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(57) Abrégé/Abstract:

This invention relates to anti-ROR1 antibodies and methods of using them in treating diseases and conditions related to ROR1 activity, e.g., cancer.

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Abstract:

This invention relates to anti-ROR1 antibodies and methods of using them in treating diseases and conditions related to ROR1 activity, e.g., cancer.

ANTI-ROR1 ANTIBODIES AND COMPOSITIONS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims priority from United States Provisional Patent Application 62/971,688, filed February 7, 2020, the disclosure of which is incorporated by reference herein in its entirety.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing that has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. The electronic copy of the Sequence Listing, created on January 26, 2021, is named 024651_WO005_SL.txt and is 73,129 bytes in size.

BACKGROUND OF THE INVENTION

[0003] Receptor tyrosine kinases (RTK) are key regulators of cellular processes such as differentiation, proliferation, survival and migration and have a role in the development and progression of cancer. Deregulation of RTKs has been linked to the initiation and progression of cancers (Du and Lovely, *Molecular Cancer* (2018) 17:58).

[0004] Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is a cell surface protein that mediates signals from its ligand, the secreted glycoprotein Wnt5a. Consistent with its role in influencing the fate of stem cells during embryogenesis, ROR1 expression is observed on invasive malignancies that revert to an embryonic transcriptional program, but is not observed on normal adult tissues, offering a favorable selectivity profile as a therapeutic target. ROR1 is commonly expressed on the malignant cells of patients with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphoblastic leukemia (CLL), mantle cell lymphoma (MCL), follicular lymphoma (FL), marginal zone lymphoma (MZL), diffuse large B-cell lymphoma (DLBCL), and Richter's transformation or Richter's syndrome (RS). ROR1 is also present on the cell surfaces of multiple solid tumors, where it appears to be a marker of cancer stem cells. Because it is not expressed to appreciable levels in healthy adult tissues, but displays high levels of expression in multiple hematological and solid tumors, ROR1 is an attractive target for tumor-specific therapy.

[0005] ROR1 presents multiple therapeutic modality possibilities, including antibody monotherapy, antibody-drug conjugates, radioconjugates, bispecific antibodies, and other multivalent biologics. Cirmtuzumab, a humanized antibody directed against ROR1, is undergoing clinical trials. A cirmtuzumab-MMAE antibody-drug conjugate is also in clinical trials.

[0006] In view of the critical role of ROR1 in cancer progression, there is a need for new and improved immune therapies that target ROR1 for treating cancer.

SUMMARY OF THE INVENTION

[0007] The present disclosure is directed to novel recombinant antibodies targeting ROR1 (e.g., human ROR1), as well as pharmaceutical compositions comprising one or more of these antibodies, and use of the antibodies and pharmaceutical compositions for treatment of cancer. Compared to currently available treatments for such cancers, including antibody treatments, it is contemplated that the antibodies of the present disclosure may provide a superior clinical response either alone or in combination with another cancer therapeutic.

[0008] In some embodiments, the present disclosure provides an anti-ROR1 antibody or an antigen-binding portion thereof, wherein the antibody comprises:

- a) a heavy chain variable domain (VH) that comprises the amino acid sequence of SEQ ID NO: 4 with at least one mutation in heavy chain (H)-CDR1, H-CDR2, or both; and a light chain variable domain (VL) that comprises the amino acid sequence of SEQ ID NO: 2;
- b) a VH that comprises the amino acid sequence of SEQ ID NO: 4; and a VL that comprises the amino acid sequence of SEQ ID NO: 2 with at least one mutation in light chain (L)-CDR1, L-CDR2, or both; or
- c) a VH that comprises the amino acid sequence of SEQ ID NO: 4 with at least one mutation in heavy chain (H)-CDR1, H-CDR2, or both; and a VL that comprises the amino acid sequence of SEQ ID NO: 2 with at least one mutation in light chain (L)-CDR1, L-CDR2, or both;

wherein the antibody or antigen-binding portion binds to the same epitope of human ROR1 as, and has a greater binding affinity for human ROR1 than, an antibody comprising a VH that comprises the amino acid sequence of SEQ ID NO: 4 and a VL that comprises the amino acid sequence of SEQ ID NO: 2. In some embodiments, the VH may further comprise a mutation at residue 110 (e.g., W110F, W110H, W110M, or W110Y), which may increase target binding, increase antibody stability, reduce aggregation, or any combination thereof.

Unless otherwise indicated, the antibody amino acid residue numbering herein is in accordance with the AHo numbering system.

[0009] In some embodiments, the present disclosure provides an anti-ROR1 antibody or an antigen-binding portion thereof, wherein said antibody comprises:

- a) a heavy chain variable domain (VH) comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from the group consisting of T32A, T32E, S69V, S69E, and N71H, or any combination thereof; and a light chain variable domain (VL) comprising the amino acid sequence of SEQ ID NO: 2;
- b) a VH comprising the amino acid sequence of SEQ ID NO: 4; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from the group consisting of A25P, K29E, K29S, T69H, T69R, L70K, and Q71T, or any combination thereof; or
- c) a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from the group consisting of T32A, T32E, S69V, S69E, and N71H, or any combination thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from the group consisting of A25P, K29E, K29S, T69H, T69R, L70K, and Q71T, or any combination thereof;

wherein the antibody or antigen-binding portion binds to the same epitope of human ROR1 as, and has a greater binding affinity for human ROR1 than, an antibody comprising a VH that comprises the amino acid sequence of SEQ ID NO: 4 and a VL that comprises the amino acid sequence of SEQ ID NO: 2.

[0010] In certain embodiments, the anti-ROR1 antibody or antigen-binding portion comprises:

- a) a VH comprising the amino acid sequence of SEQ ID NO: 4 with
 - i) one or more mutations selected from the group consisting of T32E, S69V or S69E (notated as “S69V/E”; similar notations are used throughout), and N71H, or
 - ii) the mutation T32A and one or both mutations selected from the group consisting of S69V/E and N71H, anda VL comprising the amino acid sequence of SEQ ID NO: 2;
- b) a VH comprising the amino acid sequence of SEQ ID NO: 4 with
 - i) one or more mutations selected from the group consisting of T32E, S69V/E, and N71H, or

- ii) the mutation T32A and one or both mutations selected from the group consisting of S69V/E and N71H, and
a VL comprising the amino acid sequence of SEQ ID NO: 2 with one or more mutations selected from the group consisting of A25P, K29E/S, T69H/R, L70K, and Q71T;
- c) a VH comprising the amino acid sequence of SEQ ID NO: 4 comprising the mutation T32A and
a VL comprising the amino acid sequence of SEQ ID NO: 2 with
 - i) one or more mutations selected from the group consisting of A25P, K29E/S, T69H, L70K, and Q71T, or
 - ii) the mutation T69R and one or more mutations selected from the group consisting of A25P, K29E/S, L70K, and Q71T; or
- d) a VH comprising the amino acid sequence of SEQ ID NO: 4 and
a VL comprising the amino acid sequence of SEQ ID NO: 2 with one or more mutations selected from the group consisting of A25P, K29E/S, T69H/R, L70K, and Q71T.

[0011] In certain embodiments, the anti-ROR1 antibody or antigen-binding portion comprises:

- a) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation A25P;
- b) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation T69H;
- c) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations A25P and T69R;
- d) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations K29S and T69R;
- e) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations T69R and Q71T; or

- f) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations A25P and L70K.

[0012] In some embodiments, the present disclosure provides an anti-ROR1 antibody or an antigen-binding portion thereof, comprising heavy chain (H)-CDR1-3 and light chain (L)-CDR1-3 comprising the amino acid sequences of:

- a) SEQ ID NOs: 31, 42, 89, 101, 114, and 123, respectively;
- b) SEQ ID NOs: 13, 73, 89, 101, 114, and 123, respectively;
- c) SEQ ID NOs: 13, 75, 89, 101, 114, and 123, respectively;
- d) SEQ ID NOs: 13, 80, 89, 101, 114, and 123, respectively;
- e) SEQ ID NOs: 13, 42, 89, 102, 114, and 123, respectively;
- f) SEQ ID NOs: 13, 42, 89, 103, 114, and 123, respectively;
- g) SEQ ID NOs: 13, 42, 89, 104, 114, and 123, respectively;
- h) SEQ ID NOs: 13, 42, 89, 101, 117, and 123, respectively;
- i) SEQ ID NOs: 13, 42, 89, 101, 118, and 123, respectively;
- j) SEQ ID NOs: 13, 42, 89, 101, 120, and 123, respectively;
- k) SEQ ID NOs: 29, 42, 89, 102, 114, and 123, respectively;
- l) SEQ ID NOs: 29, 42, 89, 101, 117, and 123, respectively;
- m) SEQ ID NOs: 29, 42, 89, 102, 118, and 123, respectively;
- n) SEQ ID NOs: 29, 42, 89, 103, 118, and 123, respectively;
- o) SEQ ID NOs: 29, 42, 89, 101, 147, and 123, respectively; or
- p) SEQ ID NOs: 29, 42, 89, 102, 120, and 123, respectively.

[0013] In some embodiments, an anti-ROR1 antibody or antigen-binding portion described herein may have heavy chain framework regions (FRs) 1-4 and light chain FR1-4 that comprise the amino acid sequences of SEQ ID NOs: 172-179, respectively.

[0014] In some embodiments, an anti-ROR1 antibody or antigen-binding portion described herein may be an IgG. In certain embodiments, the antibody or antigen-binding portion may comprise a human IgG1 constant region.

[0015] In some embodiments, an anti-ROR1 antibody or antigen-binding portion described herein may be a single chain variable fragment (scFv).

[0016] In some embodiments, an anti-ROR1 antibody or antigen-binding portion described herein may:

- a) bind to ROR1-transfected MEC cells with greater affinity than an antibody with a VH comprising the amino acid sequence of SEQ ID NO: 4 and a VL comprising the amino acid sequence of SEQ ID NO: 2, as assessed by flow cytometry;
- b) bind to JeKo-1 cells with greater affinity than an antibody with a VH comprising the amino acid sequence of SEQ ID NO: 4 and a VL comprising the amino acid sequence of SEQ ID NO: 2, as assessed by flow cytometry; or
- c) both a) and b).

[0017] The present disclosure also provides a pharmaceutical composition comprising an anti-ROR1 antibody or antigen-binding portion described herein and a pharmaceutically acceptable excipient. In some embodiments, the pharmaceutical composition further comprises an additional therapeutic agent such as a Bruton's tyrosine kinase (BTK) inhibitor, a B-cell lymphoma 2 (Bcl-2) inhibitor, a mammalian target of rapamycin (mTOR) inhibitor, and/or a phosphoinositide 3-kinase (PI3K) inhibitor. For example, the additional therapeutic agent may be ibrutinib, acalabrutinib, venetoclax, everolimus, sapanisertib, and/or idelalisib.

[0018] The present disclosure also provides an isolated nucleic acid molecule comprising a nucleotide sequence that encodes the heavy chain or an antigen-binding portion thereof, or a nucleotide sequence that encodes the light chain or an antigen-binding portion thereof, or both, of an anti-ROR1 antibody or antigen-binding portion described herein. Additionally, the present disclosure provides a vector comprising such an isolated nucleic acid molecule and further comprising an expression control sequence.

[0019] The present disclosure also provides a host cell comprising a nucleotide sequence that encodes the heavy chain or an antigen-binding portion thereof, and a nucleotide sequence that encodes the light chain or an antigen-binding portion thereof, of an anti-ROR1 antibody or antigen-binding portion described herein. Further, the present disclosure provides a method for producing the anti-ROR1 antibody or portion comprising providing such a host cell, culturing said host cell under conditions suitable for expression of the antibody or portion, and isolating the resulting antibody or portion.

[0020] The present disclosure also provides a bispecific binding molecule comprising the antigen-binding portion (e.g., the six CDRs, or the heavy and light chain variable domains) of an anti-ROR1 antibody described herein.

[0021] The present disclosure also provides an immunoconjugate comprising an anti-ROR1 antibody or antigen-binding portion described herein conjugated to a cytotoxic agent. In certain embodiments, the antibody comprises a heavy chain variable domain (VH) amino acid sequence of SEQ ID NO: 5 and a light chain variable domain (VL) amino acid sequence

of SEQ ID NO: 2. In certain embodiments, the antibody comprises a heavy chain variable domain (VH) amino acid sequence of SEQ ID NO: 5 and a light chain variable domain (VL) amino acid sequence of SEQ ID NO: 6.

[0022] The present disclosure also provides a method for treating cancer in a patient, comprising administering to said patient a therapeutically effective amount of an anti-ROR1 antibody or antigen-binding portion described herein, a pharmaceutical composition described herein, a bispecific binding molecule described herein, or an immunoconjugate described herein. It is understood that the antibody or antigen-binding portion, pharmaceutical composition, bispecific binding molecule, or immunoconjugate may be used for the manufacture of a medicament for treating cancer in a patient or may be for use in treating cancer in a patient. In some embodiments, the cancer is a ROR1-positive cancer. In some embodiments, the cancer is a leukemia, a lymphoma, or a solid tumor. In certain embodiments, the cancer is non-Hodgkin lymphoma (e.g., diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), marginal zone lymphoma (MZL), Burkitt's lymphoma, T cell non-Hodgkin lymphoma, or Waldenström macroglobulinemia), a non-Hodgkin lymphoma that has undergone Richter's transformation, acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), small lymphocytic leukemia (SLL), breast cancer, non-small cell lung cancer, ovarian cancer, or sarcoma. The patient may also be treated with an additional therapeutic agent, such as a Bruton's tyrosine kinase (BTK) inhibitor, a B-cell lymphoma 2 (Bcl-2) inhibitor, a mammalian target of rapamycin (mTOR) inhibitor, and/or a phosphoinositide 3-kinase (PI3K) inhibitor. In certain embodiments, the additional therapeutic agent is ibrutinib, acalabrutinib, venetoclax, everolimus, sapanisertib, or idelalisib.

[0023] The present disclosure also provides a kit comprising an anti-ROR1 antibody or antigen-binding portion described herein, a pharmaceutical composition described herein, a bispecific binding molecule described herein, or an immunoconjugate described herein.

[0024] The present disclosure also provides an article of manufacture comprising an anti-ROR1 antibody or antigen-binding portion described herein, a pharmaceutical composition described herein, a bispecific binding molecule described herein, or an immunoconjugate described herein, wherein said article of manufacture is suitable for treating cancer in a patient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] **FIG. 1** is a pair of photographs of filter lift assays. Panel A (top) shows bacterial expression of both the heavy chain (right, “HA”) and light chain (left, “K”) of the Ab1 Fab. Panel B (bottom) depicts the binding of immobilized Ab1 Fab to biotinylated human ROR1 extracellular domain (huROR1-ECD).

[0026] **FIG. 2** is a graph showing the binding of immobilized huROR1-ECD by the Ab1 Fab and by control chimeric and humanized anti-ROR1 Fabs (“ch α -ROR1 Fab” and “hu α -ROR1 Fab,” respectively) and a negative control Fab (“irrelevant Fab”).

[0027] **FIG. 3** is a representative table showing amino acid representation at each position for H-CDR1 and H-CDR2 of Ab1 after site saturation mutagenesis.

[0028] **FIG. 4** is a photograph showing a capture lift assay indicating the binding of Ab1 variants to human ROR1. The binding variants are numbered, while less intense staining variants are indicated with arrows.

[0029] **FIG. 5** is a graph showing the binding of soluble Fab (Ab1, or variants with H-CDR1 amino acid substitution T32A or T32E) to huROR1-ECD as characterized by ELISA.

[0030] **FIG. 6** is a graph showing the binding of soluble Fab (Ab1, or variants with H-CDR2 amino acid substitution S69V or S69E) to huROR1-ECD as characterized by ELISA.

[0031] **FIG. 7** is a graph showing the binding of soluble Fab (Ab1, or variants with L-CDR1 amino acid substitution A25P, K29E, or K29S) to huROR1-ECD as characterized by ELISA.

[0032] **FIG. 8** is a graph showing the binding of soluble Fab (Ab1, or variants with L-CDR2 amino acid substitution T69H, T69R, or L70K) to huROR1-ECD as characterized by ELISA.

[0033] **FIG. 9** is a pair of graphs showing the binding of soluble Fab to huROR1-ECD as characterized by ELISA. Panel A (top) shows the binding of Ab1 Fab and variants with H-CDR1 amino acid substitution T32A in combination with L-CDR1 amino acid substitution A25P, L-CDR2 amino acid substitution T69H, or L-CDR2 amino acid substitution T69R. Panel B (bottom) shows the binding of Ab1 Fab and variants with H-CDR1 amino acid substitution T32A in combination with L-CDR2 amino acid substitution T69R, L-CDR1 amino acid substitution A25P and L-CDR2 amino acid substitution T69R, L-CDR1 amino acid substitutions A25P and K29S and L-CDR2 amino acid substitution T69R, L-CDR1 amino acid substitution K29E and L-CDR2 amino acid substitution T69R, or L-CDR1 amino acid substitution K29S and L2 amino acid substitution T69R.

[0034] FIG. 10 is a graph showing binding to huROR1-ECD of Ab1 scFv or variants with H-CDR1 amino acid substitution T32A or H-CDR1 amino acid substitution T32A and L-CDR2 amino acid substitution T69R, as characterized by ELISA.

[0035] FIG. 11 is a graph showing binding to ROR1-transfected MEC cells of Ab1 Fab or variants with H-CDR1 amino acid substitution T32A or T32E or H-CDR3 amino acid substitution W110Y, as quantitated using flow cytometry.

[0036] FIG. 12 is a set of graphs showing binding to ROR1-transfected JeKo-1 cells of Ab1 Fab and variants (Panels A and B) and Ab1 scFv and variants (Panel C), as quantitated using flow cytometry.

[0037] FIG. 13 is a graph showing the binding of soluble Fab (Ab1, or variants with H-CDR3 amino acid substitution W110F, W110H, W110M, or W110Y) to huROR1-ECD as characterized by ELISA.

DETAILED DESCRIPTION OF THE INVENTION

[0038] The present disclosure provides new anti-human ROR1 antibodies that can be used to suppress ROR1 activity in a patient, such as a cancer patient. Unless otherwise stated, as used herein, “ROR1” refers to human ROR1. A human ROR1 polypeptide sequence is available under UniProt Accession No. Q01973-1 (SEQ ID NO: 181).

[0039] The term “antibody” (Ab) or “immunoglobulin” (Ig), as used herein, refers to a tetramer comprising two heavy (H) chains (about 50-70 kDa) and two light (L) chains (about 25 kDa) interconnected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable domain (VH) and a heavy chain constant region (CH). Each light chain is composed of a light chain variable domain (VL) and a light chain constant region (CL). The VH and VL domains can be subdivided further into regions of hypervariability, termed “complementarity determining regions” (CDRs), interspersed with regions that are more conserved, termed “framework regions” (FRs). Each VH and VL is composed of three CDRs (H-CDR herein designates a CDR from the heavy chain; and L-CDR herein designates a CDR from the light chain) and four FRs, arranged from amino-terminus to carboxyl-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

[0040] The assignment of amino acid numbers, and of FR and CDR regions, in the heavy or light chain may be in accordance with IMGT® definitions (EU numbering; Lefranc et al., *Dev Comp Immunol.* (2003) 27(1):55-77) or the definitions of Kabat, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, MD (1987 and 1991); Chothia & Lesk, *J. Mol. Biol.* (1987) 196:901-17; Chothia et al., *Nature* (1989) 342:878-83;

MacCallum et al., *J. Mol. Biol.* (1996) 262:732-45; or Honegger and Plückthun, *J. Mol. Biol.* (2001) 309(3):657-70 (“AHO” numbering).

[0041] In some aspects, the anti-ROR1 antibody of the present disclosure is a monoclonal antibody. In some aspects, the anti-ROR1 antibody is a humanized antibody.

[0042] In some aspects, the anti-ROR1 antibody of the present disclosure is a recombinant antibody. The term “recombinant antibody” refers to an antibody that is expressed from a cell or cell line comprising the nucleotide sequence(s) that encode the antibody, wherein said nucleotide sequence(s) are not naturally associated with the cell.

[0043] In some aspects, the anti-ROR1 antibody of the present disclosure is an isolated antibody. The term “isolated protein”, “isolated polypeptide” or “isolated antibody” refers to a protein, polypeptide or antibody that by virtue of its origin or source of derivation (1) is not associated with naturally associated components that accompany it in its native state, (2) is free of other proteins from the same species, (3) is expressed by a cell from a different species, and/or (4) does not occur in nature. Thus, a polypeptide that is chemically synthesized or synthesized in a cellular system different from the cell from which it naturally originates will be “isolated” from its naturally associated components. A protein may also be rendered substantially free of naturally associated components by isolation, using protein purification techniques well known in the art.

[0044] The term “affinity” refers to a measure of the attraction between an antigen and an antibody or an antigen-binding fragment thereof, or a related molecule such as a bispecific binding molecule. The intrinsic attractiveness of the antibody for the antigen is typically expressed as the binding affinity equilibrium constant (K_D) of a particular antibody-antigen interaction. An antibody is said to specifically bind to an antigen when the K_D is ≤ 1 mM, preferably ≤ 100 nM. A K_D binding affinity constant can be measured, e.g., by surface plasmon resonance (BIAcore™) or Bio-Layer Interferometry, for example using the IBIS MX96 SPR system from IBIS Technologies or the Octet™ system from ForteBio.

[0045] The term “epitope” as used herein refers to a portion (determinant) of an antigen that specifically binds to an antibody or a related molecule such as a bispecific binding molecule. Epitopic determinants generally consist of chemically active surface groupings of molecules such as amino acids or carbohydrate or sugar side chains and generally have specific three-dimensional structural characteristics, as well as specific charge characteristics. An epitope may be “linear” or “conformational.” In a linear epitope, all of the points of interaction between a protein (e.g., an antigen) and an interacting molecule (such as an antibody) occur linearly along the primary amino acid sequence of the protein. In a conformational epitope,

the points of interaction occur across amino acid residues on the protein that are separated from one another in the primary amino acid sequence. Once a desired epitope on an antigen is determined, it is possible to generate antibodies to that epitope using techniques well known in the art. For example, an antibody to a linear epitope may be generated, e.g., by immunizing an animal with a peptide having the amino acid residues of the linear epitope. An antibody to a conformational epitope may be generated, e.g., by immunizing an animal with a mini-domain containing the relevant amino acid residues of the conformational epitope. An antibody to a particular epitope can also be generated, e.g., by immunizing an animal with the target molecule of interest (e.g., ROR1) or a relevant portion thereof, then screening for binding to the epitope.

[0046] One can determine whether an antibody binds to the same epitope of ROR1 as or competes for binding with an antibody described herein by using methods known in the art, including, without limitation, competition assays, epitope binning, and alanine scanning. In some aspects, one allows an antibody described herein to bind to ROR1 under saturating conditions, and then measures the ability of the test antibody to bind to said antigen. If the test antibody is able to bind to said antigen at the same time as the reference antibody, then the test antibody binds to a different epitope than the reference antibody. However, if the test antibody is not able to bind to the antigen at the same time, then the test antibody binds to the same epitope, an overlapping epitope, or an epitope that is in close proximity to the epitope bound by the antibody described herein. This experiment can be performed using, e.g., ELISA, RIA, BIACORE™, SPR, Bio-Layer Interferometry or flow cytometry. To test whether an antibody described herein cross-competes with another antibody for binding to ROR1, one may use the competition method described above in two directions, i.e., determining if the known antibody blocks the test antibody and vice versa. Such cross-competition experiments may be performed, e.g., using an IBIS MX96 SPR instrument or the Octet™ system.

[0047] The term “antigen-binding portion” or “antigen-binding fragment” of an antibody, as used herein, refers to one or more portions or fragments of an antibody that retain the ability to specifically bind to an antigen (e.g., human ROR1, or a portion thereof). It has been shown that certain fragments of a full-length antibody can perform the antigen-binding function of the antibody. Examples of binding fragments encompassed within the term “antigen-binding portion” include (i) a Fab fragment: a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment: a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) an Fd fragment

consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment, which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR) capable of specifically binding to an antigen. Furthermore, although the two domains of the Fv fragment, VL and VH, are encoded by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH domains pair to form monovalent molecules known as single chain variable fragments (scFvs). Also within the present disclosure are antigen-binding molecules comprising a VH and/or a VL. In the case of a VH, the molecule may also comprise one or more of a CH1, hinge, CH2, or CH3 region. Such single chain antibodies are also intended to be encompassed within the term “antigen-binding portion” of an antibody. Other forms of single chain antibodies, such as diabodies, are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen-binding sites.

[0048] In certain aspects, an antigen-binding portion of the present disclosure is a single-chain antibody fragment comprising a variable heavy chain region and/or a variable light chain region, such as an scFv. To create an scFv, VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH domains joined by the flexible linker. In certain aspects, the flexible linker may have the amino acid sequence (Gly₄Ser)₃ (SEQ ID NO: 180).

[0049] Unless otherwise indicated, all antibody amino acid residue numbers referred to in this disclosure are those under the AHo numbering scheme.

