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Multi-specific binding proteins that bind BCMA, NKG2D and CD16, and methods of use

Abstract

Multi-specific binding proteins that bind to and kill human cancer cells are described, as well as pharmaceutical compositions and therapeutic methods useful for the treatment of cancer. The cancer can be B-cell maturation antigen (BCMA)-expressing cancer. The multi-specific binding proteins provided herein exhibit high potency and maximum lysis of target cells compared to anti-BCMA monoclonal antibodies.

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Background/Summary

RELATED APPLICATIONS (1) This application is a U.S. National Stage Application of International Patent Application No. PCT/US2019/045632, filed Aug. 8, 2019, which claims the benefit of and priority to U.S. Provisional Application No. 62/716,207, filed Aug. 8, 2018, which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

(1) The present specification is being filed with a computer readable form (CRF) copy of the Sequence Listing. The CRF entitled 14247-425-999_Sequence_Listing.txt, which was created on Jan. 20, 2021 and is 137,045 bytes in size, is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

(2) The invention relates to multi-specific binding proteins that bind to to NKG2D, CD16, and B-cell maturation antigen (BCMA). These multi-specific binding proteins exhibit high potency and maximum lysis of target cells compared to anti-BCMA monoclonal antibodies, and are useful for killing human cancer cells expressing BCMA.

BACKGROUND

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

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

(5) 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.

(6) 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.

(7) BCMA is a transmembrane protein belonging to the TNF-receptor superfamily. It specifically binds to the tumor necrosis factor (ligand) superfamily, member 13b (TNFSF13B/TALL-1/BAFF), leading to NF-1B and MAPK8/JNK activation. Its expression is restricted to the B-cell lineage and has been shown to be important for B cell development and autoimmune response. BCMA also binds to various TRAF family members, and thus may transduce signals for cell survival and proliferation. BCMA is implicated in a variety of cancers, such as multiple myeloma, lymphoma and leukemia. The present invention provides certain advantages to improve treatments for BCMA-expressing cancers.

SUMMARY

(8) The invention provides multi-specific binding proteins that bind to BCMA, e.g., BCMA on a cancer cell, and to the NKG2D receptor and CD16 receptor, expressed on, e.g., 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 embodiments, the proteins can agonize NK cells in humans, and in other species such as rodents and cynomolgus monkeys. In certain embodiments, the proteins can agonize cytotoxic T cells in humans, and in other species such as rodents and cynomolgus monkeys. In some embodiments, the proteins agonize human NK cells. In some embodiments, the proteins agonize human cytotoxic T cells. Various aspects and embodiments of the invention are described in further detail below.

(9) Accordingly, one aspect of the invention provides a protein comprising (a) a first antigen-binding site comprising a single-chain variable fragment (scFv) that binds NKG2D; (b) a second antigen-binding site that binds B-cell maturation antigen (BCMA); and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16. In certain embodiments, a protein of the present disclosure further comprises an additional antigen-binding site that binds BCMA. In certain embodiments, the second antigen-binding site of a protein described in the present disclosure is an Fab fragment that binds BCMA. In certain embodiments, the second and the additional antigen-binding site of a protein described in the present disclosure are Fab fragments that bind BCMA.

(10) In certain embodiments, the second and the additional antigen-binding site of a protein described in the present disclosure are scFvs that bind BCMA. In certain embodiments, the heavy chain variable domain of the scFv that binds NKG2D is positioned at the N-terminus or the C-terminus of the light chain variable domain of the scFv. In certain embodiments, the light chain variable domain is positioned at the N-terminus of the heavy chain variable domain of the scFv that binds NKG2D.

(11) In certain embodiments, the scFv that binds to NKG2D is linked to the antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16. In certain embodiments, the scFv that binds to NKG2D is linked to the antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16 via a hinge comprising Ala-Ser. In certain embodiments, the scFv that binds to NKG2D is linked to the C-terminus of the antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16 via a flexible linker comprising the amino acid sequence of SEQ ID NO:168. In certain embodiments, the flexible linker linking the C-terminus of the Fc domain to the N-terminus of the V.sub.L domain of the scFv that binds NKG2D (e.g., SEQ ID NO:98) has the amino acid sequence of SEQ ID NO:168. In certain embodiments, the C-terminus of the antibody Fc domain is linked to the N-terminus of the light chain variable domain of the scFv that binds NKG2D.

(12) In certain embodiments, within the scFv that binds NKG2D, a disulfide bridge is formed between the heavy chain variable domain of the scFv and the light chain variable domain of the scFv. In certain embodiments, the disulfide bridge is formed between C44 from the heavy chain variable domain and C100 from the light chain variable domain.

(13) In certain embodiments, within the scFv that binds NKG2D, the heavy chain variable domain is linked to the light chain variable domain via a flexible linker. In certain embodiments, the flexible linker comprises (GlyGlyGlyGlySer).sub.n((G4S).sub.n; SEQ ID NO:198), wherein n is an integer between 1-10. In certain embodiments, the flexible linker has the amino acid sequence of SEQ ID NO:167.

(14) In certain embodiments, the second and the additional antigen-binding site scFvs are linked to the antibody Fc domain or a portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16, via a hinge comprising Ala-Ser. In certain embodiments, the second and the additional antigen-binding site scFvs are linked to the antibody Fc domain via a hinge comprising Ala-Ser.

(15) In certain embodiments, a disulfide bridge is formed between the heavy chain variable domain and the light chain variable domain of the second antigen-binding site and/or the additional antigen-binding site. In certain embodiments, the disulfide bridge is formed between C44 from the

heavy chain variable domain and C100 from the light chain variable domain.

(16) In certain embodiments, the scFv that binds NKG2D comprises a light chain variable domain positioned at the N-terminus of a heavy chain variable domain, wherein the light chain variable domain is linked to the heavy chain variable domain of the scFv via a flexible linker comprising the amino acid sequence of SEQ ID NO:167, and scFv that binds NKG2D is linked to the antibody Fc domain via a hinge comprising Ala-Ser.

(17) In certain embodiments, a protein of the present invention comprising a first antigen-binding site comprising an scFv that binds NKG2D, comprises: (a) a heavy chain variable domain comprising complementarity-determining region 1 (CDR1), complementarity-determining region 2 (CDR2), and complementarity-determining region 3 (CDR3) sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 191, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (b) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 193, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (c) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 95, 96, and 97, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (d) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 188, 88, and 189, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 91, 92, and 93, respectively; (e) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 185, 104, and 192, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 107, 108, and 109, respectively; (f) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 185, 72, and 159, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 75, 76, and 77, respectively; (g) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 186, 80, and 187, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 83, 84, and 85, respectively; (h) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 194, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (i) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 195, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (i) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 196, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (k) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 197, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; or (l) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs:

190, 96, and 160, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; and a second and/or an additional antigen-binding site(s) that bind(s) BCMA comprise(s): (a) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 149, 150, and 151, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 153, 154, and 155, respectively; (b) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 115, 116, and 1117, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 120, 121, and 123, respectively; (c) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 125, 126, and 127, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 129, 130, and 131, respectively; (d) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 133, 134, and 135, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 137, 138, and 139, respectively; (e) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 141, 142, and 143, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 145, 146, and 147, respectively; or (f) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 115, 116, and 117, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 120, 121, and 122, respectively.

(18) In certain embodiments, a protein of the present disclosure comprises the amino acid sequence of SEQ ID NO:162.

(19) In certain embodiments, a protein of the present disclosure comprises an amino acid sequence comprising SEQ ID NO:162, SEQ ID NO:163, and SEQ ID NO:165.

(20) In certain embodiments, a protein of the present disclosure comprises an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:162.

(21) In certain embodiments, a protein of the present disclosure comprises an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:162.

(22) In certain embodiments, a protein of the present disclosure comprises an amino acid sequence at least 99% identical to the amino acid sequence of SEQ ID NO:162.

(23) In certain embodiments, a protein of the present disclosure comprises an amino acid sequence at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of SEQ ID NO:162, and further comprises SEQ ID NO:163 and SEQ ID NO:165.

(24) In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to the amino acid sequence of SEQ ID NO:94.

(25) In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:94 and a light chain variable domain at least 90% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:94 and a light chain variable domain at least 95% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure comprises a first antigen-binding site that binds NKG2D, which comprises a

heavy chain variable domain at least 98% identical to SEQ ID NO:94 and a light chain variable domain at least 98% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure comprises a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:94 and a light chain variable domain at least 99% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain identical to SEQ ID NO:94 and a light chain variable domain identical to SEQ ID NO:98.

(26) In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) to SEQ ID NO:169 and a light chain variable domain at least 90% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:169 and a light chain variable domain at least 95% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:169 and a light chain variable domain at least 98% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:169 and a light chain variable domain at least 99% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain identical to SEQ ID NO:169 and a light chain variable domain identical to SEQ ID NO:98.

(27) In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:171 and a light chain variable domain at least 90% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:171 and a light chain variable domain at least 95% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:171 and a light chain variable domain at least 98% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:171 and a light chain variable domain at least 99% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain identical to SEQ ID NO:171 and a light chain variable domain identical to SEQ ID NO:98.

(28) In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:173 and a light chain variable domain at least 90% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:173 and a light chain variable domain at least 95% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:173 and a light chain variable domain at least 98% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:173 and a light chain variable domain at least 99% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure

includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain identical to SEQ ID NO:173 and a light chain variable domain identical to SEQ ID NO:98. (29) In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:175 and a light chain variable domain at least 90% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:175 and a light chain variable domain at least 95% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:175 and a light chain variable domain at least 98% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:175 and a light chain variable domain at least 99% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain identical to SEQ ID NO:175 and a light chain variable domain identical to SEQ ID NO:98. (30) In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:177 and a light chain variable domain at least 90% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:177 and a light chain variable domain at least 95% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:177 and a light chain variable domain at least 98% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:177 and a light chain variable domain at least 99% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain identical to SEQ ID NO:177 and a light chain variable domain identical to SEQ ID NO:98. (31) In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:179 and a light chain variable domain at least 90% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:179 and a light chain variable domain at least 95% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:179 and a light chain variable domain at least 98% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:179 and a light chain variable domain at least 99% identical to SEQ ID NO:98. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds NKG2D, which comprises a heavy chain variable domain identical to SEQ ID NO:179 and a light chain variable domain identical to SEQ ID NO:98. (32) In certain embodiments, a protein of the present disclosure includes a second antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:148 and a

light chain variable domain at least 90% identical to SEQ ID NO:152. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:148 and a light chain variable domain at least 95% identical to SEQ ID NO:152. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:148 and a light chain variable domain at least 98% identical to SEQ ID NO:152. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:148 and a light chain variable domain at least 99% identical to SEQ ID NO:152. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain identical to SEQ ID NO:148 and a light chain variable domain identical to SEQ ID NO:152.

(33) In certain embodiments, a protein of the present disclosure includes a second antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:114 and a light chain variable domain at least 90% identical to SEQ ID NO:119. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:114 and a light chain variable domain at least 95% identical to SEQ ID NO:119. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:114 and a light chain variable domain at least 98% identical to SEQ ID NO:119. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:114 and a light chain variable domain at least 99% identical to SEQ ID NO:119. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain identical to SEQ ID NO:114 and a light chain variable domain identical to SEQ ID NO:119.

(34) In certain embodiments, a protein of the present disclosure includes a second antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:124 and a light chain variable domain at least 90% identical to SEQ ID NO:128. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:124 and a light chain variable domain at least 95% identical to SEQ ID NO:128. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:124 and a light chain variable domain at least 98% identical to SEQ ID NO:128. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:124 and a light chain variable domain at least 99% identical to SEQ ID NO:128. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain identical to SEQ ID NO:124 and a light chain variable domain identical to SEQ ID NO:128.

(35) In certain embodiments, a protein of the present disclosure includes a second antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:132 and a light chain variable domain at least 90% identical to SEQ ID NO:136. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which

comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:132 and a light chain variable domain at least 95% identical to SEQ ID NO:136. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:132 and a light chain variable domain at least 98% identical to SEQ ID NO:136. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:132 and a light chain variable domain at least 99% identical to SEQ ID NO:136. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain identical to SEQ ID NO:132 and a light chain variable domain identical to SEQ ID NO:136.

(36) In certain embodiments, a protein of the present disclosure includes a second antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:140 and a light chain variable domain at least 90% identical to SEQ ID NO:144. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:140 and a light chain variable domain at least 95% identical to SEQ ID NO:144. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:140 and a light chain variable domain at least 98% identical to SEQ ID NO:144. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:140 and a light chain variable domain at least 99% identical to SEQ ID NO:144. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain identical to SEQ ID NO:140 and a light chain variable domain identical to SEQ ID NO:144.

(37) In certain embodiments, a protein of the present disclosure includes a second antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to SEQ ID NO:114 and a light chain variable domain at least 90% identical to SEQ ID NO:118. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:114 and a light chain variable domain at least 95% identical to SEQ ID NO:118. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 98% identical to SEQ ID NO:114 and a light chain variable domain at least 98% identical to SEQ ID NO:118. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain at least 99% identical to SEQ ID NO:114 and a light chain variable domain at least 99% identical to SEQ ID NO:118. In certain embodiments, a protein of the present disclosure includes a first antigen-binding site that binds BCMA, which comprises a heavy chain variable domain identical to SEQ ID NO:114 and a light chain variable domain identical to SEQ ID NO:118.

(38) In certain embodiments, the protein further comprises an additional antigen-binding site that binds BCMA. In certain embodiments, the additional antigen-binding site comprises the same CDR1, CDR2, and CDR3 of heavy chain variable domain and the same CDR1, CDR2, and CDR3 of light chain variable domain of the second antigen-binding site that binds BCMA. In certain embodiments, the additional antigen-binding site comprises a heavy chain variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to the heavy chain variable domain of the second antigen-binding site that binds BCMA, and a light chain

variable domain at least 90% (e.g., at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical) identical to the light chain variable domain of the second antigen-binding site that binds BCMA. In certain embodiments, the additional antigen-binding site comprises a heavy chain variable domain identical to the heavy chain variable domain of the second antigen-binding site that binds BCMA, and a light chain variable domain identical to the light chain variable domain of the second antigen-binding site that binds BCMA.

(39) Proteins disclosed herein comprise an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16. In certain embodiments, proteins disclosed herein comprise an antibody Fc domain. The antibody Fc domain can bind CD16. In certain embodiments, proteins disclosed herein comprise a portion of an antibody Fc domain that retains the binding affinity of the antibody Fc domain to CD16, i.e., sufficient to bind CD16. In certain embodiments, proteins disclosed herein comprise a third antigen-binding site that binds CD16. In certain embodiments, the third antigen-binding site that binds CD16 comprises a Fab fragment. In certain embodiments, the third antigen-binding site that binds CD16 comprises a scFV.

(40) In certain embodiments, the scFv that binds to NKG2D is linked to the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16. In certain embodiments, the scFv that binds to NKG2D is linked to the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16 via a hinge comprising Ala-Ser. In certain embodiments, the scFv that binds to NKG2D is linked to the C-terminus of the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16 via a flexible linker. In certain embodiments, the flexible linker comprises the amino acid sequence of SEQ ID NO:168. In certain embodiments, the C-terminus of the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16 is linked to the N-terminus of the light chain variable domain of the scFv that binds NKG2D. In certain embodiments, the flexible linker linking the C-terminus of the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16, to the N-terminus of the V.sub.L domain of the scFv that binds NKG2D (e.g., SEQ ID NO:98) has the amino acid sequence of SEQ ID NO:168.

(41) In certain embodiments, proteins disclosed herein comprise an antibody Fc domain. In certain embodiments, the scFv that binds to NKG2D is linked to the antibody Fc domain. In certain embodiments, the scFv that binds to NKG2D is linked to the antibody Fc domain via a hinge comprising Ala-Ser. In certain embodiments, the scFv that binds to NKG2D is linked to the C-terminus of the antibody Fc domain via a flexible linker. In certain embodiments, the flexible linker comprises the amino acid sequence of SEQ ID NO:168. In certain embodiments, the C-terminus of the antibody Fc domain is linked to the N-terminus of the light chain variable domain of the scFv that binds NKG2D. In certain embodiments, the flexible linker linking the C-terminus of the Fc domain to the N-terminus of the V.sub.L domain of the scFv that binds NKG2D (e.g., SEQ ID NO:98) has the amino acid sequence of SEQ ID NO:168.

(42) In certain embodiments, a protein of the present disclosure includes an antibody Fc domain comprising hinge and CH2 domains of a human IgG1 antibody.

(43) In certain embodiments, a protein of the present disclosure includes an Fc domain comprising an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody. In certain embodiments, a protein of the present disclosure includes an Fc domain comprising an amino acid sequence at least 95% identical to amino acids 234-332 of a human IgG1 antibody. In certain embodiments, a protein of the present disclosure includes an Fc domain comprising an amino acid sequence at least 98% identical to amino acids 234-332 of a human IgG1 antibody. In certain embodiments, a protein of the present disclosure includes an Fc domain comprising amino acid sequence at least 90% identical to the Fc domain of human IgG1. In certain embodiments, a protein of the present disclosure includes an Fc domain comprising amino acid sequence at least 95% identical to the Fc domain of human IgG1. In certain embodiments, a protein of the present

disclosure includes an Fc domain comprising amino acid sequence at least 98% identical to the Fc domain of human IgG1. In certain embodiments, a protein of the present disclosure includes an Fc domain comprising 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, T350, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411, and K439.

(44) In certain embodiments, a protein of the present disclosure includes an Fc domain of an human IgG1 comprising Q347R, D399V, and F405T substitutions. A protein of the present disclosure includes an Fc domain comprising Q347R, D399V, and F405T substitutions, linked to an scFv that bind NKG2D.

(45) In certain embodiments, a protein of the present disclosure includes an Fc domain of an human IgG1 comprising K360E and K409W substitutions.

(46) In certain embodiments, a protein of the present disclosure includes an Fc domain comprising K360E and K409W substitutions, linked to the second antigen binding site.

(47) In certain embodiments, the first antigen-binding site binds to NKG2D with a K.sub.D of 2 to 120 nM, as measured by surface plasmon resonance. In certain embodiments, the protein binds to NKG2D with a K.sub.D of 2 to 120 nM, as measured by surface plasmon resonance.

(48) Formulations containing at least one of these proteins; cells containing at least one or more nucleic acids expressing these proteins, and methods of enhancing tumor cell death using these proteins are also provided.

(49) In further aspect of the invention, the present disclosure provides a method of treating cancer, in which a protein of the present disclosure or a formulation comprising a protein of the present disclosure is administered to a patient in need thereof. In some embodiments, the cancer expresses BCMA. In some embodiments, at least 20% of the cells of the cancer expresses BCMA. In some embodiments, at least 50% of the cells of the cancer expresses BCMA. In some embodiments, at least 80% of the cells of the cancer expresses BCMA.

(50) In certain embodiments, a protein of the present disclosure is used in treating a cancer selected from multiple myeloma, acute myelomonocytic leukemia, T cell lymphoma, acute monocytic leukemia, and follicular lymphoma.

Description

BRIEF DESCRIPTION OF THE DRAWINGS

(1) FIGS. **1**A-**1**B illustrate exemplary trispecific antibodies (TriNKET®), which include an scFv first antigen-binding site that binds NKG2D, a second antigen-binding site that binds BCMA, an additional tumor-associated antigen-binding site that binds BCMA, and a heterodimerized antibody constant region that binds CD16. These antibody formats are referred herein as F4-TriNKET®. FIG. **1**A illustrates that the two BCMA-binding sites in the Fab format. FIG. **1**B illustrates that the two BCMA-binding sites in the scFv format.

(2) FIG. **2** illustrates an exemplary TriNKET® that contains an scFv first antigen-binding site that binds NKG2D, a second antigen-binding site that binds BCMA, and a heterodimerized antibody constant region. The antibody format is referred herein as F3-TriNKET®.

(3) FIG. **3** shows BCMA-targeted TriNKET® (NKG2D-binding-F4-TriNKET®-BCMA (in short NKG2D-F4-TriNKET®-BCMA) mediates more potent lysis of BCMA positive KMS12-PE myeloma cells than anti-BCMA mAb.

(4) FIG. **4** shows BCMA-targeted TriNKET® mediates more potent lysis of BCMA positive MM.1R myeloma cells than anti-BCMA mAb.

(5) FIG. **5** shows that incubation with BCMA-targeted antibody and TriNKET® increased total surface BCMA expression stably over time on KMS12-PE myeloma cells.

