

[0006] Natural killer (NK) cells are a component of the innate immune system and make up approximately 15% of circulating lymphocytes. NK cells infiltrate virtually all tissues and were originally characterized by their ability to kill tumor cells effectively without the need for prior sensitization. Activated NK cells kill target cells by means similar to cytotoxic T cells – *i.e.*, via cytolytic granules that contain perforin and granzymes as well as via death receptor pathways. Activated NK cells also secrete inflammatory cytokines such as IFN- γ and chemokines that promote the recruitment of other leukocytes to the target tissue.

[0007] NK cells respond to signals through a variety of activating and inhibitory receptors on their surface. For example, when NK cells encounter healthy self-cells, their activity is inhibited through activation of the killer-cell immunoglobulin-like receptors (KIRs). Alternatively, when NK cells encounter foreign cells or cancer cells, they are activated via their activating receptors (*e.g.*, NKG2D, natural cytotoxicity receptors (NCRs), DNAX accessory molecule 1 (DNAM1)). NK cells are also activated by the constant region of some immunoglobulins through CD16 receptors on their surface. The overall sensitivity of NK cells to activation depends on the sum of stimulatory and inhibitory signals.

[0008] Fibroblast activation protein alpha (FAP) is a homodimeric integral membrane gelatinase belonging to the serine protease family. This protein is thought to be involved in the control of fibroblast growth or epithelial-mesenchymal interactions during development, tissue repair, and epithelial carcinogenesis. More than 90% of all human carcinomas have FAP expression on activated stromal fibroblasts. Stromal fibroblasts play an important role in the development, growth and metastasis of carcinomas. FAP is also expressed in malignant cells of bone and soft tissue sarcomas.

[0009] The present invention provides certain advantages to improve treatments for the above-mentioned cancers.

SUMMARY

[0010] The disclosure provides multi-specific binding proteins that bind to the NKG2D receptor and CD16 receptor on natural killer cells, and the tumor-associated antigen, FAP. 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 examples and examples of the disclosure are described in further detail below.

[0010a] In one aspect, the present disclosure provides a protein comprising: (a) a first antigen-binding site that binds NKG2D; (b) a second antigen-binding site that binds fibroblast activation protein (FAP); and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16.

5 **[0010b]** In another aspect, the present disclosure refers to a protein as described herein, wherein the first antigen-binding site comprises a heavy chain variable domain at least 90% identical to an amino acid sequence selected from: 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, SEQ ID NO:167, SEQ ID NO:171, SEQ ID NO: 175, SEQ ID NO:179, SEQ
10 ID NO:183, SEQ ID NO:187, and SEQ ID NO:93.

[0010c] In yet 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.

15 **[0010d]** In yet another aspect, the present disclosure refers to the use of a protein as described herein or a formulation as described herein in the manufacture of a medicament. In yet another aspect, the present disclosure refers to the use of a protein as described herein or a formulation as described herein in the manufacture of a medicament for enhancing tumor cell death, comprising exposing tumor cells and natural killer cells to an effective amount of the
20 protein. In yet another aspect, the present disclosure refers to the use of a protein as described herein or the formulation as described herein in the manufacture of a medicament for treating cancer. In yet another aspect, the present disclosure refers to the use of a protein as described herein or a formulation as described herein in the manufacture of a medicament for treating an autoimmune disease. In yet another aspect, the present disclosure refers to the use of a
25 protein as described herein or a formulation as described herein in the manufacture of a medicament for treating fibrosis.

[0011] Accordingly, in certain examples, the disclosure provides a protein that incorporates a first antigen-binding site that binds NKG2D; a second antigen-binding site that binds FAP; and an antibody fragment crystallizable (Fc) domain, a portion thereof sufficient
30 to bind CD16, or a third antigen-binding site that binds CD16.

[0012] 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.

[0013] In certain examples, the present disclosure provides multi-specific binding proteins that bind to the NKG2D receptor and CD16 receptor on natural killer cells, and FAP on cancer cells. The NKG2D-binding site can include a heavy chain variable domain at least 90% identical to an amino acid sequence selected from: 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, SEQ ID NO:167, SEQ ID NO:171, SEQ ID NO: 175, SEQ ID NO:179, SEQ ID NO:183, SEQ ID NO:187, and SEQ ID NO:93.

[0014] 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 or SEQ ID NO:151), CDR2 (SEQ ID NO:106), and CDR3 (SEQ ID NO:107 or SEQ ID NO:152) 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 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.

[0015] Alternatively, in certain examples 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 or SEQ ID NO:153), CDR2 (SEQ ID NO:44), and CDR3 (SEQ ID NO:45 or SEQ ID NO:154) 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.