Anti-ROR1 Antibodies

[0050] The present disclosure is based on the discovery that certain variant antibodies of a parent humanized anti-ROR1 antibody (“Ab1”) have superior binding affinity for human ROR1 as compared to the parent antibody, which comprises the amino acid sequences shown below (CDRs underlined in the VH and VL sequences):

Ab1 VH (SEQ ID NO: 4)

QVQLQESGPG LVKPSQTL^{SL} TCTVSGYAFT AYN^{NIH}WVRQA PGQGLEWMGS
 FDPYDGGSSY NQKFKDRLTI SKDTSKNQVV LTMTNMDPVD TATYYCARGW
 YYFDYWGHGT LVTVSS

Ab1 VL (SEQ ID NO: 2)

DIVMTQTPLS LPVTPGEPAS ISCRASKSIS KYLA^{WYQQK}P GQAPRLLIYS
 GSTLQSGIPP RFSGSGYGTDFLTINNIES EDAAYYFCQQ HDESPYTFGE GTKVEIK

Description	Sequence	SEQ ID NO
H-CDR1	GYAFTAYNIH	SEQ ID NO: 13
H-CDR2	SFDPYDGGSSYNQKFKD	SEQ ID NO: 42
H-CDR3	ARGWYYFDY	SEQ ID NO: 89
L-CDR1	RASKSISKYLA	SEQ ID NO: 101
L-CDR2	SGSTLQS	SEQ ID NO: 114
L-CDR3	QQHDESPYT	SEQ ID NO: 123
H-FR1	QVQLQESGPGLVKPSQTL ^{SL} TCTVS	SEQ ID NO: 172
H-FR2	WVRQAPGQGLEWMG	SEQ ID NO: 173
H-FR3	RLTISKDTSKNQVVLTMTNMDPVD ^T TATYYC	SEQ ID NO: 174
H-FR4	WGHGT ^L LVTVSS	SEQ ID NO: 175
L-FR1	DIVMTQTPLSLPVT ^P GPASIS	SEQ ID NO: 176
L-FR2	WYQQKPGQAPRLLIY	SEQ ID NO: 177
L-FR3	GIPPRFSGSGYGTDFLTINNIESED ^A AYYFC	SEQ ID NO: 178
L-FR4	FGEGTKVEIK	SEQ ID NO: 179

[0051] Certain therapeutic modalities may benefit from using smaller, monovalent versions of Ab1 rather than the intact IgG1 antibody. Such smaller versions may include, e.g., Fab constructs and even smaller scFv binding formats. However, a limitation of such monovalent formats is that the higher binding achieved through the bivalent interaction of IgG with cell surface ROR1 (avidity) is lost. One approach to addressing the loss of binding avidity is to engineer a higher affinity version of Ab1. Additionally, it is possible that enhanced tumor targeting and cytotoxicity could be achieved with higher affinity IgG variants of Ab1. Accordingly, the present disclosure provides novel antibodies directed against ROR1, and antigen-binding portions thereof, that are higher affinity variants of Ab1.

[0052] In some aspects, the anti-ROR1 antibody or antigen-binding portion has VH and VL amino acid sequences that comprise, in total, at least one, two, three, four, five, six, seven, or eight amino acid substitutions from the VH and VL amino acid sequences of Ab1. In certain aspects, the VH and VL amino acid sequences comprise, in total, one amino acid substitution from the VH and VL amino acid sequences of Ab1. In certain aspects, the VH and VL amino acid sequences comprise, in total, two amino acid substitutions from the VH and VL amino acid sequences of Ab1.

[0053] In some aspects, the amino acid substitution(s) are not in H-CDR3 or L-CDR3. In some aspects, the amino acid substitution(s) are in H-CDR1, H-CDR2, L-CDR1, and/or L-CDR2, or any combination thereof (e.g., H-CDR1 alone, or H-CDR1 and L-CDR2). The CDRs may be delineated by the Kabat, Chothia, IMGT, contact, or AHO method, or any combination thereof. In certain aspects, the heavy chain CDRs are delineated by a combination of Kabat and IMGT definitions. In certain aspects, the light chain CDRs are delineated by the Kabat definition.

[0054] In some aspects, the amino acid substitution(s) are in:

- H-CDR1, at residue 27, 29, 30, 31, 32, 33, 39, 40, 41, and/or 42;
- H-CDR2, at residue 57, 58, 59, 60, 65, 66, 67, 68, 69, 70, 71, 72, 73, 75, and/or 76;
- H-CDR3, at residue 107, 108, 110, 111, 136, and/or 138;
- L-CDR1, at residue 25, 29, 30, 31, 32, and/or 41;
- L-CDR2, at residue 68, 69, 70, and/or 71;
- L-CDR3, at residue 107, 108, 109, 110, 111, 135, 137, and/or 138; or
- any combination of the above;

wherein the residues are numbered according to the AHO system.

[0055] In some aspects, the amino acid substitution(s) are in:

- H-CDR1, at residue 32;
- H-CDR2, at residue 69 and/or residue 71;
- L-CDR1, at residue 25 and/or 29;
- L-CDR2, at residue 69 and/or 70; or
- any combination of the above;

wherein the residues are numbered according to the AHO system.

[0056] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises the CDR amino acid sequences, the heavy and light variable domain amino acid sequences, or the heavy and light chain amino acid sequences of Ab1 with any combination of the mutations described in Tables 1-7 of the Examples.

[0057] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises:

- a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from G27D, G27N, G27R, G27T, Y29G, Y29K, Y29S, A30I, A30P, A30R, A30T, F31I, F31L, F31V, F31W, T32A, T32D, T32E, A33M, A33S, Y39F, Y39W, N40K, N40P, N40R, I41L, I41V, H42Q, S57A, S57K, S57R, S57T, F58I, F58P, F58V, D59S, P60G, P60T, D65E, D65N, D65T, G66D, G66E, G66H, G66N, G67H, G67K,

- G67M, G67P, G67Q, G67R, G67S, G67Y, S68A, S68E, S68I, S68Q, S68T, S69E, S69K, S69V, Y70I, Y70V, N71A, N71G, N71H, N71S, Q72N, Q72P, K73C, K73S, K75A, K75G, D76V, A107T, A107V, R108G, W110C, W110F, Y111A, Y111L, F136L, Y138Q, Y138T, and Y138V, or any combination thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- a VH comprising the amino acid sequence of SEQ ID NO: 4; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E, K29S, S30A, S30F, S30H, S30P, I31V, S32A, S32I, L41M, L41V, S68G, S68R, T69H, T69R, L70H, L70K, Q71T, Q71V, Q107S, Q108A, Q108L, Q108R, H109N, H109Q, D110H, D110N, E111I, E111L, S135G, S135H, S135P, S135V, Y137T, Y137V, T138F, and T138S, or any combination thereof; or
 - a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from G27D, G27N, G27R, G27T, Y29G, Y29K, Y29S, A30I, A30P, A30R, A30T, F31I, F31L, F31V, F31W, T32A, T32D, T32E, A33M, A33S, Y39F, Y39W, N40K, N40P, N40R, I41L, I41V, H42Q, S57A, S57K, S57R, S57T, F58I, F58P, F58V, D59S, P60G, P60T, D65E, D65N, D65T, G66D, G66E, G66H, G66N, G67H, G67K, G67M, G67P, G67Q, G67R, G67S, G67Y, S68A, S68E, S68I, S68Q, S68T, S69E, S69K, S69V, Y70I, Y70V, N71A, N71G, N71H, N71S, Q72N, Q72P, K73C, K73S, K75A, K75G, D76V, A107T, A107V, R108G, W110C, W110F, Y111A, Y111L, F136L, Y138Q, Y138T, and Y138V, or any combination thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E, K29S, S30A, S30F, S30H, S30P, I31V, S32A, S32I, L41M, L41V, S68G, S68R, T69H, T69R, L70H, L70K, Q71T, Q71V, Q107S, Q108A, Q108L, Q108R, H109N, H109Q, D110H, D110N, E111I, E111L, S135G, S135H, S135P, S135V, Y137T, Y137V, T138F, and T138S, or any combination thereof.

[0058] For example, in some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises:

- a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from T32A/E, F58I/V, G66E, G67P/Q, S69E/VV, N71A/H, and Q72P, or any combination thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- a VH comprising the amino acid sequence of SEQ ID NO: 4; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E/S, T69H/R, L70H/K, and Q71T, or any combination thereof; or

- a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from T32A/E, F58I/V, G66E, G67P/Q, S69E/V, N71A/H, and Q72P, or any combination thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E/S, T69H/R, L70H/K, and Q71T, or any combination thereof.

[0059] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises:

- a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from T32A/E, S69V/E, and N71H, or any combination thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- a VH comprising the amino acid sequence of SEQ ID NO: 4; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E/S, T69H/R, L70K, and Q71T, or any combination thereof; or
- a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from T32A/E, S69V/E, and N71H, or any combination thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E/S, T69H/R, L70K, and Q71T, or any combination thereof.

[0060] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises a VL comprising the amino acid sequence of SEQ ID NO: 2, and a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from:

- a) T32A or T32E;
- b) F58I or F58V;
- c) G66E;
- d) G67P or G67Q;
- e) S69V or S69E;
- f) N71A or N71H;
- g) Q72P; or
- h) any combination of a)-g).

For example, the VH may comprise the amino acid sequence of SEQ ID NO: 4 with

- i) one or more mutations selected from T32E, S69V/E, and N71H, or
- ii) the mutation T32A and one or both mutations selected from S69V/E, and N71H (i.e., S69V; S69E; N71H; S69V and N71H; or S69E and N71H).

[0061] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from:

- a) A25P;
- b) K29E or K29S;
- c) T69H or T69R;
- d) L70H or L70K;
- e) Q71T; or
- f) any combination of a)-e).

[0062] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises:

- a) a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from:
 - i) T32A or T32E;
 - ii) F58I or F58V;
 - iii) G66E;
 - iv) G67P or G67Q;
 - v) S69V or S69E;
 - vi) N71A or N71H;
 - vii) Q72P; or
 - viii) any combination of i)-vii); and
- b) a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from:
 - i) A25P;
 - ii) K29E or K29S;
 - iii) T69H or T69R;
 - iv) L70H or L70K;
 - v) Q71T; or
 - vi) any combination of i)-v).

For example, the VH may comprise the amino acid sequence of SEQ ID NO: 4 with

- i) one or more mutations selected from T32E, S69V/E, and N71H, or
 - ii) the mutation T32A and one or both mutations selected from S69V/E and N71H,
- and the VL may comprise the amino acid sequence of SEQ ID NO: 2 with one or more mutations selected from A25P, K29E/S, T69H/R, L70K, and Q71T.

As another example, the VH may comprise the amino acid sequence of SEQ ID NO: 4 comprising the mutation T32A and the VL may comprise the amino acid sequence of SEQ ID NO: 2 with

- i) one or more mutations selected from A25P, K29E/S, T69H, L70K, and Q71T, or
- ii) the mutation T69R and one or more mutations selected from A25P, K29E/S, L70K, and Q71T.

[0063] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises:

- a) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- b) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32E, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- c) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation F58I, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- d) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation F58V, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- e) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation G66E, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- f) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation G67P, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- g) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation G67Q, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- h) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation S69E, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- i) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation S69V, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- j) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation N71A, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- k) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation N71H, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- l) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation Q72P, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- m) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation Q72P, and a VL comprising the amino acid sequence of SEQ ID NO: 2;

- n) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutations T32A and F58V, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- o) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutations T32A, F58I, G66E, and N71H, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- p) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutations T32A, G66E, S69V, and N71H, and a VL comprising the amino acid sequence of SEQ ID NO: 2;
- q) a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation A25P;
- r) a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation K29E;
- s) a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation K29S;
- t) a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation T69H;
- u) a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation T69R;
- v) a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation L70H;
- w) a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation L70K;
- x) a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation Q71T;
- y) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation A25P;
- z) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation T69H;
- aa) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation T69R;

- bb) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations A25P and T69R;
- cc) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations K29S and T69R;
- dd) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations T69R and Q71T; or
- ee) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations A25P and L70K.

[0064] In any of the above aspects, the VH may further comprise mutation W110F, W110H, W110M, or W110Y. In certain aspects, said mutation may increase target binding, increase antibody stability, reduce aggregation, or any combination thereof.

[0065] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises an H-CDR3 amino acid sequence of SEQ ID NO: 89 and an L-CDR3 amino acid sequence of SEQ ID NO: 123, and further comprises:

- an H-CDR1 amino acid sequence of SEQ ID NO: 13, 29, 31, 35, or 40;
- an H-CDR2 amino acid sequence of SEQ ID NO: 42, 47, 49, 57, 58, 63, 64, 65, 68, 73, 75, 76, 77, 78, 79, 80, 81, 82, 83, or 88;
- an L-CDR1 amino acid sequence of SEQ ID NO: 101, 102, 103, 104, 105, 107, 110, 112, or 113;
- an L-CDR2 amino acid sequence of SEQ ID NO: 114, 117, 118, 119, 120, 121, or 122; or
- any combination of the above H-CDR1, H-CDR2, L-CDR1, and L-CDR2 amino acid sequences,

wherein the antibody does not comprise heavy chain (H)-CDR1-3 amino acid sequences of SEQ ID NOs: 13, 42, and 89, respectively, and light chain (L)-CDR1-3 amino acid sequences of SEQ ID NOs: 101, 114, and 123, respectively. In certain aspects, the VH of the anti-ROR1 antibody or antigen-binding portion further comprises the FR1, FR2, FR3, and FR4 amino acid sequences of SEQ ID NOs: 172-175, respectively, and the VL further comprises the FR1, FR2, FR3, and FR4 amino acid sequences of SEQ ID NOs: 176-179, respectively.

[0066] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises an H-CDR3 amino acid sequence of SEQ ID NO: 89 and an L-CDR3 amino acid sequence of SEQ ID NO: 123, and further comprises:

- an H-CDR1 amino acid sequence of SEQ ID NO: 13, 29 or 31;
- an H-CDR2 amino acid sequence of SEQ ID NO: 42, 47, 49, 57, 63, 64, 73, 75, 78, 80, or 83;
- an L-CDR1 amino acid sequence of SEQ ID NO: 101, 102, 103, or 104;
- an L-CDR2 amino acid sequence of SEQ ID NO: 114, 117, 118, 119, 120, or 121; or
- any combination of the above H-CDR1, H-CDR2, L-CDR1, and L-CDR2 amino acid sequences,

wherein the antibody does not comprise heavy chain (H)-CDR1-3 amino acid sequences of SEQ ID NOs: 13, 42, and 89, respectively, and light chain (L)-CDR1-3 amino acid sequences of SEQ ID NOs: 101, 114, and 123, respectively. In certain aspects, the VH of the anti-ROR1 antibody or antigen-binding portion further comprises the amino acid sequences of SEQ ID NOs: 172-175 and the VL further comprises the amino acid sequences of SEQ ID NOs: 176-179.

[0067] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises heavy chain (H)-CDR1-3 and light chain (L)-CDR1-3 comprising the amino acid sequences of:

- SEQ ID NOs: 29, 42, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 42, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 47, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 49, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 57, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 63, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 64, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 73, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 75, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 78, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 80, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 83, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 102, 114, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 103, 114, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 104, 114, and 123, respectively;

- SEQ ID NOs: 13, 42, 89, 101, 117, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 101, 118, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 101, 119, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 101, 120, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 101, 121, and 123, respectively;
- SEQ ID NOs: 29, 49, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 29, 161, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 29, 166, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 102, 114, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 101, 117, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 101, 118, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 102, 118, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 103, 118, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 101, 147, and 123, respectively; or
- SEQ ID NOs: 29, 42, 89, 102, 120, and 123, respectively.

In certain aspects, the VH of the anti-ROR1 antibody or antigen-binding portion further comprises the amino acid sequences of SEQ ID NOs: 172-175 and the VL further comprises the amino acid sequences of SEQ ID NOs: 176-179.

[0068] In any of the above aspects, the H-CDR3 amino acid sequence of SEQ ID NO: 89 may be replaced by the amino acid sequence of SEQ ID NO: 94, 169, 170, or 171. In certain aspects, said replacement may increase target binding, increase antibody stability, reduce aggregation, or any combination thereof.

[0069] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises a heavy chain variable domain (VH) amino acid sequence of SEQ ID NO: 5 and a light chain variable domain (VL) amino acid sequence of SEQ ID NO: 2.

[0070] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure comprises a heavy chain variable domain (VH) amino acid sequence of SEQ ID NO: 5 and a light chain variable domain (VL) amino acid sequence of SEQ ID NO: 6.

[0071] The class of an anti-ROR1 antibody obtained by the methods described herein may be changed or switched with another class or subclass. In some aspects of the present disclosure, a nucleic acid molecule encoding VL or VH is isolated using methods well known in the art such that it does not include nucleic acid sequences encoding CL or CH, respectively. The nucleic acid molecules encoding VL or VH then are operatively linked to a nucleic acid sequence encoding a CL or CH, respectively, from a different class of

immunoglobulin molecule. This may be achieved using a vector or nucleic acid molecule that comprises a CL or CH chain, as described above. For example, an anti-ROR1 antibody that was originally IgM may be class switched to IgG. Further, the class switching may be used to convert one IgG subclass to another, e.g., from IgG₁ to IgG₂. A κ light chain constant region can be changed, e.g., to a λ light chain constant region, or vice-versa. An exemplary method for producing an antibody of the present disclosure with a desired Ig isotype comprises the steps of isolating a nucleic acid molecule encoding the heavy chain of an anti-ROR1 antibody and a nucleic acid molecule encoding the light chain of an anti-ROR1 antibody, obtaining the variable domain of the heavy chain, ligating a coding sequence for the variable domain of the heavy chain with a coding sequence for the constant region of a heavy chain of the desired isotype, expressing the light chain and the heavy chain encoded by the ligated sequence in a cell, and collecting the anti-ROR1 antibody with the desired isotype.

[0072] The anti-ROR1 antibody of the present disclosure can be an IgG, an IgM, an IgE, an IgA, or an IgD molecule, but is typically of the IgG isotype, e.g., of IgG subclass IgG₁, IgG_{2a} or IgG_{2b}, IgG₃ or IgG₄. In some aspects, the antibody is of the isotype subclass IgG₁.

[0073] In some aspects, the anti-ROR1 antibody may comprise at least one mutation in the Fc region. A number of different Fc mutations are known, where these mutations alter the antibody's effector function. For example, in many cases it will be desirable to reduce or eliminate effector function, e.g., where ligand/receptor interactions are undesired or in the case of antibody-drug conjugates. In particular aspects, the antibody may comprise an IgG constant region that includes mutations that reduce or eliminate effector function (see, e.g., Wang et al., *Protein Cell* (2018) 9(1):63-73). For example, an anti-ROR1 antibody of the present disclosure may comprise a human IgG₁ constant region with the mutation L235E, "LALA" mutations (L234A/L235A), or "LALAGA" mutations (L234A/L235A/G237A), all numbered according to the EU numbering scheme.

[0074] In some cases, it will be desirable to increase effector function, including antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) (see, e.g., Wang et al., *Protein Cell* (2018) 9(1):63-73). For example, an anti-ROR1 antibody of the present disclosure may comprise a human IgG₁ constant region with any one of the following sets of mutations to enhance ADCC: F234L/R292P/Y300L/V305I/P396L, S293D/I332E, S239D/I332E/A330L, S298A/E333A/K334A, or L234Y/L235Q/G236W/S239M/H268D/D270E/S298A in one heavy chain and D270E/K326D/A330M/K334E in the other heavy chain, all numbered according to the EU

numbering scheme. In some cases, an anti-ROR1 antibody of the present disclosure may comprise a human IgG1 constant region with the following mutations to enhance ADCP: G236A/S239D/I332E or any one set of the following mutations to enhance CDC: K326W/E333S, S267E/H268F/S324T, IgG1/IgG3 cross subclass (Natsume et al., *Cancer Res.* (2008) 68:3863-3872), or E345R/E430G/S440Y, all numbered according to the EU numbering scheme.

[0075] In some cases, it may also be beneficial to incorporate Fc mutations that increase the serum half-life of the antibody. For example, in some cases, an anti-ROR1 antibody of the present disclosure may comprise a human IgG1 constant region with either of the following sets of mutations to enhance FcRn binding at pH 6.0 and increase the antibody half-life: M252Y/S254T/T256E or M428L/N434S, all numbered according to the EU numbering scheme.

[0076] In some aspects, the anti-ROR1 antigen-binding portion of the present disclosure may be, e.g., an scFv, a Fab fragment; a F(ab')₂ fragment; an Fd fragment; an Fv fragment; a dAb fragment; a diabody, or an isolated CDR capable of specifically binding to human ROR1. In certain aspects, modified antibodies may be prepared using anti-ROR1 antibody-encoding nucleic acid molecules. For instance, “kappa bodies” (Ill et al., *Protein Eng.* (1997) 10:949-57), “minibodies” (Martin et al., *EMBO J.* (1994) 13:5303-9), “diabodies” (Holliger et al., *Proc. Natl. Acad. Sci. USA* (1993) 90:6444-8), or “Janusins” (Traunecker et al., *EMBO J.* (1991) 10:3655-3659 and Traunecker et al., *Int. J. Cancer* (Suppl.) (1992) 7:51-2) may be prepared using standard molecular biological techniques following the teachings of the specification.

[0077] In certain aspects, the anti-ROR1 antigen-binding portion is an scFv. The scFv may comprise, e.g., the six CDRs, or the VH and VL, of an anti-ROR1 antibody described herein. The VH and VL of the scFv may be joined by a flexible linker, e.g., comprising the amino acid sequence of SEQ ID NO: 180. In particular aspects, the scFv comprises the amino acid sequence of SEQ ID NO: 10 or 12.

[0078] In some aspects, the anti-ROR1 antibody or antigen-binding portion of the present disclosure has one, two, three, four, or five properties selected from:

- competes for binding with, or binds to the same epitope of human ROR1 as, Ab1;
- binds to human ROR1 with greater affinity than Ab1 (e.g., with a K_D that is less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% of the K_D of Ab1 as determined by ELISA);

- binds to human ROR1 with a K_D of 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, or 1.0 nM or less as determined by ELISA;
- binds to ROR1-transfected MEC cells with greater affinity than an antibody with a VH comprising the amino acid sequence of SEQ ID NO: 4 and a VL comprising the amino acid sequence of SEQ ID NO: 2 (e.g., Ab1) as assessed by flow cytometry; and
- binds to ROR1-transfected JeKo-1 cells with greater affinity than an antibody with a VH comprising the amino acid sequence of SEQ ID NO: 4 and a VL comprising the amino acid sequence of SEQ ID NO: 2 (e.g., Ab1) as assessed by flow cytometry.

In certain aspects, the anti-ROR1 antibody or antigen-binding portion has all of said properties.

[0079] An anti-ROR1 antibody or antigen-binding portion of the present disclosure can be derivatized or linked to another molecule (e.g., another peptide or protein). In general, the antibodies or portions thereof are derivatized such that ROR1 binding is not affected adversely by the derivatization or labeling. Accordingly, the antibodies and antibody portions of the present disclosure are intended to include both intact and modified forms of the anti-ROR1 antibodies and portions described herein. For example, an antibody or antibody portion of the present disclosure can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (e.g., to form a bispecific antibody or a diabody), a detection agent, a pharmaceutical agent, and/or a protein or peptide that can mediate association of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

[0080] One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, e.g., to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (e.g., m-maleimidobenzoyl-N-hydroxysuccinimide ester) or homobifunctional (e.g., disuccinimidyl suberate). Such linkers are available, e.g., from Pierce Chemical Company, Rockford, IL.

[0081] An anti-ROR1 antibody or antigen-binding portion thereof can also be derivatized with a chemical group such as polyethylene glycol (PEG), a methyl or ethyl group, or a carbohydrate group. These groups may be useful to improve the biological characteristics of the antibody, e.g., to increase serum half-life.

[0082] An antibody or antigen-binding portion according to the present disclosure may also be labeled. As used herein, the terms “label” or “labeled” refer to incorporation of another

molecule in the antibody. In some aspects, the label is a detectable marker, e.g., incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). In some aspects, the label or marker can be therapeutic, e.g., a drug conjugate or toxin. Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes or radionuclides (e.g., ^3H , ^{14}C , ^{15}N , ^{35}S , ^{90}Y , ^{99}Tc , ^{111}In , ^{125}I , ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, β -galactosidase, luciferase, alkaline phosphatase), chemiluminescent markers, biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags), magnetic agents, such as gadolinium chelates, toxins such as pertussis toxin, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicine, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. In some aspects, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

[0083] In certain aspects, the antibodies of the present disclosure may be present in a neutral form (including zwitter ionic forms) or as a positively or negatively-charged species. In some aspects, the antibodies may be complexed with a counterion to form a pharmaceutically acceptable salt.

Bispecific Binding Molecules

[0084] In a further aspect, the present disclosure provides a bispecific binding molecule having the binding specificity (e.g., comprising the antigen-binding portion, such as the six CDRs or the VH and VL) of an anti-ROR1 antibody described herein and the binding specificity of a second antibody. The second antibody may be, e.g., another anti-ROR1 antibody described herein, or an antibody that targets a different protein, such as a cancer antigen or another cell surface molecule whose activity mediates a disease condition such as cancer. Such bispecific binding molecules are known in the art.

