

PROTEINS BINDING NKG2D, CD16, AND HLA-E

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Provisional Patent Application No. 62/546,300, filed August 16, 2017; U.S. Provisional Patent Application No. 62/546,297, filed August 16, 2017; U.S. Provisional Patent Application No. 62/552,152, filed August 30, 2017; and U.S. Provisional Patent Application No. 62/555,114, filed September 7, 2017, the content 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 August 15, 2018, is named DFY-033WO_SL.txt and is 214,413 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 selected from EGFR, HLA-E, CCR4, and PD-L1.

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. Blood and bone marrow cancers are 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, e.g.*, WO 2016/134371 and WO 2015/095412.

SUMMARY

[0013] The disclosure provides multi-specific binding proteins that bind to the NKG2D receptor and CD16 receptor on natural killer cells, and a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1. 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. In some examples, the proteins can agonize NK cells in humans and in other species such as rodents and cynomolgus monkeys. Various aspects and embodiments of the invention are described in further detail below.

[0013a] In one aspect, the present disclosure refers to a protein comprising: (a) a first antigen-binding site that binds NKG2D; (b) a second antigen-binding site that binds HLA-E; and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16.

[0013b] In another aspect, the present disclosure refers to a formulation comprising a protein as disclosed herein and a pharmaceutically acceptable carrier.

[0013c] In yet another aspect, the present disclosure refers to a cell comprising one or more nucleic acids encoding a protein as disclosed herein.

[0013d] In yet another aspect, the present disclosure refers to 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 as disclosed herein, wherein the tumor cell expresses HLA-E.

[0013e] In yet another aspect, the present disclosure refers to a method of treating cancer, wherein the method comprises administering an effective amount of the protein as disclosed herein or the formulation as disclosed herein to a patient.

[0014] 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 a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1; and an antibody fragment crystallizable (Fc) domain, a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16. In some examples, the first antigen-binding site binds to NKG2D in humans.

[0015] 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 a single-chain variable-fragment (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. For example, the first antigen-binding site that binds NKG2D includes an antibody heavy chain variable domain and an antibody light chain variable domain. In some examples the second antigen-binding site that binds a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1 includes an antibody heavy chain variable domain and an antibody light chain variable domain. In some examples the third antigen-binding site that binds CD16 includes an antibody heavy chain variable domain and an antibody light chain variable domain. In some examples, two or more of the first antigen-binding site, the second antigen-binding site, and the third antigen-binding site include an antibody heavy chain variable domain and an antibody light chain variable domain.

[0016] In some examples, the first antigen-binding site that binds NKG2D is a single-domain antibody, for example, a V_HH fragment or a V_{NAR} fragment. In some examples, the second antigen-binding site that binds a tumor-associated antigen selected from EGFR,

HLA-E, CCR4, and PD-L1 is a single-domain antibody, for example, a V_HH fragment or a V_{NAR} fragment. In some examples the third antigen-binding site that binds CD16 is a single-domain antibody, for example, a V_HH fragment or a V_{NAR} fragment. In some examples, two or more of the first antigen-binding site, the second antigen-binding site, and the third antigen-binding site are a single-domain antibody, for example, a V_HH fragment or a V_{NAR} fragment.

[0017] In some examples an antibody heavy chain variable domain and an antibody light chain variable domain are present on the same polypeptide. For example, in some examples the first antigen-binding site that binds NKG2D includes an antibody heavy chain variable domain and an antibody light chain variable domain present on the same polypeptide. In some examples the second antigen-binding site that binds a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1 includes an antibody heavy chain variable domain and an antibody light chain variable domain present on the same polypeptide. In some examples the third antigen-binding site that binds CD16 includes an antibody heavy chain variable domain and an antibody light chain variable domain present on the same polypeptide. In some examples, two or more of the first antigen-binding site, the second antigen-binding site, and the third antigen-binding site include an antibody heavy chain variable domain and an antibody light chain variable domain present on the same polypeptide.