5 **[0016]** In certain 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
10 (SEQ ID NO:51 or SEQ ID NO:155), CDR2 (SEQ ID NO:52), and CDR3 (SEQ ID NO:53 or SEQ ID NO:156) 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
15 ID NO:56) sequences of SEQ ID NO:50.

[0017] 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%
20 (*e.g.*, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) identical to SEQ ID NO:58 respectively. 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
25 100%) identical to SEQ ID NO:59, 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: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
30 CDR1 (SEQ ID NO:111), CDR2 (SEQ ID NO:112), and CDR3 (SEQ ID NO:113) sequences of SEQ ID NO:60.

[0018] 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. 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.

[0019] 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 or SEQ ID NO:157), CDR2 (SEQ ID NO:64), and CDR3 (SEQ ID NO:65 or SEQ ID NO:158) 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.

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 or SEQ ID NO:159), CDR2 (SEQ ID NO:72), and CDR3 (SEQ ID NO:73 or SEQ ID NO:160) 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.

[0020] 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 or SEQ ID NO:161), CDR2 (SEQ ID NO:80), and CDR3 (SEQ ID NO:81 or SEQ ID NO:162) 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.

[0021] 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 or SEQ ID NO:163), CDR2 (SEQ ID NO:88), and CDR3 (SEQ ID NO:89 or SEQ ID NO:164) 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.

[0022] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:167 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:167, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:87 or SEQ ID NO:168), CDR2 (SEQ ID NO:88), and CDR3 (SEQ ID NO:169 or SEQ ID NO:170) sequences of SEQ ID NO:167. 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.

[0023] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:171 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:171, and/or incorporate amino acid sequences identical to the CDR1

(SEQ ID NO:87 or SEQ ID NO:172), CDR2 (SEQ ID NO:88), and CDR3 (SEQ ID NO:173 or 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: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.

[0024] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:175 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:175, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:87 or SEQ ID NO:176), CDR2 (SEQ ID NO:88), and CDR3 (SEQ ID NO:177 or SEQ ID NO:178) sequences of SEQ ID NO:175. 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.

[0025] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:179 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:179, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:87 or SEQ ID NO:180), CDR2 (SEQ ID NO:88), and CDR3 (SEQ ID NO:181 or 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: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.

[0026] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:183 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:183, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:87 or SEQ ID NO:184), CDR2 (SEQ ID NO:88), and CDR3 (SEQ ID NO:185

or SEQ ID NO:186) sequences of SEQ ID NO:183. 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.

[0027] In some examples, the first antigen-binding site can incorporate a heavy chain variable domain related to SEQ ID NO:187 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:187, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:87 or SEQ ID NO:188), CDR2 (SEQ ID NO:88), and CDR3 (SEQ ID NO:189 or 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: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.

[0028] 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 or SEQ ID NO:165), CDR2 (SEQ ID NO:96), and CDR3 (SEQ ID NO:97 or SEQ ID NO:166) 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.

[0029] In certain examples, the second antigen-binding site can bind to FAP and can optionally incorporate a heavy chain variable domain related to SEQ ID NO:114 and a light chain variable domain related to SEQ ID NO:118. 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:115 or SEQ ID NO:147), CDR2 (SEQ ID NO:116 or SEQ ID NO 148), and CDR3 (SEQ ID NO:117) sequences of SEQ ID

NO:114. 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:118, and/or incorporate amino acid sequences identical to the CDR1 (SEQ ID NO:119 or SEQ ID NO:149), CDR2 (SEQ ID NO:120), and CDR3 (SEQ ID NO:121) sequences of SEQ ID NO:118.

[0030] Alternatively, the second antigen-binding site binding to FAP can optionally 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.

[0031] Alternatively, the second antigen-binding site binding to FAP can optionally 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.

[0032] Alternatively, the second antigen-binding site binding to FAP can optionally incorporate a heavy chain variable domain related to SEQ ID NO:122 and a light chain variable domain related to SEQ ID NO:126. 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:123), CDR2 (SEQ ID NO:124), and CDR3 (SEQ ID NO:125) 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:126, 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:126.

5 **[0033]** 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.

10 **[0034]** 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.

