



(51) International Patent Classification:
C07K 16/28 (2006.01)

(21) International Application Number:
PCT/US2017/048721

(22) International Filing Date:
25 August 2017 (25.08.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/379,582 25 August 2016 (25.08.2016) US

(71) Applicant: **THE UNITED STATES OF AMERICA,
AS REPRESENTED BY THE SECRETARY, DE-
PARTMENT OF HEALTH AND HUMAN SERVICES**
[US/US]; Office of Technology Transfer, National Insti-
tutes of Health, 6011 Executive Boulevard, Suite 325, MSC
7660, Bethesda, Maryland 20892-7660 (US).

(72) Inventors: **DIMITROV, Dimiter S.**; 1019 Storrington
Drive, Frederick, Maryland 21702 (US). **LI, Wei**; 926
Taney Avenue, Frederick, Maryland 21702 (US). **CHEN,
Weizao**; 9378 Penrose Street, Frederick, Maryland 21704
(US).

(74) Agent: **GASE, John L.** et al.; Leydig, Voit & Mayer, LTD.,
Two Prudential Plaza, Suite 4900, 180 North Stetson Av-
enue, Chicago, Illinois 60601 (US).

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN,
HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP,
KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA,
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: HUMAN SINGLE DOMAIN ANTIBODIES TARGETING CD16A TO MEDIATE ADCC

(57) Abstract: The invention provides a polypeptide comprising an amino acid sequence with at least 85% sequence identity to the amino acid sequence of SEQ ID NO: 2 or the amino acid sequence of SEQ ID NO: 3 (e.g., SEQ ID NO: 1), as well as a fusion protein comprising the polypeptide and one or more fusion partners, wherein the one or more fusion partners optionally is joined to the polypeptide via a linker. Additionally, nucleic acids encoding the polypeptide and fusion proteins, vectors comprising the nucleic acids, cells comprising the nucleic acids or vectors, as well as compositions comprising the polypeptide, fusion proteins, nucleic acids, vectors, and/or cells, also are provided. The inventive compositions can be used in methods of inducing antibody dependent cellular cytotoxicity (ADCC) in a host (e.g., to inhibit cancer or an infectious disease, such as a viral infection).



HUMAN SINGLE DOMAIN ANTIBODIES TARGETING CD16A TO MEDIATE ADCC
CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 62/379,582, filed August 25, 2016, the entire disclosure of which is hereby incorporated by reference.

SEQUENCE LISTING

[0002] Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 31,864 Byte ASCII (Text) file named "729079_ST25.TXT," dated August 25, 2017.

BACKGROUND OF THE INVENTION

[0003] NK cells are essential components of the innate immune system and play a critical role in host immunity against infectious diseases (e.g., viral infection) and cancer. Antibody dependent cellular cytotoxicity (ADCC) is an important mechanism for NK cells mediating direct and fast killing against the virus-infected and tumor-transformed cells without any antigen pre-priming after the NK cells' ADCC receptor CD16A is activated.

[0004] The ADCC function of NK cells is highly pursued in antibody intervening immunotherapy. Bispecific antibodies with two targeting modules, one for recruiting ADCC receptors CD16A and the other for recognizing antigen, have drawn extensive attention due to the high efficacy for engaging NK cells to kill the target cells both *in vitro* and *in vivo*. Currently, several formats of these bispecific antibodies are undergoing basic and clinical research, such as Bispecific Killer Cell Engager (BiKE), bispecific diabody (BidAb) and tetra-valent, bispecific TandAb.

[0005] BiKE is composed of two tandem scFv arms connected by a flexible polypeptide linker. Examples for this class are CD16/CD33 for graft versus leukemia, acute myeloid leukemia, and myelodysplastic syndromes; CD16/CD19 for non-Hodgkin's lymphoma; CD16/CD133 for colorectal cancer cells; and CD16/HER2 for breast cancer, all of which have shown specifically high efficacy for engaging NK cells to lyse target cells. However, these BiKEs have encountered many limitations in the production process such as low

soluble expression in bacteria, laborious re-folding from the inclusion body, high by-products due to the mismatching of its sub-domains, and high heterogeneity in the final product.

[0006] Similar problems exist for BidAb, which consists of the tail-to-head heterodimer of two scFv molecule with a short linker. The production of BidAb is highly dependent on the features of its linkers and the experimental conditions, and exhibits batch-dependent effects.

[0007] Recently, the more promising format TandAb has been developed by Affimed Therapeutics AG (Heidelberg, Germany). TandAb is a tetravalent, bispecific, tandem diabody composed of the tail-to-head homodimer of the two tandem scFv connected by three (GGG)₃ linkers. One TandAb, CD16/CD30 AFM13, has shown substantial success in phase I clinical trials for patients with relapsed or refractory Hodgkin's lymphoma (clinical trial no. NCT01221571). However, TandAb likely will encounter more production problems than BidAb since it contains more sub-domains, all of which should be paired elegantly to promise a correct TandAb. Additionally, the TandAb needs to be expressed in mammalian cells rather than in bacteria, which are expensive and time consuming. Furthermore, due to its tetravalence, two for CD16A and two for CD30, TandAb has risks for non-specifically activating CD16A in the absence of CD30 and inducing side effects.

[0008] Therefore, a need exists for antibodies that circumvent the problems encountered by BiKE, BidAb, and TandAb.

BRIEF SUMMARY OF THE INVENTION

[0009] The invention provides a polypeptide that binds CD16A. In one embodiment, the polypeptide comprises an amino acid sequence with at least 85% sequence identity to the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3. In another embodiment, the polypeptide comprises SEQ ID NO: 1. Also provided is a fusion protein comprising the polypeptide and one or more fusion partners, wherein the one or more fusion partners optionally is joined to the polypeptide via a linker. Additionally, nucleic acids encoding the polypeptide and fusion proteins, vectors comprising the nucleic acids, cells comprising the nucleic acids or vectors, as well as compositions comprising the polypeptide, fusion proteins, nucleic acids, vectors, and/or cells, also are provided.

[0010] The invention also provides a method of inducing antibody dependent cellular cytotoxicity (ADCC) in a host, a method of inhibiting cancer in a host, and a method of

inhibiting an infectious disease in a host comprising administering to the host a composition comprising the polypeptide or fusion protein and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0011] Figure 1 is a schematic showing the prevalent bispecific antibody formats: BiKE, BidAb, and TandAb.

[0012] Figure 2 is a schematic showing the bispecific antibody NanoBiKE, which comprises a single domain antibody and single domain CD4 joined by a linker (GGGGS)₃ (SEQ ID NO: 17).

[0013] Figure 3 shows luciferase activity of Jurkat T-CD16A cells after incubation with BiKEs or mD1.22-Fc (Panel A). Experiments were performed in triplicate.  represents Jurkat T-CD16A cells incubated with mbk6 and 293T-gp160_{SC} cells (Jurkat T-CD16A + mbk6 + 293T-gp160_{SC}, simply denoted as mbk6 in the figure); , Jurkat T-CD16A + mbk11 + 293T-gp160_{SC} (denoted as mbk11); , Jurkat T-CD16A + mD1.22-Fc + 293T-gp160_{SC} (simply put as mD1.22-Fc); other groups are **negative controls**, in which Jurkat T-CD16A cells was either incubated with BiKEs in the absence of target cells, or incubated with BiKEs in the presence of gp160_{SC} negative 293T cells. , Jurkat T-CD16A + mbk6; , Jurkat T-CD16A + mbk6 + 293T; , Jurkat T-CD16A + mbk11; , Jurkat T-CD16A + mbk11 + 293T; , Jurkat T-CD16A + mD1.22-Fc; , Jurkat T-CD16A + mD1.22-Fc + 293T. Figure 3 also shows an analysis of secretion of IL-2 by Jurkat T-CD16A cells activated by BiKEs at 20 nM (Panel B). The results shown are from three independent experiments. Statistical tests were performed using GraphPad Prism5. Significant differences when comparing two groups were determined by Student's t test. A two-tailed p value < 0.05 was considered significant. *: p < 0.05. **: p < 0.01. ***: p < 0.001. NS: not significant.

[0014] Figure 4 shows an analysis of NK cells degranulation mediated by BiKEs or mD1.22-Fc. Degranulation was evaluated by the NK cell surface CD107a staining using the PE-CyTM5 mouse anti-human CD107a antibody. Figure 4 (Panel A) shows CD107a expression on NK cells surface. NK cells were incubated with 20 nM of BiKEs or mD1.22-Fc in the absence or presence of CHO-ZA-gp160_{SC}. The data were analyzed by the CellQuest Pro software. Figure 4 (Panel B) shows percentage of CD107a positive NK cells. The red color in the column figure denotes the controls without BiKEs and the black color accounts for the BiKEs incubation with NK cells alone, or combined with CHO-ZA cells or CHO-ZA-gp160_{SC} cells. The results are from three independent experiments. Error bars

were calculated and statistical significance was tested by using GraphPad Prism5. Significant differences among inter-groups (blue color) and intra-group (black color) were determined by two-way ANOVA and Student's t test, respectively. A p value < 0.05 was considered significant. *: p < 0.05. **: p < 0.01. ***: p < 0.001. NS: not significant.

[0015] Figure 5A shows Killing of CHO-ZA-gp160_{sc} cells in presence of NK cells. Target cells was pre-labelled by PHK26 and incubated with serially diluted BiKEs or mD1.22-Fc followed by adding of NK cells. The dead cells were labelled by PI before evaluation by FACS analysis. Experiments were performed in triplicate and the error bars denote $\pm$ SD, n=3. $\blacksquare$ represents NK cells incubated with mbk6 and CHO-ZA-gp160_{sc} cells (NK cells + mbk6 + CHO-ZA-gp160_{sc}, denoted as mbk6 in the figure); $\blacklozenge$, NK cells + mbk11 + CHO-ZA-gp160_{sc} (denoted as mbk11); $\blacktriangleleft$, NK cells + mD1.22-Fc + CHO-ZA-gp160_{sc} (denoted as mD1.22-Fc); other groups are **negative controls**, in which NK cells incubation with CHO-ZA-gp160_{sc} cells in the absence of BiKEs, or NK cells incubated gp160 negative CHO-ZA cells in the presence of BiKEs or mD1.22-Fc. $\blacklozenge$, NK cells + mD1.22-Fc + CHO-ZA; $\blacktriangleleft$, NK cells + CHO-ZA-gp160_{sc}; $\blacktriangleleft$, NK cells + mbk6 + CHO-ZA; $\blacktriangleleft$, NK cells + mbk11 + CHO-ZA.

[0016] Figure 5B shows Killing of chronically HIV-1 infected cells (8E5) by human PBMCs mediated by BiKEs. Experiments were performed by co-incubation of PBMCs with BiKE opsonized 8E5 cells and the assay was developed by detecting LDH activity using Promega CytoTox-ONE™ Homogeneous Membrane Integrity kit. The anti-MERS-CoV mAb, IgG1 m336 was used as a negative control. Experiments were performed in duplicate and the error bars denote $\pm$ SD, n=2.

[0017] Figure 6 provides a schematic illustration of examples of BiKE variants.

[0018] Figure 7 is a graph of cell killing plotted against mbk6 concentration.

DETAILED DESCRIPTION OF THE INVENTION

[0019] Embodiments of the invention provide single domain antibodies (sdAs) targeting CD16A. According to some aspects of the disclosure, the antibodies can be used to engage antibody-dependent cell-mediated cytotoxicity (ADCC). In some embodiments, the inventive sdAs are soluble, can be expressed in bacteria with high expression level, and/or easily purified by one-step Ni-NTA affinity chromatography. In addition, they can have excellent drug-related properties including being monomeric, having high solubility and

stability, and having aggregation resistance. sdAs have low risk for eliciting immunogenicity when used as therapeutics compared to those coming from animals, such as llama. Furthermore, due to the small size of the sdAs, greater tissue penetration may be achieved. The sdAs target human CD16A and can be used to generate bispecific or multispecific antibodies for treatment of infectious diseases and cancers.

[0020] A sdA, which is also known as a domain antibody (dAb) or engineered antibody domain (eAd)), is an fragment consisting of a single monomeric variable antibody domain from the heavy or light chains.

[0021] The invention provides a polypeptide (e.g., sdA) comprising, consisting essentially of, or consisting of the amino acid sequence of SEQ ID NO: 1. In a particular embodiment, the inventive polypeptide comprises, consists essentially of, or consists of the amino acid sequence of SEQ ID NO: 2 (referred to as sdA1, or D6 below) or SEQ ID NO: 3 (referred to as sdA2, or E11). sdA1 comprises the framework sequences of SEQ ID NOs: 4, 6, 8, and 10 and the complementarity determining region (CDR) sequences (e.g., CDR1, CDR2, and CDR3) of SEQ ID NOs: 5, 7, and 9. sdA2 comprises the framework sequences of SEQ ID NOs: 11, 13, 15, and 10 and the CDR sequences (e.g., CDR1, CDR2, and CDR3) of SEQ ID NOs: 12, 14, and 16.

[0022] The invention also provides a polypeptide comprising, consisting essentially of, or consisting of a variant of sdA1 or sdA2. In one embodiment, the polypeptide comprises, consists essentially of, or consists of an amino acid sequence having at least 85% (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) sequence identity to the amino acid sequence of SEQ ID NO: 2. In another embodiment, the polypeptide comprises, consists essentially of, or consists of an amino acid sequence having at least 85% (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) sequence identity to the amino acid sequence of SEQ ID NO: 3.

[0023] The invention also provides a polypeptide comprising variants of SEQ ID NO: 2 or SEQ ID NO: 3 with up to 20 (e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) additions, deletions, substitutions, or insertions. For example, the invention provides a polypeptide comprising the SEQ ID NO: 2 or SEQ ID NO: 3 with up to 10 additions, deletions, substitutions, or insertions.

[0024] In a specific embodiment, the variants of sdA1 (SEQ ID NO: 2) and sdA2 (SEQ ID NO: 3) do not contain changes to the CDRs described above (i.e., the CDR sequences are maintained without modification in the variants of sdA1 and sdA2).

[0025] The polypeptide described above, which binds to CD16A will sometimes be referred to herein as the anti-CD16A sdA.

[0026] The anti-CD16A sdA can be provided alone, or as part of a fusion protein comprising the polypeptide and one or more (e.g., two, three, four, five, or more) fusion partners.

[0027] In one embodiment, the fusion partner enhances the stability and/or potency of the polypeptide as compared to the stability or potency of the polypeptide in the absence of the fusion partner. For instance, the fusion partner can be a naturally occurring protein or fragment thereof that resists degradation or removal by endogenous mechanisms *in vivo*, thereby increasing the half-life of the fusion protein as compared to the polypeptide in the absence of the fusion protein.

