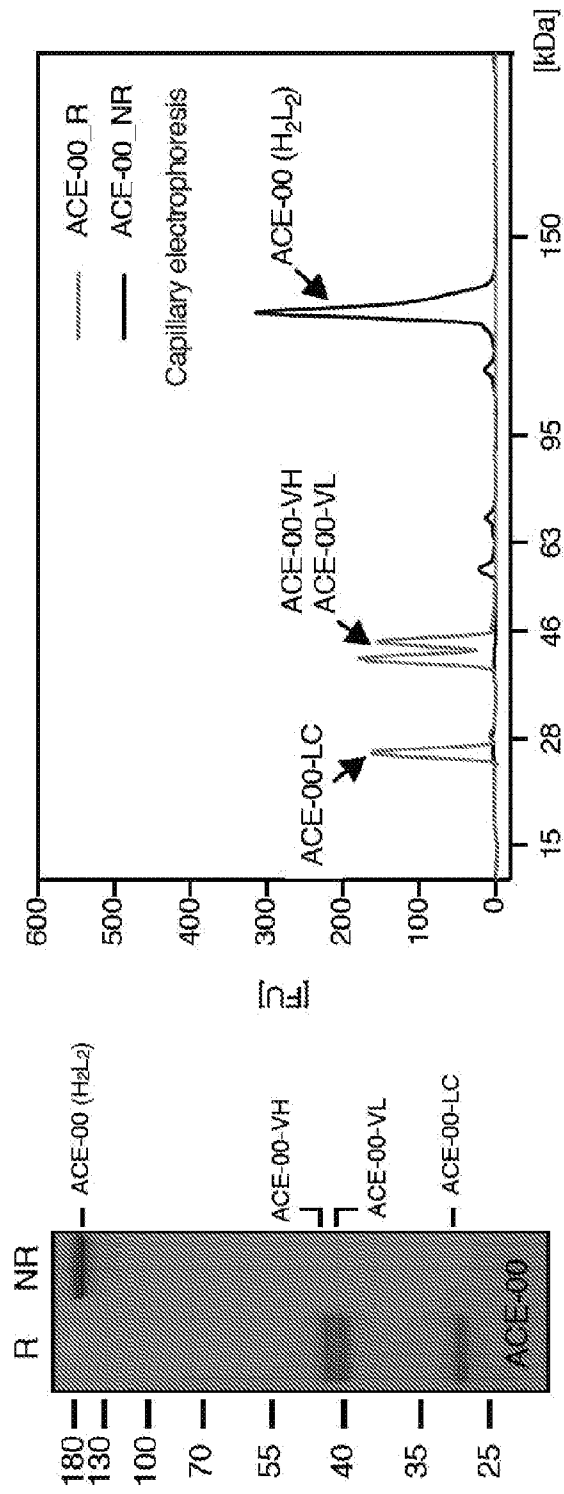


FIG. 2K

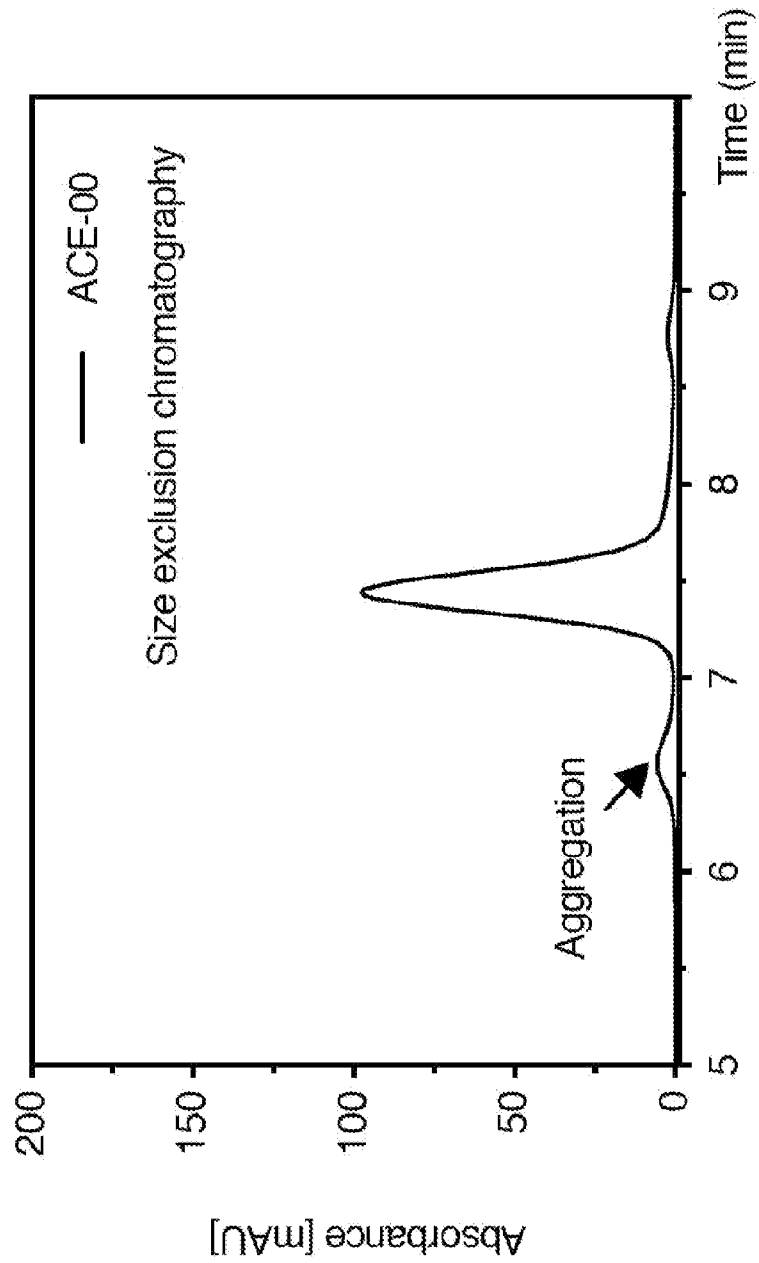


FIG. 2L

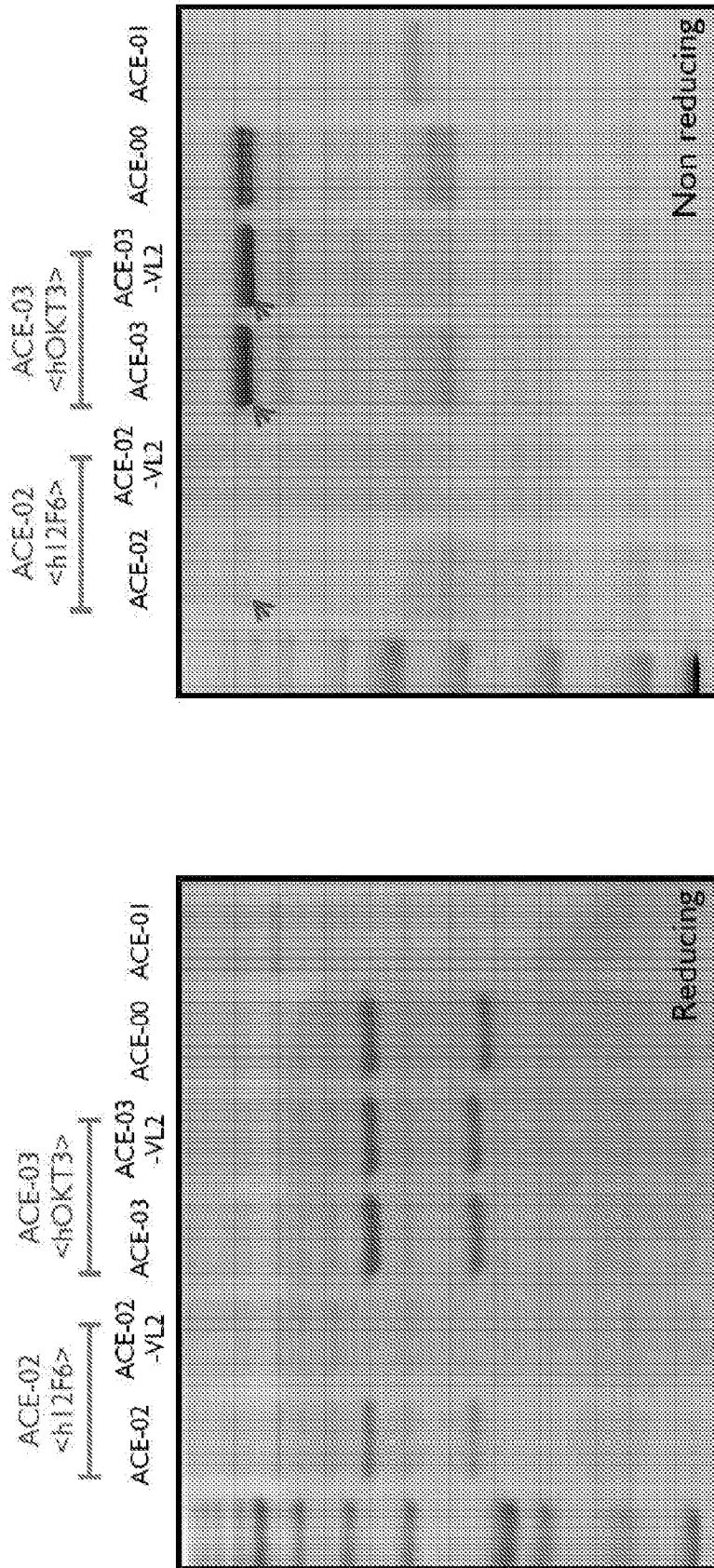


FIG. 3

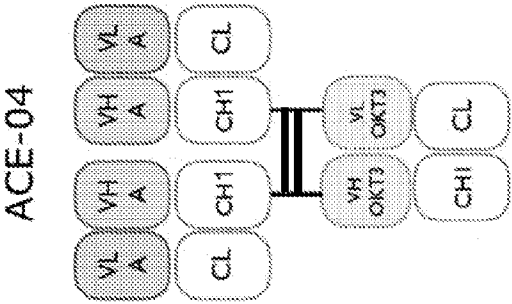


FIG. 4A

ACE-05

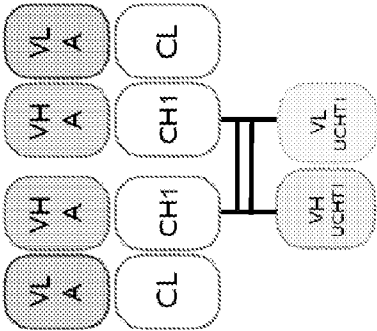


FIG. 4B

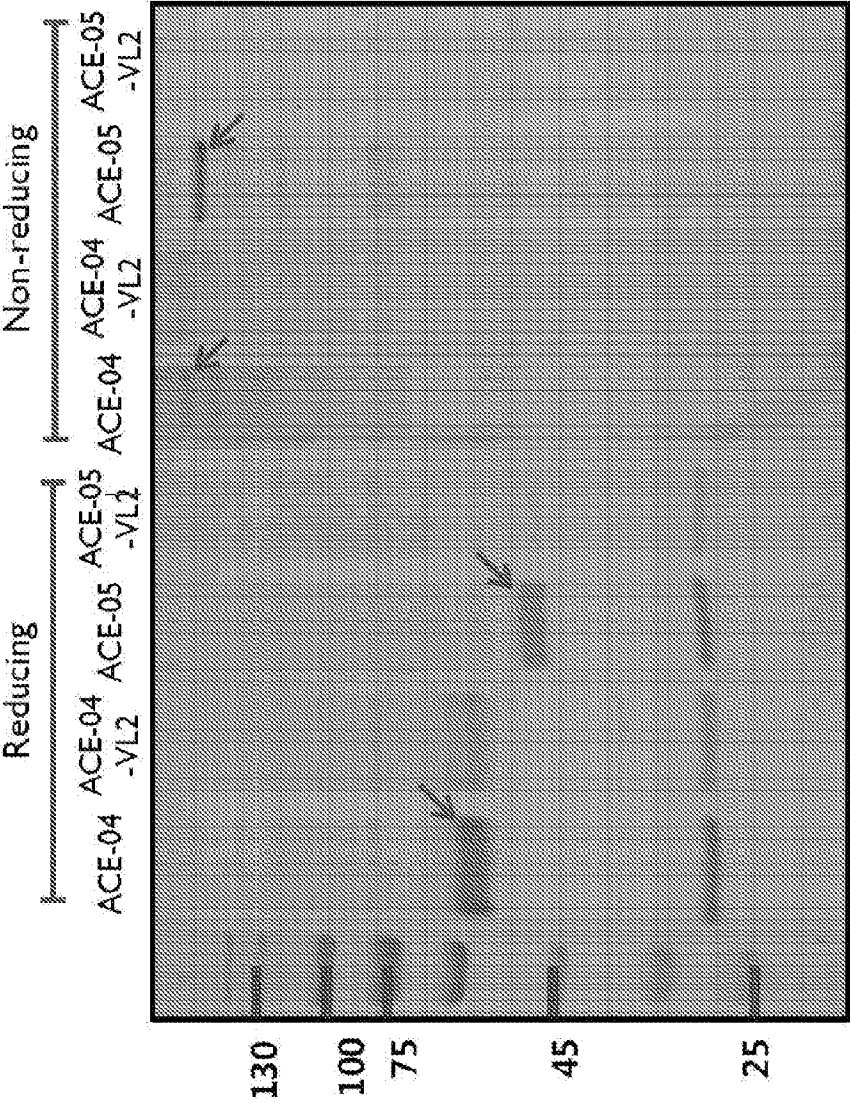
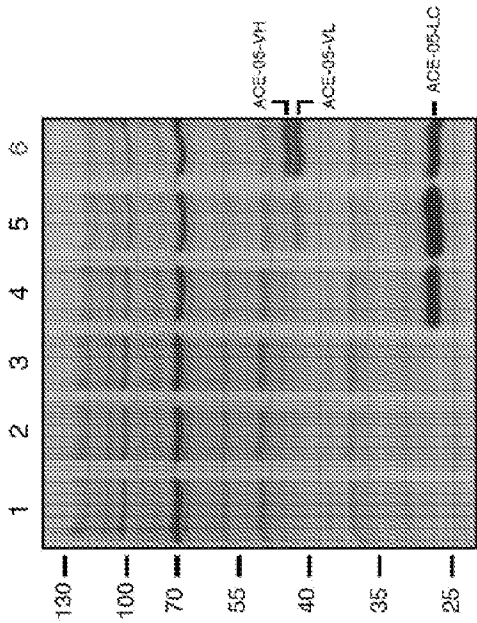


FIG. 4C



Lane	1	2	3	4	5	6
Chains of transfection	1. ACE-05-VH	1. ACE-05-VL	1. ACE-05-VH 2. ACE-05-VL	1. ACE-05-VH 2. ACE-05-LC	1. ACE-05-VL 2. ACE-05-LC	1. ACE-05-VH 2. ACE-05-VL 3. ACE-05-LC
Secretion	No	No	No	No	Yes	Yes
Potential regulatory mechanism						

