

PROTEINS BINDING NKG2D, CD16 AND NECTIN4

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application No. 62/555,110, filed September 7, 2017, and U.S. Provisional Patent Application No. 62/566,824, filed on October 2, 2017, the entire disclosure of each of which is hereby incorporated by reference in its entirety for all purposes.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on September 6, 2018, is named DFY-038WO_SL.txt and is 321,395 bytes in size.

FIELD OF THE INVENTION

[0003] The invention relates to multi-specific binding proteins that bind to NKG2D, CD16, and a tumor-associated antigen.

BACKGROUND

[0004] Cancer continues to be a significant health problem despite the substantial research efforts and scientific advances reported in the literature for treating this disease. Some of the most frequently diagnosed cancers include prostate cancer, breast cancer, lung cancer, and colorectal cancer. Prostate cancer is the most common form of cancer in men. Breast cancer remains a leading cause of death in women. Blood and bone marrow cancers are also frequently diagnosed cancer types, including multiple myelomas, leukemia, and lymphomas. Current treatment options for these cancers are not effective for all patients and/or can have substantial adverse side effects. Other types of cancer also remain challenging to treat using existing therapeutic options.

[0005] Cancer immunotherapies are desirable because they are highly specific and can facilitate destruction of cancer cells using the patient's own immune system. Fusion proteins such as bi-specific T-cell engagers are cancer immunotherapies described in the literature that bind to tumor cells and T-cells to facilitate destruction of tumor cells. Antibodies that bind to certain tumor-associated antigens and to certain immune cells have been described in the literature. See, for example WO 2016/134371 and WO 2015/095412.

to be involved in choline transport, but it has been linked with acetylcholine synthesis and transport as well as uptake of thiamine pyrophosphate, the phosphorylated form of vitamin B1. SLC44A4 is normally expressed on the apical surface of secretory epithelial cells, but it is markedly upregulated in a variety of epithelial tumors, most notably pancreatic cancer, prostate cancer, and gastric cancer.

[0015] CA19-9 is the common term for carbohydrate antigen sialyl Lewis a. It is overexpressed in cancer of the digestive organs such as pancreatic cancer, colorectal cancer, cholangiocarcinoma, and liver cancer. Therefore, it is the most frequently applied serum tumor marker for diagnosis of these above mentioned cancers.

SUMMARY

[0016] The disclosure provides multi-specific binding proteins that bind to a tumor-associated antigen (selected from any one of the antigens provided in Table 11) and to the NKG2D receptor and CD16 receptor on natural killer cells. Such proteins can engage more than one kind of NK activating receptor, and may block the binding of natural ligands to NKG2D. In certain examples, the proteins can agonize NK cells in humans, and in other species such as rodents and cynomolgus monkeys. Various aspects and exampleexamples of the disclosure are described in further detail below.

[0016a] In one aspect, the present disclosure refers to a protein comprising: (a) a first antigen-binding site that binds Natural killer group 2 member D (NKG2D); (b) a second antigen-binding site that binds Nectin cell adhesion molecule 4 (Nectin4); and (c) an antibody Fc domain or a portion thereof sufficient to bind cluster of differentiation 16 (CD16), or a third antigen-binding site that binds CD16.

[0016b] In another aspect, the present disclosure refers to a formulation comprising a protein according to any one of the preceding claims and a pharmaceutically acceptable carrier. In another aspect, the present disclosure refers to a cell comprising one or more nucleic acids encoding a protein as described herein.

[0016c] In yet another aspect, the present disclosure refers to a method of directly and/or indirectly enhancing tumor cell death, the method comprising exposing the tumor cell and a natural killer cell to a protein as described herein. In another aspect, the present disclosure refers to a method of treating cancer, wherein the method comprises administering a protein as described herein or a formulation as described herein to a patient.