[0028] Examples of suitable fusion partners include: (a) proteins from the extracellular matrix, such as collagen, laminin, integrin, and fibronectin; (b) proteins found in blood, such as serum albumin, serum albumin-binding peptide (SAbp), fibrinogen A, fibrinogen B, serum amyloid protein A, heptaglobin, protein, ubiquitin, uteroglobulin, β -2 microglobulin, plasminogen, lysozyme, cystatin C, α -1-antitrypsin, and pancreatic kypsin inhibitor; (c) immune serum proteins, such as IgE, IgG, IgM, and their fragments (e.g., Fc); (d) transport proteins, such as retinol binding protein; (e) defensins, such as β -defensin 1, neutrophil defensins 1, 2 and 3; (f) proteins found at the blood brain barrier or in neural tissues, such as melanocortin receptor, myelin, ascorbate transporter; (g) transferrin receptor specific ligand-neuropharmaceutical agent fusion proteins, brain capillary endothelial cell receptor, transferrin, transferrin receptor, insulin, insulin-like growth factor 1 (IGF 1) receptor, insulin-like growth factor 2 (IGF 2) receptor, insulin receptor; (h) proteins localized to the kidney, such as polycystin, type IV collagen, organic anion transporter Kl, Heymann's antigen; (i) proteins localized to the liver, such as alcohol dehydrogenase, G250; (j) blood coagulation factor X; (k) α -1 antitrypsin; (l) HNF 1 α ; (m) proteins localized to the lung, such as secretory component; (n) proteins localized to the heart, such as HSP 27; (o) proteins localized to the skin, such as keratin; (p) bone specific proteins, such as bone morphogenic proteins (BMPs), for example, BMP-2, -4, -5, -6, -7 (also referred to as osteogenic protein (OP-I) and -8 (OP-2); (q) tumor specific proteins, such as human trophoblast antigen, herceptin receptor, estrogen receptor, cathepsins, for example, cathepsin B (found in liver and spleen); (r) disease-specific proteins, such as antigens expressed only on activated T-cells: including LAG-3 (lymphocyte activation gene); osteoprotegerin ligand (OPGL); OX40;

metalloproteases, including CG6512 *Drosophila*, human paraplegin, human FtsH, human AFG3L2, murine ftsH; angiogenic growth factors, including acidic fibroblast growth factor (FGF-I), basic fibroblast growth factor (FGF-2), Vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), transforming growth factor- α (TGF- α), tumor necrosis factor- α (TNF- α), angiogenin, interleukin-3 (IL-3), interleukin-8 (IL-8), platelet derived endothelial growth factor (PD-ECGF), placental growth factor (PIGF), midkine platelet-derived growth factor-BB (PDGF), fractalkine; (s) stress proteins (heat shock proteins); and (t) proteins involved in Fc transport. Additional fusion partners for use in connection herewith are described in WO 2009/089295.

[0029] In one embodiment, the fusion partner is an immunoglobulin Fc region or portion thereof (e.g., the CH2 or CH3 region), especially the Fc region of a human immunoglobulin, such as a human IgG1 Fc region. Examples of an Fc region or portion thereof for use in the invention include, but are not limited to, the amino acid sequence of SEQ ID NO: 20 and SEQ ID NO: 21. CH2, CH3, or Fc regions of other Ig types, particularly human Ig types, can be used (e.g., IgG2, IgG3, IgG4). Furthermore, Fc regions or portions thereof can include any of numerous mutations known in the art to modify the properties thereof as desired (e.g., enhance or decrease effector function, improve stability, reduce recombination events, etc.).

[0030] In another embodiment, the fusion partner is an antibody or antibody fragment (e.g., Fab, scFv, dAb, or an sdA other than the inventive polypeptide). Examples of suitable antibody or antibody fragments include SEQ ID NOs: 22-26, also referred to as the m36, m36.1, m36.2, m36.4, or m36.5 antibodies, respectively, as described in U.S. Patent 8,911,728. For the inhibition of cancer, the fusion partner preferably is an antibody or antibody fragment that targets a tumor-associated antigen (e.g., PSA, CEA, MUC, and portions thereof).

[0031] In another embodiment, the fusion partner is an HIV (e.g., HIV-1 or HIV-2) envelope glycoprotein. Examples of the HIV envelope glycoprotein include gp120 and gp140. Preferably, the HIV envelope glycoprotein is HIV-1 gp120.

[0032] In a further embodiment, the fusion partner is a single domain protein of CD4. Suitable single domain proteins include SEQ ID NOs: 27 and SEQ ID NO: 28, also referred to as mD1.22 and mD1.23, as described in U.S. Patent Application Publication 2016/039904.

[0033] The anti-CD16A sdA and one or more fusion partners can be joined via a linker (i.e., a flexible molecular connection, such as a flexible polypeptide chain). The linker can be any suitable linker of any length, but is preferably at least about 15 (e.g., at least about 20, at

least about 25, at least about 30, at least about 35, at least about 40, at least about 45, at least about 50, or ranges thereof) amino acids in length. In one embodiment, the linker is an amino acid sequence that is naturally present in immunoglobulin molecules of the host, such that the presence of the linker would not result in an immune response against the linker sequence by the mammal. Examples of suitable linkers include, but are not limited to, linkers that comprise one or more (e.g., two, three, four, five, six, seven, eight, nine, or ten) G₄S motifs, such as the linkers of SEQ ID NOs: 17-19.

[0034] By way of further illustration, an example of a fusion protein includes nano-bispecific antibodies (NanoBiKE) created by fusing the anti-CD16A sdA with the single CD4 D1 domain (mD1.22) for targeting HIV-1 envelope glycoprotein (gp120) as described in the Examples. Such a construct is illustrated in Figure 2, which shows examples of fusion proteins of an sdA polypeptide (e.g., comprising any of SEQ ID NOs: 1-3 or variants thereof as described herein) with the engineered single-domain soluble human CD4 (e.g., mD1.22 (SEQ ID NO: 27)) using a flexible linker (e.g., (GGGGS)₃ flexible linker (SEQ ID NO: 17)). Different linkers can be used. In some embodiments, the sdA is fused via a linker to the N-terminal or C-terminal of the CD4. In specific embodiments, the fusion protein has the amino acid sequence of SEQ ID NO: 29, or SEQ ID NO 30, or an amino acid sequence having at least 85% (e.g., 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%) sequence identity to the amino acid sequence of SEQ ID NO: 29 or 30, or has such a sequence without respect to the linker used. In any of the above sequences, the CDRs of the sdA1 or sdA2 portion can be conserved (non-varying with respect to the CDRs of SEQ ID NOs: 5, 7, and 9 or SEQ ID NOs: 12, 14, and 16), yet the remaining sequence can have variations that do not disrupt binding to the antigens.

[0035] Other configurations of the fusion protein are illustrated in Figure 6. These configurations were designed in order to extend BiKE's in vivo half-life. The fusion protein can further comprise, for instance, a CH₂ domain linked to the single-domain CD4 protein, which can endow BiKEs with FcRn binding capacities, hence extending half-life (e.g., Fig. 6, mbk6CH₂). Also, the fusion protein can further comprise a second anti-CD16 sdA linked to the CH₂ domain, which can increase avidity binding of sdAbs to CD16A (mbk6CH₂D6).

[0036] Furthermore, the polypeptide (e.g., anti-CD16A sdA) or fusion protein can be multimerized, as for example, hetero- or homodimers, hetero- or homotrimers, hetero- or homotetramers, or higher order hetero- or homomultimers, and the invention provides such constructs. Multimerization can increase the strength of antigen binding, wherein the

strength of binding is related to the sum of the binding affinities of the multiple binding sites. In particular, cysteine residue(s) can be introduced in the amino acid sequence of the polypeptide or fusion proteins, thereby allowing interchain disulfide bond formation in a multimerized form. The homodimeric or heterodimeric (or multimeric) fusion proteins can include combinations of the same or different fusion partners, such that more than one epitope can be targeted at a time by the same construct. Such epitopes can be proximally located in the target (e.g., on the HIV target) such that the binding of one epitope facilitates the binding of the multimeric fusion proteins to the second or more epitopes. The epitopes targeted by multimeric fusion proteins also can be distally situated.

[0037] One example of such a variant is illustrated in Figure 6, wherein a fusion protein (mbk6Fc (SEQ ID NO: 31)) with human IgG1 Fc fragment has the configuration [anti-CD16A sdA]-[soluble CD4]-[CH2]-[CH3], in which the NanoBiKEs were linked to the Fc region by a (G₄S)₃ polypeptide. The two chains of the dimer are linked through disulfide bonding between the Fc regions (e.g., hinge regions of the CH₂ or CH₃ domains). All elements of the configuration are as described herein with respect to the other aspects of the invention.

[0038] The polypeptide (e.g., anti-CD16A sdA) or fusion protein can be PEGylated, or coupled to polymers of similar structure, function and purpose, to confer enhanced stability and half-life. PEGylation can provide increased half-life and resistance to degradation without a loss in activity (e.g., binding affinity) relative to non-PEGylated (e.g., antibody) polypeptides. Since PEGylation may not be advantageous with respect to some targets, in particular, those epitopes which are sterically-obstructed, the polypeptide or fusion protein should be minimally PEGylated so as not to negatively impact the accessibility to the size-restricted antigen. The polypeptide or fusion protein can be coupled to PEG or PEG-like polymers by any suitable means known in the art. Suitable PEG or PEG-like moieties can be synthetic or naturally occurring and include, but are not limited to, straight or branched chain polyalkylene, polyalkenylene or polyoxyalkylene polymers, or a branched or unbranched polysaccharide, such as a homo- or heteropolysaccharide. Preferred examples of synthetic polymers include straight or branched chain poly(ethylene glycol) (PEG), poly(propylene glycol), or poly(vinyl alcohol) and derivatives or substituted forms thereof. Substituted polymers for linkage to the domain antibodies also include substituted PEG, including methoxy(polyethylene glycol). Naturally occurring polymer moieties which can be used in

addition to or in place of PEG include, for example, lactose, amylose, dextran, or glycogen, as well as derivatives thereof.

[0039] Additional peptide sequences can be added to the fusion protein (or construct containing the fusion protein), which act to promote stability, purification, and/or detection. For example, a reporter peptide portion (e.g., green fluorescent protein (GFP), β -galactosidase, or a detectable domain thereof) can be used. Purification-facilitating peptide sequences include those derived or obtained from maltose binding protein (MBP), glutathione-S-transferase (GST), or thioredoxin (TRX). The polypeptide or fusion protein (or construct containing the fusion protein) also or alternatively can be tagged with an epitope which can be antibody purified (e.g., the Flag epitope, which is commercially available from Kodak (New Haven, Connecticut)), a hexa-histidine peptide, such as the tag provided in a pQE vector available from QIAGEN, Inc. (Chatsworth, California), or an HA tag (as described in, e.g., Wilson et al., *Cell*, 37, 767 (1984)).

[0040] The polypeptide and fusion protein can be prepared by any suitable method. For example, the polypeptide and fusion protein can be prepared by synthesizing the amino acid sequence or by expressing a nucleic acid encoding the amino acid sequence in a cell and harvesting the resulting polypeptide or fusion protein from the cell. A combination of such methods also can be used. Methods of de novo synthesizing peptides and methods of recombinantly producing peptides are known in the art (see, e.g., Chan et al., *Fmoc Solid Phase Peptide Synthesis*, Oxford University Press, Oxford, United Kingdom, 2005; *Peptide and Protein Drug Analysis*, ed. Reid, R., Marcel Dekker, Inc., 2000; *Epitope Mapping*, ed. Westwood et al., Oxford University Press, Oxford, United Kingdom, 2000; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY 2001; and Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates and John Wiley & Sons, NY, 1994).

[0041] Detectable agents, such as fluorescent compounds, also can be added to the polypeptide or fusion protein. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-1-naphthalenesulfonyl chloride, phycoerythrin and the like. The polypeptide or fusion protein also can be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When the polypeptide or fusion protein construct is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. The polypeptide or fusion protein construct also can

be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

[0042] The invention also provides a nucleic acid encoding the amino acid sequence of the polypeptide or fusion protein. The nucleic acid can comprise DNA (e.g., genomic DNA, non-genomic DNA, and cDNA) or RNA, and can be single or double stranded. Furthermore, the nucleic acid can comprise nucleotide analogues or derivatives (e.g., inosine or phosphorothioate nucleotides and the like).

[0043] The nucleic acid can be provided as part of a construct comprising the nucleic acid and elements that enable delivery of the nucleic acid to a cell, and/or expression of the nucleic acid in a cell. Such elements include, for example, expression vectors, promoters, and transcription and/or translation sequences. Suitable vectors, promoters, transcription/translation sequences, and other elements, as well as methods of preparing such nucleic acids and constructs, are known in the art (e.g., Sambrook et al., *supra*; and Ausubel et al., *supra*).

[0044] The invention further provides a recombinant vector comprising the nucleic acid. Examples of suitable vectors include plasmids (e.g., DNA plasmids), yeast (e.g., *Saccharomyces*), and viral vectors, such as poxvirus, retrovirus, adenovirus, adeno-associated virus, herpes virus, polio virus, alphavirus, baculovirus, poxvirus and Sindbis virus. When the vector is a plasmid (e.g. DNA plasmid), the plasmid can be complexed with chitosan.

[0045] When the vector is for administration to a host (e.g., mammal such as a human), the vector preferably has a low replicative efficiency in a target cell (e.g., no more than about 1 progeny per cell or, more preferably, no more than 0.1 progeny per cell are produced). Replication efficiency can readily be determined empirically by determining the virus titer after infection of the target cell.

[0046] The polypeptide and fusion protein can be administered to a host in the form of a cell comprising a nucleic acid encoding the polypeptide or fusion protein, optionally in the form of a vector. Thus, the invention also provides a cell comprising a vector or nucleic acid encoding the polypeptide or fusion protein from which the polypeptide or fusion protein desirably is secreted. Any suitable cell can be used. Examples include host cells, such as *E. coli* (e.g., *E. coli* Tb-1, TG-2, DH5 α , XL-Blue MRF' (Stratagene), SA2821, and Y1090), *Bacillus subtilis*, *Salmonella typhimurium*, *Serratia marcescens*, *Pseudomonas* (e.g., *P. aeruginosa*), *N. grassa*, insect cells (e.g., Sf9, Ea4), yeast (*S. cerevisiae*) cells, and cells derived from a mammal, including human cell lines. Specific examples of suitable

eukaryotic cells include VERO, HeLa, 3T3, Chinese hamster ovary (CHO) cells, W138 BHK, COS-7, and MDCK cells. Alternatively and preferably, cells from a mammal, such as a human, to be treated in accordance with the methods described herein can be used as host cells. In one embodiment, the cell is a human B cell.

