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(54) Title: STABILIZED SINGLE HUMAN CD4 DOMAINS AND FUSION PROTEINS

(57) Abstract: The invention provides a polypeptide comprising a single domain CD4, as well as a fusion protein comprising the single domain CD4 and one or more fusion partners. A nucleic acid encoding the polypeptide or fusion protein, as well as compositions or cells comprising the polypeptide, fusion protein, or nucleic acid also are provided.

STABILIZED SINGLE HUMAN CD4 DOMAINS AND FUSION PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 61/791,885, filed on March 15, 2013, which is incorporated by reference.

SEQUENCE LISTING

[0002] Incorporated by reference in its entirety herein is a nucleotide/amino acid sequence listing submitted concurrently herewith.

BACKGROUND OF THE INVENTION

[0003] CD4 is a nonpolymophic transmembrane glycoprotein expressed on the surface of most thymocytes and a subpopulation of matured T cells ($CD4^+$ T cells) (see Germain, *Curr. Biol., 7:* R640-R644 (1997)). It is composed of four immunoglobulin-like extracellular domains, a transmembrane segment and a cytoplasmic tail non-covalently associated with a src-family tyrosine kinase, Lck. As an important component of the immune system, CD4 functions as a co-receptor assisting the T cell receptor (TCR) on the $CD4^+$ T cells for stronger association with class II major histocompatibility complex (MHCII) on antigen-presenting cells (APCs). The association is sufficient to trigger T-cell signaling transduction resulting in activation of the $CD4^+$ T cells.

[0004] CD4 also contributes directly to the signal transduction by driving signaling cascade through its kinase-linked cytoplasmic tail. The crystal structure of human CD4-murine MHCII shows that only the first extracellular domain (D1) of CD4 makes contact with MHCII (see Wang et al., *Proc. Natl. Acad. Sci. USA, 98:* 10799-10804 (2001)). However, mutational analysis indicates that in addition to D1, other domains also affect binding to MHCII (see Moebius et al., *Proc. Natl. Acad. Sci. USA, 90:* 8259-8263 (1993)). Moreover, oligomerization of CD4 is required for stable interaction with MHCII and efficient T-cell activation (see Sakihama et al., *Proc. Natl. Acad. Sci. USA, 92:* 6444-6448 (1995)).

[0005] CD4 is also the primary receptor for HIV-1 (see Dalgleish et al., *Nature, 312:* 763-767 (1984)). HIV-1 entry is initiated by binding of the viral envelope glycoprotein (Env) gp120 to cellular receptor CD4. The interaction results in extensive conformational rearrangements of gp120 and subsequently gp41 after engagement of a coreceptor (either

CCR5 or CXCR4). The structural rearrangements of Envs and the interplay between Envs and the cellular receptor and co-receptor bring viral membrane toward target cell membrane, and eventually cause membrane fusion and viral entry. Because CD4 plays a key role in HIV-1 infection, recombinant solubly expressed CD4 (sCD4) containing either all four (T4) (see Deen et al., *Nature, 331*: 82-84 (1988)) or the first two extracellular domains (D1D2) (see Traunecker et al., *Nature, 331:* 84-86 (1988)) is a potent inhibitor of HIV-1 entry and used for crystallization alone (see Wu et al., *Nature, 387:* 527-530 (1997) and Ryu et al., *Nature, 348:* 419-429 (1990)) or with gp120 (see Kwong et al., *Nature, 393:* 648-659 (1998)).

[0006] In Sharma et al. (*Biochemistry, 44:* 16192-16202 (2005)), D1 was generated by using a mutational strategy and purified from the sonication supernatant of *E. coli*. However, the purified protein was stable only at low pH (4.0) and partially improperly folded, and had affinity several-fold lower than that of D1D2 or T4.

[0007] In Chen et al. (*J. Virol.*, 85: 9395-9405 (2011)), two stable monomeric D1 mutants, mD1.1 and mD1.2 were identified, which were significantly more soluble and bound Env gp120s more strongly (50-fold) than D1D2, neutralized a panel of HIV-1 primary isolates from different clades more potently than D1D2, induced conformation changes in gp120, and sensitized HIV-1 for neutralization by CD4-induced antibodies.

[0008] However, the desire for new D1s that are correctly folded, highly soluble and stable under physiological conditions while preserving not only binding activity and specificity but also other functions, such as induction of conformational changes in HIV-1 gp120, remains.

BRIEF SUMMARY OF THE INVENTION

[0009] The invention provides a polypeptide comprising a single domain CD4, such as SEQ ID NO: 1 or SEQ ID NO: 2, as well as a fusion protein comprising the single domain CD4 and one or more fusion partners, wherein the one or more fusion partners optionally is joined to the single domain CD4 via a linker. Additionally, constructs comprising multivalent fusion proteins are provided. Nucleic acids encoding the single domain CD4 and fusion proteins, as well as compositions or cells comprising the single domain CD4, fusion proteins, constructs, or nucleic acids, also are provided.

[0010] The invention also provides a method of inhibiting an HIV infection in a cell or a host comprising administering the polypeptide, fusion protein, or construct to the cell or host, such that the HIV infection is inhibited.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0011] Figure 1 is an amino acid sequence comparison between human single domain CD4 (D1) and mutants thereof.

[0012] Figure 2 is a depiction of constructs containing multiple fusion proteins, wherein A denotes an antibody or antibody fragment (e.g., m36.4), B denotes a single domain CD4 (e.g., the inventive polypeptide), C denotes a light chain constant region, D denotes a heavy chain constant region, and E denotes an Fc region or portion thereof. Straight lines connecting the regions denote linker sequences. The line represents optional bonds.

[0013] Figures 3A and 3B are graphs depicting the binding of the D1 mutants with two different HIV-1 envelope glycoproteins: $gp140_{Con\text{-}s}$ (A) and $gp140_{CH12.0544.2}$ (B). In each of the graphs, optical density at 450 nm is indicated on the y-axis and D1 mutant concentration (nM) is indicated on the x-axis.

[0014] Figure 4 is a graph depicting the enhancing effects of the D1 mutants on binding of CD41 antibody m36h1Fc to HIV-1 $gp140_{Con\text{-}s}$. Optical density at 450 nm is indicated on the y-axis and sCD4 concentration (nM) is indicated on the x-axis.

[0015] Figures 5A and 5B are graphs demonstrating that the D1 mutants consist primarily of β-strand secondary structures (A) and that mD1.22 has higher thermal stability than mD1.2 or mD1.23 (B). In Figure 5A, $[\Theta](103 \text{ deg cm}^2 \text{ dmol}^{-1})$ is indicated on the y-axis and wavelength (nm) is indicated on the x-axis. In Figure 5B, the fraction folded (as assayed by circular dichroism) is indicated on the y-axis and temperature (°C) is indicated on the x-axis.

[0016] Figure 6A is a graph demonstrating that the D1 mutants are monomeric (elute as one peak in size-exclusion chromatography).

[0017] Figure 6B demonstrates that that mD1.22 does not tend to aggregate at high concentrations as shown by dynamic light scattering. Percent intensity at Day 0 or Day 7 at 4 °C or 37 °C is indicated on the y-axis and the size of mD1.22 (d.nm) is indicated on the x-axis.

[0018] Figure 7 demonstrates that an Fc-fusion protein of mD1.22 (mD1.22Fc) does not bind to MHC-expressing cells (human blood B (BJAB) and T (SUP-T1) cell lines). The dark

curve contains reference cells only, while the light curve contains cells and Fc-fusion proteins.

[0019] Figure 8A is a graph demonstrating that the A55V mutant of D1D2Fc (D1D2mFc) shows increased binding to HIV-1 $gp140_{Con-s}$ as compared to D1D2Fc.

[0020] Figure 8B demonstrates that the A55V mutant of D1D2Fc (D1D2mFc) has decreased interaction with human blood B and T cell lines (BJAB and SUP-T1) relative to D1D2Fc.

[0021] Figure 9 is a graph demonstrating that the A55V mutation largely decreased binding of the CD4 D1-specific antibody D9 to mD1.22Fc and D1D2mFc, which suggests that the mutation induces conformational changes in D1. The optical density at 450 nm is indicated on the y-axis and the D9 concentration (nM) is indicated on the x-axis.

[0022] Figure 10 is a graph demonstrating that mD1.22-36.4 fusion proteins (different valences) showed much lower binding to MHC-expressing cells (human blood B (BJAB) cell line) than D1D2Fc.

[0023] Figures 11A and 11B are graphs depicting that 4Dm2m and 6Dm2m do not enhance HIV-1 infectivity to two HIV-1 primary isolates (Bal in Fig. 11A and JRFL in Fig. 11B) at low concentrations in contrast to D1D2. In each graph, percent infectivity is indicated on the y-axis and concentration (nM) is indicated on the x-axis. IgG1 m102.4 (see Zhu et al., *J. Infect. Dis., 197*: 846-853 (2008)), is a control antibody specific to Nipah and Hendra viruses that did not inhibit any of the viruses.

[0024] Figures 12A and 12B are graphs demonstrating the stability of 4Dm2m and 6Dm2m against degration in PBS and in human serum. In each graph, concentration (nM) is indicated on the y-axis and incubation time (days) is indicated on the x-axis. CD4-Ig is used as a control.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The invention provides a single domain CD4 that has the binding activity and specificity of full-length CD4 and maintains other functions, such as induction of conformational changes in HIV-1 gp120. Due to decreased molecular size, the single domain CD4 has excellent biological properties including improved binding kinetics, soluble expression in *E. coli*, higher solubility, stability and specificity, minimization of immunogenicity in animals, and better penetration into tissues, such as the densely packed

lymphoid environments (e.g., spleen, lymph node and gut) where HIV-1 mostly replicates and spreads.

[0026] The inventive single domain CD4 comprises, consists essentially of, or consists of the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2. The invention also provides a polypeptide comprising variants of SEQ ID NO: 1 or SEQ ID NO: 2 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. Preferably, the invention provides a polypeptide comprising SEQ ID NO: 1 or SEQ ID NO: 2 with up to 10 additions, deletions, substitutions, or insertions, wherein the polypeptide does not comprise SEQ ID NO: 4.

[0027] The inventive polypeptide preferably is a recombinant or synthetic polypeptide, which is non-naturally occurring.

[0028] The inventive polypeptide can be provided alone, or as part of a fusion protein comprising the polypeptide and one or more fusion partners. The fusion partner can be any suitable moiety that does not substantially inhibit the polypeptide's ability to bind its target. Desirably, 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.

[0029] 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; (1) 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-alpha (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.

[0030] 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: 7 and SEQ ID NO: 8.

[0031] In another embodiment, the fusion partner is an antibody or antibody fragment (e.g., Fab, scFv, dAb, etc.). Preferably, the antibody is a single-domain antibody (a.k.a. "domain antibody ("dAb") or "engineered antibody domain" ("eAd")), which is an antibody fragment consisting of a single monomeric variable antibody domain from the heavy or light chains. For example, the eAd can comprise SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, or SEQ ID NO: 16, also referenced herein as the m36, m36.1, m36.2, m36.4, or m36.5 antibodies, respectively.

[0032] 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.

[0033] The fusion protein comprises one or more fusion partners (e.g., two, three, four, five, or more fusion partners). For instance, the fusion protein can comprise the inventive polypeptide, an eAd (e.g., the m36, m36.1, m36.2, m36.4, or m36.5 antibodies of SEQ ID NOs: 12-16, respectively) and a stability-enhancing fusion partner, such as an immunoglobulin Fc region (e.g., human IgG1 Fc) or portion thereof (e.g., CH3).

[0034] The inventive polypeptide 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) G_4S motifs, such as the linkers of SEQ ID NOs: 9-11.

[0035] By way of further illustration, examples of fusion proteins according to the invention can have the following configuration: (first fusion partner)-(optional first linker)-(inventive polypeptide (referred to as mD1))-(optional second linker)-(optional second fusion partner). More specific illustrative examples include the following: mD1-Fc (SEQ ID NO: 17), gp120-linker-mD1, eAd-linker-mD1, mD1-linker-CH3, eAd-linker-mD1-linker-CH3, and eAd-linker-mD1-Fc.