FIG. 4D

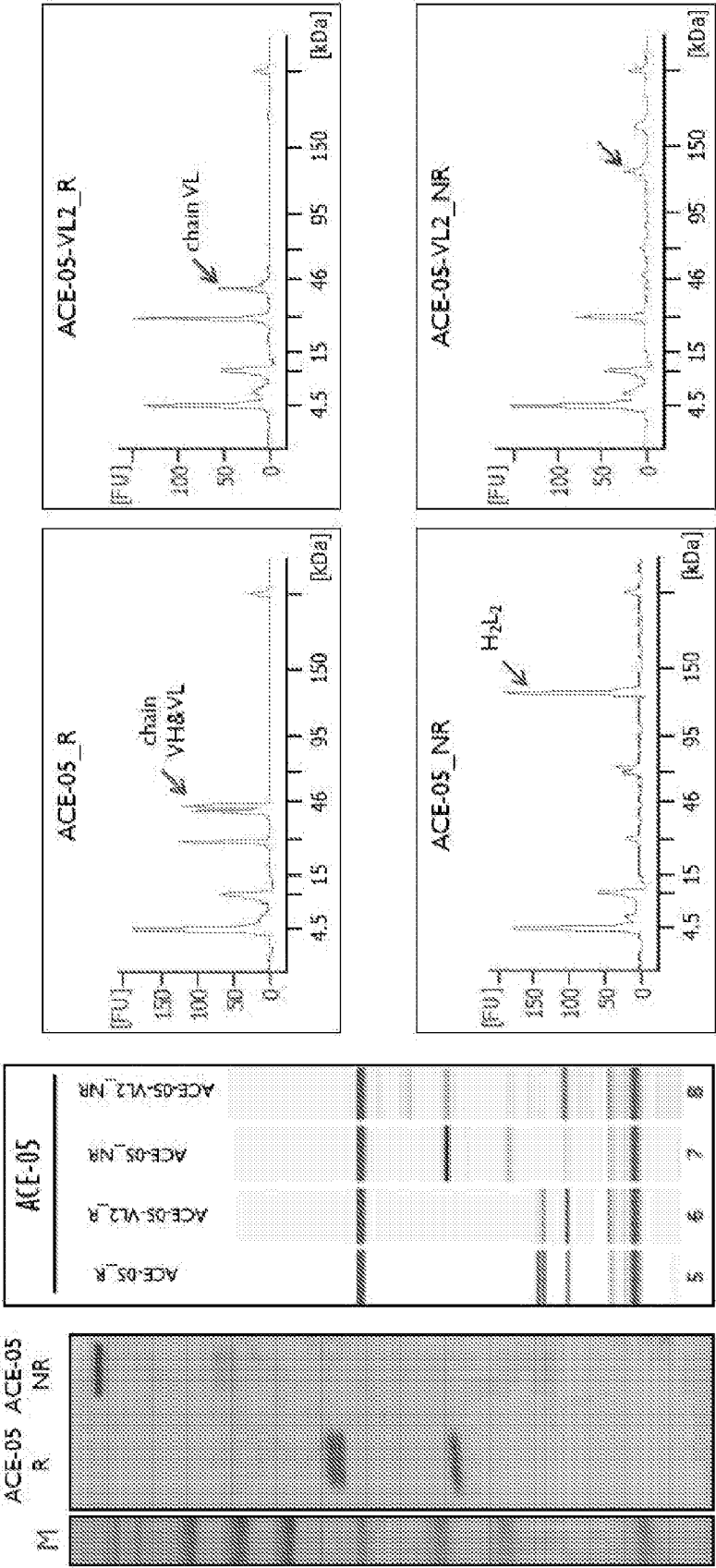


FIG. 4E

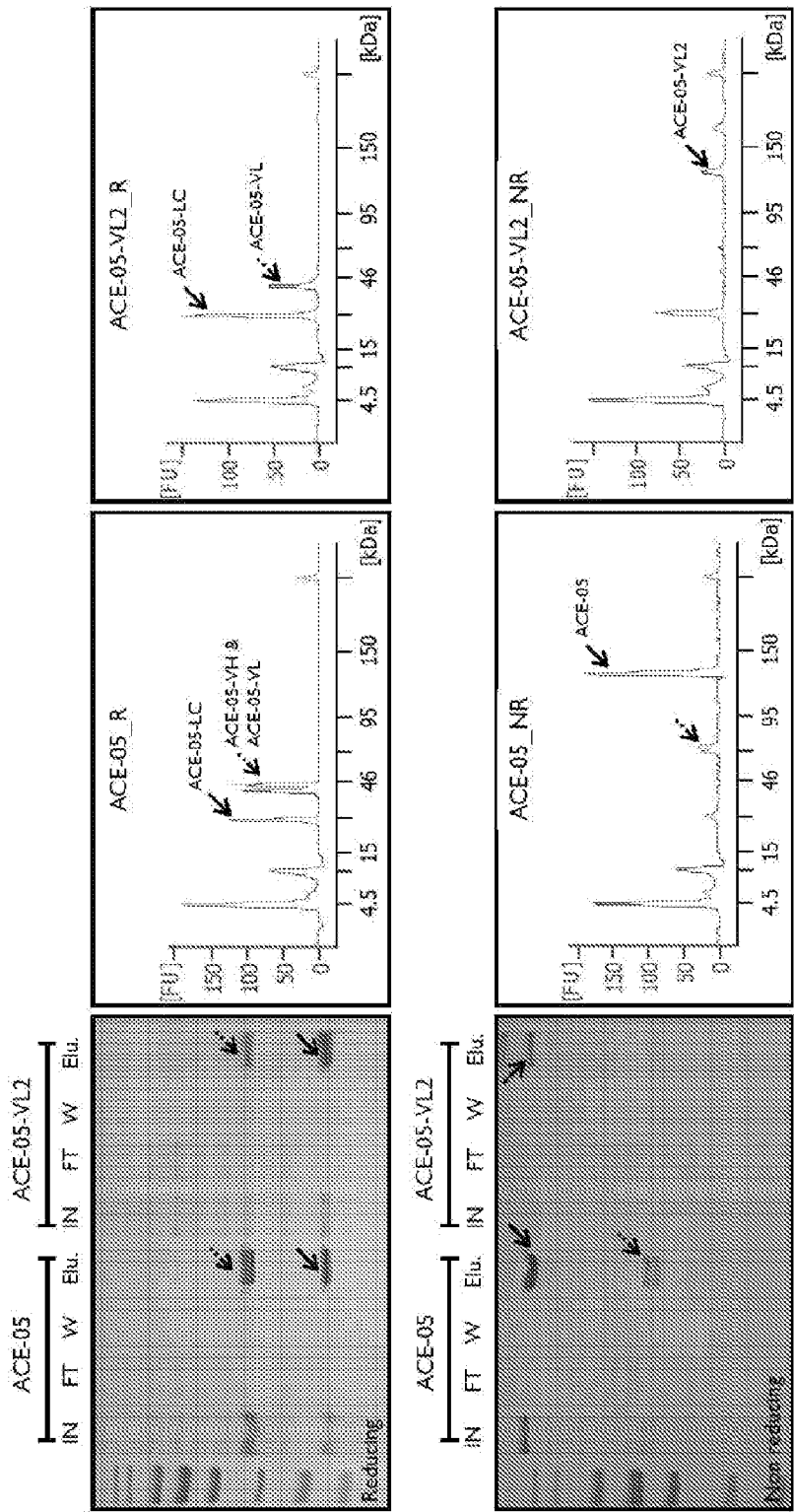


FIG. 4F

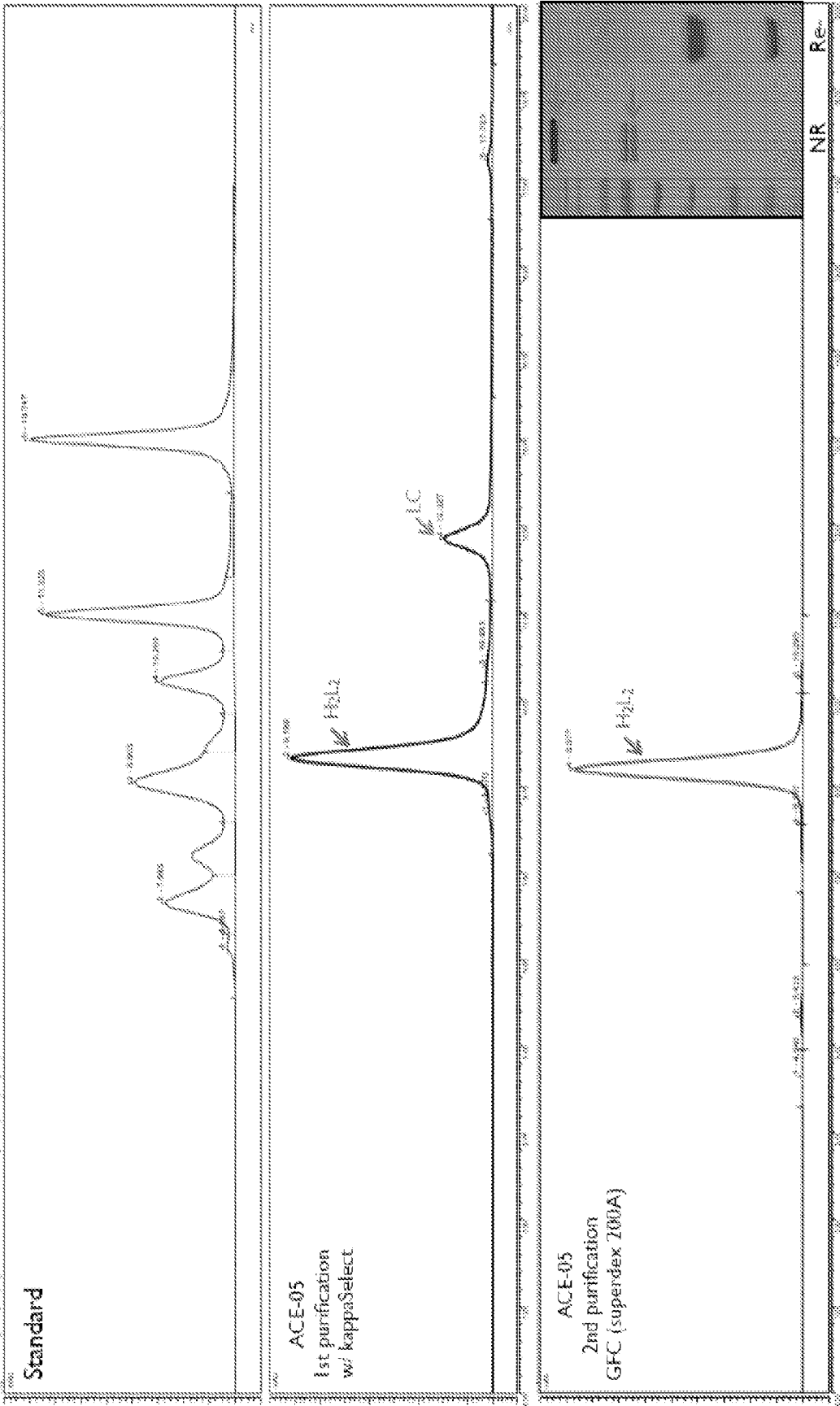


FIG. 4G

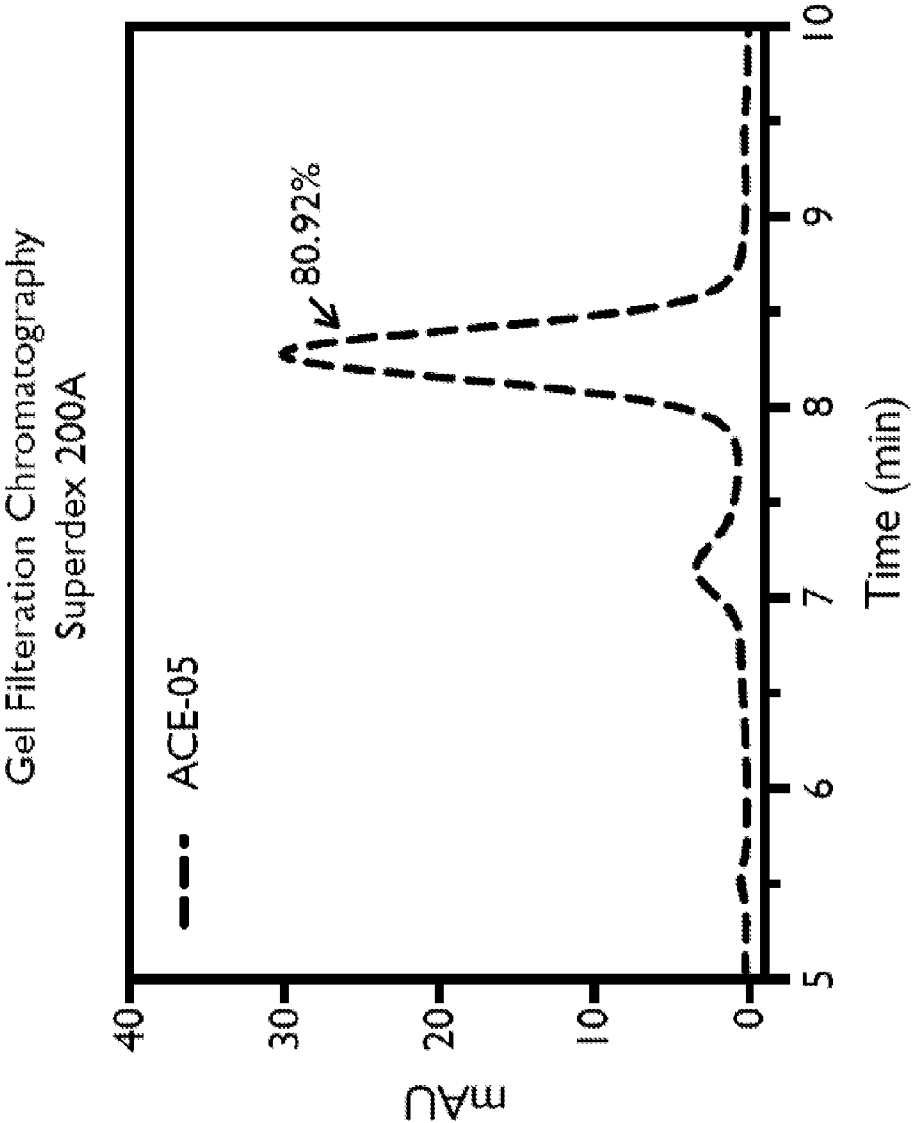


FIG. 4H

CEX HPLC

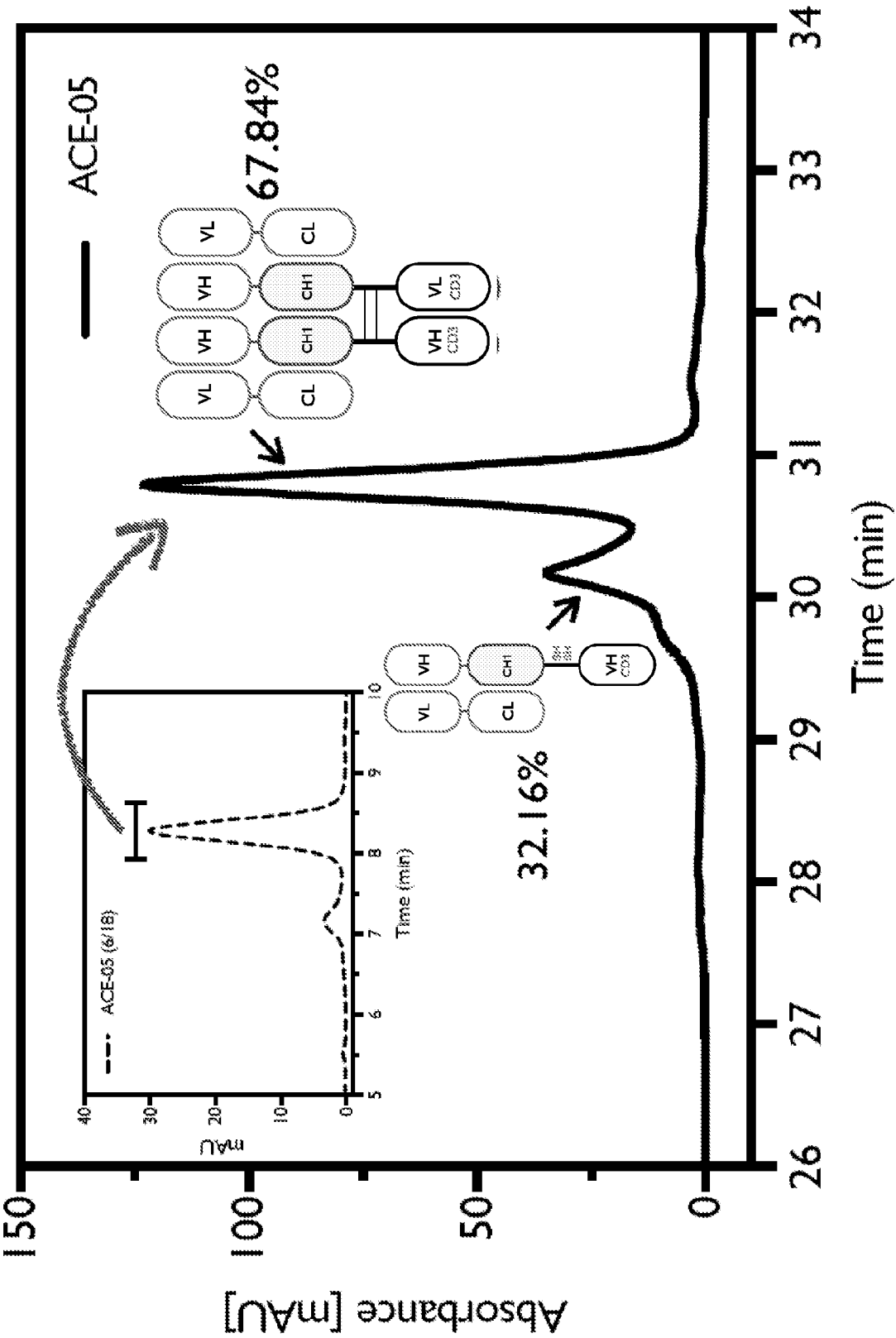
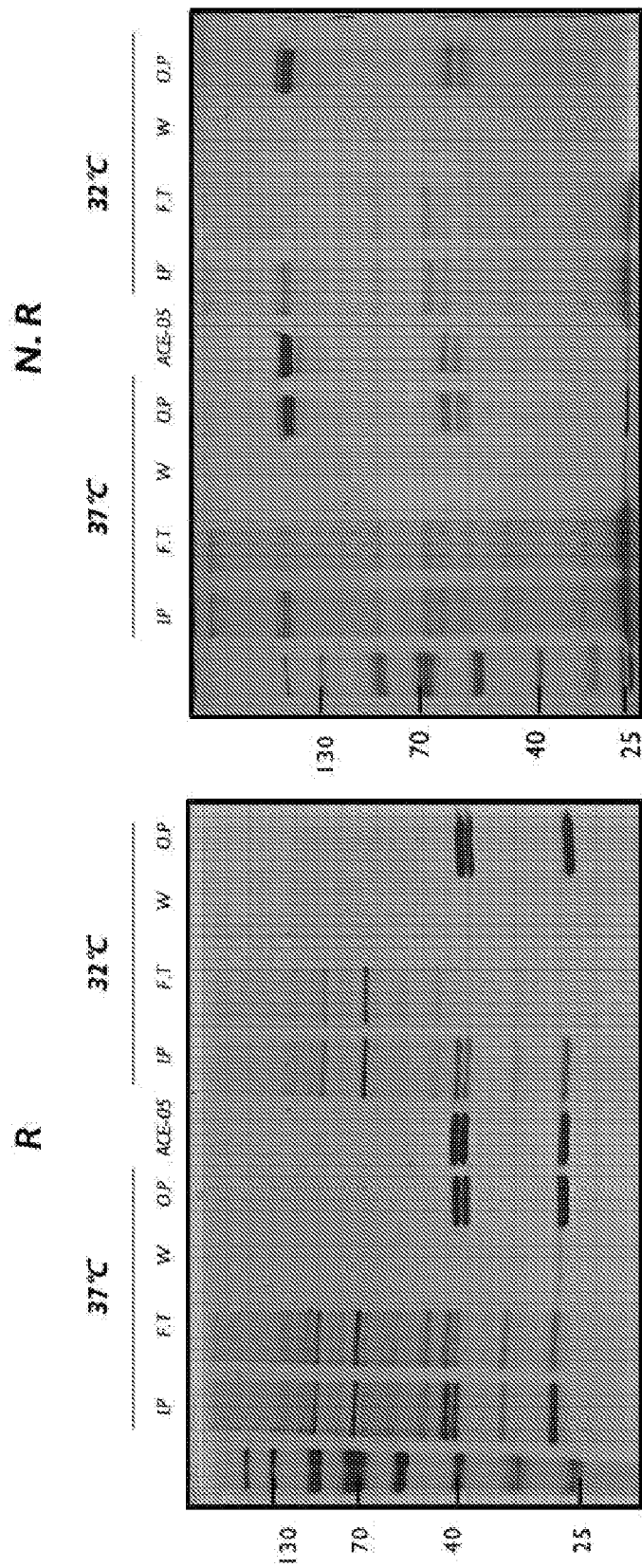
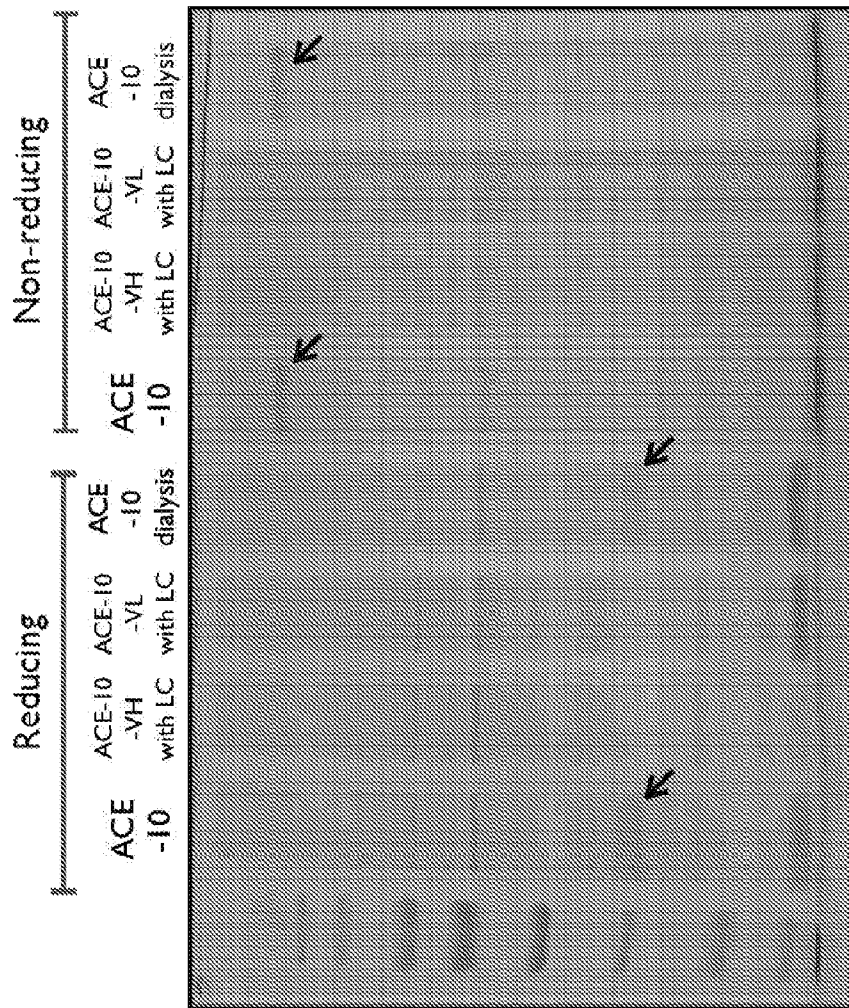
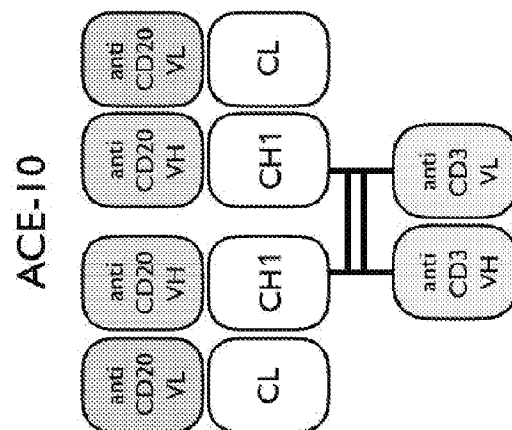


FIG. 4I



Clone	Chain	First antigen	Second antigen	Length of the GAS linker
ACE-09	ACE-09-VL	Anti-PD-L1 Ab	Anti-CD3 UCHT1	0
	ACE-09-VH	Anti-PD-L1 Ab	Anti-CD3 UCHT1	0
ACE-05	ACE-09-VL	Anti-PD-L1 Ab	Anti-CD3 UCHT1	5
	ACE-09-VH	Anti-PD-L1 Ab	Anti-CD3 UCHT1	5

