

**ANTI-OX40 ANTIBODIES AND USES THEREOF****CLAIM OF PRIORITY**

This application claims the benefit of PCT Application No. PCT/CN2024/075517, filed on February 2, 2024, PCT Application No. PCT/CN2024/092997, filed on May 14, 2024, and PCT Application No. PCT/CN2024/110179, filed on August 6, 2024. The entire contents of the foregoing are incorporated herein by reference.

**TECHNICAL FIELD**

This disclosure relates to anti-OX40 antibodies and antigen-binding protein constructs (e.g., bispecific antibodies or antigen-binding fragments thereof).

**BACKGROUND**

The T cell activation process requires TCR to recognize the MHC-peptide complex as the first signal. It also further requires co-stimulatory signals. OX40 is a class of co-stimulatory factors for T cells, which is a member of the tumor necrosis factor receptor (TNFR) superfamily, and a type I transmembrane protein. OX40 can activate the intracellular PI3K-AKT signal as well as the NFAT signal. These signals have a positive effect on the proliferation and survival of T cells. In addition, OX40 can also regulate the function and differentiation direction of T cells.

OX40 is a co-stimulatory molecule that is able to establish peripheral tolerance. It can break the immune tolerance of a tumor and restore immune surveillance. The employment of OX40 as a new target for tumor immunotherapy has already shown certain positive effects. Because of the importance of OX40 in immune response, there is a need to continue to develop anti-OX40 antibodies for various therapeutic purposes.

**SUMMARY**

This disclosure relates to anti-OX40 antibodies, antigen-binding fragment thereof, and the uses thereof.

This disclosure also relates to antigen-binding protein constructs, wherein the antigen-binding protein construct specifically binds to two distinct antigenic epitopes (e.g., OX40). In some embodiments, the multi-specific antibody (e.g., bispecific antibody) has identical light chain variable regions. In some embodiments, the multi-specific antibody (e.g., bispecific antibody) has a common light chain. The disclosure is also based on, in part, the unexpected finding that a bispecific antibody with a full OX40 ligand (OX40L) binding blocking antigen binding domain and a partial OX40 ligand (OX40L) binding blocking antigen binding domain can effectively suppress undesired immune response and can be used to treat various immune disorders, including e.g., an autoimmune disorder.

In one aspect, the disclosure is related to an antibody or antigen-binding fragment thereof that binds to OX40 (Tumor necrosis factor receptor superfamily, member 4) comprising:

5 a heavy chain variable region (VH) comprising complementarity determining regions (CDRs) 1, 2, and 3, wherein the VH CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VH CDR1 amino acid sequence, the VH CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VH CDR2 amino acid sequence, and the VH CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VH CDR3 amino acid sequence; and

10 a light chain variable region (VL) comprising CDRs 1, 2, and 3, wherein the VL CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VL CDR1 amino acid sequence, the VL CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VL CDR2 amino acid sequence, and the VL CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VL CDR3 amino acid sequence,

15 wherein the selected VH CDRs 1, 2, and 3 amino acid sequences and the selected VL CDRs 1, 2, and 3 amino acid sequences are one of the following:

(1) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

20 (2) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(3) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

25 (4) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

30 (5) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(6) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

35 (7) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively; and

(8) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 22-24, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

5 In some embodiments, the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 1-3, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to Kabat definition.

In some embodiments, the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 4-6, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to Kabat definition.

10 In some embodiments, the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 7-9, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to Kabat definition.

In some embodiments, the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 10-12, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to Kabat definition.

15 In some embodiments, the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 13-15, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to Chothia definition.

20 In some embodiments, the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 16-18, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to Chothia definition.

In some embodiments, the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 19-21, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to Chothia definition.

25 In some embodiments, the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 22-24, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to Chothia definition.

30 In one aspect, the disclosure is related to an antibody or antigen-binding fragment thereof that binds to OX40 comprising a heavy chain variable region (VH) comprising an amino acid sequence that is at least 90% identical to a selected VH sequence, and a light chain variable region (VL) comprising an amino acid sequence that is at least 90% identical to a selected VL sequence, wherein the selected VH sequence and the selected VL sequence are one of the following:

- 35 (1) the selected VH sequence is SEQ ID NO: 28, and the selected VL sequence is SEQ ID NO: 32;  
(2) the selected VH sequence is SEQ ID NO: 29, and the selected VL sequence is SEQ ID NO: 32;  
(3) the selected VH sequence is SEQ ID NO: 30, and the selected VL sequence is SEQ ID NO: 32; and  
(4) the selected VH sequence is SEQ ID NO: 31, and the selected VL sequence is SEQ ID NO: 32.

In some embodiments, the VH comprises the sequence of SEQ ID NO: 28 and the VL comprises the sequence of SEQ ID NO: 32.

In some embodiments, the VH comprises the sequence of SEQ ID NO: 29 and the VL comprises the sequence of SEQ ID NO: 32.

5 In some embodiments, the VH comprises the sequence of SEQ ID NO: 30 and the VL comprises the sequence of SEQ ID NO: 32.

In some embodiments, the VH comprises the sequence of SEQ ID NO: 31 and the VL comprises the sequence of SEQ ID NO: 32.

10 In one aspect, the disclosure is related to an antibody or antigen-binding fragment thereof that binds to OX40 comprising a heavy chain variable region (VH) comprising VH CDR1, VH CDR2, and VH CDR3 that are identical to VH CDR1, VH CDR2, and VH CDR3 of a selected VH sequence; and a light chain variable region (VL) comprising VL CDR1, VL CDR2, and VL CDR3 that are identical to VL CDR1, VL CDR2, and VL CDR3 of a selected VL sequence, wherein the selected VH sequence and the selected VL sequence are one of the following:

- 15 (1) the selected VH sequence is SEQ ID NO: 28, and the selected VL sequence is SEQ ID NO: 32;  
(2) the selected VH sequence is SEQ ID NO: 29, and the selected VL sequence is SEQ ID NO: 32;  
(3) the selected VH sequence is SEQ ID NO: 30, and the selected VL sequence is SEQ ID NO: 32; and  
(4) the selected VH sequence is SEQ ID NO: 31, and the selected VL sequence is SEQ ID NO: 32.

20 In some embodiments, the antibody or antigen-binding fragment thereof specifically binds to human or monkey OX40.

In some embodiments, the antibody or antigen-binding fragment thereof is a human antibody or antigen-binding fragment thereof, a single-chain variable fragment (scFv), and/or a multi-specific antibody (e.g., a bispecific antibody).

25 In some embodiments, the antibody or antigen-binding fragment is a human IgG1 antibody or antigen-binding fragment thereof, a human IgG2 antibody or antigen-binding fragment thereof, or a human IgG4 antibody or antigen-binding fragment thereof.

In some embodiments, the antibody or antigen-binding fragment thereof binds to two different epitopes of OX40.

30 In one aspect, the disclosure is related to an antibody or antigen-binding fragment thereof that cross-competes with the anti-OX40 antibody or antigen-binding fragment thereof as described herein.

In one aspect, the disclosure is related to an antigen-binding protein construct, comprising: a first antigen-binding domain that specifically binds to a first epitope of OX40; and a second antigen-binding domain that specifically binds to a second epitope of OX40.

35 In some embodiments, the antigen-binding protein construct comprising: a first antigen-binding domain that specifically binds to a first epitope of OX40, wherein the first antigen-binding domain is a full OX40L binding blocking antigen binding domain; and a second antigen-binding domain that

specifically binds to a second epitope of OX40, wherein the second antigen-binding domain is a partial OX40L binding blocking antigen binding domain.

In some embodiments, the first antigen-binding domain comprises a first heavy chain variable region (VH1) and a first light chain variable region (VL1); and the second antigen-binding domain  
5 comprises a second heavy chain variable region (VH2) and a second light chain variable region (VL2).

In some embodiments, the first heavy chain variable region (VH1) comprises complementarity determining regions (CDRs) 1, 2, and 3, wherein the VH1 CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VH1 CDR1 amino acid sequence, the VH1 CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VH1 CDR2 amino acid sequence, and the VH1 CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VH1 CDR3 amino acid sequence; and the first light chain variable region (VL1) comprises CDRs 1, 2, and 3, wherein the VL1 CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VL1 CDR1 amino acid sequence, the VL1 CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VL1 CDR2 amino acid sequence, and the VL1 CDR3  
10 region comprises an amino acid sequence that is at least 80% identical to a selected VL1 CDR3 amino acid sequence, wherein the selected VH1 CDRs 1, 2, and 3 amino acid sequences, the selected VL1 CDRs 1, 2, and 3 amino acid sequences are one of the following:

(1) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs:  
20 25-27, respectively;

(2) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(3) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively; and  
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(4) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the second heavy chain variable region (VH2) comprises CDRs 1, 2, and 3, wherein the VH2 CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VH2 CDR1 amino acid sequence, the VH2 CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VH2 CDR2 amino acid sequence, and the VH2 CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VH2 CDR3 amino acid sequence; and the second light chain variable region (VL2) comprises CDRs 1, 2, and 3, wherein the VL2 CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VL2 CDR1  
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amino acid sequence, the VL2 CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VL2 CDR2 amino acid sequence, and the VL2 CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VL2 CDR3 amino acid sequence, wherein the selected VH2 CDRs 1, 2, and 3 amino acid sequences, and the selected VL2 CDRs 1, 2, and 3 amino acid sequences are one of the following:

(1) the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(2) the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(3) the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively; and

(4) the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 22-24, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth

in SEQ ID NOs: 22-24, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 22-24, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the first heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 28, the first light chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 32, the second heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 30, and the second light chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 32.

In some embodiments, the first heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 28, the first light chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 32, the second heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 31, and the second light chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 32.

In some embodiments, the first heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 29, the first light chain variable region

comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 32, the second heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 30, and the second light chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 32.

5 In some embodiments, the first heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 29, the first light chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 32, the second heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 31, and the second light chain variable region comprises a sequence  
10 that is at least 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 32.

In some embodiments, the VH1 comprises the sequence of SEQ ID NO: 28 and the VL1 comprises the sequence of SEQ ID NO: 32.

In some embodiments, the VH1 comprises the sequence of SEQ ID NO: 29 and the VL1 comprises the sequence of SEQ ID NO: 32.

15 In some embodiments, the VH2 comprises the sequence of SEQ ID NO: 30 and the VL2 comprises the sequence of SEQ ID NO: 32.

In some embodiments, the VH2 comprises the sequence of SEQ ID NO: 31 and the VL2 comprises the sequence of SEQ ID NO: 32.

20 In some embodiments, the first antigen-binding domain is human or humanized; and/or the second antigen-binding domain is human or humanized.

In some embodiments, the antigen-binding protein construct is a multi-specific antibody (e.g., a bispecific antibody).

In some embodiments, the first antigen-binding domain is a single-chain variable fragment (scFv); and/or the second antigen-binding domain is a scFv.

25 In some embodiments, the first light chain variable region and the second light chain variable region are identical.

In some embodiments, the antigen-binding protein construct comprises a fragment crystallizable region (Fc region).

In some embodiments, the Fc region is afucosylated.

30 In some embodiments, the Fc region comprises YTE mutations (EU numbering: M252Y, S254T, T256E mutations).

In one aspect, the disclosure provides a nucleic acid comprising a polynucleotide encoding the anti-OX40 antibody or antigen-binding fragment thereof, or an antigen-binding protein construct as described herein.

35 In one aspect, the disclosure provides a vector comprising the nucleic acid as described herein.

In one aspect, the disclosure provides a cell comprising the vector as described herein. In some embodiments, the cell is a CHO cell or modified CHO-K1 cells.

In one aspect, the disclosure provides a cell comprising the nucleic acid as described herein.

In one aspect, the disclosure provides a method of producing an antibody or an antigen-binding  
5 fragment thereof, or an antigen-binding protein construct, the method comprising

(a) culturing the cell as described herein under conditions sufficient for the cell to produce the antibody or the antigen-binding fragment thereof, or the antigen-binding protein construct; and

(b) collecting the antibody or the antigen-binding fragment thereof, or the antigen-binding protein construct produced by the cell.

10 In one aspect, the disclosure provides an antibody-drug conjugate (ADC) comprising a therapeutic agent covalently bound to the antibody or antigen-binding fragment thereof, or the antigen-binding protein construct as described herein. In some embodiments, the therapeutic agent is a cytotoxic or cytostatic agent. In some embodiments, the therapeutic agent is MMAE or MMAF.

In one aspect, the disclosure provides a method of treating a subject having autoimmune disorders,  
15 the method comprising administering a therapeutically effective amount of a composition comprising the antibody or antigen-binding fragment thereof, the antigen-binding protein construct, or the antibody-drug conjugate as described herein, to the subject. In some embodiments, the subject has graft-versus-host disease (GvHD) or apical dermatitis. In some embodiments, the subject has received bone marrow transplantation, organ transplantation, or stem cell transplantation. In some embodiments, the subject has  
20 an acute graft-versus-host disease or a chronic graft-versus-host disease.

In some embodiments, the subject is a human.

In some embodiments, the method further comprises administering an anti-PD1 antibody to the subject.

In some embodiments, the method further comprises administering a chemotherapy to the subject.

25 In one aspect, the disclosure provides a method of reducing the GvHD score and improving survival rate, the method comprising administering a therapeutically effective amount of a composition comprising the antibody or antigen-binding fragment thereof, or the antigen-binding protein construct as described herein, to the subject.

In one aspect, the disclosure provides a method of suppressing the immune responses in a subject,  
30 the method comprising administering a therapeutically effective amount of a composition comprising the antibody or antigen-binding fragment thereof, or the antigen-binding protein construct as described herein, to the subject.

In one aspect, the disclosure provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and (a) the anti-OX40 antibody or antigen-binding fragment thereof  
35 as described herein, (b) the antigen-binding protein construct, and/or (c) the antibody-drug conjugate as described herein.

In one aspect, the disclosure provides a nucleic acid comprising a polynucleotide encoding any polypeptide as described herein. In some embodiments, the nucleic acid encodes a bispecific antibody. In some embodiments, the nucleic acid is cDNA.

5 In one aspect, the disclosure provides a vector comprising one or more of the nucleic acids described herein.

In one aspect, the disclosure provides a cell comprising the vector described herein. In some embodiments, the cell is a CHO cell or CHO-K1 cell.

In one aspect, the disclosure provides a cell comprising one or more of the nucleic acids described herein.

10 As used herein, the term “antigen-binding protein construct” is (i) a single polypeptide that includes at least two different antigen-binding domains, or (ii) a complex of two or more polypeptides (e.g., the same or different polypeptides) that together form at least two different antigen-binding domains. Non-limiting examples and aspects of antigen-binding protein constructs are described herein. Additional examples and aspects of antigen-binding protein constructs are known in the art.

15 As used herein, the term “antigen-binding domain” refers to one or more protein domain(s) (e.g., formed from amino acids from a single polypeptide or formed from amino acids from two or more polypeptides (e.g., the same or different polypeptides)) that is capable of specifically binding to an antigen. In some examples, an antigen-binding domain can bind to an antigen or epitope with specificity and affinity similar to that of naturally-occurring antibodies. In some embodiments, the antigen-binding domain can be an antibody or a fragment thereof. One example of an antigen-binding domain is an antigen-binding domain formed by a VH-VL dimer. In some embodiments, an antigen-binding domain can include an alternative scaffold. In some embodiments, the antigen-binding domain is a VHH. Non-limiting examples of antigen-binding domains are described herein. Additional examples of antigen-binding domains are known in the art. In some examples, an antigen-binding domain can bind to  
25 a single antigen.

As used herein, the term “antibody” is used herein in its broadest sense and includes certain types of protein molecules that include one or more antigen-binding domains that specifically bind to an antigen or epitope. An antibody specifically includes, e.g., intact antibodies (e.g., intact immunoglobulins), antibody fragments, bispecific antibodies, and multi-specific antibodies. One example of an antibody is a  
30 protein complex that includes two heavy chains and two light chains. Additional examples of an antibody are described herein.

As used herein, the term “full OX40L binding blocking antigen binding domain” refers to an antigen binding domain that can effectively block the binding of OX40L to the OX40 receptor. A full OX40L binding blocking antigen binding domain can have a maximum blockage over 75% as tested by  
35 flow cytometry.

As used herein, the term “partial OX40L binding blocking antigen binding domain” refers to an antigen binding domain that can partially block the binding of OX40L to the OX40 receptor. A partial OX40L binding blocking antigen binding domain can have a maximum blockage less than 75% as tested by flow cytometry.

5 As used herein, the term “multispecific antigen-binding protein construct” is an antigen-binding protein construct that includes two or more different antigen-binding domains that collectively specifically bind two or more different epitopes. The two or more different epitopes may be epitopes on the same antigen (e.g., a single polypeptide present on the surface of a cell) or on different antigens (e.g., different proteins present on the surface of the same cell or present on the surface of different cells). In  
10 some aspects, a multi-specific antigen-binding protein construct binds two different epitopes (i.e., a “bispecific antigen-binding protein construct”). In some aspects, a multi-specific antigen-binding protein construct binds three different epitopes (i.e., a “trispecific antigen-binding protein construct”). In some aspects, a multi-specific antigen-binding protein construct binds four different epitopes (i.e., a “quad-specific antigen-binding protein construct”). In some aspects, a multi-specific antigen-binding  
15 protein construct binds five different epitopes (i.e., a “quintspecific antigen-binding protein construct”). Each binding specificity may be present in any suitable valency. Non-limiting examples of multispecific antigen-binding protein constructs are described herein.

As used herein, the term “multispecific antibody” refers to an antibody that binds to two or more different epitopes. The term “bispecific antibody” refers to an antibody that binds to two different  
20 epitopes. The epitopes can be on the same antigen or on different antigens.

As used herein, a “VHH” refers to the variable domain of a heavy chain antibody. In some embodiments, the VHH is a humanized VHH.

As used herein, the term “common light chain” refers to a light chain that can interact with two or more different heavy chains, forming different antigen-binding sites, wherein these different  
25 antigen-binding sites can specifically bind to different antigens or epitopes. Similarly, the term “common light chain variable region” refers to a light chain variable region that can interact with two or more different heavy chain variable regions, forming different antigen-binding sites, wherein these different antigen-binding sites can specifically bind to different antigens or epitopes. In some embodiments, the antigen-binding construct can have a common light chain. In some embodiments, the antigen-binding  
30 construct can have a common light chain variable region.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not  
35 intended to be limiting. All publications, patent applications, patents, sequences, database entries, and

other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

5

### DESCRIPTION OF DRAWINGS

**FIG. 1** is a graph showing the blockade of the interaction between human OX40 ligand and human OX40 by anti-OX40 antibodies (1B8, 2C11, 4C8, and 3A11) evaluated by flow cytometry. The fitting curve was obtained using antibody concentration (nM) as the X-axis and the mean fluorescence intensity (MFI) as the Y-axis.

**FIG. 2** is a graph showing the blockade of the interaction between human OX40 ligand and human OX40 by anti-OX40 antibodies (1B8, 2C11, 4C8, and 3A11) evaluated by OX40 reporter cells. The fitting curve was obtained using antibody concentration (nM) as the X-axis and the relative light unit (RLU) as the Y-axis.

**FIG. 3A** is a graph showing agonistic activities of anti-OX40 antibodies (1B8, 2C11, 4C8, and 3A11; two positive control antibodies: GBR830 analog and KHK4083 analog) contrasted to the ISO using Jurkat-Luc-hOX40 reporter cells and CHO-K1-FcγRIIB cells. All anti-OX40 antibodies exhibited agonist activities when Fc-FcγRIIB cross-link existed in the co-culture condition of Jurkat-Luc-hOX40 reporter cells and CHO-K1-FcγRIIB cells.

**FIG. 3B** is a graph showing agonistic activities of all anti-OX40 antibodies (1B8, 2C11, 4C8, and 3A11; two positive control antibodies: GBR830 analog and KHK4083 analog) contrasted to the ISO using only Jurkat-Luc-hOX40 reporter cells. The agonist activities were not observed on Jurkat-Luc-hOX40 reporter cells.

**FIG. 4A** shows the binding between human OX40 protein and the anti-OX40 antibodies, including 3A11, 3A11-4C8, 3A11-2C11, and two positive control antibodies: GBR830 analog and KHK4083 analog, as determined by ELISA analysis.

**FIG. 4B** shows the binding between monkey OX40 protein and the anti-OX40 antibodies, including 3A11, 3A11-4C8, 3A11-2C11, and two positive control antibodies: GBR830 analog and KHK4083 analog, as determined by ELISA analysis.

**FIG. 5A** is a graph showing the blockade of the interaction between human OX40 ligand and human OX40 by anti-OX40 antibodies (3A11-4C8, 3A11-2C11, 1B8-2C11, and two positive control antibodies: GBR830 analog and KHK4083 analog) evaluated by flow cytometry. The fitting curve was obtained using antibody concentration (nM) as the X-axis and the mean fluorescence intensity (MFI) as the Y-axis.

**FIG. 5B** is a graph showing the blockade of the interaction between human OX40 ligand and human OX40 by anti-OX40 antibodies (3A11-4C8, 3A11-2C11, 1B8-2C11, and two positive control

antibodies: GBR830 analog and KHK4083 analog) evaluated by OX40 reporter cells. The fitting curve was obtained using antibody concentration (nM) as the X-axis and the relative light unit (RLU) as the Y-axis.

**FIG. 6A** is a graph showing agonistic activities of bispecific anti-OX40 antibodies (3A11-4C8, 3A11-2C11, and 1B8-2C11, two positive control antibodies: GBR830 analog and KHK4083 analog) contrasted to the negative control ISO using Jurkat-Luc-hOX40 reporter cells and CHO-K1-FcγRIIB cells. In the presence of Fc-FcγRIIB cross-linking, the fluorescence signals detected in GBR830 analog and KHK4083 analog groups displayed a dose-dependent response where the fluorescence signals were increased as the antibody concentration increased. However, the bispecific antibodies, 3A11-4C8, 3A11-2C11 and 1B8-2C11 exhibited mild agonistic effects only when the antibody concentration achieved to certain threshold, and these mild agonistic effects remained the same even when the antibody concentration further increased.

