MODIFIED MANA-TCES TARGETING TUMOR ANTIGENS AND ENGAGING T CELL RECEPTORS, AND METHODS OF USING THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present Application claims the benefit of priority to U.S. Provisional Application No. 63/598,464, filed on November 13, 2023, and U.S. Provisional Application No. 63/709,392, filed on October 18, 2024, the contents of each of which are hereby incorporated by reference in their entireties.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

[0002] The contents of the electronic sequence listing (MNAT_011_02WO_SeqList_ST26.xml; Size: 93,435 bytes; and Date of Creation: November 13, 2024) are herein incorporated by reference in its entirety.

BACKGROUND

[0003] One of the greatest challenges in cancer therapy is identification of targets that are highly specific to cancer or otherwise diseased cells and not present on healthy tissue. Somatic mutations in cancer are ideal targets for cancer therapy as they are uniquely expressed in cancer cells and absent from normal cells. Targeting proteins that drive tumorigenesis (broadly subdivided into oncogene proteins and tumor suppressor proteins) have added benefits. First, these mutations typically occur early during the development of the underlying cancer, thus essentially all daughter cancer cells will contain the mutation. Second, the cancer's dependence on their oncogenic-endowing capacity makes resistance less likely. Finally, driver proteins tend to have hotspot mutations shared among many patients, thus a therapy targeting a single mutation could be applied to a broad patient population. However, most of those driver proteins lie in the interior of the cells. While small molecules can target intracellular proteins, developing small molecules that can specifically inhibit the activity of a mutant driver protein and not its wild-type (WT) counterpart has remained out of reach for most of such driver proteins. Antibodies, which have the capacity to distinguish a single amino acid mutation, typically only target extracellular epitopes.

[0004] Our immune system samples the intracellular contents of cells through antigen processing and presentation. Following protein proteolysis, a fraction of the resulting peptides (can also be

referred to as epitopes) is loaded onto human leukocyte antigen (HLA) and presented on the cell surface where they serve as antigens for immune cells such as T cells, via their T cell receptors (TCRs), to distinguish self/normal versus non-self/abnormal and to initiate killing of the cells bearing non-self/abnormal antigens. For example, a virally infected cell will present viral antigens in its HLA, triggering T cells to kill that cell. Similarly, in cancer, tumor-specific mutant peptides (also called neoantigens or mutation-associated neoantigens (MANAs)) are presented in an HLA on the cell surface, referred to as Mut-pHLA complexes in the present disclosure. An estimated 10% to 20% of the epitopes created by mutant genes in cancer bind to common HLA types. In some cases, and to varying degrees, patients may mount an anti-cancer T cell response against these Mut-pHLA complexes, and checkpoint blockade antibodies can further augment this response. However, many patients, particularly those with a low mutational burden, cannot mount a sufficient anti-cancer T cell response. Improved therapies or diagnostics that specifically target Mut-pHLAs could therefore provide a truly tumor-specific method to diagnose or treat cancer.

[0005] Thus, there is a continuing need in the art to develop new methods to diagnose, monitor, and effectively treat cancer. The present invention meets these needs and offers other related advantages.

SUMMARY

[0006] The present technology provides multi-specific (*e.g.*, bispecific) molecules and compositions, as well as methods of using the same for cell therapy to diagnose and/or treat human diseases, including, for example, cancer.

[0007] In some aspects, provided are bispecific molecules comprising: (a) a first antigen binding domain that binds a tumor-specific mutant peptide presented in a peptide-human leukocyte antigen (HLA) complex (Mut-pHLA) on a target cell; and (b) a second antigen binding domain that binds a surface molecule on an effector cell, wherein the bispecific molecule further comprises a domain orientation modification, a linker modification, and one or more modifications selected from the group consisting of an Fc domain modification, a fusion protein modification, and a chemical modification, and wherein the modifications result in (i) an improved half-life, (ii) a reduced risk of complement-dependent cytotoxicity (CDC), (iii) an improved manufacturability, and/or (iv) an improved effector cell engagement of the bispecific molecule.

[0008] In some embodiments, the mutant peptide is derived from an oncogenic protein, *e.g.*, one selected from the group consisting of EGFR, IDH2, p53, KRAS, HRAS, NRAS, and CTNNB. In

some embodiments, the first antigen binding domain does not bind the mutant peptide not presented in an HLA complex or a complex that includes a wild-type version of the mutant peptide. In some embodiments, the mutant peptide comprises a peptide selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 12, 14, 16-18, 20, and 22-24.

[0009] In some embodiments, the HLA is a class I HLA selected from the group consisting of HLA-A, HLA-B, and HLA-C. In some embodiments, the HLA is HLA-A1, HLA-A2, or HLA-A3.

[0010] In some embodiments, the first antigen binding domain comprises a heavy chain variable region (Mut-pHLA V_H) and a light chain variable region (Mut-pHLA V_L). In some embodiments, the Mut-pHLA V_H comprises amino acid sequences of SEQ ID NOs: 39, 41, 43, and 45; and/or the Mut-pHLA V_L comprises amino acid sequences of SEQ ID NOs: 29, 31, 33, and 35.

[0011] In some embodiments, the mutant peptide is HMTEVVRHC (SEQ ID NO: 7), and/or the HLA is HLA-A2. In some embodiments, the Mut-pHLA V_H comprises (i) one, two, or three complementarity-determining regions (CDRs) having amino acid sequences selected from SEQ ID NOs: 40, 42, and 44; or (ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 40, 47, and 44, and/or the Mut-pHLA V_L comprises (i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 32, and 34; or (ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 37, and 34. In some embodiments, the Mut-pHLA V_H comprises an amino acid sequence of SEQ ID NO: 38 or SEQ ID NO: 46; and/or the Mut-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 28 or SEQ ID NO: 36.

[0012] In some embodiments, the mutant peptide is VVVGAVGVGK (SEQ ID NO: 17), and/or the HLA is HLA-A3. In some embodiments, the Mut-pHLA V_H comprises an amino acid sequence of SEQ ID NO: 49; and/or the Mut-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 48.

[0013] In some embodiments, the mutant peptide is ILDTAGHEEY (SEQ ID NO: 20), ILDTAGKEEY (SEQ ID NO: 22), or ILDTAGREEY (SEQ ID NO: 23), and/or the HLA is HLA-A1. In some embodiments, the Mut-pHLA V_H comprises an amino acid sequence of SEQ ID NO: 51, SEQ ID NO: 53, or SEQ ID NO: 55; and/or the Mut-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 50, SEQ ID NO: 52, or SEQ ID NO: 54.

[0014] In some embodiments, the effector cell is a T cell. In some embodiments, the surface molecule is CD3. In some embodiments, the second antigen binding domain comprises a heavy chain variable region (CD3 V_H) and light chain variable region (CD3 V_L). In some embodiments, the CD3 V_H comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 61-63; and/or the CD3 V_L comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 57-59. In some embodiments, the CD3 V_H comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 60; and/or the CD3 V_L comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 56. In some embodiments, the CD3 V_H comprises an amino acid sequence that shares at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 60; and/or the CD3 V_L comprises an amino acid sequence that shares at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 56.

[0015] In some embodiments, the bispecific molecule has one or more characteristics selected from the following: (a) a low target density of less than 2000 targets per cell, less than 1500 targets per cell, less than 1000 targets per cell, less than 500 targets per cell, less than 100 targets per cell, less than 50 targets per cell, less than 10 targets per cell, or about 1 target per cell; (b) a binding specificity as measured by half maximal effective concentration (EC50) of 0.001-3000 nM; (c) low or no cross-reactivity to proteins other than the protein from which the mutant peptide is derived; (d) a half-life of about 21 days, about 14 days, about 7 days, or about 4 days; (e) a productibility of at least about 1 g/L, at least about 2 g/L, at least about 3 g/L, at least about 4 g/L, at least about 5 g/L, at least about 10 g/L, at least about 15 g/L, at least about 20 g/L, at least about 25 g/L, or at least about 30 g/L from a cell culture; and (f) a stability at room temperature of 1-12 weeks. In some embodiments, the bispecific molecule, when engaged with the Mut-pHLA on the target cell and CD3 on the T cell, forms a connection between the target cell and the T cell of about 14 nM in length as measured from the target cell membrane to the T cell membrane. In some embodiments, the bispecific molecule has a productibility of at least 1 g/L, at least 2 g/L, at least 3 g/L, at least 4 g/L, at least 5 g/L, at least 10 g/L, at least 15 g/L, at least 20 g/L, at least 25 g/L, or at least 30 g/L from a cell culture.

[0016] In some embodiments, the domain orientation modification and linker modification comprise the bispecific molecule being configured as a single-chain diabody (scDb) in a configuration of, from N- to C-terminus order:

(i) Mut-pHLA V_L – L1 – CD3 V_H – L2 – CD3 V_L – L3 – Mut-pHLA V_H;

(ii) Mut-pHLA V_H – L1 – CD3 V_L – L2 – CD3 V_H – L3 – Mut-pHLA V_L;

(iii) Mut-pHLA V_L – L1 – CD3 V_L – L2 – CD3 V_H- – L3 – Mut-pHLA V_H;

(iv) Mut-pHLA V_H – L1 – CD3 V_H – L2 – CD3 V_L – L3 – Mut-pHLA V_L;

(v) CD3 V_L – L1 – Mut-pHLA V_H – L2 – Mut-pHLA V_L – L3 – CD3 V_H;

(vi) CD3 V_H – L1 – Mut-pHLA V_L – L2 – Mut-pHLA V_H – L3 – CD3 V_L;

(vii) CD3 V_L – L1 – Mut-pHLA V_L – L2 – Mut-pHLA V_H – L3 – CD3 V_H; or

(viii) CD3 V_H – L1 – Mut-pHLA V_H – L2 – Mut-pHLA V_L – L3 – CD3 V_L,

wherein L1, L2, and L3 are linkers. In some embodiments, the bispecific molecule is configured in a configuration of, from N- to C-terminus order, CD3 V_L – L1 – Mut-pHLA V_H – L2 – Mut-pHLA V_L – L3 – CD3 V_H. In some embodiments, L1 is about 1-10 amino acids in length, L2 is about 5-40 amino acids in length, and L3 is about 1-10 amino acids in length.

[0017] In some embodiments, the bispecific molecule further comprises an Fc domain modification, wherein the Fc domain modification comprises two chains of an Fc sequence of immunoglobulin G4 (IgG4) isotype each comprising a hinge, a CH2 domain, and a CH3 domain, and wherein one or both chains of the Fc sequence comprise one or more mutations selected from the group consisting of S228P, L235E, L336W, T366S, L368A, Y407V, and H435R in reference to a consensus full-length IgG4 sequence. In some embodiments, one chain of the Fc sequence comprises S228P, L235E, T366S, L368A, and Y407V mutations, and the other chain of the Fc sequence comprises S228P, L235E, L336W, and H435R mutations. In some embodiments, one chain of the Fc sequence comprises an amino acid sequence of SEQ ID NO: 65, and the other chain of the Fc sequence comprises an amino acid sequence of SEQ ID NO: 66. In some embodiments, one chain of the Fc sequence is connected to the first and/or second antigen binding domain at the N-terminal end of the CD3 V_L or the C-terminal end of the CD3 V_H optionally through a linker L4, wherein L4 is about 1-40 amino acids in length.

[0018] In some embodiments, the bispecific molecule further comprises a fusion protein modification, and wherein the fusion protein modification comprises a serum albumin. In some embodiments, the serum albumin is a human serum albumin (HSA), optionally wherein the HSA comprises an amino acid sequence of SEQ ID NO: 80 or SEQ ID NO: 81. In some embodiments, the serum albumin is connected to the first and/or second antigen binding domain at the N-terminal

end of the CD3 V_L or the C-terminal end of the CD3 V_H optionally through a linker L4, wherein L4 is about 1-40 amino acids in length.

[0019] In some embodiments, the bispecific molecule further comprises a chemical modification, and wherein the fusion modification comprises a polyethylene glycol (PEG) group. In some embodiments, the PEG group is connected to the first and/or second antigen binding domain at the N-terminal end of CD3 V_L, L1, L2, L3, or the C-terminal end of CD3 V_H.

[0020] In some aspects, provided are bispecific molecules comprising: (a) a first antigen binding domain that binds a tumor-specific mutant peptide derived from p53 R175H presented in a peptide-human leukocyte antigen (HLA) complex (R175H-pHLA) on a target cell; (b) a second antigen binding domain that binds CD3 on a T cell; and (c) an Fc domain of immunoglobulin G4 (IgG4) isotype comprising a first chain of an Fc sequence comprising a hinge, a CH2 domain, and a CH3 domain and a second chain of an Fc sequence comprising a hinge, a CH2 domain, and a CH3 domain, wherein the Fc domain results in (i) an improved half-life, (ii) a reduced risk of Fc-mediated cytotoxicity, (iii) and improved manufacturability, and/or (iv) an improved effector cell engagement of the bispecific molecule. In some embodiments, the mutant peptide comprises or consists of an amino acid sequence of HMTEVVRHC (SEQ ID NO: 7). In some embodiments, the HLA is HLA-A*02.01, HLA-A*02.06, or HLA-A*02.11.

[0021] In some embodiments, the bispecific molecule has one or more characteristics selected from the following: (a) a low target density of less than 2000 targets per cell, less than 1500 targets per cell, less than 1000 targets per cell, less than 500 targets per cell, less than 100 targets per cell, less than 50 targets per cell, less than 10 targets per cell, or about 1 target per cell; (b) a binding specificity as measured by half maximal effective concentration (EC50) of 0.001-3000 nM; (c) low or no cross-reactivity to proteins other than the protein from which the mutant peptide is derived; (d) a half-life of about 21 days, about 14 days, about 7 days, or about 4 days; (e) a productibility of at least about 1 g/L, at least about 2 g/L, at least about 3 g/L, at least about 4 g/L, at least about 5 g/L, at least about 10 g/L, at least about 15 g/L, at least about 20 g/L, at least about 25 g/L, or at least about 30 g/L from a cell culture; and (f) a stability at room temperature of 1-12 weeks. In some embodiments, the bispecific molecule, when engaged with the Mut-pHLA on the target cell and CD3 on the T cell, forms a connection between the target cell and the T cell of about 14 nM in length as measured from the target cell membrane to the T cell membrane. In some embodiments, the bispecific molecule, when engaged with the Mut-pHLA on the target cell

and CD3 on the T cell, forms a connection between the target cell and the T cell of about 14 nM in length as measured from the target cell membrane to the T cell membrane.

[0022] In some embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) and light chain variable region (R175H -pHLA V_L), the second antigen binding domain comprises a heavy chain variable region (CD3 V_H) and light chain variable region (CD3 V_L), and the bispecific molecule comprises, from N- to C-terminus order, CD3 V_L – L1 – R175H-pHLA V_H – L2 – R175H-pHLA V_L – L3 – CD3 V_H, wherein L1 is about 1-10 amino acids in length, L2 is about 5-40 amino acids in length, and L3 is about 1-10 amino acids in length.

[0023] In some embodiments, the R175H-pHLA V_H comprises (i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 40, 42, and 44; or (ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 40, 47, and 44, and/or the R175H-pHLA V_L comprises (i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 32, and 34; or (ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 37, and 34. In some embodiments, the R175H-pHLA V_H comprises an amino acid sequence of SEQ ID NO: 38 or SEQ ID NO: 46; and/or the R175H-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 28 or SEQ ID NO: 36.

[0024] In some embodiments, the CD3 V_H comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 61-63; and/or the CD3 V_L comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 57-59. In some embodiments, the CD3 V_H comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 60; and/or the CD3 V_L comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 56. In some embodiments, the CD3 V_H comprises an amino acid sequence that shares at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 60; and/or the CD3 V_L comprises an amino acid sequence that shares at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 56.

[0025] In some embodiments, the first chain of the Fc sequence comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 65; and/or the second chain of the Fc sequence comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 66. In some embodiments, the first chain of the Fc sequence

comprises an amino acid sequence that shares at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 65; and/or the second chain of the Fc sequence comprises an amino acid sequence that shares at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 66. In some embodiments, the first or second chain of the Fc sequence is connected to the first and/or second antigen biding domain at the N-terminal end of the CD3 V_L or the C-terminal end of the CD3 V_H optionally through a linker L4, wherein L4 is about 1-40 amino acids in length. In some embodiments, the first or second chain of the Fc sequence is connected to the C-terminal end of the CD3 V_H through L4.

[0026] In some embodiments, the bispecific molecule comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 78. In some embodiments, the bispecific molecule comprises an amino acid sequence that shares at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 78.

[0027] In some aspects, provided are bispecific molecules comprising: (a) a first polypeptide comprising a first chain of an Fc sequence comprising an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 65; and (b) a second polypeptide comprising, from N- to C-terminus order: (i) a light chain variable region derived from a first antigen binding domain that binds CD3 (CD3 V_L); (ii) a linker L1 of about 1-10 amino acids in length; (iii) a heavy chain variable region derived from a second antigen binding domain that binds a p53 R175H mutant peptide having an amino acid sequence of HMTEVVRHC (SEQ ID NO: 7) presented in a peptide-human leukocyte antigen (HLA) complex (R175H-PHLA) (R175H-pHLA V_H); (iv) a linker L2 of about 5-40 amino acids in length; (v) a light chain variable region derived from the second antigen binding domain that binds R175H-pHLA (R175H -pHLA V_L); (vi) a linker L3 of about 1-10 amino acids in length; (vii) a heavy chain variable region derived from the first antigen binding domain that binds CD3 (CD3 V_H); (viii) optionally a linker L4 of about 1-40 amino acids in length; and (ix) a second chain of an Fc sequence of immunoglobulin G4 (IgG4) isotype comprising an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 66. In some embodiments, the HLA is HLA-A*02.01, HLA-A*02.06, or HLA-A*02.11.

[0028] In some embodiments, the R175H-pHLA V_H comprises (i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 40, 42, and 44; or (ii) one, two, or three CDRs

having amino acid sequences selected from SEQ ID NOs: 40, 47, and 44, and/or the R175H-pHLA V_L comprises (i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 32, and 34; or (ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 37, and 34. In some embodiments, the R175H-pHLA V_H comprises an amino acid sequence of SEQ ID NO: 38 or SEQ ID NO: 46; and/or the R175H-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 28 or SEQ ID NO: 36.

[0029] In some embodiments, the CD3 V_H comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 61-63; and/or the CD3 V_L comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 57-59. In some embodiments, the CD3 V_H comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 60; and/or the CD3 V_L comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 56. In some embodiments, the CD3 V_H comprises an amino acid sequence that shares at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 60; and/or the CD3 V_L comprises an amino acid sequence that shares at least 80%, at least 85%, at least 90%, at least 95%, or 100% identity to SEQ ID NO: 56.

[0030] In some embodiments, the first polypeptide comprises an amino acid sequence of SEQ ID NO: 65; and/or the second polypeptide comprises an amino acid sequence of SEQ ID NO: 78.

[0031] In some aspects, provided are pharmaceutical compositions comprising a bispecific molecule according to various embodiments disclosed herein and a pharmaceutically acceptable carrier, excipient, preservative, or combination thereof.

[0032] In some aspects, provided are methods of diagnosing, treating, or preventing a disease in a subject in need thereof, the methods comprising administering to the subject a therapeutically effective amount of a bispecific molecule a pharmaceutical composition containing the same according to various embodiments disclosed herein. In some embodiments, the subject has a disease-associated protein comprising a mutant peptide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 12, 14, 16-18, 20, and 22-24.

[0033] In some embodiments, the disease is cancer. In some embodiments, the cancer is a hematological malignancy. In some embodiments, the hematological malignancy is selected from the group consisting of myeloid neoplasm, myelodysplastic syndromes (MDS), myeloproliferative/myelodysplastic syndromes, acute lymphoblastic leukemia (ALL), chronic

lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), blast crisis chronic myelogenous leukemia (bcCML), B-cell acute lymphoid leukemia (B-ALL), T-cell acute lymphoid leukemia (T-ALL), T-cell lymphoma, and B-cell lymphoma. In some embodiments, the cancer is a solid cancer. In some embodiments, the solid cancer is selected from the group consisting of pancreatic cancer, glioma, glioblastoma, astrocytoma, colorectal cancer, thyroid cancer, gastric cancer, ovarian cancer, melanoma, endometrial cancer, lung cancer, renal cancer, cervical cancer, prostate cancer, breast cancer, urothelial cancer, testicular cancer, head and neck cancer, biliary tract cancer, liver cancer, and esophageal cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] FIG. 1 depicts differing configurations of a bispecific molecule with an Fc domain modification according to various embodiments disclosed and described herein.

[0035] FIG. 2 depicts differing configurations of an Fc domain according to various embodiments disclosed and described herein. Left, a Chain-1/Chain-2 "Knob into Hole (KiH)" heterodimer configuration is shown. Middle, a Chain-1/Chain-1 Hole/Hole mis-pair having a lower isoelectric point (pI) value is shown. Right, a Chain-2/Chain-2 Knob/Knob mis-pair having a decreased protein A binding and a higher pI value is shown.

[0036] FIG. 3 shows different configurations of a bispecific molecule with an Fc domain modification in the form of an scDb-Fc according to various embodiments disclosed and described herein. The first antigen binding domain of the bispecific molecule comprises an scFv that specifically recognizes and/or binds a Mut-pHLA (comprising a heavy chain variable region, Mut-pHLA V_H, and a light chain variable region, Mut-pHLA V_L), and the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof (comprising a heavy chain variable region, CD3 V_H, and a light chain variable region, CD3 V_L). The different elements of the first and second antigen binding domains are connected through various linkers (L1-L4) as indicated. An Fc domain in a "Knob-into-Hole" configuration is shown in this figure as representative.

[0037] FIG. 4 shows an exemplary configuration of a bispecific molecule with an Fc domain modification in the form of an scDb-Fc according to an embodiment disclosed and described herein. The first antigen binding domain of the bispecific molecule comprises an scFv that specifically recognizes and/or binds a Mut-pHLA (comprising a heavy chain variable region, Mut-pHLA V_H, and a light chain variable region, Mut-pHLA V_L), and the second antigen binding

domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof (comprising a heavy chain variable region, CD3 V_H, and a light chain variable region, CD3 V_L). The Fc domain is connected to the first and/or second antigen binding domain in a configuration of, from an N- to C-terminus order, CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H-L4-Fc. The different elements of the first and second antigen binding domains and the Fc domain are connected through various linkers (L1-L4) as indicated.

[0038] FIG. 5 shows exemplary configurations of a bispecific molecule with an HSA fusion protein modification in the form of an scDb-HSA according to various embodiments disclosed and described herein. The first antigen binding domain of the bispecific molecule comprises an scFv that specifically recognizes and/or binds a Mut-pHLA (comprising a heavy chain variable region, Mut-pHLA V_H, and a light chain variable region, Mut-pHLA V_L), and the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof (comprising a heavy chain variable region, CD3 V_H, and a light chain variable region, CD3 V_L). The HSA fusion protein is connected to the first and/or second antigen binding domain in a configuration of, from an N- to C-terminus order, CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H-L4-HSA (left) or HSA-L4-CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H (right). The different elements of the first and second antigen binding domains and the HSA fusion protein are connected through various linkers (L1-L4) as indicated.

[0039] FIG. 6 shows exemplary configurations of a bispecific molecule with PEGylation modification according to various embodiments disclosed and described herein. The first antigen binding domain of the bispecific molecule comprises an scFv that specifically recognizes and/or binds a Mut-pHLA (comprising a heavy chain variable region, Mut-pHLA V_H, and a light chain variable region, Mut-pHLA V_L), and the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof (comprising a heavy chain variable region, CD3 V_H, and a light chain variable region, CD3 V_L). The different elements of the first and second antigen binding domains are connected through various linkers (L1-L4) as indicated. The PEG group is connected to the first and/or second antigen binding domain at the N-terminal end of CD3 V_L, linker 1, linker 2, linker 3, or the C-terminal end of CD3 V_H.

[0040] FIG. 7 is a schematic showing of the design of an exemplary bispecific MANA-T cell engager (MANA-TCE), MANA-TCE1, that specifically binds a p53 R175H mutant peptide (HMTEVVRHC (SEQ ID NO: 7))-HLA (HLA-A*02.01) complex. MANA-TCE1 is an

asymmetric molecule in an scDb-Fc format, with an engineered IgG4 Fc backbone, and includes two chains of polypeptides.

[0041] FIG. 8A shows an amino acid sequence map of Chain-1 of MANA-TCE1; **FIG. 8B** shows the annotated amino acid sequence of Chain-1.

[0042] FIG. 9A shows an amino acid sequence map of Chain-2 of MANA-TCE1; **FIG. 9B** shows the annotated amino acid sequence of Chain-2.

