ANTI-TROP2 ANTIGEN-BINDING MOLECULES

Technical field

The present invention relates, in general terms, to antigen-binding molecules. In particular, the present disclosure relates to antigen-binding molecules that specifically bind to human and feline TROP2.

Background

Trophoblast cell surface antigen 2 (TROP2), also known as TACSTD2 (tumor- associated calcium signal transducer 2), is a cell surface glycoprotein encoded by the TACSTD2 gene. It is expressed in various tissues, including epithelial tissues, and plays a role in cell adhesion, proliferation, and mobility. TROP2 is often overexpressed in a variety of cancers, including breast cancer, colorectal cancer, pancreatic cancer and lung cancer. Furthermore, its overexpression has been correlated with tumor progression, invasion and metastasis. The anti-TROP2 antibody, Trodelvy™ (Sacituzumab govitecan) has been recently approved by the Food and Drug Administration (FDA) for the treatment of metastatic triple-negative breast cancer and urothelial cancer in humans. However, there are a number of side effects that are associated with Trodelvy™ treatment. These include fatigue, nausea, diarrhea, neutropenia, anemia, alopecia (hair loss), vomiting, and decreased appetite.

There is therefore a need to develop new anti-TROP2 antibodies that have improved safety and efficacy profiles.

Summary

Disclosed herein is an antigen-binding molecule that specifically binds to human and feline Human Trophoblast Cell Surface Antigen 2 (TROP2).

Disclosed herein is a chimeric molecule comprising an antigen-binding molecule as defined herein and a heterologous moiety.

Disclosed herein is an isolated polynucleotide comprising a nucleic acid sequence encoding the antigen-binding molecule as defined herein.

Disclosed herein is a construct comprising a polynucleotide as defined herein in operable connection with one or more control sequences.

Disclosed herein is a host cell that contains the construct as defined herein.

Disclosed herein is a pharmaceutical composition comprising an antigen-binding molecule as defined herein or a chimeric molecule as defined herein.

Disclosed herein is an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein for use as a medicament.

Disclosed herein is a method for reducing or inhibiting proliferation and/or viability of a cancer cell, the method comprising contacting the cancer cell with a therapeutically effective amount of an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein.

Disclosed herein is a method of treating a cancer in a subject, the method comprising administering a therapeutically effective amount of an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein to the subject.

Disclosed herein is a method of treating a disease or condition associated with an undesired expression of TROP2 in a subject, wherein the method comprises administering a therapeutically effective amount of an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein to the subject.

Disclosed herein is a method of detecting the likelihood of the presence of a cancer in a subject, the method comprising determining the level of TROP2 in a sample obtained from

the subject, wherein an increased level of TROP2 as compared to a reference indicates the likelihood of the presence of a cancer in the subject.

Brief description of the drawings

Embodiments of the present invention will now be described, by way of non-limiting example, with reference to the drawings in which:

Figure 1. Screening of KP52 against various human cancers, using flow cytometry. (A) Compiled screening of KP52 against various human cancer cell lines. A threshold of 2% was defined as positive binding for all screening. (B) Representative histograms of binding by flow cytometry. Cell lines of various human breast cancer, human ovarian cancer, human pancreatic cancer, human gastric cancer, and normal human epithelial were incubated with respective mAbs and secondary antibodies conjugated with FITC. Binding (black histogram) was graded based on binding percentages after gating with a 2 - 2.1% cut-off from the negative control (light grey histogram).

Figure 2. Western blot assay. Immunoblot (IB) with KP52 shows a smear under non-reducing condition and reducing conditions reveals antigen MW of 45 and 50 kDa for both feline mammary cancer, Kelly F2, and various human cancers.

Figure 3. Determining the antigen target of KP52. KP52 antigen was successfully enriched using human cancer cell lines HCC-1937, PEA-1 and OVCAR-3. Unfortunately, Kelly F2 was not able to undergo immunoprecipitation. The mAb alone control in lane 1 is for the identification of antibody lanes in the IP lane (lane 3), while lysate only in lane 2 is to ensure no unspecific binding to sepharose beads and lysate without enrichment in lane 4 is for the identification of lysate bands. The red box indicates the band that was excised and sent for MS analysis.

Figure 4. Identification of TROP2 as the antigen target of KP52. After enrichment, bands were excised and send for mass spectrometry (MS). MS data showed to have peptide sequences (yellow highlighted) that aligns with the amino acid sequence of TROP2, with coverage > 36% for all three cancer cell lines.

Figure 5. Validation of TROP2 as the antigen target of KP52. (A) Cross IP of commercial anti-TROP2 antibody and KP52. Enrichment using both commercial anti-TROP2 antibody and KP52 were carried out and immunoblotted (IB) with either antibody. Both KP52 and commercial anti-TROP2 are enriching the same antigen band (black arrows), validating that KP52 binds to TROP2. (B) siRNA knockdown. There was a significant decrease in antigen after TROP2 siRNA knockdown (Lane 3) observed in both feline and human cancer. Immunoblotting with commercial anti-TROP2 shows similar results. Therefore, the antigen that KP52 targets is TROP2 in both feline and human cancers. GAPDH was used as loading control.

Figure 6. Sodium periodate treatment. KP52 binds to glycans on TROP2 as bands were seen in the control (lane 1) but not after treatment (lane 2). Beta-actin serves as positive control that proteins were not affected by the treatment.

Figure 7. Pronase treatment. (A) Coomassie Blue stained SDS-PAGE gel. Compared to the no treatment lane, proteins were digested after the incubation with pronase in Kelly F2, HCC-1937 and PEA-1 cell lines. (B) Dot blot. KP52 does not bind to protein backbone as dots were seen after treatment. Actin serves as a positive control to ensure all proteins have been degraded. CD15s and SSEA1 are negative controls for glycans.

Figure 8. Peptide: N-glycosidase F (PNGase F) and beta-elimination assays. Lane 1 to 3 belongs to PNGase F experiment and bands were seen after treatment, implying that KP52 does not bind to N-linked glycans. On the other hand, beta-elimination assay (lane 4 and lane 5), showed a complete abolishment of bands after sodium hydroxide treatment. This indicates that the glycan epitope of KP52 is O-linked dependent. Beta-actin is a positive control to ensure that proteins were not affected by the treatment.

Figure 9. KP52 staining of normal human tissues using immunohistochemistry (IHC). KP52 does not bind to this array of normal human tissues except salivary gland. (A) Brain (B) Salivary gland (C) Thyroid gland (D) Oesophagus (E) Stomach (F) Duodenum (G) Small intestine (H) Colon (I) Pancreas (J) Spleen (K) Liver (L) Skin (M) Testis (N) Prostate (O) Bladder (P) Peripheral nerve (Q) Uterus (R) Ovary (S) Vagina.

Figure 10. KP52 staining of malignant human tissues using immunohistochemistry

(IHC). KP52 binds to (A) oesophageal squamous cell cancer, (C) stomach adenocarcinoma, (F) squamous cell skin cancer, (H, J, K, L, M) ovarian cancer (N, O, P) breast cancer and (S, T) lung cancer. Out of 20 cores, 13 were stained. Hence, KP52 binds strongly to malignant tissues.

Figure 11. Internalisation of KP52 into cells. The controls are KP52 only (blue), pHrodo only (green) and buffer only (negative control; light grey). The purpose of the controls was to ensure that any fluorescence detected was only from the internalisation of the mAbs. In both 0 hour and 4 hours, all the controls coincide with each other, indicating that any readout was due to the internalisation of the mAbs. For KP52 + pHrodo (black), no internalisation was seen at 0 hour, but there was an increase in fluorescence at 4 hours in Kelly F2, HCC-1937 and NCI-N87. HCC-1395 is a negative binding cell line and as no fluorescence was detected in both time points, shows that KP52 is specific and must bind to cell surface antigen for internalisation to take place. KP52 is able to internalise into cancer cells and have the potential to function as an ADC.

Figure 12. KP52 ability to deliver cytotoxic payload. A secondary anti-mouse antibody conjugated to Saporin (ZAP), Mertansine (DM1) and Monomethyl auristatin E (MMAE) were used instead of direct conjugation of KP52 to cytotoxic payloads. Cells were incubated with either buffer (PBS), KP52 only, secondary drug conjugates only and KP52 + secondary-drugs. KP52 + ZAP and KP52 + DM1 showed a significant decrease in cell viability in human cancer cell lines while KP52 + MMAE had killing in HCC-1937 and PEA-1 (indicated by the orange arrows). For feline Kelly F2, only KP52 + ZAP demonstrated killing. Cells incubated with primary mAb only and cells incubated with the secondary-drugs only were comparable to no treatment. HCC-1395 is a non-binding cell line of KP52 and serves as a negative control. KP52 can function as an ADC in both feline and human cancers. Results are represented as mean +/- standard deviation of at least two independent experiments with sextuplicate wells (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, one-way ANOVA followed by Turkey post hoc test).

Figure 13. Binding of KP52-DM1 to human cancer cell lines. KP52-DM1 has similar binding to KP52 on human breast cancer HCC-1937 and HCC-1395, human ovarian cancer PEA-1 and human gastric cancer NCI-N87.

Figure 14. Cytotoxicity of KP52-DM1. (A) KP52-DM1 showed dose-dependent cytotoxicity in HCC-1937 and NCI-N87. The IC50 of KP52-DM1 was 0.97 nM for HCC-1937 and 0.99 nM for NCI-N87. For negative control, equivalent concentration of KP52 were added to the cells and no dose-dependent killing was observed in both cell lines. Values are mean +/- standard deviations of triplicates. Nonlinear regression was performed to determine the IC50 values using GraphPad Prism 9. The IC50 was similar in three independent experiments. (B) Cells were spiked with a fixed dose of 1 nM of KP52-DM1 or equivalent amount of free DM1 or KP52. KP52-DM1 showed about 50% killing in HCC-1937 and NCI-N87 but not non-binding cell line HCC-1395. KP52 had no killing for all three cell lines. Free DM1 had significant amount of killing in all the cell lines, including non-binding cell line HCC-1395. Results are represented as mean +/- standard deviation of at least two independent experiments with quadruplicate wells (**, $p < 0.01$; ***, $p < 0.001$, one-way ANOVA followed by Turkey post hoc test).

Figure 15. *In vivo* efficacy of KP52-DM1. (A) Tumour growth curve. Normalised tumour volume signifies the relative tumour volume when the tumour volume at day of randomisation is set to 1. Mice were treated twice weekly for two weeks as indicated by the black arrows. (B) Volumetric scatter plot of individual tumours on Day 14 and Day 28 after treatment. Mice treated with free DM1, KP52 and 1 mg/kg KP52-DM1 showed no significant reduction in tumour growth compared to PBS treated mice. On the other hand, mice treated with 10 mg/kg KP52-DM1 had full elimination of tumour after treatment. (C) Mean body weight of mice. Minimal change in body weight was observed in all treatment groups. Statistical significance for both tumour growth curve and mean body weight are measured by one way ANOVA followed by Turkey post hoc test using GraphPad Prism 9 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ and n.s stands for non-significant). (D) Binding of xenograft cells to KP52 and KP52-DM1 by flow cytometry. All harvested xenograft from the various treatment groups showed at least 70% of binding to KP52 and KP52-DM1.

Figure 16. KP52 and hKP52 variable heavy and light chain sequences. The complementary determining region (CDR) are highlighted in red.

Figure 17. Binding of hKP52 mAbs to various cell lines by flow cytometry. hKP52_3 and hKP52_4 (red font) had similar binding specificity to immunogen feline cancer Kelly F2 and human cancer HCC-1937 and HCC-1395 compared to parental mice KP52. On the

other hand, hKP52_1 and hKP52_2 had a 12% to 18% reduction in binding towards Kelly F2 and HCC-1937 when compared with KP52.

Figure 18: Competitive Inhibition Assay between KP52 and hKP52. (A) HCC-1937 cells were preincubated with KP52 followed by the different hKP52 and then with secondary anti-human antibody conjugated with FITC. The bar graph shows the median fluorescence intensity (MFI) and the corresponding histograms. (B) HCC-1937 cells were preincubated with the various hKP52 followed by KP52 and subsequently with secondary anti-mouse antibody conjugated with FITC. The bar graph shows the MFI and the corresponding histograms on the right. For both conditions, there was a drop in MFI for the different conditions, indicating that all four hKP52 mAbs recognises the same epitope as KP52. However, hKP52_3 and hKP52_4 had the most drop in MFI compared to hKP52_1 and hKP52_2. MFI was analysed using FlowJoTM software v10. Statistics were conducted using GraphPad Prism 9 with test being two-way ANOVA followed by Bonferroni post hoc test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to the corresponding control.

Figure 19. hKP52 has ADC function. A secondary anti-human antibody conjugated to DM1 (hDM1) was used in this indirect ADC cell-based assay. All four humanised mAbs had significant reduction of relative luminescence observed in HCC1937, PEA-1 and NCI-N87 after the treatment of hKP52 + hDM1. No killing was observed on the negative binding cell line HCC1395. Results are represented as mean +/- standard deviation of at least two independent experiments with quadruple wells (***, $p < 0.001$, one-way ANOVA followed by Turkey post hoc test was conducted using GraphPad Prism 9).

