

were originally characterized by their ability to kill tumor cells effectively without the need for prior sensitization. Activated NK cells kill target cells by means similar to cytotoxic T cells – *i.e.*, via cytolytic granules that contain perforin and granzymes as well as via death receptor pathways. Activated NK cells also secrete inflammatory cytokines such as IFN- γ and chemokines that promote the recruitment of other leukocytes to the target tissue.

[0007] NK cells respond to signals through a variety of activating and inhibitory receptors on their surface. For example, when NK cells encounter healthy self-cells, their activity is inhibited through activation of the killer-cell immunoglobulin-like receptors (KIRs). Alternatively, when NK cells encounter foreign cells or cancer cells, they are activated via their activating receptors (*e.g.*, NKG2D, NCRs, DNAM1). NK cells are also activated by the constant region of some immunoglobulins through CD16 receptors on their surface. The overall sensitivity of NK cells to activation depends on the sum of stimulatory and inhibitory signals. NKG2D is a type-II transmembrane protein that is expressed by essentially all natural killer cells where NKG2D serves as an activating receptor. NKG2D is also found on T cells where it acts as a costimulatory receptor. The ability to modulate NK cell function via NKG2D is useful in various therapeutic contexts including malignancy.

[0008] Mutations that lead to epidermal growth factor receptor (EGFR) overexpression or overactivity have been associated with a number of cancers, including non-small cell lung cancer, anal cancers, glioblastoma and epithelial tumors of the head and neck. These somatic mutations involving EGFR lead to its constant activation, which produces uncontrolled cell division. In glioblastoma a more or less specific mutation of EGFR, called EGFRvIII is often observed. Mutations, amplifications or misregulations of EGFR or family members are implicated in other solid tumors, including colorectal cancer, renal cell carcinoma, bladder cancer, cervical cancer, ovarian cancer, pancreatic cancer, and liver cancer.

[0009] Anti-EGFR monoclonal antibodies, such as cetuximab, panitumumab, necitumumab, and zalutumumab, have been developed. However, there remains a need for new and useful antibodies and related therapies for use in treatment of cancer that have greater efficacy and reduced adverse effects.

SUMMARY

[0010] The present application provides multispecific binding proteins that bind to the NKG2D receptor, CD16 receptor on natural killer cells, and EGFR. Such proteins can engage more than one kind of NK-activating receptor, and may block the binding of natural ligands to NKG2D. In one aspect, the present disclosure provides a protein comprising: (a) a first

antigen-binding site that binds NKG2D; (b) a second antigen-binding site that binds EGFR; and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16, wherein the second antigen-binding site comprises: (i) a heavy chain variable domain (VH) comprising complementarity-determining region 1 (CDR1), complementarity-determining region 2 (CDR2), and complementarity-determining region 3 (CDR3) sequences of SEQ ID NOs: 136, 146, and 138, respectively; and a light chain variable domain (VL) comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively; (ii) a VH comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 137, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively; or (iii) a VH comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 146, and 138, respectively; and a VL comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 142, respectively; wherein the VL of the second antigen-binding site comprises an F87Y substitution relative to SEQ ID NO:139, numbered under the Chothia numbering scheme. In certain ~~embodiment~~examples, the proteins can agonize NK cells in humans. In some

embodimentexamples, the proteins can agonize NK cells in humans and in other species such as rodents and cynomolgus monkeys. Formulations containing any one of the proteins disclosed herein; cells containing one or more nucleic acids expressing the proteins, and methods of enhancing tumor cell death using the proteins are also provided.

5 [0011] Accordingly, one aspectexample of the present application provides a protein comprising (a) a first antigen-binding site that binds NKG2D, (b) a second antigen-binding site that binds EGFR, and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16, wherein the second antigen-binding site comprises: (i) a heavy chain variable domain (VH) comprising complementarity-determining region 1 (CDR1), complementarity-determining region 2 (CDR2), and complementarity-determining region 3 (CDR3) sequences of SEQ ID NOs: 136, 157, and 138, respectively, and a light chain variable domain (VL) comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively; or (ii) a VH comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 146, and 138, respectively, and a VL comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 142, respectively.

[0012] In some embodimentexamples, the VH comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 157, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively.

20 [0013] In some embodimentexamples, the VH comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 146, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively.

[0014] In some embodimentexamples, the VH comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 137, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively.

25 [0015] In some embodimentexamples, the VH comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 146, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 142, respectively.

30 [0016] In some embodimentexamples, the VH comprises an amino acid sequence at least 90% identical to SEQ ID NO:145 and the VL comprises an amino acid sequence at least 90% identical to SEQ ID NO:150. In some embodimentexamples, the VH comprises the amino acid sequence of SEQ ID NO:145 and the VL comprises the amino acid sequence of SEQ ID NO:150. In some embodimentexamples, the VH comprises the amino acid sequence of SEQ ID NO:170 and the VL comprises the amino acid sequence of SEQ ID NO:171.