[0043] FIG. 10A, from top to bottom, shows the respective 3D protein structure of a peptide-HLA complex (pHLA); a MANA-TCE comprising an anti-pHLA binding domain (α-pHLA), a binding domain specific to the delta and epsilon chains of CD3 (α-CD3δε), and an IgG4 Fc domain; and a T cell receptor (TCR) comprising a TCR alpha chain, and a TCR beta chain, a CD3 gamma chain, a CD3 delta chain, and two CD3 epsilon chains. **FIG. 10B**, from left to right, shows the respective 3D protein structure of a pHLA:MANA-TCE dimer; a TCR:MANA-TCE dimer; and a pHLA:MANA-TCE:TCR trimer.

[0044] FIG. 11A shows the 3D protein structures of single-chain diabodies (scDbs) comprising an antigen binding domain derived from the V2 clone (targeting KRAS G12V; see **Table 2**) (top panels, labeled "scDB-v2") or the H2 clone (targeting p53 R175H; see Table 2) (bottom panels, labeled "scDB-h2") with various lengths of the L1 and L3 linkers. **FIG. 11B** shows the 3D protein structures of scDbs with various lengths of the L2 linker. **FIG. 11C** shows the 3D protein structure of (left panel) a native TCR on the surface of a T cell engaged with a peptide-MHC complex (pMHC) on the surface of a target cancer cell, (middle panel) a TCR engaged indirectly with the target pMHC through a scDB in the light chain-heavy chain-light chain-heavy chain (LHLH) format which binds to the CD3 component of the TCR complex and the pMHC, and (right panel) a TCR engaged indirectly with the target pMHC through a scDB in the heavy chain-light chain-heavy chain-light chain (HLHL) format which binds to the CD3 component of the TCR complex and the pMHC.

[0045] FIG. 12 shows biophysical binding characteristics of MANA-TCE1 to p53 R175H/HLA-A*02:01 pHLA monomer (left) or human CD3 delta/epsilon heterodimer (right) as measured using surface plasmon resonance (SPR).

[0046] FIGS. 13A-13B show MANA-TCE1-mediateD T cell activation and killing of tumor cells expressing p53 R175H and HLA-A2:01 with single digit nM potency.

[0047] FIG. 14 shows that MANA-TCE1 does not activate T cells in the absence of p53 R175H/HLA-A2:01 target cells.

[0048] FIG. 15 shows xScan analysis of MANA-TCE1 specificity. T2 cells were loaded with 10 uM "HMTEVVRHC" (SEQ ID NO: 7) peptide variants from the positional scanning library and co-incubated with MANA-TCE1 and Jurkat-NFAT reporter cells at an E:T ratio of 2:1 for 24 hours. T cell activation was measured by luciferase as pre manufacturer's protocol, and the mean of duplicate wells was used to plot the heatmap. Black boxes indicate the amino acids in the parental p53 R175H peptide.

[0049] FIG. 16 shows T cell reactivity to control SAOS2 (not transfected, no TFX) and SAOS2 cells transfected with full-length p53 R175H gene or full-length ZN599 gene, as assessed by flow cytometry for CD8+/CD25+ T cells.

[0050] FIG. 17 shows the half-life of MANA-TCE1 in Sprague Dawley rats as compared to an unmodified scDb (*i.e.*, without the Fc modification attached to the antigen binding regions) or an IgG4 molecule.

[0051] FIG. 18 shows plasma MANA-TCE1 concentration (nM) in hCD3Tg mice after 1 mpk i.v. dose.

[0052] FIG. 19 shows receptor occupancy (RO) of MANA-TCE1 in hCD3Tg mice after 1 mpk i.v. dose. Solid circles indicate CD3 receptor occupancy by MANA-TCE1 as % of total CD3. Open circles indicate free CD3 that is not bound to MANA-TCE1 and binds to directly labelled UCHT1.

[0053] FIG. 20 shows the concentration of MANA-TCE1 and the IgG4 monoclonal antibody in the rat serum after administration as described in Example 2.

[0054] FIG. 21 shows the change in tumor volume with time in mice treated with MANA-TCE1 or a control molecule as described in Example 3.

[0055] FIG. 22 shows the change in tumor volume with time in mice treated with a single dose of MANA-TCE1 or with multiple doses of MANA-TCE1 as described in Example 4.

[0056] FIGs. 23A-23B show the % of tumor cell death (FIG. 23A) and the concentration of IFN-γ (FIG. 23B) seen after treatment with MANA-TCE1 or a control molecule TCE isotype.

[0057] FIGs. 24A-24B show the % of tumor cell death (FIG. 24A) and the concentration of IFN-γ (FIG. 24B) seen after treatment with MANA-TCE1 or a control molecule TCE isotype.

[0058] FIG. 25 shows the activation of T-cells with HLA-A*03 transfected G12V cell line. Variants of scDb 5026 dose dependently activate T-cells upon coculture with SW-620 cells expressing HLA-A*03. 5026 variants included mutation of Tryptophan at position 340 to Histidine (5026-W340H) or Proline (5026-W340P) or mutation of Asparagine at position 166 to Tyrosine (5026-N166Y). Activity of scDbs was tested both against wild-type SW620 cells as well as SW620 cells modified to express HLA-A*03 (5026-G12V).

DETAILED DESCRIPTION

[0059] The present technology provides multi-specific (*e.g.*, bispecific) molecules and compositions, as well as methods of using the same for cell therapy to diagnose and/or treat human diseases, including, for example, cancer. In some embodiments, disclosed are bispecific molecules comprising (a) a first antigen binding domain that binds a tumor-specific mutant peptide in a peptide-human leukocyte antigen (HLA) complex (Mut-pHLA) on a target cell (*e.g.*, a cancer cell); and (b) a second antigen binding domain that binds a surface molecule on an effector cell (*e.g.*, a T cell), as well as methods of using the same for the diagnosis and/or treatment of cancers expressing the tumor-specific peptide. In a specific embodiment, disclosed is a bispecific molecule comprising (a) a first antigen binding domain that binds a Mut-pHLA on a target cell (*e.g.*, a cancer cell); and (b) a second antigen binding domain that binds CD3 on a T cell, thereby engaging the T cell with the target cancer cell and initiating T cell-mediated killing. Due to its ability to recognize tumor-specific mutant peptides or mutation-associated neoantigens (MANAs) and to engage effector T cells, the bispecific molecule can also be referred to as a MANA-T cell engager (MANA-TCE). MANA-TCEs capable of specifically targeting a Mut-pHLA have the potential of becoming a globally applicable, genetically predictable, clinically applicable, and commercially viable off-the-shelf targeted precision immune-oncology drug.

[0060] Furthermore, as explained herein, the bispecific molecules or MANA-TCEs of the present technology are modified by peptide and/or chemical modifications that result in one or more improved characteristics of the molecules such as improved potency, improved potential therapeutic index (or therapeutic window), improved half-life, reduced risk of complement-dependent cytotoxicity (CDC), improved manufacturability, and improved effector cell engagement, while maintaining functionality and specificity in the treatment of disease such as cancer.

[0061] While the present disclosure is capable of being embodied in various forms, the description below of several embodiments is made with the understanding that the present disclosure is to be

considered as an exemplification of the invention and is not intended to limit the invention to the specific embodiments illustrated. Headings are provided for convenience only and are not to be construed to limit the invention in any manner. Embodiments illustrated under any heading may be combined with embodiments illustrated under any other heading.

[0062] The use of numerical values in the various quantitative values specified in this application, unless expressly indicated otherwise, are stated as approximations as though the minimum and maximum values within the stated ranges were both preceded by the word "about." It is to be understood, although not always explicitly stated, that all numerical designations are preceded by the term "about." It is to be understood that such range format is used for convenience and brevity and should be understood flexibly to include numerical values explicitly specified as limits of a range, but also to include all individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly specified. For example, a ratio in the range of about 1 to about 200 should be understood to include the explicitly recited limits of about 1 and about 200, but also to include individual ratios, such as about 2, about 3, and about 4, and sub-ranges, such as about 10 to about 50, about 20 to about 100, and so forth. It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are known in the art.

[0063] All publications disclosed herein are incorporated by reference in their entirety. To the extent any materials incorporated by reference conflict with the present disclosure, the present disclosure controls.

Definitions

[0064] Unless otherwise specified, each of the following terms has the meaning set forth in this section.

[0065] The indefinite articles "a" and "an" denote at least one of the associated nouns and are used interchangeably with the terms "at least one" and "one or more." For example, the phrase "a module" means at least one module, or one or more modules.

[0066] The conjunctions "or" and "and/or" are used interchangeably.

[0067] In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless

otherwise indicated. The term "about", when immediately preceding a number or numeral, means that the number or numeral ranges plus or minus 10%.

[0068] The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified. Amino acid analogs refer to compounds that have the same basic chemical structure as a naturally occurring amino acid. Such analogs have modified R groups or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refer to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but that function in a manner similar to a naturally occurring amino acid.

[0069] The term "antibody" is used to denote, in addition to natural antibodies, genetically engineered or otherwise modified forms of immunoglobulins or portions thereof, including chimeric antibodies, human antibodies, humanized antibodies, or synthetic antibodies. The antibodies may be monoclonal or polyclonal antibodies. In those embodiments wherein an antibody comprises an antigen-binding portion of an immunoglobulin molecule, the antibody may include, but is not limited to, a single chain variable fragment antibody (scFv), a disulfide linked Fv, a single domain antibody (sdAb), a VHH antibody, an antigen-binding fragment F(ab), a F(ab') fragment, a $F(ab')_2$ fragment, a diabody, or a single-chain diabody (scDb). An scFv antibody is derived from a natural antibody by linking the variable regions of the heavy (V_H) and light (V_L) chains of the immunoglobulin with a short linker peptide. Similarly, a disulfide linked Fv can be generated by linking the V_H and V_L using an interdomain disulfide bond. On the other hand, sdAbs consist of only the variable region from either the heavy or light chain and usually are the smallest antigen-binding fragments of antibodies. A VHH antibody is the antigen binding fragment of heavy chain only. A diabody is a dimer of scFv fragments that consists of the V_H and V_L regions noncovalently connected by a small peptide linker or covalently linked to each other. An scDb is a derivative of a diabody by connecting the two chains by a flexible linker, transforming the heterodimeric format of a diabody into a single-chain format. The term "antigen-binding portion" may refer to an immunogenically active portion of an antibody as described that possesses the ability to specifically recognize, associate, unite, or combine with an antigen or target molecule. An antigen-binding portion includes any naturally occurring, synthetic, semi-synthetic, or recombinantly produced. Both the antibodies and antigen-binding portions thereof disclosed herein retain the ability to bind a specific antigen.

[0070] The term "antigen" as used herein refers to a molecule capable of provoking an immune response. Antigens include but are not limited to cells, cell extracts, proteins, polypeptides, peptides, polysaccharides, polysaccharide conjugates, peptide and non-peptide mimics of polysaccharides and other molecules, small molecules, lipids, glycolipids, carbohydrates, viruses and viral extracts and multicellular organisms such as parasites and allergens. The term antigen broadly includes any type of molecule which is recognized by a host immune system as being foreign. The term "neoantigen" can be used to refer to a cancer-specific antigen (*i.e.*, an antigen found on cancer cells but not on non-cancer cells) that is specifically recognized by a cognate binding molecule as described.

[0071] A "clinically effective amount," "clinically effective concentration," or "clinically effective dose" refers to a concentration or dose of a peptide, composition, or pharmaceutical composition that is shown to be effective in clinical trials or is predicted to be effective based on early phase or pre-clinical trials. In some embodiments, a "clinically effective amount" is the same as a "therapeutically effective amount." In some embodiments, a "clinically effective amount" is higher or lower than a "therapeutically effective amount." Further, the effective amount can remain constant or can be adjusted as a sliding scale or variable dose depending on the subject's response to treatment. Various factors can influence the actual effective amount used for a particular application. For example, the frequency of administration, duration of treatment, use of multiple treatment agents, route of administration, and severity of the condition may require an increase or decrease in the actual effective amount administered.

[0072] The term "codon-optimized" or "codon optimization" when referring to a nucleotide sequence is based on the discovery that the frequency of occurrence of synonymous codons (*i.e.*, codons that code for the same amino acid) in coding nucleotide is biased in different species. Such codon degeneracy allows an identical polypeptide to be encoded by a variety of nucleotide sequences. Codon optimization refers to the process of substituting certain codons in a coding nucleotide sequence with synonymous codons based on the host cell's preference without changing the resulting polypeptide sequence. A variety of codon optimization methods are known in the art, and include, for example, methods disclosed in at least U.S. Pat. Nos. 5,786,464 and 6,114,148.

[0073] The term "complementarity determining regions (CDRs)" is synonymous with "hypervariable region" or "HVR," and is known in the art to refer to sequences of amino acids within antibody variable regions, which, in general, confer antigen specificity and/or binding

affinity and are separated from one another in primary structure by framework regions (FR). In some cases, framework amino acids can also contribute to binding. In general, there are three CDRs in each variable region. Variable domain sequences can be aligned to a numbering scheme (*e.g.*, Kabat, EU, international ImMunoGeneTics information system® (IMGT®), and Aho), which can allow equivalent residue positions to be annotated and for different molecules to be compared using the Antibody Numbering and Antigen Receptor Classification (ANARCI) software tool (2016, Bioinformatics 15:298-300).

[0074] The term "conservative substitution," when referring to amino acid sequences, is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. A variety of criteria known to persons skilled in the art indicate whether an amino acid that is substituted at a particular position in a peptide or polypeptide is conservative (or similar). For example, a similar amino acid or a conservative amino acid substitution is one in which an amino acid residue is replaced with an amino acid residue having a similar side chain. Similar amino acids may be included in the following categories: amino acids with basic side chains (*e.g.*, lysine, arginine, histidine); amino acids with acidic side chains (*e.g.*, aspartic acid, glutamic acid); amino acids with uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, histidine); amino acids with nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan); amino acids with beta-branched side chains (*e.g.*, threonine, valine, isoleucine); and amino acids with aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan). Proline, which is considered more difficult to classify, shares properties with amino acids that have aliphatic side chains (*e.g.*, leucine, valine, isoleucine, and alanine). In certain circumstances, substitution of glutamine for glutamic acid or asparagine for aspartic acid may be considered a similar substitution in that glutamine and asparagine are amide derivatives of glutamic acid and aspartic acid, respectively.

[0075] The term "epitope" includes any molecule, structure, amino acid sequence or protein determinant that is recognized and specifically bound by a cognate binding molecule, such as an antibody or a T cell receptor, or other binding molecule, domain, or protein.

[0076] The term "expression" refers to the process by which a polypeptide is produced based on the encoding sequence of a nucleic acid molecule, such as a gene. The process may include transcription, post-transcriptional control, post-transcriptional modification, translation, post-translational control, post-translational modification, or any combination thereof. An expressed

nucleic acid molecule is typically operably linked to an expression control sequence (*e.g.*, a promoter).

[0077] The term "host cell" as used herein refers to a cell or microorganism targeted for genetic modification by introduction of a construct or vector carrying a nucleotide sequence for expression of a protein or polypeptide of interest.

[0078] The term "nucleic acid" or "polynucleotide" refers to a polymeric compound including covalently linked nucleotides comprising natural subunits (*e.g.*, purine or pyrimidine bases). Purine bases include adenine and guanine, and pyrimidine bases include uracil, thymine, and cytosine. Nucleic acid molecules include polyribonucleic acid (RNA) and polydeoxyribonucleic acid (DNA), which includes cDNA, genomic DNA, and synthetic DNA, either of which may be single- or double-stranded. A nucleic acid molecule encoding an amino acid sequence includes all nucleotide sequences that encode the same amino acid sequence.

[0079] The term "operably linked" refers to the association of two or more nucleic acid molecules on a single nucleic acid fragment so that the function of one is affected by the other.

[0080] The terms "peptide," "polypeptide," and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length, though a number of amino acid residues may be specified. Polypeptides may include amino acid residues including natural and/or non-natural amino acid residues. The terms also include post-expression modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. In some embodiments, the polypeptides may contain modifications with respect to a native or natural sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0081] The term "subject" refers to a mammalian subject, preferably a human. A "subject in need thereof" refers to a subject who has been diagnosed with cancer or is at an elevated risk of developing cancer. The phrases "subject" and "patient" are used interchangeably herein.

[0082] The terms "treat," "treating," and "treatment," as used herein with regard to cancer, refers to alleviating the cancer partially or entirely, inhibiting cancer cell growth, reducing the number of cancer cells, preventing the cancer, decreasing the likelihood of occurrence or recurrence of the cancer, slowing the progression or development of the cancer, or eliminating, reducing, or slowing

the development of one or more symptoms associated with the cancer. For example, "treating" may refer to preventing or slowing the existing tumor from growing larger, preventing or slowing the formation or metastasis of cancer, and/or slowing the development of certain symptoms of the cancer. In some embodiments, the term "treat," "treating," or "treatment" means that the subject has a reduced number or size of tumor compared to a subject not being administered the treatment. In some embodiments, the term "treat," "treating," or "treatment" means that one or more symptoms of the cancer are alleviated in a subject receiving the pharmaceutical compositions as disclosed and described herein, compared to a subject who does not receive such treatment.

[0083] A "therapeutically effective amount" as used herein is an amount that produces a desired effect in a subject for an indication, condition, disease, or disorder. In certain embodiments, the therapeutically effective amount is an amount that yields maximum therapeutic effect. In other embodiments, the therapeutically effective amount yields a therapeutic effect that is less than the maximum therapeutic effect. For example, a therapeutically effective amount may be an amount that produces a therapeutic effect while avoiding one or more side effects associated with a dosage that yields maximum therapeutic effect. A therapeutically effective amount for a particular composition will vary based on a variety of factors, including, but not limited to, the characteristics of the therapeutic composition (*e.g.*, activity, pharmacokinetics, pharmacodynamics, and bioavailability); the physiological condition of the subject (*e.g.*, age, body weight, sex, disease type and stage, medical history, general physical condition, responsiveness to a given dosage, and other present medications); the nature of any pharmaceutically acceptable carriers, excipients, and preservatives in the composition; and the route of administration. One skilled in the clinical and pharmacological arts will be able to determine a therapeutically effective amount through routine experimentation, namely, by monitoring a subject's response to administration of the therapeutic composition and adjusting the dosage accordingly. For additional guidance, see Remington: The Science and Practice of Pharmacy, 21st Edition, Univ. of Sciences in Philadelphia (USIP), Lippincott Williams & Wilkins, Philadelphia, PA, 2005.

[0084] The term "variable region" or "variable domain" refers to a portion of an antibody heavy or light chain that is involved in antigen binding. Variable domains of antibody heavy (V_H) and light (V_L) chains each generally comprise four generally conserved framework regions (FRs) and three complementarity determining regions (CDRs). FRs separate CDRs, such that CDRs are situated between FRs.

[0085] A "vector" refers to a DNA construct containing a nucleic acid molecule that is operably linked to a suitable control sequence capable of effecting the expression of the nucleic acid molecule in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites, and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, a virus, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself.

Bispecific Molecules and Compositions Thereof

[0086] In some aspects, provided herein are multi-specific (*e.g.*, bispecific) molecules that simultaneously engage target cells (*e.g.*, cancer cells) bearing a tumor-specific mutant peptide or antigen and effector cells (*e.g.*, T cells). In some embodiments, the bispecific molecule comprises (a) a first antigen binding domain that binds a tumor-specific mutant peptide or a mutation-associated neoantigen (MANA) presented in a peptide-human leukocyte antigen (HLA) complex (Mut-pHLA) on a target cell (*e.g.*, a cancer cell); and (b) a second antigen binding domain that binds a surface molecule on an effector cell (*e.g.*, a T cell). The bispecific molecule may have a plurality of peptide and/or chemical modifications to improve its potency, potential therapeutic index (or therapeutic window), half-life, manufacturability, and/or effector cell engagement, while maintaining the functionality and specificity in its application in cancer treatment.

[0087] In some embodiments, the bispecific molecule is a MANA-T cell engager (MANA-TCE), which binds a Mut-pHLA on target cells (*e.g.*, cancer cells) and a surface molecule (*e.g.*, CD3) on T cells, thereby bringing the T cells in close proximity to the target cancer cells to initiate T cell-mediated killing of the target cancer cells. Some aspects of a bispecific effector cell engager are described in WO2016154246, WO2018213467, and WO2021127184, each of which is incorporated by reference herein in its entirety. In various embodiments, the multi-specific (*e.g.*, bispecific) molecules of the present technology can be used to diagnose and/or treat cancers expressing the tumor-specific mutant peptide or MANA by engaging and stimulating T cells to eliminate the cancer cells.

A first antigen binding domain for Mut-pHLA complexes

[0088] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE) comprises a first antigen binding domain that recognizes and/or binds a tumor-specific mutant peptide or MANA presented in a Mut-pHLA complex on the surface of a target cell, *e.g.*, a cancer cell. As used

herein, a tumor-specific mutant peptide is a peptide derived from a modified polypeptide (*e.g.*, an oncogenic protein or a tumor suppressor protein) having a disease-causing mutation (*e.g.*, an oncogenic mutation). A mutant peptide can have one or more amino acid modifications (*e.g.*, substitutions) relative to its corresponding wild-type (WT) peptide. In the present disclosure, the terms "tumor-specific mutant peptides" and "MANAs" can be used interchangeably.

[0089] HLA is the major histocompatibility complex (MHC) in humans. HLA proteins are encoded by a large locus in the genome containing a set of closely linked polymorphic genes. The HLA gene family is divided into three subgroups, HLA class I, HLA class II, and HLA class III. Of those, HLA class I is present on all nucleated cells and directly involved in antigen presentation to cytotoxic T cells (or cytotoxic T lymphocytes (CTLs)). HLA class I complexes are heterodimers comprising a polymorphic heavy α subunit (encoded by the HLA genes) and a small invariant β2 microglobulin (B2M, encoded by the *B2M* gene outside of the HLA locus) subunit. The polymorphic α chain contains a N-terminal extracellular region composed of three domains, α1, α2, and α3; a transmembrane helix; and a short cytoplasmic tail. The α1 and α2 domains form a deep peptide-binding groove, while the α3 domain is involved in the interaction with CD8 coreceptors found on CTLs. B2M provides stability of the complex and participates in the recognition of peptide-HLA complex by CD8 coreceptor. Accordingly, a Mut-pHLA complex is a heterotrimer complex comprising a mutant peptide, an HLA molecule, and a B2M molecule. During antigen presentation, the antigen peptide or epitope is non-covalently bound to the HLA, held by several pockets of the peptide-binding groove. There are three classical HLA I genes, A, B, and C, each of which are highly polymorphic. Each HLA allele has a particular peptide-binding motif, and as a result, only certain peptides will bind to a particular HLA allele.

[0090] Cytotoxic T cells or CTLs express T cell receptors (TCRs), which recognize specific target antigens or epitopes presented by HLA complexes. They also express CD8, which interacts with HLA class I molecules and functions as a coreceptor in TCR-ligand binding and T cell activation. When a CTL's CD8 receptor docks to an HLA class I molecule, if the CTL's TCR fits the antigen or epitope within the HLA class I molecule, the CTL triggers the cell to undergo programmed cell death by apoptosis.

[0091] A tumor-specific mutant peptide or MANA can be any appropriate length. In some embodiments, the mutant peptide or MANA is from about 7 amino acids to about 25 amino acids (*e.g.*, from about 7 amino acids to about 20 amino acids, from about 7 amino acids to about 15 amino acids, from about 7 amino acids to about 14 amino acids, from about 7 amino acids to about

13 amino acids, from about 7 amino acids to about 12 amino acids, from about 7 amino acids to about 11 amino acids, from about 7 amino acids to about 10 amino acids, from about 7 amino acids to about 9 amino acids, from about 8 amino acids to about 25 amino acids, from about 8 amino acids to about 20 amino acids, from about 8 amino acids to about 15 amino acids, from about 8 amino acids to about 14 amino acids, from about 8 amino acids to about 13 amino acids, from about 8 amino acids to about 12 amino acids, from about 8 amino acids to about 11 amino acids, from about 8 amino acids to about 10 amino acids, from about 9 amino acids to about 25 amino acids, from about 9 amino acids to about 20 amino acids, from about 9 amino acids to about 15 amino acids, about 9 amino acids to about 14 amino acids, about 9 amino acids to about 13 amino acids, about 9 amino acids to about 12 amino acids, about 9 amino acids to about 11 amino acids, from about 10 amino acids to about 25 amino acids, from about 10 amino acids to about 20 amino acids, from about 10 amino acids to about 15 amino acids, from about 10 amino acids to about 14 amino acids, from about 10 amino acids to about 13 amino acids, from about 10 amino acids to about 12 amino acids, from about 11 amino acids to about 25 amino acids, from about 11 amino acids to about 20 amino acids, from about 11 amino acids to about 15 amino acids, from about 11 amino acids to about 14 amino acids, from about 11 amino acids to about 13 amino acids, from about 12 amino acids to about 25 amino acids, from about 12 amino acids to about 20 amino acids, from about 12 amino acids to about 15 amino acids, from about 12 amino acids to about 14 amino acids, from about 13 amino acids to about 25 amino acids, from about 13 amino acids to about 20 amino acids, from about 13 amino acids to about 15 amino acids, from about 14 amino acids to about 25 amino acids, from about 14 amino acids to about 20 amino acids, from about 14 amino acids to about 15 amino acids, from about 15 amino acids to about 25 amino acids, or from about 15 amino acids to about 20 amino acids) in length. For example, the mutant peptide or MANA can be about 7 amino acids, about 8 amino acids, about 9 amino acids, about 10 amino acids, about 11 amino acids, about 12 amino acids, about 13 amino acids, about 14 amino acids, about 15 amino acids, about 16 amino acids, about 17 amino acids, about 18 amino acids, about 19 amino acids, about 20 amino acids, about 21 amino acids, about 22 amino acids, about 23 amino acids, about 24 amino acids, or about 25 amino acids in length. For example, the mutant peptide or MANA is 9 amino acids or 10 amino acids in length.

