SUMMARY

[0014] The disclosure provides multi-specific binding proteins that bind to CAIX, ANO1, mesothelin, TROP2, CEA or Claudin-18.2 on a cancer cell 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 examples of the disclosure are described in further detail below.

[0015] 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 CAIX, ANO1, mesothelin, TROP2, or Claudin-18.2; and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16. In another aspect, the present disclosure refers to a formulation comprising a protein as described herein and a pharmaceutically acceptable carrier. In yet another aspect, the present disclosure refers to a cell comprising one or more nucleic acids encoding a protein as described herein. In yet another aspect, the present disclosure refers to a method of directly and/or indirectly enhancing tumor cell death, the method comprising exposing a tumor and natural killer cells to a protein as described herein. In still another aspect, the present disclosure refers to use of a protein as described herein or a formulation as described herein in the manufacture of a medicament for treating cancer.

[0015a] 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 CAIX, ANO1, mesothelin, TROP2, CEA or Claudin-18.2; 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 from 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.

[0016] In one example, the first antigen-binding site, which binds to NKG2D, incorporates 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:51), CDR2 (SEQ ID NO:52), and CDR3 (SEQ ID NO:53) sequences of SEQ ID NO:1. The heavy chain variable domain related to SEQ ID NO:1 can be coupled a variety of light chain

variable domains to form a 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, 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, and 40. 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:54), CDR2 (SEQ ID NO:55), and CDR3 (SEQ ID NO:56) 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:57), CDR2 (SEQ ID NO:58), and CDR3 (SEQ ID NO:59) sequences of SEQ ID NO:42. In other examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:43 and a light chain variable domain related to SEQ ID NO:44. 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:43, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:60), CDR2 (SEQ ID NO:61), and CDR3 (SEQ ID NO:62) sequences of SEQ ID NO:43. 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:44, 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:44. In certain examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:45 and a light chain variable domain related to SEQ ID NO:46. 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:45, 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:45. 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:46, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:69), CDR2 (SEQ ID NO:70), and CDR3 (SEQ ID NO:71) sequences of SEQ ID NO:46.

[0017] Alternatively, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:47 and a light chain variable domain related to SEQ ID NO:48, 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:47 and SEQ ID NO:48 respectively. In another example, 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, 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:49 and SEQ ID NO:50 respectively.

[0018] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:132 and a light chain variable domain related to SEQ ID NO:133. 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:132, 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:132. 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:133, 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:133.

[0019] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:140 and a light chain variable domain related to SEQ ID NO:141. 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:140, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:142), CDR2 (SEQ ID NO:143), and CDR3 (SEQ ID NO:144) sequences of SEQ ID NO:140. 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:141, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:145), CDR2 (SEQ ID NO:146), and CDR3 (SEQ ID NO:147) sequences of SEQ ID NO:141.

[0020] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:148 and a light chain variable domain related to SEQ ID NO:149. 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:148, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:150), CDR2 (SEQ ID NO:151), and CDR3 (SEQ ID NO:152) sequences of SEQ ID NO:148. 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:149, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:153), CDR2 (SEQ ID NO:154), and CDR3 (SEQ ID NO:155) sequences of SEQ ID NO:149.

[0021] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:156 and a light chain variable domain related to SEQ ID NO:157. 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:156, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:158), CDR2 (SEQ ID NO:159), and CDR3 (SEQ ID NO:160) sequences of SEQ ID NO:156. 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:157, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:161), CDR2 (SEQ ID NO:162), and CDR3 (SEQ ID NO:163) sequences of SEQ ID NO:157.

[0022] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:164 and a light chain variable domain related to SEQ ID NO:165. 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:164, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:166), CDR2 (SEQ ID NO:167), and CDR3 (SEQ ID NO:168) sequences of SEQ ID NO:164. 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:165, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:169), CDR2 (SEQ ID NO:170), and CDR3 (SEQ ID NO:171) sequences of SEQ ID NO:165.