[0036] The invention also provides a fusion protein comprising:

A-(optional linker)-C-(optional linker)-B (Formula (I))

or

B-(optional linker)-D-(optional linker)-E-(optional linker)-B (Formula (II))

wherein A denotes an antibody or antibody fragment (e.g., Fab, scFv, eAd, etc.), B denotes the inventive polypeptide (SEQ ID NO: 1 or SEQ ID NO: 2; referred to as mD1), C denotes an immunoglobulin light chain constant region (e.g., human IgG1 kappa light chain constant region), D denotes an immunoglobulin heavy chain constant region (e.g., human IgG1 heavy chain constant region), and E denotes an Fc region or a portion thereof (e.g., the Fc region from human IgG1). Specific examples include mD1.22-linker-human IgG1 heavy chain constant region-linker-mD1 and 36.4 eAd-linker-human IgG1 light chain constant region-linker-mD1.22.

[0037] Two or more of the fusion proteins can be conjugated or otherwise joined in a larger construct. For instance, two fusion proteins of Formula (I) above and two fusion proteins of Formula (II) above can be assembled into a single construct, as depicted in Figure 2 (6Dm2m). In one embodiment, the fusion protein of Formula (I) comprises SEQ ID NO: 23 and the fusion protein of Formula (II) comprises SEQ ID NO: 22.

[0038] Alternatively, two fusion proteins of Formula (II) above and two fusion proteins of A-(optional linker)-C (Formula III) can be assembled into a single construct, as depicted in Figure 2 (4Dm2m). In one embodiment, the fusion protein of Formula (III) comprises SEQ ID NO: 21 and the fusion protein of Formula (II) comprises SEQ ID NO: 20.

[0039] In another embodiment, two fusion proteins of Formula (III) above and two fusion proteins of B-(optional linker)-D-(optional linker)-E (Formula IV) can be assembled into a single construct, as depicted in Figure 2 (2Dm2m). The fusion protein of Formula (III) can comprise SEQ ID NO: 19 and the fusion protein of Formula (IV) can comprise SEQ ID NO: 18.

[0040] The individual fusion proteins can be joined in the manner typical of IgG type constructs, such as by disulfide bridges between the constant heavy and constant light regions and between the Fc regions. Two or more fusion proteins joined as a single construct desirably can provide a multivalent (bivalent, tetravalent, or even octavalent) molecule.

[0041] Thus, constructs comprising two or more (e.g., two, three, four, five, six, seven, eight, nine, ten, or more) of the inventive fusion proteins also are encompassed by the invention.

[0042] In one embodiment, the fusion protein is assembled (e.g., self-assembled) to form one of the constructs depicted in Figure 2, wherein A denotes an antibody or antibody fragment (e.g., m36.4 eAd), B denotes the inventive polypeptide (e.g., mD1.22), C denotes an immunoglobulin light chain constant region (e.g., human IgG1 kappa light chain constant region), D denotes an immunoglobulin heavy chain constant region (e.g., human IgG1 heavy chain constant region), and E denotes an Fc region (e.g., the Fc region from human IgG1).

[0043] The polypeptide and 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.

[0044] The polypeptide or fusion protein can be multimerized, as for example, hetero- or homodimers, hetero- or homotrimers, hetero- or homotetramers, or higher order hetero- or homomultimers. 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 (e.g., eAds), 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.

[0045] 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)).

[0046] The polypeptide and fusion protein (or construct containing the 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).

[0047] Conjugates comprising the polypeptide or fusion protein (or construct containing the fusion protein) of the invention conjugated to cytotoxic agents, such as chemotherapeutic agents, toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof; a small molecule toxin), radioactive isotopes (i.e., a radioconjugate), or antiviral compounds (e.g., anti-HIV compounds) also are encompassed by the invention. Alternatively, the polypeptide or fusion protein (or construct containing the fusion protein) can be co-administered with the cytotoxic agents, antiviral compounds, and the like.

[0048] The conjugates comprising cytotoxic agents (e.g., toxins) can be used to target viral (e.g., HIV, such as HIV-1) infected cells and eradicate (destroy) such cells. For example, with a conjugate comprising (i) a cytotoxic agent and (ii) an antibody or antibody fragment containing construct as described herein, the (ii) antibody or antibody fragment portion of the conjugate targets (detects) surface proteins of viral infected cells and (i) the cytotoxic agent portion of the conjugate eradicates (destroys) the targeted viral infected cells. Preferably, the cells to be targeted are HIV (e.g., HIV-1) infected cells and the conjugate detects/targets the HIV (e.g., HIV-1) envelope glycoprotein expressed on the HIV infected cells. Administration of the conjugates can be used to destroy viral (e.g., HIV, such as HIV-1) infected cells in a subject, thereby leading to successful treatment (cure) of the viral (e.g., HIV) infection in the subject. Accordingly, the invention provides a method for treating a viral infection in a subject comprising administering the inventive conjugate to the subject,

thereby treating (curing) the viral infection in the subject by destroying the viral-infected cells in the subject.

[0049] Methods for conjugating the polypeptide or fusion protein (or construct containing the fusion protein) to the cytotoxic agents, chemotherapeutic agents, toxins, antibacterial compounds, and antiviral compounds, and the like are well known in the art. For example, conjugates can be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyidithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as l,5-difluoro-2,4-dinitrobenzene).

[0050] Detectable agents, such as fluorescent compounds, also can be added to the polypeptide or fusion protein (or construct containing the fusion protein). Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-dimethylamine-l-napthalenesulfonyl 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.

[0051] The invention also provides a nucleic acid encoding the amino acid sequence of the polypeptide or fusion protein. The nucleic acid can comprise DNA or RNA, and can be single or double stranded. Furthermore, the nucleic acid can comprise nucleotide analogues or derivatives (e.g., inosine or phophorothioate nucleotides and the like).

[0052] 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*).

[0053] 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, baculorvirus, and Sindbis virus. When the vector is a plasmid (e.g. DNA plasmid), the plasmid can be complexed with chitosan.

[0054] When the vector is for administration to a host (e.g., 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.

[0055] The polypeptide, fusion protein, conjugate, or construct can be administered to a mammal 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, DH5α, XL-Blue MRF' (Stratagene), SA2821, and Y1090), *Bacillus subtilis, Salmonella typhimurium*, *Serratia marcescens*, Pseudomonas (e.g., *P. aerugenosa*), *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.

[0056] 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.

[0057] 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.

[0058] The polypeptide, fusion protein, conjugate, 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, conjugate, nucleic acid, vector, or cell to the mammal, viral infection (e.g., HIV infection) of the mammal is inhibited. The inventive method can prophylactically or therapeutically inhibit infection by any type of HIV, but preferably inhibits 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).

[0059] Additionally, the polypeptide, fusion protein, conjugate, nucleic acid, vector, or cell can be used to inhibit a broad range of viruses (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 viruses that may be treated in accordance with the invention include, but are not limited to, 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.

[0060] When provided therapeutically, the polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, cell, or composition thereof is provided at or after the diagnosis of viral (e.g., HIV) infection.

[0061] When provided prophylactically, the polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, cell, or composition thereof is provided in advance of viral (e.g., HIV) infection, such as to patients or subjects who are at risk for being exposed to a virus (e.g., HIV) or who have been newly exposed to a virus (e.g., HIV). 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 HIV. The prophylactic administration of the polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, cell, or composition thereof prevents, ameliorates, or delays viral (e.g., HIV) infection. In subjects who have been newly exposed to a virus (e.g., HIV) but who have not yet displayed the presence of the virus (as measured by PCR or other assays for detecting the virus) 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 virus or minimizes the level of the virus in the blood or other body fluid of the exposed individual.

[0062] The efficacy of the polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, cell, or composition thereof can be assessed in various ways well known to the skilled practitioner. For instance, one of ordinary skill in the art will understand that a polypeptide or fusion protein of the invention is efficacious in treating or inhibiting a viral (e.g., 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.

[0063] The polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, or cell can be formulated as a composition (e.g., pharmaceutical composition) comprising the polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, or cell and a carrier (e.g., a pharmaceutically or physiologically acceptable carrier). Furthermore, the polypeptide, fusion protein, conjugate, construct, 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.

[0064] Compositions (e.g., pharmaceutical compositions) comprising the polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, or cell can include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like.

[0065] 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.

[0066] The composition (e.g., pharmaceutical composition) can comprise more than one polypeptide, fusion protein, conjugate, construct, 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.

[0067] Suitable methods of administering a polypeptide, fusion protein, conjugate, construct, 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).

[0068] 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, 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.

[0069] 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.

[0070] 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.

[0071] 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.

[0072] 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.

[0073] The polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, or cell can be administered with a pharmaceutically acceptable carrier and can be delivered to the mammal's cells *in vivo* and/or *ex vivo* 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).

[0074] Additionally, probiotic therapies are envisioned by the present invention. Viable host cells containing the nucleic acid or vector of the invention and expressing the fusion protein or conjugate can be used directly as the delivery vehicle for the fusion protein to the desired site(s) *in vivo*. Preferred host cells for the delivery of the fusion protein or conjugate directly to desired site(s), such as, for example, to a selected body cavity, can comprise bacteria. More specifically, such host cells can comprise suitably engineered strain(s) of lactobacilli, enterococci, or other common bacteria, such as *E. coli*, normal strains of which are known to commonly populate body cavities. More specifically yet, such host cells can comprise one or more selected nonpathogenic strains of lactobacilli, such as those described by Andreu et al., *J. Infect. Dis., 171(5),* 1237-43 (1995), especially those having high adherence properties to epithelial cells (e.g., vaginal epithelial cells) and suitably transformed using the nucleic acid or vector of the invention.

[0075] If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the body according to standard protocols known in the art. The compositions can be introduced into the cells via any gene transfer mechanism, such as calcium phosphate mediated gene delivery, electroporation, microinjection, or proteoliposomes. The transduced cells then can be infused (e.g., with a pharmaceutically acceptable carrier) or homotopically transplanted back into the mammal per standard methods for the cell or tissue type. Standard methods are known for transplantation or infusion of various cells into a mammal.

[0076] 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 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, 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.

[0077] The polypeptide, fusion protein, conjugate, construct, 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 HIV infection. The efficacy of the polypeptide, fusion protein, conjugate, construct, nucleic acid, vector, cell, or composition thereof as an HIV infection inhibitor may be determined by *in vivo* or *in vitro* parameters known in the art.

[0078] Any suitable dose of the polypeptide fusion protein, conjugate, 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 viral (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).

[0079] 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 x 10^8 pfus can be administered (e.g., in a volume of about 0.5 mL).

[0080] The inventive cells can be administered to a host in a dose of between about 1 x 10^5 and 2 x 10^{11} (e.g., 1 x 10^6, 1 x 10^7, 1 x 10^8, 1 x 10^9, 1 x 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).

[0081] The polypeptide or fusion protein (or construct containing the fusion protein) 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 (or construct containing the 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)).

[0082] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

[0083] This example describes the identification of single domain CD4 (D1) with improved soluble expression, stability, and specificity.

[0084] Structure-guided cavity filling and library screening were used to identify D1 mutants with improved soluble expression, stability and specificity. The screening yielded two mutants: mD1.22 (SEQ ID NO: 1) and mD1.23 (SEQ ID NO: 2) (see Figure 1).

[0085] As depicted in Table 1, the mutants were significantly more soluble in *E. coli* periplasm and PBS than the previously identified D1 mutant (mD1.2; Chen et al., *J. Virol., 85*: 9395-9405 (2011)).

Table 1. Properties of single domain CD4 mutants.

Single Domain CD4	**Soluble Expression in *E. coli.* (mg/L)**	**Solubility in PBS (pH 7.4) (mg/mL)**
mD1.2	0.75	>92.6
mD1.22	5	175
mD1.23	6	ND

ND = not determined

EXAMPLE 2

[0086] This example demonstrates the characterization of mD1.22 and mD1.23.

[0087] The binding characteristics of mD1.2 (Chen et al., 2011, *supra*), mD1.22 (SEQ ID NO: 1), and mD1.23 (SEQ ID NO: 2) with HIV-1 gp140 were assessed at different D1 concentrations.