[0017] In some embodimentexamples, the VH comprises an amino acid sequence at least 90% identical to SEQ ID NO:135 and the VL comprises an amino acid sequence at least 90% identical to SEQ ID NO:150. In some embodimentexamples, the VH comprises the amino acid sequence of SEQ ID NO:135 and the VL comprises the amino acid sequence of SEQ ID NO:150.

[0018] In some embodimentexamples, the VH comprises an amino acid sequence at least 90% identical to SEQ ID NO:145 and the VL comprises an amino acid sequence at least 90% identical to SEQ ID NO:147. In some embodimentexamples, the VH comprises the amino acid sequence of SEQ ID NO:145 and the VL comprises the amino acid sequence of SEQ ID NO:147.

[0019] Another aspectexample of the present application protein comprising (a) a first antigen-binding site that binds NKG2D, (b) a second antigen-binding site that binds EGFR, and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16, wherein the second antigen-binding site comprises a VH comprising an amino acid sequence at least 90% identical to SEQ ID NO:135 and a VL comprising an amino acid sequence at least 90% identical to SEQ ID NO:139, wherein the VH comprises an S62R substitution relative to SEQ ID NO:135 and/or the VL comprises a D92R substitution and/or an F87Y substitution relative to SEQ ID NO:139, numbered under the Chothia numbering scheme.

[0020] In some embodimentexamples, the VH comprises an S62R substitution relative to SEQ ID NO:135, numbered under the Chothia numbering scheme. In some embodimentexamples, the VL comprises a D92R substitution relative to SEQ ID NO:139, numbered under the Chothia numbering scheme. In some embodimentexamples, the VH comprises an S62R substitution relative to SEQ ID NO:135 and the VL comprises a D92R substitution relative to SEQ ID NO:139, numbered under the Chothia numbering scheme.

[0021] In some embodimentexamples of any one of the aspectexamples above, the VL comprises an F87Y substitution relative to SEQ ID NO:139, numbered under the Chothia numbering scheme.

[0022] In some embodimentexamples, the second antigen-binding site comprises a single-chain fragment variable (scFv), and wherein the scFv comprises an amino acid sequence at least 90% identical to, or comprising, a sequence selected from the group consisting of SEQ ID NOs: 152, 154, 148, and 158.

[0023] In some embodimentexamples, the protein of the present disclosure comprises a polypeptide comprising an amino acid sequence at least 90% identical to, or comprising, a sequence selected from the group consisting of SEQ ID NO: 167, 168, and 166.

[0024] In some [embodimentexamples](#), the second antigen-binding site binds human EGFR with a dissociation constant (K_D) smaller than or equal to 5 nM, as measured by surface plasmon resonance (SPR). In some [embodimentexamples](#), the second antigen-binding site binds rhesus macaque EGFR with a dissociation constant (K_D) smaller than or equal to 6 nM, as measured by surface plasmon resonance (SPR).

[0025] In some [embodimentexamples](#), the first antigen-binding site that binds NKG2D is a Fab fragment, and the second antigen-binding site that binds EGFR is an scFv. In some [embodimentexamples](#), the first antigen-binding site that binds NKG2D is an scFv, and the second antigen-binding site that binds EGFR is a Fab fragment. In some [embodimentexamples](#), the protein further comprises an additional antigen-binding site that binds EGFR. In some [embodimentexamples](#), the first antigen-binding site that binds NKG2D is an scFv, and the second and the additional antigen-binding sites that bind EGFR are each a Fab fragment. In some [embodimentexamples](#), the first antigen-binding site that binds NKG2D is an scFv, and the second and the additional antigen-binding sites that bind EGFR are each an scFv. In some [embodimentexamples](#), the amino acid sequences of the second and the additional antigen-binding sites are identical.

[0026] In some [embodimentexamples](#), the scFv that binds NKG2D is linked to an antibody constant domain or a portion thereof sufficient to bind CD16, via a hinge comprising Ala-Ser or Gly-Ser, wherein the scFv comprises a heavy chain variable domain and a light chain variable domain. In some [embodimentexamples](#), each scFv that binds EGFR is linked to an antibody constant domain or a portion thereof sufficient to bind CD16, via a hinge comprising Ala-Ser or Gly-Ser, wherein the scFv comprises a heavy chain variable domain and a light chain variable domain. In some [embodimentexamples](#), the hinge further comprises an amino acid sequence Thr-Lys-Gly.