(6) FIG. **6** shows that incubation with BCMA-targeted antibody and TriNKET® increased total surface BCMA expression stably over time on MM.1R myeloma cells.
(7) FIG. **7** shows that extending incubation time with bivalent TriNKET® dramatically enhanced amount of TriNKET® bound to KMS12-PE myeloma cells.
(8) FIG. **8** shows that extending incubation time with bivalent TriNKET® dramatically enhanced amount of TriNKET® bound to MM.1R cells.
(9) FIG. **9** shows that bivalent TriNKET® (F4-format) outperformed bivalent BCMA-targeted mAb and monovalent TriNKET® in long-term purified NK killing assay.
(10) FIG. **10** shows BCMA-TriNKETs® retained efficacy in long-term cytotoxicity assay with fresh PBMC effector cells.
(11) FIG. **11** shows weak (below limit of detection) binding of BCMA-targeted TriNKET® to NKG2D expressed on KHYG-1 cells.
(12) FIG. **12** shows very little binding of bivalent BCMA-targeted TriNKET® (F4-format) beyond mAb Fc binding to KHYG-1 cells transduced to express CD16.
(13) FIGS. **13**A-**13**F shows insignificant binding in whole blood of BCMA-targeted TriNKET® (solid border, dark grey) beyond background (dashed border, white) to NK cells (FIG. **13**A), CD8+ T cells (FIG. **13**B), and CD4+ T cells (FIG. **13**C). Given proximity to IgG1 control (dotted border, light grey) binding to B cells (FIG. **13**D), monocytes (FIG. **13**E), and granulocytes (FIG. **13**F) is mostly Fc receptor mediated.
(14) FIG. **14** shows purity of CD8+ effector T cells and target expression. As shown, CD8+ effector T cells generated with ConA stimulation and cultured with IL-15 were of high purity (>99% of CD3+CD8+ cells), and all expressed NKG2D but not CD16.
(15) FIGS. **15**A-**15**B show cytolysis of KMS12-PE cells in DELFIA assay. DELFIA cytotoxicity assays were performed with human primary CD8+ effector T cells derived from two healthy donors and KMS12-PE target cells. FIG. **15**A depicts results for cells derived from Donor 1 and FIG. **15**B depicts results for cells derived from Donor 2. As shown, NKG2D-binding-F4-TriNKET®-BCMA enhanced lysis of KMS12-PE cells when co-cultured with activated CD8+ T cells, but not in the absence of effector cells. The parental anti-BCMA mAb or the irrelevant TriNKET® was unable to enhance lysis by CD8+ T cells from either donor.
(16) FIGS. **16**A-**16**B show human NK cell activation in the presence of BCMA positive target cell lines in the presence of anti-BCMA TriNKET® or monoclonal antibody within 4 hours. FIG. **16**A depicts results with KMS12-PE cells (low BCMA expression) as target cells.
(17) FIG. **16**B depicts results with H929 (high BCMA expression) as target cells. As shown, against both high and low BCMA expressing cells the F4-TriNKET® triggered an increase in degranulation and IFNγ production with subnanomolar EC50 value. Compared to a BCMA monoclonal antibody, the F4 TriNKET® stimulated a greater proportion of NK cells at maximum with enhanced potency against both cell lines.

DETAILED DESCRIPTION

(18) The invention provides multi-specific binding proteins that bind BCMA, NKG2D receptor, and CD16 receptor. The multi-specific binding proteins can bind a BCMA on a cancer cell and the NKG2D receptor and CD16 receptor on a natural killer cell to activate the natural killer cell. The multi-specific binding proteins can also bind a BCMA on a cancer cell and the NKG2D receptor and CD16 receptor on a cytotoxic T cell to activate the cytotoxic T cell. Provided herein are also pharmaceutical compositions comprising such multi-specific binding proteins, and therapeutic methods using such multi-specific proteins and pharmaceutical compositions, including for the treatment of cancer. Various aspects of the invention are set forth below in sections; however, aspects of the invention described in one particular section are not to be limited to any particular section.
(19) To facilitate an understanding of the present invention, a number of terms and phrases are defined below.

(20) The terms “a” and “an” as used herein mean “one or more” and include the plural unless the context is inappropriate.
(21) As used herein, the term “antigen-binding site” refers to the part of the immunoglobulin molecule that participates in antigen binding. In human antibodies, the antigen-binding site is formed by amino acid residues of the N-terminal variable (“V”) regions of the heavy (“H”) and light (“L”) chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as “hypervariable regions” which are interposed between more conserved flanking stretches known as “framework regions,” or “FR.” Thus the term “FR” refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In a human antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as “complementarity-determining regions,” or “CDRs.” In certain animals, such as camels and cartilaginous fish, the antigen-binding site is formed by a single antibody chain providing a “single domain antibody.” Antigen-binding sites can exist in an intact antibody, in an antigen-binding fragment of an antibody that retains the antigen-binding surface, or in a recombinant polypeptide such as an scFv, using a peptide linker to connect the heavy chain variable domain to the light chain variable domain in a single polypeptide. All the amino acid positions in heavy or light chain variable regions disclosed herein are numbered according to Kabat numbering.
(22) The term “tumor associated antigen” as used herein means any antigen including but not limited to a protein, glycoprotein, ganglioside, carbohydrate, lipid that is associated with cancer. Such antigen can be expressed on malignant cells or in the tumor microenvironment such as on tumor-associated blood vessels, extracellular matrix, mesenchymal stroma, or immune infiltrates.
(23) As used herein, the terms “subject” and “patient” refer to an organism to be treated by the methods and compositions described herein. Such organisms preferably include, but are not limited to, mammals (e.g., murines, simians, equines, bovines, porcines, canines, felines, and the like), and more preferably include humans.
(24) As used herein, the term “effective amount” refers to the amount of an agent (e.g., a protein of the present invention) sufficient to effect beneficial or desired results. The term when used in connection with a therapeutic agent refers an amount of such agent sufficient to provide a therapeutic benefit in the treatment of the disease or disorder or to delay or minimize one or more symptoms associated with the disease or disorder. An effective amount can be administered in one or more administrations, applications or dosages and is not intended to be limited to a particular formulation or administration route. As used herein, the term “treating” includes any effect, e.g., lessening, reducing, modulating, ameliorating or eliminating, that results in the improvement of the condition, disease, disorder, and the like, or ameliorating a symptom thereof.
(25) As used herein, the term “pharmaceutical composition” refers to the combination of an active agent with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use in vivo or ex vivo.
(26) As used herein, the term “pharmaceutically acceptable carrier” refers to any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, emulsions (e.g., such as an oil/water or water/oil emulsions), and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see e.g., Martin, Remington's Pharmaceutical Sciences, 15th Ed., Mack Publ. Co., Easton, PA [1975].
(27) As used herein, the term “pharmaceutically acceptable salt” refers to any pharmaceutically acceptable salt (e.g., acid or base) of a compound of the present invention which, upon administration to a subject, is capable of providing a compound of this invention or an active metabolite or residue thereof. As is known to those of skill in the art, “salts” of the compounds of

the present invention may be derived from inorganic or organic acids and bases. Exemplary acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts.
(28) Exemplary bases include, but are not limited to, alkali metal (e.g., sodium) hydroxides, alkaline earth metal (e.g., magnesium) hydroxides, ammonia, and compounds of formula NW.sub.4.sup.+, wherein W is C.sub.1-4 alkyl, and the like.
(29) Exemplary salts include, but are not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, undecanoate, and the like. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na.sup.+, NH.sub.4+, and NW.sub.4.sup.+ (wherein W is a C.sub.1-4 alkyl group), and the like.
(30) For therapeutic use, salts of the compounds of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases that are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.
(31) Throughout the description, where compositions are described as having, including, or comprising specific components, or where processes and methods are described as having, including, or comprising specific steps, it is contemplated that, additionally, there are compositions of the present invention that consist essentially of, or consist of, the recited components, and that there are processes and methods according to the present invention that consist essentially of, or consist of, the recited processing steps.
(32) As a general matter, compositions specifying a percentage are by weight unless otherwise specified. Further, if a variable is not accompanied by a definition, then the previous definition of the variable controls.
(33) I. Proteins
(34) The invention provides multi-specific binding proteins that bind BCMA on a cancer cell and the NKG2D receptor and CD16 receptor on natural killer cells to activate the natural killer cell. The multi-specific binding proteins are useful in the pharmaceutical compositions and therapeutic methods described herein. Binding of the multi-specific binding protein to the NKG2D receptor and CD16 receptor on natural killer cell enhances the activity of the natural killer cell toward destruction of a cancer cell. Binding of the multi-specific binding protein to BCMA on a cancer cell brings the cancer cell into proximity with the natural killer cell, which facilitates direct and indirect destruction of the cancer cell by the natural killer cell.
(35) The multi-specific binding proteins provided herein can also bind BCMA on a cancer cell and the NKG2D receptor and CD16 receptor on cytotoxic T cells to activate the cytotoxic T cell. The multi-specific binding proteins are useful in the pharmaceutical compositions and therapeutic methods described herein. Binding of the multi-specific binding protein to the NKG2D receptor and CD16 receptor on cytotoxic T cell enhances the activity of the cytotoxic T cell toward destruction of a cancer cell. Binding of the multi-specific binding protein to BCMA on a cancer cell brings the cancer cell into proximity with the cytotoxic T cell, which facilitates destruction of the cancer cell by the cytotoxic T cell.
(36) Further description of exemplary multi-specific binding proteins is provided below.

(37) The first component of the multi-specific binding proteins binds to NKG2D receptor-expressing cells, which can include but are not limited to NK cells, NKT cells, γδ T cells and CD8.sup.+αβ T cells. Upon NKG2D binding, the multi-specific binding proteins may block natural ligands, such as ULBP6 and MICA, from binding to NKG2D and activating NKG2D receptors.
(38) The second component of the multi-specific binding proteins binds to BCMA-expressing cells, which can include but are limited to multiple myeloma and B cell malignancies.
(39) The third component for the multi-specific binding proteins binds to cells expressing CD16, an Fc receptor on the surface of leukocytes including natural killer cells, cytotoxic T cells, macrophages, neutrophils, eosinophils, mast cells, and follicular dendritic cells.
(40) The multi-specific binding proteins described herein can take various formats. FIG. **1**A illustrates F4 TriNKET® having two antigen-binding sites that bind BCMA, wherein both antigen binding sites that bind BCMA are Fab fragment. The F4 TriNKET® (Fab) include an first antigen-binding site that binds NKG2D, which comprises a scFv, a second antigen-binding site that binds BCMA, an additional antigen-binding site that binds BCMA, and a heterodimerized antibody constant region that binds CD16. The F4 TriNKET® (Fab) is a heterodimeric, multi-specific antibody that includes four peptides: a first immunoglobulin heavy chain, a second immunoglobulin heavy chain and two immunoglobulin light chains (FIG. **1**A). The first immunoglobulin heavy chain includes, from N-terminus to C-terminus, a heavy chain variable domain (VH) linked to a heavy chain constant region 1 (CH1) which forms a first (VH-CH1) domain, and a first Fc (hinge-CH2-CH3) domain, wherein the first (VH-CH1) domain pairs with the first light chain to form a first Fab that binds BCMA, and wherein the (VH-CH1) domain is linked to the first Fc via either a linker or a hinge (FIG. **1**A). The second immunoglobulin heavy chain includes, from N-terminus to C-terminus, a second (VH-CH1) domain, a second Fc (hinge-CH2-CH3) domain, and a single-chain variable fragment (scFv) that is composed of a VH and a VL that pair and bind NKG2D, wherein the second Fc domain is linked via either a linker or a hinge at its N-terminus to the second (VH-CH1) domain, and via either a linker or a hinge at its C-terminus to the scFV that binds NKG2D, and wherein the second (VH-CH1) domain pairs with the second light chain to form a second Fab that binds BCMA(FIG. **1**A).
(41) The F4 TriNKET® (scFv) is a heterodimeric, multi-specific antibody that includes two peptides: a first immunoglobulin heavy chain and a second immunoglobulin heavy chain (FIG. **1**B). The first immunoglobulin heavy chain includes, from N-terminus to C-terminus, a first scFv that binds BCMA and a first Fc (hinge-CH2-CH3) domain, wherein the first scFv that binds BCMA is linked to the first Fc via either a linker or a hinge (FIG. **1**B). The second immunoglobulin heavy chain includes, from N-terminus to C-terminus, a second scFv that binds BCMA, a second Fc (hinge-CH2-CH3) domain, and a scFv that binds NKG2D, wherein the second Fc domain is linked via either a linker or a hinge at its N-terminus to the second scFv domain that binds BCMA, and via either a linker or a hinge at its C-terminus to the scFV that binds NKG2D (FIG. **1**B).
(42) TriNKETs® termed “NKG2D-binding-F4-TriNKET®-BCMA” can refer to the TriNKETs® depicted in FIG. **1**A (NKG2D-binding-F4 (Fab)-TriNKET®-BCMA) or FIG. **1**B (NKG2D-binding-F4 (scFv)-TriNKET®-BCMA). For example, the TriNKET® “A49-F4-TRINKET®-BCMA” refers to a TriNKET® that has the “NKG2D-binding-F4-TriNKET®-BCMA” format, and has a NKG-2D binding domain comprising the VH and VL of A49 (See Table 1 below).
(43) In some embodiments, the single-chain variable fragment (scFv) described above is linked to the antibody constant domain via a hinge sequence. In some embodiments, the hinge comprises amino acids Ala-Ser. In some other embodiments, the hinge comprises amino acids Ala-Ser and Thr-Lys-Gly. The hinge sequence can provide flexibility of binding to the target antigen, and balance between flexibility and optimal geometry.
(44) In some embodiments, the single-chain variable fragment (scFv) described above includes a heavy chain variable domain and a light chain variable domain. In some embodiments, the heavy chain variable domain forms a disulfide bridge with the light chain variable domain to enhance

stability of the scFv. 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 embodiments, the heavy chain variable domain is linked to the light chain variable domain via a flexible linker. Any suitable linker can be used, for example, the (G4S).sub.4 (SEQ ID NO: 164) linker. In some embodiments of the scFv, the heavy chain variable domain is positioned at the N-terminus of the light chain variable domain. In some embodiments of the scFv, the heavy chain variable domain is positioned at the C terminus of the light chain variable domain.

(45) The multi-specific binding proteins can provide bivalent or monovalent engagement of BCMA. Bivalent engagement of BCMA by the multi-specific proteins can stabilize the BCMA on cancer cell surface, and enhance cytotoxicity of NK cells towards the cancer cells. Bivalent engagement of BCMA by the multi-specific proteins can confer stronger binding of the multi-specific proteins to the cancer cells, thereby facilitating stronger cytotoxic response of NK cells towards the cancer cells, especially towards cancer cells expressing a low level of BCMA. Bivalent engagement of BCMA by the multi-specific proteins provided herein can also enhance cytotoxicity of cytotoxic T cells towards the cancer cells. Bivalent engagement of BCMA by the multi-specific proteins can confer stronger binding of the multi-specific proteins to the cancer cells, thereby facilitating stronger cytotoxic response of cytotoxic T cells towards the cancer cells.

(46) Within the Fc domain, CD16 binding is mediated by the hinge region and the CH2 domain. For example, within human IgG1, the interaction with CD16 is primarily focused on amino acid residues Asp 265-Glu 269, Asn 297-Thr 299, Ala 327-Ile 332, Leu 234-Ser 239, and carbohydrate residue N-acetyl-D-glucosamine in the CH2 domain (see, Sondermann et al., Nature, 406 (6793):267-273). Based on the known domains, mutations can be selected to enhance or reduce the binding affinity to CD16, such as by using phage-displayed libraries or yeast surface-displayed cDNA libraries, or can be designed based on the known three-dimensional structure of the interaction.

(47) In some embodiments, the antibody constant domain comprises a CH2 domain and a CH3 domain of an IgG antibody, for example, a human IgG1 antibody. In some embodiments, mutations are introduced in the antibody constant domain to enable heterdimerization with another antibody constant domain. For example, if the antibody constant domain is derived from the constant domain of a human IgG1, the antibody constant domain can comprise an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody, 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. All the amino acid positions in an Fc domain or hinge region disclosed herein are numbered according to EU numbering.

(48) In some embodiments, the antibody constant domain can comprise an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody, and differs by 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, T3661, T366L, T366M, T366K, T366W, T366S, L368E, L368A, L368D, K370S, N390D, N390E, K392L, K392M, K392V, K392F, K392D, K392E, T394F, D399R, D399K, D399V, S400K, S400R, D401K, F405A, F405T, Y407A, Y4071, Y407V, K409F, K409W, K409D, T411D, T411E, K439D, and K439E.

(49) Listed below are examples of the scFv linked to an antibody constant region that also includes mutations that enable heterodimerization of two polypeptide chains. The scFv containing a heavy chain variable domain (V.sub.H) and a light chain variable domain (V.sub.L) from NKG2D is used in preparing a multispecific protein of the present disclosure. Each sequence represents V.sub.L-(G4S).sub.4-V.sub.H-hinge (AS)-Fc containing heterodimerization mutations (underlined). V.sub.L and V.sub.H contain 100V.sub.L-44V.sub.H S-S bridge (underlined), and can be from any tumor targeting or NKG2D binding antibody. The Ala-Ser (AS, bolded & underlined) is included at the

elbow hinge region sequence to balance between flexibility and optimal geometry. In certain embodiments, an additional sequence Thr-Lys-Gly can be added to the AS sequence at the hinge. (G4S).sub.4 (SEQ ID NO: 164) linker is underlined in the sequences listed in the paragraph below.

(50) A TriNKET® of the present disclosure is a NKG2D-binding-F4-TriNKET®-BCMA, A49-F4-TriNKET®-BCMA, comprising a first polypeptide comprising the sequence of SEQ ID NO:162 (F4-BCMAFc-AJchainB-NKG2D-binding scFv), a second polypeptide comprising the sequence of SEQ ID NO:163 (Anti-BCMA HC-hinge-Fc), and a third and a fourth polypeptides each comprising the sequence of SEQ ID NO:165 (Anti-BCMA-Whole LC).

(51) The first polypeptide, i.e., F4-BCMAFc-AJchainB-NKG2D-binding scFv (SEQ ID NO:162) and the third polypeptide, i.e. Anti-BCMA-Whole LC, forms a first BCMA-targeting Fab fragment (including a heavy chain portion comprising a heavy chain variable domain (V.sub.H) (SEQ ID NO:148) and a CH1 domain, and a light chain portion comprising a light chain variable domain (SEQ ID NO:152) and a light chain constant domain). F4-BCMAFc-AJchainB-NKG2D-binding scFv comprises the heavy chain portion (VH-CH1) connected to an Fc domain (hinge-CH2-CH3), which at the C-terminus of the Fc is linked to a single-chain variable fragment (scFv) that binds NKG2D. The scFv that binds NKG2D is represented by the amino acid sequence of SEQ ID NO:161, and includes a light chain variable domain (V.sub.L) (SEQ ID NO:98) linked to a heavy chain variable domain (V.sub.H) (SEQ ID NO:94) via a (G4S).sub.4 (SEQ ID NO: 164) linker. As represented in SEQ ID NO:162, the C-terminus of the Fc domain is linked to the N-terminus of the V.sub.L (SEQ ID NO:98) domain using a short SGSGGGGS linker (SEQ ID NO:168).

(52) TABLE-US-00001 NKG2D-binding scFv (SEQ ID NO: 161)
DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQQKPGKAPKLLIYAA
SSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGVSFPRTFG  custom character GT
KVEIK*GGGGSGGGGSGGGGSGGGGS*EVQLVESGGGLVKPGGSLRLSCAASG
FTFSSYSMNWVRQAPGK  custom character
LEWVSSISSSSSYIYYADSVKGRFTISRDNAKN
SLYLQMNSLRAEDTAVYYCARGAPMGAAAGWFDPWGQGTLVTVSS F4-BCMAFc-AJchainB-NKG2D-binding scFv (SEQ ID NO: 162)
EVQLLESGGGLVQPGGSLRLSCAASGFTFSDNAMGWVRQAPGKGLEWVSAI
SGPGSSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKVLGW
FDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV
TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK
PSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRT
PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP**<u>R</u>**VYTLPP  custom character
RDEL TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL**<u>V</u>**SDGSF**<u>T</u>**LYSK
LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG*SGSGGGGS*DIQMTQ
SPSSVSASVGDRVTITCRASQGISSWLAWYQQKPGKAPKLLIYAASSLQSG
VPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGVSFPRTFG  custom character GTKVEIK*G*
*GGGSGGGGSGGGGSGGGGS*EVQLVESGGGLVKPGGSLRLSCAASGFTFSSY
SMNWVRQAPGK  custom character
LEWVSSISSSSSYIYYADSVKGRFTISRDNAKNSLYLQM
NSLRAEDTAVYYCARGAPMGAAAGWFDPWGQGTLVTVSS

(53) The scFv in the NKG2D-binding-F4-TriNKET®-BCMA includes a light chain variable domain of an NKG2D-binding site connected to a heavy chain variable domain with a (G4S).sub.4 (SEQ ID NO: 164) linker (represented as (V.sub.L(G4S).sub.4V.sub.H)). The light and the heavy variable domains of the scFv (SEQ ID NO:162) are connected as V.sub.L-(G4S).sub.4-V.sub.H; V.sub.L and V.sub.H contain 100V.sub.L-44V.sub.H S-S bridge (resulting from G100C and G44C substitutions, respectively) (cysteine residues are bold-italics-underlined). (G4S).sub.4 is the sequence in italics GGGGSGGGGSGGGGSGGGGS (SEQ ID NO:164) in SEQ ID NO:161 and

SEQ ID NO:162. The Fc domain in SEQ ID NO:162 comprises an S354C substitution, which forms a disulfide bond with a Y349C substitution in another Fc domain (SEQ ID NO: 163, described below). The Fc domain in SEQ ID NO:162 includes Q347R, D399V, and F405T substitutions.

(54) The second polypeptide, i.e. Anti-BCMA VH-CH1-Fc, and the fourth polypeptide, i.e. Anti-BCMA-Whole LC, forms a second BCMA-binding Fab fragment. Anti-BCMA VH-CH1-Fc includes a heavy chain portion comprising a heavy chain variable domain (SEQ ID NO:148) and a CH1 domain, wherein the heavy chain variable domain is connected to the CH1 domain, and the CH1 domain is connected to the Fc domain. Anti-BCMA-Whole LC includes a light chain portion comprising a light chain variable domain (SEQ ID NO:152) and a light chain constant domain.

