

CLAIMS

It is claimed:

1. An isolated bispecific antibody or antigen-binding fragment thereof comprising:

- a. a first heavy chain, H1;
- b. a second heavy chain, H2;
- c. a first light chain, L1; and
- d. a second light chain, L2;

wherein H1 and L1 form a first arm comprising a first antigen-binding domain that specifically binds a first antigen, preferably a first antigen of human origin, and

wherein H2 and L2 form a second arm comprising a second antigen-binding domain that specifically binds a second antigen, preferably a second antigen of human origin, wherein

- (a) H1 comprises a CH1 region of human IgG1, IgG2, IgG3, or IgG4; and
- (b) L1 comprises a CL region of a human kappa light chain or a human lambda light chain;

wherein the CH1 and CL regions comprise amino acid substitutions or a native amino acid at an amino acid residue corresponding to the amino acid position of SEQ ID NO:15, 21, 22, or 23 for CH1 and SEQ ID NO:19 or 24 for CL;

wherein the amino acid substitutions or the native amino acid in the CH1 and CL regions are selected from:

- (1) K133C and C220X in CH1, and F209C and C214X in CL;
- (2) S131C and C220X in CH1, and P119C and C214X in CL;
- (3) K133C and C220X in CH1, and K207C and C214X in CL;
- (4) F170C and C220X in CH1, and S176C and C214X in CL;
- (5) P171C and C220X in CH1, and S162C and C214X in CL;
- (6) V173C and C220X in CH1, and Q160C and C214X in CL;
- (7) F170C and C131X in CH1, and S176C and C214X in CL;
- (8) P171C and C131X in CH1, and S162C and C214X in CL;
- (9) V173C and C131X in CH1, and Q160C and C214X in CL;
- (10) A129C and C220X in CH1, and S121C and C214X in CL;
- (11) K133C and C220X in CH1, and I117C and C214X in CL;
- (12) C131 in CH1, and P119C and C214X in CL;
- (13) A129C and C131X in CH1, and S121C and C214X in CL;
- (14) R133C and C131X in CH1, and K207C and C214X in CL;
- (15) R133C and C131X in CH1, and I117C and C214X in CL;
- (16) R133C and C131X in CH1, and L117C and C214X in CL;

- (17) K133C and C220X in CH1, and L117C and C214X in CL;
 - (18) R133C and C131X in CH1, and F209C and C214X in CL;
 - (19) R133C and C131X in CH1, and V209C and C214X in CL; or
 - (20) K133C and C220X in CH1, and V209C and C214X in CL;
- wherein X is selected from S, A or G.

2. An isolated bispecific antibody or antigen-binding fragment thereof comprising:
 - a. a first heavy chain, H1;
 - b. a second heavy chain, H2;
 - c. a first light chain, L1; and
 - d. a second light chain, L2;

wherein H1 and L1 form a first arm comprising a first antigen-binding domain that specifically binds a first antigen, preferably a first antigen of human origin, and

wherein H2 and L2 form a second arm comprising a second antigen-binding domain that specifically binds a second antigen, preferably a second antigen of human origin, wherein

- (a) H1 comprises a CH1 region of human IgG1, IgG2, IgG3, or IgG4 and a heavy chain variable region (VH region); and
- (b) L1 comprises a CL region of a human kappa light chain or a human lambda light chain and a light chain variable region (VL region);

wherein the CH1 region, the VH region, the CL region, and the VL region comprise amino acid substitutions at an amino acid residue corresponding to the amino acid position of SEQ ID NO: 15, 21, 22, or 23 for CH1; SEQ ID NO: 13 for VH; SEQ ID NO: 19 or 24 for CL; and SEQ ID NO: 17 for VL;

wherein the amino acid substitutions in the CH1 region, the VH region, the CL region, and the VL region are selected from:

- (1) C220X in CH1, G44C in VH, C214X in CL, and G101C in VL; or
 - (2) C131X in CH1, G44C in VH, C214X in CL, and G101C in VL;
- wherein X is selected from S, A or G.

3. The isolated bispecific antibody or antigen-binding fragment thereof of claim 1 or 2, wherein the first antigen-binding domain is a CD47 binding domain.
4. The isolated bispecific antibody or antigen-binding fragment thereof of claim 3, wherein the VH region comprises an amino acid sequence of SEQ ID NO: 1, the CH1 region comprises an amino acid sequence of SEQ ID NO: 2, the VL region comprises an amino acid sequence of SEQ ID NO: 3, and the CL region comprises an amino acid sequence of SEQ ID NO: 4.
5. The isolated bispecific antibody or antigen-binding fragment thereof of any one of claims 1 to 4, wherein