[0088] ELISAs were performed with $gp140_{Con\text{-}s}$, which is a consensus gp140 designed by aligning >1,000 sequences of group M (see Liao et al., *Virology, 353*: 268-282 (2006)), and $gp140_{CH12.0544.2}$. Bound mD1.2 and the mD1.22 and mD1.23 mutants were detected by HRP-conjugated anti-hexahistidine tag antibody (Sigma-Aldrich, St. Louis, MO).

[0089] As shown in Figs. 3A and 3B, both mutants were cross-reactive against the tested gp140s.

[0090] Binding of genetically diverse HIV-1 Envs also was analyzed by surface plama resonance (SPR) on a Biacore X100 appartus (GE Healthcare) using a single-cycle approach. Briefly, purified HIV-1 gp140 was diluted in sodium acetate (pH 5.0) and immobilized directly onto a CM5 sensor chip via the standard amine coupling method. The reference cell was injected with N-hydroxysuccinimide-1-ethyl-3-(3-dimethyaminopropy)carbodiimide and ethanolamine without injection of gp140. The D1 mutants and D1D2 were diluted with running buffer HSB-EP (100 mM HEPES (ph 7.4), 1.5 M NaCl, 30 mM EDTA, 0.5%

surfactant 20). All analytes were tested at 500, 100, 20, 4, and 0.8 nM concentrations. Kinetic contants were calculated from the sensograms fitted with the monvalent binding model of the BiocoreX100 Evlauation software 2.0.

[0091] In the SPR analysis with four HIV-1 Env gp140s, mD1.22 showed affinities comparable to, or higher than, those of mD1.2 (see Table 8).

EXAMPLE 3

[0092] This example demonstrates that the D1 mutants maintain the functional activity of full-length CD4.

[0093] CD4 induces conformational changes in gp120 leading to exposure of CD4-inducible (CD4i) epitopes. To determine whether the D1 mutants induce such conformational changes, an CD4i antibody-based fusion protein, m36h1Fc (see Chen et al., *Proc. Natl. Acad. Sci. USA, 105:* 17121-17126 (2008)), was tested for binding to $gp140_{Con\text{-}s}$ in the absence or presence of mD1.2 (SEQ ID NO: 3), mD1.22 (SEQ ID NO: 1), or mD1.23 (SEQ ID NO: 2). The two-domain human CD4 (D1D2; SEQ ID NO: 5) was used a positive control and Fab b12, which is an HIV-1 broadly neutralizing antibody targeting the CD4-binding site on gp120 (see Roben et al., *J. Virol., 68*: 4821-4828 (1994)), was used as the negative control.

[0094] Binding of m36h1Fc to $gp140_{Con\text{-}s}$ was increased in the presence of the D1 mutants relative to the controls (see Fig. 4). Additionally, the binding m36h1Fc to $gp140_{Con\text{-}s}$ was increased or comparable to the binding observed in the presence of D1D2 and mD1.2, respectively (see Fig. 4).

EXAMPLE 4

[0095] This example demonstrates the further characterization of the D1 mutants.

[0096] The secondary structure and thermal stability of mD1.2 (SEQ ID NO: 3), mD1.22 (SEQ ID NO: 1), and mD1.23 (SEQ ID NO: 2) were determined by circular dichroism (CD) spectroscopy. The purified proteins were dissolved in PBS at the final concentration of 0.33 mg/mL, and the CD spectra were recorded on AVIV Model 202 CD Spectrometer (Aviv Biomedical). Wavelength spectra were recorded at 25 °C using a 0.1-cm path-length cuvette for native structure measurements. Thermal stability was measured at 216 nm by recording the CD signal in the temperature range of 25-90 °C with heating rate 1 °C/min. The temperature was recorded with an external probe sensor and the temperature inside the

microcuvette was calculated by calibration; it was about 2-3 °C (range from 1.9 to 3.8 °C for temperatures from 20 to 80 °C) lower than the one measured by the external sensor. After heating, wavelength spectra were recorded at 90 °C.

[0097] As evidenced by Fig. 5A, mD1.22 and mD1.23 consist primarily of β-strand secondary structures. mD1.22 has higher thermal stability than mD1.2 and mD1.23 (see Fig. 5B).

[0098] Similar to mD1.2, mD1.22 and mD1.23 were uniformly monomeric in PBS at pH 7.4 as determined by size-exclusion chromatography. D1D2 also was monomeric but it was not eluted as a single peak (see Fig. 6A). mD1.22 did not tend to aggregate at high concentrations (10 mg/mL) as shown by dynamic light scattering (see Fig. 6B).

EXAMPLE 5

[0099] This example demonstrates the characterization of Fc fusion proteins comprising the D1 mutants.

[0100] Fc-fusion proteins of D1D2, D1D2m (with A55V mutation; SEQ ID NO: 6), mD1.2, and mD1.22 (D1D2Fc, D1D2mFc, mD1.2Fc, and mD1.22Fc, respectively) were prepared using routine methods.

[0101] The binding of MHC-expression cells – human blood cell lines BJAB (B cell) and SUP-T1 (T cell) – was accessed for m36h1Fc (negative control), D1D2Fc, D1D2mFc, mD1.2Fc, and mD1.22Fc. As evidenced by Figs. 7 and 8B, Fc fusions of mD1.22 or D1D2m show decreased binding with MHC-expression cells relative to the other fusion proteins.

[0102] To determine if the A55V mutation results in conformational changes, interaction with a CD4 D1-specific antibody (D9) was assessed. As shown in Fig. 9, the presence of the A55V mutation in mD1.22Fc and D1D2mFc largely decreased binding with the D9 antibody. This suggests that the mutation induces conformation changes in D1. However, as noted above, the presence of the A55V mutation results in increased binding to HIV-1 gp140 (see Fig. 8A).

EXAMPLE 6

[0103] This example demonstrates the characterization of bispecific multivalent fusion proteins comprising mD1.22.

[0104] Bispecific multivalent fusion proteins of mD1.22 (SEQ ID NO: 1) and m36.4 as exemplified in Fig. 2 (2Dm2m, 4Dm2m, and 6Dm2m) were prepared. m36.4 (SEQ ID NO:

15) is an engineered single human antibody domain targeting a CD4i epitope on HIV-1 gp120.

[0105] In each of the bispecific multivalent fusion proteins, A corresponds to m36.4, B corresponds to mD1.22, C corresponds to a light chain constant region, D corresponds to a heavy chain constant region, and E corresponds to an Fc region. In particular, 2Dm2m comprises two fusion proteins comprising SEQ ID NO: 18 and two fusion proteins comprising SEQ ID NO: 19. 4Dm2m comprises two fusion proteins comprising SEQ ID NO: 20 and two fusion proteins comprising SEQ ID NO: 21. 6Dm2m comprises two fusion proteins comprising SEQ ID NO: 22 and two fusion proteins comprising SEQ ID NO: 23.

[0106] Similar to the results described in Example 5, the mD1.22-m36.4 multivalent fusion proteins showed much lower binding to MHC-expressing BJAB cells than D1D2Fc (see Fig. 10).

[0107] To determine the potency and breadth of HIV-1 neutralization by the D1 mutants, viruses pseudotyped with Envs from HIV-1 isolates representing clades A-E and using either CCR5 (R5) or CXCR4 (X4) or both (R5X4) as a coreceptor were included. Pseudoviruses were derived from 293T cells and a neutralization assay was performed in duplicate using HOS-CD4-CCR5 (for all R5 and dual tropic viruses) or HOS-CD4-CXCR4 cell lines as described in Chen et al., *Proc. Natl. Acad. Sci. USA, 105*: 17121-17126 (2008)). Luminescence was measured 48 hours post-infection and the percentage neutralization was calculated by the following formula: (1 – average RLU of inhibitor-containing wells/average RLU or virus-only wells) x 100. IC_{50} and IC_{90} of neutralization were assigned for the inhibitor concentration at which 50% and 90% neutralization were observed, respectively.

[0108] As shown in Tables 2 and 3, mD1.22 and mD1.22-containing fusion proteins neutralized HIV-1 primary isolates from several different clades. Significantly, 4Dm2m and 6Dm2m were more potent than broadly neutralizing monoclonal antibody VRC01 (see Table 3).

[0109] Additionally, mD1.22 and mD1.22-containing fusion proteins inhibited HIV-1 envelope glycoprotein-mediated cell fusion (see Tables 4 and 5). As shown in Table 5, 4Dm2m and 6Dm2m are more potent than VRC01 in inhibiting HIV-1 envelope glycoprotein mediated cell-cell fusion.

[0110] In a TZM-bl cell-based assay (see Brown et al., *Virology, 375*: 529-538 (2008)) where TZM-bl cells and HIV-1 isolates randomly selected from each clade from A to D, AE, and AG (total n=12) were used, 4Dm2m and 6Dm2m were much more potent than b12 and

CD4-Ig and about 10-fold more potent than VRC01 when the geometric means of their IC_{50}s and IC_{90}s were compared (see Table 6).

[0111] In another TZM-bl cell-based assay using a panel of pseudoviruses with Envs derived from 41 HIV-1 isolates predominantly circulating in China (see Yao et al., *J. Biol. Chem., 287*: 6788-6796 (2012); and Shang et al., *J. Biol. Chem., 286*: 14531-14541 (2011)), 4Dm2m and 6Dm2m were compared to T20 (enfuvirtide), which is an FDA-approved peptide inhibitor derived from the HIV-1 Env gp41 C-terminal heptad repeat (CHR), C34, which is another potent CHR peptide inhibitor, and CD4-Ig for neutralizing activities against the panel (see Table 7). 4Dm2m and 6Dm2m neutralized all viruses with IC_{50} geometric mean values of approximately 0.20 nM, which are about 50-fold (P=0.002, Student's paired t test), 16-fold (P<0.001, Student's pairent t test), and 200-fold (P<0.001, Student's paired t test) lower than those (11, 3.5, and 42 nM) of T20, C34, and CD4-Ig respectively (see Table 7). The superior potencies of 4Dm2m and 6Dm2m also were observed when the IC_{90}s were compared.

[0112] 4Dm2m and 6Dm2m also have been shown to not enhance HIV-1 infectivity at low concentrations, which is in contrast to D1D2 (see Fig. 11).

EXAMPLE 7

[0113] This example deomonstrates thet solublility, stability, and aggregration propensity of the bispecific multivalent fusion proteins.

[0114] To evaluate the potential for further development as drugs, several drug-related properties, including solubility, stability, and aggregation propensity were tested. 4Dm2m and 6Dm2m in PBS were concentrated to 25.0 and 25.9 mg/ml, respectively, without visible precipitation after high-speed centrifugation. They were stored at 4 °C for 2 weeks, and no additional precipitation was observed, suggesting high solubility of the proteins.

[0115] To test whether soluble aggregates of 4Dm2m and 6Dm2m formed during prolonged incubation, dynamic light scattering (DLS) was used. Proteins concentrated to 10 mg/ml in PBS were stored at 80 °C and slowly thawed on ice before measurements. The results showed that the particles of 4Dm2m and m909, a control human antibody in the IgG1 format (see Feng et al., *Arthritis Res. Ther. 13*: R59 (2011)), were predominantly small (average diameters, 16.3 and 12.7 nm, respectively). The minor aggregates of 4Dm2m disappeared within the first day of incubation at both 4 °C and 37 °C, while m909 aggregation continued to occur following the incubation at 37 °C. In comparison, about 30 to

50% of the CD4-Ig particles were large, with average diameters of approximately 100 nm, and the aggregation persisted during the whole period of incubation. 6Dm2m partially aggregated after the freeze-thaw cycle; although the aggregation was weakened following the incubation, it led to a relatively wide particle size distribution.

[0116] These results suggest that like typical IgG1s, 4Dm2m has a low aggregation propensity, which is lower than that of CD4-Ig.