(55) TABLE-US-00002 Anti-BCMA VH-CH1-Fc (SEQ ID NO: 163)

EVQLLESGGGLVQPGGSLRLSCAASGFTFSDNAMGWVRQAPGKGLEWVSAI
SGPGSSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKVLGW
FDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV
TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK
PSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRT
PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV[custom character]
TLPPSRDEL T**E**NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS**W**
LTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

(56) SEQ ID NO:163 represents the heavy chain portion of the second anti-BCMA Fab fragment, which comprises a heavy chain variable domain (SEQ ID NO:148) of a BCMA-binding site and a CH1 domain, connected to an Fc domain (hinge-CH2-CH3). The Fc domain in SEQ ID NO:163 includes a Y349C substitution, which forms a disulfide bond with an S354C substitution in the CH3 domain of the Fc linked to the NKG2D-binding scFv (SEQ ID NO:162). In SEQ ID NO:163, the Fc domain also includes K360E and K409W substitutions.

(57) SEQ ID NO:165 represents the light chain portion of a Fab fragments comprising a light chain variable domain (SEQ ID NO:152) of a BCMA-binding site and a light chain constant domain.

(58) TABLE-US-00003 Anti-BCMA-Whole LC (SEQ ID NO: 165)

EIVLTQSPGTLSLSPGERATLSCRASQSVSDEYLSWYQQKPGQAPRLLIHS
ASTRATGIPDRFSGSGSGTDFTLAISRLEPEDFAVYYCQQYGYPPDFTFGQ
GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLS
SPVTKSFNRGEC

(59) In an exemplary embodiment, the Fc domain linked to the NKG2D-binding scFv fragment comprises the mutations of K360E and K409W, and the Fc domain linked to the BCMA Fab fragment comprises matching mutations Q347R, D399V, and F405T for forming a heterodimer.

(60) In an exemplary embodiment, the Fc domain linked to the NKG2D-binding scFv includes a Y349C substitution in the CH3 domain, which forms a disulfide bond with an S354C substitution on the Fc linked to the BCMA-binding Fab fragment.

(61) The F3 TriNKET® is a heterodimeric, multi-specific antibody that includes three peptides: a first immunoglobulin heavy chain, a second immunoglobulin heavy chain and a immunoglobulin light chain (FIG. **2**). The first immunoglobulin heavy chain includes, from N-terminus to C-terminus, a scFv that binds NKG2D, and a first Fc (CH2-CH3) domain, wherein the scFv that binds NKG2D is linked to the first Fc via either a linker or a hinge (FIG. **2**). The second immunoglobulin heavy chain includes, from N-terminus to C-terminus, a (VH-CH1) domain, and a second Fc (CH2-CH3) domain, wherein the second Fc domain is linked via either a linker or a hinge at its N-terminus to the (VH-CH1) domain, and wherein the (VH-CH1) domain pairs with the light chain to form a Fab that binds BCMA (FIG. **2**). TriNKETs® termed “NKG2D-binding-F3-TriNKET®-BCMA” can refer to the TriNKETs® depicted in FIG. **2**. Another exemplary TriNKET® of the

present disclosure is NKG2D-binding-F3-TriNKET®-BCMA , sequences of which are described below (CDRs (Kabat numbering) are underlined).

(62) An exemplary NKG2D-binding-F3-TriNKET®-BCMA includes a BCMA-binding Fab fragment that includes a heavy chain portion comprising a heavy chain variable domain (SEQ ID NO:148) and a CH1 domain, and a light chain portion comprising a light chain variable domain (SEQ ID NO:152) and a light chain constant domain, wherein the heavy chain variable domain is connected to the CH1 domain, and the CH1 domain is connected to the Fc domain. NKG2D-binding-F3-TriNKET®-BCMA also comprises a NKG2D-binding scFv linked to an Fc domain (SEQ ID NO: 166).

(63) SEQ ID NO:163 represents an exemplary second immunoglobulin heavy chain of NKG2D-binding-F3-TriNKET®-BCMA as depicted in FIG. **2**, including the heavy chain portion of an anti-BCMA Fab fragment, which comprises a heavy chain variable domain (SEQ ID NO:148) of a BCMA-binding site and a CH1 domain, connected to an Fc domain. The Fc domain in SEQ ID NO:163 includes a Y349C substitution, which forms a disulfide bond with an S354C substitution in the CH3 domain of the Fc linked to the NKG2D-binding scFv (SEQ ID NO:166) for forming the NKG2D-binding-F3-TriNKET®-BCMA. In SEQ ID NO:163, the Fc domain also includes K360E and K409W substitutions.

(64) In an exemplary first immunoglobulin heavy chain of NKG2D-binding-F3-TriNKET®-BCMA, the scFv in the NKG2D-binding-F3-TriNKET®-BCMA includes a light chain variable domain of an NKG2D-binding site connected to a heavy chain variable domain with a (G4S).sub.4 linker (SEQ ID NO:164) (represented as (V.sub.L(G4S).sub.4V.sub.H)), which is linked to an Fc domain. In NKG2D-binding-F3-TriNKET®-BCMA, the light and the heavy variable domains of the scFv (SEQ ID NO:161) are connected as V.sub.L-(G4S).sub.4-V.sub.H; V.sub.L and V.sub.H contain 100V.sub.L-44V.sub.H S-S bridge (resulting from G100C and G44C substitutions, respectively) (cysteine residues are bold-italics-underlined); and V.sub.H is connected to the Fc domain via an Ala-Ser.

(65) SEQ ID NO:166 represents the full sequence of an NKG2D-binding scFv linked to an Fc domain via a hinge comprising Ala-Ser (scFv-Fc). The Fc domain linked to the scFv includes Q347R, D399V, and F405T substitutions.

(66) TABLE-US-00004 F3-NKG2D-binding scFv-Fc-AJchainB [V.sub.L(G4S).sub.4V.sub.H)] (SEQ ID NO: 166)

DIQMTQSPSSVSASVGDRVTITCRASQGISSWLAWYQQKPGKAPKLLIYAA
SSLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQGVSFPRTFG  custom character GT
KVEIK*GGGGSGGGGSGGGGSGGGGS*EVQLVESGGGLVKPGGSLRLSCAASG
FTFSSYSMNWVRQAPGK  custom character
LEWVSSISSSSSYIYYADSVKGRFTISRDNAKN
SLYLQMNSLRAEDTAVYYCARGAPMGAAAGWFDPWGQGTLVTVSSASDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK
ALPAPIEKTISKAKGQPREP**R**VYTLPP  custom character RDELTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVL**V**SDGSF**T**LYSKLTVDKSRWQQGNVFSCSVM
HEALHNHYTQKSLSLSPG

(67) In an exemplary embodiment of a NKG2D-binding-F3-TriNKET®-BCMA, the Fc domain of the first immunoglobulin heavy chain, which is linked to the NKG2D-binding scFv fragment comprises the mutations of K360E and K409W, and the Fc domain of the second immunoglobulin heavy chain, which is linked to the BCMA Fab fragment comprises matching mutations Q347R, D399V, and F405T for forming a heterodimer.

(68) In an exemplary embodiment of a NKG2D-binding-F3-TriNKET®-BCMA, the Fc domain of the first immunoglobulin heavy chain, which is linked to the NKG2D-binding scFv includes a Y349C substitution in the CH3 domain, which forms a disulfide bond with an S354C substitution

on the Fc domain of the second immunoglobulin heavy chain, which is linked to the BCMA-binding Fab fragment.

(69) The multi-specific binding proteins can bind to the NKG2D receptor-expressing cells, which can include but are not limited to NK cells, γδ T cells and CD8.sup.+αβ T cells. Upon NKG2D binding, the multi-specific binding proteins may block natural ligands, such as ULBP6 and MICA, from binding to NKG2D and activating NKG2D receptors.

(70) The multi-specific binding proteins binds to cells expressing CD16, an Fc receptor on the surface of leukocytes including natural killer cells, macrophages, neutrophils, eosinophils, mast cells, and follicular dendritic cells.

(71) A protein of the present disclosure binds to NKG2D with an affinity of K.sub.D of 10 nM or lower, e.g., about 10 nM, about 9 nM, about 8 nM, about 7 nM, about 6 nM, about 5 nM, about 4.5 nM, about 4 nM, about 3.5 nM, about 3 nM, about 2.5 nM, about 2 nM, about 1.5 nM, about 1 nM, between about 0.5 nM-about 1 nM, about 1 nM-about 2 nM, about 2 nM-3 nM, about 3 nM-4 nM, about 4 nM-about 5 nM, about 5 nM-about 6 nM, about 6 nM-about 7 nM, about 7 nM-about 8 nM, about 8 nM-about 9 nM, about 9 nM-about 10 nM, about 1 nM-about 10 nM, about 2 nM-about 10 nM, about 3 nM-about 10 nM, about 4 nM-about 10 nM, about 5 nM-about 10 nM, about 6 nM-about 10 nM, about 7 nM-about 10 nM, or about 8 nM-about 10 nM.

(72) Upon binding to the NKG2D receptor and CD16 receptor on natural killer cells, and a tumor-associated antigen on cancer cells, the multi-specific binding proteins can engage more than one kind of NK-activating receptor, and may block the binding of natural ligands to NKG2D. In certain embodiments, the proteins can agonize NK cells in humans. In some embodiments, the proteins can agonize NK cells in humans and in other species such as rodents and cynomolgus monkeys.

(73) Upon binding to the NKG2D receptor and CD16 receptor on cytotoxic T cells, and a tumor-associated antigen on cancer cells, the multi-specific binding proteins can engage more than one kind of activating receptor, and may block the binding of natural ligands to NKG2D. In certain embodiments, the proteins can agonize cytotoxic T cells in humans. In some embodiments, the proteins can agonize cytotoxic T cells in humans and in other species such as rodents and cynomolgus monkeys.

(74) NKG2D-Binding Site

(75) Table 1 lists peptide sequences of heavy chain variable domains and light chain variable domains that, in combination, can bind to NKG2D. In some embodiments, the heavy chain variable domain and the light chain variable domain are arranged in Fab format. In some embodiments, the heavy chain variable domain and the light chain variable domain are fused together to from an scFv.

(76) The NKG2D binding domains can vary in their binding affinity to NKG2D, nevertheless, they can activate NKG2D expressing cells, such as NK cells and cytotoxic T cells.

(77) Unless indicated otherwise, the CDR sequences provided in Table 1 are determined under Kabat.

(78) TABLE-US-00005 TABLE 1 Heavy chain variable region Light chain variable region Clones amino acid sequence amino acid sequence ADI-QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 27705 VYGGSFSGYYWSWIRQPPGKGLE CRASQSISSWLAWYQQKPGK WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS YCQQYNSYPITFGGGTKVEIK (SEQ ID NO: 1) (SEQ ID NO: 2) CDR1 (SEQ ID NO: 3) - GSFSGYYWS CDR2 (SEQ ID NO: 4) - EIDHSGSTNYNPSLKS CDR3 (SEQ ID NO: 5) - ARARGPWSFDP ADI-QVQLQQWGAGLLKPSETLSLTCA EIVLTQSPGTLSLSPGERATLS 27724 VYGGSFSGYYWSWIRQPPGKGLE CRASQSVSSSYLAWYQQKPG WIGEIDHSGSTNYNPSLKSRVTISV QAPRLLIYGASSRATGIPDRFS

DTSKNQFSLKLSSVTAADTAVYY GSGSGTDFTLTISRLEPEDFAV
CARARGPWSFDPWGQGTLVTVSS YYCQQYGSSPITFGGGTKVEIK (SEQ ID NO: 6)
(SEQ ID NO: 7) ADI- QVQLQQWGAGLLKPSETLSLTCA
DIQMTQSPSTLSASVGDRVTIT 27740 VYGGSFSGYYWSWIRQPPGKGLE
CRASQSIGSWLAWYQQKPGK (A40) WIGEIDHSGSTNYNPSLKSRVTISV
APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY
SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS
YCQQYHSFYTFGGGTKVEIK (SEQ ID NO: 8) (SEQ ID NO: 9) ADI-
QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 27741
VYGGSFSGYYWSWIRQPPGKGLE CRASQSIGSWLAWYQQKPGK
WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG
DTSKNQFSLKLSSVTAADTAVYY SGSGTEFTLTISSLQPDDFATY
CARARGPWSFDPWGQGTLVTVSS YCQQSNSYYTFGGGTKVEIK (SEQ ID NO: 10)
(SEQ ID NO: 11) ADI- QVQLQQWGAGLLKPSETLSLTCA
DIQMTQSPSTLSASVGDRVTIT 27743 VYGGSFSGYYWSWIRQPPGKGLE
CRASQSISSWLAWYQQKPGK WIGEIDHSGSTNYNPSLKSRVTISV
APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY
SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS
YCQQYNSYPTFGGGTKVEIK (SEQ ID NO: 12) (SEQ ID NO: 13) ADI-
QVQLQQWGAGLLKPSETLSLTCA ELQMTQSPSSLSASVGDRVTIT 28153
VYGGSFSGYYWSWIRQPPGKGLE CRTSQSISSYLNWYQQKPGQP
WIGEIDHSGSTNYNPSLKSRVTISV PKLLIYWASTRESGVPDRFSGS
DTSKNQFSLKLSSVTAADTAVYY GSGTDFTLTISSLQPEDSATYY
CARARGPWGFDPWGQGTLVTVS CQQSYDIPYTFGQGTKLEIK S (SEQ ID NO: 15)
(SEQ ID NO: 14) ADI- QVQLQQWGAGLLKPSETLSLTCA
DIQMTQSPSTLSASVGDRVTIT 28226 VYGGSFSGYYWSWIRQPPGKGLE
CRASQSISSWLAWYQQKPGK (C26) WIGEIDHSGSTNYNPSLKSRVTISV
APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY
SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS
YCQQYGSFPITFGGGTKVEIK (SEQ ID NO: 16) (SEQ ID NO: 17) ADI-
QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 28154
VYGGSFSGYYWSWIRQPPGKGLE CRASQSISSWLAWYQQKPGK
WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG
DTSKNQFSLKLSSVTAADTAVYY SGSGTDFTLTISSLQPDDFATY
CARARGPWSFDPWGQGTLVTVSS YCQQSKEVPWTFGQGTKVEIK (SEQ ID NO: 18)
(SEQ ID NO: 19) ADI- QVQLQQWGAGLLKPSETLSLTCA
DIQMTQSPSTLSASVGDRVTIT 29399 VYGGSFSGYYWSWIRQPPGKGLE
CRASQSISSWLAWYQQKPGK WIGEIDHSGSTNYNPSLKSRVTISV
APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY
SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS
YCQQYNSFPTFGGGTKVEIK (SEQ ID NO: 20) (SEQ ID NO: 21) ADI-
QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 29401
VYGGSFSGYYWSWIRQPPGKGLE CRASQSIGSWLAWYQQKPGK
WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG
DTSKNQFSLKLSSVTAADTAVYY SGSGTEFTLTISSLQPDDFATY
CARARGPWSFDPWGQGTLVTVSS YCQQYDIYPTFGGGTKVEIK (SEQ ID NO: 22)
(SEQ ID NO: 23) ADI- QVQLQQWGAGLLKPSETLSLTCA
DIQMTQSPSTLSASVGDRVTIT 29403 VYGGSFSGYYWSWIRQPPGKGLE
CRASQSISSWLAWYQQKPGK WIGEIDHSGSTNYNPSLKSRVTISV
APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY

SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS
YCQQYDSYPTFGGGTKVEIK (SEQ ID NO: 24) (SEQ ID NO: 25) ADI-
QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 29405
VYGGSFSGYYWSWIRQPPGKGLE CRASQSISSWLAWYQQKPGK
WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG
DTSKNQFSLKLSSVTAADTAVYY SGSGTEFTLTISSLQPDDFATY
CARARGPWSFDPWGQGTLVTVSS YCQQYGSFPTFGGGTKVEIK (SEQ ID NO: 26)
(SEQ ID NO: 27) ADI- QVQLQQWGAGLLKPSETLSLTCA
DIQMTQSPSTLSASVGDRVTIT 29407 VYGGSFSGYYWSWIRQPPGKGLE
CRASQSISSWLAWYQQKPGK WIGEIDHSGSTNYNPSLKSRVTISV
APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY
SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS
YCQQYQSFPTFGGGTKVEIK (SEQ ID NO: 28) (SEQ ID NO: 29) ADI-
QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 29419
VYGGSFSGYYWSWIRQPPGKGLE CRASQSISSWLAWYQQKPGK
WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG
DTSKNQFSLKLSSVTAADTAVYY SGSGTEFTLTISSLQPDDFATY
CARARGPWSFDPWGQGTLVTVSS YCQQYSSFSTFGGGTKVEIK (SEQ ID NO: 30)
(SEQ ID NO: 31) ADI- QVQLQQWGAGLLKPSETLSLTCA
DIQMTQSPSTLSASVGDRVTIT 29421 VYGGSFSGYYWSWIRQPPGKGLE
CRASQSISSWLAWYQQKPGK WIGEIDHSGSTNYNPSLKSRVTISV
APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY
SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS
YCQQYESYSTFGGGTKVEIK (SEQ ID NO: 32) (SEQ ID NO: 33) ADI-
QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 29424
VYGGSFSGYYWSWIRQPPGKGLE CRASQSISSWLAWYQQKPGK
WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG
DTSKNQFSLKLSSVTAADTAVYY SGSGTEFTLTISSLQPDDFATY
CARARGPWSFDPWGQGTLVTVSS YCQQYDSFITFGGGTKVEIK (SEQ ID NO: 34)
(SEQ ID NO: 35) ADI- QVQLQQWGAGLLKPSETLSLTCA
DIQMTQSPSTLSASVGDRVTIT 29425 VYGGSFSGYYWSWIRQPPGKGLE
CRASQSISSWLAWYQQKPGK WIGEIDHSGSTNYNPSLKSRVTISV
APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY
SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS
YCQQYQSYPTFGGGTKVEIK (SEQ ID NO: 36) (SEQ ID NO: 37) ADI-
QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 29426
VYGGSFSGYYWSWIRQPPGKGLE CRASQSIGSWLAWYQQKPGK
WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG
DTSKNQFSLKLSSVTAADTAVYY SGSGTEFTLTISSLQPDDFATY
CARARGPWSFDPWGQGTLVTVSS YCQQYHSFPTFGGGTKVEIK (SEQ ID NO: 38)
(SEQ ID NO: 39) ADI- QVQLQQWGAGLLKPSETLSLTCA
DIQMTQSPSTLSASVGDRVTIT 29429 VYGGSFSGYYWSWIRQPPGKGLE
CRASQSIGSWLAWYQQKPGK WIGEIDHSGSTNYNPSLKSRVTISV
APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY
SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS
YCQQYELYSYTFGGGTKVEIK (SEQ ID NO: 40) (SEQ ID NO: 41) ADI-
QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 29447
VYGGSFSGYYWSWIRQPPGKGLE CRASQSISSWLAWYQQKPGK (F47)
WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG
DTSKNQFSLKLSSVTAADTAVYY SGSGTEFTLTISSLQPDDFATY