[0092] The mutant peptide or MANA can be derived from any modified (*e.g.*, oncogenic) polypeptide. Examples of modified polypeptides from which the mutant peptide or MANA described herein can be derived include, without limitation, epidermal growth factor receptor (EGFR), isocitrate dehydrogenase 2 (IDH2), p53, RAS (*e.g.*, KRAS, HRAS, and RAS), and

CTNNB. The mutant peptide or MANA can contain any appropriate modification (*e.g.*, mutation) compared to the corresponding WT peptide, including, for example, one or more modifications shown in **Table 1**.

Table 1. Exemplary sequences of mutant peptides or MANAs derived from mutant proteins

Protein	Mutation	Modified Peptide	SEQ ID NO:	Wild-type Peptide	SEQ ID NO:	Peptide Codons	HLA Allele
EGFR	T790M	IMQLMPFGC	1	ITQLMPFGC	2	789-797	A2
EGFR	L858R	KITDFGRAK	3	KITDFGLAK	4	852-860	A3
IDH2	R140Q	SPNGTIQNIL	5	SPNGTIRNIL	6	134-143	B7
p53	R175H	HMTEVVRHC	7	HMTEVVRRC	8	168-176	A2
p53	R248Q	GMNQRPILTI	9	GMNRRPILTI	10	245-254	A2
p53	R248W	GMNWRPILTI	11	GMNRRPILTI	10	245-254	A2
KRAS	G12V	LVVVGAVGV	12	LVVVGAGGV	13	6-14	A2
KRAS	G12C	VVVGACGVGK	14	VVVGAGGVGK	15	7-16	A3
KRAS	G12D	VVVGADGVGK	16	VVVGAGGVGK	15	7-16	A3, A11
KRAS	G12V	VVVGAVGVGK	17	VVVGAGGVGK	15	7-16	A3, A11
KRAS	G12D	VVGADGVGK	18	VVGAGGVGK	19	8-16	A11
H/K/N RAS	Q61H	ILDTAGHEEY	20	ILDTAGQEEY	21	55-64	A1

Protein	Mutation	Modified Peptide	SEQ ID NO:	Wild-type Peptide	SEQ ID NO:	Peptide Codons	HLA Allele
H/K/N RAS	Q61K	ILDTAGKEEY	22	ILDTAGQEEY	21	55-64	A1
H/K/N RAS	Q61R	ILDTAGREEY	23	ILDTAGQEEY	21	55-64	A1
CTNNB	S45F	TTAPFLSGK	24	TTAPSLSGK	25	41-49	A3

[0093] In some embodiments, the mutant peptide or MANA comprises or consists of an amino acid sequence set forth in any one of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 12, 14, 16-18, 20, and 22-24. In some embodiments, the mutant peptide or MANA comprises or consists of an amino acid sequence set forth in any one of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 12, 14, 16-18, 20, and 22-24, with up to one, two, three, four, five, or more amino acid substitutions, deletions, or additions.

[0094] In some embodiments, the mutant peptide or MANA to be recognized by the first antigen binding domain of the bispecific molecule (*e.g.*, MANA-TCE) is presented by HLA, *i.e.*, in a peptide-HLA complex. A peptide-HLA complex usually includes a peptide antigen or epitope, an HLA protein, and a B2M protein. The HLA protein can be encoded by any appropriate HLA gene or allele. In some embodiments, especially in the case of antigen presentation to CD8+ T cells, the HLA gene can be a class I HLA (*e.g.*, HLA-A, HLA-B, or HLA-C) gene. In some embodiments, especially in the case of antigen presentation to CD4+ T cells, the HLA gene can be a class II HLA (*e.g.*, HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DQ, or HLA-DR) gene. For each HLA gene, there are a number of different alleles known. For example, a modified peptide derived from a mutant p53 polypeptide (*e.g.*, HMTEVVRHC (SEQ ID NO: 7)) can be in a complex with HLA-A2 and B2M. For another example, a modified peptide derived from a mutant H/K/N RAS polypeptide (*e.g.*, ILDTAGHEEY (SEQ ID NO: 20), ILDTAGKEEY (SEQ ID NO: 22), and ILDTAGREEY (SEQ ID NO: 23)) can be in a complex with HLA-A1 and B2M.

[0095] In some embodiments, the first antigen binding domain of the bispecific molecule (*e.g.*, MANA-TCE) can specifically recognize and/or bind a mutant peptide or MANA described herein, *e.g.*, a mutant peptide or MANA comprising or consisting of an amino acid sequence set forth in any one of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 12, 14, 16-18, 20, and 22-24. In certain of these

embodiments, the first antigen binding domain specific to a particular mutant peptide or MANA does not target (*e.g.*, does not recognize or bind) the same mutant peptide or MANA that is not presented by HLA, *i.e.*, not present in a peptide-HLA complex. In other embodiments, the first antigen binding domain specific to a particular mutant peptide or MANA does not target (*e.g.*, does not recognize or bind) the corresponding WT peptide, whether or not presented in a peptide-HLA complex.

[0096] In some embodiments, the first antigen binding domain (or any other binding domains described herein) can be any appropriate type or form of immunoglobulins, fragments, or portions thereof that retain the ability to specifically recognize and/or bind an antigen. The first antigen binding domain can be derived from a natural, engineered, or otherwise modified antibody and usually contain at least one of the complementarity determining regions (CDRs) responsible for antigen specificity. Non-limiting examples of a first antigen binding domain may include a single chain variable fragment (scFv), disulfide linked Fv, single domain antibody (sdAb), VHH, antigen-binding fragment (Fab), F(ab') fragment, $F(ab')_2$ fragment, diabody, and single-chain diabody (scDb).

[0097] In some embodiments, the first antigen binding domain (or any other binding domains described herein) may be derived from a mammalian species, for example, mice, rats, or human. Antibody variable regions can be those arising from one species, or they can be chimeric, containing segments of multiple species possibly further altered to optimize characteristics such as binding affinity or low immunogenicity. For human applications, it is desirable that the antigen binding domain has a human sequence. In the cases where the antigen binding domain is derived from an antibody from a non-human species, the antigen binding domain may be humanized to reduce immunogenicity in a human subject. For example, if a human antibody of the desired specificity is not available, but such an antibody from a non-human species is, the non-human antibody can be humanized, *e.g.*, through CDR grafting, in which the CDRs from the non-human antibody are placed into the respective positions in a framework of a compatible human antibody.

[0098] In some embodiments, the first antigen binding domain comprises an scFv that specifically recognizes and/or binds a mutant peptide or MANA presented in a peptide-HLA complex (Mut-pHLA) as described including, for example, those listed in **Table 1**. In certain of these embodiments, the first antigen binding domain can be in any appropriate format which includes at least one heavy chain variable region (Mut-pHLA V_H) and at least one light chain variable region (Mut-pHLA V_L), which can be directly or indirectly associated in any order or orientation.

In some embodiments, the Mut-pHLA V_H is N-terminal to the Mut-pHLA V_L. In some embodiments, the Mut-pHLA V_H is C-terminal to the Mut-pHLA V_L. The Mut-pHLA V_H and the Mut-pHLA V_L can be linked directly or indirectly by a linker. For example, the linker can be a Whitlow linker (GSTSGSGKPGSGEGSTKG (SEQ ID NO: 26)). For another example, a $(G_4S)_n$ (n can be a positive integer, *e.g.*, 1, 2, 3, 4, 5, or 6) linker may be used, including, *e.g.*, a $3xG_4S$ linker (GGGGSGGGGSGGGGS (SEQ ID NO: 27)). Exemplary Mut-pHLA V_H and V_L sequences that can be used for the first antigen binding domain to specifically recognize and/or bind a Mut-pHLA are provided in **Table 2** below.

Table 2. Exemplary V_H and V_L sequences for Mut-pHLAs

SEQ ID NO:	Target	Sequence	Description
28	p53 R175H-HLA-A2	DIQMTQSPSSLSASVGDRVTITC**RASQDVNTAVA**WYQQKPGKAPKLLIY**SAYFLYS**GVPSRFSGSRSGTDFTLTISSLQPEDFATYYC**QQYSRYSPV**TFGQGTKVEIK	Clone H2 targeting p53 R175H mutant peptide (SEQ ID NO: 7), V_L sequence, with **CDR1**, **CDR2**, and **CDR3** highlighted
29	p53 R175H-HLA-A2	DIQMTQSPSSLSASVGDRVTITC	H2 V_L, FR1
30	p53 R175H-HLA-A2	RASQDVNTAVA	H2 V_L, CDR1
31	p53 R175H-HLA-A2	WYQQKPGKAPKLLIY	H2 V_L, FR2
32	p53 R175H-HLA-A2	SAYFLYS	H2 V_L, CDR2
33	p53 R175H-HLA-A2	GVPSRFSGSRSGTDFTLTISSLQPEDFATYYC	H2 V_L, FR3
34	p53 R175H-HLA-A2	QQYSRYSPV	H2 V_L, CDR3
35	p53 R175H-HLA-A2	TFGQGTKVEIK	H2 V_L, FR4

SEQ ID NO:	Target	Sequence	Description
36	p53 R175H-HLA-A2	DIQMTQSPSSLSASVGDRVTITC**RASQDVNTAVA**WYQQKPGKAPKLLIY**SAY*S*LYS**GVPSRFSGSRSGTDFTLTISSLQPEDFATYYC**QQYSRYSPV**TFGQGTKVEIK	Clone H2* (H2 with *F53S* mutation), V_L sequence, with **CDR1**, **CDR2**, and **CDR3** highlighted
37	p53 R175H-HLA-A2	SAY*S*LYS	H2* F53S V_L, CDR2 (with *F53S* mutation)
38	p53 R175H-HLA-A2	EVQLVESGGGLVQPGGSLRLSCAASG**FNVYASGMH**WVRQAPGKGLEW**VAKIYPDSDYTYY**ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC**SRDSSFYYVYAM**DYWGQGTLVTVSS	H2 V_H sequence, with **CDR1**, **CDR2**, and **CDR3** highlighted
39	p53 R175H-HLA-A2	EVQLVESGGGLVQPGGSLRLSCAASG	H2 V_H, FR1
40	p53 R175H-HLA-A2	FNVYASGMH	H2 V_H, CDR1
41	p53 R175H-HLA-A2	WVRQAPGKGLEW	H2 V_H, FR2
42	p53 R175H-HLA-A2	VAKIYPDSDYTYY	H2 V_H, CDR2
43	p53 R175H-HLA-A2	ADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYC	H2 V_H, FR3
44	p53 R175H-HLA-A2	SRDSSFYYVYAM	H2 V_H, CDR3
45	p53 R175H-HLA-A2	DYWGQGTLVTVSS	H2 V_H, FR4

SEQ ID NO:	Target	Sequence	Description
46	p53 R175H-HLA-A2	EVQLVESGGGLVQPGGSLRLSCAA SG**FNVYASGMH**WVRQAPGKGLE W**VAKIYPDSD*I*TYY**ADSVKGRFTIS ADTSKNTAYLQMNSLRAEDTAVY YC**SRDSSFYYVYAM**DYWGQGTLV TVSS	Clone H2* (H2 with *Y57I* mutation), V_H sequence, with **CDR1**, **CDR2**, and **CDR3** highlighted
47	p53 R175H-HLA-A2	VAKIYPDSD*I*TYY	H2* Y57I V_H, CDR2 (with *Y57I* mutation)
48	RAS G12V-HLA-A3	DIQMTQSPSSLSASVGDRVTITCRA SQDVNTAVAWYQQKPGKAPKLLIY SASFLYSGVPSRFSGSRSGTDFTLTI SSLQPEDFATYYCQQSYYYFRPITF GQGTKVEIK	Clone V2 targeting KRAS G12V mutant peptide (SEQ ID NO: 17), V_L sequence
49	RAS G12V-HLA-A3	EVQLVESGGGLVQPGGSLRLSCAA SGFNLSYSDIHWVRQAPGKGLEWV AVVMPDSGHTNYADSVKGRFTISA DTSKNTAYLQMNSLRAEDTAVYY CSRATNIPVYAFDYWGQGTLVTVS S	Clone V2 targeting KRAS G12V mutant peptide (SEQ ID NO: 17), V_H sequence
50	RAS Q61H-HLA-A1	DIQMTQSPSSLSASVGDRVTITCRA SQDVNTAVAWYQQKPGKAPKLLIY SASFLYSGVPSRFSGSRSGTDFTLTI SSLQPEDFATYYCQQVIYYPFTFGQ GTKVEIK	Clone H1 targeting RAS Q61H mutant peptide (SEQ ID NO: 20), V_L sequence
51	RAS Q61H-HLA-A1	EVQLVESGGGLVQPGGSLRLSCAA SGFNLYSYAIHWVRQAPGKGLEWV ALLYPDYGVTSYADSVKGRFTISAD TSKNTAYLQMNSLRAEDTAVYYCS	Clone H1 targeting RAS Q61H mutant peptide (SEQ ID NO: 20), V_H sequence

SEQ ID NO:	Target	Sequence	Description
		RYRSYEYSVSSYSYSAMDYWGQG TLVTVSS	
52	RAS Q61K-HLA-A1	DIQMTQSPSSLSASVGDRVTITCRA SQDVNTAVAWYQQKPGKAPKLLIY SASFLYSGVPSRFSGSRSGTDFTLTI SSLQPEDFATYYCQQAVSYPWTFG QGTKVEIK	Clone L2 targeting RAS Q61K mutant peptide (SEQ ID NO: 22), V_L sequence
53	RAS Q61K-HLA-A1	EVQLVESGGGLVQPGGSLRLSCAA SGFNISSSGIHWVRQAPGKGLEWV AMVYGGSGYTNYADSVKGRFTISA DTSKNTAYLQMNSLRAEDTAVYY CSRWAHYSAYMDYWGQGTLVTVS S	Clone L2 targeting RAS Q61K mutant peptide (SEQ ID NO: 22), V_H sequence
54	RAS Q61R-HLA-A1	DIQMTQSPSSLSASVGDRVTITCRA SQDVNTAVAWYQQKPGKAPKLLIY SASFLYSGVPSRFSGSRSGTDFTLTI SSLQPEDFATYYCQQYSNYPLTFGQ GTKVEIK	Clone R6 targeting RAS Q61R mutant peptide (SEQ ID NO: 23), V_L sequence
55	RAS Q61R-HLA-A1	EVQLVESGGGLVQPGGSLRLSCAA SGFNVFYGSMHWVRQAPGKGLEW VAFIGPDSTYTYYADSVKGRFTISA DTSKNTAYLQMNSLRAEDTAVYY CSRDLGSAYAMDYWGQGTLVTVS S	Clone R6 targeting RAS Q61R mutant peptide (SEQ ID NO: 23), V_H sequence

[0099] In some embodiments, the first antigen binding domain is specific to p53 R175H mutant peptide (SEQ ID NO: 7) presented in a peptide-HLA complex (R175H-pHLA), which includes a heavy chain variable region (R175H-pHLA V_H) and a light chain variable region (R175H-pHLA V_L). In some embodiments, the R175H-pHLA V_H and/or R175H-pHLA V_L are derived from

clone H2 as described in WO2021127184, which is incorporated by reference herein in its entirety. In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises one or more (*e.g.*, one, two, or three) complementarity-determining regions (CDRs) having amino acid sequences selected from SEQ ID NOs: 40, 42, and 44; and/or a light chain variable region (R175H-pHLA V_L) that comprises one or more (*e.g.*, one, two, or three) CDRs having amino acid sequences selected from SEQ ID NOs: 30, 32, and 34. In some embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 38, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 38; and/or a light chain variable region (R175H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 28, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 28. In some embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 38, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 38; and/or a light chain variable region (R175H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 28, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 28.

[0100] In some embodiments, the first antigen binding domain comprises a heavy chain variable region and/or a light chain variable region modified from H2 clone by one or more amino acids for increased affinity for the target. The modification can comprise one or more amino acid mutations, substitutions, additions, or deletions in one or more of the CDRs and/or the FRs of the heavy and/or light chains. Several modified H2 sequences, including F53S and Y57I, are described in WO2023114430, which is incorporated by reference herein in its entirety. In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H; comprising a Y57I mutation compared to the original H2 heavy chain variable region sequence) that comprises one or more (*e.g.*, one, two, or three) CDRs having amino acid sequences selected from SEQ ID NOs: 40, 47, and 44; and/or a light chain variable region (R175H-pHLA V_L) that comprises one or more (*e.g.*, one, two, or three) CDRs having amino acid sequences selected from SEQ ID NOs: 30, 32, and 34. In some embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises or

consists of an amino acid sequence of SEQ ID NO: 46, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 46; and/or a light chain variable region (R175H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 28, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 28. In some embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 46, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 46; and/or a light chain variable region (R175H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 28, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 28.

[0101] In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises one or more (*e.g.*, one, two, or three) CDRs having amino acid sequences selected from SEQ ID NOs: 40, 42, and 44; and/or a light chain variable region (R175H-pHLA V_L; comprising a F53S mutation compared to the original H2 light chain variable region sequence) that comprises one or more (*e.g.*, one, two, or three) CDRs having amino acid sequences selected from SEQ ID NOs: 30, 37, and 34. In some embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 38, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 38; and/or a light chain variable region (R175H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 36, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 36. In some embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 38, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 38; and/or a light chain variable region (R175H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 36, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 36.

[0102] In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H; comprising a Y57I mutation compared to the original H2

heavy chain variable region sequence) that comprises one or more (*e.g.*, one, two, or three) CDRs having amino acid sequences selected from SEQ ID NOs: 40, 47, and 44; and/or a light chain variable region (R175H-pHLA V_L; comprising a F53S mutation compared to the original H2 light chain variable region sequence) that comprises one or more (*e.g.*, one, two, or three) CDRs having amino acid sequences selected from SEQ ID NOs: 30, 37, and 34. In some embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 46, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 46; and/or a light chain variable region (R175H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 36, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 36. In some embodiments, the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 46, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 46; and/or a light chain variable region (R175H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 36, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 36.

[0103] In some embodiments, the first antigen binding domain is specific to RAS G12V mutant peptide (SEQ ID NO: 17) presented in a peptide-HLA complex (G12V-pHLA), which includes a heavy chain variable region (G12V-pHLA V_H) and a light chain variable region (G12V-pHLA V_L). In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (G12V-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 49, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 49; and/or a light chain variable region (G12V-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 48, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 48.

[0104] In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (G12V-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 49, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 49; and/or a light chain variable region (G12V-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 48, or an

amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 48.

[0105] In some embodiments, the first antigen binding domain is specific to RAS Q61H mutant peptide (SEQ ID NO: 20) presented in a peptide-HLA complex (Q61H-pHLA), which includes a heavy chain variable region (Q61H-pHLA V_H) and a light chain variable region (Q61H-pHLA V_L). In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (Q61H-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 51, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 51; and/or a light chain variable region (Q61H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 50, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 50.

[0106] In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (Q61H-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 51, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 51; and/or a light chain variable region (Q61H-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 50, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 50.

[0107] In some embodiments, the first antigen binding domain is specific to RAS Q61K mutant peptide (SEQ ID NO: 22) presented in a peptide-HLA complex (Q61K-pHLA), which includes a heavy chain variable region (Q61K-pHLA V_H) and a light chain variable region (Q61K-pHLA V_L). In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (Q61K-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 53, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 53; and/or a light chain variable region (Q61K-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 52, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 52.

[0108] In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (Q61K-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 53, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%,

95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 53; and/or a light chain variable region (Q61K-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 52, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 52.

[0109] In some embodiments, the first antigen binding domain is specific to RAS Q61R mutant peptide (SEQ ID NO: 23) presented in a peptide-HLA complex (Q61R-pHLA), which includes a heavy chain variable region (Q61R-pHLA V_H) and a light chain variable region (Q61R-pHLA V_L). In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (Q61R-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 55, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 55; and/or a light chain variable region (Q61R-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 54, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 54.

[0110] In certain of these embodiments, the first antigen binding domain comprises a heavy chain variable region (Q61R-pHLA V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 55, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 55; and/or a light chain variable region (Q61R-pHLA V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 54, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 54.

[0111] The sequences provided herein are illustrative only and not intended to be limiting. Other heavy and/or light chain variable sequences (including CDR sequences) derived from scFvs specific to the targets provided in **Table 1** are described in WO2016154246, WO2018213467, WO2021127184, and WO2023114430, each of which is incorporated by reference herein in its entirety.

[0112] In some embodiments, the first antigen binding domain comprises a heavy chain variable region (Mut-pHLA V_H) and/or a light chain variable region (Mut-pHLA V_L) that share one or more of the FRs as described in scFvs specific to the p53 mutant peptide (*e.g.*, clone H2) or the RAS mutant peptide (*e.g.*, clone V2). By using the same framework, *i.e.*, maintaining the FRs and substituting in CDRs specific to other antigens or mutant peptides, one can construct scFvs specific to those other targets for use in the present technology. In certain of these embodiments,

the first antigen binding domain comprises a heavy chain variable region (Mut-pHLA V_H) that comprises one or more (*e.g.*, one, two, three, or four) FRs having amino acid sequences selected from SEQ ID NOs: 39, 41, 43, and 45; and/or a light chain variable region (Mut-pHLA V_L) that comprises one or more (*e.g.*, one, two, three, or four) FRs having amino acid sequences selected from SEQ ID NOs: 29, 31, 33, and 35. In some embodiments, the first antigen binding domain comprises a heavy chain variable region (Mut-pHLA V_H) that comprises amino acid sequences of SEQ ID NOs: 39, 41, 43, and 45; and/or a light chain variable region (Mut-pHLA V_L) that comprises amino acid sequences of SEQ ID NOs: 29, 31, 33, and 35.

[0113] In any of the embodiments described herein, the first antigen binding domain may comprise one or more amino acid substitutions (*e.g.*, conservative substitutions), insertions, and/or deletions in any of the exemplary amino acid sequences, including, for example, one or more (*e.g.*, one, two, or three) substitutions, insertions, and/or deletions in any of the exemplary CDR sequences. The present technology especially contemplates first antigen binding domains having one or more (*e.g.*, one, two, or three) substitutions, insertions, and/or deletions compared to a reference CDR sequence as described herein that still maintain a certain level of binding affinity to its intended target. In certain of these embodiments, the first antigen binding domain having one or more (*e.g.*, one, two, or three) substitutions, insertions, and/or deletions compared to a reference CDR sequence as described herein would maintain at least about 80% (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) of the binding affinity exhibited by the first antigen binding domain having the reference CDR sequence, when tested under same or similar conditions. For example, the first antigen binding domain having one or more (*e.g.*, one, two, or three) substitutions, insertions, and/or deletions compared to a reference CDR sequence as described herein would bind to its intended target with an affinity (K_D) of about $1x10^{-11}$-$1x10^{-7}$ M, *e.g.*, about $1x10^{-11}$-$1x10^{-8}$ M, about $1x10^{-11}$-$1x10^{-9}$ M, about $1x10^{-11}$-$1x10^{-10}$ M, about $1x10^{-10}$-$1x10^{-7}$ M, about $1x10^{-10}$-$1x10^{-8}$ M, about $1x10^{-10}$-$1x10^{-9}$ M, about $1x10^{-11}$-$1x10^{-7}$ M, about $1x10^{-9}$-$1x10^{-7}$ M, about $1x10^{-9}$-$1x10^{-8}$ M, or about $1x10^{-8}$-$1x10^{-7}$ M.

