

CLAIMS

WHAT IS CLAIMED IS:

1. A protein comprising:
 - (a) a first antigen-binding site comprising a single-chain variable fragment (scFv) that binds NKG2D;
 - (b) a second antigen-binding site that binds B-cell maturation antigen (BCMA); 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 protein comprises an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:162.
2. The protein according to claim 1 further comprising an additional antigen-binding site that binds BCMA.
3. The protein according to ~~claims 1 or~~ claim 2, wherein
 - (a) the second antigen-binding site that binds BCMA is an Fab fragment;
 - (b) the second and the additional antigen-binding site that bind BCMA are Fab fragments; or
 - (c) the second and the additional antigen-binding site that bind BCMA are scFvs.
4. The protein according to any one of claims 1-3, wherein the heavy chain variable domain of the scFv that binds NKG2D is positioned at the N-terminus or the C-terminus of the light chain variable domain of the scFv.
5. The protein according to claim 4, wherein the light chain variable domain is positioned at the N-terminus of the heavy chain variable domain of the scFv that binds NKG2D.

6. The protein according to any one of claims 1-5, wherein the scFv that binds to NKG2D is linked to the antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16.
7. The protein according to claim 6, wherein the scFv that binds to NKG2D
 - (a) is linked to the antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16 *via* a hinge comprising Ala-Ser; or
 - (b) is linked to the C-terminus of the antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16 *via* a flexible linker comprising the amino acid sequence of SEQ ID NO:168.
8. The protein according to claim 7, wherein the C-terminus of the antibody Fc domain is linked to the N-terminus of the light chain variable domain of the scFv that binds NKG2D.
9. The protein according to any one of claims 1-8, wherein within the scFv that binds NKG2D, a disulfide bridge is formed between the heavy chain variable domain of the scFv and the light chain variable domain of the scFv.
10. The protein according to claim 9, wherein the disulfide bridge is formed between C44 from the heavy chain variable domain and C100 from the light chain variable domain.
11. The protein according to any one of claims 1-10, wherein, within the scFv that binds NKG2D, the heavy chain variable domain is linked to the light chain variable domain via a flexible linker.
12. The protein according to claim 11, wherein the flexible linker comprises (GlyGlyGlyGlySer)_n (G4S)_n (SEQ ID NO:198), wherein n is an integer between 1-10.
13. The protein according to any one of claims 3-12, wherein the second and the additional antigen-binding site scFvs are linked to the antibody Fc domain or a portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16, via a hinge comprising Ala-Ser.

14. The protein according to any one of claims 3-13, wherein the second and the additional antigen-binding site scFvs are linked to the antibody Fc domain via a hinge comprising Ala-Ser.
15. The protein according to claims 13 or 14, wherein a disulfide bridge is formed between the heavy chain variable domain and the light chain variable domain of the second antigen-binding site and/or the additional antigen-binding site.
16. The protein according to claim 15, wherein the disulfide bridge is formed between C44 from the heavy chain variable domain and C100 from the light chain variable domain.
17. The protein according to any one of claims 1-16, wherein the scFv that binds NKG2D comprises a light chain variable domain positioned at the N-terminus of a heavy chain variable domain, wherein the light chain variable domain is linked to the heavy chain variable domain of the scFv via a flexible linker consisting of the amino acid sequence of SEQ ID NO:167, and the scFv that binds NKG2D is linked to the antibody Fc domain via a hinge comprising Ala-Ser.
18. The protein according to any one of claims 1-17, wherein the first antigen-binding site that binds NKG2D comprises:
- (a) a heavy chain variable domain comprising complementarity-determining region 1 (CDR1), complementarity-determining region 2 (CDR2), and complementarity-determining region 3 (CDR3) sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 191, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively;
 - (b) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 193, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively;
 - (c) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 95, 96, and 97, respectively; and

a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively;

(d) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 194, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively;

(e) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 195, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively;

(f) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 196, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively;

(g) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 197, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; or

(h) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 160, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively.

