

MULTI-FUNCTIONAL FUSION PROTEINS AND USES THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

The present application is entitled to priority under 35 U.S.C. § 119(e) to U.S.
5 Provisional Patent Application No. 62/789,212 filed January 7, 2019, which is hereby
incorporated by reference in its entirety herein.

BACKGROUND OF THE INVENTION

Multiple pathways regulate the survival of infected cells and of cancer cells, as well
10 as the activation or suppression of cells of the immune system such as T cells and Natural
Killer (NK) cells. One pathway that provides costimulatory and inhibitory second signals to T
cells is represented by the programmed death 1 (PD-1; also known as CD279) receptor and
its ligands, PD-L1 (B7-H1; CD274) and PD-L2 (B7-DC; CD273). PD-1 is a member of the
CD28/CTLA4 family that is expressed on activated, but not resting T cells (Nishimura et al.
15 (1996) Int. Immunol. 8:773). Binding of PD-1 by its ligands mediates an inhibitory signal
that results in reduced cytokine production, and reduced T cell survival (Nishimura et al.
(1999) Immunity 11:141; Nishimura et al. (2001) Science 291:319; Chemitz et al. (2004) J.
Immunol. 173:945).

The viral Macrophage Inflammatory Protein-II (vMIP-II) is a chemokine encoded by
20 human herpesvirus 8 (HHV-8) that interacts with the CC and CXC chemokine receptors,
including CCR5 and CXCR4 chemokine receptors. vMIP-II inhibition of HIV-1 entry is
mediated through CCR3, CCR5 and CXCR4, which are HIV-1 receptors for the entry of
HIV-1 into a target cell.

A complex interplay of positive and negative signals regulates T cell activation and
25 maintenance of T cell effector function. Members of the TNF ligand/TNF receptor
superfamily figure prominently in this matrix of signals, bridging cells of the immune system,
as well as with cells of other organ systems. In doing so, TNF superfamily members
contribute to both homeostasis and pathogenesis, via effects on cell survival and death,
cellular differentiation, and inflammation.

30 There remains a need for improved therapeutics for treating diseases such as cancer.
The present invention addresses this need.

SUMMARY OF THE INVENTION

As described herein, the present invention relates to a fusion protein with multipoint

molecular attachment capability and methods of use thereof.

One embodiment of the invention includes a fusion protein comprising Component A and/or Component B. Component A comprises Component Y, Component Z₂ and Component Z₃. Component B comprises Component X', Component Z₂' and Component Z₃'.

5 Another embodiment of the invention includes a fusion protein comprising Component A, Component B, and Component C. Component A comprises Component Y, Component Z₂ and Component Z₃. Component B comprises Component X', Component Z₂' and Component Z₃'. Component C comprises Component X, and Component C'L. Component Y comprises at least a portion of PD-1, Component Z₂ and Component Z₂' comprise CH₂ domains of human
10 Fc, and Component Z₃ and Component Z₃' comprise CH₃ domains of human Fc. Component X' and Component X comprise at least a portion of vMIP-II, and Component CL' comprises at least one CH₁ domain of human IgG1 kappa.

Disclosed herein is a fusion protein comprising Component A, Component B, and Component C; wherein: Component A comprising the amino acid sequence of SEQ ID NO:
15 14, which comprises in order Component Y, Component Z₂ and Component Z₃; and Component B comprising the amino acid sequence of SEQ ID NO: 49, which comprises in order Component X', Component Z₂' and Component Z₃'; and Component C comprising the amino acid sequence of SEQ ID NO: 57, which comprises in order Component X, and Component C'L, wherein Component Y comprises a portion of PD-1, Component Z₂ and
20 Component Z₂' comprise CH₂ domains of human Fc, Component Z₃ and Component Z₃' comprise CH₃ domains of human Fc, Component X' and Component X comprise a portion of vMIP-II, and Component CL' comprises at least one CH₁ domain of human IgG1 kappa, and wherein Component Z₂ is linked to Component Z₂' via a disulfide bond.

Yet another embodiment of the invention includes a method of generating a fusion
25 protein. The method comprises administering to the cell a first nucleic acid encoding human PD-1-hFcA, a second nucleic acid encoding vMIP-II-CH'-hFcB, and a third nucleic acid encoding vMIP-II-CL'.

Still another embodiment of the invention includes a fusion protein comprising the amino acid sequences of SEQ ID NO: 14, SEQ ID NO: 49, and SEQ ID NO: 57.

30 In another embodiment, the invention includes a pharmaceutical composition comprising a pharmaceutically acceptable carrier and any of the fusion proteins disclosed herein.

Disclosed herein is the fusion protein as defined herein for use in treating cancer in a patient in need of such treatment.

In another embodiment, the invention includes a method of treating a proliferative disorder in a patient. The method comprises administering a therapeutically effective amount of any of the fusion proteins disclosed herein to a patient in need of such treatment.

In other embodiments of the invention delineated herein, Component B further
5 comprises Component Z₁'. In certain embodiments, Component A further comprises Component Z₁.

In certain embodiments, the fusion protein of further comprises Component C, wherein Component C comprises Component X and Component C_L'.

In certain embodiments, the fusion protein further comprises component D, wherein
10 Component D comprises Component Q and Component C_L.

In certain embodiments, Component Y comprises a ligand domain, a receptor domain, an scFv domain or a lipocalin domain. In certain embodiments, Component Y comprises at least a portion of PD-1, CD112R, CD113 or MHC-I polypeptide-related sequence A (MICA).

In certain embodiments, the fusion protein binds PD-L1 or PD-L2.

15 In certain embodiments, Component X' comprises a virus-derived peptide, a ligand-derived, a receptor-derived peptide, or an HTS-selected peptide.

In certain embodiments, Component X' comprises at least a portion of vMIP-II. In certain embodiments, Component X' comprises V1 or V1delta.

In certain embodiments, Component X comprises a virus-derived peptide, a ligand-
20 derived, a receptor-derived peptide, or an HTS-selected peptide. In certain embodiments, Component X comprises V1 or V1delta.

In certain embodiments, the fusion protein binds CXCR4.

In certain embodiments, Component Y and Component Z₂ are connected via a hinge. In certain embodiments, Component Z₁' and Component Z₂' are connected via a hinge.

25 In certain embodiments, Component X' and Component Z₁' are connected via a linker. In certain embodiments, Component X and Component C_L are connected via a linker. In certain embodiments, Component Q and Component C_L are connected via a linker.

In certain embodiments, the fusion protein binds a receptor or ligand on an immune cell. In certain embodiments, the receptor is an Fc receptor.

30 In certain embodiments, Component X' comprises at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48.

In certain embodiments, Component Y comprises at least a portion of PD-1, TIGIT,

CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48.

5 In certain embodiments, Component X comprises at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48.

10 In certain embodiments, Component Q comprises at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48.

In certain embodiments, Component A comprises the amino acid sequence of any one of SEQ ID NOs: 14-22.

15 In certain embodiments, Component B comprises the amino acid sequence of any one of SEQ ID NOs: 32-35. In certain embodiments, Component B comprises the amino acid sequence of any one of SEQ ID NOs: 49-55.

In certain embodiments, Component A comprises the amino acid sequence of any one of SEQ ID NOs: 14-22 and Component B comprises the amino acid sequence of any one of SEQ ID NOs: 32-35.

20 In certain embodiments, Component C comprises the amino acid sequence of any one of SEQ ID NOs: 57-63.

In certain embodiments, Component B comprises the amino acid sequence of any one of SEQ ID NOs: 49-55 and Component C comprises the amino acid sequence of any one of SEQ ID NOs: 57-63.

25 In certain embodiments, the fusion protein is capable of binding i) PD-L1 or PD-L2, ii) CXCR4, and iii) an Fc receptor or ligand on an immune cell.

In certain embodiments, Component A comprises the amino acid sequence of SEQ ID NO: 29 and Component B comprises the amino acid sequence of SEQ ID NO: 30.

30 In certain embodiments, Component Y comprises the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

In certain embodiments, Component Z₂ comprises the amino acid sequence of SEQ ID NO: 12 and/or Component Z₃ comprises the amino acid sequence of SEQ ID NO: 13.

In certain embodiments, Component A comprises the amino acid sequence of SEQ ID NO: 14 or SEQ ID NO: 15.

In certain embodiments, Component B comprises the amino acid sequence of SEQ ID NO: 49.

In certain embodiments, Component X' comprises the amino acid sequence of SEQ ID NO: 37. In certain embodiments, Component X comprises the amino acid sequence of
5 SEQ ID NO: 37.

In certain embodiments, Component Z₂' comprises SEQ ID NO: 12 and/or Component Z₃' comprises SEQ ID NO: 48.

In certain embodiments, Component C_L' comprises the amino acid sequence of SEQ ID NO: 64.

10 In certain embodiments, Component B comprises the amino acid sequence of SEQ ID NO: 53.

In certain embodiments, Component C comprises the amino acid sequence of SEQ ID NO: 61.

15 In certain embodiments, Component A comprises the amino acid sequence of SEQ ID NO: 14, Component B comprises the amino acid sequence of SEQ ID NO: 53, and Component C comprises the amino acid sequence of SEQ ID NO: 61.

In certain embodiments, the method comprises wherein the first nucleic acid encodes the amino acid sequence of SEQ ID NO: 14, and/or the second nucleic acid encodes the amino acid sequence of SEQ ID NO: 49, and/or the third nucleic acid encodes the amino acid
20 sequence of SEQ ID NO: 57.

In certain embodiments, the proliferative disorder is cancer. In certain embodiments, the cancer is a solid tumor. In certain embodiments, the cancer is pancreatic cancer, breast cancer, ovarian cancer, bladder cancer, melanoma, glioblastoma, acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), multiple myeloma or colon cancer.

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BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of preferred embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the drawings embodiments which are presently
30 preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figures 1A and 1B are schematic representations of some embodiments of a fusion protein platform.

Figure 2 is a schematic representation of some embodiments of a fusion protein

platform. The figure illustrates a fusion protein targeting the Natural Killer (NK) cell:tumor cell interface.

Figure 3 is a schematic representation of a fusion protein that binds CXCR4, PD-L1 and FcγRIIIa.

5 Figure 4 is a schematic representation of the interaction with a cancer cell and with an NK cell of a fusion protein that binds CXCR4, PD-L1 and FcγRIIIa.

Figure 5 is a schematic representation of the possible interaction(s) with various types of cells of a fusion protein that binds CXCR4, PD-L1 and FcγRIIIa.

10 Figure 6 is a schematic representation of the possible interaction(s) with various types of cells of various fusion proteins.

Figure 7 is a schematic representation of the possible interaction(s) with various types of cells of various fusion proteins.

Figure 8 is a schematic representation of the possible interaction(s) with tumor-associated macrophages (M1) of various fusion proteins.

15 Figure 9 is a Coomassie blue-stained (reduced) SDS-PAGE gel of hPD-1-FcA/hFcB (SEQ ID NO: 14 plus SEQ ID NO: 36).

Figure 10 is a Coomassie blue-stained SDS-PAGE gel of hFcA/hFcB (SEQ ID NO: 23 plus SEQ ID NO: 36).

20 Figure 11 is a Coomassie blue-stained SDS-PAGE gel of hMICA-FcA/hFcB (SEQ ID NO: 22 plus SEQ ID NO: 36).

Figure 12 is a Coomassie blue-stained (reduced) SDS-PAGE gel of hFcA/hTIGIT-FcB (SEQ ID NO: 14 plus SEQ ID NO: 36), hFcA/hCD155-FcB (SEQ ID NO: 23 plus SEQ ID NO: 32), hFcA/hFcB (SEQ ID NO: 23 plus SEQ ID NO: 36), and hMICA-FcA/hTIGIT-FcB (SEQ ID NO: 22 plus SEQ ID NO: 33).

25 Figure 13 is a Coomassie blue-stained SDS-PAGE gel of hCD112R-FcA/hFcB (SEQ ID NO: 16 plus SEQ ID NO: 36).

Figure 14 is a Coomassie blue-stained SDS-PAGE gel of HA-PD-1-FcA/hCD113-FcB (SEQ ID NO: 15 plus SEQ ID NO: 35).

30 Figure 15 is a Coomassie blue-stained SDS-PAGE gel of hFcA/hCD113-FcB (SEQ ID NO: 23 plus SEQ ID NO: 33) and PD-1-hFcA/hCD113-FcB (SEQ ID NO: 14 plus SEQ ID NO: 35).

Figure 16 is a Coomassie blue-stained SDS-PAGE gel of hFcA/hCD155-FcB (SEQ ID NO: 23 plus SEQ ID NO: 32).

Figure 17 is a Coomassie blue-stained SDS-PAGE gel of hFcA/TIM-3-hFcB (SEQ ID NO: 23 plus SEQ ID NO: 34) and PD-1-hFcA/TIM-3-hFcB (SEQ ID NO: 14 plus SEQ ID NO: 34).

Figure 18 shows that hFcA/hTIGIT-FcB (SEQ ID NO: 23 plus SEQ ID NO: 33) binds
5 to cells expressing human CD155.

Figure 19 shows that hFcA/hTIGIT-FcB (SEQ ID NO: 23 plus SEQ ID NO: 33) binds to cells expressing human CD112.

Figure 20 shows that hFcA/hCD155-FcB (SEQ ID NO: 23 plus SEQ ID NO: 32) binds to cells expressing human TIGIT.

10 Figure 21 shows that hFcA/hTIGIT-FcB (SEQ ID NO: 23 plus SEQ ID NO: 33) and hFcA/hCD155-FcB (SEQ ID NO: 23 plus SEQ ID NO: 32) bind to cells expressing CD16 (FcγRIIIa).

Figure 22 shows fusion proteins separated and reduced (R) on SDS-PAGE, Coomassie gel staining. As depicted, 'A' refers to hFcA/hFcB (SEQ ID NO: 23 plus SEQ ID NO: 36), 'B' refers to PD-1-hFcA/FcB (SEQ ID NO: 14 plus SEQ ID NO: 36), 'C' refers to hFcA/VpI-CH'-FcB/ VpI-CL (SEQ ID NO: 23 plus SEQ ID NO: 53 and SEQ ID NO: 61) and 'D' refers to PD-1-hFcA/ VpI-CH'-FcB/VpI-CL (SEQ ID NO: 14 plus SEQ ID NO: 53 and SEQ ID NO: 61).

Figures 23A-23D are a series of Western Blots. Western Blot analysis was performed
20 on protein-A purified fusion proteins from conditioned media of Expi-CHO cells transfected with expression constructs as indicated in Figure 22. Observed bands were consistent with the expected sizes of PD-1-hFcA (SEQ ID NO: 14) and vMIP-II-CL' (SEQ ID NO: 57); 125 kDa for non-reduced (NR) samples and 60 kDa and 20 kDa, respectively, for/reduced (R) samples. The Western Blot was probed with (Figure 23A) antibody against PD1, (Figure
25 23B) antibody against IgG kappa light chain, (Figure 23C) antibody against IgG heavy chain, and (Figure 23D) antibody against vMIP-II.

Figure 24 shows fusion protein binding to B16 melanoma cell lines that express PD-L1 and CXCR4 or CXCR7 on their cell surface. The fusion proteins tested are (in order from top to bottom): hFcA/hFcB (SEQ ID NO: 23 plus SEQ ID NO: 36), PD-1-hFcA/FcB (SEQ
30 ID NO: 14 plus SEQ ID NO: 36), hFcA/V1Δmut-CH'-FcB/ V1Δmut-CL' (SEQ ID NO: 23 plus SEQ ID NO: 52 and SEQ ID NO: 60), hFcA/V1Δ-CH'-FcB/ V1Δ-CL' (SEQ ID NO: 23 plus SEQ ID NO: 51 and SEQ ID NO: 59), PD-1-hFcA/V1Δmut-CH'-FcB/ V1Δmut-CL' (SEQ ID NO: 14 plus SEQ ID NO: 52 and SEQ ID NO: 60) and PD-1-hFcA/V1Δ-CH'-FcB/

V1Δ-CL' (SEQ ID NO: 14 plus SEQ ID NO: 51 and SEQ ID NO: 59).

Figure 25 shows a transwell assay, measuring the inhibitory effects of fusion proteins on melanoma cell migration. Representative images from a single transwell migration assay of migrated B16-F10 cells treated with 100 ng/mL CXCL12 and fusion proteins are shown.

5 The fusion proteins tested are (in order from top to bottom): hFcA/hFcB (SEQ ID NO: 23 plus SEQ ID NO: 36), PD-1-hFcA/FcB (SEQ ID NO: 14 plus SEQ ID NO: 36), hFcA/V1Δmut-CH'-FcB/ V1Δmut-CL' (SEQ ID NO: 23 plus SEQ ID NO: 52 and SEQ ID NO: 60), hFcA/V1Δ-CH'-FcB/ V1Δ-CL' (SEQ ID NO: 23 plus SEQ ID NO: 51 and SEQ ID NO: 59), PD-1-hFcA/V1Δmut-CH'-FcB/ V1Δmut-CL' (SEQ ID NO: 14 plus SEQ ID NO: 52 and SEQ ID NO: 60) and PD-1-hFcA/V1Δ-CH'-FcB/ V1Δ-CL' (SEQ ID NO: 14 plus
10 SEQ ID NO: 51 and SEQ ID NO: 59). The assays were stained with 2% crystal violet.

Figures 26A-26B show inhibition of tumor growth mediated by the fusion protein (FP) PD1-hFcA/v1Δ-CH'-hFcB/v1Δ-CL' (SEQ ID NO: 14 plus SEQ ID NO: 51 and SEQ ID NO: 59) in the B16F10 melanoma subcutaneous model. C57BL/6 mice were treated with
15 100 µl of (10 µg/mL) FP in PBS (mice R, 2L and 2R) or PBS only (mice X or L) on days 13, 14, 15, 16 and 21 post subcutaneous inoculation with 1×10^5 B16F10 tumor cells. Tumor size over time was measured and plotted for each mouse in Figure 26A. Figure 26B shows representative images of one mouse from the PBS only treatment group (top) and PD1-hFcA/v1Δ-CH'-hFcB/v1Δ-CL' (FP) treatment group (bottom).

20 Figure 27 shows that NK cell-mediated ADCC is augmented by addition of multi-functional fusion proteins. The SKOV-3 ovarian cell line [target cell (T)] was plated in a 96-well plate at 3000 cells/well, allowed to adhere and labeled with CellTracker Red CMTPX reagent. The SKOV-3 cells were then labeled with a green fluorescence Caspase-3 reagent. The CD16.NK-92 cell line [V158 variant, i.e., effector cell (E)] was added to the wells at a
25 E:T 5:1 with the various fusion proteins at 25 mg/ml or no protein and analyzed using the Incucyte live-cell analysis system, measuring the number of fluorescent double positive (red + green) cells. Results shown depict the 20 h time point. Fusion proteins tested: High Affinity (HA)-PD-1-hFcA/FcB (SEQ ID NO: 15 plus SEQ ID NO: 36), hFcA/hCD113-FcB (SEQ ID NO: 23 plus SEQ ID NO: 35) and High Affinity (HA)-PD-1-hFcA/ hCD113-FcB (SEQ ID
30 NO: 15 plus SEQ ID NO: 35).

DETAILED DESCRIPTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention
5 pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used.

It is also to be understood that the terminology used herein is for the purpose of
10 describing particular embodiments only, and is not intended to be limiting.

The articles “a” and “an” are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

“About” as used herein when referring to a measurable value such as an amount, a
15 temporal duration, and the like, is meant to encompass variations of $\pm 20\%$ or $\pm 10\%$, more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods.

“Activation,” as used herein, refers to the state of a T cell that has been sufficiently stimulated to induce one or more of cytokine production, detectable effector functions, and
20 cellular proliferation. The term “activated T cells” refers to, among other things, T cells that manifest one or more of these activation features.

The term “antibody,” as used herein, refers to an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact
25 immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules, and can also exist as higher order multimers of such tetramers. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)₂, as well as single chain antibodies (scFv) and humanized antibodies (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual,
30 Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

The term “antibody fragment” refers to a portion of an intact antibody and refers to the antigenic determining variable regions of an intact antibody. Examples of antibody

fragments include, but are not limited to, Fab, Fab', F(ab')₂, and Fv fragments, linear antibodies, scFv antibodies, and multi-specific antibodies formed from antibody fragments.

An “antibody heavy chain,” as used herein, refers to the larger of the two types of polypeptide chains present in all antibody molecules in their naturally occurring
5 conformations. Gamma (γ), Mu (μ), Delta (δ), Alpha (α) and Epsilon (ε) heavy chains refer to the five major antibody heavy chain isotypes.