[0114] The first antigen binding domain can be monovalent (*e.g.*, containing a single antigen binding domain that recognizes/binds a single Mut-pHLA) or multivalent (*e.g.*, containing two or more antigen binding domains and simultaneously targeting two or more of the same or different Mut-pHLAs). For example, a bispecific molecule can include two antigen binding domains, a trispecific molecule can include three antigen binding domains, and a quadruspecific molecule can include four antigen binding domains, etc.

A second antigen binding domain for effector cell surface molecules

[0115] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE) comprises a second antigen binding domain that recognizes and/or binds a surface molecule expressed on an effector cell of interest (*e.g.*, a T cell). By binding both a mutant peptide or MANA on a target cell (*e.g.*, a cancer cell) and a surface molecule on an effector cell (*e.g.*, a T cell), the bispecific molecule (*e.g.*, MANA-TCE) can bring the target cell into close proximity with the effector cell, permitting the effector cell to act on the target cell, for example, to kill or induce apoptosis of the target cell. Examples of effector cells include, without limitation, T cells, natural killer (NK) cells, natural killer T (NKT) cells, B cells, plasma cells, macrophages, monocytes, microglia, dendritic cells, neutrophils, fibroblasts, and mast cells. Examples of surface molecules present on effector cells include, without limitation, CD3, CD4, CD8, CD16A, CD27, CD28, NKG2D, PD-1, CTLA-4, 4-1BB, OX40, ICOS, Fc receptors, and any other effector cell surface receptors.

[0116] In some embodiments, the effector cell is a T cell (*e.g.*, a CD8+ T cell), and the surface molecule to be recognized by the second antigen binding domain is CD3 or a subunit thereof. CD3 is a T cell coreceptor involved in activating both the cytotoxic T cell (predominantly CD8+ T cells) and T helper cells (predominantly CD4+ T cells). In mammals, CD3 may comprise a CD3 gamma chain, a CD3 delta chain, two CD3 epsilon chains, and a homodimer of CD3 zeta chains. A TCR complex is formed by the association of CD3 with a TCR. Thus, a TCR complex may be composed of a CD3 gamma chain, a CD3 delta chain, two CD3 epsilon chains, a homodimer of CD3 zeta chains, a TCR alpha chain, and a TCR beta chain. Alternatively, a TCR complex may be composed of a CD3 gamma chain, a CD3 delta chain, two CD3 epsilon chains, a homodimer of CD3 zeta chains, a TCR gamma chain, and a TCR delta chain.

[0117] The second antigen binding domain specific to CD3 or a subunit thereof can be any appropriate type or form of immunoglobulins, fragments, or portions thereof that retain the ability to specifically recognize and/or bind an antigen. The second antigen binding domain can be derived from a natural, engineered, or otherwise modified antibody and usually contain at least one of the CDRs responsible for antigen specificity. Non-limiting examples of a second antigen binding domain may include an scFv, disulfide linked Fv, sdAb, VHH, Fab, F(ab'), $F(ab')_2$ fragment, diabody, and scDb.

[0118] In some embodiments, the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof. In certain of these embodiments, the second antigen binding domain can be in any appropriate format which includes at least one

heavy chain variable region (CD3 V_H) and at least one light chain variable region (CD3 V_L), which can be directly or indirectly associated in any order or orientation. In some embodiments, the CD3 V_H is N-terminal to the CD3 V_L. In some embodiments, the CD3 V_H is C-terminal to the CD3 V_L. The CD3 V_H and the CD3 V_L can be linked directly or indirectly by a linker, *e.g.*, a Whitlow linker or a $(G_4S)_n$ (n can be a positive integer, *e.g.*, 1, 2, 3, 4, 5, or 6) linker. Exemplary CD3 V_H and V_L sequences that can be used for the second antigen binding domain to specifically recognize and/or bind CD3 are provided in **Table 3** below.

Table 3. Exemplary V_H and V_L sequences for CD3

SEQ ID NO:	Sequence	Description
56	DIQMTQSPSSLSASVGDRVTITC**RASQDIRNYLN**WYQQKPGKAPKLLIY**YTSRLES**GVPSRFSGSGSGTDYTLTISSLQPEDFATYYC**QQGNTLPWT**FGQGTKVEIK	UCHT1 v.9 targeting human CD3, V_L sequence, with **CDR1**, **CDR2**, and **CDR3** highlighted
57	RASQDIRNYLN	UCHT1 v.9 V_L, CDR1
58	YTSRLES	UCHT1 v.9 V_L, CDR2
59	QQGNTLPWT	UCHT1 v.9 V_L, CDR3
60	EVQLVESGGGLVQPGGSLRLSCAASG**YSFTGYTMN**WVRQAPGKGLEWVA**LINPYKGVSTYNQKFKD**RFTISVDKSKNTAYLQMNSLRAEDTAVYYCARS**GYYGDSDWYFDV**WGQGTLVTVSS	UCHT1 v.9 targeting human CD3, V_H sequence, with **CDR1**, **CDR2**, and **CDR3** highlighted
61	YSFTGYTMN	UCHT1 v.9 V_H, CDR1
62	LINPYKGVSTYNQKFKD	UCHT1 v.9 V_H, CDR2
63	SGYYGDSDWYFDV	UCHT1 v.9 V_H, CDR3

[0119] In some embodiments, the second antigen binding domain specific to CD3, which includes a heavy chain variable region (CD3 V_H) and a light chain variable region (CD3 V_L), is derived from UCHT1 v.9 as described in WO2023114430, which is incorporated by reference herein in its entirety. In certain of these embodiments, the second antigen binding domain comprises a

heavy chain variable region (CD3 V_H) that comprises one or more (*e.g.*, one, two, or three) CDRs having amino acid sequences selected from SEQ ID NOs: 61-63; and/or a light chain variable region (CD3 V_L) that comprises one or more (*e.g.*, one, two, or three) CDRs having amino acid sequences selected from SEQ ID NOs: 57-59. In some embodiments, the second antigen binding domain comprises a heavy chain variable region (CD3 V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 60, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 60; and/or a light chain variable region (CD3 V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 56, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 56.

[0120] In some embodiments, the second antigen binding domain comprises a heavy chain variable region (CD3 V_H) that comprises or consists of an amino acid sequence of SEQ ID NO: 60, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 60; and/or a light chain variable region (CD3 V_L) that comprises or consists of an amino acid sequence of SEQ ID NO: 56, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 56.

[0121] The sequences provided herein are illustrative only and not intended to be limiting. Other heavy and/or light chain variable sequences (including CDR sequences) derived from scFvs specific to CD3 or a subunit thereof are described in WO2021127184 and WO2023114430, each of which is incorporated by reference herein in its entirety. In some embodiments, the second antigen binding domain specific for CD3 comprises a V_H and/or V_L sequence as described elsewhere. See, *e.g.*, Rodrigues et al., Int. J. Cancer Suppl. 1992; 7:45-50; Shalaby et al., J. Exp. Med. 1992; 175:217-25; Brischwein et al., Mol. Immunol. 2006; 43:1129-43; Li et al., Immunology 2005; 116:487-98; WO2012162067; US20070065437; US20070065437; US20070065437; US20070065437; US20070065437; and US20070065437, each of which is incorporated by reference herein in its entirety.

[0122] In any of the embodiments described herein, the second antigen binding domain may comprise one or more amino acid substitutions (*e.g.*, conservative substitutions), insertions, and/or deletions in any of the exemplary amino acid sequences, including, for example, one or more (*e.g.*, one, two, or three) substitutions, insertions, and/or deletions in any of the exemplary CDR sequences. The present technology especially contemplates second antigen binding domains

having one or more (*e.g.*, one, two, or three) substitutions, insertions, and/or deletions compared to a reference CDR sequence as described herein that still maintain a certain level of binding affinity to its intended target (*e.g.*, CD3). In certain of these embodiments, the second antigen binding domain having one or more (*e.g.*, one, two, or three) substitutions, insertions, and/or deletions compared to a reference CDR sequence as described herein would maintain at least about 80% (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) of the binding affinity exhibited by the second antigen binding domain having the reference CDR sequence, when tested under same or similar conditions. For example, the second antigen binding domain having one or more (*e.g.*, one, two, or three) substitutions, insertions, and/or deletions compared to a reference CDR sequence as described herein would bind to its intended target (*e.g.*, CD3) with an affinity (K_D) of about $1x10^{-11}$-$1x10^{-7}$ M, *e.g.*, about $1x10^{-11}$-$1x10^{-8}$ M, about $1x10^{-11}$-$1x10^{-9}$ M, about $1x10^{-11}$-$1x10^{-10}$ M, about $1x10^{-10}$-$1x10^{-7}$ M, about $1x10^{-10}$-$1x10^{-8}$ M, about $1x10^{-10}$-$1x10^{-9}$ M, about $1x10^{-11}$-$1x10^{-7}$ M, about $1x10^{-9}$-$1x10^{-7}$ M, about $1x10^{-9}$-$1x10^{-8}$ M, or about $1x10^{-8}$-$1x10^{-7}$ M.

Configuration of the first and second antigen binding domains

[0123] In some embodiments, the first antigen binding domain and the second antigen binding domain of the bispecific molecule (*e.g.*, MANA-TCE) as described herein are associated, either directly or indirectly and either covalently or noncovalently, and part of one bispecific biologic. The bispecific biologic can be in any appropriate form known to a person skilled in the art, including, for example, a tandem scFv, diabody, scDb, scFv-Fc, scDb-Fc, bispecific T cell engager (BiTE), bispecific killer cell engager (BiKE), tandem diabody (TandAb), and dual affinity retargeting antibody (DART).

A. Tandem scFv

[0124] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE) is in the form of tandem scFvs, *i.e.*, the first antigen binding domain and second antigen binding domain of the bispecific molecule each comprise an scFv, and the two scFVs are connected by a linker. The tandem scFv can be in any appropriate orientation. Examples of tandem scFv orientations comprising an scFv-A (*i.e.*, the first antigen binding domain) and an scFv-B (*i.e.*, the second antigen binding domain) include, without limitation, from an N- to C-terminus order: (1) VLA-L1-VHA-L2-VLB-L3-VHB; (2) VLA-L1-VHA-L2-VHB-L3-VLB; (3) VHA-L1-VLA-L2-VLB-L3-VHB; (4) VHA-L1-VLA-L2-VHB-L3-VLB; (5) VLB-L1-VHB-L2-VLA-L3-VHA; (6) VLB-L1-VHB-L2-VHA-L3-VLA; (7) VHB-L1-VLB-L2-VLA-L3-VHA; and (8) VHB-L1-VLB-L2-VHA-L3-VLA, where L1/L3 can be a long linker and L2 can be a short linker. In some

embodiments, a short linker can be from about 1 amino acid to about 10 amino acids in length and can include any appropriate amino acids in any appropriate combination; a long linker can be from about 5 amino acids to about 40 amino acids in length and can include any appropriate amino acids in any appropriate combination. A non-limiting example of a short linker described herein is a G_4S linker, while a non-limiting example of a long linker described herein is a $3xG_4S$ linker.

[0125] In some embodiments, when the first antigen binding domain comprises an scFv that specifically recognizes and/or binds a Mut-pHLA, and the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof, the bispecific molecule may be in the form of a tandem scFv with an N- to C-terminus order of (1) Mut-pHLA V_L-L1-Mut-pHLA V_H-L2-CD3 V_L-L3-CD3 V_H; (2) Mut-pHLA V_L-L1-Mut-pHLA V_H-L2-CD3 V_H-L3-CD3 V_L; (3) Mut-pHLA V_H-L1-Mut-pHLA V_L-L2-CD3 V_L-L3-CD3 V_H; (4) Mut-pHLA V_H-L1-Mut-pHLA V_L-L2-CD3 V_H-L3-CD3 V_L; (5) CD3 V_L-L1-CD3 V_H-L2-Mut-pHLA V_L-L3-Mut-pHLA V_H; (6) CD3 V_L-L1-CD3 V_H-L2-Mut-pHLA V_H-L3-Mut-pHLA V_L; (7) CD3 V_H-L1-CD3 V_L-L2-Mut-pHLA V_L-L3-Mut-pHLA V_H, or (8) CD3 V_H-L1-CD3 V_L-L2-Mut-pHLA V_H-L3-Mut-pHLA V_L, where L1 and L3 are long linkers and L2 is a short linker as described.

B. Diabody

[0126] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE) is in the form of a diabody, *i.e.*, the first antigen binding domain and second antigen binding domain of the bispecific molecule each comprise an scFv, and the heavy and light chains of the scFVs are interconnected by a linker. The diabody can be in any appropriate orientation. Examples of diabody orientations comprising an scFv-A (*i.e.*, the first antigen binding domain) and an scFv-B (*i.e.*, the second antigen binding domain) include, without limitation, from an N- to C-terminus order: (1) VLA-L-VLB and VHA-L-VHB; (2) VLA-L-VLB and VHB-L-VHA (3) VLB-L-VLA and VHA-L-VHB; (4) VLB-L-VHA and VHB-L-VHA; (5) VLA-L-VHB and VHA-L-VLB; (6) VLA-L-VHB and VLB-L-VHA (7) VHB-L-VLA and VHA-L-VLB; (8) VHB-L-VLA and VLB-L-VHA, where L is a short linker. In some embodiments, a short linker can be from about 1 amino acid to about 10 amino acids in length and can include any appropriate amino acids in any appropriate combination.

[0127] In some embodiments, when the first antigen binding domain comprises an scFv that specifically recognizes and/or binds a Mut-pHLA, and the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof, the bispecific molecule may be in the form of a diabody with an N- to C-terminus order of (1) Mut-

pHLA V_L-L-CD3 V_L and Mut-pHLA V_H-L-CD3 V_H; (1) Mut-pHLA V_L-L-CD3 V_L and CD3 V_H-L-Mut-pHLA V_H; (3) CD3 V_L-L-Mut-pHLA V_L and Mut-pHLA V_H-L-CD3 V_H; (4) CD3 V_L-L-Mut-pHLA V_L and CD3 V_H-L-Mut-pHLA V_H; (5) Mut-pHLA V_L-L-CD3 V_H and Mut-pHLA V_H-L-CD3 V_L; (6) Mut-pHLA V_L-L-CD3 V_H and CD3 V_L-L-Mut-pHLA V_H; (7) CD3 V_H-L-Mut-pHLA V_L and Mut-pHLA V_H-L-CD3 V_L; or (5) CD3 V_H-L-Mut-pHLA V_L and CD3 V_L-L-Mut-pHLA V_H, where L is a short linker as described. In certain of these embodiments, the bispecific molecule can also be referred to as a DART.

C. scDb

[0128] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE) is in the form of an scDb, *i.e.*, the first antigen binding domain and second antigen binding domain of the bispecific molecule each comprise an scFv, and the heavy and light chains of the scFVs are connected by a linker into a single chain (see **FIG. 1**, configuration **A**). The scDb can be in any appropriate orientation. Examples of scDb orientations comprising an scFv-A (*i.e.*, the first antigen binding domain) and an scFv-B (*i.e.*, the second antigen binding domain) include, without limitation, from an N- to C-terminus order: (1) VLA-L1-VHB-L2-VLB-L3-VHA; (2) VHA-L1-VLB-L2-VHB-L3-VLA; (3) VLA-L1-VLB-L2-VHB-L3-VHA; (4) VHA-L1-VHB-L2-VLB-L3-VLA; (5) VLB-L1-VHA-L2-VLA-L3-VHB; (6) VHB-L1-VLA-L2-VHA-L3-VLB; (7) VLB-L1-VLA-L2-VHA-L3-VHB; and (8) VHB-L1-VHA-L2-VLA-L3-VLB, where L1/L3 can be a short linker and L2 can be a long linker. In some embodiments, a short linker can be from about 1 amino acid to about 10 amino acids in length and can include any appropriate amino acids in any appropriate combination; a long linker can be from about 5 amino acids to about 40 amino acids in length and can include any appropriate amino acids in any appropriate combination.

[0129] In some embodiments, when the first antigen binding domain comprises an scFv that specifically recognizes and/or binds a Mut-pHLA, and the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof, the bispecific molecule may be in the form of an scDb with an N- to C-terminus order of (1) Mut-pHLA V_L-L1-CD3 V_H-L2-CD3 V_L-L3-Mut-pHLA V_H; (2) Mut-pHLA V_H-L1-CD3 V_L-L2-CD3 V_H-L3-Mut-pHLA V_L; (3) Mut-pHLA V_L-L1-CD3 V_L-L2-CD3 V_H-L3-Mut-pHLA V_H; (4) Mut-pHLA V_H-L1-CD3 V_H-L2-CD3 V_L-L3-Mut-pHLA V_L; (5) CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H; (6) CD3 V_H-L1-Mut-pHLA V_L-L2-Mut-pHLA V_H-L3-CD3 V_L; (7) CD3 V_L-L1-Mut-pHLA V_L-L2-Mut-pHLA V_H-L3-CD3 V_H; or (8) CD3 V_H-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_L, where L1/L3 are short linkers and L2 is a long linker as described.

In certain of these embodiments, the bispecific molecule can also be referred to as a BiTE. In a particular embodiment, the bispecific molecule is in the form of an scDb with an N- to C-terminus order of CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H, L1/L3 are short linkers and L2 is a long linker as described.

Modifications to the bispecific molecules

[0130] In some embodiments, the bispecific molecule comprises modifications that result in one or more improved characteristics of the molecules such as improved potency, improved potential therapeutic index (or therapeutic window), improved half-life, reduced risk of complement-dependent cytotoxicity (CDC), improved manufacturability, and improved effector cell engagement, while maintaining functionality and specificity in the treatment of disease such as cancer. The modifications to the bispecific molecule may include domain orientation modifications, linker modifications, Fc domain modifications, and/or fusion modifications.

A. Domain orientation and/or linker modifications

[0131] In some embodiments, the bispecific molecule comprises a domain orientation and/or linker modification, *e.g.*, the first and second antigen binding domains, including their components, are arranged in such an arrangement and/or orientation as to optimize spatial configuration and engagement with the target/effector cells. These modifications include the positions of the first and second antigen binding domains relative (*e.g.*, proximal or distal) to the other part of the bispecific molecule (*e.g.*, an Fc domain, a fusion protein domain, or a chemical modification as discussed herein), as well as variations in the order of the elements (*e.g.*, heavy and light chain variable regions) withing each antigen binding domain (*e.g.*, the N- to C-terminus order of each element). These modifications may include additional amino acids (*e.g.*, linkers) between antigen binding domains and/or between elements (*e.g.*, heavy and light chain variable regions) withing antigen binding domains. In some embodiments, the first and second antigen binding domains are configured as tandem scFvs, diabodies, or scDbs, with the heavy and light chain variable regions of the first and second antigen binding domains connected via linkers of appropriate lengths, as described herein. For example, as discussed herein and demonstrated in the examples, the heavy and light chain variable regions of the first and second antigen binding domains can be connected through linkers in the form of an scDb in a particular orientation for superior spatial configuration and binding characteristics leading to optimal effector cell engagement.

B. Fc domain modification

[0132] In some embodiments, the bispecific molecule comprises an Fc domain modification, *e.g.*, the bispecific molecule further comprises an Fc domain connected to the first and/or second antigen binding domain to improve stability, extend half-life, and/or to confer the molecule Fc-mediated effector functions, to name a few. With an Fc domain modification, the bispecific molecule can be configured as scFv-Fcs or scDb-Fcs as described herein.

[0133] An Fc domain can be derived from a natural Ig molecule, *e.g.*, can include an amino acid sequence derived from an IgA (*e.g.*, IgA1 or IgA2) isotype, or an IgG (*e.g.*, IgG1, IgG2, IgG3, or IgG4) isotype. An Fc domain of IgA or IgG isotype typically contains two chains of an Fc sequence, each of which comprises a hinge, a CH2 domain, and a CH3 domain. Alternatively, a truncated Fc domain that excludes the hinge and/or the CH2 domain may be used.

[0134] In some embodiments, the Fc domain is of IgG4 isotype. In some embodiments, the two chains of the Fc domain are identical or symmetrical and comprise a natural or wild-type (WT) IgG4 Fc sequence, for example, the Fc portion (*e.g.*, comprising a hinge, a CH2 domain, and a CH3 domain) (SEQ ID NO: 64) of a consensus full-length IgG4 sequence as known to a person skilled in the art.

[0135] In some embodiments, the two chains of the Fc domain are unidentical or asymmetrical, as one or both chains of the Fc domain comprise one or more modifications (*e.g.*, amino acid mutations or substitutions in reference to the consensus full-length IgG4 sequence) to increase stability and/or half-life of the molecule and to increase heterodimerization and/or decrease homodimerization of the two chains. In some embodiments, the amino acid mutations or substitutions are selected from the group consisting of S228P, L235E, L336W, T366S, L368A, Y407V, and H435R, where the amino acid positions are in reference to the consensus full-length IgG4 sequence.

[0136] Without wishing to be bound by any theory, an S228P mutation in both chains of the Fc doamin can stabilize the hinge. An L235E mutation can be introduced to one or both of the two chains to minimize the potential for Fc gamma receptor binding and to extend half-life of the bispecific molecule. Additionally, T366S, L368A, and Y407V mutations can be introduced to one chain (*e.g.*, Chain-1) to form a "Hole" structure, and an L336W mutation can be introduced to the other chain (*e.g.*, Chain-2) to form a "Knob" structure, together resulting in a "Knob into Hole (KiH)" configuration to support asymmetric pairing or heterodimerization of the Fc domain (see **FIG. 2**). It has been found that compared to a Chain-1/Chain-2 KiH heterodimer configuration, a Chain-1/Chain-1 Hole/Hole mis-pair has lower isoelectric point (pI) value, while

a Chain-2/Chain-2 Knob/Knob mis-pair has a 2 Log decrease in protein A binding and a higher pI value, rendering these undesirable impurities easily removable from the KiH heterodimers (see **FIG. 2**). Finally, an H435R mutation can be introduced to one of the two chains (*e.g.*, Chain-2) to decrease affinity for protein A, thus minimizing affinity of mis-paired homodimers for protein A affinity chromatography and allowing for more efficient commercial purification strategies. Exemplary Fc sequences are provided in **Table 4** below.

[0137] Without being bound by a theory, it is thought that the improved manufacturability of the bispecific molecules disclosed herein is promoted by the presence of the H435R mutation. This mutation alters the PI (isoelectric point) of Chain-2. This allows for effective separation (e.g. ion-exchange chromatography, mixed mode chromatography and other separation methods that take advantage of charge) of Chain-1/Chain-1 mis-pairs/Chain-1 aggregates/Acidic Fragments and Chain-1/Chain-2 mis-pairs/Chain-2 aggregates/Basic-Fragments from the heterodimers (**FIG. 2**).

[0138] In certain of these embodiments, the Fc domain comprises a first chain of an Fc sequence comprising S228P, L235E, T366S, L368A, and Y407V mutations in reference to the consensus full-length IgG4 sequence, and/or the first chain of the Fc sequence comprises or consists of an amino acid sequence of SEQ ID NO: 65, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 65. In certain of these embodiments, the Fc domain comprises a second chain of an Fc sequence comprising S228P, L235E, L336W, and H435R mutations in reference to the consensus full-length IgG4 sequence, and/or the second chain of the Fc sequence comprises or consists of an amino acid sequence of SEQ ID NO: 66, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 66.

[0139] In certain of these embodiments, the Fc domain comprises a first chain of an Fc sequence comprising S228P, L235E, T366S, L368A, and Y407V mutations in reference to the consensus full-length IgG4 sequence, and/or the first chain of the Fc sequence comprises or consists of an amino acid sequence of SEQ ID NO: 65, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 65. In certain of these embodiments, the Fc domain comprises a second chain of an Fc sequence comprising S228P, L235E, L336W, and H435R mutations in reference to the consensus full-length IgG4 sequence, and/or the second chain of the Fc sequence comprises or consists of an

amino acid sequence of SEQ ID NO: 66, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 66.

Table 4. Exemplary Fc domain sequences

SEQ ID NO:	Sequence	Description
64	ESKYGPPCSPCPAPEFLGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREE QFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSS IEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLS LG	WT IgG4 Fc domain
65	ESKYGPPC**P**PCPAPEF**E**GGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREE QFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSS IEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSL**S**C**A**V KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL**V** SRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLS LG	IgG4 Fc domain, Chain-1, with **S228P**, **L235E**, **T366S**, **L368A**, and **Y407V** mutations highlighted
66	ESKYGPPC**P**PCPAPEF**E**GGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREE QFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSS IEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSL**W**CLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SRLTVDKSRWQEGNVFSCSVMHEALHN**R**YTQKSLSLS LG	IgG4 Fc domain, Chain-2, with **S228P**, **L235E**, and **L336W**, and **H435R** mutations highlighted

[0140] The Fc domain modification can be connected to the first and/or second antigen binding domain at any suitable location in any suitable configuration. For example, the first and/or second antigen binding domain can be connected to the Fc domain at its N-terminal end or C-terminal end optionally through a linker, where the linker may be about one to about 40 amino acids in

length. Alternatively, the linker may be omitted, and the Fc domain is directly connected to the first and/or second antigen binding domain.