[0085] In some aspects, the bispecific binding molecule comprises heavy chain (H)-CDR1-3 and light chain (L)-CDR1-3 comprising the amino acid sequences of:

- SEQ ID NOs: 29, 42, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 42, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 47, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 49, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 57, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 63, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 31, 64, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 73, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 75, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 78, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 80, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 83, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 102, 114, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 103, 114, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 104, 114, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 101, 117, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 101, 118, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 101, 119, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 101, 120, and 123, respectively;
- SEQ ID NOs: 13, 42, 89, 101, 121, and 123, respectively;
- SEQ ID NOs: 29, 49, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 29, 161, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 29, 166, 89, 101, 114, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 102, 114, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 101, 117, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 101, 118, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 102, 118, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 103, 118, and 123, respectively;
- SEQ ID NOs: 29, 42, 89, 101, 147, and 123, respectively; or
- SEQ ID NOs: 29, 42, 89, 102, 120, and 123, respectively.

In certain aspects, the bispecific binding molecule further comprises the amino acid sequences of SEQ ID NOs: 172-179.

[0086] In some aspects, the bispecific binding molecule comprises the six CDR amino acid sequences, or the heavy and light chain variable domain amino acid sequences, of Ab1 with any one or combination of the mutations found in Tables 1-7 of the Examples.

[0087] In some aspects, the bispecific binding molecule comprises VH and VL amino acid sequences of SEQ ID NO: 5 and SEQ ID NO: 2, respectively, or SEQ ID NO: 5 and SEQ ID NO: 6, respectively.

Immunoconjugates

[0088] In a further aspect, the present disclosure provides an immunoconjugate comprising an anti-ROR1 antibody or antigen-binding portion described herein conjugated to a cytotoxic agent.

Chimeric Antigen Receptor-T cells or -NK cells

[0089] The present disclosure also contemplates the use of sequences (e.g., the six CDR or VH and VL sequences) of an anti-ROR1 antibody or antigen-binding portion described herein in the preparation of a chimeric antigen receptor (CAR). The CAR may further comprise, e.g., sequences encoding either a T-cell activating function (CAR-T) or an NK-cell activating function (CAR-NK).

Nucleic Acid Molecules and Vectors

[0090] The present disclosure also provides nucleic acid molecules and sequences encoding anti-ROR1 antibodies or antigen-binding portions described herein. In some aspects, different nucleic acid molecules encode the heavy chain and light chain amino acid sequences of the anti-ROR1 antibody or antigen-binding portion. In other aspects, the same nucleic acid molecule encodes the heavy chain and light chain amino acid sequences of the anti-ROR1 antibody or antigen-binding portion. The present disclosure thus provides an isolated nucleic acid molecule comprising a nucleotide sequence that encodes the heavy chain or an antigen-binding portion thereof, or a nucleotide sequence that encodes the light chain or an antigen-binding portion thereof, or both, of an anti-ROR1 antibody or antigen-binding portion described herein.

[0091] A reference to a nucleotide sequence encompasses its complement unless otherwise specified. Thus, a reference to a nucleic acid having a particular sequence should be understood to encompass its complementary strand, with its complementary sequence. The term “polynucleotide” as referred to herein means a polymeric form of nucleotides of at least

10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single- and double-stranded forms.

[0092] In some aspects, the present disclosure provides a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 9 or 11.

[0093] In any of the above aspects, the nucleic acid molecules may be isolated. Nucleic acid molecules referred to herein as “isolated” or “purified” are nucleic acids which (1) have been separated away from the nucleic acids of the genomic DNA or cellular RNA of their source of origin; and/or (2) do not occur in nature.

[0094] The present disclosure further provides a vector comprising nucleic acid molecules that encode the heavy chain, the light chain, or both the heavy and light chains of an anti-ROR1 antibody as described herein or an antigen-binding portion thereof. In certain aspects, a vector of the present disclosure comprises a nucleic acid molecule as described herein. The vector may further comprise an expression control sequence.

[0095] The term “expression control sequence” as used herein means polynucleotide sequences that are necessary to effect the expression and processing of coding sequences to which they are ligated. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term “control sequences” is intended to include, at a minimum, all components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

[0096] In further aspects of the present disclosure, nucleic acid molecules encoding the VH and/or VL of an anti-ROR1 antibody or antigen-binding portion described herein may be “converted” to full-length antibody genes. In some aspects, nucleic acid molecules encoding the VH or VL domains are converted to full-length antibody genes by insertion into an expression vector already encoding heavy chain constant (CH) or light chain constant (CL) regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector, and/or the VL segment is operatively linked to the CL segment within the

vector. In some aspects, nucleic acid molecules encoding the VH and/or VL domains are converted into full-length antibody genes by linking, e.g., ligating, a nucleic acid molecule encoding a VH and/or VL domain to a nucleic acid molecule encoding a CH and/or CL region using standard molecular biological techniques. Nucleic acid molecules encoding the full-length heavy and/or light chains may then be expressed from a cell into which they have been introduced and the anti-ROR1 antibody or antigen-binding portion isolated.

[0097] In some aspects, the framework region(s) are mutated so that the resulting framework region(s) have the amino acid sequence of the corresponding germline gene. A mutation may be made in a framework region or constant region, e.g., to increase the half-life of the anti-ROR1 antibody. See, e.g., PCT Publication WO 00/09560. A mutation in a framework region or constant region also can be made to alter the immunogenicity of the antibody, and/or to provide a site for covalent or non-covalent binding to another molecule. According to the present disclosure, an antibody may have mutations in any one or more of the CDRs or framework regions of the variable domain or in the constant region.

Host Cells and Methods of Antibody Production

[0098] The present disclosure also provides methods for producing the antibodies and antigen-binding portions thereof described herein. In some aspects, the present disclosure provides a host cell comprising a nucleotide sequence that encodes the heavy chain or an antigen-binding portion thereof, and a nucleotide sequence that encodes the light chain or an antigen-binding portion thereof, of an anti-ROR1 antibody or antigen-binding portion described herein. In some aspects, the present disclosure relates to a method for producing an anti-ROR1 antibody or antigen-binding portion as described herein, comprising providing said host cell; culturing said host cell under conditions suitable for expression of the antibody or antigen-binding portion; and isolating the resulting antibody or antigen-binding portion. Antibodies or antigen-binding portions produced by such expression in such recombinant host cells are referred to herein as “recombinant” antibodies or antigen-binding portions. The present disclosure also provides progeny cells of such host cells, and antibodies or antigen-binding portions produced by same.

[0099] The term “recombinant host cell” (or simply “host cell”), as used herein, means a cell into which a recombinant expression vector has been introduced. By definition, a recombinant host cell does not occur in nature. The present disclosure provides host cells that may comprise, e.g., a vector as described herein. The present disclosure also provides host cells that comprise, e.g., a nucleotide sequence encoding the heavy chain or an antigen-

binding portion thereof, a nucleotide sequence encoding the light chain or an antigen-binding portion thereof, or both, of an anti-ROR1 antibody or antigen-binding portion thereof described herein. It should be understood that “recombinant host cell” and “host cell” mean not only the particular subject cell but also the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term “host cell” as used herein.

[0100] Nucleic acid molecules encoding anti-ROR1 antibodies and antigen-binding portions thereof and vectors comprising these nucleic acid molecules can be used for transfection of a suitable mammalian, plant, bacterial or yeast host cell. Transformation can be by any known method for introducing polynucleotides into a host cell. Methods for introduction of heterologous polynucleotides into mammalian cells are well known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene-mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei. In addition, nucleic acid molecules may be introduced into mammalian cells by viral vectors.

[0101] It is likely that antibodies expressed by different cell lines or in transgenic animals will have different glycosylation patterns from each other. However, all antibodies encoded by the nucleic acid molecules provided herein, or comprising the amino acid sequences provided herein are part of the present disclosure, regardless of the glycosylation state of the antibodies, and more generally, regardless of the presence or absence of post-translational modification(s).

Pharmaceutical Compositions

[0102] Another aspect of the present disclosure is a pharmaceutical composition comprising as an active ingredient (or as the sole active ingredient) an anti-ROR1 antibody or antigen-binding portion thereof of the present disclosure. The pharmaceutical composition may additionally comprise a pharmaceutically acceptable excipient. In some aspects, the pharmaceutical compositions are intended for amelioration, prevention, and/or treatment of cancer, e.g., a cancer described herein.

[0103] In some aspects, the pharmaceutical composition may comprise an anti-ROR1 antibody or antigen-binding portion thereof, and one or more additional antibodies that target one or more relevant cell surface receptors, e.g., one or more cancer-relevant receptors.

[0104] Generally, the antibodies, antigen-binding portions, bispecific binding molecules, and immunoconjugates of the present disclosure are suitable to be administered as a formulation in association with one or more pharmaceutically acceptable excipient(s), e.g., as described below.

[0105] The term “excipient” is used herein to describe any ingredient other than the compound(s) of the present disclosure. The choice of excipient(s) will to a large extent depend on factors such as the particular mode of administration, the effect of the excipient on solubility and stability, and the nature of the dosage form. As used herein, “pharmaceutically acceptable excipient” includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Some examples of pharmaceutically acceptable excipients are water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Additional examples of pharmaceutically acceptable substances are wetting agents or minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody.

[0106] Pharmaceutical compositions of the present disclosure and methods for their preparation will be readily apparent to those skilled in the art. Such compositions and methods for their preparation may be found, for example, in *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company, 1995). Pharmaceutical compositions are preferably manufactured under GMP (good manufacturing practices) conditions.

[0107] A pharmaceutical composition of the present disclosure may be prepared, packaged, or sold in bulk, as a single unit dose, or as a plurality of single unit doses. As used herein, a “unit dose” is a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[0108] Formulations of a pharmaceutical composition suitable for parenteral administration (e.g., intravenous administration) typically comprise the active ingredient combined with a pharmaceutically acceptable carrier, such as sterile water or sterile isotonic saline. Such formulations may be prepared, packaged, or sold in a form suitable for bolus administration or for continuous administration. Injectable formulations may be prepared, packaged, or sold

in unit dosage form, such as in ampoules or in multi-dose containers containing a preservative. Formulations for parenteral administration include, but are not limited to, suspensions, solutions, emulsions in oily or aqueous vehicles, pastes, and the like. Such formulations may further comprise one or more additional ingredients including, but not limited to, suspending, stabilizing, or dispersing agents. In some aspects of a formulation for parenteral administration, the active ingredient is provided in dry (i.e., powder or granular) form for reconstitution with a suitable vehicle (e.g., sterile pyrogen-free water) prior to parenteral administration of the reconstituted composition. Parenteral formulations also include aqueous solutions which may contain excipients such as salts, carbohydrates and buffering agents (preferably to a pH of from 3 to 9), but, for some applications, they may be more suitably formulated as a sterile non-aqueous solution or as a dried form to be used in conjunction with a suitable vehicle such as sterile, pyrogen-free water. Exemplary parenteral administration forms include solutions or suspensions in sterile aqueous solutions, for example, aqueous propylene glycol or dextrose solutions. Such dosage forms can be suitably buffered, if desired. Other parentally-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form, or in a liposomal preparation.

Therapeutic uses of antibodies and compositions of the present disclosure

[0109] In some aspects, an anti-ROR1 antibody or antigen-binding portion of the present disclosure is used to treat cancer, such as a ROR1-positive cancer, in a patient. The patient may be a mammal, e.g., a human. ROR1 has been shown to express across many types of tumors, including lymphomas and solid tumors. High proportions of human cancers express ROR1. For example, Zhang et al. showed that 54% of ovarian cancers, 57% of colon cancers, 77% of lung cancers, 90% of lymphomas, 89% of skin cancers, 83% of pancreatic cancers, 73% of testicular cancers, 43% of bladder cancers, 96% of uterus cancers, 90% of prostate cancers, and 83% of adrenal cancers that they examined had moderate-to-strong staining with the anti-ROR1 antibody 4A5 (Zhang et al., *Am J Pathol.* (2012) 181(6):1903-10). Daneshmanesh et al. similarly found near universal expression of ROR1 in chronic lymphocytic leukemia (CLL) and hairy cell leukemia (HCL) and varying degrees of expression in other lymphoid cancers such as mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL)/marginal zone lymphoma (MZL), follicular lymphoma (FL), chronic myeloid leukemia (CML), acute myeloid leukemia (AML), and myeloma (Daneshmanesh et al., *Leuk Lymphoma* (2013) 54(4):843-50). Multiple groups have demonstrated expression of ROR1 in a subset of B-cell acute lymphoblastic leukemia (ALL)

(see, e.g., Dave et al., *PLoS One* (2012) 7:e52655). ROR1 is also expressed in substantial proportions of cases of hepatocellular cancer (HCC) or non-small cell lung cancer (NSCLC) (U.S. Patent Publication 2018/0369406). Further, it has been shown that ROR1 expression increases in aggressive cancers and correlates with poor prognosis; thus, anti-ROR1 antibodies and antigen-binding portions of the present disclosure may be particularly well suited to treat aggressive or advanced cancers.

[0110] Thus, in certain aspects, an anti-ROR1 antibody or antigen-binding portion of the present disclosure, or a related pharmaceutical composition, bispecific antibody, or immunoconjugate, is for use in the treatment of cancer, e.g., cancers that originate in tissues such as the hematopoietic system, ovary, colon, lung, skin, pancreas, testicles, bladder, uterus, prostate, adrenal glands, intestine, brain, kidney, soft tissues, head and neck, liver, bone, bladder, breast, stomach, and cervix.

[0111] In some aspects, a cancer treatable by an anti-ROR1 antibody or antigen-binding portion described herein is a ROR1-expressing (i.e., ROR1-positive) cancer. The cancer can be identified as ROR1-expressing by any suitable method of determining gene or protein expression, for example, by histology, flow cytometry, RT-PCR, or RNA-Seq. The cancer cells used for the determination may be obtained through tumor biopsy or through collection of circulating tumor cells. In certain aspects, if an antibody-based assay such as flow cytometry or immunohistochemistry is used, ROR1-expressing cancers are any cancers with cells that show anti-ROR1 antibody reactivity greater than that of an isotype control antibody. In certain aspects, if an RNA-based assay is used, ROR1-expressing cancers are those that show an elevated level of ROR1 RNA compared to a negative control cell or cancer that does not express ROR1.

[0112] In certain aspects, anti-ROR1 antibodies and antigen-binding portions of the present disclosure are used to treat hematological malignancies (e.g., leukemias and/or lymphomas). In certain aspects, anti-ROR1 antibodies and antigen-binding portions of the present disclosure are used to treat solid tumors. The cancer to be treated may be selected from, e.g., lymphoma, small lymphocytic lymphoma, marginal zone lymphoma, marginal cell B-cell lymphoma, Burkitt's lymphoma, mantle cell lymphoma, follicular lymphoma, diffuse large B-cell lymphoma, a non-Hodgkin lymphoma that has undergone Richter's transformation, T cell non-Hodgkin lymphoma, lymphoplasmacytoid lymphoma, Waldenström macroglobulinemia, acute lymphoblastic leukemia, acute myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, T cell leukemia, osteosarcoma, Ewing sarcoma, soft tissue sarcoma, renal cell carcinoma, hepatocellular carcinoma, colon

cancer, colorectal cancer, breast cancer (e.g., HER2⁺ or ER⁺/PR⁺/HER2⁻ breast cancer), epithelial squamous cell cancer, glioblastoma, neuroblastoma, melanoma, myeloma, multiple myeloma, stomach cancer, brain cancer, lung cancer, non-small cell lung cancer, gastroesophageal cancer, pancreatic cancer, cervical cancer, ovarian cancer, liver cancer, bladder cancer, prostate cancer, testicular cancer, endometrial cancer, thyroid cancer, mesothelioma, and head and neck cancer. The cancer may be, e.g., at an early, intermediate, late, or metastatic stage, and/or may be relapsed or refractory to other therapeutics (e.g., triple negative breast cancer).

[0113] In some aspects, cancers treated by the anti-ROR1 antibodies and antigen-binding portions of the present disclosure may include, e.g., non-Hodgkin lymphoma (e.g., diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), marginal zone lymphoma (MZL), Burkitt's lymphoma, T cell non-Hodgkin lymphoma, or Waldenström macroglobulinemia), a non-Hodgkin lymphoma that has undergone Richter's transformation, acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), small lymphocytic leukemia (SLL), multiple myeloma, breast cancer, non-small cell lung cancer, ovarian cancer, mesothelioma, and sarcoma.

[0114] In some aspects, a patient to be treated with an anti-ROR1 antibody or antigen-binding portion of the present disclosure has received prior cancer treatment. In other aspects, the patient has not received prior cancer treatment. In certain aspects, the prior cancer treatment is a chemotherapeutic agent and/or another anti-ROR1 antibody or antigen-binding portion. In certain aspects, the prior cancer treatment comprises, e.g., ibrutinib, rituximab, or rituximab-CHOP.

[0115] "Treat", "treating" and "treatment" refer to a method of alleviating or abrogating a biological disorder and/or at least one of its attendant symptoms. As used herein, to "alleviate" a disease, disorder or condition means reducing the severity and/or occurrence frequency of the symptoms of the disease, disorder, or condition. Further, references herein to "treatment" include references to curative, palliative and prophylactic treatment.

[0116] An anti-ROR1 antibody or antigen-binding portion of the present disclosure may be administered in a therapeutically effective amount to a patient with a cancer described herein. "Therapeutically effective amount" refers to the amount of the therapeutic agent being administered that will relieve to some extent one or more of the symptoms of the disorder being treated. A therapeutically effective amount of an anti-cancer therapeutic may, for example, result in delayed tumor growth, tumor shrinkage, increased survival, elimination of

cancer cells, decreased disease progression, reversal of metastasis, or other clinical endpoints desired by healthcare professionals.

[0117] An anti-ROR1 antibody or antigen-binding portion of the present disclosure may be administered without additional therapeutic treatments, i.e., as a stand-alone therapy (monotherapy). Alternatively, treatment with an anti-ROR1 antibody or antigen-binding portion of the present disclosure may include at least one additional therapeutic treatment (combination therapy). In some aspects, an anti-ROR1 antibody or antigen-binding portion may be co-administered or formulated with another medication/drug for the treatment of cancer. The additional treatment may comprise, e.g., a chemotherapeutic, anti-neoplastic, or anti-angiogenic agent, a different anti-cancer antibody, and/or radiation therapy.

[0118] In certain aspects, an anti-ROR1 antibody or antigen-binding portion of the present disclosure is used in combination with an additional therapeutic agent or biologically active molecule (e.g., to treat a cancer described herein). Examples of biologically active molecules include, but are not limited to, peptides, proteins, enzymes, small molecule drugs, prodrugs, carbohydrates, imaging agents, lipids, nucleosides, radionuclides, oligonucleotides, toxins, cells, antibiotics, fungicides, anti-viral agents, anti-inflammatory agents, anti-tumor agents, cardiovascular agents, anti-anxiety agents, hormones, growth factors, steroidal agents, microbially derived toxins, and the like. In some aspects, the additional therapeutic agent is a vascular endothelial growth factor (VEGF) inhibitor, a Bruton's tyrosine kinase (BTK) inhibitor, an inhibitor of the mammalian target of rapamycin (mTOR), a phosphoinositide 3-kinase (PI3K) inhibitor, a Janus kinase/signal transducers and activators of transcription (Jak/STAT) signaling inhibitor, a B-cell lymphoma 2 (Bcl-2) inhibitor, a spleen tyrosine kinase (SYK) inhibitor, a microtubule inhibitor, an epithelial growth factor receptor (EGFR) inhibitor, a poly ADP ribose polymerase (PARP) inhibitor, an anaplastic lymphoma kinase (ALK) inhibitor, a DNA-repair inhibitor, a DNA cross-linker, a nucleoside analog, or an immunomodulatory agent. In some aspects, the additional therapeutic agent is an antibody such as rituximab (anti-CD20) or bevacizumab (anti-VEGF); a Bruton's tyrosine kinase inhibitor such as acalabrutinib or ibrutinib; an mTOR inhibitor such as sapanisertib, everolimus or BEZ235; a PI3K inhibitor such as idelalisib or buparlisib; a Jak/STAT signaling inhibitor such as ruxolitinib; a Bcl-2 inhibitor such as ABT-199/venetoclax, Bcl-2i-1, or Bcl-2i-2; a SYK inhibitor such as fostamatinib; a microtubule inhibitor such as paclitaxel or vincristine; an EGFR inhibitor such as erlotinib; a PARP inhibitor such as olaparib; an ALK inhibitor such as crizotinib; a DNA-repair inhibitor such as carboplatin; a DNA cross-linker such as oxaliplatin/cisplatin; a nucleoside analog such as gemcitabine; or

an immunomodulatory drug (IMiD) such as lenalidomide or pomalidomide. In certain aspects, the additional therapeutic agent is ibrutinib, acalabrutinib, venetoclax, Bcl-2i-1, Bcl-2i-2, everolimus, sapanisertib, idelalisib, pacritinib, buparlisib, BEZ235, ruxolitinib, fostamatinib, rituximab, lenalidomide, pomalidomide, paclitaxel, vincristine, erlotinib, crizotinib, carboplatin, oxaliplatin/cisplatin, bevacizumab, or gemcitabine.

[0119] In certain aspects, the additional therapeutic agent is selected from a Bruton's tyrosine kinase (BTK) inhibitor, a B-cell lymphoma 2 (Bcl-2) inhibitor, a mammalian target of rapamycin (mTOR) inhibitor, and a phosphoinositide 3-kinase (PI3K) inhibitor. In particular aspects, the additional therapeutic agent is selected from ibrutinib, acalabrutinib, venetoclax, everolimus, sapanisertib, and idelalisib.

[0120] In certain aspects, an anti-ROR1 antibody or antigen-binding portion is used in combination with an immune checkpoint modulator that enhances the patient's immune system (e.g., to treat a cancer as described herein). For example, the antibody or antigen-binding portion is used with an immune checkpoint inhibitor such as an antibody or antibody derivative, an antisense oligonucleotide, a small interfering RNA, an aptamer, or a peptide, targeting programmed death-ligand 1 (PD-L1, also known as B7-H1, CD274), programmed death 1 (PD-1), CTLA-4, PD-L2 (B7-DC, CD273), LAG3, TIM3, 2B4, A2aR, B7H1, B7H3, B7H4, BTLA, CD2, CD27, CD28, CD30, CD40, CD70, CD80, CD86, CD137, CD160, CD226, CD276, DR3, GAL9, GITR, HAVCR2, HVEM, IDO1, IDO2, ICOS (inducible T cell costimulator), KIR, LAIR1, LIGHT, MARCO (macrophage receptor with collagenous structure), PS (phosphatidylserine), OX-40, SLAM, TIGHT, VISTA, VTCN1, or any combination thereof.

[0121] The present disclosure also contemplates the use of sequences (e.g., the six CDR or VH and VL sequences) of an anti-ROR1 antibody or antigen-binding portion described herein in the preparation of a chimeric antigen receptor, which may be for use in CAR-T technology.

[0122] It is understood that the antibodies and antigen-binding portions thereof of the present disclosure may be used in a method of treatment as described herein, may be for use in a treatment as described herein, and/or may be for use in the manufacture of a medicament for a treatment as described herein. It is also understood that the therapies described herein may be carried out not only using the anti-ROR1 antibodies or antigen-binding portions thereof of the present disclosure, but also using the related pharmaceutical compositions, bispecific antibodies, and immunoconjugates described herein. The present disclosure also provides kits and articles of manufacture comprising the antibodies and antigen-binding

portions thereof, pharmaceutical compositions, bispecific antibodies, or immunoconjugates described herein.

Dose and Route of Administration

[0123] The antibodies or antigen-binding portions thereof of the present disclosure may be administered in an effective amount for treatment of the condition in question, i.e., at dosages and for periods of time necessary to achieve a desired result. A therapeutically effective amount may vary according to factors such as the particular condition being treated, the age, sex and weight of the patient, and whether the antibodies are being administered as a stand-alone treatment or in combination with one or more additional anti-cancer treatments.

[0124] Dosage regimens may be adjusted to provide the optimum desired response. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form, as used herein, refers to physically discrete units suited as unitary dosages for the patients/subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the present disclosure are generally dictated by and directly dependent on (a) the unique characteristics of the therapeutic agent and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

[0125] Thus, the skilled artisan would appreciate, based upon the disclosure provided herein, that the dose and dosing regimen are adjusted in accordance with methods well-known in the therapeutic arts. That is, the maximum tolerable dose can be readily established, and the effective amount providing a detectable therapeutic benefit to a patient may also be determined, as can the temporal requirements for administering each agent to provide a detectable therapeutic benefit to the patient. Accordingly, while certain dose and administration regimens are exemplified herein, these examples in no way limit the dose and administration regimen that may be provided to a patient in practicing the present disclosure.

[0126] It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated, and may include single or multiple doses. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over

time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the embodied composition. Further, the dosage regimen with the compositions of the present disclosure may be based on a variety of factors, including the type of disease, the age, weight, sex, medical condition of the patient, the severity of the condition, the route of administration, and the particular antibody employed. Thus, the dosage regimen can vary widely, but can be determined routinely using standard methods. For example, doses may be adjusted based on pharmacokinetic or pharmacodynamic parameters, which may include clinical effects such as toxic effects and/or laboratory values. Thus, the present disclosure encompasses intra-patient dose-escalation as determined by the skilled artisan. Determining appropriate dosages and regimens are well-known in the relevant art and would be understood to be encompassed by the skilled artisan once provided the teachings disclosed herein.