[0023] The second antigen binding site can bind to CAIX, ANO1, mesothelin, TROP2, CEA or Claudin-18.2. In some examples, the second antigen binding site binds to CAIX and incorporates a heavy chain variable domain and light chain variable domain that can be coupled to form a CAIX binding site. For example, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:72 and a light chain variable domain related to SEQ ID NO:73. 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:72, 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:72. 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:73, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:77), CDR2 (SEQ ID NO:78), and CDR3 (SEQ ID NO:79) sequences of SEQ ID NO:73.

[0024] Alternatively, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:80 and a light chain variable domain related to SEQ ID NO:81. 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:80, 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:80. 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:81, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:85), CDR2 (SEQ ID NO:86), and CDR3 (SEQ ID NO:87) sequences of SEQ ID NO:81.

[0025] Alternatively, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:88 and a light chain variable domain related to SEQ ID NO:89. 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:88, 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:88. 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:89, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:93), CDR2 (SEQ ID NO:94), and CDR3 (SEQ ID NO:95) sequences of SEQ ID NO:89.

[0026] In some examples, the second antigen binding site binds to ANO1 and incorporates a heavy chain variable domain and light chain variable domain that can be coupled to form an ANO1 binding site. For example, the second antigen binding site can incorporate a variety a heavy chain variable domain related to SEQ ID NO:97 and a light chain variable domain related to SEQ ID NO:98. 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:97, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:99), CDR2 (SEQ ID NO:100), and CDR3 (SEQ ID NO:101) sequences of SEQ ID NO:97. 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:98, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:102), CDR2 (SEQ ID NO:103), and CDR3 (SEQ ID NO:104) sequences of SEQ ID NO:98.

[0027] In some examples, the second antigen binding site binds to mesothelin and incorporates a heavy chain variable domain and light chain variable domain that can be coupled to form a mesothelin binding site. For example, the second antigen binding site can incorporate a variety a heavy chain variable domain related to SEQ ID NO:106 and a light chain variable domain related to SEQ ID NO:107. 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:106, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:108), CDR2 (SEQ ID NO:109), and CDR3 (SEQ ID NO:110) sequences of SEQ ID NO:106. 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:107, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:111), CDR2 (SEQ ID NO:112), and CDR3 (SEQ ID NO:113) sequences of SEQ ID NO:107. Alternatively, the second antigen binding site can incorporate a variety a heavy chain variable domain related to SEQ ID NO:114 and a light chain variable domain related to SEQ ID NO:115. 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:114, 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:115, and/or incorporate amino acid

sequences identical to the CDR1 (SEQ ID NO:119), CDR2 (SEQ ID NO:120), and CDR3 (SEQ ID NO:121) sequences of SEQ ID NO:115. Alternatively, the second antigen binding site can incorporate a variety a heavy chain variable domain related to SEQ ID NO:122 and a light chain variable domain related to SEQ ID NO:123. 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:122, 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:122. 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:123, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:127), CDR2 (SEQ ID NO:128), and CDR3 (SEQ ID NO:129) sequences of SEQ ID NO:123.

[0028] In some examples, the second antigen binding site binds to TROP2 and incorporates a heavy chain variable domain and light chain variable domain that can be coupled to form a TROP2 binding site. For example, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:172 and a light chain variable domain related to SEQ ID NO:173. 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:172, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:174), CDR2 (SEQ ID NO:175), and CDR3 (SEQ ID NO:176) sequences of SEQ ID NO:172. 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:173, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:177), CDR2 (SEQ ID NO:178), and CDR3 (SEQ ID NO:179) sequences of SEQ ID NO:173. Alternatively, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:180 and a light chain variable domain related to SEQ ID NO:181. 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:180, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:182), CDR2 (SEQ ID NO:183), and CDR3 (SEQ ID NO:184) sequences of SEQ ID NO:180. 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:181, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:185), CDR2 (SEQ ID NO:186), and CDR3 (SEQ ID NO:187) sequences of SEQ ID NO:181.