An “antibody light chain,” as used herein, refers to the smaller of the two types of polypeptide chains present in all antibody molecules in their naturally occurring
10 conformations. Kappa (κ) and lambda (λ) light chains refer to the two major antibody light chain isotypes.

By the term “synthetic antibody” as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the
15 antibody and which DNA molecule expresses an RNA encoding an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA, RNA or amino acid sequence has been obtained using synthetic nucleic acid or amino acid sequence technology which is available and well known in the art.

The term “antigen” or “Ag” as used herein is defined as a molecule that provokes an
20 immune response or binds to an immune recognition moiety such as an antibody and a T cell receptor. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan
25 will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an “antigen” as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full-length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial
30 nucleotide sequences of more than one gene, and that these nucleotide sequences are arranged in various combinations to elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a “gene” at all. It is readily apparent that an antigen can be generated, synthesized, or can be derived from a biological sample.

Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a biological fluid.

The term “anti-tumor effect” as used herein, refers to a biological effect which can be manifested by a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in the number of metastases, an increase in life expectancy, or amelioration of various physiological symptoms associated with the cancerous condition. An “anti-tumor effect” can also be manifested by the ability of the peptides, polynucleotides, cells and antibodies of the invention in prevention of the occurrence of tumor in the first place.

The term “auto-antigen” means, in accordance with the present invention, any self-antigen which is recognized by the immune system as being foreign. Auto-antigens comprise, but are not limited to, cellular proteins, phosphoproteins, cellular surface proteins, cellular lipids, nucleic acids, glycoproteins, including cell surface receptors.

The term “autoimmune disease” as used herein is defined as a disorder that results from an autoimmune response. An autoimmune disease is the result of an inappropriate and excessive response to a self-antigen. Examples of autoimmune diseases include but are not limited to, Addison's disease, alopecia areata, ankylosing spondylitis, autoimmune hepatitis, autoimmune parotitis, Crohn's disease, diabetes (Type I), dystrophic epidermolysis bullosa, epididymitis, glomerulonephritis, Graves' disease, Guillain-Barr syndrome, Hashimoto's disease, hemolytic anemia, systemic lupus erythematosus, multiple sclerosis, myasthenia gravis, pemphigus vulgaris, psoriasis, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjogren's syndrome, spondyloarthropathies, thyroiditis, vasculitis, vitiligo, myxedema, pernicious anemia, ulcerative colitis, among others.

As used herein, the term “autologous” is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

“Allogeneic” refers to a graft derived from a different animal of the same species.

“Xenogeneic” refers to a graft derived from an animal of a different species.

The term “cancer” as used herein is defined as disease characterized by the rapid and uncontrolled proliferation and/or accumulation of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers include but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer and the like. In certain embodiments, the cancer is medullary thyroid carcinoma.

The term “cleavage” refers to the breakage of covalent bonds, such as in the backbone

of a nucleic acid molecule. Cleavage can be initiated by a variety of methods, including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible. Double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, fusion polypeptides may be used for targeting cleaved double-stranded DNA.

As used herein, the term “conservative sequence modifications” is intended to refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within the CDR regions of an antibody can be replaced with other amino acid residues from the same side chain family and the altered antibody can be tested for the ability to bind antigens using the functional assays described herein.

A “disease” is a state of health of an animal wherein the animal cannot maintain homeostasis, and wherein if the disease is not ameliorated then the animal’s health continues to deteriorate. In contrast, a “disorder” in an animal is a state of health in which the animal is able to maintain homeostasis, but in which the animal’s state of health is less favorable than it would be in the absence of the disorder. Left untreated, a disorder does not necessarily cause a further decrease in the animal’s state of health.

“Effective amount” or “therapeutically effective amount” are used interchangeably herein, and refer to an amount of a compound, formulation, material, or composition, as described herein effective to achieve a particular biological result or provides a therapeutic or prophylactic benefit. Such results may include, but are not limited to, anti-tumor activity as determined by any means suitable in the art.

“Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (*i.e.*, rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription to mRNA and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

As used herein, “endogenous” refers to any material from or produced inside an organism, cell, tissue or system.

As used herein, the term “exogenous” refers to any material introduced from or produced outside an organism, cell, tissue or system.

The term “expand” as used herein refers to increasing in number, as in an increase in the number of T cells. In one embodiment, the T cells that are expanded *ex vivo* increase in number relative to the number originally present in the culture. In another embodiment, the T cells that are expanded *ex vivo* increase in number relative to other cell types in the culture. The term “*ex vivo*,” as used herein, refers to cells that have been removed from a living organism, (e.g., a human) and propagated outside the organism (e.g., in a culture dish, test tube, or bioreactor).

The term “expression” as used herein is defined as the transcription and/or translation of a particular nucleotide sequence driven by its regulatory element, such as a promoter.

“Expression vector” refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient *cis*-acting elements for expression; other elements for expression can be supplied by the host cell or in an *in vitro* expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, Sendai viruses, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

“Homologous” as used herein refers to the subunit sequence identity between two polymeric molecules, *e.g.*, between two nucleic acid molecules, such as, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in

both of the two molecules is occupied by the same monomeric subunit; *e.g.*, if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions; *e.g.*, if half (*e.g.*, five positions in a polymer ten subunits in length) of the positions in two sequences are homologous, the two sequences are 50% homologous; if 90% of the positions (*e.g.*, 9 of 10), are matched or homologous, the two sequences are 90% homologous.

“Humanized” forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies can comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321: 522-525, 1986; Reichmann et al., *Nature*, 332: 323-329, 1988; Presta, *Curr. Op. Struct. Biol.*, 2: 593-596, 1992.

“Fully human” refers to an immunoglobulin, such as an antibody, where the whole molecule is of human origin or consists of an amino acid sequence identical to a human form of the antibody.

“Identity” as used herein refers to the subunit sequence identity between two polymeric molecules particularly between two amino acid molecules, such as, between two polypeptide molecules. When two amino acid sequences have the same residues at the same positions; *e.g.*, if a position in each of two polypeptide molecules is occupied by an arginine, then they are identical at that position. The identity or extent to which two amino acid

sequences have the same residues at the same positions in an alignment is often expressed as a percentage. The identity between two amino acid sequences is a direct function of the number of matching or identical positions; *e.g.*, if half (*e.g.*, five positions in a polymer ten amino acids in length) of the positions in two sequences are identical, the two sequences are
5 50% identical; if 90% of the positions (*e.g.*, 9 of 10), are matched or identical, the two amino acids sequences are 90% identical.

The term “immunoglobulin” or “Ig,” as used herein is defined as a class of proteins, which function as antibodies. Antibodies expressed by B cells are sometimes referred to as the BCR (B cell receptor) or antigen receptor. The five members included in this class of
10 proteins are IgG, IgM, IgD, IgA, and IgE. IgG is the most common circulating antibody. IgM is the main immunoglobulin produced in the primary immune response in most subjects. It is the most efficient immunoglobulin in agglutination, complement fixation, and other antibody responses, and is important in defense against bacteria and viruses. IgD is the
immunoglobulin that has no known antibody function, but may serve as an antigen receptor.
15 IgA is the primary antibody that is present in body secretions, such as saliva, tears, breast milk, gastrointestinal secretions and mucus secretions of the respiratory and genitourinary tracts. IgE is the immunoglobulin that mediates immediate hypersensitivity by causing release of mediators from mast cells and basophils upon exposure to allergen.

The term “immune response” as used herein is defined as a cellular response to an
20 antigen that occurs when lymphocytes identify antigenic molecules as foreign and induce the formation of antibodies and/or activate lymphocytes to remove the antigen.

The term “lipocalin” as used herein is defined as a class of proteins, which function in their natural setting to transport small hydrophobic molecules such
as steroids, bilins, retinoids, and lipids. They share limited regions of sequence homology and
25 a common tertiary structure architecture comprising an eight-stranded antiparallel beta barrel with a repeated + 1 topology enclosing an internal ligand binding site. Lipocalins are found in gram negative bacteria, vertebrate cells, invertebrate cells, and in plants, and have been associated with many biological processes, among
them immune response, pheromone transport, biological prostaglandin synthesis,
30 retinoid binding, and cancer cell interactions. Lipocalins can be modified in many of the same ways described for antibody modification in order to alter or enhance their binding properties, *e.g.*, bind to a cell surface molecule and in this way block, enhance, or otherwise modify its functional properties.

As used herein, an “instructional material” includes a publication, a recording, a

diagram, or any other medium of expression which can be used to communicate the usefulness of the compositions and methods of the invention. The instructional material of the kit of the invention may, for example, be affixed to a container which contains the nucleic acid, peptide, and/or composition of the invention or be shipped together with a container
5 which contains the nucleic acid, peptide, and/or composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the compound be used cooperatively by the recipient.

“Isolated” means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not “isolated,” but the same nucleic
10 acid or peptide partially or completely separated from the coexisting materials of its natural state is “isolated.” An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

A “lentivirus” as used herein refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver
15 a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses. Vectors derived from lentiviruses offer the means to achieve significant levels of gene transfer in vivo.

By the term “modified” as used herein, is meant a changed state or structure of a
20 molecule or cell of the invention. Molecules may be modified in many ways, including chemically, structurally, and functionally. Cells may be modified through the introduction of nucleic acids.

By the term “modulating,” as used herein, is meant mediating a detectable increase or decrease in the level of a response in a subject compared with the level of a response in the
25 subject in the absence of a treatment or compound, and/or compared with the level of a response in an otherwise identical but untreated subject. The term encompasses perturbing and/or affecting a native signal or response thereby mediating a beneficial therapeutic response in a subject, preferably, a human.

In the context of the present invention, the following abbreviations for the commonly
30 occurring nucleic acid bases are used. “A” refers to adenosine, “C” refers to cytosine, “G” refers to guanosine, “T” refers to thymidine, and “U” refers to uridine.

Unless otherwise specified, a “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or an

RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

The term “operably linked” refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein coding regions, in the same reading frame.

The term “overexpressed” tumor antigen or “overexpression” of a tumor antigen is intended to indicate an abnormal level of expression of a tumor antigen in a cell from a disease area like a solid tumor within a specific tissue or organ of the patient relative to the level of expression in a normal cell from that tissue or organ. Patients having solid tumors or a hematological malignancy characterized by overexpression of the tumor antigen can be determined by standard assays known in the art.

“Parenteral” administration of an immunogenic composition includes, *e.g.*, subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, or infusion techniques.

The term “polynucleotide” as used herein is defined as a chain of nucleotides. Furthermore, nucleic acids are polymers of nucleotides. Thus, nucleic acids and polynucleotides as used herein are interchangeable. One skilled in the art has the general knowledge that nucleic acids are polynucleotides, which can be hydrolyzed into monomeric “nucleotides.” The monomeric nucleotides can be hydrolyzed into nucleosides. As used herein, polynucleotides include, but are not limited to, all nucleic acid sequences which are obtained by any means available in the art, including, without limitation, recombinant means, i.e., the cloning of nucleic acid sequences from a recombinant library or a cell genome, using ordinary cloning technology and PCR™, and the like, and by synthetic means.

As used herein, the terms “peptide,” “polypeptide,” and “protein” are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein’s or peptide’s sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both

short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. The polypeptides include natural peptides, recombinant peptides, synthetic peptides, or a combination thereof.

As used herein, the terms "fusion protein" and "chimeric protein" are used interchangeably, and refer to a compound comprised of two or more polypeptides. In some embodiments, the two or more polypeptides are covalently linked. In further embodiments, the two or more polypeptides are covalently linked by peptide bonds, linkers or disulfide bonds. Fusion proteins can be produced by a number of methods that are well known to those familiar with the art, most commonly, by introducing into a cell a vector(s) comprising a nucleic acid sequence that encodes or specifies a fusion protein amino acid sequence. Additional amino acid or polypeptides can be incorporated into a fusion protein in order to elicit additional functional properties, e.g., stability, half-life, multimerization, ease of purification. An example of such an element is a polypeptide linker.

The term "promoter" as used herein is defined as a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

A “tissue-specific” promoter is a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

5 A “signal transduction pathway” refers to the biochemical relationship between a variety of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of a cell. The phrase “cell surface receptor” includes molecules and complexes of molecules capable of receiving a signal and transmitting signal across the plasma membrane of a cell.

By the term “specifically binds,” as used herein with respect to an antibody, is meant
10 an antibody which recognizes a specific antigen, but does not substantially recognize or bind other molecules in a sample. For example, an antibody that specifically binds to an antigen from one species may also bind to that antigen from one or more species. But, such cross-species reactivity does not itself alter the classification of an antibody as specific. In another example, an antibody that specifically binds to an antigen may also bind to different allelic
15 forms of the antigen. However, such cross reactivity does not itself alter the classification of an antibody as specific. In some instances, the terms “specific binding” or “specifically binding,” can be used in reference to the interaction of an antibody, a protein, or a peptide with a second chemical species, to mean that the interaction is dependent upon the presence of a particular structure (e.g., an antigenic determinant or epitope) on the chemical species;
20 for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope “A”, the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled “A” and the antibody, will reduce the amount of labeled A bound to the antibody.

The term “subject” is intended to include living organisms in which an immune
25 response can be elicited (e.g., mammals). A “subject” or “patient,” as used therein, may be a human or non-human mammal. Non-human mammals include, for example, livestock and pets, such as ovine, bovine, porcine, canine, feline and murine mammals. Preferably, the subject is human.

As used herein, a “substantially purified” cell is a cell that is essentially free of other
30 cell types. A substantially purified cell also refers to a cell which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells. In other instances, this term refers simply to cell that have been separated from the cells with which they are naturally associated in their natural state. In some embodiments, the cells

are cultured *in vitro*. In other embodiments, the cells are not cultured *in vitro*.

A “target site” or “target sequence” refers to a genomic nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule may specifically bind under conditions sufficient for binding to occur.

5 The term “therapeutic” as used herein means a treatment and/or prophylaxis. A therapeutic effect is obtained by suppression, remission, or eradication of a disease state.

 The term “transfected” or “transformed” or “transduced” as used herein refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A “transfected” or “transformed” or “transduced” cell is one which has been transfected,
10 transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

 The term “transgene” refers to the genetic material that has been or is about to be artificially inserted into the genome of an animal, particularly a mammal and more particularly a mammalian cell of a living animal.

15 To “treat” a disease, as the term is used herein, means to reduce the frequency or severity of at least one sign or symptom of a disease or disorder experienced by a subject.

 The phrase “under transcriptional control” or “operatively linked” as used herein means that the promoter is in the correct location and orientation in relation to a polynucleotide to control the initiation of transcription by RNA polymerase and expression of
20 the polynucleotide.

 A “vector” is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses.

25 Thus, the term “vector” includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. An example of a plasmid vector is an episomal vector, wherein self-replication is driven or enhanced by regulatory elements derived from a virus, e.g., Epstein-Barr virus
30 and BK virus. Examples of viral vectors include, but are not limited to, Sendai virus vectors, adenovirus vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like.

 Ranges: throughout this disclosure, various embodiments of the invention can be presented in a range format. It should be understood that the description in range format is

merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be
5 considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Where any amino acid sequence is specifically referred to by a GENBANK®
10 accession number, the sequence is incorporated herein by reference, in part and/or in its entirety. Information associated with the accession number, such as identification of signal peptide, extracellular domain, transmembrane domain, promoter sequence and translation start, is also incorporated by reference herein, in part and/or in its entirety.

As envisioned in the present invention with respect to the disclosed compositions of
15 matter and methods, some embodiments of the invention comprise the components and/or steps disclosed herein. Some embodiments of the invention consist essentially of the components and/or steps disclosed herein. Other embodiments of the invention consist of the components and/or steps disclosed therein.

20 **Description**

Provided is a fusion protein. In some exemplary embodiments, said first component of the fusion protein blocks a chemokine receptor, e.g., CXCR4 and/or CXCR7 (via binding of a vMIP2 or V1 peptide or derivatives thereof in the fusion protein), and this blockade serves to immobilize the tumor cells and interfere with its migratory, invasive, metastatic, and other
25 tumorigenic properties; said second component of the fusion protein blocks a checkpoint inhibitor on said tumor cell, e.g., PD-L1 and/or PD-L2 (via binding of PD1 or derivatives thereof in the fusion protein), and this blockade serves to interfere with inhibition of a tumor-directed immune effector cell, e.g., an NK cell; and said third component of the fusion protein, triggers an activating receptor on the same immune effector cell, e.g., the FcγRIIIa
30 receptor (via binding of Fcγ or derivatives thereof in the fusion protein), that drives NK cell activation and promotes ADCC and ADCP.

In some exemplary embodiments, one component of the fusion protein blocks a checkpoint inhibitor on a tumor or other cell and two other components each trigger a distinct activating receptor on an immune effector cell. In some embodiments, where the checkpoint

inhibitor is on a tumor cell, the fusion protein molecularly bridges an immune effector cell and a target tumor cell. Further, the three interactions of the fusion protein, a combination of checkpoint inhibitory pathway blockade and activating receptor triggering, serve to functionally reinforce each other, all three cooperatively driving activation of said immune effector cell, e.g., an NK cell. In a preferred embodiment, the checkpoint inhibitor blocked by the fusion protein consists of PD-L1, PD-L2, CD113, CD112, CD155, or CD111, and the two activating receptors co-triggered by the fusion protein are the FcγRIIIa receptor and 4-1BB on an NK cell.

10 Fusion Proteins

Provided is fusion protein comprising Component A and/or Component B; wherein Component A comprises Component Y, Component Z₂ and Component Z₃; and wherein Component B comprises Component X', Component Z₂' and Component Z₃'. In some embodiments, Component B further comprises Component Z₁'. Thus, in some embodiments, Component B comprises Component X', Component Z₁', Component Z₂' and Component Z₃'.

In some embodiments, Component A further comprises Component Z₁. Thus, in some embodiments, Component A comprises Component Y, Component Z₁, Component Z₂ and Component Z₃.

In some embodiments, the fusion protein further comprises Component C, wherein Component C comprises Component X and Component C_L'. In further embodiments, Component C_L' comprises at least a portion of an immunoglobulin light chain. In some embodiments, the fusion protein further comprises Component D, wherein Component D comprises Component Q and Component C_L. In further embodiments, Component C_L comprises at least a portion of an immunoglobulin light chain.

In some embodiments, Component A further comprises a leader sequence. In further embodiments, the leader sequence is a human albumin leader sequence.

In some embodiments, Component B further comprises a leader sequence. In further embodiments, the leader sequence is a human albumin leader sequence.

In some embodiments, Component Y comprises a ligand domain, a receptor domain, an scFv domain or a lipocalin domain. In further embodiments, Component Y comprises at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48. In yet further

embodiments, the fusion protein binds PD-L1 or PD-L2.

In some embodiments, Component Z₁ comprises a domain of an immunoglobulin, a TNF superfamily member, a TNF-L superfamily member, a transferrin, a transferrin receptor, a human serum albumin or a lipocalin. In some embodiments, the domain of an

5 immunoglobulin is a CH1 domain. In some embodiments, the immunoglobulin is an IgG. In yet further embodiments, the immunoglobulin is an IgE.

In some embodiments, Component Z₂ comprises a domain of an immunoglobulin, a TNF superfamily member, a TNF-L superfamily member, a transferrin, a transferrin receptor, a human serum albumin or a lipocalin. In some embodiments, the domain of an

10 immunoglobulin is a CH2 domain. In some embodiments, the immunoglobulin is an IgG. In yet further embodiments, the immunoglobulin is an IgE.

In some embodiments, Component Z₃ comprises a domain of an immunoglobulin, a TNF superfamily member, a TNF-L superfamily member, a transferrin, a transferrin receptor, a human serum albumin or a lipocalin. In some embodiments, the domain of an

15 immunoglobulin is a CH3 domain. In some embodiments, the immunoglobulin is an IgG. In yet further embodiments, the immunoglobulin is an IgE.

In some embodiments, Component Z₁' comprises a domain of an immunoglobulin, a TNF superfamily member, a TNF-L superfamily member, a transferrin, a transferrin receptor, a human serum albumin or a lipocalin. In some embodiments, the domain of an

20 immunoglobulin is a CH1 domain. In some embodiments, the immunoglobulin is an IgG. In yet further embodiments, the immunoglobulin is an IgE.

In some embodiments, Component Z₂' comprises a domain of an immunoglobulin, a TNF superfamily member, a TNF-L superfamily member, a transferrin, a transferrin receptor, a human serum albumin or a lipocalin. In some embodiments, the domain of an

25 immunoglobulin is a CH2 domain. In some embodiments, the immunoglobulin is an IgG. In yet further embodiments, the immunoglobulin is an IgE.