[0117] The proteins were then assessed for their stability against degradation during 15 days of incubation with PBS (see Fig. 12A) or human serum (see Fig. 12B) at 37 °C. Incubation in PBS for 10 days caused no significant decrease in the amount of functional 4Dm2m, whereas about 20% and 30%, respectively, of 6Dm2m and CD4-Ig lost binding activity to $gp140_{Con\text{-}s}$ (see Fig. 12A). 4Dm2m began to be degraded and 6Dm2m continued to be degraded thereafter, while CD4-Ig appeared to be stable. Finally, about 90% of 4Dm2m remained functional, slightly more than those (80%) of 6Dm2m and CD4-Ig.

[0118] With human serum, levels of all proteins declined at similar rates during the first 5 days of incubation (see Fig. 12B). However, degradation of 4Dm2m and 6Dm2m became slower than that of CD4-Ig during the next 5 days of incubation. At the end of the measurement period, the level of CD4-Ig that retained binding activity was slightly higher than that for functional 4Dm2m and 6Dm2m, but the differences in the amounts were not statistically significant.

Table 2. Neutralization of pseudotyped HIV-1 by mD1.22 of different valences.

Virus	Clade	Tropism	m36.4		mD1.22		mD1.22Fc		2Dm2m		4Dm2m		6Dm2m	
			IC_{50}[a]	IC_{90}[b]	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Bal	B	R5	16 ± 1	75 ± 18	2.1 ± 0.1	5.2 ± 0.2	0.40 ± 0.00	1.9 ± 0.2	0.34 ± 0.15	0.59 ± 0.05	0.12 ± 0.01	0.36 ± 0.06	0.063 ± 0.004	0.22 ± 0.02
JRFL	B	R5	18 ± 5	106 ± 4	2.9 ± 0.4	25 ± 1	1.2 ± 0.1	25 ± 6	0.090 ± 0.008	0.59 ± 0.06	0.060 ± 0.014	0.29 ± 0.09	0.043 ± 0.004	0.21 ± 0.01

[a] Antibody concentration (nM) resulting in 50% inhibition of virus infection.

[b] Antibody concentration (nM) resulting in 90% inhibition of virus infection.

Table 3. Neutralization of HIV-1 pseudotyped with Envs from different clades by 4Dm2m, 6Dm2m, and bnmAbs.

Virus	Clade	Tropism	2G12		VRC01		4Dm2m		6Dm2m	
			IC_{50}[a]	IC_{90}[b]	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
92UG037.8	A	R5	0.53 ± 0.10	63 ± 4	0.33 ± 0.11	3.8 ± 4.3	0.051 ± 0.001	0.45 ± 0.01	0.14 ± 0.08	2.1 ± 0.6
Bal	B	R5	3.1 ± 0.0	41 ± 1	0.11 ± 0.04	0.56 ± 0.01	0.15 ± 0.04	0.50 ± 0.07	0.12 ± 0.01	0.38 ± 0.02
JRFL	B	R5	3.0 ± 0.5	13 ± 3	0.31 ± 0.21	0.82 ± 0.40	0.14 ± 0.01	0.39 ± 0.04	0.10 ± 0.01	0.43 ± 0.04
JRCSF	B	R5	0.58 ± 0.17	9.3 ± 8.0	0.28 ± 0.18	5.6 ± 2.1	0.29 ± 0.18	3.0 ± 2.9	0.21 ± 0.14	1.0 ± 0.1
AD8	B	R5	1.2 ± 0.1	18 ± 3	0.36 ± 0.13	4.3 ± 0.4	0.13 ± 0.01	0.74 ± 0.06	0.11 ± 0.01	0.60 ± 0.08
R2	B	R5	0.68 ± 0.11	4.6 ± 0.1	0.34 ± 0.01	4.4 ± 0.1	0.028 ± 0.011	0.43 ± 0.18	<0.021	0.14 ± 0.04
IIIB	B	X4	1.5 ± 0.0	9.3 ± 2.4	0.26 ± 0.01	0.83 ± 0.04	0.037 ± 0.001	0.10 ± 0.01	<0.021	0.086 ± 0.006
NL4-3	B	X4	1.3 ± 0.2	18 ± 10	0.25 ± 0.02	2.6 ± 0.6	0.094 ± 0.002	0.15 ± 0.02	0.068 ± 0.004	0.19 ± 0.02
89.6	B	R5X4	1.3 ± 1.1	43 ± 11	0.37 ± 0.10	14 ± 2	<0.021	<0.021	<0.021	<0.021
GXC	C	R5	-[c]	-	2.1 ± 0.1	6.6 ± 4.8	0.61 ± 0.36	4.0 ± 0.0	0.63 ±0.06	1.7 ± 0.1
Z2Z6	D	R5	0.70 ± 0.01	14 ± 2	0.11 ± 0.01	0.85 ± 0.14	0.14 ± 0.04	1.1 ± 0.1	0.045 ± 0.006	0.50 ± 0.00
CM243	E	R5	-	-	0.53 ± 0.11	9.5 ± 2.1	0.29 ± 0.11	1.2 ± 0.6	0.15 ± 0.05	1.8 ± 0.4
GXE	E	R5	-	-	2.0 ± 0.4	26 ± 23	0.83 ± 0.11	8.2 ± 1.3	0.83 ± 0.38	6.3 ± 5.2
Arithmetic means[d]			1.4	23	0.57	6.1	0.22	1.6	0.19	1.2
Geometric means[d]			1.2	17	0.37	3.4	0.12	0.56	0.083	0.46

[a] Antibody concentration (nM) resulting in 50% inhibition of virus infection.

[b] Antibody concentration (nM) resulting in 90% inhibition of virus infection.

[c] No significant neutralization at the highest antibody concentration (67 nM) tested.

[d] Arithmetic and geometric means were calculated for all viruses including those with values <0.021 nM, which were assigned a value of 0.01. The means for 2G12 were calculated based on the values with 10 isolates that were significantly neutralized.

Table 4. Inhibition of HIV-1 Env-mediated cell-cell fusion by mD1.22 of different valences.

Virus	Clade	Tropism	m36.4		mD1.22		mD1.22Fc		2Dm2m		4Dm2m		6Dm2m	
			IC_{50}[a]	IC_{90}[b]	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Bal	B	R5	11 ± 1	235 ± 49	21 ± 0	108 ± 4	24 ± 4	135 ± 21	21 ± 6	113 ± 39	4.1 ± 0.1	13 ± 3	3.0 ± 0.0	5.0 ± 0.8
JRFL	B	R5	128 ± 5	1075 ± 35	66 ± 1	208 ± 4	66 ± 1	>222	7.5 ± 0.0	80 ± 9	1.6 ± 0.7	11 ± 2	0.46 ± 0.07	10 ± 3

[a] Antibody concentration (nM) resulting in 50% inhibition of cell-cell fusion.

[b] Antibody concentration (nM) resulting in 90% inhibition of cell-cell fusion.

Table 5. Inhibition of HIV-1 Env-mediated cell-cell fusion by 4Dm2m, 6Dm2m, and bnmAbs.

Virus	Clade	Tropism	2G12		VRC01		4Dm2m		6Dm2m	
			IC_{50}[a]	IC_{90}[b]	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Bal	B	R5	38 ± 31	>67	2.6 ± 0.0	24 ± 2	4.1 ± 0.1	12 ± 0	2.3 ± 0.2	6.7 ± 2.2
JRFL	B	R5	14 ± 2	67 ± 5	3.1 ± 0.6	21 ± 1	3.6 ± 0.2	8.8 ± 0.4	2.3 ± 0.3	5.8 ± 1.1

[a] Antibody concentration (nM) resulting in 50% inhibition of cell-cell fusion.

[b] Antibody concentration (nM) resulting in 90% inhibition of cell-cell fusion.

Table 6. Neutralization of HIV-1 in TZM-bl cell-based assays

Virus	Clade	Tropism	IC_{50} (nM)[a]					IC_{90} (nM)[a]				
			b12[b]	VRC01[b]	D1D2Fc	4Dm2m	6Dm2m	b12	VRC01	D1D2Fc	4Dm2m	6Dm2m
KNH1088.EC5	A	R5	117	3.0	>250	0.48	1.0	>167	18	>250	18	33
KNH1144.EC1	A	R5	>167	2.2	4.8	0.081	0.11	>167	9.5	42	1.0	1.1
Bal.EC1 TC	B	R5	1.2	0.39	0.60	0.057	0.045	3.6	5.2	3.2	0.22	0.20
BZ167.ec9	B	X4	14	6.4	13	0.077	0.10	>167	31	60	0.50	0.76
GS-015.EC12	C	R5	0.085	>167	0.20	0.022	0.027	1.6	>167	3.2	0.17	0.17
PBL286.VRC36aPV	C	R5	4.0	2.1	87	2.0	2.1	19	10	>250	14	12
A07412VRC12A	D	R5	12	1.3	57	0.75	0.76	>167	6.0	>250	5.7	6.1
57128.VRC18	D	R5	2.3	>167	5.4	0.40	0.44	30	>167	41	2.8	2.6
CM240.EC1	AE	R5	82	0.66	20	0.45	0.40	>167	11	>250	3.7	3.6
N1 1046.e3 PV	AE	R5	138	4.2	183	3.5	3.1	>167	18	>250	27	30
CAM0015BBY.EC3	AG	R5	81	21	41	0.72	1.4	>167	>167	>250	7.4	7.6
55815.EC3	AG	R5	>167	0.33	6.2	0.084	0.077	>167	2.6	116	0.61	0.67
Arithmetic mean[c]			71	37	60	0.72	0.80	138	59	172	6.8	8.2
Geometric mean[c]			17	4.2	14	0.28	0.32	67	21	82	2.4	2.6

[a] The assay was performed in duplicate. Results are presented as mean without standard deviation. Means are calculated only when the difference between results for the duplicate assays was within 3-fold.

[b] The bnAbs b12 and VRC01 used in the neutralization assay are in IgG1 format.

[c] Arithmetic and geometric means were calculated for all viruses including those with values >167 nM, which were assigned a value of 200, and those with values >250, which were assigned a value of 300.

Table 7. Neutralization of HIV-1 isolates predominantly circulating in China in TZM-bl cell-based assays

Virus	Clade	Tropism	IC_{50} (nM)[a]						IC_{90} (nM)[a]					
			T20	C34	D1D2Fc	2Dm2m	4Dm2m	6Dm2m	T20	C34	D1D2Fc	2Dm2m	4Dm2m	6Dm2m
CH64	BC	R5	3.1	1.2	48	1.7	0.17	0.13	25	6.7	>250	14	1.6	0.80
CH70	BC	R5	65	7.6	7.3	0.40	0.11	0.09	>750	84	109	3.1	1.2	0.67
CH91	BC	R5	8.6	2.3	35	3.7	0.39	0.27	310	12	>250	56	10	3.5
CH110	BC	R5	4.3	0.78	28	0.93	0.11	0.09	262	6.3	>250	8.7	0.83	0.58
CH114	BC	R5	102	5.4	23	1.5	0.11	0.18	>750	25	198	19	4.0	1.9
CH117	BC	R5	2.3	0.60	27	5.4	0.33	0.22	34	3.2	>250	62	15	5.1
CH119	BC	R5	2.0	3.3	85	2.7	0.17	0.13	25	20	>250	25	3.4	1.1
CH120	BC	R5	11	4.6	104	4.3	0.33	0.27	443	39	>250	46	6.6	3.5
CNE7	BC	R5	4.3	6.4	19	1.5	0.18	0.14	58	22	161	8.1	1.8	1.2
CNE15	BC	R5	14	3.8	106	3.0	0.29	0.17	157	22	>250	20	2.6	1.3
CNE16	BC	R5	14	11	11	0.77	0.08	0.07	169	51	229	8.5	1.2	0.71
CNE20	BC	R5	5.5	3.3	65	11	0.05	0.29	41	14	>250	>100	5.3	2.9
CNE23	BC	R5	15	2.1	>250	5.4	0.49	0.48	228	18	>250	35	4.9	9.9
CNE30	BC	R5	26	14	17	0.58	0.07	0.06	285	110	211	3.6	0.44	0.31
CNE40	BC	R5	4.9	0.77	0.06	0.04	0.01	0.01	108	7.8	0.50	0.33	0.11	0.09
CNE46	BC	R5	26	6.4	99	3.8	0.27	0.21	743	115	>250	81	9.4	2.8
CNE47	BC	R5	4.9	1.9	152	1.5	0.11	0.12	42	14	>250	30	2.8	1.2
CNE49	BC	R5	9.1	2.3	21	0.40	0.05	0.05	236	12	224	2.6	0.56	0.44
CNE53	BC	R5	51	2.0	14	2.8	0.24	0.47	721	23	>250	95	5.5	12
CNE68	BC	R5	32	2.2	9.7	0.40	0.11	0.04	321	12	133	4.5	0.78	0.89
CNE1	B'	X4	1.6	0.73	197	0.47	0.11	0.22	17	6.8	>250	4.1	0.61	2.2
CNE4	B'	R5	1.7	6.3	3.9	0.40	0.11	0.13	14	23	24	0.40	2.4	0.80
CNE6	B'	R5	1.4	1.6	68	1.7	0.33	0.22	15	15	192	6.9	2.3	1.4
CNE9	B'	R5	1.7	0.87	9.9	1.1	0.22	0.22	14	5.8	63	7.0	3.2	1.7
CNE11	B'	R5	3.5	4.6	>250	1.8	0.33	0.36	36	42	>250	11	2.6	3.4
CNE14	B'	R5	9.7	3.9	105	0.80	0.17	0.18	305	17	248	4.13	2.4	1.6
CNE57	B'	X4	2.5	2.0	250	2.1	1.2	0.71	24	14	>250	>100	19	22
CNE64	B'	R5	1.9	5.1	12	1.4	0.56	0.22	16	21	113	6.5	3.2	1.5
CNE17	C	R5	42	3.2	11	2.4	0.11	0.13	676	82	231	36	2.4	0.84
CNE58	C	R5	54	2.0	>250	10	2.1	1.1	745	13	>250	>100	>28	>28
CNE65	C	R5	5.9	3.1	15	1.1	0.28	0.22	78	16	115	12	3.3	1.5
CNE66	C	R5	129	2.1	159	1.2	0.17	0.09	729	11	>250	>100	7.7	3.1
CNE3	AE	R5	5.6	7.1	>250	1.9	0.39	0.31	38	71	>250	11	2.8	2.0