[0127] An effective amount for tumor therapy may be measured by its ability to stabilize disease progression and/or ameliorate symptoms in a patient, and preferably to reverse disease progression, e.g., by reducing tumor size. The ability of an antibody, antigen-binding portion, pharmaceutical composition, bispecific binding molecule, or immunoconjugate of the present disclosure to inhibit cancer may be evaluated by *in vitro* assays, e.g., as described in the examples, as well as in suitable animal models that are predictive of the efficacy in human tumors. Suitable dosage regimens will be selected in order to provide an optimum therapeutic response in each particular situation, for example, administered as a single bolus or as a continuous infusion, and with possible adjustment of the dosage as indicated by the exigencies of each case.

[0128] The antibodies or antigen-binding portions thereof, pharmaceutical compositions, bispecific binding molecules, and immunoconjugates of the present disclosure may be administered by any method for administering peptides, proteins or antibodies accepted in the art, and are typically suitable for parenteral administration. As used herein, “parenteral administration” includes any route of administration characterized by physical breaching of a tissue of a subject and administration through the breach in the tissue, thus generally resulting in the direct administration into the blood stream, into muscle, or into an internal organ. Parenteral administration thus includes, but is not limited to, administration by injection, by application through a surgical incision, by application through a tissue-penetrating non-surgical wound, and the like. In particular, parenteral administration is contemplated to include, but is not limited to, intravenous, subcutaneous, intraperitoneal, intramuscular,

intrasternal, intraarterial, intrathecal, intraurethral, intracranial, intratumoral, and intrasynovial injection or infusions. In a particular aspect, the antibodies or antigen-binding portions, pharmaceutical compositions, bispecific binding molecules, and immunoconjugates described herein are administered intravenously.

Diagnostic Uses and Compositions

[0129] The antibodies and antigen-binding portions of the present disclosure also are useful in diagnostic processes (e.g., *in vitro*, *ex vivo*). For example, the antibodies and antigen-binding portions can be used to detect and/or measure the level of ROR1 in a sample from a patient (e.g., a tissue sample, or a body fluid sample such as an inflammatory exudate, blood, serum, bowel fluid, saliva, or urine). Suitable detection and measurement methods include immunological methods such as flow cytometry, enzyme-linked immunosorbent assays (ELISA), chemiluminescence assays, radioimmunoassays, and immunohistology. The present disclosure further encompasses kits (e.g., diagnostic kits) comprising the antibodies and antigen-binding portions described herein.

Articles of Manufacture and Kits

[0130] The present disclosure also provides articles of manufacture, e.g., kits, comprising a one or more containers (e.g., single-use or multi-use containers) containing a pharmaceutical composition of the anti-ROR1 antibody or antigen-binding portion, bispecific antibody, or immunoconjugate of the present disclosure; optionally an additional biologically active molecule (e.g., another therapeutic agent); and instructions for use. The anti-ROR1 antibody or antigen-binding portion, bispecific antibody, or immunoconjugate, and optional additional biologically active molecule, can be packaged separately in suitable packing such as a vial or ampule made from non-reactive glass or plastic. In certain aspects, the vial or ampule holds lyophilized powder comprising the anti-ROR1 antibody or antigen-binding portion/bispecific antibody/immunoconjugate and/or the additional biologically active molecule. In certain aspects, the vial or ampule holds a concentrated stock (e.g., 2x, 5x, 10x or more) of the anti-ROR1 antibody or antigen-binding portion/bispecific antibody/immunoconjugate or biologically active molecule. In certain aspects, the articles of manufacture such as kits include a medical device for administering the anti-ROR1 antibody or antigen-binding portion/bispecific antibody/immunoconjugate and/or the biologically active molecule (e.g., a syringe and a needle); and/or an appropriate diluent (e.g., sterile water and normal saline). The present disclosure also includes methods for manufacturing said articles.

Exemplary Embodiments

[0131] Non-limiting, exemplary embodiments of the present disclosure are described as follows.

1. An anti-ROR1 antibody or an antigen-binding portion thereof, wherein the antibody comprises:
 - a) a heavy chain variable domain (VH) that comprises the amino acid sequence of SEQ ID NO: 4 with at least one mutation in heavy chain (H)-CDR1, H-CDR2, or both; and a light chain variable domain (VL) that comprises the amino acid sequence of SEQ ID NO: 2;
 - b) a VH that comprises the amino acid sequence of SEQ ID NO: 4; and a VL that comprises the amino acid sequence of SEQ ID NO: 2 with at least one mutation in light chain (L)-CDR1, L-CDR2, or both; or
 - c) a VH that comprises the amino acid sequence of SEQ ID NO: 4 with at least one mutation in heavy chain (H)-CDR1, H-CDR2, or both; and a VL that comprises the amino acid sequence of SEQ ID NO: 2 with at least one mutation in light chain (L)-CDR1, L-CDR2, or both;

wherein the antibody or antigen-binding portion binds to the same epitope of human ROR1 as, and has a greater binding affinity for human ROR1 than, an antibody comprising a VH that comprises the amino acid sequence of SEQ ID NO: 4 and a VL that comprises the amino acid sequence of SEQ ID NO: 2; optionally wherein the antibody or antigen-binding portion does not comprise VH and VL amino acid sequences of SEQ ID NOs: 5 and 2, respectively, or SEQ ID NOs: 5 and 6, respectively.

2. The antibody or antigen-binding portion of embodiment 1, wherein said antibody comprises:
 - a) a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from T32A, T32E, S69V, S69E, and N71H, or any combination thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2;
 - b) a VH comprising the amino acid sequence of SEQ ID NO: 4; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E, K29S, T69H, T69R, L70K, and Q71T, or any combination thereof; or
 - c) a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from T32A, T32E, S69V, S69E, and N71H, or any combination

thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E, K29S, T69H, T69R, L70K, and Q71T, or any combination thereof;

wherein the residues are numbered according to the AHo system,

optionally wherein the antibody or antigen-binding portion does not comprise VH and VL amino acid sequences of SEQ ID NOs: 5 and 2, respectively, or SEQ ID NOs: 5 and 6, respectively.

3. The antibody or antigen-binding portion of embodiment 1, wherein said antibody comprises a VL comprising the amino acid sequence of SEQ ID NO: 2, and a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from:

- a) T32A or T32E;
- b) S69V or S69E;
- c) N71H; or
- d) any combination of a)-c);

wherein the residues are numbered according to the AHo system,

optionally wherein the antibody or antigen-binding portion does not comprise VH and VL amino acid sequences of SEQ ID NOs: 5 and 2, respectively.

4. The antibody or antigen-binding portion of embodiment 1, wherein said antibody comprises a VH comprising the amino acid sequence of SEQ ID NO: 4, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from:

- a) A25P;
- b) K29E or K29S;
- c) T69R or T69H;
- d) L70K;
- e) Q71T; or
- f) any combination of a)-e);

wherein the residues are numbered according to the AHo system.

5. The antibody or antigen-binding portion of embodiment 1, wherein said antibody comprises:

- a) a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from:
 - i) T32A or T32E;
 - ii) S69V or S69E;
 - iii) N71H; or

- iv) any combination of i)-iv); and
- b) a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from:
 - i) T69R or T69H;
 - ii) A25P;
 - iii) K29E or K29S;
 - iv) L70K;
 - v) Q71T; or
 - vi) any combination of i)-v);

wherein the residues are numbered according to the AHo system,

optionally wherein the antibody or antigen-binding portion does not comprise VH and VL amino acid sequences of SEQ ID NOs: 5 and 6, respectively.

6. The antibody or antigen-binding portion of embodiment 1, wherein said antibody comprises:
- a) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation A25P;
 - b) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation T69H;
 - c) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation T69R;
 - d) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations A25P and T69R;
 - e) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations K29S and T69R;
 - f) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations T69R and Q71T; or

- g) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations A25P and L70K;
- wherein the residues are numbered according to the AHo system.
7. The antibody or antigen-binding portion of embodiment 1, wherein said antibody comprises an H-CDR3 amino acid sequence of SEQ ID NO: 89 and an L-CDR3 amino acid sequence of SEQ ID NO: 123, and wherein said antibody further comprises:
- a) an H-CDR1 amino acid sequence of SEQ ID NO: 29 or 31;
 - b) an H-CDR2 amino acid sequence of SEQ ID NO: 73, 75, or 80;
 - c) an L-CDR1 amino acid sequence of SEQ ID NO: 102, 103, or 104;
 - d) an L-CDR2 amino acid sequence of SEQ ID NO: 117, 118, 120, or 121; or
 - e) any combination of the above H-CDR1, H-CDR2, L-CDR1, and L-CDR2 amino acid sequences,
- wherein the antibody does not comprise heavy chain (H)-CDR1-3 amino acid sequences of SEQ ID NOs: 13, 42, and 89, respectively, and light chain (L)-CDR1-3 amino acid sequences of SEQ ID NOs: 101, 114, and 123, respectively;
- optionally wherein the antibody does not comprise H-CDR1-3 amino acid sequences of 29, 42, and 89, respectively, and L-CDR1-3 amino acid sequences of 101, 114, and 123, respectively; or H-CDR1-3 amino acid sequences of 29, 42, and 89, respectively, and L-CDR1-3 amino acid sequences of 101, 118, and 123, respectively.
8. An anti-ROR1 antibody or an antigen-binding portion thereof, comprising heavy chain (H)-CDR1-3 and light chain (L)-CDR1-3 comprising the amino acid sequences of:
- a) SEQ ID NOs: 29, 42, 89, 101, 114, and 123, respectively;
 - b) SEQ ID NOs: 31, 42, 89, 101, 114, and 123, respectively;
 - c) SEQ ID NOs: 13, 73, 89, 101, 114, and 123, respectively;
 - d) SEQ ID NOs: 13, 75, 89, 101, 114, and 123, respectively;
 - e) SEQ ID NOs: 13, 80, 89, 101, 114, and 123, respectively;
 - f) SEQ ID NOs: 13, 42, 89, 102, 114, and 123, respectively;
 - g) SEQ ID NOs: 13, 42, 89, 103, 114, and 123, respectively;
 - h) SEQ ID NOs: 13, 42, 89, 104, 114, and 123, respectively;
 - i) SEQ ID NOs: 13, 42, 89, 101, 117, and 123, respectively;
 - j) SEQ ID NOs: 13, 42, 89, 101, 118, and 123, respectively;

- k) SEQ ID NOs: 13, 42, 89, 101, 120, and 123, respectively;
 - l) SEQ ID NOs: 29, 42, 89, 102, 114, and 123, respectively;
 - m) SEQ ID NOs: 29, 42, 89, 101, 117, and 123, respectively;
 - n) SEQ ID NOs: 29, 42, 89, 101, 118, and 123, respectively;
 - o) SEQ ID NOs: 29, 42, 89, 102, 118, and 123, respectively;
 - p) SEQ ID NOs: 29, 42, 89, 103, 118, and 123, respectively;
 - q) SEQ ID NOs: 29, 42, 89, 101, 147, and 123, respectively; or
 - r) SEQ ID NOs: 29, 42, 89, 102, 120, and 123, respectively.
9. The antibody or antigen-binding portion of embodiment 7 or 8, wherein said antibody or antigen-binding portion further comprises the amino acid sequences of SEQ ID NOs: 172-179.
10. An anti-ROR1 antibody or an antigen-binding portion thereof, wherein said antibody comprises a heavy chain variable domain (VH) amino acid sequence of SEQ ID NO: 5 and a light chain variable domain (VL) amino acid sequence of SEQ ID NO: 2.
11. An anti-ROR1 antibody or an antigen-binding portion thereof, wherein said antibody comprises a heavy chain variable domain (VH) amino acid sequence of SEQ ID NO: 5 and a light chain variable domain (VL) amino acid sequence of SEQ ID NO: 6.
12. The antibody of any one of embodiments 1-11, wherein the antibody is an IgG, optionally comprising a human IgG1 constant region.
13. The antibody or antigen-binding portion of any one of embodiments 1-11, wherein said antibody or portion is a single chain variable fragment (scFv).
14. The antibody or antigen-binding portion of embodiment 13, wherein said scFv comprises the amino acid sequence of SEQ ID NO: 10 or 12.
15. The antibody or antigen-binding portion of any one of embodiments 1-14, wherein said antibody or antigen-binding portion:
- a) binds to ROR1-transfected MEC cells with greater affinity than an antibody with a VH comprising the amino acid sequence of SEQ ID NO: 4 and a VL comprising the amino acid sequence of SEQ ID NO: 2, as assessed by flow cytometry;
 - b) binds to JeKo-1 cells with greater affinity than an antibody with a VH comprising the amino acid sequence of SEQ ID NO: 4 and a VL comprising the amino acid sequence of SEQ ID NO: 2, as assessed by flow cytometry; or
 - c) both a) and b).

16. A pharmaceutical composition comprising an anti-ROR1 antibody or antigen-binding portion thereof of any one of embodiments 1-15 and a pharmaceutically acceptable excipient.
17. The pharmaceutical composition of embodiment 16, further comprising an additional therapeutic agent selected from the group consisting of a Bruton's tyrosine kinase (BTK) inhibitor, a B-cell lymphoma 2 (Bcl-2) inhibitor, a mammalian target of rapamycin (mTOR) inhibitor, and a phosphoinositide 3-kinase (PI3K) inhibitor, or a combination therapy comprising the pharmaceutical composition of embodiment 16 and the aforementioned selected additional therapeutic agent.
18. The pharmaceutical composition or combination therapy of embodiment 17, wherein the additional therapeutic agent is selected from the group consisting of ibrutinib, acalabrutinib, venetoclax, everolimus, sapanisertib, and idelalisib.
19. An isolated nucleic acid molecule comprising a nucleotide sequence that encodes the heavy chain or an antigen-binding portion thereof, or a nucleotide sequence that encodes the light chain or an antigen-binding portion thereof, or both, of the anti-ROR1 antibody or antigen-binding portion of any one of embodiments 1-15.
20. The isolated nucleic acid molecule of embodiment 19, wherein said nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 9 or 11.
21. A vector comprising the isolated nucleic acid molecule of embodiment 19 or 20, wherein said vector further comprises an expression control sequence.
22. A host cell comprising a nucleotide sequence that encodes the heavy chain or an antigen-binding portion thereof, and a nucleotide sequence that encodes the light chain or an antigen-binding portion thereof, of the anti-ROR1 antibody or antigen-binding portion of any one of embodiments 1-15.
23. A method for producing an anti-ROR1 antibody or an antigen-binding portion thereof, comprising providing a host cell according to embodiment 22, culturing said host cell under conditions suitable for expression of the antibody or portion, and isolating the resulting antibody or portion.
24. A bispecific binding molecule comprising the antigen-binding portion of an anti-ROR1 antibody according to any one of embodiments 1-15.
25. An immunoconjugate comprising the anti-ROR1 antibody or antigen-binding portion of any one of embodiments 1-15 conjugated to a cytotoxic agent.
26. A method for treating cancer in a patient, comprising administering to said patient a therapeutically effective amount of the anti-ROR1 antibody or antigen-binding

- portion of any one of embodiments 1-15, the pharmaceutical composition of any one of embodiments 16-18, the bispecific binding molecule of embodiment 24, or the immunoconjugate of embodiment 25.
27. Use of the anti-ROR1 antibody or antigen-binding portion of any one of embodiments 1-15, the pharmaceutical composition of any one of embodiments 16-18, the bispecific binding molecule of embodiment 24, or the immunoconjugate of embodiment 25 for the manufacture of a medicament for treating cancer in a patient.
 28. The anti-ROR1 antibody or antigen-binding portion of any one of embodiments 1-15 for use in treating cancer in a patient.
 29. The method, use, or anti-ROR1 antibody or antigen-binding portion for use of any one of embodiments 26-28, wherein the cancer is a ROR1-positive cancer.
 30. The method, use, or anti-ROR1 antibody or antigen-binding portion for use of any one of embodiments 26-28, wherein the cancer is a leukemia, a lymphoma, or a solid tumor.
 31. The method, use, or anti-ROR1 antibody or antigen-binding portion for use of any one of embodiments 26-28, wherein the cancer is non-Hodgkin lymphoma (e.g., diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), marginal zone lymphoma (MZL), Burkitt's lymphoma, T cell non-Hodgkin lymphoma, or Waldenström macroglobulinemia), a non-Hodgkin lymphoma that has undergone Richter's transformation, acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), small lymphocytic leukemia (SLL), breast cancer, non-small cell lung cancer, ovarian cancer, or sarcoma.
 32. The method, use, or anti-ROR1 antibody or antigen-binding portion for use of any one of embodiments 26-31, wherein the patient is treated with an additional therapeutic agent.
 33. The method, use, or anti-ROR1 antibody or antigen-binding portion for use of embodiment 32, wherein the additional therapeutic agent is selected from the group consisting of a Bruton's tyrosine kinase (BTK) inhibitor, a B-cell lymphoma 2 (Bcl-2) inhibitor, a mammalian target of rapamycin (mTOR) inhibitor, and a phosphoinositide 3-kinase (PI3K) inhibitor.
 34. The method, use, or anti-ROR1 antibody or antigen-binding portion for use of embodiment 32, wherein the additional therapeutic agent is selected from the group

consisting of ibrutinib, acalabrutinib, venetoclax, everolimus, sapanisertib, and idelalisib.

35. A kit comprising the anti-ROR1 antibody or antigen-binding portion of any one of embodiments 1-15, the pharmaceutical composition of any one of embodiments 16-18, the bispecific binding molecule of embodiment 24, or the immunoconjugate of embodiment 25.
36. An article of manufacture comprising the anti-ROR1 antibody or antigen-binding portion of any one of embodiments 1-15, the pharmaceutical composition of any one of embodiments 16-18, the bispecific binding molecule of embodiment 24, or the immunoconjugate of embodiment 25, wherein said article of manufacture is suitable for treating cancer in a patient.

[0132] Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure. In case of conflict, the present specification, including definitions, will control.

[0133] Generally, nomenclature used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics, analytical chemistry, synthetic organic chemistry, medicinal and pharmaceutical chemistry, and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein.

[0134] Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Throughout this specification and aspects, the words "have" and "comprise," or variations such as "has," "having," "comprises," or "comprising," will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0135] All publications and other references mentioned herein are incorporated by reference in their entirety. Although a number of documents are cited herein, this citation does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

[0136] In order that the present disclosure may be better understood, the following examples are set forth. These examples are for purposes of illustration only and are not to be construed as limiting the scope of the present disclosure in any manner.

EXAMPLES

Example 1. Phage Expression of Ab1

[0137] In order to enable *in vitro* affinity maturation of the Ab1 antibody, DNA encoding the heavy and light chain variable regions of Ab1 was cloned into an M13 phage vector to facilitate the expression of soluble Fab in bacteria. Briefly, double-stranded DNA encoding the light chain variable region (SEQ ID NO: 1) and constant region followed by the heavy chain variable region (SEQ ID NO: 3) was synthesized (Integrated DNA Technologies). The DNA was codon-optimized for bacterial expression and was flanked with NcoI and HindIII/ApaI restriction sites as well as additional vector sequence to permit cloning either by restriction digest or by Gibson assembly. Subsequently, the DNA was cloned into an M13 phage expression vector (Huse et al., *J. Immunol.* (1992) 149(12):3914-20) that contained human CH1 domain followed by both histidine- and human influenza hemagglutinin A (HA) peptide tags to facilitate purification of the Fab (his-tag) and detection of the heavy chain (HA-tag).

[0138] The M13 phage vector was used to transform *E. coli* and generate phage encoding soluble (not fused to any phage proteins) Ab1 Fab. Subsequently, a filter lift assay was used to demonstrate expression of heavy and light chains. Briefly, dilutions of high titer phage stocks (typically 10⁶-fold) were combined with 0.35 mL of confluent *E. coli* strain XL cells containing 20 µg/mL tetracycline. The mixture was combined with 3.5 mL of 0.7% Bacto agar in Luria broth and was subsequently overlaid on 1.5% Bacto agar in Luria broth. The plate was incubated 6-8 h at 37°C at which time a nitrocellulose filter (Whatman 82-mm diameter, 0.45 µm pore size, GE Healthcare, cat. #10401116) was placed on top and the plate was incubated at 25°C for 12-15 h. The filter was removed, rinsed briefly in PBS, and was blocked by incubating in 5% milk in PBS (5% M-P) for 2 h at 25°C with constant agitation. The filter was split and probed to assess either kappa light chain or heavy chain expression. Each filter was transferred to the appropriate primary detection reagent: goat anti-human kappa, HRP conjugate (Southern Biotech, cat. #2060-05) diluted 1000-fold in 5% M-P for detection of light chain, or rat anti-HA, HRP conjugate (Roche, cat. #12013819001) diluted 1000-fold in 5% M-P for detection of heavy chain. Filters were incubated 2 h at 25°C with

constant agitation and were then washed 5 times, 2 minutes each wash with constant agitation, in PBS containing 0.05% Tween 20 (PBS-T). In order to assess expression, the filters were developed with 1-Step Ultra TMB-Blotting (Thermo Scientific, cat. #37574). Using this assay, bacterial expression of both heavy (**FIG. 1, Panel A**, right side) and kappa light (**FIG. 1, Panel A**, left side) chains was demonstrated. These data demonstrate the cloning and bacterial expression of heavy and light chains of Ab1 Fab.

[0139] It has been demonstrated previously that Ab1 binds an epitope on the extracellular domain of human ROR1 (see U.S. Patent 9,758,591). Consequently, recombinant human ROR1 protein corresponding to the extracellular domain (ECD) of human ROR1 was used for screening assays. To biotinylate the human ROR1-ECD antigen, 100 µg of the protein was resuspended in water to a final concentration of 400 µg/mL. The protein was then combined with EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific cat. #21327) at a 5:1 molar ratio and incubated at 25°C for 2 h. The reaction was quenched by addition of 31 µL of 0.75 M arginine hydrochloride and biotinylated huROR1 antigen was stored at 4°C until use.

[0140] Next, the filter lift assay was modified slightly to enable sensitive detection of the binding of soluble biotinylated antigen by Fab immobilized on the filter. In order to enhance the assay sensitivity and make the filter lift assay staining intensity more reflect Fab affinity (as opposed to expression levels), a previously described capture filter lift assay was used (Watkins, *Methods in Molecular Biology* (2001) 178:187-193). A nitrocellulose filter (Whatman 82 mm diameter, 0.45 mm pore size, GE Healthcare, Prod. #10401116) was floated on top of 10 mL of 10 µg/mL goat anti-human kappa (Southern Biotech Prod. #2060-01) for 2 h at 25°C. The filter was submersed briefly before removing and transferred to 10 mL of 5% M-P for 2 h at 25°C. The filter was removed from 5% M-P, rinsed briefly one time with PBS, and was air-dried. Subsequently, the filter was applied to the agar plates as described above. For detection of antigen binding, the filter was rinsed briefly in PBS, and transferred to biotinylated huROR1-ECD (ACROBiosystems, cat. #RO1-H522y) in 5% M-P.

[0141] Filters were incubated with 2.3 nM biotinylated ROR1 diluted in 5% M-P for 2 h at 25°C with constant agitation, and washed 5 times, 2 minutes each wash, with constant agitation in PBS-T. Alternatively, filters were washed more rapidly using a squirt bottle and sintered glass funnel for vacuum filtration. The filters were transferred to 10 mL of High Sensitivity Neutravidin, HRP conjugate (Thermo Scientific, cat. #31030) diluted 5000-fold in 1% BSA in PBS and incubated for 1 h at 25°C. The filters were then washed 5 times in PBS-

T and were developed with 1-Step Ultra TMB-Blotting. The binding of immobilized Ab1 Fab to biotinylated huROR1-ECD was demonstrated using this assay (**FIG. 1, Panel B**). These data demonstrate that the bacterially-expressed Fab is functional and that the modified filter lift assay can detect binding to huROR1 at low nanomolar concentrations.

Example 2. Expression and Quantitation of Ab1 Fab in the Periplasmic Space of *E. coli*

[0142] The Ab1 Fab was further characterized by expressing and quantitating Fab in the periplasmic space of *E. coli*. Briefly, XL bacteria were grown in 2X YT medium at 37°C until the culture reached a density of 0.9 – 1.1 at OD600. Isopropyl β -D-thiogalactoside was then added to the cells to a final concentration of 1 mM and 3.0 mL of culture was transferred to a 14 mL snap-top tube. Each tube was transfected with 25 μ L of high titer phage stock and the cultures were placed in a shaker (225 rpm) at 37°C. One hour later, the temperature was shifted to 25°C and the cultures were grown for an additional 14-16 h. The cells were collected by centrifugation at 3900 rpm for 30 min in an Eppendorf 5810R centrifuge (~3,200 x g), the supernatant was decanted and the cells were resuspended in 0.3 mL of lysis buffer (30 mM Tris, pH 8.0, 2 mM EDTA, 20% sucrose, 2 mg/mL lysozyme, 5 U/mL DNase I) and placed on ice for 15 min. The cell suspension was transferred to a 1.5 mL tube and cell debris was pelleted by centrifugation at 15,000 rpm for 15 minutes in an Eppendorf 5424 microfuge (~21,000 x g). The supernatant was removed carefully without disturbing the pellet and was stored at 4°C until use.