Figure 20. Dissociation constant (KD) of KP52 and hKP52 mAbs. Using ForteBio Octet RED96 system, the kinetic rate constants and equilibrium constants were determined. Purified TROP2 tagged with His were captured on anti-penta-HIS (HIS1K) biosensors and humanised mAbs and KP52 of varying concentrations were analysed. Although the humanised mAbs had moderate loss of affinity compared to KP52, they still had high affinities in the nanomolar range to TROP2. Amongst the humanised mAbs, hKP52_3 have the highest affinity.

Figure 21. Binding profile of KP52 and RS7 against feline mammary cancer and human cancer cell lines. (A) Representative histograms of binding by flow cytometry. (B)

Summary of binding profile comparing KP52 and RS7. The cut off value for positive binding is defined as 2% relative to the negative control. RS7 have < 20% binding to Kelly F2 cell line unlike KP52. Both mAbs have similar binding profile to human cancer cell lines. Note: negative control is represented by light grey histogram and mAb binding is represented by black.

Figure 22. Western blot assay of RS7. IB with RS7 shows a smear in non-reducing conditions with TROP2 MW between 40 to 60 kDa. No bands were seen under reducing conditions, indicating that RS7 binds to a conformational epitope.

Figure 23. Killing efficacy of KP52 and RS7 as ADC. Using ZAP as the payload, KP52 + ZAP demonstrated higher killing percentage compared to RS7 + ZAP in all the various doses tested in the three cancer cell lines, HCC1937, PEA-1 and NCI-N87. HCC-1395 is a non-binding cell line for both mAbs and serves as a negative control in this experiment.

Figure 24. Comparing RS7 and KP52 staining on malignant tissues via IHC. KP52 and RS7 have similar staining profile on the cancer tissues. (A) oesophageal squamous cell cancer, (B) stomach adenocarcinoma, (C) stomach adenocarcinoma, (D) colon adenocarcinoma, (E) liver hepatocellular carcinoma, (F) squamous cell skin cancer, (G) Testis seminoma, (H, I, J, K, L, M) ovarian cancer (N, O, P) breast cancer, (Q) oral leiomyosarcoma, (R) kidney clear cell carcinoma and (S, T) lung cancer.

Figure 25. Comparing RS7 and KP52 specificity on normal tissues via IHC. Both KP52 and RS7 binds normal salivary gland, but RS7 also stains pancreatic tissue. (A) Brain (B) Salivary gland (C) Thyroid gland (D) Oesophagus (E) Stomach (F) Duodenum (G) Small intestine (H) Colon (I) Pancreas (J) Spleen (K) Liver (L) Skin (M) Testis (N) Prostate (O) Bladder (P) Peripheral nerve (Q) Uterus (R) Ovary (S) Vagina.

Figure 26. Functionality of CAR(KP52) against antigen positive cell lines. (A) Histograms of KP52 binding by flow cytometry. Cell lines of various human lung and breast cancers were incubated with KP52 mAb and a no mAb negative control, followed by secondary antibodies conjugated with FITC. Binding (black histogram) was graded based on binding percentages after gating with a 2 - 2.1% cut-off from the negative control (light grey histogram). (B) Schematic diagram of CAR(KP52) constructs. Spacer variants of

CAR(KP52) were developed to determine the optimal CAR construct. (C-D) CAR mediated cytotoxicity against TROP2-positive cell lines via the xCELLigence platform. Cell index used as an analogue for adherent target cell growth. (C) T cells transfected with CAR(KP52) constructs displayed enhanced cytotoxicity against target cell lines, with further improvements using CAR constructs of intermediate and long spacer lengths. (D) No cytotoxicity of CAR(KP52) T cells was observed against TROP2-negative cell lines. (E-G) Co-cultures between CAR(KP52) T cells and target cell lines, analysed by flow cytometry, and normalised to equal sampling volumes. (E) Representative flow plots from day 6 of co-culture. Depicted flow plots gated on live cell populations. CD3-expression status used to distinguish T cell and tumour cell populations. (F) CAR(KP52) T cells expand upon recognition of TROP2-positive target cells, mediating their cytotoxic effect against target cells. Improved expansion and cytotoxicity were observed for intermediate and long spacer CAR variants. (G) CAR(KP52) T cells do not expand in co-cultures with TROP2-negative cell lines, mediating cytotoxicity no better than the non-transfected T cell control.

Figure 27: Epitope characterisation of KP52 on TROP2 protein (A) Structure of TROP2 protein, and its close protein family member, Epithelial Cell Adhesion Molecule (EpCAM), with the amino acid numbering indicated for the respective proteins. Legend - SP: Signal peptide; CRD: Cysteine-rich domain; TY: Thyroglobulin domain; CPD: Cysteine-poor domain; TMD: Transmembrane domain; ICD: Intracellular domain. The extracellular portion comprised of CRD, TY and CPD domains. (B) Schematic diagram of the three TROP2 mutant constructs, with the TROP2 sequences in white, and the EpCAM sequences in grey. The mutant and wild type TROP2 plasmid constructs were transfected into H1299 cell line to obtain stable over-expressing cell lines for at last 5 passages before testing. (C) Histograms of KP52 and commercial anti-TROP2 antibody binding by flow cytometry of the wildtype construct compared to the TROP2 mutant constructs. Loss of KP52 binding was observed for the Trop2-Dom1 swap construct, while the binding of a commercial anti-Trop2 was not affected. This indicated that KP52 epitope was likely within CRD. Note that the commercial anti-Trop2 antibody used is known to bind on the CPD domain, so loss of binding for the Trop2-Dom3 swap construct is expected.

Figure 28 shows the human TROP2-CRD and feline TROP2-CRD sequences. The sequence of full-length feline TROP2 protein is also provided.

Detailed description

The present disclosure teaches antigen-binding molecules that specifically bind to human and feline Human Trophoblast Cell surface Antigen 2 (TROP2).

In one embodiment, the antigen-binding molecule specifically binds to the cysteine-rich domain (CRD) in the extracellular domain of human or feline TROP2. The antigen-binding molecule may specifically bind to SEQ ID NO: 26 or SEQ ID NO: 27. Alternatively, the antigen-binding molecule may specifically bind to i) AAQDNCTCPTNKMT (SEQ ID NO: 34) DGPGGRCQCRALGSG (SEQ ID NO: 35); or iii) VDCSTLTSKC (SEQ ID NO: 36).

Without being bound by theory, it was found that KP52, a murine mAb generated against feline mammary cancer also binds to human cancers, including breast, ovarian, pancreatic, gastric, lung cancers and cholangiocarcinoma. Characterisation revealed that KP52 recognises an O-linked glycan epitope on TROP2. TROP2 is a transmembrane intracellular calcium signal transducer that is differentially expressed in many cancers and normal tissues. Interestingly, KP52 was able to differentiate between normal tissues and malignant tissues histologically. Comparing with a clinically approved biosimilar mAb, KP52 appears superior in *in vitro* efficacy, specificity, and binding. Additionally, KP52-DM1 antibody drug conjugate (ADC) shows IC50 in the nanomolar range and complete regression of tumour mass was observed after treatment to mice with human breast cancer HCC-1937 xenografts. KP52 was further humanized (hKP52) and transiently expressed in Human Embryonic Kidney (HEK) cells. The resultant hKP52 binding and functional efficacy was comparable to KP52.

By "antigen-binding molecule" is meant a molecule that has binding affinity for a target antigen. It will be understood that this term extends to immunoglobulins, immunoglobulin fragments and non-immunoglobulin derived protein frameworks that exhibit antigen-binding activity. Representative antigen-binding molecules that are useful in the practice of the present invention include antibodies and their antigen-binding fragments. The term "antigen-binding molecule" includes antibodies and antigen-binding fragments of antibodies.

In an embodiment, the antigen-binding molecule, as described herein, is conjugated to

another molecule or moiety, including functional moieties (e.g., toxins), detectable moieties (e.g., fluorescent molecules, radioisotopes), small molecule drugs and polypeptides.

The term "antibody", as used herein, is understood to mean any antigen-binding molecule or molecular complex comprising at least one complementarity determining region (CDR) that binds specifically to, or interacts specifically with, the target antigen. The term "antibody" includes full-length immunoglobulin molecules comprising two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, as well as multimers thereof (e.g., IgM). Each heavy chain comprises a heavy chain variable region (which may be abbreviated as HCVR, VH or V_H) and a heavy chain constant region. The heavy chain constant region typically comprises three domains - CH1, CH2 and CH3. Each light chain comprises a light chain variable region (which may be abbreviated as LCVR, VL, VK, V_K or V_L) and a light chain constant region. The light chain constant region will typically comprise one domain (CL1). The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, also referred to as framework regions (FR). Each VH and VL typically comprises three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. In some embodiments, the FRs of the antigen-binding molecules described herein may be identical to the FR of germline sequences of the target species (i.e., the species to which the antigen-binding molecules or antigen-binding fragments thereof, as described herein, will be administered). In some embodiments, the FR may be naturally or artificially modified. Whilst it is generally desirable that each of the FR sequences are identical to FR sequences derived from immunoglobulin molecules of the target species, including to minimize an immune response being raised against the binding molecule upon administration to a subject of the target species, in some embodiments, the antigen-binding molecule, or antigen-binding fragment thereof, may comprise one or more amino acid residues across one or more of its FR sequences that would be foreign at a corresponding position in one or more FR from the target species.

An antibody includes an antibody of any class, such as IgG, IgA, or IgM (or sub-class thereof), and the antibody need not be of any particular class. Depending on the antibody amino acid sequence of the constant region of its heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD,

IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2. The heavy-chain constant regions that correspond to the different classes of immunoglobulins are called α, δ, ε, γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known to a person skilled in the art.

As used herein, the term "complementarity determining regions" (CDRs; *i.e.*, CDR1, CDR2, and CDR3) refers to the amino acid residues of an antibody variable domain the presence of which are necessary for antigen binding. Each variable domain typically has three CDR regions identified as CDR1, CDR2 and CDR3. Each complementarity determining region may comprise amino acid residues from a "complementarity determining region" as defined for example by Kabat (i.e., about residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al.*, Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md. (1991)) and/or those residues from a "hypervariable loop" (i.e., about residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk J. Mol. Biol. 196:901-917 (1987)). In some instances, a complementarity determining region can include amino acids from both a CDR region defined according to Kabat and a hypervariable loop.

An "antigen-binding site" refers to the site, *i.e.*, one or more amino acid residues, of an antigen binding molecule which provides interaction with the antigen. For example, the antigen binding site of an antibody comprises amino acid residues from the complementarity determining regions (CDRs). A native immunoglobulin molecule typically has two antigen binding sites, a Fab molecule typically has a single antigen binding site. An antigen-binding site of an antigen-binding molecule described herein typically binds specifically to an antigen and more particularly to an epitope of the antigen.

The present disclosure also extends to antigen-binding molecules that bind specifically to TROP2 in humans and cats. In an embodiment, the antigen is human TROP2. In another embodiment, the antigen is feline TROP2. The present disclosure extends to antigen binding molecules that bind specifically to native TROP2 (*i.e.*, naturally-occurring TROP2), as well as to variants thereof. Such variants may include TROP2 molecules that differ from a

naturally-occurring (wild-type) molecule by one or more amino acid substitutions, deletions and / or insertions. Variant TROP2 molecules of this type may be naturally-occurring or synthetic (*e.g.,* recombinant) forms. It is to be understood, however, that in one embodiment, the antigen-binding molecules described herein bind specifically to a native form of TROP2, whether of a human or non-human species (such as feline TROP2).

The terms "antigen-binding fragment", "antigen-binding portion", "antigen-binding domain" and "antigen-binding site" are used interchangeably herein to refer to a part of an antigen-binding molecule that participates in antigen-binding. These terms include any naturally occurring, enzymatically obtainable, synthetic, or genetically engineered polypeptide or glycoprotein that specifically binds an antigen to form a complex.

Antigen-binding fragments of an antibody may be derived, *e.g.*, from full antibody molecules using any suitable standard techniques such as proteolytic digestion or recombinant genetic engineering techniques involving the manipulation and expression of DNA encoding antibody variable and optionally constant domains. Such DNA is known and/or is readily available from, *e.g.*, commercial sources, DNA libraries (including, *e.g.*, phage-antibody libraries), or can be synthesized. The DNA may be sequenced and manipulated chemically or by using molecular biology techniques, for example, to arrange one or more variable and/or constant domains into a suitable configuration, or to introduce codons, create cysteine residues, modify, add or delete amino acids, etc.

Non-limiting examples of antigen-binding fragments include: (i) Fab fragments; (ii) F(ab')2 fragments; (iii) Fd fragments; (iv) Fv fragments; (v) single-chain Fv (scFv) molecules; (vi) dAb fragments; and (vii) minimal recognition units consisting of the amino acid residues that mimic the hypervariable region of an antibody (*e.g.*, an isolated complementarity determining region (CDR) such as a CDR3 peptide), or a constrained FR3-CDR3-FR4 peptide. Other engineered molecules, such as domain-specific antibodies, single domain antibodies, domain-deleted antibodies, chimeric antibodies, CDR-grafted antibodies, one-armed antibodies, diabodies, triabodies, tetrabodies, minibodies, nanobodies (*e.g.* monovalent nanobodies, bivalent nanobodies, etc.), small modular immunopharmaceuticals (SMIPs), and shark variable IgNAR domains, are also encompassed within the expression "antigen-binding fragment" as used herein.