15 **[0035]** In certain examples, the protein further incorporates a fourth antigen-binding site that binds to a tumor-associated antigen, which includes any antigen that is associated with cancer. For example, the fourth antigen-binding site may bind to human epidermal growth factor receptor 2 (HER2), CD20, CD33, B-cell maturation antigen (BCMA), prostate-specific membrane antigen (PSMA), delta-like canonical notch ligand 3 (DLL3), ganglioside GD2 (GD2), CD123, anoctamin-1 (Ano1), mesothelin, carbonic anhydrase IX (CAIX), tumor-associated calcium signal transducer 2 (TROP2), carcinoembryonic antigen (CEA), claudin-18.2, receptor tyrosine kinase-like orphan receptor 1 (ROR1), trophoblast glycoprotein (5T4), glycoprotein non-metastatic melanoma protein B (GPNMB), folate receptor-alpha (FR-alpha), pregnancy-associated plasma protein A (PAPP-A), CD37, epithelial cell adhesion molecule (EpCAM), CD2, CD19, CD30, CD38, CD40, CD52, CD70, CD79b, fms-like tyrosine kinase 3 (FLT3), glypican 3 (GPC3), B7 homolog 6 (B7H6), C-C chemokine receptor type 4 (CCR4), C-X-C motif chemokine receptor 4 (CXCR4), receptor tyrosine kinase-like orphan receptor 2 (ROR2), CD133, HLA class I histocompatibility antigen, alpha chain E (HLA-E), epidermal growth factor receptor (EGFR/ERBB1), insulin-like growth factor 1-receptor (IGF1R), human epidermal growth factor receptor 3 (HER3)/ERBB3, human epidermal growth factor receptor 4 (HER4)/ERBB4, mucin 1 (MUC1), tyrosine protein kinase MET (cMET), signaling lymphocytic activation molecule F7 (SLAMF7), prostate stem cell antigen (PSCA), MHC class I polypeptide-related sequence A (MICA), MHC class I polypeptide-related sequence B (MICB), TNF-related apoptosis inducing ligand receptor 1 (TRAILR1), TNF-related apoptosis inducing ligand receptor 2 (TRAILR2), melanoma associated antigen 3 (MAGE-A3), B-lymphocyte antigen B7.1 (B7.1), B-lymphocyte antigen B7.2 (B7.2), cytotoxic T-lymphocyte associated protein 4 (CTLA4), programmed cell death

protein 1 (PD1), programmed cell death 1 ligand 1 (PD-L1), or CD25 antigen expressed on cancer cells.

[0036] 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.

[0037] 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 FAP-targeting multi-specific binding proteins include any cancer that expresses FAP, for example, infiltrating ductal carcinomas, pancreatic ductal adenocarcinoma, stomach cancer, uterine cancer, cervix cancer, colorectal cancer, breast cancer, ovarian cancer, bladder cancer, lung cancer, head and neck cancer, mesothelioma, gastric cancer, pancreatic cancer, liver cancer, endometrial cancer, neuroendocrine cancer, fibrosarcoma, malignant fibrous histiocytoma, leiomyosarcoma, osteosarcoma, chondrosarcoma, liposarcoma, synovial sarcoma, schwannoma, melanoma, and glioma.

[0038] In certain examples, the disclosure provides a method of treating an autoimmune disease 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. In certain examples the autoimmune disease is selected from the group consisting of rheumatoid arthritis, Grave's disease, Sjögren's syndrome, primary biliary cirrhosis, primary sclerosis cholangitis, and inflammatory destructive arthritis.

[0039] In certain examples, the disclosure provides a method of treating fibrosis in a patient comprising administering to a patient in need thereof a therapeutically effective amount of the multi-specific binding proteins described herein. In certain examples, the fibrosis is selected from the group consisting of idiopathic pulmonary fibrosis, renal fibrosis, hepatic fibrosis, and cardiac fibrosis.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] **FIG. 1** is a representation of a heterodimeric, multi-specific binding protein. Each arm can represent either an NKG2D-binding domain or a binding domain for FAP. The multi-specific binding protein further comprises an Fc domain or a portion thereof that binds to CD16. In some embodiments, the NKG2D-binding and FAP-binding domains can share a common light chain.

[0041] FIG. 2 is a representation of a heterodimeric, multi-specific binding protein. Either the NKG2D-binding domain or the binding domain to FAP can take an scFv format (right arm).

[0042] FIG. 3 is a line graph showing the binding affinity of NKG2D-binding domains (listed as clones) to human recombinant NKG2D in an ELISA assay.

[0043] FIG. 4 is a line graph showing the binding affinity of NKG2D-binding domains (listed as clones) to cynomolgus recombinant NKG2D in an ELISA assay.

[0044] FIG. 5 is a line graph showing the binding affinity of NKG2D-binding domains (listed as clones) to mouse recombinant NKG2D in an ELISA assay.

[0045] FIG. 6 is a bar graph showing the binding of NKG2D-binding domains (listed as clones) to EL4 cells expressing human NKG2D, measured by flow cytometry as mean fluorescence intensity (MFI) fold-over-background (FOB).