CNE5	AE	R5	2.1	5.9	94	1.8	0.67	0.53	19	63	236	15	8.7	5.9
CNE55	AE	R5	26	7.9	213	5.9	0.45	0.46	549	103	>250	42	10	5.1
CNE59	AE	R5	1.4	3.4	10	0.93	0.17	0.18	17	28	220	16	2.7	3.8
CNE107	AE	X4	3.8	2.8	135	3.7	0.52	0.89	45	32	>250	33	18	21
AE20	AE	R5	212	7.4	50	1.5	0.72	0.49	572	69	>250	10	5.8	4.1
AE03	AE	R5	304	24	>250	5.7	0.67	0.40	510	85	>250	58	12	15
YN192.31	AE	R5	42	27	155	5.7	0.89	2.4	336	225	>250	65	21	19
GX2010.36	AE	R5	255	15	12	4.7	0.11	0.13	>750	165	>250	90	18	6.8
Arithmetic mean[b]			37	5.3	94	2.6	0.34	0.32	277	42	242	38	6.2	5.0
Geometric mean[b]			11	3.5	42	1.7	0.22	0.20	123	25	195	17	3.5	2.4

[a] The assay was performed in duplicate. Results are presented as mean without standard deviation. Means are calculated only when the difference between results for the duplicate assays was within 3-fold.

[b] Arithmetic and geometric means were calculated for all viruses including those with values >750 nM, which were assigned a value of 800, those with values >250 nM, which were assigned a value of 300, those with values >100, which were assigned a value of 150, and those with values >28 nM, which were assigned a value of 30.

Table 8. Binding kinetics of mD1.2 and mD1.22 to HIV-1 gp140 as measured by SPR

HIV-1 gp140	Clade	mD1.2			mD1.22		
		K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (nM)	K_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (nM)
$Gp140_{Con-s}$	Consensus[a]	1.7×10^5	9.3×10^{-4}	5.4	1.7×10^5	7.7×10^{-4}	4.4
$Gp140_{MS}$	A	4.2×10^5	1.0×10^{-4}	0.24	3.8×10^5	7.3×10^{-5}	0.19
$Gp140_{89.6}$	B	3.4×10^5	4.0×10^{-4}	1.2	2.5×10^5	2.1×10^{-4}	0.87
$Gp140_{CH12.0544.2}$	B	1.5×10^5	1.1×10^{-4}	0.72	1.2×10^5	2.0×10^{-5}	0.17

[a] A consensus gp140 designed by aligning >1,000 sequences of HIV-1 group M.

K_a, association rate constant.

K_d, dissociation rate constant.

K_D, equilibrium dissociation constant.

[0119] The examples described herein demonstrate the identification of new D1 mutants. In particular, mD1.22 has improved soluble expression and stability, showed no measurable interaction with human blood B and T cell lines, and preserved binding and cross-reactivity with HIV-1 gp120. mD1.22-m36.4 fusion proteins have been shown to be potent HIV-1 neutralizers and multivalent mD1.22-m36.4 fusion proteins do not tend to enhance HIV-1 infectivity. Thus, the newly identified mutants can be used for HIV-1 prevention and therapy, and also can serve as valuable tools to study the mechanism of HIV-1 entry and biological functions of CD4 in immune responses.

[0120] 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.

[0121] 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.

[0122] 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.

CLAIMS:

1. A polypeptide comprising the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

2. A composition comprising the polypeptide of claim 1 and a carrier.

3. A fusion protein comprising (i) the amino acid sequence of SEQ ID NO: 1 and (ii) one or more fusion partners, wherein the one or more fusion partners optionally is fused to the amino acid sequence of (i) via a linker.

4. A fusion protein comprising (i) the amino acid sequence of SEQ ID NO: 2 and (ii) one or more fusion partners, wherein the one or more fusion partners optionally is fused to the amino acid sequence of (i) via a linker.

5. The fusion protein of claim 3 or 4, wherein the one or more fusion partners is an engineered antibody domain (eAd), an HIV envelope glycoprotein, an Fc region or portion thereof, an immunoglobulin heavy chain constant region, an immunoglobulin light chain constant region, or a combination thereof.

6. The fusion protein of claim 5, wherein the one or more fusion partners is an eAd.

7. The fusion protein of claim 6, wherein the eAd binds to an HIV envelope glycoprotein.

8. The fusion protein of claim 7, wherein the eAd comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 12-16.

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

10. The fusion protein of any one of claims 7-9 wherein the HIV envelope glycoprotein is gp120.

11. The fusion protein of any one of claims 7-10, wherein the HIV is HIV-1.

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

13. A fusion protein comprising:

A-(optional linker)-C-(optional linker)-B

or

B-(optional linker)-D-(optional linker)-E-(optional linker)-B

wherein A is an antibody or antibody fragment, B is the polypeptide of claim 1, C is an immunoglobulin light chain constant region, D is an immunoglobulin heavy chain constant region, and E is an Fc region or portion thereof.

14. The fusion protein of claim 13, wherein the antibody or antibody fragment of A comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 12-16.

15. The fusion protein of any one of claims 12-14, wherein the Fc region or portion thereof is an IgG1 Fc region.

16. The fusion protein of claim 15, wherein the Fc region or portion thereof comprises SEQ ID NO: 7 or SEQ ID NO: 8.

17. The fusion protein of any one of claims 3-16, wherein the linker comprises one or more G_4S motifs.

18. The fusion protein of any one of claims 3-17, wherein the linker comprises the amino acid sequence of SEQ ID NO: 9, SEQ ID NO: 10, or SEQ ID NO: 11.

19. A composition comprising the fusion protein of any one of claims 3-18 and a carrier.

20. A nucleic acid encoding the polypeptide of claim 1.

21. A nucleic acid encoding the fusion protein of any one of claims 3-18.

22. A recombinant vector comprising the nucleic acid of claim 20 or 21.

23. A cell comprising the nucleic acid of claim 20 or 21 or the vector of claim 22.

24. A composition comprising (i) the nucleic acid of claim 20 or 21, the vector of claim 22, or the cell of claim 23, and (ii) a carrier.

25. A construct comprising

two fusion proteins of A-(optional linker)-C (Formula (III)), and

two fusion proteins of B-(optional linker)-D-(optional linker)-E (Formula (IV)),

wherein A is an antibody or antibody fragment, B is the polypeptide of claim 1, C is an immunoglobulin light chain constant region, D is an immunoglobulin heavy chain constant region, and E is an Fc region or portion thereof.

26. The construct of claim 25, wherein the fusion protein of Formula (III) comprises SEQ ID NO: 19 and the fusion protein of Formula (IV) comprises SEQ ID NO: 18.

27. A construct comprising

two fusion proteins of A-(optional linker)-C (Formula (III)), and

two fusion proteins of B-(optional linker)-D-(optional linker)-E-(optional linker)-B (Formula (II)),

wherein A is an antibody or antibody fragment, B is the polypeptide of claim 1, C is an immunoglobulin light chain constant region, D is an immunoglobulin heavy chain constant region, and E is an Fc region or portion thereof.

28. The construct of claim 27, wherein the fusion protein of Formula (III) comprises SEQ ID NO: 21 and the fusion protein of Formula (II) comprises SEQ ID NO: 20.

29. A construct comprising

two fusion proteins of A-(optional linker)-C-(optional linker)-B (Formula (I)), and

two fusion proteins of B-(optional linker)-D-(optional linker)-E-(optional linker)-B (Formula (II)),

wherein A is an antibody or antibody fragment, B is the polypeptide of claim 1, C is an immunoglobulin light chain constant region, D is an immunoglobulin heavy chain constant region, and E is an Fc region or portion thereof.

30. The construct of claim 29, wherein the fusion protein of Formula (I) comprises SEQ ID NO: 23 and the fusion protein of Formula (II) comprises SEQ ID NO: 22.

31. A construct having a structure depicted in Figure 2, wherein A is an antibody or antibody fragment, B is the polypeptide of claim 1, C is an immunoglobulin light chain constant region, D is an immunoglobulin heavy chain constant region, E is an Fc region or portion thereof, and straight lines are optional linker sequences; and wherein C and D are optionally joined via disulfide bonds, and the two Fc regions are optionally joined via disulfide bonds.

32. A composition comprising the construct of any one of claims 25-31 and a carrier.

33. A conjugate comprising (a) the polypeptide of claim 1, the fusion protein of any one of claims 3-18, or the construct of any one of claims 25-31 and (b) a cytotoxic agent.

34. The conjugate of claim 33, wherein the cytotoxic agent is a toxin.

35. A composition comprising the conjugate of claim 33 or 34 and a carrier.

36. A method of prophylactically or therapeutically inhibiting a viral infection in a cell or host comprising administering to the cell or host (i) the polypeptide of claim 1, (ii) the fusion protein of any one of claims 3-18, (iii) the nucleic acid of claim 20 or 21, (iv) the vector of claim 22, (v) the cell of claim 23, (vi) the construct of any one of claims 25-31, (vii) the conjugate of claim 33 or 34, or (viii) a composition thereof, such that the viral infection is inhibited.

37. The method of claim 36, wherein the viral infection is an HIV infection.

38. The method of claim 37, wherein the HIV infection is an HIV-1 infection.

39. A method of eradicating viral-infected cells in a subject comprising administering the conjugate of claim 33 or 34 or the composition of claim 35 to the subject, thereby eradicating the viral-infected cells in the subject.