19. The protein according to ~~any one of claims 1-18~~ claim 18, wherein

(a) the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain at least ~~90%~~ 95% identical to the amino acid sequence of SEQ ID NO:94;

~~(b)~~ the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain at least 90% identical to SEQ ID NO:94 and a light chain variable domain at least 90% identical to SEQ ID NO:98;

~~(c)~~—the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:94 and a light chain variable domain at least 95% identical to SEQ ID NO:98;

~~(d)~~(c) the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:94 and a light chain variable domain identical to SEQ ID NO:98;

~~(e)~~(d) the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:169 and a light chain variable domain identical to SEQ ID NO:98;

~~(f)~~(e) the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:171 and a light chain variable domain identical to SEQ ID NO:98;

~~(g)~~(f) the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:173 and a light chain variable domain identical to SEQ ID NO:98;

~~(h)~~(g) the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:175 and a light chain variable domain identical to SEQ ID NO:98;

~~(i)~~(h) the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:177 and a light chain variable domain identical to SEQ ID NO:98; or

~~(i)~~(i) the first antigen-binding site that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:179 and a light chain variable domain identical to SEQ ID NO:98.

20. The protein according to any one of claims 1-19, wherein the second and/or the additional antigen-binding site(s) that bind(s) BCMA comprise(s):

(a) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 149, 150, and 151, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 153, 154, and 155, respectively;

(b) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 115, 116, and 117, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 120, 121, and 123, respectively;

(c) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 125, 126, and 127, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 129, 130, and 131, respectively;

(d) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 133, 134, and 135, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 137, 138, and 139, respectively;

(e) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 141, 142, and 143, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 145, 146, and 147, respectively;

or

(f) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 115, 116, and 117, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 120, 121, and 122, respectively.

21. The protein according to ~~any one of claims 1-20~~ claim 20, wherein the second and/or the additional antigen-binding site(s) that bind(s) BCMA comprise(s):

~~(a) a heavy chain variable domain at least 90% identical to SEQ ID NO:148 and a light chain variable domain at least 90% identical to SEQ ID NO:152;~~

~~(b)~~—a heavy chain variable domain at least 95% identical to SEQ ID NO:148 and a light chain variable domain at least 95% identical to SEQ ID NO:152;

~~(c)~~(b) a heavy chain variable domain identical to SEQ ID NO:148 and a light chain variable domain identical to SEQ ID NO:152;

~~(d)~~—a heavy chain variable domain at least 90% identical to SEQ ID NO:114 and a light chain variable domain at least 90% identical to SEQ ID NO:119;

~~(e)~~(c) a heavy chain variable domain at least 95% identical to SEQ ID NO:114 and a light chain variable domain at least 95% identical to SEQ ID NO:119;

~~(f)~~(d) a heavy chain variable domain identical to SEQ ID NO:114 and a light chain variable domain identical to SEQ ID NO:119;

~~(g)~~—a heavy chain variable domain at least 90% identical to SEQ ID NO:124 and a light chain variable domain at least 90% identical to SEQ ID NO:128;

~~(h)~~(e) a heavy chain variable domain at least 95% identical to SEQ ID NO:124 and a light chain variable domain at least 95% identical to SEQ ID NO:128;

~~(i)~~(f) a heavy chain variable domain identical to SEQ ID NO:124 and a light chain variable domain identical to SEQ ID NO:128;

~~(j)~~—a heavy chain variable domain at least 90% identical to SEQ ID NO:132 and a light chain variable domain at least 90% identical to SEQ ID NO:136;

~~(k)~~(g) a heavy chain variable domain at least 95% identical to SEQ ID NO:132 and a light chain variable domain at least 95% identical to SEQ ID NO:136;

~~(l)~~(h) a heavy chain variable domain identical to SEQ ID NO:132 and a light chain variable domain identical to SEQ ID NO:136;

~~(m)~~—a heavy chain variable domain at least 90% identical to SEQ ID NO:140 and a light chain variable domain at least 90% identical to SEQ ID NO:144;

~~(n)~~(i) a heavy chain variable domain at least 95% identical to SEQ ID NO:140 and a light chain variable domain at least 95% identical to SEQ ID NO:144;

~~(o)~~(j) a heavy chain variable domain identical to SEQ ID NO:140 and a light chain variable domain identical to SEQ ID NO:144;

~~(p)~~—a heavy chain variable domain at least 90% identical to SEQ ID NO:114 and a light chain variable domain at least 90% identical to SEQ ID NO:118;

~~(q)~~(k) a heavy chain variable domain at least 95% identical to SEQ ID NO:114 and a light chain variable domain at least 95% identical to SEQ ID NO:118; or

~~(r)~~(l) a heavy chain variable domain identical to SEQ ID NO:114 and a light chain variable domain identical to SEQ ID NO:118.