Alternatively, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:188 and a light chain variable domain related to SEQ ID NO:189. 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:189, and 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:189. Alternatively, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:190 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:190, and 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. Alternatively, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:192 and a light chain variable domain related to SEQ ID NO:193. 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:192, and 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:193.

[0029] In some examples, the second antigen binding site binds to CEA and incorporates a heavy chain variable domain and light chain variable domain that can be coupled to form a CEA binding site. For example, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:195 and a light chain variable domain related to SEQ ID NO:196. 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:197), CDR2 (SEQ ID NO:198), and CDR3 (SEQ ID NO:199) 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:196, 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:196. Alternatively, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:203 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:203, 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:203. 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:208), CDR2 (SEQ ID NO:209), and CDR3 (SEQ ID NO:210) sequences of SEQ ID NO:204. Alternatively, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:211 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:211, 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. 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:196, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:216), CDR2 (SEQ ID NO:217), and CDR3 (SEQ ID NO:218) sequences of SEQ ID NO:212.

[0030] In some examples, the second antigen binding site binds to Claudin-18.2 and incorporates a heavy chain variable domain and light chain variable domain that can be coupled to form a Claudin-18.2 binding site. For example, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:220 and a light chain variable domain related to SEQ ID NO:221. 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:220, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:222), CDR2 (SEQ ID NO:223), and CDR3 (SEQ ID NO:224) sequences of SEQ ID NO:220. 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:221, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:225), CDR2 (SEQ ID NO:226), and CDR3 (SEQ ID NO:227) sequences of SEQ ID NO:221. For example, the second antigen binding site can incorporate a heavy chain variable domain related to SEQ ID NO:228 and a light chain variable domain related to SEQ ID NO:229. 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:228, and/or incorporate amino acid sequences

identical to the CDR1 (SEQ ID NO:230), CDR2 (SEQ ID NO:231), and CDR3 (SEQ ID NO:232) sequences of SEQ ID NO:228. 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:229, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:233), CDR2 (SEQ ID NO:234), and CDR3 (SEQ ID NO:235) sequences of SEQ ID NO:229.

[0031] 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.

[0032] 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.

[0033] 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.

[0034] 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 proteins described herein. Cancers to be treated using CAIX-targeting multi-specific binding proteins include, for example, renal cell carcinoma, breast cancer, glioblastoma, head and neck cancer, gastric cancers, bladder cancer, ovarian cancer, tumors of the esophagus, lung, colon, kidney, cervix and non-small cell lung carcinoma. Cancers to be treated using mesothelin-targeting multi-specific binding proteins include, for example, mesothelioma, ovarian cancer, pancreatic cancer, non–small cell lung cancer, breast cancer, cholangiocarcinoma, gastric cancer, uterine serous carcinoma, thymic carcinoma, and acute myeloid leukemia. Cancers to be treated using ANO1-targeting multi-specific binding proteins include, for example, esophageal squamous cell cancer (ESCC), gastrointestinal stromal tumor (GIST), head and neck squamous cell carcinoma (HNSCC), pancreatic cancer, breast cancer, prostate cancer, and sarcoma. Cancers to be treated using TROP2-targeting multi-specific binding proteins include, for example, breast, lung, gastric, colorectal, pancreatic, prostatic, cervical, head-and-neck cancer, nasopharyngeal carcinoma, and ovarian carcinoma. Cancers to be treated using CEA-targeting multi-specific binding proteins

include, for example, gastrointestinal cancer, colorectal cancer, pancreatic cancer, non-small cell lung cancer and breast cancer. Cancers to be treated using claudin-18.2-targeting multi-specific binding proteins include, for example, esophageal cancer, non-small cell lung carcinoma, ovarian cancer, colon cancer, and several forms of biliary ductal carcinoma.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] **FIG. 1** is a representation of a heterodimeric, multi-specific antibody. Each arm can represent either the NKG2D-binding domain or one of the tumor associated antigen-binding

CLAIMS

1. A protein comprising:

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

(b) a second antigen-binding site that binds CAIX, ANO1, mesothelin, TROP2, or Claudin-18.2; 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 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. The protein according to claim 3, wherein the heavy chain variable domain and the light chain variable domain are present on the same polypeptide.