**FIG. 6B** is a graph showing agonistic activities of three bispecific anti-OX40 antibodies (3A11-4C8, 3A11-2C11, and 1B8-2C11, two positive control antibodies: GBR830 analog and KHK4083 analog) contrasted to the negative control ISO using only Jurkat-Luc-hOX40 reporter cells. The agonist activities were not observed on Jurkat-Luc-hOX40 reporter cells alone.

**FIGS. 7A-7C** show the GvHD score generated by monitoring weight loss, posture, activity, fur texture and skin integrity for 45 days post drug administration (see detailed criteria in Table 9) of different antibodies, indicating that the mice treated with 3A11-4C8, 3A11-2C11, 1B8-4C8, and 1B8-2C11 at different dosage exhibited a noteworthy decrease in GvHD scores compared to the mice treated with NaCl (**FIG. 7A**) and performed more pronounced effects in attenuating graft-versus-host-disease (GvHD) compared to the individual parent antibodies (**FIG 7B**) and positive controls, GBR830 analog and/or KHK4083 analog (**FIG 7C**).

**FIG. 8** is a graph showing the survival rate at 49 days post administration of different antibodies.

**FIG. 9A** is a graph showing that mice treated with 3A11-2C11 and 1B8-2C11 displayed better GvHD scores than those treated with 3A11-1B8.

**FIG. 9B** is a graph showing that mice treated with 3A11-2C11 and 1B8-2C11 displayed better mice survival rate than those treated with 3A11-1B8.

**FIG. 10** is a table showing the CDR sequences according to Kabat definition.

**FIG. 11** is a table showing the CDR sequences according to Chothia definition.

**FIG. 12** is a table showing the amino acid sequences of heavy chain variable regions of 1B8, 3A11, 2C11 and 4C8 and common light chain variable region.

**FIG. 13** is a table showing the amino acid sequences of light chain constant region, IgG1 heavy chain constant region with knob or hole, GBR830 variable regions on heavy and light chains, and KHK4083 heavy and light chain variable regions.

**FIG. 14A** is a graph showing the inhibition of cytokine release by anti-OX40 antibodies (3A11-2C11, 3A11-4C8, GBR830 analog, and KHK4083 analog) in human PBMC under the stimulation of plate-coated OKT3 and OX40L.

**FIG. 14B** is a graph showing the inhibition of cytokine release by anti-OX40 antibodies (3A11-2C11, 3A11-4C8, GBR830 analog, and KHK4083 analog) in T cells under the stimulation of plate-coated OKT3 and OX40L.

**FIG. 15A** is a graph showing the agonistic activities of anti-OX40 antibodies (3A11-2C11, GBR830 analog, and KHK4083 analog) in human PBMC under the stimulation of plate-coated OKT3 and anti-OX40 antibodies. IL-2 release was employed as the biomarker for assessing T cell activation in the condition within PBMC populations. The bispecific antibody 3A11-2C11 exhibited comparatively limited agonistic activities.

**FIG. 15B** is a graph showing the agonistic activities of anti-OX40 antibodies (3A11-2C11, GBR830 analog, and KHK4083 analog) in T cells under the stimulation of plate-coated OKT3 and anti-OX40 antibodies. IL-2 release was employed as the biomarker for assessing overall T cell activation. The bispecific antibody 3A11-2C11 exhibited comparatively limited agonistic activities.

**FIGs. 16A-16D** show the blockade of the interaction between human OX40 ligand and human OX40 by anti-OX40 antibodies 3A11-2C11, 3A11-2C11-AF, 3A11-2C11-AF-YTE, 3A11-4C8, 3A11-4C8-AF, 3A11-4C8-AF-YTE, GBR830 analog, and KHK4083 analog evaluated by flow cytometry (FACs) and OX40 reporter cells.

**FIG. 17A** is a graph showing agonistic activities of anti-OX40 antibodies (3A11-2C11, 3A11-2C11-AF, and 3A11-2C11-AF-YTE; two positive control antibodies: GBR830 analog and KHK4083-AF) contrasted to the ISO using Jurkat-Luc-hOX40 reporter cells and CHO-K1-FcγRIIB cells.

**FIG. 17B** is a graph showing agonistic activities of anti-OX40 antibodies (3A11-4C8, 3A11-4C8-AF, and 3A11-4C8-AF-YTE; two positive control antibodies: GBR830 analog and KHK4083-AF) contrasted to the ISO using Jurkat-Luc-hOX40 reporter cells and CHO-K1-FcγRIIB cells.

**FIG. 18** is a graph showing the inhibition of cytokine release by anti-OX40 antibodies (3A11-2C11, 3A11-2C11-AF, 3A11-2C11-AF-YTE, and KHK4083-AF) in T cells under the stimulation of plate-coated OKT3 and OX40L.

**FIG. 19** is a graph showing the agonistic activities of anti-OX40 antibodies (3A11-2C11, 3A11-2C11-AF, 3A11-2C11-AF-YTE and KHK4083 analog) in T cells under the stimulation of plate-coated OKT3 and anti-OX40 antibodies.

**FIGs. 20A-20D** show ADCC effects of anti-OX40 antibodies (3A11-2C11, 3A11-2C11-AF, 3A11-2C11-AF-YTE, 3A11-4C8, 3A11-4C8-AF, 3A11-4C8-AF-YTE, GBR830 analog, and KHK4083 analog) using FcR-TANK cells as effector cells and Jurkat-Luc-hOX40 as target cells. ADCC effects were evaluated under the culturing conditions with 20% heat-inactivated human AB serum or without serum.

**FIG. 21A** is a graph showing the GvHD scores of mice treated with 3A11-2C11-AF, 3A11-2C11-AF-YTE, KHK4083-AF, and GBR830 analog.

**FIG. 21B** is a graph showing the survival rate of mice treated with 3A11-2C11-AF, 3A11-2C11-AF-YTE, KHK4083-AF, and GBR830 analog.

5 **FIG. 22** shows the serum concentration of total antibody after administration to B-hFcRn mice.

**FIG. 23** shows the cytokine release of IL-2, IFN $\gamma$ , and IL6 in human PBMCs under the stimulation of plate-coated anti-OX40 antibodies (3A11-2C11, 3A11-2C11-AF, 3A11-2C11-AF-YTE, 3A11-4C8, 3A11-4C8-AF, and 3A11-4C8-AF-YTE). In the positive control group (PC), 2  $\mu$ g/mL OKT3 and 2  $\mu$ g/mL human OX40L protein were added. LPS was used as the positive control.

10 **FIG. 24A** is a graph showing the GvHD scores of mice treated with 1B8-N297A.

**FIG. 24B** is a graph showing the survival rate of mice treated with 1B8-N297A.

**FIG. 25** is a graph showing the inhibition of CD4 $^{+}$  T cell proliferation by anti-OX40 antibodies (3A11, 3A11-2C11, and KHK4083 analog) in human PBMC under the stimulation of Staphylococcal enterotoxin B (SEB).

15 **FIG. 26** is a graph showing the inhibition of CD4 $^{+}$  T cell proliferation by anti-OX40 antibodies (3A11-2C11, 3A11-2C11-AF-YTE, KHK4083 analog, and KHK4083-AF) in human PBMC under the stimulation of Staphylococcal enterotoxin B (SEB).

## DETAILED DESCRIPTION

20 The immune system can differentiate between normal cells in the body and those it sees as “foreign”, which allows the immune system to attack the foreign cells while leaving the normal cells alone. This mechanism sometimes involves proteins called immune checkpoints. Immune checkpoints are molecules in the immune system that either turn up a signal (co-stimulatory molecules) or turn down a signal.

25 Checkpoint inhibitors can prevent the immune system from attacking normal tissue and thereby preventing autoimmune diseases. Many tumor cells also express checkpoint inhibitors. These tumor cells escape immune surveillance by co-opting certain immune-checkpoint pathways, particularly in T cells that are specific for tumor antigens (Creelan, Benjamin C. “Update on immune checkpoint inhibitors in lung cancer.” Cancer Control 21.1 (2014): 80-89). Because many immune checkpoints are initiated by  
30 ligand-receptor interactions, they can be readily blocked by antibodies against the ligands and/or their receptors.

Tumor necrosis factor receptor superfamily, member 4 (TNFRSF4), also known as CD134 and OX40, is a member of the TNFR-superfamily of receptors which is not constitutively expressed on resting naïve T cells. OX40 is a secondary co-stimulatory immune checkpoint molecule, expressed after  
35 24 to 72 hours following activation; its ligand, OX40L, is also not expressed on resting antigen presenting cells, but is following their activation.

Engagement of the OX40 receptor on T-cells (*in vitro*), using anti-OX40 agonistic antibodies, directly promotes an increase in survival of different effector T-cell subsets. Moreover, the immunosuppressive subset of CD4+ T-cells called regulatory T-cells (Tregs) also express high levels of OX40. Tregs can inhibit effector T-cells through the secretion of immunosuppressive cytokines such as transforming growth factor-beta (TGFb) and interleukin-10 (IL-10). These negative regulators can be counter balanced by the stimulation of OX40 on effector T-cells and other TNFRSF co-stimulatory receptors such as 4-1BB (CD137) and glucocorticoid-induced tumor necrosis factor receptor (GITR) (CD357).

OX40 signaling influences Tregs function and impairs their suppressing ability, presumably through direct inhibition of FoxP3 expression. OX40 signaling also acts on the generation of Tregs: it strongly antagonizes TGFb and the antigen mediated- conversion of naive T-cells into FoxP3<sup>+</sup> Tregs.

As OX40 signaling strongly promotes the bioactivity of CD4+ and CD8+ T-cells and counteract Tregs functions, OX40 has been used as an immunomodulatory target for cancer immunotherapy. A detailed description regarding OX40 and its role as an immunomodulatory target for cancer immunotherapy can be found, e.g., in Aspeslagh, et al. "Rationale for anti-OX40 cancer immunotherapy." European Journal of Cancer 52 (2016): 50-66; Curti, et al. "OX40 is a potent immune-stimulating target in late-stage cancer patients." Cancer research 73.24 (2013): 7189-7198, which are incorporated herein by reference in the entirety.

While anti-OX40 antibodies have been used in cancer immunotherapy, the present disclosure shows that anti-OX40 antibodies have superior therapeutic effects in treating autoimmune related diseases (eg. graft versus host disease), possibly due to their exertion of ADCC effects and blockade between OX40 and OX40L by the Fab region. In some embodiments, even when the OX40-antibody has diminished ADCC effects, it still performs excellently due to its Fab region exerting a blocking effect on the OX40-OX40L interaction.

The present disclosure particularly shows that an antibody with a full OX40L binding blocking antigen binding domain (full blocker) and a partial OX40L binding blocking antigen binding domain (partial blocker) have superior therapeutic effects in treating autoimmune related diseases (eg. graft versus host disease) and modulating immune response. It is conceivable that the full blocker binds to an epitope on OX40 that overlaps or is proximal to the ligand binding site, whereas the partial blocker binds at a more distal epitope. When arms of such full and partial blockers pair, the partial blocker arm stabilizes the binding of the full blocker. This would be in contrast to the scenario where a bispecific antibody comprises the respective arms of two full blocker targeting OX40. It is conceivable that these two arms would bind very close to each other on OX40 by virtue of fully blocking the proximal ligand binding site and one arm could interfere with the binding of the other via any or certain combinations of the three mechanisms: (1) physical hindrance, (2) charge repulsion, and (3) even in the absence of physical

hindrance and charge repulsion, local conformational changes induced by the binding of the first full blocker arm could compromise the binding of the second full blocker arm, due to much closer proximity.

The present disclosure provides several anti-OX40 antibodies, antigen-binding fragments thereof, and antibodies with a full OX40L binding blocking antigen binding domain and a partial OX40L binding blocking antigen binding domain, and methods of using these antibodies and antigen-binding fragments. Particular, the present disclosure provides a bispecific antibody or antigen-binding fragment thereof that can simultaneously bind to two distinct epitopes (e.g., on the same or two different antigens).

In some embodiments, a bispecific antibody or antigen-binding fragment thereof can have two arms. Each arm has one heavy chain variable region and one light chain variable region, forming an antigen-binding domain (or an antigen-binding region). In some embodiments, the bispecific antibody has a common light chain. In some embodiments, one arm comprises a full OX40L binding blocking antigen binding domain and the other arm comprise a partial OX40L binding blocking antigen binding domain.

#### **Anti-OX40 Antibodies and Antigen-Binding Fragments**

In general, antibodies (also called immunoglobulins) can be made up of two classes of polypeptide chains, light chains, and heavy chains. A non-limiting antibody of the present disclosure can be an intact, four immunoglobulin chain protein that is composed of two heavy chains and two light chains. The heavy chain of the antibody can be of any isotype including IgM, IgG, IgE, IgA, or IgD or sub-isotype including IgG1, IgG2, IgG2a, IgG2b, IgG3, IgG4, IgE1, IgE2, etc. The light chain can be a kappa light chain or a lambda light chain. An antibody can comprise two identical copies of a light chain and/or two identical copies of a heavy chain. The heavy chains, which each contain one variable domain (or variable region, VH) and multiple constant domains (or constant regions), bind to one another via disulfide bonding within their constant domains to form the “stem” of the antibody. The light chains, which each contain one variable domain (or variable region, VL) and one constant domain (or constant region), each bind to one heavy chain via disulfide binding. The variable region of each light chain is aligned with the variable region of the heavy chain to which it is bound. The variable regions of both the light chains and heavy chains contain three hypervariable regions sandwiched between more conserved framework regions (FR).

These hypervariable regions, known as the complementary determining regions (CDRs), form loops that form a principal antigen binding surface of the antibody. The four framework regions largely adopt a beta-sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the beta-sheet structure. The CDRs in each chain are held in proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding region.

Methods for identifying the CDR regions of an antibody by analyzing the amino acid sequence of the antibody are well known, and a few definitions of the CDRs are commonly used. The Kabat definition is based on sequence variability, and the Chothia definition is based on the location of the structural loop

regions. These methods and definitions are described in, e.g., Martin, "Protein sequence and structure analysis of antibody variable domains," *Antibody engineering*, Springer Berlin Heidelberg, 2001. 422-439; Abhinandan, et al. "Analysis and improvements to Kabat and structurally correct numbering of antibody variable domains," *Molecular immunology* 45.14 (2008): 3832-3839; Wu, T.T. and Kabat, E.A. (1970) *J. Exp. Med.* 132: 211-250; Martin et al., *Methods Enzymol.* 203:121-53 (1991); Morea et al., *Biophys Chem.* 68(1-3):9-16 (Oct. 1997); Morea et al., *J Mol Biol.* 275(2):269-94 (Jan .1998); Chothia et al., *Nature* 342(6252):877-83 (Dec. 1989); Ponomarenko and Bourne, *BMC Structural Biology* 7:64 (2007); each of which is incorporated herein by reference in its entirety.

The CDRs are important for recognizing an epitope of an antigen. As used herein, an "epitope" is the smallest portion of a target molecule capable of being specifically bound by the antigen binding domain of an antibody. The minimal size of an epitope may be about three, four, five, six, or seven amino acids, but these amino acids need not be in a consecutive linear sequence of the antigen's primary structure, as the epitope may depend on an antigen's three-dimensional configuration based on the antigen's secondary and tertiary structure.

In some embodiments, the antibody is an intact immunoglobulin molecule (e.g., IgG1, IgG2a, IgG2b, IgG3, IgM, IgD, IgE, IgA). The IgG subclasses (IgG1, IgG2, IgG3, and IgG4) are highly conserved, differ in their constant region, particularly in their hinges and upper CH2 domains. The sequences and differences of the IgG subclasses are known in the art, and are described, e.g., in Vidarsson, et al, "IgG subclasses and allotypes: from structure to effector functions." *Frontiers in immunology* 5 (2014); Irani, et al. "Molecular properties of human IgG subclasses and their implications for designing therapeutic monoclonal antibodies against infectious diseases." *Molecular immunology* 67.2 (2015): 171-182; Shakib, Farouk, ed. *The human IgG subclasses: molecular analysis of structure, function and regulation*. Elsevier, 2016; each of which is incorporated herein by reference in its entirety.

The disclosure provides antibodies and antigen-binding fragments thereof that specifically bind to OX40. The anti-OX40 antigen-binding protein construct (e.g., bispecific antibodies) can include any antigen binding domains that are derived from these antibodies.

The disclosure provides anti-OX40 antibodies 3A11, 1B8, 2C11, 4C8, and the antibodies derived therefrom.

The CDR sequences for 3A11, and 3A11 derived antibodies (e.g., human antibodies) include CDRs of the heavy chain variable domain, SEQ ID NOs: 1-3, and CDRs of the light chain variable domain, SEQ ID NOs: 25-27 as defined by Kabat numbering. The CDRs can also be defined by Chothia system. Under the Chothia numbering, the CDR sequences of the heavy chain variable domain are set forth in SEQ ID NOs: 13-15, and CDR sequences of the light chain variable domain are set forth in SEQ ID NOs: 25-27.

The CDR sequences for 1B8, and 1B8 derived antibodies include CDRs of the heavy chain variable domain, SEQ ID NOs: 4-6, and CDRs of the light chain variable domain, SEQ ID NOs: 25-27, as

defined by Kabat numbering. Under Chothia numbering, the CDR sequences of the heavy chain variable domain are set forth in SEQ ID NOs: 16-18, and CDRs of the light chain variable domain are set forth in SEQ ID NOs: 25-27.

5 The CDR sequences for 2C11, and 2C11 derived antibodies include CDRs of the heavy chain variable domain, SEQ ID NOs: 7-9, and CDRs of the light chain variable domain, SEQ ID NOs: 25-27, as defined by Kabat numbering. Under Chothia numbering, the CDR sequences of the heavy chain variable domain are set forth in SEQ ID NOs: 19-21, and CDRs of the light chain variable domain are set forth in SEQ ID NOs: 25-27.

10 The CDR sequences for 4C8 derived antibodies include CDRs of the heavy chain variable domain, SEQ ID NOs: 10-12, and CDRs of the light chain variable domain, SEQ ID NOs: 25-27, as defined by Kabat numbering. Under Chothia numbering, the CDR sequences of the heavy chain variable domain are set forth in SEQ ID NOs: 22-24, and CDRs of the light chain variable domain are set forth in SEQ ID NOs: 25-27.

15 Furthermore, in some embodiments, the antibodies or antigen-binding fragments thereof described herein can also contain one, two, or three heavy chain variable region CDRs selected from the group of SEQ ID NOs: 1-3, SEQ ID NOs: 4-6, SEQ ID NOs: 7-9, SEQ ID NOs: 10-12, SEQ ID NOs: 13-15, SEQ ID NOs: 16-18, SEQ ID NOs: 19-21, SEQ ID NOs: 22-24; and/or one, two, or three light chain variable region CDRs selected from the group of SEQ ID NOs: 25-27.

20 In some embodiments, the antibodies can have a heavy chain variable region (VH) comprising complementarity determining regions (CDRs) 1, 2, 3, wherein the CDR1 region comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to a selected VH CDR1 amino acid sequence, the CDR2 region comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to a selected VH CDR2 amino acid sequence, and the CDR3 region comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to a selected VH CDR3 amino acid sequence, and a light chain variable region (VL) comprising CDRs 1, 2, 3, wherein the CDR1 region comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to a selected VL CDR1 amino acid sequence, the CDR2 region comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to a selected VL CDR2 amino acid sequence, and the CDR3 region comprises or consists of an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to a selected VL CDR3 amino acid sequence. The selected VH CDRs 1, 2, 3 amino acid sequences and the selected VL CDRs, 1, 2, 3 amino acid sequences are shown in **FIG. 10** (Kabat CDR) and **FIG.11** (Chothia CDR).

35 In some embodiments, the antibody or an antigen-binding fragment described herein can contain a heavy chain variable domain containing one, two, or three of the CDRs of SEQ ID NO: 1 with zero, one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 2 with zero, one or two amino acid

insertions, deletions, or substitutions; SEQ ID NO: 3 with zero, one or two amino acid insertions, deletions, or substitutions.

In some embodiments, the antibody or an antigen-binding fragment described herein can contain a heavy chain variable domain containing one, two, or three of the CDRs of SEQ ID NO: 4 with zero, one  
5 or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 5 with zero, one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 6 with zero, one or two amino acid insertions, deletions, or substitutions.

In some embodiments, the antibody or an antigen-binding fragment described herein can contain a heavy chain variable domain containing one, two, or three of the CDRs of SEQ ID NO: 7 with zero, one  
10 or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 8 with zero, one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 9 with zero, one or two amino acid insertions, deletions, or substitutions.

In some embodiments, the antibody or an antigen-binding fragment described herein can contain a heavy chain variable domain containing one, two, or three of the CDRs of SEQ ID NO: 10 with zero,  
15 one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 11 with zero, one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 12 with zero, one or two amino acid insertions, deletions, or substitutions.

In some embodiments, the antibody or an antigen-binding fragment described herein can contain a heavy chain variable domain containing one, two, or three of the CDRs of SEQ ID NO: 13 with zero,  
20 one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 14 with zero, one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 15 with zero, one or two amino acid insertions, deletions, or substitutions.

In some embodiments, the antibody or an antigen-binding fragment described herein can contain a heavy chain variable domain containing one, two, or three of the CDRs of SEQ ID NO: 16 with zero,  
25 one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 17 with zero, one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 18 with zero, one or two amino acid insertions, deletions, or substitutions.

In some embodiments, the antibody or an antigen-binding fragment described herein can contain a heavy chain variable domain containing one, two, or three of the CDRs of SEQ ID NO: 19 with zero,  
30 one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 20 with zero, one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 21 with zero, one or two amino acid insertions, deletions, or substitutions.