22. The protein according to any one of claims 1-21, wherein the antibody Fc domain comprises hinge and CH2 domains of a human IgG1 antibody.

23. The protein according to claim 22, wherein the Fc domain comprises an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody.

24. The protein according to claims 22 or 23, wherein the Fc domain comprises amino acid sequence at least 90% identical to the Fc domain of human IgG1 and differs at one or more positions selected from the group consisting of Q347, Y349, T350, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411, and K439.
25. The protein according to any one of claims 1-24, wherein the Fc domain is an Fc domain of a human IgG1 comprising Q347R, D399V, and F405T substitutions.
26. The protein according to claim 25, wherein the Fc domain comprising the substitutions is linked to the scFv that bind NKG2D.
27. The protein according to any one of claims 1-26, wherein the Fc domain is an Fc domain of a human IgG1 comprising K360E and K409W substitutions.
28. The protein according to claim 27, wherein the Fc domain comprising the substitutions is linked to the second antigen binding site.
29. The protein according to any one of claims 1-~~21 and 22~~-28 comprising
- (a) an amino acid sequence of SEQ ID NO:162;
 - (b) an amino acid sequence comprising SEQ ID NO:162, SEQ ID NO:163, and SEQ ID NO:165;
 - ~~(c) an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:162;~~
 - ~~(d)~~ (c) an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:162;
 - ~~(e)~~ (d) an amino acid sequence at least 99% identical to the amino acid sequence of SEQ ID NO:162; or

(fe) an amino acid sequence ~~at least 90%~~, at least 95%, or at least 99% identical to the amino acid sequence of SEQ ID NO:162, further comprising SEQ ID NO:163 and SEQ ID NO:165.

30. The protein according to any one of claims 1-29, wherein the protein binds to NKG2D with a K_D of 2 to 120 nM, as measured by surface plasmon resonance.

31. A protein comprising:

(a) a first antigen-binding site of an immunoglobulin molecule comprising a single-chain variable fragment (scFv) that binds NKG2D and comprises a heavy chain variable domain comprising complementarity-determining region 1 (CDR1), complementarity-determining region 2 (CDR2), and complementarity-determining region 3 (CDR3) sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 191, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively;

(b) a second antigen-binding site of an immunoglobulin molecule that binds B-cell maturation antigen (BCMA) and comprises a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 149, 150, and 151, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 153, 154, and 155, respectively;

(c) an additional antigen-binding site of an immunoglobulin molecule that binds BCMA and comprises a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 149, 150, and 151, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 153, 154, and 155, respectively; and

(d) an antibody Fc domain or a portion thereof sufficient to bind CD16.

32. A protein comprising:

- (a) a first polypeptide comprising the amino acid sequence of SEQ ID NO:162;
- (b) a second polypeptide comprising the amino acid sequence of SEQ ID NO:163; and
- (c) a third and fourth polypeptides each comprising the amino acid sequence of SEQ ID NO:165,

wherein the first polypeptide is linked to the second polypeptide via heterodimerization and at least one disulfide bond, wherein the third polypeptide is linked to the first polypeptide via a disulfide bond, and wherein the fourth polypeptide is linked to the second polypeptide via a disulfide bond.

3133. A formulation comprising the protein according to any one of the preceding claims and a pharmaceutically acceptable carrier.

3234. A cell comprising one or more nucleic acids encoding a protein according to any one of claims 1-~~30~~32.

3335. An *in vitro* or *ex vivo* method of ~~Use of the protein according to any one of claims 1-30 in the manufacture of a medicament for~~ directly and/or indirectly enhancing tumor cell death, by exposing a tumor and natural killer cell to the protein according to any one of claims 1-~~30~~32, wherein the protein mediates natural killer (NK) cell activation.

3436. Use of the protein according to any one of claims 1-~~30~~32 or the formulation according to claim ~~3133~~ in the manufacture of a medicament for treating cancer, wherein the protein mediates natural killer (NK) cell activation.

3537. The use of claim ~~3436~~, wherein the cancer is selected from the group consisting of multiple myeloma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, B cell lymphomas, and acute myeloid leukemia.

38. The use of claim 36, wherein the cancer is multiple myeloma.