[0047] Methods of introducing vectors into isolated host cells and the culture and selection of transformed host cells *in vitro* are known in the art and include the use of calcium chloride-mediated transformation, transduction, conjugation, triparental mating, DEAE, dextran-mediated transfection, infection, membrane fusion with liposomes, high velocity bombardment with DNA-coated microprojectiles, direct microinjection into single cells, and electroporation (see, e.g., Sambrook et al., *supra*, Davis et al., *Basic Methods in Molecular Biology* (1986), and Neumann et al., *EMBO J. 1*, 841 (1982)). Desirably, the cell comprising the vector or nucleic acid expresses the nucleic acid encoding the polypeptide or fusion protein such that the nucleic acid sequence is transcribed and translated efficiently by the cell.

[0048] The polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, or cell can be isolated. The term “isolated” as used herein encompasses compounds or compositions that have been removed from a biological environment (e.g., a cell, tissue, culture medium, body fluid, etc.) or otherwise increased in purity to any degree (e.g., isolated from a synthesis medium). Isolated compounds and compositions, thus, can be synthetic or naturally produced.

[0049] The polypeptide, fusion protein, nucleic acid, vector, or cell can be administered to any host (e.g., mammal, preferably a human) in need thereof. As a result of administration of the polypeptide, fusion protein, nucleic acid, vector, or cell to the mammal, antibody dependent cellular cytotoxicity is induced for the inhibition (e.g., treatment or prevention) of infectious diseases (e.g., viral infections, such as HIV infection) and cancers.

[0050] Non-limiting examples of specific types of cancers include cancer of the head and neck, eye, skin, mouth, throat, esophagus, chest, bone, lung, colon, sigmoid, rectum, stomach, prostate, breast, ovaries, kidney, liver, pancreas, brain, intestine, heart or adrenals. More particularly, cancers include solid tumor, sarcoma, carcinomas, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma,

papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, Kaposi's sarcoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, a blood-born tumor, acute lymphoblastic leukemia, acute lymphoblastic B-cell leukemia, acute lymphoblastic T-cell leukemia, acute myeloblastic leukemia, acute promyelocytic leukemia, acute monoblastic leukemia, acute erythroleukemic leukemia, acute megakaryoblastic leukemia, acute myelomonocytic leukemia, acutenonlymphocytic leukemia, acute undifferentiated leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia, hairy cell leukemia, or multiple myeloma. See, e.g., *Harrison's Principles of Internal Medicine*, Eugene Braunwald et al., eds., pp. 491 762 (15th ed. 2001).

[0051] When HIV infection is to be inhibited, the infection can be any type of HIV, but preferably is an HIV-1 and/or HIV-2 infection. The inventive method can be used to inhibit infection by any HIV group (e.g., groups M and/or O), and subtype (e.g., clades A, B, C, D, E, EA, F, and/or G).

[0052] Additionally, the polypeptide, fusion protein, nucleic acid, vector, or cell can be used to inhibit a broad range other viral infections (see, e.g., *Principles of Virology: Molecular Biology, Pathogenesis, and Control*, Flint et al., eds., ASM Press: Washington, D.C. (2000), particularly Chapter 19). Examples of viral infections that may be treated in accordance with the invention include, but are not limited to, infections by Type C and Type D retroviruses, HTLV-1, HTLV-2, FIV, FLV, SIV, MLV, BLV, BIV, equine infectious virus, anemia virus, avian sarcoma viruses, such as Rous sarcoma virus (RSV), hepatitis type A, B, C, non-A and non-B viruses, arboviruses, varicella viruses, human herpes virus (e.g., HHV-6), measles, mumps, filovirus (e.g., Ebola, such as Ebola strains Sudan, Zaire, Cote d'Ivoire, and Reston), SARS, influenza, and rubella viruses.

[0053] When provided therapeutically, the polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof is provided at or after the diagnosis of the infectious disease or cancer.

[0054] When provided prophylactically, the polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof is provided in advance of an infection (e.g., an HIV

infection) or cancer diagnosis, such as to patients or subjects who are at risk for being exposed to an infectious agent (e.g., a microbe, such as a bacteria, fungus, or virus, such as HIV), who have been newly exposed to an infectious agent, or who are at risk for developing cancer. Examples of such patients and subjects include, for example, healthcare workers, fetuses, neonates, or infants (e.g., nursing infants) whose mothers are infected or at risk for being infected, intravenous drug users, recipients of blood transfusions, blood products, or transplantation tissue, and other individuals who have been exposed to a body fluid that contains or may contain an infectious agent (e.g., HIV). The prophylactic administration of the polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof prevents, ameliorates, or delays infection (e.g., HIV) or the development of cancer. In subjects who have been newly exposed to a microbe (e.g., HIV) but who have not yet displayed the presence of the microbe (as measured by PCR or other assays for detecting the microbe) in blood or other body fluid, efficacious treatment with the polypeptide, fusion protein, conjugate, nucleic acid, vector, cell, or composition thereof partially or completely inhibits or delays the appearance of the microbe or minimizes the level of the microbe in the blood or other body fluid of the exposed individual.

[0055] The efficacy of the polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof can be assessed in various ways well known to the skilled practitioner. For instance, a person of ordinary skill in the art will understand that a polypeptide or fusion protein of the invention is efficacious in treating or inhibiting a microbial (e.g., viral, such as HIV) infection in a subject by observing that the polypeptide or fusion protein reduces viral load or delays or prevents a further increase in viral load. Viral loads can be measured by methods that are known in the art, for example, using PCR assays to detect the presence of viral (e.g., HIV) nucleic acid or antibody assays to detect the presence of viral (e.g., HIV) protein in a sample (e.g., blood or another body fluid) from a subject or patient, or by measuring the level of circulating anti-viral (e.g., anti-HIV) antibodies in the patient. Efficacy of the polypeptide or fusion protein treatment also can be determined by measuring the number of CD4+ T cells in the HIV-infected subject. A treatment that delays or inhibits an initial or further decrease in CD4+ T cells in an HIV-positive subject or patient, or that results in an increase in the number of CD4+ T cells in the HIV-positive subject, can be considered efficacious.

[0056] The inventive polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof is useful for preventing emergence of cancers, arresting progression of

cancers or eliminating cancers. More particularly, the inventive polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof can be used to prevent, inhibit or delay the development of tumors, and/or to prevent, inhibit or delay tumor migration and/or tumor invasion of other tissues (metastases) and/or to generally prevent or inhibit progression of cancer in an individual. The inventive polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof can also be used to ameliorate at least one symptom of the cancer, such as by reducing tumor burden in the individual; inhibiting tumor growth in the individual; increasing survival of the individual; and/or preventing, inhibiting, reversing or delaying progression of the cancer in the individual.

[0057] The polypeptide, fusion protein, nucleic acid, vector, or cell can be formulated as a composition (e.g., pharmaceutical composition) comprising the polypeptide, fusion protein, nucleic acid, vector, or cell and a carrier (e.g., a pharmaceutically or physiologically acceptable carrier). Furthermore, the polypeptide, fusion protein, nucleic acid, vector, cell, or composition of the invention can be used in the methods described herein alone or as part of a pharmaceutical formulation.

[0058] Compositions (e.g., pharmaceutical compositions) comprising the polypeptide, fusion protein, nucleic acid, vector, or cell can include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like.

[0059] Suitable carriers and their formulations are described in A.R. Gennaro, ed., *Remington: The Science and Practice of Pharmacy* (19th ed.), Mack Publishing Company, Easton, PA (1995). Pharmaceutical carriers, include sterile water, saline, Ringer's solution, dextrose solution, and buffered solutions at physiological pH. Typically, an appropriate amount of a pharmaceutically acceptable salt is used in the formulation to render the formulation isotonic. The pH of the formulation is preferably from about 5 to about 8 (e.g., about 5.5, about 6, about 6.5, about 7, about 7.5, and ranges thereof). More preferably, the pH is about 7 to about 7.5. Further carriers include sustained-release preparations, such as semipermeable matrices of solid hydrophobic polymers containing the fusion protein, which matrices are in the form of shaped articles (e.g., films, liposomes, or microparticles). It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of composition being administered.

[0060] The composition (e.g., pharmaceutical composition) can comprise more than one polypeptide, fusion protein, nucleic acid, vector, or cell of the invention. Alternatively, or in

addition, the composition can comprise one or more other pharmaceutically active agents or drugs. Examples of such other pharmaceutically active agents or drugs that may be suitable for use in the pharmaceutical composition include anticancer agents (e.g., chemotherapeutic drugs), antibiotics, antiviral drugs, antifungal drugs, cyclophosphamide, and combinations thereof. Suitable antiviral agents (e.g., anti-HIV agents) include, but are not limited to, nucleoside/nucleotide reverse transcriptase inhibitors (e.g., lamivudine, abacavir, zidovudine, stavudine, didanosine, emtricitabine, and tenofovir), non-nucleoside reverse transcriptase inhibitors (e.g., delavirdine, efavirenz, etravirine, and nevirapine), protease inhibitors (e.g., amprenavir, fosamprenavir, atazanavir, darunavir, indinavir, lopinavir, ritonavir, nelfinavir, saquinavir, and tipranavir), fusion or entry inhibitors (e.g., enfuvirtide and maraviroc), integrase inhibitors (e.g., raltegravir), and combination therapies thereof.

[0061] Examples of such other pharmaceutically active agents or drugs that may be suitable for use in the pharmaceutical composition include anticancer agents (e.g., chemotherapeutic or radiotherapeutic agents), antimetabolites, hormones, hormone antagonists, antibiotics, antiviral drugs, antifungal drugs, cyclophosphamide, and combinations thereof. Suitable anticancer agents include, without limitation, alkylating agents, folate antagonists, purine antagonists, pyrimidine antagonists, spindle poisons, topoisomerase inhibitors, apoptosis inducing agents, angiogenesis inhibitors, podophyllotoxins, nitrosoureas, cisplatin, carboplatin, interferon, asparaginase, tamoxifen, leuprolide, flutamide, megestrol, mitomycin, bleomycin, doxorubicin, irinotecan, taxol, geldanamycin (e.g., 17-AAG), and various anti-cancer peptides and antibodies known in the art.

[0062] Exemplary alkylating agents include, but are not limited to, nitrogen mustards (e.g., mechlorethamine, cyclophosphamide, melphalan, uracil mustard, or chlorambucil), alkyl sulfonates (e.g., busulfan), nitrosoureas (e.g., carmustine, lomustine, semustine, streptozocin, or dacarbazine). Exemplary antimetabolites include, but are not limited to, folic acid analogs (e.g., methotrexate), pyrimidine analogs (e.g., 5-fluorouracil (5-FU) or cytarabine), and purine analogs (e.g., mercaptopurine or thioguanine). Exemplary hormones and hormone antagonists include, but are not limited to, adrenocorticosteroids (e.g., prednisone), progestins (e.g., hydroxyprogesterone caproate, medroxyprogesterone acetate, and magesrol acetate), estrogens (e.g., diethylstilbestrol and ethinyl estradiol), antiestrogens (e.g., tamoxifen), and androgens (e.g., testosterone propionate and fluoxymesterone). Other exemplary agents include, but are not limited to, vinca alkaloids (e.g., vinblastine, vincristine,

or vindesine), epipodophyllotoxins (e.g., etoposide or teniposide), antibiotics (e.g., dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, or mitocycin C), enzymes (e.g., L-asparaginase), platinum coordination complexes (e.g., cis-diamine-dichloroplatinum II also known as cisplatin), substituted ureas (e.g., hydroxyurea), methyl hydrazine derivatives (e.g., procarbazine), and adrenocortical suppressants (e.g., mitotane and aminoglutethimide).

[0063] Chemotherapeutics that can be concurrently, sequentially or intermittently administered with the vectors and compositions disclosed herein include Adriamycin, Alkeran, Ara-C, Busulfan, CCNU, Carboplatinum, Cisplatinum, Cytosan, Daunorubicin, DTIC, 5-FU, Fludarabine, Hydrea, Idarubicin, Ifosfamide, Methotrexate, Mithramycin, Mitomycin, Mitoxantrone, Nitrogen Mustard, Taxol (or other taxanes, such as docetaxel), Velban, Vincristine, VP-16, Gemcitabine (Gemzar), Herceptin, Irinotecan (Camptosar, CPT-11), Leustatin, Navelbine, Rituxan STI-571, Taxotere, Topotecan (Hycamtin), Xeloda (Capecitabine), Zevelin, Enzalutamide (MDV-3100 or XTANDI™), and calcitriol. Exemplary immunomodulators and/or cytokines include, but are not limited to, AS-101 (Wyeth-Ayerst Labs.), bropirimine (Upjohn), gamma interferon (Genentech), GM-CSF (granulocyte macrophage colony stimulating factor; Genetics Institute), IL-2 (Cetus or Hoffman-LaRoche), human immune globulin (Cutter Biological), IMREG (from Imreg of New Orleans, La.), SK&F 106528, tumor necrosis factor (TNF)- α , and TNF- β .

[0064] Other agents, compositions or protocols (e.g., therapeutic protocols) that are useful for the inhibition of cancer in conjunction with the polypeptides, fusion proteins, nucleic acids, vectors, cells, and compositions of the invention include, but are not limited to, surgical resection of a tumor, radiation therapy, allogeneic or autologous stem cell transplantation, T cell adoptive transfer, and/or targeted cancer therapies (e.g., small molecule drugs, biologics, or monoclonal antibody therapies that specifically target molecules involved in tumor growth and progression, including, but not limited to, selective estrogen receptor modulators (SERMs), aromatase inhibitors, tyrosine kinase inhibitors, serine/threonine kinase inhibitors, histone deacetylase (HDAC) inhibitors, retinoid receptor activators, apoptosis stimulators, angiogenesis inhibitors, poly (ADP-ribose) polymerase (PARP) inhibitors, or immunostimulators).

[0065] The additional active agent (e.g., chemotherapeutics agent) can be administered before, concurrently with (including simultaneously), alternating with, sequentially, or after administration with the compositions disclosed herein. In certain embodiments, one or more

(e.g., 2, 3, 4, or 5) chemotherapeutic agents is administered in combination with the compositions disclosed herein.

[0066] The additional active agent can be administered alone or in a composition. The additional active agent can be formulated by inclusion in a vector (e.g., plasmid or viral vector), in liposomes (tecemotide, which is also known as STIMUVAX™, L-BLP25, or BLP25 liposome vaccine), or in nanoparticles (e.g., VERSAMUNE™ nanotechnology).