[0017] Accordingly, one example of the disclosure provides a protein that incorporates a first antigen-binding site that binds NKG2D; a second antigen-binding site that binds an antigen selected from EpCAM, Cancer Antigen 125 (CA125), sodium/phosphate cotransporter 2B (NaPi2b), Nectin cell adhesion molecule 4 (Nectin4), Fucosyl-GM1 (monosialotetrahexosylganglioside), disintegrin and metalloproteinase domain-containing protein 8 (ADAM8), disintegrin and metalloproteinase domain-containing protein 9 (ADAM9), solute carrier family 44 member 4 (SLC44A4), and sialylated Lewis a antigen (CA19-9); and an antibody Fc domain, a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16. The antigen-binding sites may each incorporate an antibody heavy chain variable domain and an antibody light chain variable domain (*e.g.* arranged as in an antibody, or fused together to form an scFv), or one or more of the antigen-binding sites may be a single domain antibody, such as a V_HH antibody like a camelid antibody or a V_{NAR} antibody like those found in cartilaginous fish.

[0018] The disclosure provides multi-specific binding proteins that bind the NKG2D receptor, CD16, and an antigen selected from EpCAM, Cancer Antigen 125 (CA125), sodium/phosphate cotransporter 2B (NaPi2b), Nectin cell adhesion molecule 4 (Nectin4), Fucosyl-GM1 (monosialotetrahexosylganglioside), disintegrin and metalloproteinase domain-containing protein 8 (ADAM8), disintegrin and metalloproteinase domain-containing protein 9 (ADAM9), solute carrier family 44 member 4 (SLC44A4), and sialylated Lewis a antigen (CA19-9).

[0019] Some proteins of the present disclosure include an Fab fragment linked to the antibody Fc domain or a portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16.

[0020] Some proteins of the present disclosure include an Fab fragment, wherein the heavy chain portion of the Fab fragment comprises a heavy chain variable domain and a CH1 domain, and wherein the heavy chain variable domain is linked to the CH1 domain.

[0021] Some proteins of the present disclosure include an Fab fragment linked to the antibody Fc domain.

[0022] In one example, the disclosure provides a protein comprising (a) a first antigen-binding site comprising an Fab fragment that binds NKG2D; (b) a second antigen-binding site comprising a single-chain variable fragment (scFv) that binds EpCAM; and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16. The present disclosure provides a protein in which the first antigen-

binding site that binds NKG2D is an Fab fragment, and the second antigen-binding site that binds a tumor-associated antigen EpCAM is an scFv.

[0023] Certain proteins described in the present disclosure include an scFv-targeting EpCAM, comprising a heavy chain variable domain and a light chain variable domain, linked to an 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 or Gly-Ala-Ser. Some proteins of the present disclosure includes an scFv-targeting EpCAM linked to an antibody Fc domain. Some proteins of the present disclosure includes a heavy chain variable domain of an scFv-targeting EpCAM, which forms a disulfide bridge with the light chain variable domain of the scFv.

[0024] Some proteins of the present disclosure include an scFv-targeting EpCAM, in which a disulfide bridge is formed between C44 from the heavy chain variable domain and C100 from the light chain variable domain.

[0025] Some proteins of the present disclosure include an scFv-targeting EpCAM linked to an antibody Fc domain, in which the light chain variable domain of the scFv is positioned at the N-terminus of the heavy chain variable domain of the scFv, and is linked to the heavy chain variable domain of the scFv via a flexible linker (GlyGlyGlyGlySer)₄ (G4S)₄ (SEQ ID NO:206), and the Fab fragment that binds NKG2D is linked to the antibody Fc domain.

[0026] Some proteins of the present disclosure include an scFv-targeting EpCAM in which the heavy chain variable domain is positioned at the N-terminus or the C-terminus of the light chain variable domain of the scFv.

[0027] Some proteins of the present disclosure include an scFv-targeting EpCAM in which the light chain variable domain is positioned at the N-terminus of the heavy chain variable domain of the scFv.