1. scFv-Fc

[0141] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE) with an Fc domain modification is in the form of an scFv-Fc, *i.e.*, the first antigen binding domain and second antigen binding domain of the bispecific molecule each comprise an scFv and are each connected to one chain of an Fc domain through a linker (see **FIG. 1**, configurations **B**, **C**, and **F**). The scFv-Fc can be dimeric, *e.g.*, one copy of the first antigen binding domain scFv is connected to one chain of the Fc domain and one copy of the second antigen binding domain scFv is connected to the other (see **FIG. 1**, configurations **F**); or can be trimeric, *e.g.*, one copy of the first (or second) antigen binding domain scFv is connected to each of the two chains of the Fc domain at one end (*e.g.*, at the N-terminal end) and one copy of the second (or first) antigen binding domain scFv is connected to one of the two chains of the Fc domain at the opposite end (*e.g.*, at the C-terminal end) (see **FIG. 1**, configurations **B** and **C**). The scFv-Fc can be in any appropriate orientation. Examples of (dimeric) scFv-Fc orientations comprising an scFv-A (*i.e.*, the first antigen binding domain) and an scFv-B (*i.e.*, the second antigen binding domain) include, without limitation, from an N- to C-terminus order: (1) VLA-L-VHA-Fc and VLB-L-VHB-Fc; (2) VHA-L-VLA-Fc and VHB-L-VLB-Fc; (3) VLA-L-VHA-Fc and VHB-L-VLB-Fc; and (4) VHA-L-VLA-Fc and VLB-L-VHB-Fc, where L can be a long linker. In some embodiments, a long linker can be from about 5 amino acids to about 40 amino acids in length and can include any appropriate amino acids in any appropriate combination. In the above examples, only dimeric scFv-Fcs are shown as illustrative; for trimeric scFv-Fcs, an additional copy of the first or second antigen binding domain scFv is connected to the Fc domain. In any of the embodiments discussed above, the Fc domain in an scFv-Fc can be at the N-terminus of the scFv instead of the C-terminus. In some embodiments, the Fc domain in an scFv-Fc can exclude the hinge and/or the CH2 domain. In some embodiments, the Fc domain in an scFv-Fc can include one or more modifications (*e.g.*, amino acid substitutions) as described to increase heterodimerization and/or to decrease homodimerization of the scFv-Fc.

[0142] In some embodiments, when the first antigen binding domain comprises an scFv that specifically recognizes and/or binds a Mut-pHLA, and the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof, the bispecific molecule may be in the form of an scFv-Fc with an N- to C-terminus order of (1) Mut-

pHLA V_L-L-Mut-pHLA V_H-Fc and CD3 V_L-L-CD3 V_H-Fc; (2) Mut-pHLA V_H-L-Mut-pHLA V_L-Fc and CD3 V_H-L-CD3 V_L-Fc; (3) Mut-pHLA V_L-L-Mut-pHLA V_H-Fc and CD3 V_H-L-CD3 V_L-Fc; and (4) Mut-pHLA V_H-L-Mut-pHLA V_L-Fc and CD3 V_L-L-CD3 V_H-Fc, where L is a long linker as described. In any of the embodiments discussed above, the Fc domain in an scFv-Fc can be at the N-terminus of the scFv instead of the C-terminus. In some embodiments, the Fc domain in an scFv-Fc can exclude the hinge and/or the CH2 domain. In some embodiments, the Fc domain in an scFv-Fc can include one or more modifications (*e.g.*, amino acid substitutions) as described to increase heterodimerization and/or to decrease homodimerization of the scFv-Fc.

2. scDb-Fc

[0143] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE) with an Fc domain modification is in the form of an scDb-Fc, *i.e.*, the first antigen binding domain and second antigen binding domain of the bispecific molecule each comprise an scFv with the heavy and light chains of the scFVs interconnected by a linker into a single chain in the form of an scDb, and the scDb is connected to an Fc domain through a linker (see **FIG. 1**, configurations **H** and **M**). The scDb-Fc can be monomeric, *i.e.*, one copy of the scDb is connected to one chain of the Fc domain (see **FIG. 1**, configuration **H**); or can be dimeric, *i.e.*, one copy of the scDb is connected to each of the two chains of the Fc domain (see **FIG. 1**, configuration **M**). The scDb-Fc can be in any appropriate orientation. Examples of (monomeric) scDb-Fc orientations comprising an scFv-A (*i.e.*, the first antigen binding domain) and an scFv-B (*i.e.*, the second antigen binding domain) include, without limitation, from an N- to C-terminus order: (1) VLA-L1-VHB-L2-VLB-L3-VHA-L4-Fc; (2) VHA-L1-VLB-L2-VHB-L3-VLA-L4-Fc; (3) VLA-L1-VLB-L2-VHB-L3-VHA-L4-Fc; (4) VHA-L1-VHB-L2-VLB-L3-VLA-L4-Fc; (5) VLB-L1-VHA-L2-VLA-L3-VHB-L4-Fc; (6) VHB-L1-VLA-L2-VHA-L3-VLB-L4-Fc; (7) VLB-L1-VLA-L2-VHA-L3-VHB-L4-Fc; and (8) VHB-L1-VHA-L2-VLA-L3-VLB-L4-Fc, where L1/L3 can be a short linker, L2 can be a long linker, and L4 is optional and can be a short or long linker. In some embodiments, a short linker can be from about 1 amino acid to about 10 amino acids in length and can include any appropriate amino acids in any appropriate combination; a long linker can be from about 5 amino acids to about 40 amino acids in length and can include any appropriate amino acids in any appropriate combination. In the above examples, only monomeric scDb-Fcs are shown as illustrative; for dimeric scDb-Fcs, a second copy of the scDb is connected to the second chain of the Fc domain. In any of the embodiments discussed above, the Fc domain in an scDb-Fc can be at the N-terminus of the scDb-Fc instead of the C-terminus. In some embodiments, the Fc domain in an scDb-Fc can exclude the hinge and/or the CH2 domain. In some embodiments, the Fc

domain in an scDb-Fc can include one or more modifications (*e.g.*, amino acid substitutions) as described to increase heterodimerization and/or to decrease homodimerization.

[0144] In some embodiments, when the first antigen binding domain comprises an scFv that specifically recognizes and/or binds a Mut-pHLA, and the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof, the bispecific molecule may be in the form of an scDb-Fc with an N- to C-terminus order of (1) Mut-pHLA V_L-L1-CD3 V_H-L2-CD3 V_L-L3-Mut-pHLA V_H-L4-Fc; (2) Mut-pHLA V_H-L1-CD3 V_L-L2-CD3 V_H-L3-Mut-pHLA V_H-L4-Fc; (3) Mut-pHLA V_L-L1-CD3 V_L-L2-CD3 V_H-L3-Mut-pHLA V_L-L4-Fc; (4) Mut-pHLA V_H-L1-CD3 V_H-L2-CD3 V_L-L3-Mut-pHLA V_L-L4-Fc; (5) CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H-L4-Fc; (6) CD3 V_H-L1-Mut-pHLA V_L-L2-Mut-pHLA V_H-L3-CD3 V_L-L3-Fc; (7) CD3 V_L-L1-Mut-pHLA V_L-L2-Mut-pHLA V_H-L3-CD3 V_H-L4-Fc; or (8) CD3 V_H-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_L-L4-Fc, where L1/L3 are short linkers, L2 is a long linker, and L4 is optional and can be a short or long linker as described (see **FIG. 3**). In certain of these embodiments, the bispecific molecule can also be referred to as a BiTE-Fc. In a particular embodiment, the bispecific molecule is in the form of an scDb-Fc with an N- to C-terminus order of CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H-L4-Fc, where L1/L3 are short linkers, L2 is a long linker, and L4 is optional and can be a short or long linker as described. In any of the embodiments discussed above, the Fc domain in an scDb-Fc can be at the N-terminus of the scDb-Fc instead of the C-terminus. In some embodiments, the Fc domain in an scDb-Fc can exclude the hinge and/or the CH2 domain. In some embodiments, the Fc domain in an scDb-Fc can include one or more modifications (*e.g.*, amino acid substitutions) as described to increase heterodimerization and/or to decrease homodimerization.

3. scFv/scDb-Fc

[0145] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE) with an Fc domain modification is in the form of an scFv/scDb-Fc, *i.e.*, a hybrid of an scFv-Fc and an scDb-Fc, according to various embodiments disclosed and described herein, where the first (or second) antigen binding domain scFv is connected to one chain of an Fc domain through a linker, and an scDb of the first and second antigen binding domains is connected to the other chain of the Fc domain through a linker (see **FIG. 1**, configuration **K**). The Fc domain in an scFv/scDb-Fc hybrid can be at the N-terminus of the scFv and/or scDb instead of the C-terminus. In some embodiments, the Fc domain in an scFv/scDb-Fc hybrid can exclude the hinge and/or the CH2 domain. In some

embodiments, the Fc domain in an scFv/scDb-Fc hybrid can include one or more modifications (*e.g.*, amino acid substitutions) as described to increase heterodimerization and/or to decrease homodimerization.

[0146] In any of the above embodiments involving an Fc domain, any appropriate Fc domain derived from a natural immunoglobin (Ig) molecule can be used. The Fc domain can include an amino acid sequence derived from an IgA (*e.g.*, IgA1 or IgA2) isotype, or an IgG (*e.g.*, IgG1, IgG2, IgG3, or IgG4) isotype. In some embodiments, an Fc domain can include an amino acid sequence that includes one or more modifications (*e.g.*, one or more amino acid substitutions) in the hinge, CH2 and/or CH3 domains to increase its stability and/or to increase or decrease its binding to one or more Fc receptors, as described herein. In some embodiments, an Fc domain to be used for the present technology can be as described elsewhere (see, *e.g.*, International Patent Application Publication No. WO 2017/134134 A1 at, for example, SEQ ID NOs: 25-32; and International Patent Application Publication No. WO 2017/134158 A1 at, for example, Table 38; and SEQ ID NOs: 25-32).

C. Fusion protein modification

[0147] In some embodiments, the bispecific molecule comprises a fusion protein modification, *e.g.*, the bispecific molecule further comprises a protein or polypeptide fused to the first and/or second antigen binding domain to improve stability, extend half-life, and/or simplify manufacturing.

[0148] In some embodiments, the fusion protein comprises a serum albumin, *e.g.*, a human serum albumin (HSA). Human serum albumin is the serum albumin found in human blood, and it is the most abundant protein in plasma. It constitutes about half of serum protein and transports hormones, fatty acids, and other compounds, buffers pH, and maintains oncotic pressure, among other functions. Exemplary HSA sequences are provided in **Table 5** below. In certain of these embodiments, the HSA fusion protein comprises or consists of an amino acid sequence of SEQ ID NO: 80 or SEQ ID NO: 81, or an amino acid sequence that is at least about 80% identical (e.g., at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 80 or SEQ ID NO: 81. In certain of these embodiments, the HSA fusion protein comprises or consists of an amino acid sequence of SEQ ID NO: 80 or SEQ ID NO: 81, or an amino acid sequence that is at least 80% identical (e.g., at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 80 or SEQ ID NO: 81.

Table 5. Exemplary HSA fusion protein sequences

SEQ ID NO:	Sequence	Description
80	DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLV NEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGE MADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFH DNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAW AVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDR ADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPAD LPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSV VLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQN LIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNL GKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDR VTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLS EKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCC KADDKETCFAEEGKKLVAASQAALGL	HSA
81	DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQSPFEDHVKLV NEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGE MADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFH DNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAA DKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAW AVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDR ADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPAD LPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSV VLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQN LIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNL GKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDR VTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLS EKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCC KADDKETCFAEEGKKLVAASQAALGL	HSA

[0149] The HSA fusion protein modification can be connected to the first and/or second antigen binding domain at any suitable location in any suitable configuration. For example, the first and/or second antigen binding domain can be connected to the HSA fusion protein at its N-terminal end or C-terminal end through a linker, where the linker may be about one to about 40 amino acids in length. Alternatively, the linker may be omitted, and the HSA fusion protein is directly connected to the first and/or second antigen binding domain.

4. scDb-HSA

[0150] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE) with an HSA fusion protein modification is in the form of an scDb-HSA, *i.e.*, the first antigen binding domain and second antigen binding domain of the bispecific molecule each comprise an scFv with the heavy and light chains of the scFVs interconnected by a linker into a single chain in the form of an scDb, and the scDb is connected to an HSA fusion protein through a linker. The scDb-HSA can be in any appropriate orientation. Examples of scDb-HSA orientations comprising an scFv-A (*i.e.*, the first antigen binding domain) and an scFv-B (*i.e.*, the second antigen binding domain) include, without limitation, from an N- to C-terminus order: (1) VLA-L1-VHB-L2-VLB-L3-VHA-L4-HSA; (2) VHA-L1-VLB-L2-VHB-L3-VLA-L4-HSA; (3) VLA-L1-VLB-L2-VHB-L3-VHA-L4-HSA; (4) VHA-L1-VHB-L2-VLB-L3-VLA-L4-HSA; (5) VLB-L1-VHA-L2-VLA-L3-VHB-L4-HSA; (6) VHB-L1-VLA-L2-VHA-L3-VLB-L4-HSA; (7) VLB-L1-VLA-L2-VHA-L3-VHB-L4-HSA; (8) VHB-L1-VHA-L2-VLA-L3-VLB-L4-HSA; (9) HSA-L4-VLA-L1-VHB-L2-VLB-L3-VHA; (10) HSA-L4-VHA-L1-VLB-L2-VHB-L3-VLA; (11) HSA-L4-VLA-L1-VLB-L2-VHB-L3-VHA; (12) HSA-L4-VHA-L1-VHB-L2-VLB-L3-VLA; (13) HSA-L4-VLB-L1-VHA-L2-VLA-L3-VHB; (14) HSA-L4-VHB-L1-VLA-L2-VHA-L3-VLB; (15) HSA-L4-VLB-L1-VLA-L2-VHA-L3-VHB; and (16) HSA-L4-VHB-L1-VHA-L2-VLA-L3-VLB, where L1/L3 can be a short linker, L2 can be a long linker, and L4 is optional and can be a short or long linker. In some embodiments, a short linker can be from about 1 amino acid to about 10 amino acids in length and can include any appropriate amino acids in any appropriate combination; a long linker can be from about 5 amino acids to about 40 amino acids in length and can include any appropriate amino acids in any appropriate combination.

[0151] In some embodiments, when the first antigen binding domain comprises an scFv that specifically recognizes and/or binds a Mut-pHLA, and the second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof, the bispecific molecule may be in the form of an scDb-HSA with an N- to C-terminus order of (1)

Mut-pHLA V_L-L1-CD3 V_H-L2-CD3 V_L-L3-Mut-pHLA V_H-L4-HSA; (2) Mut-pHLA V_H-L1-CD3 V_L-L2-CD3 V_H-L3-Mut-pHLA V_H-L4-HSA; (3) Mut-pHLA V_L-L1-CD3 V_L-L2-CD3 V_H-L3-Mut-pHLA V_L-L4-HSA; (4) Mut-pHLA V_H-L1-CD3 V_H-L2-CD3 V_L-L3-Mut-pHLA V_L-L4-HSA; (5) CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H-L4-HSA; (6) CD3 V_H-L1-Mut-pHLA V_L-L2-Mut-pHLA V_H-L3-CD3 V_L-L4-HSA; (7) CD3 V_L-L1-Mut-pHLA V_L-L2-Mut-pHLA V_H-L3-CD3 V_H-L4-HSA; (8) CD3 V_H-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_L-L4-HSA; (9) HSA-L4-Mut-pHLA V_L-L1-CD3 V_H-L2-CD3 V_L-L3-Mut-pHLA V_H; (10) HSA-L4-Mut-pHLA V_H-L1-CD3 V_L-L2-CD3 V_H-L3-Mut-pHLA V_H; (11) HSA-L4-Mut-pHLA V_L-L1-CD3 V_L-L2-CD3 V_H-L3-Mut-pHLA V_L; (12) HSA-L4-Mut-pHLA V_H-L1-CD3 V_H-L2-CD3 V_L-L3-Mut-pHLA V_L; (13) HSA-L4-CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H; (14) HSA-L4-CD3 V_H-L1-Mut-pHLA V_L-L2-Mut-pHLA V_H-L3-CD3 V_L; (15) HSA-L4-CD3 V_L-L1-Mut-pHLA V_L-L2-Mut-pHLA V_H-L3-CD3 V_H; or (16) HSA-L4-CD3 V_H-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_L, where L1/L3 are short linkers, L2 is a long linker, and L4 is optional and can be a short or long linker as described. In a particular embodiment, the bispecific molecule is in the form of an scDb-HSA with an N- to C-terminus order of CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H-L4-HSA, or HSA-L4-CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H, where L1/L3 are short linkers, L2 is a long linker, and L4 is optional and can be a short or long linker as described.

D. Chemical modification

[0152] In some embodiments, the bispecific molecule comprises a chemical modification, *e.g.*, the bispecific molecule further comprises a chemical group attached to the first and/or second antigen binding domain to improve stability, extend half-life, and/or simplify manufacturing.

[0153] In some embodiments, the chemical modification comprises a polyethylene glycol (PEG) group. As understood to a person skilled in the art, PEG refers to a range of synthetic polymers of ethylene oxide having a general formula of $H(OCH2CH2)_nOH$, where n represents the number of oxyethylene repeating units. The PEG group can be linear or branched, and in either embodiment, can have a molecular weight of about 200 g/mol to about 100,000 g/mol, *e.g.*, about 200 g/mol, about 400 g/mol, about 600 g/mol, about 800 g/mol, about 1000 g/mol, about 2000 g/mol, about 3000 g/mol, about 4000 g/mol, about 5000 g/mol, about 6000 g/mol, about 7000 g/mol, about 8000 g/mol, about 9000 g/mol, about 10,000 g/mol, about 20,000 g/mol, about 30,000 g/mol, about 40,000 g/mol, about 50,000 g/mol, about 60,000 g/mol, about 70,000 g/mol, about 80,000 g/mol, about 90,000 g/mol, or about 100,000 g/mol.

[0154] The PEG group can be connected to the first and/or second antigen binding domain at any suitable location in any suitable configuration. For example, when the first and second antigen binding domains are configured in the form of an scDb as described (*e.g.*, VLA-L1-VHB-L2-VLB-L3-VHA), the PEG group can be connected at the N-terminal end of the scDb, at the C-terminal end of the scDb, or at any of the short and/or long linkers. PEGylation (*i.e.*, attaching a PEG group to a protein or polypeptide) can happen through site specific amine or sulfydryl chemistry. For example, a $(G_4S)_n$ (n can be a positive integer, *e.g.*, 1, 2, 3, 4, 5, or 6) linker may be engineered to include a reactive cysteine or lysine residue for conjugation with a PEG group. The engineered reactive cysteine or lysine residue can be anywhere in the linker. Exemplary short and long linkers with engineered reactive cysteine or lysine residues for PEGylation are provided in **Table 6** below. The position of the engineered reactive cysteine or lysine residue in these exemplary linkers is for illustrative purposes only and can be anywhere in the sequence.

Table 6. Exemplary linker sequences for conjugation with a PEG group

SEQ ID NO:	Sequence	Description
67	GG**C**GS	Short linker with **reactive cysteine** for conjugation
68	GG**K**GS	Short linker with **reactive lysine** for conjugation
69	GD**K**DS	Short linker with **reactive lysine** for conjugation (trypsin, Lys-C, Lys-N inhibited)
70	GGGGSGG**C**GSGGGGS	Long linker with **reactive cysteine** for conjugation
71	GGGGSGG**K**GSGGGGS	Long linker with **reactive lysine** for conjugation
72	GGGGSGD**K**DSGGGGS	Long linker with **reactive lysine** for conjugation (trypsin, Lys-C, Lys-N inhibited)

[0155] In some embodiments, the bispecific molecule comprising one or more modifications as described herein, *e.g.*, domain orientation modifications, linker modifications, Fc domain modifications, and/or fusion modifications, possesses one or more characteristics as described in this section.

[0156] In some embodiments, the bispecific molecule exhibits low target density, for example, less than 2000 targets (*e.g.*, mutant peptides or MANAs presented in a peptide-HLA complex) per

cell (*e.g.*, target cancer cell), less than 1500 targets per cell, less than 1000 targets per cell, less than 500 targets per cell, less than 100 targets per cell, less than 50 targets per cell, less than 10 targets per cell, about 5 targets per cell, or about 1 target per cell, on an average basis.

[0157] In some embodiments, the bispecific molecule has high sensitivity to its binding target (*e.g.*, a mutant peptide or MANA presented in a peptide-HLA complex), for example, has a half maximal effective concentration (EC_{50}) of in the range of 0.001-3000 nM, for example, 0.01-1000 nM, 0.1-100 nM, 1-10 nM, 0.001-1 nM, 0.01-1 nM, 0.1-1 nM, 1-10 nM, 10-100 nM, 100-1000 nM, or 1000-3000 nM.

[0158] In some embodiments, the bispecific molecule has high specificity to its binding target (*e.g.*, a mutant peptide or MANA presented in a peptide-HLA complex), for example, has low or no cross-reactivity to the same mutant peptide or MANA not present in a peptide-HLA complex, has low or no cross-reactivity to the corresponding WT peptide whether or not presented in a peptide-HLA complex, or has low or no cross-reactivity to other proteins or peptides present in the human proteome (*e.g.*, as measured by X-scan analysis).

[0159] In some embodiments, the bispecific molecule has a long half-life, for example, has a half-life of about 21 days, about 14 days, about 7 days, about 4 days, about 3 days, about 2 days, or about 1 day. In some embodiments, the bispecific molecule has a half-life to enable twice every week, weekly, every other week, every three weeks, or monthly administration (*e.g.*, in a clinical setting).

[0160] In some embodiments, the bispecific molecule has high productibility, for example, has a productibility of at least about 1 g/L, at least about 2 g/L, at least about 3 g/L, at least about 4 g/L, or at least about 5 g/L, at least about 10 g/L, at least about 15 g/L, at least about 20 g/L, at least about 25 g/L, or at least about 30 g/L, *e.g.*, from a cell culture. In some embodiments, the bispecific molecule has high productibility, for example, has a productibility of at least 1 g/L, at least 2 g/L, at least 3 g/L, at least 4 g/L, or at least 5 g/L, at least 10 g/L, at least 15 g/L, at least 20 g/L, at least 25 g/L, or at least 30 g/L, *e.g.*, from a cell culture.

[0161] In some embodiments, the bispecific molecule has high stability, for example, has a stability at room temperature of 1-12 weeks, *e.g.*, about 12 weeks, about 11 weeks, about 10 weeks, about 9 weeks, about 8 weeks, about 7 weeks, about 6 weeks, about 5 weeks, about 4 weeks, about 3 weeks, about 2 weeks, about 1 week, about 10 days, about 7 days, about 5 days, about 3 days, or about 1 day.

Exemplary Bispecific Molecules

[0162] The specific embodiments provided in this section are illustrative only and not intended to be limiting. In some embodiments, the bispecific molecule comprises (a) a first antigen binding domain that binds a tumor-specific mutant peptide or MANA presented in a peptide-HLA complex (Mut-pHLA) on a target cell; and (b) a second antigen binding domain that binds CD3 or a subunit thereof on an effector T cell, wherein the bispecific molecule further comprises two or more modifications selected from the group consisting of a domain orientation modification, a linker modification, an Fc domain modification, a fusion protein modification, and a chemical modification, which result in (i) an improved half-life, (ii) a reduced risk of complement-dependent cytotoxicity (CDC), (iii) and improved manufacturability, and/or (iv) an improved effector cell engagement of the bispecific molecule.

[0163] In these embodiments, the first antigen binding domain comprises an scFv that specifically recognizes and/or binds a Mut-pHLA, which includes a heavy chain variable region (Mut-pHLA V_H) and a light chain variable region (Mut-pHLA V_L). The tumor-specific mutant peptide or MANA targeted by the first binding domain can be any one discussed and described herein or known to a person skilled in the art. For example, the mutant peptide or MANA can be "HMTEVVRHC" (SEQ ID NO: 7) derived from p53 R175H. The second antigen binding domain comprises an scFv that specifically recognizes and/or binds CD3 or a subunit thereof, which includes a heavy chain variable region (CD3 V_H) and a light chain variable region (CD3 V_L).