[0018] 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 EGFR; 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 EGFR is an scFv.

[0019] Certain proteins described in the present disclosure include an scFv, 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. Some proteins of the present disclosure includes an scFv linked to an antibody Fc domain. Some proteins of the present disclosure includes a heavy chain variable domain of an scFv, which forms a disulfide bridge with the light chain variable domain of the scFv.

[0031] Some proteins of the present disclosure include a sequence at least 90% identical to an amino acid sequence selected from SEQ ID NO:264, SEQ ID NO:265, and SEQ ID NO:266.

[0032] Some proteins of the present disclosure include a sequence at least 95% identical to an amino acid sequence selected from SEQ ID NO:264, SEQ ID NO:265, and SEQ ID NO:266.

[0033] Some proteins of the present disclosure include a sequence at least 99% identical to an amino acid sequence selected from SEQ ID NO:264, SEQ ID NO:265, and SEQ ID NO:266.

[0034] Some proteins of the present disclosure include a sequence at least 90% identical to an amino acid sequence selected from SEQ ID NO:267, SEQ ID NO:268, and SEQ ID NO:269.

[0035] Some proteins of the present disclosure include a sequence at least 95% identical to an amino acid sequence selected from SEQ ID NO:267, SEQ ID NO:268, and SEQ ID NO:269.

[0036] Some proteins of the present disclosure include a sequence at least 99% identical to an amino acid sequence selected from SEQ ID NO:267, SEQ ID NO:268, and SEQ ID NO:269.

[0037] In one example, the present disclosure provides multi-specific binding proteins that bind to the NKG2D receptor and CD16 receptor on natural killer cells, and a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1. The first antigen-binding site that binds to NKG2D includes a heavy chain variable domain at least 90% identical to an amino acid sequence selected from the amino acid sequence of: 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, SEQ ID NO:85, and SEQ ID NO:93.

[0038] 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.

10 **[0039]** 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.

15 **[0040]** 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.

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[0041] 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%
5 (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:58, respectively.

[0042] 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
10 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:134), CDR2 (SEQ ID NO:135), and CDR3 (SEQ ID NO:136) 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%)
15 identical to SEQ ID NO:60, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:137), CDR2 (SEQ ID NO:138), and CDR3 (SEQ ID NO:139) sequences of SEQ ID NO:60.

[0043] 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
20 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
25 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.

[0044] In some examples, the first antigen-binding site can incorporate a heavy chain
30 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%)

5 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.

[0045] 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.

[0046] 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.

30 **[0047]** 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.

[0048] 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.

[0049] 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.

[0050] In some examples, the second antigen-binding site can bind to EGFR and can incorporate a heavy chain variable domain related to SEQ ID NO:217 and a light chain variable domain related to SEQ ID NO:109. 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:217, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:218), CDR2 (SEQ ID NO:219), and CDR3 (SEQ ID NO:220) sequences of SEQ ID NO:217. 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:109, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:110), CDR2 (SEQ ID NO:111), and CDR3 (SEQ ID NO:112) sequences of SEQ ID NO:109.

[0066] Alternatively, the second antigen-binding site can bind to CCR4 and can incorporate a heavy chain variable related to SEQ ID NO:200 and a light chain variable domain related to SEQ ID NO:204. 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:200, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:201), CDR2 (SEQ ID NO:202), and CDR3 (SEQ ID NO:203) sequences of SEQ ID NO:200. 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:204, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:205), CDR2 (SEQ ID NO:206), and CDR3 (SEQ ID NO:207) sequences of SEQ ID NO:204.

[0067] Alternatively, the second antigen-binding site can bind to CCR4 and can incorporate a heavy chain variable related to SEQ ID NO:208 and a light chain variable domain related to SEQ ID NO:212. 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:208, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:209), CDR2 (SEQ ID NO:210), and CDR3 (SEQ ID NO:211) sequences of SEQ ID NO:208. 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:212, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:213), CDR2 (SEQ ID NO:214), and CDR3 (SEQ ID NO:215) sequences of SEQ ID NO:212.