[0067] The polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof can be used in combination with other well-known viral (e.g., HIV) therapies and prophylactic vaccines already in use. The combination of the fusion protein of the invention can generate an additive or a synergistic effect with current treatments. The polypeptide or fusion protein of the invention can be combined with other HIV and AIDS therapies and vaccines, such as highly active antiretroviral therapy (HAART), which comprises a combination of protease inhibitors and reverse transcriptase inhibitors, azidothymidine (AZT), structured treatment interruptions of HAART, cytokine immune enhancement therapy (e.g., interleukin (IL)-2, IL-12, CD40L + IL-12, IL-7, HIV protease inhibitors (e.g., ritonavir, indinavir, and nelfinavir, etc.), and interferons (IFNs)), cell replacement therapy, recombinant viral vector vaccines, DNA vaccines, inactivated virus preparations, immunosuppressive agents, such as Cyclosporin A, cyanovirin therapy (see, e.g., U.S. Patent No. 6,015,876), scytovirin therapy (see, e.g., U.S. Patent No. 7,491,798), and griffithsin therapy (see, e.g., U.S. Patent Application Publication 2009/0092557). Such therapies can be administered in the manner already in use for the known treatment providing a therapeutic or prophylactic effect (see, e.g., Silvestri et al. *Immune Intervention in AIDS*. In: *Immunology of Infectious Disease*, H.E. Kauffman, A. Sher, and R. Ahmed eds., ASM Press, Washington DC (2002)).

[0068] Suitable methods of administering a polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof to hosts are known in the art. The host can be any suitable host, such as a mammal (e.g., a rodent, such as a mouse, rat, hamster, or guinea pig, rabbit, cat, dog, pig, goat, cow, horse, primate, or human).

[0069] Administration can be topical (including ophthalmical, vaginal, rectal, intranasal, transdermal, and the like), oral, by inhalation, or parenteral (including by intravenous drip or subcutaneous, intracavity, intraperitoneal, or intramuscular injection). Topical intranasal administration refers to the delivery of the compositions into the nose and nasal passages through one or both of the nares and can comprise delivery by a spraying mechanism or droplet mechanism, or through aerosolization of the nucleic acid, vector, polypeptide, or

fusion protein. Administration of the compositions by inhalant can be through the nose or mouth via delivery by a spraying or droplet mechanism. Delivery can also be directly to any area of the respiratory system (e.g., lungs) via intubation.

[0070] Formulations for topical administration include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids, and powders. Conventional pharmaceutical carriers, aqueous, powder, or oily bases, thickeners, and the like may be necessary or desirable.

[0071] If the composition is to be administered parenterally, the administration is generally by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for suspension in liquid prior to injection, or as emulsions. Additionally, parental administration can involve the preparation of a slow-release or sustained-release system, such that a constant dosage is maintained. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives also can be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases, and the like.

[0072] Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids, or binders may be desirable.

[0073] Some of the compositions can potentially be administered as a pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids, such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base, such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases, such as mono-, di-, trialkyl, and aryl amines and substituted ethanolamines.

[0074] The polypeptide, fusion protein, nucleic acid, vector, or cell can be administered with a pharmaceutically acceptable carrier and can be delivered to the mammal's cells by a

variety of mechanisms well-known in the art (e.g., uptake of naked DNA, liposome fusion, intramuscular injection of DNA via a gene gun, endocytosis, and the like).

[0075] The exact amount of the composition required to treat a viral infection (e.g., HIV infection) will vary from mammal to mammal, depending on the species, age, gender, weight, and general condition of the mammal, the nature of the virus, the existence and extent of viral infection, the particular polypeptide, fusion proteins, nucleic acid, vector, or cell used, the route of administration, and whether other drugs are included in the regimen. Thus, it is not possible to specify an exact amount for every composition. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein. Effective dosages and schedules for administering the nucleic acid molecules, vectors, cells, polypeptides, and fusion proteins of the invention can be determined empirically, and making such determinations is within the skill in the art. The dosage ranges for the administration of the compositions are those large enough to produce the desired effect; however, the dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Dosage can vary, and can be administered in one or more (e.g., two or more, three or more, four or more, or five or more) doses daily, for one or more days. The composition can be administered before viral (e.g., HIV) infection or immediately upon determination of viral (e.g., HIV) infection and continuously administered until the virus is undetectable.

[0076] The polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof is administered to a host (e.g., mammal, such as a human) in an amount effective to prophylactically or therapeutically inhibit an infectious disease (e.g., HIV infection) or cancer. The efficacy of the polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof as an inhibitor may be determined by *in vivo* or *in vitro* parameters known in the art.

[0077] Any suitable dose of the polypeptide, fusion protein, nucleic acid, vector, cell, or composition thereof can be administered to a host. The appropriate dose will vary depending upon such factors as the host's age, weight, height, sex, general medical condition, previous medical history, and cancer or infectious disease (e.g., HIV infection) progression and can be determined by a clinician. For example, the polypeptide, fusion protein, or conjugate can be administered in a dose of about 1 µg/kg to up to 100 mg/kg of body weight or more per day (e.g., 5 µg/kg, 10 µg/kg, 50 µg/kg, 100 µg/kg, 200 µg/kg, 300 µg/kg, 400 µg/kg, 500 µg/kg, 600 µg/kg, 700 µg/kg, 800 µg/kg, 900 µg/kg, 1 mg/kg, 2 mg/kg, 5 mg/kg, 10 mg/kg, 20

mg/kg, 30 mg/kg, 40 mg/kg, 50 mg/kg, 60 mg/kg, 70 mg/kg, 80 mg/kg, 90 mg/kg, and ranges thereof) to the host (e.g., mammal, such as a human). Several doses (e.g., 1, 2, 3, 4, 5, 6, or more) can be provided (e.g., over a period of weeks or months).

[0078] When the vector is a viral vector, a suitable dose can include about 1×10^5 to about 1×10^{12} (e.g., 1×10^6 , 1×10^7 , 1×10^8 , 1×10^9 , 1×10^{10} , 1×10^{11} , and ranges thereof) plaque forming units (pfus), although a lower or higher dose can be administered to a host. For example, about 2×10^8 pfus can be administered (e.g., in a volume of about 0.5 mL).

[0079] The inventive cells can be administered to a host in a dose of between about 1×10^5 and 2×10^{11} (e.g., 1×10^6 , 1×10^7 , 1×10^8 , 1×10^9 , 1×10^{10} , and ranges thereof) cells per infusion. The cells can be administered in, for example, one to three (e.g., two) infusions. In addition to the administration of the cells, the host can be administered a biological response modifier, such as interleukin 2 (IL-2).

[0080] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

[0081] This example demonstrates the production of two human single domain antibodies (sdA1 and sdA2) targeting CD16A.

[0082] A phage library was constructed by CDR grafting onto human V_H3-23 scaffold, and simultaneously the FR regions around CDR2 were engineered to reinforce the stability of the single domain. Protein sequence alignment showed sdA1 and sdA2 share similarity of amino acids with V_H and J_H fragments deriving from the same gene family 3-23*04 for V_H and J4*02 for J_H . However, the short CDR3 loops of sdA1 and sdA2 (SEQ ID NOs: 8 and 15, respectively) result from a different D_H gene family: 3-10*01 for sdA1 and 7-27*01 for sdA2.

EXAMPLE 2

[0083] This example demonstrates the expression, purification, and characterization of sdA1 and sdA2.

[0084] sdA1 and 2 were expressed in HB2151 bacteria, and purified by one – step Ni-NTA metal affinity chromatography. SDS-PAGE under reducing and non-reducing conditions showed high purity and homogeneity for sdA1 and sdA2 after purification.

[0085] Size exclusion chromatography using Superdex 75 showed these sdAs exist as monomers in phosphate buffered solution (PBS).

[0086] In addition, dynamic light scattering (DLS) data showed sdA1 and sdA2 experienced no or little aggregation under long time incubation at 37 °C under the high concentration of 10 mg/ml buffered in PBS. The DLS data indicate these sdAs are aggregation resistant.

EXAMPLE 3

[0087] This example demonstrates the binding affinity to CD16A and NK cells of sdA1 and sdA2.

[0088] The antigen binding capacities of sdA1 and sdA1 were measured by ELISA and FACS. In the ELISA, recombinant CD16A ectodomain was coated on a 96-well plate with 50 ng/well, then the 5 fold serially diluted sdAs were added. After washing, the bound sdAs were detected by anti-Flag-HRP.

[0089] Results showed both sdA1 and sdA2 bind to CD16A with high binding affinity, with binding EC₅₀ of 0.1 nM for sdA1 and 0.3 nM for sdA2.

[0090] The results of fluorescence-activated cell sorting (FACS) flow cytometry showed sdA1 and sdA2 could bind NK cell surface associated CD16A efficiently under the concentration of 50 nM. Under the same concentration, the binding of sdA1 is even higher than that of the commercial mouse anti-CD16A IgG1.

EXAMPLE 4

[0091] This example demonstrates the production and characterization of fusion proteins comprising sdA1 and sdA2.

[0092] Three prevalent formats of bispecific antibodies for re-directing NK cells to lysis of target cells have been developed: BiKE, BidAb, and TandAb (see Fig. 1). BiKE is composed of two tandem scFv arms connected by a long flexible polypeptide linker. BidAb is consists of the tail-to-head heterodimer of two scFv molecule with a short linker. TandAb is a tetravalent, bispecific, tandem diabody composed of the tail-to-head homodimer of the two tandem scFv connected by three (GGG)₃ linkers.

[0093] All of these formats have encountered many limitations in the production process such as low soluble expression in bacteria; laborious re-folding from the inclusion body; high byproducts due to the mismatching of its subdomains; high heterogeneity in the final product.

[0094] To avoid the mismatching of VH and VL occurring in the above formats, a single domain antibody rather than scFv was used to construct a bispecific antibody (see Fig. 2). The bispecific antibody contains two single domains, one for targeting CD16A (SEQ ID NO: 2 or 3), the other for recognizing gp120 of HIV-1 (SEQ ID NO: 27), which is connected by the (GGGS)₃ (SEQ ID NO: 17). Since the resulting bispecific antibody is half the size of BIKE, it is referred to as NanoBiKE (i.e., NanoBiKE 1 contains sdA1 and NanoBiKE 2 contains sdA2). In some Examples below, NanoBiKE1 is referred to as mbk6 and NanoBiKE2 is referred to as mbk11.

[0095] NanoBiKE 1 and 2 were expressed in HB2151 bacteria, and purified by one-step Ni-NTA metal affinity chromatography. SDS-PAGE under reducing and non-reducing conditions showed high purity and homogeneity for NanoBiKE 1 and 2 after purification. The size exclusion using Superdex 75 showed these NanoBiKEs exist as monomers in PBS.

[0096] The antigen binding for NanoBiKE was detected by ELISA, FACS, and Biacore.

[0097] Biacore measurements showed that NanokBike 1 has a K_d for CD16A of 1.1 nM and NanoBike 2 has a K_d of 21 nM). As measured by ELISA, both NanoBiKEs retained high affinity (EC_{50} =1 nM) binding to pg140sc through the mD1.22 arm.

[0098] The ELISA data showed NanoBiKE 1 and NanoBiKE 2 retain the high binding affinity to CD16A as well as HIV-1 gp120 with the binding EC_{50} at sub-nanomolar concentrations. The FACS data showed these NanoBiKEs could bind to human NK cells expressing CD16A, and concomitantly bind to gp160-expressing 293 T cells (gp160 protein forms a homotrimer, and is cleaved into gp120 and gp41 by the host cell protease, furin).

EXAMPLE 5

[0099] This example demonstrates the specific activation of Jurkat T cells expressing CD16A using the NanoBIKEs.

[00100] We assessed whether the BiKEs, after engaging cell surface CD16A, could mediate functional activation of Jurkat T-CD16A cells by monitoring their activation and measuring cytokine (IL-2) secretion. In Jurkat T-CD16A cells, engagement of CD16A initiates activation signals to cell nucleus through the CD3- ζ chain, ultimately leading to de-phosphorylation of NTAFp and expression of luciferase. Thus, the activation signals could be monitored by testing luciferase activity. To compare the ADCC capacities of BiKEs and IgG1-Fc, we also measured the ADCC activation signals mediated by mD1.22-Fc, an Fc

fusion with two molecules of mD1.22. BiKEs and mD1.22-Fc can specifically activate Jurkat T-CD16A cells in the presence of 293T-gp160_{sc} cells (Figure 3, panel A). The incubation of BiKEs with Jurkat T-CD16A cells in the absence of target cells or in the presence of 293T cells non-expressing gp160 could not induce luciferase signals, demonstrating the high specificities of these BiKEs. Importantly, they can activate Jurkat T-CD16A cells at low concentrations (50% RLU ~ 0.1 nM). In this case the activity of mbk6 was higher than that of mbk11 and mD1.22-Fc indicating a correlation of binding affinity of BiKEs to CD16A (mD1.22-Fc < mbk11 < mbk6) with their functional activity.

[00101] We also measured cytokine (IL-2) secretion of Jurkat T-CD16A cells after CD16A cross-linking, which were achieved by either bivalent mouse anti-Flag antibody dimerizing BiKEs or by BiKEs bound to 293T-gp160_{sc} cells. Co-incubation of Jurkat T-CD16A cells with BiKEs opsonized 293T-gp160_{sc} cells resulted in release of IL-2 (Figure 3, panel B), which is consistent with the results obtained by using a positive control, bivalent 3G8. By contrast, the BiKEs alone or incubated with 293T cells, or 293T-gp160_{sc} alone did not induce IL-2 secretion, demonstrating the high specificity of these BiKEs. Similar to the activating signals, the higher affinity mbk6 induced more IL-2 than mbk11 ($p = 0.022$) but less than 3G8, which again indicates that there could be correlation between affinity/avidity and cytokine release activity. However, when the T cells were stimulated by 293T-gp160_{sc} cells the mbk6 was almost as effective as 3G8 suggesting a role for avidity effects. These results demonstrate that both BiKEs could specifically engage CD16A on the effector cell surface, and activate signals leading to cytokine release.

EXAMPLE 6

[00102] This example demonstrates potent NK cell-mediated killing of gp160-expressing cells by NanoBiKEs.

[00103] To test whether the NanoBiKEs could re-direct NK cells to kill gp160-expressing cells, freshly prepared human peripheral blood mononuclear cells (PBMCs) and enriched NK cells were used as effector cells and the cell killing was detected by using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega; Cat. No. G7890).

[00104] After incubation for 8 hours with an effector cell to target cell ratio of 10 : 1, PBMC and NK cells could be recruited to kill the target cells with high specificity since these effector cells could lyse the negative control 293 T cells.