In some embodiments, the antibody or an antigen-binding fragment described herein can contain a heavy chain variable domain containing one, two, or three of the CDRs of SEQ ID NO: 22 with zero,  
35 one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 23 with zero, one or two amino

acid insertions, deletions, or substitutions; SEQ ID NO: 24 with zero, one or two amino acid insertions, deletions, or substitutions.

In some embodiments, the antibody or an antigen-binding fragment described herein can contain a light chain variable domain containing one, two, or three of the CDRs of SEQ ID NO: 25 with zero, one  
5 or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 26 with zero, one or two amino acid insertions, deletions, or substitutions; SEQ ID NO: 27 with zero, one or two amino acid insertions, deletions, or substitutions.

The insertions, deletions, and substitutions can be within the CDR sequence, or at one or both terminal ends of the CDR sequence.

10 The disclosure also provides antibodies or antigen-binding fragments thereof that binds to OX40. The antibodies or antigen-binding fragments thereof contain a heavy chain variable region (VH) comprising or consisting of an amino acid sequence that is at least 80%, 85%, 90%, or 95% identical to a selected VH sequence, and a light chain variable region (VL) comprising or consisting of an amino acid  
15 sequence that is at least 80%, 85%, 90%, or 95% identical to a selected VL sequence. In some embodiments, the selected VH sequence is SEQ ID NO: 28, 29, 30, or 31 and the common VL sequence is SEQ ID NO: 32.

In some embodiments, the antibody or antigen binding fragments thereof can have 3 VH CDRs that are identical to the CDRs of any VH sequences as described herein. In some embodiments, the antibody or antigen binding fragments thereof can have 3 VL CDRs that are identical to the CDRs of any  
20 VL sequences as described herein.

In some embodiments, the antibodies, or antigen-binding fragments are full OX40L binding blocking agents. For example, they can include antigen-binding domains derived from 3A11 or 1B8. Wherein a full OX40L binding blocking agent is capable of fully competing with OX40 ligand for binding to OX40 receptor (eg. at a high concentration >50nM). In some embodiments, the antibodies, or  
25 antigen-binding fragments are partial OX40L binding blocking agents. For example, they can include antigen-binding domains derived from 2C11 or 4C8. A partial OX40L binding blocking agent is capable of partially competing with OX40 ligand for binding to OX40 receptor (eg. at a high concentration >50nM).

The disclosure also provides nucleic acid comprising a polynucleotide encoding a polypeptide  
30 comprising an immunoglobulin heavy chain or an immunoglobulin heavy chain. The immunoglobulin heavy chain or immunoglobulin light chain comprises CDRs as shown in **FIG. 10** or **FIG. 11**, or have sequences as shown in **FIG. 12**. When the polypeptides are paired with corresponding polypeptide (e.g., a corresponding heavy chain variable region or the light chain variable region), the paired polypeptides bind to OX40.

35 The anti-OX40 antibodies and antigen-binding fragments can also be antibody variants (including derivatives and conjugates) of antibodies or antibody fragments and multi-specific (e.g., bispecific)

antibodies or antibody fragments. Additional antibodies provided herein are polyclonal, monoclonal, multi-specific (e.g., bispecific), human antibodies, chimeric antibodies (e.g., human-mouse chimera), single-chain antibodies, intracellularly made antibodies (i.e., intrabodies), and antigen-binding fragments thereof. The antibodies or antigen-binding fragments thereof can be of any type (e.g., IgG, IgE, IgM, IgD, IgA, and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2), or subclass. In some embodiments,

the antibody or antigen-binding fragment thereof is an IgG antibody or antigen-binding fragment thereof. Fragments of antibodies are suitable for use in the methods provided so long as they retain the desired affinity and specificity of the full-length antibody. Thus, a fragment of an antibody that binds to OX40 will retain an ability to bind to OX40. An Fv fragment is an antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in tight association, which can be covalent in nature, for example in scFv. It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the VH-VL dimer. Collectively, the six CDRs or a subset thereof confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) can have the ability to recognize and bind antigen, although usually at a lower affinity than the entire binding site.

The antibody can also be an immunoglobulin molecule that is derived from any species (e.g., human, rodent, mouse, rat, camelid). Antibodies disclosed herein also include, but are not limited to, polyclonal, monoclonal, monospecific, polyspecific antibodies, and chimeric antibodies that include an immunoglobulin binding domain fused to another polypeptide. The antigen binding domain or antigen binding fragment is a portion of an antibody that retains specific binding activity of the intact antibody, i.e., any portion of an antibody that is capable of specific binding to an epitope on the intact antibody's target molecule. It includes, e.g., Fab, Fab', F(ab')<sub>2</sub>, and variants of these fragments. Thus, in some embodiments, an antibody or an antigen binding fragment thereof can be, e.g., a scFv, a Fv, a Fd, a dAb, a bispecific antibody, a bispecific scFv, a diabody, a linear antibody, a single-chain antibody molecule, a multi-specific antibody formed from antibody fragments, and any polypeptide that includes a binding domain which is, or is homologous to, an antibody binding domain. Non-limiting examples of antigen binding domains include, e.g., the heavy chain and/or light chain CDRs of an intact antibody, the heavy and/or light chain variable regions of an intact antibody, full length heavy or light chains of an intact antibody, or an individual CDR from either the heavy chain or the light chain of an intact antibody.

In some embodiments, the scFv has two heavy chain variable domains, and two light chain variable domains. In some embodiments, the scFv has two antigen binding region, and the two antigen binding regions can bind to the respective target antigens with different affinities.

In some embodiments, the antigen binding fragment can form a part of a chimeric antigen receptor (CAR). In some embodiments, the chimeric antigen receptor are fusions of single-chain variable fragments (scFv) as described herein, fused to CD3-zeta transmembrane- and endodomain. In some

embodiments, the chimeric antigen receptor also comprises intracellular signaling domains from various costimulatory protein receptors (e.g., CD28, 41BB, ICOS). In some embodiments, the chimeric antigen receptor comprises multiple signaling domains, e.g., CD3z-CD28-41BB or CD3z-CD28-OX40, to increase potency. Thus, in one aspect, the disclosure further provides cells (e.g., T cells) that express the chimeric antigen receptors as described herein.

In some embodiments, the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibodies) can bind to two different antigens or two different epitopes.

In some embodiments, the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibodies) described herein can be conjugated to a therapeutic agent. The antibody-drug conjugate comprising the antibody or antigen-binding fragment thereof can covalently or non-covalently bind to a therapeutic agent. In some embodiments, the therapeutic agent is a cytotoxic or cytostatic agent (e.g., monomethyl auristatin E, monomethyl auristatin F, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin, maytansinoids such as DM-1 and DM-4, dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, epirubicin, and cyclophosphamide and analogs).

Multimerization of antibodies may be accomplished through natural aggregation of antibodies or through chemical or recombinant linking techniques known in the art. For example, some percentage of purified antibody preparations (e.g., purified IgG1 molecules) spontaneously form protein aggregates containing antibody homodimers and other higher-order antibody multimers.

### Antigen Binding Protein Constructs

The present disclosure provides antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibodies). In some embodiments, the antigen-binding protein constructs target two different epitopes of OX40. In some embodiments, the antigen-binding protein construct comprises: a first antigen-binding domain that specifically binds to a first epitope of OX40; and a second antigen-binding domain that specifically binds to a second epitope of OX40.

In some embodiments, the first antigen-binding domain comprises a first heavy chain variable region (VH1) and a first light chain variable region (VL1). In some embodiments, the second antigen-binding domain comprises a second heavy chain variable region (VH2) and a second light chain variable region (VL2).

In some embodiments, the VH1 comprises CDRs 1, 2, and 3, wherein the VH1 CDR1 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a selected VH1 CDR1 amino acid sequence, the VH1 CDR2 region comprises an amino acid sequence that is at least 80%, 90%,

or 100% identical to a selected VH1 CDR2 amino acid sequence, and the VH1 CDR3 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a selected VH1 CDR3 amino acid sequence; and the VL1 comprises CDRs 1, 2, and 3, wherein the VL1 CDR1 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a selected VL1 CDR1 amino acid sequence, the VL1 CDR2 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a selected VL1 CDR2 amino acid sequence, and the VL1 CDR3 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a selected VL CDR3 amino acid sequence, wherein the selected VH1 CDRs 1, 2, and 3 amino acid sequences, the selected VL1 CDRs 1, 2, and 3 amino acid sequences are one of the following:

- 10 (1) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
- (2) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
- 15 (3) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
- (4) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
- 20 (5) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
- 25 (6) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
- (7) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
- 30 (8) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 22-24, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the VH1 comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, or 100% identical to a selected VH sequence, and the VL1 comprises an amino acid sequence

that is at least 80%, 85%, 90%, 95%, or 100% identical to a selected VL sequence, wherein the selected VH sequence and the selected VL sequence are one of the following:

- (1) the selected VH sequence is SEQ ID NO: 28, and the selected VL sequence is SEQ ID NO: 32;
- (2) the selected VH sequence is SEQ ID NO: 29, and the selected VL sequence is SEQ ID NO: 32;
- 5 (3) the selected VH sequence is SEQ ID NO: 30, and the selected VL sequence is SEQ ID NO: 32; and
- (4) the selected VH sequence is SEQ ID NO: 31, and the selected VL sequence is SEQ ID NO: 32.

In some embodiments, the VH2 comprises CDRs 1, 2, and 3, wherein the VH2 CDR1 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a selected VH2 CDR1 amino acid sequence, the VH2 CDR2 region comprises an amino acid sequence that is at least 80%, 90%,  
10 or 100% identical to a selected VH2 CDR2 amino acid sequence, and the VH2 CDR3 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a selected VH2 CDR3 amino acid sequence; and the VL2 comprises CDRs 1, 2, and 3, wherein the VL2 CDR1 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a selected VL2 CDR1 amino acid sequence, the VL2 CDR2 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a  
15 selected VL2 CDR2 amino acid sequence, and the VL2 CDR3 region comprises an amino acid sequence that is at least 80%, 90%, or 100% identical to a selected VL CDR3 amino acid sequence, wherein the selected VH2 CDRs 1, 2, and 3 amino acid sequences, the selected VL2 CDRs 1, 2, and 3 amino acid sequences are one of the following:

(1) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(2) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

25 (3) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(4) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27,  
30 respectively;

(5) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(6) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27,  
35 respectively;

(7) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively; and

(8) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 22-24, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

In some embodiments, the VH2 comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, or 100% identical to a selected VH sequence, and the VL2 comprises an amino acid sequence that is at least 80%, 85%, 90%, 95%, or 100% identical to a selected VL sequence, wherein the selected VH sequence and the selected VL sequence are one of the following:

- (1) the selected VH sequence is SEQ ID NO: 28, and the selected VL sequence is SEQ ID NO: 32;
- (2) the selected VH sequence is SEQ ID NO: 29, and the selected VL sequence is SEQ ID NO: 32;
- (3) the selected VH sequence is SEQ ID NO: 30, and the selected VL sequence is SEQ ID NO: 32; and
- (4) the selected VH sequence is SEQ ID NO: 31, and the selected VL sequence is SEQ ID NO: 32.

In some embodiments, the antigen-binding protein construct (e.g., bispecific antibody) comprises a full OX40L binding blocking antigen binding domain and a partial OX40L binding blocking antigen binding domain. It has been shown that an antigen-binding protein construct (e.g., a bispecific antibody) with a full OX40L binding blocking antigen binding domain and a partial OX40L binding blocking antigen binding domain have superior therapeutic effects in treating graft versus host disease and modulating immune response.

In some embodiments, the full OX40L binding blocking antigen binding domain have a maximum blockage over 80%, 85% or 90% as tested by flow cytometry.

In some embodiments, the partial OX40L binding blocking antigen binding domain have a maximum blockage less than 70%, 65%, 60%, 55% or 50% as tested by flow cytometry.

In some embodiments, the full OX40L binding blocking antigen binding domain have a maximum blockage over 80% and the partial OX40L binding blocking antigen binding domain have a maximum blockage less than 50%.

In some embodiments, the full OX40L binding blocking antigen binding domain have a maximum blockage over 90% and the partial OX40L binding blocking antigen binding domain have a maximum blockage less than 50%.

In some embodiments, the first antigen-binding domain is a full OX40L binding blocking antigen binding domain. In some embodiments, the VH1 and VL1 comprises CDRs derived from 1B8 or 3A11. In some embodiments, the VH1 and VL1 are derived from the VH and VL of 1B8 or 3A11. In some embodiments, the second antigen-binding domain is a partial OX40L binding blocking antigen binding domain. In some embodiments, the VH2 and VL2 comprises CDRs derived from 2C11 or 4C8. In some embodiments, the VH2 and VL2 are derived from the VH and VL of 2C11 or 4C8.

In some embodiments, the first antigen-binding domain can comprises one, two, or three heavy chain variable region CDRs derived from 3A11 or 1B8. In some embodiments, the second antigen-binding domain can comprises one, two, or three light chain variable region CDRs selected from 2C11 or 4C8.

5 Furthermore, these antigen-binding protein constructs (e.g., bispecific antibody), anti-OX40 antibodies, and antigen-binding fragments thereof can have various forms.

In some embodiments, the antigen-binding protein construct is a bispecific antibody. Bispecific antibodies can be made by engineering the interface between a pair of antibody molecules to maximize the percentage of heterodimers that are recovered from recombinant cell culture. For example, the  
10 interface can contain at least a part of the CH3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for  
15 increasing the yield of the heterodimer over other unwanted end-products such as homodimers. This method is described, e.g., in WO 96/27011, which is incorporated by reference in its entirety.

Any of the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibodies) described herein may be conjugated to a stabilizing molecule (e.g., a molecule that increases the half-life of the antibody or antigen-binding fragment thereof in a subject or  
20 in solution). Non-limiting examples of stabilizing molecules include: a polymer (e.g., a polyethylene glycol) or a protein (e.g., serum albumin, such as human serum albumin). The conjugation of a stabilizing molecule can increase the half-life or extend the biological activity of an antibody or an antigen-binding fragment *in vitro* (e.g., in tissue culture or when stored as a pharmaceutical composition) or *in vivo* (e.g., in a human).

25 The antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibodies) can also have various forms. Many different formats of antigen binding constructs are known in the art, and are described e.g., in Suurs, et al. "A review of bispecific antibodies and antibody constructs in oncology and clinical challenges," Pharmacology & therapeutics (2019), which is incorporated herein by reference in the entirety.

30 In some embodiments, the antigen-binding protein construct is a BiTe, a (scFv)<sub>2</sub>, a nanobody, a nanobody-HSA, a DART, a TandAb, a scDiabody, a scDiabody-CH3, scFv-CH-CL-scFv, a HSAbody, scDiabody-HAS, or a tandem-scFv. In some embodiments, the antigen-binding protein construct is a VHH-scAb, a VHH-Fab, a Dual scFab, a F(ab')<sub>2</sub>, a diabody, a crossMab, a DAF (two-in-one), a DAF (four-in-one), a DutaMab, a DT-IgG, a knobs-in-holes common light chain, a knobs-in-holes assembly, a  
35 charge pair, a Fab-arm exchange, a SEEDbody, a LUZ-Y, a Fcab, a κλ-body, an orthogonal Fab, a DVD-IgG, a IgG(H)-scFv, a scFv-(H)IgG, IgG(L)-scFv, scFv-(L)IgG, IgG(L,H)-Fv, IgG(H)-V,

V(H)-IgG, IgG(L)-V, V(L)-IgG, KIH IgG-scFab, 2scFv-IgG, IgG-2scFv, scFv4-Ig, Zyboby, DVI-IgG, Diabody-CH3, a triple body, a miniantibody, a minibody, a TriBi minibody, scFv-CH3 KIH, Fab-scFv, a F(ab')<sub>2</sub>-scFv2, a scFv-KIH, a Fab-scFv-Fc, a tetravalent HCAb, a scDiabody-Fc, a Diabody-Fc, a tandem scFv-Fc, an Intrabody, a dock and lock, a ImmTAC, an IgG-IgG conjugate, a Cov-X-Body, or a scFv1-PEG-scFv2.

In some embodiments, the antigen-binding protein construct can be a TrioMab. In a TrioMab, the two heavy chains are from different species, wherein different sequences restrict the heavy-light chain pairing.

In some embodiments, the antigen-binding protein construct has two different heavy chains and one common light chain. Heterodimerization of heavy chains can be based on the knob-in-holes or some other heavy chain pairing technique.

In some embodiments, CrossMAB technique can be used to produce bispecific antibodies. CrossMAB technique can be used enforce correct light chain association in bispecific heterodimeric IgG antibodies, this technique allows the generation of various bispecific antibody formats, including bi-(1+1), tri-(2+1) and tetra-(2+2) valent bispecific antibodies, as well as non-Fc tandem antigen-binding fragment (Fab)-based antibodies. These formats can be derived from any existing antibody pair using domain crossover, without the need for the identification of common light chains, post-translational processing/in vitro chemical assembly or the introduction of a set of mutations enforcing correct light chain association. The method is described in Klein et al., "The use of CrossMAB technology for the generation of bi- and multispecific antibodies." MAbs. Vol. 8. No. 6. Taylor & Francis, 2016, which is incorporated by reference in its entirety. In some embodiments, the CH1 in the heavy chain and the CL domain in the light chain are swapped.

The antigen-binding protein construct can be a Duobody. The Fab-exchange mechanism naturally occurring in IgG4 antibodies is mimicked in a controlled matter in IgG1 antibodies, a mechanism called controlled Fab exchange. This format can ensure specific pairing between the heavy-light chains.

In Dual-variable-domain antibody (DVD-Ig), additional VH and variable light chain (VL) domain are added to each N-terminus for bispecific targeting. This format resembles the IgG-scFv, but the added binding domains are bound individually to their respective N-termini instead of a scFv to each heavy chain N-terminus.

In scFv-IgG, the two scFv are connected to the C-terminus of the heavy chain (CH3). The scFv-IgG format has two different bivalent binding sites and is consequently also called tetravalent. There are no heavy-chain and light-chain pairing problem in the scFv-IgG.

In some embodiments, the antigen-binding protein construct can have an IgG-IgG format. Two intact IgG antibodies are conjugated by chemically linking the C-terminals of the heavy chains.

The antigen-binding protein construct can also have a Fab-scFv-Fc format. In Fab-scFv-Fc format, a light chain, heavy chain and a third chain containing the Fc region and the scFv are assembled. It can ensure efficient manufacturing and purification.

In some embodiments, antigen-binding protein construct can be a TF. Three Fab fragments are  
5 linked by disulfide bridges. Two fragments target the tumor associated antigen (TAA) and one fragment targets a hapten. The TF format does not have an Fc region.

ADAPTIR has two scFvs bound to each side of an Fc region. It abandons the intact IgG as a basis for its construct but conserves the Fc region to extend the half-life and facilitate purification.

Bispecific T cell Engager ("BiTE") consists of two scFvs, VLA VHA and VHB VLB on one  
10 peptide chain. It has only binding domains, no Fc region.

In BiTE-Fc, an Fc region is fused to the BiTE construct. The addition of Fc region enhances half-life leading to longer effective concentrations, avoiding continuous IV.

Dual affinity retargeting (DART) has two peptide chains connecting the opposite fragments, thus VLA with VHB and VLB with VHA, and a sulfur bond at their C-termini fusing them together. In DART,  
15 the sulfur bond can improve stability over BiTEs.

In DART-Fc, an Fc region is attached to the DART structure. It can be generated by assembling three chains, two via a disulfide bond, as with the DART. One chain contains half of the Fc region which will dimerize with the third chain, only expressing the Fc region. The addition of Fc region enhances half-life leading to longer effective concentrations, avoiding continuous IV.

In tetravalent DART, four peptide chains are assembled. Basically, two DART molecules are  
20 created with half an Fc region and will dimerize. This format has bivalent binding to both targets; thus, it is a tetravalent molecule.

Tandem diabody (TandAb) comprises two diabodies. Each diabody consists of an VHA and VLB fragment and a VHA and VLB fragment that are covalently associated. The two diabodies are linked with  
25 a peptide chain. It can improve stability over the diabody consisting of two scFvs. It has two bivalent binding sites.

The scFv-scFv-toxin includes toxin and two scFv with a stabilizing linker. It can be used for specific delivery of payload.

In modular scFv-scFv-scFv, one scFv directed against the TAA is tagged with a short  
30 recognizable peptide is assembled to a bispecific antibody consisting of two scFvs, one directed against CD3 and one against the recognizable peptide.

In ImmTAC, a stabilized and soluble T cell receptor is fused to a scFv recognizing CD3. By using a TCR, the ImmTAC is suitable to target processed, e.g., intracellular, proteins.

Tri-specific nanobody has two single variable domains (nanobodies) with an additional module  
35 for half-life extension. The extra module is added to enhance half-life.

In Trispecific Killer Engager (TriKE), two scFvs are connected via polypeptide linkers incorporating human IL-15. The linker to IL-15 is added to increase survival and proliferation of NKs.

In some embodiments, the antigen-binding protein construct is a bispecific antibody. In some embodiments, the bispecific antibody in present disclosure is designed to be 1+1 (monovalent for each epitope) and has an IgG1 subtype structure. This can reduce the avidity to cells with low expression levels of two epitopes on OX40 and increase the avidity to cells that co-express two epitopes on OX40, to achieve enhanced targeting function. Mutations S239D and/or I332E (SI mutations) can also be introduced in antibody heavy chains to enhance the antibody affinity to FcγRIIIA. Mutations M252Y, S254T, and/or T256E mutations (YTE mutations) can also be introduced in antibody heavy chains to enhance the antibody affinity to FcRn.