40. The method of claim 39, wherein the viral-infected cells are HIV-infected cells.

41. The method of claim 40, wherein the HIV-infected cells are HIV-1 infected cells.

FIGURE 1

```
                1       10        20        30        40        50        60        70        80        90       100   SEQ ID NO:
                |.........|.........|.........|.........|.........|.........|.........|.........|.........|.........|
D1              KKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQIKILGNQGSFLTKGPSKLNDRADSRRSLWDQGNFPLIIKNLKIEDSDTYICEVEDQKEEVQLLVFG      3
mD1.2           ....Y.....................N.....................................P.....................V.V.      4
mD1.22          ....Y.....................N.....V...............................P.....................V.V.      1
mD1.23          ....Y.....................N.....D...............................P.....................V.V.      2
```

FIGURE 2

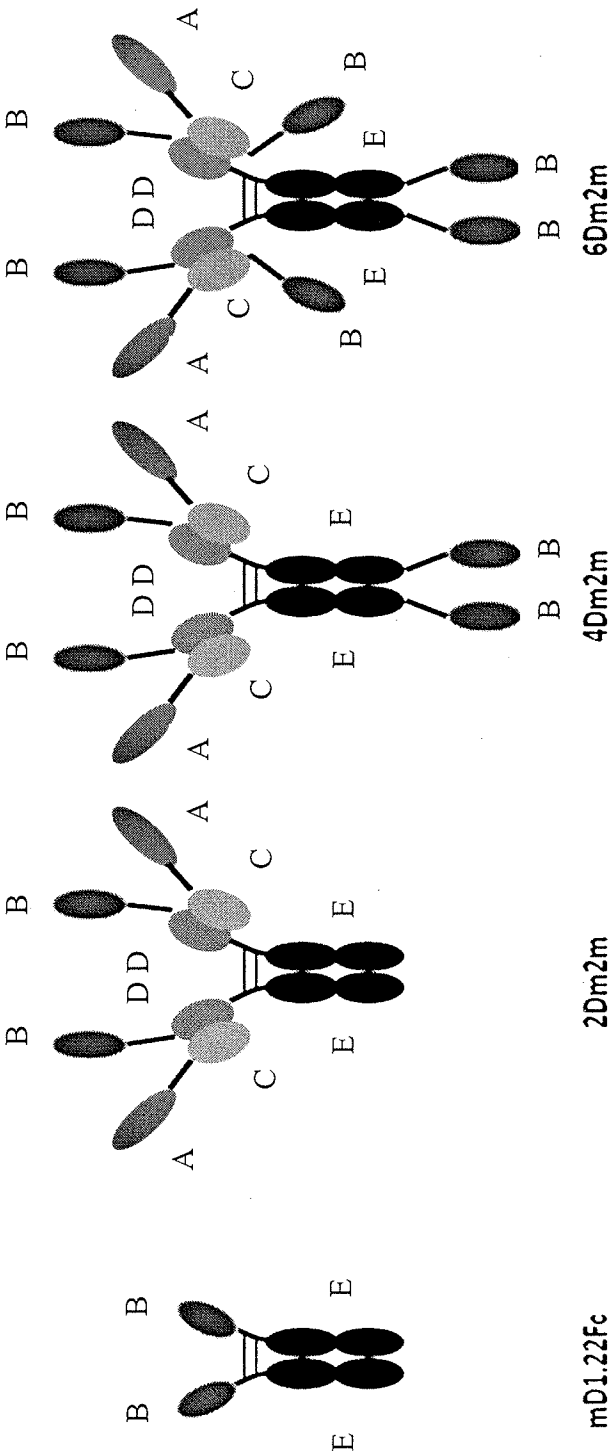


FIGURE 3

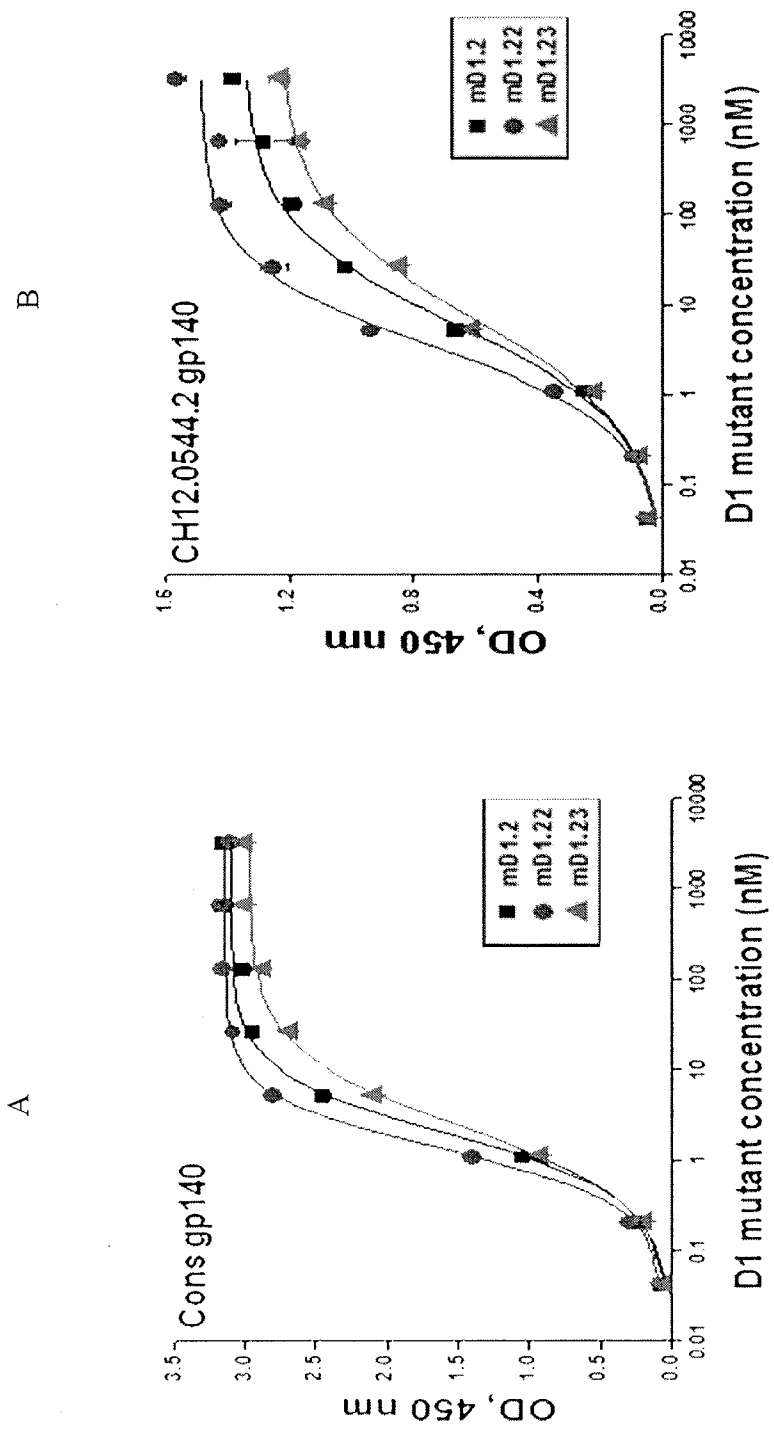
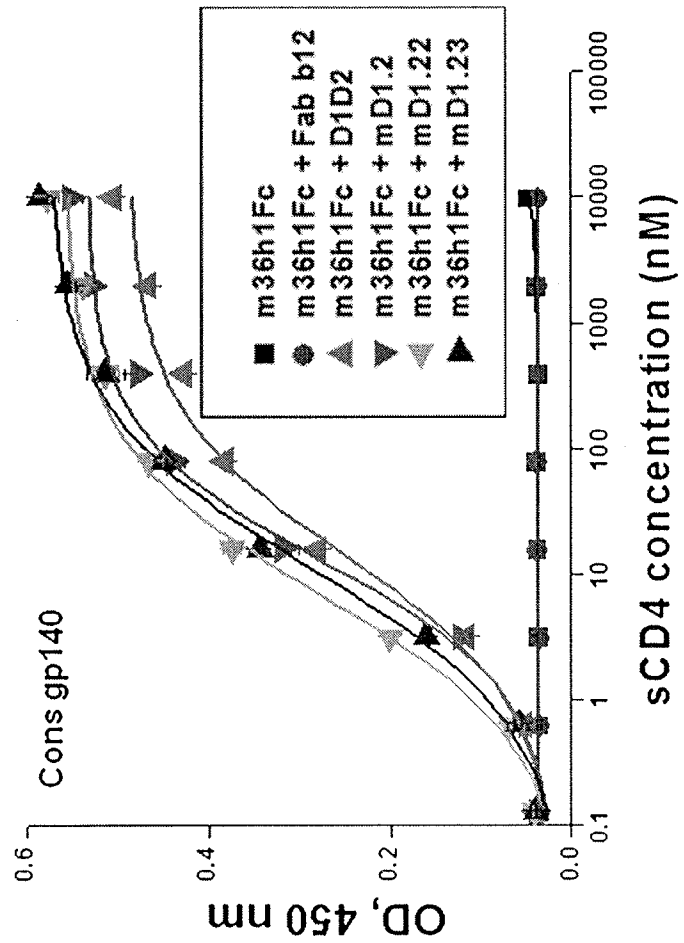

A
Cons gp140
OD, 450 nm
D1 mutant concentration (nM)
mD1.2
mD1.22
mD1.23
B
CH12.0544.2 gp140
OD, 450 nm
D1 mutant concentration (nM)
mD1.2
mD1.22
mD1.23

FIGURE 4


Cons gp140
m36h1Fc
m36h1Fc + Fab b12
m36h1Fc + D1D2
m36h1Fc + mD1.2
m36h1Fc + mD1.22
m36h1Fc + mD1.23
OD, 450 nm
0.0
0.2
0.4
0.6
0.1
1
10
100
1000
10000
100000
sCD4 concentration (nM)

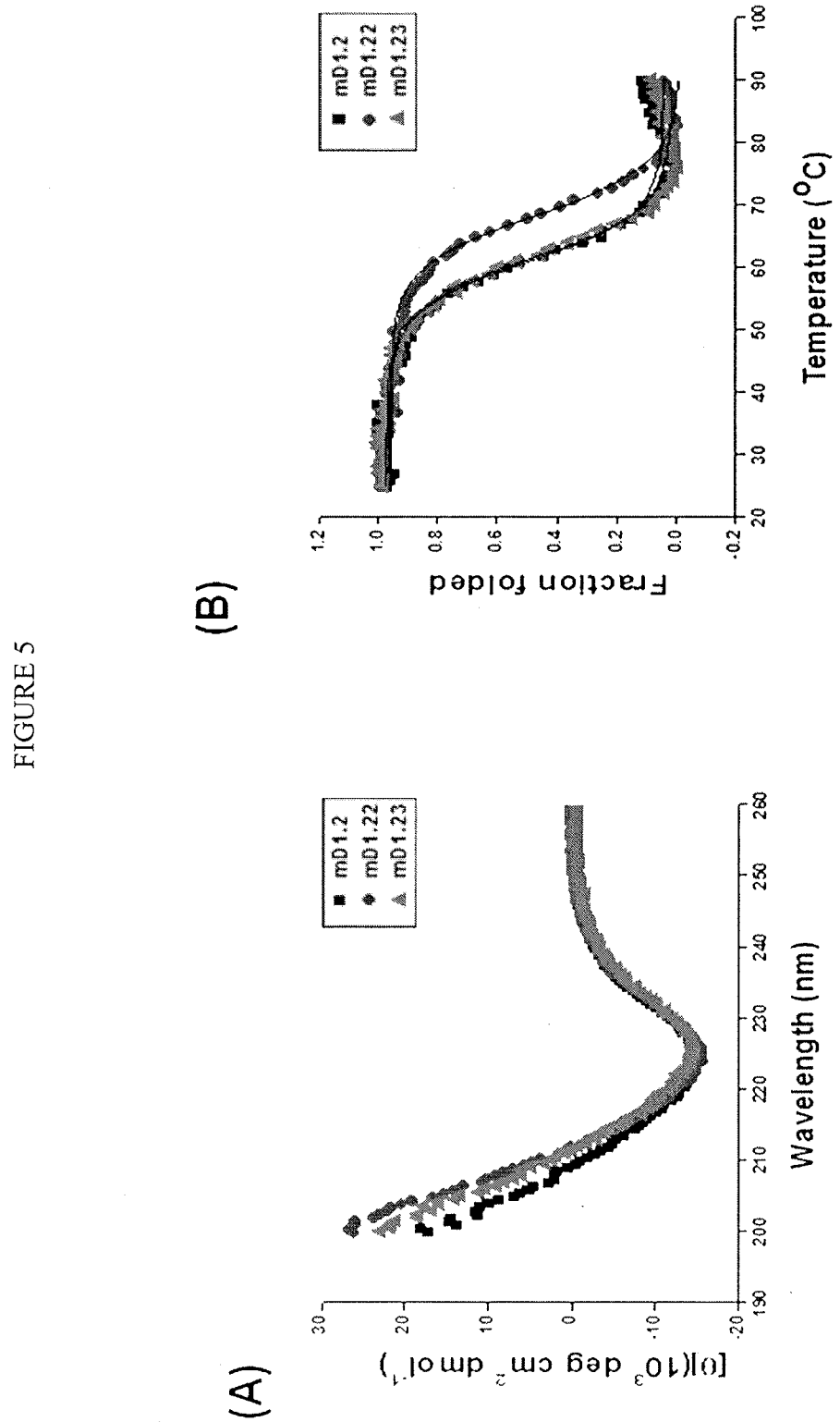

(A)
[θ](10³ deg cm² dmol⁻¹)
Wavelength (nm)
mD1.2
mD1.22
mD1.23
(B)
Fraction folded
Temperature (°C)