CARARGPWSFDPWGQGTLVTVSS YCQQYDTFITFGGGTKVEIK (SEQ ID NO: 42) (SEQ ID NO: 43) ADI- QVQLVQSGAEVKKPGSSVKVSCK DIVMTQSPDSLAVSLGERATIN 27727 ASGGTFSSYAISWVRQAPGQGLE CKSSQSVLYSSNNKNYLAWY WMGGIIPIFGTANYAQKFQGRVTI QQKPGQPPKLLIYWASTRESG TADESTSTAYMELSSLRSEDTAVY VPDRFSGSGSGTDFTLTISSLQ YCARGDSSIRHAYYYYGMDVWG AEDVAVYYCQQYYSTPITFGG QGTTVTVSS GTKVEIK (SEQ ID NO: 44) (SEQ ID NO: 48) CDR1 (SEQ ID NO: 45) - CDR1 (SEQ ID NO: 49) - GTFSSYAIS (non-Kabat) or SYAIS KSSQSVLYSSNNKNYLA (SEQ ID NO: 181) CDR2 (SEQ ID NO: 50) - CDR2 (SEQ ID NO: 46) - WASTRES GIIPIFGTANYAQKFQG CDR3 (SEQ ID NO: 51) - CDR3 (SEQ ID NO: 47) - QQYYSTPIT ARGDSSIRHAYYYYGMDV (non- Kabat) or GDSSIRHAYYYYGMDV (SEQ ID NO: 182) ADI- QLQLQESGPGLVKPSETLSLTCTV EIVLTQSPATLSLSPGERATLS 29443 SGGSISSSSYYWGWIRQPPGKGLE CRASQSVSRYLAWYQQKPGQ (F43) WIGSIYYSGSTYYNPSLKSRVTISV APRLLIYDASNRATGIPARFSG DTSKNQFSLKLSSVTAADTAVYY SGSGTDFTLTISSLEPEDFAVY CARGSDRFHPYFDYWGQGTLVTV YCQQFDTWPPTFGGGTKVEIK SS (SEQ ID NO: 56) (SEQ ID NO: 52) CDR1 (SEQ ID NO: 57) - CDR1 (SEQ ID NO: 53) - RASQSVSRYLA GSISSSSYYWG (non-Kabat) or CDR2 (SEQ ID NO: 58) - SSSYYWG (SEQ ID NO: 183) DASNRAT CDR2 (SEQ ID NO: 54) - CDR3 (SEQ ID NO: 59) - SIYYSGSTYYNPSLKS QQFDTWPPT CDR3 (SEQ ID NO: 55) - ARGSDRFHPYFDY (non-Kabat) or GSDRFHPYFDY (SEQ ID NO: 184) ADI- QVQLQQWGAGLLKPSETLSLTCA DIQMTQSPSTLSASVGDRVTIT 29404 VYGGSFSGYYWSWIRQPPGKGLE CRASQSISSWLAWYQQKPGK (F04) WIGEIDHSGSTNYNPSLKSRVTISV APKLLIYKASSLESGVPSRFSG DTSKNQFSLKLSSVTAADTAVYY SGSGTEFTLTISSLQPDDFATY CARARGPWSFDPWGQGTLVTVSS YCEQYDSYPTFGGGTKVEIK (SEQ ID NO: 60) (SEQ ID NO: 61) ADI- QVQLVQSGAEVKKPGSSVKVSCK DIVMTQSPDSLAVSLGERATIN 28200 ASGGTFSSYAISWVRQAPGQGLE CESSQSLLNSGNQKNYLTWY WMGGIIPIFGTANYAQKFQGRVTI QQKPGQPPKPLIYWASTRESG TADESTSTAYMELSSLRSEDTAVY VPDRFSGSGSGTDFTLTISSLQ YCARRGRKASGSFYYYYGMDVW AEDVAVYYCQNDYSYPYTFG GQGTTVTVSS QGTKLEIK (SEQ ID NO: 62) (SEQ ID NO: 66) CDR1 (SEQ ID NO: 63) - CDR1 (SEQ ID NO: 67) - GTFSSYAIS (non-Kabat) or SYAIS ESSQSLLNSGNQKNYLT (SEQ ID NO: 181) CDR2 (SEQ ID NO: 68) - CDR2 (SEQ ID NO: 64) - WASTRES GIIPIFGTANYAQKFQG CDR3 (SEQ ID NO: 69) - CDR3 (SEQ ID NO: 65) - QNDYSYPYT ARRGRKASGSFYYYYGMDV ADI- QVQLVQSGAEVKKPGASVKVSCK EIVMTQSPATLSVSPGERATLS 29379 ASGYTFTSYYMHWVRQAPGQGL CRASQSVSSNLAWYQQKPGQ (E79) EWMGIINPSGGSTSYAQKFQGRV APRLLIYGASTRATGIPARFSG TMTRDTSTSTVYMELSSLRSEDTA SGSGTEFTLTISSLQSEDFAVY VYYCARGAPNYGDTTHDYYYMD YCQQYDDWPFTFGGGTKVEI VWGKGTTVTVSS K (SEQ ID NO: 70) (SEQ ID NO: 74) CDR1 (SEQ ID NO: 71) - CDR1 (SEQ ID NO: 75) - YTFTSYYMH (non-Kabat) or RASQSVSSNLA SYYMH (SEQ ID NO: 185) CDR2 (SEQ ID NO: 76) - CDR2 (SEQ ID NO: 72) - GASTRAT IINPSGGST SYAQKFQG CDR3 (SEQ ID NO: 77) - CDR3 (SEQ ID NO: 73) - QQYDDWPFT ARGAPNYGDTTHDYYYMDV (non-Kabat) or GAPNYGDTTHDYYYMDV (SEQ ID NO: 159) ADI- QVQLVQSGAEVKKPGASVKVSCK EIVLTQSPGTLSLSPGERATLS 29463

ASGYTFTGYYMHWVRQAPGQGL CRASQSVSSNLAWYQQKPGQ (F63)
EWMGWINPNSGGTNYAQKFQGR APRLLIYGASTRATGIPARFSG
VTMTRDTSISTAYMELSRLRSDDT SGSGTEFTLTISSLQSEDFAVY
AVYYCARDTGEYYDTDDHGMDV YCQQDDYWPPTFGGGTKVEI WGQGTTVTVSS K
(SEQ ID NO: 78) (SEQ ID NO: 82) CDR1 (SEQ ID NO: 79) - CDR1
(SEQ ID NO: 83) - YTFTGYYMH (non-Kabat) or RASQSVSSNLA GYYMH
(SEQ ID NO: 186) CDR2 (SEQ ID NO: 84) - CDR2 (SEQ ID NO: 80) -
GASTRAT WINPNSGGTNYAQKFQG CDR3 (SEQ ID NO: 85) - CDR3 (SEQ ID
NO: 81) - QQDDYWPPT ARDTGEYYDTDDHGMDV (non- Kabat) or
DTGEYYDTDDHGMDV (SEQ ID NO: 187) ADI- EVQLLESGGGLVQPGGSLRLSCA
DIQMTQSPSSVSASVGDRVTIT 27744 ASGFTFSSYAMSWVRQAPGKGLE
CRASQGIDSWLAWYQQKPGK (A44) WVSAISGSGGSTYYADSVKGRFTI
APKLLIYAASSLQSGVPSRFSG SRDNSKNTLYLQMNSLRAEDTAV
SGSGTDFTLTISSLQPEDFATY YYCAKDGGYYDSGAGDYWGQG
YCQQGVSYPRTFGGGTKVEIK TLVTVSS (SEQ ID NO: 90) (SEQ ID NO: 86)
CDR1 (SEQ ID NO: 91) - CDR1 (SEQ ID NO: 87) - RASQGIDSWLA
FTFSSYAMS (non-Kabat) or SYAMS CDR2 (SEQ ID NO: 92) - (SEQ ID
NO: 188) AASSLQS CDR2 (SEQ ID NO: 88) - CDR3 (SEQ ID NO: 93) -
AISGSGGSTYYADSVKG QQGVSYPRT CDR3 (SEQ ID NO: 89) -
AKDGGYYDSGAGDY (non-Kabat) or DGGYYDSGAGDY (SEQ ID NO: 189) ADI-
EVQLVESGGGLVKPGGSLRLSCA DIQMTQSPSSVSASVGDRVTIT 27749
ASGFTFSSYSMNWVRQAPGKGLE CRASQGISSWLAWYQQKPGK (A49)
WVSSISSSSSYIYYADSVKGRFTIS APKLLIYAASSLQSGVPSRFSG
RDNAKNSLYLQMNSLRAEDTAV SGSGTDFTLTISSLQPEDFATY
YYCARGAPMGAAAGWFDPWGQ YCQQGVSFPRTFGGGTKVEIK GTLVTVSS (SEQ ID
NO: 98) (SEQ ID NO: 94) CDR1 (SEQ ID NO: 99) - CDR1 (SEQ ID NO:
95) - RASQGISSWLA FTFSSYSMN (non-Kabat) or SYSMN CDR2 (SEQ ID NO:
100) - (SEQ ID NO: 190) AASSLQS CDR2 (SEQ ID NO: 96) - CDR3 (SEQ
ID NO: 101) - SISSSSSYIYYADSVKG QQGVSFPRT CDR3 (SEQ ID NO: 97) -
ARGAPMGAAAGWFDP (non- Kabat) or GAPMGAAAGWFDP (SEQ ID NO: 191)
ADI- QVQLVQSGAEVKKPGASVKVSCK EIVLTQSPATLSLSPGERATLS 29378
ASGYTFTSYYMHWVRQAPGQGL CRASQSVSSYLAWYQQKPGQ (E78)
EWMGIINPSGGSTSYAQKFQGRV APRLLIYDASNRATGIPARFSG
TMTRDTSTSTVYMELSSLRSEDTA SGSGTDFTLTISSLEPEDFAVY
VYYCAREGAGFAYGMDYYYMD YCQQSDNWPFTFGGGTKVEIK VWGKGTTVTVSS
(SEQ ID NO: 106) (SEQ ID NO: 102) CDR1 (SEQ ID NO: 107) - CDR1
(SEQ ID NO: 103) - RASQSVSSYLA YTFTSYYMH (non-Kabat) or CDR2 (SEQ
ID NO: 108) - SYYMH (SEQ ID NO: 185) DASNRAT CDR2 (SEQ ID NO:
104) - CDR3 (SEQ ID NO: 109) - IINPSGGSTSYAQKFQG QQSDNWPFT CDR3
(SEQ ID NO: 105) - AREGAGFAYGMDYYYMDV or EGAGFAYGMDYYYMDV
(SEQ ID NO: 192) A49MI EVQLVESGGGLVKPGGSLRLSCA
DIQMTQSPSSVSASVGDRVTIT ASGFTFSSYSMNWVRQAPGKGLE
CRASQGISSWLAWYQQKPGK WVSSISSSSSYIYYADSVKGRFTIS
APKLLIYAASSLQSGVPSRFSG RDNAKNSLYLQMNSLRAEDTAV
SGSGTDFTLTISSLQPEDFATY YYCARGAP**I**GAAAGWFDPWGQG
YCQQGVSFPRTFGGGTKVEIK TLVTVSS (SEQ ID NO: 169) (SEQ ID NO: 98)
CDR1 (SEQ ID NO: 95) - CDR1 (SEQ ID NO: 99) - FTFSSYSMN (non-
Kabat) or SYSMN RASQGISSWLA (SEQ ID NO: 190) CDR2 (SEQ ID NO:
100) - CDR2 (SEQ ID NO: 96) - AASSLQS SISSSSSYIYYADSVKG CDR3 (SEQ
ID NO: 101) - CDR3: (SEQ ID NO: 170) - QQGVSFPRT

ARGAP**I**GAAAGWFDP (non-Kabat) or GAP**I**GAAAGWFDP (SEQ ID NO: 193)
A49MQ EVQLVESGGGLVKPGGSLRLSCA DIQMTQSPSSVSASVGDRVTIT
ASGFTFSSYSMNWVRQAPGKGLE CRASQGISSWLAWYQQKPGK
WVSSISSSSSYIYYADSVKGRFTIS APKLLIYAASSLQSGVPSRFSG
RDNAKNSLYLQMNSLRAEDTAV SGSGTDFTLTISSLQPEDFATY
YYCARGAP**Q**GAAAGWFDPWGQ YCQQGVSFPRTFGGGTKVEIK GTLVTVSS (SEQ ID
NO: 98) (SEQ ID NO: 171) CDR1 (SEQ ID NO: 99) - CDR1 (SEQ ID
NO: 95) - RASQGISSWLA FTFSSYSMN (non-Kabat) or SYSMN CDR2 (SEQ ID
NO: 100) - (SEQ ID NO: 190) AASSLQS CDR2 (SEQ ID NO: 96) - CDR3
(SEQ ID NO: 101) - SISSSSSYIYYADSVKG QQGVSFPRT CDR3 (SEQ ID NO:
172) - ARGAP**Q**GAAAGWFDP (non-Kabat) or GAP**Q**GAAAGWFDP (SEQ ID NO:
194) A49ML EVQLVESGGGLVKPGGSLRLSCA DIQMTQSPSSVSASVGDRVTIT
ASGFTFSSYSMNWVRQAPGKGLE CRASQGISSWLAWYQQKPGK
WVSSISSSSSYIYYADSVKGRFTIS APKLLIYAASSLQSGVPSRFSG
RDNAKNSLYLQMNSLRAEDTAV SGSGTDFTLTISSLQPEDFATY
YYCARGAP**L**GAAAGWFDPWGQG YCQQGVSFPRTFGGGTKVEIK TLVTVSS (SEQ ID
NO: 98) (SEQ ID NO: 173) CDR1 (SEQ ID NO: 99) - CDR1 (SEQ ID
NO: 95) - RASQGISSWLA FTFSSYSMN (non-Kabat) or SYSMN CDR2 (SEQ ID
NO: 100) - (SEQ ID NO: 190) AASSLQS CDR2 (SEQ ID NO: 96) - CDR3
(SEQ ID NO: 101) - SISSSSSYIYYADSVKG QQGVSFPRT CDR3 (SEQ ID NO:
174) - ARGAP**L**GAAAGWFDP (non-Kabat) or GAP**L**GAAAGWFDP (SEQ ID NO:
195) A49MF EVQLVESGGGLVKPGGSLRLSCA DIQMTQSPSSVSASVGDRVTIT
ASGFTFSSYSMNWVRQAPGKGLE CRASQGISSWLAWYQQKPGK
WVSSISSSSSYIYYADSVKGRFTIS APKLLIYAASSLQSGVPSRFSG
RDNAKNSLYLQMNSLRAEDTAV SGSGTDFTLTISSLQPEDFATY
YYCARGAP**F**GAAAGWFDPWGQG YCQQGVSFPRTFGGGTKVEIK TLVTVSS (SEQ ID
NO: 98) (SEQ ID NO: 175) CDR1 (SEQ ID NO: 99) - CDR1 (SEQ ID
NO: 95) - RASQGISSWLA FTFSSYSMN (non-Kabat) or SYSMN CDR2 (SEQ ID
NO: 100) - (SEQ ID NO: 190) AASSLQS CDR2 (SEQ ID NO: 96) - CDR3
(SEQ ID NO: 101) - SISSSSSYIYYADSVKG QQGVSFPRT CDR3 (SEQ ID NO:
176) - ARGAP**F**GAAAGWFDP (non-Kabat) or GAP**F**GAAAGWFDP (SEQ ID NO:
196) A49MV EVQLVESGGGLVKPGGSLRLSCA DIQMTQSPSSVSASVGDRVTIT
ASGFTFSSYSMNWVRQAPGKGLE CRASQGISSWLAWYQQKPGK
WVSSISSSSSYIYYADSVKGRFTIS APKLLIYAASSLQSGVPSRFSG
RDNAKNSLYLQMNSLRAEDTAV SGSGTDFTLTISSLQPEDFATY
YYCARGAP**V**GAAAGWFDPWGQ YCQQGVSFPRTFGGGTKVEIK GTLVTVSS (SEQ ID
NO: 98) (SEQ ID NO: 177) CDR1 (SEQ ID NO: 99) - CDR1 (SEQ ID
NO: 95) - RASQGISSWLA FTFSSYSMN (non-Kabat) or SYSMN CDR2 (SEQ ID
NO: 100) - (SEQ ID NO: 190) AASSLQS CDR2 (SEQ ID NO: 96) - CDR3
(SEQ ID NO: 101) - SISSSSSYIYYADSVKG QQGVSFPRT CDR3 (SEQ ID NO:
178) - ARGAP**V**GAAAGWFDP (non-Kabat) or GAP**V**GAAAGWFDP (SEQ ID NO:
197) A49- EVQLVESGGGLVKPGGSLRLSCA DIQMTQSPSSVSASVGDRVTIT consensus
ASGFTFSSYSMNWVRQAPGKGLE CRASQGISSWLAWYQQKPGK
WVSSISSSSSYIYYADSVKGRFTIS APKLLIYAASSLQSGVPSRFSG
RDNAKNSLYLQMNSLRAEDTAV SGSGTDFTLTISSLQPEDFATY
YYCARGAP**X**GAAAGWFDPWGQ YCQQGVSFPRTFGGGTKVEIK GTLVTVSS, wherein
X is M, L, I, V, (SEQ ID NO: 98) Q, or F CDR1 (SEQ ID NO: 99) -
(SEQ ID NO: 179) RASQGISSWLA CDR1 (SEQ ID NO: 95) - CDR2 (SEQ
ID NO: 100) - FTFSSYSMN (non-Kabat) or SYSMN AASSLQS (SEQ ID NO:
190) CDR3 (SEQ ID NO: 101) - CDR2 (SEQ ID NO: 96) - QQGVSFPRT

SISSSSSYIYYADSVKG CDR3 (SEQ ID NO: 180) - ARGAP**X**GAAAGWFDP, wherein X is M, L, I, V, Q, or F (non-Kabat) or GAP**X**GAAAGWFDP, wherein X is M, L, I, V, Q, or F (SEQ ID NO: 160)

(79) Alternatively, a heavy chain variable domain represented by SEQ ID NO: 110 can be paired with a light chain variable domain represented by SEQ ID NO:111 to form an antigen-binding site that can bind to NKG2D, as illustrated in U.S. Pat. No. 9,273,136.

(80) TABLE-US-00006 SEQ ID NO: 110
QVQLVESGGGLVKPGGSLRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAFI
RYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKDRGL
GDGTYFDYWGQGTTVTVSS SEQ ID NO: 111
QSALTQPASVSGSPGQSITISCSGSSSNIGNNAVNWYQQLPGKAPKLLIYY
DDLLPSGVSDRFSGSKSGTSAFLAISGLQSEDEADYYCAAWDDSLNGPVFG GGTKLTVL

(81) Alternatively, a heavy chain variable domain represented by SEQ ID NO:112 can be paired with a light chain variable domain represented by SEQ ID NO:113 to form an antigen-binding site that can bind to NKG2D, as illustrated in U.S. Pat. No. 7,879,985.

(82) TABLE-US-00007 SEQ ID NO: 112
QVHLQESGPGLVKPSETLSLTCTVSDDSISSYYWSWIRQPPGKGLEWIGHI
SYSGSANYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCANWDDAF
NIWGQGTMVTVSS SEQ ID NO: 113
EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIYG
ASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSPWTFGQG TKVEIK

Tumor-Associated Antigen-Binding Site

(83) The present disclosure provides a BCMA-binding site, in which the heavy chain variable domain and the light chain variable domain. In some embodiments, the BCMA-binding site is linked to the antibody Fc domain or the portion thereof sufficient to bind CD16, or the antigen-binding site that binds CD16 of the proteins disclosed herein via a hinge. The proteins disclosed herein can provide monovalent or bivalent engagement of BCMA, and have one or two BCMA-binding sites. In some embodiments, proteins disclosed herein have two BCMA-binding sites, each is linked to the antibody Fc domain or the portion thereof sufficient to bind CD16, or the antigen-binding site that binds CD16 of the proteins disclosed herein via a hinge.

(84) Table 2 lists peptide sequences of heavy chain variable domains and light chain variable domains that, in combination, can bind to BCMA.

(85) TABLE-US-00008 TABLE 2 Heavy chain variable Light chain variable Clones domain peptide sequence domain peptide sequence 1 QVQLVQSGAEVKKPGASVKV DIVMTQTPLSLSVTPGEPASIS (U.S. 14/776,649) SCKASGYSFPDYYINWVRQAP CKSSQSLVHSNGNTYLHWYL GQGLEWMGWIYFASGNSEYN QKPGQSPQLLIYKVSNRFSGVP QKFTGRVTMTRDTSSSTAYME DRFSGSGSGADFTLKISRVEAE LSSLRSEDTAVYFCASLYDYD DVGVYYCAETSHVPWTFGQG WYFDVWGQGTMVTVSS TKLEIK (SEQ ID NO: 118) (SEQ ID NO: 114) or CDR1 (SEQ ID NO: 115) - DYYIN DIVMTQTPLSLSVTPGQPASIS CDR2 (SEQ ID NO: 116) - CKSSQSLVHSNGNTYLHWYL WIYFASGNSEYNQKFTG QKPGQSPQLLIYKVSNRFSGVP CDR3 (SEQ ID NO: 117) - DRFSGSGSGTDFTLKISRVEAE LYDYDWYFDV DVGIYYCSQSSIYPWTFGQGT KLEIK (SEQ ID NO: 119) CDR1 (SEQ ID NO: 120) - KSSQSLVHSNGNTYLH CDR2 (SEQ ID NO: 121) - KVSNRFS CDR3 - AETSHVPWT (SEQ ID NO: 122) or SQSSIYPWT (SEQ ID NO: 123) 2 QIQLVQSGPELKKPGETVKISC DIVLTQSPPSLAMSLGKRATIS (PCT/US15/64269) KASGYTFTDYSINWVKRAPGK CRASESVTILGSHLIHWYQQK GLKWMGWINTETREPAYAYD PGQPPTLLIQLASNVQTGVPAR FRGRFAFSLETSASTAYLQINN FSGSGSRTDFTLTIDPVEEDDV

LKYEDTATYFCALDYSYAMD AVYYCLQSRTIPRTFGGGTKL YWGQGTSVTVSS EIK
(SEQ ID NO: 124) (SEQ ID NO: 128) CDR1 (SEQ ID NO: 125) - CDR1
(SEQ ID NO: 129) - DYSIN RASESVTILGSHLIH CDR2 (SEQ ID NO: 126) -
CDR2 (SEQ ID NO: 130) - WINTETREPAYAYDFR LASNVQT CDR3 (SEQ ID
NO: 127) - CDR3 (SEQ ID NO: 131) - DYSYAMDY LQSRTIPRT 3
QVQLVQSGAEVKKPGSSVKV DIQMTQSPSSLSASVGDRVTIT (U.S. 14/122,391)
SCKASGGTFSNYWMHWVRQ CSASQDISNYLNWYQQKPGK
APGQGLEWMGATYRGHSDTY APKLLIYYTSNLHSGVPSRFSG
YNQKFKGRVTITADKSTSTAY SGSGTDFTLTISSLQPEDFATY
MELSSLRSEDTAVYYCARGAI YCQQYRKLPWTFGQGTKLEIK
YNGYDVLDNWGQGTLVTVSS R (SEQ ID NO: 132) (SEQ ID NO: 136) CDR1
(SEQ ID NO: 133) - CDR1 (SEQ ID NO: 137) - NYWMH SASQDISNYLN
CDR2 (SEQ ID NO: 134) - CDR2 (SEQ ID NO: 138) -
ATYRGHSDTYYNQKFKG YTSNLHS CDR3 (SEQ ID NO: 135) - CDR3 (SEQ
ID NO: 139) - GAIYNGYDVLDN QQYRKLPWT 4 QLQLQESGPGLVKPSETLSLTC
SYVLTQPPSVSVAPGQTARITC (US20170051068) TVSGGSISSSSYFWGWIRQPPG
GGNNIGSKSVHWYQQPPGQA KGLEWIGSIYYSGITYYNPSLK PVVVVYDDSDRPSGIPER
SRVTISVDTSKNQFSLKLSSVT FSGSNSGNTA AADTAVYYCARHDGATAGLF
TLTISRVEAGDEAVYYCQVW DYWGQGTLVTVSS (SEQ ID DSSSDHVVFGGGTKLTVL
NO: 140) (SEQ ID NO: 144) CDR1: SSSYFWG (SEQ ID CDR1:
GGNNIGSKSVH (SEQ NO: 141) ID NO: 145) CDR2: SIYYSGITYYNPSLKS
CDR2: DDSDRPS (SEQ ID (SEQ ID NO: 142) NO: 146) CDR3:
HDGATAGLFDY (SEQ CDR3: QVWDSSSDHVV (SEQ ID NO: 143) ID NO: 147)
5 EVQLLESGGGLVQPGGSLRLS EIVLTQSPGTLSLSPGERATLS (Mab42
CAASGFTFSDNAMGWVRQAP CRASQSVSDEYLSWYQQKPG (WO2017021450))
GKGLEWVSAISGPGSSTYYAD QAPRLLIHSASTRATGIPDRFS
SVKGRFTISRDNSKNTLYLQM GSGSGTDFTLAISRLEPEDFAV
NSLRAEDTAVYYCAKVLGWF YYCQQYGYPPDFTFGQGTKV DYWGQGTLVTVSS
(SEQ ID EIK (SEQ ID NO: 152) NO: 148) CDR1: RASQSVSDEYLSW CDR1:
DNAMG (SEQ ID (SEQ ID NO: 153) NO: 149) CDR2: HSASTRAT (SEQ ID
CDR2: AISGPGSSTYYADSVKG NO: 154) (SEQ ID NO: 150) CDR3:
QQYGYPPDFT (SEQ ID CDR3: VLGWFDY (SEQ ID NO: 155) NO: 151)

(86) Alternatively, a BCMA-binding domain can include a heavy chain variable domain and light chain variable domain as listed below in 83A10 and MAB42.