In some embodiments, the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., the anti-OX40 antibody or the bispecific antibody) have a light chain constant region that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 33, and a heavy chain constant region that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to any one of SEQ ID NOs: 34 and 35.

In some embodiments, the anti-OX40 antigen-binding protein construct (e.g., antibodies, bispecific antibodies, or antibody fragments thereof) include KIH mutations and/or SI mutations. In some embodiments, the antigen-binding protein construct includes a first antigen-binding domain that specifically binds to a first epitope on OX40, and a second antigen-binding domain that specifically binds to a second epitope on OX40.

In some embodiments, the first antigen-binding domain includes a heavy chain that including one or more knob mutations (a knob heavy chain), and the second antigen-binding domain includes a heavy chain including one or more-hole mutations (a hole heavy chain). In some embodiments, the first antigen-binding domain includes a heavy chain that including one or more-hole mutations (a hole heavy chain), and the second antigen-binding domain includes a heavy chain including one or more knob mutations (a knob heavy chain).

In some embodiments, the anti-OX40 antigen-binding protein construct includes a knob heavy chain that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 34. In some embodiments, the anti-OX40 antigen-binding protein construct includes a hole heavy chain that is at least 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO: 35.

In some embodiments, the bispecific antibody or antigen-binding fragment thereof described herein has a common light chain.

### Antibody Characteristics

The anti-OX40 antigen-binding protein construct (e.g., bispecific antibodies) can include antigen-binding regions that are derived from any anti-OX40 antibody or antigen-binding fragment thereof as described herein. The anti-OX40 antibodies, antigen-binding fragments or the anti-OX40 antigen-binding protein construct (e.g., bispecific antibodies) thereof described herein can block (e.g.,  
 5 fully or partially) the binding between OX40 and OX40L.

This disclosure provides an anti-OX40 antigen-binding protein construct (e.g., bispecific antibodies), comprising: a first antigen-binding domain that specifically binds to a first epitope of OX40, wherein the first antigen-binding domain is a full OX40L binding blocking antigen-binding domain; and a  
 10 second antigen-binding domain that specifically binds to a second epitope of OX40, wherein the second antigen-binding domain is a partial OX40L binding blocking antigen-binding domain.

In some embodiments, by binding to OX40, the antibody can also promote OX40 signaling pathway and upregulates the immune response. Thus, in some embodiments, the antibodies or antigen-binding fragments thereof as described herein are OX40 agonist. In some embodiments, the activation of OX40 signaling pathway depends on Fc-FcγRIIB cross-linking or plate-coated cross-linking.  
 15

In some embodiments, the antibodies or antigen-binding fragments thereof are OX40 antagonist.

In some embodiments, the anti-OX40 antigen-binding protein construct (e.g., bispecific antibodies) thereof shows mild agonistic effects only when the antibody concentration achieved to certain threshold, and these mild agonistic effects remain the same regardless of the antibody concentration increase.

General techniques can be used to measure the affinity of an antibody for an antigen include, e.g., ELISA, RIA, and surface plasmon resonance (SPR). Affinities can be deduced from the quotient of the kinetic rate constants ( $K_D = k_{off}/k_{on}$ ). In some implementations, the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibody) can bind to OX40 (e.g., human OX40, monkey OX40, mouse OX40, and/or chimeric OX40) with a dissociation rate ( $k_{off}$ )  
 25 of less than  $0.1 \text{ s}^{-1}$ , less than  $0.01 \text{ s}^{-1}$ , less than  $0.001 \text{ s}^{-1}$ , less than  $0.0001 \text{ s}^{-1}$ , or less than  $0.00001 \text{ s}^{-1}$ . In some embodiments, the dissociation rate ( $k_{off}$ ) is greater than  $0.01 \text{ s}^{-1}$ , greater than  $0.001 \text{ s}^{-1}$ , greater than  $0.0001 \text{ s}^{-1}$ , greater than  $0.00001 \text{ s}^{-1}$ , or greater than  $0.000001 \text{ s}^{-1}$ .

In some embodiments, kinetic association rates ( $k_{on}$ ) are greater than  $1 \times 10^2/\text{Ms}$ , greater than  $1 \times 10^3/\text{Ms}$ , greater than  $1 \times 10^4/\text{Ms}$ , greater than  $1 \times 10^5/\text{Ms}$ , or greater than  $1 \times 10^6/\text{Ms}$ . In some  
 30 embodiments, kinetic association rates ( $k_{on}$ ) are less than  $1 \times 10^5/\text{Ms}$ , less than  $1 \times 10^6/\text{Ms}$ , or less than  $1 \times 10^7/\text{Ms}$ .

Affinities can be deduced from the quotient of the kinetic rate constants ( $K_D = k_{off}/k_{on}$ ). In some embodiments,  $K_D$  is less than  $1 \times 10^{-6} \text{ M}$ , less than  $1 \times 10^{-7} \text{ M}$ , less than  $1 \times 10^{-8} \text{ M}$ , less than  $1 \times 10^{-9} \text{ M}$ , or less than  $1 \times 10^{-10} \text{ M}$ . In some embodiments, the  $K_D$  is less than 30 nM, 20 nM, 15 nM, 10 nM, 9 nM,  
 35 8 nM, 7 nM, 6 nM, 5 nM, 4 nM, 3 nM, 2 nM, or 1 nM. In some embodiments,  $K_D$  is greater than  $1 \times 10^{-7} \text{ M}$ , greater than  $1 \times 10^{-8} \text{ M}$ , greater than  $1 \times 10^{-9} \text{ M}$ , or greater than  $1 \times 10^{-10} \text{ M}$ .

In some embodiments, the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibody) as described herein can increase immune response, activity of OX40, activity or number of T cells (e.g., CD8+ and/or CD4+ cells) by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2 folds, 3 folds, 5 folds, 10 folds, or 20 folds. In some embodiments, the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibody) as described herein can decrease the activity or number of Treg by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 2 folds, 3 folds, 5 folds, 10 folds, or 20 folds.

In some embodiments, the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibody) as described herein are OX40 agonist. In some embodiments, the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibody) can increase OX40 signal transduction in a target cell (e.g., T cell) that expresses OX40. In some embodiments, OX40 signal transduction is detected by monitoring NF $\gamma$ B downstream signaling.

Because the antigen-binding protein construct (e.g., bispecific antibody) binds to two distinct epitopes on OX40, thereby enhancing its binding affinity to the cells expressing OX40. Avidity can be used to measure the binding affinity of an antigen-binding protein construct to these cells. Avidity is the accumulated strength of multiple affinities of individual non-covalent binding interactions.

In some embodiments, the antibodies, the antigen-binding fragments thereof, the antigen-binding protein constructs (e.g., bispecific antibody), can bind to human OX40 or monkey OX40.

In some embodiments, the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., the anti-OX40 bispecific antibody) reduce GvHD score below 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, or 9. In some embodiments, the antibodies reduce GvHD score below 6, 6.5, 7, 7.5, 8, or 9.5. The GvHD score can be determined, e.g., at 2, 6, 8, 10, 13, 15, 17, 20, 22, 24, 27, 29, 31, 34, 36, 38, 41, 43, or 45 days after the treatment starts. The survival rate can be determined, e.g., at 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 days after the treatment starts. In some embodiments, the GvHD score is generated by monitoring weight loss, posture, activity, fur texture and skin integrity for at least 45 days post drug administration. In some embodiments, the survival rate is determined at least 49 days post treatment.

In some embodiments, the antibody, the antigen-binding fragment thereof, or the antigen-binding protein construct (e.g., bispecific antibody) has a functional Fc region. In some embodiments, effector function of a functional Fc region is antibody-dependent cell-mediated cytotoxicity (ADCC). In some embodiments, effector function of a functional Fc region is phagocytosis. In some embodiments, effector function of a functional Fc region is ADCC and phagocytosis. In some embodiments, the Fc region is human IgG1, human IgG2, human IgG3, or human IgG4. In some embodiments, one or both mutations

S239D and/or I332E (SI mutations) are introduced in antibody Fc region to enhance the antibody affinity to FcγRIIIA, thereby increasing ADCC effects. A detailed description of SI mutations can be found in U.S. Patent No. US7662925B2, which is incorporated by reference in its entirety. In some embodiments, M252Y, S254T, T256E mutations (EU numbering, YTE mutations) are introduced in antibody Fc region to enhance the antibody affinity to FcRn, thereby increasing the half-life.

In some embodiments, the antibody, the antigen-binding fragment thereof, or the antigen-binding protein construct (e.g., bispecific antibody) has an IgG1 Fc. In some embodiments, the Fc is afucosylated (e.g., expressed by CHO-S cells with FUT8 and/or SLC35c1 deletions).

In some embodiments, the antibody, the antigen-binding fragment thereof, or the antigen-binding protein construct (e.g., bispecific antibody) does not have a functional Fc region. For example, the antibodies or antigen binding fragments are Fab, Fab', F(ab')<sub>2</sub>, and Fv fragments. In some embodiments, the protein constructs as described herein have an Fc region without effector function. In some embodiments, the Fc is a human IgG4 Fc. In some embodiments, the Fc does not have a functional Fc region. For example, the Fc region has LALA mutations (L234A and L235A mutations in EU numbering), or LALA-PG mutations (L234A, L235A, P329G mutations in EU numbering).

Some other modifications to the Fc region can be made. For example, a cysteine residue(s) can be introduced into the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric fusion protein thus generated may have any increased half-life *in vitro* and/or *in vivo*.

In some embodiments, the IgG4 has S228P mutation (EU numbering). The S228P mutation prevents *in vivo* and *in vitro* IgG4 Fab-arm exchange.

In some embodiments, Fc regions are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such Fc region composition may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e.g., complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues; or position 314 in Kabat numbering); however, Asn297 may also be located about ±3 amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in Fc region sequences. Such fucosylation variants may have improved ADCC function. In some embodiments, to reduce glycan heterogeneity, the Fc region can be further engineered to replace the Asparagine at position 297 with Alanine (N297A).

#### Methods of Making Antibodies and Antigen-Binding Protein Constructs

An isolated fragment of human protein can be used as an immunogen to generate antibodies using standard techniques for polyclonal and monoclonal antibody preparation. Polyclonal antibodies can be

raised in animals by multiple injections (e.g., subcutaneous or intraperitoneal injections) of an antigenic peptide or protein. In some embodiments, the antigenic peptide or protein is injected with at least one adjuvant. In some embodiments, the antigenic peptide or protein can be conjugated to an agent that is immunogenic in the species to be immunized. Animals can be injected with the antigenic peptide or protein more than one time (e.g., twice, three times, or four times).

The full-length polypeptide or protein can be used or, alternatively, antigenic peptide fragments thereof can be used as immunogens. The antigenic peptide of a protein comprises at least 8 (e.g., at least 10, 15, 20, or 30) amino acid residues of the amino acid sequence of the protein and encompasses an epitope of the protein such that an antibody raised against the peptide forms a specific immune complex with the protein.

An immunogen typically is used to prepare antibodies by immunizing a suitable subject (e.g., human, or transgenic animal expressing at least one human immunoglobulin locus). An appropriate immunogenic preparation can contain, for example, a recombinantly-expressed or a chemically-synthesized polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or a similar immunostimulatory agent.

Polyclonal antibodies can be prepared as described above by immunizing a suitable subject with a polypeptide, or an antigenic peptide thereof (e.g., part of the protein) as an immunogen. The antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme-linked immunosorbent assay (ELISA) using the immobilized polypeptide or peptide. If desired, the antibody molecules can be isolated from the mammal (e.g., from the blood) and further purified by well-known techniques, such as protein A or protein G chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the specific antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler et al. (*Nature* 256:495-497, 1975), the human B cell hybridoma technique (Kozbor et al., *Immunol. Today* 4:72, 1983), the EBV-hybridoma technique (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96, 1985), or trioma techniques. The technology for producing hybridomas is well known (see, generally, *Current Protocols in Immunology*, 1994, Coligan et al. (Eds.), John Wiley & Sons, Inc., New York, NY). Hybridoma cells producing a monoclonal antibody are detected by screening the hybridoma culture supernatants for antibodies that bind the polypeptide or epitope of interest, e.g., using a standard ELISA assay.

Variants of the antibodies or antigen-binding fragments described herein can be prepared by introducing appropriate nucleotide changes into the DNA encoding a human, humanized, or chimeric antibody, or antigen-binding fragment thereof described herein, or by peptide synthesis. Such variants include, for example, deletions, insertions, or substitutions of residues within the amino acids sequences that make-up the antigen-binding site of the antibody or an antigen-binding domain. In a population of

such variants, some antibodies or antigen-binding fragments will have increased affinity for the target protein. Any combination of deletions, insertions, and/or combinations can be made to arrive at an antibody or antigen-binding fragment thereof that has increased binding affinity for the target. The amino acid changes introduced into the antibody or antigen-binding fragment can also alter or introduce new post-translational modifications into the antibody or antigen-binding fragment, such as changing (e.g.,  
5 increasing or decreasing) the number of glycosylation sites, changing the type of glycosylation site (e.g., changing the amino acid sequence such that a different sugar is attached by enzymes present in a cell), or introducing new glycosylation sites.

Antibodies disclosed herein can be derived from any species of animal, including mammals.  
10 Non-limiting examples of native antibodies include antibodies derived from humans, primates, e.g., monkeys and apes, cows, pigs, horses, sheep, camelids (e.g., camels and llamas), chicken, goats, and rodents (e.g., rats, mice, hamsters and rabbits), including transgenic rodents genetically engineered to produce human antibodies.

Phage display (panning) can be used to optimize antibody sequences with desired binding  
15 affinities. In this technique, a gene encoding single chain Fv (comprising VH or VL) can be inserted into a phage coat protein gene, causing the phage to "display" the scFv on its outside while containing the gene for the protein on its inside, resulting in a connection between genotype and phenotype. These displaying phages can then be screened against target antigens, in order to detect interaction between the displayed antigen binding sites and the target antigen. Thus, large libraries of proteins can be screened  
20 and amplified in a process called in vitro selection, and antibodies sequences with desired binding affinities can be obtained.

Human and humanized antibodies include antibodies having variable and constant regions derived from (or having the same amino acid sequence as those derived from) human germline immunoglobulin sequences. Human antibodies may include amino acid residues not encoded by human  
25 germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo), for example in the CDRs.

A humanized antibody typically has a human framework (FR) grafted with non-human CDRs. Thus, a humanized antibody has one or more amino acid sequence introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are  
30 typically taken from an "import" variable domain. Humanization can be essentially performed by e.g., substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. These methods are described in e.g., Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988); each of which is incorporated by reference herein in its entirety. Accordingly, "humanized" antibodies are chimeric antibodies wherein  
35 substantially less than an intact human V domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically mouse antibodies in which

some CDR residues and some FR residues are substituted by residues from analogous sites in human antibodies.

It is further important that antibodies be humanized with retention of high specificity and affinity for the antigen and other favorable biological properties. To achieve this goal, humanized antibodies can be prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved.

Identity or homology with respect to an original sequence is usually the percentage of amino acid residues present within the candidate sequence that are identical with a sequence present within the human, humanized, or chimeric antibody or fragment, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity.

In some embodiments, a mouse with a humanized heavy chain immunoglobulin locus and a humanized kappa chain immunoglobulin locus is used to generate antibodies. The heavy chain immunoglobulin locus is a region on the chromosome that contains genes for the heavy chains of antibodies. The locus can include e.g., human IGHV (variable) genes, human IGHD (diversity) genes, human IGHJ (joining) genes, and mouse heavy chain constant domain genes. The kappa chain immunoglobulin locus is a region on the chromosome that contains genes that encode the light chains of antibodies (kappa chain). The kappa chain immunoglobulin locus can include e.g., human IGKV (variable) genes, human IGKJ (joining) genes, and mouse light chain constant domain genes. A detailed description regarding RenMab™ mice can be found in PCT/CN2020/075698 or US20200390073A1, which is incorporated herein by reference in its entirety.

In some embodiments, a mouse with a humanized heavy chain immunoglobulin locus and a humanized kappa chain immunoglobulin locus is used to generate antibodies. The heavy chain immunoglobulin locus is a region on the chromosome that contains genes for the heavy chains of antibodies. The locus can include e.g., human IGHV (variable) genes, human IGHD (diversity) genes, human IGHJ (joining) genes, and mouse heavy chain constant domain genes. The kappa chain immunoglobulin locus is a region on the chromosome that contains genes that encode a common light chains. The kappa chain immunoglobulin locus can include e.g., a human IGKV (variable) gene, a human IGKJ (joining) gene, and mouse light chain constant domain genes. A detailed description regarding

RenLite™ mice can be found in PCT/CN2021/097652, which is incorporated herein by reference in its entirety.

In some embodiments, a covalent modification can be made to the antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibodies). These covalent modifications can be made by chemical or enzymatic synthesis, or by enzymatic or chemical cleavage. Other types of covalent modifications of the antibody or antibody fragment are introduced into the molecule by reacting targeted amino acid residues of the antibody or fragment with an organic derivatization agent that can react with selected side chains or the N- or C-terminal residues.

In some embodiments, antibody variants are provided having a carbohydrate structure that lacks fucose attached (directly or indirectly) to an Fc region. For example, the amount of fucose in such antibody may be from 1% to 80%, from 1% to 65%, from 5% to 65% or from 20% to 40%. The amount of fucose is determined by calculating the average amount of fucose within the sugar chain at Asn297, relative to the sum of all glycostructures attached to Asn 297 (e.g., complex, hybrid and high mannose structures) as measured by MALDI-TOF mass spectrometry, as described in WO 2008/077546, for example. Asn297 refers to the asparagine residue located at about position 297 in the Fc region (Eu numbering of Fc region residues; or position 314 in Kabat numbering); however, Asn297 may also be located about  $\pm 3$  amino acids upstream or downstream of position 297, i.e., between positions 294 and 300, due to minor sequence variations in antibodies. Such fucosylation variants may have improved ADCC function. In some embodiments, to reduce glycan heterogeneity, the Fc region of the antibody can be further engineered to replace the Asparagine at position 297 with Alanine (N297A).

In some embodiments, to facilitate production efficiency by avoiding Fab-arm exchange, the Fc region of the antibodies was further engineered to replace the serine at position 228 (EU numbering) of IgG4 with proline (S228P). A detailed description regarding S228 mutation is described, e.g., in Silva et al. "The S228P mutation prevents in vivo, and in vitro IgG4 Fab-arm exchange as demonstrated using a combination of novel quantitative immunoassays and physiological matrix preparation." *Journal of Biological Chemistry* 290.9 (2015): 5462-5469, which is incorporated by reference in its entirety.

In some embodiments, the methods described here are designed to make a bispecific antibody. Bispecific antibodies can be made by engineering the interface between a pair of antibody molecules to maximize the percentage of heterodimers that are recovered from recombinant cell culture. For example, the interface can contain at least a part of the CH3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g., tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g., alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers. This method is described, e.g., in WO 96/27011, which is incorporated by reference in its entirety.

In some embodiments, knobs-into-holes (KIH) technology can be used, which involves engineering CH3 domains to create either a “knob” or a “hole” in each heavy chain to promote heterodimerization. The KIH technique is described e.g., in Xu, Yiren, et al. "Production of bispecific antibodies in ‘knobs-into-holes’ using a cell-free expression system." *MAbs*. Vol. 7. No. 1. Taylor & Francis, 2015, which is incorporated by reference in its entirety. In some embodiments, one heavy chain has a T366W, and/or S354C (knob) substitution (EU numbering), and the other heavy chain has an Y349C, T366S, L368A, and/or Y407V (hole) substitution (EU numbering). In some embodiments, one heavy chain has one or more of the following substitutions Y349C and T366W (EU numbering). The other heavy chain can have one or more the following substitutions E356C, T366S, L368A, and Y407V (EU numbering). Furthermore, a substitution (-ppcpScp->-ppcpPcp-) can also be introduced at the hinge regions of both substituted IgG.

Bispecific antibodies can also include e.g., cross-linked or “heteroconjugate” antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin and the other to biotin. Heteroconjugate antibodies can also be made using any convenient cross-linking methods. Suitable cross-linking agents and cross-linking techniques are well known in the art and are disclosed in U.S. Patent No. 4,676,980, which is incorporated herein by reference in its entirety.

Methods for generating bispecific antibodies from antibody fragments are also known in the art. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al. (Science 229:81, 1985) describes a procedure where intact antibodies are proteolytically cleaved to generate F(ab')<sub>2</sub> fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab' TNB derivatives is then reconverted to the Fab' thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of another Fab' TNB derivative to form the bispecific antibody.

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### Recombinant Vectors

The present disclosure also provides recombinant vectors (e.g., expression vectors) that include an isolated polynucleotide disclosed herein (e.g., a polynucleotide that encodes a polypeptide disclosed herein), host cells into which are introduced the recombinant vectors (i.e., such that the host cells contain the polynucleotide and/or a vector comprising the polynucleotide), and the production of recombinant antibody polypeptides or fragments thereof by recombinant techniques.

As used herein, a “vector” is any construct capable of delivering one or more polynucleotide(s) of interest to a host cell when the vector is introduced to the host cell. An “expression vector” can deliver and expressing the one or more polynucleotide(s) of interest as an encoded polypeptide in a host cell into which the expression vector has been introduced. Thus, in an expression vector, the polynucleotide of interest is positioned for expression in the vector by being operably linked with regulatory elements such

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as a promoter, enhancer, and/or a poly-A tail, either within the vector or in the genome of the host cell at or near or flanking the integration site of the polynucleotide of interest such that the polynucleotide of interest will be translated in the host cell introduced with the expression vector.

5 A vector can be introduced into the host cell by methods known in the art, e.g., electroporation, chemical transfection (e.g., DEAE-dextran), transformation, transfection, and infection and/or transduction (e.g., with recombinant virus). Thus, non-limiting examples of vectors include viral vectors (which can be used to generate recombinant virus), naked DNA or RNA, plasmids, cosmids, phage vectors, and DNA or RNA expression vectors associated with cationic condensing agents.