FIG. 5



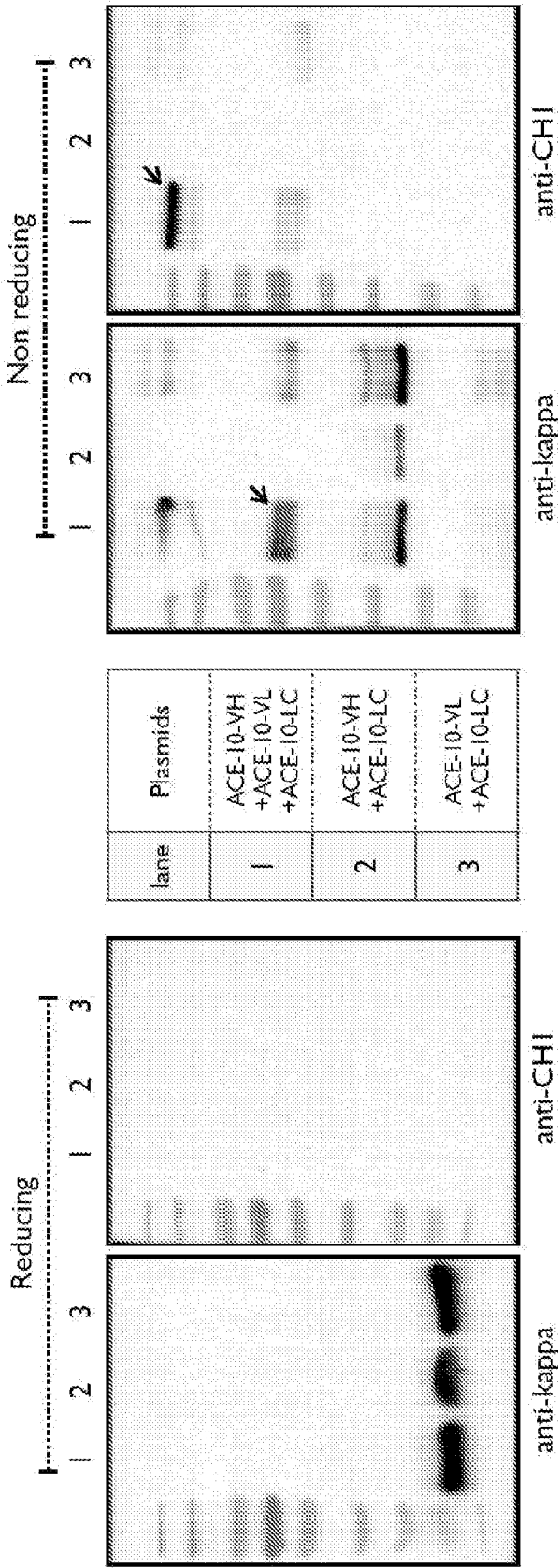
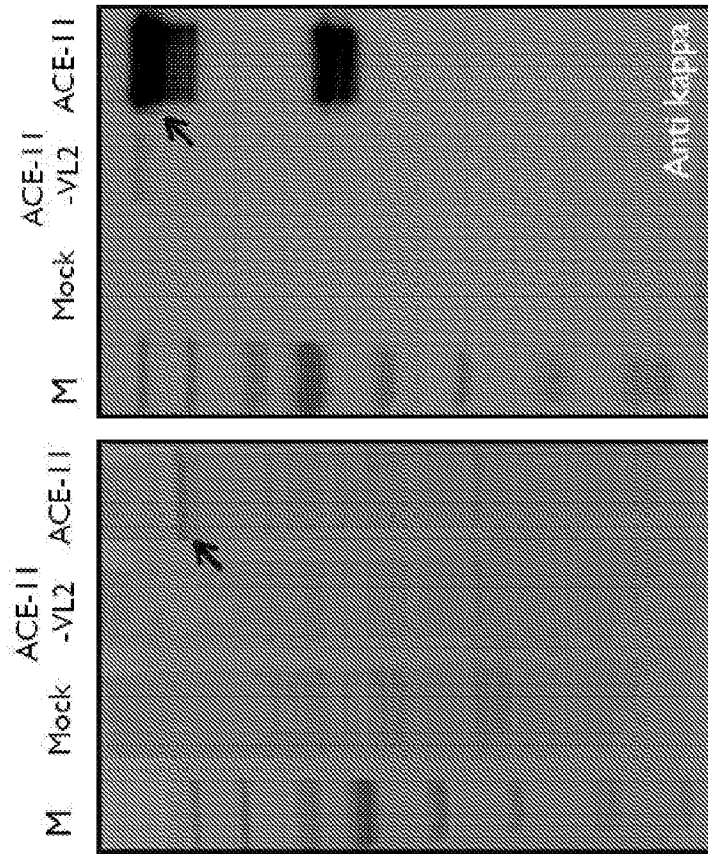
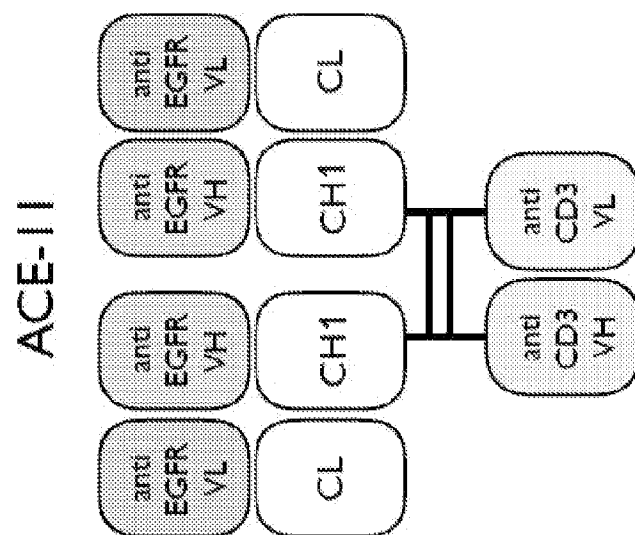
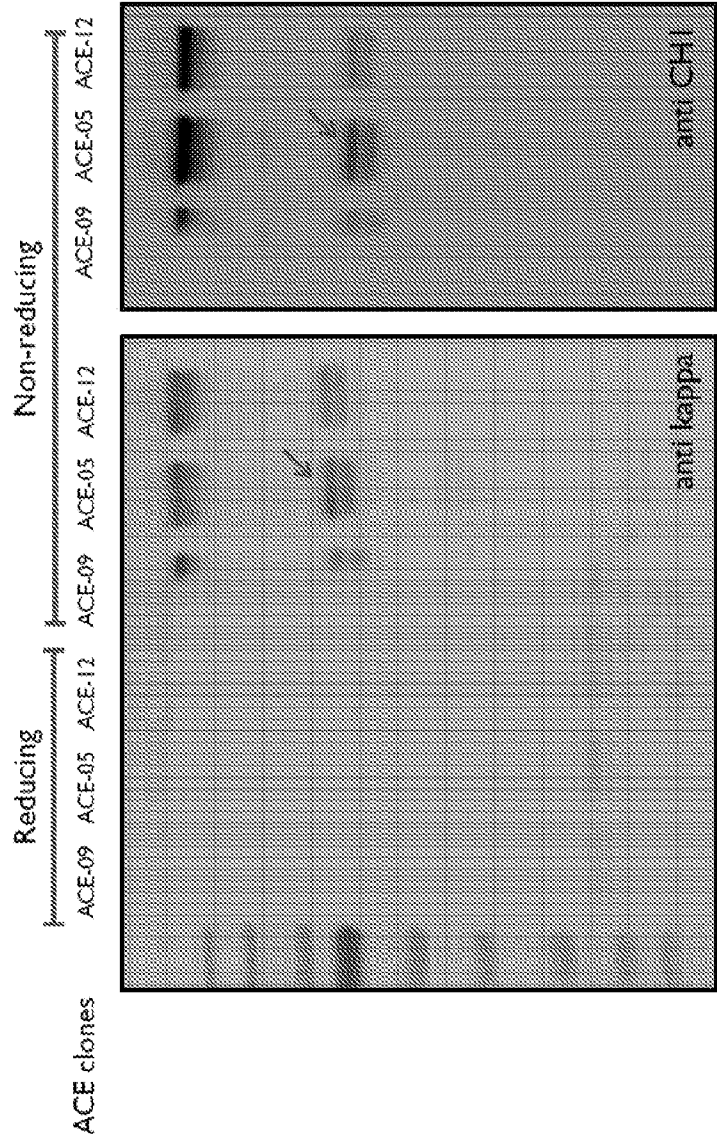


FIG. 6C





Clone	Chain	First antigen	Second antigen	Length of the GAS linker
ACE-09	ACE-09-VL	Anti-PD-L1 Ab	Anti-CD3 UCHT1	0
	ACE-09-VH	Anti-PD-L1 Ab	Anti-CD3 UCHT1	0
ACE-05	ACE-05-VL	Anti-PD-L1 Ab	Anti-CD3 UCHT1	5
	ACE-05-VH	Anti-PD-L1 Ab	Anti-CD3 UCHT1	5
ACE-12	ACE-12-VL	Anti-PD-L1 Ab	Anti-CD3 UCHT1	9
	ACE-12-VH	Anti-PD-L1 Ab	Anti-CD3 UCHT1	10

FIG. 8

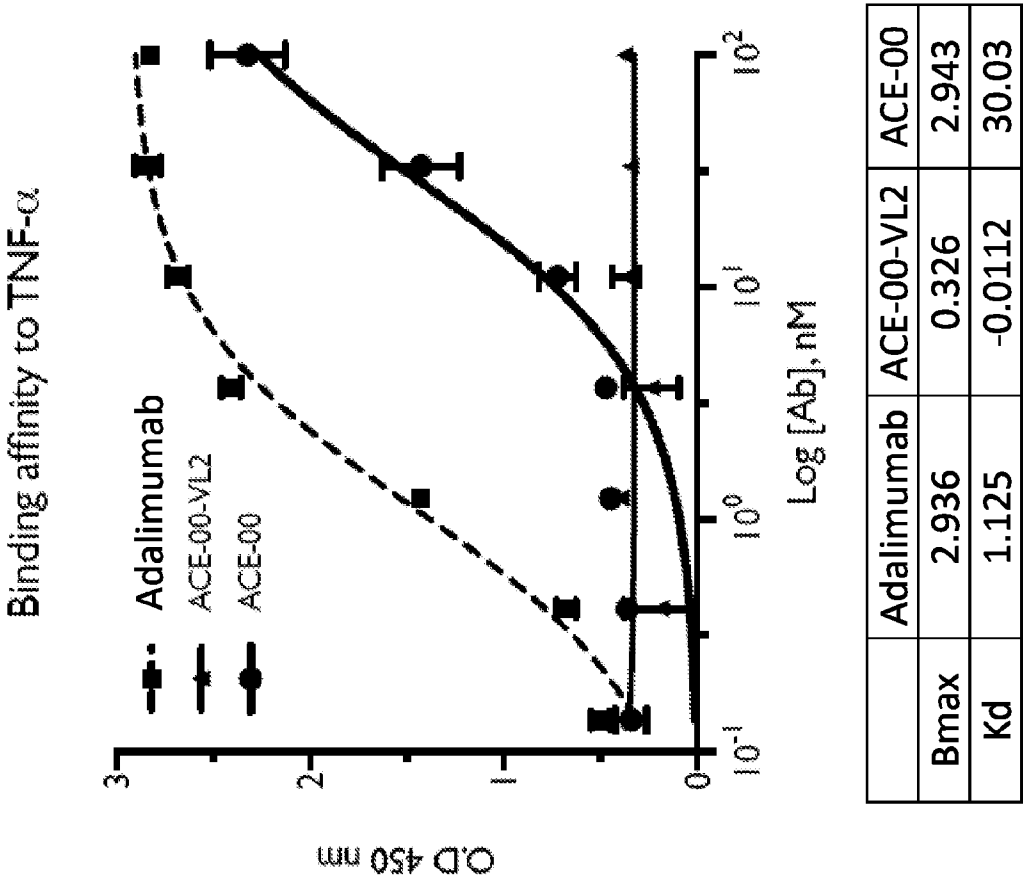


FIG. 9

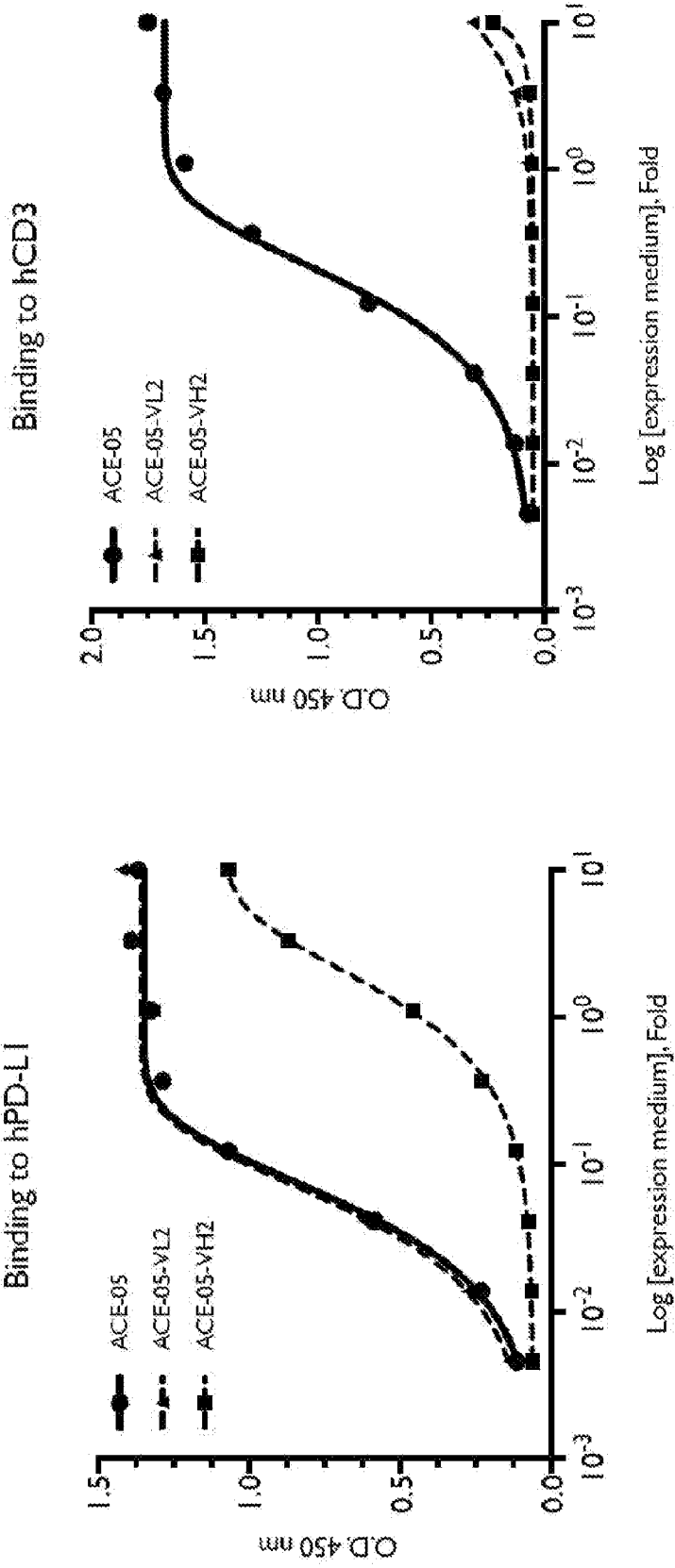


FIG. 10A

FIG. 10B

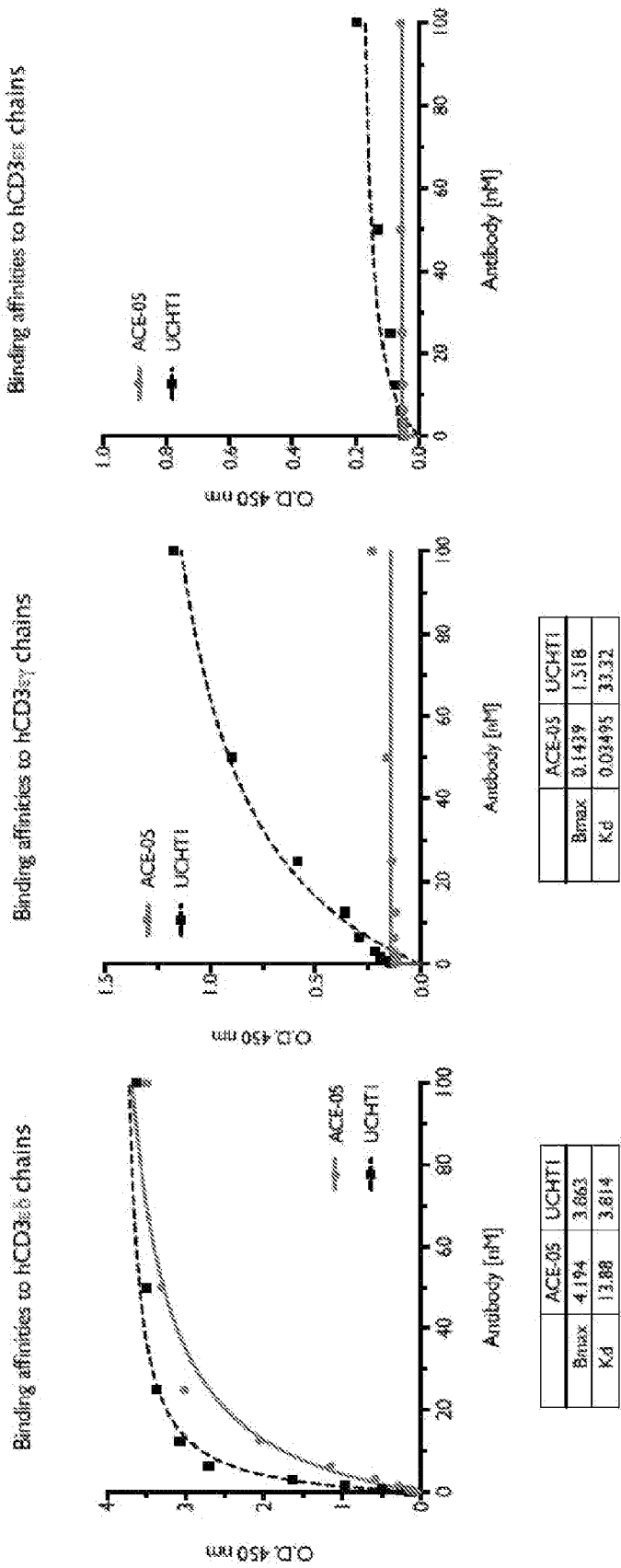
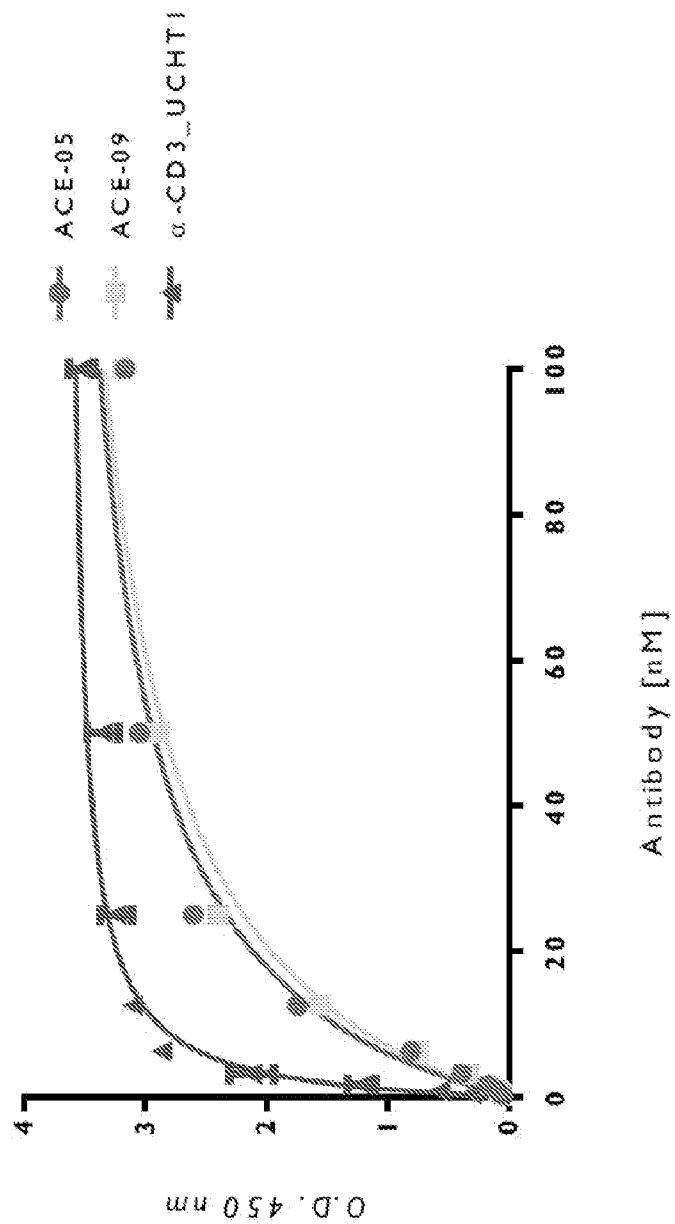