FIGURE 5

FIGURE 6

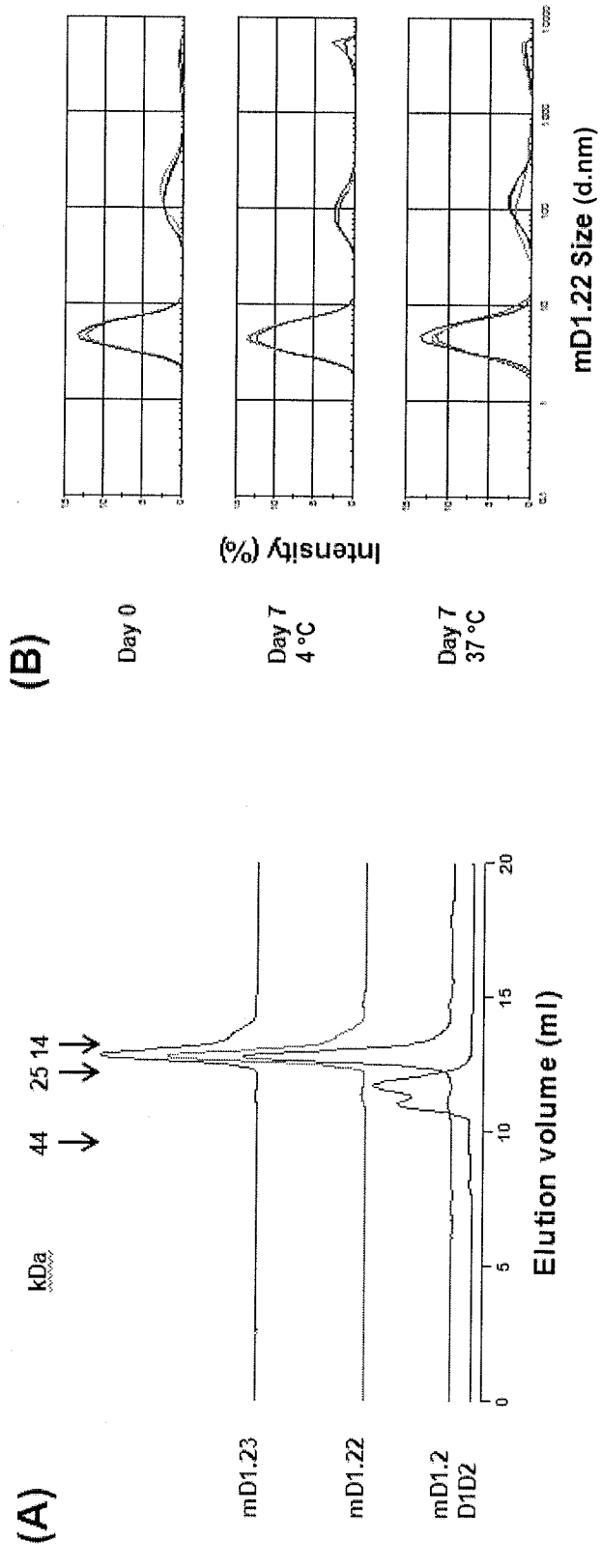

(A)
kDa
44
25 14
mD1.23
mD1.22
mD1.2
D1D2
0
5
10
15
20
Elution volume (ml)
(B)
Day 0
Day 7
4 °C
Day 7
37 °C
Intensity (%)
mD1.22 Size (d.nm)

FIGURE 7

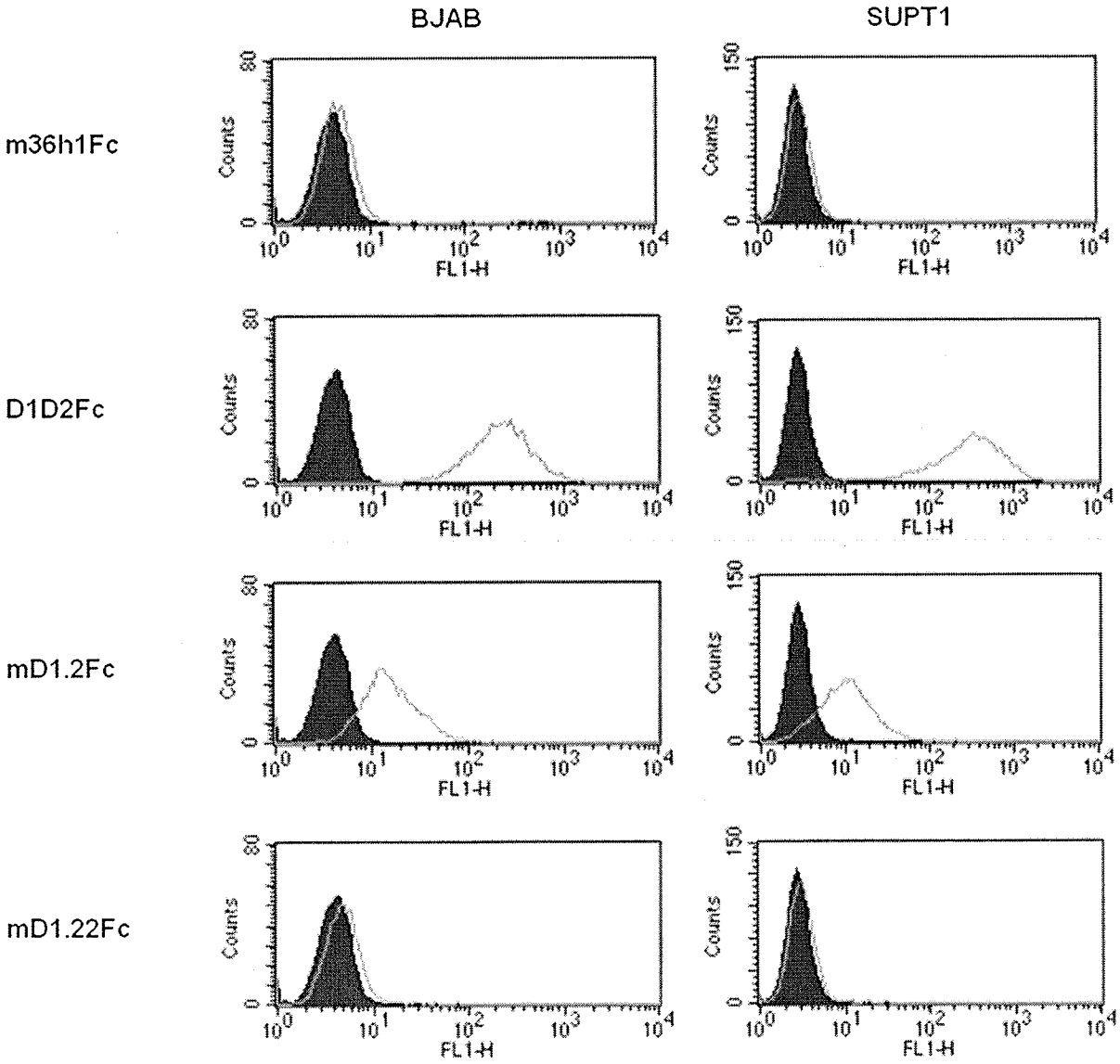


FIGURE 8

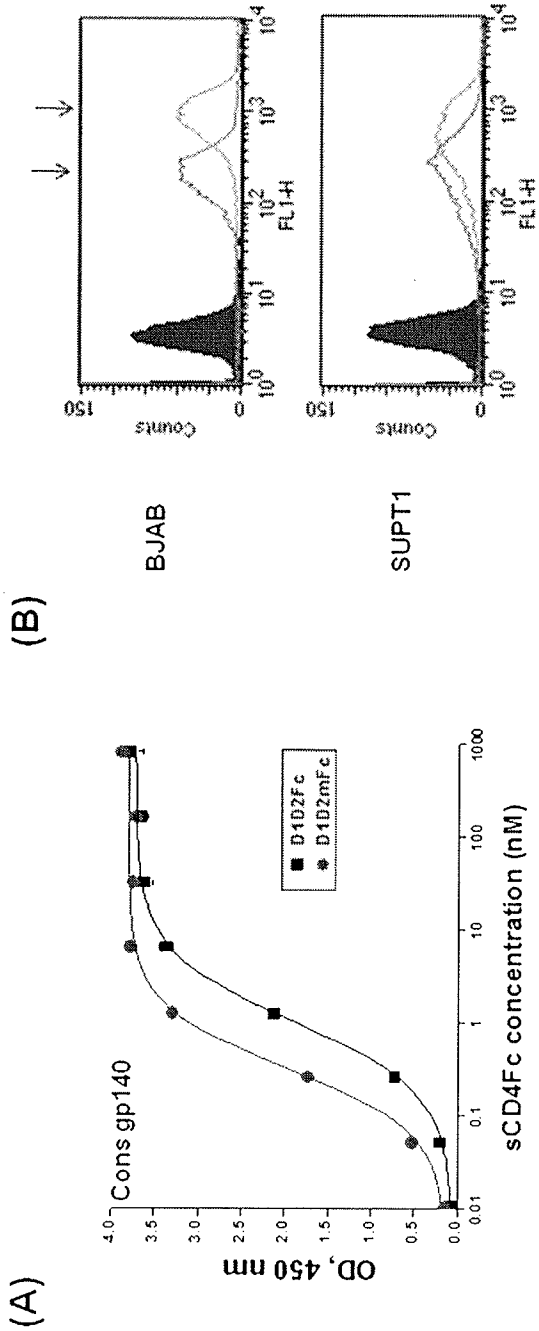

(A)
OD, 450 nm
Cons gp140
D1D2Fc
D1D2mFc
sCD4Fc concentration (nM)
(B)
BJAB
SUPT1
Counts
FL1-H

FIGURE 9

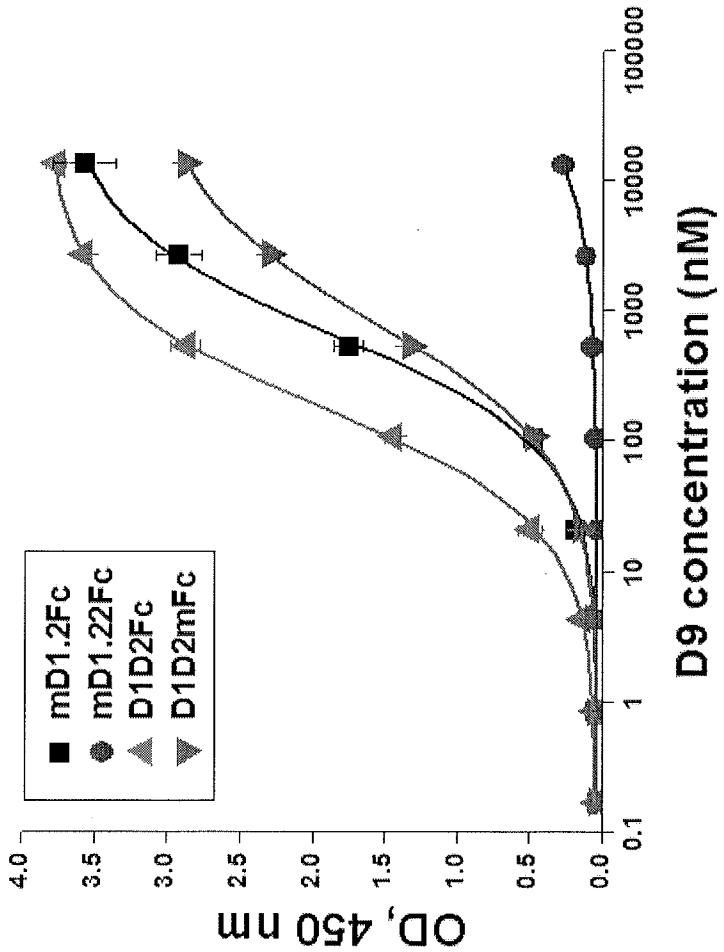

mD1.2Fc
mD1.22Fc
D1D2Fc
D1D2mFc
OD, 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.1
1
10
100
1000
10000
100000
D9 concentration (nM)

