

[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, NCRs, 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. NKG2D is a type-II transmembrane protein that is expressed by essentially all natural killer cells where NKG2D serves as an activating receptor. NKG2D is also be found on T cells where it acts as a costimulatory receptor. The ability to modulate NK cell function via NKG2D is useful in various therapeutic contexts including malignancy.

SUMMARY

[0008] In one aspect, the present disclosure refers to a protein comprising (a) a single-chain variable fragment (scFv) that binds a tumor-associated antigen, wherein the scFv comprises a heavy chain variable domain and a light chain variable domain; (b) a Fab that binds NKG2D; and (c) a first antibody constant domain and a second antibody constant domain that form a heterodimer binding CD16, wherein the scFv is linked to the N-terminus of the first antibody constant domain via a hinge comprising Ala-Ser sequence, and the Fab is linked to the N-terminus of the second antibody constant domain.

[0008a] 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.

5 **[0008b]** In a further aspect, the present disclosure refers to the protein as described herein or the formulation as described herein for use in therapy. In a further aspect, the present disclosure refers to use of the protein as described herein or the formulation as described herein in the manufacture of a medicament for treating cancer.

[0008c] In one example, the current disclosure provides an improvement on a single-chain variable fragment (scFv) that is linked to an antibody constant domain via a hinge sequence. In some examples, the hinge comprises amino acids Ala-Ser. In some other examples, the hinge comprises amino acids Ala-Ser and Thr-Lys-Gly. The scFv may include a heavy chain variable domain and a light chain variable domain. In some examples, the scFv binds NKG2D or a tumor-associated antigen. The hinge sequence provides flexibility of binding to the target antigen.

10
15

[0009] In some examples of the scFv, the heavy chain variable domain forms a disulfide bridge with the light chain variable domain. For example, a disulfide bridge can be formed between the C44 residue of the heavy chain variable domain and the C100 residue of the light chain variable domain. In some examples, the heavy chain variable domain is linked to the light chain variable domain via a flexible linker, such as (G₄S)₄ (SEQ ID NO:427). In some examples of the scFv, the heavy chain variable domain is positioned at the N terminus of the light chain variable domain. In some examples of the scFv, the heavy chain variable domain is positioned at the C terminus of the light chain variable domain.

20

[0010] In some examples, the antibody constant domain linked to the scFv is able to bind to CD16. In some examples, the antibody constant domain comprises a CH2 domain and a CH3 domain of an IgG antibody, for example, a human IgG1 antibody. In some examples, mutations are introduced in the antibody constant domain to enable heterodimerization with another antibody constant domain. For example, if the antibody constant domain is derived

25

Claims

1. A protein comprising:
 - (a) a single-chain variable fragment (scFv) that binds a tumor-associated antigen, wherein the scFv comprises a heavy chain variable domain and a light chain variable domain;
 - (b) a Fab that binds NKG2D; and
 - (c) a first antibody constant domain and a second antibody constant domain that form a heterodimer binding CD16,wherein the scFv is linked to the N-terminus of the first antibody constant domain via a hinge comprising Ala-Ser sequence, and the Fab is linked to the N-terminus of the second antibody constant domain.
2. The protein according to claim 1, wherein the heavy chain variable domain of the scFv forms a disulfide bridge with the light chain variable domain of the scFv, optionally between C44 from the heavy chain variable domain and C100 from the light chain variable domain.
3. The protein according to claim 1 or 2, wherein the heavy chain variable domain of the scFv is linked to the light chain variable domain of the scFv via a flexible linker, optionally wherein the flexible linker comprises (G₄S)₄.
4. The protein according to any one of claims 1-3, wherein the heavy chain variable domain of the scFv is positioned at the N-terminus of the light chain variable domain of the scFv.
5. The protein according to any one of claims 1-4, wherein the first antibody constant domain and the second antibody constant domain each comprise a hinge and a CH2 domain of a human IgG1 antibody.
6. The protein according to any one of claims 1-5, wherein the first antibody constant domain and the second antibody constant domain comprise an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody.
7. The protein according to any one of claims 1-6, wherein the first and second antibody constant domains comprise heterodimerization mutations.