10 In some implementations, a polynucleotide disclosed herein (e.g., a polynucleotide that encodes a polypeptide disclosed herein) is introduced using a viral expression system (e.g., vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus, or may use a replication defective virus. In the latter case, viral propagation generally will occur only in complementing virus packaging cells. Suitable systems are disclosed, for example, in Fisher-Hoch et al., 1989, Proc. Natl. Acad. Sci. USA 86:317-321; Flexner et al., 1989, Ann. N.Y. Acad  
15 Sci. 569:86-103; Flexner et al., 1990, Vaccine, 8:17-21; U.S. Pat. Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Pat. No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner-Biotechniques, 6:616-627, 1988; Rosenfeld et al., 1991, Science, 252:431-434; Kolls et al., 1994, Proc. Natl. Acad. Sci. USA, 91:215-219; Kass-Eisler et al., 1993, Proc. Natl. Acad. Sci. USA,  
20 90:11498-11502; Guzman et al., 1993, Circulation, 88:2838-2848; and Guzman et al., 1993, Cir. Res., 73:1202-1207. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., 1993, Science, 259:1745-1749, and Cohen, 1993, Science, 259:1691-1692. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads that are efficiently transported into the cells.

25 For expression, the DNA insert comprising an antibody-encoding or polypeptide-encoding polynucleotide disclosed herein can be operatively linked to an appropriate promoter (e.g., a heterologous promoter), such as the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters are known to the skilled artisan. The expression constructs can further contain sites for transcription initiation,  
30 termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs may include a translation initiating at the beginning and a termination codon (UAA, UGA, or UAG) appropriately positioned at the end of the polypeptide to be translated.

35 As indicated, the expression vectors can include at least one selectable marker. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of

appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces*, and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera Sf9* cells; animal cells such as CHO, COS, Bowes melanoma, and HK 293 cells; and plant cells. Appropriate culture mediums and conditions for the host cells described herein are known in the art.

5 Non-limiting vectors for use in bacteria include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Non-limiting eukaryotic vectors include pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other  
10 suitable vectors will be readily apparent to the skilled artisan.

Non-limiting bacterial promoters suitable for use include the *E. coli* lacI and lacZ promoters, the T3 and T7 promoters, the gpt promoter, the lambda PR and PL promoters and the trp promoter. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma  
15 virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter.

In the yeast *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. For reviews, see Ausubel *et al.* (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York, N.Y., and Grant *et al.*, *Methods Enzymol.*, 153: 516-544 (1997).

20 Introduction of the construct into the host cell can be affected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al.*, Basic Methods In Molecular Biology (1986), which is incorporated herein by reference in its entirety.

25 Transcription of DNA encoding an antibody of the present disclosure by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at base pairs 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma  
30 enhancer on the late side of the replication origin, and adenovirus enhancers.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide, or they may be heterologous signals.

35 The polypeptide (e.g., antibody) can be expressed in a modified form, such as a fusion protein (e.g., a GST-fusion) or with a histidine-tag, and may include not only secretion signals, but also additional

heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties can be added to the polypeptide to facilitate purification. Such regions can be removed prior to final  
 5 preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art.

The disclosure also provides a nucleic acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%,  
 10 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any nucleotide sequence as described herein, and an amino acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any amino acid sequence as described herein.

The disclosure also provides a nucleic acid sequence that has a homology of at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%,  
 15 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any nucleotide sequence as described herein, and an amino acid sequence that has a homology of at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% to any amino acid sequence as described herein.

In some embodiments, the disclosure relates to nucleotide sequences encoding any peptides that are described herein, or any amino acid sequences that are encoded by any nucleotide sequences as described herein. In some embodiments, the nucleic acid sequence is less than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 150, 200, 250, 300, 350, 400, 500, or 600 nucleotides. In some embodiments, the amino acid sequence is less than 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130,  
 20 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, or 400 amino acid residues.

In some embodiments, the amino acid sequence (i) comprises an amino acid sequence; or (ii) consists of an amino acid sequence, wherein the amino acid sequence is any one of the sequences as described herein.

In some embodiments, the nucleic acid sequence (i) comprises a nucleic acid sequence; or (ii) consists of a nucleic acid sequence, wherein the nucleic acid sequence is any one of the sequences as described herein.  
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To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The amino acid residues or nucleotides at  
 35 corresponding amino acid positions or nucleotide positions are then compared. When a position in the

first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid “identity” is equivalent to amino acid or nucleic acid “homology”). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, considering the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. For example, the comparison of sequences and determination of percent identity between two sequences can be accomplished using a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

The disclosure provides one or more nucleic acid encoding any of the polypeptides as described herein. In some embodiments, the nucleic acid (e.g., cDNA) includes a polynucleotide encoding a polypeptide of a heavy chain as described herein. In some embodiments, the nucleic acid includes a polynucleotide encoding a polypeptide of a light chain as described herein. In some embodiments, the nucleic acid includes a polynucleotide encoding a scFv polypeptide as described herein.

In some embodiments, the vector includes two of the nucleic acids as described herein, wherein the vector encodes the VL region and the VH region that together bind to OX40. In some embodiments, a pair of vectors is provided, wherein each vector comprises one of the nucleic acids as described herein, wherein together the pair of vectors encodes the VL region and the VH region that together bind to OX40.

### Methods of Treatment

The methods described herein include methods for treating, preventing, or reducing the risk of developing disorders associated with an abnormal or unwanted immune response, e.g., an autoimmune disorder, e.g., by affecting the functional properties of the APC cells (e.g., by blocking the interaction between OX40 and OX40L). These autoimmune disorders include, but are not limited to, Alopecia areata, lupus, ankylosing spondylitis, Meniere's disease, antiphospholipid syndrome, mixed connective tissue disease, autoimmune Addison's disease, multiple sclerosis, autoimmune hemolytic anemia, myasthenia gravis, autoimmune hepatitis, pemphigus vulgaris, Behcet's disease, pernicious anemia, bullous pemphigoid, polyarthritis nodosa, cardiomyopathy, polychondritis, celiac sprue-dermatitis, polyglandular syndromes, chronic fatigue syndrome (CFIDS), polymyalgia rheumatica, chronic inflammatory demyelinating, polymyositis and dermatomyositis, chronic inflammatory polyneuropathy, primary agammaglobulinemia, Churg-Strauss syndrome, primary biliary cirrhosis, cicatricial pemphigoid, psoriasis, CREST syndrome, Raynaud's phenomenon, cold agglutinin disease, Reiter's syndrome, Crohn's disease, Rheumatic fever, discoid lupus, rheumatoid arthritis, Cryoglobulinemia sarcoidosis, fibromyalgia, scleroderma, Grave's disease, Sjögren's syndrome, Guillain-Barre, stiff-man syndrome, Hashimoto's thyroiditis, Takayasu arteritis, idiopathic pulmonary fibrosis, temporal arteritis/giant cell arteritis, idiopathic thrombocytopenia purpura (ITP), ulcerative colitis, IgA nephropathy, uveitis, diabetes (e.g., Type I), vasculitis, lichen planus, and vitiligo. The anti-OX40 antibodies or antigen-binding fragments

thereof can also be administered to a subject to treat, prevent, or reduce the risk of developing disorders associated with an abnormal or unwanted immune response associated with cell, tissue or organ transplantation, e.g., renal, hepatic, and cardiac transplantation, e.g., graft versus host disease (GVHD), or to prevent allograft rejection. In some embodiments, the subject has dermatological disorders, liver  
5 disease (e.g., cirrhosis), Hidradenitis, experimental autoimmune encephalomyelitis. In some embodiments, the subject has renal disease, lupus, Sjogren's syndrome, ulcerative colitis, psoriasis, Allergic Dermatitis, Atopic Dermatitis, Hidradenitis suppurativa, Immune Thrombocytopenia (ITP), or other inflammatory arthritis. In some embodiments, the subject has multiple sclerosis or myasthenia gravis. In some  
10 embodiments, the subject has Crohn's disease, ulcerative colitis or type 1 diabetes. In some embodiments, the subject has autoimmune thyroid disease, Grave's disease, multiple sclerosis, psoriasis, inflammatory bowel disease (e.g., Crohn's Disease (CD) and ulcerative colitis), rheumatoid arthritis, Sjögren's syndrome, autoimmune nephritis, apical dermatitis or systemic lupus erythematosus.

The methods described herein include methods for the treatment of immune diseases, including e.g., disorders associated with Graft-versus-host disease (GvHD), an immune disorder that occurs after  
15 bone marrow or stem cell transplantation. Generally, the methods include administering a therapeutically effective number of antibodies, the antigen-binding fragments thereof, or the antigen-binding protein constructs (e.g., bispecific antibodies) as described herein, to a subject who needs, or who has been determined to need, such treatment.

As used in this context, to "treat" means to ameliorate at least one symptom of the disorder. In  
20 some embodiments, the disorder is GvHD. GvHD can manifest in various ways, depending on the severity and the organs or tissues. The common manifestations of GvHD include weight loss, dermatitis, diarrhea, and organ dysfunction (e.g., liver, lung and immune system). Severe GvHD can cause significant mortality. Thus, a treatment can result in reduced GvHD score and improved survival rate (e.g., by at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months, or by at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 years).

As used herein, the terms "subject" and "patient" are used interchangeably throughout the  
25 specification and describe an animal, human or non-human, to whom treatment according to the methods of the present invention is provided. Veterinary and non-veterinary applications are contemplated by the present invention. Human patients can be adult humans or juvenile humans (e.g., humans below the age of 18 years old). In addition to humans, patients include but are not limited to mice, rats, hamsters,  
30 guinea pigs, rabbits, ferrets, cats, dogs, and primates. Included are, for example, non-human primates (e.g., monkey, chimpanzee, gorilla, and the like), rodents (e.g., rats, mice, gerbils, hamsters, ferrets, rabbits), lagomorphs, swine (e.g., pig, miniature pig), equine, canine, feline, bovine, and other domestic, farm, and zoo animals.

In one aspect, the disclosure also provides methods for treating a cancer in a subject, methods of  
35 reducing the rate of the increase of volume of a tumor in a subject over time, methods of reducing the risk of developing a metastasis, or methods of reducing the risk of developing an additional metastasis in a

subject. In some embodiments, the treatment can halt, slow, retard, or inhibit progression of a cancer. In some embodiments, the treatment can result in the reduction of in the number, severity, and/or duration of one or more symptoms of the cancer in a subject. In some embodiments, the compositions and methods disclosed herein can be used for treatment of patients at risk for a cancer. Patients with cancer can be identified with various methods known in the art.

As used herein, by an “effective amount” is meant an amount or dosage sufficient to effect beneficial or desired results including halting, slowing, retarding, or inhibiting progression of a disease. An effective amount will vary depending upon, e.g., an age and a body weight of a subject to which the antibody, antigen binding fragment, antibody-drug conjugates, antibody-encoding polynucleotide, vector comprising the polynucleotide, and/or compositions thereof is to be administered, a severity of symptoms and a route of administration, and thus administration can be determined on an individual basis.

Effective amounts and schedules for administering the antibodies, antibody-encoding polynucleotides, antibody-drug conjugates, and/or compositions disclosed herein may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage that must be administered will vary depending on, for example, the mammal that will receive the antibodies, antibody-encoding polynucleotides, antibody-drug conjugates, and/or compositions disclosed herein, the route of administration, the particular type of antibodies, antibody-encoding polynucleotides, antigen binding fragments, antibody-drug conjugates, and/or compositions disclosed herein used and other drugs being administered to the mammal.

A typical daily dosage of an effective amount of an antibody, the antigen-binding fragment thereof, or the antigen-binding protein construct (e.g., a bispecific antibody) is 0.01 mg/kg to 100 mg/kg. In some embodiments, the dosage can be less than 100 mg/kg, 30 mg/kg, 20 mg/kg, 10 mg/kg, 9 mg/kg, 8 mg/kg, 7 mg/kg, 6 mg/kg, 5 mg/kg, 4 mg/kg, 3 mg/kg, 2 mg/kg, 1 mg/kg, 0.5 mg/kg, or 0.1 mg/kg. In some embodiments, the dosage can be greater than 10 mg/kg, 9 mg/kg, 8 mg/kg, 7 mg/kg, 6 mg/kg, 5 mg/kg, 4 mg/kg, 3 mg/kg, 2 mg/kg, 1 mg/kg, 0.5 mg/kg, 0.1 mg/kg, 0.05 mg/kg, or 0.01 mg/kg. In some embodiments, the dosage is about or at least 10 mg/kg, 9 mg/kg, 8 mg/kg, 7 mg/kg, 6 mg/kg, 5 mg/kg, 4 mg/kg, 3 mg/kg, 2 mg/kg, 1 mg/kg, 0.9 mg/kg, 0.8 mg/kg, 0.7 mg/kg, 0.6 mg/kg, 0.5 mg/kg, 0.4 mg/kg, 0.3 mg/kg, 0.2 mg/kg, or 0.1 mg/kg.

### **Pharmaceutical Compositions and Routes of Administration**

Also provided herein are pharmaceutical compositions that contain at least one (e.g., one, two, three, or four) of the antigen-binding protein constructs, antibodies (e.g., bispecific antibodies), antigen-binding fragments, or antibody-drug conjugates described herein. Two or more (e.g., two, three, or four) of any of the antigen-binding protein constructs, antibodies, antigen-binding fragments, or antibody-drug conjugates described herein can be present in a pharmaceutical composition in any combination. The pharmaceutical compositions may be formulated in any manner known in the art.

Pharmaceutical compositions are formulated to be compatible with their intended route of administration (e.g., intravenous, intraarterial, intramuscular, intradermal, subcutaneous, or intraperitoneal). The compositions can include a sterile diluent (e.g., sterile water or saline), a fixed oil, polyethylene glycol, glycerine, propylene glycol or other synthetic solvents, antibacterial or antifungal agents, such as benzyl alcohol or methyl parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like, antioxidants, such as ascorbic acid or sodium bisulfite, chelating agents, such as ethylenediaminetetraacetic acid, buffers, such as acetates, citrates, or phosphates, and isotonic agents, such as sugars (e.g., dextrose), polyalcohols (e.g., mannitol or sorbitol), or salts (e.g., sodium chloride), or any combination thereof. Liposomal suspensions can also be used as pharmaceutically acceptable carriers (see, e.g., U.S. Patent No. 4,522,811). Preparations of the compositions can be formulated and enclosed in ampules, disposable syringes, or multiple dose vials. Where required (as in, for example, injectable formulations), proper fluidity can be maintained by, for example, the use of a coating, such as lecithin, or a surfactant. Absorption of the antibody or antigen-binding fragment thereof can be prolonged by including an agent that delays absorption (e.g., aluminum monostearate and gelatin). Alternatively, controlled release can be achieved by implants and microencapsulated delivery systems, which can include biodegradable, biocompatible polymers (e.g., ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid; Alza Corporation and Nova Pharmaceutical, Inc.).

Compositions containing one or more of any of the antigen-binding protein constructs, antibodies, antigen-binding fragments, antibody-drug conjugates described herein can be formulated for parenteral (e.g., intravenous, intraarterial, intramuscular, intradermal, subcutaneous, or intraperitoneal) administration in dosage unit form (i.e., physically discrete units containing a predetermined quantity of active compound for ease of administration and uniformity of dosage).

Toxicity and therapeutic efficacy of compositions can be determined by standard pharmaceutical procedures in cell cultures or experimental animals (e.g., monkeys). One can determine the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population); the therapeutic index being the ratio of LD<sub>50</sub>:ED<sub>50</sub>. Agents that exhibit high therapeutic indices are preferred. Where an agent exhibits an undesirable side effect, care should be taken to minimize potential damage (i.e., reduce unwanted side effects). Toxicity and therapeutic efficacy can be determined by other standard pharmaceutical procedures.

Data obtained from cell culture assays and animal studies can be used in formulating an appropriate dosage of any given agent for use in a subject (e.g., a human). A therapeutically effective amount of the one or more (e.g., one, two, three, or four) antigen-binding protein constructs, antibodies or antigen-binding fragments thereof (e.g., any of the antibodies or antibody fragments described herein) will be an amount that treats the disease in a subject (e.g., a human subject identified as having cancer), or a subject identified as being at risk of developing the disease (e.g., a subject who has previously developed cancer but now has been cured), decreases the severity, frequency, and/or duration of one or

more symptoms of a disease in a subject (e.g., a human). The effectiveness and dosing of any of the antigen-binding protein constructs, antibodies or antigen-binding fragments described herein can be determined by a health care professional or veterinary professional using methods known in the art, as well as by the observation of one or more symptoms of disease in a subject (e.g., a human). Certain factors may influence the dosage and timing required to effectively treat a subject (e.g., the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and the presence of other diseases).

Exemplary doses include milligram or microgram amounts of any of the antigen-binding protein constructs, antibodies or antigen-binding fragments, or antibody-drug conjugates described herein per kilogram of the subject's weight (e.g., about 1 µg/kg to about 500 mg/kg; about 100 µg/kg to about 500 mg/kg; about 100 µg/kg to about 50 mg/kg; about 10 µg/kg to about 5 mg/kg; about 10 µg/kg to about 0.5 mg/kg; or about 0.1 mg/kg to about 0.5 mg/kg). While these doses cover a broad range, one of ordinary skill in the art will understand that therapeutic agents, including antigen-binding protein constructs, antibodies and antigen-binding fragments thereof, vary in their potency, and effective amounts can be determined by methods known in the art. Typically, relatively low doses are administered at first, and the attending health care professional or veterinary professional (in the case of therapeutic application) or a researcher (when still working at the development stage) can subsequently and gradually increase the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, and the half-life of the antibody or antibody fragment *in vivo*.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration. The disclosure also provides methods of manufacturing the antibodies or antigen binding fragments thereof, or antibody-drug conjugates for various uses as described herein.

## EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

### Example 1. Preparation and analysis of anti-OX40 antibodies

#### *Generation of anti-OX40 antibodies*

To generate antibodies against human OX40, mice (e.g., complete human heavy chain variable domain combined with a common light chain substitution *in situ*) were immunized with human OX40 Protein (ACROBiosystems Inc., Cat#: OX40-H5224, containing positions 29-216) or plasmids encoding

human OX40 protein. Before immunization, retro-orbital blood was collected as a negative control. The antibody immune response was monitored by an antigen-specific immunoassay.

When a desired immune response was achieved, antigen-specific immune cells were isolated from the immunized mice to further obtain anti-OX40 antibodies or to obtain the light chain and heavy chain variable region sequences of the anti-OX40 antibodies. For example, single cell technology (for example, using Beacon<sup>®</sup> Optofluidic System, Berkeley Lights Inc.) was used to screen and find plasma cells that secrete antigen-specific monoclonal antibodies, and reverse transcription and PCR sequencing were used to obtain antibody variable region sequences. The obtained variable region sequences were cloned into a vector containing a sequence encoding the human IgG1 constant region for antibody expression. The binding affinity of the expressed antibody to OX40 was verified using FACS.

Exemplary antibodies obtained included: 1B8, 3A11, 2C11, and 4C8. **FIG. 10** shows the CDR sequences according to Kabat definition, **FIG. 11** shows the CDR sequences according to Chothia definition. **FIG. 12** shows the heavy chain variable regions of 1B8, 3A11, 2C11, and 4C8 and the light chain common variable region.

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#### ***Binding affinity of anti-OX40 antibodies***

The affinity of the anti-OX40 antibodies to his-tagged human OX40 protein (hOX40-his, ACROBiosystems Inc., Cat#: OX0-H5224) was verified by surface plasmon resonance (SPR) using Biacore<sup>™</sup> (Biacore, Inc., Piscataway N.J.) 8K biosensor equipped with pre-immobilized Protein A sensor chips.

Purified anti-OX40 antibodies were diluted to 2 µg/mL with 10×HBS-EP+ buffer (pH=7.4) and then injected into the Biacore<sup>™</sup> 8K biosensor at 10 µL/min for 50 seconds to achieve a desired protein density (e.g., about 100 response units (RU)). The His-tagged human OX40 protein at a concentration of 200nM, 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.125 nM or 1.5625 nM was then injected at 30 µL/min for 180 seconds. Dissociation was monitored for 600 seconds. The chip was regenerated after the last injection of the titration with glycine (pH 1.5, 30 µL/min for 30 seconds).

Kinetic association rates (kon) and dissociation rates (koff) were obtained simultaneously by fitting the data globally to a 1:1 Langmuir binding model (Karlsson, R. Roos, H. Fagerstam, L. Petersson, B., 1994. *Methods Enzymology* 6. 99-110) using Biacore<sup>™</sup> 8K Evaluation Software 3.0. Affinities were deduced from the quotient of the kinetic rate constants ( $KD=koff/kon$ ).

As a person of ordinary skill in the art would understand, the same method with appropriate adjustments for parameters (e.g., antibody concentration) was performed for each tested antibody. The results for the tested antibodies are summarized in the **Table 1**.

GBR830 is a humanized IgG1 monoclonal antibody targeting OX40, which is in phase II clinical development at Ichnos Sciences for the treatment of atopic dermatitis. The VH and VL sequences of

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GBR830 are shown in SEQ ID NO: 36 and SEQ ID NO: 37, respectively. The VH and VL sequences of GBR830 were linked to the IgG1 constant region, resulting in the GBR830 analog.

Table 1

| Antibody      | Protein   | kon (1/Ms) | koff (1/s) | KD (M)   |
|---------------|-----------|------------|------------|----------|
| 1B8           | hOX40-his | 2.32E+05   | 7.47E-03   | 3.22E-08 |
| 3A11          | hOX40-his | 3.97E+05   | 6.10E-03   | 1.54E-08 |
| 2C11          | hOX40-his | 3.26E+04   | 1.05E-03   | 3.24E-08 |
| 4C8           | hOX40-his | 1.68E+05   | 2.47E-02   | 1.47E-07 |
| GBR830 analog | hOX40-his | 7.43E+04   | 9.17E-03   | 1.23E-07 |

- 5 The results demonstrated that the anti-OX40 antibodies 1B8, 3A11, 2C11, and 4C8 exhibited strong binding affinities to human OX40.