In some embodiments, Component Z₃' comprises a domain of an immunoglobulin, a TNF superfamily member, a TNF-L superfamily member, a transferrin, a transferrin receptor, a human serum albumin or a lipocalin. In some embodiments, the domain of an

30 immunoglobulin is a CH3 domain. In some embodiments, the immunoglobulin is an IgG. In yet further embodiments, the immunoglobulin is an IgE.

In some embodiments, Component X' comprises a virus-derived peptide, a ligand-derived, a receptor-derived peptide or a high-throughput screen (HTS)-selected peptide. In some embodiments, Component X' comprises a peptide sequence that binds a chemokine

receptor, a cytokine receptor, a counter-receptor for a functional ligand, an integrin, a ligand, or a part of a membrane-signaling complex. In some embodiments, Component X' comprises at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3,

5 CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48. In further embodiments, Component X' comprises at least a portion of vMIP-II. In further embodiments, Component X' comprises V1 or V1delta polypeptides. In yet further embodiments, the fusion protein binds CXCR4.

In some embodiments, Component Q comprises at least a portion of PD-1, TIGIT,
10 CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48.

In some embodiments, Component X comprises a virus-derived peptide, a ligand-derived peptide, a receptor-derived peptide or an HTS-selected peptide. In further
15 embodiments, Component X comprises V1 or V1delta polypeptides. In further embodiments, the fusion protein binds CXCR4.

In some embodiments, Component Y and Component Z₂ are connected via a hinge, for example an IgG hinge. In some embodiments, Component Z₁' and Component Z₂' are connected via a hinge, for example an IgG hinge. In some embodiments, Component X' and
20 Component Z₁' are connected via a linker. In some embodiments, Component X and Component C_L are connected via a linker.

In some embodiments, the fusion protein comprises Component A and Fc. In further embodiments, the fusion protein comprises Component A and human FcB (hFcB).

In some embodiments, the fusion protein comprises Component B and Fc. In further
25 embodiments, the fusion protein comprises Component B and human FcA (hFcA).

In some embodiments, Component A and Component B are covalently linked. In some embodiments, the covalent linkage is via a disulfide bond, or via a linker.

In some embodiments, Component A and Component B are not covalently linked. In some embodiments, Component A and Component B are held together via knobs-into-holes
30 interactions. In some embodiments, Component A and Component B comprise knobs-into-holes mutations and covalent linkage via disulfide bond. In some embodiments, Component A comprises mutations Y349C and T366W, and Component B comprises mutations D356C, T366S, L368A and Y407V ("Knobs-into-holes" mutations). The positions of mutations and alterations in these component chains are defined by the Kabat numbering convention

(Johnson, G and Wu, TT, (2001) *Nucleic Acids Res.*, 28(1), 214-18).

In some embodiments, the fusion protein comprises Component A and a domain of an immunoglobulin. In some embodiments, the immunoglobulin domain is an Fc domain.

In some embodiments, the fusion protein comprises Component B and a domain of an immunoglobulin. In some embodiments, the immunoglobulin domain is an Fc domain.

In some embodiments, Component B and Component C are covalently linked. In some embodiments, the covalent linkage is via a disulfide bond.

In some embodiments, Component B and Component C are not covalently linked.

In some embodiments, Component A and Component D are covalently linked. In some embodiments, the covalent linkage is via a disulfide bond.

In some embodiments, Component A and Component D are not covalently linked.

In some embodiments, the fusion protein binds a receptor or ligand on an immune cell.

In some embodiments, the receptor is an Fc receptor.

Also provided is a pharmaceutical composition comprising a pharmaceutically acceptable carrier and the fusion protein of any one of the preceding embodiments.

Also provided is a method of treating a proliferative disorder in a patient comprising administering a therapeutically effective amount of the fusion protein of any one of the preceding embodiments to a patient in need of such treatment. In some embodiments, the proliferative disorder is cancer. In further embodiments, the cancer is a solid tumor. In yet further embodiments, the cancer is pancreatic cancer, breast cancer, ovarian cancer, bladder cancer, melanoma, glioblastoma, acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), multiple myeloma, colon cancer, lung cancer, liver cancer, or any solid or liquid tumor type.

The present invention provides novel fusion proteins useful for treating proliferative disorders, such as cancer. On a cell expressing a receptor or ligand for Component Y and a receptor or ligand for Component X', the fusion protein of the invention may block one or both receptors or ligands. Thus, on a cell co-expressing a receptor or ligand for Component Y and a receptor or ligand for Component X', the fusion protein of the invention may lead to death, immobilization, and clearance of the tumor cell. Furthermore, on a cell expressing a receptor or ligand for Component Z₃ or Component Z₃', for instance on a Natural Killer (NK) cell, the fusion protein of the invention may trigger the receptor or ligand, and may lead to activation of the cell. Thus, the fusion protein of the present invention may mediate its activity by spanning two neighboring cells. Further, the fusion protein of the present

invention may bind three or more distinct molecules on said cells. In some embodiments, the fusion protein may act to treat a disease such as cancer by causing inhibition or a reduction in certain cells, or activation or an increase in certain cells.

5 **Component A**

Component A comprises Component Y, Component Z₂ and Component Z₃.

Component Y

In some embodiments, Component Y comprises a ligand domain, a receptor domain,
10 a scFv domain or a lipocalin domain. In further embodiments, Component Y comprises at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48.

An exemplary sequence for component Y comprises or consists of:

15 GWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFVLNWYRMSPSN
QTDKLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYLCGAISLAP
KAQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQ

(SEQ ID NO: 1) Human PD-1 extracellular domain, GENBANK® Accession No.

NM_005018.3.

20

An exemplary sequence for component Y comprises or consists of:

GWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSESFHVVWHRESPG
QTDTLAAFPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRNDSGTYVCGVISLAP
KIQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQ

25 (SEQ ID NO: 2) High affinity human PD-1 extracellular domain.

An exemplary sequence for component Y comprises or consists of:

MMTGTIETTGNISAEKGGSIILQCHLSSTTAQVTQVNWEQQDQLLAICNADLG
WHISPSFKDRVAPGPGLGLTLQSLTVNDTGEYFCIYHTYPDGTYTGRIFLEVLESSVA
30 EHGARFQIP

(SEQ ID NO: 3) human TIGIT extracellular domain, GENBANK® Accession No.

NM_173799.4.

An exemplary sequence for component Y comprises or consists of:

35 VWEKTVNTEENVYATLGSDVNLTCQTQTVGFFVQMQWSKVTNKIDLIAVYHPQYG
FYCAYGRPCESLVTFETPENGSKWTLHLRNMSCSVSGRYECMLVLYPEGIQTKIYN
LLIQTHVTADEWNSNHTIEIEINQTLIPCFQNSSSKISSEFTYAWSVENSSTDSWVLLS
KGIKEDNGTQETLISQNHLSNSTLLKDRVKLGTDYRLHLSPVQIFDDGRKFSCHIRVG

PNKILRSSTTVKVFAKPEIPVIVENNSTDVLVERRFTCLLKNVFPKANITWFDGSFLH
DEKEGIYITNEERKKGKDGFLKSVLTRVHSNKPAQSDNLTWCMALSPVPGNKVW
NISSEKITFLLGSEISSTDPPLSVTESTLDTQPSPASSVSPARYPATSSVTLDVVSALRPN
TTPQPSNSSMTTRGFNYPWTSSGTDTKKSVSRIPSETYSSSPSGAGSTLHDNVFTSTAR
5 AFSEVPTTANGSTKTNHVVHITGIVVNKPKDGM

(SEQ ID NO: 4) human CD96 extracellular domain, GENBANK® Accession No.

NM_198196.2.

An exemplary sequence for component Y comprises or consists of:

10 MGHRTLVLPWVLLTLCVTAGTPEVWVQVRMEATELSSFTIRCGFLGSGSISLV
TVSWGPNAGAGGTTLAVLHPERGIRQWAPARQARWETQSSISLILEGSGASSPCANT
TFCKKFASFPEGSWEACGSLPPSSDPGLSAPPTPAPILRAD

(SEQ ID NO: 5) human CD112R extracellular domain, GENBANK® Accession No.

NM_024070.3.

15

An exemplary sequence for component Y comprises or consists of:

EEVLWHTSVPFENMSLECVYPSMGILTQVEWFKIGTQQDSIAIFSPTHGMVI
RKPYAERVYFLNSTMASNNMTLFFRNASEDDVGYYSCSLYTYPQGTWQKVIQVVQS
20 DSFEAAVPSNSHIVSEPGKNVTLTCQPQMTWPVQAVRWEKIQPRQIDLLTYCNLVHG
RNFTSKFPRQIVSNCSHGRWSVIVIPDVTVSDSGLYRCYLQASAGENETFVMRLTVA
EGKTDNQYTLFVA

(SEQ ID NO: 6) human CD226 extracellular domain, GENBANK® Accession No.

NM_006566.3.

25

An exemplary sequence for component Y comprises or consists of:

QNLFTKDVTVIEGEVATISCQVNKSDDSVIQLLNPNRQTIYFRDFRPLKDSRFQ
LLNFSSSELKVSLTNVVISDEGRYFCQLYTDPPQESYTTITVLVPPRNLMIDIQKDTAV
EGE
30 EIEVNCTAMASKPATTIRWFKGNTELKKGKSEVEEWSDMYTVTSQMLMLKVHKEDDG
VPV
ICQVEHPAVTGNLQTQRYLEVQYKPQVHIQMTYPLQGLTREGDALELTCEAIGKPQP
V
MVTWVRVDDEMPQHAVLSGPNLFINNLNKTNDNGTYRCEASNIVGKAHSDYMLYVY
35 DPPTTIPPTTTTTTTTTTTTTTILTIITDSRAGEEGSIRAVDH

(SEQ ID NO: 7) human NECL2 extracellular domain, GENBANK® Accession No.

NM_014333.3.

An exemplary sequence for component Y comprises or consists of:

40 PIIVEPHVTA VWGKNVSLKCLIEVNETITQISWEKIHGKSSQTVA VHHHPQYGF
VQGEYQGRVLFKNYSLNDATITLHNIGFSDSGKYICKAVTFPLGNAQSSTTVTVLVEP
TVSLIKGPD SLIDGGNETVAAICIAATGKPV AHIDWEGDLGEMESTTTSFPNETATIIS
QYKLFPTRFARGRRITCVVKHPALEKDIRYSFILDIQYAPEVSVTGYDGNWVFGVGRKG

VNLKCNADANPPPFKSVWSRLDGQWPDGLLASDNTLHFVHPLTFNYSGVYICKVTN
SLGQRSDQKVIYISDPPTTTTLQPTIQWHPSTADIEDLATEPKKLPFPLSTLATIKDD
(SEQ ID NO: 8) human CD113 extracellular domain, GENBANK® Accession No.
NM_015480.3.

5

An exemplary sequence for component Y comprises or consists of:

AEPHSLRYNLTVLSWDGVSQSGFLTEVHLDGQPFLRCDRQKCRAKPQGQWA
EDVLGNKTWDRETRDLTGNGKDLRMTLAHIKDQKEGLHSLQEIRVCEIHEDNSTRSS
QHFIYDGEFLFSQNLETKEWTMPQSSRAQTLAMNVRNFLKEDAMKTKTHYHAMH
10 ADCLQELRRYLKSGVVLRRTVPPMVNVTREASEGNITVTCRASGFYPWNITLSWRQ
DGVSLSHDTQQWGDVLPDNGTYQTWVATRICQGEEQRFTCYMEHSGNHSTHPVP
SGKVLVLQSHW
(SEQ ID NO: 9) human MICA (MHC-I polypeptide-related sequence A) extracellular
domain, GENBANK® Accession No. NM_000247.3.

15

Leader Sequence

An exemplary sequence for the leader sequence comprises or consists of:

MKWVTFISLLFLFSSAYS
(SEQ ID NO: 10) human albumin leader sequence.

20

Hinge

An exemplary sequence for the hinge comprises or consists of:

EPKSSDKTHTCPPCPAPELLGG
(SEQ ID NO: 11) human IgG hinge.

25

Component Z₂

An exemplary sequence for component Z₂ comprises or consists of:

PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAK
30 (SEQ ID NO: 12) IgG1.

Component Z₃

An exemplary sequence for component Z₃ comprises or consists of:

GQPREPQVCTLPSPRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPPV
35 LQSDGSFFFLYSKLTVDKSRWQQGNVDFSCSVLHEALHSHYTKQKSLSLSPGK
(SEQ ID NO: 13) IgG1.

An exemplary sequence for component A comprises or consists of:

Human PD-1-hFcA

5 MKWVTFISLLFLFSSAYSASGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSE
SFVLNWMSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRN
DSGYLTCGAILAPKAQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQ**EPKSSDKTH**
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTI
SKAKGPREPOVCTLPSPRDELTKNOVSLWCLVKGFYPSDIAVEWESNGOPENNYK
TTPVLDSGDSFFLYSKLTVDKSRWQGNVFSCSVLHEALHSHYTOKSLSLSPGK
10 (SEQ ID NO: 14)

Italics – human albumin leader sequence

Wavy underline – human PD-1 extracellular domain; Y domain

Bold – IgG1 hinge region

15 Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

Another exemplary sequence for component A comprises or consists of:

20 High affinity human PD-1-hFcA
MKWVTFISLLFLFSSAYSASGWFLDSPDRPWNPPTFSPALLVVTEGDNATFTCSFSNTSE
SFHVVWHRESQSGQTDTLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVRARRND
SGTYVCGVISLAPKIQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQ**EPKSSDKTHT**
CPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
25 VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTIS
KAKGPREPOVCTLPSPRDELTKNOVSLWCLVKGFYPSDIAVEWESNGOPENNYKT
TPPVLDSDGDSFFLYSKLTVDKSRWQGNVFSCSVLHEALHSHYTOKSLSLSPGK
(SEQ ID NO: 15)

30 *Italics* – human albumin leader sequence

Wavy underline – high affinity human PD-1 extracellular domain; Y domain

Bold – IgG1 hinge region

Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

35

Another exemplary sequence for component A comprises or consists of:

Human CD112R-hFcA
MKWVTFISLLFLFSSAYSASMGHRTLVLWPVLLTLCVTAGTPEVWVQVRMEATELSSF
40 TIRCGFLGSGSISLTVSWGGPNGAGGTTLAVLHPERGIRQWAPARQARWETQSSISL
ILEGSGASSPCANTTFCCKFASFPEGSWEACGSLPPSSDPGLSAPPTPAPILRADE**PKSS**
DKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN
WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPA
PIEKTISKAKGPREPOVCTLPSPRDELTKNOVSLWCLVKGFYPSDIAVEWESNGOPE
45 NNYKTTTPVLDSGDSFFLYSKLTVDKSRWQGNVFSCSVLHEALHSHYTOKSLSLSP
GK
(SEQ ID NO: 16)

Italics – human albumin leader sequence

Wavy underline – human CD112R extracellular domain; Y domain

Bold – IgG1 hinge region

Double underline – IgG1 Z₂ domain

5 **Bold underline** – IgG1 Z₃ domain

Another exemplary sequence for component A comprises or consists of:

Human TIGIT-hFcA

10 MKWVTFISLLFLFSSAYSMMTGTIETTGNISAEKGGSIILQCHLSSTTAQVTQVNWEQQ
DQLLAICNADLGWHISPSFKDRVAPGPGLGLTLQSLTVNDTGEYFCIYHTYPDGTYT
GRIFLEVLESSVAEHGARFOIPEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKGPREPOVCTLPPSRDELTKNOVS
15 LWCLVKGFYPSDIAVEWESNGOPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWOOG
NVFSCSVLHEALHSHYTOKSLSLSPGK
(SEQ ID NO: 17)

Italics – human albumin leader sequence

20 Wavy underline – high affinity human TIGIT extracellular domain; Y domain

Bold – IgG1 hinge region

Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

25 Another exemplary sequence for component A comprises or consists of:

Human CD96-hFcA

*MKWVTFISLLFLFSSAYS*VWEKTVNTEENVYATLGSDVNLTCQTQTVGFFVOMOWSK
VTNKIDLIAVYHPQYGFYCAYGRPCESLVTFTETPENGSKWTLHLRNMSCSVSGRYE
30 CMLVLYPEGIQTKIYNLLIQTHVTADEWNSNHTIEIEINQTLEIPCFQNSSSKISSEFTY
AWSVENSSTDSWVLLSKGIKEDNGTQETLISQNHLSNSTLLKDRVKLGTDYRLHLSP
VQIFDDGRKFSCHIRVGPNKILRSSTTVKVFAKPEIPVIVENNSTDVLVERRFTCLLKN
VFPKANITWFDGSFLHDEKEGIYTNEERKKGDGFLELKSVLTRVHSNKPAQSDNLT
WCMALSPVPGNKVWNISSEKITELLGSEISSTDPPLSVTESTLDTQSPASSVSPARYP
35 ATSSVTLVDVSALRPNTTPQPSNSSMTTRGFNYPWTSSGTDTKKSVSRIPSETYSSSPS
GAGSTLHDNVFTSTARAFSEVPTTANGSTKTNHVHITGIVVNKPKDGMEPKSSDKT
HTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYV
DGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEK
TISKAKGPREPOVCTLPPSRDELTKNOVSLWCLVKGFYPSDIAVEWESNGOPENNY
40 KTTTPVLDSDGSFFLYSKLTVDKSRWOOGNVFSCSVLHEALHSHYTOKSLSLSPGK
(SEQ ID NO: 18)

Italics – human albumin leader sequence

Wavy underline – human CD96 extracellular domain; Y domain

45 **Bold** – IgG1 hinge region

Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

Another exemplary sequence for component A comprises or consists of:

Human CD226-hFcA

5 *MKWVTFISLLFLFSSAYSEEV*LWHTSVFPAENMSLECVYPSMGILTQVEWFKIGTQQDS
IAIFSPTHGMVIRKPYAERVYFLNSTMASNNMTLFFRNA SEDDVGYYSCLY TYPQG
TWQKVIQVVQSDSFEAAVPSNSHIVSEPGKNVTLTCQPQMTWPVQAVRWEKIQPRQI
DLLTYCNLVHGRNFTSKFPRQIVSNCSHGRWSVIVIPDVTVSDSGLYRCYLQASAGE
NETFVMRLTVAEGKTDNQYTLFVA**EPKSSDKTHTCPPCPAPELLGGPSVFLFPPKP**
10 KDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV
VSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKGOPREPOVCTLPSPRDEL
TKNOVSLWCLVKGFYPSDIAVEWESNGOPENNYKTTTPVLSDSGSFFLYSKLTVDKSR
WOQGNVFSCSVLHEALHSHYTOKSLSLSPGK
(SEQ ID NO: 19)

15 *Italics* – human albumin leader sequence

Wavy underline – human CD226 extracellular domain; Y domain

Bold – IgG1 hinge region

Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

20

Another exemplary sequence for component A comprises or consists of:

Human NECL2-hFcA

25 *MKWVTFISLLFLFSSAYSQN*LFTKDVTVIEGEVATISCQVNKSDDSVIQLLNPNRQTIYF
RDFRPLKDSRFQLLNFSSELKVSLTNVISISDEGRYFCQLYTDPPQESYTTITVLVPPR
NLMIDIQKDTAVEGEEIEVNCTAMASKPATTIRWFKGNTELKKGSEVEEWSDMYTV
TSQMLKLVHKEDDGVPVICQVEHPAVTGNLQTORYLEVQYKPQVHIQMTYPLOGLT
REGDALELTCEAIGKPQPMVMTWVRVDDEMPQHAVLSGPNLFINNLNKT DNGTYRC
EASNIVGKAHSDYMLYVYDPPTTIPPTTTTTTTTTTTTTILTIITDSRAGEEGSIRAVD
30 **HEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDP**
EVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSN
KALPAPIEKTISKAKGOPREPOVCTLPSPRDELTKNOVSLWCLVKGFYPSDIAVEWES
NGOPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQGNVFSCSVLHEALHSHYTOK
35 SLSLSPGK
(SEQ ID NO: 20)

Italics – human albumin leader sequence

Wavy underline – human NECL2 extracellular domain; Y domain

Bold – IgG1 hinge region

40 Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

Another exemplary sequence for component A comprises or consists of:

Human CD113-hFcA

45

*MKWVTFISLLFLFSSAYSASPI*IVEPHVTAVWGKNVSLKCLIEVNETITQISWEKIHGKSS
QTVAVHHPQYGFVSQGEYQGRVLFKNYSLNDATITLHNIGFSDSGKYICKAVTFPLG
NAQSSTTVTVLVEPTVSLIKGPDSLIDGGNETVAAICIAATGKPV AHIDWEGDLGEME

STTTSPNETATIISQYKLFPTRFARGRRITCVVKHPALEKDIRYSFILDIQYAPEVSVT
 GYDGNWFVGRKGVNLCNADANPPPFKSVWSRLDGQWPDGLLASDNTLHFVHPLT
 FNYSGVYICKVTNSLGQRSDQKVIYISDPPTTTTLOPTIQWHPSTADIEDLATEPKKLP
 FPLSTLATIKDDEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
 5 VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
 GKEYKCAVSNKALPAPIEKTISKAKGPREPOVCTLPPSRDELTKNOVSLWCLVKGF
 YPSDIAVEWESNGOPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQOQNVFSCSVLH
EALHSHYTOKSLSLSPGK

(SEQ ID NO: 21)

10

Italics – human albumin leader sequence

Wavy underline – human CD113 extracellular domain; Y domain

Bold – IgG1 hinge region

Double underline – IgG1 Z₂ domain

15

Bold underline – IgG1 Z₃ domain

Another exemplary sequence for component A comprises or consists of:

Human MICA-hFcA (MHC-I polypeptide-related sequence A)

20 MKWVTFISLLFLFSSAYSASAEPHSLRYNLTVLSDGSGVQSGFLTEVHLDGQPFLRCDR
 QKCRAKPQGOWAEDVLGNKTDRETRDLTGNGKDLRMTLAHIKDOKEGLHSLQEI
 RVCEIHEDNSTRSSQHFYYDGELFSLQNLETKEWTMPQSSRAOTLAMNVRNFLKED
 AMKTKTHYHAMHADCLQELRRYLKSGVVLRRTVPPMVNVTRSEASEGNITVTCRA
 SGFYFWNITLSWRQDGVSLSHDTQQWGDVLPDGNQTYQTWVATRICQGEQRFCTC
 YMEHSGNHSTHPVPSGKVLVLQSHWEPKSSDKTHTCPPCPAPELLGGPSVFLFPPK
 PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRV
 25 VSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKGPREPOVCTLPPSRDELTKNOVSLWCLVKGFYPSDIAVEWESNGOPENNYKTPPVLDSDGSFFLYSKLTVDKSR
 WOQNVFSCSVLHEALHSHYTOKSLSLSPGK

(SEQ ID NO: 22)

30

Italics – human albumin leader sequence

Wavy underline – human MICA extracellular domain; Y domain

Bold – IgG1 hinge region

Double underline – IgG1 Z₂ domain

35

Bold underline – IgG1 Z₃ domain

Human FcA

40 MKWVTFISLLFLFSSAYSASEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
 RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
 HQDWLNGKEYKCAVSNKALPAPIEKTISKAKGPREPOVCTLPPSRDELTKNOVSLW
 CLVKGFYPSDIAVEWESNGOPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQOQNV
 FSCSVLHEALHSHYTOKSLSLSPGK

(SEQ ID NO: 23)

45

Italics – human albumin leader sequence

Bold – IgG1 hinge region

Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

Component B

- 5 Component B comprises Component X', Component Z₂' and Component Z₃'. In some embodiments, Component X' comprises a virus-derived peptide, a ligand-derived peptide, a receptor-derived peptide or an HTS-selected peptide. In some embodiments, Component X' comprises at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94),
- 10 MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48.

Component X'

An exemplary sequence for component X' comprises or consists of:

- 15 WPPPGTGDVVVQAPTQVPGFLGDSVTLPCYLQVPNMEVTHVSQLTWARHGESGSM
AVFHQTQGPSYSESKRLEFVAARLGAELRNASLRMFGLRVEDEGNYTCLFVTFPQGS
RSVDIWLRLAKPQNTAEVQKVQLTGEPVPMARCVSTGGRPPAQITWHSDLGGMPN
TSQVPGFLSGTVTVTSLWILVPSSQVDGKNVTCKVEHESFEKPQLLTVNLTVYYPPEV
SISGYDNNWYLGQNEATLTCDARSNPEPTGYNWSTTMGPLPPFAVAQGAQLLIRPV
20 DKPINTTLICNVTNALGARQAELTVQVKEGPPSEHSGMSRN
(SEQ ID NO: 24) human CD155 (Polio virus receptor, PVR) extracellular domain,
GENBANK® Accession No. NM_006505.5.

An exemplary sequence for component X' comprises or consists of:

- 25 MMTGTIETTGNISAEKGGSIILQCHLSSTTAQVTQVNWEQQDQLLAICNADLGWHISP
SFKDRVAPGPGGLGLTLQSLTVNDTGEYFCIYHTYPDGTYTGRIFLEVLESSVAEHGAR
FQIP
(SEQ ID NO: 25) human TIGIT (T cell immunoreceptor with Ig and ITIM domains)
extracellular domain, GENBANK® Accession No. NM_173799.4.

30

An exemplary sequence for component X' comprises or consists of:

- LEDGYKVEVGKNAYLPCSYTLPTSGTLVPMCWGKGFCPWSQCTNELLRTDE
RNVTYQKSSRYQLKGDNLKGDVSLIKNVTLDDHGTCCRIQFPGLMNDKKLELKL
DIKAAKVTPAQTAHGDSTTASPRTLTTTERNGETQTLVTLHNNNGTKISTWADEIKD
35 SGETIR
(SEQ ID NO: 26) Mouse Tim-3 extracellular domain, GENBANK® Accession No.
NM_134250.2. Mouse TIM-3 possesses good binding to human galactin-9.

An exemplary sequence for component X' comprises or consists of:

PIIVEPHVTA VWGKNVSLKCLIEVN ETITQISWEKIHGKSSQTVAVHHPQYGF SVQGE
YQGRVLFKNYSLNDATITLHNIGFSDSGKYICKAVTFPLGNAQSSTTVTVLVEPTVSLI
5 KGPDSLIDGGNETVAAICIAATGKPV AHIDWEGDLGEMESTTTSFPNETATIISQYKLF
PTRFARGRRITCVVKHPALEKDIRYSFILD IQYAPEVSVTGYDGNW FVGRKGVNLKC
NADANPPPFKSVWSRLDGQWPDGLLASDNTLHFVHPLTFNYSGVYICKVTNSLGQR
SDQKVIYISDPPTTTTLQPTIQWHPSTADIEDLATEPKKLPFPLSTLATIKDD
(SEQ ID NO: 27) human CD113 extracellular domain, GENBANK® Accession No.
10 NM_015480.3.

Leader Sequence

An exemplary sequence for the leader sequence comprises or consists of:

MKWVTFISLLFLFSSAYS
15 (SEQ ID NO: 10) human albumin leader sequence.

Hinge

An exemplary sequence for the hinge comprises or consists of:

EPKSSDKTHTCPPCPAPELLGG
20 (SEQ ID NO: 11) human IgG hinge.

Component Z₂'

An exemplary sequence for component Z₂ comprises or consists of:

PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
25 QYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAK
(SEQ ID NO: 12) IgG1.

Component Z₃'

An exemplary sequence for component Z₃ comprises or consists of:

GQPREPQVCTLPSPRDELTKNQVSLWCLVKGFYPSDIAVEWESNGQPENNYKTTTPPV
30 LDSDGSFFLYSKLTVDKSRWQQGNV FSCSVLHEALHSHYTQKSLSLSPGK
(SEQ ID NO: 13) IgG1.

An exemplary sequence for component B comprises or consists of:

35 Human CD155-hFcB

MKWVTFISLLFLFSSAYSASWPPPGTG DVVVQAPTQVPGFLGDSVTLPCYLQVPNMEV
THVSQLTWARHGESGSMVVFHQ TQGPSYSESKRLEFVAARLGAELRNASLRMFGLR
VEDEGNYTCLFVTFPQGSRSVDIWLRLAKPQNTAEVQKVQLTGEPVPMARCVSTG

GRPPAQITWHS DLGGMPNTS QVPGF LSGTVTVTSLWILVPSSQVDGKNVTCKVEHES
FEKPQLLTVNLTVYYPPEVSISGYDNNWYLGQNEATLTCDARSNPEPTGYNWSTTM
GPLPPFAVAQGAQLLRPVDKPIINTTLCNVTNALGARQAELTVQVKEGPPSEHSGMS
RNEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHED
5 PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVS
NKALPAPIEKTISKAKGQPREPOVYTLPPSRCELTKNOVSLSCAVKGFYPSDIAVEWE
SNGOPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQOQGNVFSCSVLHEALHSHYTO
KSLSLSPGK

(SEQ ID NO: 32)

10

Italics – human albumin leader sequence

Wavy underline – human CD155 (Polio virus receptor, PVR) extracellular domain; X' domain

Bold – IgG1 hinge region

15 Double underline – IgG1 Z'2 domain

Bold underline – IgG1 Z'3 domain

Another exemplary sequence for component B comprises or consists of:

Human TIGIT-hFcB

20

MKWVTFISLLFLFSSAYSASMMTGTIETGNISAEEKGGSIILOCHLSSTTAQVTQVNWEQ
QDQLLAICNADLGWHISPSFKDRVAPGPGLGLTLOSLTVNDTGEYFCIYHTYPDGT
TGRIFLEVLESSVAEHGARFQIPEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDT
LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVL
25 TVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKGQPREPOVYTLPPSRCELTKNOV
SLSCAVKGFYPSDIAVEWESNGOPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQOQ
NVFSCSVLHEALHSHYTOKSLSLSPGK

(SEQ ID NO: 33)

30 *Italics* – human albumin leader sequence

Wavy underline – human TIGIT (T cell immunoreceptor with Ig and ITIM domains) extracellular domain; X' domain

Bold – IgG1 hinge region

Double underline – IgG1 Z'2 domain

35 Bold underline – IgG1 Z'3 domain

Another exemplary sequence for component B comprises or consists of:

Mouse TIM-3-hFcB

40

MKWVTFISLLFLFSSAYSASLEDGYKVEVGKNAYLPCSYTLPTSGTLVPMCWGKGFCP
WSQCTNELLRTDERNVITYQSSRYQLKGDNLKGDVSLIKNVTLDDHGTYYCCRIQFP
GLMNDKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTTERNGETQTLVTLHNNN
GTKISTWADEIKDSGETIREPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCAVSNKALPAPIEKTISKAKGQPREPOVYTLPPSRCELTKNOVSL
CAVKGFYPSDIAVEWESNGOPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWQOQGNV
45 FSCSVLHEALHSHYTOKSLSLSPGK

(SEQ ID NO: 34)

Italics – human albumin leader sequence

Wavy underline – mouse TIM-3 extracellular domain; X' domain

Bold – IgG1 hinge region

5 Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

Another exemplary sequence for component B comprises or consists of:

Human CD113-hFcB

10 MKWVTFISLLFLFSSAYSASPIIVEPHVTAVWGKNVSLKCLIEVNETITQISWEKIHGKSS
QTVAVHHPQYGFVSQGEYQGRVLFKNYSLNDATITLHNIGFSDSGKYICKAVTEPLG
NAQSSTTVTVLVEPTVSLIKGPDSLIDGGNETVAAICIAATGKPVAHIDWEGDLGEME
STTTSFPNETATIISQYKLFPTRFARGRRITCVMKHPALEKDIRYSFILDIQYAPEVSVT
GYDGNWFVGRKGVNLKCNADANPPPFSVWSRLDGQWPDGLLASDNTLHFVHPLT
FNYSGVYICKVTNSLGORSOKVIYISDPPTTTTLOPTIQWHPSTADIEDLATEPKKLP
15 FPLSTLATIKDDEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTC
VVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
GKEYKCAVSNKALPAPIEKTISKAKGPREPOVYTLPPSRCELTKNQVSLSCAVKGF
YPSDIAVEWESNGOPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWOOGNVFSCSVLH
EALHSHYTOKSLSLSPGK
20 (SEQ ID NO: 35)

Italics – human albumin leader sequence

Wavy underline – human CD113 extracellular domain; X' domain

Bold – IgG1 hinge region

25 Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

Human FcB

30 MKWVTFISLLFLFSSAYSASEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMIS
RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVL
HQDWLNGKEYKCAVSNKALPAPIEKTISKAKGPREPOVYTLPPSRCELTKNQVSL
CAVKGFYPSDIAVEWESNGOPENNYKTTTPVLDSDGSFFLVSKLTVDKSRWOOGNV
FSCSVLHEALHSHYTOKSLSLSPGK
(SEQ ID NO: 36)

35

Italics – human albumin leader sequence

Bold – IgG1 hinge region

Double underline – IgG1 Z₂ domain

Bold underline – IgG1 Z₃ domain

40

Component B comprising Component Z₁'

In some embodiments, Component B further comprises Component Z₁'. Thus, in some embodiments, Component B comprises Component X', Component Z₁', Component Z₂' and Component Z₃'.

In some embodiments, Component X' comprises a virus-derived peptide, a ligand-derived peptide, a receptor-derived peptide or an HTS-selected peptide. In some embodiments, Component X' comprises at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48. In further embodiments, Component X' comprises at least a portion of CD155, TIGIT, TIM-3 or CD113.

Component X'

10 An exemplary sequence for component X' comprises or consists of:

LGASWHRPDKCCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSK
DWVKKLMQQLPVTAR
(SEQ ID NO: 37) vMIP1I, GENBANK® Accession No. YP_001129362.

15 An exemplary sequence for component X' comprises or consists of:

LGASWHRPDKCCLGYQKRPLP
(SEQ ID NO: 38) V1

An exemplary sequence for component X' comprises or consists of:

20 LGASWHRPDKC~~A~~LGYQKRPLP
(SEQ ID NO: 39) V1 Δ

An exemplary sequence for component X' comprises or consists of:

25 LGASWHRPD~~A~~CALGYQKRPLP
(SEQ ID NO: 40) V1 Δ mut

An exemplary sequence for component X' comprises or consists of:

30 LGASWHRPDKCCLGYQKRPLPQVLLSSWYPTSQL
(SEQ ID NO: 41) Vp1

An exemplary sequence for component X' comprises or consists of:

LGASWHRPDKC~~A~~LGYQKRPLPQVLLSSWYPTSQL
(SEQ ID NO: 42) Vp1 Δ

35 An exemplary sequence for component X' comprises or consists of:

LGASWHRPD~~A~~CALGYQKRPLPQVLLSSWYPTSQL
(SEQ ID NO: 43) Vp1 Δ mut

Leader Sequence

An exemplary sequence for the leader sequence comprises or consists of:

MKWVTFISLLFLFSSAYS

(SEQ ID NO: 10) human albumin leader sequence.

5 Component Z₁'

An exemplary sequence for component Z₁' comprises or consists of:

SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEVTVSWNSGALTSGVHTFPAVL
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVX

(SEQ ID NO: 45)

10

Hinge

An exemplary sequence for the hinge comprises or consists of:

EPKSSDKTHTCPPCPAPELLGG

(SEQ ID NO: 11)

15

Component Z₂'

An exemplary sequence for component Z₂' comprises or consists of:

PSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE
QYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAK

(SEQ ID NO: 12)

20

Component Z₃'

An exemplary sequence for component Z₃' comprises or consists of:

GQPREPQVYTLPPSRCELTKNQVSLSCAVKGFYPSDIAVEWESNGQPENNYKTTPPV
LDSGDSFFLVSKLTVDKSRWQQGNVFSCSVLHEALHSHYTQKSLSLSPGK

(SEQ ID NO: 48)

25

An exemplary sequence for component B comprises or consists of:

vMII-CH'-hFcB

MKWVTFISLLFLFSSAYSLGASWHRPDKCCLGYQKRPLQVLLSSWYPTSQLCSKPGVI
FLTKRGRQVCADKSKDWVKLMQQLPVTARSSASTKGPSVFPLAPSSKSTSGGTA
ALGCLVKDYFPEVTVSWNSGALTSGVHTFPAVLOSSGLYSLSSVVTVPSSSLGTO
TYICNVNHKPSNTKVDKKVXEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTL
MISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRCELTKNOVS
LSCAVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGDSFFLVSKLTVDKSRWQQ
NVFSCSVLHEALHSHYTQKSLSLSPGK

(SEQ ID NO: 49)

40 *Italics* – human albumin leader sequence

Wavy underline – vMII

Wavy underline - Amino acid differences between V1, V1Δ and V1Δmut

underline – CH'

– IgG1 hinge region

Double underline – IgG1 Z'2 domain

5 **underline** – IgG1 Z'3 domain

An exemplary sequence for component B comprises or consists of:

V1-CH' - hFcB

10 MKWVTFISLLFLFSSAYSLGASWHRPDKCCLGYQKRPLPSSASTKGPSVFPLAPSSKS
TSGGTAALGCLVKDYFPEVTVSWNSGALTSGVHTFPAVLOSSGLYSLSSVVTVPS
SSLGTOTYICNVNHKPSNTKVDKKVXEPKSSDKTHTCPPCPAPELLGGPSVLEFP
PKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKGPREPOVYTLPPSRCE
LTKNQVSLSCAVKGFYPSDIAVEWESNGOPENNYKTTTPVLDSDGSFFLVSKLTVDK
15 **SRWOOGNVFSCSVLHEALHSHYTOKSLSLSPGK**
(SEQ ID NO: 50)

Italics – human albumin leader sequence

Wavy underline – V1 (V1 is the first 21 amino acids of VMIPII)

20 **Wavy underline** - Amino acid differences between V1, V1Δ and V1Δmut

underline – CH'

– IgG1 hinge region

Double underline – IgG1 Z'2 domain

25 **underline** – IgG1 Z'3 domain

An exemplary sequence for component B comprises or consists of:

V1Δ-CH' - hFcB

MKWVTFISLLFLFSSAYSLGASWHRPDKCALGYQKRPLP
30 **SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEVTVSWNSGALTSGVHTFPA**
AVLOSSGLYSLSSVVTVPSSSLGTOTYICNVNHKPSNTKVDKKVXEPKSSDKTHT
CPPCPAPELLGGPSVLEFPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTIS
KAKGPREPOVYTLPPSRCELTKNOVSLSCAVKGFYPSDIAVEWESNGOPENNYKTT
PPVLDSDGSFFLVSKLTVDKSRWOOGNVFSCSVLHEALHSHYTOKSLSLSPGK
35 (SEQ ID NO: 51)

Italics – human albumin leader sequence

Wavy underline – V1Δ (V1Δ is V1 with a C to A mutation at amino acid 11 that increases dimerization of two V1 peptides)

40 **Wavy underline** - Amino acid differences between V1, V1Δ and V1Δmut

underline – CH'

– IgG1 hinge region

Double underline – IgG1 Z'2 domain

45 **underline** – IgG1 Z'3 domain

An exemplary sequence for component B comprises or consists of:

V1Δmut-CH' - hFcB

MKWVTFISLLFLFSSAYSLGASWHRPDACALGYQKRPLP

SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEVTVSWNSGALTSGVHTF

5 **AVLOSSGLYSLSSVVTVPSSSLGTOTYICNVNHKPSNTKVDK**

KVXEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDG

VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTIS

KAKGPREPOVYTLPPSRCELTKNOVSLSCAVKGFYPSDIAVEWESNGOPENNYKTT

PPVLDSGDSFFLVSKLTVDKSRWQGNVFSCSVLHEALHSHYTOKSLSLSPGK

10 (SEQ ID NO: 52)

Italics – human albumin leader sequence

Wavy underline – V1Δmut (V1Δmut has the same C to A mutation at amino acid 11 as V1 but a K to A mutation at amino acid 9 was added that prevents receptor binding)

15 **Bold Wavy underline** - Amino acid differences between V1, V1Δ and V1Δmut

Bold underline – CH'

Bold – IgG1 hinge region

Double underline – IgG1 Z'2 domain

Bold underline – IgG1 Z'3 domain

20

An exemplary sequence for component B comprises or consists of:

Vp1-CH' - hFcB

MKWVTFISLLFLFSSAYSLGASWHRPDKCCLGYQKRPLPOVLLSSWYPTSQ

SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEVTVSWNSGALTSGVHTFPAVLOSS

25 **GLYSLSSVVTVPSSSLGTOTYICNVNHKPSNTKVDK**

KVXEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNA

KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKG

OPREPOVYTLPPSRCELTKNOVSLSCAVKGFYPSDIAVEWESNGOPENNYKTT

PPVLDSGDSFFLVSKLTVDKSRWQGNVFSCSVLHEALHSHYTOKSLSLSPGK

30 (SEQ ID NO: 53)

Italics – human albumin leader sequence

Wavy underline – Vp1 (Vp1 is the first 34 amino acids of vMIP11)

Bold Wavy underline - Amino acid differences between V1, V1Δ and V1Δmut

35 **Bold underline** – CH'

Bold – IgG1 hinge region

Double underline – IgG1 Z'2 domain

Bold underline – IgG1 Z'3 domain

An exemplary sequence for component B comprises or consists of:

Vp1Δ-CH' - hFcB

MKWVTFISLLFLFSSAYSLGASWHRPDKCALGYQKRPLPOVLLSSWYPTSQ

SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEVTVSWNSGALTSGVHTFPAVLOSS

GLYSLSSVVTVPSSSLGTOTYICNVNHKPSNTKVDK

45 **KVXEPKSSDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNA**

KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKG

OPREPOVYTLPPSRCELTKNOVSLSCAVKGFYPSDIAVEWESNGOPENNYKTT

PPVLDSGDSFFLVSKLTVDKSRWQGNVFSCSVLHEALHSHYTOKSLSLSPGK

REPOVYTLPPSRCELTKNOVSLSCAVKGFYPSDIAVEWESNGOPENNYKTPPVLDS
DGSFFLVSKLTVDKSRWQOGNVFSCSVLHEALHSHYTOKSLSLSPGK
 (SEQ ID NO: 54)

- 5 *Italics* – human albumin leader sequence
Wavy underline – Vp1Δ
Bold Wavy underline - Amino acid differences between V1, V1Δ and V1Δmut
Bold underline – CH'
Bold – IgG1 hinge region
 10 Double underline – IgG1 Z'₂ domain
Bold underline – IgG1 Z'₃ domain

- Vp1Δmut-CH' - hFcB
MKWVTFISLLFLFSSAYS LGASWHRPDACALGYQKRPLPOVLLSSWYPTSQ LSSASTK
 15 **GPSVEPLAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSGVHTFPAVLOSS**
GLYSLSSVTVPPSSSLGTOTYICNVNHKPSNTKVDKVKVXEPKSSDKTHTCPPCPA
PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNA
KTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKGQP
REPOVYTLPPSRCELTKNOVSLSCAVKGFYPSDIAVEWESNGOPENNYKTPPVLDS
 20 **DGSFFLVSKLTVDKSRWQOGNVFSCSVLHEALHSHYTOKSLSLSPGK**
 (SEQ ID NO: 55)

- Italics* – human albumin leader sequence
Wavy underline – Vp1Δmut
 25 **Bold Wavy underline** - Amino acid differences between V1, V1Δ and V1Δmut
Bold underline – CH'
Bold – IgG1 hinge region
Double underline – IgG1 Z'₂ domain
Bold underline – IgG1 Z'₃ domain
 30

Component C

In some embodiments, the fusion protein further comprises Component C, wherein Component C comprises Component X and Component C_L'.