8. The protein according to claim 7, wherein the heterodimerization mutations are located 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.
- 5 9. The protein according to claim 8, wherein the heterodimerization mutations comprise one or more substitutions selected from the group consisting of Q347E, Q347R, Y349S, Y349K, Y349T, Y349D, Y349E, Y349C, L351K, L351D, L351Y, S354C, E356K, E357Q, E357L, E357W, K360E, K360W, Q362E, S364K, S364E, S364H, S364D, T366V, T366I, T366L, T366M, T366K, T366W, T366S, L368E, L368A, L368D, K370S, N390D, N390E, K392L, 10 K392M, K392V, K392F, K392D, K392E, T394F, D399R, D399K, D399V, S400K, S400R, D401K, F405A, F405T, Y407A, Y407I, Y407V, K409F, K409W, K409D, T411D, T411E, K439D, and K439E.
10. The protein according to claim 9, wherein the heterodimerization mutations comprise Q347R, D399V, and F405T substitutions in the first antibody constant domain and K360E and 15 K409W substitutions in the second antibody constant domain.
11. The protein according to any one of claims 1-10, wherein the heavy chain variable domain (VH) of the Fab comprises a complementarity determining region (CDR) 1 sequence identical to the amino acid sequence of SEQ ID NO:95, a CDR2 sequence identical to the amino acid sequence of SEQ ID NO:96, and a CDR3 sequence identical to the amino acid sequence of 20 SEQ ID NO:97; and the light chain variable domain (VL) of the Fab comprises a CDR1 sequence identical to the amino acid sequence of SEQ ID NO:99, a CDR2 sequence identical to the amino acid sequence of SEQ ID NO:100, and a CDR3 sequence identical to the amino acid sequence of SEQ ID NO:101.
12. The protein according to claim 11, wherein the VH of the Fab comprises an amino acid 25 sequence at least 90% identical to SEQ ID NO:94, and the VL of the Fab comprises an amino acid sequence at least 90% identical to SEQ ID NO:98.

13. The protein according to claim 12, wherein the VH of the Fab comprises the amino acid sequence of SEQ ID NO:94, and the VL of the Fab comprises the amino acid sequence of SEQ ID NO:98.
14. A formulation comprising a protein according to any one of claims 1-13 and a
5 pharmaceutically acceptable carrier.
15. A cell comprising one or more nucleic acids encoding a protein according to any one of claims 1-13.
16. The protein according to any one of claims 1-13 or the formulation of claim 14 for use in therapy.
- 10 17. Use of the protein according to any one of claims 1-13 or the formulation of claim 14 in the manufacture of a medicament for treating cancer.
18. The use of claim 17, wherein the cancer is selected from the group consisting of acute myeloid leukemia, acute myelomonocytic leukemia, B cell lymphoma, bladder cancer, breast cancer, colorectal cancer, diffuse large B cell lymphoma esophageal cancer, Ewing's sarcoma,
15 follicular lymphoma, gastric cancer, gastrointestinal cancer, gastrointestinal stromal tumors, glioblastoma, head and neck cancer, melanoma, mesothelioma, multiple myeloma, myelodysplastic syndrome, renal cell carcinoma, neuroblastoma, non-small cell lung cancer, neuroendocrine tumor, ovarian cancer, pancreatic cancer, prostate cancer, sarcoma, small cell lung cancer, T cell lymphoma, testis cancer, thymic carcinoma, thyroid cancer, urothelial cancer,
20 cancer infiltrated by myeloid-derived suppressor cells, cancer with extracellular matrix deposition, cancer with a high level of reactive stroma, and cancer with neoangiogenesis.