An antigen-binding fragment of an antibody will typically comprise at least one variable domain. The variable domain may be of any size or amino acid composition and will generally comprise at least one CDR which is adjacent to or in frame with one or more framework sequences. In antigen-binding fragments having a V_H domain associated with a V_L domain, the V_H and V_L domains may be situated relative to one another in any suitable arrangement. For example, the variable region may be dimeric and contain V_H-V_H, V_H-V_L or V_L-V_L dimers. Alternatively, the antigen-binding fragment of an antibody may contain a monomeric V_H or V_L domain.

In certain embodiments, an antigen-binding fragment of an antibody may contain at least one variable domain covalently linked to at least one constant domain. Non-limiting, exemplary configurations of variable and constant domains that may be found within an antigen-binding fragment of an antibody of the present invention include: (i) V_H-C_H1; (ii) V_H-C_H2; (iii) V_H-C_H3; (iv) V_H-C_H1-C_H2; (v) V_H-C_H1-C_H2-C_H3, (vi) V_H-C_H2-C_H3; (vii) V_H-C_L; (viii) V_L-C_H1; (ix) V_L-C_H2, (x) V_L-C_H3; (xi) V_L-C_H1-C_H2; (xii) V_L-C_H1-C_H2-C_H3; (xiii) V_L-C_H2-C_H3; and (xiv) V_L-C_L. In any configuration of variable and constant domains, including any of the exemplary configurations listed above, the variable and constant domains may be either directly linked to one another or may be linked by a full or partial hinge or linker region. A hinge region may consist of at least 2 (*e.g.*, 5, 10, 15, 20, 40, 60 or more) amino acids which result in a flexible or semi-flexible linkage between adjacent variable and/or constant domains in a single polypeptide molecule. Moreover, an antigen-binding fragment of an antibody of the present disclosure may comprise a homo-dimer or hetero-dimer (or other multimer) of any of the variable and constant domain configurations listed above in non-covalent association with one another and/or with one or more monomeric V_H or V_L domain (*e.g.*, by disulfide bond(s)). A multispecific antigen-binding molecule will typically comprise at least two different variable domains, wherein each variable domain is capable of specifically binding to a separate antigen or to a different epitope on the same antigen. Any multispecific antigen-binding molecule format, including bispecific antigen-binding molecule formats, may be adapted for use in the context of an antigen-binding fragment of an antibody of the present disclosure using routine techniques available in the art.

The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antigen binding molecule to antigen. The

variable domains of the heavy chain and light chain (V_H and V_L, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three hypervariable regions (HVRs). See, *e.g.*, Kindt et al., Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007). A single V_H or V_L domain may be sufficient to confer antigen-binding specificity.

The term "constant domains" or "constant region" as used herein denotes the sum of the domains of an antibody other than the variable region. The constant region is not directly involved in binding of an antigen, but exhibits various immune effector functions.

In one embodiment, the antigen-binding molecule or antigen-binding fragment thereof is modified for compatibility with the target species. Thus, in an embodiment, the antigen-binding molecule or antigen-binding fragment thereof is humanized or felinized.

By "humanized" is meant that the antigen-binding molecule comprises an amino acid sequence that is compatible with humans, such that the amino acid sequence is unlikely to be seen as foreign by the immune system of a human subject. In an embodiment, the humanized antigen-binding molecule comprises one or more immunoglobulin framework regions derived from one or more human immunoglobulin molecules. In some embodiments, all of the framework regions of the humanized antigen-binding molecule will be derived from one or more human immunoglobulin molecules. The humanized antibody may optionally comprise an immunoglobulin heavy chain constant region derived from a human immunoglobulin molecule.

By "felinized" is meant that the antigen-binding molecule comprises an amino acid sequence that is compatible with feline, such that the amino acid sequence is unlikely to be seen as foreign by the immune system of a feline subject. In an embodiment, the felinized antigen-binding molecule comprises one or more immunoglobulin framework regions derived from one or more feline immunoglobulin molecules. In some embodiments, all of the framework regions of the felinized antigen-binding molecule will be derived from one or more feline immunoglobulin molecules. The felinized antibody may optionally comprise an immunoglobulin heavy chain constant region derived from a feline immunoglobulin molecule.

It is to be understood that the present disclosure also extends to antigen-binding molecules that are compatible with species other than human and feline. In this context, the antigen-binding molecules can be referred to as "speciesized", referring to the target species to which the molecule will be administered.

The phrase "specifically binds" or "specific binding" refers to a binding reaction between two molecules that is at least two times the background and more typically more than 10 to 100 times background molecular associations under physiological conditions. When using one or more detectable binding agents that are proteins, specific binding is determinative of the presence of the protein, in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antigen-binding molecule binds to a particular antigenic determinant, thereby identifying its presence. Specific binding to an antigenic determinant under such conditions requires an antigen-binding molecule that is selected for its specificity to that determinant. This selection may be achieved by subtracting out antigen-binding molecules that cross-react with other molecules. A variety of immunoassay formats may be used to select antigen-binding molecules (*e.g.*, immunoglobulins) such that they are specifically immunoreactive with a particular antigen. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, *e.g.*, Harlow & Lane, Antibodies, A Laboratory Manual (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Methods of determining binding affinity and specificity are also well known in the art (see, for example, Harlow and Lane, supra); Friefelder, "Physical Biochemistry: Applications to biochemistry and molecular biology" (W.H. Freeman and Co. 1976)).

"Affinity" or "binding affinity" refers to the strength of the sum total of non-covalent interactions between a single binding site of a molecule (*e.g.*, an antigen-binding molecule) and its binding partner (*e.g.*, an antigen). Unless indicated otherwise, as used herein, "binding affinity" refers to intrinsic binding affinity which reflects a 1:1 interaction between members of a binding pair *e.g.*, an antigen-binding molecule. The affinity of a molecule X for its partner Y can generally be represented by the dissociation constant (Kd), which is the ratio of dissociation and association rate constants (k_{off} and k_{on}, respectively). Thus, equivalent affinities may comprise different rate constants, as long as the ratio of the rate constants remains the same. Affinity can be measured by common methods known in the

art, including those described herein. A particular method for measuring affinity is Surface Plasmon Resonance (SPR).

The terms "polypeptide", "peptide", or "protein" are used interchangeably herein to designate a linear series of amino acid residues connected one to the other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The amino acid residues are usually in the natural "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide.

As used herein, the term "modified antibody" includes synthetic forms of antibodies which are altered such that they are not naturally occurring, e.g., antibodies that comprise at least two heavy chain portions but not two complete heavy chains (such as domain deleted antibodies or minibodies); multispecific forms of antibodies (e.g., bispecific, trispecific, etc.) altered to bind to two or more different antigens or to different epitopes on a single antigen; heavy chain molecules joined to scFv molecules and the like. ScFv molecules are known in the art and are described, *e.g.*, in U.S. Pat. No. 5,892,019. In addition, the term "modified antibody" includes multivalent forms of antibodies (*e.g.*, trivalent, tetravalent, etc., antibodies that bind to three or more copies of the same antigen).

The antigen-binding molecule may comprise a) a heavy chain variable (VH) region comprising the VHCDR1 amino acid sequence GYSFTDYIIT (SEQ ID NO: 1), the VHCDR2 amino acid sequence QIYPGSGSIYYNEKFKA (SEQ ID NO: 2) and the VHCDR3 amino acid sequence GFDYDGN (SEQ ID NO: 3); and b) a light chain variable (VL) region comprising the VLCDR1 amino acid sequence KASQDINTYLS (SEQ ID NO: 4), the VLCDR2 amino acid sequence RANRLVD (SEQ ID NO: 5) and the VLCDR3 amino acid sequence LQYDEFPLT (SEQ ID NO: 6).

The antigen-binding fragment may be an antibody or antigen-binding fragment thereof. The antibody or antigen binding fragment thereof may be a full-length antibody, a substantially intact antibody, a Fab fragment, a scFab, a Fab', a single chain variable fragment (scFv) or a one-armed antibody.

In one embodiment, the antibody or antigen-binding molecule therefore is humanized or

felinized.

The antigen-binding molecule may comprise: a) a VH region comprising an amino acid sequence having at least 70% (including at least 71% to 99% and all integer percentages therebetween) sequence identity to SEQ ID NO: 20 or 21; and b) a VL region comprising an amino acid sequence having at least 70% (including at least 71% to 99% and all integer percentages therebetween) sequence identity to SEQ ID NO: 22 or 23.

Table 1

	Protein Sequence
Heavy Chain (VH1)	QVQLVQSGAEVKKPGASVKVSCKASGYSFTDY IITWVRQATGQGLEWMGQIYPGSGSIYYNEKFK ARVTMTRNTSISTAYMELSSLRSEDTAVYFCAR GFDYDGNWGQGTLVTVSS (SEQ ID NO: 20)
Heavy Chain (VH2)	QVQLVQSGPEVKRPGASVRVSCKASGYSFTDYI ITWVRQAPGQGLEWMGQIYPGSGSIYYNEKFK ARVTLTADKSINTAYMELSSLTSEDTAVYFCARG FDYDGNWGQGTLVTVSS (SEQ ID NO: 21)
Light Chain (VL1)	DIQMTQSPSSLSASVGDRVTITCKASQDINTYLS WFQQKPGKAPKLLIYRANRLVDGVPSRFSGSG SGTDFTFTISSLQPEDIATYYCLQYDEFPLTFGG GTKVEIK (SEQ ID NO: 22)
Light Chain (VL2)	DIQMTQSPSSLSASVGDRVTITCKASQDINTYLS WFQQKPGKAPKTLIYRANRLVDGVPSRFSGSG SGTDFTFTISSLQPEDIATYYCLQYDEFPLTFGQ GTKLEIK (SEQ ID NO: 23)

The antigen-binding molecule may comprise a) a heavy chain variable region (VH) as defined herein comprising at least 70% sequence identity to at least one region other than a CDR of the VH amino acid sequence set forth in SEQ ID NO: 20 or 21 (e.g., to at least one framework region, such as 1, 2, 3 or 4 framework regions, of the VH), and b) a light chain variable region (VL) as defined herein comprising at least 70% sequence identity to at least one region other than a CDR of the VL amino acid sequence set forth in SEQ ID NO: 22 or 23 (e.g., to at least one framework region, such as 1, 2, 3 or 4 framework regions, of the VL).

The antigen-binding molecule may comprise: a) a VH as defined herein which is distinguished from the VH amino acid sequence set forth in SEQ ID NO: 20 or 21 by a

deletion, substitution or addition of one or more (e.g., 1, 2, 3, 4 or 5) amino acids in at least one region other than a CDR of the VH amino acid sequence set forth in SEQ ID NO: 20 or 21 (e.g., in at least one framework region, such as in 1, 2, 3 or 4 framework regions, of the VH), and b) a VL as defined in (1) which is distinguished from the VL amino acid sequence set forth in SEQ ID NO: 22 or 23 by a deletion, substitution or addition of one or more (e.g., 1, 2, 3, 4 or 5) amino acids in at least one region other than a CDR of the VL amino acid sequence set forth in SEQ ID NO: 22 or 23 (e.g., in at least one framework region, such as in 1, 2, 3 or 4 framework regions, of the VL).

The term "sequence identity" as used herein refers to the extent that sequences are identical on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G and I) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

The antigen-binding molecule as defined herein may comprise one or more conservative amino acid substitutions.

A "conservative amino acid substitution" is to be understood as meaning a substitution in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, which can be generally sub-classified as shown in the table "*Amino Acid Classification*", below:

AMINO ACID SUB-CLASSIFICATION

Sub-classes	Amino acids
Acidic	Aspartic acid, Glutamic acid
Basic	Noncyclic: Arginine, Lysine; Cyclic: Histidine
Charged	Aspartic acid, Glutamic acid, Arginine, Lysine, Histidine
Small	Glycine, Serine, Alanine, Threonine, Proline
Polar/neutral	Asparagine, Histidine, Glutamine, Cysteine, Serine, Threonine
Polar/large	Asparagine, Glutamine
Hydrophobic	Tyrosine, Valine, Isoleucine, Leucine, Methionine, Phenylalanine, Tryptophan
Aromatic	Tryptophan, Tyrosine, Phenylalanine
Residues that influence chain orientation	Glycine and Proline

Conservative amino acid substitution also includes groupings based on side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. For example, it is reasonable to expect that replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid will not have a major effect on the properties of the resulting variant polypeptide. Whether an amino acid change results in a functional polypeptide can readily be determined by assaying its activity.

Conservative substitutions are also shown in the table below (*EXEMPLARY AND PREFERRED AMINO ACID SUBSTITUTIONS*). Amino acid substitutions falling within the scope of the invention, are, in general, accomplished by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. After the substitutions are introduced, the variants can be

screened for their ability to bind specifically to NGF using methods known to persons skilled in the art, including those methods described elsewhere herein.