FIG. 10C



	ACE-05	ACE-09	α -CD3_UCHT1
Bmax	3.972	4.041	3.670
Kd	17.57	21.13	2.695

FIG. 11

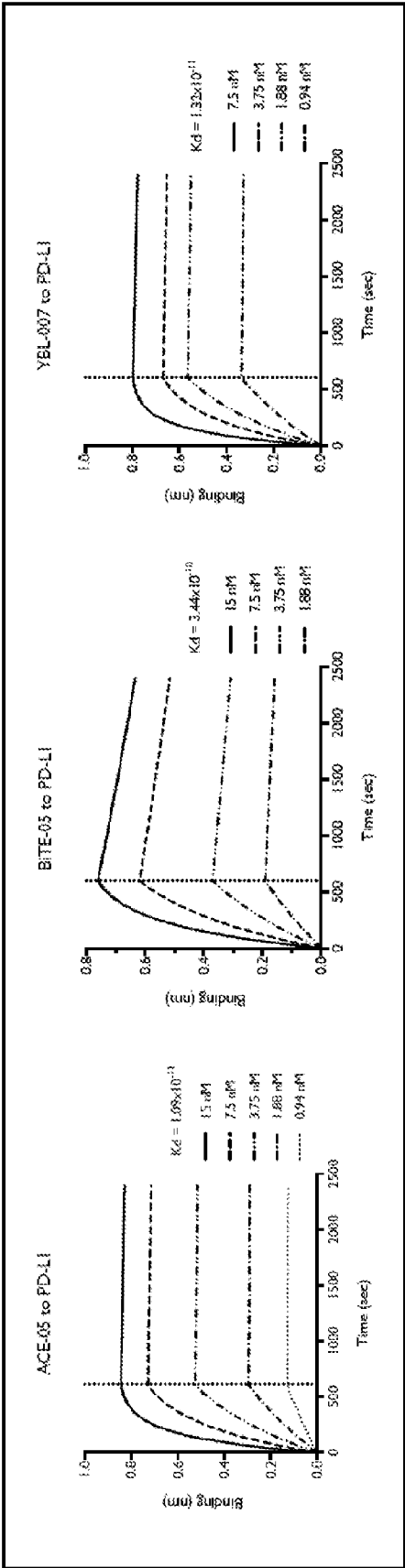


FIG. 12A

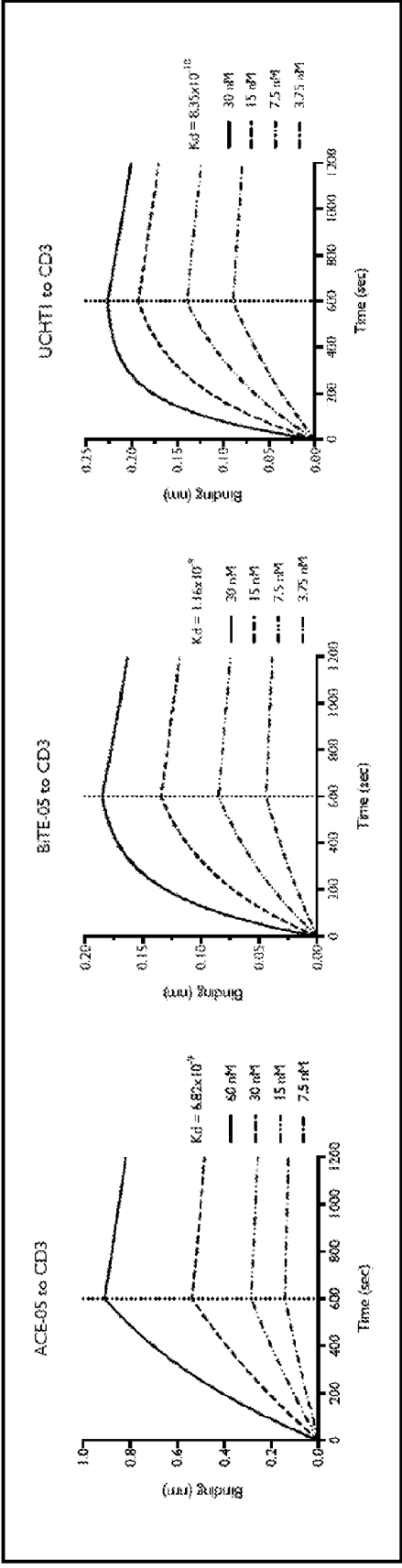
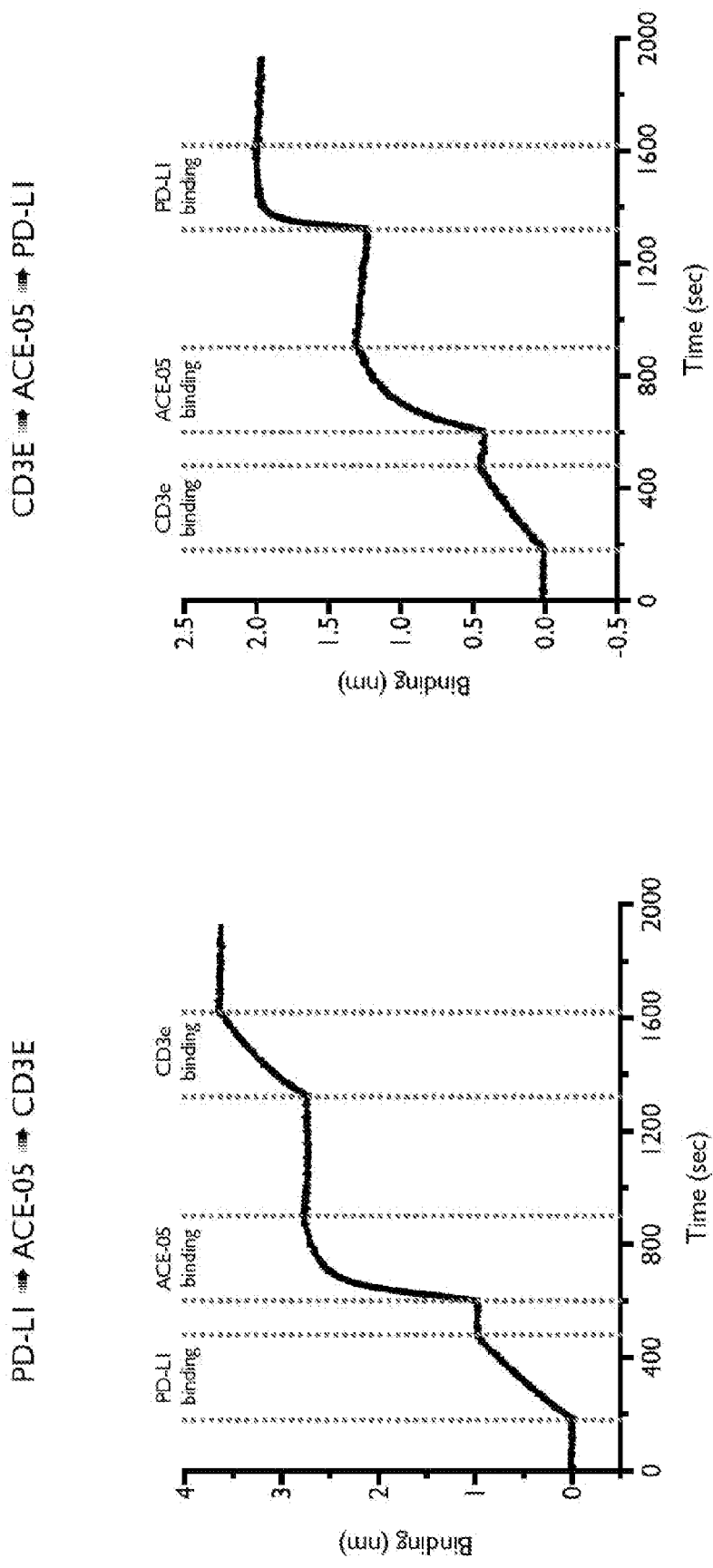


FIG. 12B

Samples	PD-L1 interaction				CD3 interaction			
	K _D	K _{on}	K _{dis}	R ²	K _D	K _{on}	K _{dis}	R ²
ACE-05	1.09×10^{-11}	9.23×10^5	1.00×10^{-5}	0.9967	6.82×10^{-9}	2.55×10^4	1.74×10^{-4}	0.9989
BITE-05	3.44×10^{-10}	2.94×10^5	1.01×10^{-4}	0.9975	1.16×10^{-9}	1.83×10^5	2.11×10^{-4}	0.9961
YBL-007 anti-PD-L1	1.32×10^{-11}	1.02×10^6	1.34×10^{-5}	0.9965	-	-	-	-
UCHT1 anti-CD3	-	-	-	-	8.35×10^{-10}	2.41×10^5	2.01×10^{-4}	0.9907