#### *Blockade assay by flow cytometry*

- 10 Blockade of the physical interaction between human OX40 ligand (hOX40L, Mouse IgG2a Fc Tag, low endotoxin, ACROBiosystems Inc., Cat#: OXL-H5250) and human OX40 by anti-OX40 antibodies were tested by flow cytometry using Jurkat-Luc-hOX40 reporter cells (transfected Jurkat-Luc cells expressing human OX40 protein).

- 15 Jurkat-Luc-hOX40 cells were seeded into a 96-well plate (cell density  $2 \times 10^5$  cells/well) and incubated. Serially diluted anti-OX40 antibodies were incubated with the cells at 4 °C for 15 minutes (the working concentrations of the tested antibodies were 10 µg/mL, 3.333 µg/mL, 1.111 µg/mL, 0.37037 µg/mL, 0.0123 µg/mL, 0.041152 µg/mL, 0.013717 µg/mL and 0.00457 µg/mL), and then human OX40 ligand (0.6 µg/mL) was added to each well. In the negative control group (NC), human OX40 ligand was replaced by PBS. ISO is an antibody with the same IgG1 subtype but targeting an unrelated target. The above 96-well plate was incubated at 4°C for 20 minutes. Then, after one wash with PBS, the cells were
- 20 incubated with the secondary antibody R-Phycoerythrin AffiniPure F(ab')<sub>2</sub> Fragment Goat Anti-Human IgG, Fcγ fragment specific (BioLegend, Cat#:109-116-098) at 1:1000 dilution and AF647 Anti-Mouse IgG Fc (BioLegend, Cat#:115-606-071) at 1:5000 dilution at 4°C for 15 minutes before flow cytometry analysis.

- 25 The mean fluorescence intensity (MFI) was determined. A fitting curve was obtained using antibody concentration (nM) as the X-axis and MFI as the Y-axis, and the maximum blockage was calculated, with the NC group as 100% and the ISO as 0. The results are shown in FIG. 1 and Table 2.

Table 2

| Antibody | Max blockage (%) |
|----------|------------------|
| 1B8      | 87.8             |
| 3A11     | 98.6             |
| 2C11     | 47.8             |
| 4C8      | 45.5             |

The results showed that 1B8 and 3A11 blocked the interaction between human OX40 and the OX40 ligand, whereas 2C11 and 4C8 only partially blocked the interaction between human OX40 and OX40 ligand even at a high concentration (e.g. > 50 nM).

5 ***Blockade assay by OX40 reporter cells***

The purpose of the experiment was to investigate whether anti-OX40 antibodies could attenuate the activation of OX40 reporter cells when exposed to human OX40 ligand (Human OX40 Ligand/TNFSF4 Protein, His Tag (active trimer) (MALS verified), ACROBiosystems Inc., Cat#: OXL-H52Q8).

10 Effector cells (Jurkat-Luc-hOX40 cells) were seeded into a 96-well plate (cell density  $5 \times 10^4$  cells/well) and incubated with serially diluted tested anti-OX40 antibodies (maximum concentration: 100  $\mu\text{g/mL}$ , 3-fold dilutions, 8 gradients) at  $37^\circ\text{C}$  for 3h, and then 25  $\mu\text{L}$  of the human OX40 ligand with a final concentration of 0.6  $\mu\text{g/mL}$  was added to each well and incubated at  $37^\circ\text{C}$  for 3h. In the negative control group (NC), human OX40 ligand was replaced by PBS. ISO is an antibody with the same IgG1  
15 subtype but targeting an unrelated target. Then, 75  $\mu\text{L}$  Bio-lite™ Luciferase Assay Reagent (Vazyme Biotech Co., Ltd., Cat#: DD1201-02-AB) was added to each well. The plate was incubated at room temperature for 5 minutes, and then placed in a luminescence detector to detect the fluorescence signal. A fitting curve was obtained using antibody concentration (nM) as the X-axis and RLU as the Y-axis. The results are shown in **FIG. 2** indicating that 1B8 and 3A11 blocked the interaction between human OX40  
20 and OX40 ligand, whereas 4C8 exhibit weak blockade, and 2C11 can't block the interaction between human OX40 and OX40 ligand.

***Jurkat-Luc-hOX40 reporter cells activation assays***

The objective of this experiment was to assess the agonistic properties of anti-OX40 antibodies  
25 on OX40 reporter cells, both in the presence and absence of cross-linking facilitated by human Fc $\gamma$ R expressing cell lines cells CHO-K1-Fc $\gamma$ RIIB.

For the condition with antibody cross-linking, basal cells CHO-K1-Fc $\gamma$ RIIB cells (Promega, Cat#: JA2251) were seeded in a 96-well plate (cell density  $4 \times 10^4$  cells/well) and incubated overnight. Effector cells Jurkat-Luc-hOX40 cells were seeded in the same 96-well plate (cell density  $5 \times 10^4$  cells/well), and  
30 then 25  $\mu\text{L}$  serially diluted anti-OX40 antibodies (the highest concentration: 30  $\mu\text{g/mL}$ , 3-fold dilutions, 8 gradients) was added to each well. ISO is an antibody with the same IgG1 subtype but targeting an unrelated target. After the incubation, 75  $\mu\text{L}$  Bio-lite™ Luciferase Assay Reagent (Vazyme Biotech Co., Ltd., Cat#: DD1201-02-AB) was added to each well. The plate was incubated at room temperature for 5 minutes, and then placed in a luminescence detector to detect the fluorescence signal.

For the condition without antibody cross-linking, effector cells Jurkat-Luc-hOX40 cells were seeded into a blank 96 well plate and subjected to the same procedure as conducted in the cross-linking experiment.

KHK4083 is a fully human afucosylated monoclonal IgG1 antibody targeting OX40, which is in phase III clinical evaluation at Kyowa Kirin and Amgen for the treatment of moderate to severe chronic atopic dermatitis. The VH and VL sequences are shown in SEQ ID NO: 38 and SEQ ID NO: 39, respectively. The VH and VL sequences of KHK4083 were linked to the IgG1 constant region, without fucose removal, resulting in the KHK4083 analog.

The findings presented in FIG.3A and FIG. 3B indicate that all anti-OX40 antibodies exhibited agonistic properties in the presence of Fc-FcγRIIB cross-linking (FIG.3A), but not in the absence of cross-linking without Fc-FcγRIIB cells (FIG. 3B).

Epitope analysis of anti-OX40 antibodies

Relative positions of target protein epitope between a pair of purified anti-OX40 antibodies were analyzed by Biolayer Interferometry (BLI) using ForteBio Octet system.

1× HBS-EP+ buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 3 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% P20, pH7.4) diluted from HBS-EP+ buffer (10×) was used as the running buffer throughout the experiment. About 5 μg/mL hOX40-his was captured at 1000 rpm/min for 200 seconds, and 300 nM of the tested antibody (Analyte 1) was injected at 1000 rpm/min to bind the ligand. Another comparison antibody (Analyte 2) was injected under the same conditions to determine whether the binding of different antibodies interfered with each other. The binding time was 180s for each antibody.

The binding value of each antibody was obtained using Data Analysis HT 12.0. To quantify the interference of one antibody binding to another, a binding ratio was calculated to compare each pair of antibodies. The binding ratio is defined as the binding value of the second antibody (analyte 2), divided by the binding value of the first antibody (analyte 1). The threshold to determine the block or non-block is 0.5. The results were summarized in the table below, which showed that four antibodies 1B8, 3A11, 2C11, and 4C8 recognize different epitopes.

Table 3

| Analyte 2 \ Analyte 1 | 4C8  | 3A11 | 2C11 | 1B8  |
|-----------------------|------|------|------|------|
| 4C8                   | 0.10 | 0.82 | 0.97 | 0.93 |
| 3A11                  | 0.79 | 0.00 | 0.93 | 0.93 |
| 2C11                  | 1.00 | 0.91 | 0.25 | 0.95 |
| 1B8                   | 0.92 | 0.91 | 0.85 | 0.03 |

Example 2. Preparation and Analysis of Anti-OX40 Bispecific Antibodies

Provided herein are bispecific antigen-binding molecules targeting two different epitopes of OX40. These antigen-binding molecules are referred to as anti-OX40 bispecific antibodies below.

*Preparation of anti-OX40 bispecific antibodies*

5           Based on the blockade assay by flow cytometry, anti-OX40 antibodies 1B8 and 3A11 are determined to be full OX40L binding blocking agents. They fully block the binding of OX40 ligand to OX40 (87.8% and 98.6% blockade respectively), whereas 2C11 and 4C8 are partial OX40L binding blocking agents that only partially interfere with the binding of OX40L to OX40 (47.8% and 45.5% blockade respectively).

10           In this example, a full OX40L binding blocking agent has a blocking effect of greater than 75% as determined by flow cytometry. Conversely, a partial OX40L binding blocking agent has a blockade of less than 75%.

          Anti-OX40 antibodies (1B8, 3A11, 2C11, and 4C8) can be paired to form bispecific antibodies. Vectors encoding the light chain and heavy chain of the antibodies were constructed. For the purposes of  
15 the subsequent studies, the bispecific antibodies were formed by pairing a full OX40L binding blocking antigen binding domain derived from e.g., antibodies 1B8 or 3A11, with a partial OX40L binding blocking antigen binding domain derived from e.g., antibodies 2C11 or 4C8.

          CHO-K1 cells or modified CHO-K1 cells (e.g. CHO-K1 cells with FUT8 and SLC35C1 deletions) were co-transfected with three vectors, including a first vector encoding the heavy chain of an anti-OX40  
20 antibody, a second vector encoding the heavy chain of another anti-OX40 antibody, and a third vector encoding the common light chain. After 14 days of culture, the cell supernatant was collected and purified by Protein A affinity chromatography.

          Various methods can be used to reduce the chance of wrong pairing between the two heavy chains. For example, knobs-into-holes mutations were introduced in the Fc regions. For example, in 3A11-2C11,  
25 the heavy chain constant region of 3A11 includes knob mutations, and the heavy chain constant region of 2C11 includes hole mutations. In 3A11-4C8, the heavy chain constant region of 3A11 includes knob mutations, and the heavy chain constant region of 4C8 includes hole mutations. In 1B8-2C11, the heavy chain constant region of 1B8 includes knob mutations, and the heavy chain constant region of 2C11 includes hole mutations. In 1B8-4C8, the heavy chain constant region of 1B8 includes knob mutations,  
30 and the heavy chain constant region of 4C8 includes hole mutations. The sequences of the light chain constant region, the heavy chain constant region with knob mutations, and the heavy chain constant region with hole mutations are shown in SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35, respectively. Exemplary bispecific antibodies obtained include: 3A11-2C11, 3A11-4C8, 1B8-2C11, 1B8-4C8, 3A11-1B8, 3A11-2C11-AF, 3A11-4C8-AF, 1B8-2C11-AF, 1B8-4C8-AF. Among them,  
35 3A11-2C11-AF, 3A11-4C8-AF, 1B8-2C11-AF, and 1B8-4C8-AF are afucosylated antibodies.

The constant region can also include some mutations. With respect to the name of the antibodies, the mutations are added to the name. For example, when YTE mutations (EU numbering: M252Y, S254T, T256E mutations) are introduced into the Fc region of 3A11-2C11, the resulting antibody is named 3A11-2C11-YTE. Exemplary antibodies obtained include 3A11-2C11-AF-YTE, 3A11-4C8-AF-YTE, 1B8-2C11-AF-YTE, and 1B8-4C8-AF-YTE. The afucosylated antibodies from the parental monoclonal antibody can also be obtained, for example, by expression through modified CHO-K1 cells (e.g., CHO-K1 cells with FUT8 and SLC35C1 deletions), and the resulting defucosylated monoclonal antibodies including 3A11-AF, 2C11-AF, 1B8-AF, and 4C8-AF. Antibodies with YTE mutations are named as: 3A11-AF-YTE, 2C11-AF-YTE, 1B8-AF-YTE, and 4C8-AF-YTE, respectively.

#### ***Binding affinity of anti-OX40 bispecific antibodies***

The binding affinity of anti-OX40 bispecific antibodies to human OX40 were verified by SPR using Biacore™ (Biacore, Inc., Piscataway N.J.) 8K biosensor equipped with pre-immobilized Protein A sensor chips.

Specifically, purified anti-OX40 bispecific antibodies were diluted to 2 µg/mL and then injected into the Biacore™ 8K biosensor at 10 µL/min for 50 seconds to achieve a desired protein density (e.g., about 100 RU). The His-tagged OX40 protein at a concentration of 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.125 nM or 1.5625 nM was then injected at 30 µL/min for 180 seconds. Dissociation was monitored for 600 seconds. The chip was regenerated after the last injection of the titration with glycine (pH 1.5, 30 µL/min for 30 seconds).

Kinetic association rates ( $k_{on}$ ) and dissociation rates ( $k_{off}$ ) were obtained simultaneously by fitting the data globally to a 1:1 Langmuir binding model using Biacore™ 8K Evaluation Software 3.0. Affinities were deduced from the quotient of the kinetic rate constants ( $KD=k_{off}/k_{on}$ ). The results for the tested antibodies are summarized in the table below.

**Table 4**

| Antibody       | Protein   | $k_{on}$ (1/Ms) | $k_{off}$ (1/s) | KD (M)   |
|----------------|-----------|-----------------|-----------------|----------|
| GBR830 analog  | hOX40-his | 7.43E+04        | 9.17E-03        | 1.23E-07 |
| KHK4083 analog | hOX40-his | 2.16E+05        | 6.81E-04        | 3.15E-09 |
| 3A11-2C11      | hOX40-his | 3.93E+05        | 3.85E-04        | 9.78E-10 |
| 3A11-4C8       | hOX40-his | 5.79E+05        | 3.77E-04        | 6.51E-10 |
| 1B8-2C11       | hOX40-his | 2.99E+05        | 2.55E-04        | 8.55E-10 |
| 1B8-4C8        | hOX40-his | 2.11E+05        | 7.86E-03        | 3.72E-08 |

The results showed that 3A11-2C11, 3A11-4C8, 1B8-2C11, and 1B8-4C8 can all bind to human OX40 with high affinity.

#### ***Binding activities of anti-OX40 bispecific antibodies***

This experiment was performed to test the binding activities of anti-OX40 antibodies to human OX40 or monkey OX40 by flow cytometry analysis and ELISA analysis.

Specifically, in the flow cytometry analysis experiment, Jurkat-Luc-hOX40 cells were transferred to a 96-well plate at a density of  $1 \times 10^5$  cells/well. Serially diluted anti-OX40 antibodies (the highest concentration: 30  $\mu\text{g/mL}$ , diluted in a 3-fold series for 12 gradients) was added to the 96-well plate, and incubated at 4°C for 30 minutes. Then, the cells were incubated with the secondary antibody Alexa Fluor® 647-conjugated AffiniPure F(ab')<sub>2</sub> Fragment Goat Anti-Human IgG, Fcγ Fragment Specific (Jackson Immuno Research Laboratories, Inc., Cat#: 109-606-170) at 4°C in the dark for 15 minutes before flow cytometry analysis. Human IgG1 protein was used as the ISO. The maximum binding was calculated, with the KHK4083 analog group as 100% and the ISO as 0. The results shown in **Table 5** demonstrated that 3A11-4C8 and 3A11-2C11 can bind to human OX40 at a higher rate than 3A11, GBR830 analog and KHK4083 analog.

**Table 5**

| Antibody       | Flow cytometry            |                          |
|----------------|---------------------------|--------------------------|
|                | EC50 ( $\mu\text{g/mL}$ ) | Relative Max binding (%) |
| 3A11-4C8       | 0.0947                    | 151.5                    |
| 3A11-2C11      | 0.1340                    | 151.3                    |
| 3A11           | 0.0473                    | 91.7                     |
| GBR830 analog  | 0.1335                    | 85.8                     |
| KHK4083 analog | 0.1253                    | 100                      |

In the ELISA analysis experiment, human OX40 protein (hOX40-his) or his-tagged monkey (*Rhesus macaque*) OX40 protein (fasOX40-his, ACROBiosystems Inc., Cat#: OX0-H5220) was added to a 96-well plate (ELISA plate) at 100  $\mu\text{L}$ /well, and then incubated overnight at 4 °C. After the incubation, the plate was washed with PBS-T buffer (PBS supplemented with Tween™ 20) 3 times. Antibody-unbound areas were blocked with 1% BSA (bovine serum albumin) for 2 hours at 37°C. Afterwards, the plate was washed with PBS-T buffer 3 times. Serially diluted tested anti-OX40 antibodies (maximum concentration: 1  $\mu\text{g/mL}$ , 4-fold dilutions, 8 gradients) were then added to each well and incubated at 37°C for 1h. After washing, Goat x-Human IgG-Fc Fragment HRP Conjugated (Bethyl Laboratories, Cat#: A80-104P) was added at 100  $\mu\text{L}$ /well to each well of the plate, and incubated at 37°C for 1 hour. After washing the plate, tetramethylbenzidine (TMB) solution was added at 100  $\mu\text{L}$ /well to the 96-well plate as the substrate. After incubating at 37°C in the dark, 50  $\mu\text{L}$  stop solution was added to each well. Luminescent signals of the plate were measured at 450 nm and 570 nm to calculate the OD value. A fitting curve was obtained using antibody concentration (ng/mL) as the X-axis and OD value as the Y-axis. Human IgG1 protein was used as the ISO. The results are shown in **FIG. 4A** and **FIG. 4B** indicating that 3A11, 3A11-4C8, and 3A11-2C11 can all bind to human OX40 and monkey OX40.

30

*Blockade assays*

Similar experimental procedures as previously described were carried out to test whether 3A11-4C8, 3A11-2C11, 1B8-2C11, GBR830 analog, or KHK4083 analog can block the interaction between human OX40 ligand and human OX40, using flow cytometry and OX40 reporter cells. The relative maximum blockage was calculated, with the the KHK4083 analog group as 100% and ISO as 0. The results are shown in **FIG. 5A**, **FIG. 5B**, and the table below, which showed that 3A11-4C8, 3A11-2C11, and 1B8-2C11 effectively completely blocked the intrinsic interaction between human OX40 and OX40 ligand.

**Table 6**

| Antibody       | Relative Max blockage (%) |                      |
|----------------|---------------------------|----------------------|
|                | Flow cytometry            | Reporter cells assay |
| 3A11-4C8       | 99                        | 97.7                 |
| 3A11-2C11      | 100                       | 101.9                |
| 1B8-2C11       | 99                        | 101.4                |
| GBR830 analog  | 72                        | 93.7                 |
| KHK4083 analog | 100                       | 100                  |

*Jurkat-Luc-hOX40 reporter cells activation assay*

For the condition with antibody cross-linking, CHO-K1-FcγRIIB cells (Promega, Cat#: JA2251) were seeded in a 96-well plate (cell density  $4 \times 10^4$  cells/well) and incubated overnight. Jurkat-Luc-hOX40 cells were seeded in the 96-well plate (cell density  $5 \times 10^4$  cells/well), and then 25 μL serially diluted anti-OX40 bispecific antibody (the highest concentration: 30 μg/mL, 3-fold dilutions, 8 gradients) was added to each well. ISO is an antibody with the same IgG1 subtype but targeting an unrelated target. After the incubation, the plate was taken out, 75 μL Bio-lite™ Luciferase Assay Reagent (Vazyme Biotech Co., Ltd., Cat#: DD1201-02-AB) was added to each well. The plate was incubated at room temperature for 5 minutes, and then placed in a luminescence detector to detect the fluorescence signal.

For the condition without antibody cross-linking, Jurkat-Luc-hOX40 cells were seeded into a blank 96 well plate and subjected to the same procedure as conducted in the cross-linking experiment.

The results are shown in **FIG. 6A** and **FIG. 6B**, which showed that in the co-culture of Jurkat-Luc-hOX40 reporter cells and CHO-K1-FcγRIIB, the fluorescence signals detected in GBR830 analog and KHK4083 analog groups were enhanced as the antibody concentration increases. These results suggested GBR830 analog and KHK4083 analog are strong agonist in the presence of Fc-FcγRIIB cross-linking. However, the bispecific antibodies, 3A11-4C8, 3A11-2C11, and 1B8-2C11 demonstrated mild agonistic effects. The agonist activities were not observed in the absence of Fc-FcγRIIB cross-linking.

**Example 3. Inhibition of T cell proliferation**

2 µg/mL anti-hCD3 antibody (clone OKT3, ACROBiosystems, Cat#: CDE-M120a) and 2 µg/mL human OX40L Protein (Human OX40 Ligand/TNFSF4 Protein, ACROBiosystems, Cat#: OXL-H52Q8) were added in a 96-well plate (50 µL/well) and incubated at 4°C overnight. After washing, 100µL human CD3+ T cells labeled with CFSE were seeded in the 96-well plate (cell density 1 × 10<sup>5</sup> cells/well), and then 100 µL of the tested anti-OX40 antibody was added to each well with the working concentrations of 30 µg/mL. In the NC1 group, plate-coated hCD3 and OX40L were replaced with PBS. In the NC2 group, only anti-hCD3 was pre-coated into wells. In the PC group, anti-hCD3 antibody and human OX40L Protein were added in the well without anti-OX40 antibody. After the incubation at 37°C in 5% CO<sub>2</sub> for 96 hours, the cells were collected for flow cytometry to detect the proliferation of hCD4+ T cells and hCD8a+ T cells. The proliferation rate of hCD4+ T cells and hCD8a+ T cells was calculated. The results are shown in the table below.