[00105] The cell killing efficacy for the NanoBiKE-activated effector cells is potently high with an EC₅₀ of ~ 200 pM for PBMC and EC₅₀ of below 50 pM for NK cells.

[00106] The cell killing experiments demonstrate the highly potent capacity of these NanoBiKEs for recruiting NK cells to kill target cells expressing HIV-1 Envelope glycoprotein.

[00107] In summary, the NanoBiKEs retain the high binding affinity to both CD16A and gp120. Importantly, they can recruit NK cells to specifically mediate ADCC killing of the HIV-1 positive cells with effective EC₅₀s below 50 pM. Therefore, the high efficacy and specificity of eliciting ADCC and the superior drugability of these NanoBiKEs mean the NanoBiKEs can be used as immunotherapeutics for treatment of HIV-1 infection. Additionally, the sdAs serve as versatile platforms for constructing multivalent, bispecific antibodies or other fusion proteins for engaging NK cells.

EXAMPLE 7

[00108] This Example demonstrates NK cells degranulation mediated by BiKEs.

[00109] BiKE-mediated degranulation of NK cells was measured in the presence of CHO-ZA-gp160_{sc} cells. NK degranulation was evaluated by monitoring CD107a and IFN γ expression levels after incubation with BiKE-opsonized target cells. To assess the specificity of these BiKEs, three negative controls were used: NK cells incubation with BiKEs in the absence of target cells, in the presence of HIV-1 Env negative cells or NK cells incubation with target cells in the absence of BiKEs. NK cells stimulated with PMA/ionomycin were used as a positive control. The CD107a staining results clearly showed that both mbk6 and mbk11 induced significant NK cell degranulation compared to the negative controls (Figure 4, panels A and B).

[00110] MbK6 degranulated NK cells more effectively than mbk11, and both BiKEs were more effective than mD1.22-Fc, which correlates with Jurkat T-CD16A cells activation and indicates that the affinity to CD16A is important for the extent of activation of CD16A-expressing effector cells. It should be also noted that the CHO-ZA-gp160_{sc} cells and CHO-ZA cells in the absence of BiKEs also could induce relatively weak NK cells degranulation, which may result from killer immunoglobulin-like receptors (KIRs) and natural cytotoxicity receptors (NCRs) recognition of Env or other ligands on target cells, which is independent on CD16A mediated NK cells engagement. Induced expression of intracellular IFN γ was also

observed although the level was not as high as the CD107a one . These results demonstrate that BiKEs can specifically engage NK cells in the presence of gp160-expressing target cells.

EXAMPLE 8

[00111] This Example shows specific killing of Env-expressing and HIV-1-infected cells mediated by mbk6 and mbk11.

[00112] Three different models were used to assess the killing activity of the new BiKEs. The first model is based on the CHO-ZA cell line, which was transfected to permanently express gp160SC. In this case, cell killing was measured by a flow cytometry-based method, where target cells were discriminated from effector cells by pre-labelling with the fluorescent dye PKH26. After incubation with BiKEs and effector cells, the dead target cells were further differentiated by PI staining. Both BiKEs and mD1.22-Fc specifically mediated cell killing by NK cells only in the presence of CHO-ZA-gp160SC cells (Figure 5A). MbK6 was most effective with maximal killing of about 50% and half of the maximal killing at the very low concentration of 100 pM. The cell killing efficacy of mbk11 was lower than that of mbk6. The mD1.22-Fc which has the lowest affinity to CD16A exhibited the lowest cell killing efficacy, which correlates with its capacity to induce NK cell degranulation. The killing activity of mbk6 was significantly reduced by D6 (at 10 nM) indicating specific killing of target cells by mbk6 (Figure 7).

[00113] BiKEs mediated killing of 8E5 cells was evaluated by using a different assay based on measuring membrane integrity. 8E5 cells are chronically HIV-1LAV-infected cells which stably express most HIV-1 structural proteins including the Env and are frequently used for modeling of chronic HIV-1 infections. In this case again mbk6 was the most effective mediator of 8E5 cell killing by PBMCs (Figure 5B). Both BiKEs were more effective than mD1.22-Fc, which correlates with their affinity and activity in all assays described above.

[00114] In another set of experiments, acutely infected T cells (CEM.NKRCCR5+ cells) were used as target cells and the cell killing assay was performed at relatively high concentrations of BiKEs and mD1.22-Fc (20 nM) by monitoring the luciferase activity of target cells. Acutely infected cells, particularly CEM cells, typically die in several days because of the infection. Accordingly, we measured killing when the cells express Env but are not yet dead. Both BiKEs as well as mD1.22-Fc mediated highly effective (about 70%) killing of the infected CEM cells by PBMCs. The BiKEs were slightly more effective than

mD1.22-Fc although not significantly likely due to saturation effects. These results suggest that these BiKEs are promising candidates for further evaluation in animal models and eventually in humans.

[00115] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[00116] The use of the terms “a” and “an” and “the” and “at least one” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term “at least one” followed by a list of one or more items (for example, “at least one of A and B”) is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[00117] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and

equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

SEQ ID NO: 1 (consensus sequence)

EVQLVESGGGLVQPGGSLRLSCAAS GFTFSX₁YG MSWVRQAPGKGLEWIGX₂
X₃X₄X₅SGGST NYNPSLKGSX₆VISRDNSKNTLYLQMNSLAEDTAX₇YYC
ARX₈X₉X₁₀X₁₁DX₁₂ WGQGTLVTVSS

X1 = N OR S

X2 = S OR E

X3 = I OR V

X4 = Y OR N

X5 = Y OR H

X6 = L OR F

X7 = T OR V

X8 = E OR V

X9 = S OR G

X10 = no amino acid or S

X11 = I or F

X12 = Y or F

SEQ ID NO: 2 (sdA1)

EVQLVESGGGLVQPGGSLRLSCAAS GFTFSNYG MSWVRQAPGKGLEWIGS
IYYSGGST NYNPSLKGSLVISRDNSKNTLYLQMNSLAEDTATYYC ARESIDY
WGQGTLVTVSS

SEQ ID NO: 3 (sdA2)

EVQLVESGGGLVQPGGSLRLSCAAS GFTFSSYG MSWVRQAPGKGLEWIGE
VNHSGGST NYNPSLKGSFVISRDNSKNTLYLQMNSLAEDTAVYYC ARVGSFDF
WGQGTLVTVSS

SEQ ID NO: 4 (sdA1 FR1)

EVQLVESGGGLVQPGGSLRLSCAAS

SEQ ID NO: 5 (sdA1 CDR1)

GFTFSNYG

SEQ ID NO: 6 (sdA1 FR2)

MSWVRQAPGKGLEWIGS

SEQ ID NO: 7 (sdA1 CDR2)

IYYSGGST

SEQ ID NO: 8 (sdA1 FR3)

NYNPSLKGSVISRDNSKNTLYLQMNSLAEDTATYYC

SEQ ID NO: 9 (sdA1 CDR3)

ARESIDY

SEQ ID NO: 10 (sdA1 and sdA2 FR4)

WGQGTLVTVSS

SEQ ID NO: 11 (sdA2 FR1)

EVQLVESGGGLVQPGGSLRLSCAAS

SEQ ID NO: 12 (sdA2 CDR1)

GFTFSSYG

SEQ ID NO: 13 (sdA2 FR2)

MSWVRQAPGKGLEWIGE

SEQ ID NO: 14 (sdA2 CDR2)

VNHSGGST

SEQ ID NO: 15 (sdA2 FR3)

NYNPSLKGSFVISRDNSKNTLYLQMNSLAEDTAVYYC

SEQ ID NO: 16 (sdA2 CDR3)

ARVGSFDF

SEQ ID NO: 17 (linker 1)

GGGGS GGGGS GGGGS

SEQ ID NO: 18 (linker 2)

GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS

SEQ ID NO: 19 (linker 3)

GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS GGGGS

SEQ ID NO: 20 (Fc region)

DKTHTCPPCPAPPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE
KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENN
YKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFSVMHEALHNHYTQKSLSLSPG
K

SEQ ID NO: 21 (CH3 region)

GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPV
LDSGDSFFLYSKLTVDKSRWQQGNVFSVMHEALHNHYTQKSLSLSPGK

SEQ ID NO: 22 (m36 amino acid sequence)

QVQLVQSGGGLVQPGGSLRLSCAASAFDFSDYEMSWVRQAPGKGLEWIGEINDSGN
TIYNPSLKSRVTISRDN SKNTLYLQMNTLRAEDTAIYYCAIYGGNSGGEYWGQGTLV
TVSS

SEQ ID NO: 23 (m36.1 amino acid sequence)

QVQLVQSGGGLVQPGGSLRLSCAASTFDFSDYEMSWVREAPGKGLEWIGEINDSGN
TIYNPSLKNRVTISRDN SKNTLYLQMNTLRAEDTAIYYCAIYGGNSGGEYWGQGTLV
TVSS

SEQ ID NO: 24 (m36.2 amino acid sequence)

QVQLVQSGGGLIQPGGSLRLSCAASAFDFSDYEMSWVRQDPGKGLEWIGEINDRGN
TIYNPSLKSRTISRDN SKNTLYLQMNTLRAEDTAIYYCAIYGGNSGGEYWGQGT
LVTVSS

SEQ ID NO: 25 (m36.4 amino acid sequence)

QVQLVQSGGGLVQPGGSLRLSCAASAFDFSDYEMSWVREAPGKGLEWIGEINDSGN
TIYNPSLKSRTISRDN SKNTLYLQMNTLRAEDTAIYYCAIYGGNSGGEYWGQGT
LVTVSS

SEQ ID NO: 26 (m36.5 amino acid sequence)

QVQLVQSGGGLVQPGGSLRLSCAASAFDFSDYEMSWVREAPGKGLEWIGEINDSGN
TIYNPSLKSRTISRDN SKNTLYLQMNTLSAEDTAIYYCAIYGGNSGGEYWGQGT
LVTVSS

SEQ ID NO: 27 (mD1.22)

KKVVYGGKGGDTVELTCTASQKKNIQFWKNSNQIKILGNQGSFLT
KGPSKLNDRVDSRRSLWDQGNFPLIKNLKPEDSDTYICEVEDQKEEVQLV
VVG

SEQ ID NO: 28 (mD1.23)

KKVVYGGKGGDTVELTCTASQKKNIQFWKDSNQIKILGNQGSFLT
KGPSKLNDRADSRRLWDQGNFPLIKNLKPEDSDTYICEVEDQKEEVQLV
VVG

SEQ ID NO: 29 (sdA1-linker1-mD1.22 fusion)

EVQLVESGGGLVQPGGSLRLSCAAS GFTFSNYG MSWVRQAPGKGLEWIGS
IYSSGGST NYNPSLKGSLVISRDNSKNTLYLQMNSLAEDTATYYC
ARESIDYWGQGT
LVTVSSGGGGSGGGGSGGGGSKKKVVYGGKGGDTVELTCTASQKKNIQFW
KNSNQIKILGNQGSFLT
KGPSKLNDRVDSRRSLWDQGNFPLIKNLKPEDSDTYICEVE
DQKEEVQLV
VVG

SEQ ID NO: 30 (sdA2-linker1-mD1.22 fusion)

EVQLVESGGGLVQPGGSLRLSCAAS GFTFSSYG MSWVRQAPGKGLEWIGE
VNHSGGST NYNPSLKG SFVISRDNSKNTLYLQMNSLAEDTAVYYC
ARVGSFDFWGQGT
LVTVSSGGGGSGGGGSGGGGSKKKVVYGGKGGDTVELTCTASQKKNIQFW

KNSNQIKILGNQGSFLT KGPSKLNDRVDSRRSLWDQGNFPLIKNLKPEDSDTYICEVE
DQKEEVQLVVVG

SEQ ID NO: 31 (mbk6Fc)

EVQLVESGGGLVQP GGSRLRLSCAASGFTFSNYGMSWVRQAP
GKGLEWIGSIYYSGSTNYNPSLKSLVTISRDN SKNTLYLQMN
SLRAEDTATYYCARESIDYWGQGTLVTVSSSGGGGSGGGGS
GGGGSKKVVYGGKKGDTVELTCTASQKKNIQFHWKNSNQIKI
LGNQGSFLT KGPSKLNDRVDSRRSLWDQGNFPLIKNLKPED
SDTYICEVEDQKEEVQLVVVG GPKTHTCPPCPAPELLGGPS
VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQ
VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF
FLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSP
GKAAARGDYKDDDDKGG LNDIFEAKIEWHE

SEQ ID NO: 32 (mbk6CH2D6)

EVQLVESGGGLVQP GGSRLRLSCAASGFTFSNYGMSWVRQAP
GKGLEWIGSIYYSGSTNYNPSLKSLVTISRDN SKNTLYLQMN
SLRAEDTATYYCARESIDYWGQGTLVTVSSSGGGGSGGGGS
GGGGSKKVVYGGKKGDTVELTCTASQKKNIQFHWKNSNQIKI
LGNQGSFLT KGPSKLNDRVDSRRSLWDQGNFPLIKNLKPED
SDTYICEVEDQKEEVQLVVVG GPKTHTCPPCPAPELLGGPS
VFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVD
GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK
CKVSNKALPAPIEKTISKAKGGGGSGGGGSGGGGSEVQLVE
SGGGLVQP GGSRLRLSCAASGFTFSNYGMSWVRQAPGKGLE
WIGSIYYSGSTNYNPSLKSLVTISRDN SKNTLYLQMNSLRAE
DTATYYCARESIDYWGQGTLVTVSSGHHHHHHHGDYKDDDD
KG

CLAIMS:

1. A polypeptide comprising any of SEQ ID NOs: 1-3, or an amino acid sequence with at least 85% sequence identity to SEQ ID NO: 2 or the amino acid sequence of SEQ ID NO: 3, optionally without variation in the CDRs; or

a single domain antibody that binds CD16A comprising (a) three CDRs comprising SEQ ID NOs 5, 7, and 9, respectively; or (b) three CDRs comprising SEQ ID NOs: 12, 14, and 16, respectively.

2. The polypeptide of claim 1, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 1.

3. The polypeptide of claim 1 or 2, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3.

4. A fusion protein comprising (i) the polypeptide of any one of claims 1-3 and (ii) one or more fusion partners, wherein the one or more fusion partners optionally is joined to the amino acid sequence of (i) via a linker.

5. The fusion protein of claim 4, wherein the one or more fusion partners is an antibody or antibody fragment, an HIV envelope glycoprotein, an Fc region or portion thereof, an immunoglobulin heavy chain constant region, an immunoglobulin light chain constant region, a single domain CD4, or a combination thereof.