35 Component X

An exemplary sequence for component X comprises or consists of:

LGASWHRPDKCCLGYQKRPLPQVLLSSWYPTSQ L C SKPGVIFLTKRGRQVCADKSK
 DWVKKLMQQLPVTAR

(SEQ ID NO: 37) vMIIPII, GENBANK® Accession No. YP_001129362.

An exemplary sequence for component X comprises or consists of:

LGASWHRPDKCCLGYQKRPLP
 (SEQ ID NO: 38) V1

40

An exemplary sequence for component X comprises or consists of:

LGASWHRPDKC**AL**GYQKRPLP
(SEQ ID NO: 39) V1Δ

An exemplary sequence for component X comprises or consists of:

5 LGASWHRPDAC**AL**GYQKRPLP
(SEQ ID NO: 40) V1Δmut

An exemplary sequence for component X comprises or consists of:

LGASWHRPDKC**CL**GYQKRPLPQVLLSSWYPTSQL
(SEQ ID NO: 41) Vp1

An exemplary sequence for component X comprises or consists of:

10 LGASWHRPDKC**AL**GYQKRPLPQVLLSSWYPTSQL
(SEQ ID NO: 42) Vp1Δ

An exemplary sequence for component X comprises or consists of:

LGASWHRPDAC**AL**GYQKRPLPQVLLSSWYPTSQL
(SEQ ID NO: 43) Vp1Δmut

15 In some embodiments, Component X and Component X' are the same. In further
embodiments, Component X and Component X' are different.

Leader Sequence

An exemplary sequence for the leader sequence comprises or consists of:

MKWVTFISLLFLFSSAYS
(SEQ ID NO: 10) human albumin leader sequence

Component C_L'

An exemplary sequence for component C_L' comprises or consists of:

20 KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESV
TEQDSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
(SEQ ID NO: 64)

An exemplary sequence for component C comprises or consists of:

25 vMIPII-CL'
MKWVTFISLLFLFSSAYSLGASWHRPDKCCLGYQKRPLPOVLLSSWYPTSQLCSKPGVI
FLTKRGRQVCADKSKDWVKKLMQQLPVTARKRTVAAPSVFIFPPSDEOLKSGTAS

**VVCLLNNFYPREAKVOWKVDNALOSGNSOESVTEODSKDSTYLSSTLTLSKAD
YEKHKVYACEVTHOGLSSPVTKSENRGEC**
(SEQ ID NO: 57)

- 5 *Italics* – human albumin leader sequence
Wavy underline – vMIPII
Bold Wavy underline - Amino acid differences between V1, V1Δ and V1Δmut
Bold underline – CL’

An exemplary sequence for component C comprises or consists of:

- 10 V1-CL’
MKWVTFISLLFLFSSAYSLGASWHRPDKCCLGYQKRPLPKRTVAAPSVFIFPPSDEOL
KSGTASVVCLLNNFYPREAKVOWKVDNALOSGNSOESVTEODSKDSTYLSSTL
TLSKADYEKHKVYACEVTHOGLSSPVTKSENRGEC
(SEQ ID NO: 58)

- 15 *Italics* – human albumin leader sequence
Wavy underline – V1
Bold Wavy underline - Amino acid differences between V1, V1Δ and V1Δmut
Bold underline – CL’

An exemplary sequence for component C comprises or consists of:

- 20 V1Δ-CL’
MKWVTFISLLFLFSSAYSLGASWHRPDKCALGYQKRPLPKRTVAAPSVFIFPPSDEOL
KSGTASVVCLLNNFYPREAKVOWKVDNALOSGNSOESVTEODSKDSTYLSSTL
TLSKADYEKHKVYACEVTHOGLSSPVTKSENRGEC
(SEQ ID NO: 59)

- 25 *Italics* – human albumin leader sequence
Wavy underline – V1Δ
Bold Wavy underline - Amino acid differences between V1, V1Δ and V1Δmut
Bold underline – CL’

- 30 An exemplary sequence for component C comprises or consists of:

- V1Δmut-CL’
MKWVTFISLLFLFSSAYSLGASWHRPDACALGYQKRPLPKRTVAAPSVFIFPPSDEOL
KSGTASVVCLLNNFYPREAKVOWKVDNALOSGNSOESVTEODSKDSTYLSSTL
TLSKADYEKHKVYACEVTHOGLSSPVTKSENRGEC
35 (SEQ ID NO: 60)

- Italics* – human albumin leader sequence
Wavy underline – V1Δmut
Bold Wavy underline - Amino acid differences between V1, V1Δ and V1Δmut
40 **Bold underline** – CL’

An exemplary sequence for component C comprises or consists of:

Vp1-CL'

MKWVTFISLLFLFSSAYSLGASWHRPDKCCLGYQKRPLPOVLLSSWYPTSQLKRTVA
APSVFIFPPSDEOLKSGTASVVCLLNNFYPREAKVOWKVDNALOSGNSOESVTE
ODSKDSTYLSSTLTLSKADYEKHKVYACEVTHOGLSSPVTKSFNRGEC

5 (SEQ ID NO: 61)

Italics – human albumin leader sequence

Wavy underline – Vp1

Bold Wavy underline - Amino acid differences between V1, V1Δ and V1Δmut

10 **Bold underline** – CL'

An exemplary sequence for component C comprises or consists of:

Vp1Δ-CL'

MKWVTFISLLFLFSSAYSLGASWHRPDKCALGYQKRPLPOVLLSSWYPTSQLKRTVA
APSVFIFPPSDEOLKSGTASVVCLLNNFYPREAKVOWKVDNALOSGNSOESVTE
ODSKDSTYLSSTLTLSKADYEKHKVYACEVTHOGLSSPVTKSFNRGEC

15 (SEQ ID NO: 62)

Italics – human albumin leader sequence

Wavy underline – Vp1Δ

20 **Bold Wavy underline** - Amino acid differences between V1, V1Δ and V1Δmut

Bold underline – CL'

An exemplary sequence for component C comprises or consists of:

Vp1Δmut-CL'

MKWVTFISLLFLFSSAYSLGASWHRPDACALGYQKRPLPOVLLSSWYPTSQLKRTVAA
PSVFIFPPSDEOLKSGTASVVCLLNNFYPREAKVOWKVDNALOSGNSOESVTEO
DSKDSTYLSSTLTLSKADYEKHKVYACEVTHOGLSSPVTKSFNRGEC

25 (SEQ ID NO: 63)

Italics – human albumin leader sequence

30 Wavy underline – Vp1Δmut

Bold Wavy underline - Amino acid differences between V1, V1Δ and V1Δmut

Bold underline – CL'

Component D

35 In some embodiments, the fusion protein further comprises Component D, wherein Component D comprises Component Q and Component CL. In some embodiments, Component Q comprises at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48.

40 An exemplary sequence for Component Q comprises or consists of SEQ ID NO: 1, 2,

3, 4, 5, 6, 7, 8, or 9. In some embodiments, Component Q and Component Y are the same. In further embodiments, Component Q and Component Y are different.

Component C_L

5 An exemplary sequence for component C_L comprises or consists of:

KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESV
TEQDSKDSITYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
(SEQ ID NO: 64)

10 Configuration of Fusion Protein

 In some embodiments of the fusion protein of the present invention, when prepared by recombinant methods described elsewhere herein, the coding sequences of the components of Component A are fused together in frame, either directly or through a linker. As used herein, the term “directly” refers to a fusion of the two components without a peptide linker in
15 between (i.e., in an expression construct, the codons encoding Component Y, Component Z₂ and Component Z₃ are contiguous). As used herein, “fused in frame” means that the expression of the fused coding sequences results in the fusion protein comprising all of the polypeptide components, e.g., in some embodiments Component A comprises all of the polypeptide components of Component Y, Component Z₂ and Component Z₃ in frame.

20 In some embodiments of the fusion protein of the present invention, when prepared by recombinant methods described elsewhere herein, the coding sequences of the components of Component B are fused together in frame, either directly or through a linker. In some embodiments in an expression construct for Component B, the codons encoding Component X', Component Z₂ and Component Z₃' are contiguous. In further embodiments, in an
25 expression construct for component B, the codons encoding Component X', Component Z₁', Component Z₂ and Component Z₃' are contiguous. In some embodiments, Component B comprises all of the polypeptide components of Component X', Component Z₂ and Component Z₃' in frame. In further embodiments, Component B comprises all of the polypeptide components of Component X', Component Z₁', Component Z₂' and Component
30 Z₃' in frame.

 In some embodiments of the fusion protein of the present invention, when prepared by recombinant methods described elsewhere herein, the coding sequences of the components of Component C are fused together in frame, either directly or through a linker. In some embodiments in an expression construct for Component C, the codons encoding Component

X and Component C_L' are contiguous. In some embodiments, Component C comprises all of the polypeptide components of Component X and Component C_L' in frame.

In some embodiments, any Component A and any Component B can be mixed and matched with each other. In some exemplary embodiments of the fusion protein of the invention, Component A and Component B are as shown in Table 1. In some embodiments, Component A and Component B from Table 1 can be mixed and matched with each other and with additional Component A and Component B options. Said additional options can also be mixed and matched with each other. Additional options of Component A or Component B may comprise at least a portion of PD-1, TIGIT, CD96, CD112R, CD113, CD155, CD111, CD112, MHC-I polypeptide-related sequence A (MICA), NKG2A (CD94), MICB, ULBP1-5, TIM-3, CD226, NECL2, CRTAM, CD80, CTLA-4, KIR2DL1/2/3, or CD48.

In some embodiments, the fusion protein comprises a Component A and hFcB. In some embodiments, the fusion protein comprises a Component B and hFcA.

In some exemplary embodiments, one component of the fusion protein blocks an inhibitory receptor, and two other components of the fusion protein each trigger distinct activating receptors. In a preferred embodiment, these three receptors are co-located on the surface of the same immune effector cell, e.g., an NK cell, and the fusion protein's three interactions, consisting of a combination of inhibitory receptor blockade and activating receptor triggering, serve to reinforce each other, all three cooperatively driving activation of the NK cell.

In other exemplary embodiments, two components of the fusion protein each block distinct inhibitory receptors, and one other component of the fusion protein triggers an activating receptor. In a preferred embodiment, these three receptors are co-located on the surface of the same immune effector cell, e.g., an NK cell, and the fusion protein's three interactions, consisting of a combination of inhibitory receptor blockade and activating receptor triggering, serve to functionally reinforce each other, all three cooperatively driving activation of the NK cell.

In yet other exemplary embodiments, three components of the fusion protein each triggers an activating receptor. In a preferred embodiment, these three receptors are co-located on the surface of the same immune effector cell, e.g., an NK cell, and the fusion protein's three interactions, all consisting of activating receptor triggering, serve to functionally reinforce each other, all three cooperatively driving activation of the NK cell.

For NK cells, an activating receptor in the aforementioned embodiments can be the FcγRIIIa receptor that drives NK cell activation and promotes antibody-dependent cellular

cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP).

Table 1-Exemplary Components A and B

Component A	Component B
Human PD-1-hFcA (SEQ ID NO: 14)	Human CD155-hFcB (SEQ ID NO: 32)
High affinity (HA) human PD-1-hFcA (SEQ ID NO: 15)	Human TIGIT-hFcB (SEQ ID NO: 33)
Human CD112R-hFcA (SEQ ID NO: 16)	Mouse TIM-3-hFcB (SEQ ID NO: 34)
Human CD113-hFcA (SEQ ID NO: 21)	Human CD113-hFcB (SEQ ID NO: 35)
Human MICA-hFcA (SEQ ID NO: 22)	
Human CD96-hFcA (SEQ ID NO: 18)	
Human TIGIT-hFcA (SEQ ID NO: 17)	
Human CD226-hFcA (SEQ ID NO: 19)	
Human NECL2-hFcA (SEQ ID NO: 20)	

5 In some exemplary embodiments, the fusion protein comprises Component B and Component C. In some embodiments, any Component B and any Component C can be mixed and matched with each other. In some embodiments, Component B and Component C are as shown in Table 2. In some embodiments, each row of Table 2 shows a pairing of a Component B and a Component C in a fusion protein.

10

Table 2

Component B	Component C
vMIP2-CH'-hFcB (SEQ ID NO: 49)	vMIP2-CL' (SEQ ID NO: 57)
V1-CH'-hFcB (SEQ ID NO: 50)	V1-CL' (SEQ ID NO: 58)
V1Δ-CH'-hFcB (SEQ ID NO: 51)	V1Δ-CL' (SEQ ID NO: 59)
V1Δmut-CH'-hFcB (SEQ ID NO: 52)	V1Δmut-CL' (SEQ ID NO: 60)
Vp1-CH'-hFcB (SEQ ID NO: 53)	Vp1-CL' (SEQ ID NO: 61)
Vp1Δ-CH'-hFcB (SEQ ID NO: 54)	Vp1Δ-CL' (SEQ ID NO: 62)
Vp1Δmut-CH'-hFcB (SEQ ID NO: 55)	Vp1Δmut-CL' (SEQ ID NO: 63)

In some embodiments, Component A and Component B are held together via disulfide bond

- stabilized knobs-into-holes interactions (KiH_s-s). In some embodiments, Component A comprises mutations Y349C and T366W (*e.g.* SEQ ID NO: 29), and Component B comprises mutations D356C, T366S, L368A and Y407V (*e.g.* SEQ ID NO: 30) (“Knobs-into-holes” mutations), enabling the enforced dimerization of Component A and Component B. The Z₁, Z₂ and Z₃ components are based upon the polypeptide amino acid backbone of the CH1, CH2 and CH3 domains of human IgG1, respectively. The positions of mutations and alterations in these component chains are defined by the Kabat numbering convention (Johnson, G and Wu, TT (2011) *Nucleic Acids Res.*, 28(1), 214-18) and are based upon the wild-type human IgG1 sequence.
- 10 Wild-type Human IgG1 (CH1, CH2 and CH3 domains) (beginning with amino acid number 118)
- A₁₁₈STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYIC₂₀₀NVNHKPSNTKVDKKVEPKSCDKTHTCPP
15 CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTY₃₀₀RVVSVLTVHLQDWLNGKEYKCKVSNKALPAPIEKTISKA
KGOPREPOVYTLPPSRDELTKNOVSLTCLVKGFYPSDIAVEWESNGOPENNYKTTTP
VLDSDGSFFLYSKLTVDKSRWQOGNVFSCSVMEALHNHYTOKSLSLSPGK₄₄₇
(SEQ ID NO: 28)
- 20 Wavy underline – IgG1 Z₁ domain
Bold – IgG1 hinge region
Double underline – IgG1 Z₂ domain
Bold underline – IgG1 Z₃ domain
- 25 In some embodiments, Component A comprises or consists of the following sequence (Y349C and T366W):
- A₁₁₈STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYIC₂₀₀NVNHKPSNTKVDKKVEPKSS*DKTHTCPP
30 CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTY₃₀₀RVVSVLTVHLQDWLNGKEYKCA**VSNKALPAPIEKTIS
KAKGOPREPOVCTLPSPSRDELTKNOVSLTCLVKGFYPSDIAVEWESNGOPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQOGNVFSCSVL***HEALHNS***YTOKSLSLSPG
K₄₄₇
(SEQ ID NO: 29)
- 35 Wavy underline – IgG1 Z₁ domain (SEQ ID NO: 67) (*see e.g.* Figure 1a)
Bold – IgG1 hinge region (SEQ ID NO: 68)
S* - Compared to wild-type, this mutation was added to the hinge region to eliminate an unpaired cysteine (C) which could bind aberrantly to another cysteine
Double underline – IgG1 Z₂ domain (SEQ ID NO: 69)
40 A** - Mutation added to reduce C1q binding (*see e.g.* Figure 1a)
Bold underline – IgG1 Z₃ domain (SEQ ID NO: 44)
L*** and S*** - Mutations added to increase FcRn binding (*see e.g.* Figure 1a)

In some embodiments, Component B comprises or consists of the following sequence (D356C, T366S, L368A and Y407V):

A₁₁₈STKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYIC₂₀₀NVNHKPSNTKVDKKVEPKSSDKTHTCPP
5 CPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTY₃₀₀RVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEKTISKA
KGOPREPOVYTLPPSRCELTKNQVSLSCAVKGFYPSDIAVEWESNGOPENNYKTTTP
VLDSGDGSFFLVSKLTVDKSRWQOGNVFSCSVLHEALHSHYTOKSLSLSPGK₄₄₇
(SEQ ID NO: 30)

10 Wavy underline – IgG1 Z₁' domain

The components shown in Tables 1 and 2 are meant to be exemplary and non-limiting.

In some exemplary embodiments, one component of the fusion protein blocks a
15 receptor on a tumor cell that contributes to said tumor cell's tumorigenicity and/or metastatic potential; a second component of the fusion protein blocks a checkpoint inhibitor on said tumor cell; and a third component of the fusion protein triggers an activating receptor on an immune effector cell. Without wishing to be bound by theory, the fusion protein serves to
20 molecularly bridge an immune effector cell and a target tumor cell, and the second and third components of the fusion protein serve to reinforce each other through a combination of blocking a checkpoint inhibitor and triggering an activating receptor on an immune effector cell, e.g., an NK cell, together cooperatively driving activation of said immune effector cell.

In a preferred embodiment, said first component of the fusion protein blocks a chemokine receptor, e.g., CXCR4 and/or CXCR7 (via binding of a vMIPII or V1 peptide or
25 derivatives thereof in the fusion protein), and this blockade serves to immobilize the tumor cells and interfere with its migratory, invasive, metastatic, and other tumorigenic properties; said second component of the fusion protein blocks a checkpoint inhibitor on said tumor cell, e.g., PD-L1 and/or PD-L2 (via binding of PD1 or derivatives thereof in the fusion protein), and this blockade serves to interfere with inhibition of a tumor-directed immune effector cell,
30 e.g., an NK cell; and said third component of the fusion protein, triggers an activating receptor on the same immune effector cell, e.g., the Fc γ RIIIa receptor (via binding of Fc γ or derivatives thereof in the fusion protein), that drives NK cell activation and promotes ADCC and ADCP. A beneficial feature of said embodiment is the same fusion protein also coordinately modulates other immune cells, beyond NK cells, with a net anti-tumor effect
35 (See Fig. 5).