FIGURE 10

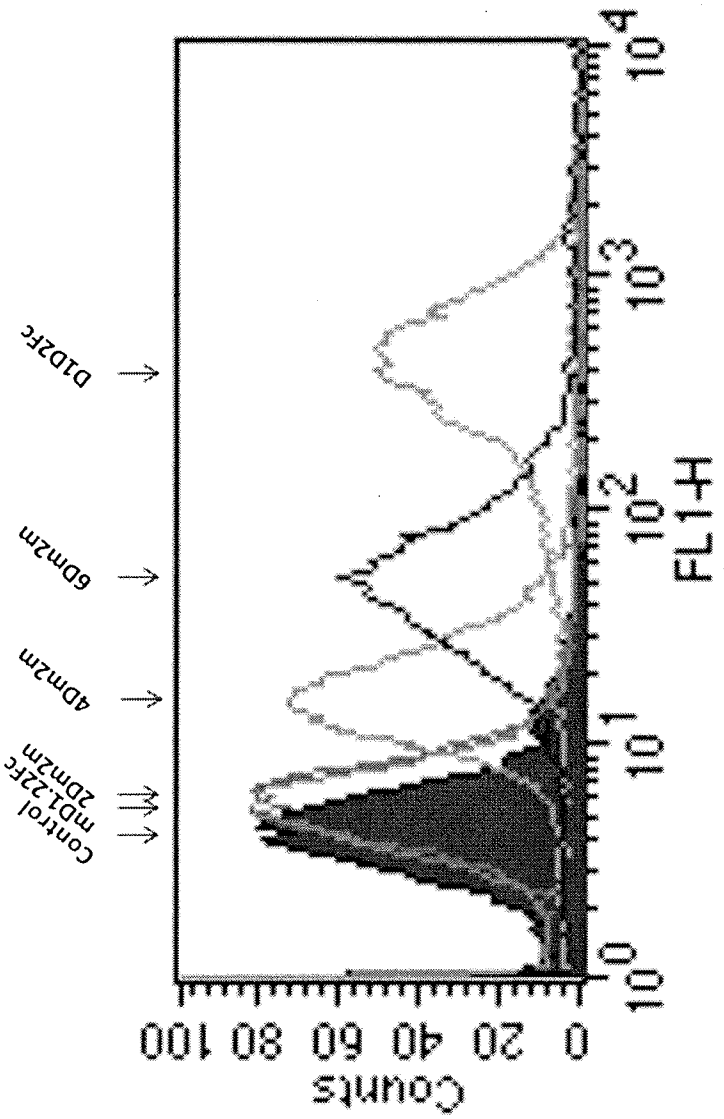
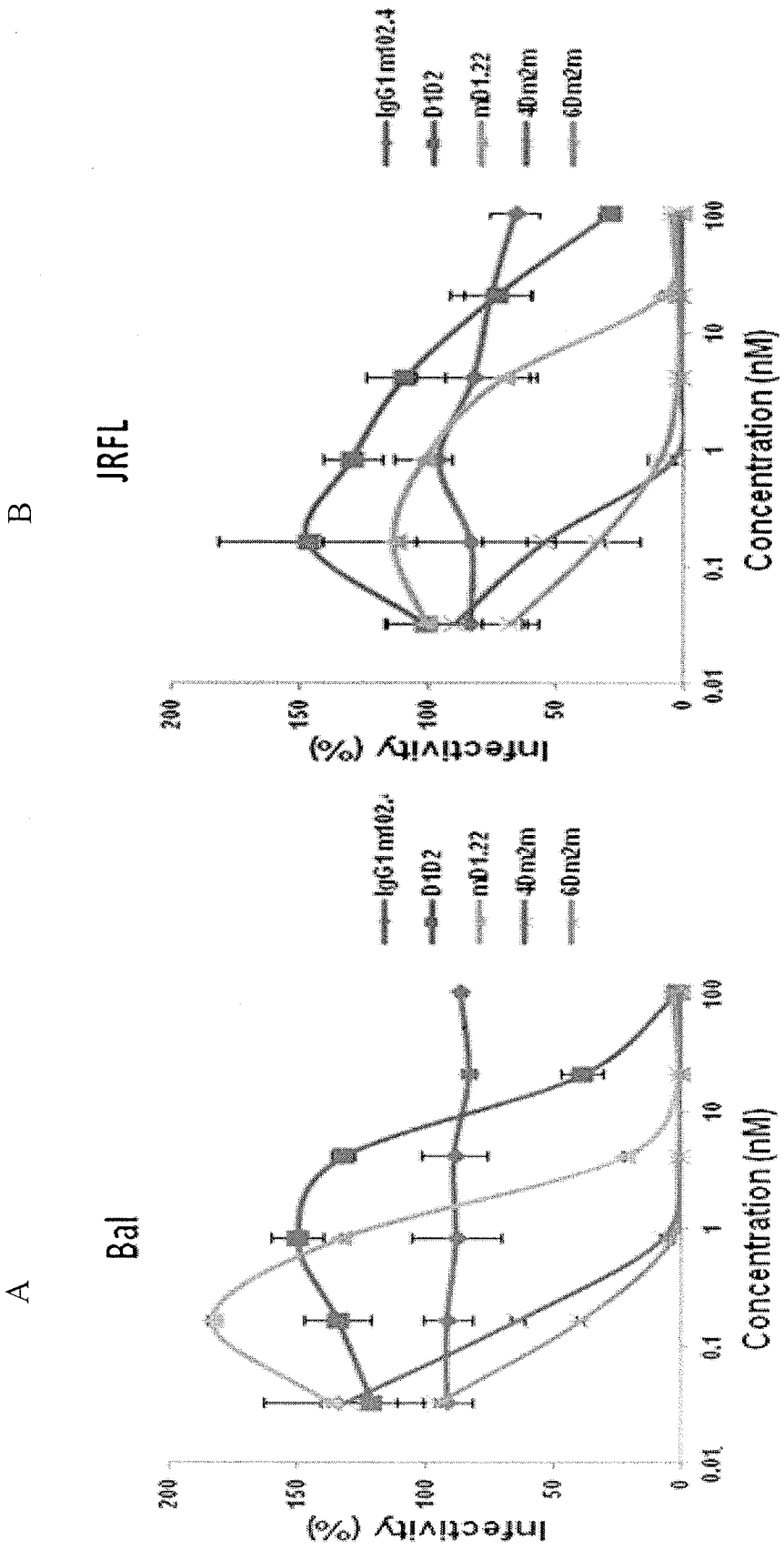

Counts
0 20 40 60 80 100
FL1-H
10^0 10^1 10^2 10^3 10^4
Control
mD1.22Fc
2DM2m
4DM2m
6DM2m
D1D2Fc

FIGURE 11


A
Bal
B
JRFL
IgG1m102.4
D1D2
m01.22
40m2m
60m2m
Infectivity (%)
Concentration (nM)
0
50
100
150
200
0.01
0.1
1
10
100

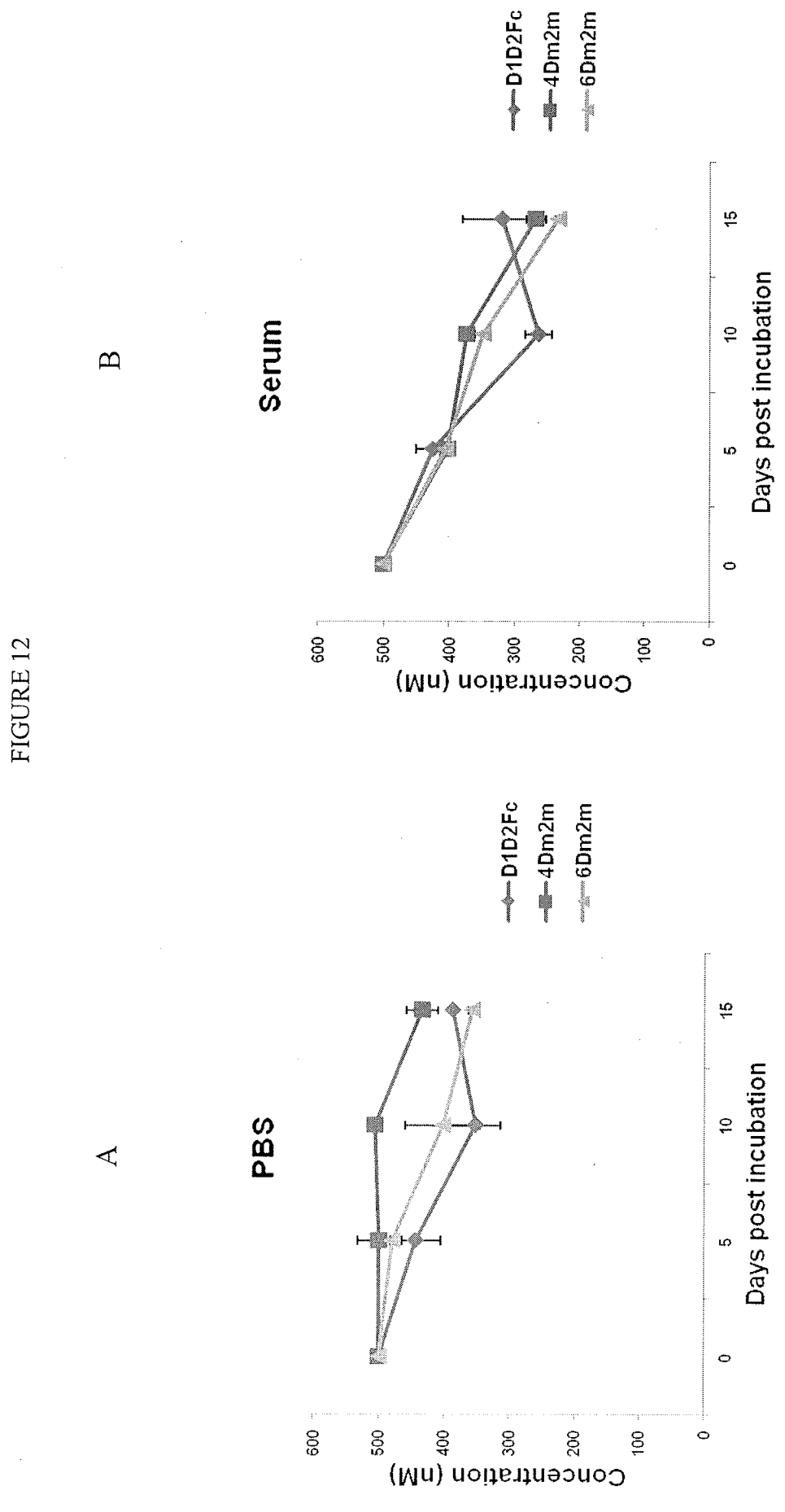

A
PBS
Concentration (nM)
600
500
400
300
200
100
0
0
5
10
15
Days post incubation
D1D2Fc
4Dm2m
6Dm2m
B
Serum
Concentration (nM)
600
500
400
300
200
100
0
0
5
10
15
Days post incubation
D1D2Fc
4Dm2m
6Dm2m

FIGURE 12