5. The protein according to claims 3 or 4, wherein the second antigen-binding site comprises a heavy chain variable domain and a light chain variable domain.

6. The 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. The 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 at least 90% identical to SEQ ID NO:1.

9. The 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:41 and a light chain variable domain at least 90% identical to SEQ ID NO:42.

10. The 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:43 and a light chain variable domain at least 90% identical to SEQ ID NO:44.

11. The 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:45 and a light chain variable domain at least 90% identical to SEQ ID NO:46.

12. The 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:47 and a light chain variable domain at least 90% identical to SEQ ID NO:48.

13. The 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:49 and a light chain variable domain at least 90% identical to SEQ ID NO:50.

14. The 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:132 and a light chain variable domain at least 90% identical to SEQ ID NO:133.

15. The 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:140 and a light chain variable domain at least 90% identical to SEQ ID NO:141.

16. The 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:148 and a light chain variable domain at least 90% identical to SEQ ID NO:149.

17. The 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:156 and a light chain variable domain at least 90% identical to SEQ ID NO:157.

18. The 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:164 and a light chain variable domain at least 90% identical to SEQ ID NO:165.

19. The protein of claim 1 or 2, wherein the first antigen-binding site is a single-domain antibody.

20. The protein of claim 19, wherein the single-domain antibody is a V_HH fragment or a V_{NAR} fragment.

21. The protein according to any one of claims 1-2 or 19-20, wherein the second antigen-binding site comprises a heavy chain variable domain and a light chain variable domain.

22. The protein according to 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.

23. The protein according to any of the preceding claims, wherein the second antigen-binding site binds CAIX and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:72 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:73.

24. The protein according to any of the preceding claims, wherein the second antigen-binding site binds CAIX and comprises:

a heavy chain CDR1 sequence identical to the amino acid sequence of SEQ ID NO:74;

a heavy chain CDR2 sequence identical to the amino acid sequence of SEQ ID NO:75; and

a heavy chain CDR3 sequence identical to the amino acid sequence of SEQ ID NO:76.

25. The protein according to claim 24, wherein the second antigen-binding site comprises:

a light chain CDR1 sequence identical to the amino acid sequence of SEQ ID NO:77;

a light chain CDR2 sequence identical to the amino acid sequence of SEQ ID NO:78; and

a light chain CDR3 sequence identical to the amino acid sequence of SEQ ID NO:79.

26. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds CAIX and comprises a heavy chain variable domain of comprising an amino acid sequence at least 90% identical to SEQ ID NO:80 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:81.

27. The protein according to any one of claims 1-22 or 26, wherein the second antigen-binding site binds CAIX and comprises:

a heavy chain CDR1 sequence identical to the amino acid sequence of SEQ ID NO:82;

a heavy chain CDR2 sequence identical to the amino acid sequence of SEQ ID NO:83; and

a heavy chain CDR3 sequence identical to the amino acid sequence of SEQ ID NO:84.

28. The protein according to claim 27, wherein the second antigen-binding site comprises:

a light chain CDR1 sequence identical to the amino acid sequence of SEQ ID NO:85;

a light chain CDR2 sequence identical to the amino acid sequence of SEQ ID NO:86; and

a light chain CDR3 sequence identical to the amino acid sequence of SEQ ID NO:87.

29. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds CAIX and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:88 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:89.