FIG. 12C



Simultaneous binding of ACE-05

FIG. 12D

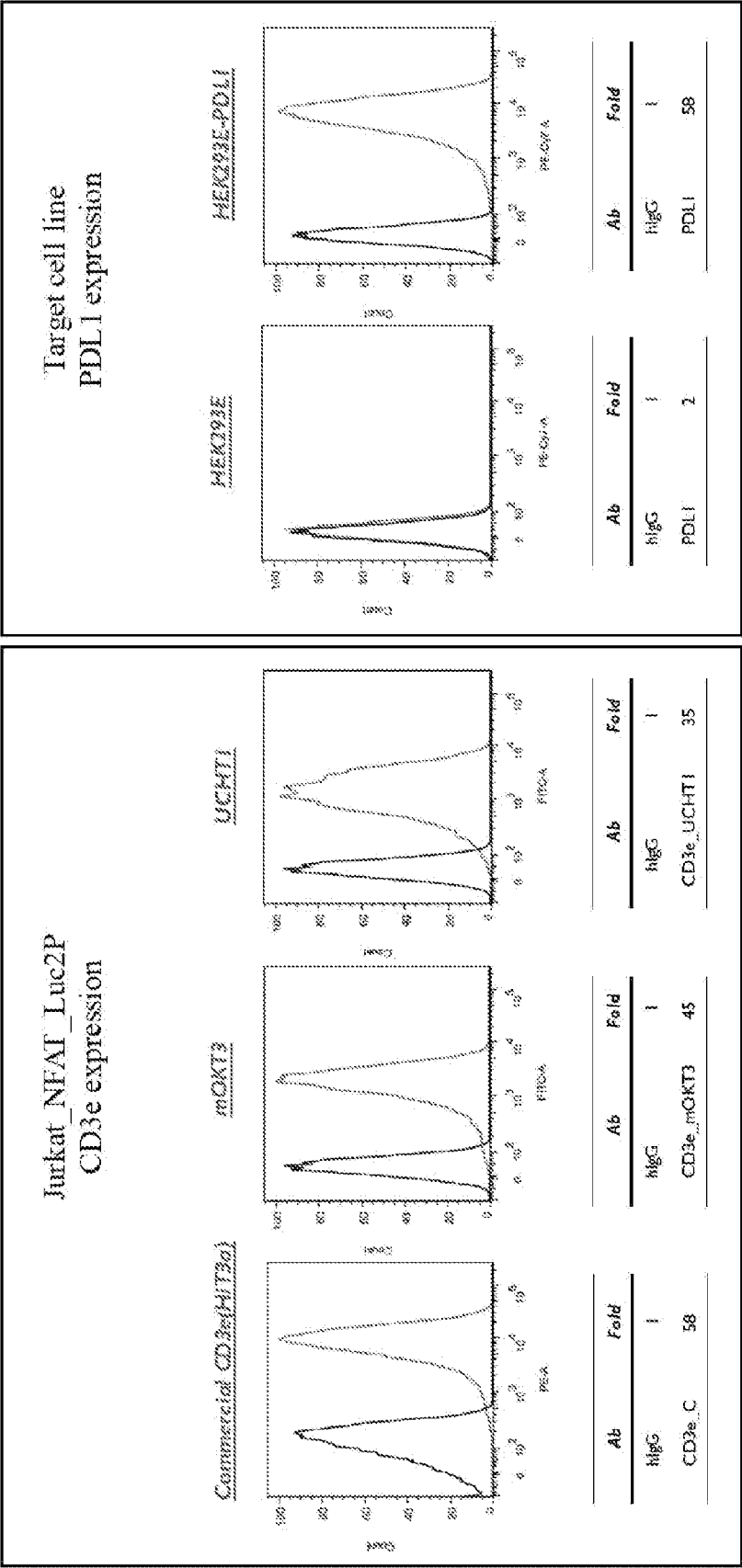


FIG. 13A

PD-L1 dependent T cell activation

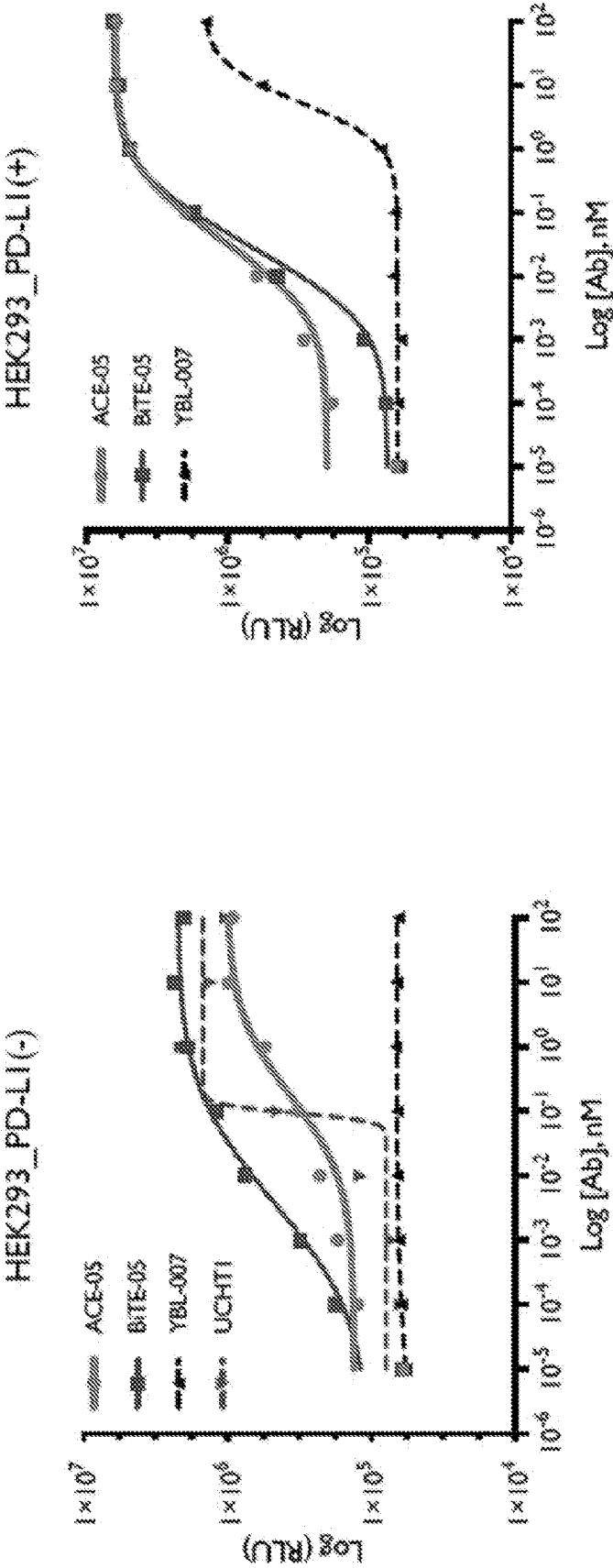


FIG. 13B

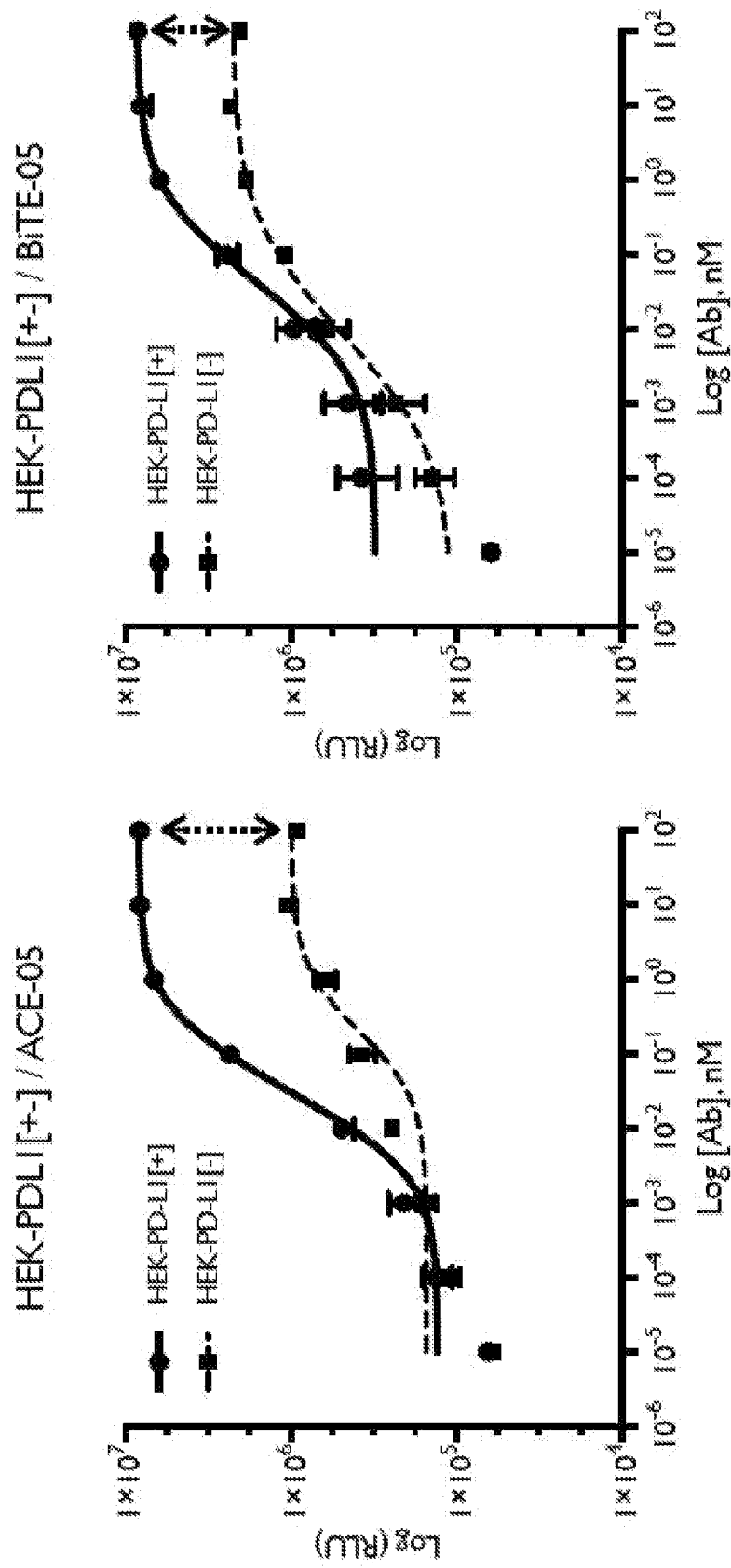


FIG. 13C

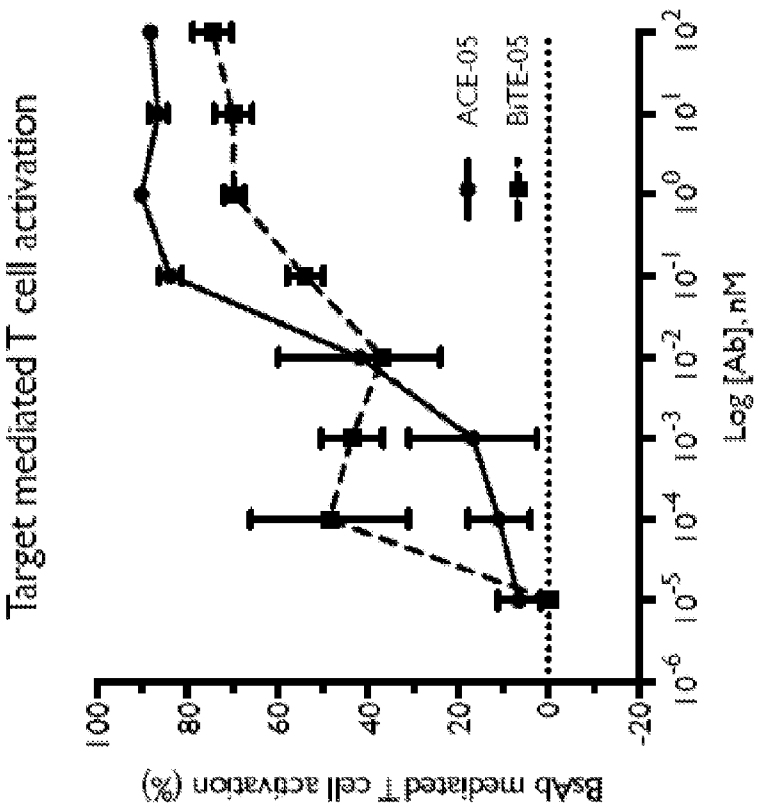


FIG. 13E

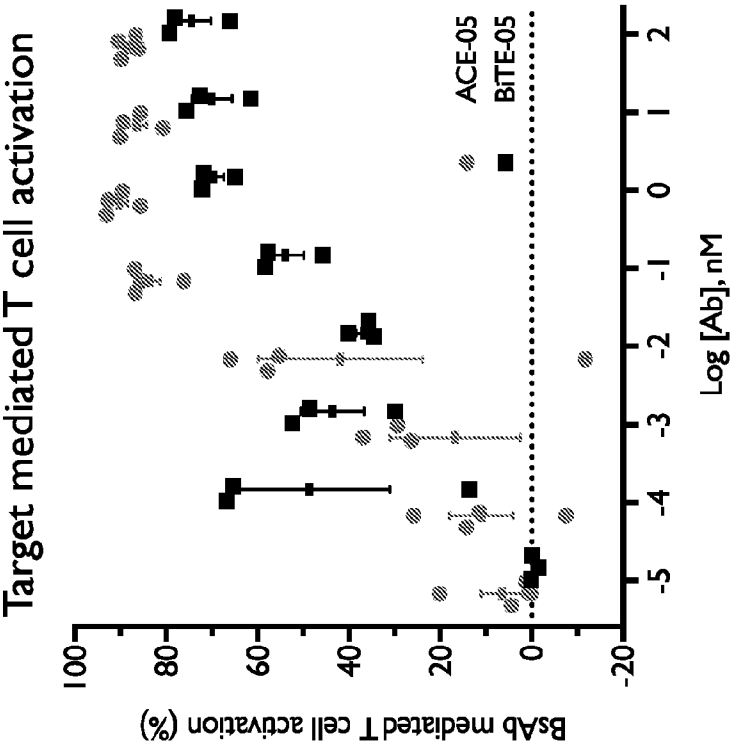


FIG. 13D

T cell response - cancer immune cycle

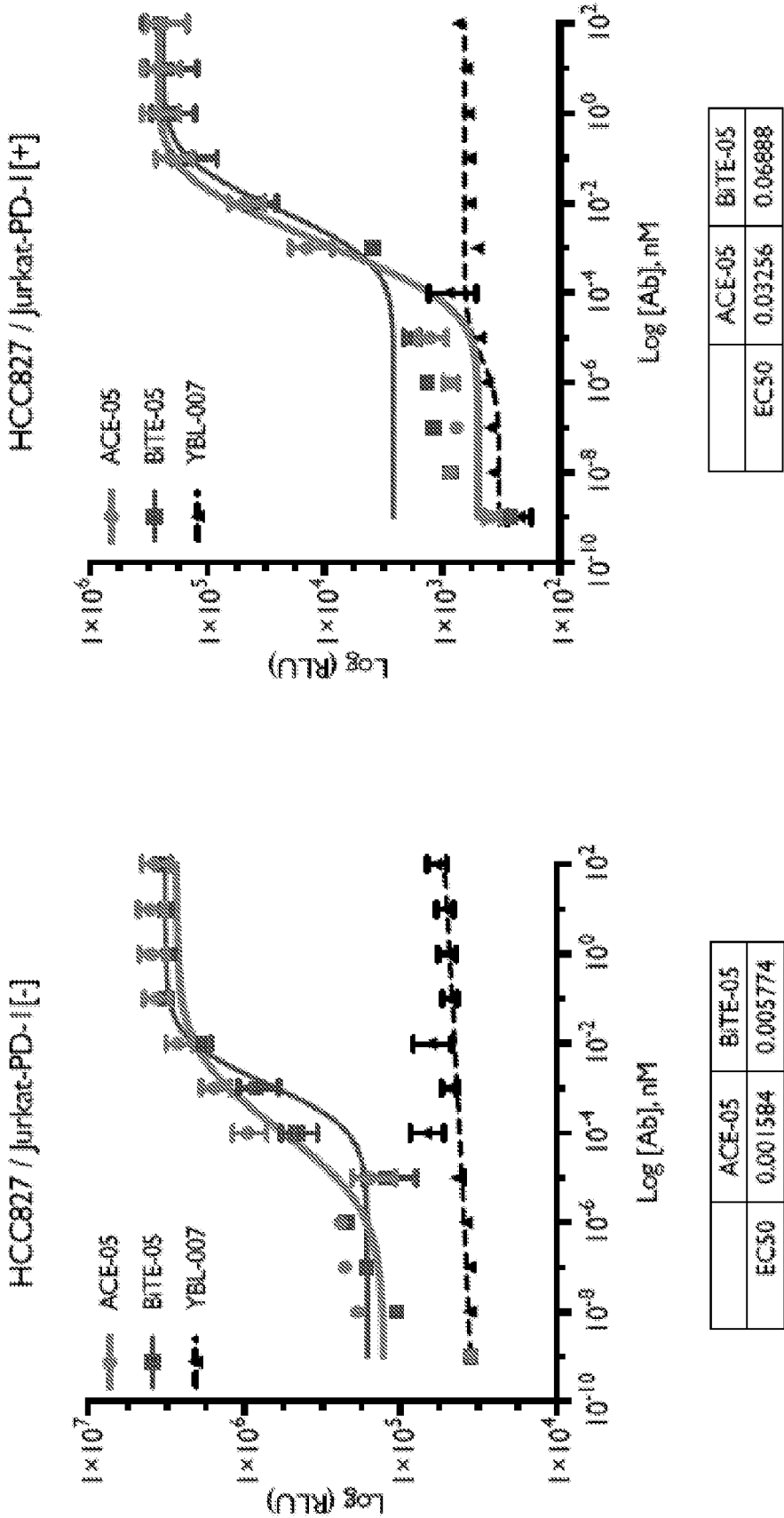


FIG. 13F

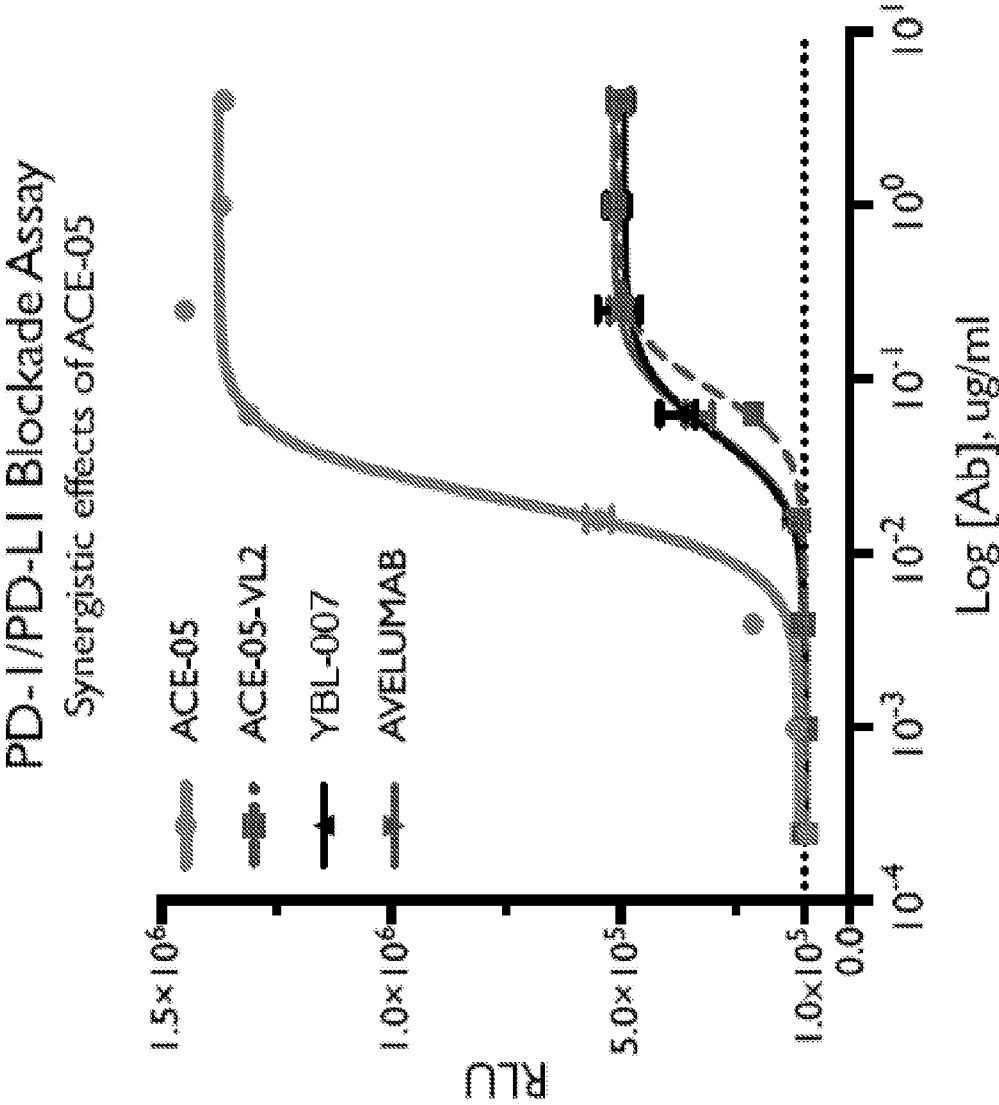


FIG. 13G

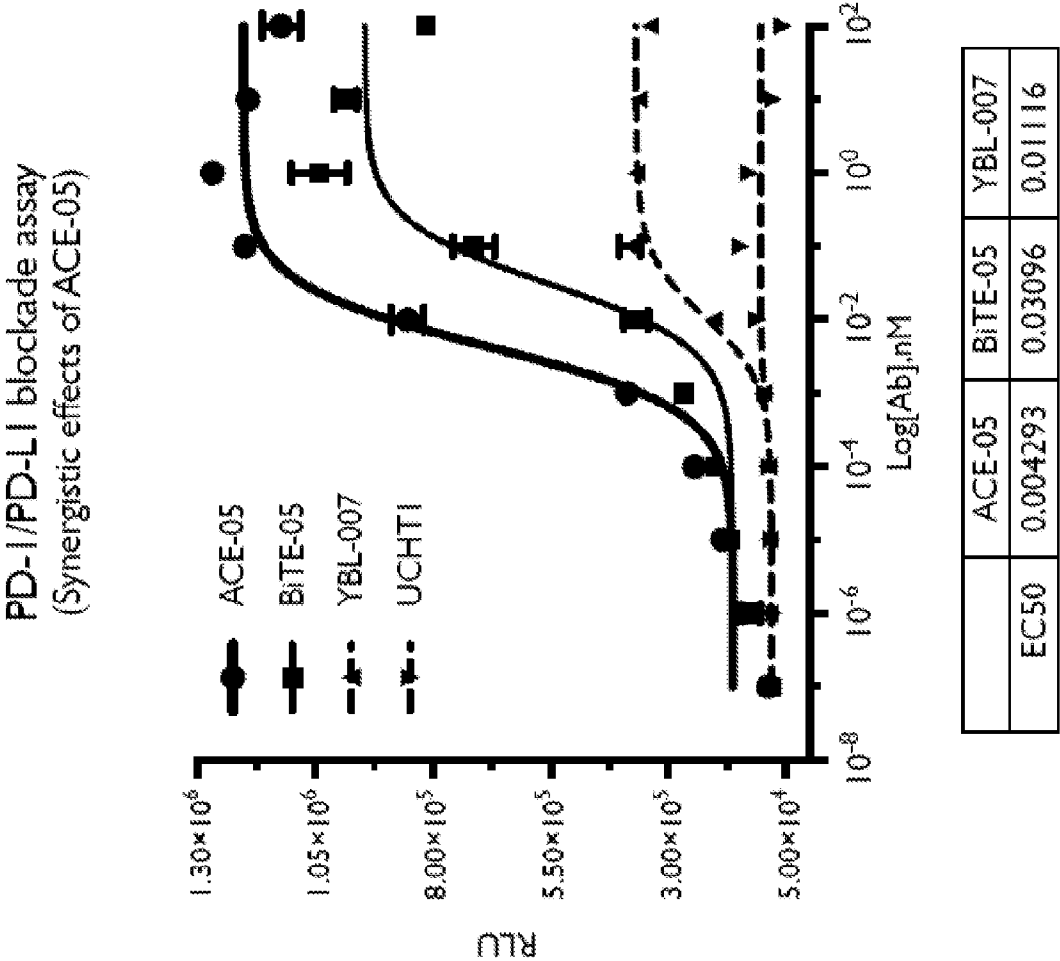


FIG. 13H

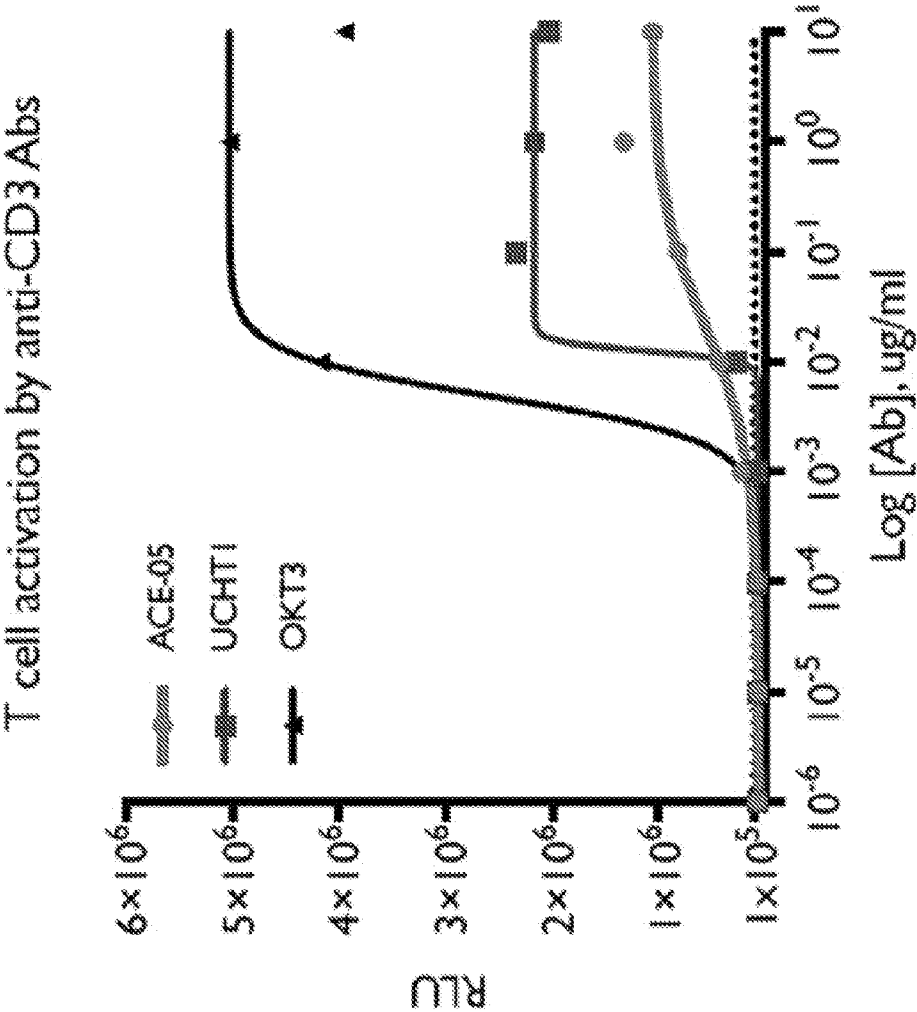


FIG. 13I

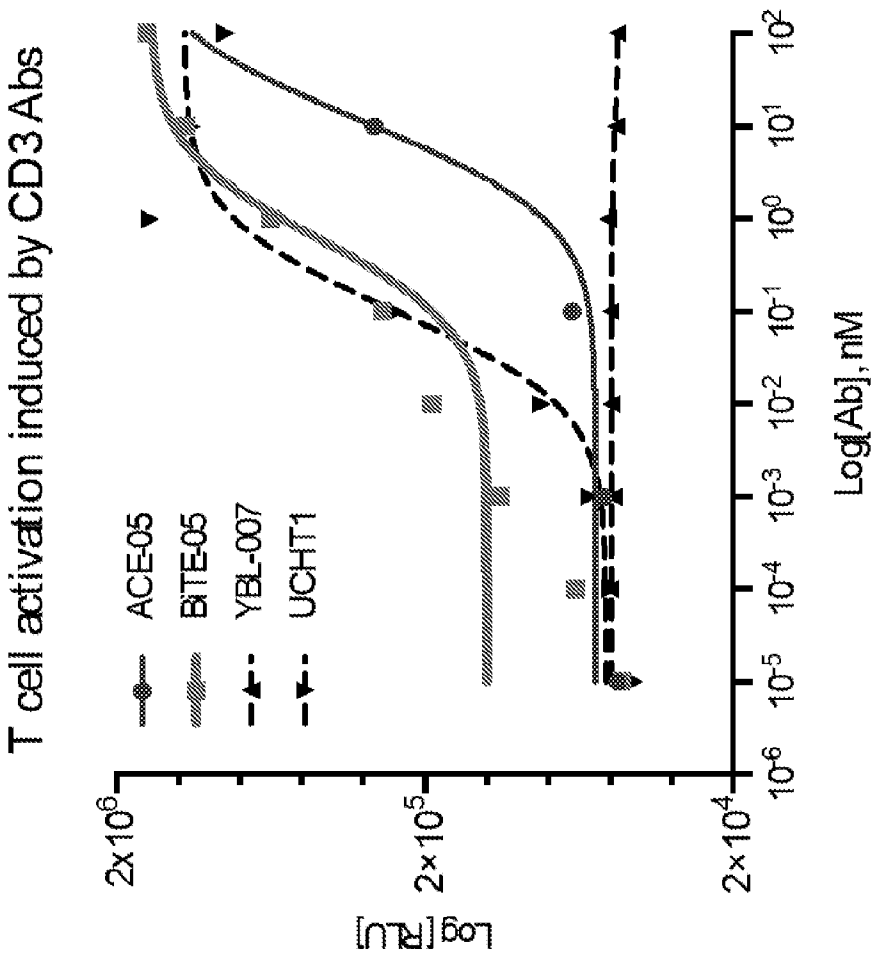