EXEMPLARY AND PREFERRED AMINO ACID SUBSTITUTIONS

Original Residue	**Exemplary Substitutions**	**Preferred Substitutions**
Ala	Val, Leu, Ile	Val
Arg	Lys, Gln, Asn	Lys
Asn	Gln, His, Lys, Arg	Gln
Asp	Glu	Glu
Cys	Ser	Ser
Gln	Asn, His, Lys,	Asn
Glu	Asp, Lys	Asp
Gly	Pro	Pro
His	Asn, Gln, Lys, Arg	Arg
Ile	Leu, Val, Met, Ala, Phe, Norleu	Leu
Leu	Norleu, Ile, Val, Met, Ala, Phe	Ile
Lys	Arg, Gln, Asn	Arg
Met	Leu, Ile, Phe	Leu
Phe	Leu, Val, Ile, Ala	Leu
Pro	Gly	Gly
Ser	Thr	Thr
Thr	Ser	Ser
Trp	Tyr	Tyr
Tyr	Trp, Phe, Thr, Ser	Phe
Val	Ile, Leu, Met, Phe, Ala, Norleu	Leu

In one embodiment, there is provided a method of producing an antigen-binding molecule, the method comprising: 1) immunizing an animal with a polypeptide from the cysteine-rich domain (CRD) of the extracellular domain of human or feline TROP2 (such as SEQ ID NO: 26 or SEQ ID NO: 27), 2) identifying and/or isolating a B cell from the animal, which binds specifically to the polypeptide; and 3) producing the antibody expressed by that B cell. In one embodiment is also an antigen-binding molecule that is obtained according to a method as defined herein.

In one embodiment, there is provided a method of producing an antigen-binding molecule, the method comprising: 1) contacting an antigen-binding molecule display library with a polypeptide from the cysteine-rich domain (CRD) of the extracellular domain of human or feline TROP2 (such as SEQ ID NO: 26 or SEQ ID NO: 27), and 2) isolating and/or selecting an antigen-binding molecule which binds specifically to the polypeptide. In one embodiment is also an antigen-binding molecule that is obtained according to a method as defined herein. The display library may be a phage, yeast or mRNA display library.

Representative antigen-binding molecules contemplated by the present disclosure include full-length immunoglobulins and antigen-binding fragments, including recombinant antigen-binding molecules, which may be monovalent or multivalent, monospecific or multispecific.

In one embodiment, the antibody or antigen-binding fragment thereof is a full-length antibody, a substantially intact antibody, a Fab fragment, scFab, Fab', a single chain variable fragment (scFv) or a one-armed antibody.

In one embodiment, the antibody has an isotype selected from the group consisting of IgG1, IgG2, IgG3, and IgG4. In one embodiment, the antibody is an IgG1 antibody. The antibody may have antibody-dependent cell-mediated cytotoxicity (ADCC) activity and can induce NK cell killing. The heavy chain constant region can be a wild-type human Fc region, or a human Fc region that includes one or more amino acid substitutions. The antibodies can have mutations that stabilize the disulfide bond between the two heavy chains of an immunoglobulin, such as mutations in the hinge region of IgG4, as disclosed in the art (e.g., Angal et al., 1993. Mol. Immunol., 30:105-08). See also, e.g., U.S. 2005/0037000. The heavy chain constant region can also have substitutions that modify the properties of the antigen-binding molecule (e.g., decrease one or more of: Fc receptor binding, antigen-binding molecule glycosylation, deamidation, binding to complement, or methionine oxidation). In some instances, the antigen-binding molecules may have mutations such as those described in U.S. Pat. Nos. 5,624,821 and 5,648,260. In some embodiments, the antigen-binding molecule is modified to reduce or eliminate effector function.

In one embodiment, the antigen-binding molecule of the present invention is a monovalent antigen-binding molecule. Non-limiting monovalent antigen-binding molecules include: a Fab fragment consisting of V_L, V_H, C_L and C_H1 domains; a Fab' fragment consisting of V_L, V_H, C_L and C_H1 domains, as well as a portion of a C_H2 domain; an Fd fragment consisting of V_H and C_H1 domains; an Fv fragment consisting of V_L and V_H domains of a single arm of an antibody; a single-chain antibody molecule (*e.g.*, scFab and scFv); a single domain antibody (dAb) fragment (Ward et al., 1989 Nature 341:544-546), which consists of a V_H domain; and a one-armed antibody, such as described in US20080063641 (Genentech) or other monovalent antibody, *e.g.*, such as described in WO2007048037 (Amgen).

In one embodiment, a monovalent antigen-binding molecule comprises an Fv fragment. The Fv fragment is the smallest unit of an immunoglobulin molecule with function in antigen-binding activities. An antigen-binding molecule in scFv (single chain fragment variable) format consists of variable regions of heavy (V_H) and light (V_L) chains, which are joined together by a flexible peptide linker that can be easily expressed in functional form in an expression host such as *E. coli* and mammalian cells, allowing protein engineering to improve the properties of scFv such as increase of affinity and alteration of specificity (Ahmed *et al.*, 2012. *Clin Dev Immunol.* 2012:980250). Representative examples of linker sequences are described in Section 4.5 *infra*. In the scFv construction, the order of the domains can be either V_H-linker-V_L or V_L-linker-V_H and both orientations can applied.

In some embodiments, the linker sequences used in scFvs are multimers of the pentapeptide GGGGS [SEQ ID NO: 29] (or G4S or Gly4Ser). Those include the 15-mer (G4S)3 (Huston *et al.*, 1988. *Proc Natl Acad Sci USA.* 85(16), 5879–83), the 18-mer GGSSRSSSSGGGGSGGGG [SEQ ID NO: 30] (Andris-Widhopf et al., "Generation of human scFv antibody libraries: PCR amplification and assembly of light- and heavy-chain coding sequences." Cold Spring Harbor Protocols, 2011(9)) and the 20-mer (G4S)4 (Schaefer *et al.*, "Construction of scFv Fragments from Hybridoma or Spleen Cells by PCR Assembly." In: Antibody Engineering, R. Kontermann and S. Dübel, Springer Verlag, Heidelberg, Germany (2010) pp. 21-44). Many other sequences have been proposed, including sequences with added functionalities, *e.g.*, an epitope tag or an encoding sequence containing a Cre-Lox recombination site or sequences improving scFv properties, often in the context of particular antibody sequences.

Cloning of the scFv is usually done by a two-step overlapping PCR (also known as Splicing by Overlap Extension or SOE-PCR), as described (Schaefer et al., 2010, supra). The V_H and V_L domains are first amplified and gel-purified and secondarily assembled in a single step of assembly PCR. The linker is generated either by overlap of the two inner primers or by adding a linker primer whose sequence covers the entire linker or more (three-fragment assembly PCR).

Single chain Fv (scFv) antigen-binding molecules may be recombinantly produced for example in *E. coli*, insect cells or mammalian host cells upon cloning of the protein coding sequence for the scFv in the context of appropriate expression vectors with appropriate translational, transcriptional start sites and, in the case of mammalian expression, a signal peptide sequence.

In one embodiment, the monovalent antigen-binding molecule comprises an Fab fragment. In an illustrative example of this type, the monovalent antigen-binding molecule is a one-armed antibody consisting or consisting essentially of a single antigen-binding fragment (Fab) and a Fc region, wherein the Fc region comprises a first and a second Fc polypeptide, and wherein the first and second Fc polypeptides are present in a complex.

Recombinant expression of Fc-containing monovalent antigen-binding molecules can often lead to undesirable bivalent, homodimer contaminants. Strategies to inhibit formation of homodimers are known including methods that introduce mutations into immunoglobulin constant regions to create altered structures that support unfavorable interactions between polypeptide chains and suppress unwanted Fc homodimer formation. Non-limiting examples of this strategy to promote heterodimerization include the introduction of knobs-into-holes (KIH) structures into the two polypeptides and utilization of the naturally occurring heterodimerization of the C_L and C_H1 domains (see, Kontermann, *supra*, pp. 1 -28 (2011) Ridgway et al., 1996. *Protein Eng.* 9(7):617-21; Atwell *et al.*, 1997. *J Mol Biol.* 270(1):26-35; as described in WO 2005/063816). These KIH mutations promote heterodimerization of the knob containing Fc and the hole containing heavy chain, improving the assembly of monovalent antibody and reducing the level of undesired bivalent antibody.

Modifications in the Fc domain of an antigen-binding molecules may also be desirable to reduce Fc receptor binding and therefore reduce the potential for FcγRIIa-mediated

activation of platelets. For example, the so-called 'LALA' double mutation (Leu234Ala together with Leu235Ala) in human IgG (including IgG1) is known to significantly impair Fc receptor binding and effector function (Lund *et al.*, 1991, *J. Immunol.* 147, 2657–2662; Lund *et al.,* 1992, *Mol. Immunol.* 29:53–59). For human IgG4, engineering mutations S228P/L235E variant (SPLE) has previously demonstrated minimal FcγR binding (Newman *et al.*, 2001, *Clin. Immunol.* 98, 164–174). Mutations in IgG1 or IgG4 Fc domains can be combined, for instance combining the LALA mutations in human IgG1 with a mutation at P329G or combining the SPLE mutation in human IgG4 with a mutation at P329G, completely abolished FcγR and C1q interactions (Schlothauer *et al.*, 2016, *Protein Eng Des. Sel.* 29, 457-466).

In one embodiment, the antigen-binding molecule (*e.g.*, a MAb or an antigen-binding fragment thereof), in which each of the IgG1 Fc chains of the antibody carries P329G, L235A, L234A (P329G LALA) mutations or each of the IgG4 Fc chains carries P329G, S228P, L235E mutations, in order to reduce or abolish any undesired cross-linking, platelet activation, or immune effector function (*e.g.*, antibody-dependent cell-meditated cytotoxicity (ADCC), phagocytosis (ADCP) and complement dependent cytotoxicity (CDC)) of the antigen-binding molecule.

In one embodiment, each of the IgG1 Fc chains of the antigen-binding molecule (or antibody) carries mutations comprising a) S239D, A330L and I332E or b) F243L, R292P, Y300L, V305I and P396L, which enhance immune effector function of the antigen-binding molecule (e.g. ADCC).

In one embodiment, the present invention contemplates monovalent antigen-binding molecules produced by co-expression of a light chain, heavy chain and a truncated Fc domain. Suitably, the heavy chain incorporates hole mutations and P329G LALA mutations, while the truncated Fc domain incorporates knob mutations and P329G LALA mutations.

Expression of the antigen-binding molecule disclosed herein can be achieved for example in bacterial (*e.g.*, *Escherichia coli*), yeast, insect or mammalian host cells upon cloning of the protein coding sequences of the constructs in the context of appropriate expression vectors with appropriate translational, transcriptional start sites, and, where appropriate, signal peptide sequences.

In one embodiment, the antigen-binding molecule is a multivalent antigen-binding molecule, non-limiting examples of which include: immunoglobulins, $F(ab')_2$, tandem scFv (taFv or $scFv_2$), scFv-Fc, diabody, dAb_2/V_HH_2, minibodies, ZIP miniantibodies, barnase-barstar dimer, knobs-into-holes derivatives, SEED-IgG, heteroFc-scFv, Fab-scFv, $Fab)_2/sc(Fab)_2$, $scFv-(TNF\alpha)_3$, scFv-Jun/Fos, Fab'-Jun/Fos, tribody, trimerbody, tribi-minibody, barnase-barstar trimer, collabody, DNL-$F(ab)_3$, $scFv_3$-C_H1/C_L, Fab-$scFv_2$, IgG-scFab, IgG-scFv, scFv-IgG, $scFv_2$-Fc, $F(ab')_2$-$scFv_2$, scDB-Fc, scDb-C_H3, Db-Fc, $scFv_2$-H/L, DVD-Ig, tandAb, scFv-dhlx-scFv, dAb_2-IgG, dAb-IgG, dAb-Fc-dAb, tetrabody, streptabody (scFv-streptavidin)$_4$, $(scFv-p53)_4$, $[sc(Fv)_2]_2$; tandem diabody (tandab) and combinations thereof.