[0028] In one example of the disclosure provides 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 EpCAM; and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16. In certain examples, a protein of the present disclosure further comprises an additional antigen-binding site that binds EpCAM. In certain examples, the second antigen-binding site of a protein described in the present disclosure is an Fab fragment that binds EpCAM. In certain examples, the second and the additional antigen-binding site of a protein described in the present disclosure are Fab fragments that bind EpCAM.

[0029] In certain examples, the second and the additional antigen-binding site of a protein described in the present disclosure are scFvs that bind EpCAM. In certain examples, 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. In certain examples, the light chain variable domain is positioned at the N-terminus of the heavy chain variable domain of the scFv that binds NKG2D.

[0030] In certain examples, 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. In certain examples, 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 *via* a hinge comprising Ala-Ser (*e.g.*, in a TriNKET that comprises an additional antigen-binding site that binds EpCAM, CA125, NaPi2b, Nectin4, Fucosyl-GM1, ADAM8, ADAM9, SLC44A4, or CA19-9) or Gly-Ala-Ser (*e.g.*, in a TriNKET that does not comprise an additional antigen-binding site that binds EpCAM, CA125, NaPi2b, Nectin4, Fucosyl-GM1, ADAM8, ADAM9, SLC44A4, or CA19-9). In certain examples, the scFv that binds to NKG2D 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 G4S. In certain examples, 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.

[0031] In certain examples, 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. In certain examples, the disulfide bridge is formed between C44 from the heavy chain variable domain and C100 from the light chain variable domain.

[0032] Some proteins of the present disclosure include a sequence selected from SEQ ID NO:210 and SEQ ID NO:211.

[0033] Some proteins of the present disclosure include an scFv linked to an antibody Fc domain, wherein the scFv linked to the antibody Fc domain is represented by a sequence selected from SEQ ID NO:208 and SEQ ID NO:209.

[0034] Some proteins of the present disclosure include a sequence of SEQ ID NO:205 and SEQ ID NO:213.

[0035] Some proteins of the present disclosure include a sequence at least 90% identical to an amino acid sequence selected from SEQ ID NO:210 and SEQ ID NO:211.

[0036] Some proteins of the present disclosure include a sequence at least 95% identical to an amino acid sequence selected from SEQ ID NO:210 and SEQ ID NO:211.

[0037] Some proteins of the present disclosure include a sequence at least 99% identical to an amino acid sequence selected from SEQ ID NO:210 and SEQ ID NO:211.

[0038] Some proteins of the present disclosure include an amino acid sequence of SEQ ID NO:203.

[0039] Some proteins of the present disclosure include an amino acid sequence of SEQ ID NO:203 and SEQ ID NO:204.

[0040] Some proteins of the present disclosure include an amino acid sequence at least 90% identical to an amino acid sequence of SEQ ID NO:203. Some proteins of the present disclosure include an amino acid sequence at least 95% identical to an amino acid sequence of SEQ ID NO:203. Some proteins of the present disclosure include an amino acid sequence at least 99% identical to an amino acid sequence of SEQ ID NO:203.

[0041] The first antigen-binding site, which binds to NKG2D, in some examples, can incorporate a heavy chain variable domain related to SEQ ID NO:1, such as by having an amino acid sequence at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:1, and/or incorporating amino acid sequences identical to the CDR1 (SEQ ID NO:105), CDR2 (SEQ ID NO:106), and CDR3 (SEQ ID NO:107) sequences of SEQ ID NO:1. The heavy chain variable domain related to SEQ ID NO:1 can be coupled with a variety of light chain variable domains to form an NKG2D binding site. For example, the first antigen-binding site that incorporates a heavy chain variable domain related to SEQ ID NO:1 can further incorporate a light chain variable domain selected from any one of the sequences related to SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, and 40. For example, the first antigen-binding site incorporates a heavy chain variable domain with amino acid sequences at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:1 and a light chain variable domain with amino acid sequences at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to any one of the sequences selected from SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, and 40.