FIG. 13J

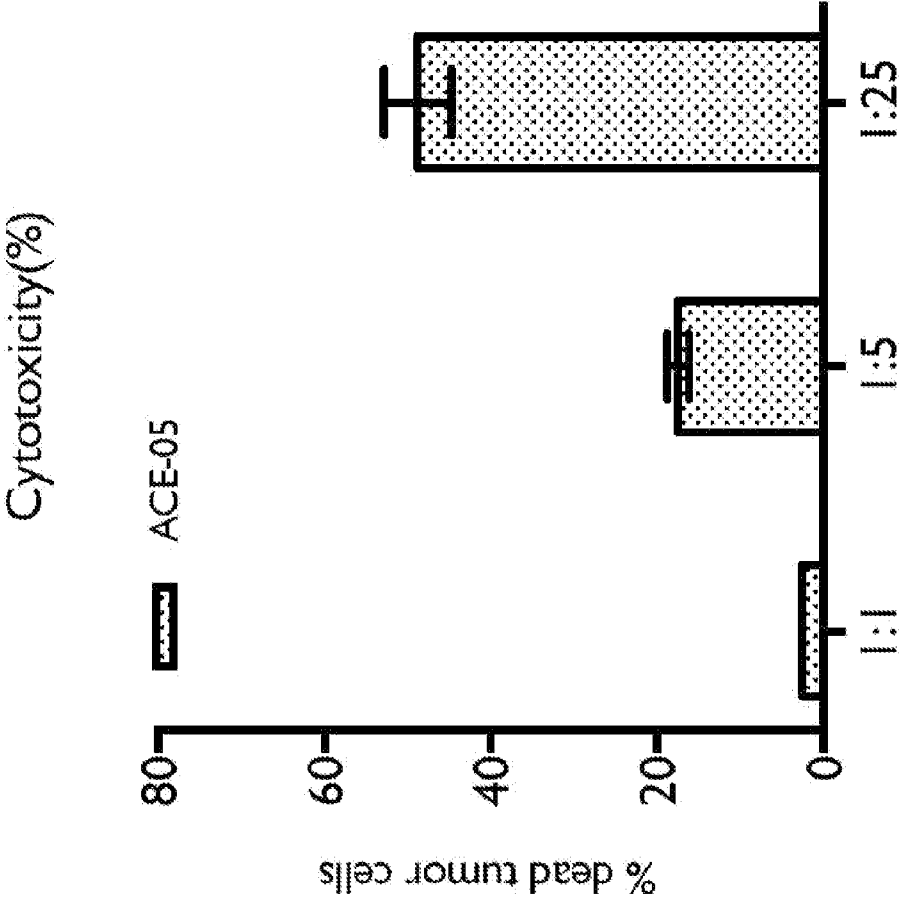


FIG. 13K

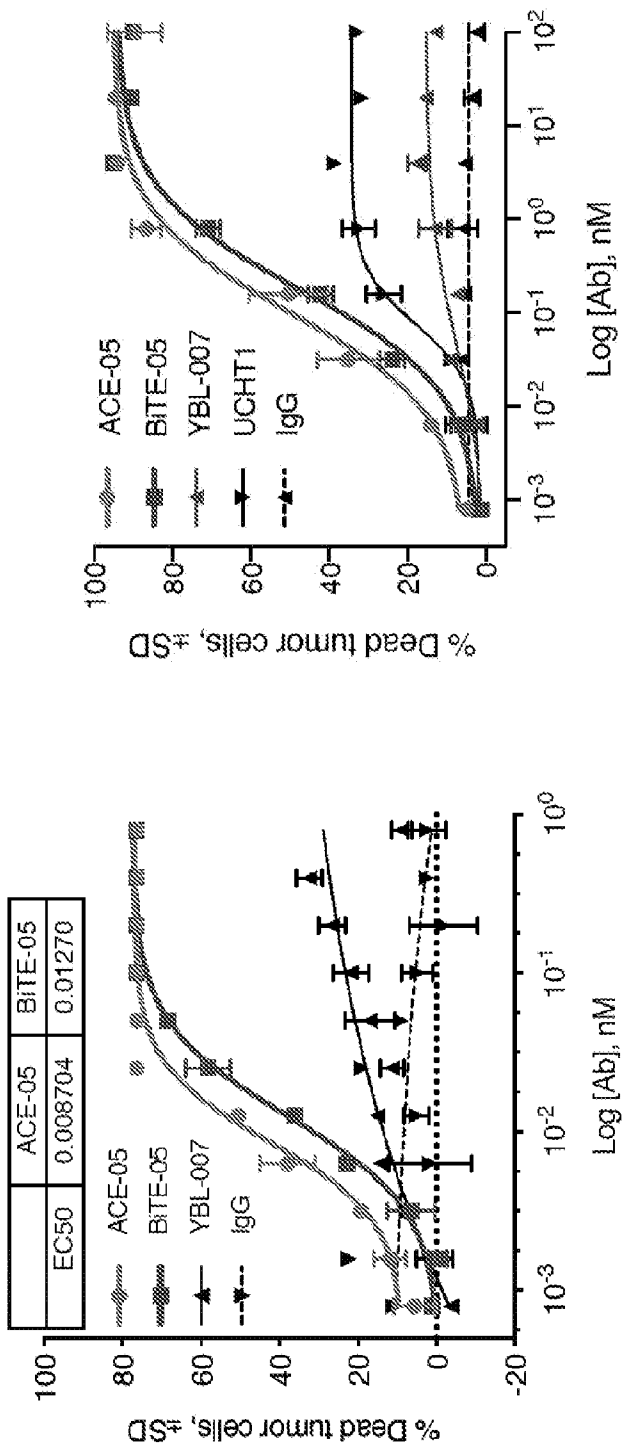


FIG. 13L

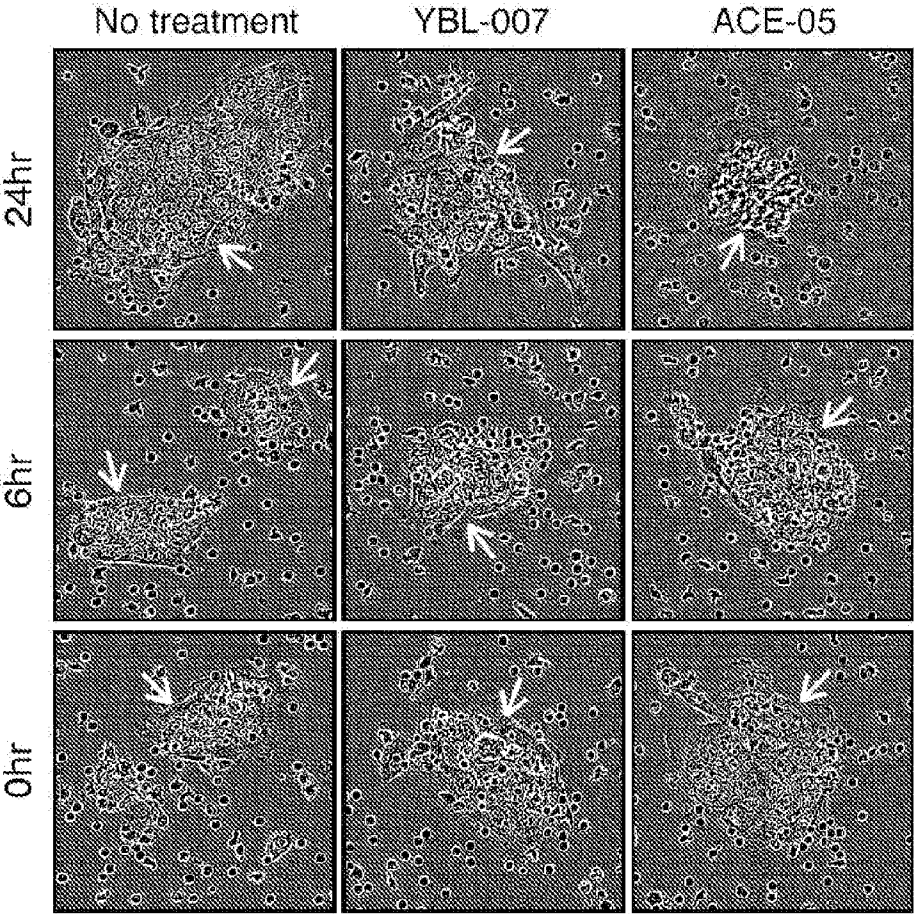


FIG. 13M

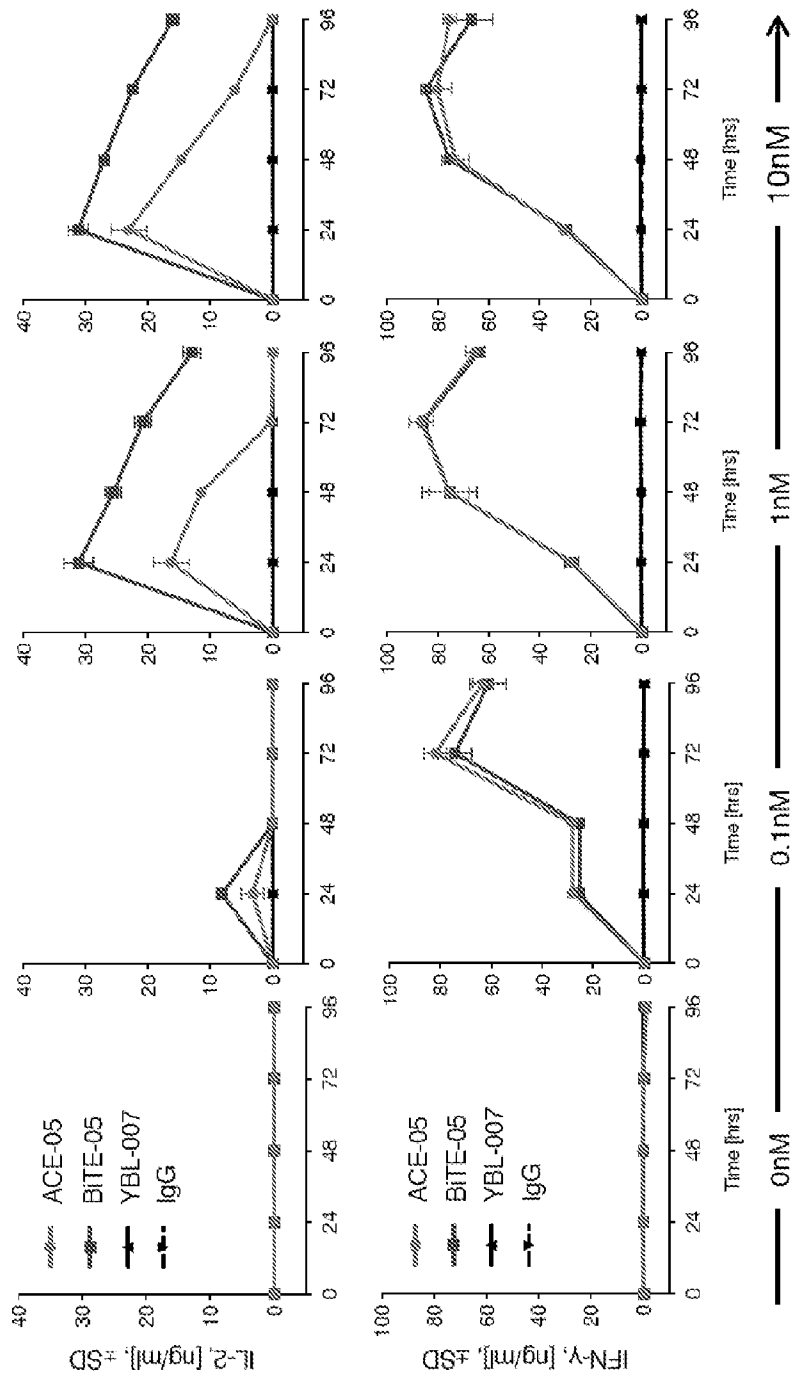


FIG. 13N

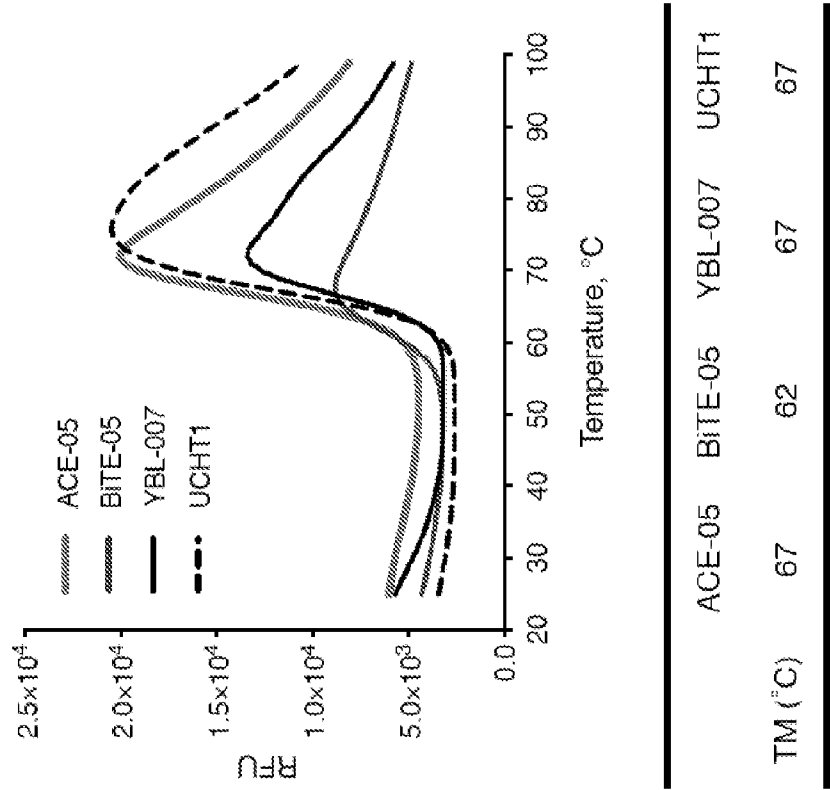


FIG. 130

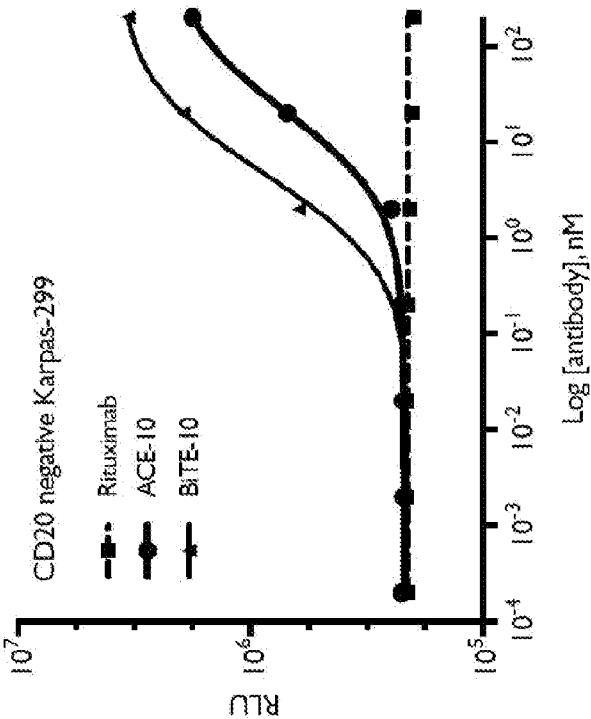
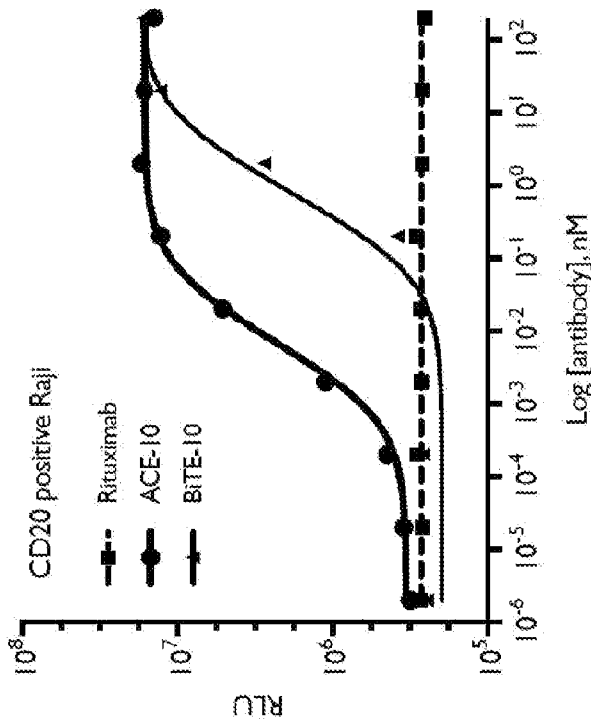


FIG. 14B



	Rituximab	ACE-10	BiTE-10
EC50	~ 8.845e-018	0.04913	7.515

FIG. 14A

ACE-II mediated T cell cytotoxicity

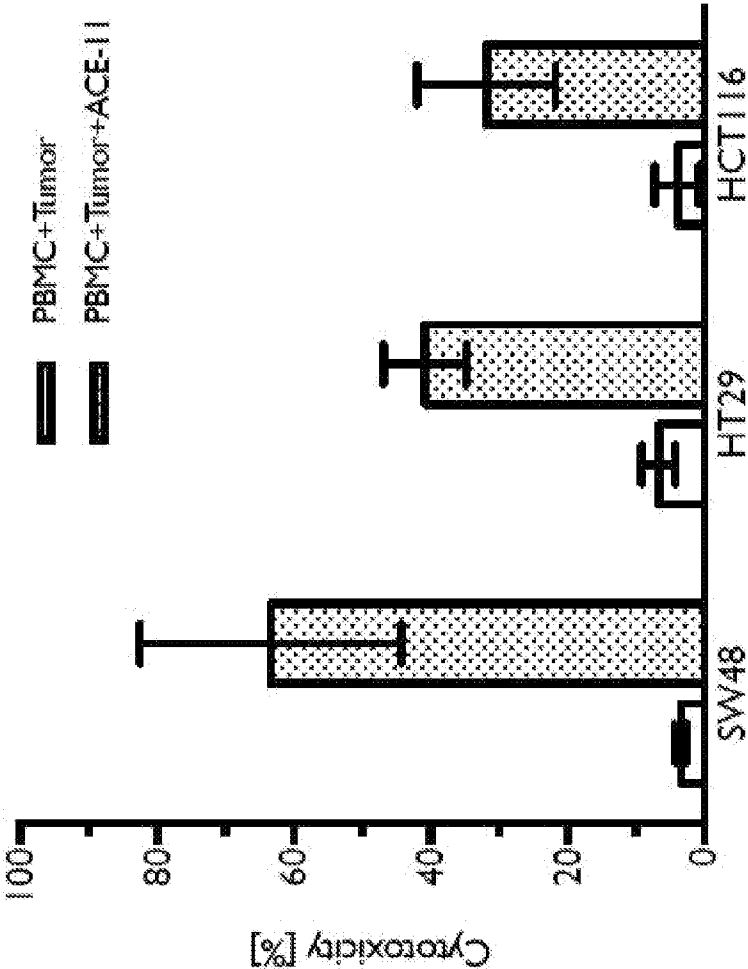


FIG. 15

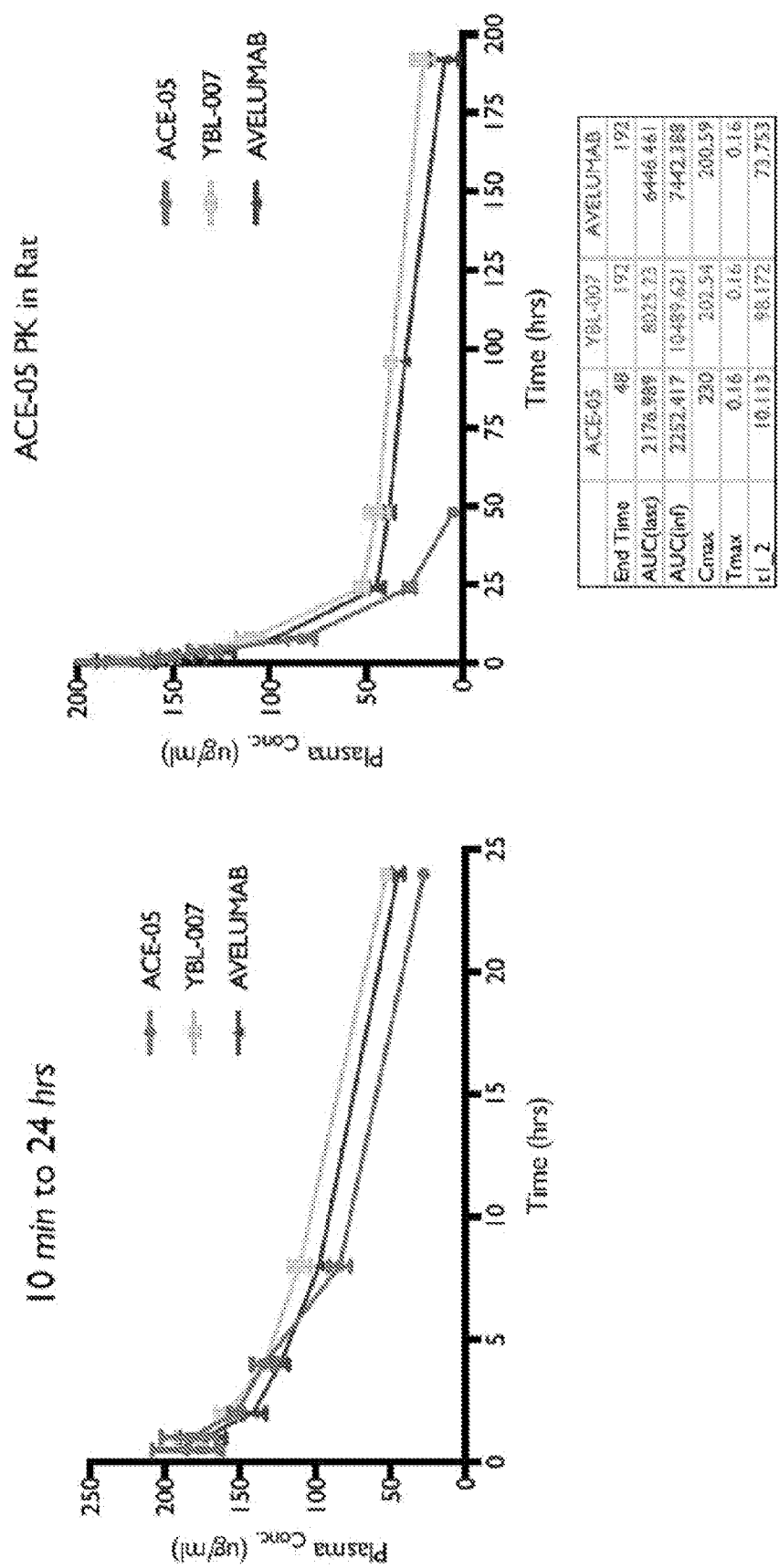
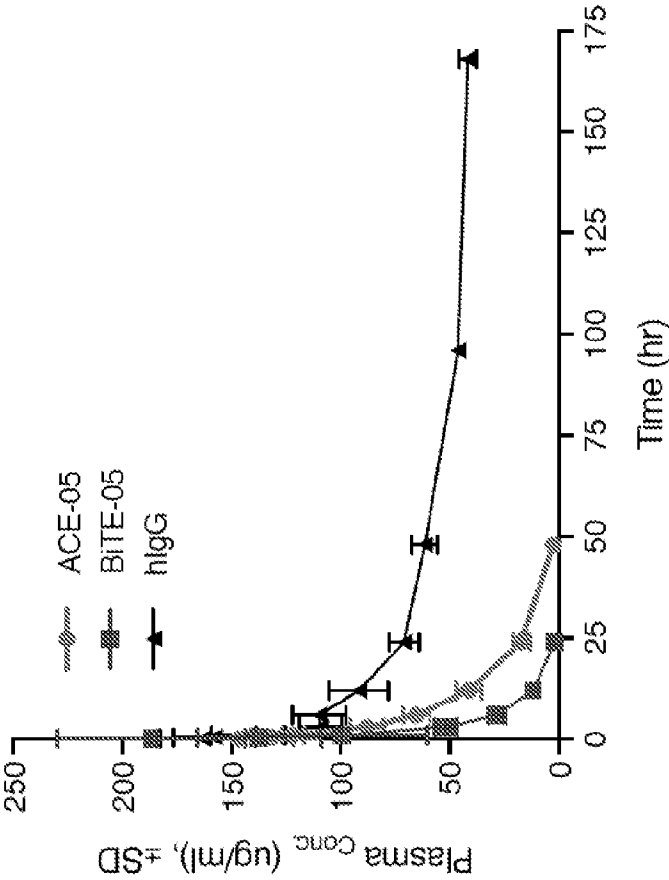