6. The fusion protein of claim 5, wherein the one or more fusion partners is a single domain CD4.

7. The fusion protein of claim 5 or 6, wherein the single domain CD4 comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 27 and 28.

8. The fusion protein of claim 5, wherein the one or more fusion partners is an antibody or antibody fragment.

9. The fusion protein of claim 6, wherein the antibody or antibody fragment binds to an HIV envelope glycoprotein.

10. The fusion protein of claim 9, wherein the antibody or antibody fragment comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 22-26.

11. The fusion protein of claim 5, wherein the one or more fusion partners is an HIV envelope glycoprotein.

12. The fusion protein of any one of claims 9-11, wherein the HIV envelope glycoprotein is gp120.

13. The fusion protein of any one of claims 9-12, wherein the HIV is HIV-1.

14. The fusion protein of claim 5, wherein the one or more fusion partners is an Fc region or portion thereof.

15. The fusion protein of claim 7 comprising SEQ ID NO: 29 or 30, or comprising an amino acid sequence having at least 85% amino acid sequence identity to the amino acid sequence of SEQ ID NO: 29, or 30, optionally without reference to the linker used and optionally with conservation of the CDRs.

16. The fusion protein of claim 4 comprising:

(a) a first anti-CD16A sdA polypeptide comprising any of SEQ ID NOs: 1-3, or an amino acid sequence with at least 85% sequence identity to SEQ ID NO: 2 or the amino acid sequence of SEQ ID NO: 3, optionally without variation in the CDRs;

(b) a single domain CD4 linked to the first polypeptide;

(c) a CH2 domain linked to the single domain CD4.

17. The fusion protein of claim 16, further comprising a second anti-CD16A sdA polypeptide comprising any of SEQ ID NOs: 1-3, or an amino acid sequence with at least 85% sequence identity to SEQ ID NO: 2 or the amino acid sequence of SEQ ID NO: 3, optionally without variation in the CDRs,

wherein the second anti-CD16A sdA polypeptide is linked to the CH2 domain, and the first and second anti-CD16A sdA polypeptides can have amino acid sequences that are the same or different.

18. The fusion protein of claim 16, further comprising a CH3 domain linked to the CH2 domain.

19. The fusion protein of any of claims 16-18, wherein the single domain CD4 is linked to the first anti-CD16A sdA polypeptide by a linker comprising a G4S motif, and the CH2 domain is linked to the single domain CD4 by a linker comprising a G4S motif.

20. The fusion protein of claim 17 or 18, wherein the single domain CD4 is linked to the first anti-CD16A sdA polypeptide by a linker comprising a G4S motif, the CH2 domain is linked to the single domain CD4 by a linker comprising a G4S motif, and the second anti-CD16A sdA polypeptide is linked to the CH2 domain by a linker comprising a G4S motif.

21. A multimer comprising two fusion proteins of any of claims 4-20.

22. A multimer comprising two fusion proteins of claim 18 joined via a linker between the CH3 domains, or by disulfide bonding between the CH2 domains.

23. A nucleic acid molecule comprising a non-genomic nucleic acid sequence that encodes the polypeptide of any one of claims 1-3 or the fusion protein of any one of claims 4-22.

24. A vector comprising the nucleic acid molecule of claim 23.

25. A cell comprising the nucleic acid molecule of claim 23 or the vector of claim 24.

26. A composition comprising:

(i) the polypeptide of any one of claims 1-3; the fusion protein of any one of claims 4-23; the nucleic acid molecule of claim 23; the vector of claim 24; or the cell of claim 25 and

(ii) a pharmaceutically acceptable carrier.

27. The composition of claim 26, wherein the composition further comprises an additional active agent.

27. A method of inducing antibody dependent cellular cytotoxicity (ADCC) in a host comprising administering a composition comprising (i) the polypeptide of any one of

claims 1-3 or the fusion protein of any of claims 4-23 and (ii) pharmaceutically acceptable carrier.

28. The method of claim 27, wherein the host has an infectious disease.

29. The method of claim 28, wherein the infectious disease is an HIV infection.

30. The method of claim 27, wherein the host has cancer.

31. A method of inhibiting cancer in a host comprising administering to the host a composition comprising (i) the polypeptide of any one of claims 1-3 or the fusion protein of any of claims 4-23 and (ii) pharmaceutically acceptable carrier, thereby inhibiting cancer in the host.

32. The method of claim 30 or 31, wherein the cancer is selected from the group consisting of cancer of the head and neck, eye, skin, mouth, throat, esophagus, chest, bone, lung, colon, sigmoid, rectum, stomach, prostate, breast, ovaries, kidney, liver, pancreas, brain, intestine, heart, or adrenals.

33. A method of inhibiting an infectious disease in a host comprising administering to the host a composition comprising (i) the polypeptide of any one of claims 1-3 or the fusion protein of claim 4 and (ii) pharmaceutically acceptable carrier, thereby inhibiting the infectious disease in the host.