In one embodiment, the multivalent antigen-binding molecule is selected from IgG-like antibodies (*e.g.*, triomab/quadroma, Trion Pharma/Fresenius Biotech; knobs-into-holes, Genentech; CrossMAbs, Roche; electrostatically matched antibodies, AMGEN; LUZ-Y, Genentech; strand exchange engineered domain (SEED) body, EMD Serono; biolonic, Merus; and Fab-exchanged antibodies, Genmab), symmetric IgG-like antibodies (*e.g.*, dual targeting (DT)-Ig, GSK/Domantis; two-in-one antibody, Genentech; crosslinked MAbs, karmanos cancer center; MAb_2, F-star; and Coy X-body, Coy X/Pfizer), IgG fusions (*e.g.*, dual variable domain (DVD)-Ig, Abbott; IgG-like bispecific antibodies, Eli Lilly; Ts2Ab, Medimmune/AZ; BsAb, ZymoGenetics; HERCULES, Biogen Idec; TvAb, Roche) Fc fusions (*e.g.*, scFv/Fc fusions, Academic Institution; SCORPION, Emergent BioSolutions/Trubion, ZymoGenetics/BMS; dual affinity retargeting technology (Fc-DART), MacroGenics; dual $(ScFv)_2$-Fab, National Research Center for Antibody Medicine) Fab fusions (*e.g.*, $F(ab)_2$, Medarex/AMGEN; dual-action or Bis-Fab, Genentech; Dock-and-Lock (DNL), ImmunoMedics; bivalent bispecific, Biotechnol; and Fab-Fv, UCB-Celltech), ScFv- and diabody-based antibodies (*e.g.*, bispecific T cell engagers (BiTEs), Micromet; tandem diabodies (Tandab), Affimed; DARTs, MacroGenics; Single-chain diabody, Academic; TCR-like antibodies, AIT, Receptor Logics; human serum albumin scFv fusion, Merrimack; and COMBODIES, Epigen Biotech), IgG/non-IgG fusions (*e.g.*, immunocytokins, EMDSerono, Philogen, ImmunGene, ImmunoMedics; superantigen fusion protein, Active Biotech; and immune mobilizing mTCR Against Cancer, ImmTAC) and oligoclonal antibodies (*e.g.*, Symphogen and Merus).

In one embodiment, the antibody is a bispecific or trispecific antibody. In one embodiment,

the antibody is a bispecific antibody.

In one embodiment, bispecific antibodies of the invention are formed using a "protuberance-into-cavity" strategy, also referred to as "knobs into holes" that serves to engineer an interface between a first and second polypeptide for hetero-oligomerization. The preferred interface comprises at least a part of the CH3 domain of an antibody constant domain. The "knobs into holes" mutations in the CH3 domain of an Fc sequence has been reported to greatly reduce the formation of homodimers (See, for example, Merchant et al., 1998, Nature Biotechnology, 16:677-681). "Protuberances" are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the protuberances are optionally created on the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). Where a suitably positioned and dimensioned protuberance or cavity exists at the interface of either the first or second polypeptide, it is only necessary to engineer a corresponding cavity or protuberance, respectively, at the adjacent interface. The protuberance and cavity can be made by synthetic means such as altering the nucleic acid encoding the polypeptides or by peptide synthesis. For further description of knobs into holes, see U.S. Patents 5,731,168; 5,807,706; 5,821,333.

A general method of preparing a heteromultimer using the "protuberance-into-cavity" strategy comprises expressing, in one or separate host cells, a polynucleotide encoding a first polypeptide that has been altered from an original polynucleotide to encode a protuberance, and a second polynucleotide encoding a second polypeptide that has been altered from the original polynucleotide to encode the cavity. The polypeptides are expressed, either in a common host cell with recovery of the heteromultimer from the host cell culture, or in separate host cells, with recovery and purification, followed by formation of the heteromultimer. In some embodiments, the heteromultimer formed is a multimeric antibody, for example a bispecific antibody.

In one embodiment, the antigen-binding molecule is a chimeric antigen receptor (CAR).

The CAR may comprise an antibody or antigen-binding fragment thereof. In one embodiment, there is provided a chimeric antigen receptor CAR comprising an antibody or

an antigen-binding fragment thereof as defined herein, a transmembrane domain, one or more co-stimulatory domains and an intracellular signaling domain.

The term "chimeric antigen receptor" or "CAR" as used herein refers to an artificial (i.e., man-made) transmembrane protein expressed on a mammalian cell comprising at least an ectodomain, a transmembrane, and an endodomain. Optionally, the CAR protein includes a "spacer" which covalently links the ectodomain to the transmembrane domain. A spacer is often a polypeptide linking the ectodomain to the transmembrane domain via peptide bonds. The CAR is typically expressed on a mammalian lymphocyte. In some embodiments, the CAR is expressed on a mammalian cell such as a T-cell or a tumor infiltrating lymphocyte (TIL). A CAR expressed on a T-cell is referred to herein as a "CAR T-cell" or "CAR-T." In some embodiments the CAR-T is a T helper cell, a cytotoxic T-cell, a natural killer T-cell, a memory T-cell, a regulatory T-cell, or a gamma delta T-cell. When used clinically in, e.g. adoptive cell transfer, a CAR-T with antigen binding specificity to the patient's tumor is typically engineered to express on a native T-cell obtained from the patient. The engineered T-cell expressing the CAR is then infused back into the patient. The CAR-T is thus often an autologous CAR-T although allogeneic CAR-T are included within the scope of the invention. The ectodomain of a CAR comprises an antigen binding region, such as an antibody or antigen binding fragment thereof (e.g. scFv), that specifically binds under physiological conditions with a target antigen, such as a tumor specific antigen. Upon specific binding a biochemical chain of events (i.e., signal transduction) results in modulation of the immunological activity of the CAR-T. Thus, for example, upon specific binding by the antigen binding region of the CAR-T to its target antigen can lead to changes in the immunological activity of the T-cell activity as reflected by changes in cytotoxicity, proliferation or cytokine production. Signal transduction upon CAR-T activation is achieved in some embodiments by the CD3-zeta chain ("CD3-z") which is involved in signal transduction in native mammalian T-cells. CAR-Ts can further comprises multiple signaling domains such as CD28, 41BB or OX40, to further modulate immunomodulatory response of the T-cell. CD3-z comprises a conserved motif known as an immunoreceptor tyrosine-based activation motif (ITAM) which is involved in T-cell receptor signal transduction.

In one embodiment, the transmembrane domain is a transmembrane domain selected from the group consisting of a T cell receptor α chain, a T cell receptor β chain, a CD3 zeta chain, a CD28, a CD3c, a CD45, a CD4, a CD5, a CD8, a CD9, a CD16, a CD22, a CD33, a CD37,

aCD64, a CD80, a CD86, a CD134, a CD137, an ICOS, a CD154 a KIR2D, a NKG2D and a GITR.

In one embodiment, the co-stimulatory domain is a co-stimulatory domain selected from the group consisting of a CD27, CD28, CD40, CD40L, a 4-1BB, a GITR, an ICOS-1, a CD27, an OX-40, Toll-like receptor (TLR), DAP10, DAP12 or 2B4.

In one embodiment, the activating domain comprises a CD3 zeta activating domain.

The CAR may comprise an scFv (V_L-linker-V_H or V_H-linker-V_L), scFv-CH_3 (V_L-linker-V_H-Hinge-CH_3 or V_H-linker-V_L-Hinge-CH_3) or scFv-Fc (V_L-linker-V_H-Hinge-CH_2-CH_3 or V_H-linker-V_L-Hinge-CH_2-CH_3). The scFv-Fc may be linked to a transmembrane domain, a co-stimulatory domain (such as CD28) and an activating domain (such as CD3 zeta activating domain).

Table 2

CAR(KP52)-[short]	MLLLVTSLLLCELPHPAFLLIPDIKMTQSPSSMYASLGEI VTITCKASQDINTYLSWFQQKPGKSPKTLIYRANRLVD GVPSRFSGSGSGQDYFLTISSLEDQDMGIYYCLQYDEFP LTFGAGTKLELKRGGGGSDYKDDDDKGGGGSQVQLQ QSGPELVRPGASVRVSCKASGYSFTDYIITWVKQRTGQ GLEWIGQIYPGSGSIYYNEKFKAKATLTADKSSNTAYM HLSSLTSEDSAVYFCSRGFDYDGNWGQGTTLTVSSESK YGPPCPPCPMFWVLVVVGGVLACYSLLVTVAFIIFWVR SKRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAA YRSGGGRVKFSRSADAPAYQQGQNQLYNELNLGRREE YDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKM AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR (SEQ ID NO: 31)
CAR(KP52)-[intermediate]	MLLLVTSLLLCELPHPAFLLIPDIKMTQSPSSMYASLGEI VTITCKASQDINTYLSWFQQKPGKSPKTLIYRANRLVD GVPSRFSGSGSGQDYFLTISSLEDQDMGIYYCLQYDEFP

	LTFGAGTKLELKRGGGGSDYKDDDDKGGGGSQVQLQ QSGPELVRPGASVRVSCKASGYSFTDYIITWVKQRTGQ GLEWIGQIYPGSGSIYYNEKFKAKATLTADKSSNTAYM HLSSLTSEDSAVYFCSRGFDYDGNWGQGTTLTVSSESK YGPPCPPCPGQPREPQVYTLPPSQEEMTKNQVSLTCLV KGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLS LGKMFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSR GGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSG GGRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDV LDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEA YSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR (SEQ ID NO: 32)
CAR(KP52)-[long]	MLLLVTSLLLCELPHPAFLLIPDIKMTQSPSSMYASLGEI VTITCKASQDINTYLSWFQQKPGKSPKTLIYRANRLVD GVPSRFSGSGSGQDYFLTISSLEDQDMGIYYCLQYDEFP LTFGAGTKLELKRGGGGSDYKDDDDKGGGGSQVQLQ QSGPELVRPGASVRVSCKASGYSFTDYIITWVKQRTGQ GLEWIGQIYPGSGSIYYNEKFKAKATLTADKSSNTAYM HLSSLTSEDSAVYFCSRGFDYDGNWGQGTTLTVSSESK YGPPCPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTC VVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFQ STYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEK TISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGF YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRL TVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK MFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRGGH SDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSGGGRV KFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRR GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPP R (SEQ ID NO: 33)

Provided herein is an isolated polynucleotide encoding a CAR as defined herein. Also provided herein is a vector comprising the polynucleotide as defined herein.

Also provided herein is an engineered cell comprising a vector as defined herein. The engineered cell may be an immune cell. The immune cell may be a T cell, Natural Killer T (NKT) cell or an NK cell.

In one embodiment, there is provided a method of preparing an engineered immune cell, the method comprising introducing the vector as defined herein into an immune cell. The method may comprise a prior step of obtaining an immune cell from a subject.

Disclosed herein is a chimeric molecule comprising an antigen-binding molecule as defined herein and a heterologous moiety.

As used herein, a "chimeric" molecule is one which comprises one or more unrelated types of components or contain two or more chemically distinct regions which can be conjugated to each other, fused, linked, translated, attached via a linker, chemically synthesized, expressed from a nucleic acid sequence, etc. For example, a peptide and a nucleic acid sequence, a peptide and a detectable label, unrelated peptide sequences, and the like. In embodiments in which the chimeric molecule comprises amino acid sequences of different origin, the chimeric molecule includes (1) polypeptide sequences that are not found together in nature (*i.e.*, at least one of the amino acid sequences is heterologous with respect to at least one of its other amino acid sequences), or (2) amino acid sequences that are not naturally adjoined. For example, a "chimeric" antibody" as used herein refers to an antibody in which a portion of the heavy and/or light chain is derived from a particular source or species, while the remainder of the heavy and/or light chain is derived from a different source or species.

In one embodiment, the heterologous moiety is a detectable moiety, a half-life extending moiety or a therapeutic moiety.

Detectable moieties contemplated by the present invention include for example any species known in the art that is appropriate for diagnostic detection, including in vitro detection and in vivo imaging. The detectable moiety may be, for example, a fluorophore, a radionuclide

reporter, a metal-containing nanoparticle or microparticle, an ultrasound contrast agent (e.g., a nanobubble or microbubble) or an optical imaging dye. This also includes contrast particles visible in magnetic resonance imaging (MRI) and magnetic particle imaging (MPI). Fluorophores can be detected and/or imaged, for example, by fluorescence polarization, fluorescence-activated cell sorting and fluorescence microscopy, which may or may not be in combination with electrospray ionization-mass spectrometry (ESI-MS) detection, as well as fluorescence emission computed tomography (FLECT) imaging. Radionuclide reporters can be detected and imaged by radionuclide (nuclear) detection, such as, for example, single-photon emission computed tomography (SPECT), positron emission tomography (PET) or scintigraphic imaging. Metal-containing nanoparticles or microparticles may be detected using optical imaging, including MRI, which is typically used with paramagnetic nanoparticles or microparticles, and MPI, which is generally used with superparamagnetic particles. Ultrasound contrast agents can be detected using ultrasound imaging including contrast-enhanced ultrasound (CEU).

The detectable label may also be an enzyme-substrate label. The enzyme may generally catalyze a chemical alteration of the chromogenic substrate that can be measured using various techniques. For example, the enzyme may catalyze a chemical alteration of the chromogenic substrate that can be measured using the various techniques. For example, the example may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light that can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (e.g., firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases (e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as unease and xanthine oxidase), lactoperoxidase, microperoxidase, and the like.

Examples of enzyme-substrate combinations include, for example:

1) Horseradish peroxidase (HRPO) utilizes hydrogen peroxide to oxidize a dye precursor (e.g.,orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));
2) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and
3) β-D-galactosidase (β-D-Gal) with a chromogenic substrate (e.g., p-nitrophenyl-β-D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl-β-D-galactosidase.

In another embodiment of the invention, the antigen-binding molecule need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the antigen-binding molecule. The antigen-binding molecule of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, immunohistochemistry and immunoprecipitation assays.