[0046] FIG. 7 is a bar graph showing the binding of NKG2D-binding domains (listed as clones) to EL4 cells expressing mouse NKG2D, measured by flow cytometry as mean fluorescence intensity (MFI) fold-over-background (FOB).

[0047] FIG. 8 is a line graph showing the binding affinity of NKG2D-binding domains (listed as clones) for recombinant human NKG2D-Fc in a competitive binding assay with NKG2D's natural ligand ULBP-6.

[0048] FIG. 9 is a line graph showing the binding affinity of NKG2D-binding domains (listed as clones) for recombinant human NKG2D-Fc in a competitive binding assay with NKG2D's natural ligand, MICA.

[0049] FIG. 10 is a line graph showing the binding affinity of NKG2D-binding domains (listed as clones) for recombinant mouse NKG2D-Fc in a competitive binding assay with NKG2D's natural ligand, Rae-1 delta.

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 fibroblast activation protein (FAP); 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 according to claim 1, wherein the first antigen-binding site binds to NKG2D in humans.
3. The protein according to claim 1 or 2, wherein the first antigen-binding site comprises a heavy chain variable domain and a light chain variable domain present on the same polypeptide.
4. The protein according to claims 3, wherein the second antigen-binding site also comprises a heavy chain variable domain and a light chain variable domain present on the same polypeptide.
5. The protein according to claim 4, 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.
6. A protein according to any one of claims 1 to 5, wherein the first antigen-binding site comprises a heavy chain variable domain at least 90% identical to an amino acid sequence selected from: 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, SEQ ID NO:167, SEQ ID NO:171, SEQ ID NO: 175, SEQ ID NO:179, SEQ ID NO:183, SEQ ID NO:187, and SEQ ID NO:93.
7. The protein according to any one of claims 1 to 5, wherein the first antigen-binding site comprises:

- a. 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;
 - b. 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;
 - c. a heavy chain variable domain at least 90% identical to SEQ ID NO:57 and a light chain variable domain at least 90% identical to SEQ ID NO:58;
 - d. a heavy chain variable domain at least 90% identical to SEQ ID NO:59 and a light chain variable domain at least 90% identical to SEQ ID NO:60;
 - e. a heavy chain variable domain at least 90% identical to SEQ ID NO:61 and a light chain variable domain at least 90% identical to SEQ ID NO:62;
 - f. a heavy chain variable domain at least 90% identical to SEQ ID NO:69 and a light chain variable domain at least 90% identical to SEQ ID NO:70;
 - g. a heavy chain variable domain at least 90% identical to SEQ ID NO:77 and a light chain variable domain at least 90% identical to SEQ ID NO:78;
 - h. a heavy chain variable domain at least 90% identical to SEQ ID NO:85, SEQ ID NO:167, SEQ ID NO:171, SEQ ID NO: 175, SEQ ID NO:179, SEQ ID NO:183, or SEQ ID NO:187, and a light chain variable domain at least 90% identical to SEQ ID NO:86;
 - i. a heavy chain variable domain at least 90% identical to SEQ ID NO:93 and a light chain variable domain at least 90% identical to SEQ ID NO:94;
 - j. a heavy chain variable domain at least 90% identical to SEQ ID NO:101 and a light chain variable domain at least 90% identical to SEQ ID NO:102; or
 - k. a heavy chain variable domain at least 90% identical to SEQ ID NO:103 and a light chain variable domain at least 90% identical to SEQ ID NO:104.
8. The protein according to claim 1 or 2, wherein the first antigen-binding site is a single-domain antibody.

9. The protein according to claim 8, wherein the single-domain antibody is a V_{HH} fragment or a V_{NAR} fragment.

10. The protein according to any one of claims 1, 2, 8, or 9, wherein the second antigen-binding site comprises a heavy chain variable domain and a light chain variable domain present on the same polypeptide.

11. The protein according to any one of claims 1 to 10, wherein the second antigen-binding site comprises:

a. 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:118;

b. a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:131 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:135;

c. a heavy chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:139 and a light chain variable domain comprising an amino acid sequence at least 90% identical to SEQ ID NO:143; or

d. 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:126.