[0164] In these embodiments, the bispecific molecule comprises a linker modification and/or a domain orientation modification, *e.g.*, the heavy and light chain variable regions of the first and second antigen binding domains are connected through linkers in the form of an scDb in an orientation of, from an N- to C-terminus order, CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H, where L1 is about 1-10 amino acids, e.g., 1-9 amino acids, 1-8 amino acids, 1-7 amino acids, 1-6 amino acids, or 1-5 amino acids in length, L2 is about 5-40 amino acids, e.g., 5-25 amino acids, 10-20 amino acids, 12-18 amino acids, or 14-17 amino acids in length, and L3 is about 1-10 amino acids, e.g., 1-9 amino acids, 1-8 amino acids, 1-7 amino acids, 1-6 amino acids, or 1-5 amino acids in length. A non-limiting example of L1 and/or L3 is a G_4S linker, while a non-limiting example of L2 is a $3xG_4S$ linker. As shown in the examples, this particular arrangement and orientation of the antigen binding domains has proven to exhibit superior spatial configuration and binding characteristics leading to optimal effector cell engagement.

[0165] In certain of these embodiments, the bispecific molecule as described above further comprises an Fc domain modification, *e.g.*, an Fc domain is connected to the scDb of the first and second antigen binding domains to improve the stability, half-life, and/or Fc-mediated effector functions of the bispecific molecule. The Fc domain may be of an IgG (*e.g.*, IgG4) isotype and contain two chains of an Fc sequence, each of which comprises a hinge, a CH2 domain, and a CH3 domain. The Fc domain may also contain one or more modifications (*e.g.*, amino acid mutations or substitutions in reference to the WT IgG4 sequence) as described to increase stability and/or half-life of the molecule and to increase heterodimerization and/or decrease homodimerization of the two chains. In some embodiments, the Fc domain comprises a first chain of an Fc sequence comprising or consisting of an amino acid sequence of SEQ ID NO: 65, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 65; and/or a second chain of an Fc sequence comprising or consisting of an amino acid sequence of SEQ ID NO: 66, or an amino acid sequence that is at least about 80% identical (*e.g.*, at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 66. In some embodiments, the Fc domain comprises a first chain of an Fc sequence comprising or consisting of an amino acid sequence of SEQ ID NO: 65, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 65; and/or a second chain of an Fc sequence comprising or consisting of an amino acid sequence of SEQ ID NO: 66, or an amino acid sequence that is at least 80% identical (*e.g.*, at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO: 66. The Fc domain may be connected to the first and/or second antigen binding domain in a configuration of, from an N- to C-terminus order, CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H-L4-Fc (see **FIG. 4**), where L1, L2, and L3 are as described, and L4 is optional and can be about 1-40 amino acids in length (*e.g.*, a G_4S linker).

[0166] In certain of these embodiments, the bispecific molecule as described above further comprises an HSA fusion protein modification, *e.g.*, an HSA fusion protein is connected to the scDb of the first and second antigen binding domains to improve stability, extend half-life, and/or simplify manufacturing. The HSA fusion protein may be connected to the first and/or second antigen binding domain in a configuration of, from an N- to C-terminus order, CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H-L4-HSA, or HSA-L4-CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H (see **FIG. 5**), where L1, L2, and L3 are as described, and L4 is optional and can be about 1-40 amino acids in length.

[0167] In certain of these embodiments, the bispecific molecule as described above further comprises a PEGylation modification, *e.g.*, a linear or branched PEG group is connected to the scDb of the first and second antigen binding domains to improve stability, extend half-life, and/or simplify manufacturing. The PEG group can be connected to the scDb (*i.e.*, CD3 V_L-L1-Mut-pHLA V_H-L2-Mut-pHLA V_L-L3-CD3 V_H) at the N-terminal end, at the C-terminal end, or at any of the linkers L1-L3 (see **FIG. 6**), for example, through site specific amine or sulfydryl chemistry. For example, L1 and L2 can be a G_4S linker, and L3 can be a $3xG_4S$ linker, any one of which may be engineered to include a reactive cysteine or lysine residue for conjugation with a PEG group. See **Table 5** for exemplary short and long linkers with engineered reactive cysteine or lysine residues for PEGylation.

Antibody-Drug Conjugates and Compositions

[0168] In some aspects, provided herein are antibody drug conjugates (ADCs) comprising a bispecific molecule (*e.g.*, MANA-TCE) according to various embodiments disclosed herein attached (*e.g.*, covalently or non-covalently) to a therapeutic agent. The therapeutic agent can be any suitable therapeutic agent including, for example, an anti-cancer agent. Non-limiting examples of anti-cancer agents that can be attached to a bispecific molecule (*e.g.*, MANA-TCE) described herein include such as monomethyl auri statin E (MMAE), monomethyl auri statin F (MMAF), maytansine, mertansine/emtansine (DM1), ravtansine/soravtansine (DM4), SN-38, calicheamicin, D6.5, dimeric pyrrol obenzodiazepines (PBDs), a-amantin (AAMT), PNU-159682, ricin, pseudomonas exotoxin A, diphtheria toxin, and gelonin.

[0169] In some aspects, provided herein are pharmaceutical compositions comprising a bispecific molecule (*e.g.*, MANA-TCE) according to various embodiments disclosed herein. The pharmaceutical composition may further comprise one or more pharmaceutically acceptable carriers, excipients, preservatives, or a combination thereof. A "pharmaceutically acceptable carrier or excipient" refers to a pharmaceutically acceptable material, composition, or vehicle that is involved in carrying or transporting a compound of interest from one tissue, organ, or portion of the body to another tissue, organ, or portion of the body. For example, the carrier or excipient may be a liquid or solid filler, diluent, excipient, solvent, or encapsulating material, or some combination thereof. Each component of the carrier or excipient must be "pharmaceutically acceptable" in that it must be compatible with the other ingredients of the formulation. It also must be suitable for contact with any tissue, organ, or portion of the body that it may encounter, meaning that it must not carry a risk of toxicity, irritation, allergic response, immunogenicity, or

any other complication that excessively outweighs its therapeutic benefits. Non-limiting examples of such carriers or excipients include water, saline, Ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used.

[0170] In some embodiments, the pharmaceutical composition can be formulated (*e.g.*, injectable, lyophilized, liquid formulations, or oral formulations) to be compatible with its intended route of administration. Examples of routes of administration include oral administration, extracorporeal administration, parenteral administration, intravenous administration, subcutaneous administration, intralesional administration (*e.g.*, injection into tumors), and by administration into biological spaces infiltrated by tumors (*e.g.*, intraspinal administration, intracerebellar administration, intraperitoneal administration, intralymphatic administration, intranodal administration, and/or pleural administration). For example, a pharmaceutical composition provided herein can be administered systemically by oral administration or by intravenous administration (*e.g.*, injection or infusion). Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol, or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates, or phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes, or multiple-dose vials made of glass or plastic.

[0171] In some embodiments, the pharmaceutical composition can be co-formulated in the same dosage unit or can be individually formulated in separate dosage units. The term "dosage unit" herein refers to a portion of a pharmaceutical composition that contains an amount of a therapeutic agent suitable for a single administration to provide a therapeutic effect. Such dosage units may be administered one to a plurality (*e.g.*, 1 to about 10, 1 to 8, 1 to 6, 1 to 4, or 1 to 2) of times per day, or as many times as needed to elicit a therapeutic response.

Kits

[0172] In some aspects, provided herein are kits comprising a bispecific molecule (*e.g.*, MANA-TCE), an ADC, or a pharmaceutical composition containing the same, according to various embodiments disclosed herein. The kit can include any of bispecific molecules, ADCs, and/or pharmaceutical compositions described herein, either mixed together or individually packaged.

The bispecific molecules, ADCs, and/or pharmaceutical compositions described herein can be packaged separately into discrete vials, bottles, or other containers, and can be packaged in appropriate ratios and/or amounts to facilitate use or administration.

[0173] The kit can include instructions for administering those bispecific molecules, ADCs, and/or pharmaceutical compositions. Such instructions can provide the information described throughout this application. The bispecific molecules, ADCs, and/or pharmaceutical compositions can be provided within any of the kits in the form of a delivery device. Alternatively, a delivery device can be separately included in the kits, and the instructions can describe how to assemble the delivery device prior to administration to a subject.

[0174] Any of the kits can also include syringes, catheters, scalpels, sterile containers for sample or cell collection, diluents, pharmaceutically acceptable carriers, and the like. The kits can provide other factors such as any of the supplementary factors or drugs described herein for the compositions in the preceding section or other parts of the application.

Therapeutic Methods

[0175] In some aspects, provided are methods for diagnosing, preventing, and/or treating a disease or condition in a subject in need thereof. Subjects who are suitable for the compositions and/or methods of the present disclosure include individuals (*e.g.*, mammalian subjects, such as humans, non-human primates, domestic mammals, experimental non-human mammalian subjects such as mice, rats, etc.) having a disease-associated mutation in a polypeptide including, for example, any of the mutations as described herein including, for example, those listed in **Table 1** in EGFR, IDH2, p53, RAS (*e.g.*, KRAS, HRAS, and RAS), and CTNNB. In some embodiments, the subject is a human.

[0176] In some aspects, provided are methods for diagnosing a subject having or suspected of having a disease or condition associated with a mutation in a polypeptide including, for example, any of the mutations as described herein including, for example, those listed in **Table 1**. In some embodiments, the disease or condition is cancer. In some embodiments, the cancer expresses one or more mutant peptides or MANAs as described herein (*e.g.*, p53 R175H peptide or MANA). The method entails obtaining a biological sample from the subject and contacting the biological sample with a bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, according to various embodiments disclosed herein to detect the absence or presence of cancer cells expressing the mutant peptides or MANAs. The biological sample can be any sample suitable to one skilled in the art for diagnostic purposes and can include, for

example, a blood sample, a bone marrow sample, a tissue sample, and/or a cell sample, *e.g.*, obtained from a biopsy procedure from a tumor site. The subject can be identified as having cancer when a biological sample obtained from the subject has the one or more mutant peptides or MANAs as detected by the bispecific molecule.

[0177] In some aspects, provided are methods for treating and/or preventing a disease or condition associated with a mutation in a polypeptide including, for example, any of the mutations as described herein including, for example, those listed in **Table 1**. In some embodiments, the disease or condition is cancer. In some embodiments, the cancer expresses one or more mutant peptides or MANAs as described herein (*e.g.*, p53 R175H peptide or MANA) or involves or is dependent on the signaling of the corresponding oncogenic protein (*e.g.*, p53 R175H-dependent). The method entails administering to the subject a therapeutically effective amount or a clinically effective amount of a bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, according to various embodiments disclosed herein. The subject can be a mammal, for example, without limitation, primates (*e.g.*, humans and non-human primates such as chimpanzees, baboons, or monkeys), dogs, cats, pigs, sheep, rabbits, mice, and rats. In some embodiments, the subject is a human.

[0178] A "therapeutically effective amount" as used herein is an amount that produces a desired effect in a subject for an indication, condition, disease, or disorder. In certain embodiments, the therapeutically effective amount is an amount that yields maximum therapeutic effect. In other embodiments, the therapeutically effective amount yields a therapeutic effect that is less than the maximum therapeutic effect. For example, a therapeutically effective amount may be an amount that produces a therapeutic effect while avoiding one or more side effects associated with a dosage that yields maximum therapeutic effect. A therapeutically effective amount for a particular composition will vary based on a variety of factors, including, but not limited to, the characteristics of the therapeutic composition (*e.g.*, activity, pharmacokinetics, pharmacodynamics, and bioavailability); the physiological condition of the subject (*e.g.*, age, body weight, sex, disease type and stage, medical history, general physical condition, responsiveness to a given dosage, and other present medications); the nature of any pharmaceutically acceptable carriers, excipients, and preservatives in the composition; and the route of administration. One skilled in the clinical and pharmacological arts will be able to determine a therapeutically effective amount through routine experimentation, namely, by monitoring a subject's response to administration of the therapeutic composition and adjusting the dosage accordingly. A "clinically effective amount," "clinically effective concentration," or "clinically effective dose" refers to a concentration or dose of a

peptide, composition, or pharmaceutical composition that is shown to be effective in clinical trials or is predicted to be effective based on early phase or pre-clinical trials. In some embodiments, a "clinically effective amount" is the same as a "therapeutically effective amount." In some embodiments, a "clinically effective amount" is higher or lower than a "therapeutically effective amount." Further, the effective amount can remain constant or can be adjusted as a sliding scale or variable dose depending on the subject's response to treatment. Various factors can influence the actual effective amount used for a particular application. For example, the frequency of administration, duration of treatment, use of multiple treatment agents, route of administration, and severity of the condition may require an increase or decrease in the actual effective amount administered. For additional guidance, see Remington: The Science and Practice of Pharmacy, 21st Edition, Univ. of Sciences in Philadelphia (USIP), Lippincott Williams & Wilkins, Philadelphia, PA, 2005.

[0179] In some embodiments, the cancer is a hematological malignancy or blood cancer. Non-limiting examples of hematological malignancies include myeloid neoplasm, myelodysplastic syndromes (MDS), myeloproliferative/myelodysplastic syndromes, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), blast crisis chronic myelogenous leukemia (bcCML), B-cell acute lymphoid leukemia (B-ALL), T-cell acute lymphoid leukemia (T-ALL), T-cell lymphoma, and B-cell lymphoma.

[0180] In some embodiments, the cancer is a solid cancer. Non-limiting examples of solid cancers include pancreatic cancer, glioma, glioblastoma, astrocytoma, colorectal cancer, thyroid cancer, gastric cancer, ovarian cancer, melanoma, endometrial cancer, lung cancer, renal cancer, cervical cancer, prostate cancer, breast cancer, urothelial cancer, testicular cancer, head and neck cancer, biliary tract cancer, liver cancer, esophageal cancer, and other types of solid cancers.

[0181] It is within the purview of one of ordinary skill in the art to select a suitable administration route, such as oral administration, extracorporeal administration, parenteral administration, intravenous administration, subcutaneous administration, intralesional administration (*e.g.*, injection into tumors), and by administration into biological spaces infiltrated by tumors (*e.g.*, intraspinal administration, intracerebellar administration, intraperitoneal administration, intralymphatic administration, intranodal administration, and/or pleural administration). For treating a subject in need thereof, the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical

composition containing the same, can be administered continuously or intermittently, for an immediate release, controlled release, or sustained release.

[0182] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, is administered to the subject in a range of from about 0.00001 mg/kg to about 30 mg/kg, from about 0.0001 mg/kg to about 30 mg/kg, from about 0.001 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from 0.1 mg/kg to about 10 mg/kg, from 0.1 mg/kg to about 3 mg/kg, for example, at a dose of about 0.00001 mg/kg, about 0.00005 mg/kg, about 0.0001 mg/kg, about 0.0005 mg/kg, about 0.001 mg/kg, about 0.005 mg/kg, about 0.01 mg/kg, a about 0.05 mg/kg, bout 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1 mg/kg, about 2 mg/kg, about 3 mg/kg, about 4 mg/kg, about 5 mg/kg, about 6 mg/kg, about 7 mg/kg, about 8 mg/kg, about 9 mg/kg, about 10 mg/kg, about 15 mg/kg, about 20 mg/kg, about 25 mg/kg, or about 30 mg/kg. In some embodiments, a single dose or multiple doses may be administered to a subject. In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, is administered once or multiple times a day.

[0183] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, is administered to the subject once a day, twice a day, three times a day, or four times a day for a period of about 1 day, about 2 days, about 3 days, about 5 days, about 7 days, about 10 days, about 2 weeks, about 3 weeks, about 4 weeks, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 1 year, about 2 years, about 3 years, about 4 years, about 5 years, or more than about 5 years. In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, is administered every day, every other day, 3 times a week, every third day, weekly, biweekly (*i.e.*, every other week), every third week, monthly, every other month, every third month, every fourth month, every fifth month, every sixth month, every ninth month, every year, every 18 months, every 2 years, every 5 years, every 10 years, or every 20 years. In some embodiments, the dose regimens listed above could be repeated after a period of about 1 week, about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 1 year, about 2 years, about 3 years, about 4 years, about 5 years, or more than about 5 years. In some embodiments, the schedule of administration is a hybrid of these periods, for example, the bispecific molecule

(*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, is administered a number of times a week and that pattern is repeated a number of times a month every month or every second, third, fourth, fifth, or sixth month, and treatment according to that pattern is continued for part of a year to several years, as set out above. In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, is administered in a cycle of a number or administrations over a week or two weeks, and the cycle is repeated at spaced intervals over a number of months or years, as set out above. In some embodiments treatment is continued until disease is eliminated, until no further improvement is achieved, or as long as the disease does not progress. In some embodiments, a cancer within a subject to be treated can be monitored to evaluate the effectiveness of the treatment using any appropriate method known to a skilled artisan. For example, imaging techniques or laboratory assays can be used to assess the number of cancer cells and/or the size of a tumor present within the subject. The same can also be used to assess the location of cancer cells and/or a tumor present within the subject.

[0184] In some embodiments, the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, is administered over a predetermined time period. Alternatively, the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, is administered until a particular therapeutic benchmark is reached. In some embodiments, the methods provided herein further include a step of evaluating one or more therapeutic benchmarks in a biological sample, such as, but not limited to, the level of a cancer biomarker, to determine whether to continue administration of the treatment.

[0185] In some embodiments, the method further entails administering one or more other cancer therapies such as surgery, immunotherapy, radiotherapy, and/or chemotherapy to the subject sequentially or simultaneously.

[0186] In some embodiments, the methods further comprise administering the subject a pharmaceutically or clinically effective amount of one or more additional anti-cancer agents to obtain improved or synergistic therapeutic effects. In some embodiments, the one or more additional anti-cancer agents are selected from the group consisting of an immunotherapy agent, a chemotherapy agent, and a biologic agent. Examples of anti-cancer agents include, without limitation, platinum compounds (*e.g.*, a cisplatin or carboplatin), taxanes (*e.g.*, paclitaxel, docetaxel, or an albumin bound paclitaxel such as nab-paclitaxel), altretamine, capecitabine, cyclophosphamide, etoposide (vp-16), gemcitabine, ifosfamide, irinotecan (cpt-11), liposomal

doxorubicin, melphalan, pemetrexed, topotecan, vinorelbine, luteinizing-hormone-releasing hormone (LHRH) agonists (*e.g.*, goserelin and leuprolide), anti-estrogens (*e.g.*, tamoxifen), aromatase inhibitors (*e.g.*, letrozole, anastrozole, and exemestane), angiogenesis inhibitors (*e.g.*, bevacizumab), poly(ADP)-ribose polymerase (PARP) inhibitors (*e.g.*, olaparib, rucaparib, and niraparib), radioactive phosphorus, anti-CTLA-4 antibodies, anti-PD-1 antibodies, anti-PD-L1 antibodies, IL-2 and other cytokines, other bispecific antibodies, and any combinations thereof. In some embodiments, the subject was administered the one or more additional anti-cancer agents before administration of the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same. In some embodiments, the subject is co-administered the one or more additional anti-cancer agents and the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same. In some embodiments, the subject was administered the one or more additional anti-cancer agents before after administration of the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same. As one of ordinary skill in the art would understand, the one or more additional anti-cancer agents and the bispecific molecule (*e.g.*, MANA-TCE), or a pharmaceutical composition containing the same, can be administered to a subject in need thereof one or more times at the same or different doses, depending on the diagnosis and prognosis of the subject. One skilled in the art would be able to combine one or more of these therapies in different orders to achieve the desired therapeutic results.

EXAMPLES

Example 1. Exemplary MANA-TCE specific to p53 R175H

[0187] In this example, a bispecific MANA-T cell engager (MANA-TCE), referred to as MANA-TCE1, that specifically binds a p53 R175H mutant peptide-HLA (HLA-A*02.01) complex (pHLA) and CD3 was generated and tested. MANA-TCE1 is an asymmetric molecule engineered to target "HMTEVVRHC" (SEQ ID NO: 7) derived from the p53 R175H oncoprotein. It was designed in an scDb-Fc format, with an engineered IgG4 Fc backbone. As shown in **FIG. 7**, the expressed protein of MANA-TCE1 includes two polypeptide chains. Each chain contains an Fc sequence having a hinge, a CH2 domain, and a CH3 domain. Chain-1 is designed to complement and pair with Chain-2 in a "Knob into Hole" format. Chain-1 contains only the Fc domain, while Chain-2 contains the Fc domain as well as a R175H-pHLA binding domain (anti-pHLA) and a CD3 binding domain (anti-CD3) N-terminal to the Fc domain. Both chains of the Fc domain

contain amino acid substitutions from the wild-type sequence that provide stability, serum half-life extension, and ease of manufacturability.

[0188] Chain-1 is 247 amino acids long inclusive of a leader sequence which is cleaved off, and the final polypeptide is 228 amino acids long. The Fc domain amino acid sequence is derived from a consensus IgG4 Fc sequence, with the following mutations (numbers in mutation names are based on a consensus full-length IgG sequence; the amino acid position in reference to the final Chain-1 polypeptide is indicated in parentheses):

S228P (a.a. 9): designed to stabilize the hinge;

L235E (a.a. 17): designed to minimize the potential for Fc gamma receptor binding;

T366S (a.a. 148): forms a "Hole" structure in the "Knob into Hole" configuration to support asymmetric heterodimer pairing;

L368A (a.a. 150): forms a "Hole" structure in the "Knob into Hole" configuration to support asymmetric heterodimer pairing; and

Y407V (a.a. 189): forms a "Hole" structure in the "Knob into Hole" configuration to support asymmetric heterodimer pairing.

[0189] Chain-2 is 735 amino acids long inclusive of a leader sequence which is cleaved off, and the final polypeptide is 716 amino acids long. In additional to an Fc domain, Chain-2 contains a first antigen binding domain (scFv) specific to R175H-pHLA, which includes a heavy chain variable region (R175H-pHLA V_H) and a light chain variable region (R175H-pHLA V_L), and a second antigen biding domain (scFv) specific to CD3, which includes a heavy chain variable region (CD3 V_H) and a light chain variable region (CD3 V_L), both of which are N-terminal to the Fc domain. The heavy and light chain variable regions of the first and second antigen binding domains and the Fc domain are connected through linkers in the form of an scDb-Fc in an orientation of, from an N- to C-terminus order, CD3 V_L–L1– R175H-pHLA V_H–L2–R175H-pHLA V_L–L3–CD3 V_H–L4–Fc. The R175H-pHLA binding domain is derived from a sequence described in WO2023114430; the CD3 binding domain is derived from the UCHT1 v.9 sequence also described in WO2023114430; the Fc domain amino acid sequence is derived from a consensus IgG4 Fc sequence, with the following mutations (numbers in mutation names are based on a consensus full-length IgG sequence; the amino acid position in reference to the final Chain-1 polypeptide is indicated in parentheses):

Y57I (a.a. 165): designed to support improved binding to the peptide-HLA complex;

S228P (a.a. 497): designed to stabilize the hinge

L235E (a.a. 505): designed to minimize the potential for Fc gamma receptor binding;

T366W (a.a. 636): forms a "Knob" structure in the "Knob into Hole" configuration to support asymmetric heterodimer pairing; and

H435R (a.a. 705): designed to decrease affinity for protein A and minimize affinity of mis-paired Chain-2 homodimers for protein A affinity chromatography and increased pI, allowing for more efficient commercial purification strategies.

[0190] Detailed maps and sequences of Chain-1 and Chain-2 are provided in **FIGS. 8A-8B**, **9A-9B**, and **Table 7**.