[0042] Alternatively, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:41 and a light chain variable domain related to SEQ ID NO:42. For example, the heavy chain variable domain of the first antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:41, and/or incorporate amino acid sequences identical to the CDR1

(SEQ ID NO:43), CDR2 (SEQ ID NO:44), and CDR3 (SEQ ID NO:45) sequences of SEQ ID NO:41. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:42, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:46), CDR2 (SEQ ID NO:47), and CDR3 (SEQ ID NO:48) sequences of SEQ ID NO:42.

[0043] In other examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:49 and a light chain variable domain related to SEQ ID NO:50. For example, the heavy chain variable domain of the first antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:49, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:51), CDR2 (SEQ ID NO:52), and CDR3 (SEQ ID NO:53) sequences of SEQ ID NO:49. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:50, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:54), CDR2 (SEQ ID NO:55), and CDR3 (SEQ ID NO:56) sequences of SEQ ID NO:50.

[0044] Alternatively, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:57 and a light chain variable domain related to SEQ ID NO:58, such as by having amino acid sequences at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:57 and at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:58, respectively.

[0045] In another example, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:59 and a light chain variable domain related to SEQ ID NO:60. For example, the heavy chain variable domain of the first antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:59, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:109), CDR2 (SEQ ID NO:110), and CDR3 (SEQ ID NO:111) sequences of SEQ ID NO:59. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:60, and/or incorporate amino acid sequences identical to the CDR1

(SEQ ID NO:112), CDR2 (SEQ ID NO:113), and CDR3 (SEQ ID NO:114) sequences of SEQ ID NO:60.

[0046] The first antigen-binding site, which binds to NKG2D, in some examples, can incorporate a heavy chain variable domain related to SEQ ID NO:61 and a light chain variable domain related to SEQ ID NO:62. For example, the heavy chain variable domain of the first antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:61, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:63), CDR2 (SEQ ID NO:64), and CDR3 (SEQ ID NO:65) sequences of SEQ ID NO:61. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:62, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:66), CDR2 (SEQ ID NO:67), and CDR3 (SEQ ID NO:68) sequences of SEQ ID NO:62.

[0047] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:69 and a light chain variable domain related to SEQ ID NO:70. For example, the heavy chain variable domain of the first antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:69, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:71), CDR2 (SEQ ID NO:72), and CDR3 (SEQ ID NO:73) sequences of SEQ ID NO:69. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:70, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:74), CDR2 (SEQ ID NO:75), and CDR3 (SEQ ID NO:76) sequences of SEQ ID NO:70.

[0048] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:77 and a light chain variable domain related to SEQ ID NO:78. For example, the heavy chain variable domain of the first antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:77, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:79), CDR2 (SEQ ID NO:80), and CDR3 (SEQ ID NO:81) sequences of SEQ ID NO:77. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:78, and/or incorporate amino acid sequences identical to the CDR1

(SEQ ID NO:82), CDR2 (SEQ ID NO:83), and CDR3 (SEQ ID NO:84) sequences of SEQ ID NO:78.

[0049] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:85 and a light chain variable domain related to SEQ ID NO:86. For example, the heavy chain variable domain of the first antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:85, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:87), CDR2 (SEQ ID NO:88), and CDR3 (SEQ ID NO:89) sequences of SEQ ID NO:85. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:86, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:90), CDR2 (SEQ ID NO:91), and CDR3 (SEQ ID NO:92) sequences of SEQ ID NO:86.

[0050] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:93 and a light chain variable domain related to SEQ ID NO:94. For example, the heavy chain variable domain of the first antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:93, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:95), CDR2 (SEQ ID NO:96), and CDR3 (SEQ ID NO:97) sequences of SEQ ID NO:93. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:94, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:98), CDR2 (SEQ ID NO:99), and CDR3 (SEQ ID NO:100) sequences of SEQ ID NO:94.

[0051] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:101 and a light chain variable domain related to SEQ ID NO:102, such as by having amino acid sequences at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:101 and at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:102, respectively.