[0143] In order to quantitate Fab expression, a 96-well Costar-3366 plate was coated with 50 μ L/well of 2 μ g/mL sheep anti-human Fd (Southern Biotech, cat. #2046-01) in PBS overnight at 4°C. The plate was washed three times with PBS containing 0.05% Tween 20 (PBS-T) and 50 μ L/well of sample dilutions was added. Sample dilutions were performed with PBS-T. A standard curve was generated using human Fab (Rockland, cat. #009-01015) diluted serially 2-fold, beginning at 500 ng/mL. The plates were incubated 1 h at 25°C, washed three times with PBS-T, and incubated with 50 μ L/well of anti-kappa HRP conjugate (Southern Biotech, cat. #2060-05), diluted 10,000-fold in PBS-T for 1 h at 25°C. The plate was washed three times with PBS-T, and developed with 50 μ L/well 1-Step Ultra TMB-ELISA (Thermo Scientific, cat. #34028). The reaction was terminated by the addition of 2 N H₂SO₄ and the A450 was quantitated using a Spectramax plate reader.

[0144] Ab1 Fab expressed well in bacteria. Fab isolated from the periplasmic fraction of 3 mL cultures of *E. coli* ranged from 18 μ g/mL to 188 μ g/mL (average = 84 +/- 67 μ g/mL, n =

10). For comparison, two unrelated Fab clones that also bind the extra-cellular domain of huROR1, one humanized and one chimeric, expressed 9.26 +/- 2.16 µg/mL and 5.13 +/- 0.39 µg/mL, respectively.

[0145] The previously described filter lift assay provides a visual, qualitative assessment of antigen binding (Example 1). In order to assess the ROR1 binding activity of Ab1 Fab in a more quantitative fashion, an ELISA assay was developed. A 96-well Costar-3366 plate was coated with 50 µL/well of 1 µg/mL human ROR1-ECD (ACROBiosystems, cat. #RO1-H522y) in PBS overnight at 4°C. The plate was rinsed once with PBS-T and blocked with 100 µL/well of 5% M-P for 1 h at 25°C. Fab samples were serially diluted 3-fold using 5% M-P and 50 µL/well was incubated for 1 h at 25°C. The plate was washed three times with PBS-T and 50 µL/well anti-human kappa, and HRP conjugate (Southern Biotech, cat. #2060-05) diluted 10,000-fold in 5% M-P was added for 1 h at 25°C. In certain assays (extended wash format), the plate was placed in large volumes (up to 1 L) of PBS-T and incubated, with mixing, for 2-5 h prior to the addition of anti-human kappa, HRP conjugate. The plate was washed three times with PBS-T, and developed with 50 µL/well 1-Step Ultra TMB-ELISA (Thermo Scientific, cat. # 34028). The reaction was terminated by the addition of 2 N H₂SO₄ and the A450 was determined using a Spectramax plate reader.

[0146] The Ab1 Fab bound immobilized huROR1-ECD in a concentration-dependent manner (**FIG. 2**). The K_D in this assay format was 3.0 nM, while the K_D of the chimeric and humanized control ROR1 Fabs were 9.7 nM and >125 nM, respectively. No binding of irrelevant Fab was detected in this assay. The K_D of Ab1 Fab in this assay was consistent with the modified filter lift assay, in which binding was detected with low nanomolar biotinylated huROR1-ECD. Collectively, these data demonstrate the expression of functional Ab1 Fab in bacteria and support the use of the phage expression system for affinity maturation of the antibody.

Example 3. *In vitro* Affinity Maturation of Ab1

[0147] In the absence of structural information regarding the interaction of Ab1 with huROR1, a comprehensive mutagenesis strategy was employed for affinity maturation. Specifically, site saturation mutagenesis was performed at every position of every CDR and the variants were screened for binding to soluble ROR1-ECD. For these studies, the AHo numbering scheme was used and the heavy chain CDRs were delineated by the combination of Kabat and IMGT definitions while the light chain CDRs were delineated by the Kabat

definition. For simplicity, CDR libraries are named according to the chain (H, heavy chain or L, light chain) and the CDR number (1, 2, or 3). The libraries of heavy chain CDR3 (H3) and light chain CDR3 (L3) were created by Kunkel mutagenesis using degenerate NNK oligonucleotides at each scan position. To minimize the frequency of WT clones (non-mutated template) in the library, the target CDR was deleted from the Ab1 template prior to mutagenesis. The libraries of heavy chain CDR1 (H1), CDR2 (H2), light chain CDR1 (L1) and CDR2 (L2) were also built on deletion templates but were synthesized differently. Instead of using NNK degenerate oligonucleotides, nineteen non-wild-type codons were introduced at each position. Sequencing of the libraries demonstrated relatively equivalent representation of amino acids at each position and exclusion of wild type and termination codons. An example of the representation at each position of H1 and H2 is shown in **FIG. 3**. Following synthesis of the site saturation libraries, two different screening strategies were employed. In some cases, each CDR position was screened as a single library, while in other cases the positional site saturation libraries within a CDR were pooled and the entire CDR library was screened.

[0148] All libraries were initially screened by capture lift to identify variants with improved binding. The capture lift assay, described in Example 1, enables the filter lift assay to discern variants that bind antigen more strongly based on the staining intensity. In order to determine the optimal concentration of antigen to use for screening, biotinylated huROR1-ECD was titrated on a capture lift of Ab1 Fab. Binding to Ab1 was clearly visible at antigen concentrations ≥ 23 nM and the signal was diminished at lower huROR1 concentrations. Based on these results, library screening was performed at 2.3 nM as this concentration stained with a lower intensity, providing an opportunity to identify higher affinity, more intensely staining variants in the first round of screening. In addition, for more stringent screening, filters were probed with lower concentrations of antigen and/or development of the filter was terminated earlier, before the Ab1 staining was visible. Ab1 variants displayed a range of binding strengths, as demonstrated by the varying intensity of plaques on the lift. For example, a representative L3 library lift is shown in **FIG. 4**. In this example, approximately 250 library variants were plated. Twelve variants clearly bound huROR1 (**FIG. 4**, numbered). Less intense staining variants are indicated with arrows and other variants did not stain at all under these conditions. Exemplary clones identified on the capture lifts (those displaying the most intense signals) were picked, grown in culture and the DNA was sequenced. Using this approach, mutations that demonstrated huROR1 binding were identified in every CDR. Ab1 variants that bound huROR1-ECD under these conditions

are summarized in Tables 1-7 (heavy chain CDR1, CDR2a, CDR2b, CDR3 and light chain CDR1, CDR2, and CDR3, respectively). The H2 CDR is 17 amino acids in length and the data from screening this library is split into two tables, Table 2 and Table 3.

Table 1. H-CDR1 (H1)

Residue Number & Amino Acid										ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
27	29	30	31	32	33	39	40	41	42	WT	Variant		
G	Y	A	F	T	A	Y	N	I	H			GYAFTAYNIH	13
D												DYAFTAYNIH	14
N										3.2	8.4	NYAFTAYNIH	15
R												RYAFTAYNIH	16
T												TYAFTAYNIH	17
	G									3.2	7.1	GGAFTAYNIH	18
	K											GKAFTAYNIH	19
	S											GSAFTAYNIH	20
		I										GYIFTAYNIH	21
		P								3.2	12	GYPFTAYNIH	22
		R										GYRFTAYNIH	23
		T								3.2	16	GYTFTAYNIH	24
			I							3.2	5.8	GYAITAYNIH	25
			L									GYALTAYNIH	26
			V							3.2	8.3	GYAVTAYNIH	27
			W									GYAWTAYNIH	28
				A						6.0	1.5	GYAFAAYNIH	29
				D								GYAFDAYNIH	30
				E						6.0	3.3	GYAFEAYNIH	31
					M					3.2	15	GYAFTMYNIH	32
					S					3.2	11	GYAFTSYNIH	33
						F				3.2	5.7	GYAFTAFNIH	34
						W				3.2	3.3	GYAFTAWNIH	35
							K					GYAFTAYKIH	36
							P			3.2	40	GYAFTAYPIH	37
							R			3.2	43	GYAFTAYRIH	38
								L				GYAFTAYNLH	39
								V		3.2	3.5	GYAFTAYNVH	40
									Q			GYAFTAYNIQ	41

Table 2. H-CDR2a (residues 57 – 67) (H2)

Residue Number & Amino Acid								ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
57	58	59	60	61	65	66	67	WT	Variant		
S	F	D	P	Y	D	G	G			SFDPYDGGSSYNQKFKD	42
A								1.4	3.6	AFDPYDGGSSYNQKFKD	43
K										KFDPYDGGSSYNQKFKD	44
R										RFDPYDGGSSYNQKFKD	45
T										TFDPYDGGSSYNQKFKD	46
	I							1.6	1.8	SIDPYDGGSSYNQKFKD	47
	P									SPDPYDGGSSYNQKFKD	48
	V							1.6	0.9	SVDPYDGGSSYNQKFKD	49
		S								SFSPYDGGSSYNQKFKD	50
			G							SFDGYDGGSSYNQKFKD	51
			T							SFDTYDGGSSYNQKFKD	52
					E			1.2	4.9	SFDPYEGGSSYNQKFKD	53
					N					SFDPYNGGSSYNQKFKD	54
					T					SFDPYTGGSSYNQKFKD	55
						D		1.6	2.2	SFDPYDDGSSYNQKFKD	56
						E		1.3	0.9	SFDPYDEGSSYNQKFKD	57
						H		1.3	1.4	SFDPYDHGSSYNQKFKD	58
						N		1.3	2.2	SFDPYDNGSSYNQKFKD	59
							H			SFDPYDGHSSYNQKFKD	60
							K	1.3	2.3	SFDPYDGKSSYNQKFKD	61
							M			SFDPYDGMSSYNQKFKD	62
							P	1.2	0.6	SFDPYDGPSSYNQKFKD	63
							Q	1.2	<1.0	SFDPYDQGSSYNQKFKD	64
							R	1.3	1.7	SFDPYDGRSSYNQKFKD	65
							S			SFDPYDGSSSYNQKFKD	66
							Y	5.7	6.3	SFDPYDGYSSYNQKFKD	67

Table 3. H-CDR2b (residues 68 – 76)(H2)

Residue Number & Amino Acid									ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
68	69	70	71	72	73	74	75	76	WT	Variant		
S	S	Y	N	Q	K	F	K	D			SFDPYDGGSSYNQKFKD	42
A									1.3	1.2	SFDPYDGGASYNQKFKD	68
E											SFDPYDGGESYNQKFKD	69
I											SFDPYDGGISYNQKFKD	70
Q											SFDPYDGGQSYNQKFKD	71
T									5.7	6.4	SFDPYDGGTSYNQKFKD	72
	E								1.3	1.2	SFDPYDGGSEYNQKFKD	73
	K								5.7	46	SFDPYDGGSKYNQKFKD	74
	V								1.3	1.0	SFDPYDGGSVYNQKFKD	75
		I							1.4	1.7	SFDPYDGGSSINQKFKD	76

		V							1.6	1.5	SFDPYDGGSSVNQKFKD	77
			A						1.6	1.3	SFDPYDGGSSYAQKFKD	78
			G						1.2	1.5	SFDPYDGGSSYGQKFKD	79
			H						1.6	1.1	SFDPYDGGSSYHQKFKD	80
			S						1.2	1.2	SFDPYDGGSSYSQKFKD	81
				N					1.2	1.3	SFDPYDGGSSYSNKFAD	82
				P					1.2	1.0	SFDPYDGGSSYSPKFKD	83
					C						SFDPYDGGSSYNQCFKD	84
					S						SFDPYDGGSSYNQSFKD	85
							A				SFDPYDGGSSYNQKFKD	86
							G				SFDPYDGGSSYNQKFGD	87
								V	1.3	1.8	SFDPYDGGSSYNQKFKV	88

Table 4. H-CDR3 (H3)

Residue Number & Amino Acid									CDR SEQ	SEQ ID NO:
107	108	109	110	111	135	136	137	138		
A	R	G	W	Y	Y	F	D	Y	ARGWYYFDY	89
T									TRGWYYFDY	90
V									VRGWYYFDY	91
	G								AGGWYYFDY	92
			C						ARGCYFFDY	93
			F						ARGFYFFDY	94
				A					ARGWAYFDY	95
				L					ARGWLYFDY	96
						L			ARGWYYLDY	97
								Q	ARGWYYFDQ	98
								T	ARGWYYFDT	99
								V	ARGWYYFDV	100

Table 5. L-CDR1 (L1)

Residue Number & Amino Acid											ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
24	25	26	29	30	31	32	39	40	41	42	WT	Variant		
R	A	S	K	S	I	S	K	Y	L	A			RASKSISKYLA	101
	P										1.5	0.7	RPSKSISKYLA	102
			E								1.5	1.2	RASESISKYLA	103
			S								1.5	0.9	RASSSISKYLA	104
				A							1.5	1.5	RASKAISKYLA	105
				F							8.1	18	RASKFISKYLA	106
				H							1.5	1.4	RASKHISKYLA	107
				P							8.1	16	RASKPISKYLA	108
					V						1.5	2.8	RASKSVSKYLA	109
						A					1.0	1.1	RASKSIKYLK	110
						I					1.0	1.9	RASKSIKYLK	111
								M			1.0	1.3	RASKSISKYMA	112

								V	1.0	1.1	RASKSISKYVA	113
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Table 6. L-CDR2 (L2)

Residue Number & Amino Acid							ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
58	67	68	69	70	71	72	WT	Variant		
S	G	S	T	L	Q	S			SGSTLQS	114
		G					1.0	1.6	SGGTLQS	115
		R					8.1	11	SGRTLQS	116
			H				2.9	1.5	SGSHLQS	117
			R				2.9	2.1	SGSRLQS	118
				H			2.9	2.3	SGSTHQS	119
				K			2.9	1.5	SGSTKQS	120
					T		1.5	1.1	SGSTLTS	121
					V		1.0	1.3	SGSTLVS	122

Table 7. L-CDR3 (L3)

Residue Number & Amino Acid									CDR SEQ	SEQ ID NO:
107	108	109	110	111	135	136	137	138		
Q	Q	H	D	E	S	P	Y	T	QQHDESPYT	123
S									SQHDESPYT	124
	A								QAHDESPYT	125
	L								QLHDESPYT	126
	R								QRHDESPYT	127
		N							QQNDESPYT	128
		Q							QQQDESPYT	129
			H						QQHHESPYT	130
			N						QQHNESPYT	131
				I					QQHDISPYT	132
				L					QQHDLSPYT	133
					G				QQHDEGPYT	134
					H				QQHDEHPYT	135
					P				QQHDEPPYT	136
					V				QQHDEVPYT	137
							T		QQHDESPTT	138
							V		QQHDESPVT	139
								F	QQHDESPYF	140
								S	QQHDESPYS	141

[0149] Certain variants that displayed higher intensity staining on the capture lifts were further characterized by ELISA, using soluble Fab obtained from periplasmic preps, as described in Example 2. Titration of soluble Fab against huROR1-ECD enabled a more precise comparison of the relative affinities of variants. In addition, Ab1 was included on

every ELISA plate to control for assay variability. Certain mutations that increased the binding of Ab1 to huROR1-ECD in this assay format were identified. For example, mutation of heavy chain CDR1 residue T32 to either alanine (H1 T32A) or glutamic acid (H1 T32E) increased the binding of Ab1 by approximately 4-fold or 2-fold, respectively (**FIG. 5**). Similarly, affinity-enhancing mutations were identified in heavy chain CDR2 (**FIG. 6**), light chain CDR1 (**FIG. 7**) and light chain CDR2 (**FIG. 8**). The relative affinities measured in this way are summarized in **Tables 1-7**. These experiments identified multiple mutations from different CDRs of both heavy and light chains that maintain or improve the affinity of Ab1.

Example 4. Synthesis, Expression and Screening of Combinatorial Libraries

[0150] In order to further optimize the affinity of Ab1, the beneficial mutations identified from screening the CDR site saturation libraries were tested in various combinations with the H1 T32A mutation. In the first step, beneficial mutations from L1 or L2 or H2 were combined with the H1 T32A to create three libraries (**Table 8**, Library 1, 2 and 3, respectively). The unique combinations of mutations present in each library are summarized in **Table 9** (H1 T32A x L1 mutations), **Table 10** (H1 T32A x L2 mutations) and **Table 11** (H1 T32A x H2 mutations).

[0151] In a parallel approach, a template comprising the best heavy chain variant, T32A, and the light chain variant L1 A25P, was used to evaluate combinations with beneficial mutations from L2 (**Table 8**, Library 4). The unique combinations of mutations present in this library are summarized in **Table 12** (H1 T32A + L1 A25P x L2 mutations).

[0152] Additionally, a template comprising the best heavy chain variant, T32A, and the light chain variant L2 T69R, was used to evaluate combinations with beneficial mutations from L1 (**Table 8**, Library 5). The unique combinations of mutations present in this library are summarized in **Table 13** (H1 T32A + L2 T69R x L1 mutations).

[0153] Subsequently, the libraries were screened by capture lift to identify the strongest binding variants. The identity of the variants was determined by DNA sequencing and the relative affinities were characterized using an ELISA (described in Example 2) modified to enable further discrimination between the highest affinity variants. Specifically, following the initial incubation with soluble Fab, the plates were washed for a prolonged time (>1 h) in large volumes of PBS-T. The plates were then developed as described in Example 2.

[0154] Alteration of the assay conditions enables the binding of high affinity variants to be distinguished from one another, but precludes the direct comparison of EC50 values to screening data obtained under different assay parameters (Example 3). For each library, the

binding of the variants was compared to the appropriate clone, corresponding to the template the library was built upon. For example, variants from libraries built upon H1 T32A were compared to clone H1 T32A in order to identify further improvements in binding affinity.

[0155] Screening of the first three libraries, all built upon the H1 T32A template, identified multiple combinations that further increased binding affinity over variant H1 T32A (summarized in **Table 9**, **Table 10**, and **Table 11**). For example, screening of Library 1 identified the combination of L1 A25P with H1 T32A (**FIG. 9, Panel A**, compare open circles with closed squares), while screening of library 2 identified the combinations of L2 T69H or L2 T69R with H1 T32A (**FIG. 9, Panel A**, compare open squares or open triangles, respectively, with closed squares).

[0156] In addition to the simple combinations of two mutations described above, variants containing >2 mutations were also identified. For example, combining light chain mutations L1 A25P and L2 T69R with H1 T32A led to a substantial improvement in binding (**FIG. 9, Panel B**, compare open triangles with open circles). Likewise, the combination of L1 K29S and L2 T69R with H1 T32A also improved binding relative to H1 T32A alone (**FIG. 9, Panel B**, compare inverted closed triangles with open circles).

[0157] Collectively, these data demonstrate that individual mutations identified through screening of site saturation CDR libraries can be combined to further improve affinity. Additionally, multiple variants with distinct sequences and higher affinity than Ab1 or H1 T32A were identified by this approach.

Table 8. Combinatorial libraries

Library	Template	Library CDR	Mutations	Unique Diversity
1	H1 T32A	L1	A25P, K29E, K29S	5
2	H1 T32A	L2	T69R, T69H, L70K, Q71T	11
3	H1 T32A	H2	F58I, F58V, G66E, S69V, N71H	23
4	H1 T32A + L1 A25P	L2	T69R, T69H, L70K, Q71T	11
5	H1 T32A + L2 T69R	L1	A25P, K29E, K29S	5

Table 9. Library 1 (L1 Variants on H1 T32A template)

Residue Number & Amino Acid											ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
24	25	26	29	30	31	32	39	40	41	42	Template	Variant		
R	A	S	K	S	I	S	K	Y	L	A			RASKSISKYLA	101
	P										3.0	1.8	RPSKSISKYLA	102
			E								2.7	3.3	RASESISKYLA	103
			S								2.7	2.1	RASSSISKYLA	104
	P		E										RPSESISKYLA	142

	P		S										RPSSSISKYLA	143
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Table 10. Library 2 (L2 Variants on H1 T32A template)

Residue Number & Amino Acid							ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
58	67	68	69	70	71	72	Template	Variant		
S	G	S	T	L	Q	S			SGSTLQS	114
			H				3.0	2.3	SGSHLQS	117
			R				3.0	1.1	SGSRLQS	118
				K				1.6	SGSTKQS	120
					T				SGSTLTS	121
			H	K				1.8	SGSHKQS	144
			H		T				SGSHLTS	145
			R	K					SGSRKQS	146
			R		T			1.1	SGSRLTS	147
				K	T			1.6	SGSTKTS	148
			H	K	T			1.4	SGSHKTS	149
			R	K	T				SGSRKTS	150

Table 11. Library 3 (H2 Variants on H1 T32A template)

Residue Number & Amino Acid											ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
58	59	60	61	65	66	67	68	69	70	71	Template	Variant		
F	D	P	Y	D	G	G	S	S	Y	N			SFDPYDGGSSYNQKFKD	42
I											3.6	4.5	SIDPYDGGSSYNQKFKD	47
V											3.6	3.2	SVDPYDGGSSYNQKFKD	49
					E						1.6	2.7	SFDPYDEGSSYNQKFKD	57
								V			2.7	3.3	SFDPYDGGSVYNQKFKD	75
									H		2.7	2.1	SFDPYDGGSSYHQKFKD	80
I					E						3.6	5.0	SIDPYDEGSSYNQKFKD	151
I								V			3.6	8.5	SIDPYDGGSVYNQKFKD	152
I									H		1.6	2.2	SIDPYDGGSSYHQKFKD	153
V					E						1.6	3.0	SVDPYDEGSSYNQKFKD	154
V								V					SVDPYDGGSVYNQKFKD	155
V									H		1.6	2.4	SVDPYDGGSSYHQKFKD	156
					E			V					SFDPYDEGSVYNQKFKD	157
					E				H				SFDPYDEGSSYHQKFKD	158
								V	H				SFDPYDGGSVYHQKFKD	159
I					E			V					SIDPYDEGSVYNQKFKD	160
I					E				H		3.6	3.4	SIDPYDEGSSYHQKFKD	161
I								V	H				SIDPYDGGSVYHQKFKD	162
V					E			V			1.6	8.0	SVDPYDEGSVYNQKFKD	163
V					E				H				SVDPYDEGSSYHQKFKD	164
V								V	H		1.6	3.9	SVDPYDGGSVYHQKFKD	165
					E			V	H		3.6	3.2	SFDPYDEGSVYHQKFKD	166
I					E			V	H				SIDPYDEGSVYHQKFKD	167
V					E			V	H				SVDPYDEGSVYHQKFKD	168

Table 12. Library 4 (L2 Variants on H1 T32A + L1 A25P template)

Residue Number & Amino Acid							ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
58	67	68	69	70	71	72	Template	Variant		
S	G	S	T	L	Q	S			SGSTLQS	114
			H				1.8	2.3	SGSHLQS	117
			R				1.8	2.4	SGSRLQS	118
				K				2.1	SGSTKQS	120
					T			2.9	SGSTLTS	121
			H	K					SGSHKQS	144
			H		T				SGSHLTS	145
			R	K					SGSRKQS	146
			R		T				SGSRLTS	147
				K	T				SGSTKTS	148
			H	K	T				SGSHKTS	149
			R	K	T				SGSRKTS	150

Table 13. Library 6 (L1 Variants on H1 T32A + LR2 T69R template)

Residue Number & Amino Acid											ELISA K _D (nM)		CDR SEQ	SEQ ID NO:
24	25	26	29	30	31	32	39	40	41	42	Template	Variant		
R	A	S	K	S	I	S	K	Y	L	A			RASKSISKYLA	101
	P										2.3	2.1	RPSKSISKYLA	102
			E								2.3	4.8	RASESISKYLA	103
			S								2.3	2.1	RASSSISKYLA	104
	P		E										RPSESISKYLA	142
	P		S								2.3	3.9	RPSSSISKYLA	143

Example 5. Design, Expression and Characterization of Ab1 scFv Variants

[0158] Intact IgG antibodies have prolonged serum half-life due to FcRn receptor mediated recycling. The prolonged circulation time leads to elevated blood levels and exposure of healthy organs to the antibody, attributes that may be detrimental when developing imaging agents, radiotherapeutics, or antibody drug conjugate (ADC) therapies. In contrast, smaller antibody constructs have shorter circulation time *in vivo* due to the lack of the Fc region and their smaller size enables better tissue penetration. Shorter circulation time and better tissue penetration are beneficial attributes for tumor imaging (Mendler et al., *mAbs* (2015) 7(1):96-109). However, one potential drawback to the use of small, monovalent antibody constructs for tumor targeting is that they tend to diffuse out of the tumor as the plasma concentration drops due to the lack of avidity effect (multivalent binding). Thus, the affinity of monovalent antibodies for tumor cell surface antigen has a direct impact on tumor accumulation. The higher affinity Ab1 variants described in Examples 3 and 4 may overcome the limitation

associated with using lower affinity monovalent antibody constructs, smaller single domain constructs, or bispecific antibody constructs with a single ROR1 targeting arm.

[0159] Certain higher affinity variants described in Examples 3 and 4 were used to create scFv format antibodies. Briefly, scFvs were designed with a pelB leader sequence to direct periplasmic expression, followed by the VH sequence, a 20-amino acid flexible linker consisting of (Gly₄Ser) x 3 (SEQ ID NO: 180), and the VL sequence. In addition, human influenza hemagglutinin (HA) peptide and FLAG peptide tags were added to the carboxy-terminus of the constructs to enable purification and/or quantitation of the scFv protein. The parental Ab1 and two variants, H1 T32A and H1 T32A + L2 T69R, were expressed and characterized.