34. The method of claim 33, wherein the infectious disease is an HIV infection.

35. The fusion protein of claim 4 comprising SEQ ID NO: 29, 30, or 32.

36. The multimer of claim 21 comprising SEQ ID NO: 31.

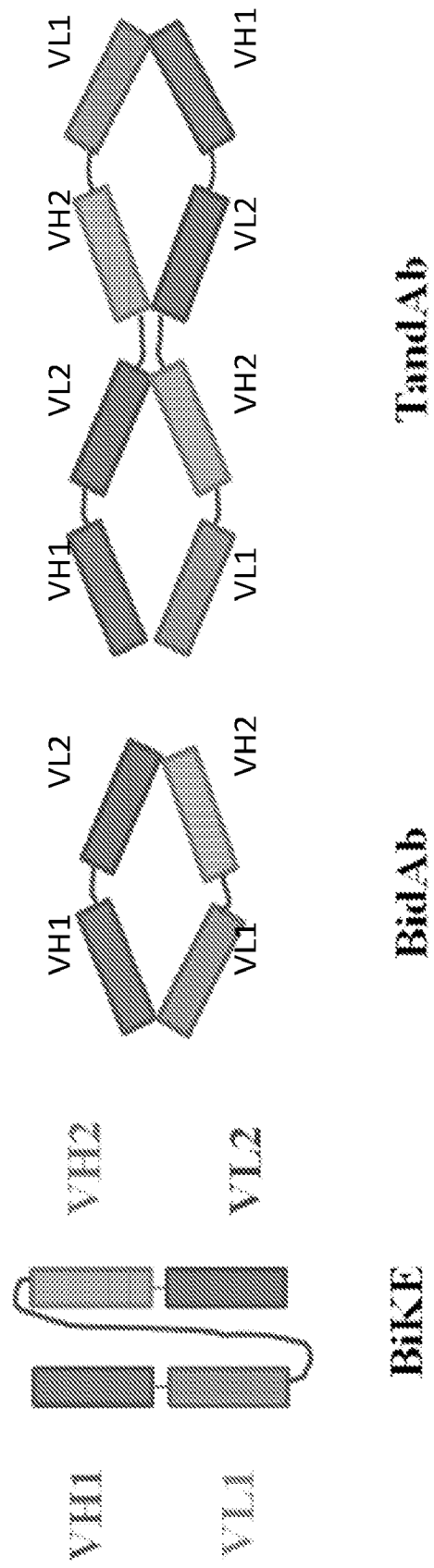


FIG. 1

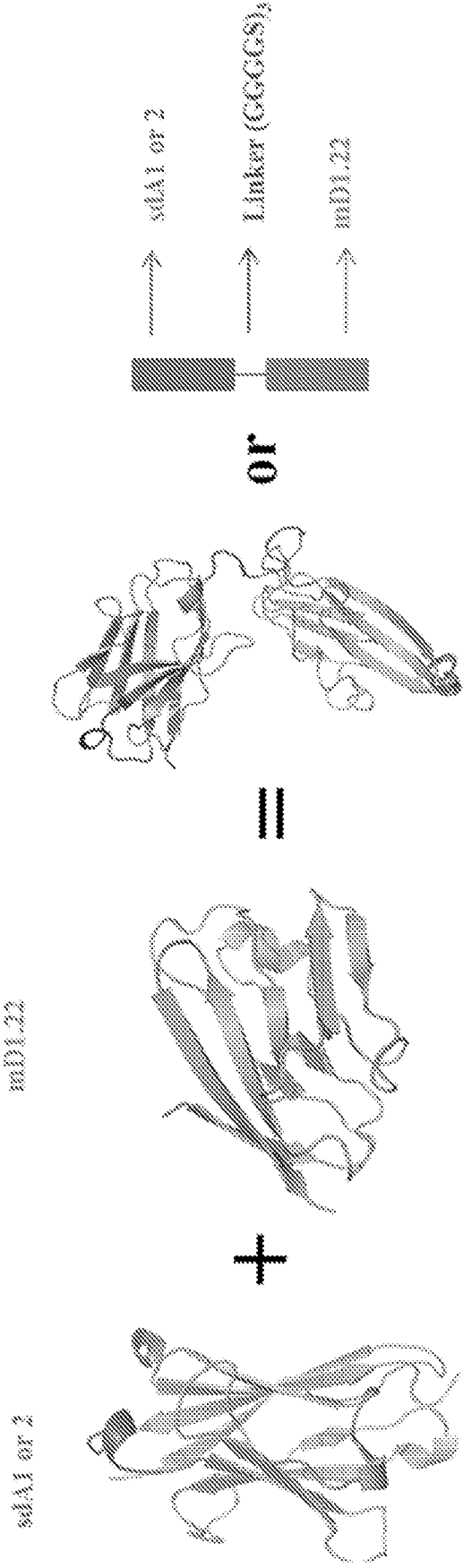


FIG. 2

3/10

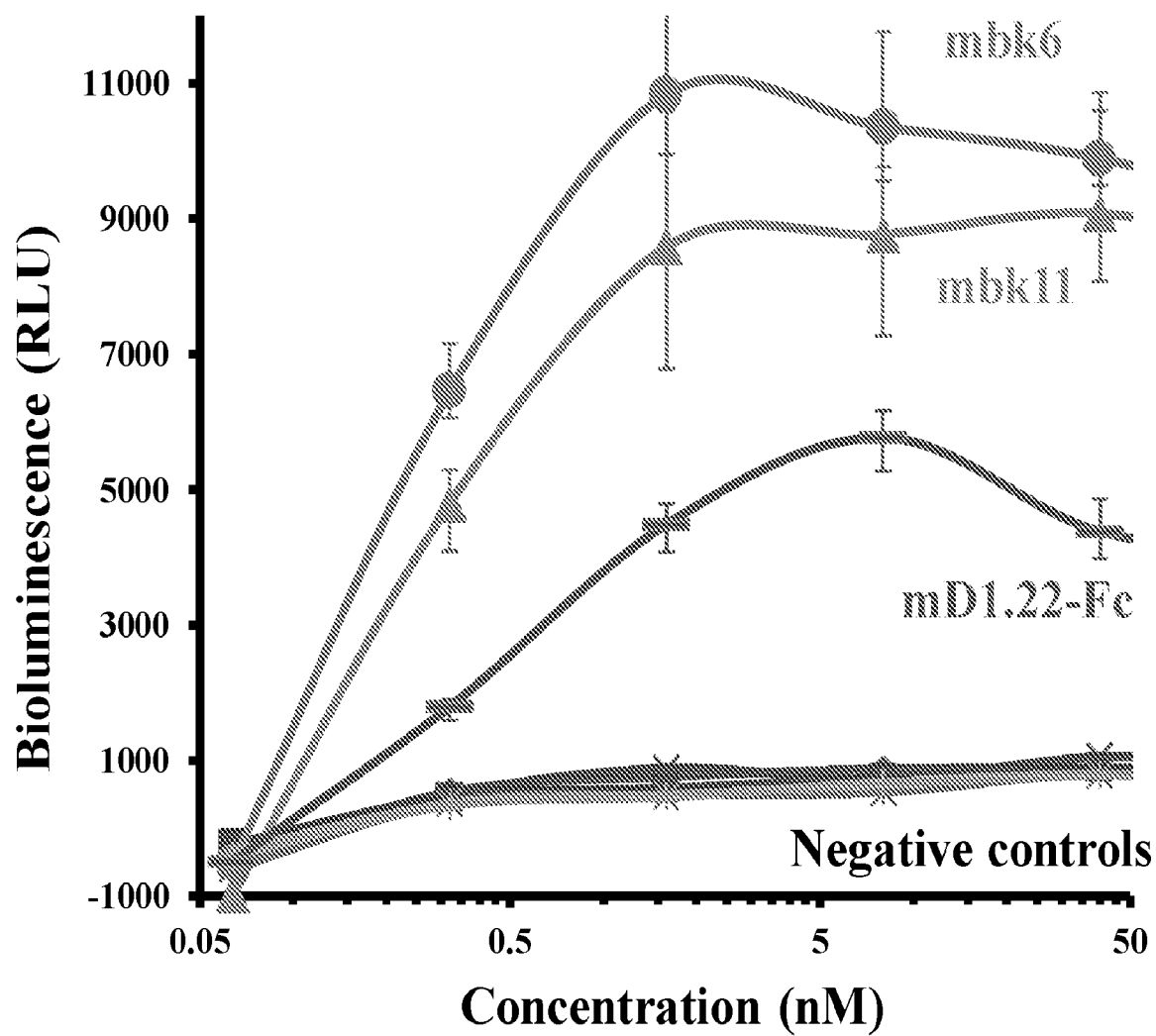


FIG. 3A

4/10

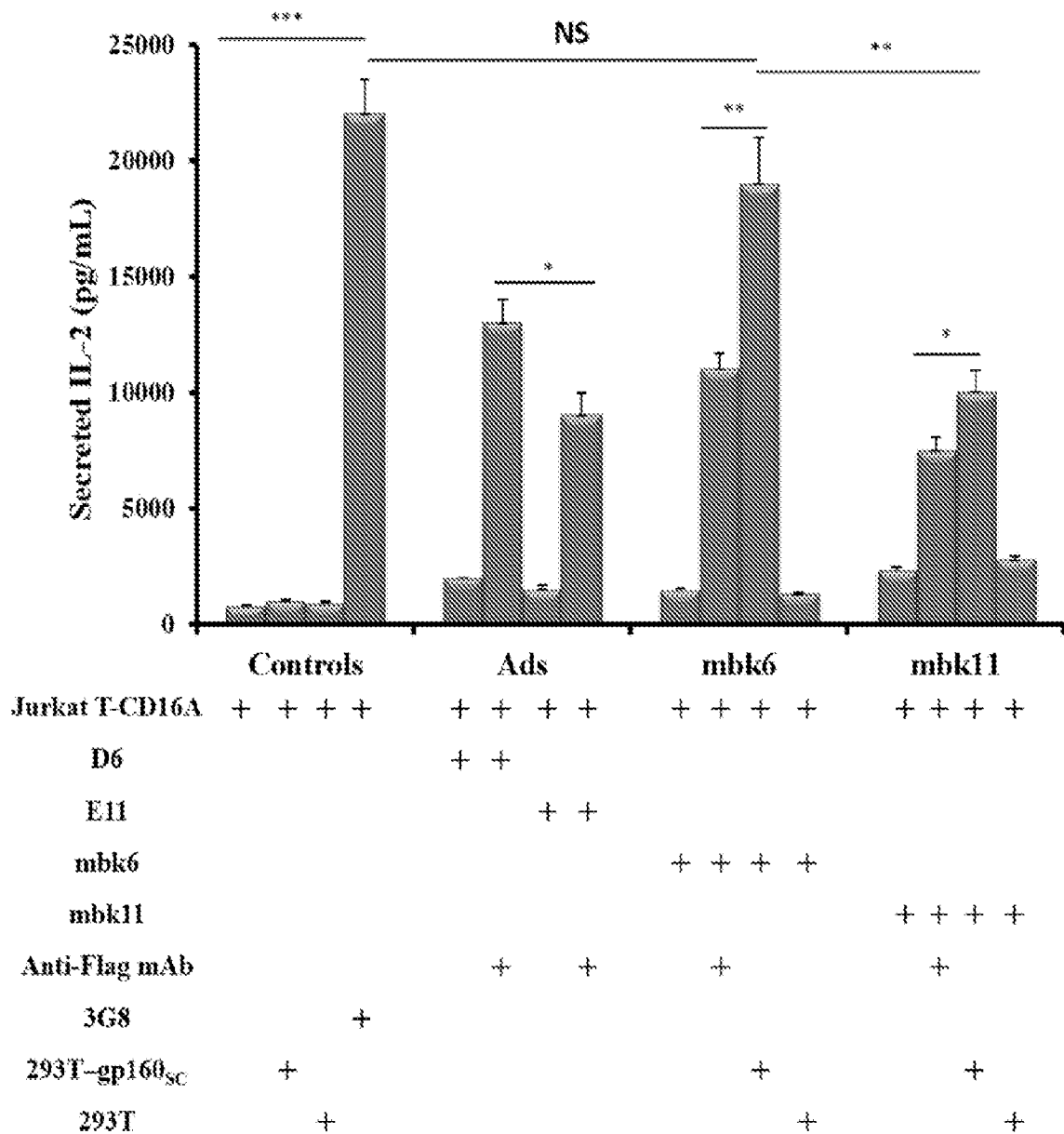


FIG. 3B

5/10

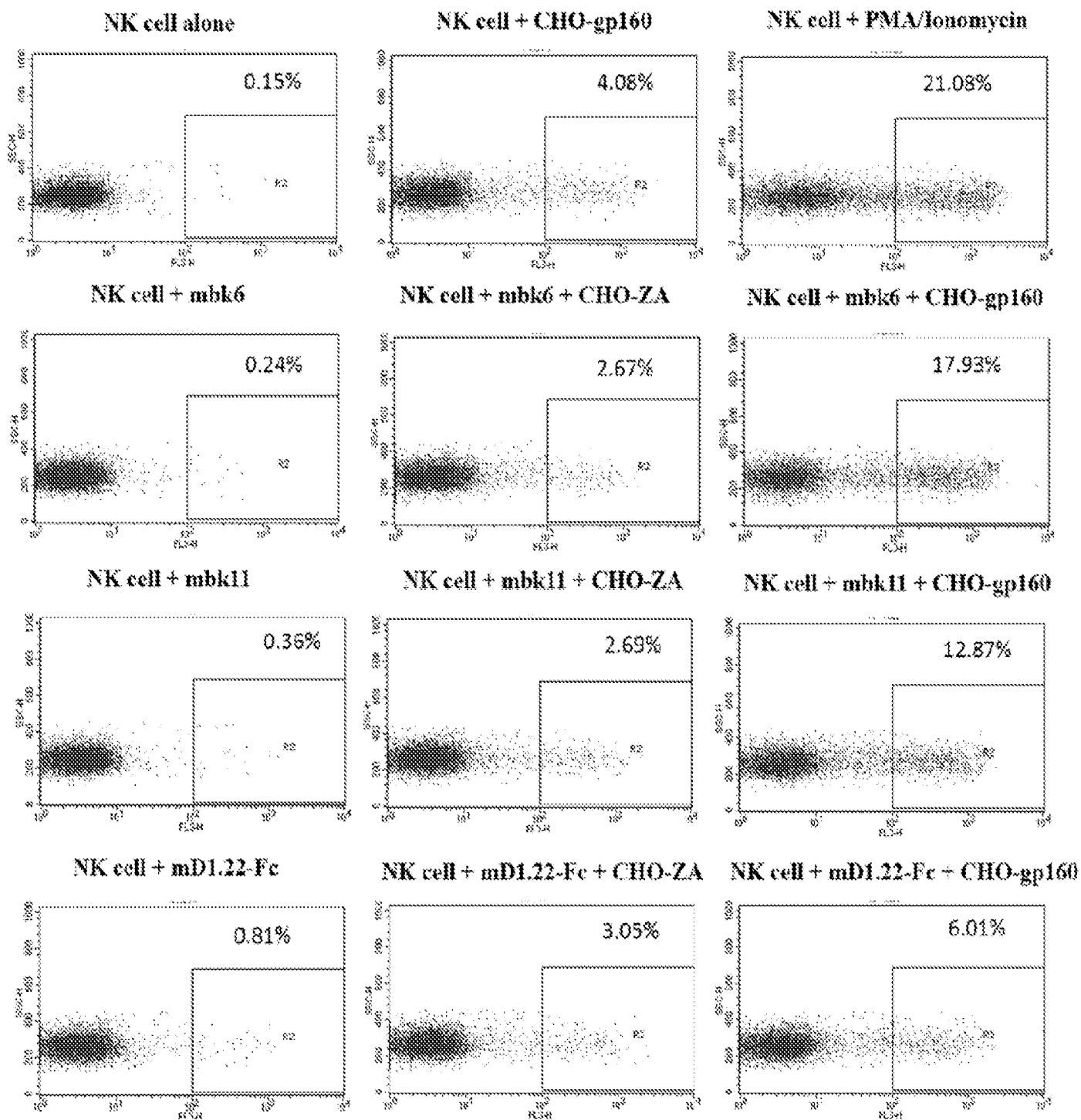


FIG. 4A

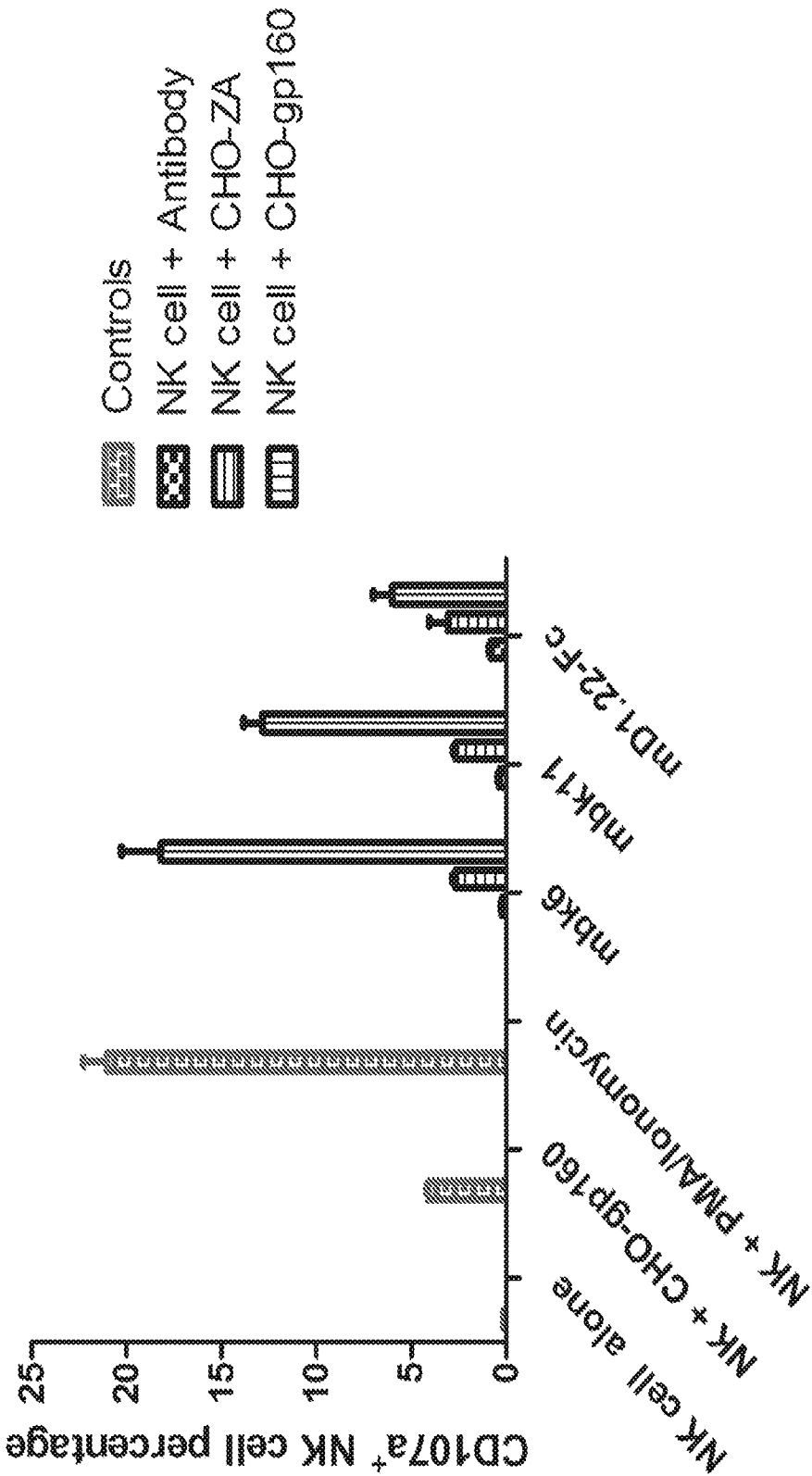


FIG. 4B

7/10

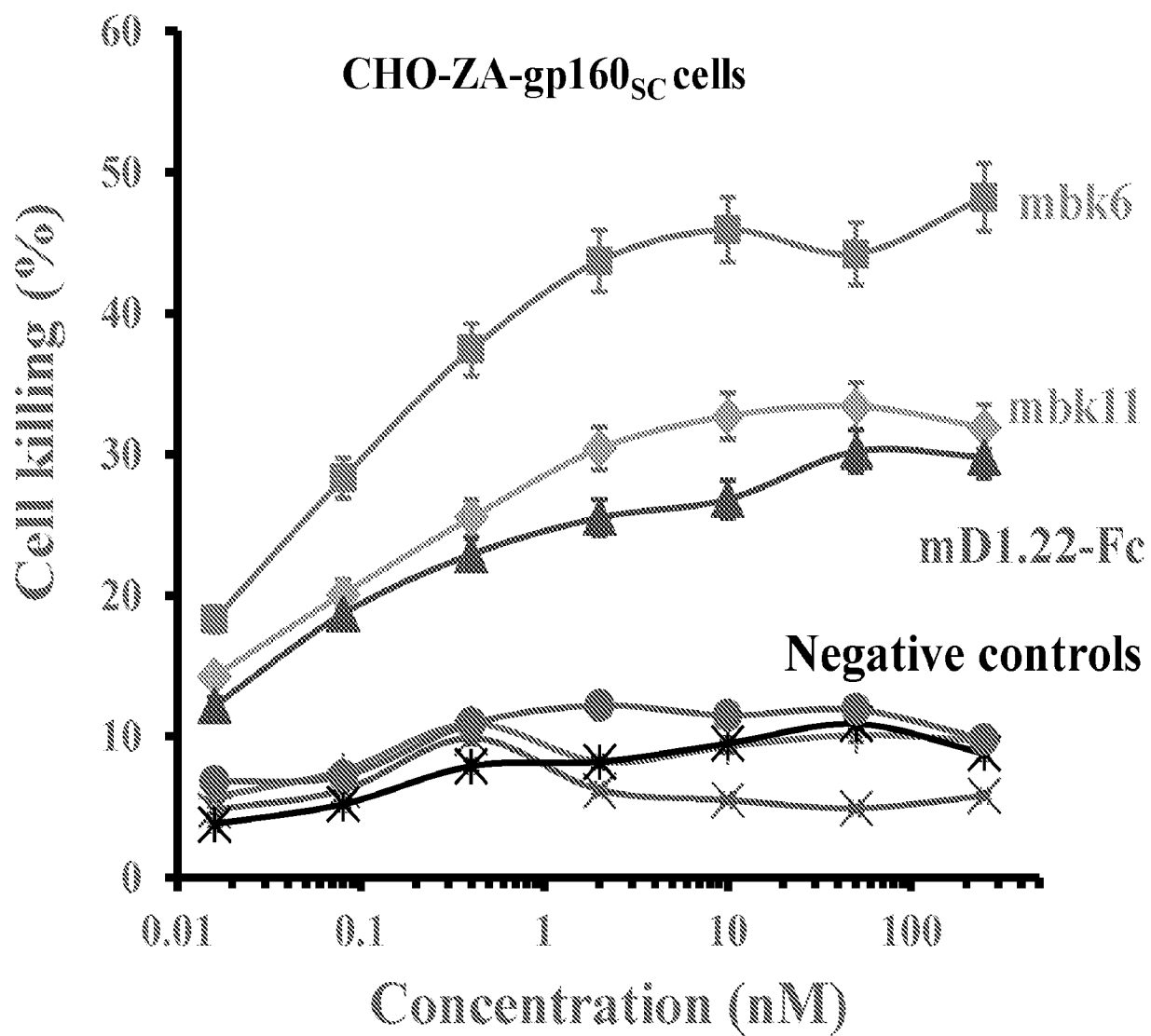
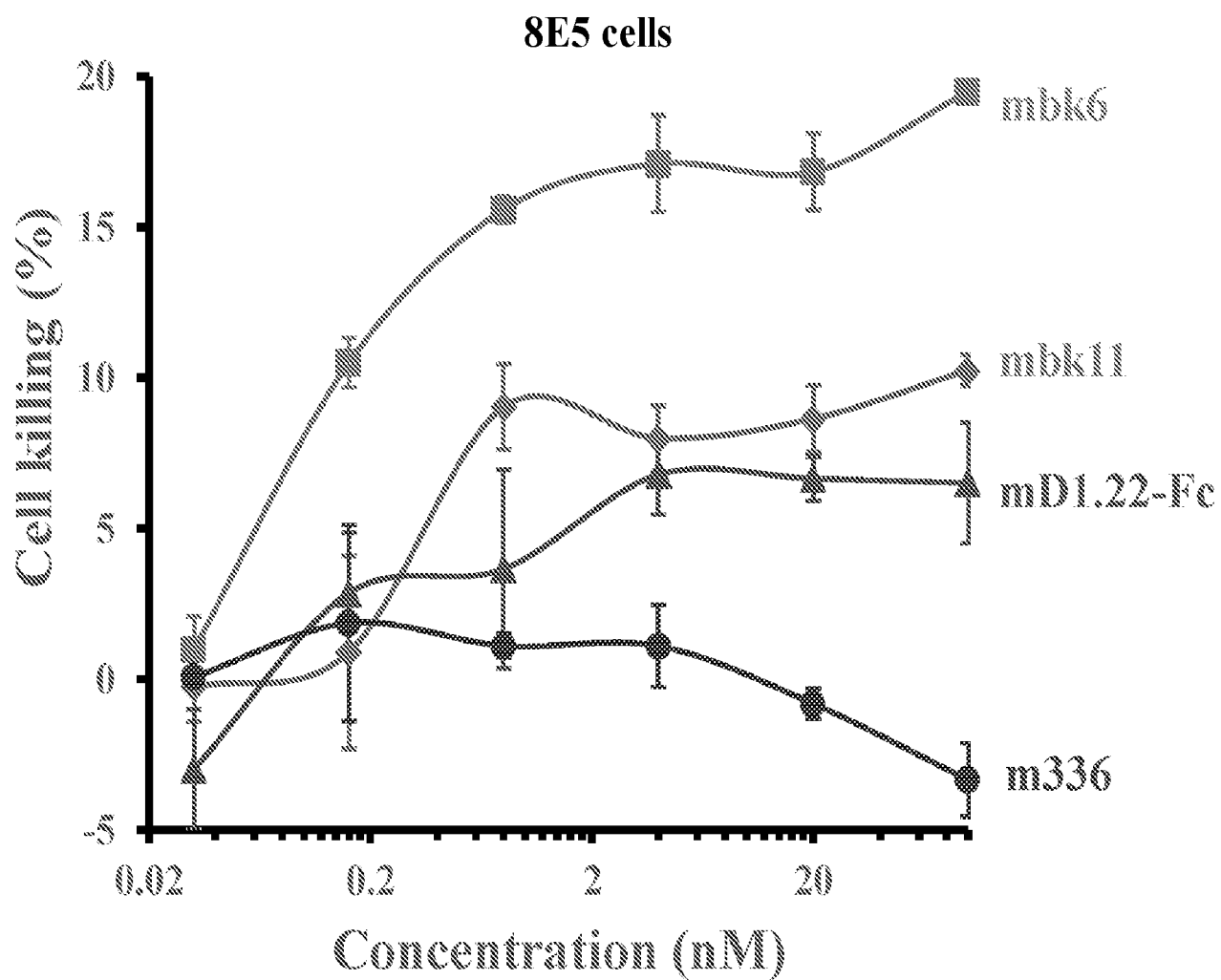
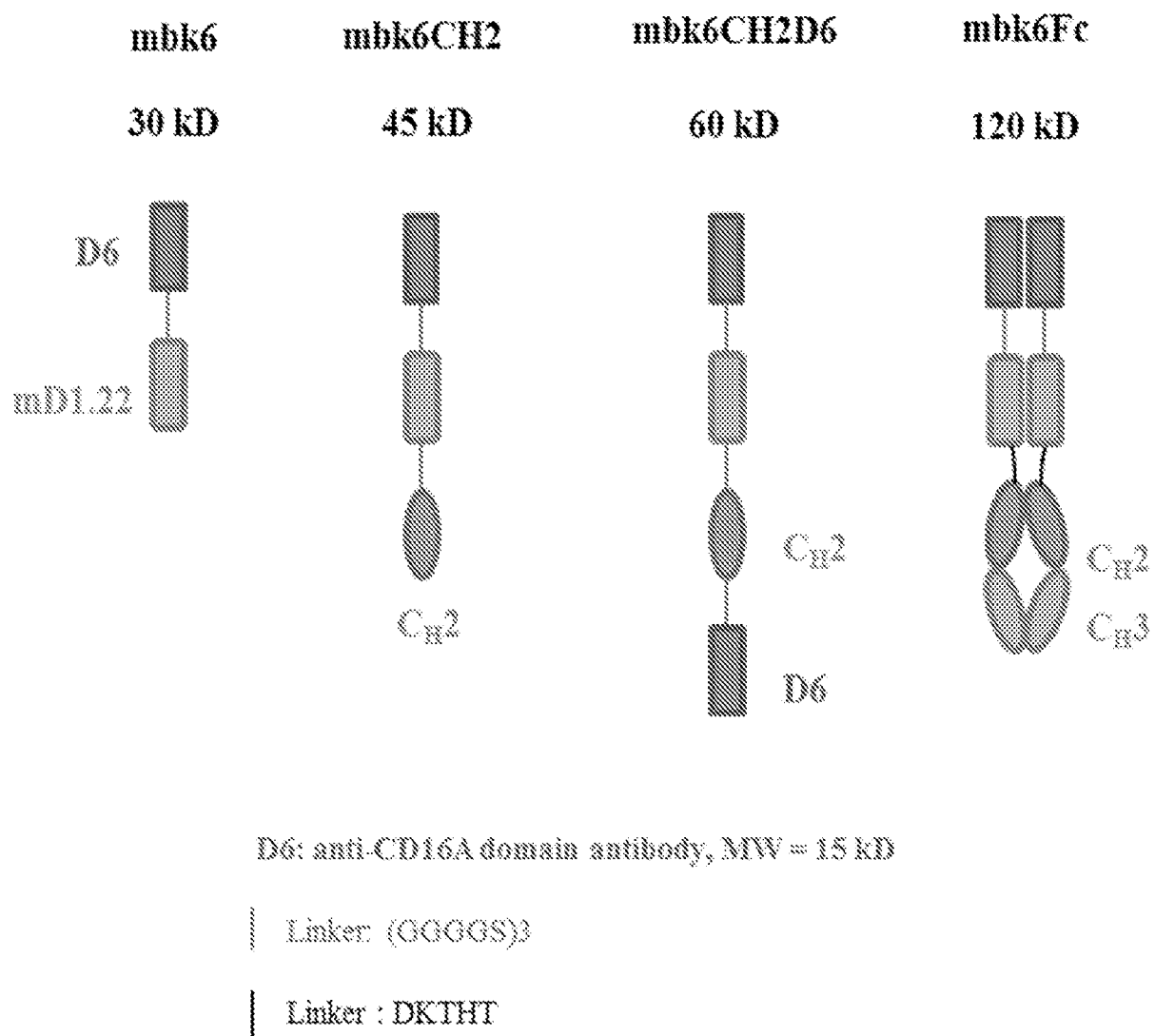


FIG. 5A

8/10

**FIG. 5B**

9/10

**FIG. 6**

10/10

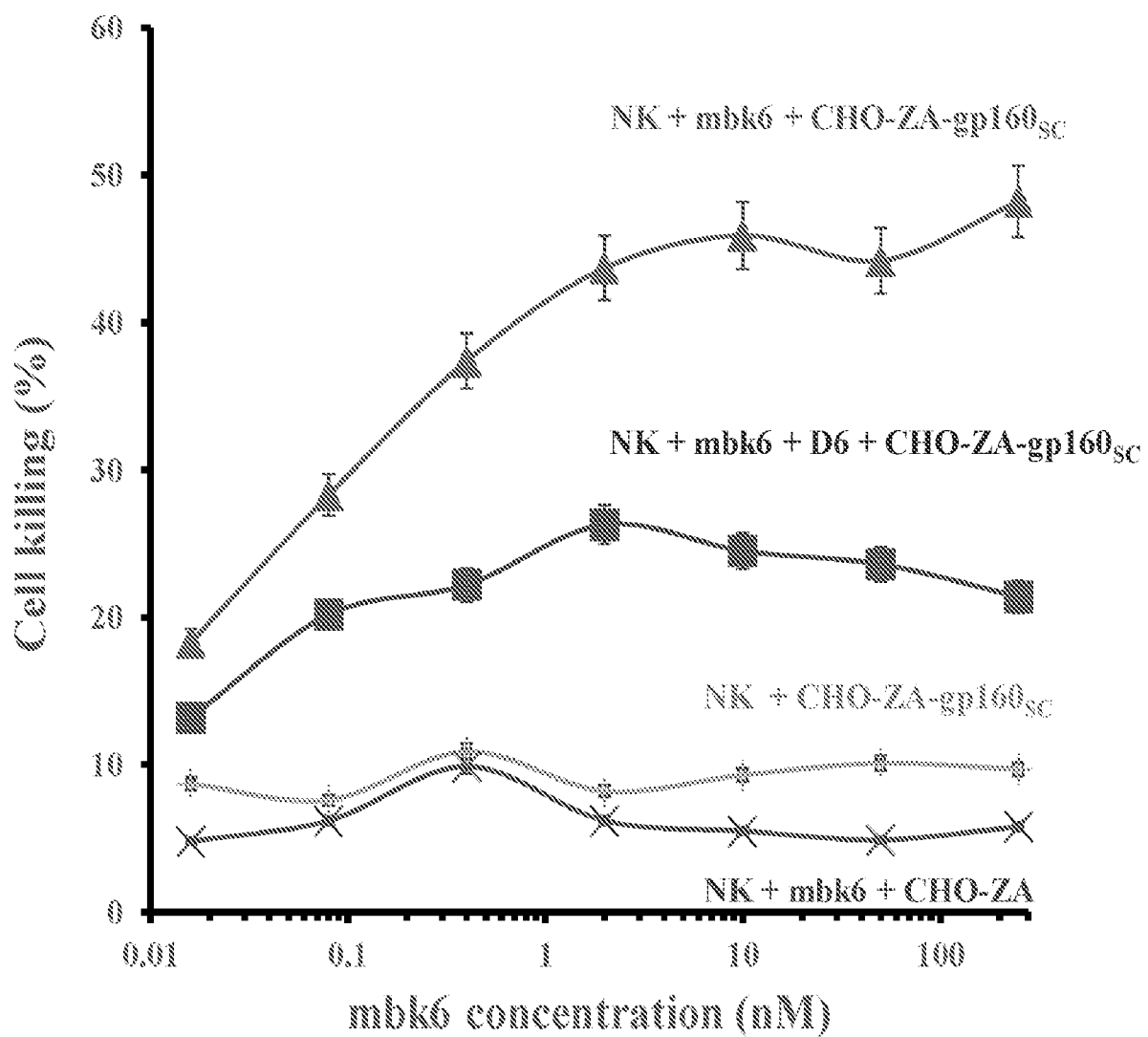


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/048721

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/048721

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EP0-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/163519 A1 (DUTALYS [AT]; BECKMANN ROLAND [AT]) 6 December 2012 (2012-12-06) the whole document, in particular SEQ ID NOs: 1, 3, 5, 11, 13, 15, 17 ----- -/--	1,23-25



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

24 October 2017

Date of mailing of the international search report

30/10/2017

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Bayer, Annette

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/048721

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GHISLAINE BEHAR ET AL: "Isolation and characterization of anti-Fc[gamma]RIII (CD16) llama single-domain antibodies that activate natural killer cells", PROTEIN ENGINEERING, DESIGN AND SELECTION JANUARY 2008 OXFORD UNIVERSITY PRESS GB,, vol. 21, no. 1, 1 January 2008 (2008-01-01), pages 1-10, XP002646408, DOI: 10.1093/PROTEIN/GZM064 [retrieved on 2007-12-11] page 2, right-hand column, paragraph 3 page 5, right-hand column, paragraph 2 - page 9, right-hand column, paragraph 4; figure 1	1,2,4-36