[0027] In some [embodimentexamples](#), within the scFv that binds NKG2D, the heavy chain variable domain of the scFv forms a disulfide bridge with the light chain variable domain of the scFv. In some [embodimentexamples](#), within each scFv that binds EGFR, the heavy chain variable domain of the scFv forms a disulfide bridge with the light chain variable domain of the scFv. In some [embodimentexamples](#), the disulfide bridge is formed between C44 of the heavy chain variable domain and C100 of the light chain variable domain, numbered under the Kabat numbering scheme. In some [embodimentexamples](#), within the scFv that binds NKG2D, the heavy chain variable domain is linked to the light chain variable domain via a flexible linker. In some [embodimentexamples](#), within each scFv that binds EGFR, the heavy chain variable domain is linked to the light chain variable domain via a flexible linker. In some [embodimentexamples](#), the flexible linker comprises (G4S)₄ (SEQ ID NO: 119).

[0028] In some embodimentexamples, within the scFv that binds NKG2D, the heavy chain variable domain is positioned at the C-terminus of the light chain variable domain. In some embodimentexamples, within each scFv that binds EGFR, the heavy chain variable domain is positioned at the C-terminus of the light chain variable domain. In some embodimentexamples, within the scFv that binds NKG2D, the heavy chain variable domain is positioned at the N-terminus of the light chain variable domain.

[0029] In some embodimentexamples, within each scFv that binds EGFR, the heavy chain variable domain is positioned at the N-terminus of the light chain variable domain. In some embodimentexamples, the Fab fragment that binds NKG2D is not positioned between an antigen-binding site and the Fc or the portion thereof. In some embodimentexamples, no Fab fragment that binds EGFR is positioned between an antigen-binding site and the Fc or the portion thereof.

[0030] In some embodimentexamples, the first antigen-binding site that binds NKG2D comprises a VH comprising CDR1, CDR2, and CDR3 comprising the amino acid sequences of SEQ ID NOs: 81, 82, and 112, respectively; and a VL comprising CDR1, CDR2, and CDR3 comprising the amino acid sequences of SEQ ID NOs: 86, 77, and 87, respectively. In some embodimentexamples, the first antigen-binding site that binds NKG2D comprises a VH comprising CDR1, CDR2, and CDR3 comprising the amino acid sequences of SEQ ID NOs: 81, 82, and 97, respectively; and a VL comprising CDR1, CDR2, and CDR3 comprising the amino acid sequences of SEQ ID NOs: 86, 77, and 87, respectively. In some embodimentexamples, the VH of the first antigen-binding site comprises an amino acid sequence at least 90% identical to SEQ ID NO:95, and the VL of the first antigen-binding site comprises an amino acid sequence at least 90% identical to SEQ ID NO:85. In some embodimentexamples, the VH of the first antigen-binding site comprises the amino acid sequence of SEQ ID NO:95, and the VL of the first antigen-binding site comprises the amino acid sequence of SEQ ID NO:85.

[0031] In some embodimentexamples, the antibody Fc domain comprises a hinge and a CH2 domain. In some embodimentexamples, the antibody Fc domain is a human IgG1 antibody Fc domain. In some embodimentexamples, the antibody Fc domain or the portion thereof comprises an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody or SEQ ID NO:118.

[0032] In some embodimentexamples, at least one polypeptide chain of the antibody Fc domain comprises one or more mutations, relative to SEQ ID NO:118, at one or more positions selected from Q347, Y349, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411, and K439, numbered according to the EU numbering system. In some embodimentexamples, at least one polypeptide chain

of the antibody Fc domain comprises one or more mutations, relative to SEQ ID NO:118, selected from Q347E, Q347R, Y349S, Y349K, Y349T, Y349D, Y349E, Y349C, L351K, L351D, L351Y, S354C, E356K, E357Q, E357L, E357W, K360E, K360W, Q362E, S364K, S364E, S364H, S364D, T366V, T366I, T366L, T366M, T366K, T366W, T366S, L368E, L368A, L368D, K370S, N390D, N390E, K392L, K392M, K392V, K392F, K392D, K392E, T394F, D399R, D399K, D399V, S400K, S400R, D401K, F405A, F405T, Y407A, Y407I, Y407V, K409F, K409W, K409D, T411D, T411E, K439D, and K439E, numbered according to the EU numbering system.