FIG. 16A



	ACE-05	BITE-05	hIgG
t1/2	8.4h	3.87h	90.5h

FIG. 16B

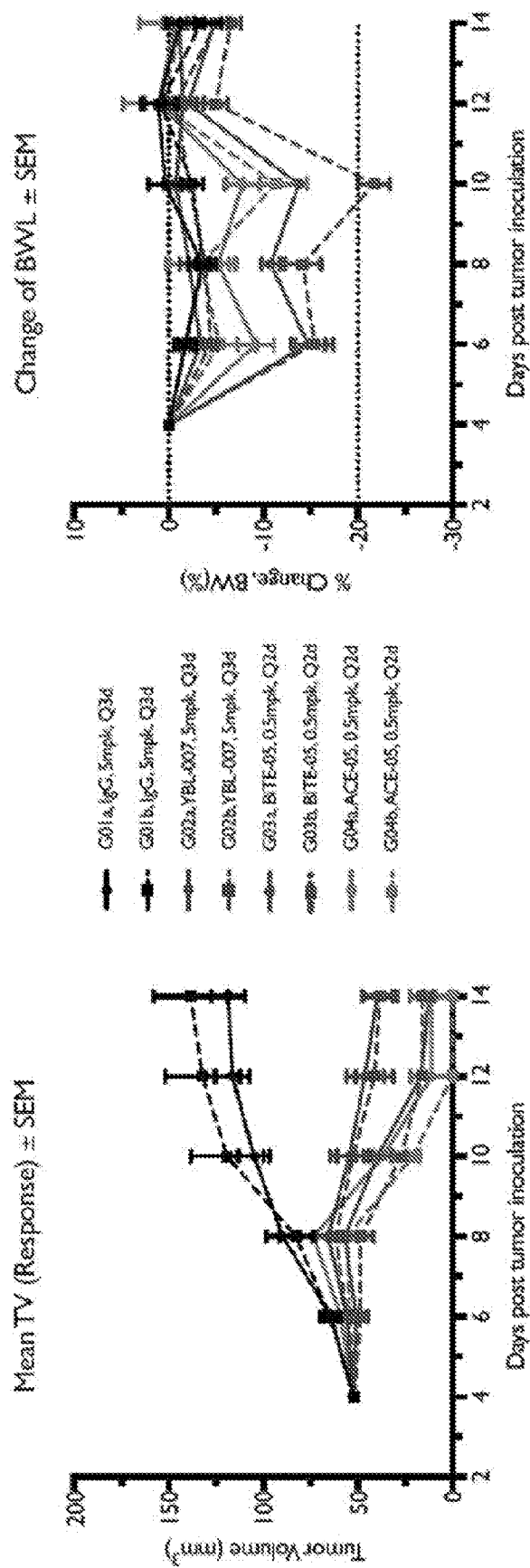


FIG. 17A

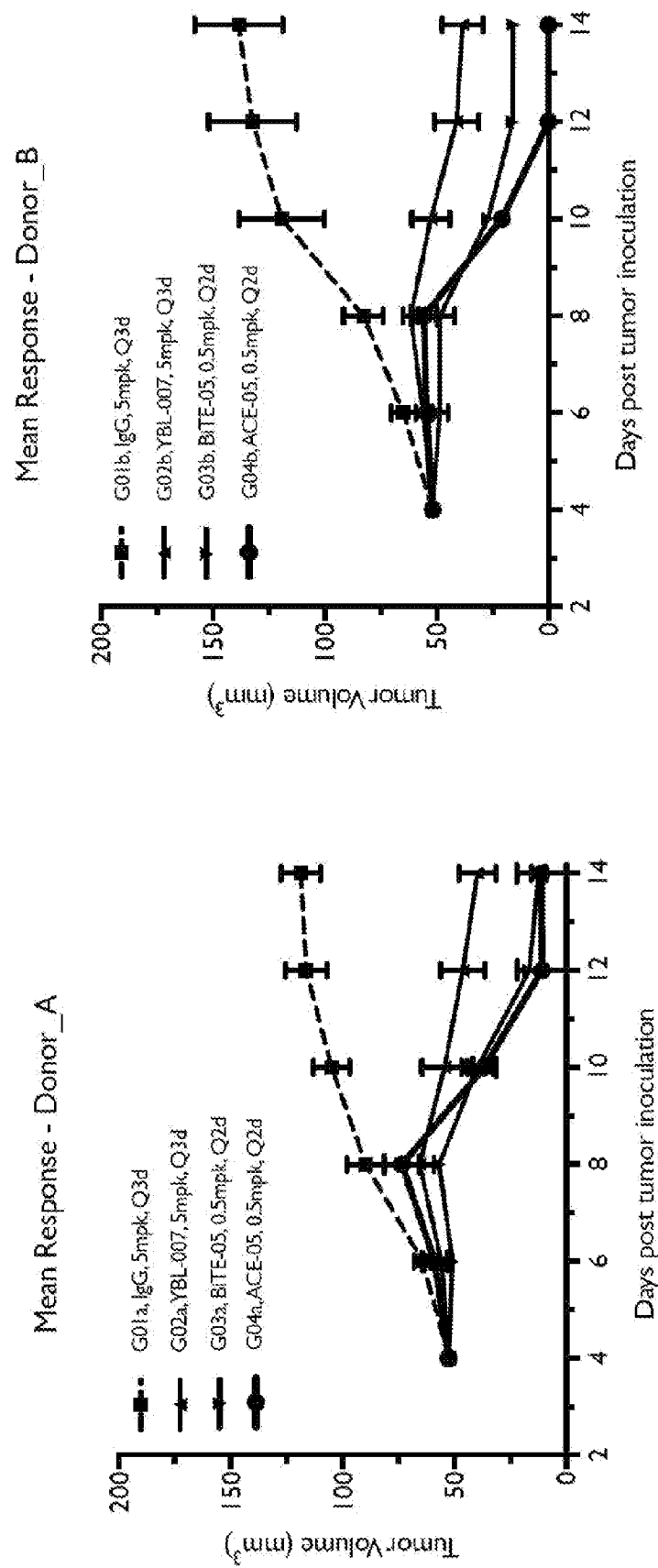


FIG. 17B

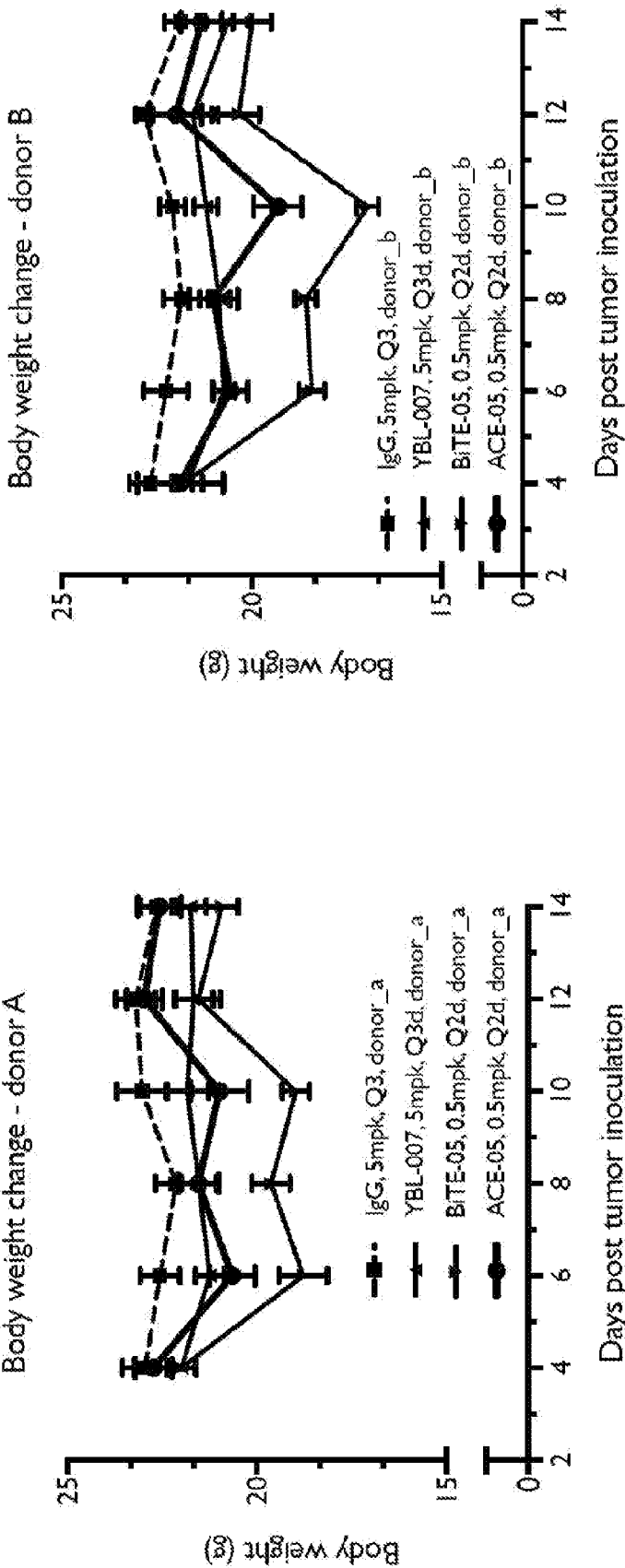


FIG. 17C

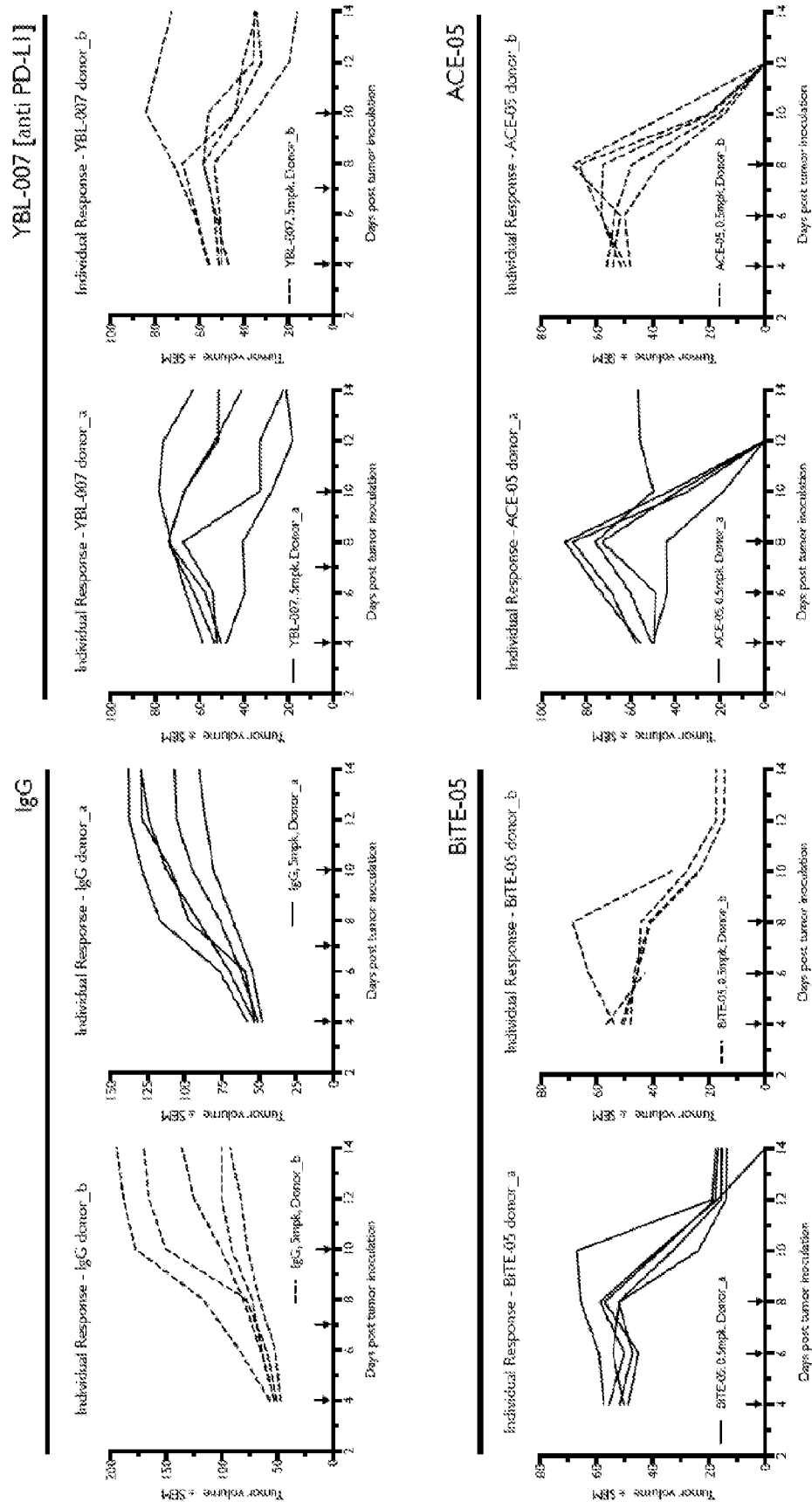


FIG. 17D

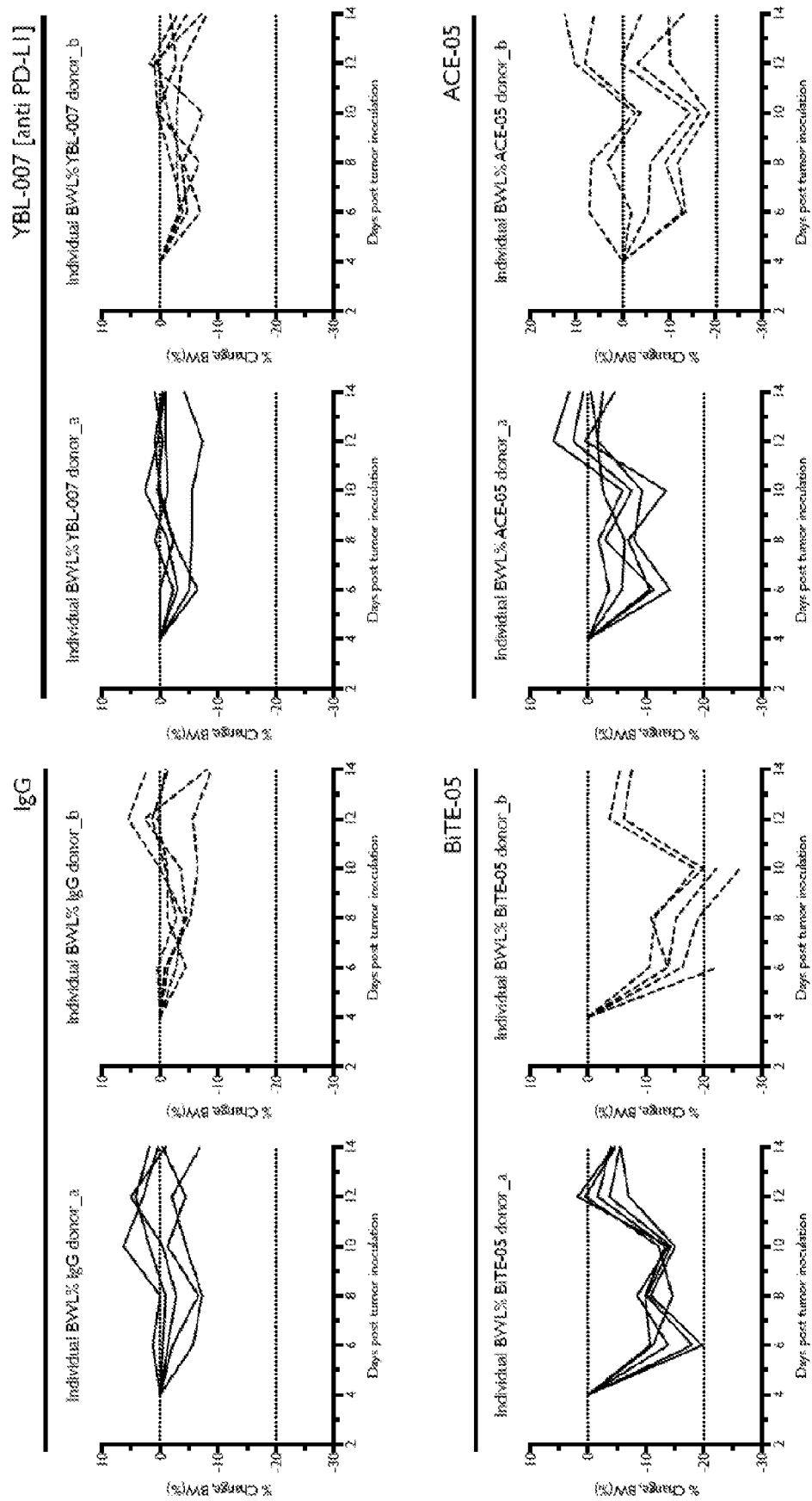


FIG. 17E

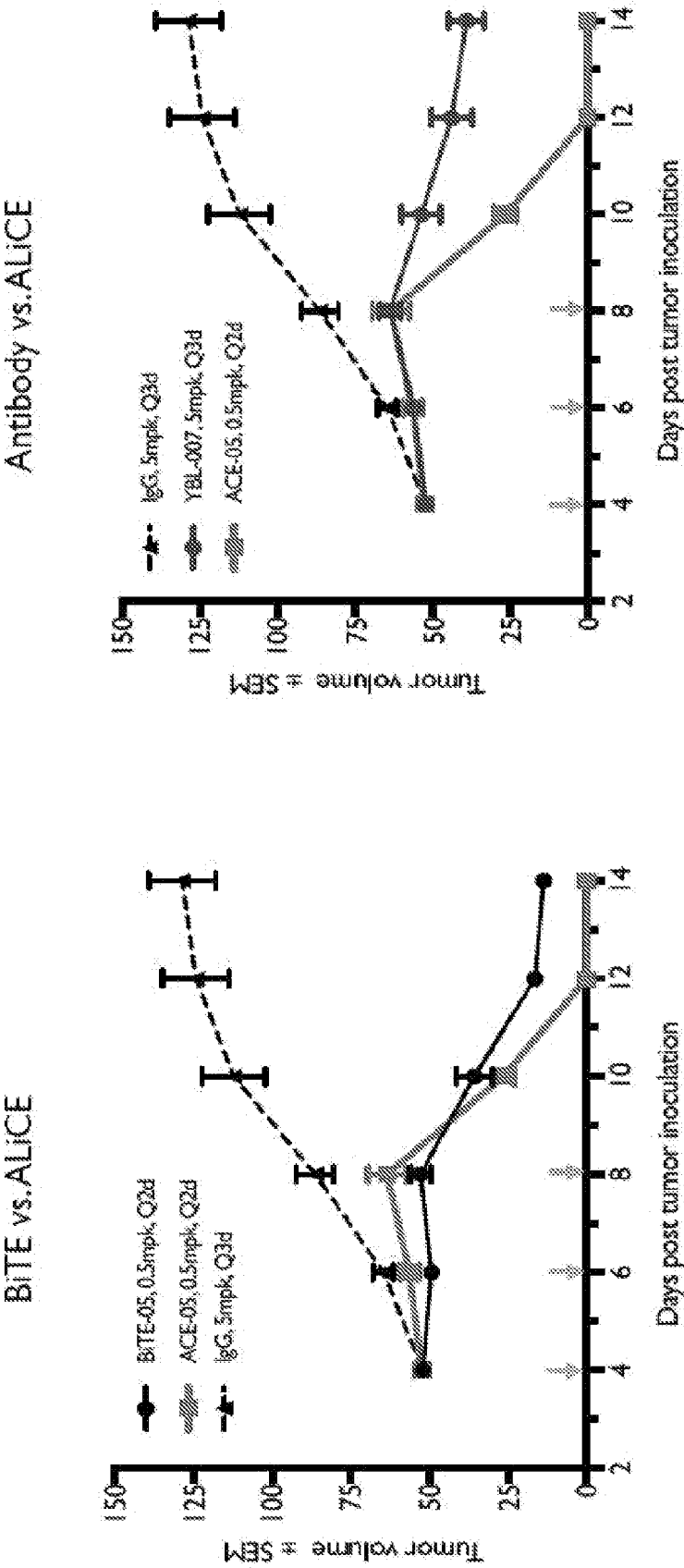


FIG. 17F

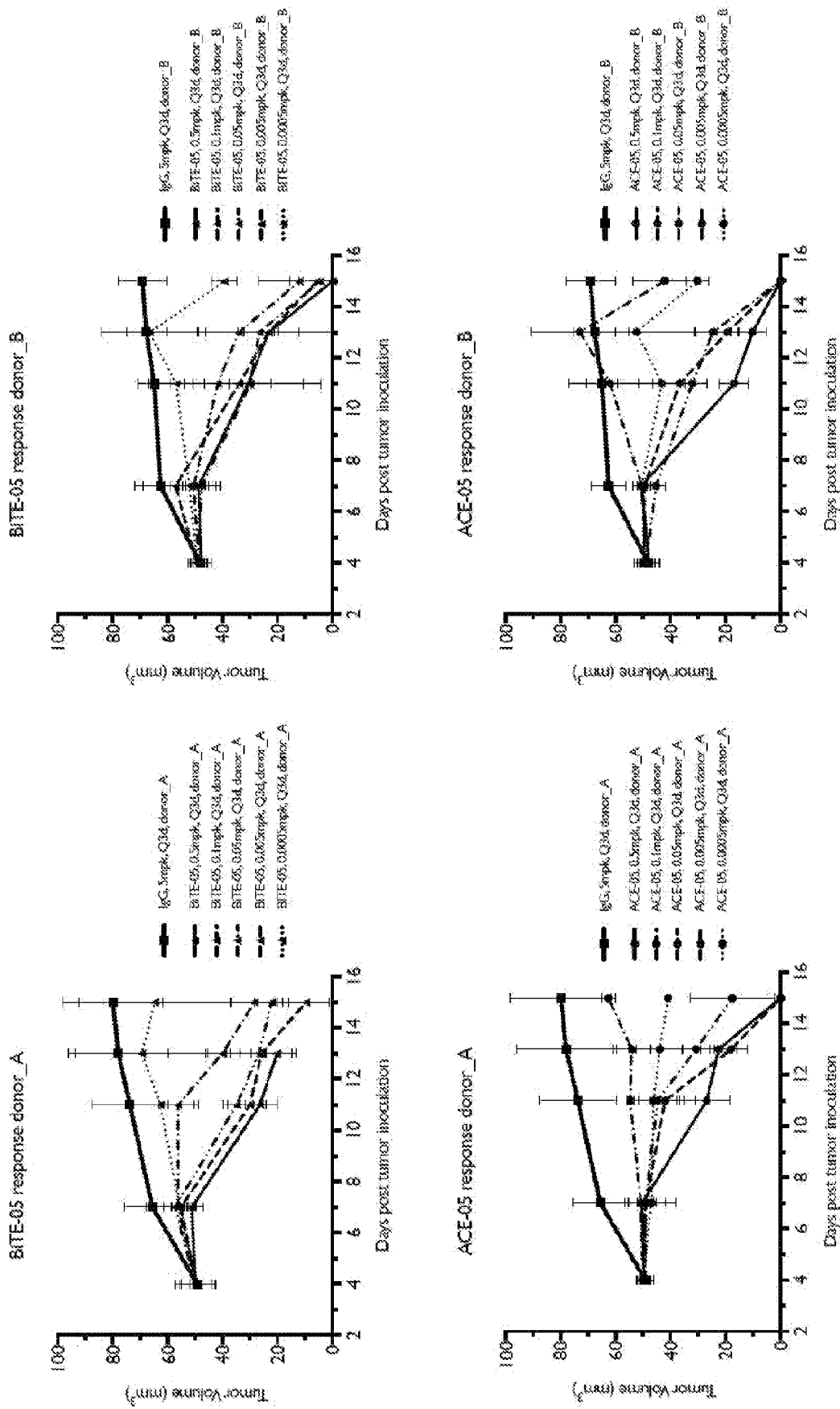


FIG. 18A

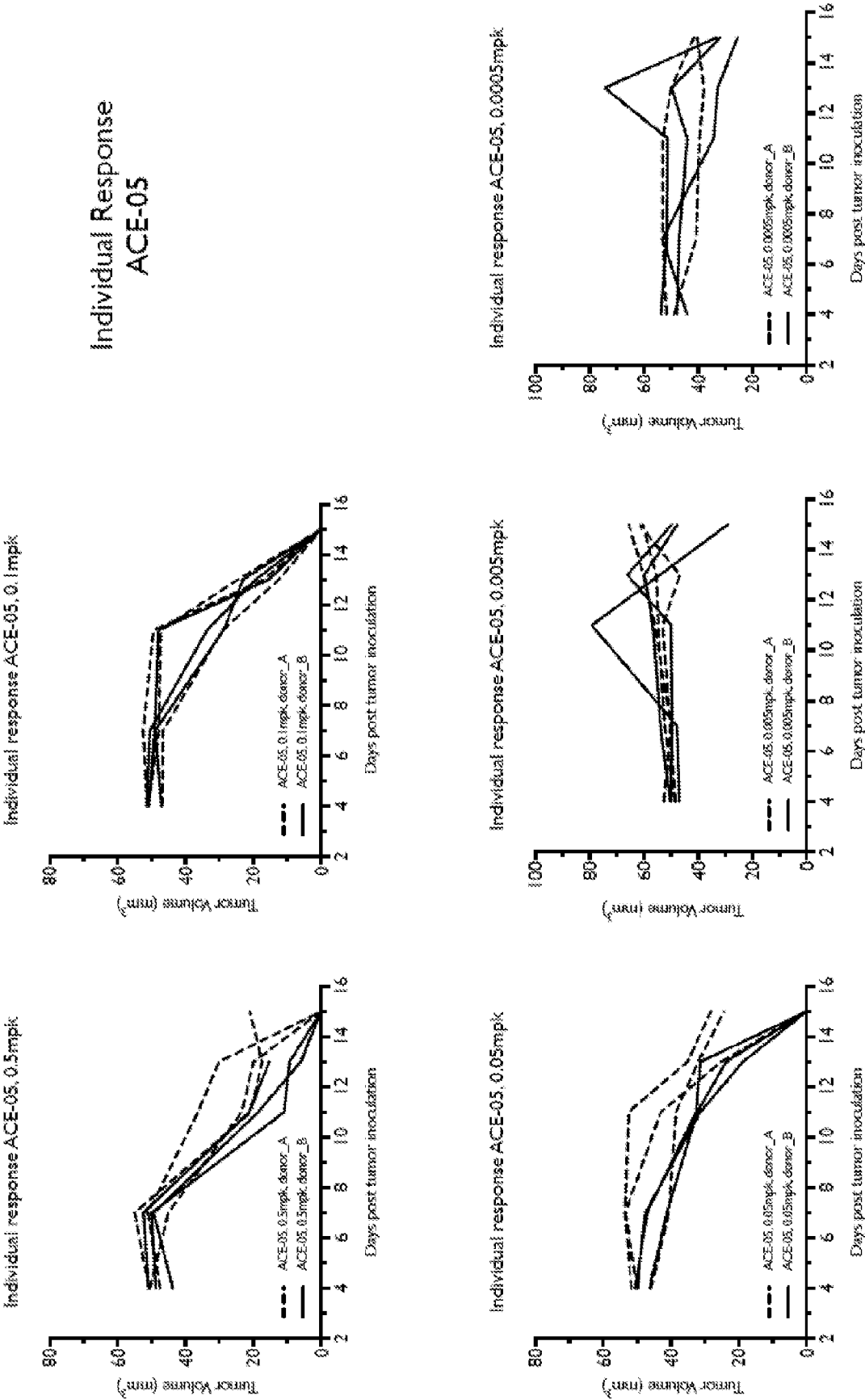


FIG. 18B

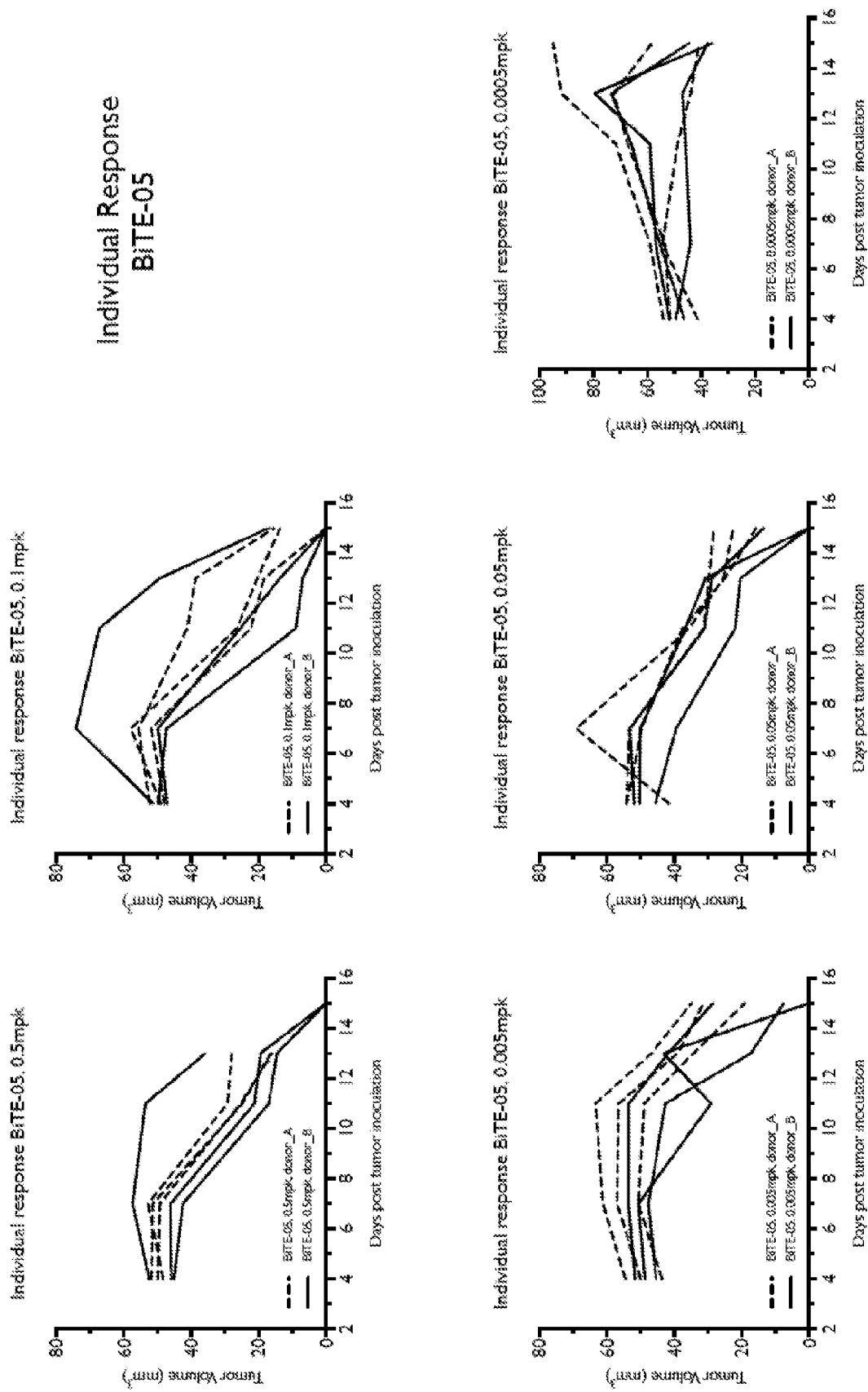
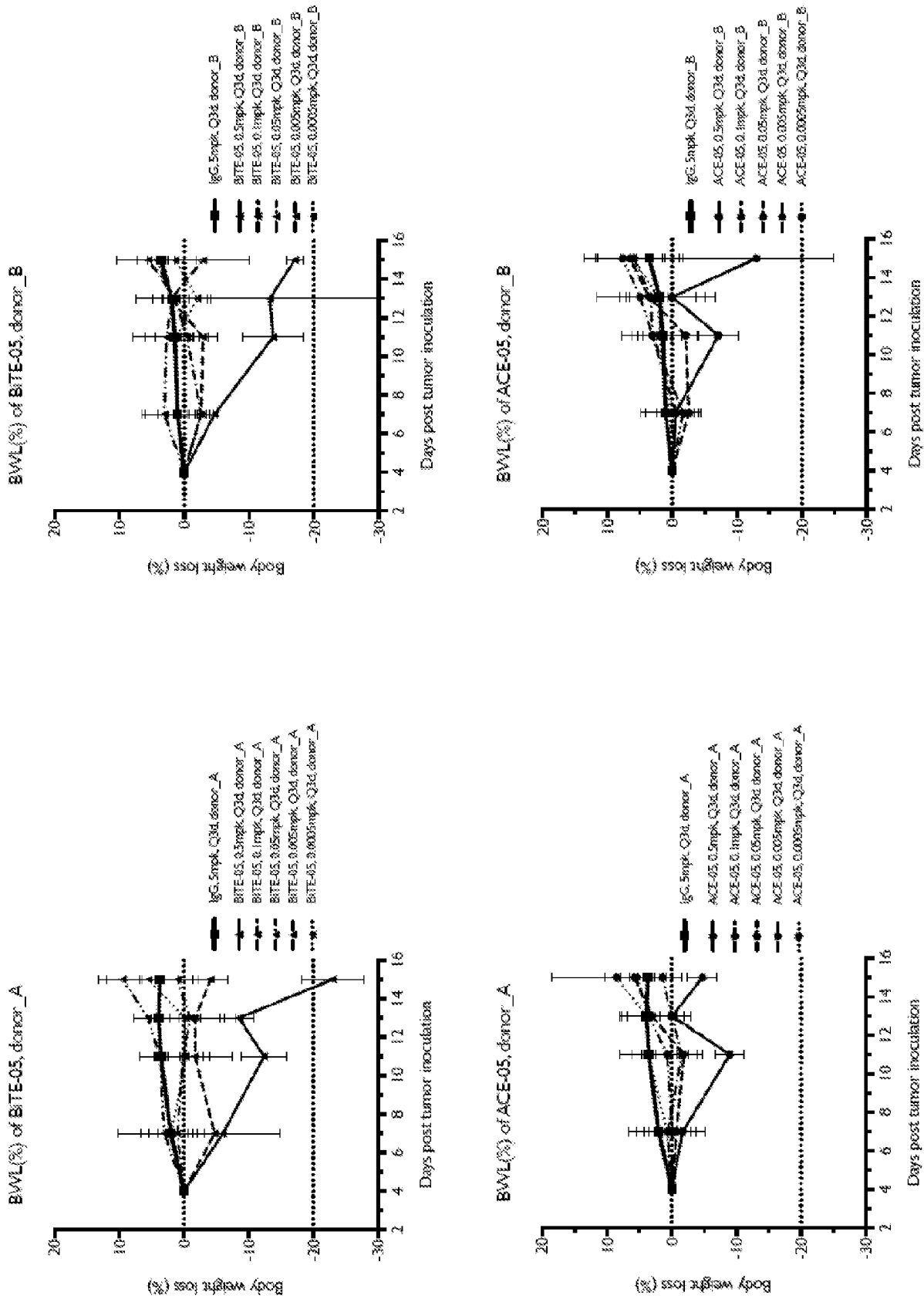


FIG. 18C



Body weight loss (%) of ACE-05 and BiTE-05 treated group

FIG. 18D

A. CLASSIFICATION OF SUBJECT MATTER**C07K 16/28(2006.01)i, C07K 16/30(2006.01)i, C07K 16/46(2006.01)i, A61P 35/00(2006.01)i, A61K 39/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 16/28; A61K 39/395; C07K 16/24; C07K 16/30; C07K 16/46; A61P 35/00; A61K 39/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & keywords: binding molecule, antibody Fab region, VH, CH1, VL, CL, Fv, immune cell, PD-L1, CD3

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2016-0108118 A1 (METABOLIC ENGINEERING LABORATORIES CO., LTD.) 21 April 2016 See paragraph [0038]; and figure 1.	1-9, 19-24, 55-145 , 159-161, 163-165 , 167-169
A	SCHOONJANS, R. et al., `Fab Chains As an Efficient Heterodimerization Scaffold for the Production of Recombinant Bispecific and Trispecific Antibody Derivatives`, The Journal of Immunology, 2000, Vol.165, pp.7050-7057 See figure 1.	1-9, 19-24, 55-145 , 159-161, 163-165 , 167-169
A	WEIDLE, U. H. et al., `The Intriguing Options of Multispecific Antibody Formats for Treatment of Cancer`, Cancer Genomics & Proteomics, 2013, Vol.10, pp.1-18 See figure 1.	1-9, 19-24, 55-145 , 159-161, 163-165 , 167-169
A	KONTERMANN, R. E. et al., `Bispecific antibodies`, Drug Discovery Today, July 2015, Vol.20, No.7, pp.838-847 See figure 2.	1-9, 19-24, 55-145 , 159-161, 163-165 , 167-169