[0052] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:103 and a light chain variable domain related to SEQ

ID NO:104, such as by having amino acid sequences at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:103 and at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:104, respectively.

[0053] In some examples, the second antigen-binding site can bind to EpCAM and can incorporate a heavy chain variable domain related to SEQ ID NO:115 and a light chain variable domain related to SEQ ID NO:119. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:115, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:116), CDR2 (SEQ ID NO:117), and CDR3 (SEQ ID NO:118) sequences of SEQ ID NO:115. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:119, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:120), CDR2 (SEQ ID NO:121), and CDR3 (SEQ ID NO:122) sequences of SEQ ID NO:119.

[0054] In some examples, the second antigen-binding site can bind to EpCAM and can incorporate a heavy chain variable domain related to SEQ ID NO:123 and a light chain variable domain related to SEQ ID NO:127. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:123, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:124), CDR2 (SEQ ID NO:125), and CDR3 (SEQ ID NO:126) sequences of SEQ ID NO:123. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:127, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:128), CDR2 (SEQ ID NO:129), and CDR3 (SEQ ID NO:130) sequences of SEQ ID NO:127.

[0055] In some examples, the second antigen-binding site can bind to EpCAM and can incorporate a heavy chain variable domain related to SEQ ID NO:131 and a light chain variable domain related to SEQ ID NO:135. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:131, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:132), CDR2 (SEQ ID NO:133), and CDR3 (SEQ ID NO:134) sequences of SEQ ID NO:131. Similarly, the light chain variable domain

of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:135, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:136), CDR2 (SEQ ID NO:137), and CDR3 (SEQ ID NO:138) sequences of SEQ ID NO:135.

[0056] In some examples, the second antigen-binding site can bind to EpCAM and can incorporate a heavy chain variable domain related to SEQ ID NO:139 and a light chain variable domain related to SEQ ID NO:143. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:139, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:140), CDR2 (SEQ ID NO:141), and CDR3 (SEQ ID NO:142) sequences of SEQ ID NO:139. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:143, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:144), CDR2 (SEQ ID NO:145), and CDR3 (SEQ ID NO:146) sequences of SEQ ID NO:143.

[0057] In some examples, the second antigen-binding site can bind to CA125 and can incorporate a heavy chain variable domain related to SEQ ID NO:155 and a light chain variable domain related to SEQ ID NO:159. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:155, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:156), CDR2 (SEQ ID NO:157), and CDR3 (SEQ ID NO:158) sequences of SEQ ID NO:155. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:159, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:160), CDR2 (SEQ ID NO:161), and CDR3 (SEQ ID NO:162) sequences of SEQ ID NO:159.

[0058] In some examples, the second antigen-binding site can bind to CA125 and can incorporate a heavy chain variable domain related to SEQ ID NO:163 and a light chain variable domain related to SEQ ID NO:167. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:163, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:164), CDR2 (SEQ ID NO:165), and CDR3 (SEQ ID NO:166) sequences of SEQ ID NO:163. Similarly, the light chain variable domain

of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:167, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:168), CDR2 (SEQ ID NO:169), and CDR3 (SEQ ID NO:170) sequences of SEQ ID NO:167.

[0059] In some examples, the second antigen-binding site can bind to NaPi2b and can incorporate a heavy chain variable domain related to SEQ ID NO:171 and a light chain variable domain related to SEQ ID NO:175. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:171, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:172), CDR2 (SEQ ID NO:173), and CDR3 (SEQ ID NO:174) sequences of SEQ ID NO:171. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:175, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:176), CDR2 (SEQ ID NO:177), and CDR3 (SEQ ID NO:178) sequences of SEQ ID NO:175.

[0060] In some examples, the second antigen-binding site can bind to Nectin4 and can incorporate a heavy chain variable domain related to SEQ ID NO:179 and a light chain variable domain related to SEQ ID NO:183. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:179, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:180), CDR2 (SEQ ID NO:181), and CDR3 (SEQ ID NO:182) sequences of SEQ ID NO:179. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:183, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:184), CDR2 (SEQ ID NO:185), and CDR3 (SEQ ID NO:186) sequences of SEQ ID NO:183.