[0033] In some embodimentexamples, one polypeptide chain of the antibody heavy chain constant region comprises one or more mutations, relative to SEQ ID NO:118, at one or more positions selected from Q347, Y349, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, K392, T394, D399, S400, D401, F405, Y407, K409, T411 and K439; and the other polypeptide chain of the antibody heavy chain constant region comprises one or more mutations, relative to SEQ ID NO:118, at one or more positions selected from Q347, Y349, L351, S354, E356, E357, S364, T366, L368, K370, N390, K392, T394, D399, D401, F405, Y407, K409, T411, and K439, numbered according to the EU numbering system. In some

embodimentexamples, one polypeptide chain of the antibody heavy chain constant region comprises K360E and K409W substitutions relative to SEQ ID NO:118; and the other polypeptide chain of the antibody heavy chain constant region comprises Q347R, D399V and F405T substitutions relative to SEQ ID NO:118, numbered according to the EU numbering system.

[0034] In some embodimentexamples, one polypeptide chain of the antibody heavy chain constant region comprises a Y349C substitution relative to SEQ ID NO:118; and the other polypeptide chain of the antibody heavy chain constant region comprises an S354C substitution relative to SEQ ID NO:118, numbered according to the EU numbering system.

[0035] Another aspectexample of the present application provides a protein comprising (a) a first polypeptide comprising the amino acid sequence of SEQ ID NO:167, (b) a second polypeptide comprising the amino acid sequence of SEQ ID NO:164, and (c) a third polypeptide comprising the amino acid sequence of SEQ ID NO:165.

[0036] Another aspectexample of the present application provides a protein comprising (a) a first polypeptide comprising the amino acid sequence of SEQ ID NO:168, (b) a second polypeptide comprising the amino acid sequence of SEQ ID NO:164, and (c) a third polypeptide comprising the amino acid sequence of SEQ ID NO:165.

[0037] Another aspectexample of the present application provides a protein comprising (a) a first polypeptide comprising the amino acid sequence of SEQ ID NO:166, (b) a second

polypeptide comprising the amino acid sequence of SEQ ID NO:164, and (c) a third polypeptide comprising the amino acid sequence of SEQ ID NO:165.

[0038] Another aspect of the present application provides a formulation comprising a protein as disclosed herein and a pharmaceutically acceptable carrier.

5 [0039] Another aspect of the present application provides a cell comprising one or more nucleic acids encoding a protein as disclosed herein. In some embodimentexamples, the cell comprising one or more nucleic acids encoding a protein comprising an amino acid sequence of SEQ ID NO: 167 or SEQ ID NO: 169.

10 [0040] Another aspectexample of the present application provides a purified protein as disclosed herein. In some embodimentexamples, the protein is purified using a method selected from the group consisting of: centrifugation, depth filtration, cell lysis, homogenization, freeze-thawing, affinity purification, gel filtration, ion exchange chromatography, hydrophobic interaction exchange chromatography, and mixed-mode chromatography.

15 [0041] Another aspectexample of the present application provides a method of enhancing tumor cell death, comprising exposing the tumor cell and a natural killer cell to an effective amount of a protein as disclosed herein or a formulation as disclosed herein, wherein the tumor cell expresses EGFR.

20 [0042] In yet another aspect, the present disclosure provides a use of a protein as described herein in the manufacture of a medicament for treating a cancer. Another aspectexample of the present application provides a method of treating cancer, wherein the method comprises administering to a patient in need thereof an effective amount of a protein as disclosed herein or a formulation as disclosed herein. In some embodimentexamples, the cancer is a solid tumor. In some embodimentexamples, the cancer is lung cancer, breast
25 cancer, kidney cancer, colorectal cancer, gastric cancer, brain cancer, glioma, bladder cancer, head and neck cancer, bladder cancer, pancreatic cancer, and liver cancer, cervical cancer, ovarian cancer or prostate cancer. In some embodimentexamples, the cancer expresses EGFR.

30 [0043] These and other aspectexamples and advantages of the TriNKETs described in the present application are illustrated by the following figures, detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0044] **FIG. 1** is a representation of a heterodimeric, multispecific binding antibody, *e.g.*, a trispecific binding protein (TriNKET). Each arm can represent either the NKG2D-binding domain, or the binding domain corresponding to a tumor-associated antigen. In some
5 embodiments, the NKG2D binding domain and the tumor-associated antigen binding domains can share a common light chain.