(87) 83A10 Heavy Chain Variable Domain (SEQ ID NO: 157):

(88) TABLE-US-00009

EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVS

CDR1

AISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK CDR2VSS

VLGWFDYWGQGTLVT CDR3

83A10 Light Chain Variable Domain (SEQ ID NO:158):

(89) TABLE-US-00010

EIVLTQSPGTLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLIY

CDR1

GASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGYPPDFTF

CDR2

CDR3 GQGTKVEIK

(90) Alternatively, novel antigen-binding sites that can bind to BCMA can be identified by screening for binding to the amino acid sequence defined by SEQ ID NO:156.

(91) TABLE-US-00011 SEQ ID NO: 156
MLQMAGQCSQNEYFDSLLHACIPCQLRCSSNTPPLTCQRYCNASVTNSVKG
TNAILWTCLGLSLIISLAVFVLMFLLRKINSEPLKDEFKNTGSGLLGMANI
DLEKSRTGDEIILPRGLEYTVEECTCEDCIKSKPKVDSDHCFPLPAMEEGA
TILVTTKTNDYCKSLPAALSATEIEKSISAR
(92) Within the Fc domain, CD16 binding is mediated by the hinge region and the CH2 domain. For example, within human IgG1, the interaction with CD16 is primarily focused on amino acid residues Asp 265-Glu 269, Asn 297-Thr 299, Ala 327-Ile 332, Leu 234-Ser 239, and carbohydrate residue N-acetyl-D-glucosamine in the CH2 domain (see, Sondermann et al, Nature, 406 (6793):267-273). Based on the known domains, mutations can be selected to enhance or reduce the binding affinity to CD16, such as by using phage-displayed libraries or yeast surface-displayed cDNA libraries, or can be designed based on the known three-dimensional structure of the interaction.
(93) The assembly of heterodimeric antibody heavy chains can be accomplished by expressing two different antibody heavy chain sequences in the same cell, which may lead to the assembly of homodimers of each antibody heavy chain as well as assembly of heterodimers. Promoting the preferential assembly of heterodimers can be accomplished by incorporating different mutations in the CH3 domain of each antibody heavy chain constant region as shown in U.S. Ser. No. 13/494,870, U.S. Ser. No. 16/028,850, U.S. Ser. No. 11/533,709, U.S. Ser. No. 12/875,015, U.S. Ser. No. 13/289,934, U.S. Ser. No. 14/773,418, U.S. Ser. No. 12/811,207, U.S. Ser. No. 13/866,756, U.S. Ser. No. 14/647,480, and U.S. Ser. No. 14/830,336. For example, mutations can be made in the CH3 domain based on human IgG1 and incorporating distinct pairs of amino acid substitutions within a first polypeptide and a second polypeptide that allow these two chains to selectively heterodimerize with each other. The positions of amino acid substitutions illustrated below are all numbered according to the EU index as in Kabat.
(94) In one scenario, an amino acid substitution in the first polypeptide replaces the original amino acid with a larger amino acid, selected from arginine (R), phenylalanine (F), tyrosine (Y) or tryptophan (W), and at least one amino acid substitution in the second polypeptide replaces the original amino acid(s) with a smaller amino acid(s), chosen from alanine (A), serine (S), threonine (T), or valine (V), such that the larger amino acid substitution (a protuberance) fits into the surface of the smaller amino acid substitutions (a cavity). For example, one polypeptide can incorporate a T366W substitution, and the other can incorporate three substitutions including T366S, L368A, and Y407V.
(95) An antibody heavy chain variable domain of the invention can optionally be coupled to an amino acid sequence at least 90% identical to an antibody constant region, such as an IgG constant region including hinge, CH2 and CH3 domains with or without CH1 domain. In some embodiments, the amino acid sequence of the constant region is at least 90% identical to a human antibody constant region, such as an human IgG1 constant region, an IgG2 constant region, IgG3 constant region, or IgG4 constant region. In some other embodiments, the amino acid sequence of the constant region is at least 90% identical to an antibody constant region from another mammal, such as rabbit, dog, cat, mouse, or horse. One or more mutations can be incorporated into the constant region as compared to human IgG1 constant region, for example at Q347, Y349, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411 and/or K439. Exemplary substitutions include, for example, Q347E, Q347R, Y349S, Y349K, Y349T, Y349D, Y349E, Y349C, T350V, L351K, L351D, L351Y, S354C, E356K, E357Q, E357L, E357W, K360E, K360W, Q362E, S364K, S364E, S364H, S364D, T366V, T3661, T366L, T366M, T366K, T366W, T366S, L368E, L368A, L368D, K370S, N390D, N390E, K392L, K392M, K392V, K392F, K392D, K392E, T394F, T394W, D399R, D399K, D399V, S400K, S400R, D401K, F405A, F405T, Y407A, Y4071, Y407V, K409F, K409W, K409D, T41ID, T411E, K439D, and K439E.

(96) In certain embodiments, mutations that can be incorporated into the CH1 of a human IgG1 constant region may be at amino acid V125, F126, P127, T135, T139, A140, F170, P171, and/or V173. In certain embodiments, mutations that can be incorporated into the Cκ of a human IgG1 constant region may be at amino acid E123, F116, S176, V163, S174, and/or T164.
(97) Amino acid substitutions could be selected from the following sets of substitutions shown in Table 3.
(98) TABLE-US-00012 TABLE 3 First Polypeptide Second Polypeptide Set 1 S364E/F405A Y349K/T394F Set 2 S364H/D401K Y349T/T411E Set 3 S364H/T394F Y349T/F405A Set 4 S364E/T394F Y349K/F405A Set 5 S364E/T411E Y349K/D401K Set 6 S364D/T394F Y349K/F405A Set 7 S364H/F405A Y349T/T394F Set 8 S364K/E357Q L368D/K370S Set 9 L368D/K370S S364K Set 10 L368E/K370S S364K Set 11 K360E/Q362E D401K Set 12 L368D/K370S S364K/E357L Set 13 K370S S364K/E357Q Set 14 F405L K409R Set 15 K409R F405L
(99) Alternatively, amino acid substitutions could be selected from the following sets of substitutions shown in Table 4.
(100) TABLE-US-00013 TABLE 4 First Polypeptide Second Polypeptide Set 1 K409W D399V/F405T Set 2 Y349S E357W Set 3 K360E Q347R Set 4 K360E/K409W Q347R/D399V/F405T Set 5 Q347E/K360E/K409W Q347R/D399V/F405T Set 6 Y349S/K409W E357W/D399V/F405T
(101) Alternatively, amino acid substitutions could be selected from the following set of substitutions shown in Table 5.
(102) TABLE-US-00014 TABLE 5 First Polypeptide Second Polypeptide Set 1 T366K/L351K L351D/L368E Set 2 T366K/L351K L351D/Y349E Set 3 T366K/L351K L351D/Y349D Set 4 T366K/L351K L351D/Y349E/L368E Set 5 T366K/L351K L351D/Y349D/L368E Set 6 E356K/D399K K392D/K409D
(103) Alternatively, at least one amino acid substitution in each polypeptide chain could be selected from Table 6.
(104) TABLE-US-00015 TABLE 6 First Polypeptide Second Polypeptide L351Y, D399R, D399K, T366V, T366I, T366L, T366M, N390D, S400K, S400R, Y407A, N390E, K392L, K392M, K392V, K392F Y407I, Y407V K392D, K392E, K409F, K409W, T411D and T411E
(105) Alternatively, at least one amino acid substitutions could be selected from the following set of substitutions in Table 7, where the position(s) indicated in the First Polypeptide column is replaced by any known negatively-charged amino acid, and the position(s) indicated in the Second Polypeptide Column is replaced by any known positively-charged amino acid.
(106) TABLE-US-00016 TABLE 7 First Polypeptide Second Polypeptide K392, K370, K409, or K439 D399, E356, or E357
(107) Alternatively, at least one amino acid substitutions could be selected from the following set of in Table 8, where the position(s) indicated in the First Polypeptide column is replaced by any known positively-charged amino acid, and the position(s) indicated in the Second Polypeptide Column is replaced by any known negatively-charged amino acid.
(108) TABLE-US-00017 TABLE 8 First Polypeptide Second Polypeptide D399, E356, or E357 K409, K439, K370, or K392
(109) Alternatively, amino acid substitutions could be selected from the following set in Table 9.
(110) TABLE-US-00018 TABLE 9 First Polypeptide Second Polypeptide T350V, L351Y, T350V, T366L, F405A, and Y407V K392L, and T394W
(111) Alternatively, or in addition, the structural stability of a hetero-multimeric protein may be increased by introducing S354C on either of the first or second polypeptide chain, and Y349C in the opposing polypeptide chain, which forms an artificial disulfide bridge within the interface of the two polypeptides.
(112) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody

constant region differs from the amino acid sequence of an IgG1 constant region at position T366, and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of T366, L368 and Y407.

(113) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of T366, L368 and Y407, and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at position T366.

(114) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of E357, K360, Q362, S364, L368, K370, T394, D401, F405, and T411 and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of Y349, E357, S364, L368, K370, T394, D401, F405 and T411.

(115) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of Y349, E357, S364, L368, K370, T394, D401, F405 and T411 and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of E357, K360, Q362, S364, L368, K370, T394, D401, F405, and T411.

(116) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of L351, D399, S400 and Y407 and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of T366, N390, K392, K409 and T411.

(117) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of T366, N390, K392, K409 and T411 and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of L351, D399, S400 and Y407.

(118) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of Q347, Y349, K360, and K409, and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of Q347, E357, D399 and F405.

(119) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of Q347, E357, D399 and F405, and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of Y349, K360, Q347 and K409.

(120) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of K370, K392, K409 and K439, and wherein the

amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of D356, E357 and D399.
(121) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of D356, E357 and D399, and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of K370, K392, K409 and K439.
(122) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of L351, E356, T366 and D399, and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of Y349, L351, L368, K392 and K409.
(123) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of Y349, L351, L368, K392 and K409, and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region at one or more positions selected from the group consisting of L351, E356, T366 and D399.
(124) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by an S354C substitution and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by a Y349C substitution.
(125) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by a Y349C substitution and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by an S354C substitution.
(126) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by K360E and K409W substitutions and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by 0347R, D399V and F405T substitutions.
(127) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by 0347R, D399V and F405T substitutions and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by K360E and K409W substitutions.
(128) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by a T366W substitutions and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T366S, T368A, and Y407V substitutions.
(129) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T366S, T368A, and Y407V substitutions and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by a

T366W substitution.
(130) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T350V, L351Y, F405A, and Y407V substitutions and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T350V, T366L, K392L, and T394W substitutions.
(131) In some embodiments, the amino acid sequence of one polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T350V, T366L, K392L, and T394W substitutions and wherein the amino acid sequence of the other polypeptide chain of the antibody constant region differs from the amino acid sequence of an IgG1 constant region by T350V, L351Y, F405A, and Y407V substitutions.
(132) The multi-specific proteins described above can be made using recombinant DNA technology well known to a skilled person in the art. For example, a first nucleic acid sequence encoding the first immunoglobulin heavy chain can be cloned into a first expression vector; a second nucleic acid sequence encoding the second immunoglobulin heavy chain can be cloned into a second expression vector; a third nucleic acid sequence encoding the immunoglobulin light chain can be cloned into a third expression vector; and the first, second, and third expression vectors can be stably transfected together into host cells to produce the multimeric proteins.
(133) To achieve the highest yield of the multi-specific protein, different ratios of the first, second, and third expression vector can be explored to determine the optimal ratio for transfection into the host cells. After transfection, single clones can be isolated for cell bank generation using methods known in the art, such as limited dilution, ELISA, FACS, microscopy, or Clonepix.
(134) Clones can be cultured under conditions suitable for bio-reactor scale-up and maintained expression of the multi-specific protein. The multispecific proteins can be isolated and purified using methods known in the art including centrifugation, depth filtration, cell lysis, homogenization, freeze-thawing, affinity purification, gel filtration, ion exchange chromatography, hydrophobic interaction exchange chromatography, and mixed-mode chromatography.
(135) II. Characteristics of the Multi-Specific Proteins
(136) A multi-specific binding protein of the present disclosure (e.g., NKG2D-binding-F4-TriNKET®-BCMA or NKG2D-binding-F3-TriNKET®-BCMA), which includes an NKG2D-binding scFV and a BCMA-binding domain are more effective in reducing tumor growth and killing cancer cells. For example, a multi-specific binding protein of the present disclosure that targets BCMA-expressing tumor/cancer cells is more effective than an anti-BCMA monoclonal antibody MAB42. A TriNKET® of the present disclosure NKG2D-binding-F4-TriNKET*-BCMA is more effective in promoting NK-mediated cell lysis of a human cancer cell line expressing BCMA than an anti-BCMA monoclonal antibody MAB42.
(137) NKG2D-binding-F4-TriNKET®-BCMA shows weak binding to cells expressing NKG2D. However, the multi-specific binding proteins described herein including an NKG2D-binding domain (e.g., NKG2D-binding-F4-TriNKET®-BCMA or NKG2D-binding-F3-TriNKET®-BCMA) exhibit a significant advantage in potency and maximum lysis of target cells compared to MAB42 anti-BCMA mAb.
(138) Accordingly, compared to monoclonal antibodies, the multi-specific binding proteins described herein (e.g., NKG2D-binding-F4-TriNKET®-BCMA or NKG2D-binding-F3-TriNKET®-BCMA) are advantageous in treating BCMA-expressing cancers.
(139) III. Therapeutic Applications
(140) Proteins disclosed herein can be used to activate cytotoxic T cells or natural killer cells. In some embodiments, provided herein are methods of activing a cytotoxic T cell by exposing the cytotoxic T cell to a protein disclosed herein. In some embodiments, provided herein are methods of activing a natural killer cell by exposing the natural killer cell to a protein disclosed herein.
(141) Accordingly, provided herein are methods of enhancing tumor cell death by exposing tumor

cells to a protein disclosed herein in the presence of cytotoxic T cells or natural killer cells. In some embodiments, provided herein are methods of enhancing tumor cell death by exposing tumor cells to a protein disclosed herein in the presence of cytotoxic T cells. In some embodiments, provided herein are methods of enhancing tumor cell death by exposing tumor cells to a protein disclosed herein in the presence of natural killer cells.

(142) Provided herein are also methods of enhancing immune response against BCMA-expressing cancer cells in a subject by administering a protein disclosed herein or a formulation disclosed herein to the subject.

(143) The invention provides methods for treating cancer using a multi-specific binding protein described herein and/or a pharmaceutical composition described herein. The methods may be used to treat a variety of cancers by administering to a patient in need thereof a therapeutically effective amount of a multi-specific binding protein described herein. In some embodiments, cancers that can be treated by proteins disclosed herein express BCMA.

(144) The therapeutic method can be characterized according to the cancer to be treated. For example, in certain embodiments, the cancer is breast, ovarian, esophageal, bladder or gastric cancer, salivary duct carcinoma, salivary duct carcinomas, adenocarcinoma of the lung or aggressive forms of uterine cancer, such as uterine serous endometrial carcinoma.

(145) In certain other embodiments, the cancer to be treated by a multi-specific binding protein described herein and/or a pharmaceutical composition described herein is brain cancer, breast cancer, cervical cancer, colon cancer, colorectal cancer, endometrial cancer, esophageal cancer, leukemia, lung cancer, liver cancer, melanoma, ovarian cancer, pancreatic cancer, rectal cancer, renal cancer, stomach cancer, testicular cancer, or uterine cancer. In yet other embodiments, the cancer is a squamous cell carcinoma, adenocarcinoma, small cell carcinoma, melanoma, neuroblastoma, sarcoma (e.g., an angiosarcoma or chondrosarcoma), larynx cancer, parotid cancer, bilary tract cancer, thyroid cancer, acral lentiginous melanoma, actinic keratoses, acute lymphocytic leukemia, acute myeloid leukemia, adenoid cystic carcinoma, adenomas, adenosarcoma, adenosquamous carcinoma, anal canal cancer, anal cancer, anorectum cancer, astrocytic tumor, bartholin gland carcinoma, basal cell carcinoma, biliary cancer, bone cancer, bone marrow cancer, bronchial cancer, bronchial gland carcinoma, carcinoid, cholangiocarcinoma, chondosarcoma, choriod plexus papilloma/carcinoma, chronic lymphocytic leukemia, chronic myeloid leukemia, clear cell carcinoma, connective tissue cancer, cystadenoma, digestive system cancer, duodenum cancer, endocrine system cancer, endodermal sinus tumor, endometrial hyperplasia, endometrial stromal sarcoma, endometrioid adenocarcinoma, endothelial cell cancer, ependymal cancer, epithelial cell cancer, Ewing's sarcoma, eye and orbit cancer, female genital cancer, focal nodular hyperplasia, gallbladder cancer, gastric antrum cancer, gastric fundus cancer, gastrinoma, glioblastoma, glucagonoma, heart cancer, hemangiblastomas, hemangioendothelioma, hemangiomas, hepatic adenoma, hepatic adenomatosis, hepatobiliary cancer, hepatocellular carcinoma, Hodgkin's disease, ileum cancer, insulinoma, intaepithelial neoplasia, interepithelial squamous cell neoplasia, intrahepatic bile duct cancer, invasive squamous cell carcinoma, jejunum cancer, joint cancer, Kaposi's sarcoma, pelvic cancer, large cell carcinoma, large intestine cancer, leiomyosarcoma, lentigo maligna melanomas, lymphoma, male genital cancer, malignant melanoma, malignant mesothelial tumors, medulloblastoma, medulloepithelioma, meningeal cancer, mesothelial cancer, metastatic carcinoma, mouth cancer, mucoepidermoid carcinoma, multiple myeloma, muscle cancer, nasal tract cancer, nervous system cancer, neuroepithelial adenocarcinoma nodular melanoma, non-epithelial skin cancer, non-Hodgkin's lymphoma, oat cell carcinoma, oligodendroglial cancer, oral cavity cancer, osteosarcoma, papillary serous adenocarcinoma, penile cancer, pharynx cancer, pituitary tumors, plasmacytoma, pseudosarcoma, pulmonary blastoma, rectal cancer, renal cell carcinoma, respiratory system cancer, retinoblastoma, rhabdomyosarcoma, sarcoma, serous carcinoma, sinus cancer, skin cancer, small cell carcinoma, small intestine cancer, smooth muscle cancer, soft tissue cancer, somatostatin-secreting tumor,

spine cancer, squamous cell carcinoma, striated muscle cancer, submesothelial cancer, superficial spreading melanoma, T cell leukemia, tongue cancer, undifferentiated carcinoma, ureter cancer, urethra cancer, urinary bladder cancer, urinary system cancer, uterine cervix cancer, uterine corpus cancer, uveal melanoma, vaginal cancer, verrucous carcinoma, VIPoma, vulva cancer, well differentiated carcinoma, or Wilms tumor.

(146) In certain other embodiments, the cancer to be treated by a multi-specific binding protein described herein and/or a pharmaceutical composition described herein is non-Hodgkin's lymphoma, such as a B-cell lymphoma or a T-cell lymphoma. In certain embodiments, the non-Hodgkin's lymphoma is a B-cell lymphoma, such as a diffuse large B-cell lymphoma, primary mediastinal B-cell lymphoma, follicular lymphoma, small lymphocytic lymphoma, mantle cell lymphoma, marginal zone B-cell lymphoma, extranodal marginal zone B-cell lymphoma, nodal marginal zone B-cell lymphoma, splenic marginal zone B-cell lymphoma, Burkitt lymphoma, lymphoplasmacytic lymphoma, hairy cell leukemia, or primary central nervous system (CNS) lymphoma. In certain other embodiments, the non-Hodgkin's lymphoma is a T-cell lymphoma, such as a precursor T-lymphoblastic lymphoma, peripheral T-cell lymphoma, cutaneous T-cell lymphoma, angioimmunoblastic T-cell lymphoma, extranodal natural killer/T-cell lymphoma, enteropathy type T-cell lymphoma, subcutaneous panniculitis-like T-cell lymphoma, anaplastic large cell lymphoma, or peripheral T-cell lymphoma.