In one embodiment, the chimeric molecule comprises at least one heterologous moiety that is a "half-life extending moiety". Half-life extending moieties, can comprise, for example, (i) XTEN polypeptides; (ii) Fc; (iii) albumin, (iv) albumin binding polypeptide or fatty acid, (v) the C-terminal peptide (CTP) of the 13 subunit of human chorionic gonadotropin, (vi) PAS; (vii) HAP; (viii) transferrin; (ix) polyethylene glycol (PEG); (x) hydroxyethyl starch (HES), (xi) polysialic acids (PSAs); (xii) a clearance receptor or fragment thereof which blocks binding of the chimeric molecule to a clearance receptor; (xiii) low complexity peptides; (xiv) or any combinations thereof. In some embodiments, the half-life extending moiety comprises an Fc region. In other embodiments, the half-life extending moiety comprises two Fc regions fused by a linker. Exemplary heterologous moieties also include, e.g., FcRn binding moieties (e.g., complete Fc regions or portions thereof which bind to FcRn), single chain Fc regions (scFc regions, e.g., as described in U.S. Publ. No. 20080260738, WO 2008/012543 and WO 2008/1439545), or processable scFc regions. In some embodiments, a heterologous moiety can include an attachment site for a non-polypeptide moiety such as polyethylene glycol (PEG), hydroxyethyl starch (HES),

In one embodiment, the therapeutic moiety is a toxin. The toxin may, for example, be auristatin E, mertansine (DM-1), saporin, Exatecan (DX8951), gemcitabine, irinotecan, SN-38, etoposide, vinblastine, pemetrexed, docetaxel, paclitaxel, platinum agents (for example, cisplatin, oxaliplatin or carboplatin), vinorelbine, capecitabine, mitoxantrone, ixabepilone,

eribulin, 5-fluorouracil, trifluridine or tipiracil). In one embodiment, the toxin is mertansine (DM-1).

In one embodiment, the ADC conjugation is succinimidyl4-(n-maleimidomethyl)cyclohexane-1-carboxylate-mertansin (SMCC-DM1). This may be done by coupling the lysine residues of the antigen-binding molecule with succinimide group on the toxin-linker

Disclosed herein is an isolated polynucleotide comprising a nucleic acid sequence encoding the antigen-binding molecule as defined herein.

In one embodiment, there is provided an isolated polynucleotide comprising a nucleic acid sequence encoding the chimeric antigen receptor as defined herein.

The term "polynucleotide" or "nucleic acid" are used interchangeably herein to refer to a polymer of nucleotides, which can be mRNA, RNA, cRNA, cDNA or DNA. The term typically refers to polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA.

Also disclosed herein is a vector that comprises a nucleic acid encoding the antigen-binding molecule as described herein.

By "vector" is meant a nucleic acid molecule, preferably a DNA molecule derived, for example, from a plasmid, bacteriophage, or virus, into which a nucleic acid sequence may be inserted or cloned. A vector preferably contains one or more unique restriction sites and may be capable of autonomous replication in a defined host cell including a target cell or tissue or a progenitor cell or tissue thereof, or be integrable with the genome of the defined host such that the cloned sequence is reproducible. Accordingly, the vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a linear or closed circular plasmid, an extrachromosomal element, a mini-chromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the

genome and replicated together with the chromosome(s) into which it has been integrated. A vector system may comprise a single vector or plasmid, two or more vectors or plasmids, which together contain the total DNA to be introduced into the genome of the host cell, or a transposon. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vector may also include a selection marker such as an antibiotic resistance gene that can be used for selection of suitable transformants. Examples of such resistance genes are well known to those of skill in the art.

Disclosed herein is a construct comprising a polynucleotide as defined herein in operable connection with one or more control sequences.

The term "construct" refers to a recombinant genetic molecule including one or more isolated nucleic acid sequences from different sources. Thus, constructs are chimeric molecules in which two or more nucleic acid sequences of different origin are assembled into a single nucleic acid molecule and include any construct that contains (1) nucleic acid sequences, including regulatory and coding sequences that are not found together in nature (i.e., at least one of the nucleotide sequences is heterologous with respect to at least one of its other nucleotide sequences), or (2) sequences encoding parts of functional RNA molecules or proteins not naturally adjoined, or (3) parts of promoters that are not naturally adjoined. Representative constructs include any recombinant nucleic acid molecule such as a plasmid, cosmid, virus, autonomously replicating polynucleotide molecule, phage, or linear or circular single stranded or double stranded DNA or RNA nucleic acid molecule, derived from any source, capable of genomic integration or autonomous replication, comprising a nucleic acid molecule where one or more nucleic acid molecules have been operably linked. Constructs of the present invention will generally include the necessary elements to direct expression of a nucleic acid sequence of interest that is also contained in the construct, such as, for example, a target nucleic acid sequence or a modulator nucleic acid sequence. Such elements may include control elements or regulatory sequences such as a promoter that is operably linked to (so as to direct transcription of) the nucleic acid sequence of interest, and often includes a polyadenylation sequence as well. Within certain embodiments of the invention, the construct may be contained within a vector. In addition to the components of the construct, the vector may include, for example, one or more selectable markers, one or more origins of replication, such as prokaryotic and eukaryotic

origins, at least one multiple cloning site, and/or elements to facilitate stable integration of the construct into the genome of a host cell. Two or more constructs can be contained within a single nucleic acid molecule, such as a single vector, or can be containing within two or more separate nucleic acid molecules, such as two or more separate vectors. An "expression construct" generally includes at least a control sequence operably linked to a nucleotide sequence of interest. In this manner, for example, promoters in operable connection with the nucleotide sequences to be expressed are provided in expression constructs for expression in an organism or part thereof including a host cell. For the practice of the present invention, conventional compositions and methods for preparing and using constructs and host cells are well known to one skilled in the art, see for example, Molecular Cloning: A Laboratory Manual, 3rd edition Volumes 1, 2, and 3. J. F. Sambrook, D. W. Russell, and N. Irwin, Cold Spring Harbor Laboratory Press, 2000.

By "control element", "control sequence", "regulatory sequence" and the like, as used herein, mean a nucleic acid sequence (e.g., DNA) necessary for expression of an operably linked coding sequence in a particular host cell. The control sequences that are suitable for prokaryotic cells for example, include a promoter, and optionally a cis-acting sequence such as an operator sequence and a ribosome binding site. Control sequences that are suitable for eukaryotic cells include transcriptional control sequences such as promoters, polyadenylation signals, transcriptional enhancers, translational control sequences such as translational enhancers and internal ribosome binding sites (IRES), nucleic acid sequences that modulate mRNA stability, as well as targeting sequences that target a product encoded by a transcribed polynucleotide to an intracellular compartment within a cell or to the extracellular environment.

Disclosed herein is a host cell that contains the construct as defined herein.

The terms "host", "host cell", "host cell line" and "host cell culture" are used interchangeably and refer to cells into which exogenous nucleic acid has been introduced, including the progeny of such cells. Host cells include "transformants" and "transformed cells", which include the primary transformed cell and progeny derived therefrom without regard to the number of passages. Progeny may not be completely identical in nucleic acid content to a parent cell, but may contain mutations. Mutant progeny that have the same function or biological activity as screened or selected for in the originally transformed cell

are included herein. A host cell is any type of cellular system that can be used to generate the antigen binding molecules of the present invention. Host cells include cultured cells, *e.g.*, mammalian cultured cells, such as CHO cells, BHK cells, NS0 cells, SP2/0 cells, YO myeloma cells, P3X63 mouse myeloma cells, PER cells, PER.C6 cells or hybridoma cells, yeast cells, insect cells, and plant cells, to name only a few, but also cells comprised within a transgenic animal, transgenic plant or cultured plant or animal tissue. The host cell may also be a mammalian immune cell, such as a T cell, Natural Killer T (NKT) cell or an NK cell. The host cell may also be referred to an engineered cell.

In one embodiment, there is provided an immune cell comprising a construct as defined herein.

Disclosed herein is a pharmaceutical composition comprising an antigen-binding molecule as defined herein or a chimeric molecule as defined herein.

By "pharmaceutically acceptable carrier" is meant a pharmaceutical vehicle comprised of a material that is not biologically or otherwise undesirable, i.e., the material may be administered to a subject along with the selected active agent without causing any or a substantial adverse reaction. Carriers may include excipients and other additives such as diluents, detergents, coloring agents, wetting or emulsifying agents, pH buffering agents, preservatives, and the like.

Representative pharmaceutically acceptable carriers include any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives {*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient(s), its use in the pharmaceutical compositions is contemplated.

The pharmaceutical compositions may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and

infusible solutions), dispersions or suspensions, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Suitable pharmaceutical compositions may be administered intravenously, subcutaneously or intramuscularly. In some embodiments, the compositions are in the form of injectable or infusible solutions. A preferred mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). In specific embodiments, the pharmaceutical composition is administered by intravenous infusion or injection. In other embodiments, the pharmaceutical composition is administered by intramuscular or subcutaneous injection.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. In the subject invention, pharmaceutically acceptable carriers include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Other common parenteral vehicles include sodium phosphate solutions, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the like. Preservatives and other additives can also be present such as for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like.

More particularly, pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In such cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and will preferably be

preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin and/or by the maintenance of the required particle size. In specific embodiments, an agent of the present disclosure may be conjugated to a vehicle for cellular delivery. In these embodiments, the agent may be encapsulated in a suitable vehicle to either aid in the delivery of the agent to target cells, to increase the stability of the agent, or to minimize potential toxicity of the agent. As will be appreciated by a skilled artisan, a variety of vehicles are suitable for delivering an agent of the present disclosure. Non-limiting examples of suitable structured fluid delivery systems may include nanoparticles, liposomes, microemulsions, micelles, dendrimers and other phospholipid-containing systems. Methods of incorporating agents of the present disclosure into delivery vehicles are known in the art. Although various embodiments are presented below, it will be appreciated that other methods known in the art to incorporate an antigen-binding molecule, as described herein, into a delivery vehicle are contemplated.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. An antigen-binding molecule of the present disclosure can be administered on multiple occasions. Intervals between single dosages can be daily, weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of modified polypeptide or antigen in the patient. Alternatively, the antigen-binding molecule can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the polypeptide in the patient.

It may be advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutically acceptable carrier. The specification for the dosage unit forms of the invention are dictated by and directly

dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Dosages and therapeutic regimens of the antigen-binding molecule can be determined by a skilled artisan. In certain embodiments, the antigen-binding molecule is administered by injection (*e.g.*, subcutaneously or intravenously) at a dose of about 0.01 to 40 mg/kg, *e.g.*, 0.01 to 0.1 mg/kg, *e.g.*, about 0.1 to 1 mg/kg, about 1 to 5 mg/kg, about 5 to 25 mg/kg, about 10 to 40 mg/kg. The dosing schedule can vary from *e.g.*, once a week to once every 2, 3, or 4 weeks.

It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

Disclosed herein is an antigen-binding molecule as defined herein, a chimeric molecule as define herein, a host cell as defined herein, or a pharmaceutical composition as defined herein for use as a medicament.

Disclosed herein is a method for reducing or inhibiting proliferation and/or viability of a cancer cell, the method comprising contacting the cancer cell with a therapeutically effective amount of an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein or a pharmaceutical composition as defined herein.

In one embodiment, provided herein is an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein or a pharmaceutical composition as defined herein for use in reducing or inhibiting proliferation and/or viability of a cancer cell.

In one embodiment, provided herein is the use of an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein or a

pharmaceutical composition as defined herein in the manufacture of a medicament for reducing or inhibiting proliferation and/or viability of a cancer cell.

The terms “cancer” and “cancerous” refer to or describe the physiological condition in mammals that is typically characterized in part by unregulated cell growth. As used herein, the term “cancer” refers to non-metastatic and metastatic cancers, including early stage and late stage cancers. By “non-metastatic” is meant a cancer that remains at the primary site and has not penetrated into the lymphatic or blood vessel system or to tissues other than the primary site. The term "metastatic cancer" refers to cancer that has spread or is capable of spreading from one part of the body to another. Generally, a non-metastatic cancer is any cancer that is a Stage 0, I, or II cancer, and occasionally a Stage III cancer. A metastatic cancer, on the other hand, is usually a stage IV cancer.

The term "cancer" includes but is not limited to, breast cancer, large intestinal cancer, lung cancer, small cell lung cancer, gastric (stomach) cancer, liver cancer, blood cancer, bone cancer, pancreatic cancer, skin cancer, head and/or neck cancer, cutaneous or intraocular melanoma, uterine sarcoma, ovarian cancer, rectal or colorectal cancer, anal cancer, colon cancer, fallopian tube carcinoma, endometrial carcinoma, cervical cancer, vulval cancer, squamous cell carcinoma, vaginal carcinoma, Hodgkin's disease, non-Hodgkin's lymphoma, esophageal cancer, small intestine cancer, endocrine cancer, thyroid cancer, parathyroid cancer, adrenal cancer, soft tissue tumor, urethral cancer, penile cancer, prostate cancer, chronic or acute leukemia, lymphocytic lymphoma, bladder cancer, kidney cancer, ureter cancer, renal cell carcinoma, renal pelvic carcinoma, CNS tumor, glioma, astrocytoma, glioblastoma multiforme, primary CNS lymphoma, bone marrow tumor, brain stem nerve gliomas, pituitary adenoma, uveal melanoma (also known as intraocular melanoma), testicular cancer, oral cancer, pharyngeal cancer or a combination thereof.