WHAT IS CLAIMED IS:

1. A protein comprising:

(a) a first antigen-binding site that binds NKG2D;

(b) a second antigen-binding site that binds EGFR; and

5 (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16,

wherein the second antigen-binding site comprises:

10 (i) a heavy chain variable domain (VH) comprising complementarity-determining region 1 (CDR1), complementarity-determining region 2 (CDR2), and complementarity-determining region 3 (CDR3) sequences of SEQ ID NOs: 136, 157, and 138, respectively; and a light chain variable domain (VL) comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively; ~~or~~

15 (ii) a VH comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 137, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively; or

(iii) a VH comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 146, and 138, respectively; and a VL comprising CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 142, respectively;

20 ~~2. The protein of claim 1, wherein the VH comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 157, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively.~~

~~3. The protein of claim 2, where the VH comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 146, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively.~~

25 ~~4. The protein of claim 2, wherein the VH comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 137, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 151, respectively.~~

5. ~~The protein of claim 1, wherein the VH comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 136, 146, and 138, respectively; and the VL comprises CDR1, CDR2, and CDR3 sequences of SEQ ID NOs: 140, 141, and 142, respectively.~~

6. ~~A protein comprising:~~

- ~~—(a)— a first antigen-binding site that binds NKG2D;~~
- ~~—(b)— a second antigen-binding site that binds EGFR; and~~
- ~~—(c)— an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16;~~

~~wherein the second antigen-binding site comprises a VH comprising an amino acid sequence at least 90% identical to SEQ ID NO:135 and a VL comprising an amino acid sequence at least 90% identical to SEQ ID NO:139,~~

~~wherein the VH comprises an S62R substitution relative to SEQ ID NO:135 and/or the VL comprises a D92R substitution and/or an F87Y substitution relative to SEQ ID NO:139, numbered under the Chothia numbering scheme.~~

7. ~~The protein of claim 6, wherein the VH comprises an S62R substitution relative to SEQ ID NO:135, numbered under the Chothia numbering scheme.~~

8. ~~The protein of claim 6, wherein the VL comprises a D92R substitution relative to SEQ ID NO:139, numbered under the Chothia numbering scheme.~~

9. ~~The protein of claim 6, wherein the VH comprises an S62R substitution relative to SEQ ID NO:135 and the VL comprises a D92R substitution relative to SEQ ID NO:139, numbered under the Chothia numbering scheme.~~

10. ~~The protein of any one of claims 1-9, wherein the VL of the second antigen-binding site comprises an F87Y substitution relative to SEQ ID NO:139, numbered under the Chothia numbering scheme.~~

11. ~~The protein of any one of claims 1-3, and 6-10, wherein:~~

~~(i) the second antigen-binding site is as defined in claim 1(i) and the VH comprises an amino acid sequence at least 90% identical to SEQ ID NO:145 and the VL comprises an amino acid sequence at least 90% identical to SEQ ID NO:150, optionally~~

- ~~12. The protein of any one of claims 1-3, and 6-11, wherein (a) the VH comprises the amino acid sequence of SEQ ID NO:145 and the VL comprises the amino acid sequence of SEQ ID NO:150, or (b) the VH comprises the amino acid sequence of SEQ ID NO:170 and the VL comprises the amino acid sequence of SEQ ID NO:171;~~
- 5 ~~13. The protein of any one of claims 1, 2, 4, 6, 8, and 10,~~
- ~~(ii) the second antigen-binding site is as defined in claim 1(ii) and wherein~~ the VH comprises an amino acid sequence at least 90% identical to SEQ ID NO:135 and the VL comprises an amino acid sequence at least 90% identical to SEQ ID NO:150, optionally
- 10 ~~14. The protein of any one of claims 1, 2, 4, 6, 8, 10, and 13,~~ wherein the VH comprises the amino acid sequence of SEQ ID NO:135 and the VL comprises the amino acid sequence of SEQ ID NO:150; or
- ~~15. The protein of any one of claims 1, 5-7, and 10, wherein~~
- 15 ~~(iii) the second antigen-binding site is as defined in claim 1(iii) and~~ the VH comprises an amino acid sequence at least 90% identical to SEQ ID NO:145 and the VL comprises an amino acid sequence at least 90% identical to SEQ ID NO:147, optionally
- ~~16. The protein of any one of claims 1, 5-7, 10, and 15,~~ wherein the VH comprises the amino acid sequence of SEQ ID NO:145 and the VL comprises the amino acid sequence of SEQ ID NO:147.
- 20 ~~17. The protein of any one of claims 1-16 or 2,~~ wherein the second antigen-binding site comprises a single-chain fragment variable (scFv), and wherein the scFv comprises an amino acid sequence at least 90% identical to a sequence selected from the group consisting of SEQ ID NOs: 152, 154, and 148, ~~and 158;~~ optionally
- ~~18. The protein of any one of claims 1-3, 6-12, and 17, wherein the scFv comprises an amino acid sequence at least 90% identical to SEQ ID NO:152.~~
- 25 ~~19. The protein of any one of claims 1-3, 6-12, 17, and 18,~~ wherein the scFv comprises ~~an~~ the amino acid sequence of SEQ ID NO:152;
- ~~20. The protein of any one of claims 1, 2, 4, 6, 8, 10, 13, 14, and 17, wherein the scFv comprises an amino acid sequence at least 90% identical to SEQ ID NO:154.~~