In some exemplary embodiments, one component of the fusion protein blocks a checkpoint inhibitor on a tumor or other cell and the other two components each trigger a

distinct activating receptor on an immune effector cell. Where the checkpoint inhibitor is on a tumor cell, the fusion protein in effect serves to molecularly bridge an immune effector cell and a target tumor cell. Further, the fusion protein's three interactions, a combination of checkpoint inhibitory pathway blockade and activating receptor triggering, serve to functionally reinforce each other, all three cooperatively driving activation of said immune effector cell, e.g., an NK cell. In a preferred embodiment, the checkpoint inhibitor blocked by the fusion protein consists of PD-L1, PD-L2, CD113, CD112, CD155, or CD111, and the two activating receptors co-triggered by the fusion protein are the Fc γ RIIIa receptor and 4-1BB on an NK cell (see Fig. 6).

10 In some exemplary embodiments, one component of the fusion protein blocks a checkpoint inhibitor on a tumor or other cell; a second component of the fusion protein blocks a coinhibitory receptor for the same or a different checkpoint inhibitor on an immune effector cell; and a third component of the fusion protein triggers an activating receptor on an immune effector cell. Where the checkpoint inhibitor is on a tumor cell, the fusion protein in effect serves to molecularly bridge an immune effector cell and a target tumor cell, and the fusion protein's three interactions, a combination of checkpoint inhibitory pathway blockade and activating receptor triggering, serve to functionally reinforce each other, all three cooperatively driving activation of said immune effector cell, e.g., an NK cell. In a preferred embodiment, the checkpoint inhibitor blocked by the first component of the fusion protein consists of PD-L1, PD-L2, CD113, CD112, CD155, or CD111; the coinhibitory receptor on an NK cell blocked by the fusion protein consists of PD-1, TIGIT, CD96, or CD112R; and the activating receptor triggered by the fusion protein is the Fc γ RIIIa receptor the same NK cell (see Fig. 7).

25 In some exemplary embodiments, one component of the fusion protein blocks a 'don't eat me' inhibitory receptor on a tumor-associated macrophage of the M1 type, thereby unleashing its anti-tumor phagocytic and other activities; two other components of the fusion protein each trigger a distinct activating receptor on said tumor-associated macrophage. The fusion protein's three interactions, a combination of blocking a macrophage inhibitory pathway and triggering separate activating receptors on the macrophage, serve to functionally reinforce each other, all three cooperatively driving activation of the tumor-associated macrophage and promoting its anti-tumor functions. In a preferred embodiment, the 'don't eat me' receptor blocked by the first component of the fusion protein is SIRP α ; the activating receptors triggered by the fusion protein are CD40 and the Fc γ RIIIa receptor on the same

macrophage (see Fig. 8, left panel).

In some exemplary embodiments, one component of the fusion protein blocks a 'don't eat me' inhibitory receptor on a tumor-associated macrophage of the M1 type, thereby unleashing its anti-tumor phagocytic and other activities; a second component of the fusion protein blocks a distinct inhibitory receptor on said macrophage; and a third component of the fusion protein triggers an activating receptor on said tumor-associated macrophage. The fusion protein's three interactions, a combination of blocking two macrophage inhibitory pathways and triggering an activating receptor on the macrophage, serve to functionally reinforce each other, all three cooperatively driving activation and/or anti-tumor effector functions of the tumor-associated macrophage. In a preferred embodiment, the 'don't eat me' receptor blocked by the first component of the fusion protein is SIRP α ; the inhibitory receptor blocked by the second component of the fusion protein is PD-1; and the activating receptor triggered by the fusion protein is the Fc γ RIIIa receptor (see Fig. 8, right panel).

Preferred embodiments of the fusion protein of the present invention comprise a cytokine, or a portion or derivative thereof, which can be incorporated in Component A, Component B, Component C and/or Component D. This includes a wide range of cytokines well known to those familiar with the art, which fall into a number of different classes, e.g., interleukins, tumor necrosis factors, interferons, colony-stimulating factors, and others; have been ascribed various functions, with a range of activating or inhibitory properties, e.g., adaptive immunity, pro-inflammatory signaling, anti-inflammatory signaling, stem cell modulation and differentiation, chemotaxis, phagocytosis, cytotoxicity, and anti-viral effects; and have been associated with a range of immune and non-immune cell targets, e.g., B cells, T cells, NK cells, macrophage/monocytes, dendritic cells, bone marrow stromal cells, stem cells, fibroblasts, endothelial cells, and epithelial cells. Preferred embodiments comprise cytokines linked to adaptive immunity (e.g., IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, GM-CSF); pro-inflammatory signaling [e.g., IL-1 family (IL-1, IL-18, IL-33, IL-36); IL-6 family (IL-6, IL-11, IL-31, CNTF, CT-1, LIF, OPN, OSM); TNF α family (TNF α , TNF β , BAFF, APRIL); IL-17 family (IL-17A-F, IL-25); Type I IFN family (IFN α , IFN β , IFN κ , Limitin); Type II IFN family (IFN γ); and Type III IFN family (IFN λ 1/IL-29), IFN λ 2/IL-28A, IFN λ 3/IL-28B)]; and anti-inflammatory signaling [IL-12 family (IL-12, IL-23, IL-27, IL-35); and IL-10 family (IL-10, IL-19, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29)]. See Turner, Mark D., et al.: "Cytokines and Chemokines: At the Crossroads of Cell Signaling and Inflammatory Disease", *Biochimica et Biophysica Acta* 1843 (2014) 2563-2582.

vMIP-II

In some embodiments, a component of the fusion protein of the invention may comprise vMIP-II or a variant thereof. The viral Macrophage Inflammatory Protein-II (vMIP-II) is a chemokine that interacts with the CC and CXC chemokine receptors, including the CCR5 and CXCR4 chemokine receptors. CCR5 and CXCR4 are the principal coreceptors required for cell entry of human immunodeficiency virus (HIV-1). CXCR4 may also be found on cancer cells, e.g. tumor cells. vMIP-II, a chemokine encoded by human herpesvirus 8 (HHV-8) (Moore, P.S., et al., Science, 274:1739-1744, 1996) displays diverse interactions with both CC and CXC chemokine receptors and inhibits HIV-1 entry mediated through CCR3, CCR5, and CXCR4. *See* U.S. Patent Publication No. 2003/0220482, which is hereby incorporated by reference in its entirety. vMIP-II also binds CXCR7, which like CXCR4, has been implicated in tumorigenesis. V1 (aa 1-21 of vMIP-II), and its related DV1 (D amino acid isomer) display antagonistic activity against CXCR4 and CXCR7, but not against CCR5.

vMIP inhibits binding of CXCL12, the natural ligand. In some embodiments, the Δ signifies a mutation at the 12th amino acid that causes dimerization between the two peptides of the B and C component. In some embodiments, the Δ mut signifies a mutation at the 10th amino acid that prevents binding of the peptide, acting as a negative control.

PD-1

In some embodiments, a component of the fusion protein of the invention may comprise PD-1 or a variant thereof. PD-1 (Programmed cell death protein 1), also known as CD279, is a protein on the surface of cells that has a role in regulating the response of the immune system to the cells. PD-1 down-regulates the immune system and promotes self-tolerance by suppressing T cell inflammatory activity. Without wishing to be bound by theory, PD-1 acts as an immune checkpoint through at least two mechanisms. PD-1 promotes apoptosis of antigen-specific T cells in lymph nodes. PD-1 also reduces apoptosis in regulatory T cells. PD-1 binds the ligands PD-L1 and PD-L2, which are members of the B7 family. PD-L1 and PD-L2 are expressed on the surface of some tumor cells. PD-L1 expressed on tumor cells engages PD-1 on effector T cells and NK cells and inhibits their function. Thus, PD-L1 expressed on tumor cells inhibits effector T cell anti-tumor activity.

In some embodiments, the PD-1 variant of the fusion protein of the invention is high affinity PD-1. Native PD-1 has a relatively low affinity for its ligands, PD-L1 and PD-L2. Higher affinity variants of the PD-1 ectodomain would serve as greater competitive antagonists for its ligands. PD-1 contact residues between human PD-1 and PD-L1 were

mutated and then assessed for binding. High affinity PD-1 described herein has ten mutated amino acids that result in enhanced affinity for PD-L1 of greater than 10,000-fold. For further details, see Maute et al., PNAS, 112(47): 6506-6514, 2015.

CD112R

5 In some embodiments, a component of the fusion protein of the invention may comprise CD112R or a variant thereof. CD112R is expressed on T cells and NK cells and inhibits activating responses. CD112, widely expressed on antigen-presenting cells and tumor cells, is the ligand for CD112R. CD112R competes with CD226, a coinhibitory receptor, for binding to CD112. Without wishing to be bound by theory, disrupting the CD112R-CD112
10 interaction may increase T cell response. Human CD112R contains a single extracellular IgV domain. The CD112R fusion protein variant described herein consists of the entire ectodomain of human CD112R linked to the hinge, CH2 and CH3 domains of human IgG1. CD112R and variants may bind to its ligand, CD112, and serve as a competitive antagonist for native CD112R on NK cells and T cells, preventing inhibitory signaling.

15 CD113

 In some embodiments, a component of the fusion protein of the invention may comprise CD113 or a variant thereof. CD113, also known as poliovirus receptor-related 3 (PVRL3) or nectin-3, is a member of the immunoglobulin superfamily which forms part of adherens junctions. CD113 has been shown to interact, without limitation, with MLLT4,
20 PARD3 and PTPRM. In addition, CD113 engages TIGIT, CD111, CD112, CD155 and itself. The CD113 fusion protein variant described herein consists of the entire extracellular domain of native human CD113 linked to the hinge, CH2 and CH3 domains of human IgG1. Fusion proteins containing CD113 may serve as a competitive antagonist for native TIGIT on NK cells and T cells, preventing inhibitory signaling. Alternatively, CD113 fusion proteins may
25 bind to CD112, CD155 and/or CD111 and block their engagement with the inhibitory receptors CD112R, TIGIT and CD96, respectively, restoring NK cell and T cell cytotoxicity and cytokine production.

MICA

 In some embodiments, a component of the fusion protein of the invention may
30 comprise MHC class I polypeptide-related sequence A (MICA) or a variant thereof. MICA is a cell surface glycoprotein encoded by the MICA gene located within MHC locus. MICA is not associated with β 2-microglobulin nor does it bind peptides as conventional MHC class I molecules do. Without wishing to be bound by theory, MICA may act as a stress-induced

ligand for the NKG2D receptor. MICA is broadly recognized by NK cells, $\gamma\delta$ T cells, and CD8+ $\alpha\beta$ T cells that express NKG2D on their cell surface. Effector cytolytic responses of T cells and NK cells against tumor cells expressing MICA are initiated as a result of NKG2D-MICA binding. In some embodiments, the MICA variant consists of the entire MICA ectodomain linked to the hinge, CH2 and CH3 domains of human IgG1. MICA triggering of NKG2D signaling on NK cells and cytotoxic T cells is an important mediator of anti-tumor activity.

CD155

In some embodiments, a component of the fusion protein of the invention may comprise CD155 or a variant thereof. CD155 is a type I transmembrane glycoprotein in the immunoglobulin superfamily. In humans, CD155 is encoded by the poliovirus receptor (PVR) gene. CD155 is involved in the establishment of intercellular adherens junctions between epithelial cells. The external domain of CD155 mediates cell attachment to the extracellular matrix molecule vitronectin, while its intracellular domain interacts with the dynein light chain Tctex-1/DYNLT1. In addition, CD155 engages NK cell inhibitory receptors, TIGIT and CD96, limiting NK cell cytotoxicity, and the activating receptor CD226 (DNAM-1). Fusion proteins containing the extracellular domain of CD155 may bind to TIGIT or CD96 on NK and T cells and serve as a competitive antagonist for endogenous CD155 expressed by tumor cells or antigen presenting cells (APC). CD155-containing fusion proteins may also bind to the costimulatory receptor, CD226, and induce NK cell-mediated lysis of tumor targets.

TIGIT

In some embodiments, a component of the fusion protein of the invention may comprise TIGIT or a variant thereof. In some embodiments, the TIGIT is mouse TIGIT. In further embodiments, the TIGIT is human TIGIT. In some embodiments, the TIGIT variant comprises the entire TIGIT extracellular IgV-like domain linked to the hinge, CH2 and CH3 domains of human IgG1.

TIGIT, also known as T cell immunoreceptor with Ig and ITIM domains, is an immune receptor present on some T cells and NK cells. TIGIT is also known as WUCAM or Vstm3. TIGIT binds to CD155(PVR) on cells such as dendritic cells (DCs) and macrophages with high affinity, and also to CD112(PVRL2) with lower affinity. TIGIT is a checkpoint inhibitor and is over expressed on tumor antigen-specific (TA-specific) CD8+ T cells and CD8+ tumor infiltrating lymphocytes (TILs) from individuals with cancer, e.g. melanoma.

Without wishing to be bound by theory, blockade of TIGIT may lead to increased cell proliferation, cytokine production, and degranulation of tumor antigen-specific CD8⁺ T cells and TIL CD8⁺ T cells. Fusion proteins containing TIGIT may bind to its receptors CD155(PVR) and CD112(PVRL2) and block their interaction with TIGIT on NK cells.

- 5 Disrupting the interaction of TIGIT with its ligands on cancer cells will restore NK cell cytotoxic activity and cytokine production.

TIM-3

- In some embodiments, a component of the fusion protein of the invention may comprise TIM-3 or a variant thereof. T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), also known as Hepatitis A virus cellular receptor 2 (HAVCR2), is a protein that in humans is encoded by the *HAVCR2* gene. HAVCR2 is a cell surface molecule expressed on IFN γ producing CD4⁺ Th1 and CD8⁺ Tc1 cells. TIM-3 expression has also been detected in Th17 cells, regulatory T-cells, and innate immune cells (dendritic cells, NK cells, monocytes). TIM-3 is an immune checkpoint and mediates T cell exhaustion. Without
- 10 wishing to be bound by theory, TIM-3 is upregulated in tumor infiltrating lymphocytes (TIL) in several cancers, including but not limited to lung, gastric, head and neck, schwannoma, melanoma and follicular B-cell non-Hodgkin lymphoma. In some embodiments, the TIM-3 variant comprises the entire TIM-3 ectodomain, including the N-terminal IgV-like domain, linked to the hinge, CH2 and CH3 domains of human IgG1. Fusion proteins containing TIM-
- 15 3 may bind to its natural ligands, Galectin-9, Ceacam-1 and Phosphatidyl serine, and block their interaction with endogenous TIM-3 expressed on NK cells, T cells and APC, reversing T cell exhaustion and restoring NK cell cytotoxicity and cytokine production.

Tumor cell receptor targets

- In some embodiments, the fusion protein, or one or more components thereof, binds
- 25 to a tumor cell receptor target. In some embodiments, the fusion protein, or one or more components thereof, prevents binding of a ligand to a tumor cell receptor target. In some embodiments, the tumor cell receptor targets comprise, without limitation, chemokine receptors, notch receptors, immune checkpoint inhibitors, and tumor vasculature ligands and receptors. In some embodiments, the chemokine receptor comprises CXCR4, CCR10 or CCR7. In some embodiments, the immune checkpoint inhibitor comprises PD-L1 or PD-L2.
- 30 In some embodiments, the tumor vasculature target comprises α v β 3, α v β 5, CD13 (aminopeptidase N), a target of an NGF motif peptide, or Tie2 (receptor for Angiopoietin-2).

Immune cell receptor targets

In some embodiments, the immune cell receptor target is CD40. In some
embodiments, the fusion protein, or one or more components thereof comprises agonist CD40
scFv. In further embodiments, the immune cell receptor target is SIRP α . In some
embodiments, the fusion protein, or one or more components thereof comprises antagonist
5 SIRP α peptide. In some embodiments, the immune cell receptor target is 4-1BB. In some
embodiments, the fusion protein, or one or more components thereof comprises agonist 4-
1BB scFv or agonist 4-1BB peptide.

In some embodiments, the immune cell receptor target is CD96. In some
embodiments, a component of the fusion protein of the invention may comprise CD96 or a
10 variant thereof. In some embodiments, a component of the fusion protein of the invention
may comprise an antagonist or an agonist of CD96.

In some embodiments, a component of the fusion protein of the invention may
comprise CD226 or a variant thereof. In some embodiments, a component of the fusion
protein of the invention may comprise an antagonist or an agonist of CD226.

15 In some embodiments, a component of the fusion protein of the invention may
comprise TIM-3 or a variant thereof. In some embodiments, a component of the fusion
protein of the invention may comprise an antagonist or an agonist of TIM-3.

In some embodiments, the immune cell receptor target is CD111. In some
embodiments, a component of the fusion protein of the invention may comprise CD111 or a
20 variant thereof. In some embodiments, a component of the fusion protein of the invention
may comprise an antagonist or an agonist of CD111.

In some embodiments, the immune cell receptor target is CD112. In some
embodiments, a component of the fusion protein of the invention may comprise CD112 or a
variant thereof. In some embodiments, a component of the fusion protein of the invention
25 may comprise an antagonist or an agonist of CD112.

In some embodiments, the immune cell receptor target is CD113. In some
embodiments, a component of the fusion protein of the invention may comprise CD113 or a
variant thereof. In some embodiments, a component of the fusion protein of the invention
may comprise an antagonist or an agonist of CD113.

30 In some embodiments, the immune cell receptor target is CD115. In some
embodiments, a component of the fusion protein of the invention may comprise CD115 or a
variant thereof. In some embodiments, a component of the fusion protein of the invention
may comprise an antagonist or an agonist of CD115.

In some embodiments, the immune cell receptor target is TIGIT. In some embodiments, a component of the fusion protein of the invention may comprise TIGIT or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of TIGIT.

5 In some embodiments, the immune cell receptor target is KIR2DL1/2/or 3 or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise KIR2DL1/2/or 3 or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of KIR2DL1/2/or 3.

10 In some embodiments, the immune cell receptor target is HLA-C. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of HLA-C. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of HLA-C.

15 In some embodiments, the immune cell receptor target is NKG2A (CD94) or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise NKG2A (CD94) or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of NKG2A (CD94) or a variant thereof.

20 In some embodiments, the immune cell receptor target is HLA-E. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of HLA-E. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of HLA-E.

25 In some embodiments, the immune cell receptor target is 2B4 or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise 2B4 or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of 2B4 or a variant thereof.

30 In some embodiments, the immune cell receptor target is CD48 or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of CD48. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of CD48 or a variant thereof.

 In some embodiments, the immune cell receptor target is NKG2D or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise NKG2D or a variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of NKG2D or a variant thereof.

 In some embodiments, the immune cell receptor target is MICA/B or ULBP1 or a

variant thereof. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of MICA/B or ULBP1. In some embodiments, a component of the fusion protein of the invention may comprise an antagonist or an agonist of MICA/B or ULBP1 or a variant thereof.

5 In any one of the preceding embodiments, the immune cell is an NK cell, a T cell, a dendritic cell (DC), an antigen-presenting cell (APC), a macrophage, or a tumor-associated macrophage (M1).

Immune cell receptor or ligand

10 In some embodiments, the fusion protein, or one or more components thereof, binds to an immune cell receptor or ligand. In some embodiments, binding to the immune cell receptor or ligand results in NK cell activation (cytokine production (IFN γ and TNF), antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP)). In some embodiments, the receptor is an Fc receptor. In some
15 embodiments, binding to the Fc receptor results in NK cell activation (ADCC and ADCP). In some embodiments, the Fc receptor is an Fc γ , Fc ϵ , Fc α , Fc μ , or Fc δ receptor. In some embodiments, the immune cell receptor or ligand is a member of the TNF superfamily or a receptor thereof, a member of the TNF-L superfamily or a receptor thereof, transferrin or a receptor thereof, human serum albumin or a receptor thereof, or a member of the lipocalin structural family or a receptor thereof.