- (a) the second arm comprising H2 and L2 does not comprise the amino acid substitutions of the first arm comprising H1 and L1;
 - (b) the two heavy chains H1 and H2 each comprise a VH region, a CH1 region, and a Fc region (containing CH2 and CH3 regions), wherein the VH regions have different amino acid sequences;
 - (c) the two heavy chains H1 and H2 each comprise a VH region, a CH1 region, and a Fc region (containing CH2 and CH3 regions), wherein the CH1 regions have different amino acid sequences;
 - (d) the two heavy chains H1 and H2 each comprise a VH region, a CH1 region, and a Fc region (containing CH2 and CH3 regions), wherein the Fc regions have different amino acid sequences;
 - (e) the two light chains L1 and L2 each comprise a VL region and a CL region, wherein the VL regions have different amino acid sequences; and/or
 - (f) the two light chains L1 and L2 each comprise a VL region and a CL region, wherein the CL regions have different amino acid sequences.
6. The isolated bispecific antibody or antigen-binding fragment thereof of claim 5, wherein H1 and H2 form a heterodimer.
7. The isolated bispecific antibody or antigen-binding fragment thereof of any one of claims 1 to 6, wherein
- (a) the VH region of H1 and the VL region of L1 have a Q39E and a Q38K substitution mutation, respectively, and the VH region of H2 and the VL region of L2 have a Q39K and a Q38E substitution mutation, respectively; or
 - (b) the VH region of H1 and the VL region of L1 have a Q39K and a Q38E substitution mutation, respectively, and the VH region of H2 and the VL region of L2 have a Q39E and a Q38K substitution mutation, respectively.
8. The isolated bispecific antibody or antigen-binding fragment thereof of any one of claims 1 to 7, wherein the isolated bispecific antibody or antigen-binding fragment is an anti-CD47/anti-FR α bispecific antibody or antigen-binding fragment thereof, wherein the first antigen-binding domain specifically binds CD47, preferably human CD47, and the second antigen-binding domain specifically binds folate receptor α (FR α), preferably human FR α .
9. The isolated bispecific antibody or antigen-binding fragment thereof of any one of claims 1 to 8, wherein
- (a) the first antigen-binding domain has the VH sequence of SEQ ID: 13 and VL sequence of SEQ ID: 17, and the second antigen-binding domain has the VH sequence of SEQ ID: 33 and VL sequence of SEQ ID: 35; or

- (b) the first antigen-binding domain has the VH sequence of SEQ ID: 13 and VL sequence of SEQ ID: 17, and the second antigen-binding domain has the VH sequence of SEQ ID: 14 and VL sequence of SEQ ID: 18.
10. The isolated bispecific antibody or antigen-binding fragment thereof of claim 8 or 9, wherein the anti-CD47/anti-FR α bispecific antibody or antigen-binding fragment thereof is capable of blocking binding of signal regulatory protein alpha (SIRP α) to CD47 on cancer cells that express both FR α and CD47, inducing macrophage-mediated phagocytosis of cancer cells that express both FR α and CD47, and/or binding cancer cells that express both FR α and CD47 with minimal to undetectable binding to human red blood cells (RBCs).
 11. An isolated nucleic acid encoding the bispecific antibody or antigen-binding fragment of any one of claims 1 to 10.
 12. A vector comprising the isolated nucleic acid of claim 11.
 13. A host cell comprising the vector of claim 12.
 14. A pharmaceutical composition, comprising the isolated bispecific antibody or antigen-binding fragment thereof of any one of claims 1 to 10 and a pharmaceutically acceptable carrier.
 15. A method of targeting FR α and CD47 that are both expressed on a cancer cell surface in a subject in need thereof, blocking the binding of SIRP α to CD47 on cancer cells that express both FR α and CD47 in a subject in need thereof, inducing macrophage-mediated phagocytosis of cancer cells that express both FR α and CD47 in a subject in need thereof, binding cancer cells that express both FR α and CD47 with minimal to undetectable binding to human red blood cells (RBCs) in a subject in need thereof, and/or treating cancer in a subject in need thereof, comprising administering to the subject a pharmaceutical composition comprising the isolated anti-CD47/anti-FR α bispecific antibody or antigen-binding fragment thereof of any one of claims 8 to 10 and a pharmaceutically acceptable carrier, optionally the cancer is selected from the group consisting of a lung cancer, a gastric cancer, an esophageal cancer, a bile duct cancer, a cholangiocarcinoma, a colon cancer, a hepatocellular carcinoma, a renal cell carcinoma, a bladder urothelial carcinoma, a metastatic melanoma, a breast cancer, an ovarian cancer, a cervical cancer, a head and neck cancer, a pancreatic cancer, a glioma, a glioblastoma, and other solid tumors, and a non-Hodgkin's lymphoma (NHL), an acute lymphocytic leukemia (ALL), a chronic lymphocytic leukemia (CLL), a chronic myelogenous leukemia (CML), a multiple myeloma (MM), an acute myeloid leukemia (AML), and other liquid tumors.
 16. A method of producing the bispecific antibody or antigen-binding fragment thereof of any one of claims 1 to 10, comprising culturing a cell comprising a nucleic acid encoding the bispecific antibody or antigen-binding fragment thereof under conditions to produce the

bispecific antibody or antigen-binding fragment thereof, and recovering the bispecific antibody or antigen-binding fragment thereof from the cell or culture.

17. A method of producing a pharmaceutical composition comprising the bispecific antibody or antigen-binding fragment thereof of any one of claims 1 to 10, comprising combining the bispecific antibody or antigen-binding fragment thereof with a pharmaceutically acceptable carrier to obtain the pharmaceutical composition.