~~21. The protein of any one of claims 1, 2, 4, 6, 8, 10, 13, 14, 17, and 20, wherein the scFv comprises an amino acid sequence of SEQ ID NO:154., or~~

~~22. The protein of any one of claims 1, 5-7, 10, and 15-17, wherein the scFv comprises an amino acid sequence at least 90% identical to an amino acid sequence of SEQ ID NO:148.~~

~~23. The protein of any one of claims 1, 5-7, 10, 15-17, and 22, wherein the scFv comprises an amino acid sequence of SEQ ID NO:148.~~

~~24.~~ The protein of any one of claims 1-~~23~~, wherein the protein comprises a polypeptide comprising an amino acid sequence at least 90% identical to a sequence selected from the group consisting of SEQ ID NO: 167, 168, and 166.; optionally

~~25. The protein of any one of claims 1-3, 6-12, 17-19, and 24, wherein the polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:167.~~

~~26. The protein of any one of claims 1-3, 6-12, 17-19, 24, and 25, wherein the polypeptide comprises an the amino acid sequence of SEQ ID NO:167.~~

~~27. The protein of any one of claims 1, 2, 4, 6, 8, 10, 13, 14, 17, 20, 21, and 24, wherein the polypeptide comprises an amino acid sequence at least 90% identical to SEQ ID NO:168.~~

~~28. The protein of any one of claims 1, 2, 4, 6, 8, 10, 13, 14, 17, 20, 21, 24, and 27, wherein the polypeptide comprises an amino acid sequence of SEQ ID NO:168., or~~

~~29. The protein of any one of claims 1, 5-7, 10, 15-17, and 22-24, wherein the polypeptide comprises an amino acid sequence at least 90% identical to an amino acid sequence of SEQ ID NO:166.~~

~~30. The protein of any one of claims 1, 5-7, 10, 15-17, 22-24, and 29, wherein the scFv comprises an amino acid sequence of SEQ ID NO:166.~~

~~31.~~ The protein of any one of claims 1-~~30~~, wherein the second antigen-binding site binds human EGFR with a dissociation constant (K_D) smaller than or equal to 5 nM, as measured by surface plasmon resonance (SPR).; and/or

~~32. The protein of any one of claims 1-31, wherein~~ the second antigen-binding site binds rhesus macaque EGFR with a dissociation constant (K_D) smaller than or equal to 6 nM, as measured by surface plasmon resonance (SPR).

5 ~~336. The protein of any one of claims 1-16, 312, and 325, wherein the first antigen-binding site that binds NKG2D is a Fab fragment, and the second antigen-binding site that binds EGFR is an scFv.~~

~~34. The protein of any one of claims 1-16, 31, and 32, wherein the first antigen-binding site that binds NKG2D is an scFv, and the second antigen-binding site that binds EGFR is a Fab fragment.~~

10 ~~35. The protein of any one of claims 1-16, 31 and 32, further comprising an additional antigen-binding site that binds EGFR.~~

~~36. The protein of claim 35, wherein the first antigen-binding site that binds NKG2D is an scFv, and the second and the additional antigen-binding sites that bind EGFR are each a Fab fragment.~~

15 ~~37. The protein of claim 35, wherein the first antigen-binding site that binds NKG2D is an scFv, and the second and the additional antigen-binding sites that bind EGFR are each an scFv.~~

~~38. The protein of any one of claims 35-37, wherein the amino acid sequences of the second and the additional antigen-binding sites are identical.~~

20 ~~39. The protein of any one of claims 34 and 36-38, wherein the scFv that binds NKG2D is linked to an antibody constant domain or a portion thereof sufficient to bind CD16, via a hinge comprising Ala-Ser or Gly-Ser, wherein the scFv comprises a heavy chain variable domain and a light chain variable domain.~~

25 ~~40. The protein of any one of claims 33 and 37-39, wherein each scFv that binds EGFR is linked to an antibody constant domain or a portion thereof sufficient to bind CD16, via a hinge comprising Ala-Ser or Gly-Ser, wherein the scFv comprises a heavy chain variable domain and a light chain variable domain.~~

~~41. The protein of claim 39 or 40, wherein the hinge further comprises an amino acid sequence Thr-Lys-Gly.~~

~~42. The protein of any one of claims 34 and 36-41, wherein within the scFv that binds NKG2D, the heavy chain variable domain of the scFv forms a disulfide bridge with the light chain variable domain of the scFv.~~

~~43~~7. The protein of ~~any one of claims 33 and 37-42~~6, wherein within each scFv that binds EGFR₇;

(i) the heavy chain variable domain of the scFv forms a disulfide bridge with the light chain variable domain of the scFv₇; optionally