12. The protein according to any one of claims 1 to 10, wherein the second antigen-binding site comprises:

a. CDR1, CDR2, and CDR3 sequences of a heavy chain variable domain and a light chain variable domain selected from the group consisting of SEQ ID NO:114 and 118, 131 and 135, 139 and 143, and 122 and 126, respectively;

b. a heavy chain CDR1 amino acid sequence of SEQ ID NO:147;
a heavy chain CDR2 amino acid sequence of SEQ ID NO:148;

- a heavy chain CDR3 amino acid sequence of SEQ ID NO:117;
 - a light chain CDR1 amino acid sequence of SEQ ID NO:149;
 - a light chain CDR2 amino acid sequence of SEQ ID NO:120; and
 - a light chain CDR3 amino acid sequence of SEQ ID NO:121;
- c. a heavy chain CDR1 amino acid sequence of SEQ ID NO:123;
- a heavy chain CDR2 amino acid sequence of SEQ ID NO:124;
 - a heavy chain CDR3 amino acid sequence of SEQ ID NO:125;
 - a light chain CDR1 amino acid sequence of SEQ ID NO:127;
 - a light chain CDR2 amino acid sequence of SEQ ID NO:128; and
 - a light chain CDR3 amino acid sequence of SEQ ID NO:129;
- d. a heavy chain CDR1 amino acid sequence of SEQ ID NO:132;
- a heavy chain CDR2 amino acid sequence of SEQ ID NO:133;
 - a heavy chain CDR3 amino acid sequence of SEQ ID NO:134;
 - a light chain CDR1 amino acid sequence of SEQ ID NO:136;
 - a light chain CDR2 amino acid sequence of SEQ ID NO:137; and
 - a light chain CDR3 amino acid sequence of SEQ ID NO:138; or
- e. a heavy chain CDR1 amino acid sequence of SEQ ID NO:140;
- a heavy chain CDR2 amino acid sequence of SEQ ID NO:141;
 - a heavy chain CDR3 amino acid sequence of SEQ ID NO:142;
 - a light chain CDR1 amino acid sequence of SEQ ID NO:144;
 - a light chain CDR2 amino acid sequence of SEQ ID NO:145; and
 - a light chain CDR3 amino acid sequence of SEQ ID NO:146.
13. The protein according to any one of claims 1 to 3 or 6 to 9, wherein the second antigen-binding site is a single-domain antibody.
14. The protein according to claim 13, wherein the second antigen-binding site is a V_HH fragment or a V_{NAR} fragment.
15. The protein according to any one of claims 1 to 14, wherein the protein comprises a portion of an antibody Fc domain sufficient to bind CD16, wherein the antibody Fc domain comprises a hinge and CH2 domains of a human IgG1 antibody.

16. The protein according to claim 15, wherein (i) the antibody Fc domain comprises a hinge and CH2 domains of a human IgG1 antibody or (ii) the Fc domain comprises an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody.
17. The protein according to claim 16, 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.
18. A formulation comprising a protein according to any one of claims 1 to 19 and a pharmaceutically acceptable carrier.
19. A cell comprising one or more nucleic acids encoding a protein according to any one of claims 1 to 17.
20. Use of a protein according to any one of claims 1 to 17 or a formulation according to claim 18 in the manufacture of a medicament.
21. Use of a protein according to any one of claims 1 to 17 or a formulation according to claim 18 in the manufacture of a medicament for enhancing tumor cell death, comprising exposing tumor cells and natural killer cells to an effective amount of the protein.
22. Use of a protein according to any one of claims 1 to 17 or the formulation according to claim 18 in the manufacture of a medicament for treating cancer.
23. The use according to claim 22, wherein the cancer to be treated is selected from the group consisting of infiltrating ductal carcinoma, pancreatic ductal adenocarcinoma, stomach cancer, uterine cancer, cervical cancer, colorectal cancer, breast cancer, ovarian cancer, bladder cancer, lung cancer, mesothelioma, gastric cancer, pancreatic cancer, head and neck cancer, liver cancer, endometrial cancer, neuroendocrine cancer, fibrosarcoma, malignant fibrous histiocytoma, leiomyosarcoma, osteosarcoma, chondrosarcoma, liposarcoma, synovial sarcoma, schwannoma, melanoma, and glioma.

24. Use of a protein according to any one of claims 1 to 17 or a formulation according to claim 18 in the manufacture of a medicament for treating an autoimmune disease.
25. The use according to claim 24, wherein the autoimmune disease is selected from the group consisting of rheumatoid arthritis, Grave's disease, Sjögren's syndrome, primary biliary cirrhosis, primary sclerosis cholangitis, and inflammatory destructive arthritis.
26. Use of a protein according to any one of claims 1 to 17 or a formulation according to claim 18 in the manufacture of a medicament for treating fibrosis.
27. The use according to claim 26, wherein the fibrosis is selected from the group consisting of idiopathic pulmonary fibrosis, renal fibrosis, hepatic fibrosis, and cardiac fibrosis.