(147) In certain embodiments, the cancer to be treated by a multi-specific binding protein described herein and/or a pharmaceutical composition described herein is diffuse large B-cell lymphoma (DLBCL). In certain embodiments, the DLBCL is germinal center B-cell (GCB) DLBCL. In certain embodiments, the DLBCL is activated B-cell (ABC) DLBCL

(148) In certain embodiments, the cancer to be treated by a multi-specific binding protein described herein and/or a pharmaceutical composition described herein is multiple myeloma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, B cell lymphomas, or acute myeloid leukemia. In certain embodiments, the cancer is multiple myeloma. In certain embodiments, the cancer is chronic lymphocytic leukemia. In certain embodiments, the cancer is acute myeloid leukemia.

(149) The cancer to be treated can be characterized according to the presence of a particular antigen expressed on the surface of the cancer cell. In certain embodiments, the cancer cell can expresses one or more of the following in addition to BCMA: CD2, CD19, CD20, CD30, CD38, CD40, CD52, CD70, EGFR/ERBB1, IGF1R, HER3/ERBB3, HER4/ERBB4, MUC1, cMET, SLAMF7, PSCA, MICA, MICB, TRAILR1, TRAILR2, MAGE-A3, B7.1, B7.2, CTLA4, and PD1.

(150) IV. Combination Therapy

(151) Another aspect of the invention provides for combination therapy. A multi-specific binding protein described herein can be used in combination with additional therapeutic agents to treat cancer.

(152) Exemplary therapeutic agents that may be used as part of a combination therapy in treating cancer, include, for example, radiation, mitomycin, tretinoin, ribomustin, gemcitabine, vincristine, etoposide, cladribine, mitobronitol, methotrexate, doxorubicin, carboquone, pentostatin, nitracrine, zinostatin, cetrorelix, letrozole, raltitrexed, daunorubicin, fadrozole, fotemustine, thymalfasin, sobuzoxane, nedaplatin, cytarabine, bicalutamide, vinorelbine, vesnarinone, aminoglutethimide, amsacrine, proglumide, elliptinium acetate, ketanserin, doxifluridine, etretinate, isotretinoin, streptozocin, nimustine, vindesine, flutamide, drogenil, butocin, carmofur, razoxane, sizofilan, carboplatin, mitolactol, tegafur, ifosfamide, prednimustine, picibanil, levamisole, teniposide, improsulfan, enocitabine, lisuride, oxymetholone, tamoxifen, progesterone, mepitiostane, epitiostanol, formestane, interferon-alpha, interferon-2 alpha, interferon-beta, interferon-gamma (IFN-γ), colony stimulating factor-1, colony stimulating factor-2, denileukin diftitox, interleukin-2, luteinizing hormone releasing factor and variations of the aforementioned agents that may exhibit differential binding to its cognate receptor, or increased or decreased serum half-life.

(153) For certain cancers, e.g., multiple myeloma, the additional therapies can be one or more of lenalidomide, pomalidomide, thalidomide, bortezomib, dexamethasone, cyclophosphamide, doxorubicin, carfilzomib, iaxizomib, cisplatin, doxorubicin, etoposide, an anti-CD38 antibody such as daratumumab, panobinostat, and elotuzumab, either alone, in one of the combinations listed above, or in any other combination.
(154) An additional class of agents that may be used as part of a combination therapy in treating cancer is immune checkpoint inhibitors. Exemplary immune checkpoint inhibitors include agents that inhibit one or more of (i) cytotoxic T lymphocyte-associated antigen 4 (CTLA4), (ii) programmed cell death protein 1 (PD1), (iii) PDL1, (iv) LAG3, (v) B7-H3, (vi) B7-H4, and (vii) TIM3. The CTLA4 inhibitor ipilimumab has been approved by the United States Food and Drug Administration for treating melanoma.
(155) Yet other agents that may be used as part of a combination therapy in treating cancer are monoclonal antibody agents that target non-checkpoint targets (e.g., herceptin) and non-cytotoxic agents (e.g., tyrosine-kinase inhibitors).
(156) Yet other categories of anti-cancer agents include, for example: (i) an inhibitor selected from an ALK Inhibitor, an ATR Inhibitor, an A2A Antagonist, a Base Excision Repair Inhibitor, a Bcr-Abl Tyrosine Kinase Inhibitor, a Bruton's Tyrosine Kinase Inhibitor, a CDC7 Inhibitor, a CHK1 Inhibitor, a Cyclin-Dependent Kinase Inhibitor, a DNA-PK Inhibitor, an Inhibitor of both DNA-PK and mTOR, a DNMT1 Inhibitor, a DNMT1 Inhibitor plus 2-chloro-deoxyadenosine, an HDAC Inhibitor, a Hedgehog Signaling Pathway Inhibitor, an IDO Inhibitor, a JAK Inhibitor, a mTOR Inhibitor, a MEK Inhibitor, a MELK Inhibitor, a MTH1 Inhibitor, a PARP Inhibitor, a Phosphoinositide 3-Kinase Inhibitor, an Inhibitor of both PARP1 and DHODH, a Proteasome Inhibitor, a Topoisomerase-II Inhibitor, a Tyrosine Kinase Inhibitor, a VEGFR Inhibitor, and a WEE1 Inhibitor; (ii) an agonist of OX40, CD137, CD40, GITR, CD27, HVEM, TNFRSF25, or ICOS; and (iii) a cytokine selected from IL-12, IL-15, GM-CSF, and G-CSF.
(157) Proteins of the invention can also be used as an adjunct to surgical removal of the primary lesion.
(158) The amount of multi-specific binding protein and additional therapeutic agent and the relative timing of administration may be selected in order to achieve a desired combined therapeutic effect. For example, when administering a combination therapy to a patient in need of such administration, the therapeutic agents in the combination, or a pharmaceutical composition or compositions comprising the therapeutic agents, may be administered in any order such as, for example, sequentially, concurrently, together, simultaneously and the like. Further, for example, a multi-specific binding protein may be administered during a time when the additional therapeutic agent(s) exerts its prophylactic or therapeutic effect, or vice versa.
(159) V. Pharmaceutical Compositions
(160) The present disclosure also features pharmaceutical compositions that contain a therapeutically effective amount of a protein described herein. The composition can be formulated for use in a variety of drug delivery systems. One or more physiologically acceptable excipients or carriers can also be included in the composition for proper formulation. Suitable formulations for use in the present disclosure are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed., 1985. For a brief review of methods for drug delivery, see, e.g., Langer (Science 249:1527-1533, 1990).
(161) The intravenous drug delivery formulation of the present disclosure may be contained in a bag, a pen, or a syringe. In certain embodiments, the bag may be connected to a channel comprising a tube and/or a needle. In certain embodiments, the formulation may be a lyophilized formulation or a liquid formulation. In certain embodiments, the formulation may freeze-dried (lyophilized) and contained in about 12-60 vials. In certain embodiments, the formulation may be freeze-dried and 45 mg of the freeze-dried formulation may be contained in one vial. In certain embodiments, the about 40 mg-about 100 mg of freeze-dried formulation may be contained in one

vial. In certain embodiments, freeze dried formulation from 12, 27, or 45 vials are combined to obtained a therapeutic dose of the protein in the intravenous drug formulation. In certain embodiments, the formulation may be a liquid formulation and stored as about 250 mg/vial to about 1000 mg/vial. In certain embodiments, the formulation may be a liquid formulation and stored as about 600 mg/vial. In certain embodiments, the formulation may be a liquid formulation and stored as about 250 mg/vial.

(162) The protein could exist in a liquid aqueous pharmaceutical formulation including a therapeutically effective amount of the protein in a buffered solution forming a formulation.

(163) These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as-is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably between 5 and 9 or between 6 and 8, and most preferably between 7 and 8, such as 7 to 7.5. The resulting compositions in solid form may be packaged in multiple single dose units, each containing a fixed amount of the above-mentioned agent or agents. The composition in solid form can also be packaged in a container for a flexible quantity.

(164) In certain embodiments, the present disclosure provides a formulation with an extended shelf life including the protein of the present disclosure, in combination with mannitol, citric acid monohydrate, sodium citrate, disodium phosphate dihydrate, sodium dihydrogen phosphate dihydrate, sodium chloride, polysorbate 80, water, and sodium hydroxide.

(165) In certain embodiments, an aqueous formulation is prepared including the protein of the present disclosure in a pH-buffered solution. The buffer of this invention may have a pH ranging from about 4 to about 8, e.g., from about 4.5 to about 6.0, or from about 4.8 to about 5.5, or may have a pH of about 5.0 to about 5.2. Ranges intermediate to the above recited pH's are also intended to be part of this disclosure. For example, ranges of values using a combination of any of the above recited values as upper and/or lower limits are intended to be included. Examples of buffers that will control the pH within this range include acetate (e.g., sodium acetate), succinate (such as sodium succinate), gluconate, histidine, citrate and other organic acid buffers.

(166) In certain embodiments, the formulation includes a buffer system which contains citrate and phosphate to maintain the pH in a range of about 4 to about 8. In certain embodiments the pH range may be from about 4.5 to about 6.0, or from about pH 4.8 to about 5.5, or in a pH range of about 5.0 to about 5.2. In certain embodiments, the buffer system includes citric acid monohydrate, sodium citrate, disodium phosphate dihydrate, and/or sodium dihydrogen phosphate dihydrate. In certain embodiments, the buffer system includes about 1.3 mg/mL of citric acid (e.g., 1.305 mg/mL), about 0.3 mg/mL of sodium citrate (e.g., 0.305 mg/mL), about 1.5 mg/mL of disodium phosphate dihydrate (e.g., 1.53 mg/mL), about 0.9 mg/mL of sodium dihydrogen phosphate dihydrate (e.g., 0.86), and about 6.2 mg/mL of sodium chloride (e.g., 6.165 mg/mL). In certain embodiments, the buffer system includes 1-1.5 mg/mL of citric acid, 0.25 to 0.5 mg/mL of sodium citrate, 1.25 to 1.75 mg/mL of disodium phosphate dihydrate, 0.7 to 1.1 mg/mL of sodium dihydrogen phosphate dihydrate, and 6.0 to 6.4 mg/mL of sodium chloride. In certain embodiments, the pH of the formulation is adjusted with sodium hydroxide.

(167) A polyol, which acts as a tonicifier and may stabilize the antibody, may also be included in the formulation. The polyol is added to the formulation in an amount which may vary with respect to the desired isotonicity of the formulation. In certain embodiments, the aqueous formulation may be isotonic. The amount of polyol added may also be altered with respect to the molecular weight of the polyol. For example, a lower amount of a monosaccharide (e.g., mannitol) may be added, compared to a disaccharide (such as trehalose). In certain embodiments, the polyol which may be used in the formulation as a tonicity agent is mannitol. In certain embodiments, the mannitol concentration may be about 5 to about 20 mg/mL. In certain embodiments, the concentration of mannitol may be about 7.5 to 15 mg/mL. In certain embodiments, the concentration of mannitol

may be about 10-14 mg/mL. In certain embodiments, the concentration of mannitol may be about 12 mg/mL. In certain embodiments, the polyol sorbitol may be included in the formulation.
(168) A detergent or surfactant may also be added to the formulation. Exemplary detergents include nonionic detergents such as polysorbates (e.g., polysorbates 20, 80 etc.) or poloxamers (e.g., poloxamer 188). The amount of detergent added is such that it reduces aggregation of the formulated antibody and/or minimizes the formation of particulates in the formulation and/or reduces adsorption. In certain embodiments, the formulation may include a surfactant which is a polysorbate. In certain embodiments, the formulation may contain the detergent polysorbate 80 or Tween 80. Tween 80 is a term used to describe polyoxyethylene (20) sorbitanmonooleate (see Fiedler, Lexikon der Hifsstoffe, Editio Cantor Verlag Aulendorf, 4th ed., 1996). In certain embodiments, the formulation may contain between about 0.1 mg/mL and about 10 mg/mL of polysorbate 80, or between about 0.5 mg/mL and about 5 mg/mL. In certain embodiments, about 0.1% polysorbate 80 may be added in the formulation.
(169) In embodiments, the protein product of the present disclosure is formulated as a liquid formulation. The liquid formulation may be presented at a 10 mg/mL concentration in either a USP/Ph Eur type I 50R vial closed with a rubber stopper and sealed with an aluminum crimp seal closure. The stopper may be made of elastomer complying with USP and Ph Eur. In certain embodiments vials may be filled with 61.2 mL of the protein product solution in order to allow an extractable volume of 60 mL. In certain embodiments, the liquid formulation may be diluted with 0.9% saline solution.
(170) In certain embodiments, the liquid formulation of the disclosure may be prepared as a 10 mg/mL concentration solution in combination with a sugar at stabilizing levels. In certain embodiments the liquid formulation may be prepared in an aqueous carrier. In certain embodiments, a stabilizer may be added in an amount no greater than that which may result in a viscosity undesirable or unsuitable for intravenous administration. In certain embodiments, the sugar may be disaccharides, e.g., sucrose. In certain embodiments, the liquid formulation may also include one or more of a buffering agent, a surfactant, and a preservative.
(171) In certain embodiments, the pH of the liquid formulation may be set by addition of a pharmaceutically acceptable acid and/or base. In certain embodiments, the pharmaceutically acceptable acid may be hydrochloric acid. In certain embodiments, the base may be sodium hydroxide.
(172) In addition to aggregation, deamidation is a common product variant of peptides and proteins that may occur during fermentation, harvest/cell clarification, purification, drug substance/drug product storage and during sample analysis. Deamidation is the loss of NH.sub.3 from a protein forming a succinimide intermediate that can undergo hydrolysis. The succinimide intermediate results in a 17 dalton mass decrease of the parent peptide. The subsequent hydrolysis results in an 18 dalton mass increase. Isolation of the succinimide intermediate is difficult due to instability under aqueous conditions. As such, deamidation is typically detectable as 1 dalton mass increase. Deamidation of an asparagine results in either aspartic or isoaspartic acid. The parameters affecting the rate of deamidation include pH, temperature, solvent dielectric constant, ionic strength, primary sequence, local polypeptide conformation and tertiary structure. The amino acid residues adjacent to Asn in the peptide chain affect deamidation rates. Gly and Ser following an Asn in protein sequences results in a higher susceptibility to deamidation.
(173) In certain embodiments, the liquid formulation of the present disclosure may be preserved under conditions of pH and humidity to prevent deamination of the protein product.
(174) The aqueous carrier of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a liquid formulation. Illustrative carriers include sterile water for injection (SWFI), bacteriostatic water for injection (BWFI), a pH buffered solution (e.g., phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.

(175) A preservative may be optionally added to the formulations herein to reduce bacterial action. The addition of a preservative may, for example, facilitate the production of a multi-use (multiple-dose) formulation.
(176) Intravenous (IV) formulations may be the preferred administration route in particular instances, such as when a patient is in the hospital after transplantation receiving all drugs via the IV route. In certain embodiments, the liquid formulation is diluted with 0.9% Sodium Chloride solution before administration. In certain embodiments, the diluted drug product for injection is isotonic and suitable for administration by intravenous infusion.
(177) In certain embodiments, a salt or buffer components may be added in an amount of 10 mM-200 mM. The salts and/or buffers are pharmaceutically acceptable and are derived from various known acids (inorganic and organic) with “base forming” metals or amines. In certain embodiments, the buffer may be phosphate buffer. In certain embodiments, the buffer may be glycinate, carbonate, citrate buffers, in which case, sodium, potassium or ammonium ions can serve as counterion.
(178) A preservative may be optionally added to the formulations herein to reduce bacterial action. The addition of a preservative may, for example, facilitate the production of a multi-use (multiple-dose) formulation.
(179) The aqueous carrier of interest herein is one which is pharmaceutically acceptable (safe and non-toxic for administration to a human) and is useful for the preparation of a liquid formulation. Illustrative carriers include sterile water for injection (SWFI), bacteriostatic water for injection (BWFI), a pH buffered solution (e.g., phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.
(180) The protein of the present disclosure could exist in a lyophilized formulation including the proteins and a lyoprotectant. The lyoprotectant may be sugar, e.g., disaccharides. In certain embodiments, the lyoprotectant may be sucrose or maltose. The lyophilized formulation may also include one or more of a buffering agent, a surfactant, a bulking agent, and/or a preservative.
(181) The amount of sucrose or maltose useful for stabilization of the lyophilized drug product may be in a weight ratio of at least 1:2 protein to sucrose or maltose. In certain embodiments, the protein to sucrose or maltose weight ratio may be of from 1:2 to 1:5.
(182) In certain embodiments, the pH of the formulation, prior to lyophilization, may be set by addition of a pharmaceutically acceptable acid and/or base. In certain embodiments the pharmaceutically acceptable acid may be hydrochloric acid. In certain embodiments, the pharmaceutically acceptable base may be sodium hydroxide.
(183) Before lyophilization, the pH of the solution containing the protein of the present disclosure may be adjusted between 6 to 8. In certain embodiments, the pH range for the lyophilized drug product may be from 7 to 8.
(184) In certain embodiments, a salt or buffer components may be added in an amount of 10 mM-200 mM. The salts and/or buffers are pharmaceutically acceptable and are derived from various known acids (inorganic and organic) with “base forming” metals or amines. In certain embodiments, the buffer may be phosphate buffer. In certain embodiments, the buffer may be glycinate, carbonate, citrate buffers, in which case, sodium, potassium or ammonium ions can serve as counterion.
(185) In certain embodiments, a “bulking agent” may be added. A “bulking agent” is a compound which adds mass to a lyophilized mixture and contributes to the physical structure of the lyophilized cake (e.g., facilitates the production of an essentially uniform lyophilized cake which maintains an open pore structure). Illustrative bulking agents include mannitol, glycine, polyethylene glycol and sorbitol. The lyophilized formulations of the present invention may contain such bulking agents.
(186) A preservative may be optionally added to the formulations herein to reduce bacterial action. The addition of a preservative may, for example, facilitate the production of a multi-use (multiple-

dose) formulation.
(187) In certain embodiments, the lyophilized drug product may be constituted with an aqueous carrier. The aqueous carrier of interest herein is one which is pharmaceutically acceptable (e.g., safe and non-toxic for administration to a human) and is useful for the preparation of a liquid formulation, after lyophilization. Illustrative diluents include sterile water for injection (SWFI), bacteriostatic water for injection (BWFI), a pH buffered solution (e.g., phosphate-buffered saline), sterile saline solution, Ringer's solution or dextrose solution.
(188) In certain embodiments, the lyophilized drug product of the current disclosure is reconstituted with either Sterile Water for Injection, USP (SWFI) or 0.9% Sodium Chloride Injection, USP. During reconstitution, the lyophilized powder dissolves into a solution.
(189) In certain embodiments, the lyophilized protein product of the instant disclosure is constituted to about 4.5 mL water for injection and diluted with 0.9% saline solution (sodium chloride solution).
(190) Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.
(191) The specific dose can be a uniform dose for each patient, for example, 50-5000 mg of protein. Alternatively, a patient's dose can be tailored to the approximate body weight or surface area of the patient. Other factors in determining the appropriate dosage can include the disease or condition to be treated or prevented, the severity of the disease, the route of administration, and the age, sex and medical condition of the patient. Further refinement of the calculations necessary to determine the appropriate dosage for treatment is routinely made by those skilled in the art, especially in light of the dosage information and assays disclosed herein. The dosage can also be determined through the use of known assays for determining dosages used in conjunction with appropriate dose-response data. An individual patient's dosage can be adjusted as the progress of the disease is monitored. Blood levels of the targetable construct or complex in a patient can be measured to see if the dosage needs to be adjusted to reach or maintain an effective concentration. Pharmacogenomics may be used to determine which targetable constructs and/or complexes, and dosages thereof, are most likely to be effective for a given individual (Schmitz et al., Clinica Chimica Acta 308: 43-53, 2001; Steimer et al., Clinica Chimica Acta 308: 33-41, 2001).
(192) In general, dosages based on body weight are from about 0.01 g to about 100 mg per kg of body weight, such as about 0.01 g to about 100 mg/kg of body weight, about 0.01 g to about 50 mg/kg of body weight, about 0.01 g to about 10 mg/kg of body weight, about 0.01 g to about 1 mg/kg of body weight, about 0.01 g to about 100 g/kg of body weight, about 0.01 g to about 50 g/kg of body weight, about 0.01 g to about 10 g/kg of body weight, about 0.01 g to about 1 g/kg of body weight, about 0.01 g to about 0.1 g/kg of body weight, about 0.1 g to about 100 mg/kg of body weight, about 0.1 g to about 50 mg/kg of body weight, about 0.1 g to about 10 mg/kg of body weight, about 0.1 g to about 1 mg/kg of body weight, about 0.1 g to about 100 g/kg of body weight, about 0.1 g to about 10 g/kg of body weight, about 0.1 g to about 1 g/kg of body weight, about 1 g to about 100 mg/kg of body weight, about 1 g to about 50 mg/kg of body weight, about 1 g to about 10 mg/kg of body weight, about 1 g to about 1 mg/kg of body weight, about 1 g to about 100 g/kg of body weight, about 1 g to about 50 g/kg of body weight, about 1 g to about 10 g/kg of body weight, about 10 g to about 100 mg/kg of body weight, about 10 g to about 50 mg/kg of body weight, about 10 g to about 10 mg/kg of body weight, about 10 g to about 1 mg/kg of body weight, about 10 g to about 100 g/kg of body weight, about 10 g to about 50 g/kg of body weight, about 50 g to about 100 mg/kg of body weight, about 50 g to about 50 mg/kg of body weight, about 50 g to about 10 mg/kg of body weight, about 50 g to about 1 mg/kg of body weight, about 50 g to about 100 g/kg of body weight, about 100 g to about 100 mg/kg of body weight, about 100 g to about 50 mg/kg of body weight, about 100 g to about 10 mg/kg of body weight, about 100 g to about 1

mg/kg of body weight, about 1 mg to about 100 mg/kg of body weight, about 1 mg to about 50 mg/kg of body weight, about 1 mg to about 10 mg/kg of body weight, about 10 mg to about 100 mg/kg of body weight, about 10 mg to about 50 mg/kg of body weight, about 50 mg to about 100 mg/kg of body weight.