~~44. The protein of claim 42 or 43,~~ wherein the disulfide bridge is formed between C44 of the heavy chain variable domain and C100 of the light chain variable domain, numbered under the Kabat numbering scheme₇; and/or

~~45. The protein of any one of claims 34 and 36-44, wherein within the scFv that binds NKG2D, the heavy chain variable domain is linked to the light chain variable domain via a flexible linker.~~

~~46. The protein of any one of claims 33 and 37-45, wherein within each scFv that binds EGFR₇,~~

(ii) the heavy chain variable domain is linked to the light chain variable domain via a flexible linker₇; optionally

~~47. The protein of claim 45 or 46,~~ wherein the flexible linker comprises (G₄S)₄ amino acid sequence set forth in (SEQ ID NO:119)₇; and/or

~~48. The protein of any one of claims 34 and 36-47, wherein within the scFv that binds NKG2D, the heavy chain variable domain is positioned at the C-terminus of the light chain variable domain.~~

~~49. The protein of any one of claims 33 and 37-48, wherein within each scFv that binds EGFR₇,~~

(iii) the heavy chain variable domain is positioned at the C-terminus of the light chain variable domain.

~~50. The protein of any one of claims 34 and 36-47, wherein within the scFv that binds NKG2D, the heavy chain variable domain is positioned at the N-terminus of the light chain variable domain.~~

~~51. The protein of any one of claims 33, 37-48, and 50 wherein within each scFv that binds EGFR, the heavy chain variable domain is positioned at the N-terminus of the light chain variable domain.~~

~~52. The protein of any one of claims 33, 40-41, 43-44, 46-47, 49 and 51, wherein the Fab fragment that binds NKG2D is not positioned between an antigen-binding site and the Fc or the portion thereof.~~

~~53. The protein of any one of claims 34, 36, 38-39, 41-42, 44-45, 47-48, and 50, wherein no Fab fragment that binds EGFR is positioned between an antigen-binding site and the Fc or the portion thereof.~~

~~548.~~ The protein according to any one of claims 1-~~53~~7, wherein the first antigen-binding site that binds NKG2D comprises a VH comprising CDR1, CDR2, and CDR3 comprising the amino acid sequences of SEQ ID NOs: 81, 82, and 112, respectively; and a VL comprising CDR1, CDR2, and CDR3 comprising the amino acid sequences of SEQ ID NOs: 86, 77, and 87, respectively.

~~559.~~ The protein ~~according to~~ of any one of claims ~~541-7~~, wherein the first antigen-binding site that binds NKG2D comprises a VH comprising CDR1, CDR2, and CDR3 comprising the amino acid sequences of SEQ ID NOs: 81, 82, and 97, respectively; and a VL comprising CDR1, CDR2, and CDR3 comprising the amino acid sequences of SEQ ID NOs: 86, 77, and 87, respectively.

~~5610.~~ The protein of claim ~~54-8~~ or ~~559~~, wherein the VH of the first antigen-binding site comprises an amino acid sequence at least 90% identical to SEQ ID NO:95, and the VL of the first antigen-binding site comprises an amino acid sequence at least 90% identical to SEQ ID NO:85, optionally

~~57. The protein of any one of claims 54-56,~~ wherein the VH of the first antigen-binding site comprises the amino acid sequence of SEQ ID NO:95, and the VL of the first antigen-binding site comprises the amino acid sequence of SEQ ID NO:85.

~~58~~11. The protein according to any one of claims 1-~~57~~10, wherein the antibody Fc domain comprises a hinge and a CH2 domain.

~~59. The protein of any one of claims 1-58, wherein the antibody Fc domain is of~~ a human IgG1 antibody Fc domain, optionally

~~60. The protein of claim 59,~~ wherein the antibody Fc domain or the portion thereof comprises an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody or SEQ ID NO:118.

~~61. The protein of claim 59 or 60, wherein at least one polypeptide chain of the antibody Fc domain comprises one or more mutations, relative to SEQ ID NO:118, at one or more positions selected from Q347, Y349, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411, and K439, numbered according to the EU numbering system.~~

~~62. The protein of any one of claims 59-61, wherein at least one polypeptide chain of the antibody Fc domain comprises one or more mutations, relative to SEQ ID NO:118, selected from Q347E, Q347R, Y349S, Y349K, Y349T, Y349D, Y349E, Y349C, L351K, L351D, L351Y, S354C, E356K, E357Q, E357L, E357W, K360E, K360W, Q362E, S364K, S364E, S364H, S364D, T366V, T366I, T366L, T366M, T366K, T366W, T366S, L368E, L368A, L368D, K370S, N390D, N390E, K392L, K392M, K392V, K392F, K392D, K392E, T394F, D399R, D399K, D399V, S400K, S400R, D401K, F405A, F405T, Y407A, Y407I, Y407V, K409F, K409W, K409D, T411D, T411E, K439D, and K439E, numbered according to the EU numbering system.~~