[0061] In some examples, the second antigen-binding site can bind to fucosyl-GM1 and can incorporate a heavy chain variable domain related to SEQ ID NO:187 and a light chain variable domain related to SEQ ID NO:191. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:187, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:188), CDR2 (SEQ ID NO:189), and CDR3 (SEQ ID NO:190) sequences of SEQ ID NO:187. Similarly, the light chain variable domain

of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:191, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:192), CDR2 (SEQ ID NO:193), and CDR3 (SEQ ID NO:194) sequences of SEQ ID NO:191.

[0062] In some examples, the second antigen-binding site can bind to SLC44A4 and can incorporate a heavy chain variable domain related to SEQ ID NO:195 and a light chain variable domain related to SEQ ID NO:199. For example, the heavy chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:195, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:196), CDR2 (SEQ ID NO:197), and CDR3 (SEQ ID NO:198) sequences of SEQ ID NO:195. Similarly, the light chain variable domain of the second antigen-binding site can be at least 90% (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:199, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:200), CDR2 (SEQ ID NO:201), and CDR3 (SEQ ID NO:202) sequences of SEQ ID NO:199.

[0063] In some examples, the second antigen binding site incorporates a light chain variable domain having an amino acid sequence identical to the amino acid sequence of the light chain variable domain present in the first antigen binding site.

[0064] In some examples, the protein incorporates a portion of an antibody Fc domain sufficient to bind CD16, wherein the antibody Fc domain comprises hinge and CH2 domains, and/or amino acid sequences at least 90% identical to amino acid sequence 234-332 of a human IgG antibody.

[0065] Some proteins of the present disclosure bind to NKG2D with a K_D of 10 nM or weaker affinity.

[0066] Formulations containing one of these proteins; cells containing one or more nucleic acids expressing these proteins, and methods of enhancing tumor cell death using these proteins are also provided.

[0067] Another example of the disclosure provides a method of treating cancer in a patient. The method comprises administering to a patient in need thereof a therapeutically effective amount of the multi-specific binding protein described herein. Exemplary cancers for treatment using the multi-specific binding proteins include, for example, head and neck cancer, ovarian cancer, bladder cancer, breast cancer, colorectal cancer, prostate cancer, gastric cancer, liver cancer, esophageal cancer, and lung cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

[0068] **FIG. 1** is a representation of a heterodimeric, multi-specific antibody (a trispecific binding protein (TriNKET)). Each arm can represent either the NKG2D-binding domain, or the tumor associated antigen-binding domain. In some embodiments, the NKG2D- and the tumor associated antigen- binding domains can share a common light chain.

[0069] **FIG. 2** is a representation of a heterodimeric, multi-specific antibody. Either the NKG2D-binding domain or the tumor associated antigen-binding domain can take the scFv format (right arm).

[0070] **FIG. 3** are line graphs demonstrating the binding affinity of NKG2D-binding domains (listed as clones) to human recombinant NKG2D in an ELISA assay.

[0071] **FIG. 4** are line graphs demonstrating the binding affinity of NKG2D-binding domains (listed as clones) to cynomolgus recombinant NKG2D in an ELISA assay.

[0072] **FIG. 5** are line graphs demonstrating the binding affinity of NKG2D-binding domains (listed as clones) to mouse recombinant NKG2D in an ELISA assay.