Table 7. Sequences of MANA-TCE1

SEQ ID NO:	Sequence	Description
73	MEWSWVFLFFLSVTTGVHSESKYGPPCPPCPAPEFEGG PSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFN WYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQD WLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYT LPPSQEEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPE NNYKTTPPVLDSDGSFFLVSRLTVDKSRWQEGNVFSCS VMHEALHNHYTQKSLSLSLG	Chain-1 sequence (inclusive of leader sequence)
65	ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPE VTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREE QFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSS IEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLSCAV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLV SRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLS LG	Chain-1 sequence (exclusive of leader sequence)
74	ESKYGPPCPPCP	Chain-1, Fc domain, hinge sequence

SEQ ID NO:	Sequence	Description
75	APEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQE DPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK	Chain-1, Fc domain, CH2 sequence
76	GQPREPQVYTLPPSQEEMTKNQVSLSCAVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLVSRLTVDKSR WQEGNVFSCSVMHEALHNHYTQKSLSLSLG	Chain-1, Fc domain, CH3 sequence
77	MEWSWVFLFFLSVTTGVHSDIQMTQSPSSLSASVGDR VTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLES GVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQQGNTLP WTFGQGTKVEIKGGGGSEVQLVESGGGLVQPGGSLRL SCAASGFNVYASGMHWVRQAPGKGLEWVAKIYPDSD ITYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAV YYCSRDSSFYYVYAMDYWGQGTLVTVSSGGGGSGGG GSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNT AVAWYQQKPGKAPKLLIYSAYFLYSGVPSRFSGSRSGT DFTLTISSLQPEDFATYYCQQYSRYSPVTFGQGTKVEIK GGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSFTGY TMNWVRQAPGKGLEWVALINPYKGVSTYNQKFKDRF TISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYYGDS DWYFDVWGQGTLVTVSSGGGGSESKYGPPCPPCPAPE FEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREP QVYTLPPSQEEMTKNQVSLWCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEG NVFSCSVMHEALHNRYTQKSLSLSLG	Chain-2 sequence (inclusive of leader sequence)
78	DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQ KPGKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISSL QPEDFATYYCQQGNTLPWTFGQGTKVEIKGGGGSEVQ	Chain-2 sequence (exclusive of leader sequence)

SEQ ID NO:	Sequence	Description
	LVESGGGLVQPGGSLRLSCAASGFNVYASGMHWVRQAPGKGLEWVAKIYPDSDITYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRDSSFYYVYAMDYWGQGTLVTVSSGGGGSGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSAYFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQYSRYSPVTFGQGTKVEIKGGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQAPGKGLEWVALINPYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYYGDSDWYFDVWGQGTLVTVSSGGGGSESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNRYTQKSLSLSLG	
56	DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISSLQPEDFATYYCQQGNTLPWTFGQGTKVEIK	Chain-2, CD3 V_L sequence
79	GGGGS	Chain-2, L1 (G_4S linker) sequence
46	EVQLVESGGGLVQPGGSLRLSCAASGFNVYASGMHWVRQAPGKGLEWVAKIYPDSD/TYYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRDSSFYYVYAMDYWGQGTLVTVSS	Chain-2, R175H-pHLA V_H sequence
27	GGGGSGGGGSGGGGS	Chain-2, L2 (3xG_4S linker) sequence

SEQ ID NO:	Sequence	Description
28	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQ QKPGKAPKLLIYSAYFLYSGVPSRFSGSRSGTDFTLTISS LQPEDFATYYCQQYSRYSPVTFGQGTKVEIK	Chain-2, R175H-pHLA V_L sequence
79	GGGGS	Chain-2, L3 (G_4S linker) sequence
60	EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWV RQAPGKGLEWVALINPYKGVSTYNQKFKDRFTISVDK SKNTAYLQMNSLRAEDTAVYYCARSGYYGDSDWYFD VWGQGTLVTVSS	Chain-2, CD3 V_H sequence
79	GGGGS	Chain-2, L4 (G_4S linker) sequence
74	ESKYGPPCPPCP	Chain-2, Fc domain, hinge sequence
75	APEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQE DPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSV LTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK	Chain-2, Fc domain, CH2 sequence
76	GQPREPQVYTLPPSQEEMTKNQVSLWCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSR WQEGNVFSCSVMHEALHNRYTQKSLSLSLG	Chain-2, Fc domain, CH3 sequence

[0191] The individual 3D protein structures of the MANA-TCE and its binding targets pHLA and CD3 are shown in **FIG. 10A**. **FIG. 10B** shows the 3D protein structures of when the MANA-TCE is engaged with the pHLA, with the delta and epsilon chains of CD3, and with both the pHLA and CD3, respectively. When engaged with both the pHLA and CD3, the pHLA:MANA-TCE:TCR trimer was in an optimal configuration to bridge the TCR complex and the pHLA so that the distance between them resembles that of a TCR naturally engaged with its binding ligand.

[0192] In the process of optimizing the MANA-TCE structure to arrive at its final form, various designs, including different linker lengths, scDB configurations, and inclusion of an Fc domain were tested. As shown in **FIG. 11A**, the L1/L3 linkers could be as short as 1 amino acid long,

which simply brought the adjacent two domains closer. However, when L1/L3 linkers were too long, for example, around 10-12 amino acids long, the scDB would misfold into unexpected conformation. Meanwhile, as shown in **FIG. 11B**, extending the length of the L2 linker to as long as 40 amino acids did not impact scDb folding. But if too short, for example, around 3-5 amino acids long, L2 would limit the folding of the domains that it connects. Moreover, two different configurations of the scDb were tested: one in a light chain-heavy chain-light chain-heavy chain (LHLH) format and the other in a heavy chain-light chain-heavy chain-light chain (HLHL) format (**FIG. 11C**). The LHLH format, which was employed in MANA-TCE1, engaged the target peptide-MHC complex (pMHC) and CD3 in such a way to bring the T cell and the target cancer cell to a distance similar to that created by a native T-cell receptor (TCE) engaged with its pMHC binding ligand (which is about 14 nm as measured from the T cell membrane to the target cell membrane), resulting in an optimal configuration of the interacting proteins while exposing the alpha and beta chains of the TCR to enable its native target binding. Conversely, the HLHL format engaged the CD3 and pMHC in a different way so that the T cell and the target cancer cell were not in the same orientation as that of a natively engaged T cell/cancer cell pair.

[0193] The optimized MANA-TCE1 was first tested for its binding affinities for its intended ligands, the p53 R175H/HLA-A*02:01 pHLA complex and human CD3 delta/epsilon chains, via surface plasmon resonance (SPR). P53 R175H/HLA-A*02:01 pHLA monomer or human CD3 delta/epsilon heterodimer were used as ligands to immobilize onto a CM5 chip using standard amine coupling chemistry in 10 mM sodium acetate buffer at pH 5.0. HBS-P (10 mM HEPES pH 7.4, 150 mM NaCl, 0.05% v/v/ surfactant P20) was used as the immobilization running buffer. Overnight kinetics were performed in the presence of HBS-P. Two 20 s pulses of 2M NaCl followed by one 20 s pulse of glycine pH 1.5 was used for surface regeneration. A flow rate of 50 uL/min was used for drug injections. Drug injections were performed in duplicate at 6 different concentrations from 100 nM down to 3.125 nM over the p53 R175H pHLA or CD3 immobilized surfaces. Sonograms from the kinetics were evaluated using a 1:1 kinetics fitting model. **FIG. 12**, shows the binding affinities of MANA-TCE1 to p53 R175H/HLA-A*02:01 pHLA monomer (left) and human CD3 delta/epsilon heterodimer (right).

[0194] Next, the abilities of MANA-TCE1 to mediate T cell activation and killing of tumor cells expressing p53 R175H and HLA-A*02:01 were tested. *In vitro* potency of MANA-TCE1 was assessed by co-culture of T cells and p53 R175H/HLA-A*02:01 KMS-26 cells and measurement of cytotoxicity and cytokine secretion. Briefly, peripheral blood mononuclear cells (PBMCs) were cultured with anti-human CD3 antibody (OKT3, BioLegend, 317347) at 15 ng/ml for three

days. T cells were cultured in RPMI-1640 with 10% FBS, 50 ug/ml gentamicin, 100 IU/ml recombinant human IL-2 (Sigma Aldrich, GF333), and 5 ng/ml recombinant human IL-7 (BioLegend, 581908) for 10 days in RPMI 10% FBS plus IL2 and IL-7. Activated T cells were co-cultured with target cells at 2:1 effector cell: target cell (E:T) ratio for 72 hours in RPMI 10% FBS for 2 days. Cytotoxicity was evaluated using Cell Titer Glo (Promega, G7570) according to manufacturer's protocol and analyzed by normalizing to the zero-drug condition as 0% cell death. IFNgamma production was measured by ELISA (R&D systems, DIF50C). **FIG. 13A** shows effective killing of KMS-26 cells by activated T cells (left panel) and production of IFNgamma (right panel) at single digit nM concentration. Similarly, from PBMCs from five donors, **FIG. 13B** again shows effective killing of KMS-26 cells and production of cytokines IFNgamma, IL2 and Granzyme B. PBMCs from 5 donors were co-cultured with KMS-26 cells and MANA-TCE1 for 3 days, and killing of target cells was assessed by flow cytometry using GFP labeled KMS-26 cells and counting beads (cat# 01-1234-42) according to manufacturer's protocol. Data was normalized to the 0 nM drug condition as 0% cell death. IFNg, IL2 and Granzyme B were assessed by MSD (cat# K151AEM-2). In the absence of target cells, there is no detectable IFNgamma, IL-2, or Granzyme B production.

[0195] To further confirm whether MANA-TCE1-mediated T cell activation is target-dependent, off-target T cell activation by MANA-TCE1 was assessed by co-culture of PBMCs from 8 different donors with MANA-TCE1 and with or without target p53 R175H/HLA-A*02:01 KMS-26 cells, with SAOS2 WT cells (HLA-A*02:01/p53 WT), or with SAOS2 R175H cells (HLA-A*02:01/p53 R175H). Effector and target cells were co-cultured at a 3:1 ratio for 72 hours in RPMI 10% FCS. Cells were harvested and assessed for T cells activation by staining with CD8 and CD25. T cell activation was measured by the percentage of CD8+ T cells that express CD25 by gating on single cells, live, CD8+ T cells. As shown in **FIG. 14**, MANA-TCE1-mediated T cell activation is dependent on the presence of p53 R175H and HLA-A*02:01 on target cells.

[0196] Next, the specificity of MANA-TCE1 was examined. Scanning mutagenesis was used to identify peptides in the human proteome with which the scDb portion of MANA-TCE1 might cross-react. *See* Harper J *et al.*, PLoS One (2018) 13(10):e0205491, doi: 10.1371/journal.pone.0205491. A peptide library was generated by systemically substituting amino acids at each position of the target p53 R175H peptide (HMTEVVRHC (SEQ ID NO: 7)) with each of the remaining 19 common amino acids. T2 cells loaded with each of the 171 variant peptides were then used to assess T cell activation by measuring Jurkat-NFAT activation (Promega J1601 as per manufacturers protocol) (**FIG. 15**).

[0197] A monomer binding motif was generated using 20% target peptide reactivity as a cut off for amino acids in each position: [ACFGHIKLMNQRSTVWY]-[LM]-[ACT]-[DEHNWY]-[AITV]-[ILMNSTV]-R-H-[ACGILMTV] (SEQ ID NO: 82). A search of this motif was then carried out using the UniProtKB human protein data base using ScanProsite yielding 2 proteins that contain peptides that may be presented by HLA-A*02:01 and recognized by MANA-TCE1: LLAYAVRHT (SEQ ID NO: 83) gene OTUL and YMADVIRHM (SEQ ID NO: 84) gene ZN599. LLAYAVRHT (SEQ ID NO: 83) showed no activity in the T2 assay. YMADVIRHM (SEQ ID NO: 84) showed a low level of activity in the T2 assay, and therefor full-length ZN599 was transfected into SAOS2 cells. Briefly, p53 R175H or ZN599 genes linked to GFP using a T2A linker were cloned into pcDNA3.4 vector (GeneArt). SAOS2 cells were transfected with plasmids at 70-80% confluency using Lipofectamine 3000 (Thermo Fisher Scientific, L3000015) and incubated at 37°C overnight in T75 flasks. Transfected cells were cocultured with PBMCs and MANA-TCE1 for 72 hours, after which T cell activation was assessed by flow cytometry for CD8+/CD25+ T cells. As shown in **FIG. 16**, T cell activation was observed by cells expressing p53 R175H full-length protein but not by cells expressing ZN599 full-length protein, suggesting that MANA-TCE1 is highly specific to its target.

[0198] Next, the stability of MANA-TCE1 was examined *in vivo*. Sprague Dawley rats were dosed i.v. with 3 mpk MANA-TCE1, a scDb, or a control human IgG4. Exposure was determined by MSD using CD3 capture, biotinylated protein L probing and detection with streptavidin-SULFO-tag. As shown in **FIG. 17**, inclusion of an Fc domain increased the overall half-life of the MANA-TCE molecule to a level similar to an IgG, as compared to an unmodified scDb molecule.

[0199] Finally, pharmacokinetic and receptor occupancy characteristics of MANA-TCE1 were tested in mice. Human CD3Tg mice (Genoway cat# ICP38) n=3 per group per timepoint were dosed i.v. with 1 mpk MANA-TCE1. Mice were sacrificed at 1, 3, 7, 14, 21, and 28 days, and blood was collected by cardiac puncture and analyzed for exposure and receptor occupancy (RO). Serum exposure was measured by IgG4 (human) isotyping AlphaLISA Detection Kit (Revvity cat# AL310C) as per manufacturer's protocol. RO was measured by flow cytometry of fixed whole blood. Briefly, 100 ul of whole blood was lyse/fixed as per manufacturers protocol (ThermoFisher cat# 00-5333-57) and then incubated for 10 min at room temp with mouse FcR block (Milteny cat# 130-092-575), followed by staining with anti-CD3-AF647 (clone CD3-12 BioRad cat# MCA1477A647), anti-CD3-pacific blue (clone UCHT1 Biolegend cat# 980004), anti-mouse CD4-BV605 (Biolegend cat# 100451), anti-mouse CD8-PerCP-Cy5 (Biolegend cat#

100734), and anti-human IgG4-PE (Southern Bio# 9200-09). CD4+ and CD8+ T cells were gated, gMFI for anti-human IgG4-PE was used to define bound CD3, and gMFI for anti-CD3-pacific blue was used to define free CD3. Receptor occupancy was normalized to 100% bound by pre-addition of 100 ug/mL drug to blood and incubation at 37°C for 30 minutes, and 0% bound using blood from non-dosed mice, using the following formula:

$$RO = \left(\frac{MFI3-MFI2}{MFI1-MFI2}\right) \times 100,$$

wherein MFI1 is 100% bound, MFI2 is 0% bound, and MFI3 is experimental sample. **FIG. 18** shows plasma concentration of MANA-TCE1 in mice up to 28 days after initial i.v. injection at 1 mpk dose. **FIG. 19** shows RO in hCD3Tg mice after 1 mpk i.v. dose.

[0200] Taken together, these data suggest that MANA-TCE1 is highly sensitive and specific to its target. When engaged with its target peptide-HLA complex and CD3, MANA-TCE1 can bring together T cells and target cancer cells in an optimal configuration, similar to that achieved by a T cell naturally engaged with its target through the TCR/pHLA complex. It can also induce T cell activation and specific killing of target cancer cells bearing the p53 R175H mutant peptide in single digit nM range. Finally, it exhibits *in vivo* stability and half-life resembling those of an immunoglobulin, rendering it an ideal candidate for therapeutic applications.

Example 2

[0201] A single 3 mg/kg intravenous dose of MANA-TCE1, an IgG4 monoclonal antibody, or a scDb control were administered to Sprague Dawley rats via a jugular catheter based on individual animal body weights. The scDb control does not have linker 4 or any part of the Fc (no hinge, no CH2 domain, no CH3 domain). Serum from dosed rats was collected at days -1 and 0, and 1, 2, 4, 8, 24, 72, 168, 240, 336, 504, and 672 hours post-administration. The concentration of the scDb in the rat serum was measured using a custom Meso Scale Discovery assay using recombinant human CD3 for capture and biotinylated Protein-L and Streptavidin SULFO-TAG reagents for detection. Values at each time point were interpolated from standard curves. The concentration of MANA-TCE1 and the IgG4 monoclonal antibody in the rat serum was measured using an IgG4 ALPHAlisa kit (Revvity, Inc.) and plotted as shown in **FIG. 20**.

Example 3

[0202] NSG MHC I/II DKO mice (The Jackson Laboratory) were engrafted IV with 10^7 human PBMCs. After 3 weeks of PBMC engraftment, mice were randomized based on the percentage of human $CD45^+$ cells in the peripheral blood and SC injected with 5 x 10^6 KMS-26 tumor cells in

their right flank. At the same time of tumor implantation, mice were dosed IV with 0.1, 0.3, or 1 mg/kg of MANA-TCE1 (or 1 mg/kg of TCE isotype control). A second and third IV dose of drug was given on day 7 and 14 after tumor implantation, respectively. Tumors were measured using digital calipers. The tumor growth curves shown in **FIG. 21** show that average tumor volume ± SEM (n = 8 mice/group) is markedly lower upon administration of MANA-TCE1 in a dose-dependent manner.

Example 4

[0203] NSG MHC I/II DKO mice (The Jackson Laboratory) were engrafted IV with 10^7 human PBMCs. After 3 weeks of PBMC engraftment, mice were randomized based on the percentage of human $CD45^+$ cells in the peripheral blood and SC injected with 5 x 10^6 KMS-26 tumor cells in their right flank. Once tumors reached ~100 mm^3 (day 9 post-tumor inoculation), mice were dosed IV with 1 mg/kg of MANA-TCE1. The Q1W dosing group continued to receive 1 mg/kg of MANA-TCE1 once weekly. Tumors were measured using digital calipers. The tumor growth curves shown in **FIG. 22** show that the average tumor volume ± SEM (n = 8 mice/group) is lower for the Q1W dosing group as compared to the single dose group. However, both groups show a dramatic reduction in tumor volume compared to the control group.

Example 5

[0204] HLA-A*02:01$^+$ adenocarcinoma organoids obtained from the NCI PDMR bank (expressing $p53^{R175H}$ **(FIGs. 23A and 23B)** or $p53^{WT}$ **(FIGs. 24A and 24B)**) were co-cultured with PBMCs at a 10:1 ratio and treated with either MANA-TCE1 or a control molecule, TCE isotype control, in which the anti-pHLA domain was replaced with a non-specific variable domain. After a 5-day coculture, cytotoxicity was assessed by comparing the number of live organoid cells in the drug-treated cocultures to those in the untreated cocultures as measured by flow cytometry. The IFN-γ concentration in the coculture supernatant was measured using the Meso Scale Disovery V-plex IFN-g kit. These data show that the administration of MANA-TCE1 specifically targets cells with mutated p53 as compared to cells with wild type p53.

[0205] Sequence of control molecule, TCE isotype control:

[0206] Chain-2:

DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKPGKAPKLLIYYTSRLESGVPS
RFSGSGSGTDYTLTISSLQPEDFATYYCQQGNTLPWTFGQGTKVEIKGGGGSQVTLRES
GPALVKPTQTLTLTCTFSGFSLSTSGMSVGWIRQPPGKALEWLADIWWDDKKDYNPSL

KSRLTISKDTSKNQVVLKVTNMDPADTATYYCARSMITNWYFDVWGAGTTVTVSSGG GGSGGGGSGGGGSDIQMTQSPSTLSASVGDRVTITCKSQLSVGYMHWYQQKPGKAPKL LIYDTSKLASGVPSRFSGSGSGTEFTLTISSLQPDDFATYYCFQGSGYPFTFGGGTKLEIK GGGGSEVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQAPGKGLEWVALIN PYKGVSTYNQKFKDRFTISVDKSKNTAYLQMNSLRAEDTAVYYCARSGYYGDSDWYF DVWGQGTLVTVSSGGGGSESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNG KEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLWCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALH NRYTQKSLSLSLG (SEQ ID NO: 85);

[0207] Chain 1:
ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWY VDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTI SKAKGQPREPQVYTLPPSQEEMTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTT PPVLDSDGSFFLVSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLG (SEQ ID NO: 65)

Example 6

[0208] Methods: SW620 (KRas-G12V mutant) cell line was modified to express HLA-A*03. T-cells were cocultured with modified or wild type SW620 cell line in the presence of vehicle or increasing amounts of scDbs for 72 hrs. The ratio of T-cells to SW620 cells was 2:1 at the start of the experiment. Following the incubation, expression of CD25 on CD8+ T-cells was analyzed using FACS.

Name	scDb	pHLAre Chain 2	pHLAre Chain 1
5026 parental	DIQMTQSPSSLSASVGD RVTITCRASQDIRNYLN WYQQKPGKAPKLLIYY TSRLESGVPSRFSGSGS GTDYTLTISSLQPEDFA TYYCQQGNTLPWTFGQ GTKVEIKGGGGSQVQL VQSGAEVKKPGASVKV SCKASGYSFTGYYMH WVRQAPGQGLEWMG	DIQMTQSPSSLSASVGDRVTITC RASQDIRNYLNWYQQKPGKAP KLLIYYTSRLESGVPSRFSGSGS GTDYTLTISSLQPEDFATYYCQ QGNTLPWTFGQGTKVEIKGGG GSQVQLVQSGAEVKKPGASVK VSCKASGYSFTGYYMHWVRQ APGQGLEWMGWIIPNSGVTNY GQKFQGRLTMTTDTSTNTAYM DLSSLRSEDTAVYYCGRGVSG	

	WIIPNSGVTNYGQKFQ GRLTMTTDTSTNTAYM DLSSLRSEDTAVYYCG RGVSGLDVWGQGTMV TVSSGGGGSGGGGSGG GGSEIVMTQSPATLSVS PGERATLSCRASQSVSS NLAWYQQKPGQAPRL LIYGASTRATGIPDRFS GSGSGTEFTLTISSLQSE DFAVYYCQQYNNWPS WTFGQGTKVEIKGGGG SEVQLVESGGGLVQPG GSLRLSCAASGYSFTGY TMNWVRQAPGKGLEW VALINPYKGVSTYNQK FKDRFTISVDKSKNTAY LQMNSLRAEDTAVYYC ARSGYYGDSDWYFDV WGQGTLVTVSS (SEQ ID NO: 86)	LDVWGQGTMVTVSSGGGGSG GGGSGGGGSEIVMTQSPATLSV SPGERATLSCRASQSVSSNLAW YQQKPGQAPRLLIYGASTRATG IPDRFSGSGSGTEFTLTISSLQSE DFAVYYCQQYNNWPSWTFGQ GTKVEIKGGGGSEVQLVESGG GLVQPGGSLRLSCAASGYSFTG YTMNWVRQAPGKGLEWVALI NPYKGVSTYNQKFKDRFTISVD KSKNTAYLQMNSLRAEDTAVY YCARSGYYGDSDWYFDVWGQ GTLVTVSSGGGGSESKYGPPCP PCPAPEFEGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPR EEQFNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKGLPSSIEKT ISKAKGQPREPQVYTLPPSQEE MTKNQVSLWCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLD SDGSFFLYSRLTVDKSRWQEG NVFSCSVMHEALHNRYTQKSL SLSLG (SEQ ID NO: 87)	
5026 W340P	DIQMTQSPSSLSASVGD RVTITCRASQDIRNYLN WYQQKPGKAPKLLIYY TSRLESGVPSRFSGSGS GTDYTLTISSLQPEDFA TYYCQQGNTLPWTFGQ GTKVEIKGGGGSQVQL VQSGAEVKKPGASVKV SCKASGYSFTGYYMH WVRQAPGQGLEWMG WIIPNSGVTNYGQKFQ GRLTMTTDTSTNTAYM DLSSLRSEDTAVYYCG RGVSGLDVWGQGTMV TVSSGGGGSGGGGSGG	DIQMTQSPSSLSASVGDRVTITC RASQDIRNYLNWYQQKPGKAP KLLIYYTSRLESGVPSRFSGSGS GTDYTLTISSLQPEDFATYYCQ QGNTLPWTFGQGTKVEIKGGG GSQVQLVQSGAEVKKPGASVK VSCKASGYSFTGYYMHWVRQ APGQGLEWMGWIIPNSGVTNY GQKFQGRLTMTTDTSTNTAYM DLSSLRSEDTAVYYCGRGVSG LDVWGQGTMVTVSSGGGGSG GGGSGGGGSEIVMTQSPATLSV SPGERATLSCRASQSVSSNLAW YQQKPGQAPRLLIYGASTRATG IPDRFSGSGSGTEFTLTISSLQSE	