[0160] scFv constructs were expressed and isolated from the periplasmic space using the same protocol that was used for Fabs (Example 2). In order to quantitate the relative levels of scFv expression of the three variants, a 96-well Costar-3366 plate was coated with 50 µl/well of 2 µg/mL anti-FLAG-unlabeled (Rockland, cat. #600-401-383) in PBS overnight at 4°C. The plate was washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 100 µL of 5% M-P. The plate was washed once, then 50 µl/well of sample dilutions was added. Sample dilutions were performed with 5% M-P. The plates were incubated 1 h at 25°C, washed three times with PBS-T, and incubated with 50 µl/well of anti-HA HRP conjugate (Roche, cat. #12013819001), diluted 1,000-fold in 5% M-P for 1 h at 25°C. The plate was washed three times with PBS-T, and developed with 50 µl/well 1-Step Ultra TMB-ELISA (Thermo Scientific, Prod. # 34028, Lot # SF2405221). The reaction was terminated by the addition of 2 N H₂SO₄ and the A450 was determined using a Spectramax plate reader.

[0161] Next, the binding of the isolated scFv variants to huROR1-ECD was assessed by ELISA as described in Example 2. A 96-well Costar-3366 plate was coated with 50 µl/well of 1 µg/mL human ROR-1 (AcroBiosystems, cat. #R01H522Y) in PBS overnight at 4°C. The plate was washed three times with PBS-T and blocked with 100 µL of 5% M-P. The plate was washed once, then 50 µl/well of 2-fold serial dilutions of sample was added. Sample dilutions were performed with 5% M-P. The plates were incubated 1 h at 25°C, washed three times with PBS-T, and incubated with 50 µl/well of anti-HA HRP conjugate (Roche, cat. #12013819001), diluted 1,000-fold in 5% M-P for 1 h at 25°C. The plate was washed three times with PBS-T and developed with 50 µl/well 1-Step Ultra TMB-ELISA (Thermo Scientific, Prod. # 34028, Lot # SF2405221). The reaction was terminated by the

addition of 2 N H₂SO₄ and the A450 was determined using a Spectramax plate reader. In order to compare the relative binding strength of the scFv variants, the expression levels as determined using the scFv ELISA described above were normalized to the Ab1 parental construct. Similar to the Fab variants, H1 T32A + L2 T69R bound most strongly, followed by H1 T32A, and Ab1 (**FIG. 10**, open circles, open squares, closed circles, respectively). These data demonstrate that the scFv format preserved the enhanced affinity observed in the Fab format.

Example 6. Binding of Ab1 Fab and scFv Variants to Live Cells

[0162] The *in vitro* affinity maturation of Ab1 used recombinant huROR1-ECD for screening. In order to verify that the improvements in binding to the recombinant huROR1 protein would translate to improved tumor cell binding, select variants were characterized for binding to live ROR1-transfected MEC cells and/or JeKo-1 cells. The variants analyzed are summarized in **Table 14**. The JeKo-1 mantle cell lymphoma cell line has previously been demonstrated to express human ROR1 (Yang et al., *PLoS One* (2011) **6(6)**:e21018).

Table 14. Fab and scFv variants characterized for binding to live cells

Clone Format	Mutations	
	Heavy Chain	Light Chain
Fab, scFv	WT	WT
Fab, scFv	H1 T32A	WT
Fab, scFv	H1 T32A	L2 T69R
Fab	H1 T32E	WT
Fab	H3 W110Y	WT
Fab	H1 T32A	L1 K29S
Fab	H1 T32A	L2 T69R, Q71T
Fab	H1 T32A + H2 N71H	WT
Fab	H1 T32A	L1 A25P + L2 T69R
Fab	H1 T32A	L1 A25P + L2 L70K
Fab	H1 T32A	L1 K29S + L2 T69R

[0163] ROR1 binding was quantitated using flow cytometry and 2.5E5 ROR1-transfected MEC cells or JeKo-1 cells for each concentration of Fab variant. The cells were placed in 50 μ L of PBS with 2% FBS. Next, the cells were diluted with an equal volume of 2X stock of the test Fab variant and the cells and antibody were co-incubated on ice for 20 min. The cells were then washed 3 times with 300 μ L of FACS buffer and were resuspended in 100 μ L of a

70-fold dilution of anti-HA.11-PE conjugate, clone 16B12 (BioLegend, cat. #901517) and incubated on ice with reduced light for 20 min. Subsequently, the cells were washed 3 times with 300 μ L of FACS buffer and fixed with 2% paraformaldehyde for 10 min at 25°C. The cells then were washed 2 times with 300 μ L of FACS buffer and analyzed on a Miltenyi MACSQuant Analyzer.

[0164] Initially, three anti-ROR1 Fab variants, each differing from the parental antibody by a single amino acid, were characterized for binding to human ROR1 using live ROR1-transfected MEC cells. Two of the variants, H1 T32A and H1 T32E, displayed higher affinity than the parental Fab (**FIG. 11**, open circles and squares versus closed circles) while the other variant, H3 W110Y, displayed lower affinity (**FIG. 11**, triangles). The binding profiles of the variants on live ROR1-transfected MEC cells were consistent with the binding profiles obtained using recombinant huROR1-ECD and an ELISA (see Example 3, **FIG. 5**). No binding of any of the variants was detected when control empty vector transfected MEC cells were used (not shown), demonstrating the specificity of the variants for ROR1. These data demonstrate that the affinity improvements identified using recombinant huROR1-ECD are reflective of the binding to ROR1 on live ROR1-transfected cells for these variants.

[0165] Next, additional variants were characterized for binding to the JeKo-1 tumor cell line. JeKo-1 cells express lower levels of ROR1 (~13,000 copies/cell) than the ROR1-transfected MEC cells (~56,000 copies/cell). All Fab variants tested bound JeKo-1 cells more tightly than the parental Ab1 Fab (**FIG. 12, Panel A, FIG. 12, Panel B**). Variant H1 T32E binding to JeKo-1 cells was not characterized. In addition, five of the seven combinatorial variants bound more tightly than the H1 T32A variant (**FIG. 12, Panel A, FIG. 12, Panel B**). Combining L1 K29S or H2 N71H with H1 T32A did not appear to further improve binding to live cells (**FIG. 12, Panel B**, compare triangles with open circles). In summary, the improved binding of variants to recombinant huROR1-ECD in ELISA format reflected improved binding to tumor cells.

[0166] Finally, the binding of Ab1, Ab1.1 and Ab1.2 scFv constructs to JeKo-1 cells was also characterized. Similar to Fab variant binding to JeKo-1 cells (**FIG. 12, Panel A, FIG. 12, Panel B**) and variant binding to recombinant huROR1-ECD, as determined by ELISA (**FIG. 9, Panel A**, and **FIG. 10**), the combinatorial scFv variant H1 T32A + L2 T69R bound most tightly, followed by scFv variant H1 T32A (**FIG. 12, Panel C**). Both variants bind significantly stronger than the parental Ab1 scFv (**FIG. 12, Panel C**). Thus, the improved binding of Fab variants was preserved as the mutations were tested in the alternative, smaller scFv format.

[0167] Collectively, these data demonstrate the affinity maturation of Ab1. The improved affinity was >10-fold, as determined by multiple screening formats. Typically, avidity (bivalent interaction) can increase the apparent binding affinity of antibodies by up to 10-fold. Thus, the affinity maturation of Ab1 led to the identification of smaller, monovalent constructs that may bind tumor cells *in vivo* as avidly as the bivalent Ab1 IgG.

Example 7. Identification and Engineering of Potential Sequence Liabilities.

[0168] In order to facilitate the clinical development of Ab1 it is desirable for the antibody to express at high levels, have low product heterogeneity, and be stable over long periods of time (reviewed in Jarasch et al., *J Pharm Sci.* (2015) 104(6):1885-98). Product heterogeneity and stability are greatly impacted by chemical degradation, fragmentation, and aggregation. One example of a potential degradation event is the oxidation of solvent-exposed methionine and tryptophan residues. Previously, it has been demonstrated that oxidation may lead to loss of potency and/or aggregation. For these reasons, the sequence of Ab1 was evaluated for potential developability issues using the Therapeutic Antibody Profiler (Raybould et al., *Proc Natl Acad Sci USA* (2019) 116(10):4025-30). The analysis identified four residues potentially susceptible to oxidation: light chain M4 and W43 and heavy chain W110 and W139. Residues M4 and W43 of the light chain and W139 of the heavy chain are human germline amino acids associated with the antibody framework region and consequently, were not altered in order to avoid the introduction of potential immunogenic sequences. In contrast, heavy chain CDR3 is solvent-exposed and often involved in antigen binding. Therefore, oxidation of W110 might reduce target binding, reduce antibody stability, and/or induce aggregation. Given the potential liability of oxidation of W110, all nineteen non-wild-type amino acids were isolated from the H3 library, confirmed by DNA sequencing, and soluble Fab was expressed and isolated from the periplasm of *E. coli*, as described in Example 2. The binding of variants to huROR1-ECD was characterized by ELISA, as described in Example 2. Mutation of W110 significantly impacted binding of Ab1 to ROR1. Little or no detectable binding was observed with A, E, G, K, L, N, Q, R, S and T substitutions, while significantly impaired binding was observed with C, D, I, P and V substitutions. Substitution with F, Y, M and H reduced binding by 10- to 23-fold (**FIG. 13**). The affinities of the most active W110 variants are summarized in **Table 15**.

Table 15. Identification of most active H3 W110 variants

Residue Number & Amino Acid									ELISA K _d (nM)	CDR SEQ	SEQ ID NO:
107	108	109	110	111	135	136	137	138			
A	R	G	W	Y	Y	F	D	Y	7.0	ARGWYYFDY	89
			F						96	ARGFYFDY	94
			H						110	ARGHYFDY	169
			M						160	ARGMYFDY	170
			Y						68	ARGYYFDY	171

[0169] While the substitution of very large hydrophobic phenylalanine (F) or neutral tyrosine (Y) for the very large hydrophobic tryptophan (W) was not entirely unexpected, the substitution of histidine (H) or methionine (M) was more surprising. Although these four substitutions reduce binding by 10- to 23-fold it is possible that one (or more) of these mutations may be combined with the higher affinity variants described in Examples 3 and 4 to generate a variant that is both more stable and higher affinity than Ab1.

Table 15. Sequence Descriptions

SEQ ID NO:	DESCRIPTION
1	Ab1 VL nucleotide sequence
2	Ab1 VL amino acid sequence
3	Ab1 VH nucleotide sequence
4	Ab1 VH amino acid sequence
5	H1 T32A VH amino acid sequence
6	L2 T69R VL amino acid sequence
7	Ab1 scFv nucleotide sequence
8	Ab1 scFv amino acid sequence
9	H1 T32A Ab1 scFv nucleotide sequence
10	H1 T32A Ab1 scFv amino acid sequence
11	H1 T32A/L2 T69R Ab1 scFv nucleotide sequence
12	H1 T32A/L2 T69R Ab1 scFv amino acid sequence
13-41	H1 variant amino acid sequences (see Table 1)
42-67	H2a variant amino acid sequences (see Table 2)
68-88	H2b variant amino acid sequences (see Table 3)
89-100	H3 variant amino acid sequences (see Table 4)
101-113	L1 variant amino acid sequences (see Table 5)
114-122	L2 variant amino acid sequences (see Table 6)
123-141	L3 variant amino acid sequences (see Table 7)
142	L1 A25P/K29E amino acid sequence (see Table 9)
143	L1 A25P/K29S amino acid sequence (see Table 9)
144-150	L2 double and triple variant amino acid sequences (see Table 10)
151-168	H2 double and triple variant amino acid sequences (see Table 11)
169-171	H3 W110 variant amino acid sequences (see Table 15)
172	Ab1 H-FR1 amino acid sequence
173	Ab1 H-FR2 amino acid sequence
174	Ab1 H-FR3 amino acid sequence
175	Ab1 H-FR4 amino acid sequence
176	Ab1 L-FR1 amino acid sequence
177	Ab1 L-FR2 amino acid sequence
178	Ab1 L-FR3 amino acid sequence
179	Ab1 L-FR4 amino acid sequence
180	linker (Gly ₄ Ser) ₃
181	human ROR1 amino acid sequence

SEQUENCES**SEQ ID NO: 1 (Ab1 VL DNA)**

GATATTGTTATGACTCAAACCTCCGTTGTCTTTACCGGTAACGCCGGGTGAGCCCGCATCCAT
 TTCTTGCCGTGCCTCAAAATCTATCTCTAAGTACCTGGCTTGGTATCAGCAAAAAGCCGGGTC
 AGGCACCTCGCTTGTTAATTTACTCGGGGTCTACACTGCAATCTGGCATCCCGCCGCGTTTC
 TCAGGGAGCGGATACGGCACGGATTTACCTTAACTATCAACAACATCGAATCTGAAGACGC
 CGCCTACTACTTTTGCCAACAGCATGATGAGTCACCTTACACTTTCGGTGAGGGAACGAAGG
 TTGAAATCAAA

SEQ ID NO: 2 (Ab1 VL peptide)

DIVMTQTPLSLPVTGPGEPAISCRASKSISKYLAWYQQKPGQAPRLLIYSGSTLQSGIPPRF
 SGSGYGTDFTLTINNIESEDAAYYFCQQHDESPYTFGEGTKVEIK

SEQ ID NO: 3 (Ab1 VH DNA)

CAGGTTACGCTTCAGGAATCTGGTTCCTGCTTTAGTGAAACCTAGTCAGACATTAAGTCTTAC
 ATGCACAGTTTCTGGCTATGCCTTCACGGCATATAACATCCACTGGGTACGCCAAGCCCCAG
 GCCAGGGACTGGAATGGATGGGCAGCTTCGATCCCTATGATGGCGGTAGTTCGTACAACCAG
 AAGTTCAAGGATCGCCTTACTATCTCAAAGGATACCTCGAAGAATCAGGTGGTCCTTACTAT
 GACAAATATGGATCCAGTGGACACTGCCACATACTATTGCGCCCGTGGGTGGTACTATTTTG
 ACTACTGGGGCCACGGCACTTTAGTGACGGTCTCCTCC

SEQ ID NO: 4 (Ab1 VH peptide)

QVQLQESGPGLVKPSQTLSTLTCTVSGYAFTAYNIHWVRQAPGQGLEWMGSFDPYDGGSSYNQ
 KFKDRLTISKDTSKNQVVLMTNMDPVDATYYCARGWYFDYWGHGTLVTVSS

SEQ ID NO: 5 (H1 T32A Ab1 VH peptide)

QVQLQESGPGLVKPSQTLSTLTCTVSGYAFAAYNIHWVRQAPGQGLEWMGSFDPYDGGSSYNQ
 KFKDRLTISKDTSKNQVVLMTNMDPVDATYYCARGWYFDYWGHGTLVTVSS

SEQ ID NO: 6 (L2 T69R Ab1 VL peptide)

DIVMTQTPLSLPVTGPGEPAISCRASKSISKYLAWYQQKPGQAPRLLIYSGSRLQSGIPPRF
 SGSGYGTDFTLTINNIESEDAAYYFCQQHDESPYTFGEGTKVEIK

SEQ ID NO: 7 (Ab1 scFv DNA)

CAGGTTACGCTTCAGGAATCTGGTTCCTGGTTTAGTGAAACCTAGTCAGACATTAAGTCTTAC
 ATGCACAGTTTCTGGCTATGCCTTCACGGCATATAACATCCACTGGGTACGCCAAGCCCCAG
 GCCAGGGACTGGAATGGATGGGCAGCTTCGATCCCTATGATGGCGGTAGTTCGTACAACCAG
 AAGTTCAAGGATCGCCTTACTATCTCAAAGGATACCTCGAAGAATCAGGTGGTCCTTACTAT
 GACAAATATGGATCCAGTGGACACTGCCACATACTATTGCGCCCGTGGGTGGTACTATTTTG
 ACTACTGGGGCCACGGCACTTTAGTGACGGTCTCCTCCGGAGGTGGCGGCTCGGGTGGGGGT
 GGTAGTGGAGGAGGAGGCAGCGCAGATATTGTTATGACTCAAACCTCCGTTGTCTTTACCGGT
 AACGCCGGGTGAGCCCGCATCCATTTCTTGCCGTGCCTCAAAATCTATCTCTAAGTACCTGG
 CTTGGTATCAGCAAAAAGCCGGGTGAGGCACCTCGCTTGTTAATTTACTCGGGGTCTACACTG
 CAATCTGGCATCCCGCCGCGTTTCTCAGGGAGCGGATACGGCACGGATTTACCTTAACTAT
 CAACAACATCGAATCTGAAGACGCCGCCTACTACTTTTGCCAACAGCATGATGAGTCACCTT
 AACTTTTCGGTGAGGGAACGAAGGTTGAAATCAAACGGGATTACAAAGATGACGACGATAAG
 GGAAGCCAGGAAGTGGTAAACCCGGCTCCGGAAAGCCGGGAAGCGGCAACCTGGTTCTTA
 CCCGTACGATGTACCTGACTACGCTTCA

SEQ ID NO: 8 (Ab1 scFv peptide)

QVQLQESGPGLVKPSQTLSTCTVSGYAFTAYNIHWVRQAPGQGLEWMGSFDPYDGGSSYNQ
 KFKDRLTISKDTSKNQVVLMTNMDPVDATYYCARGWYYFDYWGHGTLVTVSSGGGGSGGG
 GSGGGGSADIVMTQTPLSLPVTGPGEPAISCRASKSISKYLAWEYQKPGQAPRLLIYSGSTL
 QSGIPPRFSGSGYGTDFTLTINNIESEDAAYYFCQQHDESPYTFEGEGTKVEIKRDYKDDDDK
 GKPGSGKPGSGKPGSGKPGSYPYDVPDYAS

SEQ ID NO: 9 (H1 T32A Ab1 scFv DNA)

CAGGTTTCAGCTTCAGGAATCTGGTCCTGGTTTGTAGTGAAACCTAGTCAGACATTAAGTCTTAC
 ATGCACAGTTTCTGGCTATGCCTTCGCAGCATATAACATCCACTGGGTACGCCAAGCCCCAG
 GCCAGGGACTGGAATGGATGGGCAGCTTCGATCCCTATGATGGCGGTAGTTTCGTACAACCAG
 AAGTTCAAGGATCGCCTTACTATCTCAAAGGATACCTCGAAGAATCAGGTGGTCCTTACTAT
 GACAAATATGGATCCAGTGGACACTGCCACATACTATTGCGCCCGTGGGTGGTACTATTTTG
 ACTACTGGGGCCACGGCACTTTAGTGACGGTCTCCTCCGGAGGTGGCGGCTCGGGTGGGGGT
 GGTAGTGGAGGAGGAGGCAGCGCAGATATTGTTATGACTCAAACCTCCGTTGTCTTTACCGGT
 AACGCCGGGTGAGCCCGCATCCATTTCTTGCCGTGCCTCAAATCTATCTCTAAGTACCTGG
 CTTGGTATCAGCAAAAGCCGGGTGAGGCACCTCGCTTGTTAATTTACTCGGGGTCTACACTG
 CAATCTGGCATCCCGCCGCGTTTCTCAGGGAGCGGATACGGCACGGATTTACCTTAACTAT
 CAACAACATCGAATCTGAAGACGCCGCCTACTACTTTTGCCAACAGCATGATGAGTCACCTT
 AACTTTTCGGTGAGGGAACGAAGGTTGAAATCAAACGGGATTACAAAGATGACGACGATAAG
 GGGAAAGCCAGGAAGTGGTAAACCCGGCTCCGGAAAGCCGGGAAGCGGCAAACCTGGTTCTTA
 CCCGTACGATGTACCTGACTACGCTTCA

SEQ ID NO: 10 (H1 T32A Ab1 scFv peptide)

QVQLQESGPGLVKPSQTLSTCTVSGYAFAAYNIHWVRQAPGQGLEWMGSFDPYDGGSSYNQ
 KFKDRLTISKDTSKNQVVLMTNMDPVDATYYCARGWYYFDYWGHGTLVTVSSGGGGSGGG
 GSGGGGSADIVMTQTPLSLPVTGPGEPAISCRASKSISKYLAWEYQKPGQAPRLLIYSGSTL
 QSGIPPRFSGSGYGTDFTLTINNIESEDAAYYFCQQHDESPYTFEGEGTKVEIKRDYKDDDDK
 GKPGSGKPGSGKPGSGKPGSYPYDVPDYAS

SEQ ID NO: 11 (H1 T32A/L2 T69R Ab1 scFv DNA)

CAGGTTTCAGCTTCAGGAATCTGGTCCTGGTTTGTAGTGAAACCTAGTCAGACATTAAGTCTTAC
 ATGCACAGTTTCTGGCTATGCCTTCGCAGCATATAACATCCACTGGGTACGCCAAGCCCCAG
 GCCAGGGACTGGAATGGATGGGCAGCTTCGATCCCTATGATGGCGGTAGTTTCGTACAACCAG
 AAGTTCAAGGATCGCCTTACTATCTCAAAGGATACCTCGAAGAATCAGGTGGTCCTTACTAT
 GACAAATATGGATCCAGTGGACACTGCCACATACTATTGCGCCCGTGGGTGGTACTATTTTG
 ACTACTGGGGCCACGGCACTTTAGTGACGGTCTCCTCCGGAGGTGGCGGCTCGGGTGGGGGT
 GGTAGTGGAGGAGGAGGCAGCGCAGATATTGTTATGACTCAAACCTCCGTTGTCTTTACCGGT
 AACGCCGGGTGAGCCCGCATCCATTTCTTGCCGTGCCTCAAATCTATCTCTAAGTACCTGG
 CTTGGTATCAGCAAAAGCCGGGTGAGGCACCTCGCTTGTTAATTTACTCGGGGTCTCGGCTG
 CAATCTGGCATCCCGCCGCGTTTCTCAGGGAGCGGATACGGCACGGATTTACCTTAACTAT
 CAACAACATCGAATCTGAAGACGCCGCCTACTACTTTTGCCAACAGCATGATGAGTCACCTT
 AACTTTTCGGTGAGGGAACGAAGGTTGAAATCAAACGGGATTACAAAGATGACGACGATAAG
 GGGAAAGCCAGGAAGTGGTAAACCCGGCTCCGGAAAGCCGGGAAGCGGCAAACCTGGTTCTTA
 CCCGTACGATGTACCTGACTACGCTTCA

SEQ ID NO: 12 (H1 T32A/L2 T69R Ab1 scFv peptide)

QVQLQESGPGLVKPSQTLSTCTVSGYAFAAYNIHWVRQAPGQGLEWMGSFDPYDGGSSYNQ
 KFKDRLTISKDTSKNQVVLMTNMDPVDATYYCARGWYYFDYWGHGTLVTVSSGGGGSGGG
 GSGGGGSADIVMTQTPLSLPVTGPGEPAISCRASKSISKYLAWEYQKPGQAPRLLIYSGSRL

QSGIPPRFSGSGYGTDFTLTINNIESEDAAYYFCQQHDESPYTFGEGTKVEIKRDYKDDDDK
GKPGSGKPGSGKPGSGKPGSYPYDVPDYAS

SEQ ID NO: 181 (human ROR1)

MHRPRRRGTRPPLLALLAALLLAARGAAAQETELSVSAELVPTSSWNISSELNKS SYLTLD
PMNNITTS LGQTAE LHCKVSGNPPPTIRWFKNDAPVVQEPRRLSFRSTIYGSRLRIRNLDTT
DTGYFQCVATNGKEVVSSTGVL FVKFGPPPTASPGYSDEYEEDGFCQPYRGIACARFIGNRT
VYMESLHMQGEIENQITAAFTMIGTSSHLSDKCSQFAIPSLCHYAFPYCDETSSV PKPRDLC
RDECEILENVLCQTEYIFARSNPMILMRLKLPNCEDLPQPESPEAANCIRICIPMADPINKN
HKCYNSTGV DYRGTVSVTKSGRQCQPWNSQYPHTHTFTALRFPPELNGGHSYCRNPGNQKEAP
WCFTLDENFKSDLCDIPACDSKDSKEKNKMEILYILVPSVAIPLAIAL LFFFICVCRNNQKS
SSAPVQROPKHVRGQNVEMSM LNAYKPKSKAKELPLSAVRFMEELGECAFGKIYKGHLYLPG
MDHAQLVAIKTLKDYN NPQQWTEFQQEASLMAELHHPNIVCLLGAVTQE QPVCMLFEYINQG
DLHEFLIMRSPHSDVGCSSDEDGTVKSSLDHGDFLHIAIQIAAGMEYLSSHFFVHKDLAARN
ILIGEQLHVKISDLGLSREIYSADYYRVQSKSLPIRWMPEAIMYGKFSSDS DIWSFGVVL
WEI FSFGLQPYYGFSNQEV IEMVRKRQLLPCSEDCPPRMYS LMTECWNEIPSRRPRFKDIHV
RLRSWEGLSSHTSSTTPSGGNATTQT TSLSASPVSNL SNPRYPNYMFPSQGITPQGQIAGFI
GPPIPQNQRFIPINGYPIPPGYAAFPAAHYQPTGPPRVIQHCPPPKSRSPSSASGSTSTGHV
TSLPSSGSNQEANIPLLPHMSIPNHPGGMGITVFGNKSQKPYKIDSKQASLLGDANIHGHT
SMISAEL

CLAIMS

What is claimed is:

1. An anti-ROR1 antibody or an antigen-binding portion thereof, wherein said antibody comprises:

- a) a heavy chain variable domain (VH) comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from T32A, T32E, S69V, S69E, and N71H, or any combination thereof; and a light chain variable domain (VL) comprising the amino acid sequence of SEQ ID NO: 2;
- b) a VH comprising the amino acid sequence of SEQ ID NO: 4; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E, K29S, T69H, T69R, L70K, and Q71T, or any combination thereof; or
- c) a VH comprising the amino acid sequence of SEQ ID NO: 4 with a mutation selected from T32A, T32E, S69V, S69E, and N71H, or any combination thereof; and a VL comprising the amino acid sequence of SEQ ID NO: 2 with a mutation selected from A25P, K29E, K29S, T69H, T69R, L70K, and Q71T, or any combination thereof;

wherein the residues are numbered according to the AHo system and wherein the antibody or antigen-binding portion binds to the same epitope of human ROR1 as, and has a greater binding affinity for human ROR1 than, an antibody comprising a VH that comprises the amino acid sequence of SEQ ID NO: 4 and a VL that comprises the amino acid sequence of SEQ ID NO: 2.