20 Linkers

 In some embodiments, the components of the fusion protein of the invention may be optionally connected via a peptide linker. The residues for the linker may be selected from naturally occurring amino acids, non-naturally occurring amino acids, and modified amino acids. The linker will typically connect the carboxy terminus of the first component to the
25 amino terminus of the second component. The linker may alter the distance between the two structural components of the fusion protein, as well as alter the flexibility of this region. The linker may comprise any number of amino acids. The linker may thus comprise, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53,
30 54, 55, 56, 57, 58, 59, 60, or more amino acids. In some embodiments, the linker may be composed of from 3 to 60 amino acid residues, from 3 to 40 amino acid residues, from 3 to 30 amino acid residues, from 3 to 24 amino acid residues, from 3 to 18 amino acid residues, or from 3 to 15 amino acid residues. The linker may comprise, for example, a repeating sub-

sequence of 2, 3, 4, 5 or more amino acid residues, comprising 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more repeats of the sub-sequence.

Linkers may be naturally-occurring sequences or designed sequences. Peptide linkers useful in the fusion protein of the invention include, but are not limited to, glycine linkers, glycine-rich linkers, serine-glycine linkers, and the like. A glycine-rich linker comprises at least about 50% glycine and preferably at least about 60% glycine. In one embodiment, the linker comprises the amino acid sequence Gly-Ser, or repeats thereof. See, e.g. Huston, et al., *Methods in Enzymology*, 203:46-88 (1991). In another embodiment, the linker comprises the amino acid sequence Gly-Lys, or repeats thereof. See, e.g., Whitlow et al., *Protein Eng.*, 6:989 (1993). In another embodiment, the linker comprises the amino acid sequence Gly-Gly-Ser or repeats thereof. In another embodiment, the linker comprises the amino acid sequence Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 29), or repeats thereof. In certain specific embodiments, the linker comprises the amino acid sequence Gly-Gly-Gly-Ser-Gly-Gly-Gly-Ser (SEQ ID NO: 30). In certain embodiments, the linker contains from 2 to 12 repeats of Gly-Gly-Ser or Gly-Gly-Gly-Ser or Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 29). See U.S. Pat. No. 6,541,219 for examples of peptide linkers. In one embodiment, the linker may comprise the sequence GDPLVTAASVLEFGGSGGGSEGGGSEGGGSEGGGSDI (SEQ ID NO: 31).

Linkers are useful for separating the two components of the fusion protein to enable proper folding of the components, to reduce potential steric problems, and/or to contribute to optimal receptor binding. The skilled artisan is familiar with the design and selection of peptide linkers. See, for instance, Robison *et al.*, 1998, *Proc. Natl. Acad. Sci. USA* 95:5929-5934. Automated programs are also available for peptide linker design (e.g., Crasto *et al.*, 2000, *Protein Engineering* 13:309-312).

Optional other elements

The fusion protein optionally may also include further elements apart from Component A, Component B, Component C and/or Component D. Such further elements may include, without limitation: an initiator methionine, a signal peptide, an antigen polypeptide, a trimerization domain, a higher order multimerization domain, and a purification tag, such as His-6. An exemplary purification tag is ASHHHHHHM (SEQ ID NO: 46). In an embodiment, the fusion protein of the invention comprises an optional trimerization domain.

Fusion proteins of the invention optionally comprise a signal peptide. Signal peptides can be varied according to the needs of the user, the expression system, and other factors, as would be understood by one skilled in the art. Signal peptides are well known in the art, and

any desired signal peptide can be used, including those recognized/predicted by publicly available signal peptide recognition software known to those skilled in the art.

In some embodiments, the fusion protein of the invention comprises a hinge region which allows for flexibility between components. See Lobner et al. (2016) Immunol.

- 5 Reviews 270:113-131. In some embodiments, Component Y and Component Z₂ are connected via a hinge, for example an IgG hinge. In some embodiments, Component Z₁' and Component Z₂' are connected via a hinge, for example an IgG hinge.

- 10 In some embodiments, a N-linked glycan is attached to an Asn on Component A and/or Component B. In some embodiments, a N-linked glycan is attached to Asn297 on Component A and/or Component B. Without wishing to be bound by theory, this may promote FcR binding and may promote structural integrity and thermal stability of the fusion protein. See Arnold et al. (2007) Annu Rev Immunol 25:21-50.

- 15 In some embodiments, Component A and/or Component B comprise a K to A mutation. In some embodiments, Component A and/or Component B comprise a K322A mutation. Without wishing to be bound by theory, this may reduce C1q binding and complement-mediated lysis. See Idusogie et al. (2000) J. Immunol 164:4178-4184.

- 20 In some embodiments, Component A and Component B comprise knobs-into-holes mutations. In some embodiments, Component A comprises mutations Y349C and T366W, and Component B comprises mutations D356C, T366S, L368A and Y407V ("Knobs-into-holes" mutations). Without wishing to be bound by theory, this may promote heterodimerization over homodimerization. See Merchant et al. (1998) Nature Biotech. 16:677-681.

- 25 In some embodiments, Component A and/or Component B comprise mutations that increase binding to neonatal Fc receptor (FcRn). In some embodiments, Component A and/or Component B comprises mutations M428L and N434S. Without wishing to be bound by theory, this may increase binding to neonatal Fc receptor (FcRn) on various cells, prolonging serum half-life. See Kuo and Aveson (2011) mAbs 3:422-430.

Trimerization domains

- 30 Trimerization domains are well known in the art. Non-limiting examples of trimerization domains suitable as a heterologous trimerization domain in the fusion protein of the invention include: the GCN4 leucine zipper (Harbury et al., 1993, "A switch between two-, three-, and four-stranded coiled coils in GCN4 leucine zipper mutants," Science 262(5138):1401-7); a 35 amino-acid sequence from lung surfactant protein (Hoppe et al., 1994, "A parallel three stranded alpha helical bundle at the nucleation site of collagen triple-

helix formation” FEBS letters 344(2-3):191-5); short, repeating heptad sequences from collagen (McAlinden et al., 2003, “Alpha-helical coiled-coil oligomerization domains are almost ubiquitous in the collagen superfamily,” J Biol Chem. 278(43):42200-7. Epub 2003 Aug 14.); and the bacteriophage T4 fibrin “foldon” (see, e.g., Miroshnikov et al., 1998, “Engineering trimeric fibrous proteins based on bacteriophage T4 adhesins,” Protein Eng. 11(4):329-32). Exemplary trimerization domains are also disclosed in U.S. Patent Nos. 6,911,205 and 8,147,843, and U.S. Patent Publication Pub. 2010/0136032. An exemplary trimerization sequence is the T4 “foldon” having the sequence:

GYIPEAPRDGQAYVRKRGEWVLLSTFL (SEQ ID NO: 47). Another exemplary trimerization domain is from thrombospondin-1 and has the sequence: VTTLQDSIRKVTENKELANELRR (SEQ ID NO: 56).

Modification

The invention encompasses variants of the fusion proteins described herein. While in general it is desirable for variants to show enhanced ability for binding to a given molecule, in some embodiments, variants may be designed with slightly reduced activity as compared to other fusion proteins of the invention, for example in instances in which one would purposefully want to attenuate activity. Furthermore, variants or derivatives can be generated that would have altered multimerization properties.

Preferably, variants or derivatives of the fusion proteins of the present invention maintain the hydrophobicity/hydrophilicity of the amino acid sequence.

In additional embodiments, the fusion protein of the invention is a variant and/or derivative of the amino acid sequence shown in SEQ ID NO: 14, 15, 16, 17, 18, 19, 20, 21, 22, 32, 33, 34, 35, 49, 50, 51, 52, 53, 54, 55, 65, 66, 67, 68, 69, 70, or 71. In one embodiment, variants of the fusion proteins of the present invention will have at least 80% or greater sequence identity or homology, as those terms are understood in the art, to SEQ ID NO: 14, 15, 16, 17, 18, 19, 20, 21, 22, 32, 33, 34, 35, 49, 50, 51, 52, 53, 54, 55, 65, 66, 67, 68, 69, 70, or 71, more preferably at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or even 99% sequence identity SEQ ID NO: 14, 15, 16, 17, 18, 19, 20, 21, 22, 32, 33, 34, 35, 49, 50, 51, 52, 53, 54, 55, 65, 66, 67, 68, 69, 70, or 71.

The invention also provides chemical modification of a fusion protein of the invention. Non-limiting examples of such modifications may include but are not limited to aliphatic esters or amides of the carboxyl terminus or of residues containing carboxyl side chains, O-acyl derivatives of hydroxyl group-containing residues, and N-acyl derivatives of the amino-terminal amino acid or amino-group containing residues, e.g., lysine or arginine.

Other derivatives of the fusion proteins of the present invention include incorporation of unnatural amino acid residues, or phosphorylated amino acid residues such as phosphotyrosine, phosphoserine or phosphothreonine residues. Other potential modifications include sulfonation, biotinylation, or the addition of other moieties, particularly those which have molecular shapes similar to phosphate groups.

Derivatives also include polypeptides modified by glycosylation. These can be made by modifying glycosylation patterns during synthesis and processing in various alternative eukaryotic host expression systems, or during further processing steps. Methods for producing glycosylation modifications include exposing the fusion proteins to glycosylating enzymes derived from cells that normally carry out such processing, such as mammalian glycosylation enzymes. Alternatively, deglycosylation enzymes can be used to remove carbohydrates attached during production in eukaryotic expression systems. Additionally, one can also modify the coding sequence so that glycosylation site(s) are added or glycosylation sites are deleted or disabled. Furthermore, if no glycosylation is desired, the proteins can be produced in a prokaryotic host expression system.

Variants and/or derivatives of the fusion proteins of the invention can be prepared by chemical synthesis or by using site-directed mutagenesis (Gillman *et al.*, *Gene* 8:81 (1979); Roberts *et al.*, *Nature* 328:731 (1987) or Innis (Ed.), 1990, *PCR Protocols: A Guide to Methods and Applications*, Academic Press, New York, N.Y.) or the polymerase chain reaction method (PCR; Saiki *et al.*, *Science* 239:487 (1988)), as exemplified by Daugherty *et al.*, *Nucleic Acids Res.* 19:2471 (1991)) to modify nucleic acids encoding the complete receptors.

In additional embodiments, the fusion proteins of the present invention may further comprise one or more additional polypeptide domains added to facilitate protein purification, to increase expression of the recombinant protein, or to increase the solubility of the recombinant protein. Such purification/expression/solubility facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath, 1992, *Protein Expr Purif* 3:0.26328 1), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, Wash.). The inclusion of a cleavable linker sequence such as Factor Xa or enterokinase (Invitrogen, San Diego, Calif.) between the purification domain and the fusion of Components A and B is useful to facilitate purification.

Fusion expression vectors include pGEX (Pharmacia, Piscataway, N.J.), pMAL (New

England Biolabs, Beverly, Mass.) and pRITS (Pharmacia, Piscataway, N.J.) which fuse glutathione S transferase (GST), maltose B binding protein, or protein A, respectively, to the target recombinant protein. EBV, BKV, and other episomal expression vectors (Invitrogen) can also be used. In addition, retroviral and lentiviral expression vectors can also be used.

- 5 Furthermore, any one of a number of in vivo expression systems designed for high level expression of recombinant proteins within organisms can be invoked for producing the fusion proteins specified herein.

As discussed above, a fusion protein of the present invention may contain a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host
10 cells), expression and/or secretion of the fusion protein can be increased through use of a heterologous signal sequence. Signal sequences are typically characterized by a core of hydrophobic amino acids, which are generally cleaved from the mature protein during secretion in one or more cleavage events. Such signal peptides contain processing sites that allow cleavage of the signal sequence from the mature proteins as they pass through the
15 secretory pathway. Thus, the invention pertains to the described polypeptides having a signal sequence, as well as to polypeptides from which the signal sequence has been proteolytically cleaved (*i.e.*, the cleavage products).

In order to enhance stability and/or reactivity, the fusion protein of the present invention can also be modified to incorporate one or more polymorphisms in the amino acid
20 sequence resulting from natural allelic variation. Additionally, D-amino acids, non-natural amino acids or non-amino acid analogues can be substituted or added to produce a modified fusion protein within the scope of this invention.

The amino acid sequences of the present invention may be produced by expression of a nucleotide sequence coding for same in a suitable expression system.

25 In addition, or in the alternative, the fusion protein itself can be produced using chemical methods to synthesize the desired amino acid sequence, in whole or in part. For example, polypeptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high-performance liquid chromatography (*e.g.*, Creighton (1983) *Proteins: Structures And Molecular Principles*, WH Freeman and Co, New York N.Y.). The
30 composition of the synthetic polypeptides may be confirmed by amino acid analysis or sequencing (*e.g.*, the Edman degradation procedure). Additionally, the amino acid sequence of a fusion protein of the invention, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with a sequence from other subunits, or any part thereof, to produce a variant polypeptide.

Assays for measuring the biological activity of any homolog, derivative or variant of any fusion protein of the present invention are well known in the art.

Activity and Utility

In one embodiment, the fusion proteins of the present invention reduce or prevent a
5 tumor cell from migrating, infiltrating neighboring tissues, and/or metastasizing to distant sites, in effect immobilizing said cell. In another embodiment, the fusion proteins of the present invention reduce or prevent a tumor cell from evading phagocytosis by a phagocytotic cell, such as a macrophage, while also promoting apoptosis and/or immune destruction of the tumor cell. In other embodiments, the fusion proteins of the invention
10 reduce or prevent a tumor cell from evading phagocytosis by a phagocytotic cell, while promoting apoptosis of a neighboring tumor cell. Thus, the fusion proteins of the present invention promote tumor cell destruction by any one of a number of mechanisms.

PD-1 ligands or receptors are expressed on a wide range of tumor cells, such a solid tumor cells. Thus, in one embodiment, the invention provides a method of treating a
15 proliferative disorder by administering a therapeutically effective amount of a fusion protein of the invention to a subject diagnosed with a proliferative disorder.

The fusion proteins according to the invention may be administered to individuals (such as mammals, including animals and humans) afflicted with a cellular proliferative disorder such as cancer, and malignant and benign tumors. In a particular embodiment of the
20 invention, the individual treated is a human.

The fusion proteins are believed effective against a broad range of tumor types, including but not limited to the following: ovarian cancer; cervical cancer; breast cancer; prostate cancer; testicular cancer, lung cancer, renal cancer; colorectal cancer; skin cancer; brain cancer; leukemia, including acute myeloid leukemia, chronic myeloid leukemia, acute
25 lymphoid leukemia, and chronic lymphoid leukemia.

More particularly, cancers that may be treated by the compounds, compositions and methods of the invention include, but are not limited to, the following:

cardiac cancers, including, for example sarcoma, e.g., angiosarcoma, fibrosarcoma, rhabdomyosarcoma, and liposarcoma; myxoma; rhabdomyoma; fibroma; lipoma and
30 teratoma;

lung cancers, including, for example, bronchogenic carcinoma, e.g., squamous cell, undifferentiated small cell, undifferentiated large cell, and adenocarcinoma; alveolar and bronchiolar carcinoma; bronchial adenoma; sarcoma; lymphoma; chondromatous hamartoma; and mesothelioma;

- gastrointestinal cancer, including, for example, cancers of the esophagus, e.g., squamous cell carcinoma, adenocarcinoma, leiomyosarcoma, and lymphoma; cancers of the stomach, e.g., carcinoma, lymphoma, and leiomyosarcoma; cancers of the pancreas, e.g., ductal adenocarcinoma, insulinoma, glucagonoma, gastrinoma, carcinoid tumors, and
- 5 vipoma; cancers of the small bowel, e.g., adenocarcinoma, lymphoma, carcinoid tumors, Kaposi's sarcoma, leiomyoma, hemangioma, lipoma, neurofibroma, and fibroma; cancers of the large bowel, e.g., adenocarcinoma, tubular adenoma, villous adenoma, hamartoma, and leiomyoma;
- genitourinary tract cancers, including, for example, cancers of the kidney, e.g.,
- 10 adenocarcinoma, Wilm's tumor (nephroblastoma), lymphoma, and leukemia; cancers of the bladder and urethra, e.g., squamous cell carcinoma, transitional cell carcinoma, and adenocarcinoma; cancers of the prostate, e.g., adenocarcinoma, and sarcoma; cancer of the testis, e.g., seminoma, teratoma, embryonal carcinoma, teratocarcinoma, choriocarcinoma, sarcoma, interstitial cell carcinoma, fibroma, fibroadenoma, adenomatoid tumors, and
- 15 lipoma;
- liver cancers, including, for example, hepatoma, e.g., hepatocellular carcinoma; cholangiocarcinoma; hepatoblastoma; angiosarcoma; hepatocellular adenoma; and hemangioma;
- bone cancers, including, for example, osteogenic sarcoma (osteosarcoma),
- 20 fibrosarcoma, malignant fibrous histiocytoma, chondrosarcoma, Ewing's sarcoma, malignant lymphoma (reticulum cell sarcoma), multiple myeloma, malignant giant cell tumor chordoma, osteochondroma (osteochondrogenous exostoses), benign chondroma, chondroblastoma, chondromyxofibroma, osteoid osteoma and giant cell tumors;
- nervous system cancers, including, for example, cancers of the skull, e.g., osteoma,
- 25 hemangioma, granuloma, xanthoma, and osteitis deformans; cancers of the meninges, e.g., meningioma, meningiosarcoma, and gliomatosis; cancers of the brain, e.g., astrocytoma, medulloblastoma, glioma, ependymoma, germinoma (pinealoma), glioblastoma multiform, oligodendroglioma, schwannoma, retinoblastoma, and congenital tumors; and cancers of the spinal cord, e.g., neurofibroma, meningioma, glioma, and sarcoma;
- 30 gynecological cancers, including, for example, cancers of the uterus, e.g., endometrial carcinoma; cancers of the cervix, e.g., cervical carcinoma, and pre-tumor cervical dysplasia; cancers of the ovaries, e.g., ovarian carcinoma, including serous cystadenocarcinoma, mucinous cystadenocarcinoma, unclassified carcinoma, granulosa-thecal cell tumors, Sertoli-Leydig cell tumors, dysgerminoma, and malignant teratoma; cancers of the vulva,

e.g., squamous cell carcinoma, intraepithelial carcinoma, adenocarcinoma, fibrosarcoma, and melanoma; cancers of the vagina, e.g., clear cell carcinoma, squamous cell carcinoma, botryoid sarcoma, and embryonal rhabdomyosarcoma; and cancers of the fallopian tubes, e.g., carcinoma;

5 hematologic cancers, including, for example, cancers of the blood, e.g., acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, myeloproliferative diseases, multiple myeloma, and myelodysplastic syndrome, Hodgkin's lymphoma, non-Hodgkin's lymphoma (malignant lymphoma) and Waldenström's macroglobulinemia, angioimmunoblastic T-cell lymphoma (AITL), chronic lymphocytic
10 leukemia (CLL), acute nonlymphocytic leukemia, chronic lymphocytic leukemia, acute granulocytic leukemia, chronic granulocytic leukemia, monocytic leukemia, myeloblastic leukemia, myelocytic leukemia, myeloid granulocytic leukemia, myelomonocytic leukemia, Naegeli leukemia, plasma cell leukemia, plasmacytic leukemia, promyelocytic leukemia, Rieder cell leukemia, Schilling's leukemia, stem cell leukemia, subleukemic leukemia, acute
15 promyelocytic leukemia, adult T-cell leukemia, aleukemic leukemia, aleukocythemic leukemia, basophilic leukemia, blast cell leukemia, bovine leukemia, chronic myelocytic leukemia, leukemia cutis, embryonal leukemia, undifferentiated cell leukemia, eosinophilic leukemia, Gross' leukemia, hairy-cell leukemia, hemoblastic leukemia, hemocytoblastic leukemia, histiocytic leukemia, stem cell leukemia, acute monocytic leukemia, leukopenic
20 leukemia, lymphatic leukemia, lymphoblastic leukemia, lymphocytic leukemia, lymphogenous leukemia, lymphoid leukemia, lymphosarcoma cell leukemia, mast cell leukemia, megakaryocytic leukemia and micromyeloblastic leukemia;

 skin cancers, including, for example, malignant melanoma, basal cell carcinoma, squamous cell carcinoma, Kaposi's sarcoma, moles dysplastic nevi, lipoma, angioma,
25 dermatofibroma, keloids, psoriasis; and

 adrenal gland cancers, including, for example, neuroblastoma.