30. The protein according to any one of claims 1-22 or 29, wherein the second antigen-binding site binds CAIX and comprises:

a heavy chain CDR1 sequence identical to the amino acid sequence of SEQ ID NO:90;

a heavy chain CDR2 sequence identical to the amino acid sequence of SEQ ID NO:91; and

a heavy chain CDR3 sequence identical to the amino acid sequence of SEQ ID NO:92.

31. The protein according to claim 30, wherein the second antigen-binding site comprises:

a light chain CDR1 sequence identical to the amino acid sequence of SEQ ID NO:93;

a light chain CDR2 sequence identical to the amino acid sequence of SEQ ID NO:94; and

a light chain CDR3 sequence identical to the amino acid sequence of SEQ ID NO:95.

32. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds ANO1 and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:97 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:98.

33. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds mesothelin and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:106 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:107.

34. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds mesothelin and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:114 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:115.

35. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds mesothelin and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:122 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:123.

36. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds TROP2 and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:172 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:173

37. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds TROP2 and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:180 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:181.

38. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds TROP2 and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:188 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:189.

39. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds TROP2 and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:190 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:191.

40. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds TROP2 and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:192 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:193.

41. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds claudin-18.2 and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:220 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:221.

42. The protein according to any one of claims 1-22, wherein the second antigen-binding site binds claudin-18.2 and comprises a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:228 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:229.

43. A protein according to any one of claims 1-4 or 8-20, wherein the second antigen-binding site is a single-domain antibody.

44. The protein of claim 43, wherein the second antigen-binding site is a V_HH fragment or a V_{NAR} fragment.

45. A protein according to any one of the preceding claims, wherein the protein comprises a portion of an antibody Fc domain sufficient to bind CD16, wherein the antibody Fc domain comprises hinge and CH2 domains.

46. A protein according to claim 45, wherein the antibody Fc domain comprises hinge and CH2 domains of a human IgG1 antibody.

47. A protein according to claim 45 or 46, wherein the Fc domain comprises an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody.

48. A protein according to any one of claims 45-47, 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.

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

50. A cell comprising one or more nucleic acids encoding a protein according to any one of claims 1-48.

51. A method of directly and/or indirectly enhancing tumor cell death, the method comprising exposing a tumor and natural killer cells to a protein according to any one of claims 1-48.

52. Use of a protein according to any one of claims 1-48 or a formulation according to claim 49 in the manufacture of a medicament for treating cancer.

53. The use of claim 52, wherein the second antigen binding site of the protein binds CAIX, and the cancer to be treated is selected from the group consisting of renal cell carcinoma, breast

cancer, glioblastoma, head and neck cancer, gastric cancers, bladder cancer, ovarian cancer, tumors of the esophagus, lung, colon, kidney, cervix and non-small cell lung carcinoma.

54. The use of claim 52, wherein the second antigen binding site of the protein binds mesothelin, and the cancer to be treated is selected from the group consisting of mesothelioma, ovarian cancer, pancreatic cancer, non-small cell lung cancer, breast cancer, cholangiocarcinoma, gastric cancer, uterine serous carcinoma, thymic carcinoma, and acute myeloid leukemia.

55. The use of claim 52, wherein the second antigen binding site of the protein binds ANO1, and the cancer to be treated is selected from the group consisting of esophageal squamous cell cancer (ESCC), gastrointestinal stromal tumor (GIST), head and neck squamous cell carcinoma (HNSCC), pancreatic cancer, breast cancer, prostate cancer, and sarcoma.

56. The use of claim 52, wherein the second antigen binding site of the protein binds TROP2, and the cancer to be treated is selected from the group consisting of breast, lung, gastric, colorectal, pancreatic, prostatic, cervical, head-and-neck cancer, nasopharyngeal carcinoma, and ovarian carcinoma.

57. The use of claim 52, wherein the second antigen binding site of the protein binds claudin-18.2, and the cancer to be treated is selected from the group consisting of esophageal cancer, non-small cell lung carcinoma, ovarian cancer, colon cancer, and several forms of biliary ductal carcinoma.