(193) Doses may be given once or more times daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the targetable construct or complex in bodily fluids or tissues. Administration of the present invention could be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, intracavitary, by perfusion through a catheter or by direct intralesional injection. This may be administered once or more times daily, once or more times weekly, once or more times monthly, and once or more times annually.

(194) The description above describes multiple aspects and embodiments of the invention. The patent application specifically contemplates all combinations and permutations of the aspects and embodiments.

EXAMPLES

(195) The invention now being generally described, will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and is not intended to limit the invention.

Example 1—Primary Human NK Cell Cytotoxicity Assay

(196) Peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood buffy coats using density gradient centrifugation. Isolated PBMCs were washed and prepared for NK cell isolation. NK cells were isolated using a negative selection technique with magnetic beads, purity of isolated NK cells was typically >90% CD3-CD56+. Isolated NK cells were rested overnight, rested NK cells were used the following day in cytotoxicity assays.

(197) DELFIA Cytotoxicity Assay:

(198) Human cancer cell lines expressing BCMA were harvested from culture, cells were washed with HBS, and were resuspended in growth media at 10.sup.6/mL for labeling with BATDA reagent (Perkin Elmer AD0116). Manufacturer instructions were followed for labeling of the target cells. After labeling cells were washed 3× with HBS, and were resuspended at 0.5-1.0×10.sup.5/mL in culture media. To prepare the background wells an aliquot of the labeled cells was put aside, and the cells were spun out of the media. 100 μl of the media were carefully added to wells in triplicate to avoid disturbing the pelleted cells. 100 μl of BATDA labeled cells were added to each well of the 96-well plate. Wells were saved for spontaneous release from target cells, and wells were prepared for max lysis of target cells by addition of 1% TRITON™-X. Monoclonal antibodies or a TriNKET® against BCMA (NKG2D-binding-F4-TriNKET®-BCMA) were diluted in culture media, and 50 μl of diluted mAb or the TriNKET® were added to each well. Rested NK cells were harvested from culture, cells were washed, and were resuspended at 10.sup.5-2.0×10.sup.6/mL in culture media depending on the desired E:T ratio. 50 μl of NK cells were added to each well of the plate to make a total of 200 μl culture volume. The plate was incubated at 37° C. with 5% CO2 for 2-3 hours before developing the assay.

(199) After culturing for 2-3 hours, the plate was removed from the incubator and the cells were pelleted by centrifugation at 200 g for 5 minutes. 20 μl of culture supernatant was transferred to a clean microplate provided from the manufacturer, 200 μl of room temperature europium solution was added to each well. The plate was protected from the light and incubated on a plate shaker at 250 rpm for 15 minutes. The plate was read using either Victor 3 or SpectraMax i3X instruments. % Specific lysis was calculated as follows: % Specific lysis=((Experimental release− Spontaneous release)/(Maximum release− Spontaneous release))*100%.

(200) FACS-Based Long-Term Cytotoxicity Assay:

(201) Human cancer cell lines expressing BCMA and transduces to stably express NucLight Green (Essen BioScience 4475) after puromycin selection were harvested from culture spun down, and

resuspended at 10.sup.5/mL in culture media. 100 μl of target cells was added to each well of a 96-well plate. NKG2D-binding-F4-TriNKET®-BCMA TriNKET® was diluted in culture media and 50 μl of each was added to duplicate wells. Purified human NKs rested overnight were harvested from culture, washed, and resuspended at 4×10.sup.5/mL in culture media. For a 1:1 effector cell:target cell (E:T) ratio, 50 μl of NK cells was added to all wells with the exception of target-only controls, which received 100 μl of culture media. For use of freshly processed PBMCs as effectors, an E:T ratio of 10:1 was instead used. The plate was incubated at 37° C. with 5% CO.sub.2 for 30 hours.

(202) After co-culture, cells were stained, fixed and analyzed by flow cytometry. Remaining target cells were detected with strong shifts in the FITC channel, with dead cells excluded with viability staining. The number of green events was exported and % killing calculated by comparison to target-only control samples. Counting beads were included to ensure recorded volumes were comparable.

Example 2—Assessment of TriNKET® Binding to NKG2D Positive Cells

(203) Binding of TriNKETs® in Human Whole Blood

(204) 100 μl of heparinized human whole blood was added to each tube/well. Directly labeled TriNKET® (NKG2D-binding-F4-TriNKET®-BCMA) or mAb was added directly into whole blood, a mixture of directly conjugated mAbs was also added for immunophenotyping, and samples were incubated at room temperature for 20 minutes. For directly labeled NKG2D-binding-F4-TriNKET®-BCMA or mAbs, after incubation 2 mL of 1×RBC lysis/fixation buffer was added to each sample. Samples were incubated 15 minutes at room temperature. Samples were washed once after lysis, then prepared for analysis.

(205) FIG. **3** and FIG. **4** show human NK cell lysis of BCMA-positive target cell lines in the presence of anti-BCMA TriNKET® (NKG2D-binding-F4-TriNKET®-BCMA) or an anti-BCMA monoclonal antibody, within 2 hours. KMS12-PE cells (FIG. **3**) and MM.1R cells (FIG. **4**), which has a low and high BCMA expression, respectively, were used as target cells. NKG2D-binding-F4-TriNKET®-BCMA demonstrated sub-nanomolar EC.sub.50 values against both KMS12-PE and MM.1R cells. Compared to an anti-BCMA monoclonal antibody (MAB42), NKG2D-binding-F4-TriNKET®-BCMA provided greater maximum specific lysis and potency against both cell lines (KMS12-PE cells (FIG. **3**) and MM.1R cells (FIG. **4**)).

(206) BCMA Surface Stabilization by TriNKETs®

(207) KMS12-PE or MM.1R cells were incubated with an anti-BCMA monoclonal antibody (MAB42), bivalent TriNKET® (NKG2D-binding-F4-TriNKET®-BCMA), or monovalent TriNKET® (A49-DB-TriNKET®-BCMA). A49-DB-TriNKET®-BCMA is a TriNKET® in which the first antigen-binding site comprises an Fab that binds NKG2D and the second antigen-binding site comprises an Fab that binds BCMA, each connected to an Fc domain, forming a bi-valent antibody (WO2018/148566).

(208) To assess total surface BCMA, a saturating concentration of 100 g/mL was used, whereas 100 ng/mL was selected to investigate sub-saturation surface stabilization. Each sample was divided into thirds, with an aliquot each placed on ice for 20 minutes, at 37° C. for 2 hours or 37° C. for 24 hours. After the incubation period cells were washed and bound TriNKET® was detected using an anti-human IgG secondary antibody. After staining the cells were fixed and stored at 4° C., all samples were analyzed at the end of the study.

(209) TriNKETs® Stabilize Surface BCMA

(210) FIG. **5** shows staining of surface BCMA on KMS12-PE cells with A49-DB-TriNKET®-BCMA or BCMA monoclonal antibody (MAB42), after incubation for the indicated time. Both the BCMA mAb and TriNKET® were able to stabilize surface BCMA rapidly after incubation and sustain increased expression over a 24-hour period. FIG. **6** shows that the same effect was observed on the innately higher BCMA expressing cell line MM.1R.

(211) A notable improvement in BCMA target cell binding with longer incubation times was also

observed at sub-saturating concentrations of A49-DB-TriNKET®-BCMA and anti-BCMA mAb (MAB42). FIG. **7** shows avid binding provided by the anti-BCMA mAb (MAB42) and the bivalent TriNKET® (NKG2D-binding-F4-TriNKET®-BCMA) facilitated a rapid and sustained increase in binding to BCMA on KMS12-PE cells while with the monovalent TriNKET® (A49-DB-TriNKET®-BCMA) only limited improvement in biding was observed. FIG. **8** shows a similar pattern on MM.1R cells.

Example 3—Bivalent TriNKETs® Mediate Superior Long-Term Cytotoxicity

(212) The ability of purified human NKs to deplete BCMA-expressing KMS12-PE cells in the presence of a bivalent TriNKET® (NKG2D-binding-F4-TriNKET®-BCMA) was compared with that of an anti-BCMA monoclonal antibody MAB42. FIG. **9** shows rested NK-mediated depletion of KMS12-PE cells by purified human NK cells (E:T ratio of 1:1), as detected by flow cytometry after 20 hours. Bivalent BCMA TriNKET® (NKG2D-binding-F4-TriNKET®-BCMA) resulted in more potent killing than either monoclonal antibody or monovalent TriNKET™ (A49-DB-TriNKET®-BCMA). Using PBMCs at a 10:1 E:T ratio rather than purified NKs yielded similar results (FIG. **10**). Compared to either TriNKET® format, the anti-BCMA mAb provided reduced maximum killing and potency with both effector cell types.

(213) BCMA TriNKET Possesses Extremely Weak Binding Interaction with NKG2D on Cells

(214) The KHYG-1 human NK cell line was used to assess NKG2D binding of TriNKET® NKG2D-binding-F4-TriNKET®-BCMA. KHYG-1 cells transduced to express CD16-F158V were used to investigate the contribution of Fc CD16 binding. TriNKETs® were diluted, and were incubated with KHYG-1 cells. Binding of the TriNKET® was detected using a fluorophore conjugated anti-human IgG secondary antibody. Cells were analyzed by flow cytometry and Median Fluorescence Intensity ("MFI") reported.

(215) The ability of the TriNKETs® to bind NKG2D-expressing cells was investigated. As shown in FIG. **11**, virtually no binding of TriNKETs® (NKG2D-binding-F4-TriNKET®-BCMA and NKG2D-binding-F3-TriNKET®-BCMA) was observed to KHYG-1 cells, which express NKG2D but not CD16. In contrast, when the context of KHYG-1 cells were transduced to express the high affinity variant of CD16, the NKG2D-binding-F4-TriNKET®-BCMA was able to bind the cells at a level only marginally higher MFI than the anti-BCMA monoclonal antibody MAB42 (FIG. **12**). However, the NKG2D-binding-F3-TriNKET®-BCMA was able to bind to the CD16 expressing KHYG-1 cells at a higher MFI (FIG. **12**). That the TriNKETs® did not bind to NKG2D expressing cells was further evident by the inability of the TriNKETs® to bind NKG2D positive NK cells (FIG. **13**A) or CD8+ T cells (FIG. **13**B) in whole blood. TriNKETs® were able to bind B cells (FIG. **13**D), monocytes (FIG. **13**E) and granulocytes (FIG. **13**F) in whole blood at a level comparable to the IgG1 control binding.

Example 4—TriNKETs® Triggered CD8+ T Cell Lysis of BCMA+ Tumor Cells

(216) Primary Human CD8+ T Cell Cytotoxicity Assay:

(217) Primary human CD8+ effector T cell generation: Human PBMCs were isolated from human peripheral blood buffy coats using density gradient centrifugation. Isolated PBMCs were stimulated with 1 μg/ml Concanavalin A (ConA) at 37° C. for 18 hr. Then ConA was removed and cells were cultured with 25 unit/ml IL-2 at 37° C. for 4 days. CD8+ T cells were purified using a negative selection technique with magnetic beads, then cultured in media containing 10 ng/ml IL-15 at 37° C. for 6-13 days.

(218) Primary human CD8+ effector T cell characterization: Human CD8+ effector T cells generated above were analyzed by flow cytometry for CD8+ T cell purity as well as NKG2D and CD16 expression. Cells were stained with fluorophore conjugated antibodies against CD3, CD8, NKG2D and CD16, then analyzed by flow cytometry.

(219) Short-term CD8+ effector T cell DELFIA cytotoxicity assay: Human multiple myeloma KMS12-PE cells expressing a target of interest, BCMA, were harvested from culture. Cells were washed and resuspended in growth media at 106/mL for labeling with BATDA reagent (Perkin

Elmer AD0116). Manufacturer instructions were followed for labeling of the target cells. After labeling cells were washed three times with HBS, and were resuspended at 0.5×10.sup.5/mL in culture media. 100 μl of BATDA labeled cells were added to each well of the 96-well plate. Wells were saved for spontaneous release from target cells, and wells were prepared for max lysis of target cells by addition of 1% TRITON™-X. TriNKETs® and mAb were diluted in culture media and added to the plate at 50 L/well. CD8+ effector T cells were harvested from culture, washed, and resuspended at 5×10.sup.6/mL in culture media (E:T ratio=50:1). Then 50 μl of CD8+ T cells was added to each well of the plate to make a total of 200 μl culture volume. The plate was incubated at 37° C. with 5% CO.sub.2 for 3.5 hrs before developing the assay. After incubation, the plate was removed from the incubator and the cells were pelleted by centrifugation at 500 g for 5 minutes. Then 20 μl of culture supernatant was transferred to a clean microplate provided from the manufacturer, 200 μl of room temperature europium solution was added to each well. The plate was protected from the light and incubated on a plate shaker at 250 rpm for 15 minutes. Plate was read using SpectraMax i3X instruments.

% Specific lysis was calculated as follows:% Specific lysis=((Experimental release−Spontaneous release)/(Maximum release−Spontaneous release))*100%

Characterization of CD8+ Effector T Cells Used in Cytotoxicity Assay

(220) As shown in FIG. **14**, CD8+ effector T cells generated with ConA stimulation and cultured with IL-15 were of high purity (>99% of CD3+CD8+ cells), and all expressed NKG2D but not CD16.

(221) NKG2D-Binding-F4-TriNKET-BCMA Enhanced Lysis of KMS12-PE Cells when Co-Cultured with Activated CD8+ T Cells

(222) Cytolysis of KMS12-PE cells in DELFIA assay: 60 nM of NKG2D-binding-F4-TriNKET®-BCMA, anti-BCMA mAb, or irrelevant TriNKET® was added in cultures of KMS12-PE target cells in the presence or absence of IL-15-stimulated CD8+ T cells from Donor 1 (FIG. **15**A) and Donor 2 (FIG. **15**B). Activated CD8+ T cells co-cultured with KMS12-PE cells in the absence of TriNKETs*/mAbs were included as background T cell killing.

(223) FIGS. **15**A-**15**B show the results of DELFIA cytotoxicity assays with human primary CD8+ effector T cells derived from two healthy donors and KMS12-PE target cells. As shown, NKG2D-binding-F4-TriNKET®-BCMAenhanced lysis of KMS12-PE cells when co-cultured with activated CD8+ T cells, but not in the absence of effector cells. The parental anti-BCMA mAb or the irrelevant TriNKET® was unable to enhance lysis by CD8+ T cells from either donor.

Example 5—TriNKETs® Stimulated NK Cell Activation

(224) Co-culture activation of human purified NK cells: Human cancer cell lines expressing BCMA were harvested from culture, and cells were adjusted to 1×10.sup.6 cells/mL. TriNKET®/mAbs were diluted in culture media. Rested NK cells were harvested from culture and washed. Purified NK cells were resuspended at 1×10.sup.6 cells/mL for a 1:1 E:T. All co-cultures were supplemented with hIL-2, Brefeldin-A, monensin and fluorophore-conjugated anti-CD107a and incubated for 4 hrs. Intracellular staining of live NK cells was achieved after fixation using permeabilization/wash buffer and fluorophore-conjugated anti IFNγ.

(225) FIGS. **16**A-**16**B show human NK cell activation in the presence of BCMA positive target cell lines in the presence of anti-BCMA TriNKET® or monoclonal antibody within 4 hours. In FIG. **16**A, KMS12-PE cells (low BCMA expression) were used as target cells. As shown, BCMA-targeted TriNKET® mediated more significant activation of human NK cells in co-culture with BCMA positive KMS12-PE myeloma cells than anti-BCMA mAb. In FIG. **16**B, H929 (high BCMA expression) were used as target cells. As shown, BCMA-targeted TriNKET® mediated more significant activation of human NK cells in co-culture with BCMA positive H929 myeloma cells than anti-BCMA mAb. Thus, against both high and low BCMA expressing cells the F4-TriNKET® triggered an increase in degranulation and IFNγ production with subnanomolar EC50 value. Compared to a BCMA monoclonal antibody, the F4 TriNKET® stimulated a greater

proportion of NK cells at maximum with enhanced potency against both cell lines.

EXEMPLARY EMBODIMENTS

(226) Embodiment 1: A protein comprising: (a) a first antigen-binding site comprising a single-chain variable fragment (scFv) that binds NKG2D; said scFv that binds NKG2D comprising a heavy chain variable domain and a light chain variable domain; (b) a second antigen-binding site that binds B-cell maturation antigen (BCMA); and (c) an antibody Fc domain or a portion thereof sufficient to bind CD16, or a third antigen-binding site that binds CD16.

(227) Embodiment 2: A protein according to embodiment 1 further comprising an additional antigen-binding site that binds BCMA.

(228) Embodiment 3: The protein according to embodiment 1 or 2, wherein the second antigen-binding site that binds BCMA is an Fab fragment.

(229) Embodiment 4: The protein according to any one of embodiments 1-3, wherein the second and the additional antigen-binding site that bind BCMA are Fab fragments.

(230) Embodiment 5: The protein according to embodiment 1 or 2, wherein the second and the additional antigen-binding site that bind BCMA are scFvs, each comprising a heavy chain variable domain and a light chain variable domain.

(231) Embodiment 6: The protein according to any one of embodiments 1-5, wherein the heavy chain variable domain of the scFv that binds NKG2D is positioned at the N-terminus or the C-terminus of the light chain variable domain of the scFv that binds NKG2D.

(232) Embodiment 7: The protein according to embodiment 6, wherein the light chain variable domain of the scFv that binds NKG2D is positioned at the N-terminus of the heavy chain variable domain of the scFv that binds NKG2D.

(233) Embodiment 8: The protein according to any one of embodiments 1-7, wherein the scFv that binds to NKG2D is linked to the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16.

(234) Embodiment 9: The protein according to embodiment 8, wherein the scFv that binds to NKG2D is linked to the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16 via a hinge comprising Ala-Ser.

(235) Embodiment 10: The protein according to embodiment 8, wherein the scFv that binds to NKG2D is linked to the C-terminus of the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16 via a flexible linker comprising the amino acid sequence of SEQ ID NO:168.

(236) Embodiment 11: The protein according to embodiment 10, wherein the C-terminus of the antibody Fc domain is linked to the N-terminus of the light chain variable domain of the scFv that binds NKG2D.

(237) Embodiment 12: The protein according to any one of embodiments 1-11, wherein within the scFv that binds NKG2D, a disulfide bridge is formed between the heavy chain variable domain and the light chain variable domain of the scFv that binds NKG2D.

(238) Embodiment 13: The protein according to embodiment 12, wherein the disulfide bridge is formed between C44 from the heavy chain variable domain and C100 from the light chain variable domain.

(239) Embodiment 14: The protein according to any one of embodiments 1-13, wherein, within the scFv that binds NKG2D, the heavy chain variable domain is linked to the light chain variable domain via a flexible linker.

(240) Embodiment 15: The protein according to embodiment 14, wherein the flexible linker comprises (GlyGlyGlyGlySer)n (SEQ ID NO:198), wherein n is an integer between 1-10.

(241) Embodiment 16: The protein according to any one of embodiments 5 to 15, wherein the second and the additional antigen-binding site scFvs are each linked to the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16, via a hinge comprising Ala-Ser.

(242) Embodiment 17: The protein according to any one of embodiments 5 to 16, wherein the second and the additional antigen-binding site scFvs are linked to the antibody Fc domain via a hinge comprising Ala-Ser.
(243) Embodiment 18: The protein according to embodiment 16 or 17, wherein a disulfide bridge is formed between the heavy chain variable domain and the light chain variable domain of the second antigen-binding site, the additional antigen-binding site, or both.
(244) Embodiment 19: The protein according to embodiment 18, wherein the disulfide bridge is formed between C44 from the heavy chain variable domain and C100 from the light chain variable domain of the second antigen-binding site, the additional antigen-binding site, or both.
(245) Embodiment 20: The protein according to any one of embodiments 1 to 19, wherein the light chain variable domain of the scFv that binds NKG2D is positioned at the N-terminus of a heavy chain variable domain of the scFv that binds NKG2D, wherein the light chain variable domain of the scFv that binds NKG2D is linked to the heavy chain variable domain of the scFv that binds NKG2D via a flexible linker consisting of the amino acid sequence of SEQ ID NO:167, and the scFv that binds NKG2D is linked to the antibody Fc domain via a hinge comprising Ala-Ser.
(246) Embodiment 21: The protein according to any one of embodiments 1-20, wherein the scFv that binds NKG2D comprises: (a) a heavy chain variable domain comprising complementarity-determining region 1 (CDR1), complementarity-determining region 2 (CDR2), and complementarity-determining region 3 (CDR3) sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 191, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (b) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 193, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (c) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 95, 96, and 97, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (d) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 188, 88, and 189, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 91, 92, and 93, respectively; (e) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 185, 104, and 192, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 107, 108, and 109, respectively; (f) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 185, 72, and 159, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 75, 76, and 77, respectively; (g) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 186, 80, and 187, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 83, 84, and 85, respectively; (h) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 194, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (i) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 195, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101,

respectively; (i) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 196, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; (k) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 197, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively; or (1) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 190, 96, and 160, respectively; and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 99, 100, and 101, respectively.

(247) Embodiment 22: Embodiment The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain at least 90% identical to the amino acid sequence of SEQ ID NO:94.