[0068] In some examples, the light chain variable domain of the first antigen-binding site includes an amino acid sequence identical to the amino acid sequence of the light chain variable domain of the second antigen-binding site. For example, in some examples, the light chain variable domain of the first antigen-binding site that binds NKGD2 includes an amino acid sequence identical to the amino acid sequence of the light chain variable domain of the second antigen-binding site that binds a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1.

[0069] In some examples, the protein incorporates a portion of an antibody Fc domain sufficient to bind CD16, wherein the antibody Fc domain comprises a hinge and a CH2 domain, for example, a hinge and a CH2 domain of a human IgG antibody. In some

examples, the antibody Fc domain includes amino acid sequences at least 90% identical to amino acid sequence 234-332 of a human IgG antibody. In some examples, the antibody Fc domain includes an amino acid sequence at least 90% identical to the Fc domain of human IgG1 and the amino acid sequence of the antibody Fc domain differs 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, K439.

[0070] Formulations containing any one of the proteins described herein, cells containing one or more nucleic acids expressing the proteins, and methods of enhancing tumor cell death using the proteins are also provided. In some examples, the disclosure provides a formulation that includes a protein described herein and a pharmaceutically acceptable carrier. For example, in some examples, the formulation includes a protein that incorporates a first antigen-binding site that binds NKG2D; a second antigen-binding site that binds a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1; and an antibody Fc domain, a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16, and a pharmaceutically acceptable carrier. In some examples, the disclosure provides a cell containing one or more nucleic acids that express a protein that incorporates a first antigen-binding site that binds NKG2D; a second antigen-binding site that binds a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1; and an antibody Fc domain, a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16. In some examples, the disclosure provides a method of enhancing tumor cell death by exposing tumor cells and natural killer cells to an effective amount of a protein described herein, where the tumor cells express EGFR, HLA-E, CCR4, or PD-L1. For example, provided herein is a method of enhancing tumor cell death by exposing a tumor cell and a natural killer cell to an effective amount of a protein that incorporates a first antigen-binding site that binds NKG2D; a second antigen-binding site that binds a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1; and an antibody Fc domain, a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16, where the tumor cell expresses the tumor-associated antigen to which the second antigen-binding site of the protein binds (*e.g.*, EGFR, HLA-E, CCR4, or PD-L1).

[0071] Another example of the disclosure provides a method of treating cancer in a patient. The method comprises administering to a patient, for example, a patient in need thereof, a therapeutically effective amount of a multi-specific binding protein described herein or a formulation that includes a therapeutically effective amount of a multi-specific binding

protein described herein. For example, in some examples, the method of treating cancer includes administering to a patient, for example, a patient in need of treatment, a formulation that includes a therapeutically effective amount of a multi-specific binding protein described herein and a pharmaceutically acceptable carrier.

5 **[0072]** In some examples, the method of treating cancer includes administering to a patient, for example, a patient in need of treatment, a therapeutically effective amount of a protein that incorporates a first antigen-binding site that binds NKG2D; a second antigen-binding site that binds a tumor-associated antigen selected from EGFR, HLA-E, CCR4, and PD-L1; and an antibody Fc domain, a portion thereof sufficient to bind CD16, or a third
10 antigen-binding site that binds CD16.