A	DIGIANDOMENICO, A. et al., `A multifunctional bispecific antibody protects against Pseudomonas aeruginosa`, Science Translational Medicine, 12 November 2014, Vol.6, Issue 262, 262ra155, pp.1-12 See figure 1.	1-9, 19-24, 55-145 , 159-161, 163-165 , 167-169



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 August 2019 (12.08.2019)

Date of mailing of the international search report

12 August 2019 (12.08.2019)

Name and mailing address of the ISA/KR

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2019/052896

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YANG, F. et al., `Bispecific Antibodies as a Development Platform for New Concepts and Treatment Strategies`, International Journal of Molecular Sciences, 2017, Vol.18, 48, pp.1-21 See figure 2.	1-9, 19-24, 55-145 , 159-161, 163-165 , 167-169
A	WO 2018-026248 A1 (Y-BIOLOGICS INC.) 8 February 2018 See the whole document.	1-9, 19-24, 55-145 , 159-161, 163-165 , 167-169

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2019/052896

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 153-158, 162, 166, 170
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 153-158, 162, 166, 170 are directed to a treatment method of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and Rule 39.1(iv), to search.
2. ☒ Claims Nos.: 11-18, 27-54, 147-152, 154-158
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 11-18, 27-54, 147-152, 154-158 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).
3. ☒ Claims Nos.: 10, 25, 26, 146, 153
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/IB2019/052896

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2016-0108118 A1	21/04/2016	AU 2014-269287 A1 AU 2014-269287 B2 CA 2913118 A1 CN 105263964 A EP 3000827 A1 HK 1223112 A1 JP 2016-520595 A KR 10-1671955 B1 KR 10-2015-0010919 A PH 12015502601 A1 US 10087248 B2 WO 2014-189306 A1 WO 2014-189306 A8	10/12/2015 25/08/2016 27/11/2014 20/01/2016 30/03/2016 21/07/2017 14/07/2016 07/11/2016 29/01/2015 29/02/2016 02/10/2018 27/11/2014 03/12/2015
WO 2018-026248 A1	08/02/2018	AU 2017-306506 A1 CA 3032783 A1 EP 3495390 A1 KR 10-2018-0016320 A	21/03/2019 08/02/2018 12/06/2019 14/02/2018