2. The anti-ROR1 antibody or antigen-binding portion of claim 1, wherein said antibody comprises:

- a) a VH comprising the amino acid sequence of SEQ ID NO: 4 with
 - i) one or more mutations selected from T32E, S69V/E, and N71H, or
 - ii) the mutation T32A and one or both mutations selected from S69V/E and N71H, anda VL comprising the amino acid sequence of SEQ ID NO: 2;
- b) a VH comprising the amino acid sequence of SEQ ID NO: 4 with
 - i) one or more mutations selected from T32E, S69V/E, and N71H, or

- ii) the mutation T32A and one or both mutations selected from S69V/E and N71H, and
a VL comprising the amino acid sequence of SEQ ID NO: 2 with one or more mutations selected from A25P, K29E/S, T69H/R, L70K, and Q71T;
 - c) a VH comprising the amino acid sequence of SEQ ID NO: 4 comprising the mutation T32A and
a VL comprising the amino acid sequence of SEQ ID NO: 2 with
 - i) one or more mutations selected from A25P, K29E/S, T69H, L70K, and Q71T, or
 - ii) the mutation T69R and one or more mutations selected from A25P, K29E/S, L70K, and Q71T; or
 - d) a VH comprising the amino acid sequence of SEQ ID NO: 4 and
a VL comprising the amino acid sequence of SEQ ID NO: 2 with one or more mutations selected from A25P, K29E/S, T69H/R, L70K, and Q71T.
3. The antibody or antigen-binding portion of claim 1 or 2, wherein said antibody comprises:
- a) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation A25P;
 - b) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutation T69H;
 - c) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations A25P and T69R;
 - d) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations K29S and T69R;
 - e) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations T69R and Q71T; or

- f) a VH comprising the amino acid sequence of SEQ ID NO: 4 with the mutation T32A, and a VL comprising the amino acid sequence of SEQ ID NO: 2 with the mutations A25P and L70K.
4. An anti-ROR1 antibody or an antigen-binding portion thereof, comprising heavy chain (H)-CDR1-3 and light chain (L)-CDR1-3 comprising the amino acid sequences of:
- a) SEQ ID NOs: 31, 42, 89, 101, 114, and 123, respectively;
 - b) SEQ ID NOs: 13, 73, 89, 101, 114, and 123, respectively;
 - c) SEQ ID NOs: 13, 75, 89, 101, 114, and 123, respectively;
 - d) SEQ ID NOs: 13, 80, 89, 101, 114, and 123, respectively;
 - e) SEQ ID NOs: 13, 42, 89, 102, 114, and 123, respectively;
 - f) SEQ ID NOs: 13, 42, 89, 103, 114, and 123, respectively;
 - g) SEQ ID NOs: 13, 42, 89, 104, 114, and 123, respectively;
 - h) SEQ ID NOs: 13, 42, 89, 101, 117, and 123, respectively;
 - i) SEQ ID NOs: 13, 42, 89, 101, 118, and 123, respectively;
 - j) SEQ ID NOs: 13, 42, 89, 101, 120, and 123, respectively;
 - k) SEQ ID NOs: 29, 42, 89, 102, 114, and 123, respectively;
 - l) SEQ ID NOs: 29, 42, 89, 101, 117, and 123, respectively;
 - m) SEQ ID NOs: 29, 42, 89, 102, 118, and 123, respectively;
 - n) SEQ ID NOs: 29, 42, 89, 103, 118, and 123, respectively;
 - o) SEQ ID NOs: 29, 42, 89, 101, 147, and 123, respectively; or
 - p) SEQ ID NOs: 29, 42, 89, 102, 120, and 123, respectively.
5. The antibody or antigen-binding portion of claim 4, wherein the heavy chain framework regions (FRs) 1-4 and the light chain FR1-4 of said antibody or antigen-binding portion comprise the amino acid sequences of SEQ ID NOs: 172-179, respectively.
6. The antibody of any one of claims 1-5, wherein the antibody is an IgG, optionally comprising a human IgG1 constant region.
7. The antibody or antigen-binding portion of any one of claims 1-5, wherein said antibody or portion is a single chain variable fragment (scFv).

8. The antibody or antigen-binding portion of any one of claims 1-7, wherein said antibody or antigen-binding portion:
- a) binds to ROR1-transfected MEC cells with greater affinity than an antibody with a VH comprising the amino acid sequence of SEQ ID NO: 4 and a VL comprising the amino acid sequence of SEQ ID NO: 2, as assessed by flow cytometry;
 - b) binds to JeKo-1 cells with greater affinity than an antibody with a VH comprising the amino acid sequence of SEQ ID NO: 4 and a VL comprising the amino acid sequence of SEQ ID NO: 2, as assessed by flow cytometry; or
 - c) both a) and b).
9. An isolated nucleic acid molecule comprising a nucleotide sequence that encodes the heavy chain or an antigen-binding portion thereof, or a nucleotide sequence that encodes the light chain or an antigen-binding portion thereof, or both, of the anti-ROR1 antibody or antigen-binding portion of any one of claims 1-8.
10. A vector comprising the isolated nucleic acid molecule of claim 9, wherein said vector further comprises an expression control sequence linked operatively to the isolated nucleic acid molecule.
11. A host cell comprising a nucleotide sequence that encodes the heavy chain or an antigen-binding portion thereof, and a nucleotide sequence that encodes the light chain or an antigen-binding portion thereof, of the anti-ROR1 antibody or antigen-binding portion of any one of claims 1-8.
12. A method for producing an anti-ROR1 antibody or an antigen-binding portion thereof, comprising providing the host cell of claim 11, culturing said host cell under conditions suitable for expression of the antibody or portion, and isolating the resulting antibody or portion.
13. A bispecific binding molecule comprising the antibody or antigen-binding portion of any one of claims 1-8.

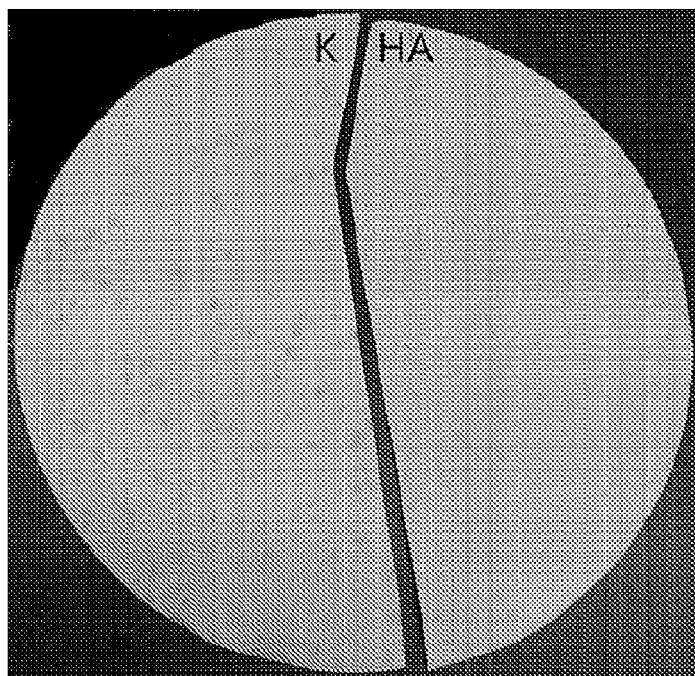
14. An immunoconjugate comprising the anti-ROR1 antibody or antigen-binding portion of any one of claims 1-8 conjugated to a cytotoxic agent.
15. An immunoconjugate comprising an anti-ROR1 antibody or an antigen-binding portion thereof conjugated to a cytotoxic agent, wherein said antibody comprises a heavy chain variable domain (VH) amino acid sequence of SEQ ID NO: 5 and a light chain variable domain (VL) amino acid sequence of SEQ ID NO: 2.
16. An immunoconjugate comprising an anti-ROR1 antibody or an antigen-binding portion thereof conjugated to a cytotoxic agent, wherein said antibody comprises a heavy chain variable domain (VH) amino acid sequence of SEQ ID NO: 5 and a light chain variable domain (VL) amino acid sequence of SEQ ID NO: 6.
17. A pharmaceutical composition comprising the anti-ROR1 antibody or antigen-binding portion thereof of any one of claims 1-8, the bispecific binding molecule of claim 13, or the immunoconjugate of any one of claims 14-16, and a pharmaceutically acceptable excipient.
18. A method for treating cancer in a patient in need thereof, comprising administering to said patient a therapeutically effective amount of the anti-ROR1 antibody or antigen-binding portion of any one of claims 1-8, the bispecific binding molecule of claim 13, the immunoconjugate of any one of claims 14-16, or the pharmaceutical composition of claim 17.
19. Use of the anti-ROR1 antibody or antigen-binding portion of any one of claims 1-8, the bispecific binding molecule of claim 13, or the immunoconjugate of any one of claims 14-16 for the manufacture of a medicament for treating cancer in a patient in need thereof.
20. The anti-ROR1 antibody or antigen-binding portion of any one of claims 1-8, the bispecific binding molecule of claim 13, the immunoconjugate of any one of claims 14-16, or the pharmaceutical composition of claim 17, for use in treating cancer in a patient in need thereof.

21. The method, use, or anti-ROR1 antibody or antigen-binding portion, bispecific binding molecule, immunoconjugate, or pharmaceutical composition for use of any one of claims 18-20, wherein the cancer is a ROR1-positive cancer.
22. The method, use, or anti-ROR1 antibody or antigen-binding portion, bispecific binding molecule, immunoconjugate, or pharmaceutical composition for use of any one of claims 18-21, wherein the cancer is a leukemia, a lymphoma, or a solid tumor.
23. The method, use, or anti-ROR1 antibody or antigen-binding portion, bispecific binding molecule, immunoconjugate, or pharmaceutical composition for use of any one of claims 18-22, wherein the cancer is non-Hodgkin lymphoma, optionally diffuse large B-cell lymphoma (DLBCL); follicular lymphoma (FL); mantle cell lymphoma (MCL); marginal zone lymphoma (MZL); Burkitt's lymphoma; T cell non-Hodgkin lymphoma; Waldenström macroglobulinemia; a non-Hodgkin lymphoma that has undergone Richter's transformation; acute myeloid leukemia (AML); acute lymphoblastic leukemia (ALL); chronic lymphocytic leukemia (CLL); small lymphocytic leukemia (SLL); breast cancer; non-small cell lung cancer; ovarian cancer; or sarcoma, optionally Ewing's sarcoma.
24. The method, use, or anti-ROR1 antibody or antigen-binding portion, bispecific binding molecule, immunoconjugate, or pharmaceutical composition for use of any one of claims 18-23, wherein the patient is treated with an additional therapeutic agent.
25. The method, use, or anti-ROR1 antibody or antigen-binding portion, bispecific binding molecule, immunoconjugate, or pharmaceutical composition for use of claim 24, wherein the additional therapeutic agent is selected from the group consisting of a Bruton's tyrosine kinase (BTK) inhibitor, a B-cell lymphoma 2 (Bcl-2) inhibitor, a mammalian target of rapamycin (mTOR) inhibitor, and a phosphoinositide 3-kinase (PI3K) inhibitor.
26. The method, use, or anti-ROR1 antibody or antigen-binding portion, bispecific binding molecule, immunoconjugate, or pharmaceutical composition for use of claim 25, wherein the additional therapeutic agent is selected from the group consisting of ibrutinib, acalabrutinib, venetoclax, everolimus, sapanisertib, and idelalisib.

27. A kit comprising the anti-ROR1 antibody or antigen-binding portion of any one of claims 1-8, the bispecific binding molecule of claim 13, the immunoconjugate of any one of claims 14-16, or the pharmaceutical composition of claim 17.

28. An article of manufacture comprising the anti-ROR1 antibody or antigen-binding portion of any one of claims 1-8, the bispecific binding molecule of claim 13, the immunoconjugate of any one of claims 14-16, or the pharmaceutical composition of claim 17, wherein said article of manufacture is suitable for treating cancer in a patient in need thereof.

A



B

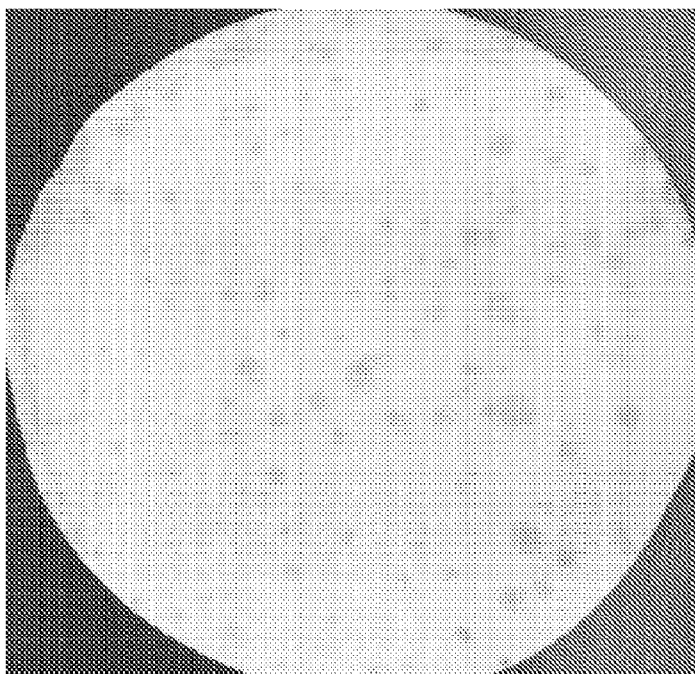


FIG. 1

2/14

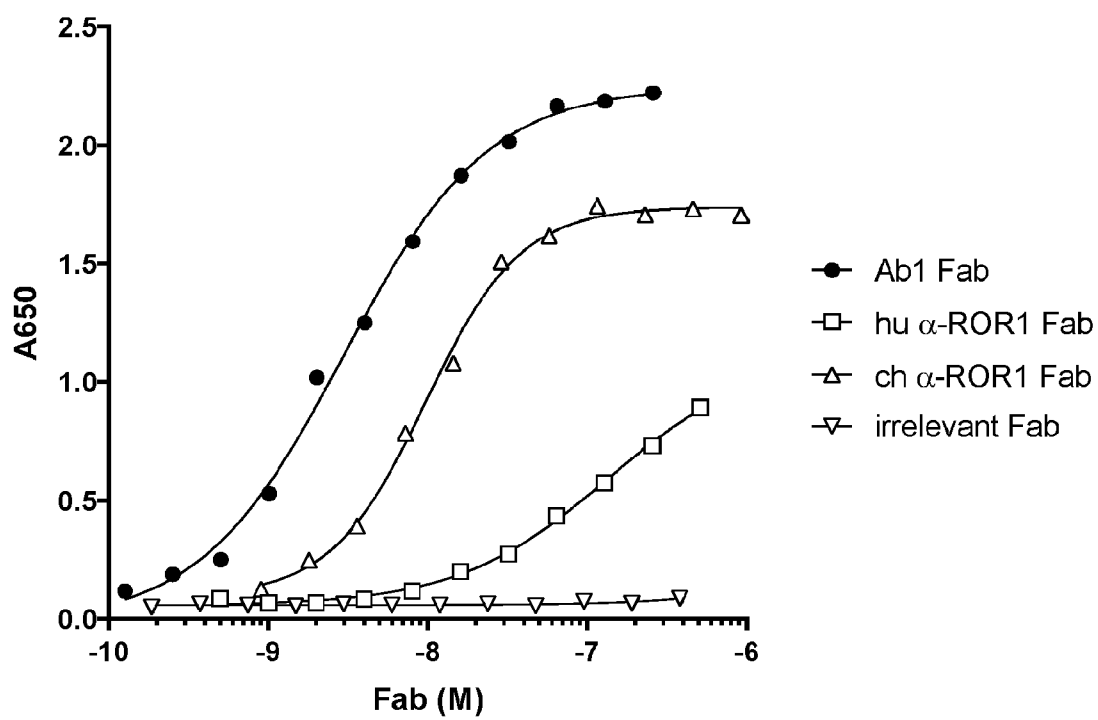


FIG. 2

3/14

CDR	WT AA	Residue	% Variant Amino Acid									
			Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile
HCDR1	Gly	27	3.21%	5.71%	14.64%	19.64%	5.54%	2.68%	5.71%	0.00%	5.71%	1.43%
	Tyr	29	2.07%	3.53%	8.30%	11.83%	7.26%	7.68%	7.68%	2.90%	3.73%	4.77%
	Ala	30	0.00%	3.87%	9.16%	12.42%	2.44%	5.70%	10.18%	6.72%	4.48%	4.89%
	Phe	31	4.11%	5.28%	14.87%	11.74%	3.72%	2.74%	4.50%	7.63%	7.63%	3.13%
	Thr	32	5.76%	2.62%	12.04%	8.38%	0.92%	5.89%	7.98%	5.10%	4.97%	3.80%
	Ala	33	0.00%	3.90%	6.93%	11.69%	5.41%	3.90%	7.14%	7.79%	3.46%	4.76%
	Tyr	39	5.15%	6.47%	6.62%	6.03%	3.24%	6.91%	9.85%	6.18%	4.85%	4.71%
	Asn	40	6.85%	4.32%	0.00%	8.93%	4.76%	6.55%	6.70%	8.48%	0.89%	4.61%
	Ile	41	1.60%	3.91%	13.14%	9.06%	4.26%	4.62%	6.57%	7.64%	6.93%	0.00%
	His	42	8.91%	8.00%	0.00%	0.00%	0.00%	12.18%	13.27%	0.00%	0.00%	0.00%
HCDR2	Ser	57	4.60%	5.11%	6.81%	8.01%	3.58%	2.73%	8.01%	7.50%	3.58%	6.30%
	Phe	58	3.72%	2.97%	4.34%	9.29%	3.97%	4.71%	7.19%	5.82%	5.33%	5.08%
	Asp	59	3.87%	3.78%	7.03%	0.00%	6.13%	3.24%	5.23%	8.56%	4.32%	5.86%
	Pro	60	7.01%	3.01%	4.34%	4.51%	4.51%	5.84%	5.51%	4.34%	3.34%	7.51%
	Tyr	61	4.00%	6.06%	6.06%	5.13%	6.98%	7.49%	4.31%	4.00%	3.70%	6.88%
	Asp	65	5.31%	3.84%	7.78%	0.00%	4.13%	5.61%	5.81%	6.99%	4.04%	5.12%
	Gly	66	2.92%	7.78%	7.29%	8.91%	4.38%	7.46%	6.97%	0.00%	5.35%	3.89%
	Gly	67	4.40%	5.28%	8.06%	8.21%	4.84%	7.92%	7.92%	0.00%	5.87%	5.57%
	Ser	68	5.50%	3.42%	5.50%	6.98%	1.93%	5.50%	5.35%	6.84%	5.79%	7.28%
	Ser	69	5.19%	4.82%	4.17%	6.21%	3.34%	5.00%	6.12%	5.93%	3.06%	6.77%
	Tyr	70	4.61%	3.36%	3.86%	5.11%	2.62%	3.24%	4.98%	5.35%	3.86%	17.06%
	Asn	71	5.79%	3.64%	0.00%	6.28%	5.29%	4.63%	9.26%	6.78%	2.81%	6.45%
	Gln	72	5.33%	2.36%	4.59%	6.33%	4.34%	0.00%	7.07%	9.06%	3.35%	5.58%
	Lys	73	5.60%	4.96%	5.36%	7.69%	6.16%	3.84%	7.29%	6.33%	4.32%	5.52%
	Phe	74	3.10%	5.22%	4.73%	7.83%	5.55%	5.87%	5.87%	5.87%	4.73%	4.40%
	Lys	75	7.91%	3.91%	3.91%	4.89%	5.33%	3.47%	5.51%	5.96%	3.29%	4.80%
	Asp	76	6.00%	5.03%	5.91%	0.00%	2.65%	6.70%	7.50%	7.41%	5.29%	5.29%

FIG.3

CDR	WT AA	Residue	% Variant Amino Acid											
			Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val	Ter	
HCDR1	Gly	27	2.50%	3.75%	2.68%	1.96%	5.54%	11.07%	2.68%	2.50%	1.96%	1.07%	0.00%	
	Tyr	29	1.45%	8.51%	4.36%	2.49%	3.53%	4.98%	6.85%	5.19%	0.00%	2.90%	0.00%	
	Ala	30	4.28%	6.11%	3.67%	2.04%	5.09%	6.11%	2.85%	3.87%	1.02%	5.09%	0.00%	
	Phe	31	3.91%	4.11%	4.11%	0.00%	3.52%	6.85%	2.35%	3.33%	3.33%	3.13%	0.00%	
	Thr	32	2.09%	10.99%	4.84%	1.31%	7.59%	7.72%	0.00%	4.32%	1.05%	2.62%	0.00%	
	Ala	33	1.73%	6.06%	6.28%	1.95%	4.76%	4.76%	7.79%	5.19%	1.52%	4.98%	0.00%	
	Tyr	39	2.65%	6.32%	3.38%	2.79%	4.41%	6.47%	5.29%	5.15%	0.00%	3.24%	0.29%	
	Asn	40	2.38%	9.67%	5.21%	2.38%	6.85%	7.89%	4.46%	2.53%	3.27%	3.27%	0.00%	
	Ile	41	1.60%	8.17%	3.02%	4.44%	3.55%	5.86%	6.75%	4.97%	2.31%	1.60%	0.00%	
	His	42	0.91%	17.09%	11.45%	0.00%	9.64%	0.00%	9.64%	4.91%	0.00%	3.82%	0.18%	
HCDR2	Ser	57	1.02%	7.50%	3.41%	6.64%	2.04%	0.00%	5.11%	8.18%	6.30%	3.58%	0.00%	
	Phe	58	2.60%	5.95%	4.09%	0.00%	3.22%	5.70%	5.08%	7.93%	7.93%	5.08%	0.00%	
	Asp	59	3.06%	6.40%	5.14%	5.32%	2.16%	8.11%	6.04%	5.14%	5.77%	4.77%	0.09%	
	Pro	60	5.34%	4.67%	6.51%	4.67%	0.00%	3.84%	8.35%	3.51%	4.17%	9.02%	0.00%	
	Tyr	61	3.59%	6.88%	2.67%	4.31%	4.62%	7.49%	6.37%	5.34%	0.00%	4.00%	0.10%	
	Asp	65	3.05%	4.92%	3.64%	4.63%	6.00%	8.96%	8.56%	3.84%	5.22%	2.46%	0.10%	
	Gly	66	2.59%	7.62%	3.57%	3.08%	3.73%	5.51%	4.54%	2.76%	7.29%	4.38%	0.00%	
	Gly	67	2.05%	8.80%	3.37%	2.49%	1.47%	8.65%	5.43%	3.52%	2.64%	3.52%	0.00%	
	Ser	68	4.16%	5.65%	4.16%	5.05%	2.23%	0.00%	5.05%	6.24%	4.75%	8.32%	0.30%	
	Ser	69	3.06%	6.12%	7.78%	4.73%	1.85%	0.00%	4.91%	9.55%	5.38%	5.84%	0.19%	
	Tyr	70	3.99%	5.48%	5.85%	7.35%	1.74%	4.11%	3.99%	4.11%	0.00%	9.34%	0.00%	
	Asn	71	4.63%	7.27%	5.62%	4.30%	2.31%	7.60%	5.45%	4.30%	3.64%	3.97%	0.00%	
	Gln	72	3.47%	4.34%	6.82%	3.60%	6.70%	7.07%	5.21%	3.47%	4.59%	6.45%	0.25%	
	Lys	73	5.52%	0.00%	4.72%	3.12%	2.24%	7.21%	4.72%	3.68%	4.56%	7.13%	0.00%	
	Phe	74	3.43%	5.87%	4.89%	0.00%	3.59%	4.89%	2.94%	6.36%	7.67%	7.18%	0.00%	
	Lys	75	5.60%	0.00%	7.47%	3.91%	4.44%	6.13%	7.82%	5.78%	3.20%	6.67%	0.00%	
Asp	76	2.03%	3.79%	3.70%	5.56%	5.47%	7.23%	7.67%	3.88%	3.70%	5.11%	0.09%		

FIG.3 (cont.)