**Table 7**

| Antibody       | Proliferation (%) |                |
|----------------|-------------------|----------------|
|                | hCD4+ T cells     | hCD8a+ T cells |
| GBR830 analog  | 15.0              | 21.4           |
| KHK4083 analog | 7.6               | 21.5           |
| 3A11-4C8       | 7.1               | 21.4           |
| 3A11-2C11      | 6.6               | 19.5           |
| 1B8-2C11       | 7.0               | 20.7           |
| NC1            | 2.0               | 4.8            |
| NC2            | 16.8              | 44.2           |
| PC             | 64.4              | 81.4           |

The results showed that 3A11-4C8, 3A11-2C11, and 1B8-2C11 inhibited T cell proliferation more effectively than the controls GBR830 analog and KHK4083 analog.

In another experiment, the inhibition activity of anti-OX40 bispecific antibody was tested by SEB assay. Specifically, human PBMCs (1×10<sup>5</sup> cells/well) were distributed in a 96-well round-bottom plate and labelled with Cell Trace™ Violet (CTV, Invitrogen, Cat#: C34557). Staphylococcal enterotoxin B (SEB), as a T cell receptor stimulus, at a final concentration of 0.1 µg/mL and series dilutions of anti-OX40 antibodies (the highest concentration: 133 nM, 5-fold dilutions, 8 gradients) were added to the wells. Plates were incubated for 72 hours at 37°C in a CO<sub>2</sub> incubator. T cell proliferation was assessed by flow cytometry by gating on CD4+ population and enumeration of CTV low cells. Human IgG1 was used as an ISO control.

As shown in **FIG. 25**, 3A11-2C11 functionally blocked SEB-stimulated CD4+ T cell proliferation. In addition, anti-OX40 monoclonal antibody 3A11 exhibited comparable inhibitory activity on T cell proliferation as the positive control KHK4083 analog.

In another experiment,  $1 \times 10^5$  CellTrace™ Violet stained PBMCs were added to wells of 96-well plate, followed by serial diluted anti-OX40 antibodies. SEB and heat-inactivated human AB serum were then added with final concentrations of 0.1 ug/mL and 5% to make up 200 uL/well of total volume. After incubation at 37°C for 72 hours, cells were pelleted and washed before being stained for live/dead cells.

5 After live/dead staining, cells were blocked with Fc blocking agent and stained for CD4 before being fixed with PFA (paraformaldehyde) and run on a FASC machine. Duplicate wells were run for each dose point. ISO and no SEB controls were run for each donor. Percent of proliferation of CD4+ cells was obtained by FACS analysis and normalized to respective ISO values from the same donor, which were defined as 100% activity.

10 The results are shown in **FIG. 26**, in which 3A11-2C11 inhibited SEB-mediated T cell proliferation significantly more potently than KHK4083 analog in both afucosylated format and IgG1 format.

#### Example 4. Immunosuppressive potency in GvHD Mouse Model

15 The immunosuppressive potency of the antibodies was tested in a xenogeneic GvHD model based on the reconstitution of B-NDG mice (Biocytogen Pharmaceuticals (Beijing) Co., Ltd., Cat#: B-CM-002) with human PBMCs.

The B-NDG mice were randomly placed into different groups based on body weight. The treatment groups (G2-G15) were randomly selected for treatment with antibodies by intravenous (i.v.) on the same day. The negative control group mice (G1) were injected with an equal volume of 0.9% sodium chloride injection (NaCl). Two days after grouping, the mice in G1-G15 were irradiated and about  $5 \times 10^6$  human peripheral blood mononuclear cells (PBMC) were intravenously injected into the mice. The intravenous administration was performed once a week for 7 continuous weeks. Details are shown in the table below.

25

**Table 8**

| Group | No. of mice | Antibody       | Dosage  | Route | Total No. of administration |
|-------|-------------|----------------|---------|-------|-----------------------------|
| G1    | 6           | NaCl           | -       | i.v.  | 7                           |
| G2    | 6           | 3A11-4C8       | 10mg/kg | i.v.  | 7                           |
| G3    | 6           | 3A11-4C8       | 1mg/kg  | i.v.  | 7                           |
| G4    | 6           | 3A11-2C11      | 10mg/kg | i.v.  | 7                           |
| G5    | 6           | 3A11-2C11      | 1mg/kg  | i.v.  | 7                           |
| G6    | 6           | 1B8-4C8        | 10mg/kg | i.v.  | 7                           |
| G7    | 6           | 1B8-4C8        | 1mg/kg  | i.v.  | 7                           |
| G8    | 6           | 1B8-2C11       | 10mg/kg | i.v.  | 7                           |
| G9    | 6           | 1B8-2C11       | 1mg/kg  | i.v.  | 7                           |
| G10   | 6           | 3A11           | 10mg/kg | i.v.  | 7                           |
| G11   | 6           | 2C11           | 10mg/kg | i.v.  | 7                           |
| G12   | 6           | 1B8            | 10mg/kg | i.v.  | 7                           |
| G13   | 6           | 4C8            | 10mg/kg | i.v.  | 7                           |
| G14   | 6           | KHK4083 analog | 10mg/kg | i.v.  | 7                           |
| G15   | 6           | GBR830 analog  | 10mg/kg | i.v.  | 7                           |

The mice were weighted and scored three times per week for GvHD symptoms including weight loss, posture, activity, fur texture and skin integrity on a scale from 0 to 3 (Details are shown in the table below). Animals were ethically sacrificed when any of the following conditions were met: GvHD score equals or greater than 7 or weight loss over 30% and without improvement within one week. GvHD score was generated by adding these five parameters together, and the results are shown in **FIG. 7A-7C**. **FIG. 8** showed the survival mice rate at 49 days post administration.

Table 9

| Criteria       | Score 0 | Score 1                             | Score 2                                          | Score 3 (only applicable for humane endpoint) |
|----------------|---------|-------------------------------------|--------------------------------------------------|-----------------------------------------------|
| Weight loss    | <10%    | 10%~20%                             | >20%                                             | Continuous >30% for 1 week                    |
| Posture        | Normal  | Hunching noted only at rest         | Severe hunching impairs movement                 | /                                             |
| Activity       | Normal  | Mild to moderate decrease           | Severer decrease; no response to any stimulation | /                                             |
| Fur texture    | Normal  | Mild to moderate ruffling/hair loss | Severe ruffling and hair loss                    | /                                             |
| Skin integrity | Normal  | Scaling of paws and/or tail         | Obvious areas of denuded skin                    | /                                             |

The results showed that the mice treated with 3A11-4C8, 3A11-2C11, 1B8-4C8, and 1B8-2C11 exhibited a noteworthy decrease in GvHD scores compared to the mice treated with NaCl. The GvHD scores in these anti-OX40 antibody groups were also lower than those observed in mice treated with the individual parent antibody and GBR830 analog and/or KHK4083 analog. Furthermore, 3A11-4C8, 3A11-2C11, 1B8-4C8 and 1B8-2C11 treatments significantly improved mouse survival rate in contrast to GBR830 analog and/or KHK4083 analog. In addition, body weight in groups of 3A11-4C8, 3A11-2C11, 1B8-4C8 and 1B8-2C11 tended to recover better than GBR830 analog group (not shown). These results suggested bispecific antibodies 3A11-4C8, 3A11-2C11, 1B8-4C8 and 1B8-2C11 performed more pronounced effects in attenuating graft-versus-host-disease (GvHD) compared to the individual parent antibodies and positive controls, GBR830 analog and/or KHK4083 analog. These effects of bispecific antibodies in reducing GvHD also exhibited a dose-dependent pattern.

In another similar experiment, the immunosuppressive potency of the antibodies 3A11-1B8 (G3), 3A11-2C11 (G4), 1B8-2C11 (G5) were tested. The negative control group mice (G2) were injected with an equal volume of 0.9% sodium chloride injection (NaCl) instead of the anti-OX40 antibody. The blank control group (BC, G1) mice were only injected with 0.9% NaCl. The intravenous administration at a dose of 10mg/kg was performed once a week for 6 continuous weeks. **FIG. 9A** showed that 3A11-2C11 and 1B8-2C11 treatments improved GvHD outcomes more effectively than 3A11-1B8. **FIGs. 9B** showed that 3A11-2C11 and 1B8-2C11 treatments improved the survival mice rate more effectively than

3A11-1B8. In addition, body weight in groups of 3A11-2C11 and 1B8-2C11 tended to recover better than 3A11-1B8 (data not shown).

In another similar experiment, the immunosuppressive potency of the antibody 1B8-N297A (G3) was tested. 1B8-N297A was obtained by introducing the N297A mutation into the IgG1 Fc region of 1B8.

5 Mice in the blank control group (BC, G1) mice were only injected with 0.9% NaCl, while those in the negative control group mice (G2) were injected with an equal volume of 0.9% NaCl instead of the anti-OX40 antibody. Intravenous administration at a dose of 10mg/kg was performed once a week for 5 continuous weeks. The results presented in **FIG. 24A** and **FIG. 24B** show that compared to mice treated with NaCl, those treated with 1B8-N297A significantly reduced GvHD scores and had improved mouse survival rates-indicating that 1B8-N297A performed pronounced effects in the GvHD model. These findings suggest that despite the N297 mutation, which is known to potentially reduce ADCC activity by affecting the interaction between an antibody's Fc region and Fc $\gamma$  receptors on effector cells such as natural killer cells, it appears that 1B8-N297A still exhibits superior performance in a GvHD model due to its Fab region's critical role.

#### 15 **Example 5. Inhibition of cytokine release in T cells or human PBMC**

A 96-well plate were pre-coated with 2  $\mu$ g/mL anti-human CD3 antibody (clone OKT3, ACROBiosystems, Cat#: CDE-M120a) and 2  $\mu$ g/mL human OX40L Protein (ACROBiosystems, Cat#: OXL-H52Q8) and incubated at 4°C overnight. After washing, human PBMC ( $1.5 \times 10^5$  cells/mL) or T cells ( $8 \times 10^4$  cells/mL) were added to the 96-well plate at 100 $\mu$ L/well, and then the tested anti-OX40 antibodies were added to each well at the working concentration of 10  $\mu$ g/mL, 3-fold dilutions, 5-6 gradients as indicated. After the incubation for 96 hours(PBMC) or 72 hours(T cells), the supernatants were collected for IL-2 ELISA detection using the IL2 Human uncoated ELISA Kit (Invitrogen, Cat#: 88-7025-88). Human IgG1 protein (Sino Biological, Cat#:HG1K) was used as the ISO. The results are shown in **FIG. 14A** and **FIG. 14B**, indicating that 3A11-2C11 and 3A11-4C8 can reduce cytokine levels in T cells or human PBMC under the stimulation of anti-hCD3 antibody and OX40L.

#### **Example 6. Lack of agonistic properties of anti-OX40 antibodies in T cells or human PBMC**

To prepare the assay setup, a 96-well plate were coated with 2  $\mu$ g/mL anti-human CD3 antibody (clone OKT3, ACROBiosystems, Cat#: CDE-M120a) and tested anti-OX40 antibodies. The tested antibodies were diluted to a working concentration of 30  $\mu$ g/mL, and further diluted in a 10-fold manner to create 4 gradients. The plate was then incubated at 4°C overnight. After washing, human PBMC ( $1.5 \times 10^5$  cells/mL) or T cells ( $8 \times 10^4$  cells/mL) were added to the 96-well plate at 100 $\mu$ L/well. After the incubation for 48 hours (PBMC) or 72 hours (T cells), the supernatants were collected for IL-2 ELISA detection using the IL2 Human Uncoated ELISA Kit. In the positive control group (PC), the tested anti-OX40 antibody was replaced with 2  $\mu$ g/mL (equals to 40nM) of human OX40L protein

(ACROBiosystems, Cat#: OXL-H52Q8). The results are shown in **FIG. 15A** and **FIG. 15B**, indicating that 3A11-2C11 can only weakly promote cytokine levels in human T cells or PBMC, which is far less than that induced by OX40L. This suggests that compared to OX40L, 3A11-2C11 only exhibit a weak activation effect. In addition, 3A11-2C11 exhibited a weaker activation effect than KHK4083 analog.

5

#### **Example 7. Analysis of defucosylated anti-OX40 antibodies**

##### ***Binding affinity of defucosylated anti-OX40 bispecific antibodies***

The affinity of the defucosylated anti-OX40 antibodies to hOX40-his or fasOX40-his was verified using the similar experimental procedures as previously described. The results are shown in the table below which demonstrated that 3A11-2C11, 3A11-2C11-AF, 3A11-2C11-AF-YTE, 3A11-4C8, 3A11-4C8-AF, and 3A11-4C8-AF-YTE all exhibited strong binding affinities to both human and monkey OX40.

The afucosylated antibody from the KHK4083 analog was obtained, for example, by expression through modified CHO-K1 cells (e.g., CHO-K1 cells with FUT8 and SLC35C1 deletions). The resulting defucosylated antibody was named as KHK4083-AF.

15

**Table 10**

| <b>Antibody</b>  | <b>Protein</b> | <b>kon (1/Ms)</b> | <b>koff (1/s)</b> | <b>KD (M)</b> |
|------------------|----------------|-------------------|-------------------|---------------|
| KHK4083-AF       | hOX40-his      | 2.43E+05          | 6.98E-04          | 2.88E-09      |
|                  | fasOX40-his    | 1.64E+05          | 1.23E-03          | 7.47E-09      |
| 3A11-2C11        | hOX40-his      | 5.02E+05          | 3.18E-04          | 6.34E-10      |
|                  | fasOX40-his    | 2.06E+05          | 1.87E-04          | 9.05E-10      |
| 3A11-2C11-AF     | hOX40-his      | 5.25E+05          | 3.73E-04          | 7.11E-10      |
|                  | fasOX40-his    | 2.21E+05          | 1.76E-04          | 7.96E-10      |
| 3A11-2C11-AF-YTE | hOX40-his      | 5.35E+05          | 8.99E-04          | 1.68E-09      |
|                  | fasOX40-his    | 1.96E+05          | 4.89E-04          | 2.50E-09      |
| 3A11-4C8         | hOX40-his      | 6.90E+05          | 3.12E-04          | 4.51E-10      |
|                  | fasOX40-his    | 3.50E+05          | 8.93E-04          | 2.55E-09      |
| 3A11-4C8-AF      | hOX40-his      | 8.78E+05          | 5.01E-04          | 5.70E-10      |
|                  | fasOX40-his    | 3.88E+05          | 1.01E-03          | 2.60E-09      |
| 3A11-4C8-AF-YTE  | hOX40-his      | 7.35E+05          | 8.84E-04          | 1.20E-09      |
|                  | fasOX40-his    | 3.27E+05          | 1.39E-03          | 4.24E-09      |

##### ***Blockade assays***

The blockade of the interaction between human OX40 ligand and human OX40 by defucosylated anti-OX40 antibodies was verified using flow cytometry and OX40 reporter cells, following the same experimental procedures as previously described. The results are shown in **FIGs. 16A-16D**.

The results demonstrated that 3A11-2C11, 3A11-2C11-AF, 3A11-2C11-AF-YTE, 3A11-4C8, 3A11-4C8-AF, and 3A11-4C8-AF-YTE all effectively blocked the intrinsic interaction between human OX40 and OX40 ligand at a significantly higher max blocking rate than the positive control GBR830 analog.

25

#### *Jurkat-Luc-hOX40 reporter cells activation assay*

The Jurkat-Luc-hOX40 reporter cells activation assay was carried out to verify whether the defucosylated anti-OX40 antibodies exhibit OX40-OX40L agonistic effects, using the same experimental procedures as previously described. The results are shown in **FIG. 17A** and **FIG. 17B** indicating that 3A11-2C11, 3A11-2C11-AF, 3A11-2C11-AF-YTE, 3A11-4C8, 3A11-4C8-AF, and 3A11-4C8-AF-YTE demonstrated mild agonistic activities when Fc-FcγRIIB cross-linking existed, while GBR830 analog and KHK4083-AF exhibited strong agonistic effects. The agonist activities were not observed in the absence of Fc-FcγRIIB cross-linking (results not shown).

#### *Inhibition of cytokine release in T cells*

The inhibition of cytokine release by defucosylated anti-OX40 antibodies in T cells under the stimulation of OKT3 and OX40L was verified using the similar experimental procedures as previously described.

The results are shown in **FIG. 18**, indicating that 3A11-2C11, 3A11-2C11-AF, and 3A11-2C11-AF-YTE can reduce cytokine levels in T cells under the stimulation of anti-CD3 antibody and OX40L.

#### *Lack of agonistic properties of defucosylated anti-OX40 antibodies in T cells*

The agonistic properties of defucosylated anti-OX40 antibody 3A11-2C11, 3A11-2C11-AF, and 3A11-2C11-AF-YTE in T cells under the stimulation of OKT3 and OX40 was verified using the same experimental procedures as described in **Example 6**.

The results are shown in **FIG. 19**, indicating that 3A11-2C11, 3A11-2C11-AF, and 3A11-2C11-AF-YTE can weakly promote cytokine levels in human T cells, which is far less than that induced by OX40L. This suggests 3A11-2C11, 3A11-2C11-AF, and 3A11-2C11-AF-YTE demonstrated comparatively weaker T cell activation effects and exhibited a weaker activation effect in comparison to KHK4083-AF.

#### **Example 8. ADCC effects of defucosylated anti-OX40 antibodies**

Jurkat-Luc-hOX40 cells and FcR-TANK cells (CD16a (158 V)-overexpressed NK92MI cells) were seeded in a 96-well plate, with a cell density of  $2 \times 10^5$  cells/well for each type of cell. Anti-OX40 antibodies were diluted to a working concentration of 0.5 μg/mL then added followed by 1:3 dilution, along with 50 μL of medium or 40 μL of heat-inactivated human AB serum (Gimibio, Cat#:100-512) with 10 μL of medium and incubated at 37°C for 5h. The cell supernatant was collected through centrifugation to detect lactate dehydrogenase (LDH) activity using the Invitrogen CyQUANT™ LDH Cytotoxicity Assay (Fisher Scientific, Cat#:C20301). The results are shown in **FIGs. 20A-20D**, in which

3A11-2C11-AF and 3A11-2C11-AF-YTE exhibited higher ADCC effects than 3A11-2C11. Similarly, 3A11-4C8-AF and 3A11-4C8-AF-YTE also exhibited higher ADCC effects than 3A11-4C8.

**Example 9. Immunosuppressive potency of defucosylated anti-OX40 antibodies in GvHD Mouse Model**

The immunosuppressive potency of the antibodies was tested using the same experimental procedure as previously described. B-NDG mice were randomly assigned to different groups based on body weight. The treatment groups (G1-G4) were randomly selected for treatment with antibodies by intravenous (i.v.) on the same day. The negative control group of mice (G5) were injected with an equal volume of 0.9% NaCl. Two days after grouping, the mice in G1-G5 were irradiated and about  $1 \times 10^7$  human PBMC were intravenously injected into the mice. The blank control group (BC, G6) mice were only injected with 0.9% NaCl. The intravenous administration at a dose of 10mg/kg was performed once a week for 6 continuous weeks. Details are shown in the table below.

**Table 11**

| Group | No. of mice | Antibody         | Dosage  | Route | Total No. of administration |
|-------|-------------|------------------|---------|-------|-----------------------------|
| G1    | 6           | 3A11-2C11-AF     | 10mg/kg | i.v.  | 6                           |
| G2    | 6           | 3A11-2C11-AF-YTE | 10mg/kg | i.v.  | 6                           |
| G3    | 6           | KHK4083-AF       | 10mg/kg | i.v.  | 6                           |
| G4    | 6           | GBR830 analog    | 10mg/kg | i.v.  | 6                           |
| G5    | 6           | NaCl             | —       | i.v.  | 6                           |
| G6    | 6           | —                | —       | i.v.  | 6                           |

The results are shown in **FIG. 21A** and **FIG. 21B**. **FIG. 21A** showed that 3A11-2C11-AF and 3A11-2C11-AF-YTE treatments improved GvHD outcomes more effectively than GBR830 analog or KHK4083-AF. **FIGs. 21B** showed that 3A11-2C11-AF and 3A11-2C11-AF-YTE treatments improved the survival rate of mice more effectively than GBR830 analog or KHK4083-AF.

**Example 10. Pharmacokinetic Profiles**

The pharmacokinetic clearance rates of the anti-OX40 antibodies were determined in B-NDG mice (Biocytogen Pharmaceuticals (Beijing) Co., Ltd., Cat#: 110001). Specifically, the B-hFcRn mice were randomly assigned to different groups(6 mice per group) based on body weight and the treatment groups (G1-G7) were randomly selected for treatment with antibodies by intravenous (i.v.) on the same day. The intravenous administration at a dose of 10mg/kg was performed(1 administration in total). Details are shown in the table below. Blood samples of mice in group G1-G7 were collected at 0 day, 1 day, 2 days, 5 days, 10 days, 14 days, 21 days, 24 days, 35 days and 42 days after administration. These collected samples were used to detect the total antibody levels in serum by sandwich ELISA.