In one embodiment, the cancer cell is a solid or haematological cancer cell.

The term “solid cancer” may refer to one or more of breast cancer, large intestinal cancer, lung cancer, small cell lung cancer, gastric (stomach) cancer, gastrointestinal cancer, liver cancer, bone cancer, pancreatic cancer, skin cancer, head and/or neck cancer, cutaneous or intraocular melanoma, uterine sarcoma, ovarian cancer, rectal or colorectal cancer, anal cancer, colon cancer, fallopian tube carcinoma, endometrial carcinoma, cervical cancer,

vulval cancer, squamous cell carcinoma, vaginal carcinoma, esophageal cancer, small intestine cancer, endocrine cancer, thyroid cancer, parathyroid cancer, adrenal cancer, soft tissue tumor, urethral cancer, penile cancer, prostate cancer, bladder cancer, kidney cancer, ureter cancer, renal cell carcinoma, renal pelvic carcinoma, CNS tumor, glioma, astrocytoma, glioblastoma multiforme, primary CNS lymphoma, bone marrow tumor, brain stem nerve gliomas, pituitary adenoma, uveal melanoma (also known as intraocular melanoma), testicular cancer, oral cancer, pharyngeal cancer, sarcomas or a combination thereof.

The term "haematological cancer' may refer to one or more of leukemia, lymphoma, Chronic Myeloproliferative Disorders, Langerhans Cell Histiocytosis, Multiple Myeloma/Plasma Cell Neoplasm, Myelodysplasia Syndromes, Myelodysplastic/Myeloproliferative Neoplasms or a combination thereof. In some embodiments, leukemia is any one or more of Acute Lymphoblastic Leukemia (ALL), Acute Myeloid Leukemia (AML), Chronic Lymphocytic Leukemia (CLL), Chronic Myelogenous Leukemia (CML), Hairy Cell Leukemia (HCL) or a combination thereof. In some embodiments, lymphoma is any one or more of AIDS-Related Lymphoma, Cutaneous T- Cell Lymphoma, Hodgkin Lymphoma, Mycosis Fungoides, Non-Hodgkin Lymphoma, Primary Central Nervous System Lymphoma, Sezary Syndrome, T-Cell Lymphoma, Cutaneous, Waldenstrom Macroglobulinemia, B cell lymphoma or a combination thereof.

In one embodiment, the cancer is a metastatic cancer. The cancer may be a refractory or a relapsed cancer. The cancer may be a TROP2-expressing cancer. The cancer may be a cancer associated with undesired expression of TROP2.

Disclosed herein is a method of treating a cancer in a subject, the method comprising administering a therapeutically effective amount of an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein to the subject.

Disclosed herein is a method of treating a disease or condition associated with an undesired expression of TROP2 in a subject, wherein the method comprises administering a therapeutically effective amount of an antigen-binding molecule as defined herein, a

chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein to the subject.

In one embodiment, provided herein is an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein for use in treating a cancer in a subject. In one embodiment, provided herein is an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein for use in treating a disease or condition associated with an undesired expression of TROP2 in a subject.

In one embodiment, provided herein is the use of an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein in the manufacture of a medicament for treating a cancer in a subject. In one embodiment, provided herein is the use of an antigen-binding molecule as defined herein, a chimeric molecule as defined herein, a host cell as defined herein, or a pharmaceutical composition as defined herein in the manufacture of a medicament for treating a disease or condition associated with an undesired expression of TROP2 in a subject.

The term "treating" as used herein may refer to (1) delaying the appearance of one or more symptoms of the condition; (2) inhibiting the development of the condition or one or more symptoms of the condition; (3) relieving the condition, *i.e.*, causing regression of the condition or at least one or more symptoms of the condition; and/or (4) causing a decrease in the severity of the condition or of one or more symptoms of the condition.

The terms "subject", "patient", "host" or "individual" used interchangeably herein, refer to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject, for whom therapy or prophylaxis is desired. Suitable vertebrate animals that fall within the scope of the invention include, but are not restricted to, any member of the subphylum Chordata including primates (*e.g.*, humans, monkeys and apes, and includes species of monkeys such as from the genus Macaca (*e.g.*, cynomolgus monkeys such as Macaca fascicularis, and/or rhesus monkeys (Macaca mulatta)) and baboon (Papio ursinus), as well as marmosets (species from the genus Callithrix), squirrel monkeys (species from

the genus Saimiri) and tamarins (species from the genus Saguinus), as well as species of apes such as chimpanzees (Pan troglodytes)), rodents (*e.g.*, mice rats, guinea pigs), lagomorphs (*e.g.*, rabbits, hares), bovines (*e.g.*, cattle), ovines (*e.g.*, sheep), caprines (*e.g.*, goats), porcines (*e.g.*, pigs), equines (*e.g.*, horses), canines (*e.g.*, dogs), felines (*e.g.*, cats), avians (*e.g.*, chickens, turkeys, ducks, geese, companion birds such as canaries, budgerigars etc.), marine mammals (*e.g.*, dolphins, whales), reptiles (snakes, frogs, lizards etc.), and fish. In one embodiment, the subject is a feline subject. In another embodiment, the subject is a human subject.

The methods as disclosed herein may comprises the administration of a "therapeutically effective amount" of an agent (e.g. an antigen-binding molecule, a chimeric molecule, a polynucleotide, a construct, a vector, a host cell or a pharmaceutical composition) to a subject. As used herein the term "therapeutically effective amount" includes within its meaning a non-toxic but sufficient amount of an agent or compound to provide the desired therapeutic effect. The exact amount required will vary from subject to subject depending on factors such as the species being treated, the age and general condition of the subject, the severity of the condition being treated, the particular agent being administered and the mode of administration and so forth. Thus, it is not possible to specify an exact "effective amount". However, for any given case, an appropriate "effective amount" may be determined by one of ordinary skill in the art using only routine experimentation.

Disclosed herein is a method of detecting the likelihood of the presence of a cancer in a subject, the method comprising determining the level of TROP2 in a sample obtained from the subject, wherein an increased level of TROP2 as compared to a reference indicates the likelihood of the presence of a cancer in the subject.

The method may comprise contacting the sample with an antigen-binding molecule as defined herein or a chimeric molecule as defined herein to determine the level of TROP2 in the sample.

Samples herein may be tissue samples, biological fluids, or cultures derived from tissue or fluid samples.

In one embodiment, the sample is a bodily fluid or a liquid biopsy. Bodily fluids include but

are not limited to peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, bronchoalveolar lavage fluid, semen (including prostatic fluid), Cowper's fluid or pre-ejaculatory fluid, female ejaculate, sweat, fecal matter, tears, cyst fluid, pleural fluid, peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates and other lavage fluids. In one embodiment, the sample is a blood, serum or plasma sample.

In some embodiments, the sample is a cancer sample. As used herein, a "cancer sample" refers to a sample derived from a cancerous cell or tissue. Cancer samples would typically contain cancer cells, circulating tumor cells (CTCs), and/or cellular material (e.g., cellular vesicles, secretions or debris) derived from cancer cells or CTCs.

The reference may be TROP2 expression level in a sample from a subject of the same species without cancer, or an average expression level in samples from a population of subjects of the same species (e.g., of varying ages, ethnic backgrounds and genders) without cancer. The reference may be TROP2 expression level in a non-cancerous tissue sample from the same subject. Alternatively, the reference may be the expression level in a sample from the same subject before the suspected onset of a cancer. Reference values may be TROP2 mRNA or protein levels. The reference values can be a predetermined value and can be stored in a database and used as a reference in subsequent analyses. In one embodiment, methods herein comprise the step of comparing the level of TROP2 expression in the sample to the reference.

The measured expression level of a gene or protein may first be normalised before comparison with a reference. Normalisation is typically used to control for unwanted biological variation. In a non-limiting example, biological variation can result from some feature of the subject or the sample collection that is not relevant to the methods of the present disclosure, such as variations created by collecting samples at different times of the day and variations due to the age or gender of the subject.

As used herein, the term "increase" or "increased" with reference to a biomarker refers to a statistically significant and measurable increase in the biomarker as compared to a reference.

The increase may be an increase of at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 100%.

As used herein, the term "decrease" or "decreased" with reference to a biomarker refers to a statistically significant and measurable decrease in the biomarker as compared to a reference. The decrease may be a decrease of at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90%.

Methods herein may be combined with other biomarkers or other clinical methods to provide a more comprehensive prediction of treatment outcome. Such biomarkers may include non-genetic factors such as demographic or clinical variables, non-limiting examples of which include gender, age, lifestyle (e.g., diet, frequency of physical activity, alcohol intake, tobacco use), physiological parameters (e.g., body mass index, blood pressure), family history of disease, clinical history of cancer and/or other comorbid diseases or conditions, and other diagnostic indications of cancer (e.g., levels of other biomarkers, medical imaging). Clinical methods may include imaging methods and histopathological analyses of biopsy samples.

Methods herein may be performed prior to, during or after a cancer therapy, and may be performed on multiple occasions over a period of time.

As used herein, "and/or" refers to and encompasses any and all possible combinations of one or more of the associated listed items, as well as the lack of combinations when interpreted in the alternative (or).

As used in this application, the singular form "a," "an," and "the" include plural references unless the context clearly dictates otherwise. For example, the term "an agent" includes a plurality of agents, including mixtures thereof.

Throughout this specification and the statements which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will

be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Throughout this specification and the statements which follow, unless the context requires otherwise, the phrase "consisting essentially of", and variations such as "consists essentially of" will be understood to indicate that the recited element(s) is/are essential i.e. necessary elements of the invention. The phrase allows for the presence of other non-recited elements which do not materially affect the characteristics of the invention but excludes additional unspecified elements which would affect the basic and novel characteristics of the method defined.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications, which fall within the spirit and scope. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Unless otherwise defined, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs.

Certain embodiments of the invention will now be described with reference to the following examples which are intended for the purpose of illustration only and are not intended to limit the scope of the generality hereinbefore described.

EXAMPLES

Characterisation of KP52

Leveraging on the Antibody Development Platform (ADP) in BTI, KP52, an IgG2a mouse mAb was generated using an in-house derived feline mammary cancer (Kelly F2) as immunogen. In addition to recognising feline mammary cancer, KP52 also exhibited cross-reactivity against pan-human cancers including breast cancer, lung cancer, pancreatic cancer, gastric cancer, ovarian cancer and cholangiocarcinoma (Figure 1). Using western blot analysis, a smear was observed between 40 to 60 kDa in molecular weight (MW) under non-reducing conditions. Under reducing conditions, KP52 binding was not affected and revealed a doublet with MW of about 45 and 50 kDa for human cancers and feline mammary cancer Kelly F2 (Figure 2).

The antigen target of KP52 was identified to be Trophoblast cell surface antigen 2 (TROP2) through immunoprecipitation-coupled with mass spectrometry (IP-MS) (Figure 3 and Figure 4) and further validated with cross probing with commercial anti-TROP2 antibody (Figure 5A) and siRNA knockdown (Figure 5B). As TROP2 is a heavily glycosylated protein with four N-linked glycosylation sites in the extracellular domain, KP52 was subjected to a series of glycan studies to determine if it binds to a glycosylated motif on TROP2. Firstly, periodate (Figure 6) oxidises carbohydrate moiety of glycoproteins and the disruption affects binding of mAbs if such confirmation is needed for binding. The abolishment of KP52 binding to TROP2 in Kelly F2, and various human cancer cell lines indicates that glycan is important for KP52 binding. Next, pronase assay (Figure 7) was used to determine if KP52 binds to the protein backbone of TROP2. KP52 was still able to recognise TROP2 even after treatment. Hence both periodate and pronase assays shows that KP52 binds to glycans. Hence, chemical and enzymatic reactions were used to determine if KP52 recognises O-linked glycan or N-linked glycans (Figure 8). The ability of KP52 to detect the protein band after enzyme Peptide: N-glycosidase F treatment, indicates that KP52 does not bind to N-linked glycans. On the contrary, bands were not observed after chemical treatment to remove O-linked glycans. Therefore, KP52 recognises O-linked glycans of TROP2. Further characterisation of KP52's binding on TROP2 has identified that epitope is residing within the Cysteine-Rich Domain (CRD) in the extracellular domain of the protein (Figure 27).

TROP2 is a type I transmembrane glycoprotein that functions as an intracellular calcium signal transducer. It is upregulated in many cancers and is associated with increased cancer

metastasis, aggressiveness and poor disease prognosis. Hence, TROP2 is an attractive target for therapy. However, TROP2 is also expressed in many normal tissues such as breast, salivary gland, skin, pancreas and lungs. Hence, the specificity of KP52 to normal and malignant tissues were tested on fresh frozen tissue array. For normal tissues (Figure 9), immunoreactivity were comparable to the negative control stain, except for salivary gland (Figure 9B). Downstream preclinical in vitro and in vivo toxicology assessment can be carried out to determine if the binding of salivary gland results in adverse toxicity in patients. Instead, KP52 binds very strongly to wide variety of malignant tissues, with 13/20 (65%) cores stained (Figure 10). Therefore, KP52 is highly tumour specific with minimal binding to normal tissues.