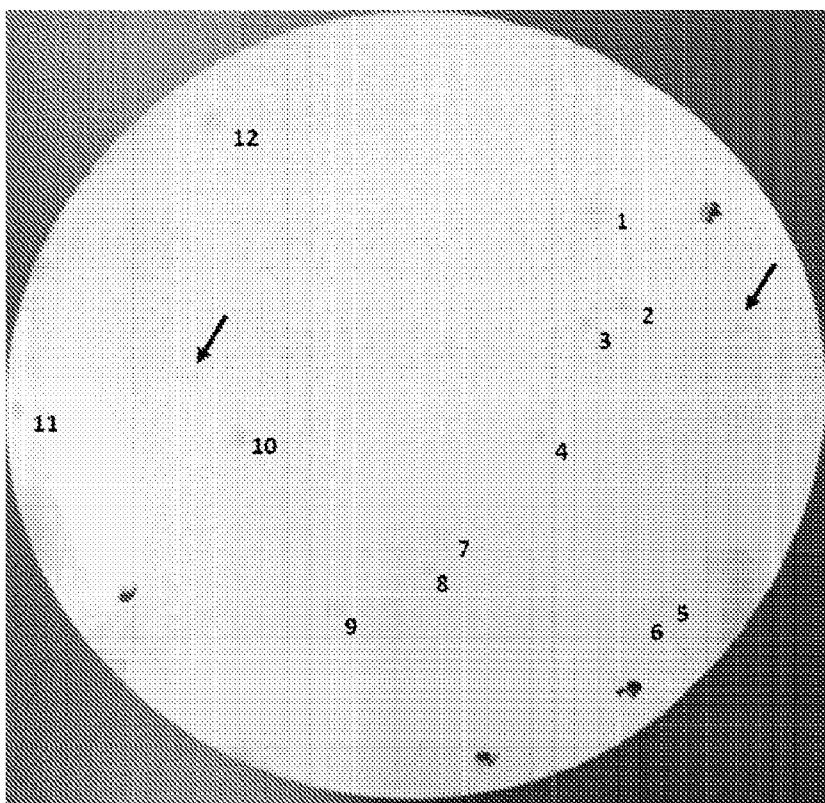


FIG. 4

6/14

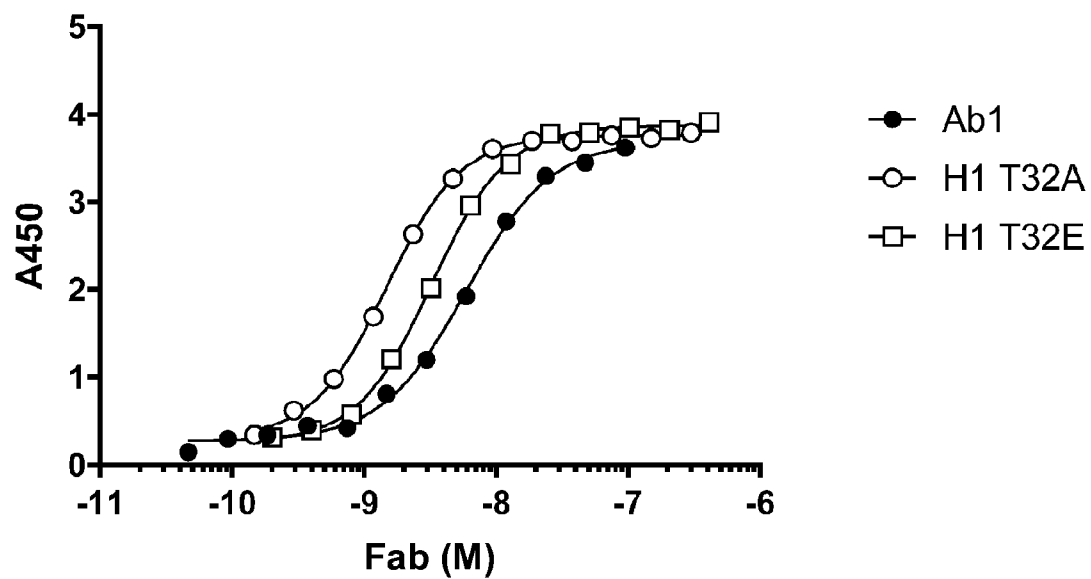


FIG. 5

7/14

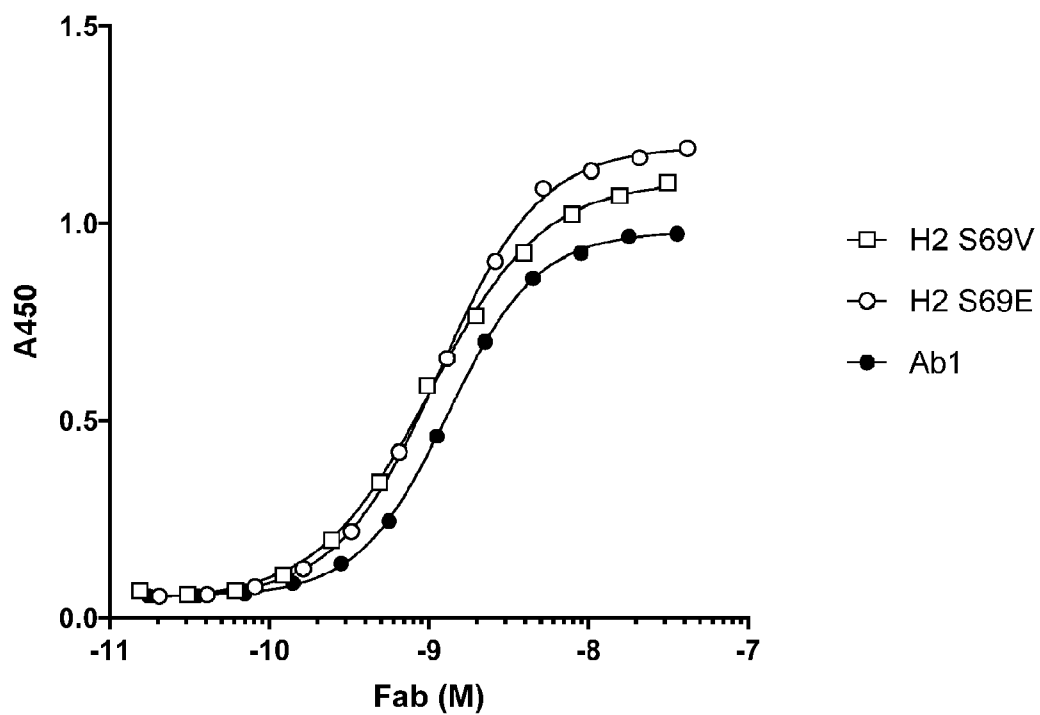


FIG. 6

8/14

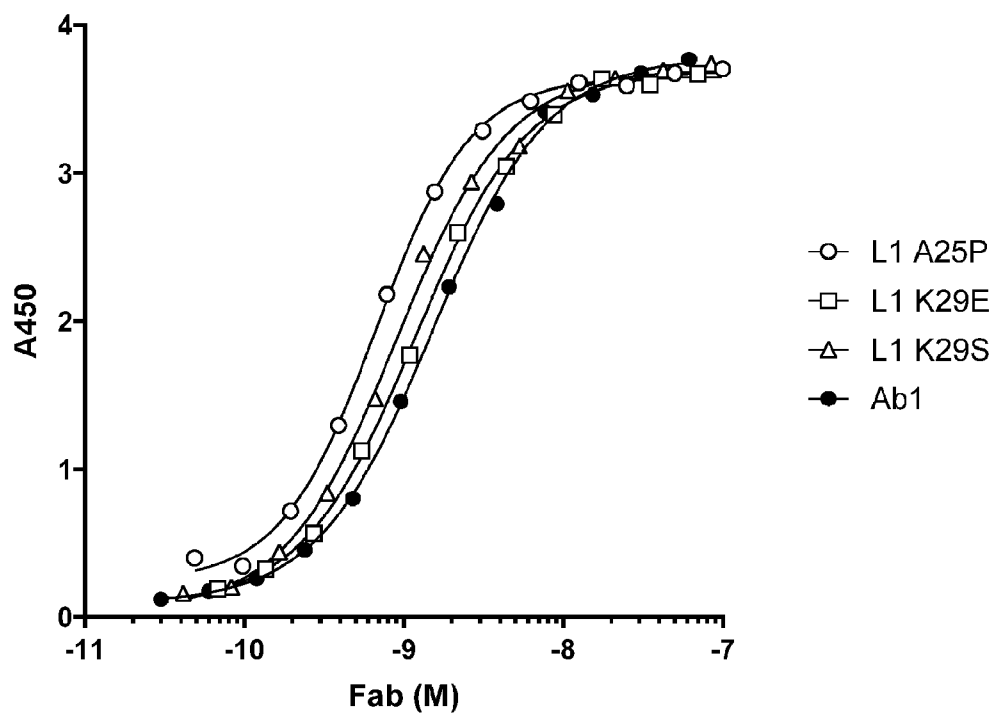


FIG. 7

9/14

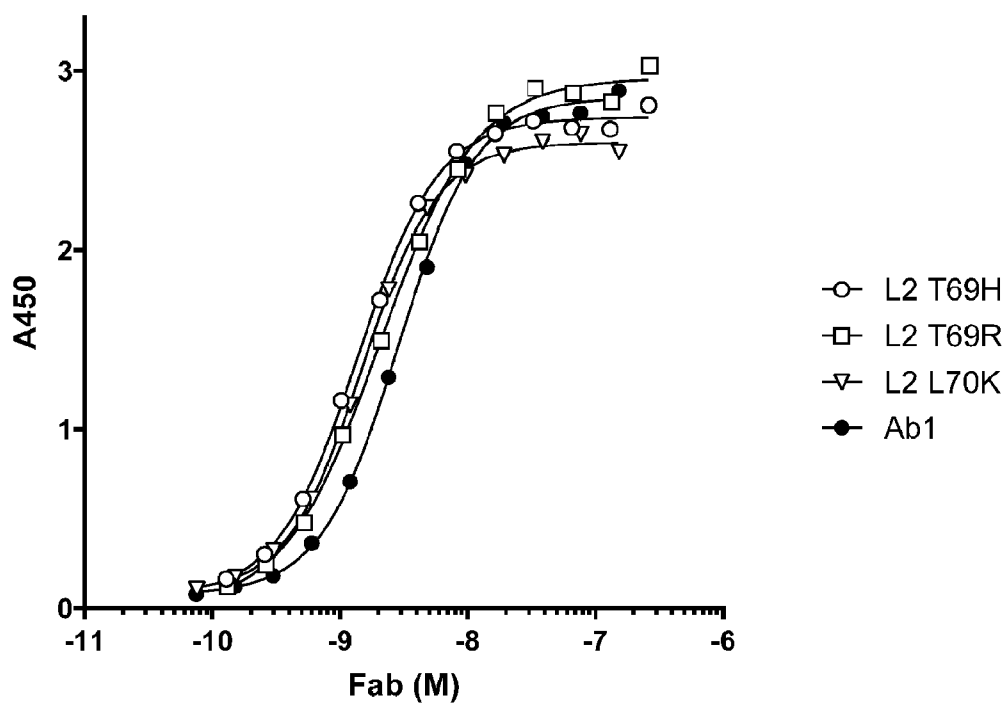


FIG. 8

10/14

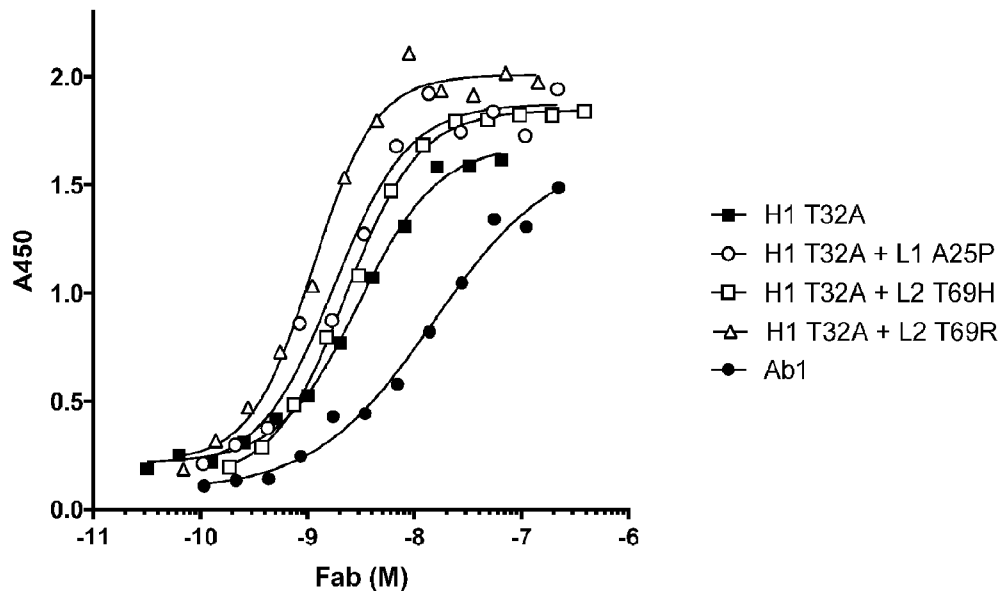
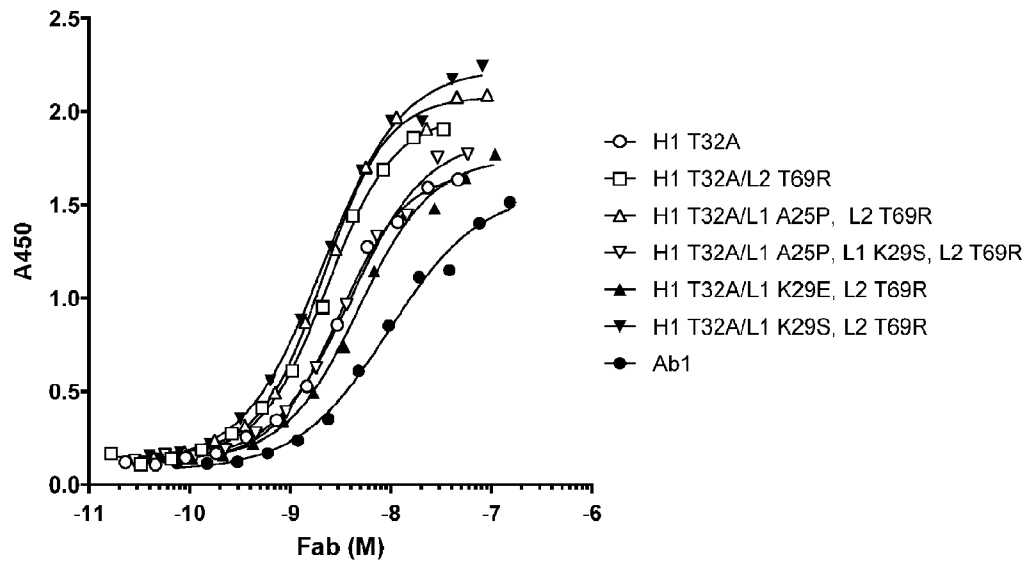
A**B**

FIG. 9

11/14

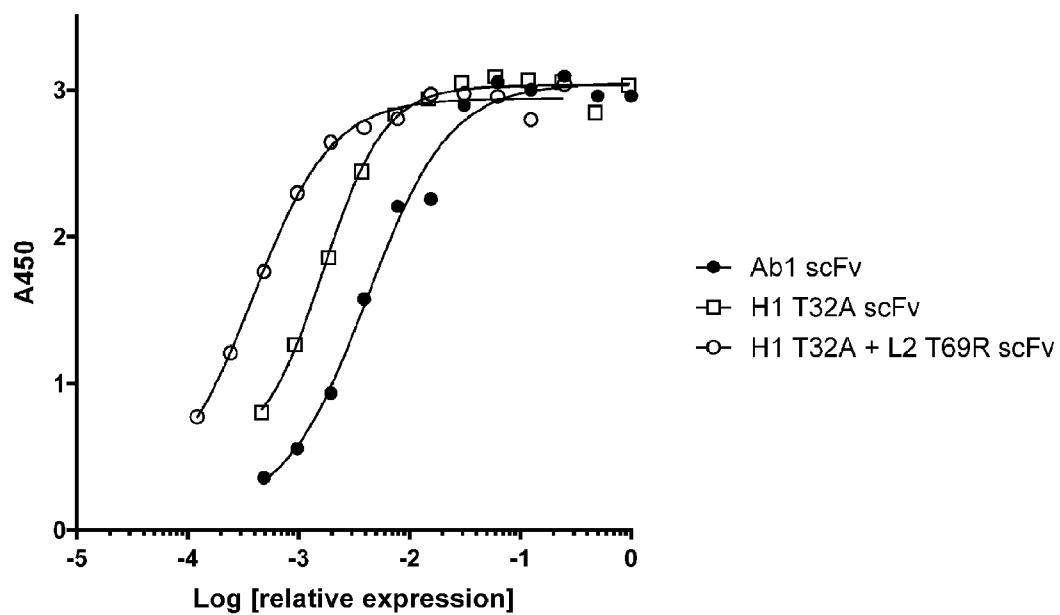


FIG. 10

12/14

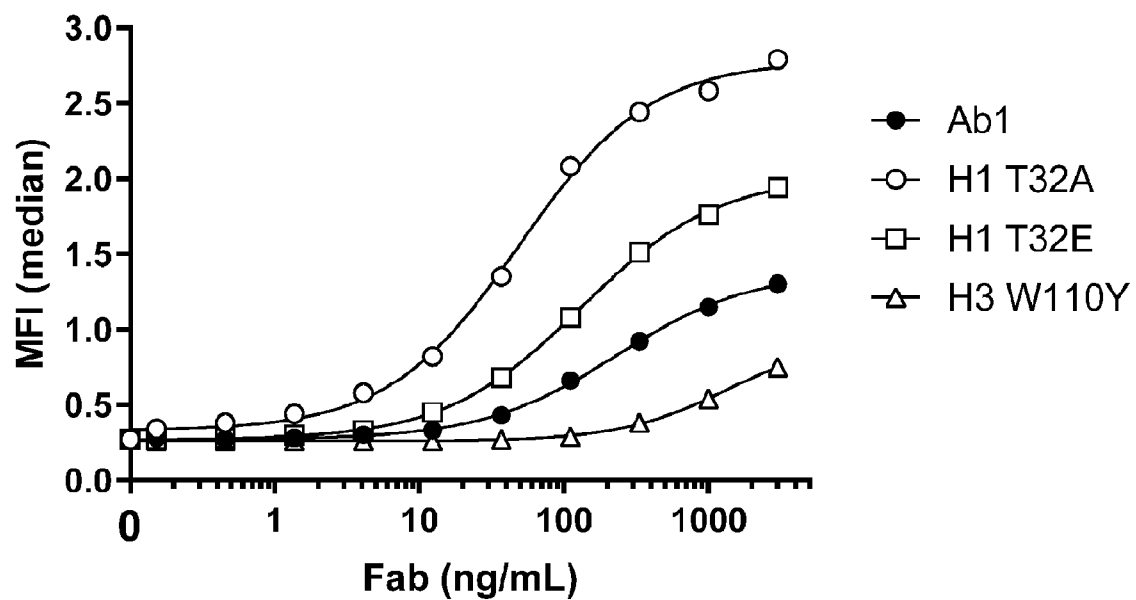
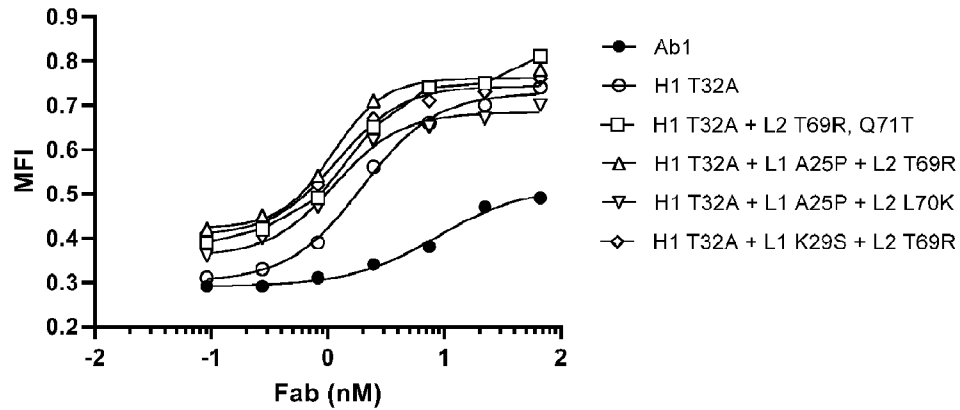
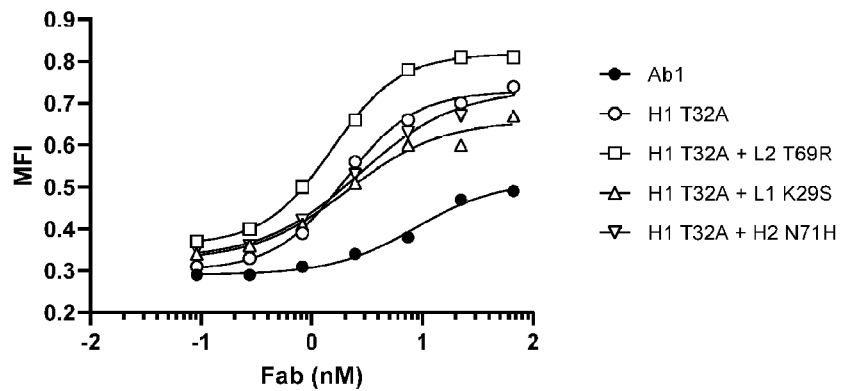
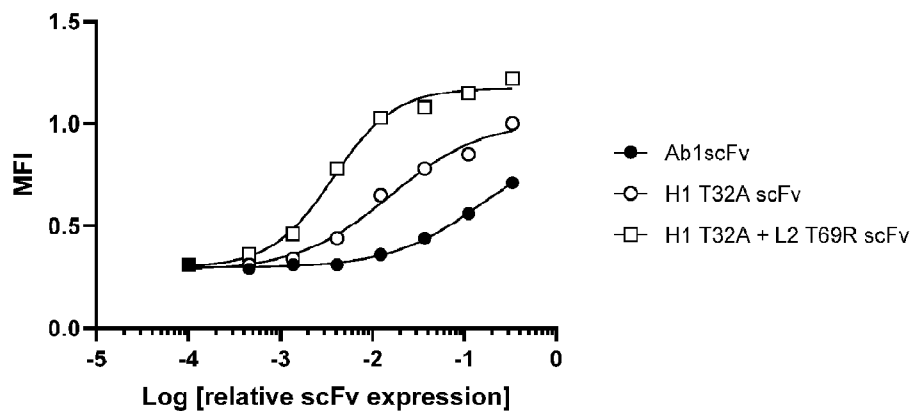


FIG. 11

13/14

A**B****C****FIG. 12**

14/14

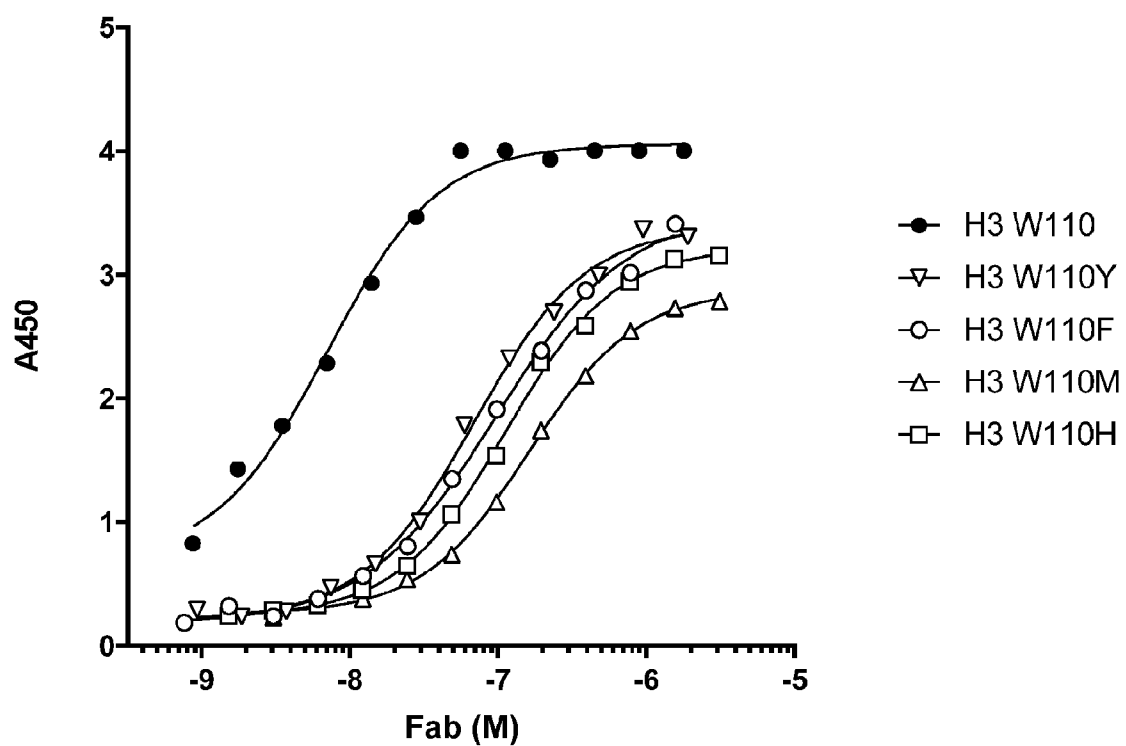


FIG. 13