 More particular examples of such cancers include kidney or renal cancer, breast cancer, colon cancer, rectal cancer, colorectal cancer, lung cancer including small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung and squamous carcinoma of
30 the lung, squamous cell cancer (e.g. epithelial squamous cell cancer), cervical cancer, ovarian cancer, prostate cancer, liver cancer, bladder cancer, cancer of the peritoneum, hepatocellular cancer, gastric or stomach cancer including gastrointestinal cancer, gastrointestinal stromal tumors (GIST), pancreatic cancer, head and neck cancer, glioblastoma, retinoblastoma,

astrocytoma, thecomas, arrhenoblastomas, hepatoma, hematologic malignancies including non-Hodgkins lymphoma (NHL), multiple myeloma and acute hematologic malignancies, endometrial or uterine carcinoma, endometriosis, fibrosarcomas, choriocarcinoma, salivary gland carcinoma, vulval cancer, thyroid cancer, esophageal carcinomas, hepatic carcinoma, anal carcinoma, penile carcinoma, nasopharyngeal carcinoma, laryngeal carcinomas, Kaposi's sarcoma, melanoma, skin carcinomas, Schwannoma, oligodendroglioma, neuroblastomas, rhabdomyosarcoma, osteogenic sarcoma, leiomyosarcomas, urinary tract carcinomas, thyroid carcinomas, Wilm's tumor, as well as B-cell lymphoma (including low grade/follicular non-Hodgkin's lymphoma (NHL); small lymphocytic (SL) NHL; intermediate grade/follicular NHL; intermediate grade diffuse NHL; high grade immunoblastic NHL; high grade lymphoblastic NHL; high grade small non-cleaved cell NHL; bulky disease NHL; mantle cell lymphoma; AIDS-related lymphoma; and Waldenstrom's Macroglobulinemia); chronic lymphocytic leukemia (CLL); acute lymphoblastic leukemia (ALL); acute myelogenous leukemia (AML); Hairy cell leukemia; chronic myeloblastic leukemia; and post-transplant lymphoproliferative disorder (PTLD), as well as abnormal vascular proliferation associated with phakomatoses, edema (such as that associated with brain tumors), and Meigs' syndrome. "Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

Cancers may be solid tumors that may or may not be metastatic. Cancers may also occur, as in leukemia, as a diffuse tissue. Thus, the term "tumor cell", as provided herein, includes a cell afflicted by any one of the above identified disorders.

In a preferred embodiment, the cancer is a solid tumor. In preferred embodiments, the cancer is one of pancreatic cancer, breast cancer, ovarian cancer, bladder cancer, melanoma and glioblastoma.

In another embodiment, the cancer is a hematologic cancer. In preferred embodiments, the hematological cancer is one of acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML).

Pharmaceutical Compositions and Dosing Regimens

Administration of the compositions of the invention is typically parenteral, by subcutaneous, intravenous, intramuscular, or intraperitoneal injection, or by infusion or by any other acceptable systemic method. In a preferred embodiment, administration is by subcutaneous injection. In another preferred embodiment, administration is by intravenous infusion, which may typically take place over a time course of about 1 to 5 hours. In addition, there are a variety of oral delivery methods for administration of therapeutic proteins, and

these can be applied to the therapeutic fusion proteins of this invention.

Often, treatment dosages are titrated upward from a low level to optimize safety and efficacy. Generally, daily dosages will fall within a range of about 0.01 to 20 mg protein per kilogram of body weight. Typically, the dosage range will be from about 0.1 to 5 mg protein per kilogram of body weight. Various modifications or derivatives of the fusion proteins, such as addition of polyethylene glycol chains (PEGylation), may be made to influence their pharmacokinetic and/or pharmacodynamic properties.

To administer the fusion protein by other than parenteral administration, it may be necessary to coat the protein with, or co-administer the protein with, a material to prevent its inactivation. For example, protein may be administered in an incomplete adjuvant, co-administered with enzyme inhibitors or in liposomes. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.*, 1984, *J. Neuroimmunol.* 7:27).

Although the compositions of the invention can be administered in simple solution, they are more typically used in combination with other materials such as carriers, preferably pharmaceutically acceptable carriers. Useful pharmaceutically acceptable carriers can be any compatible, non-toxic substance suitable for delivering the compositions of the invention to a patient. Sterile water, alcohol, fats, waxes, and inert solids may be included in a carrier. Pharmaceutically acceptable adjuvants (buffering agents, dispersing agents) may also be incorporated into the pharmaceutical composition. Generally, compositions useful for parenteral administration of such drugs are well known; *e.g.*, *Remington's Pharmaceutical Science*, 17th Ed. (Mack Publishing Company, Easton, Pa., 1990). Alternatively, compositions of the invention may be introduced into a patient's body by implantable drug delivery systems (Urquhart *et al.*, 1984, *Ann. Rev. Pharmacol. Toxicol.* 24:199).

Therapeutic formulations may be administered in many conventional dosage formulations. Formulations typically comprise at least one active ingredient, together with one or more pharmaceutically acceptable carriers. Formulations may include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration.

The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. *See, e.g.*, Gilman *et al.* (eds.) (1990), *The Pharmacological Bases of Therapeutics*, 8th Ed., Pergamon Press; and *Remington's Pharmaceutical Sciences*, *supra*, Easton, Pa.; Avis *et al.* (eds.) (1993)

Pharmaceutical Dosage Forms: Parenteral Medications, Dekker, N.Y.; Lieberman *et al.* (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets*, Dekker, N.Y.; and Lieberman *et al.* (eds.) (1990), *Pharmaceutical Dosage Forms: Disperse Systems*, Dekker, N.Y.

In additional embodiments, the present invention contemplates administration of the
5 fusion proteins by gene therapy methods, e.g., administration of an isolated nucleic acid
encoding a fusion protein of interest. The protein building blocks (e.g., Component A and
Component B) of the fusion protein of the present invention have been well-characterized,
both as to the nucleic acid sequences encoding the proteins and the resultant amino acid
sequences of the proteins. Engineering of such isolated nucleic acids by recombinant DNA
10 methods is well within the ability of one skilled in the art. Codon optimization, for purposes
of maximizing recombinant protein yields in particular cell backgrounds, is also well within
the ability of one skilled in the art. Administration of an isolated nucleic acid encoding the
fusion protein is encompassed by the expression “administering a therapeutically effective
amount of a fusion protein of the invention.” Gene therapy methods are well known in the art.
15 *See, e.g.*, WO96/07321 which discloses the use of gene therapy methods to generate
intracellular antibodies. Gene therapy methods have also been successfully demonstrated in
human patients. *See, e.g.*, Baumgartner *et al.*, 1998, *Circulation* 97: 12, 1114-1123, and more
recently, Fatham, 2007, “A gene therapy approach to treatment of autoimmune diseases,”
Immun. Res. 18:15-26; and U.S. Pat. No. 7,378,089, both incorporated herein by reference.
20 *See also* Bainbridge *et al.*, 2008, “Effect of gene therapy on visual function in Leber's
congenital Amaurosis,” *N Engl Med* 358:2231-2239; and Maguire *et al.*, 2008, “Safety and
efficacy of gene transfer for Leber's congenital Amaurosis,” *N Engl J Med* 358:2240-8.
There are two major approaches for introducing a nucleic acid encoding the fusion protein
(optionally contained in a vector) into a patients cells: *in vivo* and *ex vivo*. For *in vivo*
25 delivery, the nucleic acid is injected directly into the patient, usually at the site where the
fusion protein is required. For *ex vivo* treatment, the patient's cells are removed, the nucleic
acid is introduced into these isolated cells and the modified cells are administered to the
patient either directly or, for example, encapsulated within porous membranes which are
implanted into the patient (*see, e.g.*, U.S. Pat. Nos. 4,892,538 and 5,283,187). There are a
30 variety of techniques available for introducing nucleic acids into viable cells. The techniques
vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro*, or *in*
vivo in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into
mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell
fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. Commonly used

vectors for ex vivo delivery of the gene are retroviral and lentiviral vectors.

Preferred in vivo nucleic acid transfer techniques include transfection with viral vectors such as adenovirus, Herpes simplex I virus, adeno-associated virus), lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example), naked DNA, and transposon-based expression systems. For review of the currently known gene marking and gene therapy protocols see Anderson *et al.*, *Science* 256:808-813 (1992). *See also* WO 93/25673 and the references cited therein.

“Gene therapy” includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Oligonucleotides can be modified to enhance their uptake, e.g. by substituting their negatively charged phosphodiester groups by uncharged groups. Fusion proteins of the present invention can be delivered using gene therapy methods, for example locally in tumor beds, intrathecally, or systemically (e.g., via vectors that selectively target specific tissue types, for example, tissue-specific adeno-associated viral vectors). In some embodiments, primary cells (such as lymphocytes or stem cells) from the individual can be transfected ex vivo with a gene encoding any of the fusion proteins of the present invention, and then returning the transfected cells to the individual's body.

“Treating” or “treatment” refers to therapeutic treatment, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. A subject is successfully “treated” if: after receiving a therapeutic amount of a fusion protein of the invention according to the methods of the present invention, the subject shows observable and/or measurable reduction in or absence of one or more signs and symptoms of the particular disease. For example, for cancer, reduction in the number of cancer cells or absence of the cancer cells; reduction in the tumor size; inhibition (*i.e.*, slow to some extent and preferably stop) of tumor metastasis; inhibition, to some extent, of tumor growth; increase in length of remission, and/or relief to some extent, one or more of the symptoms associated with the specific cancer; reduced morbidity and mortality, and improvement in quality of life issues. Reduction of the signs or symptoms of a disease may also be felt by the patient. Treatment can achieve a complete response, defined as disappearance of all signs of cancer, or a partial response, wherein the size of the tumor is decreased, preferably by more than 50%, more preferably by 75%. A patient is also considered treated if the patient experiences a stabilization of disease. These parameters for assessing successful treatment and improvement in the disease are readily measurable by routine procedures familiar to a

physician of appropriate skill in the art.

In the context of treatment for cancer, the fusion proteins of the present invention can optionally be administered to a patient in combination with other chemotherapeutic agents. Suitable chemotherapeutic agents include, for example, alkylating agents such as thiotepa and
5 cyclophosphamide (CYTOXANTM); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide,
10 mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabycin, caminomycin, carzinophilin, chromomycins, dactinomycin,
15 daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin,
20 trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid;
25 aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziqune; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK.RTM; razoxane; sizofiran; spirogermanium; tenuazonic
30 acid; triaziquone; 2,2',2''-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (TAXOL.RTM, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (TAXOTERE.RTM, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum

analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); Ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylomithine (DMFO); retinoic acid; esperamicins; capecitabine; and
5 pharmaceutically acceptable salts, acids or derivatives of any of the above.

Other chemotherapeutic agents further include anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and anti-androgens such as
10 flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

Other tumor cytotoxic agents that can be used in combination with a fusion protein of the invention are themselves fusion proteins. Exemplars of tumor cytotoxic fusion proteins are CTLA-4-FasL and Fn14-TRAIL, which, as cis loop-back proteins, can act by creating
15 auto-apoptotic signaling loops at the surface of tumor cells. *See* U.S. Patent Nos 7,569,663; 8,329,657 and 8,039,437, each of which is incorporated by reference in its entirety. In turn, those cis loop-back proteins incorporating a TRAIL component can be administered in combination with chemotherapeutic agents that are able to sensitize tumor cells to TRAIL and overcome TRAIL resistance, such as proteasome inhibitors and histone deacetylase
20 (HDAC) inhibitors, cycloheximide, imatinib mesylate and other protein tyrosine kinase inhibitors, 17-allylamino-17-demethoxygeldanamycin, arsenic trioxide and X-linked Inhibitors of Apoptosis Protein small molecule antagonists; and pharmaceutically acceptable salts, acids or derivatives of any of these.

Additional information on the methods of cancer treatment is provided in U.S. Pat.
25 No. 7,285,522, incorporated by reference in its entirety.

The practice of the invention is illustrated by the following non-limiting examples. The invention should not be construed to be limited solely to the compositions and methods described herein, but should be construed to include other compositions and methods as well. One of skill in the art will know that other compositions and methods are available to perform
30 the procedures described herein.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are well within the purview of the skilled artisan. Such techniques are explained fully in the literature, such as,

“Molecular Cloning: A Laboratory Manual”, fourth edition (Sambrook, 2012);
“Oligonucleotide Synthesis” (Gait, 1984); “Culture of Animal Cells” (Freshney, 2010);
“Methods in Enzymology” “Handbook of Experimental Immunology” (Weir, 1997); “Gene
Transfer Vectors for Mammalian Cells” (Miller and Calos, 1987); “Short Protocols in
5 Molecular Biology” (Ausubel, 2002); “Polymerase Chain Reaction: Principles, Applications
and Troubleshooting”, (Babar, 2011); “Current Protocols in Immunology” (Coligan, 2002).
These techniques are applicable to the production of the polynucleotides and polypeptides of
the invention, and, as such, may be considered in making and practicing the invention.
Particularly useful techniques for particular embodiments will be discussed in the sections
10 that follow.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental
15 examples. These examples are provided for purposes of illustration only, and are not intended
to be limiting unless otherwise specified. Thus, the invention should in no way be construed
as being limited to the following examples, but rather, should be construed to encompass any
and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can,
20 using the preceding description and the following illustrative examples, make and utilize the
compounds of the present invention and practice the claimed methods. The following
working examples therefore, specifically point out the preferred embodiments of the present
invention, and are not to be construed as limiting in any way the remainder of the disclosure.

25 Example 1-Fusion Protein Binding

Fusion protein constructs and expression.

The expression plasmid constructs for these fusion proteins were generated using gene
fragments synthesized de novo (ThermoFisher Scientific) for each of the desired
ligand/receptor components, linked to synthesized gene fragments of the hinge, CH2 and
30 CH3 domains of either the A or B chains (*i.e.* Z₂, Z₃, Z₂' or Z₃' domains). The individual
components were spliced together by polymerase chain reaction (PCR) using primers at the
ends containing restriction sites for cloning into the expression plasmid, pCEP4, an EBV
episomal expression vector (originally developed in the Tykocinski laboratory) that replicates
extra-chromosomally at high copy numbers. At the time of gene synthesis, the DNA

fragments are codon optimized for expression in Chinese hamster ovary suspension (CHO-S) cells. Fusion proteins were produced by transient co-transfection of an A chain construct and a B chain construct into ExpiCHO-S shake flask cultures using TransIT-Pro reagent (MIRUS) at 37° C for 24 h, then incubated at 32° C for 8-10 total days. Proteins were purified from conditioned culture supernatant by mixing with Protein A agarose resin at 6° C overnight and then collected and eluted with a non-denaturing neutral pH elution buffer (PIERCE). Each fusion protein was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for size and integrity.

10 *Fusion protein binding studies.*

Ligand constructs expressing the full-length cDNA for human CD155, T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), CD112 and CD16 (Fc γ RIIIa) were generated by PCR and cloned into the expression plasmid pcDNA3.1+ (ThermoFisher Scientific). Each expression construct was transfected into CHO-S cells with Lipofectamine 3000 (ThermoFisher) and stable transfectants selected by the addition of G418. Ligand expression was determined by flow cytometry following immunostaining with the appropriate fluorochrome-conjugated anti-ligand antibody (black line) or the appropriate fluorochrome-conjugated isotype control antibody (gray line and fill). Data were analyzed using FCSalyzer software. For fusion protein binding studies, fusion proteins were added to either non-transfected CHO-S cells as control (gray line and fill) or CHO-S transfectants expressing corresponding ligands and incubated for 1 h at 6° C, then washed, and immunostained with Cy5-conjugated anti-human IgG, Fc γ -specific antibody (black line) and analyzed by flow cytometry. *See* Figures 18-20. For CD16 binding studies, TIGIT or CD155 containing fusion proteins were incubated with either non-transfected CHO-S cells as control (gray line and fill) or CD16-expressing CHO-S transfectants and detected by immunostaining with APC- or PE-conjugated antibody recognizing TIGIT or CD155, respectively (black line). *See* Figure 21.

Example 2-Fusion Protein Binding Assays and Migration Assays

30 *Protein gel analysis*

Fusion Proteins were separated and reduced (R) or non-reduced (NR) on 12% SDS-PAGE gel and stained for 12 hours in PageBlue Protein Staining Solution (Thermo Scientific). *See* Figure 22.

Immunoblot analysis

Fusion proteins were then quantitated by Pierce BCA Protein Assay Kit (Thermo Fisher) and loaded on 12% SDS-PAGE gels. Proteins were electrotransferred onto Immobilon-P
5 membranes (EMD Millipore, Billerica, MA) and incubated with (A) Ab against PD1 and (B) Ab against IgG light chain primary antibodies for 12 h in Odyssey blocking buffer (Licor, Lincoln, NE). The corresponding secondary antibodies were used at 1:10,000 dilutions (Santa Cruz). Immunoblots were scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences, model #9120). *See Figure 23.*

10

Fusion protein binding studies.

CXCR4/7 and PD1+ melanoma cell lines, (A) YUMMER1.7 cells or (B) B16, were incubated with the indicated fusion proteins for 1 h at 6° C, then washed, and immunostained with Cy5-conjugated anti-human IgG, Fcγ-specific antibody and analyzed by flow cytometry
15 (solid black line), control sample incubated without fusion protein (filled grey line) as shown in Figure 24.

Transwell Assay.

Transwell inserts were inserted in 24-well companion plates (Corning, cat. #353504). Cell
20 suspensions were seeded in top invasion chambers at 25,000 cells/chamber. DMEM with 100ng/mL of CXCL12 was added to each bottom well as chemoattractant. Samples were incubated at 37°C for 24 hours to allow for cell migration. Non-invading cells on the apical surface of the insert were removed with cotton swabs, and cells that had migrated to the lower surface of the supports were stained using 2% crystal violet. *See Figure 25.*

25

Example 3- TriTouch-101 induces melanoma regression

Inhibition of tumor growth was induced by TriTouch-101 PD1hFcA*vMIP1ICH-hFcB*CL (FP) *in vivo*. The B16F10 melanoma subcutaneous model was used to demonstrate the induction of melanoma regression by PD1hFcA*vMIP1ICH-hFcB*CL (FP). Briefly,
30 BL16 mice were treated with 100 µl of (10ug/mL) FP (mice R, 2L and 2R) or PBS (Mice X or L), 5 times on days 13, 14, 15, 16 and 21 days post-subcutaneously inoculated with 1×10^5 B16F10 cells. Tumor sizes were measured (Figure 26A). Representative images of 1 mouse from each treatment group, (X top and 2R bottom) are depicted in Figure 26B. These

in vivo data showing TriTouch-101 induces melanoma regression, go along with previous data showing that this fusion protein inhibits melanoma cells in a migration assay (Figure 25)

5 Example 4- NK modulating TriTouch protein species

ADCC was augmented by addition of multi-functional fusion proteins (Figure 27). The SKOV-3 ovarian cell line was plated in a 96-well plate at 3000 cells/well, allowed to adhere and labeled with CellTracker Red CMTPX reagent. The SKOV-3 cells were then labeled with a green fluorescence Caspase-3 reagent. The CD16.NK-92 cell line (V158
10 variant) was added to the wells at a E:T 5:1 with the various fusion proteins at 25 mg/ml or no protein and analyzed using the Incucyte live-cell analysis system, measuring the number of fluorescent double positive (red + green) cells . Results shown depict the 20 h time point (Figure 27).

15 Other Embodiments

The recitation of a listing of elements in any definition of a variable herein includes definitions of that variable as any single element or combination (or subcombination) of listed elements. The recitation of an embodiment herein includes that embodiment as any
20 single embodiment or in combination with any other embodiment or portions thereof.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be
25 construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A fusion protein comprising Component A, Component B, and Component C;
wherein:

5 Component A comprising the amino acid sequence of SEQ ID NO: 14, which
comprises in order Component Y, Component Z₂ and Component Z₃; and

 Component B comprising the amino acid sequence of SEQ ID NO: 49, which comprises
in order Component X', Component Z₂' and Component Z₃'; and

10 Component C comprising the amino acid sequence of SEQ ID NO: 57, which comprises
in order Component X, and Component C'_L, wherein

 Component Y comprises a portion of PD-1,

 Component Z₂ and Component Z₂' comprise CH2 domains of human Fc,

 Component Z₃ and Component Z₃' comprise CH3 domains of human Fc,

 Component X' and Component X comprise a portion of vMIP-II, and

15 Component C'_L comprises at least one CH1 domain of human IgG1 kappa, and
wherein Component Z₂ is linked to Component Z₂' via a disulfide bond.

2. The fusion protein of claim 1, wherein the fusion protein is capable of binding i)
PDL1 or PD-L2, ii) CXCR4, and iii) an Fc receptor or ligand on an immune cell.

20

3. The fusion protein of any one of claims 1-2 for use in treating cancer in a patient in
need of such treatment.