**Table 12**

| Group | No. of mice | Antibody | Dosage | Route |
|-------|-------------|----------|--------|-------|
|-------|-------------|----------|--------|-------|

|    |   |                  |         |      |
|----|---|------------------|---------|------|
| G1 | 6 | 3A11-2C11        | 10mg/kg | i.v. |
| G2 | 6 | 3A11-2C11-AF     | 10mg/kg | i.v. |
| G3 | 6 | 3A11-2C11-AF-YTE | 10mg/kg | i.v. |
| G4 | 6 | 3A11-4C8         | 10mg/kg | i.v. |
| G5 | 6 | 3A11-4C8-AF      | 10mg/kg | i.v. |
| G6 | 6 | 3A11-4C8-AF-YTE  | 10mg/kg | i.v. |
| G7 | 6 | KHK4083-AF       | 10mg/kg | i.v. |

Briefly, Goat Anti-Human IgG antibody (Bioss Antibodies, Cat#: bs-0297G) was diluted to a final concentration of 2000 ng/mL, added to a 96-well plate (ELISA plate) at 100  $\mu$ L/well, and then incubated overnight at 2-8 °C. After the incubation, the plate was washed with PBS-T buffer (PBS supplemented with Tween™ 20) 4 times. Antibody-unbound areas were blocked with 2% BSA (bovine serum albumin) for 2 hours at 37°C. Afterwards, the plate was washed with PBS-T buffer 4 times. After washing, 100  $\mu$ L of blocking buffer (1% BSA) was added to each well and incubated with the sample at 37°C for 1 hour. After washing the plate using a plate washer, Peroxidase AffiniPure F(ab')<sub>2</sub> Fragment Goat Anti-Human IgG, Fc $\gamma$  fragment specific (Jackson ImmunoResearch Inc., Cat#: 109-036-098) was added at 100  $\mu$ L/well to each well of the plate, and incubated at 37°C for 1 hour for determining the concentration of total antibody. After washing the plate, tetramethylbenzidine (TMB) solution was added at 100  $\mu$ L/well to the 96-well plate as the substrate. After incubating at room temperature in the dark, 100  $\mu$ L stop solution (Beyotime, Cat#: P0215) was added to each well. Luminescent signals of the plate were measured at 450 nm and 630 nm to calculate the concentrations. The absorbance value and corresponding concentration of the calibration sample prepared by each test product was used to create a standard curve with four parameters (i.e.,  $T_{1/2}$ ,  $C_{max}$ ,  $AUC_{0-42day}$ , and CL). The standard curve was used to calculate the antibody concentration of each serum sample. A drug concentration-time curve was created using the calculated sample concentration at each time point. Phoenix™ WinNolin 8.3 was used to calculate the pharmacokinetic parameters.

The results are shown in the table below and **FIG. 22**, which showed that 3A11-2C11-AF-YTE and 3A11-4C8-AF-YTE exhibited longer half-lives compared to KHK4083-AF.

Table 13

| Antibody                                                                                                                                   | $T_{1/2}$ (Day) | $C_{max}$ ( $\mu$ g/mL) | $AUC_{0-last}$ (day* $\mu$ g/mL) | CL (mL/day/kg) |
|--------------------------------------------------------------------------------------------------------------------------------------------|-----------------|-------------------------|----------------------------------|----------------|
| 3A11-2C11                                                                                                                                  | 12.209          | 193.421                 | 1156.850                         | 7.919          |
| 3A11-2C11-AF                                                                                                                               | 7.092           | 208.972                 | 1083.900                         | 9.090          |
| 3A11-2C11-AF-YTE                                                                                                                           | 17.956          | 192.898                 | 1928.831                         | 4.351          |
| 3A11-4C8                                                                                                                                   | 6.477           | 216.344                 | 982.208                          | 1139.374       |
| 3A11-4C8-AF                                                                                                                                | 7.844           | 264.608                 | 1443.276                         | 7.650          |
| 3A11-4C8-AF-YTE                                                                                                                            | 16.486          | 252.514                 | 2107.203                         | 4.102          |
| KHK4083-AF                                                                                                                                 | 7.995           | 225.673                 | 1298.030                         | 7.708          |
| $C_{max}$ : Max Concentration;<br>$AUC_{0-last}$ : Area Under the Curve from Time Zero to Last Quantifiable Concentration<br>CL: Clearance |                 |                         |                                  |                |

**Example 11. Cytokine release assay in PBMCs**

The cytokine release assay was conducted to validate the potential risk of anti-OX40 antibodies in inducing adverse pro-inflammatory clinical reactions, thereby predicting their safety.

5 The tested anti-OX40 antibody was added to a 96-well plate at a working concentration of 10 µg/mL or 100 µg/mL and incubated overnight at 4°C. After washing, human PBMC ( $1 \times 10^6$  cells/mL) were added to each well of the 96-well plate at a volume of 100µL/well. After a 48 hours incubation period, the supernatants from the wells were collected to detect IL-2, IFNγ, and IL6 using ELISA. In this experiment, the positive control (PC) consisted of 2 µg/mL anti-human CD3 antibody (clone OKT3, ACROBiosystems, Cat#: CDE-M120a) and 2 µg/mL human OX40L protein (ACROBiosystems, Cat#: 10 OXL-H52Q8). These components were utilized as T cell activators and were pre-coated onto the wells. Additionally, 1 µg/mL Lipopolysaccharides (LPS) (Sino Biological, Cat#: HG1K) served as the second positive control to induce macrophage activation.

The results, as depicted in **FIG. 23**, indicate that 3A11-2C11-AF and 3A11-2C11-AF-YTE all did not stimulate human PBMCs to release IL-2, IFNγ, or IL6. This observation indicates that these 15 anti-OX40 antibodies potentially possess a reduced likelihood of directly activating T cells or monocytes, which are the predominant cell populations involved in triggering cytokine release events. Consequently, these antibodies demonstrated promising safety profiles.

**OTHER EMBODIMENTS**

20 It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

**WHAT IS CLAIMED IS:**

1. An antibody or antigen-binding fragment thereof that binds to OX40 (Tumor necrosis factor receptor superfamily, member 4) comprising:
  - a heavy chain variable region (VH) comprising complementarity determining regions (CDRs) 1, 2, and 3, wherein the VH CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VH CDR1 amino acid sequence, the VH CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VH CDR2 amino acid sequence, and the VH CDR3 region comprises an amino acid that is at least 80% identical to a selected VH CDR3 amino acid sequence; and
  - a light chain variable region (VL) comprising CDRs 1, 2, and 3, wherein the VL CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VL CDR1 amino acid sequence, the VL CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VL CDR2 amino acid sequence, and the VL CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VL CDR3 amino acid sequence,
- wherein the selected VH CDRs 1, 2, and 3 amino acid sequences and the selected VL CDRs, 1, 2, and 3 amino acid sequences are one of the following:
  - (1) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
  - (2) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
  - (3) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
  - (4) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
  - (5) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
  - (6) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(7) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively; and

5 (8) the selected VH CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 22-24, respectively, and the selected VL CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

2. The antibody or antigen-binding fragment thereof of claim 1, wherein the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 1-3, respectively, and the VL comprises  
10 CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to the Kabat numbering scheme.
3. The antibody or antigen-binding fragment thereof of claim 1, wherein the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 4-6, respectively, and the VL comprises  
15 CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to the Kabat numbering scheme.
4. The antibody or antigen-binding fragment thereof of claim 1, wherein the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 7-9, respectively, and the VL comprises  
20 CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to the Kabat numbering scheme.
5. The antibody or antigen-binding fragment thereof of claim 1, wherein the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 10-12, respectively, and the VL comprises  
25 CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to the Kabat numbering scheme.
6. The antibody or antigen-binding fragment thereof of claim 1, wherein the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 13-15, respectively, and the VL comprises  
30 CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to the Chothia numbering scheme.
7. The antibody or antigen-binding fragment thereof of claim 1, wherein the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 16-18, respectively, and the VL comprises  
35 CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to the Chothia numbering scheme.

8. The antibody or antigen-binding fragment thereof of claim 1, wherein the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 19-21, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to the Chothia numbering scheme.
9. The antibody or antigen-binding fragment thereof of claim 1, wherein the VH comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 22-24, respectively, and the VL comprises CDRs 1, 2, 3 with the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, according to the Chothia numbering scheme.
10. An antibody or antigen-binding fragment thereof that binds to OX40 comprising a heavy chain variable region (VH) comprising an amino acid sequence that is at least 90% identical to a selected VH sequence, and a light chain variable region (VL) comprising an amino acid sequence that is at least 90% identical to a selected VL sequence, wherein the selected VH sequence and the selected VL sequence are one of the following:
- (1) the selected VH sequence is SEQ ID NO: 28, and the selected VL sequence is SEQ ID NO: 32;
  - (2) the selected VH sequence is SEQ ID NO: 29, and the selected VL sequence is SEQ ID NO: 32;
  - (3) the selected VH sequence is SEQ ID NO: 30, and the selected VL sequence is SEQ ID NO: 32; and
  - (4) the selected VH sequence is SEQ ID NO: 31, and the selected VL sequence is SEQ ID NO: 32.
11. The antibody or antigen-binding fragment thereof of claim 10, wherein the VH comprises the sequence of SEQ ID NO: 28 and the VL comprises the sequence of SEQ ID NO: 32.
12. The antibody or antigen-binding fragment thereof of claim 10, wherein the VH comprises the sequence of SEQ ID NO: 29 and the VL comprises the sequence of SEQ ID NO: 32.
13. The antibody or antigen-binding fragment thereof of claim 10, wherein the VH comprises the sequence of SEQ ID NO: 30 and the VL comprises the sequence of SEQ ID NO: 32.
14. The antibody or antigen-binding fragment thereof of claim 10, wherein the VH comprises the sequence of SEQ ID NO: 31 and the VL comprises the sequence of SEQ ID NO: 32.
15. An antibody or antigen-binding fragment thereof that binds to OX40 comprising a heavy chain variable region (VH) comprising VH CDR1, VH CDR2, and VH CDR3 that are identical to VH CDR1, VH CDR2, and VH CDR3 of a selected VH sequence; and a light chain variable region (VL)

comprising VL CDR1, VL CDR2, and VL CDR3 that are identical to VL CDR1, VL CDR2, and VL CDR3 of a selected VL sequence, wherein the selected VH sequence and the selected VL sequence are one of the following:

- (1) the selected VH sequence is SEQ ID NO: 28, and the selected VL sequence is SEQ ID NO: 32;
  - 5 (2) the selected VH sequence is SEQ ID NO: 29, and the selected VL sequence is SEQ ID NO: 32;
  - (3) the selected VH sequence is SEQ ID NO: 30, and the selected VL sequence is SEQ ID NO: 32; and
  - (4) the selected VH sequence is SEQ ID NO: 31, and the selected VL sequence is SEQ ID NO: 32.
16. The antibody or antigen-binding fragment thereof of any one of claims 1-15, wherein the antibody or  
10 antigen-binding fragment thereof specifically binds to human or monkey OX40.
  17. The antibody or antigen-binding fragment thereof of any one of claims 1-16, wherein the antibody or antigen-binding fragment is a human antibody or antigen-binding fragment thereof.
  - 15 18. The antibody or antigen-binding fragment thereof of any one of claims 1-17, wherein the antibody or antigen-binding fragment is a single-chain variable fragment (scFV).
  19. The antibody or antigen-binding fragment thereof of any one of claims 1-17, wherein the antibody or antigen-binding fragment thereof is a multi-specific antibody (e.g., a bispecific antibody).
  - 20 20. The antibody or antigen-binding fragment thereof of claim 19, wherein the antibody or antigen-binding fragment thereof binds to two different epitopes of OX40.
  21. An antibody or antigen-binding fragment thereof that cross-competes with the antibody or  
25 antigen-binding fragment thereof of any one of claims 1-20.
  22. A nucleic acid comprising a polynucleotide encoding a polypeptide comprising:
    - (1) an immunoglobulin heavy chain or a fragment thereof comprising a heavy chain variable region (VH) comprising complementarity determining regions (CDRs) 1, 2, and 3 comprising the  
30 amino acid sequences set forth in SEQ ID NOs: 1-3, respectively, and wherein the VH, when paired with a light chain variable region (VL) comprising the amino acid sequence set forth in SEQ ID NO: 32, binds to OX40;
    - (2) an immunoglobulin light chain or a fragment thereof comprising a light chain variable region (VL) comprising complementarity determining regions (CDRs) 1, 2, and 3 comprising the  
35 amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, and wherein the VL, when paired

with a heavy chain variable region (VH) comprising the amino acid sequence set forth in SEQ ID NO: 28, binds to OX40;

5 (3) an immunoglobulin heavy chain or a fragment thereof comprising a VH comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 4-6, respectively, and wherein the VH, when paired with a VL comprising the amino acid sequence set forth in SEQ ID NO: 32, binds to OX40;

10 (4) an immunoglobulin light chain or a fragment thereof comprising a VL comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, and wherein the VL, when paired with a VH comprising the amino acid sequence set forth in SEQ ID NO: 29, binds to OX40;

(5) an immunoglobulin heavy chain or a fragment thereof comprising a VH comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 7-9, respectively, and wherein the VH, when paired with a VL comprising the amino acid sequence set forth in SEQ ID NO: 32, binds to OX40;

15 (6) an immunoglobulin light chain or a fragment thereof comprising a VL comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, and wherein the VL, when paired with a VH comprising the amino acid sequence set forth in SEQ ID NO: 30, binds to OX40;

20 (7) an immunoglobulin heavy chain or a fragment thereof comprising a VH comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 10-12, respectively, and wherein the VH, when paired with a VL comprising the amino acid sequence set forth in SEQ ID NO: 32, binds to OX40;

25 (8) an immunoglobulin light chain or a fragment thereof comprising a VL comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 25-27, respectively, and wherein the VL, when paired with a VH comprising the amino acid sequence set forth in SEQ ID NO: 31, binds to OX40;

30 (9) an immunoglobulin heavy chain or a fragment thereof comprising a VH comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 13-15, respectively, and wherein the VH, when paired with a VL comprising the amino acid sequence set forth in SEQ ID NO: 32, binds to OX40;

(11) an immunoglobulin heavy chain or a fragment thereof comprising a VH comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 16-18, respectively, and wherein the VH, when paired with a VL comprising the amino acid sequence set forth in SEQ ID NO: 32, binds to OX40;

35 (13) an immunoglobulin heavy chain or a fragment thereof comprising a VH comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 19-21, respectively, and

wherein the VH, when paired with a VL comprising the amino acid sequence set forth in SEQ ID NO: 32, binds to OX40; or

- (15) an immunoglobulin heavy chain or a fragment thereof comprising a VH comprising CDRs 1, 2, and 3 comprising the amino acid sequences set forth in SEQ ID NOs: 22-24, respectively, and  
5 wherein the VH, when paired with a VL comprising the amino acid sequence set forth in SEQ ID NO: 32, binds to OX40.

23. The nucleic acid of claim 22, wherein the VH when paired with a VL specifically binds to human or monkey OX40, or the VL when paired with a VH specifically binds to human or monkey OX40.  
10
24. The nucleic acid of claim 22 or 23, wherein the immunoglobulin heavy chain or the fragment thereof is a human immunoglobulin heavy chain or a fragment thereof.
25. The nucleic acid of any one of claims 22-24, wherein the nucleic acid encodes a single-chain variable  
15 fragment (scFv).
26. The nucleic acid of any one of claims 22-25, wherein the nucleic acid is cDNA.
27. An antigen-binding protein construct, comprising: a first antigen-binding domain that specifically  
20 binds to a first epitope of OX40, wherein the first antigen-binding domain is a full OX40L binding blocking antigen binding domain; and a second antigen-binding domain that specifically binds to a second epitope of OX40, wherein the second antigen-binding domain is a partial OX40L binding blocking antigen binding domain.
- 25 28. The antigen-binding protein construct of claim 27, wherein the first antigen-binding domain comprises a first heavy chain variable region (VH1) and a first light chain variable region (VL1); and the second antigen-binding domain comprises a second heavy chain variable region (VH2) and a second light chain variable region (VL2).
- 30 29. The antigen-binding protein construct of claim 28, wherein  
the first heavy chain variable region (VH1) comprises complementarity determining regions (CDRs) 1, 2, and 3, wherein the VH1 CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VH1 CDR1 amino acid sequence, the VH1 CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VH1 CDR2 amino acid sequence, and  
35 the VH1 CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VH1 CDR3 amino acid sequence; and

the first light chain variable region (VL1) comprises CDRs 1, 2, and 3, wherein the VL1 CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VL1 CDR1 amino acid sequence, the VL1 CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VL1 CDR2 amino acid sequence, and the VL1 CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VL CDR3 amino acid sequence,

wherein the selected VH1 CDRs 1, 2, and 3 amino acid sequences, the selected VL1 CDRs 1, 2, and 3 amino acid sequences are one of the following:

(1) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(2) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(3) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively; or

(4) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

30. The antigen-binding protein construct of claim 28 or 29, wherein

the second heavy chain variable region (VH2) comprises CDRs 1, 2, and 3, wherein the VH2 CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VH2 CDR1 amino acid sequence, the VH2 CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VH2 CDR2 amino acid sequence, and the VH2 CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VH2 CDR3 amino acid sequence; and

the second light chain variable region (VL2) comprises CDRs 1, 2, and 3, wherein the VL2 CDR1 region comprises an amino acid sequence that is at least 80% identical to a selected VL2 CDR1 amino acid sequence, the VL2 CDR2 region comprises an amino acid sequence that is at least 80% identical to a selected VL2 CDR2 amino acid sequence, and the VL2 CDR3 region comprises an amino acid sequence that is at least 80% identical to a selected VL2 CDR3 amino acid sequence,

wherein the selected VH2 CDRs 1, 2, and 3 amino acid sequences, and the selected VL2 CDRs 1, 2, and 3 amino acid sequences are one of the following:

(1) the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

(2) the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

5 (3) the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively; or

(4) the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 22-24, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.

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31. The antigen-binding protein construct of any one of claims 28-30, wherein

(1) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

15 (2) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 1-3, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

20 (3) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

25 (4) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 13-15, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 22-24, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

30 (5) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 7-9, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;

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- (6) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 4-6, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 10-12, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively;
- (7) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 19-21, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively; or
- (8) the selected VH1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 16-18, respectively, and the selected VL1 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively, and the selected VH2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 22-24, respectively, and the selected VL2 CDRs 1, 2, 3 amino acid sequences are set forth in SEQ ID NOs: 25-27, respectively.
32. The antigen-binding protein construct of any one of claims 28-31 wherein the first heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 28, the first light chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 32, the second heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 30, and the second light chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 32.
33. The antigen-binding protein construct of any one of claims 28-31, wherein the first heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 28, the first light chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 32, the second heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 31, and the second light chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 32.
34. The antigen-binding protein construct of any one of claims 28-31, wherein the first heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 29, the first light chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 32, the second heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 30, and the second light chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 32.

35. The antigen-binding protein construct of any one of claims 28-31, wherein the first heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 29, the first light chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 32, the second heavy chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 31, and the second light chain variable region comprises a sequence that is at least 80%, 85%, 90%, or 95% identical to SEQ ID NO: 32.
36. The antigen-binding protein construct of any one of claims 27-35, wherein the antigen-binding protein construct is a multi-specific antibody (e.g., a bispecific antibody).
37. The antigen-binding protein construct of any one of claims 27-35, wherein the first antigen-binding domain is a single-chain variable fragment (scFv); and/or the second antigen-binding domain is a scFv.
38. The antigen-binding protein construct of any one of claims 28-37, wherein the first light chain variable region and the second light chain variable region are identical.
39. The antigen-binding protein construct of any one of claims 27-38, wherein the antigen-binding protein construct comprises a fragment crystallizable region (Fc region).
40. The antigen-binding protein construct of claim 39, wherein the Fc region is afucosylated.
41. The antigen-binding protein construct of any one of claims 39 or 40, wherein the Fc region comprises YTE mutations (EU numbering: M252Y, S254T, T256E mutations).
42. A vector comprising one or more of the nucleic acids of any one of claims 22-26, a nucleic acid encoding the antibody or antigen-binding fragment thereof of any one of claims 1-21, or a nucleic acid encoding the antigen-binding protein construct of any one of claims 27-41.
43. A cell comprising the vector of claim 42.
44. The cell of claim 43, wherein the cell is a CHO cell or CHO-K1 cell.

45. A cell comprising one or more of the nucleic acids of any one of claims 22-26, a nucleic acid encoding the antibody or antigen-binding fragment thereof of any one of claims 1-21, or a nucleic acid encoding the antigen-binding protein construct of any one of claims 27-41.
- 5 46. A method of producing an antibody or an antigen-binding fragment thereof, or an antigen-binding protein construct, the method comprising
- (a) culturing the cell of any one of claims 43-45 under conditions sufficient for the cell to produce the antibody or the antigen-binding fragment thereof, or the antigen-binding protein construct; and
- 10 (b) collecting the antibody or the antigen-binding fragment thereof, or the antigen-binding protein construct produced by the cell.
47. An antibody-drug conjugate comprising a therapeutic agent covalently bound to:
- (a) the antibody or antigen-binding fragment thereof of any one of claims 1-21; or
- 15 (b) the antigen-binding protein construct of any one of claims 27-41.
48. The antibody drug conjugate of claim 47, wherein the therapeutic agent is a cytotoxic or cytostatic agent.
- 20 49. A method of treating a subject having autoimmune disorders, the method comprising administering a therapeutically effective amount of a composition comprising the antibody or antigen-binding fragment thereof of any one of claims 1-21, or the antigen-binding protein construct of any one of claims 27-41, to the subject.
- 25 50. The method of claim 49, wherein the subject has graft-versus-host disease (GvHD) or apical dermatitis.
51. The method of claim 49, wherein the subject has an acute graft-versus-host disease or a chronic graft-versus-host disease.
- 30 52. The method of any one of claims 49-51, wherein the subject is a human.
53. A method of reducing the GvHD score and improving survival rate, the method comprising administering a therapeutically effective amount of a composition comprising the antibody or antigen-binding fragment thereof of any one of claims 1-21, or the antigen-binding protein construct of any one of claims 27-41, to the subject.
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54. A method of suppressing the immune responses in a subject, the method comprising administering a therapeutically effective amount of a composition comprising the antibody or antigen-binding fragment thereof of any one of claims 1-21, or the antigen-binding protein construct of any one of claims 27-41, to the subject.
55. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and
- (a) the antibody or antigen-binding fragment thereof of any one of claims 1-21;
  - (b) the antigen-binding protein construct of any one of claims 27-41; or
  - (c) the antibody-drug conjugate of any one of claims 47 or 48.