	GGSEIVMTQSPATLSVS PGERATLSCRASQSVSS NLAWYQQKPGQAPRL LIYGASTRATGIPDRFS GSGSGTEFTLTISSLQSE DFAVYYCQQYNNWPS PTFGQGTKVEIKGGGG SEVQLVESGGGLVQPG GSLRLSCAASGYSFTGY TMNWVRQAPGKGLEW VALINPYKGVSTYNQK FKDRFTISVDKSKNTAY LQMNSLRAEDTAVYYC ARSGYYGDSDWYFDV WGQGTLVTVSS (SEQ ID NO: 88)	DFAVYYCQQYNNWPSPTFGQG TKVEIKGGGGSEVQLVESGGG LVQPGGSLRLSCAASGYSFTGY TMNWVRQAPGKGLEWVALIN PYKGVSTYNQKFKDRFTISVDK SKNTAYLQMNSLRAEDTAVYY CARSGYYGDSDWYFDVWGQG TLVTVSSGGGGSESKYGPPCPP CPAPEFEGGPSVFLFPPKPKDTL MISRTPEVTCVVVDVSQEDPEV QFNWYVDGVEVHNAKTKPRE EQFNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKGLPSSIEKTI SKAKGQPREPQVYTLPPSQEEM TKNQVSLWCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDS DGSFFLYSRLTVDKSRWQEGN VFSCSVMHEALHNRYTQKSLS LSLG (SEQ ID NO: 89)	
5026 W340H	DIQMTQSPSSLSASVGD RVTITCRASQDIRNYLN WYQQKPGKAPKLLIYY TSRLESGVPSRFSGSGS GTDYTLTISSLQPEDFA TYYCQQGNTLPWTFGQ GTKVEIKGGGGSQVQL VQSGAEVKKPGASVKV SCKASGYSFTGYYMH WVRQAPGQGLEWMG WIIPNSGVTNYGQKFQ GRLTMTTDTSTNTAYM DLSSLRSEDTAVYYCG RGVSGLDVWGQGTMV TVSSGGGGSGGGGSGG GGSEIVMTQSPATLSVS PGERATLSCRASQSVSS NLAWYQQKPGQAPRL LIYGASTRATGIPDRFS GSGSGTEFTLTISSLQSE	DIQMTQSPSSLSASVGDRVTITC RASQDIRNYLNWYQQKPGKAP KLLIYYTSRLESGVPSRFSGSGS GTDYTLTISSLQPEDFATYYCQ QGNTLPWTFGQGTKVEIKGGG GSQVQLVQSGAEVKKPGASVK VSCKASGYSFTGYYMHWVRQ APGQGLEWMGWIIPNSGVTNY GQKFQGRLTMTTDTSTNTAYM DLSSLRSEDTAVYYCGRGVSG LDVWGQGTMVTVSSGGGGSG GGGSGGGGSEIVMTQSPATLSV SPGERATLSCRASQSVSSNLAW YQQKPGQAPRLLIYGASTRATG IPDRFSGSGSGTEFTLTISSLQSE DFAVYYCQQYNNWPSHTFGQ GTKVEIKGGGGSEVQLVESGG GLVQPGGSLRLSCAASGYSFTG YTMNWVRQAPGKGLEWVALI NPYKGVSTYNQKFKDRFTISVD	

	DFAVYYCQQYNNWPS HTFGQGTKVEIKGGGG SEVQLVESGGGLVQPG GSLRLSCAASGYSFTGY TMNWVRQAPGKGLEW VALINPYKGVSTYNQK FKDRFTISVDKSKNTAY LQMNSLRAEDTAVYYC ARSGYYGDSDWYFDV WGQGTLVTVSS (SEQ ID NO: 90)	KSKNTAYLQMNSLRAEDTAVY YCARSGYYGDSDWYFDVWGQ GTLVTVSSGGGGSESKYGPPCP PCPAPEFEGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSQEDPE VQFNWYVDGVEVHNAKTKPR EEQFNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKGLPSSIEKT ISKAKGQPREPQVYTLPPSQEE MTKNQVSLWCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLD SDGSFFLYSRLTVDKSRWQEG NVFSCSVMHEALHNRYTQKSL SLSLG (SEQ ID NO: 91)	
5026 N166Y	DIQMTQSPSSLSASVGD RVTITCRASQDIRNYLN WYQQKPGKAPKLLIYY TSRLESGVPSRFSGSGS GTDYTLTISSLQPEDFA TYYCQQGNTLPWTFGQ GTKVEIKGGGGSQVQL VQSGAEVKKPGASVKV SCKASGYSFTGYYMH WVRQAPGQGLEWMG WIIPYSGVTNYGQKFQ GRLTMTTDTSTNTAYM DLSSLRSEDTAVYYCG RGVSGLDVWGQGTMV TVSSGGGGSGGGGSGG GGSEIVMTQSPATLSVS PGERATLSCRASQSVSS NLAWYQQKPGQAPRL LIYGASTRATGIPDRFS GSGSGTEFTLTISSLQSE DFAVYYCQQYNNWPS WTFGQGTKVEIKGGGG SEVQLVESGGGLVQPG GSLRLSCAASGYSFTGY TMNWVRQAPGKGLEW	DIQMTQSPSSLSASVGDRVTITC RASQDIRNYLNWYQQKPGKAP KLLIYYTSRLESGVPSRFSGSGS GTDYTLTISSLQPEDFATYYCQ QGNTLPWTFGQGTKVEIKGGG GSQVQLVQSGAEVKKPGASVK VSCKASGYSFTGYYMHWVRQ APGQGLEWMGWIIPYSGVTNY GQKFQGRLTMTTDTSTNTAYM DLSSLRSEDTAVYYCGRGVSG LDVWGQGTMVTVSSGGGGSG GGGSGGGGSEIVMTQSPATLSV SPGERATLSCRASQSVSSNLAW YQQKPGQAPRLLIYGASTRATG IPDRFSGSGSGTEFTLTISSLQSE DFAVYYCQQYNNWPSWTFGQ GTKVEIKGGGGSEVQLVESGG GLVQPGGSLRLSCAASGYSFTG YTMNWVRQAPGKGLEWVALI NPYKGVSTYNQKFKDRFTISVD KSKNTAYLQMNSLRAEDTAVY YCARSGYYGDSDWYFDVWGQ GTLVTVSSGGGGSESKYGPPCP PCPAPEFEGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSQEDPE	

	VALINPYKGVSTYNQK FKDRFTISVDKSKNTAY LQMNSLRAEDTAVYYC ARSGYYGDSDWYFDV WGQGTLVTVSS (SEQ ID NO: 92)	VQFNWYVDGVEVHNAKTKPR EEQFNSTYRVVSVLTVLHQDW LNGKEYKCKVSNKGLPSSIEKT ISKAKGQPREPQVYTLPPSQEE MTKNQVSLWCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLD SDGSFFLYSRLTVDKSRWQEG NVFSCSVMHEALHNRYTQKSL SLSLG (SEQ ID NO: 93)	
pHLAre common Chain-1			ESKYGPPCPPCPAPEFEGGPSVF LFPPKPKDTLMISRTPEVTCVV VDVSQEDPEVQFNWYVDGVE VHNAKTKPREEQFNSTYRVVS VLTVLHQDWLNGKEYKCKVS NKGLPSSIEKTISKAKGQPREPQ VYTLPPSQEEMTKNQVSLSCA VKGFYPSDIAVEWESNGQPEN NYKTTPPVLDSDGSFFLVSRLT VDKSRWQEGNVFSCSVMHEAL HNHYTQKSLSLSLG (SEQ ID NO: 94)

[0209] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the present technology, suitable methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[0210] From the foregoing, it will be appreciated that specific embodiments of the invention have been described herein for purposes of illustration, but that various modifications may be made without deviating from the scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

What is claimed is:

1. A bispecific molecule comprising:

(a) a first antigen binding domain that binds a tumor-specific mutant peptide presented in a peptide-human leukocyte antigen (HLA) complex (Mut-pHLA) on a target cell; and

(b) a second antigen binding domain that binds a surface molecule on an effector cell,

wherein the bispecific molecule further comprises a domain orientation modification, a linker modification, and one or more modifications selected from the group consisting of an Fc domain modification, a fusion protein modification, and a chemical modification, and

wherein the modifications result in (i) an improved half-life, (ii) a reduced risk of complement-dependent cytotoxicity (CDC), (iii) an improved manufacturability, and/or (iv) an improved effector cell engagement of the bispecific molecule.

2. The bispecific molecule of claim 1, wherein the mutant peptide is derived from an oncogenic protein.

3. The bispecific molecule of claim 2, wherein the oncogenic protein is selected from the group consisting of EGFR, IDH2, p53, KRAS, HRAS, NRAS, and CTNNB.

4. The bispecific molecule of claim 2, wherein the first antigen binding domain does not bind the mutant peptide not presented in an HLA complex or a complex that includes a wild-type version of the mutant peptide.

5. The bispecific molecule of any one of claims 1-4, wherein the mutant peptide comprises a peptide selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 12, 14, 16-18, 20, and 22-24.

6. The bispecific molecule of any one of claims 1-5, wherein the HLA is a class I HLA selected from the group consisting of HLA-A, HLA-B, and HLA-C.

7. The bispecific molecule of claim 6, wherein the HLA is HLA-A1, HLA-A2, or HLA-A3.

8. The bispecific molecule of any one of claims 1-7, wherein the first antigen binding domain comprises a heavy chain variable region (Mut-pHLA V_H) and a light chain variable region (Mut-pHLA V_L).

9. The bispecific molecule of claim 8, wherein the Mut-pHLA V_H comprises amino acid sequences of SEQ ID NOs: 39, 41, 43, and 45; and/or the Mut-pHLA V_L comprises amino acid sequences of SEQ ID NOs: 29, 31, 33, and 35.

10. The bispecific molecule of claim 9, wherein the mutant peptide is HMTEVVRHC (SEQ ID NO: 7), and/or the HLA is HLA-A2.

11. The bispecific molecule of claim 10, wherein:

the Mut-pHLA V_H comprises

(i) one, two, or three complementarity-determining regions (CDRs) having amino acid sequences selected from SEQ ID NOs: 40, 42, and 44; or

(ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 40, 47, and 44, and/or

the Mut-pHLA V_L comprises

(i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 32, and 34; or

(ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 37, and 34.

12. The bispecific molecule of claim 11, wherein the Mut-pHLA V_H comprises an amino acid sequence of SEQ ID NO: 38 or SEQ ID NO: 46; and/or the Mut-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 28 or SEQ ID NO: 36.

13. The bispecific molecule of claim 9, wherein the mutant peptide is VVVGAVGVGK (SEQ ID NO: 17), and/or the HLA is HLA-A3.

14. The bispecific molecule of claim 13, wherein the Mut-pHLA V_H comprises an amino acid

sequence of SEQ ID NO: 49; and/or the Mut-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 48.

15. The bispecific molecule of claim 9, wherein the mutant peptide is ILDTAGHEEY (SEQ ID NO: 20), ILDTAGKEEY (SEQ ID NO: 22), or ILDTAGREEY (SEQ ID NO: 23), and/or the HLA is HLA-A1.

16. The bispecific molecule of claim 15, wherein the Mut-pHLA V_H comprises an amino acid sequence of SEQ ID NO: 51, SEQ ID NO: 53, or SEQ ID NO: 55; and/or the Mut-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 50, SEQ ID NO: 52, or SEQ ID NO: 54.

17. The bispecific molecule of any one of claims 1-16, wherein the effector cell is a T cell.

18. The bispecific molecule of claim 17, wherein the surface molecule is CD3.

19. The bispecific molecule of claim 18, wherein the second antigen binding domain comprises a heavy chain variable region (CD3 V_H) and light chain variable region (CD3 V_L).

20. The bispecific molecule of claim 19, wherein the CD3 V_H comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 61-63; and/or the CD3 V_L comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 57-59.

21. The bispecific molecule of claim 20, wherein the CD3 V_H comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 60; and/or the CD3 V_L comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 56.

22. The bispecific molecule of any one of claims 1-21, wherein the bispecific molecule has one or more characteristics selected from the following:

(a) a low target density of less than 2000 targets per cell, less than 1500 targets per

cell, less than 1000 targets per cell, less than 500 targets per cell, less than 100 targets per cell, less than 50 targets per cell, less than 10 targets per cell, or about 1 target per cell;

(b) a binding specificity as measured by half maximal effective concentration (EC50) of 0.001-3000 nM;

(c) low or no cross-reactivity to proteins other than the protein from which the mutant peptide is derived;

(d) a half-life of about 21 days, about 14 days, about 7 days, or about 4 days;

(e) a productibility of at least about 1 g/L, at least about 2 g/L, at least about 3 g/L, at least about 4 g/L, at least about 5 g/L, at least about 10 g/L, at least about 15 g/L, at least about 20 g/L, at least about 25 g/L, or at least about 30 g/L from a cell culture; and

(f) a stability at room temperature of 1-12 weeks.

23. The bispecific molecule of any one of claims 18-22, wherein the bispecific molecule, when engaged with the Mut-pHLA on the target cell and CD3 on the T cell, forms a connection between the target cell and the T cell of about 14 nM in length as measured from the target cell membrane to the T cell membrane.

24. The bispecific molecule of any one of claims 19-23, wherein the domain orientation modification and linker modification comprise the bispecific molecule being configured as a single-chain diabody (scDb) in a configuration of, from N- to C-terminus order:

(i) Mut-pHLA V_L – L1 – CD3 V_H – L2 – CD3 V_L – L3 – Mut-pHLA V_H;

(ii) Mut-pHLA V_H – L1 – CD3 V_L – L2 – CD3 V_H – L3 – Mut-pHLA V_L;

(iii) Mut-pHLA V_L – L1 – CD3 V_L – L2 – CD3 V_H- – L3 – Mut-pHLA V_H;

(iv) Mut-pHLA V_H – L1 – CD3 V_H – L2 – CD3 V_L – L3 – Mut-pHLA V_L;

(v) CD3 V_L – L1 – Mut-pHLA V_H – L2 – Mut-pHLA V_L – L3 – CD3 V_H;

(vi) CD3 V_H – L1 – Mut-pHLA V_L – L2 – Mut-pHLA V_H – L3 – CD3 V_L;

(vii) CD3 V_L – L1 – Mut-pHLA V_L – L2 – Mut-pHLA V_H – L3 – CD3 V_H; or

(viii) CD3 V_H – L1 – Mut-pHLA V_H – L2 – Mut-pHLA V_L – L3 – CD3 V_L,

wherein L1, L2, and L3 are linkers.

25. The bispecific molecule of claim 24, wherein the bispecific molecule is configured in a

configuration of, from N- to C-terminus order, CD3 V_L – L1 – Mut-pHLA V_H – L2 – Mut-pHLA V_L – L3 – CD3 V_H.

26. The bispecific molecule of claim 25, wherein L1 is about 1-10 amino acids in length, L2 is about 5-40 amino acids in length, and L3 is about 1-10 amino acids in length.

27. The bispecific molecule of claim 25 or 26, wherein the bispecific molecule further comprises an Fc domain modification, wherein the Fc domain modification comprises two chains of an Fc sequence of immunoglobulin G4 (IgG4) isotype each comprising a hinge, a CH2 domain, and a CH3 domain, and wherein one or both chains of the Fc sequence comprise one or more mutations selected from the group consisting of S228P, L235E, L336W, T366S, L368A, Y407V, and H435R in reference to a consensus full-length IgG4 sequence.

28. The bispecific molecule of claim 27, wherein one chain of the Fc sequence comprises S228P, L235E, T366S, L368A, and Y407V mutations, and the other chain of the Fc sequence comprises S228P, L235E, L336W, and H435R mutations.

29. The bispecific molecule of claim 28, wherein one chain of the Fc sequence comprises an amino acid sequence of SEQ ID NO: 65, and the other chain of the Fc sequence comprises an amino acid sequence of SEQ ID NO: 66.

30. The bispecific molecule of any one of claims 27-29, wherein one chain of the Fc sequence is connected to the first and/or second antigen binding domain at the N-terminal end of the CD3 V_L or the C-terminal end of the CD3 V_H optionally through a linker L4, wherein L4 is about 1-40 amino acids in length.

31. The bispecific molecule of claim 25 or 26, wherein the bispecific molecule further comprises a fusion protein modification, and wherein the fusion protein modification comprises a serum albumin.

32. The bispecific molecule of claim 31, wherein the serum albumin is a human serum albumin (HSA), optionally wherein the HSA comprises an amino acid sequence of SEQ ID NO: 80 or SEQ ID NO: 81.

33. The bispecific molecule of claim 31 or 32, wherein the serum albumin is connected to the first and/or second antigen binding domain at the N-terminal end of the CD3 V_L or the C-terminal end of the CD3 V_H optionally through a linker L4, wherein L4 is about 1-40 amino acids in length.

34. The bispecific molecule of claim 25 or 26, wherein the bispecific molecule further comprises a chemical modification, and wherein the fusion modification comprises a polyethylene glycol (PEG) group.

35. The bispecific molecule of claim 34, wherein the PEG group is connected to the first and/or second antigen binding domain at the N-terminal end of CD3 V_L, L1, L2, L3, or the C-terminal end of CD3 V_H.

36. A bispecific molecule comprising:

(a) a first antigen binding domain that binds a tumor-specific mutant peptide derived from p53 R175H presented in a peptide-human leukocyte antigen (HLA) complex (R175H-pHLA) on a target cell;

(b) a second antigen binding domain that binds CD3 on a T cell; and

(c) an Fc domain of immunoglobulin G4 (IgG4) isotype comprising a first chain of an Fc sequence comprising a hinge, a CH2 domain, and a CH3 domain and a second chain of an Fc sequence comprising a hinge, a CH2 domain, and a CH3 domain,

wherein the Fc domain results in (i) an improved half-life, (ii) a reduced risk of Fc-mediated cytotoxicity, (iii) and improved manufacturability, and/or (iv) an improved effector cell engagement of the bispecific molecule.

37. The bispecific molecule of claim 36, wherein the mutant peptide comprises or consists of an amino acid sequence of HMTEVVRHC (SEQ ID NO: 7).

38. The bispecific molecule of claim 36 or 37, wherein the HLA is HLA-A*02.01, HLA-A*02.06, or HLA-A*02.11.

39. The bispecific molecule of any one of claims 36-38, wherein the bispecific molecule has one or more characteristics selected from the following:

(a) a low target density of less than 2000 targets per cell, less than 1500 targets per cell, less than 1000 targets per cell, less than 500 targets per cell, less than 100 targets per cell, less than 50 targets per cell, less than 10 targets per cell, or about 1 target per cell;

(b) a binding specificity as measured by half maximal effective concentration (EC50) of 0.001-3000 nM;

(c) low or no cross-reactivity to proteins other than the protein from which the mutant peptide is derived;

(d) a half-life of about 21 days, about 14 days, about 7 days, or about 4 days;

(e) a productibility of at least about 1 g/L, at least about 2 g/L, at least about 3 g/L, at least about 4 g/L, at least about 5 g/L, at least about 10 g/L, at least about 15 g/L, at least about 20 g/L, at least about 25 g/L, or at least about 30 g/L from a cell culture; and

(f) a stability at room temperature of 1-12 weeks.

40. The bispecific molecule of any one of claims 36-39, wherein the bispecific molecule, when engaged with the Mut-pHLA on the target cell and CD3 on the T cell, forms a connection between the target cell and the T cell of about 14 nM in length as measured from the target cell membrane to the T cell membrane.

41. The bispecific molecule of any one of claims 36-40, wherein:

the first antigen binding domain comprises a heavy chain variable region (R175H-pHLA V_H) and light chain variable region (R175H -pHLA V_L),

the second antigen binding domain comprises a heavy chain variable region (CD3 V_H) and light chain variable region (CD3 V_L), and

the bispecific molecule comprises, from N- to C-terminus order, CD3 V_L – L1 – R175H-pHLA V_H – L2 – R175H-pHLA V_L – L3 – CD3 V_H, wherein L1 is about 1-10 amino acids in length, L2 is about 5-40 amino acids in length, and L3 is about 1-10 amino acids in length.

42. The bispecific molecule of claim 41, wherein:

the R175H-pHLA V_H comprises

(i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 40, 42, and 44; or

(ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 40, 47, and 44, and/or

the R175H-pHLA V_L comprises

(i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 32, and 34; or

(ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 37, and 34.

43. The bispecific molecule of claim 42, wherein the R175H-pHLA V_H comprises an amino acid sequence of SEQ ID NO: 38 or SEQ ID NO: 46; and/or the R175H-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 28 or SEQ ID NO: 36.

44. The bispecific molecule of any one of claims 41-43, wherein the CD3 V_H comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 61-63; and/or the CD3 V_L comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 57-59.

45. The bispecific molecule of claim 44, wherein the CD3 V_H comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 60; and/or the CD3 V_L comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 56.

46. The bispecific molecule of any one of claims 36-45, wherein the first chain of the Fc sequence comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 65; and/or the second chain of the Fc sequence comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 66.

47. The bispecific molecule of any one of claims 36-46, wherein the first or second chain of the Fc sequence is connected to the first and/or second antigen biding domain at the N-terminal end of the CD3 V_L or the C-terminal end of the CD3 V_H optionally through a linker L4, wherein L4 is about 1-40 amino acids in length.

48. The bispecific molecule of claim 47, wherein the first or second chain of the Fc sequence is connected to the C-terminal end of the CD3 V_H optionally through L4.

49. The bispecific molecule of claim 48, wherein the bispecific molecule comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 78.

50. A bispecific molecule comprising:

(a) a first polypeptide comprising a first chain of an Fc sequence comprising an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 65; and

(b) a second polypeptide comprising, from N- to C-terminus order:

(i) a light chain variable region derived from a first antigen binding domain that binds CD3 (CD3 V_L);

(ii) a linker L1 of about 1-10 amino acids in length;

(iii) a heavy chain variable region derived from a second antigen binding domain that binds a p53 R175H mutant peptide having an amino acid sequence of HMTEVVRHC (SEQ ID NO: 7) presented in a peptide-human leukocyte antigen (HLA) complex (R175H-PHLA) (R175H-pHLA V_H);

(iv) a linker L2 of about 5-40 amino acids in length;

(v) a light chain variable region derived from the second antigen binding domain that binds R175H-pHLA (R175H -pHLA V_L);

(vi) a linker L3 of about 1-10 amino acids in length;

(vii) a heavy chain variable region derived from the first antigen binding domain that binds CD3 (CD3 V_H);

(viii) optionally a linker L4 of about 1-40 amino acids in length; and

(ix) a second chain of an Fc sequence of immunoglobulin G4 (IgG4) isotype comprising an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 66.

51. The bispecific molecule of claim 50, wherein the HLA is HLA-A*02.01, HLA-A*02.06, or HLA-A*02.11.

52. The bispecific molecule of claim 50 or 51, wherein:

the R175H-pHLA V_H comprises

(i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 40, 42, and 44; or

(ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 40, 47, and 44, and/or

the R175H-pHLA V_L comprises

(i) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 32, and 34; or

(ii) one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 30, 37, and 34.

53. The bispecific molecule of claim 52, wherein the R175H-pHLA V_H comprises an amino acid sequence of SEQ ID NO: 38 or SEQ ID NO: 46; and/or the R175H-pHLA V_L comprises an amino acid sequence of SEQ ID NO: 28 or SEQ ID NO: 36.

54. The bispecific molecule of any one of claims 50-53, wherein the CD3 V_H comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 61-63; and/or the CD3 V_L comprises one, two, or three CDRs having amino acid sequences selected from SEQ ID NOs: 57-59.

55. The bispecific molecule of claim 54, wherein the CD3 V_H comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 60; and/or the CD3 V_L comprises an amino acid sequence that shares at least about 80%, at least about 85%, at least about 90%, at least about 95%, or 100% identity to SEQ ID NO: 56.

56. The bispecific molecule of any one of claims 50-55, wherein the first polypeptide comprises an amino acid sequence of SEQ ID NO: 65; and/or the second polypeptide comprises an amino acid sequence of SEQ ID NO: 78.

57. A pharmaceutical composition comprising the bispecific molecule of any one of claims 1-56 and a pharmaceutically acceptable carrier, excipient, preservative, or combination thereof.

58. A method of diagnosing, treating, or preventing a disease in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the bispecific molecule of any one of claims 1-56, or the pharmaceutical composition of claim 57.

59. The method of claim 58, wherein the subject has a disease-associated protein comprising a mutant peptide sequence selected from the group consisting of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 12, 14, 16-18, 20, and 22-24.

60. The method of claim 58 or 59, wherein the disease is cancer.

61. The method of claim 60, wherein the cancer is a hematological malignancy.

62. The method of claim 61, wherein the hematological malignancy is selected from the group consisting of myeloid neoplasm, myelodysplastic syndromes (MDS), myeloproliferative/myelodysplastic syndromes, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), blast crisis chronic myelogenous leukemia (bcCML), B-cell acute lymphoid leukemia (B-ALL), T-cell acute lymphoid leukemia (T-ALL), T-cell lymphoma, and B-cell lymphoma.

63. The method of claim 60, wherein the cancer is a solid cancer.

64. The method of claim 63, wherein the solid cancer is selected from the group consisting of pancreatic cancer, glioma, glioblastoma, astrocytoma, colorectal cancer, thyroid cancer, gastric cancer, ovarian cancer, melanoma, endometrial cancer, lung cancer, renal cancer, cervical cancer, prostate cancer, breast cancer, urothelial cancer, testicular cancer, head and neck cancer, biliary tract cancer, liver cancer, and esophageal cancer.