In terms of functionality, KP52 can function as an antibody-drug conjugate (ADC) (Figure 12). Using secondary anti-mouse Ig ADC that are conjugated to different cytotoxic payload, mainly saporin (ZAP), mertansine (DM1) and monomethyl auristatin E (MMAE). As seen in Figure 12, there was a decrease in cell viability (orange arrows) compared to the control groups. Additionally, there were no cytotoxicity seen in the negative binding cell line, HCC-1395. This shows that KP52 is required to bind to cell surface before drugs are internalised into cells and exert their killing effect. Therefore, KP52 can function as an ADC in both feline mammary cancer and various human cancers.

Direct conjugation of KP52 to DM1 (KP52-DM1)

As proof of concept, KP52 was directly conjugated to DM1 payload, using non-cleavable SMCC linker (KP52-DM1). This conjugation was conducted by Moradec LLC. The drug to antibody ratio (DAR) determined by A252:A280 is 3.1. The binding of KP52-DM1 in Kelly F2 and human cancer cell lines was retained (Figure 13). Additionally, the IC50 of KP52-DM1 was in the nanomolar range of 0.99nM and 0.97nM for NCI-N87 and HCC1937 respectively (Figure 14). The efficacy of KP52-DM1 was further demonstrated in *in vivo* HCC-1937 breast cancer xenograft model (Figure 15). Mice were randomised into five different treatment groups of five mice each when xenografts were reached approximately 100mm^3. The different treatment groups are 1mg/kg KP52-DM1, 10mg/kg KP52-DM1, equivalent moles of KP52 only and free DM1 of the highest dose of ADC, and buffer (PBS) control. The mice were treated twice weekly for two weeks by intravenous tail vein injections. Mice treated with KP52 alone, free DM1 and 1mg/kg KP52-DM1 did not have

reduction in tumour growth as their growth were comparable to control. However, mice treated with 10mg/kg KP52-DM1 had complete tumour regression after the fourth dose and mice remained tumour free till the end of study (Figure 15A). This dose was well tolerated in mice, with no reduction in weight loss or toxicity observed (Figure 15C). Tumours that did not regress were harvested when they were more than $1000mm^3$ or ulcerated. These harvested xenografts were processed for flow cytometry and had more than 70% binding with KP52 and KP52-DM1 (Figure 15D). This means that mice treated with 1mg/kg ADC dosage was too low to confer cytotoxic effect and not due to loss of antigen expression. Therefore, with the right dose, KP52-DM1 is able to bind to antigen target on the xenograft and exert its killing effect, resulting in complete elimination of tumour.

Humanisation of KP52 (hKP52)

Since KP52 have demonstrated great potential to function as an ADC *in vitro* and *in vivo*, it was further developed to minimise the risk of immunogenicity through humanisation. Firstly, the sequence of KP52 was obtained through molecular genetic techniques. Subsequently, the complementarity determining region (CDR) were grafted onto human IgG1 frameworks. The sequences were then put through in silico sequence liabilities and immunogenicity checks. Finally, two variable heavy chains (VH1 and VH2) and two variable light chains (VL1 and VL2) sequences were obtained (Figure 16). These sequences were then cloned into an expression vector and in order to determine the best sequence, various permutations of the heavy and light chains were transiently transfected into HEK293 cells. For simplicity, VH1/VL1 is known as hKP52_1, VH1/VL2 as hKP52_2, VH2/VL1 as hKP52_3 and VH2/VL2 as hKP52_4. These hKP52 mAbs underwent various tests to determine the best humanised sequence.

Firstly, the binding of the four hKP52 mAbs were tested on immunogen Kelly F2, human breast cancer HCC-1937 and non-binding HCC-1395 cell lines (Figure 17). Unlike hKP52_1 and hKP52_2, the binding specificities for hKP52_3 and hKP52_4 were comparable to parental KP52 on Kelly F2 and HCC-1937. Like KP52, all four mAbs were negative binders to HCC-1395. Based on competitive binding assay, KP52 and all hKP52 recognises the same epitope as parental KP52 (Figure 18). Additionally, using indirect ADC assay with hDM1 as the secondary payload, all hKP52 mAbs had significant amount of killing in all cell lines except non-binding HCC-1395 which served as negative control (Figure 19). The affinity of

the mAbs were also conducted (Figure 20), with all hKP52 mAbs having affinity (KD) in the nanomolar range towards TROP2. The mAb with the highest affinity to TROP2 is hKP52_3.

Comparing KP52 with a biosimilar mouse version of approved anti-TROP2 mAb, RS7

Sacituzumab govitecan is an ADC composed of human anti-TROP2 mAb hRS7, linked to a topoisomerase I inhibitor, SN-38, via a cleavable pH-sensitive CL2A linker (1-3). Using a parental murine version of Sacituzumab govitecan known as RS7, it was compared with KP52 and there were some differences that set them apart.

Firstly, KP52 and RS7 were evaluated in binding assays against feline mammary cancer and various human cancer cell lines using flow cytometry (Figure 21). RS7 and KP52 have similar binding intensity to human cancer cell lines. However, RS7 have weak binding to Kelly F2, compared to KP52 which have a strong binding. This suggest that the epitope of RS7 is not conserved in feline mammary cancer and may not be suitable for comparative oncology, unlike KP52.

To further ascertain that KP52 and RS7 are differentiated from each other, western blot analysis was conducted (Figure 22). RS7 have a conformational epitope as no bands were detected by RS7 in the reducing condition. This was supported an earlier study whereby chemical and enzymatic treatments with western blot studies suggest that RS7 antigenic determinant is composed of conformational-dependent peptide. Conversely, KP52 recognises a glycan epitope that is only partially affected under reducing conditions. Hence, although both mAbs bind to TROP2, they are discrete and distinct from each other.

Since KP52 functions as an ADC and RS7 was developed into an ADC, the cytotoxicity of both mAbs as an ADC were compared. Using the indirect ADC assay with secondary mAb conjugated to ZAP as the payload, KP52 + ZAP was more efficacious compared to RS7 + ZAP in all the various doses tested (Figure 23).

Subsequently, the specificity of RS7 was compared with KP52 on fresh frozen tissue array, using immunohistochemistry. KP52 and RS7 bind to similar number of malignant tissues with comparable staining intensity (Figure 24). On the other hand, KP52 recognises normal

salivary gland tissues, but RS7 binds to normal salivary gland and pancreas (Figure 25). However, the FDA and EMA have stated that hRS7 binds to normal tissues with membranal staining including salivary gland and pancreas. But, there were no specific safety concerns identified (5, 6). Moreover, the adverse effect of Sacituzumab govitecan in clinical trials were related to gastrointestinal tract and haematological toxicity but unrelated to salivary glands (7). Hence, the binding of KP52 to salivary gland tissues is inconsequential.

Demonstration of KP52 functionality as a CAR-T cell therapeutic

In vitro functionality of KP52 as a CAR construct in T cells has also been demonstrated against two cell lines, PC-9 and HCC1937 (Figure 26). T cells transfected with CAR(KP52) constructs have shown cell cytotoxicity in the two target cell lines, and CAR-T cell expansion displayed after 6 days of co-culture. TROP2 negative cells (BT474 and H1299) were not affected, demonstrating specificity only towards TROP2 expressing cancer cell lines.

Conclusion

In conclusion, using a comparative oncology approach to identify a unique conserved antigen in feline mammary cancer and pan-human cancers, have clearly supported our hypothesis that there is conservation of antigens between companion animal (CA) cancers and human cancers. Furthermore, using an ADC-based therapy, to target this unique O-linked glycan on TROP2 have demonstrated killing efficacy *in vitro* and *in vivo*. Additionally, KP52 was superior in terms of binding, specificity and efficacy compared to commercially approved mAb. Although only the humanisation development of KP52 was reviewed, KP52 could also be developed for feline mammary cancer treatment. Thus, this comparative oncology approach could benefit both the veterinary and human medicine, by targeting a novel antigen target.

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CLAIMS

1. An antigen-binding molecule that specifically binds to human and feline Human Trophoblast Cell Surface Antigen 2 (TROP2).

2. The antigen-binding molecule of claim 1, wherein the antigen-binding molecule specifically binds to the cysteine-rich domain (CRD) in the extracellular domain of human or feline TROP2.

3. The antigen-binding molecule of claim 2, wherein the antigen-binding molecule specifically binds to SEQ ID NO: 26 or SEQ ID NO: 27.

4. The antigen-binding molecule of any one of claims 1 to 3, wherein the antigen-binding molecule comprises:
   a) a heavy chain variable (VH) region comprising the VHCDR1 amino acid sequence GYSFTDYIIT (SEQ ID NO: 1), the VHCDR2 amino acid sequence QIYPGSGSIYYNEKFKA (SEQ ID NO: 2) and the VHCDR3 amino acid sequence GFDYDGN (SEQ ID NO: 3); and
   b) a light chain variable (VL) region comprising the VLCDR1 amino acid sequence KASQDINTYLS (SEQ ID NO: 4), the VLCDR2 amino acid sequence RANRLVD (SEQ ID NO: 5) and the VLCDR3 amino acid sequence LQYDEFPLT (SEQ ID NO: 6).

5. The antigen-binding molecule of any one of claims 1 to 4, wherein the antigen-binding molecule is an antibody or antigen-binding fragment thereof.

6. The antigen-binding molecule of claim 5, wherein the antibody or antigen binding fragment thereof is a full-length antibody, a substantially intact antibody, a Fab fragment, a scFab, a Fab', a single chain variable fragment (scFv) or a one-armed antibody.

7. The antigen-binding molecule of any one of claims 1 to 4, wherein the antigen-binding molecule is a chimeric antigen receptor (CAR).

8. The antigen-binding molecule of any one of claims 1 to 7, wherein the antibody or antigen-binding molecule therefore is humanized or felinized.

9. The antigen-binding molecule of any one of claims 1 to 8, wherein the antigen-binding molecule comprises:
a) a VH region comprising an amino acid sequence having at least 70% sequence identity to SEQ ID NO: 20 or 21; and
b) a VL region comprising an amino acid sequence having at least 70% sequence identity to SEQ ID NO: 22 or 23.

10. A chimeric molecule comprising an antigen-binding molecule according to any one of claims 1 to 9 and a heterologous moiety.

11. The chimeric molecule of claim 10, wherein the heterologous moiety is a detectable moiety, a half-life extending moiety or a therapeutic moiety.

12. The chimeric molecule of claim 11, wherein the therapeutic moiety is a toxin.

13. The chimeric molecule of claim 12, wherein the toxin is Mertansine (DM1),

14. An isolated polynucleotide comprising a nucleic acid sequence encoding the antigen-binding molecule according to any one of claims 1 to 9.

15. A construct comprising a polynucleotide of claim 14 in operable connection with one or more control sequences.

16. A host cell that contains the construct of claim 15.

17. The host cell of claim 16, wherein the host cell is a NK cell or a T cell.

18. A pharmaceutical composition comprising an antigen-binding molecule according to any one of claims 1 to 9 or a chimeric molecule according to any one of claims 10 to 13.

19. An antigen-binding molecule according to any one of claims 1 to 9, a chimeric molecule according to any one of claims 10 to 13, a host cell of claim 16 or 17, or a pharmaceutical composition according to claim 18 for use as a medicament.

20. A method for reducing or inhibiting proliferation and/or viability of a cancer cell, the method comprising contacting the cancer cell with a therapeutically effective amount of an antigen-binding molecule according to any one of claims 1 to 9, a chimeric molecule according to any one of claims 10 to 13, a host cell of claim 16 or 17, or a pharmaceutical composition according to claim 18.

21. A method of treating a cancer in a subject, the method comprising administering a therapeutically effective amount of an antigen-binding molecule according to any one of claims 1 to 9, a chimeric molecule according to any one of claims 10 to 13, a host cell of claim 16 or 17, or a pharmaceutical composition according to claim 18 to the subject.

22. The method of claim 21, wherein the cancer is a TROP2 expressing cancer.

23. The method of claim 21 or 22, wherein the cancer is breast cancer, lung cancer, pancreatic cancer, gastrointestinal cancer, ovarian cancer or cholangiocarcinoma.

24. The method of any one of claims 21 to 23, wherein the subject is a human or feline subject.

25. A method of treating a disease or condition associated with an undesired expression of TROP2 in a subject, wherein the method comprises administering a therapeutically effective amount of an antigen-binding molecule according to any one of claims 1 to 9, a chimeric molecule according to any one of claims 10 to 13, a host cell of claim 16 or 17, or a pharmaceutical composition according to claim 18 to the subject.

26. A method of detecting the likelihood of the presence of a cancer in a subject, the method comprising determining the level of TROP2 in a sample obtained from the subject, wherein an increased level of TROP2 as compared to a reference indicates

the likelihood of the presence of a cancer in the subject.

27. The method of claim 26, wherein the sample is a cell, tissue or blood sample.

28. The method of claim 26 or 27, wherein the method comprises contacting the sample with an antigen-binding molecule according to any one of claims 1 to 9 or a chimeric molecule according to any one of claims 10 to 13 to determine the level of TROP2 in the sample.