X	----- C. ROZAN ET AL: "Single-Domain Antibody-Based and Linker-Free Bispecific Antibodies Targeting Fc RIII Induce Potent Antitumor Activity without Recruiting Regulatory T Cells", MOLECULAR CANCER THERAPEUTICS, vol. 12, no. 8, 1 August 2013 (2013-08-01), pages 1481-1491, XP055156453, ISSN: 1535-7163, DOI: 10.1158/1535-7163.MCT-12-1012 abstract page 1484, right-hand column, paragraph 1 - page 1485, left-hand column, paragraph 1; figure 1 page 1486, left-hand column, paragraph 1 page 1486, right-hand column, paragraph 3 - page 1487, right-hand column, paragraph 1 page 1489, left-hand column, paragraph 2 - page 1490, right-hand column, paragraph 1	1,2,4-36
A	----- WINKLER K ET AL: "Changing the antigen binding specificity by single point mutations of an anti-p24 (HIV-1) antibody", THE JOURNAL OF IMMUNOLOGY, THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol. 165, no. 8, 15 October 2000 (2000-10-15), pages 4505-4514, XP002579393, ISSN: 0022-1767 abstract discussion and general conclusion; figures 3-7	1-36
A	----- WO 2014/150748 A2 (US HEALTH [US]) 25 September 2014 (2014-09-25) paragraphs [0009], [0010], [0031], [0040]; claims 1-41; examples 1-6	4-36
	----- -/--	

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/048721

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	LI WEI ET AL: "Identification of high-affinity anti-CD16A allotype-independent human antibody domains", EXPERIMENTAL AND MOLECULAR PATHOLOGY, vol. 101, no. 2, 3 October 2016 (2016-10-03), pages 281-289, XP029818570, ISSN: 0014-4800, DOI: 10.1016/J.YEXMP.2016.10.001 the whole document	1-36
X,P	WEI LI ET AL: "One-domain CD4 Fused to Human Anti-CD16 Antibody Domain Mediates Effective Killing of HIV-1-Infected Cells", SCIENTIFIC REPORTS, vol. 7, no. 1, 22 August 2017 (2017-08-22), XP055417861, DOI: 10.1038/s41598-017-07966-3 the whole document	1-36

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2017/048721

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2012163519 A1	06-12-2012	EP 2726504 A1	07-05-2014
		US 2014213459 A1	31-07-2014
		WO 2012163519 A1	06-12-2012

WO 2014150748 A2	25-09-2014	CN 105209057 A	30-12-2015
		EP 2968453 A2	20-01-2016
		US 2016039904 A1	11-02-2016
		WO 2014150748 A2	25-09-2014