(248) Embodiment 23: The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain at least 90% identical to SEQ ID NO:94 and a light chain variable domain at least 90% identical to SEQ ID NO:98.

(249) Embodiment 24: The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain at least 95% identical to SEQ ID NO:94 and a light chain variable domain at least 95% identical to SEQ ID NO:98.

(250) Embodiment 25: The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:94 and a light chain variable domain identical to SEQ ID NO:98.

(251) Embodiment 26: The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:169 and a light chain variable domain identical to SEQ ID NO:98.

(252) Embodiment 27: The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:171 and a light chain variable domain identical to SEQ ID NO:98.

(253) Embodiment 28: The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:173 and a light chain variable domain identical to SEQ ID NO:98.

(254) Embodiment 29: The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:175 and a light chain variable domain identical to SEQ ID NO:98.

(255) Embodiment 30: The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:177 and a light chain variable domain identical to SEQ ID NO:98.

(256) Embodiment 31: The protein according to any one of embodiments 1-21, wherein the scFv that binds NKG2D comprises a heavy chain variable domain identical to SEQ ID NO:179 and a light chain variable domain identical to SEQ ID NO:98.

(257) Embodiment 32: The protein according to any one of embodiments 1-31, wherein the second antigen-binding site that binds BCMA comprises: (a) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 149, 150, and 151, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 153, 154, and 155, respectively; (b) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 115, 116, and 1117, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 120, 121, and 123, respectively; (c) a heavy chain variable

domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 125, 126, and 127, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 129, 130, and 131, respectively; (d) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 133, 134, and 135, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 137, 138, and 139, respectively; (e) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 141, 142, and 143, respectively, and a light chain variable domain comprising CDR 1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 145, 146, and 147, respectively; or (f) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 115, 116, and 117, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 120, 121, and 122, respectively.

(258) Embodiment 33: The protein according to any one of embodiments 1-32, wherein the second antigen-binding site that binds BCMA comprises: (a) 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:152; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:148 and a light chain variable domain at least 95% identical to SEQ ID NO:152; or (c) a heavy chain variable domain identical to SEQ ID NO:148 and a light chain variable domain identical to SEQ ID NO:152.

(259) Embodiment 34: The protein according to any one of embodiments 1-32, wherein the second antigen-binding site that binds BCMA comprises: (a) a heavy chain variable domain at least 90% identical to SEQ ID NO:114 and a light chain variable domain at least 90% identical to SEQ ID NO:119; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:114 and a light chain variable domain at least 95% identical to SEQ ID NO:119; or (c) a heavy chain variable domain identical to SEQ ID NO:114 and a light chain variable domain identical to SEQ ID NO:119.

(260) Embodiment 35: The protein according to any one of embodiments 1-32, wherein the second antigen-binding site that binds BCMA comprises: (a) a heavy chain variable domain at least 90% identical to SEQ ID NO:124 and a light chain variable domain at least 90% identical to SEQ ID NO:128; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:124 and a light chain variable domain at least 95% identical to SEQ ID NO:128; or (c) a heavy chain variable domain identical to SEQ ID NO:124 and a light chain variable domain identical to SEQ ID NO:128.

(261) Embodiment 36: The protein according to any one of embodiments 1-32, wherein the second antigen-binding site that binds BCMA comprises: (a) 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:136; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:132 and a light chain variable domain at least 95% identical to SEQ ID NO:136; or (c) a heavy chain variable domain identical to SEQ ID NO:132 and a light chain variable domain identical to SEQ ID NO:136.

(262) Embodiment 37: The protein according to any one of embodiments 1-32, wherein the second antigen-binding site that binds BCMA comprises: (a) 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:144; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:140 and a light chain variable domain at least 95% identical to SEQ ID NO:144; or (c) a heavy chain variable domain identical to SEQ ID NO:140 and a light chain variable domain identical to SEQ ID NO:144.

(263) Embodiment 38: The protein according to any one of embodiments 1-32, wherein the second antigen-binding site that binds BCMA comprises: (a) a heavy chain variable domain at least 90% identical to SEQ ID NO:114 and a light chain variable domain at least 90% identical to SEQ ID NO:118; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:114 and a light chain variable domain at least 95% identical to SEQ ID NO:118; or (c) a heavy chain variable domain identical to SEQ ID NO:114 and a light chain variable domain identical to SEQ ID NO:118.

(264) Embodiment 39: The protein according to any one of embodiments 2-38, wherein the additional antigen-binding site that binds BCMA comprises: (a) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 149, 150, and 151, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 153, 154, and 155, respectively; (b) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 115, 116, and 1117, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 120, 121, and 123, respectively; (c) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 125, 126, and 127, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 129, 130, and 131, respectively; (d) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 133, 134, and 135, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 137, 138, and 139, respectively; (e) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 141, 142, and 143, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 145, 146, and 147, respectively; or (f) a heavy chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 115, 116, and 117, respectively, and a light chain variable domain comprising CDR1, CDR2, and CDR3 sequences represented by the amino acid sequences of SEQ ID NOs: 120, 121, and 122, respectively.

(265) Embodiment 40: The protein according to any one of embodiments 2-38, wherein the additional antigen-binding site that binds BCMA comprises: (a) 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:152; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:148 and a light chain variable domain at least 95% identical to SEQ ID NO:152; or (c) a heavy chain variable domain identical to SEQ ID NO:148 and a light chain variable domain identical to SEQ ID NO:152.

(266) Embodiment 41: The protein according to any one of embodiments 2-38, wherein the additional antigen-binding site that binds BCMA comprises: (a) a heavy chain variable domain at least 90% identical to SEQ ID NO:114 and a light chain variable domain at least 90% identical to SEQ ID NO:119; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:114 and a light chain variable domain at least 95% identical to SEQ ID NO:119; or (c) a heavy chain variable domain identical to SEQ ID NO:114 and a light chain variable domain identical to SEQ ID NO:119.

(267) Embodiment 42: The protein according to any one of embodiments 2-38, wherein the additional antigen-binding site that binds BCMA comprises: (a) a heavy chain variable domain at least 90% identical to SEQ ID NO:124 and a light chain variable domain at least 90% identical to SEQ ID NO:128; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:124 and a light chain variable domain at least 95% identical to SEQ ID NO:128; or (c) a heavy chain

variable domain identical to SEQ ID NO:124 and a light chain variable domain identical to SEQ ID NO:128.

(268) Embodiment 43: The protein according to any one of embodiments 2-38, wherein the additional antigen-binding site that binds BCMA comprises: (a) 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:136; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:132 and a light chain variable domain at least 95% identical to SEQ ID NO:136; or (c) a heavy chain variable domain identical to SEQ ID NO:132 and a light chain variable domain identical to SEQ ID NO:136.

(269) Embodiment 44: The protein according to any one of embodiments 2-38, wherein the additional antigen-binding site that binds BCMA comprises: (a) 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:144; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:140 and a light chain variable domain at least 95% identical to SEQ ID NO:144; or (c) a heavy chain variable domain identical to SEQ ID NO:140 and a light chain variable domain identical to SEQ ID NO:144.

(270) Embodiment 45: The protein according to any one of embodiments 2-38, wherein the additional antigen-binding site that binds BCMA comprises: (a) a heavy chain variable domain at least 90% identical to SEQ ID NO:114 and a light chain variable domain at least 90% identical to SEQ ID NO:118; (b) a heavy chain variable domain at least 95% identical to SEQ ID NO:114 and a light chain variable domain at least 95% identical to SEQ ID NO:118; or (c) a heavy chain variable domain identical to SEQ ID NO:114 and a light chain variable domain identical to SEQ ID NO:118.

(271) Embodiment 46: The protein according to any one of embodiments 1-45, wherein the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16, is an antibody Fc domain comprising hinge and CH2 domains of a human IgG1 antibody.

(272) Embodiment 47: The protein according to any one of embodiments 1-45, wherein the antibody Fc domain or the portion thereof sufficient to bind CD16, or the third antigen-binding site that binds CD16, is an antibody Fc domain comprising an amino acid sequence at least 90% identical to amino acids 234-332 of a human IgG1 antibody.

(273) Embodiment 48: The protein according to embodiment 46 or 47, wherein the antibody Fc domain comprises amino acid sequence at least 90% identical to the Fc domain of human IgG1, differing at one or more positions selected from the group consisting of Q347, Y349, T350, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411, and K439.

(274) Embodiment 49: The protein according to embodiment 48, wherein the antibody Fc domain is an Fc domain of an human IgG1 comprising Q347R, D399V, and F405T substitutions.

(275) Embodiment 50: The protein according to embodiment 49, wherein the antibody Fc domain is linked to the scFv that binds NKG2D.

(276) Embodiment 51: The protein according to embodiment 48, wherein the Fc domain is an Fc domain of an human IgG1 comprising K360E and K409W substitutions.

(277) Embodiment 52: The protein according to embodiment 51, wherein the Fc domain is linked to the second antigen binding site.

(278) Embodiment 53: The protein according to any one of embodiments 1-33 and 46-52 comprising the amino acid sequence of SEQ ID NO:162.

(279) Embodiment 54: The protein according to any one of embodiments 1-33 and 46-52 comprising an amino acid sequence comprising SEQ ID NO:162, SEQ ID NO:163, and SEQ ID NO:165.

(280) Embodiment 55: The protein according to any one of embodiments 1-33 and 46-52

comprising an amino acid sequence at least 90% identical to the amino acid sequence of SEQ ID NO:162.
(281) Embodiment 56: The protein according to any one of embodiments 1-33 and 46-52 comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:162.
(282) Embodiment 57: The protein according to any one of embodiments 1-33 and 46-52 comprising an amino acid sequence at least 99% identical to the amino acid sequence of SEQ ID NO:162.
(283) Embodiment 58: The protein according to any one of embodiments 1-33 and 46-52 comprising an amino acid sequence at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of SEQ ID NO:162, further comprising SEQ ID NO:163 and SEQ ID NO:165.
(284) Embodiment 59: A protein according to any one of embodiments 1-58, wherein the protein binds to NKG2D with a KD of 2 to 120 nM, as measured by surface plasmon resonance.
(285) Embodiment 60: A protein according to any one of embodiments 1-59, wherein the protein activates natural killer cells or cytotoxic T cells upon binding.
(286) Embodiment 61: A formulation comprising a protein according to any one of the preceding embodiments and a pharmaceutically acceptable carrier.
(287) Embodiment 62: A cell comprising one or more nucleic acids encoding a protein according to any one of embodiments 1-60.
(288) Embodiment 63: A method of enhancing cell death in a tumor, comprising exposing the tumor to a protein according to any one of embodiments 1-60, in the presence of natural killer cells or cytotoxic T cells.
(289) Embodiment 64: A method of treating cancer in a subject, comprises administering a protein according to any one of embodiments 1-60 or a formulation according to embodiment 61 to the subject.
(290) Embodiment 65: The method of embodiment 64, wherein the cancer is selected from the group consisting of multiple myeloma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, B cell lymphomas, and acute myeloid leukemia.
(291) Embodiment 66: The method of embodiment 64 or 65, wherein the cancer expresses BCMA.
(292) Embodiment 67: A method of agonizing a cytotoxic T cell comprising exposing the cytotoxic T cell to a protein according to any one of embodiments 1-60.
(293) Embodiment 68: A method of agonizing a natural killer cell comprising exposing the natural killer cell to a protein according to any one of embodiments 1-60.
INCORPORATION BY REFERENCE
(294) The entire disclosure of each of the patent documents and scientific articles referred to herein is incorporated by reference for all purposes.
EQUIVALENTS
(295) The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are intended to be embraced therein.

Claims

1. A protein comprising: (a) a first antigen-binding site comprising a single-chain variable fragment (scFv) that binds NKG2D and comprises a heavy chain complementarity-determining region 1 (CDR1) sequence comprising the amino acid sequence of SEQ ID NO:95, a heavy chain

complementarity-determining region 2 (CDR2) sequence comprising the amino acid sequence of SEQ ID NO:96, a heavy chain complementarity-determining region 3 (CDR3) sequence comprising the amino acid sequence of SEQ ID NO:97, a light chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO:99, a light chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO: 100, and a light chain CDR3 sequence comprising the amino acid sequence of SEQ ID NO:101; (b) a second antigen-binding site that binds B-cell maturation antigen (BCMA) and comprises a heavy chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO:149, a heavy chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO: 150, a heavy chain CDR3 sequence comprising the amino acid sequence of SEQ ID NO:151, a light chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO:153, a light chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO:154, and a light chain CDR3 sequence comprising the amino acid sequence of SEQ ID NO:155; (c) an additional antigen-binding site that binds BCMA and comprises a heavy chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO: 149, a heavy chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO: 150, a heavy chain CDR3 sequence comprising the amino acid sequence of SEQ ID NO:151, a light chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO:153, a light chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO:154, and a light chain CDR3 sequence comprising the amino acid sequence of SEQ ID NO:155; and (d) an antibody Fc domain that binds CD16 or a portion thereof that binds CD16, wherein the portion of the antibody Fc domain that binds CD16 comprises an amino acid sequence at least 90% identical to amino acid residues 234-332 of a human IgG1 antibody, numbered according to EU numbering.

2. The protein of claim 1, wherein the second antigen-binding site and the additional antigen-binding site are Fab fragments.

3. The protein of claim 1, wherein the heavy chain variable region of the scFv of the first antigen-binding site is positioned at the C-terminus of the light chain variable region of the scFv of the first antigen-binding site.

4. The protein of claim 1, wherein the N-terminus of the light chain variable region of the scFv of the first antigen-binding site is linked to the C-terminus of the antibody Fc domain that binds CD16 or a portion thereof that binds CD16 via a linker.

5. The protein of claim 4, wherein the linker comprises the amino acid sequence of SEQ ID NO:168.

6. The protein of claim 1, wherein a disulfide bridge is formed between the heavy chain variable region of the scFv of the first antigen-binding site and the light chain variable region of the scFv of the first antigen-binding site.

7. The protein of claim 1, wherein the heavy chain variable region of the scFv of the first antigen-binding site is linked to the light chain variable region of the scFv of the first antigen-binding site via a linker, wherein the linker comprises the amino acid sequence of (GlyGlyGlyGlySer).sub.n (SEQ ID NO: 198), and wherein n is an integer between 1-10.

8. The protein of claim 2, wherein the C-terminus of each of the second antigen-binding site and the additional antigen-binding site is linked to the antibody Fc domain that binds CD16 or a portion thereof that binds CD16 via a hinge.

9. The protein of claim 8, wherein the hinge comprises Ala-Ser.

10. The protein of claim 1, wherein the scFv of the first antigen-binding site comprises the amino acid sequence of SEQ ID NO:161.

11. The protein of claim 1, wherein the scFv of the first antigen-binding site comprises a heavy chain variable region amino acid sequence at least 99% identical to SEQ ID NO:94 and a light chain variable region amino acid sequence at least 99% identical to SEQ ID NO:98.

12. The protein of claim 1, wherein the second antigen-binding site and the additional antigen-binding site each comprises a heavy chain variable region at least 99% identical to SEQ ID NO:148

and a light chain variable region at least 99% identical to SEQ ID NO:152.
13. The protein of claim 1, wherein the antibody Fc domain is an Fc domain of a human IgG1 antibody comprising amino acid substitutions for forming a heterodimer.
14. The protein of claim 1 comprising an amino acid sequence at least 95% identical to the amino acid sequence of SEQ ID NO:162.
15. A pharmaceutical composition comprising the protein of claim 1 and a pharmaceutically acceptable carrier.
16. A cell comprising one or more nucleic acids encoding the protein of claim 1.
17. A method of directly and/or indirectly enhancing tumor cell death, wherein the method comprises exposing the tumor cell and a natural killer cell to the protein of claim 1.
18. A method of treating cancer, wherein the method comprises administering the protein of claim 1 to a patient.
19. The method of claim 18, wherein the cancer is selected from the group consisting of multiple myeloma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, B cell lymphomas, and acute myeloid leukemia.
20. The protein according to claim 1, wherein the antibody Fc domain comprises a mutation at one or more positions that is Q347, Y349, T350, L351, S354, E356, E357, K360, Q362, S364, T366, L368, K370, N390, K392, T394, D399, S400, D401, F405, Y407, K409, T411, or K439, numbered according to EU numbering.
21. The protein according to claim 1, wherein the antibody Fc domain comprises Y349C, K360E and K409W substitutions, numbered according to EU numbering.
22. The protein according to claim 1, wherein the antibody Fc domain comprises S354C, Q347R, D399V, and F405T substitutions, numbered according to EU numbering.
23. A nucleic acid encoding a polypeptide of the protein according to claim 1, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:162, SEQ ID NO:163, or SEQ ID NO:165.
24. A method of producing the protein according to claim 1, wherein the method comprises: (a) culturing a host cell under conditions suitable for expression of the protein, wherein the host cell comprises a first nucleic acid encoding a first polypeptide comprising the amino acid sequence of SEQ ID NO:162, a second nucleic acid encoding a second polypeptide comprising the amino acid sequence of SEQ ID NO: 163, and a third nucleic acid encoding a third polypeptide comprising the amino acid sequence of SEQ ID NO:165; and (b) isolating and purifying the protein.
25. A protein comprising: (a) a first antigen-binding site comprising a single-chain variable fragment (scFv) that binds NKG2D and comprises a heavy chain complementarity-determining region 1 (CDR1) sequence comprising the amino acid sequence of SEQ ID NO:190, a heavy chain complementarity-determining region 2 (CDR2) sequence comprising the amino acid sequence of SEQ ID NO:96, a heavy chain complementarity-determining region 3 (CDR3) sequence comprising the amino acid sequence of SEQ ID NO:191, a light chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO:99, a light chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO:100, and a light chain CDR3 sequence comprising the amino acid sequence of SEQ ID NO:101; (b) a second antigen-binding site that binds B-cell maturation antigen (BCMA) and comprises a heavy chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO:149, a heavy chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO: 150, a heavy chain CDR3 sequence comprising the amino acid sequence of SEQ ID NO:151, a light chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO:153, a light chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO:154, and a light chain CDR3 sequence comprising the amino acid sequence of SEQ ID NO: 155; (c) an additional antigen-binding site that binds BCMA and comprises a heavy chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO: 149, a heavy chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO: 150, a heavy chain CDR3 sequence comprising the amino acid

sequence of SEQ ID NO:151, a light chain CDR1 sequence comprising the amino acid sequence of SEQ ID NO:153, a light chain CDR2 sequence comprising the amino acid sequence of SEQ ID NO:154, and a light chain CDR3 sequence comprising the amino acid sequence of SEQ ID NO: 155; and (d) an antibody Fc domain that binds CD16 or a portion thereof that binds CD16, wherein the portion of the antibody Fc domain that binds CD16 comprises an amino acid sequence at least 90% identical to amino acid residues 234-332 of a human IgG1 antibody, numbered according to EU numbering.

26. A protein comprising: (a) a first antigen-binding site comprising a single-chain variable fragment (scFv) that binds NKG2D and comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:94, and a light chain variable region comprising the amino acid sequence of SEQ ID NO:98, wherein the heavy chain variable region of the scFv further comprises one amino acid substitution relative to the amino acid sequence of SEQ ID NO:94 located in a framework region of the heavy chain variable region of the scFv, and wherein the light chain variable region of the scFv further comprises one amino acid substitution relative to the amino acid sequence of SEQ ID NO:98 located in a framework region of the light chain variable region of the scFv; (b) a second antigen-binding site that binds B-cell maturation antigen (BCMA) and comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:148, and a light chain variable region comprising the amino acid sequence of SEQ ID NO:152, wherein the heavy chain variable region further comprises no amino acid substitution or one amino acid substitution relative to the amino acid sequence of SEQ ID NO:148 located in a framework region of the heavy chain variable region, and wherein the light chain variable region further comprises no amino acid substitution or one amino acid substitution relative to the amino acid sequence of SEQ ID NO:152 located in a framework region of the light chain variable region; (c) an additional antigen-binding site that binds BCMA and comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO:148, and a light chain variable region comprising the amino acid sequence of SEQ ID NO:152, wherein the heavy chain variable region further comprises no amino acid substitution or one amino acid substitution relative to the amino acid sequence of SEQ ID NO:148 located in a framework region of the heavy chain variable region, and wherein the light chain variable region further comprises no amino acid substitution or one amino acid substitution relative to the amino acid sequence of SEQ ID NO:152 located in a framework region of the light chain variable region; and (d) an antibody Fc domain that binds CD16 or a portion thereof that binds CD16, wherein the portion of the antibody Fc domain that binds CD16 comprises an amino acid sequence at least 90% identical to amino acid residues 234-332 of a human IgG1 antibody, numbered according to EU numbering.

27. The protein of claim 26, wherein the one substitution in the heavy chain variable region of the scFv of the first antigen-binding site and the one substitution in the light chain variable region of the scFv of the first antigen-binding site form a disulfide bond.

28. The protein of claim 27, wherein the one substitution in the heavy chain variable region of the scFv of the first antigen-binding site is G44C numbered according to Kabat numbering, and the one substitution in the light chain variable region of the scFv of the first antigen-binding site is G100C numbered according to Kabat numbering.

29. A protein comprising: (a) a first polypeptide comprising the amino acid sequence of SEQ ID NO: 162; (b) a second polypeptide comprising the amino acid sequence of SEQ ID NO: 163; and (c) a third and fourth polypeptides each comprising the amino acid sequence of SEQ ID NO: 165.

30. The protein according to claim 29, wherein the first polypeptide is linked to the second polypeptide via heterodimerization and at least one disulfide bond, wherein the third polypeptide is linked to the first polypeptide via at least one disulfide bond, and wherein the fourth polypeptide is linked to the second polypeptide via at least one disulfide bond.