~~63. The protein of any one of claims 59-62, wherein one polypeptide chain of the antibody heavy chain constant region comprises one or more mutations, relative to SEQ ID NO:118, at one or more positions selected from Q347, Y349, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, K392, T394, D399, S400, D401, F405, Y407, K409, T411 and K439; and the other polypeptide chain of the antibody heavy chain constant region comprises one or more mutations, relative to SEQ ID NO:118, at one or~~

~~more positions selected from Q347, Y349, L351, S354, E356, E357, S364, T366, L368, K370, N390, K392, T394, D399, D401, F405, Y407, K409, T411, and K439, numbered according to the EU numbering system.~~

5 ~~6412. The protein of claim 6311, wherein the antibody Fc domain is a human IgG1 antibody Fc domain comprising two polypeptide chains, wherein~~

~~(i) one of the polypeptide chains of the antibody heavy chain constant region comprises K360E and K409W substitutions relative to SEQ ID NO:118; and the other polypeptide chain of the antibody heavy chain constant region comprises Q347R, D399V and F405T substitutions relative to SEQ ID NO:118, numbered according to the EU numbering system., and/or~~

10 ~~65. The protein of claim 63 or 64, wherein~~

~~(ii) one of the polypeptide chains of the antibody heavy chain constant region comprises a Y349C substitution relative to SEQ ID NO:118; and the other polypeptide chain of the antibody heavy chain constant region comprises an S354C substitution relative to SEQ ID NO:118, numbered according to the EU numbering system.~~

15 ~~6613. A The protein of claim 1, comprising:~~

~~(i) (a) a first polypeptide comprising the amino acid sequence of SEQ ID NO:167;~~

~~(b) a second polypeptide comprising the amino acid sequence of SEQ ID NO:164; and~~

~~(c) a third polypeptide comprising the amino acid sequence of SEQ ID NO:165.;~~

20 ~~67. A protein comprising:~~

~~(ii) (a) a first polypeptide comprising the amino acid sequence of SEQ ID NO:168;~~

~~(b) a second polypeptide comprising the amino acid sequence of SEQ ID NO:164; and~~

~~(c) a third polypeptide comprising the amino acid sequence of SEQ ID NO:165.; or~~

~~68. A protein comprising:~~

25 ~~(iii) (a) a first polypeptide comprising the amino acid sequence of SEQ ID NO:166;~~

(b) a second polypeptide comprising the amino acid sequence of SEQ ID NO:164; and

(c) a third polypeptide comprising the amino acid sequence of SEQ ID NO:165.

~~69~~14. A formulation comprising a protein according to any one of ~~the preceding~~ claims 1-13 and a pharmaceutically acceptable carrier.

5 ~~70~~15. A cell comprising one or more nucleic acids encoding a protein of any one of claims 1-~~68~~13.

~~71. A method of enhancing tumor cell death, the method comprising exposing the tumor cell and a natural killer cell to an effective amount of the protein of any one of claims 1-68 or the formulation according to claim 69, wherein the tumor cell expresses EGFR.~~

10 ~~72~~16. ~~A method of treating a cancer, wherein the method comprises administering to a patient in need thereof an effective amount~~ Use of the a protein of any one of claims 1-~~68~~13 in the manufacture of a medicament for treating a cancer~~or the formulation according to claim 69.~~

~~73. The method of claim 72, wherein the cancer is a solid tumor.~~

15 ~~74. The method of claim 72 or 73, wherein the cancer is lung cancer, breast cancer, kidney cancer, colorectal cancer, gastric cancer, brain cancer, glioma, bladder cancer, head and neck cancer, bladder cancer, pancreatic cancer, and liver cancer, cervical cancer, ovarian cancer or prostate cancer.~~

~~75. The method of any one of claims 72-74, wherein the cancer expresses EGFR.~~

20 ~~76. A cell comprising one or more nucleic acids encoding a protein comprising an amino acid sequence of SEQ ID NO:167.~~

~~77. A cell comprising one or more nucleic acids encoding a protein comprising an amino acid sequence of SEQ ID NO:169.~~

~~78. A protein of any one of claims 1-68, wherein the protein is a purified protein.~~

25 ~~79. The protein of claim 78, wherein the protein is purified using a method selected from the group consisting of: centrifugation, depth filtration, cell lysis, homogenization, freeze-~~

thawing, affinity purification, gel filtration, ion-exchange chromatography, hydrophobic interaction-exchange chromatography, and mixed-mode chromatography.