CLAIMS

1. A protein comprising:
 - (a) a first antigen-binding site that binds Natural killer group 2 member D (NKG2D);
 - (b) a second antigen-binding site that binds Nectin cell adhesion molecule 4 (Nectin4); and
 - (c) an antibody Fc domain or a portion thereof sufficient to bind cluster of differentiation 16 (CD16), or a third antigen-binding site that binds CD16.
2. The protein of claim 1, wherein the first antigen-binding site binds to NKG2D in humans and non-human primates.
3. The protein of claim 1 or 2, wherein the first antigen-binding site comprises a heavy chain variable domain and a light chain variable domain.
4. A protein according to claim 3, wherein the heavy chain variable domain and the light chain variable domain are present on the same polypeptide.
5. A protein according to claim 3 or 4, wherein the second antigen-binding site comprises a heavy chain variable domain and a light chain variable domain.
6. A protein according to claim 5, wherein the heavy chain variable domain and the light chain variable domain of the second antigen-binding site are present on the same polypeptide.
7. A protein according to claim 5 or 6, wherein the light chain variable domain of the first antigen-binding site has an amino acid sequence identical to the amino acid sequence of the light chain variable domain of the second antigen-binding site.
8. A protein according to any one of the preceding claims, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to an amino acid sequence selected from: SEQ ID NO:85, SEQ ID NO:1, SEQ ID NO:41, SEQ ID NO:49, SEQ ID NO:57, SEQ ID NO:59, SEQ ID NO:61, SEQ ID NO:69, SEQ ID NO:77, and SEQ ID NO:93.

9. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain at least 90% identical to SEQ ID NO:85 and a light chain variable domain at least 90% identical to SEQ ID NO:86.
10. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:41 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:42.
11. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:49 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:50.
12. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:57 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:58.
13. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:59 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:60.
14. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:61 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:62.
15. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:69 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:70.

16. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:77 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:78.
17. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:93 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:94.
18. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:101 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:102.
19. A protein according to any one of claims 1-7, wherein the first antigen-binding site comprises a heavy chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:103 and a light chain variable domain amino acid sequence at least 90% identical to SEQ ID NO:104.
20. The protein of any one of claims 1-4, wherein the first antigen-binding site is a single-domain antibody.
21. The protein of claim 20, wherein the single-domain antibody is a V_HH fragment or a V_{NAR} fragment.
22. A protein of any one of claims 1-4 or 20-21, wherein the second antigen-binding site comprises a heavy chain variable domain and a light chain variable domain.
23. A protein of claim 22, wherein the heavy chain variable domain and the light chain variable domain of the second antigen-binding site are present on the same polypeptide.
24. A protein of any one of claims 1 or 8-19, wherein the second antigen-binding site comprises a heavy chain variable domain comprising an amino acid sequence at least 90%

identical to SEQ ID NO:179 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:183.

25. The protein of any one of claims 1, 8-19, or 24, wherein the second antigen-binding site comprises:

- a heavy chain CDR1 comprising the amino acid sequence of SEQ ID NO:180;
- a heavy chain CDR2 comprising the amino acid sequence of SEQ ID NO:181;
- a heavy chain CDR3 comprising the amino acid sequence of SEQ ID NO:182;
- a light chain CDR1 comprising the amino acid sequence of SEQ ID NO:184;
- a light chain CDR2 comprising the amino acid sequence of SEQ ID NO:185; and
- a light chain CDR3 comprising the amino acid sequence of SEQ ID NO:186.

26. A protein according to any one of claims 1-24, wherein the antibody Fc domain comprises hinge and CH2 domains of a human IgG1 antibody.

27. A protein of claim 26, wherein the Fc domain comprises an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody.

28. A protein of claim 27, wherein the Fc domain comprises an 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, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411, and K439.

29. A protein according to any one of claims 1-27, wherein the protein binds to NKG2D with a K_D of 10 nM or weaker affinity.

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

31. A cell comprising one or more nucleic acids encoding a protein according to any one of claims 1-29.

32. A method of directly and/or indirectly enhancing tumor cell death, the method comprising exposing the tumor cell and a natural killer cell to a protein according to any one of claims 1-29.

33. A method of treating cancer, wherein the method comprises administering a protein according to any one of claims 1-29 or a formulation according to claim 30 to a patient.

34. The method of claim 33, wherein the cancer is selected from the group consisting of bladder cancer, breast cancer, ovarian cancer, pancreatic cancer, colorectal cancer, and lung cancer.