[0073] Exemplary cancers to be treated using the multi-specific binding proteins include adult T-cell lymphoma/leukemia, anaplastic large cell lymphoma, a B cell malignancy, bladder cancer, chronic lymphocytic leukemia, cervical cancer, colorectal cancer, cutaneous T cell lymphoma, gastric cancer, glioblastoma, glioma, head and neck cancer, Hodgkin's
15 lymphoma, leukemia, liver cancer, lung cancer, lymphoma, a mature T/natural killer (NK) cell neoplasm, melanoma, multiple myeloma, non-Hodgkin's lymphoma, non-small cell lung cancer, ovarian cancer, pancreatic cancer, peripheral T cell lymphoma, renal cancer, renal cell carcinoma, a sarcoma, and thymoma. In some examples, the second antigen-binding site of the protein binds EGFR, and the cancer to be treated is head and neck cancer, colorectal
20 cancer, non-small cell lung cancer, glioma, renal cell carcinoma, bladder cancer, cervical cancer, ovarian cancer, pancreatic cancer, or liver cancer. In some examples, the second antigen-binding site of the protein binds HLA-E, and the cancer to be treated is lymphoma, head and neck cancer, bladder cancer, cervical cancer, lung cancer, renal cancer, melanoma, colorectal cancer, ovarian cancer, glioblastoma, or a sarcoma. In some examples, the second
25 antigen-binding site of the protein binds PD-L1, and the cancer to be treated is lymphoma, leukemia, multiple myeloma, head and neck cancer, bladder cancer, cervical cancer, lung cancer, renal cancer, melanoma, colorectal cancer, ovarian cancer, glioblastoma, a sarcoma, or gastric cancer. In some examples, the second antigen-binding site of the protein binds CCR4, and the cancer to be treated is adult T-cell lymphoma/leukemia, leukemia, peripheral
30 T cell lymphoma, cutaneous T cell lymphoma, chronic lymphocytic leukemia, a B cell malignancy, non-Hodgkin's lymphoma, Hodgkin's lymphoma, anaplastic large cell lymphoma, a mature T/natural killer (NK) cell neoplasm, thymoma, gastric cancer, or renal cell carcinoma.

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 HLA-E; and
 - (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16.
2. The protein of claim 1, wherein the first antigen-binding site binds to human and cynomolgus monkey NKG2D.
3. The protein of any one of claims 1 or 2, wherein the first antigen-binding site comprises a heavy chain variable domain and a light chain variable domain.
4. The protein of 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. The protein of 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 claims 1-7, 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 amino acid sequence at least 90% identical to SEQ ID

NO:85 and a light chain variable domain amino acid sequence 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 claim 1 or 2, wherein the first antigen-binding site comprises a single-domain antibody.
21. The protein of claim 20, wherein the single-domain antibody of the first antigen-binding site comprises a V_{HH} fragment or a V_{NAR} fragment.
22. A protein according to any one of claims 1-2, 20, or 21, wherein the second antigen-binding site comprises a heavy chain variable domain and a light chain variable domain.
23. The protein of claim 21, 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 according to any one of claims 1-7, wherein the second antigen-binding site comprises a single-domain antibody.

25. The protein of claim 24, wherein the single-domain antibody of the second antigen-binding site comprises a V_HH fragment or a V_{NAR} fragment.
26. A protein according to any one of claims 1-24, wherein the antibody Fc domain comprises a hinge and a CH2 domain.
27. The protein of claim 25, wherein the antibody Fc domain comprises a hinge and a CH2 domain of a human IgG1 antibody.
28. The protein of claim 26 or 27, wherein the Fc domain comprises an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody.
29. The protein of claim 28, 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.
30. A protein according to any one of claims 1-29, wherein the protein binds to NKG2D with a K_D of 10 nM or weaker affinity.
31. A formulation comprising a protein according to any one of the preceding claims and a pharmaceutically acceptable carrier.
32. A cell comprising one or more nucleic acids encoding a protein according to any one of claims 1-29.
33. 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 according to any one of claims 1-29, wherein the tumor cell expresses HLA-E.
34. A method of treating cancer, wherein the method comprises administering an effective amount of the protein according to any one of claims 1-29 or the formulation according to claim 31 to a patient.

35. The method of claim 34, wherein the cancer is selected from the group consisting of lymphoma, head and neck cancer, bladder cancer, cervical cancer, lung cancer, renal cancer, melanoma, colorectal cancer, ovarian cancer, glioblastoma, and a sarcoma.